THE GENETIC PROFILES OF *TIF1* AND *TIF2* DUPLICATE GENES

BY

VERONICA VENTURI

A thesis

Submitted to the Victoria University of Wellington

in fulfilment of the requirements for the degree of

Doctor of Philosophy in

Cell and Molecular Bioscience

Victoria University of Wellington

2012

Abstract

As one of the key steps in protein synthesis, translation initiation is subjected to multi-level regulation which is achieved via diverse mechanisms. The cell adjusts protein synthesis accordingly to its status and environment. The degree of contribution of the processes involved in the regulation of translation initiation is still poorly understood.

The first part of this study focuses on identifying mechanisms of regulation in a translationally deficient yeast system, impaired by the loss of one or the other of the *TIF1/2* duplicate genes, which together code for the eukaryotic initiation factor 4A (eIF4A). A major finding of this research is related to the functional competences associated with the two duplicate members of the gene pair. Although the genetic profile associated with *TIF1* highlights a connection with transcriptional process, the majority of transcription-translation inter-talk is allocated with *TIF2*, along with a dense network of genetic interactions surrounding the SAGA complex. *TIF2* is also the only paralog devoted to interactions with a substantial group of functionally related genes involved in early meiotic gene expression. Protein degradation in the global control of protein synthesis represents a fundamental process and accounts for diverse points of control, in particular through ubiquitination/deubiquitination. This research concludes that functional turnover of proteins and the translation/transcription inter-talk emerges as the most significant contributors to the sophisticatedly regulated translational regulation,

The second part of this study aims to determine the extent of similarity and divergence between the *TIF1* and *TIF2* paralogs. Growth of their individual

deletion strains was challenged under different chemical and environmental conditions with the intent to explore the relative contributions of each duplicate in response to an extend range of perturbations. The pair of duplicates appeared convincingly robust in coping with these adversities under disparate cellular contexts, thus suggesting a highly conserved and backed-up genetic network.

One of the primary treatments made use of lithium, a condition which was hoped to help, along with furthering our understanding of the *TIF1* and *TIF2* networks, in formulating an explanation on how augmented translation initiation overcomes lithium toxicity. Although this approach did not return results that could be used to address this unresolved topic, evaluation of genetic inhibition and suppression was highly instructive regarding the mechanisms of response triggered upon lithium/galactose stress. Regulation and synchronization of basic cellular processes were affected: emphasis brought on aspects of cell communication highlighted mechanisms articulated by kinase enzymes and the importance of repression of cell cycle progression in control of protein synthesis. Data from the screen also indicated the stress that combined lithium/galactose treatment places on central metabolic pathways, for instance those between the Leloir, gluconeogenesis, and trehalose pathways.

Acknowledgements

A PhD is a long journey and, by the end of it, you come to the realization that other things were taking place in your life while the majority of your experiments were failing. To me it has been incredibly tough and I do know in my heart I would be nowhere without my supervisor Associate Professor Paul Teesdale-Spittle. Paul you are my hero. Thanks for supporting me in rough times, even when that meant saying nothing at all. Thanks for dealing with my quick mood swings and slow timing, for which when it comes to deadlines “tomorrow equals next week” but when I need something “tomorrow means now”. Thank you for teaching me how to write and for un-stiffing my brain. I felt spoilt on a daily basis by having the only supervisor that comes with a specialization in etymology and knows everything about linguistics...and who complimented my new haircut the day I decided to wear contacts instead of the usual glasses. Sometimes I seem to forget that, for how bright you are, you still have the wrong chromosome. I truly enjoyed our time together. I nourish the hope that our mentors will influence the scientists we will be tomorrow and I know integrity and respect will always set my path. For how scary as it sounds, I would start another PhD tomorrow to be your student again.

As everything appears to be “duplicated” in this thesis, I would like to express my gratitude to another Paul: Professor Paul Atkinson, you are the most irreverent academic I have ever met and I love you to bits. Thanks for giving me a home away from home and helping me to get a house in that little paradise named Eastbourne, the most beautiful place where I have ever lived. From you I have

learnt that it is OK to go straight to the point as often it will save you time later and that networking is important in any of its forms.

Back in my dark chemistry days, thanks to my co-supervisors Associate Professor Peter Northcote and Dr Joanne Harvey for having me in their labs; also thanks to Dr Joanna Wojnar, who helped solving any early settlement issues, from the card access to the leaking chromatographic column; although dated in time, your help has not been forgotten. A special mention to the most fashionable scientists Victoria University has ever known: Dr Fern Kelly and Dr John TV-star Watt, thanks for being lovely people to be around and for taking showers daily despite being chemists. Finally, thanks to Master-to-the-power-of-two Jeremy Robinson for being an insanely loyal and caring friend all the way.

I would have never made it in the lab without Dr Dave Maass and his unrivalled knowledge of molecular biology; the fact he is such a sexist is only condoned by his baking of the number one pumpkin pie in the world. A great amount of gratitude also goes to Dr James Matthews for spending time trying to teach me science and to his family for opening me their house and for the most decadent Brit style soirées. I also want to say a big thank you to Associate Professor Bill Jordan for keeping me on my toes when it comes to genetic networks and for always having nice and reassuring words. Along with Bill, a big thanks to the Proteomics Group for being such good friends across the corridor and to Professor John Miller and Associate Professor Anne La Flamme for letting me utilise their facilities.

To Miss Natelle Master Quek, the porcelain face on the other side of my desk and the most generous and sincere person on the planet, I rate our friendship as the most beautiful thing of my PhD years, even surpassing the genetic networks. I

feel we shared so many cool things and we set common milestones for our lives: I am now able to order my weak frothy cappuccino in Spanish and it is not for anyone to be the promo faces of the NZ Sign Language class for three years in a row. I am addicted to your sense of humour and I love that you love me the way I am. I have a debt of gratitude to Miss Christina Roberts, who single-handedly managed my thesis submission while I was overseas; thanks for helping me so many times and for being a fantastic friend. Thanks to Dr-to-be Peter Bircham for helping me with anything I needed and for sharing audacious recipes. I wonder what I am going to do now when the computer freezes or I have no clue what to cook for dinner. A special thank to Miss I-have-got-too-many-degrees-to-frame Juliana Brito for contributing remarkably to my lab work, for bringing a twist of Brazilian flavour in our lab, for introducing me to fried pastels, and for making me feel a bit less crazy. I will never forget the awkward environmental friendly showers masterminded by horrible Dr-to-be-in-a-click Ploi Yibmantasiri: this department has got a lot to learn from you when it comes to green policies. Also thanks for keeping up the mood of the troops. Thanks to Rose for helping me in the lab with all those annoying little things that I could have sorted out myself but I was too lazy to and for being that grip with reality in that crazy zoo named Chem Gen. It is has also been great fun to share the lab with Dr-before-than-anyone-else Yee, who made me feel lazy every single day of my PhD and happy-go-lucky Dora, who first departed the shores of Chemical Genetics, leading the way for the girls' pile exodus. Wish we could turn back time. Anyone else in the lab, thanks with all my heart: I am sure at some stage you have helped me with something.

I finally would like to knowledge my parents for supporting me all through this journey with their words and their bank accounts, for encouraging me in any crazy idea I have, for being the most liberal parents I could have wished, and for being always happy for me no matter what I do.

List of Figures

Figure 1-1. Section of a phylogenetic tree showing relationships among the sequenced fungal genomes.....	27
Figure 1-2. Duplicate regions (blocks) of chromosomes X and XI	29
Figure 2-1. SGA methodology.	66
Figure 2-2. An example of relationship between pathways identifiable through an SGA	68
Figure 2-3. A network representation of synthetic lethal interactions associated with yeast chromosome instability (CIN) genes found having human homologs mutated in cancer.....	71
Figure 2-4. Summary diagram of SGA methodology.....	101
Figure 2-5. Construction of <i>tif1Δ::natR</i> query strain via switching method for non-essential genes	113
Figure 2-6. <i>tif1Δ::natR</i> and <i>tif2Δ::natR</i> query strains diagnosis PCR.	114
Figure 2-7. An example of a <i>tif1Δ::natR</i> SGA plate.	117
Figure 2-8. Linkage genes for <i>TIF1</i> gene on chromosome XI.	118
Figure 2-9. Linkage groups.	119
Figure 2-10. An example of <i>tif2Δ::natR</i> SGA plate.....	120
Figure 2-11. Linkage genes for <i>TIF2</i> gene on chromosome X.	121
Figure 2-12. <i>LYP1</i> and <i>CAN1</i> linkage groups.....	122
Figure 2-13. BiNGO-generated <i>tif2Δ</i> GO components.....	129
Figure 2-14: BiNGO-generated <i>tif1Δ</i> GO processes	129
Figure 2-15. BiNGO-generated <i>tif2Δ</i> processes.....	130
Figure 2-16. BiNGO-generated <i>tif2Δ</i> processes.....	131
Figure 2-17. A schematic representation of a tetrad dissection	136
Figure 2-18. Small scale SGA plate	140

Figure 2-19. <i>TetO₂</i> -“off” promoter cassette.....	141
Figure 2-20. <i>tetO₂</i> promoter associated to a selective marker (<i>kanMX4</i>)	142
Figure 2-21. Assessment of repression of the titrable <i>tetO₂</i> -“off” promoter in <i>tif1Δ::natR tetO₂-tif2</i> strains.....	143
Figure 2-22. Manual spot assay	144
Figure 2-23. <i>GAL</i> cassette 1% agarose gel	145
Figure 2-24. Hypothetical genotype of a successful transformant strain with insertion of <i>GAL</i> over-expression promoter.....	146
Figure 2-25: Cytoscape generated genetic network surrounding the deletions of <i>TIF1</i> and <i>TIF2</i> genes.....	149
Figure 2-26. Vacuolar protein sorting	153
Figure 2-27. Partial representation of ESCRT machinery components’ subset of interactions	158
Figure 2-28. SAGA-related mechanisms of transcription.....	174
Figure 3-1. Galactose pathway.	218
Figure 3-2. An example of lithium/galactose assay plate.....	244
Figure 3-3. An example of spot assay plate containing 30 mM LiCl.....	245
Fig 3-4. BiNGO-generated illustration of over-represented GO function terms for sensitive strains upon lithium/galactose treatment.	252
Fig 3-5. BiNGO-generated illustration of over-represented GO function terms for sensitive strains upon lithium/galactose treatment.	253
Fig 3-6. BiNGO-generated illustration of over-represented GO process terms for sensitive strains upon lithium/galactose treatment.	254
Fig 3-7. BiNGO-generated illustration of over-represented GO process terms for sensitive strains upon lithium/galactose treatment.	255
Fig 3-8. BiNGO-generated representation of over-represented GO process terms for sensitive strains upon lithium/galactose treatment.....	256
Fig 3-9. BiNGO-generated illustration of over-represented GO process terms for resistant strains upon lithium/galactose treatment.	257

Fig 3-10. BiNGO-generated illustration of over-represented GO process terms for resistant strains upon lithium/galactose treatment.	258
Figure 3-11. Rapamycin spot assay	259
Figure 3-12. Diagram of the CWI cascade.....	263
Figure 3-13. The TCA cycle.	286
Figure 3-14. Interplay of the TCA and glyoxylate cycles (Kolkman, 2005).	287
Figure 3-15. Trehalose pathway.	296
Figure 3-16. Galactose utilization in gluconeogenesis.	299
Figure 3-17. Bypass suppressor mechanism.....	301
Figure 3-18. Calcineurin-mediated regulation of ion homeostasis in budding yeast.....	317

Map of Tables

Table 2-1. List of linkage genes surrounding <i>TIF1</i> (YKR059W).	119
Table 2-2. List of linkage genes surrounding <i>TIF2</i> (YJL138C)	122
Table 2-3. Genes genetically interacting with <i>TIF1</i>	124
Table 2-4. Genes genetically interacting with <i>TIF2</i>	124
Table 2-5. Genes genetically interacting with both <i>TIF1</i> and <i>TIF2</i>	125
Table 2-6. GO term enriched for biological processes in genes interacting with <i>TIF2</i>	127
Table 2-7. GO term enriched for components in genes interacting with <i>TIF2</i> ...	127
Table 2-8. Genetic interactions revealed by this study reported against the ones annotated in the existent literature.....	134
Table 2-9. Filtered hits from the primary screen were further confirmed via RSA and tetrad dissection.	137
Table 2-10. Genetic interactions for <i>TIF1</i> utilizing the Open Biosystem library	138
Table 2-11. Genetic interactions for <i>TIF2</i> utilizing the Open Biosystem library	139
Table 3-1. Scoring of sensitivity to lithium/galactose	239
Table 3-2. Interactions reported by this study upon 30 mM LiCl/galactose treatment.....	246
Table 3-3. GO term function over-represented for deletion strains sensitive to lithium/galactose treatment.....	247
Table 3-4. GO term process over-represented for deletion strains sensitive to lithium/galactose treatment.....	249
Table 3-5. GO process terms over-represented for deletion strains resistant to lithium/galactose treatment.....	251

List of Abbreviations

4E-BPs	4E-binding proteins
BINGO	Biological Networks Gene Ontology
BLASTP	Basic Local Alignment Search Tool Program
BSA	Bovine serum albumin
CCE	Capacitative calcium entry
CG	Cystine guanine
ChIP	ChiP-chip -chromatin immunoprecipitation
clonNAT	Nourseothricin-dihydrogen sulfate
CS	Chitin synthases
CWI	Cell wall integrity
CYGD	Comprehensive Yeast Genome Database
d	Day
dd water	Double distilled water
DDC	Duplication-degeneration-complementation
DEAD box	D-E-A-D (Asp-Glu-Ala-Asp) motif
DMA	Deletion mutant array
DMSO	Dimethyl sulfoxide
dNTP	Deoxy-nucleotide triphosphates
DO	Drop-out
DTT	Dithiothreitol
DUBm	Deubiquitinating module
DUBs	Deubiquitinating enzymes
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
ERMES	ER-mitochondria encounter structure
ESCRT	Endosomal sorting complex required for transport
ETC	Electron transfer chain
F-1,6P2	Fructose-1,6-bisphosphate
F-6P	β -D-fructose-6-phosphate
FBPase	Fructose-1,6-bisphosphatase
FDA	American Food and Drug Administration
FOB	Ferrioxamine B
G418	Geneticin
Gal-1P	Galactose-1-phosphate
GALE	UDP-glucose-4-epimerase
GALT	Galactose-1-phosphate uridylyltransferase
GDC	Glycine decarboxylase complex
GFP	Green fluorescent protein
GK	Galactokinase enzyme
Glu-1-P	Glucose-1-phosphate
Glu-6-P	Glucose-6-phosphate

GNA	Glucose Nutrient Agar
GO	Gene ontology
GRAS	Generally Recognized As Safe
GSK-3 β	Glycogen synthase kinase 3 β
GTP	Guanosine-5'-triphosphate
H	Histone
HH	Hereditary hemochromatosis
HOG	High osmolarity glycerol
IM	Inositol monophosphate
IM	Inner mitochondrial membrane
IMPases	Inositol monophosphatases
Ino80-C	INO80 complex
IPPases	Inositol polyphosphate 1-phosphatases
IRES	Internal Ribosome Entry Site
<i>kanR</i>	Kanamycin resistant
KO	Knockout
LB	Luria-Bertani
LG	Linkage group
MAPK	Mitogen-activated protein kinase
MDN	Mutation during non-functionality
MS	Mass spectrum
MSG	Monosodium glutamate
MVB	Multivesicular body
MVE	Multivesicular endosomal
mya	Million years ago
<i>NatR</i>	Nourseothricin resistant
NER	Nucleotide excision repair
NMD	Non-sense mediated decay
NPD	Non-parental ditype
NT2	New terminator
OD	Optical density
OST	Oligosaccharyl transferase complex
PD	Parental ditype
PEG	Polyethylene glycol
PGM	Phosphoglucomutase
PTC	Premature termination codon
PtdIns3P	Phosphatidylinositol-3-phosphate
QTL	Quantitative trait loci
Rap	Rapamycin
RNA Pol	RNA Polymerase
RSA	Random Spore Analysis
SAGA	Spt-Ada-Gcn5 acetyltransferase
SAGE	Serial Analysis of Gene Expression
SC	Synthetic Complete
SD	Synthetic Dropout
SESA	SGA Experiment Set Analyser

SGA	Synthetic genetic array
SGal	Synthetic galactose
SGD	<i>Saccharomyces</i> genome database
SL	Synthetic lethality
SLAM	Synthetic Lethality Analysis by Microarray
SLIK	SAGA-like complex
SMP	Synaptotagmin-like, mitochondrial and lipid-binding protein)
SS	Synthetic sickness
SSD	Short segmental duplication
STRE	Stress response element
T	Tetrad
TAP	Tandem Affinity Purification
TBE	Tris/Borate/Ethylenediaminetetraacetic acid
TCA	Tricarboxylic acid cycle
TE	Tris-Ethylenediamine Tetraacetic Acid
<i>tet</i>	Tetracycline promoter
THF	Tetrahydrofuran
TOR	Target or rapamycin
TPP	Trehalose-6-phosphate phosphatase
TPS	Trehalose-6-phosphate synthase
Tris	Tris(hydroxymethyl)aminomethane
UASrpg	Upstream activation sequence of ribosome protein genes
UBDs	Ubiquitin Binding Domains
UDP	Uracil-diphosphate
UDP-Glc	UDP-glucose
UIM	Ubiquitin-interacting motif
UPR	Unfolded protein response
UTR	Untranslated region
WGD	Whole genome duplication
YDS	Yeast Deletion Set
YEASTRACT	Yeast Search for Transcriptional Regulators And Consensus Tracking
yFACT	Yeast facilitates chromatin transcription
YKO	Yeast Knock Out
YPD	Yeast Peptone Dextrose
YT	Yeast extract/Tryptone)

Contents

1. Chapter 1	22
1.1. Introduction.....	22
1.2. Duplication of the <i>Saccharomyces cerevisiae</i> genome	26
1.2.1. Yeast taxonomy.....	26
1.2.2. Evidence for duplication.....	27
1.3. Different fates of duplicated genes	30
1.4. Post-duplication evolutionary-driven functional divergence and retention	35
1.4.1. What if Ohno was wrong?	36
1.4.2. Contemporary knowledge.....	38
1.5. Genetic and systemic robustness	40
1.5.1. Robustness and Evolution	41
1.5.2. Do duplicates genes really play a role in genetic robustness?	42
1.6. The <i>TIF1</i> and <i>TIF2</i> genes.....	44
1.7. The promoter regions.....	45
1.8. eIF4A and the DEAD-box helicases family	46
1.8.1. Eukaryotic Translation Initiation.....	48
1.9. Aims and objectives	51
2. Chapter 2	52
2.1. Introduction.....	52
2.2. Yeast as a model organism	56
2.3. Yeast as a model for human diseases	57
2.4. Yeast in the investigation of translation initiation.....	58
2.5. Overview of studies in yeast.....	59
2.6. The Yeast Deletion Set	60
2.7. Generation and understanding of a genetic network.....	62
2.7.1. Genetic Interactions.....	62
2.7.2. Synthetic Genetic Analysis (SGA)	65
2.7.3. Genetic Networks.....	69
2.7.3.1. The network inside the network.....	72
2.8. Aims and objectives	74
2.9. Methods	75
2.9.1. Yeast	75
2.9.1.1. SGA.....	75
2.9.1.2. tetO promoter.....	76

2.9.1.3.	GAL over-expression promoter	76
2.9.2.	Bacteria.....	76
2.9.2.1.	Plasmids	77
2.9.3.	Stock solutions	78
2.9.4.	Growth media	79
2.9.4.1.	Bacteria.....	79
2.9.4.2.	Yeast.....	80
2.9.5.	Growth Conditions	81
2.9.5.1.	Liquid growth	81
2.9.5.2.	Solid growth media.....	82
2.10.	Instruments	83
2.10.1.	Robotic pinning system	83
2.11.	Bacterial transformation.....	83
2.12.	<i>E. coli</i> plasmid preparation	84
2.13.	Transformation of yeast <i>Saccharomyces cerevisiae</i>	85
2.13.1.	Transformation of intact yeast cells treated with lithium ions.....	85
2.13.2.	High efficiency transformation	86
2.14.	Genomic DNA purification and extraction	87
2.14.1.	Purification.....	87
2.14.2.	RNA degradation	88
2.14.3.	DNA extraction with phenol/chloroform/isoamylalcohol.....	88
2.14.4.	Ethanol precipitation.....	88
2.15.	Polymerase chain reaction (PCR)	89
2.15.1.	Enzymes and cycle conditions	89
2.15.2.	Amplification of selective marker cassettes	90
2.15.3.	Confirmation PCR.....	91
2.15.3.1.	Yeast colony PCR.....	91
2.15.3.2.	PCR on genomic DNA (gDNA).....	91
2.16.	DNA electrophoresis	91
2.17.	Serial spot dilution assay	92
2.18.	Query strain construction - Non-essential gene switching method.....	92
2.18.1.	Bacterial growth and plasmid purification	93
2.18.2.	Restriction enzyme digestion.....	93
2.18.3.	Transformation	93
2.18.4.	Confirmation PCR.....	94
2.18.5.	Diploids constructions - Micromanipulation.....	94

2.18.6.	Assay for pheromone production.....	95
2.18.7.	Sporulation and Mating type selection	96
2.18.8.	Synthetic Genetic Array (SGA) Analysis	97
2.19.	Scoring genetic interactions.....	102
2.19.1.	Gene ontology	104
2.20.	Validation of putative interactions	105
2.20.1.	Tetrad analysis	105
2.20.1.1.	Random Spore Analysis (RSA).....	106
2.20.1.2.	Tetrad dissection.....	107
2.20.2.	Small scale SGA independent examination	108
2.21.	Modulable promoter	108
2.21.1.	PCR amplification of “tet” promoter cassette	109
2.21.2.	Transformation	109
2.21.3.	Assessment of repression (tetrability)	109
2.22.	Over expression promoter	110
2.22.1.	Generation of <i>tif1Δ::URA</i> and <i>tif2Δ::URA</i> strains.....	110
2.22.1.1.	Marker switching.....	110
2.22.2.	PCR amplification of <i>GAL</i> over expression cassette.....	110
2.22.3.	Transformation	111
2.22.4.	Assessment of over expression	111
2.23.	Results.....	112
2.24.	Query strain construction via non-essential gene switching method 112	
2.24.1.	PCR Diagnosis	114
2.24.2.	Assay for pheromone production.....	115
2.24.3.	Mating type selection and haploid status selection.....	116
2.25.	<i>tif1Δ</i> SGA	116
2.26.	<i>tif2 Δ</i> SGA	120
2.27.	Genetic interactions	122
2.28.	Gene Ontology analysis.....	125
2.29.	The Biological Networks Gene Ontology.....	128
2.30.	Scoring genetic interactions.....	131
2.31.	Validation of Putative Interactions	135
2.31.1.	Tetrad analysis	135
2.31.1.1.	Random Spore Analysis (RSA).....	135
2.31.1.2.	Tetrad dissection.....	135
2.31.2.	Open Biosystems library small scale SGA.....	137

2.32.	Tetracycline modulable promoter	140
2.32.1.	Transformation and assessment of repression-tetrability	141
2.32.2.	Spot dilution assay	143
2.33.	<i>GAL</i> over-expression promoter	144
2.33.1.	Plasmid purification and amplification PCR.....	144
2.33.2.	Transformation	145
2.34.	Discussion	147
2.35.	The biology behind the interactions	147
2.36.	<i>TIF1</i> genetic profile.....	149
2.37.	<i>TIF2</i> genetic profile.....	169
2.38.	<i>TIF1</i> and <i>TIF2</i> genes functional profiling.....	189
2.39.	Summary.....	197
2.40.	Redundancy in genetic networks	203
2.41.	SGA as a tool for evolutionary studies.....	205
2.42.	Validation of the SGA study	208
2.42.1.	Small scale SGA.....	208
2.42.2.	Tetrad analysis	209
2.42.3.	Potential and limitation of an SGA approach.....	209
2.43.	Significance of the promoter regions.....	211
2.43.1.	Tet-titratable promoter.....	212
2.43.2.	Galactose over-expression promoter	213
3.	Chapter 3	215
3.1.	Introduction.....	215
3.2.	Galactose and the Leloir pathway	217
3.3.	Calcium homeostasis.....	221
3.4.	The TOR pathway.....	223
3.5.	Lithium	225
3.6.	Yeast as a model for neurodegenerative disorders	227
3.7.	Further therapeutic and commercial benefits.....	228
3.8.	Chemical genetic screening.....	229
3.9.	Aim and objectives	232
3.10.	Methods.....	234
3.11.	Yeast strains	234
3.11.1.	Lithium experiment.....	234
3.11.2.	Different environmental and chemical challenges.....	235
3.12.	Growth media.....	235

3.12.1.	Stock solutions.....	235
3.12.2.	Solid growth.....	236
3.12.2.1.	Lithium experiment.....	236
3.12.2.2.	Different environmental and chemical challenges	236
3.12.3.	Liquid growth.....	236
3.13.	Lithium investigation.....	237
3.13.1.	Preliminary experiments.....	237
3.13.1.1.	Pilot study.....	237
3.13.2.	Chemical genetic screen.....	237
3.13.3.	Data analysis.....	237
3.13.4.	Spot Assay	238
3.13.4.1.	Scoring system	239
3.13.5.	Dealing with incongruencies	240
3.13.6.	Analysis	240
3.14.	Environmental challenges.....	240
3.14.1.	24-well plate sensitivity assessment spot assay	241
3.14.2.	Serial spot dilution assay.....	241
3.14.2.1.	Rapamycin.....	241
3.14.2.2.	Environmental challenges	241
3.15.	Results.....	242
3.15.1.	Chemical genetic profile of lithium.....	242
3.15.2.	Spot Assay	244
3.16.	Gene Ontology Analysis.....	246
3.17.	BiNGO Analysis.....	251
3.18.	Different environmental conditions.....	259
3.19.	Discussion	260
3.20.	Cell signaling.....	260
3.20.1.	Kinases.....	260
3.21.	Cell wall organization and biogenesis	262
3.22.	Endocytosis	267
3.23.	Chitin	270
3.24.	Cell cycle	274
3.25.	Response to stress.....	276
3.26.	Chromatin organization , histone , transcription	280
3.27.	Mitochondria	282
3.27.1.	Calcium.....	282

3.27.2.	The TCA cycle	284
3.27.3.	Cytochrome c oxidase	290
3.27.4.	Additional mitochondrial gene deletions	292
3.28.	Trehalose	295
3.29.	Gluconeogenesis.....	297
3.30.	Suppression of lithium/galactose toxicity.....	300
3.30.1.	Ubiquitination.....	302
3.30.2.	Galactose metabolism	304
3.30.3.	Glycosylation	311
3.31.	Calcium homeostasis	314
3.32.	The TOR pathway	321
3.33.	Targets of lithium	322
3.34.	Implications for lithium's mode of action and neuroprotective agents 324	
3.35.	This study screen versus Zhao's and Hillenmeyer's.....	327
3.36.	Summary.....	329
3.37.	Different environmental challenges	331
4.	Chapter 4	333
4.1.	Summary	333
4.2.	Future directions.....	339
5.	Appendix.....	342
5.1.	Negative genetic interactions identified in <i>tif1</i> SGA.....	342
5.2.	Positive genetic interactions identified in <i>tif1</i> SGA	352
5.3.	Negative genetic interactions identified in <i>tif2</i> SGA.....	353
5.4.	Positive genetic interactions identified in <i>tif2</i> SGA	364
5.5.	Primers.....	365
5.6.	Lithium/galactose sensitive deletion strains	367
5.7.	Lithium/galactose suppressor deletion strains	387
6.	References.....	391

1. Chapter 1

1.1. Introduction

The capability of a biological system to successfully respond and adapt to a range of environmental challenges represents the key to success for survival and is manifested through new variation and evolutionary novelty. The genome can be subjected to a number of mutations that could potentially affect it, including gene duplication. Differently from a point mutation or a chromosome rearrangement, the duplication process displays the unique characteristic of affecting genomic functionality while concurrently enhancing mutational robustness.

The Japanese scientist S. Ohno has made a major contribution to the subject of gene duplication. His ahead-of-time views and landmark statements gained him a place of honour in the field of genome evolution. The legacy of his contribution still lives on, with scientists occasionally referring to duplicated genes derived by genome duplication as “ohnologs” (Wolfe, 2000). Nevertheless, discussion of the significance and implications of genome duplication events had previously been addressed and has an extensive, if often neglected, history, as discussed below.

At the beginning of the past century, studies on meiosis in pollen conducted by Y. Kuwada revealed that maize was an ancient tetraploid. This was recognised on the basis of the observation of two set of paralogous chromosomes in the *Zea mays* karyotype (Kuwada, 1911). Almost two decades later, L. Stadler reported that polyploids of some species of cereal grains, such barley and oats,

are less prone to irradiation than their related diploids (Stadler, 1929). This latter piece of scientific work is frequently underestimated; undoubtedly, it is deserving of recognition for the novel connection suggested between stress, environmental circumstances, and adaptability of the system, especially in relation to the benefits of gene duplication. Further speculation on the advantage of duplication events came with the publication of *The Causes of Evolution* (Haldane, 1932); in this book, emphasis was given to the potential malleability of the genome as being a source of genes capable of bearing mutations without harmful consequences for the organism. As a corollary of this consideration, organisms that display various copies of genes should be less prone to lethal mutations.

Additional instances of underrated contributions are also works from: A. Serebrovsky (1938), who pioneered an argument nowadays known as the subfunctionalization model (**section 1.3**) by envisioning a pre-duplication multi-task gene whose functions may become reallocated; C. Metz (1947) and his acknowledgement of the duplication of chromosome sections as a key feature in evolution and S. Stephens (1951), who asserted that increments in the number of genetic loci provides an ideal pathway for a system to evolutionarily progress. Although containing elements of imprecision, these and other considerations fuelled interest for further investigation into gene duplication.

Only occasionally diverted by a passionate period of militancy in the Communist Party, J. Haldane, by this time better known in the scientific community as one of the founders of the discipline of population genetics, marked his return to evolutionary studies in 1957 with *The cost of natural selection* (Haldane, 1957). In this publication the author re-evaluated the subject

of gene duplication closely, although in different terms. His contribution resulted in crucial reflections on the so-called “cost of substitution”, referring to a scenario in which an advantageous mutation needs to be fixed in the genome and its contribution increased in terms of number of copies, driven by evolutionary progression. Following this principle, newly mutated genes take the place of some pre-existent ones, which will end up being lost. However, remarks Haldane, there is a limit to the speed at which this process can occur and this is what would intrigue him for his lifetime. Whilst not addressing the theme of gene duplication directly, Haldane’s intuition provided inspirational scientific ground for scientists to come, as attested by further interpretations of his popular and well accredited theory that turned into a “dilemma” (Van Valen, 1963).

Almost concurrently, the evolutionary biologist M. Kimura referred to the loss of pre-existent genes in favour of new ones generated by duplication and successive up-take of non-lethal mutations as the “substitutional load” (Kimura, 1960). Together with colleague T. Maruyama, he introduced models for the study of genetic drift, contributing amply to the controversy over genetic load (the cost in genetic deaths to the population of replacing one allele by another -a mutation- in the course of evolutionary change) throughout the 1960s. Attributed to Kimura is also the neutral theory of molecular evolution (Kimura, 1983) that ascribes a predominant role to mutational pressure and genetic drift in terms of contribution to evolutionary changes, focussing on molecular rather than phenotypic level. This controversial model was received by some as an argument aimed at diminishing the role attributed to natural selection by Darwin's theory of evolution. Although the neutral model acknowledges a predominant role to random drift, most evolutionary biologists agree that the

two hypothesis are compatible, as Kimura feverishly defended in his 1986 publication: "The theory does not deny the role of natural selection in determining the course of adaptive evolution", (Kimura, 1986).

It was not until the publication of *Evolution by Gene Duplication* (Ohno, 1970), that the concept of gene duplication was no longer confined to theoretical speculations among evolutionists but saw its contribution broadly acknowledged in biology. S. Ohno (1928–2000) was a scientist of multifaceted capacities and indeed the image of Ohno emerging from this monograph is of a sharply minded man and a scientist of visionary talent. In the preface of his book *Evolution by Gene Duplication* he wrote:

Had evolution been entirely dependent upon natural selection, from a bacterium only numerous forms of bacteria would have emerged. The creation of metazoans, vertebrates, and finally mammals from unicellular organisms would have been quite impossible, for such big leaps in evolution required the creation of new gene loci with previously nonexistent function. Only the cistron that became redundant was able to escape from the relentless pressure of natural selection. By escaping, it accumulated formerly forbidden mutations to emerge as a new gene locus.

At this stage Ohno was persuaded by the potential of raw genomic material as forceful means and proposed it as the source through which new functions or new genes could be generated. The time had come for scientists to combine molecular genetics tools with modern biology theories and thereby to explore the critical contribution of duplication to evolution.

1.2. Duplication of the *Saccharomyces cerevisiae* genome

1.2.1. Yeast taxonomy

The number of species of fungi described so far amounts to approximately 80,000, however existing fungal species are estimated to be in the order of millions (Hedges, 2002). The ordinary classification based on morphology divides fungi into five phyla: Ascomycota represents the largest phylum and is characterized by the presence of the ascus, a specific structure that surrounds the spores (James, 2006), a product of the mitotic event. Within the Ascomycota two prominent subphyla are identified: Pezizomycotina (to which the hyphal fungus *Neurospora crassa* belongs) and Saccharomycotina (accounting yeast such *Candida albicans* and *Saccharomyces cerevisiae*). Genomes of all the species belonging to Saccharomycotina are ascribable to four main clusters; the most well known and extensively investigated in terms of genome sequencing is referred to as the “*Saccharomyces* complex” (Kurtzman & Robnett, 2003) and comprises almost exclusively species from the genera *Saccharomyces* and *Kluyveromyces* (**Figure 1-1**).

Figure 1-1. Section of a phylogenetic tree showing relationships among the sequenced fungal genomes. *Saccharomyces* complex - the group of species sharing duplication of the whole genome (WGD) - clade and *Saccharomyces sensu stricto* (van der Walt, 1970) are annotated. The numbers indicated the bootstrap values associated to each node. Adapted from Fitzpatrick (2006).

Contemporary yeast classification accounts for an extensive variety of approaches, depending on whether the categorization is pheno- or genotypically determined.

1.2.2. Evidence for duplication

Gene duplication can take place as a single-gene phenomenon or be limited to duplication of short chromosomal segments (SSD, short segment duplication); cases of aneuploidy also occur with duplication of entire chromosomes up to the more drastic case of whole genome duplication (WGD). Given the ubiquity of duplication events among different species, scientists attempted to express how often such a phenomenon takes place: the probability of gene duplication during evolution has been described in terms of gene duplicability intended as the rate of mutation producing duplicate genes and the

probability that the duplicates are fixed and retained in the genome (He & Zhang, 2005a). In recent times gene duplication has also been annotated as continuing (Lynch & Conery, 2000; Friedman & Hughes, 2003), highlighting how this process incessantly happens in the genomes of all cellular organisms.

The credit for advancing the hypothesis of the polyploid origin of the yeast genome goes to M. Smith during his study on *S. cerevisiae* histone gene loci (Smith, 1987). The outcome of his research pointed to a diploidization or fusion of two ancient fungi as the probable origin of the modern yeast *S. cerevisiae* genome. With the most plausible scenarios likely to be either subsequent duplications or a single *in toto* genome duplication followed by translocations. Genetic sequencing studies and comparative genomics have assessed these possibilities. Over time, significant contributions echoed with the model proposed by Ohno, lending credibility to the WGD hypothesis. Results of a BLASTP analysis conducted by Wolfe and Shields (1997) using a spatial map-based approach on the baker's yeast genome revealed 55 segments of duplicated regions: 50 out of the 55 so-called blocks displayed the same orientation with respect to the centromere.

Figure 1-2. Duplicate regions (blocks) of chromosomes X and XI. Adapted from Wolfe & Shields (1997).

As an example, three defined duplicate regions on chromosomes X and XI are represented as diagonal clusters of genes in **Figure 1-2**; as a result of random post-WGD individual gene deletions and/or reciprocal translocations, singletons are scattered in between the duplicate pairs. The authors interpreted this result as proof that these genome segments were generated in a single duplication process, followed by translocations among duplicate chromosomes as, if each single region was the product of an independent duplication event, their orientation would be expected to be random. A similar model in which most, but not all, of the original chromosomes were duplicated (aneuploidy) seems less

likely but cannot be ruled out entirely. The investigators concluded that baker's yeast is the outcome of the WGD event that occurred after divergence of *S. cerevisiae* from *Kluyveromyces lactis* about 100 million years ago (mya). The duplication of many genomic regions both intrachromosomally and interchromosomally -including genes and nongenic segments- suggests that duplication is a major attribute of *S. cerevisiae* genome evolution.

Supporting evidence (Kellis, 2004) confirms a WGD, believed to have taken place 8 to 10 mya, as the most accredited hypothesis. As a result of this event, the number of genes of *S. cerevisiae* genome doubled from a diploid number of 5,000 to a tetraploid of 10,000. WGD is regarded as a cataclysmic event in which an enormous amount of genetic material became available for evolution to forge, followed by a massive gene loss (85% of the copies were deleted). Subsequent chromosomal rearrangements and translocations in the genome progressively shaped the obvious outcome of the duplication event, leaving behind a faint genomic footprint. The existing *S. cerevisiae* genome, accounting for about 5,800 genes, still encloses many conserved paralogs as the inheritance of that gene duplication.

1.3. Different fates of duplicated genes

In general, a eukaryote duplicate gene has a half life of just several million years (Lynch & Conery, 2000) and a chance of being lost varying from 50% to 92% (Wagner, 2001). A number of models have been proposed to account for the functional divergence and retention of duplicate genes, mechanisms of which are still partially unknown.

Four possible fates await a duplicated gene:

- i. Both copies persist
- ii. One copy is deleted
- iii. One copy functionally diverges
- iv. One copy becomes a pseudogene

i. Both copies persist

Since complete redundancy is not favoured by evolution (Kitano, 2004), retention of two gene copies over evolutionary periods strongly depends on the degree of redundant function(s). This may apply to the case of two perfectly redundant gene copies, where any damaging mutational effects on one copy can be extensively buffered by activity of the other gene copy (Fisher, 1935). It has also been suggested that a gene may be maintained not because it is intrinsically useful, but simply as this deletion would result in a dosage imbalance, relative to key partner genes, which may also be held in duplication. Such impairment of the dosage equilibrium would eventually trigger the engagement of very expensive cellular processes, like alleviating mechanisms of dosage-compensation. These considerations represent the rationale behind the gene dosage hypothesis (deVries, 1901) and a possible explanation for the preservation of two copies of the same gene. Safeguarding of both duplicates has additionally been postulated in the case of protein products needed in significant amounts and/or those that are essential for survival of an organism (e.g., increased dosage can be beneficial for ribosomal RNA and histone genes).

ii. One copy is deleted

The most likely outcome of duplication sees one copy of the gene being lost. One of the mechanisms accounting for such a tendency is referred to as non-functionalization, a process in which one paralog is deprived of its function and is eventually lost. Such a mechanism appears not to contribute in terms of adding novelty and variation for evolution as it leads to the restoration of both pre-duplication genotype and phenotype.

iii. One copy functionally diverges

If a gene pair undergoes either neofunctionalization or subfunctionalization, then both duplicates will be maintained by selection; the presence of partial redundancy, however, may result in one or both genes experiencing an altered selective regime relative to the ancestral single-copy state.

iiia. Neofunctionalization

S. Ohno proposed that after duplication one copy of the gene becomes functionally redundant and free to accumulate mutations without any lethal phenotypical consequence. Occasionally, such mutations can by chance pre-adapt the gene product for a new function, allowing it to “emerge” as a new gene (Ohno, 1973). Neofunctionalization means the retention of both gene copies and confers an advantage as both genotype and phenotype can be altered.

iiib. Subfunctionalization

R. Jensen (1976) first introduced the hypothesis that two genes with separate functions may evolve, after duplication events, if the original functions are subdivided among the duplicate pairs. According to his theory, the gene's ancestral functions are partitioned between the two daughter genes, resulting in a re-establishment of the original phenotype accompanied by a variation in the genotype. This model was introduced by L. Orgel (1977), who proposed sharing of an original function as the way diversification takes place in multigene families. This proposal attracted the interest of scientists over decades, with notable further investigations by R. Jensen and G. Byng (1981) and A. Hughes (1994). Re-allocation of pre-duplication functions among paralogs after duplication has been referred to as “subfunctionalization” and took shape in the duplication-degeneration-complementation (DDC) mathematical model proposed by Force and colleagues (Force, 1999, Lynch & Force, 2000). DDC attributes complementary degenerative mutations in regulatory elements for controlling the expression of duplicated genes, leading to portioning of ancestral gene functions. If any selective pressure acts on this mechanism, the evolutionary advantage of subfunctionalization is presumably to allow individual optimisation of the newly separated functions, although it could simply represent the most likely outcome for complexly regulated genes.

iiic. Sub-neo-functionalization

As both neofunctionalization and subfunctionalization have failed to satisfactorily describe genome configuration of genetic interactions singularly, a more complex scenario known as “sub-neo-functionalization” has also been

theorized (He & Zhang, 2005). Elements of previously proposed models are combined: in addition to changes at the amino acid sequence level, an important aspect of subfunctionalization of duplicate genes is likely to involve differences in expression patterns. The two paralogs' expression patterns will diverge as a result of modifications in *cis*-regulatory elements, which allow them to bind different sets of transcription factors. According to this theory, a conspicuous number of paralogs have endured rapid subfunctionalization followed by prolonged neofunctionalization (He & Zhang, 2005). More recent work done by Hittinger and Carroll on duplication and divergence of *GAL* genes and their contribution to genetic novelty (Hittinger & Carroll, 2007) provided experimental evidence in support of this model.

iv. One copy becomes a pseudo-gene (Pseudogenization)

After duplication the most likely gene fate is loss, with the majority of the duplicates degenerating into pseudogenes (Jacq, 1977), DNA sequences displaying all characteristics of ordinary genes, including exon structures and promoter sequences. Although generally believed to execute no function, several pseudogenes have been described since their discovery and some of them have been surprisingly reported to affect other functions (e.g., the courtship behaviour of flies, Kaessmann, 2009). Where duplication has arisen through retroposition or retroduplication (Kaessmann, 2009), the duplicate is particularly susceptible to decay mechanisms and their fate is often to become a pseudogene.

A possible scenario may involve a copy of a functional gene acquiring mutations that cause it to become non-functional. Nevertheless, pseudogenes may have previously held a prior role: some duplicate pairs have been retained

in the functional genome for a time, before being turned into pseudogenes. Pseudogenization may be triggered by changes in genetic background and/or environment variation, in this latter case it would be more appropriate to refer to pseudogenization by neutral selection. This process renders a formerly functional gene into one that is no longer operating, leading to an easing of the negative selection exercised on the gene itself; as a consequence, mutations disrupting the gene are fixed by genetic drift, and the gene can assume the appearance of a pseudogene.

1.4. Post-duplication evolutionary-driven functional divergence and retention

Not all duplicate genes survive in an organism, nor have they the same probability to be retained; the forces addressing their process of functionalization and eventual retention in the genome are still largely unknown. Natural selection represents a key element, according to Ohno's view. Over an early period following the duplication event, one of the paralogs would benefit from the absence of selective pressure, leaving the duplicate free to accumulate mutational events with no disruptive phenotypical consequences for the organism. It is through such a mechanism that acquisition of new function(s) takes place. Studies of molecular evolution also suggest that during this period of less selective pressure, shortly after duplication, "new" duplicates can bear accumulation of changes, with around 10-fold more amino acid variations than paralogs duplicated in more remote times, and are more tolerant to nucleotide changes than their single copy counterparts (Lynch & Conery, 2000). However,

the assumption that the duplicate will benefit from a “genetic drift free” status for the rest of its lifetime should be discarded in consideration of the purifying selection that operates on the gene copy, as long as it is expressed. This argument leads to the consideration that paralogs are not entirely freed from constraint (Hughes, 1994). Whilst Ohno’s hypothesis permits neofunctionalization as the only mechanism through which duplicates can escape being lost and are thus enduringly retained, this model has nowadays revealed its inadequacy in representing the entirety of duplicate gene pairs. Alternative mechanisms are additionally proposed to take place, as reviewed in **section 1.3**. In referring to yeast duplicate gene pairs, paralogs are often implied as arising from WGD, however SSD paralogs deserve a further elucidation as their divergence and eventual retention is underpinned by distinctive selective constraints. For this reason, differences are present between WGD and SSD in the post-duplication time period. Following the dosage balance hypothesis, WGD-derived duplicate genes are preferentially maintained whilst SSD paralogs, perceived as a perturbation of the stoichiometric balance of the system, are rarely retained (Papp, 2003a).

1.4.1. What if Ohno was wrong?

Over the past two decades, although inspiring and very popular, Ohno’s theory has been surrounded by raising doubts, leading evolutionist biologists to formulate alternative hypotheses. J. Piatigorsky and G. Wistow are likely to have inspired most of them, by pioneering the concept of gene sharing (Piatigorsky & Wistow, 1989), a scenario in which a protein displays at least two distinct functions. Shortly after, the duo of scientists emphasized their view in the iconic

paper *The recruitment of crystallins: new functions precede gene duplication* (Piatigorsky & Wistow, 1991). The notion of gene sharing was here refined and the idea that duplication was not an essential pre-requisite to gain a new function authenticated as theory.

Over time, a persuasive body of research ended up contradicting Ohno's paradigm when considered in *stricto sensu* and the mutation during non-functionality (MDN) model, a scenario in which post-duplication gene copy is redundant and free to accumulate mutations (substitutions) at random, began inexorably to loose followers. The downfalls of Ohno's theory can be perceived from work published by A. Hughes, who underlined how duplicate pairs are inexorably under the pressure of purifying selection and so are far from being free to accumulate random mutations (Hughes, 1994). This outspoken scientist also embraced the theory of gene sharing and laid emphasis on how studies on multi-gene families (Piatigorsky & Wistow, 1991) were pointing at positive (Darwinian) selection as a possible mechanism underlying the existence of related proteins with distinct functions. Hughes was simply fuelling an already emergent current of thought which was persuaded that gene sharing could indeed precede duplication events. All together these considerations questioned the importance of duplicate genes' contribution as prerequisite for formation of new proteins and, by the beginning of the 21st century, Ohno's view had acquired an unexpected anachronistic connotation.

Disregarding the dispute, interrogatives of critical importance for understanding the functioning and impact of gene duplication have not yet been answered: "which copy evolves a new function?" and "which forces influence or drive this process?". Ohno postulated that mutations arising during the period of

relaxed selection pressure drive the duplicate gene to either non -or neo-functionalization.

1.4.2. Contemporary knowledge

Several years of exciting research has gone past since Ohno's first proposals; significant theoretical and empirical advancements have been achieved, revolutionizing the meaning of what we call a "gene". Innovative approaches and methodologies, such as combined efforts of genome sequencing, comparative genomics and studies on chromosome morphology, have been crucial in the acquisition of knowledge surrounding the factors that influence paralogs' outcomes. Their destiny has been shown to be affected by genetic aspects including redundancy (Piskur, 2007) and expression dosage (Conant & Wolfe, 2006). It is nowadays extensively accepted that the characteristics associated with retention of a duplicate can be quite diverse, including the modality the modality through which the gene has ended up in the genome. For example, a WGD-derived duplicated copy may be preferentially retained on the basis of the gene dosage balance hypothesis (Lynch & Conery, 2000), as it is essential for the dosage equilibrium to be preserved. Gene function also plays a role. For example, cyclins, myosins, protein kinases, pheromones and transcription factors are annotated as the preferentially retained pairs of genes in *S. cerevisiae* (Wolfe & Shields, 1997).

Outside of WGD events, a number of additional factors which affect the tendency for duplication to occur are now recognised:

- 1) The species to which the gene of interest belongs. The duplication rate is different between species with humans and worms being faster than yeast and other species (Gregory, 2005);

2) the gene evolutionary rate. Rapidly evolving genes undergo duplication less often than those with low rate of evolution: the more slowly evolving copy may be under stronger selective constraint, whilst the faster evolving copy may be under more relaxed selective constraint (Conant & Wagner 2003; Zhang, 2003). Several recent studies have shown that duplicate genes in general tend to evolve more slowly (Davis & Petrov 2004; Jordan, 2004), indicating strong purifying selection on duplicate proteins, presumably to conserve its ancestral sequence and function;

3) the length and structure of the gene. Long genes appear to be duplicated less often than short genes (Gregory, 2005).

Contemporary knowledge has deepened our understanding of gene duplication, enhancing comprehension of the advantage of such an apparently expensive phenomenon. Little is known, though, about the rules that govern post-duplication modifications of gene interactions or whether these latter affect the fate of duplicate genes. Fascinating questions regarding how two copies initially identical and affected by the same mutational forces embark on different evolutionary pathways remain yet to be answered. Whilst we are left yearning for enlightenment about the evolutionary impact of duplicated genes, it seems likely that participation of short-term mechanisms may be required to guarantee the long-term advantages granted by the retention of both paralogs (Lynch & Katju, 2004).

1.5. Genetic and systemic robustness

The phenomenon through which malfunction in one component of the organism does not result into a failure of the whole system, thanks to the intervention of another constituent, is known as robustness. The genome of the yeast *S. cerevisiae* displays a high degree of gene dispensability with the organism able to bear a deletion of a predominant fraction of Open Reading Frames (ORFs) with no evident phenotypical consequences on the system under laboratory conditions. Only 18% of the genes have been found to be essential under experimental conditions in rich glucose-containing medium (Giaever, 2002), aligning findings in yeast with similar ones in bacteria (Kobayashi, 2003) and worms (Kamath, 2003). However, this does not mean that only such a small percentage of genes are involved in essential process but it represents the extent of robustness inherent in the system. Some of the so-called non-essential genes are believed to play roles in essential processes, with a portion of non-essential genes defined essential under specific conditions.

The high level of redundancy in the genome has for a long time been ascribed to the presence of duplicate genes, where a direct backup compensation from one of the paralogs assists the counterpart when this latter is impaired. In other words, functional redundancy between paralogs may lead to genetic robustness. This characteristic makes baker's yeast an appropriated organism for studying this topic.

Investigations into the biological properties and contribution to robustness of duplicates have been conducted with different approaches. So far, the most informative attempts have resulted in examinations based on physical

interactions (Baudot, 2004; Musso, 2007), metabolic networks (Papp, 2004) and single-gene deletion phenotypes (Gu, 2003). This congruent body of studies has given evidence for functional similarities among WGD and SSD pairs of duplicates, supporting the hypothesis that there is an advantage in preserving a functional overlap, disregarding the nature of the duplication event. However, yeast duplicate pairs derived from WGD appear to be more dispensable (with the exception of ribosomal protein genes) than duplicates generated through other mechanisms (Lin, 2007). The contribution of paralogs to genetic robustness has been estimated and WGD pairs appeared more likely to buffer each other than SSD pairs -39% versus 18%- (Li, 2010), showing indeed a more pronounced buffering strength. This observation may have implications in the case of a duplicate under strong functional constraint, which reduces the rate of functional divergence between WGD paralogs.

Redundancy caused by duplication is not the only source of robustness against mutations: an important contribution comes from the network system that arises from metabolic pathways, regulatory and feed-back networks (Wagner, 2000). Such coordinated effort is better known as system robustness, where networks of interactions display a certain degree of back up, even if their elements and elementary processes themselves do not.

1.5.1. Robustness and Evolution

It is interesting to consider how evolution contributes to the robustness of a biological system and how robustness and function interrelate. Is cellular robustness a well articulated strategy that the organism engages to avoid the detrimental effects of deleterious mutations (e.g., genetic robustness) or does it

arise from such mutations? Currently, there is uncertainty about whether there is pressure to maintain robustness or whether it is simply a random event. The robustness expressed as the ability of a system to formulate novel and effective solutions, it is currently viewed as facilitating evolutionary innovations or, at the very least, a chance for their occurrence. Phenotypic diversity, which provides options to be tested against stressors, can be summarized in the form of evolutionary innovation that goes under the name of “evolvability” (Kirschner & Gerhart, 1998). Gene duplication causes robustness that, in turn facilitates evolvability. The requirement for variation that results in non-lethal phenotypes hints at an intimate link between evolvability and mutational robustness (Wagner, 2008). The survival of the system seems to be correlated with its capacity to find a compromise between robustness and adaptability; this key requirement has to be met to allow long- term survival in an environment that presents incessant new challenges. Approaching the topic from a population’s perspective, the outcome of a highly robust system will guarantee that fewer mutations have deleterious effects, leading to a disfavoured phenotype. As a result of that, a greater diversity of genotypes will arise in a population of robust individuals and such diversity, although itself not always accompanied by phenotypical change, may facilitate the emergence of new phenotypes (Ciliberti, 2007; Wagner, 2008).

1.5.2. Do duplicates genes really play a role in genetic robustness?

The contribution of paralogs to mechanisms of genetic robustness has been established by an exhaustive investigation from Gu and colleagues (Gu, 2003), with later studies showing direct backup compensation accounting only

for a quarter of the system robustness (Ihmels, 2007). Duplicates have been shown to possess significantly higher probability of functional compensation than singletons and a higher probability of having a severe fitness defect when the copy with higher expression level is deleted. Finding that a less severe fitness consequence is associated with deletion of a duplicate gene rather than a singleton gene has been challenged by studies done by He & Zhang (2006), which suggest that it could partially be traced back to the fact that duplicates are intrinsically less important than singletons, suggesting thus an overvaluation of the duplicate genes' contribution to mechanisms of genetic robustness. Furthermore, a high correlation between the frequency of compensation and the similarity in sequence of two duplicates has also been reported: duplicated genes that are highly similar in sequences perform better at compensating for each other than duplicates whose sequences (and presumably functions) have diverged further (Gu, 2003).

Whilst recent studies confirmed that functional overlap between duplicate genes is supported by genetic redundancy (DeLuna, 2008, Li, 2010), a number of paralogs still fail to display backup capacity. In many cases, the buffering ability of the duplicates, the strength of which is strictly tied to the extent of mutations in their background and proportional to their divergence time (Li, 2010), appears to have partially been lost. The observation that duplicate pairs often fail to genuinely back up for each other is confirmed by the lack of similarity between interactions profiles of the paralogs (Ihmels, 2007). Such a transitory status profile of functional compensation between duplicated pairs indicates that they do not contribute heavily towards functional redundancy, as might be expected from reliable mechanisms of cellular

robustness. Overall, it seems that gene duplication does not represent the major route to robustness; instead interactions among 'unrelated' genes provide robustness against mutations by participation in regulatory networks (Wagner, 2000).

1.6. The *TIF1* and *TIF2* genes

S. cerevisiae gene *TIF1* (Translation Initiation Factor) was isolated as a suppressor of a missense mutation in the mitochondrial gene *oxi2* (Linder & Slonimski, 1988) and described to code for a 395 amino acid protein. In association with this discovery, a second gene -*TIF2*- almost identical to *TIF1*, except for five silent substitutions within the ORF was also reported (Linder & Slonimski, 1988). *TIF1* and *TIF2* genes, respectively located on chromosome XI and X (Muller, 1989), are functionally redundant under normal growth conditions (Linder & Slonimski, 1989); protein sequence comparison showed they both specify for the same protein (Linder & Slonimski, 1989) which shares 81% of homology with the mouse eukaryotic initiation factor 4A (eIF4A). The role of the *TIF1* gene is confirmed by the presence of two sequences resembling the upstream activating sequence UASrpg -upstream activation sequence of ribosome protein genes- (Teem, 1984) in its 5' region. Such a feature is characteristic of the promoter regions of yeast genes coding for elements of the protein synthesis machinery. Interestingly, although the coding regions of the two *TIF* genes are almost identical, no homology can be found in regions surrounding the two ORFs, as their 5' and 3' untranslated regions are completely different. This is also the case with other duplicated genes involved in translation and may serve to regulate their expression. Intriguingly, *TIF1* and *TIF2* duplicate

pair retained absolute protein product identity. Both genes are characterized by high expression and similar mRNA size, with *TIF2*, however, showing the greater abundance of both mRNA and protein (Prat, 1990).

1.7. The promoter regions

Promoter regions appear to be of particular interest in the study of duplicate genes and have been addressed in several investigations. Regulatory regions of paralogs have been reported able to diverge, allowing expansion of functionality as well as changes in expression levels (Louis, 2007). As a result of these changes, novel or complementary organism functions could arise from differential regulatory mutations (Hellsten, 2007).

The ability of a paralog to undergo transcriptional reprogramming upon deletion or mutation of its counter-part has been shown to be crucial when it comes to compensation (Kafri, 2005). Thus, it is not important whether two genes are co-expressed under normal conditions but rather that one of the paralogs can be expressed at a satisfactory level, should a functional copy of its counterpart to be no longer available (Kafri, 2005). The same study revealed that dispensable paralogs have highly correlated expression in a few conditions only (Kafri, 2005), while a congruent body of evidence (Zhang, 2004; Gu, 2005) corroborated the belief that coding sequence divergence and expression divergence are correlated, although this correlation is generally weak. A later investigation led by Hughes and Friedman, highlighted that sharing of transcription factors tends to decrease with increased divergence in coding

sequence. Their data also showed significantly higher conservation of transcription factors by paralogs arising from duplicated genomic blocks than in other duplicated gene pairs. In addition, genes in duplicated regions also showed significantly greater conservation when coding sequences were compared (Hughes & Friedman, 2007).

The promoter regions of *TIF1* and *TIF2* genes bind to the same and different transcription factors, possibly as a result of diverse acquired mutations and/or changes induced by adaptation to challenges. The *TIF1* gene has been annotated as having eight documented transcription factors, while the number rises to thirty-one, if the potential ones are also taken into account; the *TIF2* gene displays five documented and thirty-one potential transcription factors respectively (yeastract.com). The two duplicate genes appear to share just two of the documented transcription factors Met4p and Sfp1p. Such differentiated use of promoters provides a crucial contribution to the functions of duplicated genes and is regarded as one of the mechanisms by which diversity at the gene expression level is achieved. The resultant flexibility of both qualitative and quantitative gene expression regulation may represent the outcome of a post-duplication event in which genes have become functionally differentiated in a multi-dimensional process, with different paralogs enduring differentiation in different ways (Hughes & Friedman, 2007).

1.8. eIF4A and the DEAD-box helicases family

The DEAD-box family of proteins owe its name to the occurrence of a D-E-A-D (Asp-Glu-Ala-Asp) motif, extensively conserved among helicases (Linder,

1989); this motif is required for ATP binding and hydrolysis and is found in the archetypal family of RNA helicases eIF4A. RNA helicases are crucial for translation initiation and in general for translational control. Their involvement is reported in almost every step of RNA metabolism, such as transcription, pre-mRNA splicing, nuclear export, RNA transport, mRNA decay, ribosome biogenesis, and translation initiation. (Hernandez & Vazquez-Pianzola, 2005). DEAD-box proteins are ATP-dependant RNA helicases and RNA-dependant ATPases, able to transfer the energy generated from hydrolysis of ATP into unwinding activity on RNA secondary structure or to separate RNA strands (Linder & Lasko, 2006).

The eukaryotic translation Initiation Factor 4A (eIF4A) was originally purified from rabbit reticulocyte lysate (Grifo, 1982); the same study also revealed the interaction between eIF4A and ATP, mediated through an ATP-stimulated binding of mRNA. The interaction between eIF4A and RNA binding was confirmed by Abramson (1987). Whilst isolated eIF4A is a non-processive bi-directional RNA helicase, when part of the eIF4F complex it becomes processive and 5' - 3' uni-directional (Rogers, 2001) and its weak helicase function increased by eIF4B, an RNA binding protein (Altmann, 1995). eIF4A is recruited at the mRNA 5'-untranslated region (5'-UTR) by eIF4G, a scaffolding protein, which directly interacts with both the 5'-UTR and eIF4E, the eukaryote initiation translation initiation factor 4E. mRNA secondary structures in the 5'-UTR are resolved by eIF4A, facilitating the formation of the 43S pre-initiation complex and ultimately ribosome recruitment and scanning to an AUG start codon (Sonenberg & Hinnebusch, 2009). Translation of mRNAs that display a greater degree of secondary structures has a higher requirement for eIF4A, both

in vivo and *in vitro* (Svitkin, 2001). Interestingly, mRNAs as short as 8 nucleotides have been shown to be still dependent on eIF4A activity, suggesting an additional role(s) for this complex protein (Blum, 1992).

Translation is a key step in the processes that leads from the genetic code to the synthesis of a functioning protein and ultimately to life; for this reason it does not come as a surprise that proteins involved in its regulation and performance are extensively conserved. The eIF4A family is critical for the process of translation to initiate in human (Gingras, 1999) as in yeast (Linder & Slonimski, 1989), being the most abundant translation initiation factor in both species (von der Haar & McCarthy, 2002). In humans it is referred to as eIF4AI/II while in yeast it is known as Tif1p and Tif2p. A third form, eIF4AIII, is annotated in humans, although unable to compensate for the eIF4AI/II activity *in vitro* or *in vivo* (Ferraiuolo, 2004). Instead it plays a key role in non-sense mediated decay (NMD). An rRNA processing (Fal1p) is found in yeast (Kressler, 1997), which has a similar homology to Tif1/2p as eIF4AIII does to eIF4AI/II in humans. However, the two proteins do not appear to be functional homologues despite the acronym FAL standing for “Four A Like”. While the process of translation initiation is highly conserved in both humans and yeast, there are minor divergences. The process of translation initiation is overviewed in the next section.

1.8.1. Eukaryotic Translation Initiation

Translation initiation is a conserved mechanism that occurs in most cells primarily through the cap-dependant mechanism, which functions through the 5'-methyl guanine cap, a structure that marks mRNAs. Extensive studies done in *S. cerevisiae* have revealed how complexly regulated this process is (Hinnebusch

& Liebman, 1991). Recognition and recruitment of the G5'ppp5'N structure is done by eIF4E (7-methyl guanine cap-binding protein) which, in association with eIF4G and eIF4A, form the eIF4F complex. While the process of translation initiation is highly conserved in both humans and yeast, there are minor differences. One of these is that human eIF4G1 displays two binding sites for eIF4A (Korneeva, 2001), whilst yeast eIF4G contains only one (Li, 2001). The cap-dependent translation process involves the engagement of numerous eukaryotic initiation factors (eIFs) and is dependent on eIF4A, as secondary structures in the 5' UTR require RNA-helicase activity for the initiation process to begin (de la Cruz, 1999). After recruitment, the 40S subunit scans the mRNA for the initiation codon, association with the 60S subunit eventually takes place, and translation begins.

The major point of control for cap-dependent translation seems to be the association of the 40S subunit with the eIF2•GTP•Met-tRNA_i ternary complex, which ultimately permits successful scanning. It has been shown that Gcn2p, a kinase activated under particular conditions of stress, phosphorylates eIF2 and mediates the inhibition of translation *via* a complex interchange of AUG recognition and eIF2-GTP status (Hinnebusch, 1996). A further point of regulation is the level of interaction of the small ribosomal subunit to the 5' end of the mRNA: in this case, control is mainly achieved through modulation of eIF4E affinity for the cap structure by phosphorylation (Scheper & Proud, 2002) and inhibition of the eIF4E-eIF4G interaction (Lin, 1994; Gingras, 1999). Ultimately, an important mode of control has to be ascribed to the presence of secondary structures in the 5' UTR, as these are inhibitory for translation both *in vivo* and *in vitro* (Pelletier & Sonenberg, 1985; Baim & Sherman, 1988). In this

context, RNA helicases, specifically eIF4A, function by smoothening the landing path for the translation-competent small ribosome subunit and thereby achieving a form of control on the translation process.

A second mechanism, known as cap-independent translation initiation, does not require the ribosome to start scanning from the 5' end of the mRNA cap to the start codon. It relies instead on the presence of an Internal Ribosome Entry Site (IRES), often distant from the cap structure. The intervention of IRES trans-acting factors permits the ribosome to bypass the need for the scanning process: the ribosome is loaded onto the transcript, independent of the 5'-UTR and cap structure, and finally trafficked to the start site. There is a variable requirement for an eIF4A contribution in this process (Jackson, 2010). In yeast, IRES-containing transcripts are not particularly ubiquitous but have been reported in genes involved in filamentous growth induced in response to nutrient starvation (Fernandez, 2001). In eukaryotes, cap-independent translation has also been revealed to be important in conditions of apoptosis and stress-induced responses (López-Lastra, 2005).

In summary, there are a number of important questions that remain unanswered about the role of the duplicate genes *TIF1* and *TIF2* in yeast, and why these have been evolutionarily restrained to provide an identical protein. A number of complementary techniques will be required to address these questions.

1.9. Aims and objectives

The aim of this research is to develop an understanding of the evolutionary benefits underlying the conservation of a single protein product (eIF4A, referred to below as Tifp, the protein product of the *TIF1* and *TIF2* genes) formed from a whole genome duplication event.

To achieve this, different complementary approaches have been employed to understand the different roles of *TIF1* and *TIF2* in the context of translation initiation in yeast:

- The first part of the study is an exhaustive analysis of the genetic surroundings of the *TIF1* and *TIF2* genes, by using deletions of identified genes as a probe in a wide-scale gene-to-function oriented approach.
- The second part of this research utilizes chemicals and environmental challenges to complement *TIF1* and *TIF2* genome-wide screen results, specifically to formulate an explanation on how augmented translation initiation overcomes Li⁺ toxicity.
- A global interpretative picture of wide-scale information is finally assembled in a network context, where the duplicate nature of *TIF1* and *TIF2* genes is analysed to unveil the evolutionary path they have gone through as paralogs.

2. Chapter 2

This chapter describes a detailed analysis of a robustly replicated and validated synthetic genetic array (SGA) analysis of the genetic network of the *TIF1* and *TIF2* genes. Analysis of the results should provide evidence towards the global aim of understanding evolutionary benefits underlying conservation of a single protein product (eIF4A, referred to below as Tifp, the protein product of the *TIF1* and *TIF2* genes) from the two genes.

2.1. Introduction

Yeast has played a significant role in human civilization for a very long time as an essential component for domestic activities such as baking, brewing and wine-making. The use of this resourceful organism for human purposes has been dated to 7400-7000 years ago, with the earliest evidence ascribed to the Neolithic Era: traces of the calcium salt of tartaric acid and the resin of the *Pistacia* (terebinth) tree identified on narrow-necked pottery specimens hint to these findings as the first evidence of wine making. Tartaric acid is found in great amounts only in grapes and it is believed that the *Pistacia* resin was included as antibacterial preservative. Man's first experience with yeast possibly arose from a process that did not require an inoculum of the organism, as yeast is present in the fruit and able to carry out fermentation.

Archaeological and chemical records from lower Mesopotamia unveiled how Sumerian tribes, who ruled the region around 4000 to 3000 B.C., were also engaged in production of beer and wine. A mounting body of evidence such as pictograms, a seal representing Ninkasi (the goddess of brewing), beer recipes,

and the presence of calcium oxalate on manufactured vessels of specific shape strongly suggests that Sumerians were the first civilized culture carrying on brewing and wine-making, as a constitutive part of their customs. Analysis on a DNA sample isolated from a presumptive wine jug found in Abydos (Egypt) and dated around 3150 B.C., showed a close sequence match for the modern yeast *S. cerevisiae* (Cavalieri, 1988).

Historians and scientists have also contemplated the hypothesis that around 1000 B.C., Ancient Egyptians were able to “isolate” the active yeast, culture it and directly introduce it into their dough, designating it as the first form of the domesticated organism. Once again, speculation has been raised on whether yeast present in the air as a natural contaminant may have triggered the fermentation process, causing the mix of flour and water to leaven.

Studies on the genus *Saccharomyces* date back to 1837, when German physiologist T. Schwann experimentally showed that alcoholic fermentation is dependent on the presence of a bubble-giving living organism, yeast. He correctly annotated it as a fungus, and called it in German “Zuckerpilz”: sugar fungus. The following year, *S. cerevisiae* became the first yeast species to be described: Julius Meyen translated the German “Zuckerpilz” into Latin *Saccharomyces*, assigning to the organism its biological name. He named it after *Saccharomyces* meaning sugar moulds and *cerevisiae* meaning beer from the Gaelic word *kerevigia* or the old French word *cervoise*.

In more recent times, yeast boasts a history of successful applications while technological advancements and research have given rise to its extensive use in different industrial fields such alcoholic beverages, baked goods and food additives. Following Pasteur’s work and the development of microbiological

techniques, specialized strains were introduced and evolution partially interrupted, as such strains are separated out from the evolutionary pool on which natural selection acts on. Industrial usage of yeast relies on specialized strains of *S. cerevisiae* that have been selected for a specific process and are not easily interchangeable.

Yeast metabolism is of particular interest and its energetic cost is covered through the employment of different substrates, primarily sugars, while the presence or absence of oxygen in the environment generates two types of metabolism: in the condition of aerobiosis, oxygen is used to oxidise glucose with release of carbon dioxide, water and energy; this route, known as a respiration, allows for the whole potential energy in glucose to be released. In the absence of oxygen (anaerobiosis), yeast is still able to utilize sugars for the production of energy necessary for its survival, however with a compromised efficiency. This metabolic process, described by L. Pasteur as fermentation, is dramatically less resource-efficient in terms of releasing potential biochemical energy, as only a partial oxidation of glucose into alcohol and carbon dioxide is completed. This ability to grow under anaerobic conditions is unlike the majority of other fungi, which display an exclusively aerobic metabolism. As gene duplication took place prior to the WGD, it can be hypothesised that such an event facilitated the acquiring of novel and distinctive competences, including the ability to survive in anaerobic environmental conditions.

Fermentation denotes a characteristic ability of members of *Saccharomyces yeast* and is represented in their ability to convert sugar into alcohol. There is a great variety in the landscape of production of beer, wine, and

distilled spirits which reflects the use of different sub-strains of *S. cerevisiae*, however all those different sub-strains can be ascribed to one, *S. cerevisiae*. Whilst alcohol production for recreational use embodies the most popular application of the fermentation process, fermentation is not just about hangovers! Distinctive metabolic features of members of the Fungi kingdom have shown potential for application in the degradation of hazardous and organic waste, mainly through the construction of genetically engineered yeasts (Loper, 1988). The use of *S. cerevisiae* as a biocatalyst to generate electricity in microbial fuel cells has also been explored: in a recent publication, Raghavulu and colleagues presented encouraging data for a yeast-based fuel cell to be developed as a remedy for high strength wastewaters with coupled generation of power (Raghavulu, 2011). The yeast fermentation derived ethanol represents, together with the transesterification derived biodiesel, one of the so-called first generation biofuels used to produce ethanol for the biofuel industry. The construction of novel genetically modified yeast strains, less sensitive to the ill-effects of their own produced alcohol, has been accomplished by Stephanopoulos' lab (Alper, 2006); the implications of this revolutionary methodology are considered to be enormous as the process could lead to boosting the efficiency of generating ethanol-based fuels from corn and plant wastes. Furthermore, of particular appeal for the pharmaceutical industry, *S. cerevisiae* can also be employed in the manufacture of steroids: a patent deposited by R. Spagnoli and others in 2010 is based upon genetically modified yeast strains able to carry out steroid synthesis, through their own fermentation process (Spagnoli, 2010).

2.2. Yeast as a model organism

The yeast *Saccharomyces cerevisiae* is a round-ovoid single-celled organism of 5–10 μm in diameter, belonging to the family of Saccharomycetaceae (phylum Ascomycota, kingdom Fungi). It contains 16 characterized chromosomes that range in sizes between 200 to 2,200 Kb and its completed sequence was determined in 1996 (Goffeau, 1996), uncovering a 12,068 Kb genome, with 5885 ORFs encoding for potential proteins. Unlike other unicellular organisms, the yeast genome is quite compact, with genes representing 72% of the total sequence. Easy to manipulate, a short life cycle with a doubling time of 90 minutes, assessed tractability and a relatively small genome, represent only few of the characteristics featured by this powerful organism. Baker's yeast is historically remembered as the first eukaryotic genome to be fully sequenced (Goffeau, 1996) and transformed by plasmids (Beggs, 1978). It also holds a high rate of homologous recombination that allows precise manipulation of the genome for the construction of gene disruptions and allele-specific replacements; its stability both in haploid and diploid state renders it suitable for limitless experimental approaches. Its maintenance and growth are also inexpensive and it holds a designation GRAS (Generally Recognized As Safe) assigned by the American Food and Drug Administration (FDA). In addition, broad shared knowledge in annotated information about ORFs and their protein products is available via regularly updated public databases such as *Saccharomyces* genome Database (SGD) (www.yeastgenome.org/), Yeast Search for Transcriptional Regulators And Consensus Tracking (YEASTRACT) (www.yeasttract.com/), YeastMine (yeastmine.yeastgenome.org/) and MIPS Comprehensive Yeast Genome

Database (CYGD) (mips.helmholtz-muenchen.de/genre/proj/yeast/). With the genome sequence completed, nowadays the challenge is pairing yeast genes with their function(s) and potential human homologs; elucidated knowledge on this topic would allow more ambitious approaches to genetic therapies and drug discovery and treatment strategies and overall enhance our comprehension of disease states etiology.

2.3. Yeast as a model for human diseases

Research on genetic diseases has undeniably benefited from the availability of diverse experimental organisms: *S. cerevisiae* contributes remarkably to the understanding of genes linked to human disease, while displaying the potential for further groundbreaking discoveries.

“Each time I have identified an intriguing aspect of the cancer problem, I have found that it could be approached more effectively in the simpler eukaryotic cell, Saccharomyces cerevisiae, than the human cell.”

L. Hartwell, 2001 Nobel Prize in Physiology or Medicine, Nobel lecture.

Prof. Hartwell who conducted pioneering studies on the mechanisms of cancer biology in *S. cerevisiae* (Hartwell, 2002) emphasizes a concept already quite popular among his colleagues: the beauty of yeast lies in its simplicity.

The life cycle of this resourceful organism reflects an ordinary eukaryotic one, characterized by a cell cycle that undergoes G₁, S, G₂, and M phases (Williamson, 1965) and fundamental checkpoints for damaged DNA have also been reported (Harrison & Haber, 2006). Baker's yeast reproduces by budding, with the bud growing in size during the cell cycle (Hartwell, 1970), an element which provides an indication of the progression of the cycle. The high degree of conservation between yeast and higher eukaryotes is not confined to basic elements of cell structure but it is also extended to molecular pathways (signal transduction and life span, for example), making *S. cerevisiae* a valuable model organism for studying human disorders. At least 31% of proteins encoded by yeast genes have human homologues and conversely nearly 50% of human genes implicated in heritable disease display yeast homologues (Hartwell, 2004). Today's efforts are concentrated around potentially modulatable conserved biomolecular pathways, motivated by the vision to better understand, prevent or delay diseases such cancer, diabetes, cardiovascular and neurodegenerative disorders.

2.4. Yeast in the investigation of translation initiation

The yeast *S. cerevisiae* has proved an appropriate organism in which to study translation initiation. For the past three decades genetic analyses have largely contributed to the identification of genes involved in this process. Although eIF4A was first reported in mammals (Grifo, 1982) and plants (Seal, 1985), it was through work done in yeast that P. Linder and colleagues (Linder & Slonimski, 1989) encouraged further investigations. Yeast eIF4A mutants were latterly employed in experiments aimed to confirm the ATPase dependence of

the eIF4A function (Blum, 1989) and the finding that yeast eIF4E cap-binding protein can be complemented by the same protein sourced from other organisms, such as the fruit fly (*Drosophila melanogaster*) or mouse (*Mus musculus*), (Altmann, 1989; Hernandez, 2005) represented a milestone in recognizing the “authenticity” of yeast in the study of translation initiation. Discovery of yeast eIF4B was also done in *S. cerevisiae* by screening cDNA libraries with anti-cap binding complex antibodies (Altmann, 1993) and performing genetic screens (Coppolecchia, 1993); yeast eIF4B RNA-annealing activity was further elucidated (Altmann, 1995). Studies of proteomics and genetic analysis done in *S. cerevisiae* also highlighted the engagement of Ded1p in the splicing process, as a component of the spliceosome (Stevens, 2002) and established its role as initiation factor (de La Cruz, 1997) respectively. Finally, elucidation of the ribosome-associated scanning mechanism has been successfully pioneered in yeast by F. Sherman, whose studies showed that the first AUG is used as start codon to initiate translation (Sherman, 1980), while T. Donahue’s group later identified genes required for initiator codon selection (Cigan, 1988). Several translation factors discoveries have been achieved through analysis of suppressor genes: eIF2 α (Cigan, 1989), eIF4 β (Donahue, 1988), eIF2 γ and eIF5 (Huang, 1997) and eIF1 (Yoon & Donahue, 1992) were all found playing a major role in the scanning and AUG recognition processes; once again, yeast was the organism of choice.

2.5. Overview of studies in yeast

Studies on protein-protein interactions such as two-hybrid screens (Young, 1998) and Tandem Affinity Purification (TAP) (Puig, 2001) have seen

yeast utilized successfully, often paired with mass spectrometry (Gavin, 2002). The first global protein localization analysis was also achieved through fluorescent labelling applications in *S. cerevisiae* (Huh, 2003). Informative analyses on RNA levels have been conducted through microarrays (reviewed in Schulze & Downward, 2011) and Serial Analysis of Gene Expression (SAGE) (Velculescu, 1997) and transcription factor-dependent gene regulation was pioneered in ChIP-chip -chromatin immunoprecipitation (ChIP) coupled with microarray (chip)- experiments by Lee and colleagues (Lee, 2002). A multiplicity of approaches have been developed for investigations at the gene level, ranging from footprinting (Densmore, 1991), random mutagenesis (Ernst & Richman, 1989), targeted gene deletions, including Synthetic Genetic Array (SGA) (Tong, 2001) to Synthetic Lethality Analysis by Microarray (SLAM) (Ooi, 2006).

Yeast's features offer potential that was recognized by a group of scientists as an opportunity to apply functional genomics approaches in a comprehensive, genome-wide fashion. Such innovation initiated the way for a range of straightforward, but at the same time sophisticated, applications and was followed by opened availability and commercialization of several yeast libraries. The *S. cerevisiae* variety of collections, such as knock-out, over expression, modulatable promoters, green fluorescent protein (GFP), etc has been received with enormous enthusiasm by the scientific community, endorsing the ascent of yeast in the study of genetics.

2.6. The Yeast Deletion Set

Among the aforementioned libraries, the Yeast Deletion Set (YDS) is one of the most successful and vastly used. The most utilized YDS features 4,800 *S.*

cerevisiae non-essential single deletion strains conveniently arranged in a format designed to undergo high throughput screening. The essential feature of this array, containing deletions of almost the entirety of non essential ORFs, is the replacement of each single gene with a *KanR* gene cassette which confers resistance to the aminoglycoside antibiotic Geneticin (G418) (Winzeler, 1999).

A bonus feature was introduced into the yeast collection through elegant work done by R. Davis's laboratory with the creation of a molecular barcode that is introduced along with the resistance cassette and enables an unambiguous identification of the specific deletion strain. Each deleted ORF is flanked by a pair of 20-nucleotide TAGs (UPTAG and DOWNTAG) edged by sites amplifiable by universal primers; the advancement achieved in micro-array analyses thanks to this is incommensurable.

The YDS, also known as Yeast Knock Out (YKO) set, is available in different formats covering combinations of both *Mata* and *Mata* mating types and homo and heterozygous conditions (Winzeler, 1999). Essential genes are only available in *Mata* and *Mata* heterozygous diploid sets. The haploid state has proven useful for identifying recessive deletions as they are easily recovered; on the other hand, a diploid state allows genetic analysis, including tests of complementation, recombination and epistasis. The *Mata* haploid YDS collection, a kind gift from C. Boone's lab (University of Toronto, Canada), was employed in this research thesis as an investigative tool in 'classic' SGA studies (Tong, 2001), while the homozygous diploid library purchased from Thermo Scientific-Open Biosystems represented the YKO set of choice in chemical genetic screens and assays for scoring fitness defects in response to a broad spectrum of growth conditions, environmental challenges and drug treatments.

As phenotypic investigation resulting from gene deletions has become increasingly regarded as one of the most powerful ways to determine gene functions, the YDS has found applications in a wide range of research approaches to address this topic: synthetic genetic array, chemical genomic screening, dosage synthetic lethality, genetic suppression of lethality, synthetic haplo-insufficiency, and physiological stress screening are among the most popular.

2.7. Generation and understanding of a genetic network

The availability of resources embodied by genome-wide arrays became translated into a valuable opportunity to investigate deletions in an extensive range of high throughput assays (Tong, 2001; Jasnon & Korona, 2007), which ultimately generated such large data sets that a new method for representing the great collection of annotated interactions was required. The task was not an easy one as the approach was required to represent a simplified portrait of the connections, while maintaining the essential complexity of the information revealed. Networks proved to be a valuable means in representing gene interactions and promptly made data analysis more accessible, unlocking a novel way of thinking about genetic interactions. Our understanding of such connections greatly benefited from network representations and their consequential impact from a biological point of view has gradually been unveiled (Merico, 2009).

2.7.1. Genetic Interactions

In organisms, gene functions appear coordinated in a cellular environment rather than through isolated one-to-one interactions. When

simultaneous perturbation of at least two genes results in a deviation from the expected neutral phenotype, a genetic interaction is observed. Such connections are extremely important when it comes to identifying relationships of a functional nature among genes (or their encoded protein products), as they suggest an associated function between the interacting genes. Genetic interactions tend to occur between genes involved in functionally related pathways, with the nature of the interactions strongly indicative of the type of interaction (Boone, 2007), and are therefore highly informative of how cellular processes are conducted.

Genetic interactions can extensively be interpreted through the study of epistasis, described as the interaction between two or more genes to control a single phenotype. Fitness, and specifically “growth”, represents the phenotype considered in the majority of investigations, including research presented here. In the context of duplicated genes, epistasis can be considered as the capacity of a gene to buffer the phenotypic consequences of mutation of its sister gene; epistatic evidence has also successfully been processed with the aim of recapitulating the interactions lying behind genes that share a common function. (St Onge, 2007). A relationship of positive epistasis between two genes suggests the two genes lie inside the same pathway and possess a suppression (or within-pathway) connection. A diminishing (or alleviating) interaction is reported when the fitness defect of the double mutant phenotype is less severe than expected. On the other hand, negative genetic interactions are displayed by double mutants whose fitness is lower than expected for the joint effect of the single mutant growth phenotypes; negative epistasis is described as aggravating when it decreases the fitness and underlines the involvement of separate

pathways that feed into a common output. While positive epistasis would seem an always-winning response in overcoming inconvenient conditions, natural selection favours removing deleterious mutations, thereby increasing the overall fitness of a population. Thus, positive epistasis needs to be used parsimoniously in a biological system context.

Nowadays we define as synthetic (or synergistic) interaction the case of a double mutant displaying a more severe growth phenotype than expected from the two deletions on their own and *synthetic lethality* (SL), an acute representation of it. The process later called SL was initially described by geneticist C. Bridges in his *The origin of variation* (Bridges, 1922), during his studies on *D. melanogaster*. T. Dobzhansky (1946) appears to have first utilized the term, where the choice of the word '*synthetic*' sought the specific intent to evoke its Greek meaning, as the combination of two "entities" to form something new. SL represents a severe case of genetic interaction corresponding to two single mutations that, whilst viable on their own, result in a lethal phenotype when deleted in combination. What do these interactions mean in the context of biological systemic connections? When two genes show a SL interaction, it often indicates that the genes products impinge on the same essential function, such that under dual deletion one pathway can no longer functionally compensate for the defects in the other. This phenomenon is also known as buffering and accounts for the functional redundancy that characterizes biological systems. Similarly, if a combination of deletions results in a not-lethal growth impaired phenotype, we call this genetic interaction *synthetic sickness*; this type of synthetic fitness interaction underlies an important, although non-essential, genetic interaction.

Although particularly popular in the context of loss of function mutants, SL can also be mimicked by other types of genetic interactions such those generated by exposure to different environmental conditions and chemicals. This research thesis covers both typical (SGA, chapter 2) and less conventional (environmental challenges and chemicals, chapter 3) SL-like interactions.

2.7.2. Synthetic Genetic Analysis (SGA)

One of the most effective ways of investigating a biological system is indeed a “controlled”, guided perturbation, complemented by observation of the differences between perturbed versus native state. Making a gene non functional by deleting it and studying the consequence of the deletion is one way of achieving this. An equally informative approach involves inactivation of two genes at the same time and analysis of their genetic interactions with the purpose of uncovering phenotypes otherwise hidden by alternative mechanisms (e.g., robustness) (Tong, 2001). This scenario may include the case of a knockout (KO) gene displaying a relatively weak phenotype, but for other reasons the gene is assumed to affect the process of interest. A redundant gene or pathway backing up the system may exist, as suggested by the extent of functional redundancy that characterizes the yeast genome (Hartman, 2001). One way to overcome this redundancy is looking for a second-site mutation that alters somehow the phenotype of interest, either enhancing or suppressing it.

Synthetic Genetic Analysis (SGA) represents an effective solution for the systematic construction of double KOs (or double mutants) and enables a comprehensive analysis of SS/SL interactions. A typical SGA screen consists of mating of the yeast deletion library (**section 2.6**) with a query mutation of

interest to the investigation. Taking advantage of the mating type specificity of a promoter carried by the query strain, consecutive selection steps are undertaken, ultimately generating the meiotic progeny carrying both mutations (Figure 2-1).

Figure 2-1. SGA methodology. a. A *MAT α* strain carries a query mutation linked to a dominant selectable marker (represented as a filled black circle), such as the nourseothricin-resistance marker *natMX*, and the SGA reporter *can1 Δ ::MFA1pr-HIS3* (in which *MFA1pr-HIS3* is integrated into the genome such that it deletes the ORF of the *CAN1* gene, which normally confers sensitivity to canavanine). b. This query strain is crossed to an ordered array of *MAT α* deletion mutants (*xxx Δ*). In each of these deletion strains, a single gene is disrupted by the insertion of a dominant selectable marker, such as the kanamycin-resistance (*kanR*) module (the disrupted gene is represented as a filled blue circle). b. The resultant heterozygous diploids are transferred to a medium with reduced carbon and nitrogen to induce sporulation and the formation of haploid

meiotic spore progeny. c. Spores are transferred to a synthetic medium that lacks histidine, which allows for selective germination of *MATa* meiotic progeny because these cells express the SGA reporter *can1Δ:: MFA1pr-HIS3*. To improve this selection, canavanine, which selects for *can1Δ* and kills *CAN1* cells, is included in the selection medium. d. The *MATa* meiotic progeny are transferred to a medium that contains kanamycin, which selects for single mutants, equivalent to the original array mutants and double mutants. e. An array of double mutants is selected on a medium that contains both nourseothricin and kanamycin. Adapted from Boone (2007).

SGA is conveniently accompanied by a scoring system and valuable information is revealed through the study of synthetic lethal/sick interactions and positive growth effects. All together, these features make SGA a quick assay for capturing an authentic, although blurred, picture of the kind of processes the gene of interest is involved in.

If both mutations are null alleles and occur in non-essential genes, the ordinary interpretation is that these genes function in parallel cellular pathways that impinge on a shared essential function. This model is often referred to as the “between-pathway” model and usually reflects some bidirectionality in their genetic redundancy, as that each pathway compensates for defects in the other. Further insights on cellular mechanisms can be gained from an adequate knowledge of the gene function and/or the pathway the gene’s product is involved. This is for instance the case of a synthetic-lethal interaction that underlies a unidirectional compensation: one pathway normally prevents a potentially harmful cellular event that can be corrected by another pathway, but not vice versa (Boone, 2007). Data generated through this experimental approach are likely to highlight genes encoding components of a functionally related pathway or interacting protein complexes. As basic processes are

frequently associated with viability, which is intrinsically tied to fitness, the SGA methodology offers the opportunity for a wide variety of biological processes to be assessed. Whereas the activity of a given pathway is required for cellular fitness when a gene is functionally compromised, components of this pathway are expected to be identified in the SGA, as shown in **Figure 2-2**.

Figure 2-2. An example of relationship between pathways identifiable through an SGA; the potential genetic interactions are represented by lines. Adapted from Boone (2007).

The identification and better characterization of several biochemical pathways has been substantially contributed to by the SGA experimental approach: extensive mapping of genetic interactions in *S. cerevisiae* was made possible through the identification of mutations in genes that either enhance or suppress the phenotype of mutation in a known gene (Tong, 2004).

As negative genetic interactions are often taken as evidence of a relationship of a functional nature, their interpretation can help in estimating the

extent of redundancy between genes. In the specific case of genes holding a duplicate counterpart, SGA-generated interactions offer an additional means to assess functional associations and differences between duplicate pairs as the number of negative interactions is often indicative of functional similarities and/or relationships between paralogs.

The SGA approach does have limitations, as inter-laboratory variability and the occurrence of mechanisms of compensation (e.g., functional redundancy), which might affect the screen's results and cause only a fraction of the genetic interactions which characterized the query gene network to be revealed. These are discussed more fully in **section 2.42.3**.

2.7.3. Genetic Networks

The intricate bundle of genetic interactions generated through genome-wide studies is readily displayed in network representations, where cellular mechanisms can be investigated thanks to efficient visualization and regularly updated databases of known orthologues and interactions. A network is generally described as a system in which a set of elements can be represented by nodes and relationships between the elements by edges (or links), indicating coupling or relationships of some nature. In a genetic network context, a gene is represented as an entity characterized by its interactions; the neighbourhood of genes surrounding it (**Figure 2-3**). The potential to be uncovered is enormous as a full investigation can be covered spacing from the small scale (single KO or specific gene subsets) to the big picture (network of networks), in which the gene and its associated role(s) is/are considered as constituent of a more complex system.

Such a comprehensive representation of biological systems aligns with the current awareness of genetic diseases. Testimony to such complexity exists in the occurrence of disease states caused by rare combinations of genetic defects, despite the existence of different alleles within the human population. Partial contribution of several genes, often acting in unrelated biochemical pathways, has been acknowledged as a crucial component of such complexity. As emerged from an increasing numbers of quantitative trait loci (QTL) investigations (Schadt, 2003), there is a burning need to achieve a higher resolution of genetic interaction mapping, motivated by the intent to understand disease states at the genetic level.

Interpretation of networks appears crucial as it frequently offers a view into the functional organization of the organism of interest. Dilemmas such as the influence of genotypes over phenotypes in biological systems can be investigated in their entirety through the interpretation of genetic networks and the valuable information underlying them. A gene network appears like a “small world” surrounded by condensed local neighbourhoods (Tong, 2004) where a positive correlation is held between gene’s centrality in interaction networks and the severity of KO effects (Jeong, 2001).

Figure 2-3. A network representation of synthetic lethal interactions associated with yeast chromosome instability (CIN) genes found having human homologs mutated in cancer Adapted from Yuen (2007).

Although these types of interactions have extensively been investigated through the use of baker's yeast, the concept of genetic interaction itself is still being developed, as it remains to be resolved which degree of comparability we can assign to protein-protein interactions versus genetic interactions. The majority of protein products display a promiscuous nature and engage in physical interactions with several other proteins. Two proteins in the same region of the genetic network are likely to share a physical interaction (Tong 2001; 2004) and proteins with many physical interactions are also highly connected from a genetic point of view (Ozier, 2003).

Data generated *via* SGA analysis can be effectively represented via networks. In studies such as the one to be presented below, a SL genetic interaction between a gene deletion of interest and a knock-out library gene is represented as an edge in the network, as the condition that the phenotype

generated by the combined perturbation is greater than the sum of the two individual perturbations (Lehner, 2006).

2.7.3.1. The network inside the network

Although our understanding of the mechanisms of evolution among post-duplication genetic interactions is still limited, we are at least now able to predict complex network topologies based on underpinning experimental observations. This is an advance from earlier basic network characterized by a limited set of components (Presser, 2008). The plasticity and adaptability of a network is partially thought to be supported by duplication events, where new components provide evolutionary capacity within the network system (Aury, 2006). Such features render genetic networks valuable sources of information, with some power to predict function: clustering networks of an uncharacterized gene with known genes' ones of for example, is a very useful way to inform speculation about functional associations (Tong, 2004). Furthermore, the potential for merging networks generated through different data sets, approaches, and organisms in a high-throughput fashion is regarded as highly enlightening for the understanding of cellular and evolutionary processes. Integration of physical interactions maps with networks representative of genetic connections has been proven a validated investigative approach to reveal pathways (Kelley & Ideker, 2005).

As genes functions are extensively conserved among organisms, it is a fair expectation for their genetic interactions to be conserved as well, This has turned out not to be the case: it emerged instead that networks, and not single gene connections, tend to be conserved in their topology as well in their

organization, and then only to a limited extent. Such a scenario, in which the network system is partially conserved whilst the specific genetic interactions are not, points towards the extent of system robustness.

2.8. Aims and objectives

To understand fundamental characteristics of the *TIF1* and *TIF2* duplicate genes (e.g., functional divergences) and the reason behind the retention of their identical protein product. In order to achieve this, a phenotypic investigation will be achieved through SGA analysis.

2.9. Methods

2.9.1. Yeast

All strains of *S. cerevisiae* used in this study were kindly gifted from C. Boone (University of Toronto, Canada) or purchased from Thermo Scientific-Open Biosystems (Huntsville, AL, USA) as part of the YKO *MATa* or YKO *MATα* Strain Collection (Winzeler, 1999). The yeast strains are derived from parental strain SC118 and bear a genotype of *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* in a BY4741 background (Brachmann, 1998).

2.9.1.1. SGA

tif1Δ::natR and *tif2Δ::natR* query strains construction were constructed via a non-essential gene switching method (**method 2.18**). Starting strains *tif1Δ::kanR* and *tif2Δ::kanR* (Boone's *Mata* library) underwent replacement of the *kanR* cassette with the *natR* cassette, amplified from p4339 plasmid *MX4-natR* switcher cassette.

Y8205, the SGA starting strain into which query mutations can be introduced by switching protocol, was provided by the C. Boone laboratory (University of Toronto, Canada) as part of an SGA kit. It bears an α mating type and a genotype *can1Δ:STE2pr-Sp_HIS5 lyp1Δ:STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0* and was mated to *tif1Δ::natR* and *tif2Δ::natR Mata* to provide the query strain with the genetic background necessary for SGA selection.

The control (or surrogate “wild-type”) strain of choice for this study was the *his3Δ::kanR* strain, where the *HIS3* gene has been replaced with the cassette conferring resistance to the antibiotic G418. This is believed to have no impact on growth, given that BY4741 display histidine autotrophy.

2.9.1.2. **tetO promoter**

tif1Δ::natR tetTIF2 and *tif2Δ::natR tetTIF1* strains were constructed utilizing *tif1Δ::natR* and *tif2Δ::natR* as starting strains, in which generated *tetO₂* PCR (**method 2.15.2**) product was inserted via transformation (**method 2.18.3**). Regulation of activity of the *tetO₂* promoter is achieved through addition of doxycycline to the medium in concentration higher than 10 µg/ml (Belli, 1998b).

2.9.1.3. **GAL over-expression promoter**

tif1Δ::URA GAL1pr-TIF2, *tif2Δ::URA GAL1pr-TIF1*, *tif1Δ::URA GALLpr-TIF2*, *tif2Δ::URA GALLpr-TIF1*, *tif1Δ::URA GALSpr-TIF2*, *tif2Δ::URA GALSpr-TIF1* over-expression strains were generated utilizing *tif1Δ::natR* and *tif2Δ::natR* as starting strains by marker switching (**method 2.13.1**) with the use of the *CaURA3MX4* cassette amplified by plasmid pAG60; this was followed by insertion of *GAL* derived PCR products via transformation (**method 2.13.2**).

All the aforementioned *S. cerevisiae* yeast strains were stored at -80 °C in YPD containing 20% glycerol.

2.9.2. **Bacteria**

Escherichia coli DH5α (Maximum Efficiency DH5α Competent cells, Invitrogen, Carlsbad, CA) were used for transformation by yeast plasmid DNA. Bacteria strains were grown at 37 °C in LB (Luria-Bertani) or YT (Yeast extract/Tryptone) media (see **method 2.9.4.1** for media composition) and stored at -80 °C in one of the above media containing 20% glycerol.

2.9.2.1. Plasmids

Plasmid p4339, constructed by amplifying *natMX* with primers (5'-ACATGGAGGCCCGAGAATACCC-3' and 5'-CAGTATAGCGACCAGCATTAC-3') followed by insertion of the product into pCRII-TOPO (Invitrogen) at the unique EcoRI site, was gifted from C. Boone's laboratory (University of Toronto, Canada) as part of the SGA kit. This plasmid can be used to switch *kanMX4* deletion alleles to *natMX4* alleles by cutting with EcoRI.

Plasmid pCM224, bearing a *tetO₂* (Belli, 1998) promoter-substitution cassette with associated *kanMX4* selectable marker, was purchased from Euroscarf (Frankfurt, Germany) as part of the three sets of plasmids for tetracycline dependent expression of gene products. pCM224 allows promoter cassette substitution of the promoter of the native chromosomal ORF of interest *via* one-step PCR integration and regulation of the expression of the promoter by tetracycline in the medium.

Plasmid pAG60, bearing a *C. albicans URA3* ORF that confers uracil prototrophy to transform auxotrophic yeasts (Goldstein, 1999), was purchased from Euroscarf (Frankfurt, Germany) as part of the DEL-MARKER-SET. The pAG60 plasmid allows the substitution of selective resistance cassettes *via* marker switching.

Galactose driven over-expression plasmids pYM-N23, pYM-N27, pYM-N31 (respectively carrying *GAL1*, *GALL*, and *GALS* cassettes with associated *natNT2* selectable marker) were purchased from Euroscarf (Frankfurt, Germany) as part of a tool-box (Janke, 2001).

All the aforementioned plasmids were stored at -20 °C.

2.9.3. Stock solutions

Antibiotics stock solutions were prepared as following:

Ampicillin (Sigma-Aldrich, Auckland, New Zealand) was dissolved in water at concentration of 100 mg/ml, filter-sterilized, stored in aliquots at 4 °C and added to the medium to a final concentration of 100 µg/ml.

G418 (Geneticin, Invitrogen, Carlsbad, CA, USA) was dissolved in water at 200 mg/ml, filter-sterilized, stored in aliquots at 4 °C and added to the medium to a final concentration of 200 µg/ml.

clonNAT (nourseothricin, Werner BioAgents, Jena, Germany) was dissolved in water at 100 mg/ml, filter-sterilized, stored in aliquots at 4 °C and added to the medium to a final concentration of 100 µg/ml.

Canavanine (L-canavanine sulfate salt, Sigma-Aldrich, Auckland, New Zealand) was dissolved in water at 100 mg/ml, filter-sterilized, stored in aliquots at 4 °C and added to the medium to a final concentration of 50 mg/l.

Thialysine (S-(2-aminoethyl)-L-cysteine hydrochloride, Sigma-Aldrich, Auckland, New Zealand) was dissolved in water at 100 mg/ml, filter-sterilized, stored in aliquots at 4 °C and added to the medium to a final concentration of 50mg/l.

Doxycycline (Sigma-Aldrich, Auckland, New Zealand) was dissolved in distilled, autoclaved water at final concentration of 10 mM, filter-sterilized, stored in aliquots at -20 °C in dark tubes, and added to the medium at appropriate concentration, as listed in **method 2.21.1**.

Carbon source stock solutions were prepared as following:

Glucose (Dextrose, Fisher Scientific, Auckland, New Zealand) was dissolved in water at concentration of 40% (w/v). The solution was autoclaved and stored at room temperature and added to the medium to a final concentration of 2% (w/v).

Galactose (Fisher Scientific, Auckland, New Zealand) was dissolved in water at concentration of 20% (w/v). The solution was filter-sterilized and stored at 4°C and added to the medium to a final concentration of 2% (w/v).

2.9.4. Growth media

The amino acids and monosodium glutamate (MSG, L-glutamic acid sodium salt hydrate) were purchase from Sigma-Aldrich (Auckland, New Zealand); Bacto Peptone, Bacto Triptone, Bacto Yeast Extract, and Bacto Yeast Nitrogen Base were purchase from DIFCO (Detroit, MI, USA).

2.9.4.1. Bacteria

All bacteria were cultured at 37 °C overnight in one of the following media supplemented with 100 µg/ml of ampicillin.

Luria-Bertani (LB)

LB medium was prepared according to the following recipe: 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, and 10 g NaCl were mixed into 800 ml of dd water and the pH adjusted to 7.5 with 1 M NaOH solution. The volume was finally adjusted to 1 l with dd water, the medium sterilized by autoclaving and stored at room temperature.

Yeast extract/Tryptone (YT)

YT medium was prepared according to the following recipe: 16 g Bacto Tryptone, 10 g Bacto Yeast Extract, and 5 g NaCl were mixed in 900 ml of dd

water. The pH was adjusted to 7.0 with NaOH 1 M solution and taken to a final volume of 1 l with dd water. The solution was then sterilized by autoclaving and stored at room temperature.

2.9.4.2. Yeast

All yeast strains were cultured according to standard yeast methods (Amberg, 2005) in one of the following media:

Yeast Peptone Dextrose (YPD)

YP medium was prepared according the following recipe: 8 g yeast extract, 16 g Bacto Peptone, and 0.096 g adenine were mixed into 760 ml of dd water and sterilized by autoclaving; 40 ml of 40% glucose solution was added and antibiotics at appropriate concentrations when required.

Synthetic Complete (SC)

“Amino acids” supplement powder mixture for Synthetic Complete (SC) media contains: 3 g adenine, 2 g uracil, 2 g inositol, 0.2 g *para*-aminobenzoic acid, 2 g alanine, 2 g arginine, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g isoleucine, 10 g leucine, 2 g lysine, 2 g methionine, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tyrosine, 2 g tryptophan, and 2 g valine.

Drop-out (DO) powder mixture is a combination of the above listed ingredients minus the appropriate supplement; 2 g of the DO powder mixture is used per litre of medium.

Synthetic Complete (SC) medium was prepared as follows: 1.7 g of Bacto Yeast Nitrogen Base (without amino acids or ammonium sulphate), 1 g of MSG, and 2 g of “amino acid supplement power mix” (DO – His/Arg/Lys) were mixed

into 760 ml of dd water and sterilized by autoclaving; 40 ml of 40% glucose solution was added and antibiotics at appropriate concentrations if required.

If galactose was used instead of glucose, the same amounts of ingredients were mixed into 720 ml of dd water and sterilized by autoclaving; 80 ml of galactose 20% solution was added and antibiotics at appropriate concentrations if required.

Synthetic Dropout (SD)

SD -URA (synthetic dropout missing uracil): as for SC medium but without uracil in the “amino acid mix”; glucose or galactose was added at final concentration of 2% (w/v).

SGal -URA: as for SD-URA with the following modifications

Sporulation medium

For efficient sporulation yeast diploids were brought into sporulation in the following medium: 8 g potassium acetate, 0.8 g Bacto Yeast Extract, glucose 0.4 g, and 0.08 g amino acid sporulation mix, were mixed into 800 ml of dd water and sterilized by autoclaving.

2.9.5. Growth Conditions

2.9.5.1. Liquid growth

Deletion strains from Boone and Open Biosystems libraries

All *xxxΔ::kanR* yeast strains sourced from one of the arrays were cultured in YPD medium supplemented with G418 at a final concentration of 200 µg /ml.

Query strains

tif1Δ::natR and *tif2Δ::natR* queries strains were cultured in YPD medium supplemented with clonNAT at a final concentration of 100 µg /ml.

Tet-promoter strains

tif1Δ::natR tetTIF2 and *tif2Δ::natR tetTIF1* strains were cultured in YPD medium supplemented with G418 and with or without doxycycline.

GAL promoter strains

tif1Δ::URA and *tif2Δ::URA* strains were cultured in SD -Ura ; *tif1Δ::URA GAL1TIF2* and *tif2Δ::URA GAL1TIF1* over-expression strains were cultured in SD -URA or SGal -URA supplemented with clonNAT at a final concentration of 100 µg /ml.

2.9.5.2. Solid growth media

YPD

As for liquid medium with addition of 16 g of agar. Approximately 15 ml poured onto Petri dishes or 35 ml poured onto PlusPlates (Singer Instruments, Somerset, UK).

SC

As for liquid medium with addition of 16 g of agar. Approximately 15 ml poured onto Petri dishes or 35 ml poured onto PlusPlates (Singer Instruments, Somerset, UK).

SD - xxx amino acid

As for liquid medium with addition of 16 g of agar. Approximately 15 ml poured onto Petri dishes or 35 ml poured onto PlusPlates (Singer Instruments, Somerset, UK).

Glucose Nutrient Agar (GNA) pre-sporulation medium

GNA was prepared as following: 3 g of Bacto Peptone, 3 g of Bacto Yeast, and 20 g of agar were added to 750 ml of dd water and mixed together; 250 ml of 40% glucose were added after autoclaving and approximately 15 ml of medium

poured onto Petri dishes. Storing of GNA medium is not advised due to the particularly high concentration of sugar that makes it prone to contamination.

Sporulation medium

As for liquid sporulation medium with addition of 16 gr of agar. Approximately 15 ml poured onto Petri dishes or 35 ml poured onto PlusPlates.

2.10. Instruments

2.10.1. Robotic pinning system

SGA experiments were performed with the use of RoToR HDA (Singer Instruments, Somerset, UK), a compact bench top robot for manipulation of high density arrays of yeast (or bacteria). It allows reagents sets such as a deletion mutant collection to be utilised for large-scale, synthetic genetic array, phenotypic and chemical-genetic analysis.

The Singer RoToR uses plastic replica plating RePads HDA (Singer Instruments, Somerset, UK) and supports liquid pinning to and from 96 and 384 well microtiter plates and agar pinning at densities of 96, 384, 768, and 1536.

RePads were cleaned and sterilized following a cycle of 1) soaking in detergent and water, for 2 h, 2) water rinsing, 3) bleach solution soaking for 45 min, 4) water rinsing, and 5) sterilization with 80% ethanol.

2.11. Bacterial transformation

DH5 α *E. coli* competent cells were purchased by Invitrogen and bacterial transformation performed according to the manufacturer's general guidelines with minor modifications. A 1.5 ml microcentrifuge tube containing competent cells was allowed to thaw on ice, and then the cells gently mixed with a pipette

tip. From this, 25 µl aliquots were taken out for each intended transformation and transferred into a 1.5 ml microcentrifuge tube. One to 5 µl (1-10 ng) of DNA of interest was added to the cells and gently mixed (making sure to not pipette up and down) followed by incubation on ice for 30 min and heat shock for 20 s in a 42 °C water bath without shaking. Tubes were then placed in ice for 2 min and 475 µl of 30 °C pre-warmed LB medium added to each tube. Final incubation of the tubes was conducted at 37 °C for 1 h in a shaking incubator at 225 rpm. Aliquots of 20, 100 and 200 µl (it is recommended to plate different volumes of the transformation mix to ensure that at least one plate will have well-spaced colonies) of transformation mix were plated onto selective plates (LB containing 100 µg/ml of ampicillin) pre-warmed to 37 °C to select for the transformants containing the DNA of interest, followed by incubation of the plates at 37°C overnight.

2.12. *E. coli* plasmid preparation

Isolation and purification of plasmid DNA from DH5α *E. coli* competent cells was performed at room temperature with Zyppy™ Plasmid Miniprep kit (Zymo Research, Orange, CA, USA). Following the manufacture instructions, 600 µl of bacterial culture grown in LB medium overnight was transferred to a 1.5 ml centrifuge tube together with 100 µl of 7X Lysis Buffer (blue) and mixed by inverting the tube 4-6 times. Within 2 min, 350 µl of cold Neutralization Buffer was added and mixed thoroughly (the sample turned yellow when the neutralization was complete and a yellowish precipitate formed). The sample was inverted an additional 2-3 times to ensure complete neutralization and centrifuged at 11,000-16,000 *xg* for 2-4 min. About 900 µl of supernatant was

then transferred into the provided Zymo-spin TM column, paying attention to avoid disturbing the cell debris pellet. The column was placed into a collection tube and centrifuged at 11,000-16,000 $\times g$ for 15 s, the flow-through discarded and the column placed back into the same collection tube. Two hundred μ l of Endo-Wash buffer was utilized to wash the column, followed by centrifugation at 11,000-16,000 $\times g$ for 15 s. A second washing was performed by adding 400 μ l of Zippy Wash Buffer to the column, followed by centrifugation at 11,000-16,000 $\times g$ for 30 s. The column was then transferred into a clean 1.5 ml centrifuge tube and 30 μ l of dd water, added directly to the column matrix and let stand for 1 min. Finally, the sample underwent centrifugation at 11,000-16,000 $\times g$ for 30 s to elute the plasmid DNA.

2.13. Transformation of yeast *Saccharomyces cerevisiae*

Transformation of *S. cerevisiae* strains was performed using one of the two following methods:

2.13.1. Transformation of intact yeast cells treated with lithium ions

The following protocol, adjusted from H. Ito (1983), was kindly provided by Dr. D. Maass (Chemical Genetics Laboratory, Victoria University of Wellington, New Zealand; personal communication).

Single yeast colonies were inoculated in 50 ml of YPD medium containing the appropriate antibiotic and incubated overnight at 30 °C in a shaking incubator. Once the cells reached an OD₆₀₀ in 0.8 and 1, they were spun for 5 min at 2500 rpm in a microcentrifuge. The supernatant was then discarded and the cells washed twice with 10 ml 0.1 M LiAc and resuspended in 500 μ l of 0.1 M

LiAc. Single stranded salmon sperm DNA (90 mg/ml in dd water) was boiled for ten minutes and consequently placed on ice. Ninety μ l of cells, 10 μ l of the prepared salmon sperm DNA and 20 μ L of *natR* PCR product were added to a microcentrifuge tube and mixed, then incubated at 30 °C for 15 min. Six hundred μ l Li-PEG solution (1.2 ml 90% PEG, 0.15 ml of dd water and 0.15 ml 1 M LiAc) was added and the cells were incubated at 30 °C for 30 min, at which time 68 μ l DMSO was added and the mixture was incubated at 42 °C for 15 min with the intent of causing heat shock. The cells were centrifuged at 1000 rpm for 2 min in a microcentrifuge, the supernatant removed and the pellet resuspended in 800 μ L of YPD medium and incubated at 30 °C for 4 h. Finally, the tube was centrifuged at 2000 rpm, the supernatant was removed and the pellet resuspended in 200 μ l of YPD medium. Different amounts of cell suspension (50, 100, 200 μ l) were plated onto YPD Petri dishes containing clonNAT (100 μ g/ml) to select for the resistant transformants.

2.13.2. High efficiency transformation

Transformation of *S. cerevisiae* was performed using the LiAc/single stranded carrier DNA/PEG method (Gietz & Schiestl, 2007) with minor modifications.

Prior to transformation, a single colony of each strain to be transformed was inoculated into 5 ml of YPD media and incubated overnight at 30 °C in a shaking incubator at 200 rpm. The next day the culture was diluted to an OD₆₀₀ of 0.05 in YPD media and returned to the incubator until the OD₆₀₀ had reached 0.8–1. A volume of culture corresponding to 1×10^8 cells was removed; the cells recovered by centrifugation at 3000 xg , washed three times with dd water and

resuspended in 360 µl of transformation mix (240 µl PEG 50% (w/v), 36 µl 1 M LiAc, 50 µl single stranded salmon sperm DNA (2 mg/ml), 34 µl dd water containing approximately 1 µg of plasmid DNA). This was incubated at 42 °C for 40 min, followed by micro-centrifugation at 13,000 *xg* for 1 min, after which the transformation mix was removed. The cells were washed with 1 ml of warm YPD and aliquots of two, 20, and 200 µl spread onto Petri dishes containing SD agar supplemented with clonNAT (100 µg/ml), or G418 (200 µg/ml) and clonNAT (100 µg/ml), depending on the strain, to select for successful transformants.

2.14. Genomic DNA purification and extraction

2.14.1. Purification

Purification of DNA was carried out using the MasterPure™ Yeast DNA Purification Kit (Epicenter Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. Yeast cells were pelleted from a saturated 1.5 ml culture by centrifugation in a microcentrifuge at 10,000 rpm for 2-5 min. Three hundred µl of Yeast Cell Lysis Solution was added to the aforementioned microcentrifuge tube and suspended by pipetting repeatedly. The suspended cells were incubated at 65 °C for 15 min. The sample was kept on ice for 5 min, followed by addition of 150 µl of MPC Protein Precipitation Reagent and vortexed for 10 s. The cellular debris was pelleted by centrifugation in a microcentrifuge for 10 min at ≥10,000 rpm. The supernatant was then removed by pipetting and discarding and the pellet containing the DNA washed with 0.5 ml of 70% isopropanol. The ethanol was carefully removed by pipetting and discarding and the DNA pellet briefly centrifuged to remove any remaining ethanol. Each DNA sample was dissolved in 35 µl of TE buffer (1 mM EDTA, 10

mM Tris-HCl pH 7.5) and maintained at 60 °C on a heat block for 10 min to make sure it reached dissolution and then stored at 4 °C.

2.14.2. RNA degradation

One µl of 5 µg/µl RNase A (from MasterPure™ Yeast DNA Purification Kit, Epicenter Biotechnologies, Madison, WI, USA) was added, followed by incubation at 37 °C for 30 min.

2.14.3. DNA extraction with phenol/chloroform/isoamylalcohol

The sample was made up to 500 µl with dd water; in the fume hood, 500 µl of phenol/chloroform/isoamylalcohol (25:24:1) was added to the sample, vortexed for 30 s, followed by microcentrifugation at 16000 *xg* for 10 min. The upper aqueous layer (containing DNA) was then removed and 1 volume (500 µl) of chloroform was added to it, then vortexed and microcentrifuged at 16000 *xg* for 10 min. Finally, the upper aqueous layer was removed (approx 400 µl) and conserved for ethanol precipitation.

2.14.4. Ethanol precipitation

Ethanol precipitation of DNA was performed by adding 1 ml of 96% ethanol and 40 µl of 3 M sodium acetate (pH 5.3), followed by inversion to mix (making sure to not pipette) and stored at -20 °C. After 25 min the tube was spun for 10 min at 16000 *xg*, the supernatant removed and washed with 70% ethanol. The resulting pellet was then re-dissolved in 35 µl of TE buffer and incubated a

65 °C for 10 min to allow complete dissolution. This sample of DNA was employed as a pure genomic template in PCR reactions and/or stored at 4 °C.

2.15. Polymerase chain reaction (PCR)

2.15.1. Enzymes and cycle conditions

All the oligonucleotides used in this study were purchased from Invitrogen, diluted in dd water to a concentration in the order of picomolar and stored at 4 °C.

All the PCRs performed were run with the use of one of the following polymerase enzymes:

HotStartTaq polymerase enzyme

The HotStartTaq DNA polymerase kit (Qiagen) was utilized in conventional PCRs as it reduces the generation of non-specific products and primer artefacts. In this method the polymerase enzyme is chemically modified and made afunctional; the enzyme is reactivated by preheating the reaction mix at 94–95 °C for 9–12 min. This can be problematic, as high temperature reactivation can result in depurination of the DNA template (Lindahl & Nyberg, 1972), reducing the quality of the products produced. In addition, the long reactivation time increases the length of the PCR reaction and lowers throughput.

For a 25 µl reaction, the PCR mix was set up as following: 2.5 µl of 10X buffer, 0.5 µl of dNTP (20 mM), 0.5 µl forward primer, 0.5 µl reverse primer, 1 µl of 1:50 dd water dilution of DNA template, HotStartTaq DNA polymerase 0.25 µl,

19.75 µl of dd water. In case of a PCR product with a high CG content, 10% DMSO supplemented the reaction.

The following cycling conditions were used, unless differently stated: 15 min initial denaturation at 94 °C (as required for initial activation of the HotStartTaq DNA polymerase), followed by 30 cycles of 1 min at 94 °C (denaturation), 1 min at 52 °C (annealing), 1 min/Kb 72°C (extension), and 5 min final extension at 72 °C.

Platinum High Fidelity polymerase enzymes

Whenever a PCR reaction proved troublesome, a high fidelity (or low error rate) PCR was undertaken using the Platinum® *Taq* DNA Polymerase High Fidelity.

For a 25 µl reaction, the PCR mix was set up as following: 2.5 µl of 10X buffer, 1 µl of 50 mM MgCl₂, 2.5 mM dNTP, forward primer 10 µM 0.5 µl, reverse primer 10µM 0.5 µl, 1 µl of DNA template, Platinum Taq 0.1 µl, dd water 17.5 µl.

The following conditions were utilized: initial denaturation 2 min at 94 °C, followed by 30-40 cycles of denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 68 °C for 1 min/Kb, followed by a final extension at 68 °C for 15 min.

2.15.2. Amplification of selective marker cassettes

PCR amplification of selective marker cassettes from plasmids was performed using the Quiagen HotStartTaq DNA polymerase kit according to *Methods in Yeast Genetics 2005* (Laboratory Course Manual, Edition Cold Spring Harbour). Cycling conditions are stated in **method 2.15.1**.

2.15.3. Confirmation PCR

The correct genomic integration of DNA of interest was confirmed by PCR diagnosis using two pairs of primers. One of the two following techniques was utilized:

2.15.3.1. Yeast colony PCR

A 2-day streaked single colony was picked and treated with 50 µl of 1mg/ml Zymolase (Zymo Research, Irvine, CA, USA) in water and incubated at 37 °C for 30 min. The product of the lysis was used as template for the confirmation PCR, following the HotStartTaq DNA polymerase PCR conditions modified as follows: 35 cycles of denaturation and final extension was conducted for 10 min.

2.15.3.2. PCR on genomic DNA (gDNA)

Purified gDNA obtained according to **method 2.14** was employed as template in a diagnosis PCR using the HotStartTaq DNA polymerase conditions modified as follows: 15 minutes at 95 °C, 9 cycles of 95 °C for 30 s, 54 °C for 30 s, 68 °C 2 min followed by 25 cycles of 95 °C 30 s, 54 °C 30 s, 68 °C 2 min plus 10 s each cycle. A final 10 minute extension at 68 °C was also performed to complete any partially extended primers.

2.16. DNA electrophoresis

All DNA electrophoresis was performed according to standard molecular biology methods (Ausubel, 1987). Electrophoresis was performed in 1% or 2% agarose gel according to the sizes of the expected product, and run in TBE buffer (89 mM Boric Acid, 2 mM EDTA disodium dihydrate, 89 mM Tris Base pH 8.3) to which 0.5 µg/ml ethidium bromide was added. All samples were dissolved and mixed in a 5:1 ratio with 5x sample loading buffer (30% glycerol (v/v), 0.25%

(w/v) bromophenol blue, 0.25% (w/v) cyanol xylene) prior to loading onto the gel. Electrophoresis was performed at a constant 100 V, and agarose gels were visualized on a transilluminator (UVIttec Limited, Cambridge, United Kingdom) under UV light at 365 nm.

2.17. Serial spot dilution assay

Yeast strains were inoculated into YPD or SD medium with the appropriate antibiotic or drug and grown overnight under shaking at 30 °C. The overnight culture was diluted to an OD₆₀₀ of approximately 0.05, returned to the shaking incubator and grown to an OD₆₀₀ of 1. The culture was transferred to a sterile 96-well plate and serially diluted ten-fold with the same medium used for culturing at least 6 times. A multi-channel pipette was used to apply 3 µl of each dilution of the culture onto YPD or SD agar plates containing the appropriate antibiotic or drug of interest; after absorption by the agar the plate was incubated at 30 °C (Parsons, 2004). Photographic records were obtained every 24 h for 5 d.

2.18. Query strain construction - Non-essential gene switching method

SGA requires the mating of deletion mutant array (DMA), available commercially, to a query strain that needs to be constructed. The non-essential singular nature of *TIF1* and *TIF2* genes made them suitable for undergoing SGA investigation with the final intent of generating *TIF1* and *TIF2* null mutants as query strains. Since SGA depends on successful mating, the DMA and the query

strains must harbour different selectable markers. The antibiotic cassette in the query strains was swapped from the *kanMX4* of DMA to the marker *natMX4* via a non-essential gene switching method following an established protocol (Boone & Tong, 2006) with minor modifications as follows:

2.18.1. Bacterial growth and plasmid purification

E. coli bacterium containing p4339 *natMX4* switcher was cultured into 3 ml of YT supplemented with ampicillin (1/1000) of at 37 °C overnight and purified according to **method 2.12**.

2.18.2. Restriction enzyme digestion

A 30 µl aliquot of p4339 plasmid was digested in a mix containing 4 µl of 10x buffer, 1 µl of bovine serum albumin (BSA) buffer 100x, 2 µl of EcoRI enzyme and 3 µl of dd water. Digestion was carried out overnight at 37 °C.

2.18.3. Transformation

MATa *tif1Δ::KanR* and *tif2Δ::KanR* strains, belonging to Boone's haploid deletion library, were transformed according to **method 2.13.1**, using EcoRI-cut p4339 as a template. EcoRI sites flank the *MX4* cassette, and these enable switching of *kanMX4* cassette (selection associated to original strain) with *natMX4* obtained from the p4339 plasmid. Transformation mix was plated onto YPD supplemented with clonNAT and grown at 30 °C for 2 d to select for growth of meiotic progeny carrying the gene deletion mutation (*xxxΔ::natR*). Colonies were then re-streaked onto dishes containing medium supplemented with either clonNAT or G418 to ensure authenticity of the resistance towards nourseothricin and loss of resistance to geneticin, respectively. A pair of such verified *tif1Δ::natR*

and *tif2Δ::natR* transformants: 1) underwent DNA Yeast genomic DNA purification (**method 2.14**) and was utilized as template in diagnosis PCR (**method 2.15.3.2**) and 2) was then employed in the construction of diploids.

2.18.4. Confirmation PCR

Correct integration of the NAT cassette in the *tif1Δ* and *tif2Δ* query strains was confirmed *via* PCR (**method 2.15.3**). Yeast colony PCR (**method 2.15.3.1**) was first followed but, due to unsatisfactory gel electrophoresis, PCR on genomic DNA (**method 2.15.3.2**) was utilized instead. The transformed genomic DNA (gDNA) obtained according to **method 2.14** was used as template in combination with two pairs of primers with homology for either *TIF1* (#26 and #27) or *TIF2* sequences (#28 and #29) which flank each integration site and an internal (#3 and #4) *natR* cassette primers (**appendix 5.5 table A-1**). The resulting PCR product was assessed following **method 2.16**.

2.18.5. Diploids constructions - Micromanipulation

Construction of diploids was achieved *via* dissection microscopy by individually crossing *tif1Δ::natR* and *tif2Δ::natR* *Mata* newly-generated strains to Y8205 *Mata*, a starting strain which confers the genetic background for SGA. Both *tif1Δ::kanR* and Y8205 inocula were placed parallel to each other on an YPD Petri dish and single haploid cells picked up from both sample strips and mated on the plate. Following an identical procedure, mating was separately repeated for *tif2Δ::natR* X Y8205 strains. Plates containing the products of the mating processes (*tif1Δ::natR* X Y8205 and *tif2Δ::natR* X Y8205) were incubated at 30 °C for 48 h.

2.18.6. Assay for pheromone production

An assay for pheromone production was conducted in order to assess the veracity of the diploids constructed via micromanipulation. The two tester strains of choice for this assay were SY2014 (*MAT α ste3 Δ 306::LEU2 sst2 Δ*) and SY2625, (*MAT α bar1 Δ*) (Sprague & Herskowitz, 1981; Chan & Otte, 1982). A small number of cells were picked using an autoclaved fire wooden splint from 12 diploid colonies grown on each mating plate generated via **method 2.18.5** and dissolved in 100 μ l of dd water into a 96 well plate. Liquid to agar transfer was done via Singer robot in 96 pin format; liquid diploid cultures were pinned onto PlusPlates (Singer Instruments, Somerset, UK) containing the following solid media:

1. YPD (control)
2. YPD supplemented with clonNat
3. YPD “blue” plate with layer of SY-2014
4. YPD “blue” plate with a layer of SY-2625

Plates 3 and 4 were obtained as follows:

i. “Blue” testing plates were prepared adding citrate to a final concentration of 0.1 M and methylene blue 0.03% (w/v) to autoclaved YPD agar medium, which was then pH adjusted to 4.5.

ii. 800 μ l aliquots of 1:5 and 1:10 dilutions of SY2014 and SY2625 overnight cultures were transferred onto the plates and a layer (“lawn”) of yeast was created with the help of plating beads.

Plating beads were then washed and kept in 70% ethanol at 4 °C.

Plates 1 and 2 were incubated at 30 °C for 36 h, whilst plates 3 and 4 were grown at room temperature for the same length of time, as incubation at the lower temperature has shown to give larger halos.

Strains were scored through visual inspection by two independent investigators and annotated as genuine diploids if satisfying all 4 the following conditions:

1. Growth in YPD
2. Growth in YPD plus clonNAT
3. No growth in YPD “blue” plate with layer of SY 2014
4. No growth in YPD “blue” plate with a layer of SY 2625

2.18.7. Sporulation and Mating type selection

Confirmed diploids were then induced to sporulate by transferring them onto solid medium with low level of carbon and nitrogen (**method 2.9.4.2**). After 5 days, a small amount of spores was suspended in 50 µl of dd water; this latter sample and a further 1:10 dilution were plated onto plates containing SD –(Leu, Lys, Arg) medium supplemented with canavanine and thialysine, which selects for *MAT α* a mating type. The *MAT α* meiotic progeny were then replicated onto plates contains clonNAT, which selects for growth of meiotic progeny that carries the gene deletion mutation (*xxx Δ ::natR*). Successful colonies were picked, grown overnight, stored at -80 °C in YPD medium containing glycerol 20% and utilized as “query” strains.

2.18.8. Synthetic Genetic Array (SGA) Analysis

The ability of budding yeast to exist in a haploid form makes it usable in genetic investigations, such as identification of synthetic lethal interactions. We performed a Synthetic Genetic Array (SGA) analysis, an approach that enables mass-mating construction of double mutants with the purpose of investigating the effects of a deletion by growth phenotype assessment (**Figure 2-4**).

i. Deletion Mutant Array (DMA)

The Yeast Deletion Set (YDS) utilized in this study is the Boone's YKO *MATa* haploid strain collection, which features 5,800 *S. cerevisiae* single deletion strains conveniently arrayed in 14 plates designed to undergo high-throughput screening. Each plate contains 56 genes represented as quadruplicate, framed by a border of controls and a number of auxotrophic mutants as key indicators of correct performance of the SGA process. The entire knock out array has a *MATa* mating type in a BY4741 background and a genotype of *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*. Each non essential gene target is represented by a *MATa* haploid strain that undergoes mating with *MATα* query strain *tif1Δ* or *tif2Δ* generating an extensive array of diploids that will be sporulated and then selected using a 'SGA reporter' that allows selection only for progeny bearing a *MATa* mating type. Double mutants are then selected and scored.

i-b. Construction and maintenance of Deletion Mutant Array (DMA)

The entire collection of haploid *MATa* yeast deletion strains or DMA was grown on plates containing solid YPD medium in 384 colony type formats and

subsequently arrayed onto 14 YPD plates in a final 1536 colonies reproducing a quadruplet format of 384 source strains, using the Singer RoToR robot (Somerset, UK). G418 antibiotic was always present in the YPD medium at a concentration of 200 µg/ml to ensure the selective pressure on the DMA strains. The DMA can be stored at 4 °C for up to two months and utilized several times.

ii. Query strains

tif1Δ::natR and *tif2Δ::natR* query strains were streaked out from glycerol frozen stock and after two days a single colony was picked and cultured in 5 ml liquid YPD overnight supplemented with clonNAT at 100 µg/ml. The saturated cultures were poured over YPD plates with the intent to form a lawn from which the Singer RoToR (Somerset, UK) would pin to array into 384 colony format. The query strain set was grown for two days at 30 °C, robotically re-arrayed into a 1536 colony format onto plates containing solid YPD plus clonNAT (100 µg/ml). The re-formatted yeast deletion query strains were grown for two additional days at 30 °C.

iii. Mating of haploid query strain with DMA

The *tif1Δ::natR* and *tif2Δ::natR* query strains were first pinned onto 14 fresh 1536 colony on YPD agar plates. Next, the DMA was pinned on top of each colony of the 1536 query strain colonies, using the mating program on the Singer RoToR (Somerset, UK). The cells were incubated at 30 °C overnight for 2 d to allow for diploid formation.

iv. *MATa/a* diploid selection

The progenies resulting from the mating were pinned onto YPD plus G418/clonNAT agar plates to allow for selection of diploid colonies carrying both the query and DMA gene deletions and incubated for 2 d at 30 °C.

v. Sporulation of double mutant diploid

Diploids were pinned onto sporulation medium which has low carbon and nitrogen source. Plates were incubated for 5 to 7 d at 22 °C to allow cells to undergo efficient sporulation.

vi. *MATa* haploid selection

MATa haploid spores were selected by pinning the resulting progeny onto SD – (His/Arg/Lys) plus canavanine (50 mg/l)/thialysine (50 mg/l). The progeny were incubated at 30 °C for 2 d. Plates were pinned for a second round on the same medium as above and incubated at 30 °C for 1 d to reduce the possibility of *MATa/a* diploid cells resulting in a false positive.

In order to carry out SGA, it was necessary to ensure that parental haploid and *MATa/a* diploids were not selected using specific reporters that allow the carrying strain to grow in the following selective steps.

vii. *MATa-kanR* meiotic progeny selection

MATa progeny carrying the *kanR* marker were selected for by pinning the plates onto SD-(His/Arg/Lys) plus canavanine (50 mg/l)/thialysine (50 mg/l), followed by incubation at 30°C for 2 d.

viii. *MATa-kanR-natR* meiotic progeny selection

Both DMA and query gene were selected for by pinning the progenies resulting from vii onto SD-(His/Arg/Lys) supplemented with canavanine/thialysine/G418 (200 µg /ml)/clonNAT (100 µg /ml), followed by incubation at 30 °C for 2 d. The final selected progenies correspond to a haploid *MATa* double mutant containing both the query gene deletion and one of the ~5000 non-essential gene deletions featured in the DMA.

Figure 2-4. Summary diagram of SGA methodology. Query strain and YDS, both carrying a different antibiotic marker, undergo mating followed by sporulation. Taking advantage of the promoter carried by the query strain, meiotic progenies selected through consecutive steps to identified double mutants, (Tong, 2001).

2.19. Scoring genetic interactions

Genetic interactions were inferred from reduced (or increased) colony growth in which double mutants grew less (or more) than the expected combination of parental phenotypes. Images of 1536-colony plates were analysed by sequential steps including gridding, segmenting and digitizing. Colony sizes were recorded using ColonyHT software (Collins, 2006) and then uploaded into a MySQL database, containing the standardised known colony position of each gene in the yeast DMA.

The fitness of each double deletion mutant strain was determined by SESA (SGA Experiment Set Analyser; developed by C. Jack, Chemical Genetics VUW), software which analyses the images of the final *MATa* double mutant SGA plates. The SGA results were compared against a control set of other SGA results, *ura3Δ::natR* (*MATα ura3Δ::natR can1Δ::STE2pr-HIS5 lyp1Δ*), acting as a control screen. The genetic background of *tif1Δ::natR* and *tif2Δ::natR* query strains included a *URA3* deletion, therefore the deletion of *TIF1* or *TIF2* represents the only variable in genetic background accountable between the query strain and control. Comparison of *tif1Δ* or *tif2Δ* SGA results to the *ura3Δ* SGA results allows for the filtering of non-specific genetic interactions (so-called “frequent flyers”). Additionally, any hits that have appeared in 3 or more previous independent SGA screens in the VUW laboratory are deemed to be frequent flyers and are discounted from the results. The frequent flyer hits are usually genes involved in mating or in the sporulation process and therefore not particularly informative to the assay. Finally, the linkage genes (the genes that are in close physical proximity to the query gene locus) are also discounted as the genes around the

query locus are expected to show no growth due to the decreased ability of genetic recombination to occur between these loci. This pattern of no growth of loci flanking the query gene was used as circumstantial confirmation of the query gene.

Fitness is regarded as a key element in the interpretation of an SGA experiment and factors potentially affecting growth should be taken into consideration. Yeast colonies surrounding empty spots or occupying lateral positions in the array plate show the tendency to grow bigger and faster than deletion strain colonies spotted in the middle of the plate, almost certainly due to an increased access to nutrients in the medium. To ensure that there are no dual deletion mutant strains that experience growth advantages from positioning at the plate edge, a two colony wide border of *his3Δ::kanR* was added into the original array.

A robust statistical algorithm was used to score interactions in high – throughput fashion, utilising colony sizes as element for assessing epistatic interactions using the SESA software analysis. This combines replicate experiment and control data sets that were performed under the same growth conditions. Plate-to-plate variation was controlled through calculation of relative colony growth by taking the ratio of a mutant colony area to the median control colony internal bordered area on each plate, where each plate contained 144 *his3Δ* mutants as controls (border strain). Experimental colony growth ratios were then compared to control plates where 1536-colony plates of the DMA as an SGA using *ura3Δ* as a query gene (Tong, 2001). Controls comprised 8 independent *ura3Δ* SGAs and experimental SGAs comprised 4 independent experiments. To improve statistical robustness against pinning errors and random growth

defects, robust statistics of the form $\text{median} \pm 3 \cdot \text{MAD}$ (median absolute deviation) were used. In SESA, confidence intervals were compared as per the (directional) standard method, forming each confidence interval by smoothed bootstrapping (two-tailed, $\alpha < 0.01$, $r = 1500$), where the bandwidth of the smoothing function (Epanechnikov kernel) (Silverman, 1990) was inversely proportional to the number of independent replicates (Schenker, 2001). Such bootstrapping data were sampled from all available data from either control or experimental sets to better resolve the true distribution function (Cameron Jack, personal communication).

Overall, SESA software identified synthetic sick and lethal mutants as well as enhancers, assigning a degree of reliability based on comparison to the set of controls. A text file list of significant results was outputted and interactions classified in three different types of 'hit' groups: possible (indicating the lowest index of reliability, sometimes due to unreliability of the control set), probable, and definitive (deletions whose genetic interaction with the query strain has been determined with the highest degree of probability). Results were ultimately manually audited to confirm software reliability.

2.19.1. Gene ontology

Gene Ontology (GO) is a controlled vocabulary used to describe the biology of a gene product in any organism. GO annotations allows comparison of functional, process or cellular localisation associations between gene products (Ashburner, 2000; Gene Ontology Consortium, 2004). Statistically significant changes in GO term distribution within a set of hits compared to that of the non-essential genome suggests enrichment in the number of genes involved in that

particular process. BiNGO (Maere, 2005), a plug-in ideated for Cytoscape (Cline, 2007) and the yeast GO Slim Mapper (Cherry, 1998) were used for functional analysis of SGA and chemical genetic hit genes. The fold change differences in distribution of GO terms for the genes representing the deletions of interest were compared to the one of the whole non-essential genome.

2.20. Validation of putative interactions

A number of criteria were applied to diminish the noise in the SGA screens. Gene deletion strains that are annotated as “wild-type SL” in Boone’s array were removed from the final list of interactions as these deletions appear to generate a growth defect phenotype independently from the deletions they are paired with. Dubious ORFs were also removed, unless they overlapped partially or totally with an ORF of annotated function. Deletions that appear in more than 3 SGA experiments based on unrelated query strains performed in our laboratory were excluded as well. Linkage groups surrounding the deletion of *TIF1* or *TIF2* genes are fully reported in **sections 2.25** and **2.26** and underwent the filtering process as well.

2.20.1. Tetrad analysis

Tetrad analysis (Rizet & Engelmann, 1949) allows the unambiguous identification of individual meiotic products and it was the technique of choice for the validation of *tif1Δ* and *tif2Δ* SGA generated interactions. The tetrad analysis technique was applied to confirm synthetic lethal interactions identified by SESA as “definitive” hits between the deletion strain of interest and other deletions featured into the array.

$\Delta xxx::kanR$ strains indicated by SESA as “definite” or “probable” hits were streaked out from the Boone library frozen glycerol stock onto Petri dishes containing YPD supplemented with G418 (200 $\mu\text{g/ml}$). Two-day single $\Delta xxx::kanR$ colony were picked and mated with the query strain Y8205 $tif1\Delta::natR$ and/or $tif2\Delta::natR$ onto YPD plates in a chevron shape and incubated at 30 °C for 2 days. The plates were then replicated onto YPD plates supplemented with clonNAT (100 $\mu\text{g/ml}$), G418 (200 $\mu\text{g/ml}$) or clonNAT/G418 plates to select for diploid cells. The diploids resulting from the mating were replicated onto plates containing rich pre-sporulation GNA medium.

2.20.1.1. Random Spore Analysis (RSA)

Deletion strains reported by SESA as “probable” initially underwent Random Spore Analysis (RSA), a technique invented by G. Magni (1963) which allows many more spores to be examined than in tetrad analysis; however, a relatively high transformation efficiency is recommended (> 50%) when carrying out RSA.

After 2 days of growth on solid GNA plates generated as **method 2.20.1** were patched onto SPO solid media and grown for 5 d. A small amount of spores (~ the size of a pinprick) was inoculated in 1 ml of sterile water, mixed well and incubated at 30 °C for 30 min, after being treated with 50 μl of Zymolase (0.5% in 1 M sorbitol), with the intent of weakening the asci and promoting the release of the spores.

The product of the lysis was plated as following: 20 μl on SD – His/Arg/Lys + canavanine/thialysine; 40 μl on SD–His/Arg/Lys + canavanine/thialysine/G418; 40 μl on SD–His/Arg/Lys + canavanine/thialysine/clonNAT; 80 μl on SD–His/Arg/Lys +

canavanine/thialysine/G418/clonNAT and incubated at 30 °C for 2 days. The number of colonies on the double-drug selection was scored against the single-drug selections.

2.20.1.2. Tetrad dissection

Optimal conditions for sporulation are no more than 18 h in GNA, a cell concentration around 3×10^7 cell/ml (but not less) and cultures' growth at 25 °C for 5-7 d.

After approximately 16 h of growth on GNA solid medium, $\frac{3}{4}$ of the yeast patch generated through **method 2.20.1** was scraped and inoculated into 2.5 ml of sporulation rich medium (as for **methods 2.9.4.2** liquid sporulation medium) at a concentration of at least 3×10^7 cells/ml. Spore germination was induced at 25 °C under rotation for 5 to 7 d, period after which samples were microscopically examined for tetrads and eventually returned to the incubator for few more days whenever the sporulation efficiency was not satisfactory.

After a period of incubation at 25 °C variable from 5 to 8 d, a 50 µl aliquot of the cultures containing spores was spun down in a microcentrifuge and the supernatant removed. The pelleted cells were then treated with 50 µl of Zymolase (0.5% in 1 M sorbitol) and incubated at 30 °C for 10 min, with the intent of weakening the asci for the upcoming tetrad dissection.

Approximately 15 mm from the edge of the Petri dish containing a thin YPD solid medium slab, a line was drawn and 15 µl of the sporulated cells were spread along the line on the plate in such a way that the inoculum would be in the field of a dissection microscope (Singer Instruments, Somerset, UK) and over the micro-manipulation needle.

With the use of a dissection microscope (Singer Instruments, Somerset, UK), the spores underwent dissection on the surface of a Petri dish using a dissecting needle which enables the separation of the 4 spores. Each ascus was micromanipulated to give a line of four isolated spores, separated by about 3-5 mm. The spores were incubated until full formation of the colonies at 30 °C for two days. After this period of time pictures were taken and plates were replicated onto YPD clonNAT and YPD G418 plates for two days followed by interpretation of the type of interaction.

2.20.2. Small scale SGA independent examination

Open Biosystems *MATa* deletion strains of interest (*xxxΔ::kanR*) were streaked out from array glycerol frozen stock onto Petri dishes containing YPD solid medium supplemented with G418. Single colonies were picked and manually arrayed in 96-well plate containing YPD liquid medium plus G418 and grown at 30 °C. Saturated strains were manually arrayed creating an external border of *his3Δ::kanR* and robotically pinned onto YPD medium supplemented with G418, followed by 2-d incubation at 30 °C. A standard SGA (**method 2.18.8**) was conducted utilizing *tif1Δ::natR* and *tif2Δ::natR* as query strains against the newly generated array. Scoring of sick or lethal mutants was carried out by visual inspections of two independent investigators.

2.21. Modulable promoter

Insertion of *tetO₂* “off” promoter cassette -associated to a G418 resistance cassette- was carried out in *tif1Δ::natR* and *tif2Δ::natR* strains. The “*tet*”

modulable promoter system was developed in the group of E. Herrero (Lleida, Spain) and continued in Manchester (Yen, 2003).

2.21.1. PCR amplification of “tet” promoter cassette

The plasmid pCM224 was purified according to **method 2.12** and utilized as template in PCR (**method 2.15.2**) to amplify PCR products with primers that have flanking regions overlapping with *TIF1* (#79;#80) or *TIF2* (primers #81;#82) as listed in **appendix 5.5 table A-2**. PCR products were verified based on size following **method 2.16**.

2.21.2. Transformation

Transformation of *tif1Δ::natR* and *tif2Δ::natR* strains was carried on utilizing the *tetO₂* promoter (and *KanR* associated cassette) PCR products following **method 2.18.3**. Selection was done on plates containing both clonNAT and G418 antibiotics to ensure for presence of both *NatR* cassette (query strain) and *KanR* cassette (*tetO₂* promoter); successful colonies were velvet transferred onto Petri dishes containing clonNAT, G418, and doxycycline at concentrations of 50/100/150/200 µg/ml to assess for repressibility.

2.21.3. Assessment of repression (tetrability)

Serial spot dilution assays were conducted according to **method 2.17**.

Yeast strains were inoculated into YPD or SD medium supplemented with 200 µg/ml G418 and 100 µg/ml clonNAT. Strains were serially diluted into a 96 well plate and spotted onto YPD or SD agar plates containing 200 µg/ml G418 and 100 µg/ml clonNAT, with or without doxycycline at concentration of 200 µM. Eventual repression of growth due to the inhibition of the off promoter in the presence of doxycycline was monitored over a period of 5 d.

2.22. Over expression promoter

Insertion of *GAL* over expression promoter –associated to *NatR* cassette– was conducted in *tif1Δ::URA* and *tif2Δ::URA* strains.

2.22.1. Generation of *tif1Δ::URA* and *tif2Δ::URA* strains

Plasmid pAG60 was purified according to **method 2.12** and utilized in PCR (**method 2.15.2**) to amplify PCR products with primers (#83; #84) that have flanking regions overlapping with the universal *MX4* cassette (**appendix 5.5 table A-4**).

2.22.1.1. Marker switching

Transformation of *tif1Δ::natR* and *tif2Δ::natR* strains was carried on with *URA3MX4* PCR products amplified from pAG60, following **method 2.13.1** to generate *tif1Δ::URA* and *tif2Δ::URA*. Final selection was done on plates containing SD-URA medium to ensure for acquired auxotrophy. Successful colonies were re-tested in clonNAT containing medium to confirm the loss of the *NatR* cassette present in the starting strain.

2.22.2. PCR amplification of *GAL* over expression cassette

The plasmids pYM-N23, pYM-N27, pYM-N31 (respectively carrying *GAL1*, *GALL*, and *GALS* cassettes) were purified according to **method 2.12** and utilized as template in PCR (**method 2.15.2**) to amplify PCR products with primers that have flanking regions overlapping with *TIF1* (#40;#41) and *TIF2* (#42;#43) (**appendix 5.5 table A-3**). PCR products were verified based on size following **method 2.16**.

2.22.3. Transformation

Colonies obtained through marker switching (**method 2.22.1.1**) were transformed according to **method 2.13.2** utilizing the PCR products generated in **method 2.22.2**. Final selection was done on plates containing SGal-URA medium supplemented with clonNAT.

2.22.4. Assessment of over expression

Yeast strains generated through **method 2.22.2** were tested for repression of growth in both liquid and solid glucose containing media: a small number of cells were i) streaked out on SD-URA medium supplemented with clonNAT and ii) inoculated in SD-URA medium supplemented with clonNAT. Growth was monitored for 5 d.

2.23. Results

To understand similarity and diversity between *TIF1* and *TIF2* paralogs and investigate the contribution of their respective promoter regions, a study on the genetic surrounding of the duplicate genes *TIF1* and *TIF2* has been undertaken. The genetic interactions of *TIF1* and *TIF2* genes were established using the SGA technique which has been described in **section 2.18.8**. This chapter reports the construction of the query strains *tif1Δ::natR* and *tif2Δ::natR* and the epistatic genetic interactions which characterize them.

2.24. Query strain construction via non-essential gene switching method

The strains *MATα tif1Δ::natR* and *tif2Δ::natR* were constructed according to **method 2.18** as summarized in **Figure 2-5**.

Figure 2-5. Construction of *tif1Δ::natR* query strain via switching method for non-essential genes. p4339 plasmid carries the *natMX4* cassette utilized for marker switched *MATa tif1::kanR* into *MATa tif1::natR*. This strain is mated with SGA starting strain Y8025, sporulated and finally tested in the appropriate selection media to select for the query strain *MATa tif1Δ::natR*. Adapted from Tong & Boone (2005).

2.24.1. PCR Diagnosis

The correct insertion of the *natMX4* cassette into the Y8205 SGA starting strain was confirmed using genomic *tif1Δ::natR* and *tif2Δ::natR* DNA in combination with flanking primers to generate diagnostic PCR products as shown in **Figure 2-6**. Hot Start was the polymerase of choice: this PCR method reduces the generation of non-specific products and primer artefacts. This chemically modified afunctional polymerase is reactivated by preheating the reaction mix at 94–95 °C for 9–12 min. Confirmation PCR products of the expected size were obtained for *tif1Δ::natR* and *tif2Δ::natR*, as shown in **Figure 2-6**.

Figure 2-6. *tif1Δ::natR* and *tif2Δ::natR* query strains diagnosis PCR. 1% agarose gel showing corrected integration of the *natMX4* cassette, replacing *TIF1* and *TIF2* ORFs. Lane 1: *tif1Δ::natR*, confirmation forward (control), lane 2: *tif1Δ::natR* confirmation reverse (control), lane 3: *tif1Δ::natR* confirmation forward (~600 bp), lane 4: *tif1Δ::natR* confirmation reverse (~750 bp),

lane 5: *tif2Δ::natR* confirmation forward (control), lane 6: *tif2Δ::natR* confirmation reverse (control), lane 7: *tif2Δ::natR* conf forward (~650 bp), lane 8: *tif2Δ::natR* confirmation reverse (~800 bp), lane 9: 1 Kb plus ladder.

2.24.2. Assay for pheromone production

To ensure the authenticity of the diploids constructed through micromanipulation, the halo assay for pheromone production (Sprague & Herskowitz, 1981) was used. This assay relies on the use of special pheromone tester strains (SY2014 and SY2625) that are hypersensitive to pheromones. A strain secreting pheromone will inhibit growth of a tester strain resulting in a clear area of no-growth surrounding the strain grown as patch (halo).

Diploids generated via micromanipulation following **method 2.18.5** are not expected to mate with either SY2014 or SY2625 and were considered genuine, if fulfilling all three the following requirements:

1. No halo in plate with layer of SY2014 (*MAT α*).
2. No halo in plate with a layer of SY2625 (*MAT α*).
3. Growth in plate supplemented with clonNAT.

Out of the 12 diploid liquid cultures examined for each *TIF* gene, 4 were identified as genuine for *tif1Δ*xY8205 and 4 for *tif2Δ*xY8205; 2 out of the 4 diploid clones were chosen for each of the *TIF* genes to be utilized in the next section.

2.24.3. Mating type selection and haploid status selection

Diploid strains were selected for the appropriate mating type (*MAT α*) and haploid status by plating spore onto synthetic medium:

1. Lacking –Leu, which allows for selective germination of *MAT α* meiotic progeny as only these cells express the *MFA1pr-LEU2* reporter;
2. Containing canavanine and thialysine, which allow for selective germination of meiotic progeny that carry the *can1 Δ* (*can1 Δ ::MFA1pr-HIS3*) and *lyp1 Δ* markers.

Two recessive markers conferring drug resistance, *can1 Δ* and *lyp1 Δ* , were introduced, into the query strain. The *CAN1* gene encodes an arginine permease that allows canavanine, a toxic analog for arginine, to enter and kill cells (Kitagawa & Tomiyama, 1929; Sychrova & Chevallier, 1993). Similarly, the *LYP1* gene encodes a lysine permease that allows thialysine, a toxic analog for lysine, to enter and kill cells (Kitagawa, 1929; Sychrova & Chevallier, 1993). Including *can1 Δ* and *lyp1 Δ* into the query strain ensures that *MAT α /a* diploid cells that could be present due to mitotic crossover events (Tong & Boone, 2006) are killed by canavanine and thialysine, because they carry a wild type copy of the *CAN1* and *LYP1* genes.

The *MAT α* meiotic progeny were then tested for resistance to clonNAT to ensure *tif1 Δ ::natR* and *tif2 Δ ::natR* strains clones had been generated.

2.25. *tif1 Δ* SGA

The *tif1 Δ* query strain underwent SGA analysis and was mass mated with other deletion strains of the non-essential set as described in **method 2.18.8** generating a genome-wide set of interactions. SESA software identified 65

probable genetic interactions (62 negative and 3 positive interactions) and 47 definitive (42 negative and 5 positive) as shown in **sections 5.1** and **5.2** of the appendix.

A typical SGA fingerprint pattern was observed in *tif1Δ* SGA data analysis and displayed in **Figure 2-7**. Chromosomally contiguous sets of genes form linkage groups (LG) (**Figure 2-8**), having a reduced tendency towards crossover during meiosis. When a *KanR* marked deletion is close to the *NatR* marked query gene (*YKR059W*, *TIF1* locus), genetic linkage means that progeny will carry one or other of the selection marker but not both. Under the dual nat/kan selection these strains are lost, the appearance of synthetic lethality (SL). Whilst not representing SL they do represent a useful indication that the SGA worked correctly being centred around the locus of the selection marker. The LG for *tif1Δ::natR* appeared in all 4 SGA screens, further confirmation that *TIF1* gene was the one replaced with the *natMX4* cassette (*tif1Δ::natR*) (**Table 2-1**).

Figure 2-7. An example of a *tif1Δ::natR* SGA plate (plate 8), where the linkage group pattern of *TIF1* gene (indicated by the green arrow) is present. A section of the *his3Δ::kanR* border is also shown in yellow.

A *his3Δ::kanR* border frames the array plate inside which deletion of a given gene is represented as quartets of replicates. The alternating pattern of growing/not growing quartets arises as a result of our library array pattern in which contiguous genes from one chromosome are interspersed in alternating fashion with those from another chromosome (**Figure 2-7**).

Figure 2-8. Linkage genes for *TIF1* gene on chromosome XI.

<i>ORF</i>	<i>Gene</i>
<i>YKR047W</i>	
<i>YKR051W</i>	
<i>YKR052C</i>	<i>MRS4</i>
<i>YKR053C</i>	<i>YSR3</i>
<i>YKR054C</i>	<i>DYN1</i>
<i>YKR055W</i>	<i>RHO4</i>
<i>YKR056W</i>	<i>TRM2</i>
<i>YKR057W</i>	<i>RPS21A</i>
<i>YKR058W</i>	<i>GLG1</i>
<i>YKR059W</i>	<i>TIF1</i>
<i>YKR060W</i>	<i>UTP30</i>
<i>YKR061W</i>	<i>KTR2</i>
<i>YKR064W</i>	
<i>YKR065C</i>	<i>FMP18</i>

<i>YKR066C</i>	<i>CCP1</i>
<i>YKR067W</i>	<i>GPT2</i>
<i>YKR078W</i>	
<i>YKR082W</i>	<i>NUP133</i>
<i>YKR096W</i>	
Table 2-1. List of linkage genes surrounding <i>TIF1</i> (<i>YKR059W</i>).	

As further confirmation that the SGA worked correctly, the LG of the *CAN1* and *LYP1* genes (**method 2.24.3**) were also identified in all 4 screens (**Figure 2-9** and **Figure 2-12**).

Figure 2-9. Linkage groups. Left: plate 11 of *tif1Δ::natR* SGA, where the linkage group pattern of *LYP1* gene is present. Right: plate 4 of *tif1Δ::natR* SGA, where the linkage group pattern of *CAN1* gene is present.

2.26. *tif2* Δ SGA

The *tif2* Δ SGA resulted in 52 probable genetic interactions (49 negative and 3 positive) and 58 definitive (51 positive and 7 positive) as reported in **tables 5.3** and **5.4** of the appendix.

As for *tif1* Δ , the typical SGA fingerprint pattern was observed in *tif2* Δ SGA. In the case of the *TIF2* gene (**Figure 2-11**), the LG is quite extended (**Table 2-2**) and only a section of the region is highlighted in **Figure 2-10**.

Figure 2-10. An example of *tif2::natR* SGA plate (plate 7), where the linkage group pattern of *TIF2* gene (indicated by the green arrow) is present. The pattern is quite extended; the region immediately surrounding the *TIF2* locus is bordered.

Figure 2-11. Linkage genes for *TIF2* gene on chromosome X.

ORF	Gene
<i>YJL119C</i>	
<i>YJL126W</i>	<i>NIT2</i>
<i>YJL128C</i>	<i>PBS2</i>
<i>YJL130C</i>	<i>URA2</i>
<i>YJL132W</i>	
<i>YJL133W</i>	<i>MRS3</i>
<i>YJL134W</i>	<i>LCB3</i>
<i>YJL135W</i>	
<i>YJL136C</i>	<i>RPS21B</i>
<i>YJL137C</i>	<i>GLG2</i>
<i>YJL138C</i>	<i>TIF2</i>
<i>YJL139C</i>	<i>YUR1</i>
<i>YJL141C</i>	<i>YAK1</i>
<i>YJL142C</i>	
<i>YJL144W</i>	
<i>YJL145W</i>	<i>SFH5</i>
<i>YJL146W</i>	<i>IDS2</i>
<i>YJL147C</i>	
<i>YJL157C</i>	<i>FAR1</i>

<i>YJL159W</i>	<i>HSP150</i>
<i>YJL160C</i>	
<i>YJL162C</i>	<i>JJJ2</i>
<i>YJL164C</i>	<i>TPK1</i>
<i>YJL170C</i>	<i>ASG7</i>
<i>YJL175W</i>	
<i>YJL204C</i>	<i>RCY1</i>
Table 2-2. List of linkage genes surrounding <i>TIF2 (YJL138C)</i>	

As for *tif1Δ::nat* SGA, the LG of the *CAN1* and *LYP1* genes (**method 2.24.3**) were also identified in all 4 *tif2Δ::nat* SGA screens (**Figure 2-12**).

Figure 2-12. *LYP1* and *CAN1* linkage groups. Left: plate 11 of *tif2Δ::natR* SGA, where the linkage group pattern of *LYP1* gene is present; Right: plate 4 of *tif2Δ::natR* SGA, where the linkage group pattern of *CAN1* gene is present.

2.27. Genetic interactions

The filtering process described in section **method 2.20** narrowed down the lists of genes that genetically interact with *TIF1* and/or *TIF2* to a number of fifteen negative genetic interactions for *TIF1* gene, 14 (13 and 1 positive) for

TIF2 gene, and 15 (14 and 1 positive) interactions common to both paralogs. Complete lists of SESA-generated definitive and probable genetic interactions for *TIF1* and *TIF2* genes are reported in **sections 5.1, 5.2, 5.3, and 5.4** of the appendix; here below are annotated only the deletions of genes from the “probable” and “definitive” SESA-generated hits that resulted from the filtering process described in **method 2.19**.

When the query strain (*tif1Δ::natR* or *tif2Δ::natR*, in this study) is crossed to an identical one (*tif1Δ::kanR* or *tif2Δ::kanR*) in the array, the formation of double mutant cannot occur as the haploid cells carry a single copy of each allele; therefore, *tif1Δ* and *tif2Δ* appear to be synthetic lethal with themselves as well as being synthetic lethal with each other and for this reason they do not appear in the results.

ORF	Gene	Biological process
<i>YOR355W</i>	<i>GDS1</i>	aerobic respiration
<i>YJR075W</i>	<i>HOC1</i>	cell wall mannoprotein biosynthetic process
<i>YGL168W</i>	<i>HUR1</i>	DNA replication
<i>YGL253W</i>	<i>HXK2</i>	glycolysis
<i>YGL016W</i>	<i>KAP122</i>	protein import into nucleus
<i>YOR221C</i>	<i>MCT1</i>	aerobic respiration, fatty acid metabolism
<i>YLL006W</i>	<i>MMM1</i>	mitochondrial genome maintenance, protein import into mitochondrial outer membrane, phospholipid transport
<i>YMR080C</i>	<i>NAM7</i>	ATP-dependent RNA helicase activity, RNA-dependent ATPase activity
<i>YER162C</i>	<i>RAD4</i>	nucleotide-excision repair, proteasomal ubiquitin-dependent protein catabolic process
<i>YLR448W</i>	<i>RPL6B</i>	ribosomal large subunit assembly
<i>YGR118W</i>	<i>RPS23A</i>	regulation of translational fidelity
<i>YLL039C</i>	<i>UBI4</i>	protein ubiquitination

ORF	Gene	Biological process
<i>YNR006W</i>	<i>VPS27</i>	protein targeting to vacuole, late endosome to vacuole transport
<i>YDR080W</i>	<i>VPS41</i>	vacuolar protein processing
<i>YFL013W-A</i>	<i>YFL013W-A</i>	dubious ORF which overlaps with <i>IES1/YFL013C</i>
Table 2-3. Genes genetically interacting with <i>TIF1</i> .		

ORF	Gene	Biological process
<i>YGR097W</i>	<i>ASK10</i>	cellular response to oxidative stress
<i>YER164W</i>	<i>CHD1</i>	regulation of transcription by chromatin organization
<i>YOR030W</i>	<i>DFG16</i>	protein processing
<i>YNL255C</i>	<i>GIS2</i>	positive regulation of translation
<i>YOR358W</i>	<i>HAP5</i>	carbon catabolite activation of transcription from RNA polymerase II promoter
<i>YHL027W</i>	<i>RIM101</i>	fungal-type cell wall biogenesis, response to pH
<i>YBL027W</i>	<i>RPL19B</i>	cytoplasmic translation
<i>YGL244W</i>	<i>RTF1</i>	histone H2B ubiquitination, regulation of transcription for RNA polymerase II promoter
<i>YPL047W</i>	<i>SGF11</i>	histone deubiquitination, regulation of transcription for RNA polymerase II promoter
<i>YNL257C</i>	<i>SIP3</i>	positive regulation of transcription from RNA polymerase II promoter
<i>YEL065W</i>	<i>SIT1</i>	cellular iron ion homeostasis
<i>YMR223W</i>	<i>UBP8</i>	histone deubiquitination
<i>YEL057C</i>	<i>YEL057C</i>	unknown
<i>YGL024W</i>	<i>YGL024W</i>	dubious ORF which overlaps with <i>PGD1/YGL025C</i>
<i>YGR122W</i>	<i>YGR122W</i>	negative regulation of transcription from RNA polymerase II promoter
<i>YPR044c</i>	<i>YPR044c</i>	unknown; dubious ORF which overlaps with <i>RPL43A/YPR043W</i>
Table 2-4. Genes genetically interacting with <i>TIF2</i> .		

ORF	Gene	Biological process
<i>YDL074C</i>	<i>BRE1</i>	histone monoubiquitination
<i>YLR062C</i>	<i>BUD28</i>	dubious ORF which overlaps with <i>RPL22A</i> , cellular bud site selection
<i>YER061C</i>	<i>CEM1</i>	fatty acid biosynthetic process
<i>YER092W</i>	<i>IES5</i>	unknown
<i>YGL219C</i>	<i>MDM34</i>	mitochondrion organization, phospholipid transport
<i>YDL002C</i>	<i>NHP10</i>	double-strand break repair
<i>YMR154C</i>	<i>RIM13</i>	protein processing
<i>YOR275C</i>	<i>RIM20</i>	invasive growth in response to glucose limitation, proteolysis, response to pH
<i>YGL045W</i>	<i>RIM8</i>	invasive growth in response to glucose limitation, ubiquitin-dependent endocytosis
<i>YGL147C</i>	<i>RPL9A</i>	cytoplasmic translation
<i>YCL010C</i>	<i>SGF29</i>	positive regulation of transcription from RNA polymerase II promoter
<i>YOL052C</i>	<i>SPE2</i>	spermine/spermidine biosynthetic process
<i>YKL081W</i>	<i>TEF4</i>	translational elongation
<i>YGL212W</i>	<i>VAM7</i>	vesicle fusion, vacuole fusion, non-autophagic
<i>YGL046W</i>	<i>YGL046W</i>	merged ORF, does not encode a discrete protein; it was merged with an adjacent ORF into a single reading frame, designated <i>YGL045W</i>
Table 2-5. Genes genetically interacting with both <i>TIF1</i> and <i>TIF2</i> .		

2.28. Gene Ontology analysis

Analysis of GO terms, describing the molecular function, biological process, and cellular component of gene products, was applied to the three following set of data: *tif1Δ* SGA hits, SGA *tif2Δ* hits, and hits common to *tif1Δ* and *tif2Δ* SGA.

Significant interactions were reported for *tif1Δ* in only one of the GO categories; the process GO term associated to cell organization and biogenesis

returned a p-value of 0.042 for the following group of genes: *YJR075W*, *YGL168W*, *YGL253W*, *YGL016W*, *YLL006W*, *YMR080C*, *YER162C*, *YLR448W*, *YGR118W*, *YNR006W*, and *YDR080W*. Information on further processes mediated by the protein products encoded by genes showing genetic interactions with *TIF1* ORF will be revised in the next section with the use of a different analysis.

A manual analysis revealed a number of mitochondria-related hits (*YOR358W*, *YGL253W*, *YOR355W*, *YOR221C*, and *YLL006W*) among the groups of genes interacting with *TIF1*. Further research showed however, that both *mct1Δ* (*YOR221C*) and *mmm1Δ* (*YLL006W*) are annotated as “petite” and thus have the tendency to harbour slow growing and/or small colonies, and so their appearance in the SESA interpretation of growth phenotype is likely to relate to this, rather than any genuine genetic interaction.

Genes populating the genetic surrounding of *TIF2* encode proteins that function in transcription regulator activity (*YGR097W*, *YER164W*, *YOR358W*, *YHL027W*, *YGL244W*, and *YNL257C* (p-value 0.002)), RNA Pol II transcription elongation factor activity (*YER164W* and *YGL244W* (p-value 0.004)), and transcription regulation regulator activity (*YER164W* and *YGL244W* (p-value 0.016)). Genes populating the genetic surrounding of *TIF2* encode proteins that function in processes such as regulation of transcription from RNA Pol II promoter, transcription, DNA-dependent, and RNA biosynthetic process as reported in **Table 2-6**. As is common for such analyses, many of the processes reported as significant are actually nested terms, with both global and more detailed GO terms reported. For example, both transcription, DNA-dependent and the more global term RNA biosynthetic process are reported for the same set of genes.

Biological process	p-value	ORF
transcription	5.2E-04	<i>YGR097W, YER164W, YOR358W, YHL027W, YGL244W, YPL047W, YNL257C, YGR122W</i>
transcription from RNA polymerase II promoter	0.002	<i>YER164W, YHL027W, YGL244W, YPL047W, YNL257C, YGR122W</i>
RNA biosynthetic process	0.032	<i>YER164W, YHL027W, YGL244W, YPL047W, YNL257C, YGR122W</i>
transcription, DNA-dependent	0.032	<i>YER164W, YHL027W, YGL244W, YPL047W, YNL257C, YGR122W</i>
Table 2-6. GO term enriched for biological processes in genes interacting with <i>TIF2</i> .		

Protein products of genes which hold a SL with *TIF2* display preferential localization in the SAGA complex and nucleosome, as reported in **Error! eference source not found..**

Component	p-value	ORF
SAGA complex	0.6E-04	<i>YER164W, YPL047W, YMR223W</i>
nucleoplasm part	1.2E-04	<i>YGR097W, YER164W, YOR358W, YGL244W, YPL047W, YMR223W</i>
nucleoplasm	1.7E-04	<i>YGR097W, YER164W, YOR358W, YGL244W, YPL047W, YMR223W</i>
nuclear lumen	0.001	<i>YGR097W, YER164W, YOR358W, YGL244W, YPL047W, YMR223W</i>
histone acetyltransferase complex	0.001	<i>YER164W, YPL047W, YMR223W</i>
transcription factor complex	0.002	<i>YER164W, YOR358W, YPL047W, YMR223W</i>
membrane-enclosed lumen	0.013	<i>YGR097W, YER164W, YOR358W, YGL244W, YPL047W, YMR223W</i>
organelle lumen	0.013	<i>YGR097W, YER164W, YOR358W, YGL244W, YPL047W, YMR223W</i>
DNA-directed RNA polymerase II, holoenzyme	0.02	<i>YGR097W, YGL244W</i>
transcription elongation factor complex	0.03	<i>YER164W, YGL244W</i>
Table 2-7. GO term enriched for components in genes interacting with <i>TIF2</i> .		

For what concerns hits common to both *tif1Δ* and *tif2Δ* SGA, only the GO process term associated with protein processing returned a statistically significant enrichment with an associated p-value of 0.006 for the following genes: *YMR154C*, *YOR275C*, and *YGL045W*.

2.29. The Biological Networks Gene Ontology

The Biological Networks Gene Ontology (BiNGO) tool is an open-source Java application used to determine which GO terms are significantly over-represented in a set of genes (Maere, 2005), and it was used for further analysis, graphic representation and determination of associated statistics on the full GO terms data set generated. Over-representation of these terms is reported with associated statistical significance generated according to the hypergeometric test; the most significant GO clusters for genes interacting with one of the paralogs only will be discussed in this section.

As anticipated by the GO component analysis, a substantial fraction of genes whose deletion leads to SL with *tif2Δ* are confirmed to be involved in nuclear activities: this takes place via participation in the (Spt-Ada-Gcn5-Acetyltransferase) SAGA complex (*SGF11*, *UBP8*, *CHD1* and, *SGF29*) and histone acetyltransferase (SAGA-like) SLIK complex, as *UBP8* and *CHD1* genes play an ambivalent role in both complexes (**Figure 2-13**).

Figure 2-13. BiNGO-generated *tif2Δ* GO components. BiNGO-generated *tif2Δ* GO components showing preferential localization of genes interacting with *TIF2* for the SAGA and SLIK complexes, histone acetyltransferase and nucleoplasm (hypergeometric test).

When analysed through BiNGO, a main cluster among *TIF1* interacting genes (*RIM8*, *YGL046W*, *RIM20*, *RIM13*, and *VPS41*) corresponded to an overrepresentation for protein processing and maturation. A minor contribution also came from *RPS23A*, *RPL6B*, and *NAM7* in regulation of translation and *HXX2*, *RPS23A* and, *NAM7* in RNA metabolic process, as shown in **Figure 2-14**.

Figure 2-14: BiNGO-generated *tif1Δ* GO processes. BiNGO-generated *tif1Δ* GO processes showing processes over-represented for genes interacting with *TIF1*: protein maturation and processing and post-transcriptional regulation of gene expression, regulation of translation and cellular protein metabolic processes (hypergeometric test). Colours shown reflect p-values and follow the colour coding shown in Figure 2.13.

Differently, *tif2Δ* hits displayed a number of gene subsets significantly over-represented for transcription (*SGF11*, *BRE1*, *UBP8*, *ASK10*, *RTF1*, *CHD1*, *SGF29*, *HAP5*, *RIM101*, and, *NHP10*) and gene expression, (*RIM8*, *SIT1*, *YGL046W*, *BRE1*, *ASK10*, *RIM101*, *DFG16*, *NHP10*, *RPL9A*, *SGF11*, *TEF4*, *RIM20*, *UBP8*, *RTF1*, *TIF1*, *RIM13*, *CHD1*, *SGF29*, *HAP5* and, *RPL19B*), regulation of both the above (in particular in DNA- and RNA Pol II polymerase-dependent), along with processing and maturation of proteins (*RIM8*, *YGL046W*, *RIM20*, *RIM13*, *RIM101*, and *DFG16*) (**Figure 2-15**). A robust group of genes annotated as functioning in histone modifications (*SGF11*, *BRE1*, *UBP8*, *RTF1* and, *SGF29*) -in particular ubiquitination-deubiquitination- and chromatin modification/organization (*SGF11*, *IES5*, *BRE1*, *UBP8*, *RTF1*, *CHD1*, *SGF29* and, *NHP10*) and remodelling (*SIP3*, *YGR122W*, *SGF11*, *BRE1*, *CHD1*, *RIM101*, and *NHP10*) was also annotated (**Figure 2-16**).

Figure 2-15. BiNGO-generated *tif2Δ* processes. BiNGO-generated *tif2Δ* processes showing processes over-represented for genes interacting with *TIF2*: gene expression and transcription

and regulation of the above, protein processing and maturation, and regulation of transcription from RNA Pol II promoter-(hypergeometric test).

Figure 2-16. BiNGO-generated *tif2Δ* processes. BiNGO-generated *tif2Δ* processes showing processes over-represented for genes interacting with *TIF2*: chromatin organization, modification, and remodelling, histone mono/ubiquitination and deubiquitination, protein monoubiquitination and deubiquitination, and histone modification (hypergeometric test).

2.30. Scoring genetic interactions

This study confirmed 2 genetic interactions annotated in the literature for *TIF1* (*GDS1* and *NAM7*) and 2 for *TIF2* (*SGF11* and *UBP8*) respectively; it also

endorsed a previously reported physical interaction for *TIF2* with *GIS2*, as shown in **Table 2-8**.

Gene name	This study		Other studies		References
	<i>tif1Δ</i>	<i>tif2Δ</i>	<i>tif1Δ</i>	<i>tif2Δ</i>	
<i>ASK10</i>		√			
<i>BRE1</i>	√	√			
<i>BUD28</i>	√	√			
<i>CEM1</i>	√	√			
<i>CHD1</i>		√			
<i>DFG16</i>		√			
<i>GDS1</i>	√		√	√	Costanzo, 2010 (genetic interaction)
<i>GIS2</i>		√	√	√	Sammons, 2011 (physical interaction)
<i>HAP5</i>		√			
<i>HOC1</i>	√				
<i>HUR1</i>	√				
<i>HXK2</i>	√				
<i>IES5</i>	√	√			
<i>KAP122</i>	√				
<i>MCT1</i>	√				
<i>MDM34</i>	√	√			
<i>MMM1</i>	√				

Gene name	This study		Other studies		References
<i>NAM7</i>	√		√	√	Wilmes, 2008 (genetic interaction)
<i>NHP10</i>	√	√			
<i>RAD4</i>	√				
<i>RIM101</i>		√			
<i>RIM13</i>	√	√			
<i>RIM20</i>	√	√			
<i>RIM8</i>	√	√			
<i>RPL19B</i>		√			
<i>RPL6B</i>	√				
<i>RPL9A</i>	√	√			
<i>RPS23A</i>	√				
<i>RTF1</i>		√			
<i>SGF11</i>		√		√	Costanzo, 2010 (genetic interaction)
<i>SGF29</i>	√	√			
<i>SIP3</i>		√			
<i>SIT1</i>		√			
<i>SPE2</i>	√	√			
<i>TEF4</i>	√	√			
<i>UBI4</i>	√			√	Peng, 2003 (physical interaction)

Gene name	This study		Other studies		References
<i>UBP8</i>		✓	✓	✓	Lee, 2011 (physical interaction); Costanzo, 2010 (genetic interaction), <i>tif1Δ</i> ; Costanzo, 2010 (genetic interaction); <i>tif2Δ</i>
<i>VAM7</i>	✓	✓			
<i>VPS27</i>	✓				
<i>VPS41</i>	✓				
<i>YEL057C</i>		✓			
<i>YFL013W-A</i>	✓				
<i>YGL024W</i>		✓			
<i>YGL046W</i>	✓	✓			
<i>YGR122W</i>		✓			
<i>YPR044C</i>		✓			
Table 2-8. Genetic interactions revealed by this study reported against the ones annotated in the existent literature.					

The results obtained from this study suggest a very high level of noise exists among genome-wide screens and a scrupulous follow-up analysis is advised to confirm extended data sets generated from these experiments. The variability observed among laboratories also represents a further element of variability that is hard to manage. Rigorous confirmation of SL hits has been undertaken in this study as described below.

2.31. Validation of Putative Interactions

2.31.1. Tetrad analysis

2.31.1.1. Random Spore Analysis (RSA)

Random Spore Analysis (RSA) (Dixon, 2008) was performed on yeast strains identified as synthetic lethal or sick or suppressors by SESA; however, independent visual examination by two different investigators lead to inconclusive assessment of sensitivity in the tested strains.

2.31.1.2. Tetrad dissection

As the large majority of genetic interactions could not be recapitulated via RSA, double mutants of interest were tested through tetrad dissection, as a more reliable and precise technique to assess genuine genetic relationships. The patterns of growth of the tetrads allow determination of the segregation type: examples of linkage types – parental ditype (PD), non-parental ditype (NPD) and tetratype (T) – are illustrated in **Figure 2-17**.

Figure 2-17. A schematic representation of a tetrad dissection. Diploids cells are induced in sporulation and the product of their meiotic progeny dissected with a micromanipulator microscope. Single spores are replicate plated on selective media and spore viability is then assessed. Different growth patterns reflecting PD, NPD, and T linkage types are observed for the dissected tetrads; an example tetrad type is shown framed by the green box.

Two interactions were confirmed through this analysis for *tif1Δ*, 7 for *tif2Δ* and 7 for both paralogs. A number of strains (8) could not be confirmed due to low sporulation process or to spores failing to grow even in the non selective medium.

Hits verified RSA	Hits verified tetrad dissection	Hits not verified	Hits not tested
<i>NHP10, VPS27</i>	<i>VPS27, ASK10, RIM101, RTF1, SGF11, UBP8, YGR122W, CEM1, IES5, NHP10, SPE2, SGF29, VAM7, RIM20, CHD1, HXX2</i>	<i>KAP122, SIP3, SIT1, BRE1, GIS2, UBI4, VPS41, DFG16</i>	<i>GDS1, HOC1, HUR1, MCT1, MMM1, NAM7, RAD4, RPL6B, RPS23A, YFL013W-A, HAP5, RPL19B, YEL057C, YGL024W, BUD28, TEF4, YGL046W, RPL9A, MDM34, RIM13, RIM8, YPR044C</i>

Table 2-9. Filtered hits from the primary screen were further confirmed via RSA and tetrad dissection. A group of hits could not be verified due to difficulties with sporulation efficiency or slow/no growth of the dissected spores and a further group was not tested, including the petite strains shown in bold.

2.31.2. Open Biosystems library small scale SGA

An independent evaluation of the SGA hits generated through Boone's array was carried on utilizing Open Biosystems deletion set, which features the same deletion strains arrayed in Boone's collection. This study investigated the phenotypic interactions between the query strains of interest and any deletion identified by SESA in the previously SGA screens.

Independent visual inspection carried out by two investigators identified 12 deletions as SL for *tif1Δ* and 14 for *tif2Δ*, and 17 SS for *tif1Δ* and 14 for *tif2Δ* respectively among the 188 tested separately against both *tif1Δ::natR* and *tif2Δ::natR*. While a large fraction of them had been previously identified by using Boone's array, it has to be noticed that a few deletions identified in this screen were not available in the original library.

<i>tif1Δ</i> SGA			
SL	Boone's	SS	Boone's
ARP8	√	<i>BUD31</i>	√
<i>CSE2</i>	√	<i>CAF1</i>	n/a
INO4		<i>CPA2</i>	√
PRS3	n/a	<i>GRE3</i>	
RIM13	√	<i>HAP5</i>	
RPL37B		<i>HIS2</i>	√
SRB8	√	<i>HOC1</i>	√
STE20	√	<i>LYS5</i>	√
TSR2		<i>RAD4</i>	√
UBP3		<i>RCY1</i>	√
UTH1	√	<i>RIM21</i>	√
YJL175W		<i>SIT1</i>	
		<i>SNF5</i>	√
		<i>TIF1</i>	√
		<i>TIF2</i>	√
		<i>TIF4631</i>	n/a
		<i>UTP30</i>	√

Table 2-10. Genetic interactions for *TIF1* utilizing the Open Biosystem library. SL (bold) and SS (green) interactions shared by the two paralogs. The interactions common to the two genes although with different degrees of impairment on growth are also indicated (red), along with the deletion strains not available (n/a) in the primary screen.

<i>tif2Δ</i> SGA			
<i>SL</i>	<i>Boone's</i>	<i>SS</i>	<i>Boone's</i>
ARP8	√	<i>BUD31</i>	√
<i>ASE1</i>		<i>CAF1</i>	n/a
<i>DHH1</i>		<i>CPA2</i>	√
<i>GRE3</i>		<i>CSE2</i>	
<i>HOC1</i>		<i>HAP5</i>	√
<i>INO4</i>		<i>HIS2</i>	√
<i>PRS3</i>	n/a	<i>LYS5</i>	√
RIM13	√	<i>RAD4</i>	
RPL37B		<i>RCY</i>	√
SRP8	√	<i>SIT1</i>	√
STE20	√	<i>TIF1</i>	√
TSR2		<i>TIF2</i>	√
UTH1		<i>TIF4631</i>	n/a
YJL175W	√	<i>YKR070W</i>	√

Table 2-11. Genetic interactions for *TIF2* utilizing the Open Biosystem library. SL (bold) and SS (green) interactions shared by the two paralogs. The interactions common to the two genes although with different degrees of impairment on growth are also indicated (red), along with the deletion strains not available (n/a) in the primary screen.

A general trend among the genetic interactions within the Open Biosystem confirmation assay was observed, with combinations of double mutants harbouring synthetic sick progeny rather than aggravated SL. Strikingly, even the combination of *tif1Δ tif2Δ* dual deletion mutant, which by nature should be a non-viable double mutant, appeared only partially impaired in growth, and so were the linkage groups flanking the deletions of interest and previously reported interactions. A certain degree of confidence in this small scale SGA is provided by “control” interactions included in the array that, as expected, harboured defecting growth double mutants. The growth defect arising from the combination of *tif2Δ* with *tif4631Δ* (a homolog of human eIF4G) or m-RNA decapping stimulator *dhh1Δ*, is shown in **Figure 2-18**.

Figure 2-18. Small scale SGA plate. SGA plate showing SL *dhh1Δ tif2Δ* (framed in blue) and SS *tif4631Δ tif2Δ* (framed in orange).

2.32. Tetracycline modifiable promoter

Amplification of the *tetO₂*-“off” promoter cassette from plasmid pCM24 was conducted in a temperature gradient PCR with primers designed to have flanking regions to the *TIF1* or *TIF2* genome sequence (#79;#80, #81;#82, listed in **appendix 5.5 table A-2**). A PCR fragment of expected size was obtained as shown in **Figure 2-19**.

Figure 2-19. *TetO₂*-“off” promoter cassette. 1% agarose gel showing PCR products *tetO₂* promoter cassette -with associated *kanR* cassette- amplified from pCM224 of expected size 4,000 bp (~3,800 bp plus ~ 80 bp of primer flanking on each side). Lane 1: 56.7°C, lane 2: 59.1 °C, lane 3: 60.4 °C, lane 4: 61.7 °C, lane 5: 62.9°C , lane 6: ladder1 Kb plus.

2.32.1. Transformation and assessment of repression-tetrability

The *tetO₂*-“off” promoter cassette, with its associated *KanR* marker, was then transformed into *tif1Δ::natR* and *tif2Δ::natR* strains and the transformant mixture selected onto Petri dishes containing YPD supplemented with clonNAT and G418 to test for the presence of both resistance markers. Colonies which successfully grow will have the genome modified as illustrated in **Figure 2-20**.

Figure 2-20. *tetO₂* promoter associated to a selective marker (*kanMX4*). *tetO₂* promoter associated to a selective marker (*kanMX4*) was integrated in the genome to drive expression of the *TIF2* gene in a *tif1Δ::natR* strain.

The clonNAT-kan resistant colonies were then tested for doxycycline-induced repression on YPD and SD plates containing 50, 100, 150 and 200 µg/ml of doxycycline. No repression was observed for strains streaked out onto low and medium concentrations of doxycycline, whilst an effect on growth was seen at the highest concentration. Out of 12 successful transformant colonies tested, only one (clone #2) appeared to have integrated the *tetO₂*-“off” cassette in a way that makes the strain modulable in the expression of *TIF2* in *tif1Δ* background, at least in SD medium (**Figure 2-21**). While clone #4 seemed to display a growth defect as well, it should be noticed that it appears to be a “slow grower” even in plain SD medium. No repression was observed for any of the 9 *tif2Δ::natR tetO₂*. *tif1* transformants colonies tested.

Figure 2-21. Assessment of repression of the titrable *tetO₂*-“off” promoter in *tif1Δ::natR tetO₂.tif2* strains. Clones #1-4 were streaked out on SD (b) and YPD (d) media containing 200 μg of doxycycline and on correspondent control dishes. Clones #1 and #4 showed repressed growth in the presence of the drug, but in SD medium only.

2.32.2. Spot dilution assay

Sensitive strains were 1 in 10 serially diluted in a 96-well plate and spotted both manually (**Figure 2-22**) and robotically (not shown) onto YPD or SD media containing 200 μg/ml of doxycycline.

Figure 2-22. Manual spot assay. Lines 1-4: *Δtif1Δ::natR tetO₂-tif2* clones #2-a, #2-b, #2-c, #2-d; line 5: *tif1Δ::natR ire1Δ::kanR* (control) in A) SD containing clonNAT and G418 and B) SD clonNAT, G418, and doxycycline 200 µg/ml.

2.33. *GAL* over-expression promoter

2.33.1. Plasmid purification and amplification PCR

Amplification of the *GAL* promoter cassettes from plasmid pYM-N23, pYM-N27, pYM-N31, was conducted in a PCR with primers designed to have flanking regions to the *TIF1* or *TIF2* genome sequence (#40;#41, #42;#43 listed in **appendix 5.5 table A-3**); PCR fragments of expected size were obtained as shown in **Figure 2-23**.

Figure 2-23. *GAL* cassette 1% agarose gel showing in lane 1) pYM-N23, 2) pYM-N27, 3) pYM-N31 purified plasmids (~4,000 bp), *GALI* from pYM-N23 with primers for *TIF1* promoter, 5) *GALI* from pYM-N23 with primers for *TIF2* region, 6) *GALL* from pYM-N27 with primers for *TIF1* promoter, 7) *GALL* from pYM-N27 with primers for *TIF2* promoter, 8) *GALS* from pYM-N31 with primers for *TIF1* promoter, 9) *GALS* from pYM-N31 with primers for *TIF2* promoter (~ 1,800 bp). The three *GAL* cassette plasmids were from a toolbox (Janke, 2004). Choice of polymerase was based on your product based on the fidelity, specificity, and target length for required application.

2.33.2. Transformation

A *GAL* over-expression promoter cassette, with its associated *KanR* marker, was then transformed into *tif1Δ::URA* and *tif2Δ::URA* strains and the transformant mixture selected onto Petri dishes containing SGal –URA supplemented with clonNAT and G418 to test for the presence of both resistance markers which select for the construct illustrated in **Figure 2-24**. No successful transformants were harboured through this approach.

Figure 2-24. Hypothetical genotype of a successful transformant strain with insertion of *GAL* over-expression promoter. Hypothetical genotype of a successful transformant strain in which *GAL* over-expression promoter associated to a selective marker (*natNT2*) was integrated in the genome to drive expression of one *TIF2* gene in a *tif1Δ::URA* strain.

A further attempt to generate a strain for the over-expression of *TIF1* or *TIF2* genes was undertaken via construction of diploids strains that could possibly better cope with the initial drastic adjustment required by the transformation conditions. For reasons which are strain-dependent however, the mating of diploids was not achievable due to an unusual, but not unprecedented, case in which the yeast behaves as an opposite mating type and hence does not mate with the expected opposite mating type as it recognizes as its own.

2.34. Discussion

An investigation into aspects of gene duplication will also be addressed through enquiry into *TIF1* and *TIF2* genetic interactions, revealing insights on the degree of buffering existent between this pair of duplicates and the degree of functional differences between these closely related genes.

The genetic networks surrounding the *TIF1* and *TIF2* paralogs have shown to represent several aspects into the organization of a cellular system in which biological processes are coordinated by gene regulation and signal transductions along with physical protein interactions.

A crucial characteristic of a networked system is related to its ability of remodelling upon perturbations, in a process known as plasticity. To be able to re-draw the network connections, even if only to a limited extend, is one of the key for survival for biological systems, which through this re-wiring are able to deal with variations that challenge the stability of some nodes in the network.

2.35. The biology behind the interactions

The current study presents two highly challenging features: firstly that the duplicate genes under investigation (*TIF1* and *TIF2*) encode an identical common protein and secondly this protein is involved in a fundamental cellular process (translation). As seen in studies of duplicate genes in metabolic pathways (Harrison, 2007; Szappanos, 2011), the protein product encoded by one or both paralogs can be tracked and its involvement in cellular processes straightforwardly revealed. There are a few classes of duplicates, such

mitochondrial-related genes and other ORFs involved in processes for which a promptly measurable metabolic outcome is not readily available. Finally, always to be considered is the possibility that interactions may often be masked by functional and/or systemic redundancy.

TIF1 and *TIF2* are indeed two atypical genes: their common encoded protein plays a role in the limiting step of protein synthesis, participating in a broad variety of processes as availability of nutrients, glucose-signalling, mTOR pathway (Montero-Lomeli, 2002; Proud, 2002), to cite some and the equivalent genes in mammalian systems are involved with the cellular dysregulation associated with cancer (Mamane, 2006). The identical protein product encoded by the *TIF1* and *TIF2* paralogs is an element that further complicates the study of their duplicate nature and compels for an articulated and systematic genetic approach, with the intent to satisfactorily fulfil the gaps that protein focussed studies were unable to. In a SGA, interactions are probed through mating of a strain harbouring deletion of a query gene (in this case either *tif1Δ* or *tif2Δ*) with other deletion strains. In the investigation of SGA-generated genetic interactions, the primary focus is on synthetic sick and lethal phenotypes, as the most severe display of genetic interaction. Deviation from an expected wild-type phenotype reflects a common involvement of the two genes in a functional process. Interaction can be rationalized as: 1) A direct effect on the production of a required protein; 2) A redundancy associated with regulation of a certain function in which contribution of either one of the two genes is essential (Tong, 2001).

Representation of data via a Cytoscape diagram (**Figure 2-25**) showed a common involvement for the two paralogs in response to stress and translation-

related processes. Genes holding a genetic interaction with *TIF1* only function in vesicular trafficking and mitochondrial activities, while transcription-related processes as regulation of RNA Pol II enzyme and ubiquitination/deubiquitination of the histone structure appear to govern *TIF2* genetic network.

Figure 2-25: Cytoscape generated genetic network surrounding the deletions of *TIF1* and *TIF2* genes.

2.36. *TIF1* genetic profile

Pathways critical for the fitness of yeast following deletion of *TIF1* gene were revealed by interactions with genes involved in the up keeping and regulation of the vesicular trafficking (*VPS27*) and vacuolar sorting (*VPS41*), processes that hold a certain degree of coordination with the ubiquitination-deubiquitination pathway (*RAD4* and *UBI4*). Some elements of chromatin remodelling (*YFL013W-A*) and transcription progression and regulation (*KAP122*

and *BRE1*) were also identified, along with a genetic link (*NAM7*) to the NMD machinery.

As trafficking of lipids and proteins among cellular organelles is critically dependent on vesicular transport (Rothman & Orci, 1992), any alteration of this pathway can potentially act as prompt trigger for cellular stress, mainly as a result of altered membrane lipid composition and pile up of unusual amounts of mislocalized proteins (Nelson & Lemmon, 1993). Rapid and specific attenuation of translation initiation in vesicular deficient strains has been studied by an extensive work done by Deloche and colleagues, that reported unexpected genetic interactions between Tif3 (yeast eIF4B ortholog) and proteins involved in different aspects of vesicular trafficking. As Tif3p/eIF4B promotes the functions of Tifp/eIF4A (Hernández, 2005) so that KO of TIF3 could reasonably be expected to somewhat mimic KO of one of *TIF1* or *TIF2*. The authors first raised the question whether a link in between the initiation step of protein synthesis and vesicular transport, two apparently remotely distant processes, might exist. Polysome profiling of a collection of temperature sensitive mutants showed that initiation of translation is rapidly inhibited by a deficient vesicular transport along the secretory and vesicular pathway, whilst it is unaffected by a transport defect in the biosynthetic pathway (Deloche, 2004).

Efficient transport, aimed to dynamically fulfil cellular requests with a prompt delivery of proteins and lipids to subcellular organelles, is not the only task that vesicular mediated trafficking undertakes in eukaryotes. Prior sorting into vesicles is also required for delivery of membrane proteins to proteolytic structures, where sorting of membrane proteins is triggered by mono-

ubiquitination and subsequent delivery of cargoes to Multivesicular Bodies (MVBs) vesicles. Upon fusion of MVBs with the vacuole (commonly referred to as lysosome) and consequent lysis of the cargo, the membrane proteins excluded from the MVBs can potentially be recycled, mainly via the trans-Golgi.

The vacuolar membrane protein Vps41p is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex and its contribution is essential for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport (**Figure 2-26**). The cellular environment seems to rapidly adapt to such a block in vesicular transport at the plasma membrane level, by controlling protein synthesis with a rate of inhibition that has been measured at 30% (Deloche, 2004). Speculation could be made on whether translational control in response to vesicular impairment in the case of *vps41Δ* takes place via *TIF1* network and when *TIF2* is the only functional paralog, translation initiation cannot be properly tuned. Moreover, as endosomal structures represent the hub where proteins can be redirected to the plasma membrane saving them from degradation, the unavailability of the retro-Golgi transport may impair recycling of components directed to proteolytic structures, such as MVBs and lysosomes. As precursors of lysosomal enzymes are transported via vesicles as well, missed delivery of such components to the degradative structure may result in inefficient proteolytic activity. Cellular death may occur as result of an anomalous trafficking that involves, for example, membrane proteins being wrongly conducted to the lysosome compartment to undergo degradation. In the case of the viable *vps41Δ* strain, however, this loss in trafficking must be aggravated by disruption of translation. Furthermore, MVBs, also known as multivesicular endosomal (MVE), are endocytic intermediates that

contain molecules previously internalised through endocytosis. Given that MVBs also receive cargo from the trans-Golgi, it is understandable how intraluminal vesicles have an important task as storage vehicles for transmembrane proteins that are meant to be released from the cell in a regulated manner.

Along with *vps41Δ*, an additional gene deletion related to vesicular trafficking was identified in this SGA, *VPS27*, the gene encoding for Vps27p membrane-associated protein, holds a negative genetic interaction with *TIF1* which could be rationalized following the same reasoning as for *vps41*. A further mechanism, although, may implicate *VPS27* gene, in virtue of its recently established role in the endosomal sorting complex required for transport (ESCRT) complex. As with the majority of vacuolar protein sorting (Vps) proteins, Vps27p is important for endosomal maturation and transport of proteins to the lysosome (Raymond, 1992). The categorization of Vps27p as component of the ESCRT-0 complex, along with Hse1, is quite recent (Williams & Urbe, 2007), following biochemical and genetic studies that suggested this complex recruits the previously characterized ESCRT-I (Bilodeau, 2003). ESCRT complexes are recruited from the cytoplasm to the endosomal membrane to engage in a widely connected network of interactions. As the first member of ESCRT pathway, ESCRT-0 mediates the recognition of mono-ubiquitinated proteins and their entry in the pathway. Both monoubiquitinated cargo and the ESCRT-I complex are recruited by Vps27p and this interaction appears to facilitate transferring of ubiquitinated cargo to ESCRT-I (Bilodeau, 2003) from which it is transferred to ESCRT-II and finally to ESCRT-III. At this last step deubiquination of loaded proteins is required (Amerik, 2000) for their deposition into intraluminal vesicles formed by the invagination and budding off

of endosomal membranes to form MVBs (Gruenberg & Stenmark, 2004, for review) (**Figure 2-26**).

Figure 2-26. Vacuolar protein sorting. Deletion strains found sensitive to lithium/galactose treatment correspond to genes coding Vps27p and Vps41p respectively, as shown. EE: early endosome; LE: late endosome; MVB: multivesicular body; ER: endoplasmatic reticulum. (Reviewed in Hurley, 2010).

A link between trafficking and ubiquitin has been established since the discovery that many ESCRT components display Ubiquitin Binding Domains (UBDs) that contribute to their ability to sort ubiquitinated membrane proteins. Chains of ubiquitin appear to constitute the operative sorting signal recognized by ESCRT UBDs (Paiva, 2009) that activates the transport of ubiquitinated proteins into the MVBs pathway (Babst, 2005). Specifically, Vps27p contains an ubiquitin-interacting motif (UIM) identified in several regulators of endocytosis as well as of MVBs (Hofmann & Falquet, 2001) and is required for binding of a single ubiquitin molecule and internalization of proteins (Shih, 2002). The

human Vps27p ortholog Hrs is also required for sorting of a transferrin-receptor-ubiquitin fusion protein to degradative pathway (Raiborg, 2003).

The ESCRT machinery does not appear to be required either for coordination in the early step of endocytosis of ubiquitinated receptors nor in the processing at the early endosome level. Whilst ESCRT involvement has often been narrowed to MVBs formation, this study results may suggest a revised role for its component Vps27p. Intriguing findings that UIM-containing proteins might interact with ubiquitinated cargo at the plasma or early endosome membrane (Shih, 2002) and thereby mediate its sorting into endocytic or intraluminal vesicles endorse this scenario. UIM-Ub interaction is, however, characterized by a low affinity binding and the K_d associated with this interaction is in the micromolar order, presumably strong enough to mediate recognition for sorting but weak enough to allow dissociation for future processing. Additionally, as the VPS27 human ortholog has been shown to be able to engage other UIM-containing proteins into complexes with potential sorting function, a model in which such large conformations bind to multi-ubiquitinated containing receptor complexes has been proposed (Raiborg, 2003). Under this scenario, *VPS27* presence is required for the formation of the UIM-containing proteins or multi-ubiquitinated-containing complexes as they aggravate the cellular phenotype, already challenged by the translation initiation diminution driven by the UPR response and/or stress. A further issue of spatial nature is also imaginable in which equilibrium and activity of the aforementioned complexes are perturbed by the loss of sterical control on interaction with cargoes and/or sorting components. Finally, although not entirely excludible, a model in which Vps27p-UIMs act as a powerful trigger for

monoubiquitination instead as a sorting “decision-maker” seems less likely: as monoubiquitination is also invoked by other independent signals, the role of *VPS27* in such context is expected to be non-essential.

Additionally, in virtue of their UBDs, ESCRT complexes can also be ubiquitinated themselves by a process termed “coupled ubiquitination” (Haglund & Stenmark, 2006); such an event has been proposed to drive critical aspects of ESCRT function by altering intermolecular interactions between ESCRT proteins that bear UBDs, or triggering cargo release. Such ability is quite remarkable as ESCRT-0 and -I regulate ESCRT complex activity by modulating its own ubiquitination thanks to their ability to associate with ubiquitin-ligase and deubiquitinating enzymes (Ren, 2007). This specific task might be particularly prominent in the ESCRT-0 complex component *VPS27*; a characteristic that renders the encoding gene essential under specific conditions, such as when associated with defects in translation.

Very recent work from Stringer and Piper (2011) showed that strains harbouring *VPS27* genes encoding Vps27p lacking ubiquitinatable lysine residues are not ubiquitinated however still functional in MVBs cargo sorting (Stringer & Piper, 2011) suggesting that ubiquitination of the ESCRT apparatus is not required for its ability to sort monoubiquitinated cargo into the MVBs (Stringer & Piper, 2011). Questions should be raised as to whether the role of ESCRT-0 has been appropriately annotated and on what kind of regulation allows MVBs formation and functioning if ubiquitination is not the focal one. Interestingly, Vps27p is enriched at the endosomal membrane by virtue of its interaction with phosphatidylinositol-3-phosphate (PtdIns3P) lipid-binding domain (Katzmann, 2002) and is the only member of a list of protein candidates

for ubiquitin-binding receptors (Shih, 2002) that contains a zinc finger FYVE motif. In consideration of this, it is tempting to speculate that such PtdIns3P binding motif might be needed to regulate or be regulated by interactions with other proteins or lipids.

In situations of stress, UPR represents the cellular workhorse for coping with misfolded proteins in the ER (Schroder & Kaufman, 2005): this response is accountable for the up-regulation of the expression of certain genes that are needed for eukaryotic cells' survival. These upregulated genes are involved in folding, mainly chaperones and folding factors, and in ubiquitin/proteasome degradation. Transcriptional activation of the aforementioned genes appears quite demanding and, while the cell equips for such event, a temporary arrest in translation initiation takes place during early stages of UPR, mediated by phosphorylation of eukaryotic initiation factor 2alpha (eIF2alpha). It is inferred that continued synthesis and accumulation of misfolded proteins are in this way prevented. Decreased translation is coupled with an arrest in ubiquitin/proteasomal degradation with the intent to avoid a fast depletion of essential proteins through degradation (Shenkman, 2007). Short-lived E3 ubiquitin ligases are believed to be among the multiple factors that govern the ubiquitin/proteasomal pathway (Glickman & Ciechanover, 2002) as these enzymes become depleted upon the inhibition of protein synthesis. This abrogates ubiquitination, as has been shown for p53 (Shenkman, 2007). The ubiquitination step has been proposed as the most affected stage in ubiquitin/proteasomal degradation occurring after a block in protein synthesis and it is likely to be the only affected step (Shenkman, 2007), based on the observation that the protein synthesis inhibitor cycloheximide leads to a

depression of ubiquitinated proteins, but achieve this through inhibition or degradation of ubiquitin conjugates (Hershko, 1982). Once E3 ligases are depleted, their substrates become stabilized and this may apply to a vast number of cellular substrates (Shenmkman, 2007). Interestingly, ubiquitination, of the ESCRT-0 complex, largely mediated by the E3 Ub ligase Rsp5p, has been proposed as a means of assessing the role of ESCRT ubiquitination. (Stringer & Piper, 2011). The specificity of Rsp5p as ligase is, however, quite broad, as required for ubiquitination of a variety of membrane proteins at multiple compartments. No genetic interactions between *RSP5* and either *VPS27* or *HSE1* genes are reported in the literature; Vps27p does not appear to hold any interaction of physical nature with Rsp5p either, whilst physical interactions of Rsp5p with Hse1p have been independently reported by two different research groups (Bowers, 2004; Ren, 2007). This network arrangement (**Figure 2-27**) suggests that the physical interaction that Vps27p holds with Hse1p (Bilodeau, 2003; Gavin, 2006) may be sufficient for Rsp5p to control ESCRT-0 as a complex via indirect control on Hse1p, whilst translation initiation exhibits control on *VPS27*. It is hard to state whether this happens with or without the involvement of the ESCRT-I component Vps23p, which both Vps27p and Hse1p share a physical interaction with (Bilodeau, 2003; Bowers, 2004). Results reported in **Table 2-3** show a genetic interaction of *TIF1* gene with *VPS27* that is not mutual to *HSE1*. Altogether, this set of interactions suggests that, although sharing functional duties in the ESCRT-0 complex, Vps27p and Hse1p may have different roles and significance on their own. This results further open to question whether ESCRT complex function does require both elements of the complex to perform the sorting of ubiquitinated cargo into endosomal membrane

invaginations and the degree of competences assigned between the two complex subunits. A genetic interaction of *TIF1* with E3 ligase, *NAM7*, will be discussed later in this chapter.

Figure 2-27. Partial representation of ESCRT machinery components' subset of interactions. *TIF1-VPS27* (this study), *Vps23p-Hse1p* (Bowers, 2004), *Vps27p-Hse1p* (Gavin, 2002), *Vps27p-Vps23p* (Bowers, 2004), and *Hse1p-Rps5p* (Bowers, 2004).

Strikingly, deletion mutants of no other ESCRT encoding genes appeared in our screen as SL in combination with either *tif1Δ* or *tif2Δ* query strains. This result could find an explanation in the described role of *VPS27* as gatekeeper for the pathway. The role of ESCRT-0 in first recruiting both mono-ubiquitinated cargo and the ESCRT-I may be accompanied by activation of the signal that ultimately leads to the formation of MVBs. The input that triggers the invagination and budding off of endosomal membranes could be sent, among other locations, from the start of the pathway and be under the expertise of *VPS27*. As a result of the cellular stress generated by the vesicular trafficking

disequilibrium, Tifp -encoded by *TIF2* gene only - becomes target of more intense ubiquitination and an increasing number of Tif proteins undergo degradation, in a mechanism of response masterminded by the system to slow down protein synthesis via translation initiation and regulation of its own factors. An alternative scenario may have to do with Vps27p sorting of mono-ubiquitinated proteins and the impaired functionality which arises from the aforementioned proteins not being able to be loaded on ESCRT complex.

The lack of proper sorting has an undoubted impact on degradation and recycling, leading to some protein products and/or ubiquitin tags no longer being available in physiologically required concentrations. Such a situation may be somewhat overcome by an increase in the rate of protein synthesis, using the higher throughput of protein production to provide sufficient copies of required proteins to overcome inefficiencies in protein handling processes. This type of response would represent an extreme cellular attempt to overcome stress and it is likely to be tolerated by the organism for a limited time only, as not all the recycling can be processed through the proteasome structure and the cell will end up dying due to lack of fundamental components derived from recycling and possibly by inefficient ubiquitination of mislocalized and/or unfolded proteins. In other words, such scenario not only would trigger the UPR but it would be also accompanied by a great number of unfolded proteins in need to be degraded, as result of a paucity of ubiquitin tags. In the case of *vps27Δ tif1Δ* double mutant strain, the translational rate, which is dependent on the amount of Tif protein encoded (Hilbert, 2011), may not be adequate to successfully process such request as the translational process is already weakened by the absence of one of the paralogs.

Finally, the role of the ESCRT complex in vesicle formation from endosomal membranes and biogenesis of MVBs impacts indirectly on cell signalling through membrane receptors which would normally be regulated through endocytosis. It is inferred that ESCRT complex function in modulating such signals is highly important, as identified by several studies that show the deregulation of cell signalling in ESCRT impaired strains (Michelet, 2010). This link with cell communication is supported by data relating to *D. melanogaster* Vps proteins that hints at different functions for vesicular proteins beside MVBs formation (Herz, 2009). Although not confirmed in a mouse model, studies in fruit fly suggest that mammalian ESCRT members may have tumor-suppressor properties (Herz & Bergmann, 2009). This is of particular interest given that dysregulation of translational events has been associated with carcinogenesis (Zimmer, 2000) and an increased effort is directed towards tumor-suppressor strategies during the early stages of carcinogenesis (Tani, 2000).

As indicated above, dysregulation of ubiquitination is one reasonable explanation of the SL of the *vps27Δ tif1Δ* double mutant. The possibility of given further credit since accessibility of ubiquitin precursors and ubiquitinated protein turnover appears also to be essential for proper cellular fitness as combinations of mutants lacking genes as *UBI4* or *RAD4* lead to SL in a background lacking *TIF1* gene.

UBI4 encodes for ubiquitin, a 76 aminoacid protein that conjugated to proteins, labelling them for selective degradation via the ubiquitin-26S proteasome system. While the lethal phenotype recorded in absence of *UBI4* contribution may simply reflects its essential role in the cellular stress response,

it might also indicate how crucial ubiquitination of the cargo is, as it triggers for its endocytic internalization (Kolling, 1994). Although internalization of proteins is a selective and complex process that relies on both ubiquitin dependent and independent circuit signals, it may be possible that the ubiquitin-dependent holds a contribution of greater importance and the lethal phenotype is likely to occur due to a missing or confusing internalization signal. In response to stress, Tifp becomes ubiquitinated (Tagwerker, 2006) leading to its degradation so the cell can decrease protein synthesis. For Tif2p to be ubiquitinated though, the cell needs the ubiquitin tag that, in stress conditions, may see its output increased following *UBI4* over expression. The SL phenotype observed for *ubi4Δtif2Δ* is likely to be ascribed to the fine balance that governs essential biological processes, as the synthesis of proteins is needed to produce ubiquitin, which subsequently can label Tifp to decrease protein synthesis itself. It is understood that a source of non-ubiquitinated Tifp can be found in stress granule formations, with the purpose to restart translation after relief of stress.

RAD4 encodes for Rad4p, a protein which works with Rad23p in recognizing and binding damaged DNA during nucleotide excision repair (NER), a major pathway deputy to repair an extensive range of DNA lesions. Specifically, Rad4p binds to the damaged double helix, where it makes the location available for other DNA repair enzymes, when encountering a helical distortion. Error-free DNA represents a pre-requisite for the cell to accurately transcribe genes and, whilst DNA damage occurs at a relatively low rate in the human genome, lesions in critical genes (e.g., tumor-suppressor genes) can impair cellular functionality and such dysregulation is associated with a predisposition to cancer. The underlying principle beneath *rad4Δ tif1Δ* synthetic lethality can be rationalized

in consideration of the unambiguous link established between DNA damage and protein synthesis: global inhibition of protein synthesis, accompanied by re-programming of translation, follows UV radiation initiated DNA damage (Deng, 2002; Wu, 2002). A reduction of the global rate of protein synthesis induced by UV DNA damage is accompanied by an increase in phosphorylation of the translation initiation factor eukaryotic initiation factor α (eIF2 α).

Mutations of XPC (the human homologue of yeast *RAD4*) or impaired NER activity lead to genomic instability and carcinogenesis, as established for *Xeroderma pigmentosum*, a hereditary disease that exhibits a strong susceptibility for skin cancer (Hanawalt, 2003). Until recent and ground-breaking work from Li and colleagues (2010) suggested a role for Rad4p in ubiquitin/proteasome-mediated protein turnover, the cellular function of Rad4p was believed to be restricted to DNA repair. Substrates that accumulate in *rad4 Δ* mutants are found to be ubiquitinated, suggesting that Rad4p acts as a regulator of a post-ubiquitination step via its binding to the proteasome structure and ubiquitin conjugates, while these unfavourable phenotypes associated with *XPC* mutations were previously exclusively attributed to their incapability of repairing DNA.

Finally, together with Rad23p, Rad4p has also been annotated to play a role in the proteolytic process; this aspect of *RAD4* regulatory contribution, however, is suspected to be accessorial as several proteasomal substrates maintained their normal degradation in *rad4 Δ* mutant strains. For this reason, no SL interaction should be expected in cells lacking this particular aspect of *RAD4* regulation. It is of interest to report that, although Rad4p and Rad23p are believed to work together and share common substrates, only deletion of *RAD4*

was identified as SL in combination with *tif1Δ*. Moreover, the *rad23Δ* strain displayed a suppression-like phenotype in both *TIF1* and *TIF2* KO screens, however statistically not significant enough to be trusted. If this was true, it would further suggest a different role and regulation for the two genes, although sharing a common function.

Among the *tif1Δ* SGA SL hits, deletions of genes functioning in the translation initiation process were also identified. *NAM7* encodes for Nam7p (Upf1p), part of the triad of Upf proteins involved in NMD and functioning in a complex as core of the NMD machinery. Specifically, Upf1p is a cytoplasmic ATP-dependent RNA helicase which is required for functional NMD (Czaplinki, 1995), a surveillance system that eliminates transcripts with premature termination codons (PTC) (Chang, 2007). Ubf proteins assemble on polysomes for recognition of aberrant mRNAs; as transcripts are housed during their transcription on the ribosomal structure that helps processing them, the ribosome stalling on a PTC is a sufficient trigger for the NMD response to be activated with consequent destruction of the mRNA (Brognia & Wen, 2009). At least two surveillance steps have been suggested during the NMD process: the first involves Upf1p to be docked to polysomes, independently of Upf2p; the second takes place via Upf2p-dependent association of Upf3p with polysomes (Atkin, 1997). Interpretation of this SGA data makes it tempting to speculate that translational initiation control is linked to Upf1p exclusively.

Alongside its established function as an Upf2p binding protein, Upf1p exploits an additional role by virtue of its RING domain that mediates an interaction with E2 Ubc3p. It was inferred that Upf1p contains an E3 ligase

activity, and Upf1p-mediated ubiquitin modifications may be involved in the signalling pathway from the recognition of PTC to NMD (Takahashi, 2008). This additional feature of Upf1p can be related to the previously discussed mechanism that affects ubiquitin/proteasomal degradation upon a block in protein synthesis in concern to vesicular damage.

Very recent work from Z. Zhang (2010) reported that mRNAs targeted by NMD are also suppressed at the translational level. The low translational efficiency is reported as the result of multiple features acting in concert, including the translational initiation rate, 5' secondary structures, weak initiation sites and low translational elongation speed. It is currently unknown, however, whether this low translational efficiency is a feature evolved by the cell to allow suppression of translation or whether transcripts with low transcriptional efficiency are simply more prone to be targeted by NMD. ORFs bound by more Upf1p have been reported as more translationally repressed, although not significantly (Zhang, 2010) and it was discovered that Upf1p can target normal mRNAs to cytoplasmic foci known as P-bodies (Sheth & Parker, 2006) suggesting an attempt to defer translation. Given the potential involvement of NMD factors in translational repression, NMD control may be considered as a part of a more expanded system that coordinates with delay or impediment of the translational process. This system has to hold a certain degree of connection and plasticity, since the effectiveness of NMD degradation activity has been shown to be limited and highly condition-dependent (Zetoune, 2008), for instance NMD is largely absent when ribosomal movement across mRNA is slow, as seen during starvation (Mendell & Sharifi, 2004). It is also conceivable that inefficient translation could prompt for transcripts to attract the NMD machinery.

Finally, a reflection should be made on the extent of post-transcriptional regulation in yeast in comparison to other higher organisms in consideration of the fact that yeast displays a paucity of introns that limits the option of alternative splicing. Although still worthwhile a comparison, yeast presents dissimilarities in the organization of mRNA surveillance compared to other higher organisms, perhaps the most significant being NMD: in yeast this process occurs both during export and inside the cytoplasm and it is not constrained to the pioneer round of translation (Johansson, 2007).

A few genes, deletions of which were reported as SL with *tif1Δ*, have protein products that act as structural constituent of the ribosomal structure. *RPS23a*, for example, encodes for the ribosomal protein 28 (rp28) of the small (40S) ribosomal subunit and its connection with translational accuracy has been established (Synetos, 1996). An almost identical protein product to Rsp23ap, Rps23b, exists, however it does only appear as a “possible” hit in either *tif1Δ* or *tif2Δ* SGA. This result makes difficult to state whether the accurateness associated to translation is highly detailed and *RSP23a* and *RSP23b* undergo different regulation or the different severity in their growth is principally to be ascribed to specific strain differences. Other ribosome-related hits were: 1) *RPL6b* which encodes for a protein component of the large (60S) ribosomal subunit and binds to the 5.8S rRNA, one of the four ribosomal RNAs and 2) *BUD28* which associated open reading frame is believed to be dubious; however, is 98% overlapping with the verified gene *RPL22A* is involved in ribosome biosynthesis/biogenesis falls in with work from Linder’s laboratory which showed how rapid repression of translation is not governed by repression of

ribosome biosynthesis (Deloche, 2004). It is interesting to notice that *RPL22B* has far fewer associated interactions, at least for high throughput screens, than *RPL22A*: nearly all *RPL22B*'s physical interactions are also found with *RPL22A*.

As cellular stress evokes the UPR, an imbalance in Ca^{2+} equilibrium takes place in the cell and it may have consequences on diverse cellular compartments, from Golgi-ER to endosomes and lysosome structures. Cellular Ca^{2+} signalling is tightly regulated and Ca^{2+} has a number of intracellular stores, such as the ER, where Ca^{2+} is stored in high quantities compared to the cytosol, which is characterized by a low Ca^{2+} concentration. It is conceivable that genes involved into transport of Ca^{2+} into Golgi structures could then be essential under certain conditions. This may be the case of *HUR1* gene, deletion of which generated SL phenotype when paired with *tif1Δ* query strain, which has not annotated function. The overlap of its ORF with *PMR1*, however, may help in elucidating its cellular task as *PMR1* encodes for a high affinity $\text{Ca}^{2+}/\text{Mn}^{2+}$ P-type ATPase required for Ca^{2+} and Mn^{2+} transport into the Golgi compartment. *PMR1* involvement has been annotated in Ca^{2+} dependent protein sorting and processing, with mutations in human homolog ATP2C1 reported to trigger acantholytic skin condition Hailey-Hailey disease (Hu, 2000).

Also ascribable to the Golgi context is the assessment of SL between *tif1Δ* and *hoc1Δ*, this latter gene encoding for an α -1,6-mannosyltransferase involved in cell wall mannan biosynthesis. Once again, a genetic interaction has been reported for *HOC1* only, among the five components of the Golgi-localized complex Hoc1p is part of. The observed SL for *hoc1Δ tif1Δ* double mutant can be rationalized as caused by a general impairment of protein synthesis. However,

being the remaining components of the complex verified “misses”, it seems appropriate to avoid over-interpretation of this result.

A small subset of mitochondrial-related hits was also identified: the first, *MTC1*, encodes for a predicted malonyl-CoA ACP transferase, a putative component of a type-II mitochondrial fatty acid synthase that produces intermediates for phospholipid remodelling. *mct1* mutant strains display respiratory-deficiency and are characterized by an inability to utilize non-fermentable substrates (e.g., glycerol) for growth (Torkko, 2001). Such impaired strains, also known as ‘petite’, have the tendency to harbour smaller colonies compared to cells equipped with a fully functional respiratory system. The gene *MMM1* encodes for the ER-resident protein Mmm1p (Maintenance of mitochondrial morphology). This latter protein is part of the ER-mitochondria encounter structure (ERMES) complex and its deletion leads to respiratory impairment. Discussion on its involvement with the translational initiation process will be extensively discussed in **section 2.38** along with *MMM2*, another constituent of the same complex. The third SGA hit, *GDS1*, encodes for a protein of unknown function, required for growth on glycerol and is detected in highly purified mitochondria (Reinders, 2006). Interestingly, *gds1* strains exhibit an abnormal cell cycle progression in G₁ phase (Sopko, 2006). The genetic connection between *TIF1* and *GDS1* genes could be rationalized in consideration of the G₁ block on the cell cycle that under stress conditions can be triggered by inhibition of protein synthesis (Zetterberger, 1995) and the UPR. There is convincing evidence, that protein synthesis is required to complete some

regulatory steps in the cell cycle as, for example, initiation of DNA synthesis and mitosis are particularly sensitive to protein synthesis inhibitors.

KAP122 gene, previously known as *PDR6*, mediates a nuclear import pathway. Kap122p is responsible for transporting the Toa1p-Toa2p complex (large and small subunit of the transcription factor IIA (TFIIA)) into the nucleus, where Kap122p shuttles its substrate by binding to nucleoporins Nup1p and Nup2p (Titov & Blobel, 1999). Whilst not affected in their cell viability, *kap122Δ* strains show a preferential cytoplasmic localization of both large (Toa1p) and small (Toa2p) subunits of the complex, indicating that Kap122p activity is required for shuttling of such components into the nucleus compartment, however alternative mechanisms must exist to back up for either Kap122p or TFIIA function (Titov & Blobel, 1999). Transcription factor TFIIA is a nuclear protein involved in the RNA polymerase II-dependent transcription of DNA and required for all transcription events that use RNA polymerase II. The genetic connections among RNA polymerase II, DNA transcription and translation initiation will be more extensively discussed in **section 2.37**, however the importance of this interaction deserves a mention as it gives evidence for a direct control on gene activation, that proceeds directly on the transport of Toa1p and Toa2p TFIIA subunits. Finally, although Kap122p role is established, ambiguity remains about the exact order and modality through which Kap122p joins its interacting components and subsequently shuttle into the nucleus. Although this research study is unable to provide clarification, speculation can be made in based on the observation that deletions of genes encoding for other associated components (i.e., Wtm1p and Wtm2p) did not lead to SL in this SGA screen.

Finally, *HXK2* encodes for a hexokinase isoenzyme 2 that catalyzes phosphorylation of glucose in the cytosolic compartment. During the initial irreversible step in the cellular metabolism of glucose, the action of the three enzymes Hxk1p, Hxk2p and Glk1p is required for the phosphorylation of glucose molecules at carbon C6 (Walsh, 1983). Whilst all three enzymes participate in the uptake of glucose (Bisson & Fraenkel, 1983), a major role is attributed to Hxk2p, at least during glucose phosphorylation *in vivo*, where it works as the predominant isoenzyme during growth on glucose (Walsh, 1983). The *HXK2* gene is expressed when yeast cells are grown on a medium containing a fermentable carbon source as glucose, fructose or mannose. Upon shift of the cell to a non-fermentable carbon source, *HXK2* gene is repressed and *HXK1* and *GLK1* de-repressed (Rodriguez, 2001). The genetic interaction that characterized *HXK2* and *TIF1* genes could be related to the response triggered by glucose limitation in mammalian cells, where an increase in the phosphorylation of eIF2 α occurs leading to a decrease in translation initiation (Vazquez de Aldana & Hinnebusch, 1994).

2.37. *TIF2* genetic profile

TIF2 genetic profile revealed an explicit involvement of this paralog in the regulation at different stages along the transcriptional process. *TIF2* participation takes place via a highly interconnected genetic network of SAGA components (*CHD1*, *UBP8* and *SGF11*) via interactions that involve histone H2B ubiquitination/deubiquitination cycles (*UBP8* and *BRE1*), the chromatin remodelling process and assembly and regulation of RNA Pol II (*ASK10*, *RTF1*,

SIP3 and *YGL024W*). Regulation of early meiotic gene expression (*DFG16*, *RIM101*, *YEL057C*, and *YGR122W*) is a further influential element.

The SAGA complex mediates histone modification of gene promoters to enhance transcriptional activation and facilitate elongation by deubiquitinating histones downstream of RNA Pol II. The most critical among transcriptional-related steps appears to be the ubiquitination-deubiquitination status of H2B histone, governed to an extent by SAGA interactions. SAGA (Spt-Ada-Gcn5 acetyltransferase), a chromatin-modifying complex, acts as transcriptional co-activator of a number of cellular processes (Baker & Grant, 2007); its coordination is known to happen through histone post-translational modifications, as SAGA takes part in processes such as acetylation, and deubiquitination, thereby controlling gene activation and repression (Vikki, 2008). While ubiquitination-related processes have been extensively studied, an increasing interest has arisen around regulation of chromatin dynamics and gene expression articulated via counteracting of ubiquitination enzymatic activity on specific targets. As part of the SAGA deubiquitination module (DUBm), crucial players in the ubiquitin-proteasome system as well as in autophagy are the DeUBiquitinating enzymes (DUBs), one of the largest classes of proteases. Among them, Ubp8p is localized to sites of interaction with other SAGA components and together with Bre1p watches over the detailed dynamic of ubiquitination/deubiquitination of H2B histone. Genetic interactions of an opposite nature are associated with deletions of *UBP8* (negative interaction) and *BRE1* (positive interaction) in *tif2Δ* SGA: this results is likely to reflect the genes' respective roles: Ubp8 SAGA-associated protein is required for deubiquitination

(Henry, 2003) of H2B, while Bre1p, an E3 ubiquitin ligase for Rad6p, coordinates the ubiquitination of H2B, contributing in this way to optimal gene activation.

In case of cellular stress, with the aim to decrease the biosynthetic workload on the ER, the UPR inhibits the initial stages protein synthesis, for example, via degradation of protein products, among them even Tifp (Tagwerker, 2006). As more proteins are processed through the proteasome an increased need for ubiquitin tags arises and there is pressure on the other structures to release ubiquitin tags. This suggests that under certain cellular circumstances, ubiquitin associated to histonic structures can be targeted for recycling and made available for other processes. When the cellular system functions with a faulting production of proteins, the ubiquitin itself becomes scarce because of lack of newly synthesised ubiquitin, exacerbating the tags' availability issue. Furthermore, there is a possibility that with a shortfall in protein synthesis the protein products with short half lives (including those prone to ubiquitination) become lost. Although purely speculative, different scenarios could be conceived: As the protein products with short half lives (including those prone to ubiquitination) become lost there is the possibility that proteins associated with the histones become degraded because of their ubiquitination but then cannot be replaced. Alternatively, due to the general lack of cellular ubiquitin, once the ubiquitination process has taken place, it cannot be re-established. Following this reasoning, the loss of histone ubiquitination might ultimately lead to changes in expression that allowed a cell to cope with the stress of depleted protein synthesis: this is the rationale behind the issue with loss of *UBP8* gene. A cross-talk between translational and transcriptional processes can be envisioned and when translation is delayed by UPR, transcription of some genes may have to be

adjusted accordantly. This may be actuated, among other mechanisms, by affecting Ubp8p, which established function in deubiquination of H2B is likely to be one of the most critical in managing and maintaining of transcriptional processes, as it is ultimately necessary for transcriptional elongation to proceed. There may be the possibility that Ubp8p becomes over expressed in response to faulty translation, shifting the equilibrium of the transcriptional process towards the elongation phase. In the case of *ubp8Δ tif1Δ* double mutant, cellular death may occur as in the absence of Tif1p and Ubp8p contributions, the cell lacks the intercommunication that enables it to adjust the transcriptional process quickly enough. Given the physical interaction (affinity capture MS) annotated for Tif1p and Ubp8p (Lee, 2011), a “direct” communication between Tif1p and Ubp8p might be proposed. Although *TIF1* and *TIF2* genes encode an identical protein product (Tifp), no physical interaction has been reported for Tif2p-Ubp8p. A modest importance, however, should be given to this observation, in consideration of the promiscuity of protein-protein interactions. This thesis additionally postulates that Ubp8p might be required for actioning some of the changes required by loss of Tif1p. Furthermore, without Ubp8p, there is a cellular shortage of ubiquitin, and this cannot be replenished in the situation of decreased Tifp.

Moreover, *UBP8* has been linked to mRNA export, for example it has been shown that loss of *SGF73*, the gene that activates Ubp8p-deubiquination activity, results in a defected export of *GAL1* mRNA. The cell could try to overcome this fault in the mRNA export system by increasing translation, for example of specific mRNAs that are kept in reserve for occasions in which the export of new mRNAs cannot function at full range.

Factors that influence H2B ubiquitination have been identified and point to a series of events involved in H2B during transcription. In this scenario Bre1p and Rad6p (this latter gene is not among this study's SGA hits) have been proposed to first recruit to their promoters and then associate RNA Pol II as it begins the process of transcription. It may be possible that the deletion of *TIF2* is received by the system as a signal of a decrease in protein synthesis and *BRE1* could over express in response to this stimulus inducing methylation and consequentially transcription initiation. When translation struggles, Bre1p (and perhaps Rad6p) take action and ubiquitinate more H2B ultimately augmenting gene activation and so transcription initiation activity. In the absence of Bre1p activity, a great amount of H2B may undergo RNA Pol II-mediated elongation. This could imply will be more expression from the histone genes *HTB1* and/or 2 and possibly elongation of *HTB1* or 2 beyond the normal termination. As a result, an increased amount of H2B would go through elongation. The resultant anomalous number of transcripts, which are unrelated to the transcription signalling, may be deleterious for the cell (**Figure 2-28**). However, it appears that Bre1p role remains to be fully elucidated as, for example, Rad6p requires Bre1p for its recruitment independently from H2B ubiquitination status (Kao, 2004).

Figure 2-28. SAGA-related mechanisms of transcription. Genes interacting with *TIF2* (this study) are bordered in orange.

Altogether, all these considerations assert the importance of Bre1p/Ubp8p control on chromatin status and ultimately suggest different levels of intervention on the dialogue between transcription and translation initiation. It is concluded from this research study that when translation initiation needs to be modulated, a way to control transcription concurrently is to have a regulatory network surrounding the most powerful genes in affecting the histone status.

Ubiquitination and histone methylation play complementary roles in the regulation of transcription, operating under different timescales. Although a so-called cross-talk between H2B ubiquitination and other histone modifications

has been established, studies indicated that in the case of H3 methylation, the connection is unidirectional as mutations affecting H2B affect H3 methylation but not vice versa (Briggs, 2002). This organization is confirmed in our data through the paucity of genes involved in the H3 methylation process genetically associated to the translation initiation process. Both *UBP8* and *BRE1* genes share the involvement in the regulation of histone H3 methylation, with yeast Ubp8p additionally annotated as part of the SLIK complex and its activity required for differentially regulating H3 methylation at some SAGA promoters. The cell benefits from the simplicity of an extended signalling regulation designed around the H2B histone as its status holds an indirect and efficient control on H3. Furthermore, whilst histone ubiquitination is rapidly reversed by DUB enzymes and allows quick control, histone methylation can remain stable long after transcription ends, maintaining the chromatin in a more readily accessible conformation for the transcription machinery (Ng, 2003).

Deletions of *UBP8* affects only *TIF2* deletion, whilst deletion of *BRE1* produces suppressor-like phenotypes in both *TIF1* and *TIF2* KO screens. This difference suggests a differential and fine regulation on the ubiquitination and deubiquitination processes, which is not totally surprising given the different regulation associated to single enzymatic steps in the ubiquitination-deubiquitination process. The observation that histone deubiquitination may be the result of an altered equilibrium in the ubiquitin cycle has been reported in a previous study, although the observation was confined to the stress caused by impairment of the proteasome structure (Dantuma, 2006).

Strikingly, no genetic interaction was found among genes that encode for other de-ub H2B enzymes. An explanatory example may be the viable phenotype

associated with *tif2Δ ubp10Δ* strain. Although Ubp8p and Ubp10p perform the same post-translational modification, they work on different pools of ubH2B (Schulze, 2011); this result aligns with a proposed separated role for *UBP10*, likely in silencing and telomere maintenance.

Structural studies also revealed as Ubp8p does not work alone but controlled by its associated factors. Induced allosteric changes within Ubp8p regulate its activity by binding to other partners, supposedly other components of the DUBm such the identified SL Sgf11p (Ingvarsdottir, 2005). This deduction is supported by the findings that, as an isolate recombinant protein, Ubp8p is inactive (Lee, 2005) and its ZnF-UBP domain -which is required to bind to SAGA- is unable to bind free ubiquitin (Ingvarsdottir, 2005). SAGA integrity is required for Ubp8 deubiquitination activity, as mutations disrupting the complex (for example *sgf11Δ*) increase the level of ubH2B (Powell, 2004). As a lethal phenotype has been reported for both *SGF11* and *UBP8* genes, Sgf11p may be accounted as one of the functionally interacting Ubp8p partners. Sgf11p is annotated as an integral subunit of SAGA histone acetyltransferase complex that allows regulation of transcription of a subset of genes. It has recently been identified as part of the SAGA DUBm (Lee, 2005) where it plays a role in the Ubp8p association with SAGA and H2B deubiquitination. As expected, strains lacking *SGF11* encounter problems in the recruitment and association of Ubp8p with SAGA, which becomes difficult or impossible; the SL phenotype generated by combination of *sgf11Δ* and *tif2Δ* is not necessarily to be attributed to a direct interaction this specific pair of mutants as there is the possibility that this lethal phenotype is the result of an indirect effect of *sgf11* preventing Ubp8p from interacting with SAGA properly. In this scenario Ubp8p is present and potentially

functional but it cannot be associated, showing how important Sgf11p contribution is in regulating the association of Ubp8p with SAGA complex, as it likely changes the conformation allowing the ubiquitin on.

Work from independent groups reported higher eukaryote orthologues for both yeast *UBP8* and *SGF11*. The human gene USP22 (yeast *UBP8*) is of particular interest since studies identified it as component of an 11-gene signature associated with prognosis of diverse types of cancer (Glinsky, 2005); more recent work done in McMahon's laboratory further showed how USP22 functions as a subunit of human SAGA (hSAGA) and it is required for cell-cycle progression, with its depletion leading to arrest of the cell cycle in G₁ phase (Zhang, 2008). Supported by a convincing body of evidence (Liu, 2003; Kurabe, 2007), Zhang's results align with the hypothesis that hSAGA is linked mechanistically to human cancer and, in this scenario, USP22 could regulate tumorigenesis by inducing histone modification. It has been argued though, that being as USP22 is the only component of the hSAGA complex reported among the 11 ORFs listed in the oncogenic signature, and this gene's effect could be SAGA-independent (Rodriguez-Navarro, 2009).

Yeast *SGF11* and *UBP8* genes are believed to be part of the same DUBm that also includes *SUS1*. Whilst both *SGF11* and *UBP8* deletion strains were reported in this study as holding genetic interactions with *TIF2*, the deletion of *SUS1* strain was unavailable in our yeast collection and consequentially participation of this particular module in translational control could not be tested. Nevertheless, literature research revealed genetic interactions between *SUS1* and *TIF2* (Costanzo, 2010) and a physical interaction with Tif4631p (Cuenca Bono, 2010). Interestingly the association of this module is under the

competence of *SGF73* deletion, however, with the dual *tif2Δ sgf73Δ* is viable. This result suggests that *SGF73*'s role is limited to the cooperation with other components of SAGA and their correct assembling, however, it may have little to do with regulation of protein synthesis.

Elucidation on the exact correlation among *SGF73*, *SUS1*, *UBP8* and *TIF2* may be worthwhile as it could potentially be informative on the proposed *UBP8* role in mRNA export. A direct role in mRNA export can be hypothesised for genes (e.g., *GAL1*) for which an mRNA export defect has been noted (Gwizdek, 2006) that are localized to the nuclear periphery. Loss of *SGF73*, the gene that activates Ubp8p deubiquitination activity, results in a defect in the export of mRNA of *GAL1*. This may reflect the control actuated on translation via different directions, for example mRNA export. There could be a defect in translation that may be overcome by increasing the export of mRNAs or vice versa. There could be a defect in the export of mRNAs which is overcome by increasing translation of the remaining mRNAs, as it cannot export at full range; this is the case, for instance, for specific mRNAs that the cell has in reserve ("translation on demand") (Brockmann, 2007). It may reflect a mechanism of regulation of protein synthesis for conditions under which mRNA export from the nucleus is compromised.

Finally, although SAGA accounts for two enzymatic activities, only deletion of *UBP8* resulted in a SL with *tif2Δ*, whilst the deletion of *GCN5*, gene through which SAGA controls acetylation, was not synthetically lethal with *tif2Δ*. This result endorses the versatility of SAGA as highly competent machinery in which different sub-modules can co-exist. Different modules of SAGA are required for different stages of gene activation, as such complex accounts for distinct units that collaborate to maintain structural integrity, genomic

recruitment and interaction with the transcription machinery (Koutelou, 2010). Genetic interactions between *TIF2* and some of SAGA components reveal a dense specialized genetic network of SAGA elements surrounding the *TIF2* gene, which ultimately has critical implications in translation initiation and protein synthesis.

Modifications involving H2B chromatin, in particular its ubiquitination, appear to be part of a highly dynamic regulatory circuitry, as an alternating temporal cycle of H2B ubiquitination followed by deubiquitination is required for optimal gene activation. Gene control by DUBs may involve a wide range of distinct mechanisms, among them the control on the level of subcellular localization of key transcription factors in response to signalling. An emerging theory suggests that associated partner proteins control the activity and specificity of DUBs and selective DUBs can be targeted to specific genomic loci by transcription factors. This representation is in accordance with the discussed expanded role of DUBs as crucial regulators of chromatin dynamic and gene expression due to their counteracting ubiquitin enzymatic activity on specific targets. Results from this SGA screen suggest a strong control of transcription actuated through selected components of the SAGA complex only.

Fuelling the interest on the relevance of SAGA transcriptional-regulated mechanisms is the finding that *CHD1* gene -deletion of which is SL with *TIF2* query strain has been shown to be involved in chromatin remodelling, gene expression and transcriptional elongation. Pray-Grant and colleagues identified it in a mass spectrometry study as a component of SLIK (SAGA-like complex) (Pray-Grant, 2005) as Chd1p physically interacts with the SAGA and SLIK complexes and binds histone methylated tail peptides (Pray-Grant, 2005). Chd1p

is the only yeast member among the Chd family of proteins that contain two N-terminal chromodomains, a central helicase/ATPase domain and a DNA binding domain at the C-terminus. More recently, an additional role for this gene was proposed (Biswas, 2008) in which Chd1p opposes to the function of the positive transcription factor yFACT (facilitates chromatin transcription) (Orphanides, 1999). The yFACT complex promotes RNA Pol II transcription and plays a role in regulating transcription (Ausubel, 1987) and DNA replication (Wittmeyer, 1999), together with a regulatory role on TATA binding protein (TBP) during the initiation step of transcription (Biswas, 2005). Transcription initiation by RNA polymerase II is dependent on a set of transcription factors, including the TBP, which recognize the core promoter and facilitate initiation from the correct start site (Conaway, 1998) on which Chd1p and yFACTp have opposing roles in regulating TBP binding at promoters. Chd1p is thought to promote formation of inhibitory chromatin (Robinson & Schultz, 2003), in this way controlling chromatin assembly.

This SGA screen also brought attention on the genetic surroundings of *RTF1* and *CHD1* genes, as both deletions resulted in SL phenotypes when paired with *tif2Δ*. *S. cerevisiae* Paf1 complex colocalizes with RNA Pol II during transcription elongation and it is required for the normal expression of a subset of genes (Krogan, 2002). Mutations in components of the Paf1 complex, including RNA polymerase II-associated protein Rtf1p, cause a loss of H2B ubiquitination (Ng, 2003). Noticeably, *RTF1* was the only one among five components of the Paf1p complex to be identified as hit in this research SGA screens. The fact that *RTF1* is annotated as the most significant contributor in regulating modifications such as H2B ubiquitination and H3 methylation (Mueller, 2004; Porter, 2005)

may give the reason for this finding. Furthermore, monoubiquitination of H2B by Rad6p-Bre1p (discussed previously in the text) is eliminated in strains lacking Rtf1p or the Paf1 complex; such modification appears of fundamental importance as it represents the pre-requisite for H3 methylation and suggests a control of H2B ubiquitination on H3.

A physical interaction between Chd1p and Rtf1p has been described and it is ultimately thought to promote association with active ORFs (Simic, 2003). With Chd1p functioning as a component of SAGA and SILK complexes, the interaction between Rtf1p and Chd1p may serve to synchronize the activities of histone-modifying enzymes. Additionally, the involvement of Rtf1p in processes such as histone modification, recruitment of Chd1p and, to minor extent, telomeric silencing suggests that Rtf1p influences on transcription may be actuated via alteration of chromatin structure. Although yet not backed up by experimental evidence, the intriguing possibility that Rtf1p mediates effects on chromatin structure by recruiting Chd1p to active ORFs cannot be entirely ruled out: in this scenario Rtf1p would stabilize Chd1p association by promoting methylation of H3 histone. As both *CHD1* and *RTF1* hold a negative genetic interaction with *TIF2*, questions could be raised on the nature of the genes that could be regulated in this way and whether translation gets a say on which subsequent mRNAs are recruited.

Three additional SL hits linked to RNA Pol II activity were also identified, suggesting an inter-control of the translational machinery on gene expression, possibly mediated via activity of the aforementioned enzyme. The first ORF, *YGL024W*, has been reported as dubious; however, it partially/completely

overlaps the verified gene (PolyGlutamine Domain) *PGD1* (Fisk, 2006). Pgd1p, is a subunit of the RNA Pol II mediator complex, associated with core polymerase subunits to form the RNA Pol II holoenzyme and regarded as essential for basal and activated transcription (Guglielmi, 2004). Deletion of the *PGD1* gene itself is not available in Boone's YDS library and for this reason it could not be tested. *YGL024W* is a direct target of Cyc8p-Tup1p transcriptional corepressor, a duo of protein that functions in a complex to mediate repression of a certain subset of genes (for instance, those involved in osmotic stress and DNA response damage) (Lee, 2000). Such co-repression appears to be important as Cyc8p-Tup1p can suppress transcriptional activity by packing nucleosomes into repressive chromatin structures (Papamichos-Chronakis, 2000).

A second gene, *ASK10*, encoding for a RNA Pol II holoenzyme component, was identified in this SGA. Ask10p becomes phosphorylated in response to oxidative stress in a MAP kinase-dependent fashion and it functions in activation of the aquaglyceroporin Fps1p, an aquaporine protein that regulates the plasma membrane permeability via its glycerol channel, upon changes in cellular osmolarity environment (Page, 1996). Ask10p-mediated function appears quite important as the MAP kinase pathway transmits an oxidative stress signal to the RNA Pol II holoenzyme through phosphorylation of Ask10p itself (Beese, 2009). Interesting in the context of duplicate genes is the observation that *ASK10*, also known as *RGC2*, has a paralog, *RGC1* (Beese, 2009), which did not display any lethal or sick growth phenotype associated with its deletion, when paired with a deficient translation system.

Finally, *HAP5* encodes for a subunit of the heme-activated, glucose-repressed Hap2/3/4/5 CCAAT-binding complex, a transcriptional activator and

global regulator of respiratory gene expression, also required for assembly and DNA binding activity of the complex (McNabb, 1995). It contributes to RNA Pol II sequence-specific DNA binding and is involved in positive regulation of the transcriptional process (McNabb, 1997).

In summary, a number of genes reported in this study hold genetic interactions with *TIF2* gene associated with the function of the RNA polymerase II enzyme. The described SL interactions are believed to arise from a lack of cellular ubiquitin that cannot be replenished in a faulty translational cellular background. Further evidence of cross-talk between transcription and translation are endorsed by observed genetic interactions between *TIF2* and the SAGA complex: this latter acts as one the main mediators of histone modifications on gene promoters, ultimately enhancing the transcriptional activation and facilitating the elongation by deubiquitinating histones downstream of RNA Pol II. Furthermore, the ubiquitination-deubiquitination status of the H2B histone emerges as critical among transcriptional-related steps in addressing mechanism of gene expression that are required to be activated upon certain type of cellular challenges.

TIF2 holds a negative genetic interaction with *RIM101*, a gene first identified in baker's yeast as a positive regulator of early meiotic gene expression (Su & Mitchell, 1993) and lately shown to behave as a positive regulator of haploid invasive growth (Li & Mitchell, 1997). Annotated literature also describes *RIM101* alkaline-pH-induced gene expression competence and its role in general ion homeostasis (Lamb, 2001). The ability of Rim101p to be proteolitically processed to its active form and to act as a repressor at the

negative regulatory element (NRE) depends on well characterized components of the Rim signalling pathway and requires *DFG16*, *YGR122W* and components of the ESCRT trafficking pathway (Rothfels, 2005). Supporting the idea that Rim101p signalling pathway has an important involvement, both *DFG16* and *YGR122W* deletions appear along with *RIM101* in the screen as ultra sensitive deletions SL associated with *tif2* query strain. Ygr122wp, a non-ESCRT protein, is identified as contributor to Rim101 processing (Rothfels, 2005) and regulation (Xu & Mitchell, 2001); Dfg16p is a probable multiple transmembrane protein, involved in diploid invasive and pseudohyphal growth upon nitrogen starvation and it is required for accumulation of processed Rim101p. Since these genetic interactions are exclusive for *tif2Δ*, the intercommunication between translation initiation and Rim101 signalling pathway seems to be allocated to one of the *TIF* paralogs exclusively. Interestingly, deletions of genes involved in the proteolytic activation of Rim101p were identified as common between the two paralogs (further discussed in **section 2.38**). Lastly, the *YEL057C* ORF, deletion of which resulted in a SL phenotype, encodes for a protein of partially unknown function and it is annotated as involved in meiotic gene expression as target of (Unscheduled Meiotic gene Expression) UME6 (Williams, 2002), a key transcriptional regulator of early meiotic genes that supports coupling metabolic responses to nutritional cues with initiation and progression of meiosis.

As it might be expected a gene directly involved in translation-related processes was also identified in *tif2* SGA; surprisingly its annotation describes it as part of the translation initiation cap-independent process: *GIS2* encodes for a translational activator for mRNAs with an IRES, cytoplasmic Gis2p associates

with the eukaryotic ribosome and acts as regulator of cap-independent translation. Gis2p functions are evolutionarily preserved in higher eukaryotes, as part of a conserved mechanism to control translation of specific mRNA molecules that contain an IRES (Tsvetanova, 2010). Its human ortholog gene, ZNF9, has been reported to be involved in myotonic dystrophy type 2 (Sammons, 2011).

Although under certain conditions, inhibition of protein synthesis may seem a counterintuitive cellular choice, this choice could be explained by the differential regulation of mRNA, as reported under specific stress conditions (Spriggs, 2008). The fact that the translational process can be adjusted and its connections rewired allows translation of selective proteins and relies on upstream ORFs in the 5' untranslated region of such mRNAs. Quite often encoding for protein products essential to cellular stress response are translated using alternative mechanisms of translation initiation, such as IRES (Bushell, 2006). It is nowadays accepted that specific mRNAs are able to escape the overall inhibition of protein synthesis (Bushell, 2006) and a contingent of these mRNAs.

A genome-wide study revealed the existence of a physical interaction between Gis2p and Sro9p (Tarassov, 2008). Sro9p, first discovered as multi copy suppressor of an RNA export defect, was later shown to associate with polysomes (Sobel & Wolin, 1999); in particular Sro9p associates with nascent transcripts and forms part of an mRNP complex that is exported to the cytoplasm, where it (Sro9p) then acts as a regulator of translation (Rother, 2010). Although no genetic interaction was identified between *TIF2* and *SRO9* genes, the physical interaction between Gis2p and Sro9p makes wonder whether a correlation between mRNA export and translation initiation may signal through this interaction, possibly through *GIS2*. Integration of a signal between

translation initiation and mRNA transport from *GIS2* to *SRO9* may be essential as, in absence of Gis2p, Sro9p struggles to counteract the mRNA export defect with the amount of mRNAs shuttled to the cytoplasm dramatically decreased; translation initiation is enhanced to make the most of the mRNAs already present. In a strain missing the *TIF2* gene, translation initiation cannot be augmented sufficiently, either because this signal is dealt with by *TIF2* only and *TIF1* is unspecialized to perform this type of signal processing as it misses the network of interactions of its counterpart gene and/or because more protein product is encoded by *TIF2* transcripts and although Tif1p production may be enhanced, it is still not satisfactory.

Finally, this screen identified that deletion of the *SIT1* gene, encoding for a ferrioxamine B (FOB) transporter member of the ARN family of proteins (Lesuisse, 1998), as phenotypically suppressor when paired with *tif2Δ* query strain. All organisms require iron as it is essential in diverse biological processes, for instance respiration and oxidation-reduction pathways. At physiological pH, the majority of this element is presented in an insoluble ferric form (Fe^{3+}) and accessible for cellular use. Excess of iron inside the cell promotes the formation of (reactive oxygen species) ROS, with potential damage to DNA, lipids, and proteins. Organisms have developed different mechanism to take up and transport iron; *S. cerevisiae* has two different pathways functioning in the absorption of this element: the first one is the reductive iron up taking pathway (see Philpott, 2006 for more detailed reading); the second one relies on siderophore-iron complexes, iron chelators that bind ferric ion with strong affinity (de Freitas, 2003). Among these latter, the Arnp family of transporters -Arn1p,

Arn2p, Arn3p (alias Sit1p) and Arn4p- functions in the uptake of the siderophore-iron complexes; each of the Arn proteins is specialized for conveying different kinds of siderophores. In particular, Sit1p exhibits specificity for both ferroamine, including FOB and ferricrocin, and cells deleted of *SIT1* gene become unable to take up FOB. Sit1p is potentially phosphorylated by Cdc28p and physically interacts with Ubi4p (Erpapazoglou, 2008) while *SIT1* holds a negative genetic interaction with *VPS27* (Aguilar, 2010), deletions of which both appeared as SL in this thesis screens.

Functional trafficking of membrane-bound metal transporters is not only essential for protein function in order to maintain cellular ion homeostasis but, it also represents a notable feature of post-translational regulation. Aft1p, a major regulator of the so-called iron regulon, is located in the nucleus in the absence of iron, where it induces transcription of certain genes but, as soon as the metal concentration is sufficient for the cellular needs, Aft1p is exported to the cytosolic compartment with associated reduction of expression of the previously induced target genes. Sit1p domains have been suggested to interact with Aft1p, holding thus an effect on ubiquitination of specific sites and guiding this way the intracellular trafficking and degradation of Sit1p (Jeong, 2009). Interestingly Aft1p is also one of the “documented” transcription factors for the *TIF3* gene (Harbison, 2004). Deletion of *SIT1* could lead to some level of iron deficiency and this could result in upregulation of genes sharing the Aft1p transcription factor, *TIF3* among them. As a result, *TIF3* (eIF4B) expression increases and the phenotype of eIF4A deficiency is suppressed.

Noticeably, Aft1p is also one of the “documented” transcription factors for the *TIF3* gene (Harbison, 2004). Given that, as a siderophore-dependent

facilitator, Sit1p functions in what is considered a high affinity iron uptake pathway (Kosman, 2003), its participation might be particularly important for ensuring the physiological iron uptake. Such iron deficiency caused by the loss of Sit1p contribution could result in upregulation of genes sharing the Aft1p transcription factor, *TIF3* among them. As a result, *TIF3* (eIF4B) expression increases and enhances Tifp activity (as introduced in **section 1.8**): in this case, the phenotype expected from an inefficient translation, is suppressed. As no genetic interaction was observed for *SIT1-TIF1* combination, the different phenotypes observed may reflect the different regulation of the two paralogs and could be dictated by a preferential relationship between *TIF3* and *TIF2* genes, through which the cell finds more beneficial to upregulate *TIF2* in combination with this type of event.

Recent studies have link UPR activation with cellular iron metabolism, as suggested by the modulation of ferroportin and ferritin H expression resulting from ER insults (Olivera, 2009). Data from the above publication suggest that the mRNA enrichment observed for both ferroportin and ferritin encoding genes in cells affected by ER stress could represent an attempt to bypass the intracellular deposition of free iron, either through its sequestration or export, respectively. The functional intersection between ER stress and iron homeostasis has been excellently reviewed by Olivera and colleagues (2010): dysregulation of iron metabolism emerged from studies into hereditary hemochromatosis (HH) type 1, one of the most common genetic disorders in northern European populations. (Pietrangelo, 2004) that evolves from a defective regulation of iron absorption. The hypothesis that diverse factors could modify this genetically determined disease was hinted by the outstanding phenotypic heterogeneity reported among

patients (Olynyk, 1999). Modifiers of the clinical traits of HH disorder have been investigated (Cruz, 2008), and the UPR appears to be a potential candidate, which could be probed in further studies.

2.38. *TIF1* and *TIF2* genes functional profiling

This research study revealed a number of common synthetic sick/lethal interactions between *TIF1* and *TIF2* genes, underlying functional competences shared by the duplicate pairs. Evidence is given for functional redundancy between the two paralogs with their full co-participation prominently represented in processes such as response to alkaline pH, whilst cellular responsiveness associated with processes such as vesicular trafficking and precise segments of transcription appear to be rather exclusive for each duplicate.

As reported for both *tif1Δ* and *tif2Δ* SGAs separately, few deletions of genes involved in chromatin-related processing were also identified as SL hits mutual to the two paralogs. Among them, the *SGF29* gene has been only recently properly annotated thanks to work done by Bian and colleagues (2011), which showed through an extensive crystallography/*in vivo*/*in vitro* approach how Sgf29p recognizes methylated H3 histone to recruit SAGA to its targets sites and intervening in this way in acetylation of H3. Both genetic and physical interactions are annotated in the literature for *SGF29*/Sgf29p and *CHD1*, *SGF11*, and *UBP8* (all reported as SL in *tif2Δ* SGA) and their protein products. As previously discussed for the SAGA-related hit *sgf11Δ*, the functional connection

suggested by this genetic interaction, could find its explanation in an inter-talk between translation initiation and transcription mediated via SAGA components.

Additional control on non-histonic chromatin is also suggested by lethal phenotypic outcomes that arise from combination of *TIF1* and *TIF2* KO with *nhp10Δ* and *ies5Δ* strains. *NHP10* encodes for a protein related to mammalian high mobility group proteins and is annotated as a likely component of the chromatin-remodelling INO80 complex (Ino80-C). It is also believed to hold a role in DNA repair as Nhp10p preferentially binds DNA ends, protecting them from exonucleatic cleavage. The second gene, *IES5*, encodes for a protein that associates with the Ino80-C under low-salt conditions. Although the role of chromatin remodelling in the context of translational control has been discussed in the previous chapter, a reflection should be made on the nature of chromatin-related processes that involve the duplicate pairs together rather than alone. It appears that the competence shared by both duplicates in the matter of chromatin remodelling is limited to stress responses such as DNA repair (*NHP10*) and low-salt conditions (*IES5*), with no hints to genetic connections between translation and histone ubiquitination status, as observed for *TIF2* genetic profile. Although Ino80-C shares a common core of conserved subunits in yeast and higher eukaryotes, where it contributes to a general variety of chromatin-dependent related processes including DNA repair and DNA replication and transcription (Conaway & Conaway, 2009), studies suggest that this complex diverged substantially during evolution acquiring new subunits with apparently species-specific functions.

Interestingly, Ino80-C interacts with the early NER damage recognition complex Rad4-Rad23 (previously encountered in **section 2.36**) and is recruited

to chromatin by Rad4 in a UV damage-dependent fashion; a modulation of chromatin structure is believed to happen during excision repair, although the exact mechanism is still unknown. Recent publication from McHugh's laboratory showed that *INO80* functions in the same genetic pathway as the NER machinery (Sarkar, 2010) and is required for the restoration of chromatin structure after repair. Chromatin becomes a target of extensive disruption to allow efficient NER, however, its structure is restored soon after the repairing stage –the "access–repair–restore model"– (Dinant, 2008), possibly as a result of targeted translation of selective mRNAs that cause the synthesis of chromatin remodelling proteins to increase (Bushell, 2006).

Of particular interest, given the considerations made regarding the equilibrium of the lysosome compartment as previously discussed for the *tif1Δ* SGA, is the SL reported for both *TIF1* and *TIF2* KO strains when paired with *ydr455cΔ*, a dubious ORF which overlaps with *YDR456w* (alias *VPS44*). Vps44p is a Na⁺/H⁺ and K⁺/H⁺ exchanger, required for intracellular sequestration of Na⁺ and K⁺ and located in the vacuole and late endosome compartments, where it is needed for osmotolerance to hypertonic shock and vacuolar fusion.

The lysosome represents an important compartment for Ca²⁺ signalling (Galione & Churchill, 2002) and is characterized by concentrations of this cation comparable to those in the ER (Miyawaki, 1997). This degradative structure is filled with Ca²⁺ via the action of not completely understood ion transporting ATPase and/or solute channels. Ca²⁺ transport could be directed against the proton gradient or involve a mechanism in which Ca²⁺ and Na⁺ transports are coupled (Na⁺/H⁺ and Na⁺/Ca²⁺ transporters). Given Vps44p role in vacuolar

function, its ion exchanging nature, and also considering that the mechanism for which Ca^{2+} enters the lysosome is proton-dependent (Christensen, 2002), it is tempting to propose Ydr455cp as one of the channels coupled with Ca^{2+} transport into lysosomes.

Ca^{2+} accumulation in late endosomal and lysosomal structures is critical for the cell functioning as normal endocytic function is coupled with lysosomal Ca^{2+} homeostasis; Ca^{2+} release, on the other hand, represents both requirement and trigger for the final step in the fusion between docked endosomes and lysosomes complex assembly (Pfeffer, 1999). The majority of Ca^{2+} is released into the cytosol compartment from early endosomes in response to the initiation of acidification of the endolysosomal system.

Deletions of three genes (*RIM13*, *RIM20*, and *RIM8*) belonging to the RIM family of genes were reported as SL in combination with both *TIF1* and *TIF2* KOs: these ORFs all share an involvement in the signalling pathway in the response to alkaline pH via their participation to the proteolytic activation of Rim101p. Two of three deletions also display interactions with components of the ESCRT pathway: Rim20p cooperates with ESCRT-III subunit Snf7p while Rim8p interrelates with ESCRT-I subunits Stp22p and Vps28p. The third hit, Rim13p, is a calpain-like cysteine protease involved in proteolytic activation of Rim101p in response to alkaline pH. This subset of genes suggests an affiliation between pH response and MVBs formation that could further enrich the network of interactions revealed so far for *TIF1* paralog in concern to vesicular damage.

Environmental pH impacts cellular growth, differentiation, and viability. For this reason an evolutionary development that generates a response system

that intervenes to counteract the effects of external alkalinity should be favoured, given the debilitating nature of disruption of membrane proton gradients for the cell. Such gradients normally supply energy for nutrient and ion transport (Van der Rest, 1995). It is quite remarkable that both paralogs conserved an interaction with alkaline response genes as, this particular subset of ORFs exhibits unique attributes that distinguishes it from other growth limitation conditions. This group does not correspond entirely to other sets of starvation or stress-induced genes; furthermore, alkaline response genes are not consistently induced by ordinary physiological triggers such as stationary phase, heat shock, or lack of calcineurin. In other words, the response to alkaline pH may be due to a combination of stress and starvation response, which shares essential functional implications with *TIF1* and *TIF2* pair of duplicates.

The genetic interactions between mitochondrial-related genes and *TIF1* paralog has been commented on in **section 2.36**: whilst interaction of *MMM1*, the gene encoding for the ER-resident Mmm1p component of the ERMES, seems to be mediated via *TIF1* gene only, both duplicates show a functional connection with the *MDM34* (Mitochondrial distribution and morphology, alias *MMM2*) gene, the product of which is an outer membrane protein that is also a component of the ERMES, which is discussed in the paragraph below. While it is arguable that the sensitivity of the revelation system may be marginally inexact when dealing with petite or slow growing strains and, there is thus the possibility that the observed genetic interaction is not exclusive for *TIF1* and *TIF2* genes, it is encouraging to find the deletion of *YGL218W*, an ORF overlapping to *MDM34* as

SL in the paralogs screens, although it needs to be noticed that *ygl218wΔ* itself has been annotated as petite.

Differently from the majority of the organelles, mitochondria do not exchange phospholipids via vesicular transport: an association between ER and mitochondria is necessary for the exchange to take place (Achleitner, 1999). Recently, a complex named ERMES -ER-mitochondria encounter structure- has been described: it consists of proteins located both at the mitochondrial and ER sides and acts as a molecular connector between these two cellular compartments, where is required for efficient inter-organelle phospholipid exchange. Given the extent degree of lipid trafficking between ER and mitochondria outer membrane, the location of the ERMES appears critical; this exchange has been suggested to be mediated by the synaptotagmin-like, mitochondrial and lipid-binding protein (SMP) domains of the ERMES subunits themselves (Kopeck, 2010), which are capable to assemble into structurally well-defined complexes, establishing this way a lipophilic, tubular path between the two membranes. Kornmann and colleagues (2009) showed through genome-wide mapping of genetic interactions that the components of the ESCRT complex (Mmm1p/Mdm10p/Mdm12p/Mdm34p) are functionally connected to phospholipid biosynthesis and Ca^{2+} -signalling genes. Their work satisfactorily showed that phospholipid biosynthesis is impaired in mutant cells, where the ESCRT complex localized into foci, thus suggesting that distinct sites of intimate contact between ER and mitochondria help the exchange of both Ca^{2+} and phospholipids in between the organelles (Kornmann, 2009). Such a connection between ER and mitochondria might have relevance in buffering and controlling of both cytosolic and mitochondrial Ca^{2+} concentrations (Pinton, 2008). In virtue

of ERMES tether function in connecting the ER and mitochondria and allowing transport of phospholipids between these compartments, the disruption of the complex, which likely takes place in the tested ERMES deficient KO strains would cause some components of the complex to partition with the ER, whilst others would end up on the mitochondrial side. The Ca^{2+} deposited in the ER is depleted following grave stress conditions, and the cellular response upon perturbation of ER Ca^{2+} homeostasis involves, among the others, the suppression at the initiation step of protein synthesis. At the same time, serious stresses recall an enormous amount of Ca^{2+} into mitochondria, increasing the influx of the bivalent cation into the respiratory organelles; this latter physiological response is believed to trigger the production of free radicals, the opening of the mitochondrial permeability transition (MPT) pore -identified as an inducer of cell death (Matsumoto, 1999)-, and perturbations of energy required for cellular metabolism. Deletions of *MDM10* and *MDM12* are annotated as 'wild-type SL' in Boone's SGA set and for this reason not highly informative, as these types of deletion strains, although being viable on their own, are prone to SL with the majority of the other deletions present in the array.

Another identified interaction was with *CEM1*, a gene encoding for a mitochondrial β -keto-acyl synthase that holds a possible role in fatty acid synthesis and it is required for mitochondrial respiration. The fact that no corroborating deletions were reported as sensitive in this study, along with the 'petite' annotation which labelled this strain, make us wondering whether the SL observed may be caused or augmented by a respiratory system impaired in the first place.

As previously identified in both *tif1Δ* and *tif2Δ* screens separately, some translation-related processes appear as SL in both duplicates' SGAs: *TEF4* encodes for Tef4p, a gamma subunit of translational elongation factor eEF1B, that stimulates the binding of aminoacyl-tRNA to ribosomes by releasing eEF1A (Tef1p/Tef2p) from the ribosomal complex. *RPL9a* gene encodes for a protein component of the large (60S) ribosomal subunit.

One SL hit that seems to not find placement into the functional groups annotated so far is reported below is *VRP1*, which encodes for actin-associated Vrp1, a protein involved in cytoskeletal organization and cytokinesis. Loss of Vrp1p leads to a partial loss of cortical actin patch polarization during the cell cycle (Donnelly, 1993) and it has been related to mammalian Wiskott-Aldrich syndrome protein interacting protein.

Although the actin cytoskeleton and the translation machinery are considered to be separate cellular complexes, growing evidence supports overlapping regulation of the two systems. In fact, the eukaryotic translation elongation factor 1A (eEF1A), other actin binding proteins, and actin mutants affect translation initiation through the actin cytoskeleton. A model has emerged in which actin and the cytoskeleton may play important regulatory functions in protein synthesis, providing a scaffold for translational components including mRNAs, polyribosomes, and translation factors. The report of *TIF1* and *TIF2* interaction with *VPR1* could be interpreted as a direct supporting evidence of the regulation of protein synthesis by actin cytoskeleton components, Perturbations of the actin cytoskeleton in mammalian cells have been shown to induce profound effects on protein synthesis (Gross & Kinzy, 2007) and cases in which

the inhibition of translation initiation is accompanied by the depolymerization of the actin cytoskeletons of yeast cells *in vivo* systems following glucose deprivation have also been reported (Uesono, 2004). Although the actin cytoskeleton is believed to provide a scaffold for the translational apparatus, it is rather mysterious how such depolymerization would result in a reduction of the translational process. The fact that different constituents of the translation machinery interact with the actin cytoskeleton hints to an effect potentially mediated by steric inhibition. Translation initiation factors such as eIF2, eIF3, eIF4A (Tif1/2p), and eIF4B have been shown to associate with the cytoskeleton (Howe & Hershey, 1984). It is therefore conceivable that, upon actin depolymerization, one or more of these constituents lose their spatial arrangements and cannot properly interact with the ribosomal structure (Gross & Kinzy, 2007).

Cytokinesis, the process in which Vrp1p is annotated to participate, requires protein synthesis to complete cell separation (Thanabalu & Munn, 2001). Indeed, protein synthesis is required to complete a number of regulatory steps in the cell cycle, for instance after G₁ (Burke, & Church, 1991) and to complete the G₂ phase and enter mitosis (Goebi & Byers, 1988). Furthermore, initiation of DNA synthesis and mitosis are particularly sensitive to protein synthesis inhibitors (Schvartzman & Van't Hof, 1982).

2.39. Summary

Translation can be regulated at different levels and via diverse procedures (Sonnenberg & Dever, 2003): some of them involve general responses, whilst others rely on specific mechanisms of translational regulation. The cell

may select from several processes to adjust protein synthesis accordingly to the cellular status, these include: mRNA abundance, translational rate through regulation of ribosome density/occupancy and protein specific degradation. As emerged from this research, mRNA concentration and export, ribosome assembling, degradation of selective proteins, and levels of transcription are, to different extents, role-players in regulation of translation initiation. Different cellular contexts and environments may require quite different, and sometimes apparently convoluted, mechanisms of translational regulation.

An intimate connection between transcription and translation has long been suspected for a long time (Hinnebusch, 2005; Topisirovic, 2011). This is despite the unambiguous compartmentalisation of the two processes, as transcription of DNA into mRNA takes place in the nucleus, while translation of the mRNA occurs in the cytoplasm, where the transcript is also ultimately degraded. In prokaryotes, coupling of these two fundamental cellular processes has been acknowledged (Chen & Zubay, 1983); nevertheless the existence of a tied mechanism in eukaryotes has only recently been demonstrated in *S. cerevisiae* (Harel-Sharvitl, 2010). Transcription and translation become unveiled as inter-communicative through a sophisticated network of physical interactions, as extensively shown in annotated literature (Keene, 2001; Halbeisen, 2008) along with the wealth of genetic interactions described in this study. Interestingly, the functional competences relate to the two members of the duplicates on the duplicate pair separately. The *tif1Δ* genetic profile highlights a connection with transcription mediated via the direct formation of the TFIIA transcription subunit, a nuclear protein required for all RNA Pol II-dependent transcription events. A major finding of this work, however, is that the majority

of transcription-translation inter-talk is allocated to *TIF2* duplicate: the translational initiation process appears tightly connected to control of gene expression via RNA Pol II assembly and functionality, along with a dense network of genetic interactions surrounding the SAGA complex. *TIF2* also appears unequivocally the only paralog devoted to interactions with a substantial group of functionally related genes involved in early meiotic gene expression. Altogether the genetic portrait of *TIF1* and *TIF2* genes speaks for a mutual control between transcription and translation aimed to reinforce the control of the two processes singularly.

During cellular stresses, however, some genes can be efficiently transcribed whilst translation is suppressed or the translation products are rapidly degraded. In either case, the cell explores alternative ways to establish higher protein concentrations bypassing the need for transcribing additional mRNAs. It is possible then, that for proteins with little post-transcriptional regulation inside a certain compartment or functional module, their protein level will be more likely to be correlated to the corresponding mRNA concentrations. Changes in the translational state and stability may affect a quite broad range of mRNAs, as a positive correlation between mRNA high expression levels and its tendency to be highly translated has been reported (Beyer, 2004). When environmental signals require a quick cellular response, protein expression, regulation via altering transcription may occasionally be too slow. Prepared for such events, the cell constitutively maintains a sufficient level of mRNA, but it blocks translation, until the protein product is effectively required (Hinnebush & Natharajan, 2002). Under this scenario, ribosomal components could bind mRNA without actively translating the message (Khun, 2001). Stalling of the ribosome

at internal sites in an mRNA will also induce cleavage and degradation (Doma & Parker, 2006). For a given mRNA species there is a negative correlation between translation activity and degradation, suggesting that a way to decrease translation is to diminish mRNA abundance.

The correlation between mRNA and protein abundance is profoundly affected by protein turnover (Pratt, 2002), as degradation of proteins in the global control of protein synthesis represents a fundamental process and it accounts for diverse points of potential control, as also suggested by this study's SGAs data. Ubiquitin appears as a common denominator in degradative processes taking place in both the proteasome and MVBs vesicles, by generation of poly- and mono-ubiquitin tagged aggregates, respectively. Both degradative pathways are represented in this study's genetic investigation of translation.

The interplay between a functional vesicular system and rate of protein synthesis for appropriate cellular up-keep during changes in cellular metabolism has also been reiterate by this study's findings, whether this implies delivery of components to subcellular organelles or trafficking directed towards the degradative pathway. In particular this latter pathway seems to be playing an important role, with the kinetics of lysosomal degradation of a particular membrane protein tightly correlated with the extent of its ubiquitination (Hicke & Dunn, 2003). Although ubiquitination can itself initiate a sorting pathway directed to proteolytic structures at a variety of compartments, multiple rounds of ubiquitination must be undertaken for a competent delivery of a given cargo protein to be delivered to MVBs/lysosomal degradative structures from the ESCRT. An articulated regulation of MVBs should, however, not come as a surprise, given the conspicuous number of fundamental biological processes this

pathway is required for, such as developmental signalling, antigen presentation, cell growth control, and even the budding of certain viruses (reviewed in Katzmann, 2002). Such coordination of MVBs is likely to reflect its need to respond to cellular changes quickly. In consideration of the subdivision of roles required for the different ESCRT complexes, results of this study hint at the coordination of the ubiquitination grouping process, performed by ESCRT-0, as the most relevant step in terms of interconnections with the translational system. Contrarily, the ESCRT-I and -II complexes that respectively promote budding of the vesicles from the plasma membrane and finalize the scission of the vesicles do not seem to interact directly with translation.

The mRNA surveillance mechanism NMD was represented in this study, although not prominently; this connection could be expected in consideration of the role of NMD and the fact that it has already been linked to other stages of translation, for example through the Ubf proteins, which are involved in the process of translation (Czaplinki, 1995).

Finally, it is critically important to comment on the relative paucity of interactions reported for genes involved in ribosome biosynthesis/biogenesis in the *tif1Δ* and *tif2Δ* SGAs. This result seems to fall in with work from Linder's laboratory which showed how rapid repression of translation is not governed by repression of ribosome biosynthesis, at least in secretory-deficient pathways (Deloche, 2004).

Interpretation of data associated with transcription-related subsets of genes provides evidence in support of an expanded role for the DUB enzymes as crucial regulators of chromatin dynamic and gene expression, likely due to their counteracting ubiquitinating enzymatic activity on specific targets. In this

scenario DUBs assist in the inter-talk between transcription and protein synthesis, with the recruitment of modules that involve selected components of the SAGA complex. Functional turnover of proteins is, to a certain extent, regulated by the balance between post-translational modifications, in particular ubiquitination/deubiquitination, indicating that this latter could be a general and predominant mechanism to regulate the stability of cellular proteins. In particular H2B histone ubiquitination may be of particular relevance; behind its requirement in early steps of transcription initiation and RNA Pol II-mediated elongation (Xiao, 2005), this modification appears to embody a prominent task in the ubiquitin balance, which ultimately has repercussions on the whole cell. In other words, modifications of H2B status may imply further consequences in modulation of chromatin dynamics and gene transcription. As optimal transcription of some genes requires complete cycles of H2B ubiquitination and deubiquitination. H2B ubiquitination takes place in the early stage of the transcriptional event; it becomes clear that the context of ubiquitination within the nucleosome is critical in arbitrating the outcome mediated by this modification.

Free ubiquitin is present in very small quantities and there is a physiological requirement for it to be restocked continually, as ubiquitin is used in both cytoplasmic and nuclear processes. Under certain conditions of stress, there is a cellular demand for more free ubiquitin to be incorporated in poly-ubiquitinated substrates, which will then be directed to the proteasome structure: this happens at the cost of mono-ubiquitinated histones resulting in chromatin remodelling and altered transcription (reviewed in Groothuis, 2006). The choice of targeting histone ubiquitin seems very intuitive in consideration of

the fact that H2A and H2B histones represent the sites where major pools of ubiquitin are conjugated in physiological conditions (Zhang, 2003), whilst less significant amounts are employed in processes such as endocytosis and MVBs formation. The change in the ubiquitin pools must be sensed by the nucleus by affecting the histone-ubiquitin status in a concerted mechanism that directs the redistribution of ubiquitin from the nuclear compartment to the cytosolic one.

This study has given an insight on the dynamics of translation regulation, which emerges as highly interconnected, this is understandable given the importance of protein synthesis as a cellular process. Supported by a previous hypothesis which suggest that eIF4Ap function is necessary even when no melting activity of secondary structures is required (Linder, 2003), this research thesis suggests that helicase activity may not be the only mechanism eIF4A protein participates, some of which still not clearly resolved in the literature.

In certain circumstances the challenge may be overcome by allocating new competences to genes to achieve this remodelling. This seems to be reflected in the variety of genes identified in these SGA screens, where the observed genetic interactions are to be attributed to either their direct involvement in the function, such as the production of a protein or an intermediate or indirectly, through regulation of a common function.

2.40. Redundancy in genetic networks

Although use of ORF deletions is a well-established way to investigate a system of connected genes (Tong, 2001), the interpretation of the resultant networks can often be far from simple. Redundancy represents a severe

complication in interpreting genetic networks; nonetheless it is a phenomenon which requires greater understanding, as its functional significance cannot be disengaged from the cellular system's organism. As introduced in **section 1.5**, redundancy is often associated with robustness but, what do we really mean when we refer to robustness of living systems? The term tends to be rather context-specific in biology and its use should be accompanied by a precise indication as robustness of the state, robustness of the adaptability, etc.... Functional and systemic robustness is a feature of systematic gene deletion experiments, such as the SGAs in this research, where a combination of mutants shows the absence of a phenotype due, for instance, to the presence of a counterpart duplicate gene. Both functional and systemic robustness are described in *TIF1* and *TIF2* genetic networks, with references of physical interactions but also regulatory connections. The goal of the research presented here was to investigate the networks associated with translation initiation. It soon became evident that the network displayed a relatively limited level of redundancy. Erosion of backup capacities has been described as an unavoidable process (Li, 2010); the extent to which their ancestral genes managed to conserve the ancestral functional redundancy of the *TIF1* and *TIF2* genes has been conserved appears rather limited. This observation can be explained by the theory that duplicate pairs exhibit a functional backup ability which is limited to certain conditions and environments (Flintoft, 2007) and full functional redundancy between duplicate pairs is generally not mandatory. Indeed, the *TIF1* and *TIF2* paralogous still share an essential subset of functions to achieve genetic buffering. It may be informative to consider *GLG1* (neighbouring gene to *TIF1*) and *GLG2* (neighbouring gene to *TIF2*) a duplicate pair which share 43% identity

between duplicates. Given neighbouring relationship of *TIF1/2* and *GLG1/2* has been preserved, these pairs of paralogs went through the same duplication event, suggesting that although there has been opportunity to diverge for *TIF1* and *TIF2*, they have resisted change.

The average number of interactions per query strain has been reported to be thirty-four per screen and except for very few examples, the majority of the strains place around this value (VanderSluis, 2010). The genetic network surrounding *TIF1* and *TIF2* genes KO presents a more limited number of genetic interactions: the absence of phenotypic consequences could indicate robustness of the genetic network supported by gene redundancy and/or regulatory mechanisms able to reprogram the activity of the network nodes (Lesne, 2008) in the attempt to dynamically adapt to that specific condition.

It is concluded that reliability of a cellular network is not necessarily due to the physical duplication of its components as it appears to be dependent on how well-coordinated the duplicate genes are between each other, along with the extent of connections within their own networks. This research shows how specific the interactions of singular paralogs are within their neighbourhoods of interacting genes and it strongly suggests that for some functional processes, the contribution of a specific paralog in controlling an essential function cannot be substituted as the responsiveness of the two duplicates is not interchangeable.

2.41. SGA as a tool for evolutionary studies

This investigation shed light on the evolutionary benefit to retain the duplicate nature of *TIF1* and *TIF2* paralogs. Apart from what can be considered a

relatively limited overlap, the two genes appear to have developed different cellular roles; remnants of their ancestral interactions have been preserved along their evolutionary paths, however, and range from response to alkaline pH to chromatin remodelling-related processes.

The different genetic surroundings discovered for *TIF1* and *TIF2* paralogs showed how, within the same function, different competences can be developed and maintained in terms of interactions with particular subsets of genes. This appears true in the merged genetic network of the investigated pair of duplicates for the processes of translation and transcription. Although it is difficult to state with confidence whether *TIF1* and *TIF2* genes are exclusively retained for functional compensation or the presence of both help in fulfilling the requirement for dosage balance, data arising from this study suggests that functional compensation exists for these WGD duplicate pair, at least under certain conditions. It is conceivable that in some cases gene duplication could merely serve to increase the number of gene copies and thus the quantity of protein product produced, as seen in previous studies (Hughes & Friedman, 2007). This feature of genetic profiles will be further investigated in the next chapter through an experimental approach that challenges the pair of paralogs in diverse environmental conditions.

Genetic interactions identified as common for the pair of duplicates, strongly suggests that the duplicates' functions used to be allocated in a single ancestral gene and it was only after duplication that the roles have been split. Although it appears less likely, one or more functions may have been performed by a different gene that was replaced or such function/s did not exist. (Wolfe & Shields 1997). It remains arduous to determine which evolutionary pathway

TIF1 and *TIF2* genes have endured, however, because of the prominence of transcription-related hits in *tif2* SGA, it would be tempting to speculate that ORF *YJL138C*, also known as *TIF2*, is in fact the ancestral *TIF* gene. In the cellular interest, it seems conceivable that the majority of the expertise for transcription is to be preserved in the original gene copy. Endorsing this hypothesis is the finding that genetic connections with a subset of ORFs involved in the early meiotic gene expression are univocally allocated to *TIF2* gene. This latter result further infers that, in terms of regulation, the duplicates can respond to different types of circuits. On the other side, under the rationale that adaptation is the new copy's key for survival, the opposite hypothesis is as much intriguing and envisions the possibility that, being gifted the transcriptional system, the new copy could have directly adjusted the level of transcription, in a way that possibly eased its evolutionary survival. As for others paralogs, in the case of *TIF1* and *TIF2*, uncertainty remains on whether the fate of duplicate pairs could, to some extent, be influenced by their own gene interactions.

A surprising outcome of studying *tif1Δ* and *tif2Δ* query strains was the wealth of information not found in the reported genetic interactions, including the ESCRT-0 complex, DUB modules, and ribosomal proteins, to cite some. Investigation on the modality through which the paralogs have split their functions may highlight a specialization of their roles not only between the paralogs but also regarding other genes that genetically interact with at least one of the duplicates.

2.42. Validation of the SGA study

2.42.1. Small scale SGA

This experiment was aimed to be an independent evaluation of the previously performed SGA. In this screen the mutants indicated by SESA as genetically interacting with one or both duplicates were manually arrayed from the Open Biosystems collection to generate an ad hoc YDS. This validation was expected to reproduce the combinations of double mutants highlighted by the SESA analysis in a more selective setting, where the number of “false positive” should be decreased. In this more thoughtfully selection wild type SL and frequent flyers strains were removed.

Whilst the outcome of this experiment was at first glance surprising as very limited overlap between the results of the two SGA analyses was observed, this finding may be ascribed to a technical practicality. Whilst Boone’s set is used regularly and so thrown several times, the Open Biosystems library is not in such regular use and for this reason better conserved. Several consecutive thawing/re-freezing procedures may have influenced the viability of cells in the Boone’s array; the more robust set of strains in the Open Biosystems library may be less prone to synthetic sickness, giving a different profile to the potentially more fragile Boone’s array. It is our laboratory’s procedure to utilize daughter libraries of the Boone’s array with the aim of minimizing the described effect, this occurrence is not entirely to be ruled out. Although coherent with observation of linkage analysis and annotated literature, weak, the genetic interactions observed with the Open Biosystems were weak and this study will consider the results obtained with the Boone’s array only, as the yeast show better growth characteristics.

2.42.2. Tetrad analysis

With the aim to confirm genes identified in the Boone's SGA, RSA was used to ascertain the genotype of meiotic products, however, the indications obtained from the RSA were extremely ambiguous (**section 2.31.1.1**). This may be in some measure ascribed to the nature of *S. cerevisiae* spores to stick to one other, characteristic that makes dispersion of the spores and the assessment of their growth, quite devious in comparison to *S. pombe* (Lundblad & Struhl, 2010).

Seeking for a more rigorous verification, the genes of interest were tested through tetrad dissection. Not all the selected strains returned the SL suggested by the primary screen, a fraction of them due to low sporulation efficiency, an essential factor for the successful accomplishment of tetrad dissection and/or inviability of the spores.

2.42.3. Potential and limitation of an SGA approach

The SGA approach has proven useful in characterizing the function of the two duplicate genes *TIF1* and *TIF2* and also for uncovering interactions between the genes of interest and others (Tong & Boone, 2006). The use of SGA methodology in the investigation of the genetic surrounding of a gene holding a duplicate counterpart can be enormously informative; however it still encounters a number of limitations and problems. As in a "singleton" gene SGA screen, the phenotype revealed for the KO of interest is likely to portray not merely the absence of the query gene but also the response of the whole cell system to the lack of that gene. Mechanisms associated with such a response have been suggested to engage in altered regulation of other genes and re-allocation of metabolic fluxes (Fischer, 2003a; Kafri, 2005). A study on plasticity

of genetic interactions, conducted by a joint effort in between the Universities of Manchester and Oxford, revealed how numerous *S. cerevisiae* genes contribute to fitness under some but not all lab-reproducible environmental conditions, leading to speculation on the number of genes that still remain to be linked to a particular function or pathway due to the non-appearance of a detectable phenotype under certain environmental circumstances (Harrison, 2007). Moreover, in duplicate gene deletion screens the occurrence of compensation mechanisms (e.g., functional redundancy, gene-dosage effect) cannot be excluded and results should be considered only partially informative of the array of interactions characterizing the query gene network. As humorously stated by A. Meyer in his *Duplication, duplication* view essay (Meyer, 2003), ‘such genetic redundancy is a headache for researchers’ as while trying to determine the effects of a missing gene, the system faces the issue of other genes (and pathways) buffering the genome against such adverse conditions. Not the entire set of possible lethal interactions will be represented, with the biological system functionally backed up by other interacting genes possibly involved in alternative pathways. Peculiarly, the genetic panorama neighbouring *TIF1* and *TIF2* genes is even more intricate as a result of their common protein product (Tifp): removing one gene leaves the system with the contribution given by the other.

Furthermore, there are indeed some genes whose deletions suffer in the first place: if a yeast deletion strain presents a “weak” phenotype to start with, combination with another such strain could arise synthetic sick or lethal double mutants. In other words, the eventual no growth phenotype could be ascribed to an inability to tolerate further insult which may, or may not, have an

underpinning functional relationship. Despite having many advantages, such as large-scale and high throughput identification of synthetic lethal interactions, SGA also has several downsides. The percentage of false negatives (Tong, 2004) is estimated to be approximately 17%. A further way of confirming the interactions highlighted by SGA is advised. Tetrad analysis is often conducted in order to confirm the interactions by looking at the segregation patterns of the genes. Overall, successful outcome of our and others' studies (Tong, 2001; 2004) reinforce the concept that studying of mutant phenotypes is a fundamental method for understanding gene function.

Given the problems associated with the SGA technique identified above (e.g., false positives, pathway compensation to mask true interactions and sickness or lethality arising without underlying functional relationships), it is advisable to design complementary experiments that can compensate and/or integrate SGA-derived results, adding pieces of information to our network of interactions. Experiments might include, over expression constructs, environmental challenges, and the use of chemicals and consideration of promoter systems. This latter approach is described below.

2.43. Significance of the promoter regions

As previously mentioned, the *TIF1* and *TIF2* genes present essentially the same ORF, with the exception of very few substitutions; this particular feature suggests that differences in their genetic interactions are likely to be derived from their regulation. The promoter for a gene whose protein product is involved in an essential cellular process, such as eIF4A, will have to be able to respond to a diverse range of cellular requirements. It is possible that a single promoter

sequence would be unable to carry effectively a full range of transcriptional controls. In these circumstances, the retention of identical duplicates bearing differential promoter or terminator sequences becomes evolutionarily advantageous. This highlights the importance of the promoter regions and associated transcription factors. In this context, Kafri (2005) showed that dispensable paralogs have highly correlated expression in a few conditions but not in most (Kafri, 2005). This it is not important whether two genes are co-expressed under normal conditions but rather that at least one of the paralogs is expressed at an adequate level under a diverse array of conditions, which could include the loss of functionality of one of the paralogous genes (Kafri, 2005).

2.43.1. Tet-titratable promoter

Outcomes of this experiment, even if limited, underline the relevance of the promoter regions in *TIF1* and *TIF2* pair of duplicates. Whilst insertion of a promoter cassette into a strain bearing a deletion for one of *TIF* genes is technically achievable, insertion of the *tetO₂*-“off” cassette in the region upstream the coding region of either resulted in slowly growing mutants that were therefore reliable for undergoing SGA analysis. This implies that the promoter regions of *TIF1* and *TIF2* are very delicate and highly important for the cellular well-being. A salient result is the finding that repression by doxycycline is different depending on whether rich (YPD) or minimal (SD) medium was used. This observation recalls an effect previously observed in Hillenmeyer’s study (2008) in which *tif1Δ* and *tif2Δ* strains scored, which growth in drug-free SC media among the top 10 conditions inducing sensitivity (for fairness it needs to

be pointed out that this phenomenon was observed utilizing an homozygous YDS).

In this study, the small number of *tetO₂*-off strains in which repression of the promoter worked properly was quite low. It remains interesting to observe how different clones showed different degrees of repression in presence of doxycycline. As the regulation of this system is dependent on two elements -the inducible promoter and the transactivator- there is the possibility that the regulation is troubled by interferences relating these two elements, a fault that could become exacerbated in a translationally impaired *tifΔ* background.

Although the concentration of doxycycline required for partially repressing the transformed strain was higher than the one utilized in the original paper, this would not represent a major issue. It is widely accepted that doxycycline is innocuous to yeast as not only are there no obvious phenotypic effects observed when it is added to growth media to control promoter expression but also this drug has no significant effect on global transcription levels (Wishart, 2005).

Although the issue of the promoter possibly being refractory to recombination should have been fixed with attent design of the flanking regions, there is still the possibility that the insertion of the promoter cassette itself leads to lethality, for instance by mis-expressing the gene when the *tetO₂* promoter is “on”. Furthermore, there is the possibility that the insertion of the *tetO₂* cassette may be sterically problematic.

2.43.2. Galactose over-expression promoter

Insertion of the *GAL* overexpressing cassette in place of the native promoter resulted in the disruption of the activity and regulation of the “wild-type” *TIF1* and *TIF2* genes promoters. This experiment did not lead to the generation of viable constructs as explained in **section 2.33**.

It is well understood that isolated studies on system single components have a limited investigative value and are uninformative on the complexity surrounding biological systems, whilst more valuable information is revealed by investigating the connections between constituents of the system that will ultimately display the phenotype associated with a particular deletion or condition. This study contributes to the understanding of the mechanisms of duplication and attempts to elucidate evolutionary processes acting on the *S. cerevisiae* genome. Future studies of systems biology, such as population genomics and genome evolution, are likely to see the budding yeast as the organism of choice. The genetic profiles generated in this research may provide even greater value when integrated with other experimental outcomes, contributing this way to the reconstruction and assembly of connection patterns among networks. Integration of non-essential genome SL results with protein-protein interaction analyses, and microarray mRNA profiling could enable construction of a highly wired network, which would be advantageous not only for evolutionary studies but also for the understanding of higher eukaryotes' biology and genetic diseases traits.

Further investigations into the robustness of the translation initiation process with respect to a given class of perturbations will be discussed in the next chapter.

3. Chapter 3

This chapter describes the use of the chemical and environmental perturbation, primarily treatment with the lithium ions, to complement the *TIF1* and *TIF2* genome-wide screen results presented in Chapter 2. In addition to furthering our understanding of the *TIF1* and *TIF2* networks, it was hoped that the study might formulate an explanation on how augmented translation initiation overcomes lithium toxicity. Finally, a global interpretative picture of wide-scale information is assembled in a network context, where the duplicate nature of *TIF1* and *TIF2* genes is analysed to unveil the evolutionary path they have gone through as paralogs.

3.1. Introduction

Discussion on the significance of the genetic interactions considered in the previous chapter has shed light on the differences and similarities between the pair of duplicate genes *TIF1* and *TIF2*. Due to these paralogs' essential nature, an exhaustive understanding of their genetic connections is difficult to resolve by exclusively genetic means, as the double mutant is not viable. A set of complementary studies is required to augment the knowledge generated through the SGA screen and this was partially addressed in the previous chapter with the use of promoter-modulable constructs and by testing the pair of duplicates through a range of chemical and environmental challenges. This third chapter will focus on the exploration of Tifp function in consideration of the interactions highlighted by chemical perturbation with the lithium ion. Although it was not the first intended focus of this research, the study described in the

current chapter has the potential to provide information relating to the mode-of-action and toxicity of the lithium ion itself.

The essentiality of translation initiation is reflected in the genetic networks of *TIF1* and *TIF2*, which encompass many and distant cellular processes. The extent of these connections is so vast that, for some of the cellular events described in the literature in which initiation of translation has been established to be involved, the exact mechanisms remain to be elucidated. Still enigmatic, for instance, is the finding that the translation process is inhibited by the chemical LiCl at the initiation step (Montero-Lomeli, 2002). In yeast this appears to be true only when galactose is the sole carbon source, as revealed by a polysome analysis showing an accumulation of 80S monosomes for yeast cells incubated with 6 mM LiCl in galactose (Montero-Lomeli, 2002); immunoblot analysis data from the same study also showed Tifp did not sediment with the ribosomal subunits. Over-expression of the *TIF2* gene, however, was shown to overcome protein synthesis inhibition and restore Tifp sedimentation with the initiation complex, in this way conferring lithium tolerance in a galactose containing medium (Montero-Lomeli, 2002). The same research group had previously shown that, in *S. cerevisiae*, lithium toxicity is highly dependent on the carbohydrate source utilized to ensure growth (Masuda, 2001): when glucose is the carbon source of choice, the IC₅₀ for lithium is 100 mM, whilst it lowers to 6 mM, if galactose is utilized instead. To elucidate whether the inhibition of protein synthesis in cells grown in media containing lithium and galactose takes place through the same mechanism established for glucose starvation (Martinez-Pastor & Estruch, 1996), Masuda's group studied whether both experimental conditions lead to the dissociation of Tifp from the preinitiation complexes.

Interestingly, glucose starvation still leads to a loss of polysomes but not to dissociation of Tifp from the preinitiation complexes, strongly suggesting that the translational response to lithium treatment in galactose-grown cells is different from the response to glucose starvation; this statement is further validated by the observation that overexpression of Tifp does not reverse the inhibition on initiation of translation under glucose starvation (Montero-Lomeli, 2002).

3.2. Galactose and the Leloir pathway

The utilization of galactose, a simple hexose sugar and the carbon source of choice for this study, is more complicated than glycolysis. The galactose metabolic pathway was revealed by the biochemist L. Leloir (1906-1987), whose studies on carbohydrate metabolism gained him the Nobel Prize in Chemistry in 1970, in part for his significant contribution to the elucidation of the genetic disease galactosemia.

The process Leloir helped elucidate is named after him and occurs through several steps (**Figure 3-1**) which ultimately allow galactose to enter glycolysis by its conversion to glucose-1-phosphate (Glu-1-P).

Figure 3-1. Galactose pathway. Adapted from Masuda (2001).

Initially, galactose is phosphorylated by the ATP-dependent galactokinase enzyme (GK) (encoded in yeast by the *GAL1* gene) to produce galactose-1-phosphate (Gal-1-P); this is then processed by galactose-1-phosphate uridylyltransferase (GALT) (encoded in yeast by the *GAL7* gene) which catalyses the exchange of the phosphate of Gal-1-P and the UDP-group of UDP-glucose, leading to concurrent production of glucose-1-phosphate and UDP-galactose. The Glu-1-P produced by the transferase reaction is a substrate for the phosphoglucomutase (PGM) enzyme (encoded in yeast by the yeast genes *PGM1* and *PGM2*) which reversibly converts it into glucose-6-phosphate (Glu-6-P), while the other transferase product, UDP-galactose, is epimerized to UDP-glucose by UDP-glucose-4-epimerase (GALE) (encoded in yeast by the *GAL10* gene).

The importance of the PGM-catalyzed step is crucial as it ultimately allows the transformed galactose to be utilized in the generation of cellular energy and consequently PGM is an essential enzyme for galactose metabolism (Boles, 1994). It is at this step along the Leloir pathway that the action of lithium affects the cellular system: the ion is toxic to yeast when grown in galactose medium, mainly due to the inhibition of PGM (**Figure 3-1**) an enzyme which lithium inhibits in yeast (IC_{50} about 0.2 mM) and also in human (IC_{50} about 1.5 mM) (Masuda, 2001). The yeast paralogous genes *PGM1* and *PGM2* are responsible for encoding these key isoform enzymes, Pgm1p and Pgm2p, with Pgm2p accounting for 90% of the total PGM activity (Oh & Hopper, 1990). A third isoform of Pgm has recently been named: following experimental evidence which suggests that *YMR278W* ORF encodes a protein product with phosphoglucomutase activity that takes part in the interconversion of Glu-1-P and Glu-6-P, the aforementioned ORF was named Pgm3p (Tiwari, 2008). In general, lithium inhibits the activity of enzymes by competitively displacing magnesium from its binding site (Ray, 1978): this is also the case with the inositol phosphatases and fructose 1,6-bisphosphatase enzymes, that all share a magnesium-binding domain (York, 1995). Although, the magnesium-binding domain of both Pgm1p and Pgm2p (collectively referred as Pgm) differ from these enzymes, lithium still competitively displaces the bivalent cation from its binding site on Pgm, thus reducing its enzymatic activity (Masuda, 2001).

Galactose toxicity is found in humans as an autosomal, recessive genetic disorder named galactosemia. *S. cerevisiae* is an appropriate model system for studying galactose toxicity (Metha, 1999) and galactosemia (Riehm, 2001), and an increased understanding of galactose metabolism may further lead to a

better comprehension of the metabolic events resulting in galactose-induced toxicity, an effect observed both in yeast strains and galactosemic patients. Several forms of this disease exist, all sharing an impairment of the normal processing of galactose; the most common and severe of which is caused by cells with genetic defects in the Gal-1-P uridyltransferase GALT (yeast *GAL7*) and is known as classic galactosemia (type I). Mutations in the galactokinase GALK1 (yeast *GAL1*) and the epimerase GALE (yeast *GAL10*) encoding genes lead to galactosemia types II and III respectively. The clinical outcomes of this disease range in severity and it can lead to poor growth, hepatomegaly and/or jaundice; symptoms which disappear if the patient is kept on a galactose-free diet. However, there is the possibility that these individuals will suffer life-threatening neurological disturbances and possibly premature ovarian failure. For long time Gal-1-P was believed to be the only toxic intermediated (de Jongh, 2008) but evidence has been collected and it seems that P_{gmp} inhibition by Gal-1-P has a role in toxicity, however, the ratio between the concentration of Gal-1-P and galactitol together with the accumulation of galactitol could be the cause of the toxic effects instead, at least according to a study undertaken in mice (Ning, 2001).

Yeast strains lacking the major isoform of phosphoglucomutase (*PGM2*) accumulates a high level of Glu-1-P and this mimics phenotypes resulting from an altered Ca²⁺ homeostasis, when galactose is utilized as the carbon source (Fu, 2000), such as an increase in Ca²⁺ uptake and accumulation and sensitivity to high environmental Ca²⁺ levels. Unlike in mammals, a Glu-6-P transporter able to stimulate Ca²⁺ sequestrating into ER or Golgi has not yet been identified in yeast; Glu-1-P, however, has been shown to plays a role in yeast Ca²⁺ homeostasis (Fu,

2000). An extensive work done by Aiello and colleagues showed convincing evidence for a coupling mechanism between the cellular Ca^{2+} homeostasis and the levels of Glu-6-P and Glu-1-P in *S. cerevisiae* (Aiello, 2002). This demonstrated that Ca^{2+} homeostasis defects arising in the *pgm2Δ* strain are specific to accumulated Glu-1-P; furthermore, it was shown that an increased synthesis of Glu-1-P and not the sugar nucleotides derived, UDP-Glu, influences Ca^{2+} homeostasis and that although Glu-6-P has an effect on this process, it is distinct from the one involving Glu-1-P (Aiello, 2002).

3.3. Calcium homeostasis

Calcium homeostasis in eukaryotic cells is a complex process involving the regulated internalization and sequestration of calcium ions into a variety of locales. The basic mechanisms that mammals and yeast use to carry out this process are similar, and the cytosolic Ca^{2+} concentration in both types of cells is normally in the range of 50-200 nM (Iida, 1990; Miseta, 1999). In yeast, 90% of Ca^{2+} is stored in the vacuole (Halachmi & Eilam, 1989) where Ca^{2+} is complexed with vacuolar polyphosphate in a relatively stable form referred to as the non-exchangeable Ca^{2+} pool. This name is derived from the fact that vacuolar Ca^{2+} exchanges with the environment more slowly than Ca^{2+} located in either the cytosol or other intracellular compartments (Cunningham & Fink, 1994b). Along with the vacuole, the ER and Golgi are also important for maintaining proper intracellular Ca^{2+} homeostasis in yeast. In these organelles the concentration of Ca^{2+} is carefully maintained to ensure the proper folding and processing of proteins transported through the secretory pathway. In mammals, the ER serves as a store for a readily mobilized source of Ca^{2+} for use in Ca^{2+} signalling. Upon

stimulus, ER Ca^{2+} stores can be released to generate a momentary increase in the cytosolic Ca^{2+} concentration which will then activate various signalling pathways (Putney, 1992). In non-excitabile cells, the release of Ca^{2+} from the ER has been proposed to lead to a store depletion signal and consequential influx of Ca^{2+} cations across the plasma membrane in a process known as capacitative Ca^{2+} entry (CCE). CCE amplifies the transitory increase in cytosolic Ca^{2+} , initiated by its release from the ER, and ultimately serves as a source of cytosolic Ca^{2+} for refilling the depleted ER Ca^{2+} store (Parekh & Penner, 1997). The variation in the Ca^{2+} concentration in the cytosol generated upon a stimulus activates a variety of Ca^{2+} -sensing proteins such as calmodulin and calcineurin, which ultimately leads to the induction of various downstream signal transduction pathways (Putney, 1992).

Loss of *PGM2*, the gene that contributes most to the phosphoglucomutase activity in *S. cerevisiae* (see **section 3.2**), results in a significant increase in the cellular Glu-1-P:Glu-6-P ratio when cells are grown in a medium containing galactose as the carbon source. Importantly, this imbalance in metabolites was shown to also cause a six- to nine-fold increase in cellular Ca^{2+} accumulation. Lithium inhibition of Pmgp causes a similar elevation of cellular Ca^{2+} in medium containing galactose, but not glucose, as sole carbon source (Csutora, 2005); this lithium-induced Ca^{2+} over-accumulation has been shown to be significantly reduced in a yeast strain lacking the vacuolar Ca^{2+} -ATPase Pmc1p, suggesting that lithium inhibition of Pmgp results in an increased Glu-1-P:Glu-6-P ratio, which is associated with an accelerated rate of Ca^{2+} uptake by the vacuolar compartment, mediated by the Ca^{2+} -ATPase Pmc1p (Csutora, 2005).

3.4. The TOR pathway

Sit4p, a putative Ser-Thr phosphatase, was successfully cloned and, as shown for Tifp, when over-expressed confers lithium tolerance in galactose medium (Masuda, 2001) and it also appears to have a protective effect on translation initiation from lithium-induced inhibition (Montero-Lomeli, 2002). Although both inhibition of glycolysis by lithium and glucose deprivation lead to inhibition of protein synthesis (Ashe, 2000; Montero-Lomeli, 2002), only the first mechanism seems to be associated with *SIT4*, as overexpression induces recovery of protein synthesis when cells are cultured in galactose in the presence of lithium but not in a glucose-deprived environment (Montero-Lomeli, 2002). These results suggest that each treatment could be responsible for inducing a separate mechanism of inhibition of protein synthesis.

In baker's yeast, the type 2A-like protein phosphatase catalytic subunit of Sit4p functions together with its regulatory subunit Tap42p downstream of the rapamycin-sensitive TOR (Target Of Rapamycin) complex 1 (TORC1) to mediate several Tor signalling events and to control mechanisms of responses to nutrient levels and events required for the physiological G₁ to S phase cell cycle transition and budding (Fernandez-Sarabia, 1992). Sit4p-mediated TOR signalling functions via Sap-Sit4 complexes and helps in coordinating both transcription and translation mechanisms that connect cellular growth to the availability of amino acids (Rohde, 2004). The TOR pathway is known to have overall effects on the translation machinery of both mammalian and yeast cells, as amino acid starvation or use of the TOR inhibitor rapamycin (Rap) quickly inhibits the synthesis of the ribosomal components.

A link between TOR-mediated signalling and lithium sensitivity has also been established by studies on both *S. cerevisiae* and *C. albicans*, where deletion of one of the components of the TORC1 complex, Tco89p, induces sensitivity to both lithium and Rap (Zheng, 2007); this latter drug has been shown to inhibit translation initiation indirectly by inhibiting TOR. This results in the phosphorylation of numerous translation initiation factors, which ultimately prevents cap-dependant translation initiation in an indirect way (Crespo & Hall, 2002). This particular aspect of inhibition of the translation initiation process has been recently regarded with increasing interest in the use of specific cap-dependant translation inhibitors as anti-neoplastic drugs, given the over-expression of numerous eIF4F components revealed in a number of cancer conditions (Lindqvist & Pelletier, 2009), and the role of translation initiation as the major point of translational control (Sonenberg & Hinnebusch, 2009). One of the hypothesised mechanisms of translational regulation goes through the phosphorylation of the cap binding protein eIF4E which plays an important role in regulating its affinity for the cap. On the other hand, eIF4E binding proteins (4E-BPs, which are Eap1p and Caf20p in yeast) bind to eIF4E and thereby abolish the interactions between eIF4E and eIF4G thus blocking ribosome recruitment to the mRNA (Poulin, 1998). In mammalian systems, the binding of 4E-BPs to eIF4E is regulated by phosphorylation: hypophosphorylated 4E-BPs bind to eIF4E whilst hyperphosphorylation blocks their binding to eIF4E. Many different extracellular signals have been shown to influence the hyperphosphorylated status of 4E-BPs (Gingras, 1999) such as hormones, growth factors and mitogens, nutrient deprivation and certain types of stresses such as treatment with the immunosuppressant Rapamycin. The conserved TOR signalling pathway appears

central to the regulation of 4E-BP phosphorylation (Teleman, 2005) as TOR is activated in response to a variety of stimuli that eventually lead to phosphorylation of 4E-BPs and other factors promoting cap-dependent translation (see **sections 1.8.1** and **2.4** for an overview of translation initiation).

3.5. Lithium

Lithium was discovered in 1817 by Swedish chemist J. Arfwedson, during analysis of the mineral petalite he was conducting in J. Berzelius' lab; it was Berzelius who gave the mineral its name after the Greek word 'lithos' which means 'stone', as it was found to be quite abundant in a large number of igneous rocks and many mineral springs.

Although we cannot state with accuracy when lithium was first used for medical purposes, historical evidence suggests that between the 5th and 2nd centuries B.C. Roman and Greek physicians would treat manic patients with alkaline waters available in loco, waters which are now known to contain very high levels of lithium salts (Kline, 1969). It was the Australian psychiatrist J. Cade in the mid-20th century who first discovered the role of lithium in controlling bipolar disorder, while running experiments with a compound he believed was the cause of manic behaviours: the uric acid. To prove his theory, Cade injected this substance into guinea pigs and chose lithium urate for its high solubility. Surprisingly, administration of lithium had a calming effect on the animals, instead of the expected increased excitation. Following experiments on both guinea pigs and humans proved that lithium had an effect on mania-depressive (or bipolar) disorder. Cade published the results of his work in the landmark

paper, *Lithium salts in the treatment of psychotic excitement* (1949) and shortly after, the psychopharmacologist R. Fieve was pioneering lithium clinical trials in the United States. By the late 1960s, the interest in this medication for bipolar disorders treatment among the world medical community was very solid and by the 1970s the FDA had approved lithium as a prescription medication.

Bipolar disorder is a severe psychiatric condition with a strong genetic component and unknown pathophysiology with clinical manifestations afflicting 5% of the general population (Sümegei, 2008). It results in heavy social and economical costs on society. Current medications used for its treatment are associated with variable rates of efficacy and often intolerable side effects. Although lithium is nowadays the oldest mood stabilizer available on the pharmaceutical market and its exact mechanism(s) remain(s) unknown, it is still commonly used clinically either alone or in combination with valproic acid or carbamazepine. Lithium is efficacious in the maintenance phase treatment of bipolar disorder, with evidence supporting an additional beneficial effect which prevents recurrence of mania; it is however less clear, if lithium prevents depressive episodes in bipolar disorder (Young, 2006). Lithium use has also been proposed for treatment of other neurodegenerative conditions by virtue of its stabilizing neuroprotective effect (Csutora, 2005), confirmed by evidence in the literature that links lithium and other mood stabilizers to an enhancement of the expression of the antiapoptotic gene BCL-2 (Zarate, 2006), suggesting a likely neuro-protective role for this medication. Its antimanic, antidepressive, and potentially anti-suicidal effects could make it suitable for the treatment of an expanded set of neurological and neurodegenerative diseases (Yu, 2012). This approach is endorsed by the encouraging finding that lithium exploits a

protective effect on neurons against β -amyloid-induced neurodegeneration by preventing the enhanced phosphorylation of tau proteins (Alvarez, 1999), and thus it could find an application in the Alzheimer's disease treatment strategy. Furthermore, lithium may be applicable to Huntington's disease therapy as it has been shown that it robustly protects neurons in the striatum (Chung, 1999). As lithium hits several targets, an extensive understanding of how this drug interacts with them is urgently needed to successfully integrate current knowledge on lithium's mode of action; this would also be beneficial to address issues like combinatorial therapies, development of strategic lithium-alternative medications, the role of BCL-2 in lithium's effects and amelioration of the side effects.

3.6. Yeast as a model for neurodegenerative disorders

The study of neurodegenerative disorders relied for a long time on post-mortem neuropathologic observations until findings of mutations and misfolded proteins responsible for diseases encouraged molecular genetic probes, promoting the development of cellular and animal models. The high degree of conservation in cellular processes between yeast and higher eukaryotes has lead to the use of this simple organism as a reliable system for studying basic mechanisms underlying human diseases (**section 2.3**). The possibility of modelling neurodegenerative conditions has also been explored, unveiling the enormous potential of *S. cerevisiae* to provide insights into cellular pathways underlying such pathologies and their mode of action, along with elucidation of the toxicity of the drugs utilized in their treatments. Limitations exist in the use of yeast for such studies. The most obvious among all is the investigation of

aspects of multicellularity and cell-cell interactions, such as immuno-inflammatory responses and diverse kinds of transmissions as synaptic signals and glia-neuron connections. Even though molecular processes may appear distant between human and *S cerevisiae*, and it can be argued that some mammalian functions have diversified or indeed never had a yeast counterpart, this monocellular organism is still representative of the majority of cellular mechanisms possessed by neuronal cells. This is particularly true for yeast cells in stationary phase that are not dividing and therefore experience a similar context to the brain neurons (Chen, 2005). Neuronal cells cannot attenuate cellular insults (for instance, the accumulation of damaged proteins, oxidative stress, proteasome impairment and the build-up of mutations) and neither can yeast; the effects of a challenge cannot be diluted out via cellular division by either organism.

3.7. Further therapeutic and commercial benefits

Great advancement in the understanding of the genetic and biochemical pathways underlying neurodegenerative disorders has been achieved (reviewed in Bertram & Tanzi, 2005), however, in certain cases the treatments for these pathologies are still not satisfactory and the side effects associated with the therapies are highly undesirable. The identification of specific mechanisms involving a drug of interest is of extreme importance to resolve whether side effects are caused as a consequence of interactions with the primary target(s) or arise from off-target interactions. Elucidation of this extremely complicated scenario would consequently make any attempt of modifying and improving the performance of a drug more feasible.

Although there are many pathways held in common between all yeast strains, and indeed between yeast and other eukaryotes, there are metabolic differences in the production and utilization of the substrates between different strains and organisms. For example, elucidation of the regulatory underlying mechanisms underlying energy metabolism is crucial for understanding the general principles of carbohydrate regulation but also it is of valuable for the biotechnology industry, where capabilities of specific yeast strains could be taken advantage of. Insights into galactose metabolism in *S. cerevisiae* may open opportunities for designing strains that have improved galactose uptake, which is relevant for the industrial uses that need to enhance growth in galactose containing medium (Ha, 2011) and increase yields to ultimately optimize large scale processes.

3.8. Chemical genetic screening

There is diversity in the way in which a biological phenomenon is investigated using genetic approaches and the choice of a particular screen will determine the perspective from which the cellular process of interest will be explored. With the purpose of achieving a global understanding of a complex biological process, such as translation initiation, a set of complementary approaches has been used along with a genome-wide screen on genes KO (chapter 2). Chemicals and environmental conditions have been used as modulators of gene activity (Parson, 2004, 2006; Hillenmeyer, 2008). Budding yeast is particularly suitable for applications such chemical genetic probes on a genome-wide scale, where targeting of the gene at the protein level is achieved

by using small molecules to induce phenotypic changes in organisms (**section 2.5**).

The field of chemical genetics is based on the principle that small molecules can mimic the effects of genetic mutations: for example, a loss-of-function mutation in the gene encoding a compound target may be mimicked by an inhibitory compound and vice versa. Deletion strains identified in a chemical genetic screen as sensitising to a compound will correspond to loss of genes encoding proteins which function in pathways that buffer the cell from the growth inhibitory effect of the compound. This information about pathways that are required to buffer an inhibitory effect may be especially useful when the specific target(s) of the compound are unknown (Parsons, 2004, 2006). This comparison is not, however, an aim of this research as, *TIF1* and *TIF2*, the genes investigated through the SGA (chapter 2) are not known targets of lithium.

Interactions between lithium and particular cellular processes revealed through a chemical genetic screen should help to elucidate the relationship between this drug's mode-of-action, toxicity and translation initiation. The fundamental principle assumed in the study of chemical genetics (Parsons, 2004; 2006; Hillenmeyer, 2008) is that a small perturbagen molecule can specifically bind to a gene product and disturb its function(s), mimicking a mutation in the corresponding gene. In other words, interactions of an epistatic nature between a mutant and a small molecule can be defined and further utilized to characterize genetic interaction networks.

Although the *TIF1* and *TIF2* genes are not targets of lithium, over-expression of the *TIF2* gene has been shown to protect yeast cells from this drug's toxic effect, as introduced in **section 3.1**. Lithium was thus the compound

of choice to investigate the processes associated with the Tifp proteins, as the lithium ion is known to interact with translation initiation. It would have been difficult to obtain such a level of understanding of Tifp function by limiting our investigation to a gene-based perturbation only as presented in chapter 2. A side benefit from use of lithium to probe the Tifp is that additional meaningful information could emerge from this experimental approach in regards to the mechanism and toxicity of lithium.

3.9. Aim and objectives

The aim of the work presented in this chapter is to improve further our understanding of the evolutionary benefits underlying conservation of a single protein product eIF4A (referred to below as Tifp, the protein product of the *TIF1* and *TIF2* genes) through chemical and environmental perturbation, complementing the genetic approaches presented in chapter 2.

In addition, as the lithium ion was selected as a key perturbant, it was hoped that the study might formulate an explanation on how augmented translation initiation overcomes lithium toxicity and provide information on the detailed mechanism of action of lithium ion treatment. Finally, a global interpretative picture of wide-scale information is assembled in a network context, where the duplicate nature of *TIF1* and *TIF2* genes is analysed to unveil the evolutionary path they have gone through as paralogs.

To achieve these aims, a set of experiments will be undertaken:

- Assess a concentration of the lithium ion that provides a modest growth defect in wild type strains with galactose as carbon source.
- In a high throughput assay, treat the YDS with lithium ions in galactose at chosen concentrations, and identify sensitive and resistant deletion mutants.
- Validate the high throughput assay results of selected deletion mutants, and thereby reveal pathways associated with lithium/galactose activity.

- Undertake a pilot study of to identify perturbing conditions that may be useful in evaluating the differences in use and regulation of the *TIF1* and *TIF2* genes.
- Compare the functional redundancy and robustness of *TIF1* and *TIF2* paralogs, emphasizing any similarity or specificity in their regulation.

3.10. Methods

This section reports materials and methods for a first set of experimental procedures involving the use of lithium to assess mechanisms of translation initiation involved in resistance to this drug and a second investigation into diverse environmental and chemical challenges, aimed to test the robustness of the duplicate genes of interest.

3.11. Yeast strains

3.11.1. Lithium experiment

Two sets of non-essential *S. cerevisiae* deletion strains were utilized in this investigation: the first set was kindly gifted by C. Boone's laboratory (**method 2.9.1**), while the second one was purchased from Thermo Scientific-Open Biosystems (Huntsville, AL, USA) as a part of the YKO *MATa* homozygous diploid strain collection (Winzeler, 1999). Each deletion strain holds a *MATa* mating type in a BY4741 background and a genotype of *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*. The wild-type surrogate strain for this study is the *his3Δ::kanR* strain, which is used as replacement of the *HIS3* gene with the kanamycin resistance cassette has no significant effect on the fitness since BY4741 is auxotrophic, but provides a suitable control for experiments in which all deletion strains have a gene of interest replaced by a *KanR*.

pTIF2tif1Δ strain carries *TIF2* gene driven by an over-expression *GAL11 2* micron plasmid promoter in a *tifΔ::URA* background (this study) and was kindly donated by Dr J. Matthews, Chemical Genetics laboratory, Victoria University of Wellington.

3.11.2. Different environmental and chemical challenges

The deletion strains tested under a range of experimental conditions include *MAT α tif1 Δ ::natR* and *MAT α tif2 Δ ::natR* (both generated in this study) and wild type *MAT α Y8025* (**method 2.9.1**).

3.12. Growth media

Yeast strains utilized in this experimental section were grown in one of the following media: YPD, SD and SC (**method 2.9.4.2**); YPGal and SGal media were also prepared following the same procedure but replacing the glucose carbon source with the same concentration of galactose.

3.12.1. Stock solutions

Lithium chloride 1 M stock solution was prepared dissolving lithium chloride powder in an appropriate amount of dd water, autoclaved and stored at room temperature.

Rapamycin (LC Laboratories) 0.1 mM, tunicamycin (Merck) 12 mM, cycloheximide (Sigma) 35 mM, DTT (Merck, Whitehouse Station, NJ, USA) 1 M, and caffeine (Sigma) 100 mM stock solutions were prepared by dissolving the drug in an appropriate amount of dd water (rapamycin, cycloheximide, DTT, and caffeine) or DMSO (tunicamycin) and then filter sterilizing and storing at -4 °C. ClonNAT and G418 were prepared and stored as described in **method 2.9.3**; acetic acid 1 M, hydrochloric acid 1 M, hydrogen peroxide 1 M, sodium hydroxide 1 M, magnesium chloride 1 M and ethanol 90 % were stored at room temperature.

3.12.2. Solid growth

3.12.2.1. Lithium experiment

The two sets of deletion strains utilized for the lithium chemical genetic screen and further spot assays were grown and maintained according to **method 2.18.8 i-b.**

After autoclaving, agar was cooled to 50 °C and LiCl was added from stock to both YPGal and YPD plates at final concentrations of 7, 15, and 30 mM. Lithium-free YPD and YPGal control plates were also prepared.

Magnesium chloride was added to media as for lithium at a final concentration of 7, 15 and 30 mM.

3.12.2.2. Different environmental and chemical challenges

After autoclaving, agar was cooled to 50 °C and the drug of choice was added from stock at the concentration listed in **method 3.14.**

3.12.3. Liquid growth

All the strains utilized in these experiments were grown in YPD, YPGal, SD, or SGal medium supplemented with 200 µg/ml of G418 or 100 µg/ml of clonNAT, if required.

3.13. Lithium investigation

3.13.1. Preliminary experiments

3.13.1.1. Pilot study

A pilot test to determine a window of working concentrations was undertaken. A few plates from Boone's KO collection were pinned on YPGal medium containing LiCl at the final concentration of 7 mM, 15 mM, and 30 mM. Pinned colonies were incubated for 5 d during which photographic records were taken to assess the concentration of drug that inhibits colony growth.

3.13.2. Chemical genetic screen

Boone's KO collection was pinned on PlusPlates (Singer Instruments, Somerset, UK) containing YPGal and LiCl at final concentrations of 7, 15, and 30 mM. The same array was also pinned onto drug-free YPGal plates acting as controls.

3.13.3. Data analysis

Following a 48-h incubation at 30 °C, the sensitivity or enhancement of growth of each deletion strain featured in the Boone's array was assayed by comparison to controls using Colony HT and SESA software (**method 2.19**). The program SESA generated a list of gene deletions strains whose growth was perturbed by lithium treatment in presence of galactose as sole carbon source compared to their control lithium-free medium. Outcome includes a degree of statistical confidence grouped for simplicity under headings in "definitive",

“probable”, and “possible” hits; visual assessment was also carried on plates to confirm the results generated by the software.

3.13.4. Spot Assay

Sensitive and suppressor deletion strains indicated by SESA as “probable” and “definite” hits underwent further testing through spot assays along with a set of additional selected deletions strains. These additional strains were selected on the basis of: i) Ambiguity in the original screen relating to the visual assessment of the plate image and/or results reported by the software SESA; ii) a functional association with other deletions highlighted by the primary screen supported by literature; iii) a functional interaction held by one or more genes outlined in the first screen, for example genes which are part of the same complex; and vi) visual assessment identifying a phenotypic change in a deletion listed as “possible” hit (generally not considered in data analysis due to its low statistical significance).

Selected yeast strains were individually grown at 30 °C into 96-well plates containing YPGal liquid medium supplemented with G418. Saturated cultures were diluted in a 96-well plate containing YPGal medium using a BioTek robotic liquid handler (Winooski, VT, USA). The liquid-to-solid phase transfer of the diluted strains was robotically achieved utilizing a Singer RoToR (Somerset, UK) that spots strains from the 96-well plates onto an YPGal agar plates supplemented with concentrations of LiCl of 7, 15 or 30 mM and onto corresponding lithium-free YPGal control plates. The plates were incubated for five days; during this time photographic records were taken to assess growth variations according to scoring system described below (**section 3.13.4.1**).

Each plate contained the following as control strains: sensitive strain (*cnb1Δ::kan*), suppressor-like strain (*bre5Δ::kan*), over-expression strain (*pTIF2tif1Δ*), and “wild- type” surrogate strain (*his3Δ::kan*). The assay was routinely performed twice for each KO strain of interest, however, a subset of deletion strains underwent a further third test, as later explained in **method 3.13.5**.

3.13.4.1. Scoring system

Deletion strains showing growth impairment or enhanced growth were scored for sensitivity or resistance according to the following scoring system: The number of the 4 dilution spots growing at each concentration was visually determined and scored according to the table below. This allowed deletion strains to be characterized into lithium “ultra-sensitive” (score 0-1.5) and “sensitive” (score 2-3.5). Strains with enhanced growth on lithium treatment had scores ranging from 5 to 8 (**Table 3-1**).

Spots growing on 15 mM lithium/galactose	Spots growing on 30 mM lithium/galactose	Score
●○○○	○○○○	1
●●○○	○○○○	2
●●●○	○○○○	3
●●●●	○○○○	4
●●●●	●○○○	5
●●●●	●●○○	6
●●●●	●●●○	7
●●●●	●●●●	8

Table 3-1. Scoring of sensitivity to lithium/galactose. ● represents growth of a spot equivalently to control, ○ represents an absence of a dilution spot where growth was observed in the control. Partial growth of a dilution spot was interpolated to the nearest 0.5, such that less than 0.25 growth for a spot gained no score, 0.25-0.75 added a score of 0.5 and greater than 0.75 growth was scored as an additional 1.

3.13.5. Dealing with incongruences

A limited number of strains were selected for re-testing due to one of the following: i) the spot assay outcome was in contrast with the result obtained from the primary screen, ii) contrasting outcomes existed between the first and the second spot assay (e.g., strains showing sensitivity in one assay whilst being associated with resistance or unaltered phenotype in the other), iii) strains did not grow due to technical issues (e.g., missed pinning). These strains were re-streaked from frozen stock following repetition of **method 3.13.4**; the results were analyzed as for **method 3.13.4.1** and merged with the previously generated set.

3.13.6. Analysis

The overall resulting interactions were grouped according to the molecular function description gene ontology (**method 2.19.1**), along with a manually curated functional classification.

3.14. Environmental challenges

Single colonies of yeast strains listed in **method 3.11.2** were inoculated into 10 ml of liquid YPD and SD media supplemented with 200 µg/ml G418 or 100 µg/ml of clonNAT when required and grown overnight with shaking at 30 °C. The following morning the overnight cultures were diluted to an OD₆₀₀ of approximately 0.05, returned to the shaking incubator and grown to an OD₆₀₀ of 1. Serial dilutions were used to spot the cultures according to the following protocols:

3.14.1. 24-well plate sensitivity assessment spot assay

Diluted cultures were spotted on 24-well plates containing YPD, SD, YPGal, and SGal solid media supplemented with drugs and relevant controls (DMSO or dd water) to identify a window of sensitivity for each compound, as follows: i) caffeine, from 1 μ M to 100 mM; ii) cycloheximide, from 50 nM to 200 nM; iii) DTT, from 3 mM to 7.5 mM; iv) rapamycin, from 1.5 nM to 250 nM; and v) tunicamycin, from 0.6 mM to 1.2 mM over 5 dilution spots.

The plates were incubated at 30 °C and photographic records taken every day for 5 d. Growth was assessed comparing the deletion strains with the appropriate control strain.

3.14.2. Serial spot dilution assay

3.14.2.1. Rapamycin

If a change was observed upon the treatment performed according to **method 3.14.1**, the condition was further tested through a spot assay over a more focussed concentration range. Diluted cultures were spotted following **method 2.17** on YPD or SD agar plates containing rapamycin at the final concentrations of 3 nM and 5 nM.

3.14.2.2. Environmental challenges

Supplementary environmental challenges were also tested specifically: i) 2.5% acetic acid in YPD, SD, YPGal, SGal and SC media; ii) 2% and 4% glycerol in YP and SC media, iii) 2.5% ethanol in YP and SC media; iv) 1 M sorbitol in YPD, SD, YPGal, and SGal media; v) medium adjusted to pH 3 with hydrochloric acid in YPD, SD, YPGal, and SGal media; vi), medium adjusted to pH 10 with sodium

hydroxide in YPD, SD, YPGal, and SGal media; vii) Growth in minimal medium (SC medium); viii) Growth at non-optimal temperature (37°C) in YPD, SD, YPGal, and SGal media. With the exception of condition viii), the plates were incubated at 30 °C and photographic records taken every day for 5 d.

3.15. Results

3.15.1. Chemical genetic profile of lithium

In order to achieve a comprehensive analysis on the role of the translational initiation process in the relief of lithium toxicity in *S. cerevisiae*, the non-essential homozygous diploid gene knock-out collection was screened in rich media containing lithium at different concentrations, utilizing galactose as the only carbon source.

The primary assay consisted of screening Boone's YDS of ~4,480 strains grown on YPGal supplemented with 7, 15, and 30 mM LiCl. SESA, the software utilized for an analysis of growth and the previously described SGA (**method 2.30**) releases results with a degree of statistical confidence and groups them as “definitive”, “probable”, and “possible” hits. As a general trend, deletions that showed phenotypic changes in medium supplemented with 30 mM LiCl also showed concordant phenotypes when the concentration of drug was halved. In the 15 mM screen, 29 out of the 29 sensitive definitive interactions indicated by SESA were also accounted in the higher concentration screen. Only 2 deletions were identified as suppressors in the 15 mM screen: one of them (*ypt7Δ*) was also noted in the 30 mM LiCl screen results, while the other (*mub1Δ*) was a unique hit for the 15 mM screen. For this latter working concentration, SESA also

generated a “probable” results list of 107 sensitive deletion strains: whilst 92 of them were correspondently reported as sensitive in the 30 mM experiment. No suppressor-like hits were reported for “probable” results under 15 mM LiCl treatment. On the basis of these observations this study intends to refer to the 30 mM data, utilizing the 15 mM and 7 mM generated datasets as further elements of comparison, particularly in cases of uncertainty.

This first experiment identified 138 “definitive” hits, i.e., strains with gene deletions that cause yeast cells to become sensitive to 30 mM of LiCl in YPGal medium. In this set, 81 deletions displayed a growth defect (sensitive), while the remaining 57 were described as suppressor phenotypes. A second subset of 172 deletions was also reported by the software as “probable” and accounted for 154 sensitive and 18 suppressor phenotypes.

Figure 3-2. An example of lithium/galactose assay plate. An example of assay plate highlighting an example of sensitive strain *chs5Δ::kanR* (red circle) and suppressor strain *gal2Δ::kanR* (green circle) represented as a quartet of identical deletions in YPGal medium supplemented with 30 mM LiCl.

3.15.2. Spot Assay

Among the “definite” hits, 78 out of the 81 sensitives were further tested through a spot assay and so were 48 out of the 57 suppressors. In the “probable” group, 135 out of the 154 sensitive hits underwent spot assay, along with 12 out of 18 suppressor deletion strains. Spot assay confirmed 25 out of the 78 sensitive definite hits and 34 out of the 135 sensitive probable tested. For the suppressors, the spot assay recapitulated 19 out of the 48 “definitive” and 4 out of the 12 “probable” hits tested. The spot assay was also done on 154 deletion strains, which were manually selected on the basis of principles described in **method**

3.13.4. The spot assay confirmed 25 out of the 154 manually inserted strains as sensitive.

Figure 3-3. An example of spot assay plate containing 30 mM LiCl. An example of spot assay plate showing the basic array set up, comprising control strains included on each plate, namely: a) over-expression plasmid *pTIF2tif1Δ*, b) *rcn1Δ::kanR* sensitive strain, c) *bre5Δ::kanR* suppressor strain, and d) *his3Δ::kanR* wild-type surrogate strain. Examples of strains scored as sensitive (*snf5Δ::kanR*) in red, e) suppressor (*hur1Δ::kanR*) in green, and f) dismissed as no phenotypic change observed (*pex11Δ::kanR*) in blue are also indicated.

As described in **method 3.13.5**, following the two spot assays, a small subset of 49 strains was selected for re-testing. This third spot assay confirmed a further 24 sensitive and 2 suppressor hits and these results were merged with the previously generated ones. Two strains (*gal80Δ* and *snf5Δ*) still gave conflicting results after this assay, and were no further evaluated.

The complete lists of deletion strains indicated by SESA as sensitive or suppressor upon lithium/galactose which were confirmed through the spot

assay are reported in **sections 5.6** and **5.7** of the appendix. Altogether, the final number of interactions reported in the experimental condition of 30 mM LiCl in YPGal was of 108 for sensitives and 25 for resistants, as reported below.

Phenotype	Primary assay	Spot dilution assay	Total confirmed
Sensitive	81/4,480 (1.8%) definitive 154/4,480 (3.4%) probable	25/78 (32%) definitive 34/135 (25%) probable Plus 24/49 (49%) re-test 25/154 (16%) selected	108
Suppressor	57/4,480 (1.3%) definitive 18/4,480 (0.4%) probable	19/48 (40%) definitive 4/12 (33%) probable Plus 2/49 (4.1%) re-test	25
Table 3-2. Interactions reported by this study upon 30 mM LiCl/galactose treatment.			

3.16. Gene Ontology Analysis

A GO terms analysis, as previously described in **method 2.19.1** was used. When molecular function was used as a classification criterion, SGD mapper results (Error! Reference source not found.) reveal that sensitivity to lithium/galactose treatment is engendered by deletion of genes that are significantly (p-value <0.05) enriched for phosphotransferase/kinase activities. These are subclassified as follows: phosphotransferase activity and alcohol group as acceptor (15), kinase activity (15), transferase activity, transferring phosphorus-containing groups (15), a subset of the above genes' group (12) functioning in protein kinase activity and protein serine/threonine kinase

activity (8). This subset was further divided into protein threonine/tyrosine kinase activity (4) and MAP kinase kinase (3).

Function	p-value	ORF
phosphotransferase activity, alcohol group as acceptor	6.45E-05	<i>YJL187C, YOR231W, YPL140C, YJL155C, YNL307C, YHL007C, YIL035C, YJR049C, YHR030C, YJL128C, YJL095W, YGR092W, YLR113W, YHR025W, YHR079C</i>
kinase activity	2.4E-04	<i>YJL187C, YOR231W, YPL140C, YJL155C, YNL307C, YHL007C, YIL035C, YJR049C, YHR030C, YJL128C, YJL095W, YGR092W, YLR113W, YHR025W, YHR079C</i>
protein threonine/tyrosine kinase activity	3.4E-04	<i>YOR231W, YPL140C, YNL307C, YJL128C</i>
protein kinase activity	6.8E-04	<i>YJL187C, YOR231W, YPL140C, YNL307C, YHL007C, YIL035C, YHR030C, YJL128C, YJL095W, YGR092W, YLR113W, YHR079C</i>
protein serine/threonine kinase activity	0.008	<i>YNL307C, YHL007C, YIL035C, YHR030C, YJL095W, YGR092W, YLR113W, YHR079C</i>
transferase activity, transferring phosphorus- containing groups	0.009	<i>YJL187C, YOR231W, YPL140C, YJL155C, YNL307C, YHL007C, YIL035C, YJR049C, YHR030C, YJL128C, YJL095W, YGR092W, YLR113W, YHR025W, YHR079C</i>
MAP kinase kinase activity	0.011	<i>YOR231W, YPL140C, YJL128C</i>
Table 3-3. GO term function over-represented for deletion strains sensitive to lithium/galactose treatment.		

Additionally, SGD mapper process results showed statistically significant (p-value <0.005) over-representation for deletions sensitive to the same experimental conditions in the following, often overlapping, terms: response to

stimulus (34), cell communication (18), signal transduction (17), phosphorylation (13), protein amino acid phosphorylation (12), response to stress (23), response to abiotic stimulus (11), response to osmotic stress (9). Further GO process terms associated to a lower level of significance (p-value <0.05) were response to acid (4), cellular carbohydrate metabolic process (14), phosphate metabolic process (13), phosphorus metabolic process (13), and carbohydrate metabolic process (14). As is clear in Error! Reference source not found. many of these GO process terms are interconnected, often with one term being a subset of another, and their appearance in the ontological list presented in the table is often driven by the same set of genes. For example, some processes all appear as separate terms in the process output, despite being clear subsets of each other, and with only minor differences between them. Clearly the generic GO processes such as these are less informative than the more deeply nested terms such as endoplasmic reticulum unfolded protein response. It is these more informative terms that prove most useful in disentangling the basis of the interactions between lithium/galactose treatment and yeast cell survival. It is these terms which will be considered in the discussion below.

Process	p-value	ORF
response to stimulus	3.52E-05	YKL190W, YML057W, YLR332W, YOL109W, YHL027W, YDR388W, YIL017C, YLL043W, YNL080C, YJL101C, YNL307C, YDR359C, YNL136W, YHL007C, YIL035C, YHR030C, YJL128C, YJL095W, YER177W, YLR113W, YER118C, YCL032W, YPL089C, YER117W, YMR037C, YDR074W, YDR001C, YHR104W, YOL089C, YCR009C, YIL101C, YJL201W, YHR079C, YFL031W

cell communication	6.31E-05	YKL190W, YKL159C, YML057W, YOR231W, YPL140C, YHL007C, YHR030C, YJL128C, YJL095W, YER177W, YER118C, YCL032W, YOL081W, YPL089C, YMR037C, YPR040W, YHR079C, YFL031W
signal transduction	8.62E-05	YKL190W, YKL159C, YML057W, YOR231W, YPL140C, YHL007C, YHR030C, YJL128C, YJL095W, YER177W, YER118C, YCL032W, YOL081W, YPL089C, YPR040W, YHR079C, YFL031W
phosphorylation	1.9E-04	YOR231W, YPL140C, YNL307C, YHL007C, YIL035C, YHR030C, YJL128C, YJL095W, YNL052W, YIL111W, YGR092W, YLR113W, YHR079C
protein amino acid phosphorylation	3.4E-04	YOR231W, YPL140C, YNL307C, YHL007C, YIL035C, YHR030C, YJL128C, YJL095W, YGR092W, YLR113W, YHR079C
response to stress	0.002	YLR332W, YOL109W, YDR388W, YNL080C, YJL101C, YNL307C, YDR359C, YNL136W, YIL035C, YJL128C, YER177W, YLR113W, YER118C, YCL032W, YMR037C, YDR074W, YDR001C, YHR104W, YOL089C, YCR009C, YIL101C, YHR079C, YFL031W
response to abiotic stimulus	0.003	YLR332W, YHL027W, YDR388W, YJL101C, YJL128C, YLR113W, YER118C, YCL032W, YMR037C, YOL089C, YCR009C
response to osmotic stress	0.005	YLR332W, YDR388W, YJL128C, YLR113W, YER118C, YCL032W, YMR037C, YOL089C, YCR009C
response to acid	0.007	YLR332W, YHR030C, YJL095W, YPL089C
cellular carbohydrate metabolic process	0.01	YNL233W, YBR023C, YLR330W, YIL017C, YJL155C, YKL127W, YMR105C, YER177W, YOR136W, YIL125W, YDR074W, YDR001C, YHR104W, YHR079C
phosphate metabolic process	0.012	YOR231W, YPL140C, YNL307C, YHL007C, YIL035C, YHR030C, YJL128C, YJL095W, YNL052W, YIL111W, YGR092W, YLR113W, YHR079C
phosphorus metabolic process	0.012	YOR231W, YPL140C, YNL307C, YHL007C, YIL035C, YHR030C, YJL128C, YJL095W, YNL052W, YIL111W, YGR092W, YLR113W, YHR079C
carbohydrate metabolic process	0.032	YNL233W, YBR023C, YLR330W, YIL017C, YJL155C, YKL127W, YMR105C, YER177W, YOR136W, YIL125W, YDR074W, YDR001C, YHR104W, YHR079C
Table 3-4. GO term process over-represented for deletion strains sensitive to lithium/galactose treatment.		

Protein products of genes corresponding to deletion sensitive to lithium/galactose treatment displayed a cellular localization significantly (p-value <0.05,) enriched for the calcineurin complex (*YKL190W*, *YKL159C*, and *YML057W*) and the site of polarized growth (*YJL187C*, *YOR231W*, *YNL233W*, *YBR023C*, *YHL007C*, *YHR030C*, *YGR238C*, *YGR092W*, *YER118C*, and *YBL047C*).

For what concerns the resistant strains, GO analysis conducted on the basis of functional and localization similarities did not return significant enrichment. According to the same source, galactose metabolic process (3) is significantly (p-value <0.005) over-represented among strains identified as suppressor for the lithium/galactose treatment, along with galactose transport (2) and glycosylation related processes (4), as shown in **Table 3-5**.

Biological process	p-value	ORF
primary metabolic process	3.5E-04	<i>YGL168W</i> , <i>YGL167C</i> , <i>YBR020W</i> , <i>YLR081W</i> , <i>YPL248C</i> , <i>YML048W</i> , <i>YOR002W</i> , <i>YOR067C</i> , <i>YGR078C</i> , <i>YNR051C</i> , <i>YER151C</i> , <i>YDR092W</i> , <i>YNL323W</i> , <i>YNL123W</i> , <i>YAL023C</i> , <i>YMR263W</i> , <i>YHR041C</i> , <i>YMR039C</i> , <i>YOR304W</i> , <i>YLR372W</i> , <i>YER111C</i>
galactose metabolic process	0.002	<i>YBR020W</i> , <i>YLR081W</i> , <i>YPL248C</i>
metabolic process	0.003	<i>YGL168W</i> , <i>YGL167C</i> , <i>YBR020W</i> , <i>YLR081W</i> , <i>YPL248C</i> , <i>YML048W</i> , <i>YOR002W</i> , <i>YOR067C</i> , <i>YGR078C</i> , <i>YNR051C</i> , <i>YER151C</i> , <i>YDR092W</i> , <i>YNL323W</i> , <i>YNL123W</i> , <i>YAL023C</i> , <i>YMR263W</i> , <i>YHR041C</i> , <i>YMR039C</i> , <i>YOR304W</i> , <i>YLR372W</i> , <i>YER111C</i>
galactose transport	0.006	<i>YBR020W</i> , <i>YLR081W</i>

cellular metabolic process	0.01	<i>YGL168W, YGL167C, YBR020W, YLR081W, YPL248C, YML048W, YOR002W, YOR067C, YGR078C, YNR051C, YER151C, YDR092W, YNL323W, YAL023C, YMR263W, YHR041C, YMR039C, YOR304W, YLR372W, YER111C</i>
macromolecule metabolic process	0.017	<i>YGL168W, YGL167C, YBR020W, YLR081W, YPL248C, YML048W, YOR002W, YOR067C, YGR078C, YNR051C, YER151C, YDR092W, YAL023C, YMR263W, YHR041C, YMR039C, YOR304W</i>
biopolymer glycosylation	0.022	<i>YGL167C, YOR002W, YOR067C, YAL023C</i>
protein amino acid glycosylation	0.022	<i>YGL167C, YOR002W, YOR067C, YAL023C</i>
glycoprotein biosynthetic process	0.026	<i>YGL167C, YOR002W, YOR067C, YAL023C</i>
glycoprotein metabolic process	0.028	<i>YGL167C, YOR002W, YOR067C, YAL023C</i>
Table 3-5. GO process terms over-represented for deletion strains resistant to lithium/galactose treatment.		

3.17. BiNGO Analysis

Both sensitive and resistant strains datasets generated through **methods 3.13** were further analysed using BiNGO, as described in **section 2.29**; over-representation of GO terms is reported with associated statistical significance generated according to the hypergeometric test.

A main cluster among deletions sensitive to lithium/galactose treatment emerged for genes which protein products functionally serve as part of the oxido-reductase activity, particularly if acting on the heme group of donors, with oxygen as acceptor (p-value 2.0E-03) and heme-copper terminal oxidase activity (p-value 0.002) both which activities converge in the cytochrome c oxidase activity (p-value 2.0E-03). Furthermore, phosphoglucomutase enzymatic activity (p-value 5.20E-06) was also found to be effected, as shown in **Fig 3-4**.

Fig 3-4. BiNGO-generated illustration of over-represented GO function terms for sensitive strains upon lithium/galactose treatment.

An over-representation was as well observed for kinase activity with serine/threonine (1.43E-07 p-value) and serine/threonine/tyrosine specificity (p-value 2.97E-06), and for MAPK kinase (p-value 2.05E-05) and MAP kinase activity (p-value 3.0E-03) (**Fig 3-5**).

Fig 3-5. BiNGO-generated illustration of over-represented GO function terms for sensitive strains upon lithium/galactose treatment.

The processes shown to be the most affected by lithium treatment in galactose as sole carbon source is cell wall organization and biogenesis (p-value 8.07E-08), although this latter function is considerably less represented. In addition, cytokinetic (p-value 6.11E-04), barrier septum formation (p-value 5.76E-06), and external encapsulating structure organization (p-value 1.44E-06) functional cluster were also highlighted (**Fig 3-6**).

Fig 3-6. BiNGO-generated illustration of over-represented GO process terms for sensitive strains upon lithium/galactose treatment.

Again, in line with the GO term analysis described above, a convincing subnetwork of related processes can be described for genes associated to the signalling pathway (p-value $1.84\text{E-}09$): signal transmission (p-value $1.60\text{E-}04$) in particular if mediated by signal transmission via phosphorylation event (p-value $7.15\text{E-}05$), and transduction (p-value $1.42\text{E-}04$) eventually converge into the intracellular protein kinase (p-value $7.15\text{E-}05$) which operates via the MAPKKK cascade (p-value 0.004). An extended network branch showed that response to ER stress (p-value $1.68\text{E-}07$) and consequent driven UPR (p-value $2.06\text{E-}08$) processes are negatively affected by lithium/galactose treatment; in particular, among the intracellular signalling pathways, the ER nucleus signalling pathway (p-value $1.87\text{E-}10$) was characterized by an high enrichment (**Fig 3-7**). This illustrates the value of the BiNGO analysis in highlighting the relatedness between terms, particularly their sub-dependency on each other, which is not presented within the crude GO analysis, as discussed above.

Fig 3-7. BiNGO-generated illustration of over-represented GO process terms for sensitive strains upon lithium/galactose treatment.

A last cluster was noticed for lithium sensitive hits: the protein amino acid phosphorylation (p-value 9.44E-07) process appeared over-represented along with the converging branches of phosphorus (p-value 4.53E-05) and phosphate (p-value 3.68E-05) metabolic processes which contribute to phosphorylation (7.93E-07) on one side and the protein modification processes, in particular post-translational protein modification (p-value 6.87E-05) on the other (**Fig 3-8**).

Fig 3-8. BiNGO-generated representation of over-represented GO process terms for sensitive strains upon lithium/galactose treatment.

For what concerns deletion strains that showed tolerance to lithium, the function-based classification was not particularly informative on specific categories, with the exception of a small cluster indicating transferase activity of hexosyl and glycosyl groups (p-value 2.13E-04).

The process-based categorization, however, returned a wealth of information on the processes in whose genes' deletion resistance in to lithium/galactose are involved: the most represented correspond to galactose metabolic process (p-value 9.32E-06), a number of nested terms relating to glycosylation (p-value 1.66E-04), protein amino acid N-linked glycosylation (p-value 8.85E-04), glycoprotein (p-value 2.16E-04) and oligosaccharides (p-value

3.18. Different environmental conditions

Among the vast set of environmental and chemical challenges against which *TIF1* and *TIF2* deletion strains have been tested (**method 3.14**), only one condition returned an appreciable phenotypic change. *MATa tif1Δ::kanR* displayed sensitivity to Rap treatment at both 3 nM and 5 nM in both rich (YPD) and minimal (SD) media but this was not the case for the same strain of opposite mating type nor the same strain carrying a different antibiotic cassette, as shown in **Figure 3-11**.

Figure 3-11. Rapamycin spot assay. Spot assay showing from the top: *MATa Δtif1::kanR*, *MATa Δtif2::kanR*, *MATa Δhis3::kanR* (WT surrogate strain), *MATα Δtif1::natR*, *MATα Δtif2::natR*, *MATα* Y7092 (WT) on YPD supplemented with RapA 5nM (right) and DMSO control (left) showing sensitivity to RapA for *MATa tif1Δ::kanR* exclusively.

3.19. Discussion

Although SGA is a very powerful technique, a downside of it is that each mutant is forced to adapt to the loss of a particular gene, and compensatory events that can obscure the true effect of the original gene knockout cannot be ruled out. A way to attempt a resolution of this issue is to design complementary experiments that can integrate with the data from the SGA and thereby add pieces of information to the network of interactions, such as the use of promoter systems (chapter 2), chemicals, and environmental challenges (current chapter).

3.20. Cell signaling

Cell signaling functions as part of a complex system of communication that governs and synchronizes basic cellular activities; its associated GO term process was considerably over-represented among lithium sensitive strains and, as described in **sections 3.16** and **3.17**, contributions from different regulatory proteins' mechanisms were observed. The high number of hits is indicative of the ubiquitous nature, importance and interconnections of these signalling pathways. Phosphorylation-mediated signalling governs the convincing cluster of related processes described for genes associated with signal transmission and transduction. Phosphorylation of proteins by kinase enzymes is important in signal transduction and regulation of cellular activity, such as cell division.

3.20.1. Kinases

Considered the key regulators of cell function, a substantial number of protein kinases have been reported to be effected by lithium action. Because

kinases instigate sweeping effects on cells, their activity is often regulated via phosphorylation, by binding of activator or inhibitor proteins, and/or small molecules. Different kinases mediate different signals and, according to GO term classification (**sections 3.16** and **3.17**) the enzymes affected by lithium treatment are to be identified in a substantial group of protein serine/threonine kinases.

As many kinases use Mg^{2+} as a cofactor, it is likely that lithium interferes with the activity of these enzymes due to its competitive nature in occupying magnesium binding sites. As introduced in **section 3.2**, lithium binds to one of its targets, the enzyme P_{gmp}, by virtue of its ability to displace magnesium and compete in this way for the binding site; the same rationale could apply to the hypersensitivity observed upon lithium/galactose treatment in strains bearing a deletion of a kinase, and so the importance of one or more other kinases has been increased.

One very important group of protein kinases are the mitogen-activated protein kinases (MAPK), which are activated by extracellular ligands that in turn activate cognate transmembrane receptors and/or proteins associated with them, whilst their inactivation is usually due to signals that inactivate the phosphatase controlling a given MAP kinase. Although the sensitivity of strains lacking kinase activity in this study has been rationalized with a general mechanism based on substrate competition, the consistent group of deletions which has been highlighted by the screens suggests that something more may need to be said about the Pck1p kinase pathway, its signalling along the cell wall

integrity (CWI) route and the MAPK cascade which it initiates; this topic will be discussed in **section 3.21**.

Kinase-mediated cell signalling is also a main regulatory component of stress response pathways, such as the High Osmolarity Glycerol (HOG) response pathway, which mediates cellular adaptation to hyperosmotic stress. Roles and activities of kinases involved in these routes will be discussed in **section 3.25**.

3.21. Cell wall organization and biogenesis

When confronting stressors of different natures, yeast cells face challenges that impact on their ability to adapt and ultimately survive and ensure growth. Changes at a cellular level are achieved through regulation of gene expression of selected genes, enabling the cell to endure unfavourable environments; this mechanism requires a coordinated effort from several pathways, the most significant of which is undertaken by the cell wall. As yeast is a unicellular organism, its cell wall is the primary means through which the cell defends itself from environmental adversities, assisted by stress sensors that advertise any damage inflicted and coordinate the repair process by activating the appropriate signalling pathway(s). Among eukaryotes the cell wall is a cellular structure exclusive to fungi. Knowledge of the organization of signalling proteins involved in cell wall-related response mechanisms is valuable for understanding the intricate signalling and pathways' inter-talk in *S. cerevisiae*. Among several signalling pathways contributing to the cell wall maintenance, the cell wall integrity (CWI) pathway is primarily accountable for addressing the response to insults and coordinating changes to the wall structure. A precise implication of the CWI pathway in responding to lithium/galactose treatment

has been revealed by this research study, identifying the majority of the components of the pathway, from the cell surface sensor (Mid2p) which triggers CWI activation, through the MAPKs (Bck1p, Mkk1p, Mkk2p, and Slt2p) that propagate the signal to the ultimate transcription factor (Rlm1p), as shown in **Figure 3-12**.

Figure 3-12. Diagram of the CWI cascade. The Mid2p sensor binds Rom2p that activates Rho1p; this latter initiates the PKC pathway following activation of Pkc1p. The PKC pathway includes the MAPK cascade (Bck1p, Mkk1p/2p, Slt2p, and Slt2p) which ultimately leads to the activation of the transcription factor Rlm1p. Zeo1p, a peripheral membrane protein acting as a regulator of the CWI cascade is also shown. The genes affected by lithium/galactose treatment are indicated with a red thick. Additional stress sensors exist and are capable of activating the CWI pathway,

although not represented in this diagram; for a detailed review on this topic the reader is referred to Fuchs & Mylonakis, 2009.

A family of cell surface sensors is responsible for detecting the status of the cell wall and initiating a signalling response in the event of any compromise of cell wall integrity, this includes Wsc1p, Wsc2p and Wsc3p (Verna, 1997) and Mid2p and Mtl1p (Levin, 2005). Interestingly, this research screen identified only the *mid2Δ* strain as sensitive to lithium/galactose treatment and, although the deletions of Wsc family strains were all tested in the primary screen, none of them returned appreciable changes in phenotype. The *mtl1Δ* was brought to attention by the primary screen but spot assay results did not confirm its sensitivity to the drug/carbon source treatment. Among the aforementioned cell wall stress sensors, Wsc1p and Mid2p are considered the most important and serve with partially overlapping roles in CWI signalling; in this study, however, different phenotypes were displayed by their KO strains. This result finds correspondence with published data showing how *MID2* and *WSC1* genes respond differently to different stimuli (Rodicio & Heinisch, 2010). The finding that only one of the sensors was impaired emphasizes the existence of a sharp specificity among cellular membrane sensors, although co-existing with an extensive and robust redundancy. In contrast, the failure to find sensitivity in either the *wsc2Δ* or sensitivity in either the *wsc2Δ* or *wsc3Δ* strains presumably arises from the ability of these partially redundant sensor transducers to cover for each other's absence.

The O-mannosylation of Mid2p and Wsc1p is regarded as important to their own stability (Philip & Levin, 2001), in fact, reduced O-mannosylation

leads to incorrect proteolytic processing of these proteins, which in turn results in impaired activation of the PKC pathway and finally causes cell death in the absence of osmotic stabilization (Lommel, 2004). More recently, an N-glycan of Mid2p was found to be specifically and directly involved in Mid2p sensing, both the extent of the N-linked glycan and its distance from the plasma membrane affect Mid2p function. In line with this, Mid2p lacking N-glycosylation fails to perceive cell wall challenges (Hutzler, 2008). These observations demonstrate the importance of glycosylation for CWI sensing and in turn, its contribution to cell wall biogenesis and polarized growth, at least in yeast. In consideration of the fact that the concentration of lithium tested in this screen it is not high enough to be considered a strong stress inducer, this study suggests an alternative proposition in which the reason for finding hits related to the CWI pathway may not lie in cell wall stress but rather in a glycosylation issue, which gives some form of feedback in accordance with minor cell wall stress.

The cell surface stress sensor Mid2p, a verified hit in the screen, is responsible for detecting the cell wall status and communicating to Rom2p, a small G-protein which triggers a set of effectors, Rho1p among them. The chemical genetic technique used here is unable to provide an assessment of phenotypic changes for KO of genes encoding components of the so-called “upstream region” of the CWI pathway as both *RHO1* and *PKC1* are essential genes, so their deletion strains are not available in the homozygous deletion set. Furthermore, *ROM2* has a duplicate, *ROM1*, which has at least partially overlapping functions, so its deletion generates an unreliable weakly sensitive phenotype. Rho1p consequently turns on the Pkc1-mediated pathway through the phosphorylation of Pkc1p, which transmits the signal via the MAPK cascade.

Pkc1p transmits the signal through the MAPK cascade, components of which all display indisputable sensitivity to lithium/galactose treatment. The target of Pkc1p phosphorylation is Bck1p, a MAPK kinase kinase (MAPKKK), that functions in the regulation of the MAPK kinases (MAPKKs) Mkk1p and Mkk2p. Differently from other yeast species in which only one enzyme isoform has been described, the baker's yeast accounts for two kinases (Mkk1p and Mkk2p), and strains bearing deletions of either were adversely affected by lithium/galactose treatment. This result can be rationalized given the high degree of functional redundancy these protein share, although it implies that deletion of either gene leads to a situation where insufficient Mkk1/2 protein is available to provide the level of signalling required under lithium/galactose stress. The signal from Mkk1p and Mkk2p is received by the MAPK Slt2p, a kinase that in *S. cerevisiae* is activated by an extensive subset of stimuli (Torres, 1991; Costigan & Snyder, 1994). Slt2p phosphorylation activity ultimately activates the transcription factor Rlm1p. Altogether these results emphasize the importance of the CWI pathway in budding yeast and align with emerging literature that proposes a more significant involvement of CWI for the *S. cerevisiae* species compared to other yeasts.

This study concludes that all expected components of the Pkc1-mediated CWI pathway display sensitivity to lithium treatment when galactose is used as sole carbon source, suggesting that an involvement of this pathway in supporting the response. This latter may be correlated to TOR2, which is known to interact with elements of the CWI such as Rho1p and in this fashion modulate Pkc1p (Nonaka, 1995).

Furthermore, results indicated that deletion of *ZEO1*, the protein product of which is a regulator of the CWI (Green, 2003), also leads to sensitivity under lithium/galactose treatment. *ZEO1* is a gene that encodes a peripheral membrane protein that interacts with the cytoplasmatic domain of the previously described Mid2p. This result allows speculation on whether Zeo1p, could also play an active role in the response mechanism activated by lithium in the outer cellular membrane, possibly through a conformational change imposed on Mid2p, which would activate the cascade of CWI response. Findings from the screens reported here hint to a scenario in which Zeo1p acts as a sensor for lithium, forwarding the message down the CWI pathway and possibly integrating this message to be addressed to other pathways as well.

A better characterization of CWI pathway represents a steady improvement in consideration of the relevant roles the pathway retains to have in fungi pathogenic for humans, such as *C. albicans*, and its application in design of antifungal drugs.

3.22. Endocytosis

As extensively discussed in **section 2.36**, sorting and internalization of cell surface cargo into vesicles budding from the plasma membrane is achieved via endocytosis and is characterized by a complex network of highly connected proteins. Two deletion strains relevant to this process have been identified among the sensitive hits: *RVS161* and *RVS167* genes encode for actin-associated proteins with roles in endocytosis and actin cytoskeleton organization. Rvs161p and Rvs167p are recruited to the bud tip during polarized growth (Bon, 2000)

and are collectively referred to as the *RVS* genes or Rvs proteins (Rvsps) below. These genes are homologs of mammalian amphiphysins, a protein family that has been postulated to play a key role in clathrin-mediated endocytosis of synaptic vesicles. Amphiphysins play important roles in mammals and insects, having pleiotropic functions in clathrin-mediated endocytosis and the regulation of membrane dynamics, perhaps through the actin cytoskeleton (reviewed in Zhang & Zehhof, 2002).

In *S. cerevisiae*, the endocytic defects discovered in Rvsp mutants have been interpreted as supporting evidence for a role of amphiphysins in clathrin-mediated endocytosis; however, the role for Rvsps in endocytosis may present differences from that proposed for vertebrates. To further complicate the matter, remarkable differences exist in the clathrin-mediated endocytosis in yeast compared to other multicellular organisms (Baggett & Wendland, 2001) where clathrin is absolutely required for receptor-mediated endocytosis and cellular viability, whereas deletion of the homolog proteins in budding yeast only moderately reduces them. These observations suggest that the endocytotic defects caused by deletion of *RVS* genes may be the result of a different mechanism, rather than directly assisting vesicle fission. Congruent results support the model in which Rvs167p and Rvs161p are required for normal cortical actin cytoskeleton organization which, in turn, is coupled to endocytosis and membrane dynamics through signalling (Schafer, 2002). A correct stoichiometry of cytoskeletal components has long time been suspected as a crucial requirement for cell viability, at least in yeast (Drubin, 1988; Magdolen, 1993); it was only recently, however, that a connection was uncovered between the actin cytoskeleton and the translational machinery (Gross & Kinzy, 2007).

Although these two cellular processes appear very distant, a total reduction of translation, eventually accompanied by accumulation of the 80S ribosome, was observed in strains carrying a mutation or a null allele of genes encoding actin or actin-regulating proteins. Both Rvs161p and Rvs167p proteins show a considerable number of physical interactions (97 and 312 respectively) strongly limiting the relevance of speculations. However in the context of this overall study, it is interesting to note that among the Rvs161p annotated physical interactions, the most relevant to be recognized is with Cdc33p the cytoplasmic mRNA cap binding protein and translation initiation factor eIF4E, the RNA helicase Ded1p (introduced in **section 2.4**), and Met4p, a transcription factor for several translation related genes, including *TIF1*, *TIF2*, *TIF3*, and *TIF4631*, along with a number of ribosomal proteins and few ubiquitin/de-ubiquitin proteins. For what concerns Rvs161p, which has 97 physical interactions, among them Tor1p, Tor2p, and Psb2p (sensitive deletion identified in this screen). The finding of this pair of genes may also be considered in the light of the impairment of the phosphorylation events observed (**section 3.20**). Phosphorylation also acts as a regulator of interactions between endocytic proteins as such modification allows disassembling of endocytic complexes to prepare for the next round of cargo sorting and vesicle formation (Smythe & Ayscough, 2003).

The TOR pathway acts an important signalling component by linking external signals to the cytoskeletal machinery in a variety of cell types and organisms. A connection between Tor2p and the CWI pathway (**section 3.21**) also suggests a link to actin/cytoskeleton related processes, with the serine/threonine kinase Pkc1p being the Rho1p effector which mediates Tor2p signalling to the actin cytoskeleton (Helliwell, 1998).

Strengthening the connection with the Rvsps proteins is the suppressor phenotype exhibited by deletion of the *SUR4* gene upon lithium/galactose treatment; this is supported by the positive genetic interaction in combination with *pgm2Δ* observed by an independent group (Szappanos, 2011). Sur4p is involved in sphingolipid biosynthesis (Schneider, 1999) and is annotated as suppressor of *rvs161* and *rvs167*, both of them identified in this study as sensitive deletions. As *RVS161* and *RVS167* genes code actin patch components, this suppression is likely to be mediated by an effect on the sphingolipid synthesis (Desfarges, 1993; Young, 2002).

3.23. Chitin

The polysaccharide chitin is an important structural component of many fungi; in particular the yeast cell wall is mainly composed of glucan and mannan, together with a minor content (about 2%) of chitin and proteins; the majority of chitin is however found at the site of bud formation and bud scars (Tkacz & Lampent, 1972).

When stress conditions cause alterations to the wall structure, the cell activates several responses as part of the so-called “compensatory mechanism” (Popolo, 2001), among these is an increase in chitin content. In *S. cerevisiae*, chitin content has been found to increase greatly as a compensatory stress mechanism in response to mutations that lead to a weakening of the cell wall, such as in mannoprotein, β -1,3-glucan and β -1,6-glucan synthesis KO mutants (Popolo, 1997; Valdivieso, 2000). Other changes in the structure and/or composition are known to be borne by the cell wall by virtue of its dynamic

nature; these amendments co-operate in the repair of the damaged cell wall and ultimately allow the cell to cope with a variety of environmental stresses.

Given the importance of chitin in surviving environmental stress, it is no surprise that loss of chitin leads to sensitivity to lithium treatment. The synthesis of this long-chain polymer of N-acetylglucosamine is directly controlled by three distinct chitin synthases (CS) I, II, and III, each characterized by different enzymatic activity (reviewed in Henar Valdivieso, 1999). CSIII provides the majority of cell wall chitin, whilst CSI and II are produced as zymogens that are presumably activated only at need. For this reason, loss of functionality within the CSIII system leads the sensitivity profile found in this study.

As reported in **section 3.16**, strains bearing deletions of genes encoding for members of the CSIII subfamily (*bni4Δ*, *chs3Δ*, and *chs5Δ*) are debilitated in their growth in lithium/galactose medium. The *BNI4* gene encodes a bud neck localized scaffold protein, which is required for localization of CSIII by interacting with septin neck filaments and Chs4p, a regulatory subunit of CSIII. The same Bni4p protein is also involved in targeting protein phosphatase 1 (Glc7p) to the bud neck before bud emergence and its tasks also account for the assembly of the chitin ring, the septum formation and the maintenance of bud neck integrity. Bni4p localization is regulated during the cell cycle, with the protein located on the exterior of the septin ring before budding and on the mother side of the collar after budding (Kozubowski, 2005). *CHS3* encodes the CSIII catalytic subunit, which promotes the transfer of N-acetylglucosamine to chitin and it is required for synthesis of the majority of cell wall chitin, the chitin ring during bud emergence, and spore wall chitosan. Exporting of Chs3p from the Golgi structure to the plasma membrane is under the expertise of Chs5p, a

component of the exomer complex encoded by *CHS5*. Although this is not applicable to the whole set of chitin-related deletions, it is interesting to note that *CHS5* has also been reported to share a negative genetic interaction with *PGM1* (Costanzo, 2010). In line with the above results, deletion of *SHE1*, whose corresponding protein localizes to the bud neck in a ring-shaped structure (Wong, 2007), also leads to sensitivity.

Although data from this research show the involvement of chitin in the response to lithium treatment as uncontroversial, it remains to elucidate whether the impact on chitin is because of a role of chitin in lithium exclusion or whether lithium inhibits a different component of the cell wall, in which case chitin's contribution would be required to compensate for the damage caused. In this context, chitin's function would become essential. A similar instance has been previously verified for the antifungal agent echinocandin, an inhibitor of $\beta(1,3)$ -glucan synthesis in *C. albicans* (Onishi, 2000) in which treatments increasing the chitin content of the cell wall reduced the efficacy of echinocandins. Although the mechanism is not fully understood, this supposition has been endorsed by previous work done by Walker and colleagues (2008) that proposed that it is likely to occur either by selection of a sub-population of naturally occurring chitin-rich cells, and/or by induction of the cell wall compensatory mechanisms that activate chitin synthesis.

As extensively elucidated in *C. albicans*, the Ca^{2+} -calcineurin route plays a key role in activating CS enzymes as part of the compensatory mechanism -at least of *CaChs3p* (Class IV), which corresponds to *S. cerevisiae* Chs3p (Class IV) (Munro, 2007)- as one of the signal transduction pathways that function in this response, along with HOG and PKC routes. *CaMkc1*, corresponds to yeast Slt2p

(a sensitive hit discussed in **section 3.21**) is among the proteins that take part in this mechanism. In consideration of the wealth of lithium sensitive Ca^{2+} -calcineurin-related hits identified in this study (**sections 3.24** and **3.31**), parallels could be drawn between *C. albicans* and *S. cerevisiae* with respect to signalling pathways regulation.

Although purely speculative, the possibility that the reason for finding this pathway may not lie as much in cell wall stress as in a problem with glycosylation may be considered. This event could provide some form of feedback inducing minor cell wall stress. A study undertaken in the fungus *A. fumigatus*, on the complex mechanisms of cell wall biosynthesis, recently revealed that the major compensatory mechanism induced by repression of N-glycosylation causes an activation of the UPR, which may subsequently trigger an up-regulation in the expression of cell wall protein and chitin (Li, 2011).

In summary, under lithium/galactose stress, the yeast cell responds by activating several mechanisms, among these the increase in chitin synthesis rate and content is one of the most significant. It is important to consider the role of chitin not only as a minor component of the cell wall but also as component of support for neck and cellular budding which may be crucial for cell surviving in certain situations. How Ca^{2+} signalling influences this and what extent of connection it holds with other processes remains to be elucidated.

3.24. Cell cycle

Complicated mechanisms of Ca^{2+} -dependent regulation are known to take place during the cell cycle, in which a Ca^{2+} signalling pathway inactivates the cell-cycle at the G_2 phase through two different route branches, one of them involving calcineurin, the other acting on the Slt2p MAPK cascade. Interestingly, *SLT2*, deletion of which was identified as leading to sensitivity to lithium/galactose in this screen (**section 3.21**), is also reported to share a negative genetic interaction with *PGM1* (Costanzo, 2010), endorsing the rationale that any deletion that is SL with *PGM1/2* deletion could hold the same type of genetic interaction with the “Pgmp inhibition” condition.

Genetic and molecular analysis of the Ca^{2+} -dependent cell cycle regulation has revealed an elaborate mechanism for the calcineurin-dependent regulation of the G_2/M transition, in which calcineurin activates the protein kinase Swe1p. *SWE1* gene deletion resulted in sensitivity to lithium/galactose treatment. Calcineurin elevates *SWE1* expression at the transcriptional level and this is important for its activation leading to G_2 cell cycle inhibition and polarized bud growth (Mizunuma, 2004). Interestingly, Ca^{2+} -induced phenotypes were also reverted by deletion of Bck1p kinase (**section 3.21**) (Mizunuma, 1998), suggesting that calcineurin and the MAPK Bck1p function together in the Ca^{2+} -dependent regulation of the cell cycle and morphogenesis. Swe1p is regulated by calcineurin in the cell cycle G_2/M transition and at several levels such transcription, post-translational modifications and degradation. The physiological importance for cell cycle regulation by Ca^{2+} -dependent Swe1p activation is not completely understood, but this mechanism seems to be

required for the yeast response to an altered membrane since Ca^{2+} -dependent G_2 cell cycle inhibition is induced in response to a membrane stretching stress that appears to trigger the activation of a Ca^{2+} signal (Mizunuma, 2004). Upon activation of the morphogenesis checkpoint that monitors the actin cytoskeleton and the septin ring in the G_1 and early S phases, G_2 cell cycle inhibition is induced by activation of Swe1p (Lew & Reed, 1995). However Ca^{2+} -dependent Swe1p activation seems to take place by a mechanism that is distinctive from the morphogenesis checkpoint (McMillan, 1999).

Interestingly, deletion of *XPB1*, a gene coding for a transcriptional repressor that binds to promoter sequences of the cyclin genes, was identified in this screen, raising the number of hits related to repression of cell cycle progression. Strikingly, among the very limited number of physical interactions (9) annotated for Xbp1p, one of them (protein-RNA; Colomina, 2008) is with the RNA binding protein Whi3p. The progression of cell cycle upon lithium/galactose treatment may then also be regulated by processes related to Whi3p, whose corresponding gene deletion was identified as sensitive in the screen. Although this finding is coherent with other hits identified in this study, it needs to be remarked that a conspicuous number of genetic/physical interactions are annotated for *WHI3*/Whi3p. This protein plays a role in monitoring of cellular fate and dose-dependent regulation of the critical cell size required for passage through START. This form of control is achieved by Whi3p RNA binding protein activity sequestering the mRNA of a G_1 cyclin involved in cell cycle progression (Cln3p) in foci structures localized in the cytoplasm.

Whilst the discovery that both *XPB1* and *WHI3* deletions sensitise to the lithium/galactose condition, this may be coincidental. Xbp1 is a transcription

factor that the YEASTRACT (Yeast Search for Transcriptional Regulators And Consensus Tracking) database records to regulate more than 400 genes supported by documented, direct evidence. Loss of any of these interactions could explain the sensitivity of the *XPB1* deletion strain. The *WHI3* deletion strain sensitivity could also have an alternative explanation: Whi3p is an RNA binding protein and a study by Colomina et al (2008) shows that Whi3p targets a preponderance of mRNAs associated with membrane and exocytic proteins. It is salient to note that this group highlighted genetic interactions between *WHI3* and the CWI pathway, commenting that “cell wall integrity is compromised in Whi3-deficient cells” (Colomina, 2008).

3.25. Response to stress

Whilst the cell has to face a broad range of environmental and chemical insults, the elements which function in the pathways related to these responses are present in a limited number. For this reason, when coping with environmental adversities specific cellular mechanisms can enable the same genes to participate in more than one response pathway. It is then not surprising to find a number of sensitising deletions correspond to genes (*EOS1*, *GRE3*, *GSH1*, *HOG1*, *MID2*, *MSN2*, *PBS2*, *SHO1*, *STE20*, and *STE50*) that operate in responding to diverse stress conditions, as chemical stimuli, osmotic stress, oxidative stress and response to heat among the lithium/galactose sensitive hits; a substantial subset of these genes co-participate in regulating the response to more than one challenge. This scenario simply reflects the cellular organization in adjusting the system after insults: the ability of certain genes to be modulated by an appropriately tuned signal enables these gene products to multifunction in

different pathways. This needs to be borne in mind when interpreting results generated through GO term analysis as data of this nature does not necessarily mean that lithium is involved in all these particular routes, as in the case of response to heat stress.

Although the set of genes reported in the mechanisms of response to stress will not be extensively discussed, it is interesting to mention the sensitive phenotype observed for deletion of *MSN2*, which encodes for Msn2p, a zinc finger transcriptional activator required for transcriptional induction mediated through the stress response element (STRE) (Martinez-Pastor, 1996). The transcription factor activity of Msn2p, together with the related Msn4p, controls the general stress response in budding yeast (Martinez-Pastor, 1996) via regulation of the expression of more than a thousand genes. The result that deletion of *MSN4* was not identified in the screen could find its rationale in what it is currently known about these two ORFs: *MSN4* gene expression is itself Msn2p/Msn4p-dependent and is induced by stress, while *MSN2* expression appears constitutive (Gasch, 2000). Msn2p and Msn4p share 41% identity and are similar in size (Estruch & Carlson, 1993); they are largely, but not completely, functionally redundant and there is increasing awareness that the individual regulatory contributions of these transcription factors could differ for specific genes and under particular stress conditions (Estruch, 2000 for review). Several genes have their expression regulated by Msn2p (and Msn4p) in response to diverse stresses, including heat shock, osmotic shock, oxidative stress, low pH, glucose starvation, sorbic acid, and high ethanol concentrations by binding to the STRE element, located in the promoters of these genes (Martinez-Pastor, 1996). Interestingly, proteomics (Boy-Marcotte, 1998) and microarray (Chua, 2006)

evidence show that *PGM2* and *PGM3* are amongst the genes targeted by Msn2p/4p. Under non-stress conditions, Msn2p and Msn4p are localized in the cytoplasm (Gorner, 1998); the regulation behind this localization is in some measure regulated by TOR signalling through the 14-3-3 protein (accounting for Bmh1p and Bmh2p, in *S. cerevisiae*), which acts as a cytoplasmic anchoring partner for both Msn2/4p (Beck & Hall, 1999). Upon stress, however, they display a periodic nucleocytoplasmic shuttling behaviour, as both Msn2p and Msn4p become hyperphosphorylated and relocalized to the nucleus (Jacquet, 2003). There is also evidence that Bmh1p/2p may suppress growth inhibition by rapamycin (Bertram, 1998). This screen reports deletion of *BMH1*, but not *BMH2*, as a verified lithium/galactose sensitive hit: Bmh1p is the major isoform, so it is possible that the extent of its contribution is reflected in the deletion strain's ability to grow under the condition tested.

Deletions of key genes in the osmoregulation system, which is responsible for active control of the cellular water balance, lead to enhanced sensitivity in the presence of by LiCl. Although the molarity value utilized in this study is not high enough to invoke an osmotic stress (Brewster, 1993) a number of stress-related annotated hits were reported and speculation could be made about lithium being able to induce an ion stress at lower concentrations than other ions. The osmoregulatory system in the yeast *S. cerevisiae* has been extensively investigated and the crucial contribution to its osmoregulation is the production and accumulation of glycerol, a mechanism which is partly controlled by the high osmolarity glycerol (HOG) signalling system (reviewed in O'Rourke, 2002). Deletion of *HOG1* gene, which encodes a MAPK with mammalian homolog p38

involved in inflammatory and stress responses (Raingeaud, 1995), was observed as sensitive to lithium/galactose in this study. Hog1p kinase activity is integral to the osmoregulatory signal transduction cascade, which ultimately affects gene expression, progression through G₁ and G₂ phases of the cell cycle, and cellular ion levels in response to hyperosmotic stress (reviewed in Correia, 2010). Whilst a conspicuous number of interactions records are annotated for *HOG1*/Hog1p making the interpretation of this hit rather difficult, the lithium sensitive interaction (this study) reported for *PBS2* may provide the ground for more solid speculation. The MAPKK Pbs2p (Brewster, 1993) phosphorylates Hog1p, that once activated translocates to the nucleus with following induction of a specific transcriptional program. Distinct pathways are known to function in the Hog mediated adaptation response that leads to the activation of several MAPK cascades mediated by three distinct osmosensors (Alepuz, 2001): the first, Sho1p, was identified as sensitive hit in the screen and principally functions in the adaptation to high salt conditions by activating the Hog signalling pathway. Although functioning in a Sho1p-mediated parallel pathway, deletion of the Msb2p osmosensor did not result in a strain sensitive to lithium/galactose treatment, while deletion of the third osmosensor Sln1p could not be tested as it is an essential gene and therefore not available in the YDS. The fact that both deletion of *HOG1* and *PBS2* were identified supports the extent of their known relationship: Pbs2p serves both as a scaffold and a kinase in the Hog pathway, in order to prevent “crosstalk” activation between pathways, scaffold molecules and indeed the kinases themselves, coordinate a set of interactions that ensure the fidelity of each signalling cascade.

Phosphorylation of target proteins by Hog1p can lead to the recruitment of other transcription factors that recruit the transcription machinery; however Hog1p has also been shown to directly recruit RNA Pol II (Alepez, 2003). Interaction between Hog1p and the histone deacetylase Rpd3p targets the Rpd3p/Sin3p/Sap30p (this last deletion was identified as a suppressor in this study) complex to the promoters of osmotic stress genes, resulting in chromatin modification and transcription initiation (De Nadal, 2004).

In summary, deletion of genes *MSN2*, *BMH1*, *HOG1*, and *SHO1*, all lead to sensitivity to lithium/galactose; contrarily deletion of *SAP30* lead to enhanced growth. These genes work in concert in the response to stress pathway, and implicate it in yeast's ability tolerate these conditions. Although the working concentrations of LiCl employed in this study do not justify a full osmostress response, a branch of this highly connected network and this pathway partly seems to assist cellular survival under these conditions.

3.26. Chromatin organization , histone , transcription

A great variety of stresses are sensed by specific conserved signal transduction pathways, which usually conclude in the modulation of protein kinase activities, such as the previously described MAPKs (Gustin, 1998) and TOR (Martin & Hall, 2005). Independent studies have elucidated the complexity behind the mechanism through which these signalling kinases function, showing the requirement not only for the modulation of specific transcription factors but also for the steady association that takes place between the kinase and the chromatin of the regulated gene (Chow & Davis, 2006). Once tied to the

chromatin of inducible genes, Hog1p (**section 3.25**) undergoes activation and can favour transcription by recruiting chromatin complexes (De Nadal, 2004).

Deletions of genes encoding for subunits of the histone acetyltransferase (*EAF1/VID21* and *EAF7*), deacetylase (*RCO1*) and methyltransferase (*SET2*) enzymes were reported among the lithium sensitive hits, along with a subset of KOs corresponding to genes encoding for proteins functioning in chromatin remodelling (*ARP8*, *IES5*, and *SWP82*). Transcriptional-related processes are also represented with transcription factors (*HAL9* and *SEF1*) and repressors (*XBP1*) and a component of the SAGA complex (*SGF29*). Although there is one gene hit (*NNF2*) that interacts with Rpb8p, a subunit shared by yeast RNA Pol I, II, and III (Briand, 2001), the majority of the connections links to RNA Pol II only, suggesting it as the most influential subunit to support re-programmed transcription, following post-stress adjustment to lithium/galactose treatment. The findings of genes involved in chromatin remodelling are thus explainable in terms of re-programming transcription after an environmental challenge, mechanism that is under the influence of the extensive action of Hog1p and other pathways. The direct recruitment of Hog1p to osmoreponsive promoters by specific transcription factors, such as the previously discussed Msn2p (**section 3.25**), seems to be stimulating the recruitment of RNA Pol II (Alepez, 2003). Furthermore, it is understood that proper transcription is also promoted by recruitment of the Rpd3S, a complex with histone deacetylase function; deletions of one of the genes (*RCO1*) encoding for an essential subunit of this complex appears among the sensitive hits. Once again it is through Hog1p that Rpd3S complex gets recruited to specific promoters (Proft, 2006) and following Hog1p-mediated chromatin modification, it can properly initiate transcription (de

Nadal, 2004), suggesting a form of regulation of initiation of transcription by Hog1p through chromatin modification. Altogether the finding of genes involved in chromatin remodelling sensitive to lithium/galactose treatment falls in line with the model of a more complex and intimate role for the regulation of transcription initiation by signalling kinases.

A possible explanation for the observed interactions could lie in the ability of lithium to fill some of the binding sites destined to different metals. The roles of metal ion cofactors are not interchangeable and may be accompanied by a loss in the enzyme's activity (an example in Job, 1988). Even a partial substitution/lack of the required metal could be enough to impair the proper transcriptional rate and efficiency: this is because certain coactivators enhance transcription initiation by stabilizing the formation of the RNA polymerase holoenzyme, thus enabling faster clearance of the promoter and/or certain ions are required for optimal DNA binding of certain transcription factors (Zhang, 2003).

3.27. Mitochondria

The use of *S. cerevisiae* growing in the fermentative state allows for the analysis of mitochondrial defects otherwise lethal in mammalian cells.

3.27.1. Calcium

The mechanisms of signalling and uptake for Ca^{2+} in the respiratory compartment have attracted interest since scientists intuited that Ca^{2+} trafficking not only has to do with processes in the cytoplasmatic compartment (**section 3.31**), but with mitochondrial functions as well. Work done by Campanella and

colleagues (2004) revealed that mitochondrial Ca^{2+} uptake influences the kinetic properties of the cytoplasmic Ca^{2+} concentration and that, within the respiratory organelles, a Ca^{2+} -mediated signal can trigger crucial and diverse effects, among them the induction of apoptosis (Campanella, 2004).

The ability to accumulate, retain and promptly release Ca^{2+} is a fundamental function of mitochondria, which also able to handle the high amount of Ca^{2+} found in the cytosolic compartment upon certain stress conditions. For the exogenous Ca^{2+} to be accumulated inside the mitochondria, an electrogenic carrier that facilitates the cation transport across the inner mitochondrial membrane (IM) into the matrix is required. This mechanism takes place via a co-transport that leads to the accumulation of inorganic phosphates, which can form precipitate-like formations in the matrix of mitochondria (reviewed in Chalmers & Nicholls, 2004). Reduction in the ability to transport phosphate in the mitochondria would also lead to a reduction in Ca^{2+} cation storage: this may explain the sensitivity to lithium/galactose treatment that arises from deletion of the mitochondrial phosphate carrier gene *MIR1*.

A functionally redundant protein has been identified for Mir1p, at least in *S. cerevisiae* and *A. thaliana* (Hamel, 2004): phosphate carrier isoform 2 (Pic2p) is constitutively less abundant than Mir1p under normal conditions, but it still participates in the delivery of phosphate to the mitochondria. The *pic2Δ* strain does not appear among the screen hits: this may be simply explained by the fact that Pic2p expression has been so far shown to be induced at high temperature (Hamel, 2004) only, and this protein may be required under specific stress conditions, which presumably not include lithium induced stress.

Deletion of *MMM1*, a gene previously encountered in the SGA study (**section 2.36**), also lead to sensitivity to lithium/galactose treatment. Its encoded product, Mmm1p, an ER integral membrane protein, is considered a component of ERMES (previously discussed in **section 2.38**), a complex with putative roles in the promotion of inter-organellar Ca^{2+} and phospholipid exchange. Ca^{2+} released from the ER storage passes through the membrane into the mitochondria where elevated intramitochondrial concentrations of Ca^{2+} can stimulate the activity of the TCA cycle, thereby boosting energy production in the mitochondria, as discussed in the next section.

Extensive studies have shown that in the mitochondrial matrix Ca^{2+} promotes the activity of NADH production by pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), and α -ketoglutarate dehydrogenase (α -KGDH) (reviewed in McCormack, 1990), thus enhancing the electron flow through the electron transport chain (ETC) and increasing ATP production (Jouaville, 1999). More than one gene usually encodes for proteins functioning in these three enzymatic activities, two of them corresponding to KOs (*idh1Δ* and *kgd1Δ*) were reported as lithium sensitive in this screen, and will be discussed in the next section.

3.27.2. The TCA cycle

As discussed in the previous section, Ca^{2+} uptake can impact on mitochondrial functions as well as cytoplasmic ones. The major targets of the mitochondrial Ca^{2+} import pathway are the dehydrogenases of the tricarboxylic acid (TCA) cycle, enzymes which are rate limiting to the cycle and are all upregulated by Ca^{2+} -dependent processes (Maechler & Wollheim, 2000).

The first dehydrogenase functioning in the Krebs' cycle, Idhp, is part of the isocitrate dehydrogenase system, provided by *IDH1* and *IDH2* genes (this latter is a sensitive hit in this screen) and catalyzes the first NADH-yielding reaction of the cycle through the oxidation of isocitrate to α -ketoglutarate. The following step of the TCA cycle involves the oxidative decarboxylation of α -ketoglutarate to form succinyl-CoA, accompanied by a production of NADH (**Figure 3-14**). This metabolic step is catalyzed by α -KGDH enzyme, whose components are encoded by the genes *KGD1*, *KGD2*, and *LPD1*. A very recent metabolomics investigation, aimed to elucidate a carbon source dependent model of transcriptional regulation in respiration, was undertaken (Fendt & Sauer 2010). While it is known that *S. cerevisiae* displays various degrees of respiration depending on the carbon source, the aforementioned study showed that a specific respiratory contribution to cellular energy production exists: this was almost absent on glucose and mannose supplemented media, intermediate in the presence of galactose, and rather high in pyruvate containing medium (Fendt & Sauer, 2010). What was observed for the galactose containing medium condition aligns with previous assertions that carbon sources such as galactose permit yeast to undergo simultaneous respiration and fermentation (Herrero, 1985). In galactose supplemented medium, a 2.7-fold increase was reported for Kgd1p, whilst no change was observed for Kgd2p. In the same way, Idh2p showed an increment of 2.5-fold versus an only slightly augmented Idh1p (Fendt & Sauer, 2010). These findings may give the reason for the results presented in this investigation, where only loss of one gene for each duplicate pair was found to lead to sensitivity, reflecting their importance in response to galactose. In particular, it should be noticed that of the genes mentioned in Fendt & Sauer's

study, only the *kgd1Δ* strain exhibited a growth defect upon lithium/galactose treatment in the study presented here, as shown in **Figure 3-13**.

Figure 3-13. The TCA cycle. Genes encoding proteins functioning in the TCA, indicated in red are lithium sensitive hits, whilst in green were not tested as not present in the YDS, although not essential.

The TCA cycle is required for the provision of bio-precursors, independent of the carbon source. Yeast is able to interplay the TCA and the glyoxylate cycles (**Figure 3-14**) in a way that, if one of the pathways is malfunctioning, the other can be utilized as a back up (Kolkman, 2005). In the glyoxylate cycle, two molecules of acetyl-CoA are converted into oxaloacetate, bypassing in this way the TCA cycle reactions in which CO_2 is released. The glyoxylate cycle is essential during growth on C2 compounds for the synthesis of all cellular compounds with three or more carbon atoms, for example for the biosynthesis of amino acids and nucleotides (Cozzone, 1998).

Figure 3-14. Interplay of the TCA and glyoxylate cycles (Kolkman, 2005).

The identified hits (*KGD1* and *IDH2*) are associated to the process of conversion of isocitrate (from both glyoxylate and TCA pathways) to alpha-ketoglutarate (in TCA cycle) and on to succinyl-CoA (in TCA cycle), as shown in the figure above. **Error! Reference source not found.** This could imply a malfunctioning, or even a block, of the glyoxylate cycle, presumably after the isocitrate-yielding step (since the conversion oxaloacetate to citrate to isocitrate is common to both pathways). The metal-activate enzyme isocitrate lyase (encoded by *ICL1/2* genes) catalyzes the reversible cleavage of isocitrate into succinate and glyoxylate. This enzyme has been suggested to require Mg^{2+} at determined concentrations, at least in *Pinus pinea* (Giacchetti, 1988). There is no conclusive evidence in the literature regarding *S. cerevisiae* which describes whether the true substrate of Iclp is the free isocitrate or the Mg-isocitrate complex. In saying so, if what observed in other organisms is conserved to yeast, the possibility of a block of the glyoxylate pathway should be considered, a scenario which would explain the finding of any sensitive hits in the TCA cycle. The lack of a sensitive hit from *MDH1* deletion may come down to the ability of

the cell to extricate itself with a functional Mdh2p as perhaps, albeit cytosolic, it may obtain malate from mitochondria to cytoplasm. The lack of appearance of some gene deletions in this screen might find its rationale in the fact that the protein products encoded by these genes are not required for survival by the organism, at least under this carbon source condition. It seems a reasonable assumption that formation of succinate could be essential for the cell, for instance in the formation of amino acids. The enzymes shared among the TCA and glyoxylate cycles are not dramatically down-regulated under glucose repression and/or anaerobic repression (Wales, 1980), while the Sdhp's succinate dehydrogenase (Sdh1p/2p/3p/4p) and fumarase (Fum1p) are indeed repressed. In this particular case, under anaerobic/fermentative conditions, the organism is able to tolerate the loss of these proteins' contributions as, cultured in galactose medium, yeast is able to successfully combine fermentation and respiration (Fendt & Sauer, 2010).

Under normal conditions of anaerobic or glucose repression, which exhausts Kgdp's enzymatic activity (Wales, 1980), the cell can still obtain succinate via the glyoxylate pathway, supporting the notion that the biological system does seem to be focussed on acquisition of succinate. It is conceivable that, if the glyoxylate cycle is not functioning properly (for instance due to the inhibition of isocitrate lyase somewhere along this pathway, like isocitrate lyase), then the cell will require the TCA cycle to obtain succinate. When the TCA cycle's output is compromised by deletions, the cell has no chance to survive, a fate that is reflected in the impaired growth of these mutants. This hypothesis could be easily addressed in future studies by adding succinate to the medium,

with the expectation that addition of extra substrate will lead to rescue of the lethal phenotype observed.

As discussed in the previous section, Ca^{2+} mobilization across cellular compartments appears crucial for the cell to store, deploy or dispose of any surplus Ca^{2+} . Elevated intramitochondrial concentrations of Ca^{2+} can stimulate the activity of the TCA cycle, thereby boosting energy production in the respiratory organelles. In consideration of the described interplay between the TCA and the glyoxylate cycles, it is conceivable that any weakening of TCA-generated contribution could potentially result in a lethal phenotype. This includes the hits related to NADH contribution, which is critical for the mitochondrial respiratory chain functioning and to boost energy. Interestingly, the genes corresponding to the sensitive deletions observed in this study share a common Ca^{2+} signalling regulation. Along with Idhp and Kgdh, the TCA cycle also relies on the contribution of a third dehydrogenase for accomplishing the production of NADH: the malate dehydrogenase Mdh1p. Further studies may elucidate whether there is a Ca^{2+} -connection with the regulation of TCA cycle, which is partially dependent on local energy demands and where ATP and NADH function as regulators. Hypotheses could be drawn, although of purely speculative nature, on the consequences of an imprecise regulation as a result of dysfunctional Ca^{2+} signals; this latter directly or indirectly affected by lithium may be responsible for the generation of conflicting signals. This could perhaps be the case for the TCA cycle, whose functions may run too slowly relative, for instance, to the β -oxidation process.

Results discussed in this section offer an example of cellular benefits acquired via genome duplication. Due to its outstanding importance for the cell's survival, the TCA cycle is one of the central pathways of metabolism, and it is not surprising that so many partially redundant genes have not been swept away by selection, should the yeast's adaptive strategies be to provide robustness and adaptability for its central metabolism. This is true in general for the central carbon metabolism, as shown by the high number of retained duplicates associated to the same enzymatic activity (Kuepfer, 2005; Maltsev, 2005). Uninterrupted flow of energy, precursor metabolites and utilizable energy traffic along the metabolic pathways of central metabolism via glycolysis and the TCA cycle is essential, the proper functioning is ensured by a variety of adaptive mechanisms. One of the most significant mechanisms appears to be gene duplication, an event which has produced a number of isozymes which functioning under variable environmental and physiological conditions.

3.27.3. Cytochrome c oxidase

Cytochrome c oxidase is a large mitochondrial transmembrane protein that provides the proton motive force to drive synthesis of ATP and other fundamental cellular functions. As the last enzyme of the respiratory ETC, cytochrome c oxidase is located in the inner mitochondrial membrane where it accepts an electron from each of four cytochrome c molecules, and then transfers them to a single oxygen molecule to finally convert molecular oxygen into water. In the process, cytochrome c oxidase additionally translocates four protons across the membrane, helping to establish an electrochemical potential difference which leads to a transmembrane proton gradient utilized by ATP

synthase to synthesize ATP. Deletions of genes that encode for proteins with roles in the structure (*COX5A* and *COX5B*) and assembly (*COX14*) of this enzyme resulted in strains that were sensitive to lithium/galactose treatment. *COX5A* and *COX5B* genes encode for subunit Va and Vb of cytochrome c oxidase respectively. Cox5Ap, which is predominantly expressed during aerobic growth, is the terminal member of the ETC placed on the mitochondrial inner membrane, while its isoform Vb (Cox5Bp) is expressed during anaerobic growth. These are the only two of eight cytochrome oxidase c subunits encoded by nuclear genes that becomes differently expressed according to aerobic/anaerobic conditions of the surrounding environment. It may appear unusual that deletions of other Coxp were not affected as any of the genes encoding cytochrome c oxidase subunits (with exception of *cox14Δ*, discussed below), and any of the multiple genes required for expression or assembly of the subunits is associated with a decrease (in the most severe cases even a block) in respiratory growth (yeastgenome). This apparently odd result could be explained by consideration of the fact that, in addition to catalytic cofactors, the matrix portion-associated peripheral subunit Cox5b contains a bound Zn^{2+} ion, while an Mg^{2+} ion is found at the interface of Cox1p and Cox2p subunits (Stiburek, 2006). Given the similarity between Cox5ap and Cox5bp proteins, which share a 63% identity, it is conceivable that if Cox5bp binds Zn^{2+} , so could/should Cox5ap; this consideration appears well justified by the size of the Zn^{2+} ionic radius (88 pm, c.f. Li^+ at 90 and Mg^{2+} at 86). This study hypothesizes that lithium may be causing a mild functional disruption of Cox5p, such that loss of either Cox5ap or Cox5bp could lead to the observed growth defect. *COX14* encodes a mitochondrial membrane protein involved in assembling cytochrome c oxidase, and the *cox14Δ* strain was found to be lithium

sensitive. This protein is also involved in the translational regulation of Cox1p and in the association with complex IV assembly intermediates and complex III/complex IV supercomplexes. The impairment displayed by both assembling process and functionality of the complex IV upon lithium/galactose treatment echoes the notion that a paucity of cytochrome c oxidase is likely to occur as a consequence of the long term lithium pharmacological treatment. In fact, recent research from Hroudova and colleagues (2010) regarding the *in vitro* effects of some antidepressants and mood stabilizers showed those medications inhibit the activities of respiratory ETC complexes, with complexes I (NADH hydrogenase) and IV (cytochrome c oxidase) being the most affected (Hroudova, 2011).

3.27.4. Additional mitochondrial gene deletions

Mitochondria possess a residual genome which heavily depends on factors encoded by the nuclear genome for both their maintenance and expression. A number of deletion strains whose genes encode proteins involved in up-holding the mitochondrial genome were reported as lithium sensitive: *HMI1* whose product is the mitochondrial inner membrane localized ATP-dependent DNA helicase Hmi1p (Lee, 1999), *RIM1*, which encodes for the single-stranded DNA-binding protein Rim1p (Van Dyck, 1992), and *PET130* whose corresponding protein has been suggested to have a role in respiratory growth (Reinders, 2006).

Loss of *GCV2* leads to sensitivity under lithium/galactose treatment. This gene encodes the P subunit of the mitochondrial glycine decarboxylase complex (GDC) (Sinclair, 1996), a multienzyme complex that catalyses the reversible

oxidative cleavage of glycine into carbon dioxide and ammonia. Thanks to the GDC, budding yeast can grow on glycine as sole nitrogen source and is also able to convert glycine to serine via a reaction catalyzed by the same complex (Sinclair & Dawes, 1995). The GDC has three additional subunits known as H-, L-, and T-proteins: but deletion of genes responsible for these proteins were either not available in the YDS (*GCV3*) or did not lead to sensitivity to lithium/galactose (*GCV1* and *LPD1*) Gcv2p has been shown to be regulated by levels of 5,10-methylene-THF (substrate) in the cytoplasmic compartment; as this enzyme acts together with several co-enzymes, which rely on Mg^{2+} as a cofactor which lithium competes with, this may explain this interaction.

Furthermore, *POT1*, the gene coding the Pot1p thiolase enzyme catalyzing the last step of the β -oxidation (the cleavage of 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA); is a lithium sensitive hit. β -Oxidation is the process by which fatty acids, in the form of acyl-CoA molecules, are broken down in mitochondria and/or in peroxisomes with generation of acetyl-CoA, which is then principally fed into the TCA cycle (discussed in **section 3.27.2**). It is extremely difficult to comment on the sensitivity of *pot1 Δ* strain, in consideration of the fact that *POT1* shares around 50 negative genetic interactions and appears as the only hit in its pathway.

An additional interaction was reported for *CBR1*, which encodes a microsomal cytochrome b reductase; mutation in a conserved NADH binding domain of the human ortholog results in type I methemoglobinemia (Nussenzveig, 2006). Only two genes have been manually curated in SGD as being SL with *CBR1* (Tiedje, 2007). These are *NCP1* (a P450 reductase) and *CLA4* (Cdc42p-activated signal transducing kinase of the PAK family). Lithium could

lead to loss of Cla4p kinase function as discussed in **section 3.20.1** which, from a cellular prospective, is equivalent to *CLA4* deletion. This could underlie the sensitivity of the *CBR1* strain upon lithium treatment.

Three further genes (*AIM34*, *YBL059W*, and *YGL057C*) were additionally identified. Although their corresponding protein products have no annotated function, they all share a common mitochondrial localization. In particular *AIM34* holds a negative genetic interaction (Costanzo, 2010) with *MCK1*, a protein kinase involved in regulating entry into meiosis, which has been found sensitive in this screen and discussed in **section 3.33**. *YBL059W* holds a negative genetic interaction with the *TOR2* gene, while *YGL057C* encodes a protein of unknown function and null mutants exhibit a respiratory growth defect and synthetic interactions with one of the prohibitin encoding genes (*PHB1*). Although the precise molecular function of the Phb complex is not clear, a role as chaperone for respiratory chain proteins or as a general scaffold required for optimal mitochondrial morphology and function are suspected (Nijtmans, 2000).

Noticeably, none of the discussed mitochondria-related hits seem to be associated with a diauxic shift effect, in fact no common hit can be found when comparing the hits revealed by this study to the ones reported by a proteomics study on diauxic shift (Ohlmeier, 2004). Furthermore, no correspondence has been noted between hits presented in this study and the proteins found in increased or decreased abundance in Kolkman's proteomic study (2005).

This study indicates mitochondrial activities as critical in responding to lithium treatment in medium containing galactose as sole-carbon-source. Important contributions have been identified from diverse processes, these

being: mitochondrial Ca^{2+} traffic, Krebs' cycle, and ETC, along with additional activities, as reported in **section 3.27.4**.

3.28. Trehalose

This screen also reported sensitivity for deletion strains lacking production of proteins related to trehalose, a reserve disaccharide found in yeast (reviewed in Gancedo, 2004) and a wide variety of organisms including bacteria, insects, and plants. In *S. cerevisiae*, trehalose (α,α -1,1-diglucose), is among the major storage carbohydrates available to the cell upon stresses triggered by heat, ethanol, osmotic, and oxidative stresses (Pereira, 2001). Trehalose biosynthesis takes place through a two-step process in which glucose 6-phosphate and UDP-glucose are first converted by trehalose-6-phosphate synthase (TPS), encoded by *TPS1*, into α,α -trehalose-6-phosphate, which is then converted with water into trehalose and phosphate by trehalose-6-phosphate phosphatase (TPP), encoded by *TPS2* (Francois & Parrou, 2001), this latter a sensitive deletion in this study (**Figure 3-15**). Tps1p and Tps2p are part of the α,α -trehalose-phosphate synthase complex (Pereira, 2001) together with the regulatory proteins Tps3p and Tsl1p, and all four of proteins are co-induced under stress conditions, and corepressed by the Ras-cAMP pathway (Winderickx, 1996). Dysfunctions in strains carrying a full deletion of either *TPS1* or *TPS2* have been investigated: both KOs result in a growth defect on fermentable carbon sources, specifically *tps1 Δ* resulted in loss of both TPS activity and trehalose biosynthesis (Bell, 1992), whereas *tps2 Δ* displayed temperature sensitivity and loss of TPP activity

(De Virgilio, 1993). A plausible explanation is that most strains build a trehalose reserve from before their final pinning onto lithium/galactose. This reserve may help them through the initial response to the new lithium/galactose environment and provide a boost to cell numbers. Strains that cannot use trehalose become disadvantaged, and so are seen as sensitive.

Figure 3-15. Trehalose pathway. Gene deletions sensitive to lithium/galactose treatment are indicated with a red box.

Although the trehalose biosynthetic pathway may appear distant and little related to the main utilized pathways, in fact it can affect glycolysis as one of its intermediates, trehalose-6-phosphate, inhibits hexokinase activity, which restricts the influx of sugars to glycolysis during the switch to fermentative metabolism (Hohmann, 1996). Trehalose is a storage carbohydrate that can either be synthesized by the cell itself or obtained from the external environment, and then converted by trehalase enzymes with the use of water to obtain two glucose molecules (Francois & Parrou, 2001). *S. cerevisiae* has two

trehalase enzymes, an acid trehalase encoded by *ATH1* (Alizadeh & Klionsky, 1996) and a neutral trehalase encoded by *NTH1* (Kopp, 1993). *NTH1* is a sensitive hit in this screen and is induced by various stresses including exposure to heat, hydrogen peroxide, or cycloheximide (Zahringer, 1997) and its protein product is a cytoplasmic homodimer required for the hydrolysis of intracellular trehalose (Kopp, 1993). Deletion of *NTH1* results in complete loss of neutral trehalase activity, accumulation of internal trehalose (**Figure 3-15**). The rationale reasoned for *tps2Δ*, could be applied to this interaction as well. Interestingly, Nth1p shares a physical interaction (Gavin, 2002) with Bmh2p, the 14-3-3 protein already encountered in **section 3.25**; this leads to the suspicion that this interaction might underlie the same cellular connection and thus be under TOR signalling regulation.

3.29. Gluconeogenesis

Gluconeogenesis is a highly regulated cellular process by which yeast can produce glucose from alternative nutrient sources such as acetate, amino acids, lactate, and glycerol. *S. cerevisiae* senses and adapts to nutrient changes in its environment by altering the transcriptional profile and undertaking modifications of the protein complement within the cell.

For example, when glucose repression is lifted, galactose can be imported and metabolised to produce glucose and derivatives for glycolysis, the TCA cycle and pentose phosphate pathway, as shown in **Figure 3-16**. D-Galactose is imported into the cell via Gal2p and the pathway begins with the production of α -D-galactose-1-phosphate (Gal-1P), catalysed by Gal1p. Gal-1P is converted to

UDP-Gal which is isomerised by Gal10p to UDP-glucose (UDP-Glc), which under the action of Gal7p forms α -D-glucose-1-phosphate (Glc-1P). Glc-1P is isomerised to Glc-6P by the phosphoglucomutase enzymes (Pgm1p, 2p and 3p; collectively Pgmp) which are known targets of lithium as previously discussed. Phosphoglucose isomerase (Pgi1p) interconverts Glc-6P and β -D-fructose-6-phosphate (F-6P), which in turn is converted to its 1,6-bisphosphate (F-1,6P₂) and on to glyceraldehyde-3-phosphate. F-6P and glyceraldehyde-3P are key metabolites, ultimately providing for entry to key parts of the pentose phosphate pathway (e.g., the synthesis of ribose, purine and pyrimidine metabolism) and the TCA cycle respectively. Under lithium inhibition of Pgmp, levels of these metabolites will be reduced, and any deletion that exacerbates this situation is likely to lead to a growth defect.

Figure 3-16. Galactose utilization in gluconeogenesis.

Enzymes that would be most expected to have an effect the balance of F-6P and glyceraldehyde-3P are those in the surrounding equilibria. However, Pgi1p and Fba1p are encoded for by essential genes, so were not assessed in this study. For example, deletion of *FBP26*, the gene coding for F-2,6-bisphosphatase, an enzyme required for glucose metabolism resulted in a sensitive strain in this screen. The product of its enzymatic reaction is F-2,6P, a metabolite that allosterically affects the activity of the enzymes phosphofructokinase 1 (PFK-1) (encoded by *PFK1* and *PFK2*) and F-1,6-bisphosphatase (FBPase-1) (encoded by *FBP1*) to regulate glycolysis and gluconeogenesis (**Figure 3-16**), however the major effect of this deletion might be that regulatory branch to F-2,6P is now a

one-way siphon and there is a resultant loss of the key metabolites, F-6P and glyceraldehyde-3P.

An additional pair of genes involved with degradation of F-1,6 biphosphate was scored as sensitive in the assay: *VID22* and *VID28*. These two genes produce proteins that are recognised as being important in the degradation of F-1,6-biphosphatase (Fbp1p, FBPase). In particular, the Vid proteins are major mediators of the degradation of FBPase on long term growth in glucose deficient media (Hung, 2004). FBPase is important in the gluconeogenesis pathway, where it converts F-1,6-biphosphate into F-6-P. In this sense, the loss of these Vid proteins enhances the lithium mediated reduction in the flux through the glycolysis pathway, leading to higher levels of FBPase activity or lowering the forward flux through the pathway. This leads to further reduction in the production of key metabolites such as glyceraldehyde-3-phosphate and pyruvate and a reduced flux into the TCA cycle and subsequent inhibition of growth. Finally, it is not completely to be ruled out that the organism may utilize its stock of F-6-P too rapidly, depleting in this way the cellular reserve.

3.30. Suppression of lithium/galactose toxicity

In order to explain the suppression of lithium/galactose toxicity, it is helpful to understand the underlying mechanisms of suppression. Depending on the allele and gene specificity associated with suppressors, diverse mechanisms have been inferred for what is known as “second-site suppressors” (Prelich, 1999): i. Dosage suppression where a protein product that stabilizes the mutant

phenotypes is expressed at high levels. This was elegantly shown by Chew et al (2010) in the stabilization of a Tbp1E186D mutant protein and suppression of the TBP1E186D mutant strain defect, following the overexpression of the deubiquitinating enzyme Ubp3p. **ii.** Interaction suppression where deleterious effects of a mutation in one protein causes a loss of a key interaction with a partner. A suppressing mutation in the partner can restore the interaction. This form of suppression is not observed with deletion mutants. The analogous situation for deletion set screens is where a second deletion relieves a stress induced by a first deletion as described below. **iii.** A bypass suppressor activates an alternative pathway to the wild-type pathway (an example in Hardy, 1997). It is pathway-specific and rescues null allele (**Figure 3-17**).

Figure 3-17. Bypass suppressor mechanism. A bypass suppressor activates an alternative pathway to the wild type pathway. Adapted from Forsburg (2001).

The “dosage suppression” mechanism does not seem likely to describe the outcome of this study, however there is a limited potential for dosage suppression via loss of regulatory proteins (such as those associated with

ubiquitination), which could lead to enhanced abundance of their target proteins. The second mechanism describes the so-called “interaction suppressor”. This mechanism appears even less likely than dosage suppression, given our study partner lithium/galactose condition (intended as the first mutation) with gene deletions. By far the most likely source of suppressor deletion mutants are those which fall into the third category above: “interaction suppression”. In this scenario, deletion mutants may force the yeast cell to shift metabolic flux away from pathways that are problematic in the presence of lithium/galactose and towards others which permit enhanced growth or survival.

It has to be pointed out that the study of suppressor strains has limitations: as reflected by the small number of interactions reported, not all the KOs will be tractable for suppression as in certain cases the strains simply revert the phenotype back to a wild type level, rather than leading to enhanced growth. The deletion strains leading to suppression of lithium/galactose toxicity, and indeed to enhanced growth on this medium, initially appear to fall into two main categories: those associated with de-ubiquitination and those associated with galactose metabolism. However, they appear to coalesce on the single process – galactose metabolism – as discussed below.

3.30.1. Ubiquitination

Ubiquitination is an important process which impacts or is involved in a vast variety of cellular events. As previously discussed in **section 2.37**, together with enzymes functioning in ubiquitination, there is much current interest in deubiquitination mechanisms, as they appear to be as important in the contribution of the homeostasis of the cell as the original ubiquitination event.

The data generated in this study indicated functional correlations for lithium sensitive genes separately involved in both ubiquitination and its reverse process: the sensitive deletion strain (*bul1Δ*) is a component of an ubiquitin ligase complex, whereas the two strong suppressor strains (*bre5Δ* and *ubp3Δ*) are part of a deubiquitination complex. The opposite phenotypes exhibited by these deletions under lithium/galactose treatment reflect the opposite functions of their corresponding genes.

As indicated in **section 3.21**, two deubiquitination-related hits were identified in the screen: *BRE5* and *UBP3* genes, the protein products from which function in a deubiquitination complex where the ubiquitin protease, Ubp3p requires the presence of its cofactor Bre5p to cleave ubiquitin tags from their substrates (Cohen, 2003). The proteins and processes that are controlled by the Ubp3/Bre5p complex are only now becoming clear and include the processes of: ribophagy (autophagy specifically of ribosomes, where the de-ubiquitination actually destabilises ribosomes)(Beau, 2008); telomeric silencing; maintenance of the cell wall; global transcriptional regulation (Bilsland, 2007); and validated protein regulation of: Sec23p (a component of the COPII complex essential for the anterograde transport between the ER and Golgi (Cohen, 2003)) and Tbp1p (also known as Spt15p, a TATA binding protein and component of the general transcription factor, TFIID (Chew, 2010)). Activity against Sec23p appears to be also under the control of Cdc48p (Ossareh-Nazari, 2010). Unfortunately both *SEC23* and *CDC48* are essential genes and so their involvement in lithium/galactose sensitivity could not be probed by this analysis.

Despite this range of target proteins and processes, it is reasonable to presume that the regulation of Tbp1p is especially important in the context of

growth of lithium/galactose. Chew et al (2010) have elegantly shown that Ubp3p-mediated stabilisation of Tbp1p is required for activation of *GAL1* by Gal4p and indeed Tbp1p is recruited to the *GAL1* promoter upon galactose induction. This level of control of the galactose response is sufficiently profound that the *ubp3Δ* strain was shown to have a 20-fold reduction in the activation of *GAL1* and is unable to grow on galactose under anaerobic conditions. This points to an expectation of reduced viability in response to disruption of the Ubp3p/Bre5p-mediated de-ubiquitination pathway, however the reverse is in fact true under the conditions of this screen – the individual deletions are recorded as suppressors in the lithium/galactose experiment. Unexpected as this may be at first glance, it is noticeable that deletion of *GAL1* and *GAL4* also lead to a suppressor phenotype, so loss of Tbp1p regulation, removal of Gal4p and deletion of the *GAL1* gene all lead to the same result, as would be expected, as discussed below.

3.30.2. Galactose metabolism

Carbohydrate metabolism makes nutrients available for the cell via anabolic processes that in yeast, as for other organisms, are also mediated by enzymes that act in regulated metabolic pathways. Genes coding for enzymes functioning in carbohydrate metabolic processes are extensively represented in the lithium screen results, as shown in **section 3.16**.

Metabolism of galactose, which is not the ideal or usual carbon source for yeast, is under the competence of enzymes encoded by a family of *GAL* genes that are repressed under growth in glucose while their expression is 1000-fold increased when glucose is absent (Johnston, 1987). As reported in **section 3.16**,

a cohesive group of *GAL* genes (*GAL1*, *GAL2*, and *GAL4*) were among those deletion strains which express a suppressor phenotype upon lithium/galactose treatment.

The gene *GAL1* encodes for Gal1p, a galactokinase, that phosphorylates α -D-galactose to α -D-galactose-1-phosphate in the first step of galactose catabolism (Schell & Wilson, 1977); the same protein is also required for inducible high-affinity galactose uptake (Ramos, 1989), and can function as a weak transcriptional regulator in the absence of Gal3p (Schell & Wilson, 1977). Deletions of both *GAL1* and *GAL3* genes were identified by SESA software as suppressor hits but, whilst *gal1 Δ* was confirmed in all the further screens, *gal3 Δ* failed to reproduce a similar phenotype in the spot dilution assays. Although it is commonly found that deletions identified in the first high-throughput primary assay are the results of experimental noise, it is possible that loss of sensitivity in the spot dilution assay with *GAL3* deletion is an artefact. This possibility is raised because *GAL1* and *GAL3* share 90% amino acid similarity, the protein products Gal1p and Gal3p are not completely functionally redundant. In the context of gene duplication, the *GAL1* and *GAL3* pair deserves mention as not only it presents evidence of an intimate evolutionary relationship, but it also represents an exemplar case of gene duplication followed by sub-neo-functionalization. It is likely that a pre-duplication bifunctional protein held two activities that then became separated and through this process the loss of galactokinase activity made it possible for Gal3p to acquire specialized regulatory activity, as hinted at the finding that the re-insertion of only two amino acids was sufficient to confer galactokinase activity to the transcriptional regulator Gal3p (Platt, 2000). In the closely related yeast *K. lactis* there is no trace of a *GAL3*-equivalent gene and

Gal1p acts as both galactokinase and regulator of its own activity (Meyer, 1991). Differently, in *S. cerevisiae*, there have been reported cases of mutants lacking *GAL3* expression in which Gal1p seemed to take over the regulatory role of Gal3p (reviewed in Dittmar, 2010) and the circuit through which this pair of *GAL* paralogs respond to lithium mechanism/s could possibly be among them. However, given the interaction with Upb3p/Bre5p and the lack of validation on spot dilution assay, it is safest to assume that the action on lithium is directed towards pathways associated to the galactokinase activity, which is a function specific to Gal1p. The Gal1p galactokinase enzyme is conserved from bacteria to humans and mutations in the hGALK1, the human functional ortholog of yeast *GAL1*, is known to lead to galactokinase deficiency (also known as galactosemia type II), an hereditary metabolic disease introduced in **section 3.2**. Although this form of the disorder is less common and is not associated with life-threatening conditions compared to galactosemia type I, it still causes cataract formation in infants due to galactitol accumulation (reviewed in Holden, 2004).

The gene *GAL2* encodes a high affinity galactose permease (Tschopp, 1986): Gal2p is an integral plasma membrane protein belonging to a superfamily of sugar transporters functionally and is structurally similar to sugar transporters identified in humans (Nehlin, 1989). When cells are grown on glucose-containing media, *GAL2* is negatively regulated at both the transcriptional level and protein degradation, this latter occurring through ubiquitination, followed by endocytosis and vacuolar proteolysis (Horak & Wolf, 1997). The secretion of certain hexose transporters, including Gal2p is promoted by the ER localized integral membrane protein Gsf2p. Importantly, deletion of *GSF2* was verified as leading to suppression in this study.

The *GAL* genes, including the above discussed *GAL1* and *GAL2*, are regulated in a coordinate fashion in the absence of glucose at the level of transcription in response to galactose by the combined engagement of Gal3p, Gal4p, and Gal80p (Platt & Reece, 1998). Although these proteins' action is believed to be concerted, results of this study results argue that a level of regulation via Gal4p exclusively, as the *gal3* and *gal80* deletions did not return enhanced growth when combined with a lithium/galactose treatment as shown by converging studies (Masuda, 2008 and this study). *GAL4* encodes for a DNA-binding transcriptional regulator required for the activation of the majority of *GAL* genes in response to galactose induction and it also functions in the activation of *GAL1* expression (Griggs & Johnston, 1991). When cells are grown in the presence of galactose, Gal3p senses the carbon source and enters the nucleus where it binds to transcriptional repressor Gal80p, blocking any further interactions between this protein and Gal4p (Wightman, 2008). Active Gal4p can then recruit several RNA polymerase II related complexes, such as SAGA (extensively discussed in **section 2.37**) and the Mediator complex, which is required for the regulated transcription of nearly all RNA polymerase II-dependent genes in baker's yeast (reviewed in Björklund & Gustafsson, 2005). Activation by Gal4p relies on an acidic C-terminal region that both stimulates transcription and also binds Gal80p (Ma & Ptashne, 1987); in addition, this acidic activating domain interacts with subunits of the 19S regulatory particle of the 26S proteasome (reviewed in Traven, 2006) and is a site of monoubiquitination (Archer, 2008). Mechanisms of inhibition and relief of this inhibitory effect on Gal4p differ according to the carbon source; the reader is directed to Peng & Hopper (2002) and Lohr (1995) for more detailed reading.

Assessment of the phenotype associated with deletion of *GAL80* upon lithium/galactose treatment has given mixed results (as reported in **section 3.15.2**) and therefore it remains difficult to state the phenotype associated with it and comment further on this type of mechanism, however it is worth noticing that Masuda (2008) did not observe a reversion of toxicity in the *gal80Δ* strain.

Although initially surprising, the finding that some of the *GAL* mutant strains tested were associated with enhanced growth in galactose as the primary carbon source has a reasonably obvious explanation. It is essential to remember that to be noted as a suppressor, a deletion strain has to grow better in lithium/galactose than the parental strain does. However, impaired growth may be observed relative to growth on galactose without lithium, for example. It has been noted in the literature that a small number of intermediary metabolites that lie between galactose import and the step catalysed by phosphoglucomutase (Pgm1p and Pgm2p), an enzyme that is known to be inhibited by lithium, are deleterious to growth. Thus, as discussed below, Pgm1/2p inhibition leads to a reduced flux through the Leloir pathway and a build up of these metabolites (de Jongh, 2008; Masuda, 2008). Loss of galactose import or processing means that these toxic metabolites are not formed, and the yeast can grow satisfactorily (if not particularly well) on the basis of other carbon sources in the YPGal media and/or on internal trehalose stores. Work undertaken in *S. cerevisiae* shows that lithium mimics a “galactosemia-like” phenotype, almost certainly as a consequence of its inhibitory effect on Pgm, a key enzyme in galactose metabolism (Masuda, 2001) This thesis study’s results strongly endorse this model.

A congruent body of evidence demonstrates that the *GAL* genes are essential in galactose metabolism: for example, *gal1Δ* mutant strains are usually defective in galactose growth (Bhat, 1990) and Gal2p permease activity is required for galactose catabolism, and its loss renders cells unable to utilize galactose as a sole carbon source (Douglas & Condie, 1954). Despite this, these *GAL* deletions grew unequivocally better than relative controls in lithium/galactose media and consistently reproduced the same suppressor-like phenotypes, in primary and spot dilution spot assays. That the effect observed is not an artefact is further evidences by the congruent set of genes discussed (*GAL1*, *GAL2*, *GAL4*, *UBP3*, *BRE5*, and potentially *GAL3*).

These findings assume that Gal-1-P, a phosphorylated intermediate of the Leloir pathway, is one of the toxic compounds and are also consistent with the direct correlation showed between the severity of lithium-induced impairment of growth and the galactose concentration in the medium (Masuda, 2008).

Overall, there is unanimity of results on the phenotypes exhibited by the majority of the *gal* mutants tested in this study and Masuda (2008): deletions of *GAL1* and *GAL4* were suppressors and *GAL7*, *GAL10* were not, unfortunately we cannot further speculate on *gal80Δ* related phenotype for reasons discussed above. The same publication, however, informed that deletion of *GAL2* was unable to reverse the galactosemic-like phenotype; in disagreement with this observation, the *gal2Δ* was found to be a suppressor strain in this study. In a lithium-free environment, the deletion of this gene, reduces, but does not inhibit galactose uptake entirely, suggesting a reason why its removal fails to revert the galactosemic-like phenotype in the previously discussed paper. Clearly under conditions used here, the deletion of *GAL2* was sufficient to lower the amount of

growth inhibitory metabolites to the point where a suppressor phenotype was observed. The difference between the results presented here and these of Masuda (2008) may well lay in their inclusion of 2% glycerol in the growth media, with possible downstream consequences on pathways such as respiration.

Alleviation of the toxic effect is also to be expected for any pathway that can stop galactose from entering into the Leloir pathway, as this will ultimately help cellular growth. This is proven by the same Masuda and colleagues (2008), who showed that over-expressing *GRE3*, an aldose reductase that transforms galactose into galactitol, also reverts the galactosemia-like phenotype. As more galactitol is accumulated, less Gal-1-P is formed, with benefit for cellular fitness. Astonishingly, in this study *gre3Δ* was a sensitizing deletion, suggesting that further investigation is required to explain this result.

Independent studies on intracellular metabolite profiles of *S. cerevisiae* strains with improved or impaired growth on galactose, (de Jongh, 2008) suggest that other metabolites and/or unbalance of metabolic fluxes are also likely to cause cellular toxicity. According to de Jongh's investigation, it is the combination of the ratio between Gal-1-P concentration and Pgmp activity, and the elevated intracellular galactitol concentration that is critical for galactose related toxicity. Although this latter study conceives that Gal-1-P might cause a negative effect through inhibition of Pgmp, the toxic intermediate Gal-1-P is not the sole cause of galactose induced toxicity, as suggested by further identified toxic metabolite in the pathway, UDP-Gal.

The research presented in chapter 3 concludes that with *PGM1/2* inhibited by lithium/galactose treatment, Gal-1-P and UDP-Gal build up, leading

to slow growth. Blockage in the production of those, either by stopping the *GAL* pathway engaging by *GAL4* deletion, or by deletion of *GAL1* and thereby stopping formation of Gal-1P or by indeed preventing galactose entering the cells (*gal2Δ*) implies that no toxic metabolite is produced.

3.30.3. Glycosylation

During N-linked glycosylation of proteins, two molecules of N-acetylglucosamine, nine molecules of mannose, and three of glucose are sequentially assembled on a lipid carrier molecule forming an oligosaccharide chain. A 14-residue oligosaccharide core is brought together and subsequently transferred to an asparagine residue on nascent polypeptide chains in the ER. As proteins progress through the Golgi apparatus, the oligosaccharide cores are shortened or extended to generate a diverse array of glycosylated proteins (reviewed in Herscovics & Orlean, 1993). In addition to those highlighted above relating, a further subset of suppressor-behaving deletion strains was identified in this screen upon lithium/galactose treatment; those involved in N-glycosylation. These were as the duplicate pair *ALG6* and *ALG8*, and the *OST5* gene, which bridges connections with the secretory pathway.

In the lumen of the ER, the α -1,3-glucosyltransferase Alg6p catalyses the addition of the first molecule of glucose to the oligomannose core (Burda, 1999), followed by Alg8p (Stagljar, 1994) and Alg10p (Burda & Aebi, 1998) glucosyltransferase enzymes, that add the second and third glucoses, respectively, to the growing oligosaccharide chain. Deletion of *ALG10* was identified in the primary screen as associated to the lowest range of statistic

significance (not shown) and it did not undergo spot assays; this deletion still showed a coherent, although statistically unreliable, suppressor phenotype at all of the tested concentrations of lithium in galactose medium.

Once the 14-sugar branched oligosaccharide is assembled, the oligosaccharyl transferase complex (OST complex) relocates it from the lipid carrier to the consensus glycosylation acceptor site, an asparagine residue. The complex includes nine protein subunits: Ost1p, Ost2p, Ost3p, Ost4p, Ost5p, Ost6p, Stt3p, Swp1p, and Wbp1p, all of which are integral membrane proteins of the ER (reviewed in Herscovics & Orlean, 1993), as mentioned above, *ost5Δ* was associated to a suppressor phenotype in this screen. Ost5p, along with other OST components, is not essential for the viability of the cell; however it is still required for the optimal activity of the complex both *in vivo* and *in vitro* (Knauer & Lehle, 1999b; Yan & Lennarz, 2005). Because more than 70% of all proteins processed by this pathway are N-glycosylated (Dempski & Imperiali, 2002), the OST complex plays a critical role at the initial step of the secretory pathway; this is because the addition and subsequent processing of the carbohydrate chains is crucial for the folding, stability, and maturation of several glycoproteins. Significantly, the OST complex has been described as the “gatekeeper” of the secretory pathway (Dempski & Imperiali, 2002) and interacts with the Sec61p pore complex (Chavan, 2005) involved in protein import into the ER.

At first glance, it appears strikingly odd that only one (Ost5p) out of the 9 complex subunit was identified in the screen; primarily this is likely to be due to the nature of the other members of the complex. *OST1*, *OST2*, *STT3*, *SWP1*, and *WBP1* are essential, and were therefore not available for screening in this experiment. *OST3* and *OST6* are noted to produce proteins that have overlapping

function, such that the individual gene deletions have little associated phenotype (Karaoglu, 1995).

Ost5p was initially identified in a synthetic lethal screen in *alg5* mutant cells (Reiss, 1997). Individually, deletions of either *OST5* or *ALG5* genes have only mild phenotypes, but deletion of both confers a synthetic temperature-sensitive glycosylation defect at 30° C (Reiss, 1997). A congruent genetic relationship of SL nature has been described for this pair of genes (Schuldiner, 2005; Costanzo, 2010). A potential cause for the identification of *OST5* in this screen could be that, whilst the loss of individual functionality is insufficient to cause a growth defect, it is sufficient to cause a growth benefit under lithium/galactose treatment. In the context of the other suppressor strains observed, this option seems rather parsimonious. In this model, a reduction in glycosylation leads to a corresponding loss of active cell surface glycosylated transporters. In the lithium/galactose context, any reduction in the transport of Li⁺ (relieving its intracellular effects including Pgmp inhibition) or of galactose (nothing the suppressing effects from deletion of *GAL2*) would lead to enhanced growth. *S. cerevisiae* sugar carriers of glucose, galactose, and maltose have been identified as glycoproteins (Lagunas, 1986), although glycosylation of Gal2p has not been established beyond all reasonable doubt (Tschopp, 1986; Ramos, 1989). A number of ion channels (Shi & Trimmer, 1999) and nuclear pore complexes (Belanger, 2005) are also glycosylated. In this model, complete ablation of glycosylation and the secretory pathway may be unnecessary (and indeed may be deleterious), all that would be required is a reduction in lithium import (so as to relieve Pgmp inhibition) and/or of galactose import (to ensure that levels of proposed toxic metabolites discussed in **section 3.30.2** do not accumulate). If

Gal2p is glycosylated, than loss of glycosylation could lead to less galactose to be available, then reversing the toxic effect and leading to the suppressor phenotype observed.

Although this study concludes that the reduction of lithium or galactose transport leads to enhanced growth and the observed suppressor phenotype is the most likely underpinning of the effect, an additional rather speculative proposition could be explored. Numerous cell surface proteins are modified by N-linked glycosylation, including a number of receptors important for growth regulation. The extent of their glycosylation affects their surface expression and cellular growth or differentiation (Lau, 2007; Fang, 2010). In an *algΔ* background strain, in the presence of lithium/galactose, the signalling pathway may be faulty, following its strict requirement for glucose-containing oligosaccharides, as indicators of N-glycosylation performance. Under the glucose-limiting conditions of this screen, it is possible that a signalling defect of this kind would lead to improper TOR regulation, and a resultant inability to modulate cellular processes, including protein synthesis.

3.31. Calcium homeostasis

In *S. cerevisiae* the majority of the cellular responses to hyper-osmotic stress is regulated by two interconnected pathways involving the MAPK Hog1p, discussed in **section 3.25**, and calcineurin, a Ca²⁺/calmodulin-regulated protein and a major player in Ca²⁺-dependent eukaryotic signal transduction pathways. Following a stimulus there is variation in the Ca²⁺ concentration in the cytosol. This activates a variety of Ca²⁺ sensing proteins such as calmodulin and calcineurin, which ultimately leads to the induction of various downstream

signal transduction pathways (reviewed in Kraus & Heitman, 2003). The extended regulation of calcineurin-mediated route has extensively been exposed in this study by its regulation of specific components of the cell cycle (**section 3.24**) and by playing a key role in activating CS enzymes as part of the compensatory mechanism (**section 3.23**). As several of the SL-like phenotypes observed upon lithium/galactose treatment have been linked to the competitive nature of the lithium ion, it has to be mentioned that, in yeast cells, a Mg^{2+} depletion leads to enhanced cellular Ca^{2+} concentration, which will then activate the Crz1p/calcineurin pathway, crucial for this organism to cope with the Mg^{2+} depletion stress (Wiesenberger, 2007). It is conceivable that, if the loss of Mg^{2+} ion is recognised in response to loss of activity of an Mg^{2+} -dependent protein, then lithium could block that activity and generate an Mg^{2+} -deficient response.

Calcineurin type 2B phosphatase consists of a catalytic subunit, calcineurin A, and a regulatory subunit, calcineurin B; all eukaryotic organisms possess one or more genes for each subunit, which have been identified in yeast, insects, and mammals (Guerini & Klee, 1989). In budding yeast, the catalytic subunit of calcineurin is encoded by two genes (*CNA1/CMP1* and *CNA2/CMP2*), whilst only one gene (*CNB1*) is accountable for the B subunit. Interestingly, this study's screen identified only one of the two genes encoding the catalytic subunit of calcineurin A, *CMP2*, along with *CNB1*, which encodes for the regulatory Ca^{2+} -binding subunit calcineurin B, but no interaction was found for the isoform encoded by *CNA1/CMP1*. *cmp1Δ* was part of the manually picked set of genes that went into the follow-up spot dilution assays, and was confirmed as not sensitive, suggesting it was not simply lost through noise in the more high-throughput screening approach. A further calcineurin-related sensitive hit was reported:

RCN1 encodes a protein whose levels are believed to fine tune calcineurin signalling in yeast and for this Rcn1p is involved in calcineurin regulation during Ca^{2+} signalling. Increasing interest has arisen around the *RCN1* gene, following the discovery that it has conserved structural and functional similarities to *H. sapiens* DSCR1, and it is therefore likely that the mammalian Rcn1p-related proteins, calciressins, could modulate calcineurin signalling in humans, potentially contributing to disorders such as Down syndrome (Kingsbury & Cunningham, 2000).

In order to maintain Ca^{2+} homeostasis, the contribution of proteins found in different cellular compartments varies little, however the contribution to surveillance and maintenance of this cation balance may be more variable, as emerged in this screen. Ca^{2+} homeostasis affects vacuole, Golgi and ER compartments, which are subjected to calcineurin regulation via the stress-response transcription factor Crz1p (**Figure 3-18**), required for the induction of genes encoding the vacuolar Pmc1p and secretory Pmr1p Ca^{2+} -pumps (Cunningham & Fink 1994; Stathopoulos & Cyert, 1997). This latter deletion was identified as a suppressor in this screen. There is the possibility that deletion is causing a reduction in the secretory pathway activity as discussed for the *ALG* and *OST* deletions, hence the suppressor effect.

Along with Pmc1p, Ca^{2+} vacuolar intake also happens via Vcx1p (not shown), whose activity is still under calcineurin regulation, although in a Crz1p-independent manner: The functional relationship between these two vacuolar Ca^{2+} transporters is rather complex: calcineurin activation stimulates Pmc1p function, whilst it inhibits Vcx1p's through a post-translational mechanism (Cunningham & Fink, 1996).

Figure 3-18. Calcineurin-mediated regulation of ion homeostasis in budding yeast.

The high affinity $\text{Ca}^{2+}/\text{Mn}^{2+}$ P-type ATPase encoded by *PMR1* and localized on the plasma membrane, is required for both cations' transport into the Golgi (Miseta, 1999), and it was identified as a suppressor in this study. Pmr1p belongs to family of transporters known as Secretory Pathway Ca^{2+} -ATPases, which are found in *C. elegans*, *D. melanogaster*, and mammals (Culotta, 2005) and plays a role in maintaining the Ca^{2+} luminal concentration required for proper glycosylation and processing of proteins in the Golgi compartment (Antebi & Flink, 1992). Both Ca^{2+} and Mn^{2+} are necessary for proper processing and trafficking of polypeptides through the secretory pathway, but each with a different role: whilst Ca^{2+} is required to sustain protein sorting, Mn^{2+} is needed for protein glycosylation, a process represented in this screen (**section 3.30.3**). In this context it is possible that the *PMR1* deletion leads to loss of efficient

glycosylation and secretion of key cell surface proteins, which might include Gal2p and pumps which might import lithium. As previously discussed, this might allow reduced galactose import and/or lower intracellular concentrations of lithium which would then lead to the suppressor phenotype observed with other strains working in this pathway.

Deletion of the gene *HUR1* also resulted in a suppressor phenotype. This gene codes a protein of unknown function; however, its appearance in this screen is likely to be due to its overlap with the suppressor *PMR1* gene. The deletion of these two genes causes suppression, so the inability to transport Ca^{2+} into the Golgi leads to enhanced growth in lithium/galactose environment.

PMR1 and *PMC1* undergo a regulation driven by calcineurin (**Figure 3-18**) so the observed phenotype may have something to do with the specific compartment where the Ca^{2+} in excess is accumulated. It is likely to be a response driven from the Golgi compartment, as hinted at by the wild-type like phenotype observed for deletion of vacuolar Ca^{2+} pumps genes *PMC1* and *VCX1*.

Lithium-induced Ca^{2+} over-accumulation is reduced in yeast strains lacking the vacuolar Ca^{2+} -ATPase contribution of Pmc1p (Kellermayer, 2003), a result that lead the authors to conclude that lithium inhibition of Pgmp results in an increased Glu-1-P to Glu-6-P ratio, which consequently translates into an augmented rate of vacuolar Ca^{2+} uptake mediated by the Ca^{2+} -ATPase Pmc1p. This led us to expect a change in sensitivity on *PMC1* deletion, the *pmc1Δ* was selected to undergo spot assay. Contrary to our expectations, it still did not return a change in growth and we are left to conclude that the difference in the strains' backgrounds or the specific lithium/galactose treatment used in this

study. It is worth recognising that other cellular compartments can compensate for the reduced capability to uptake Ca^{2+} by the vacuole (Miseta, 1999). The diverse contributions of cellular compartments are possibly dictated by their own ability to take up this cation.

A depletion of secretory pathway Ca^{2+} stores is caused by the mutation or deletion in *PMR1* region and subsequently leads to improper folding and processing of proteins that transit through ER and Golgi (Antebi & Fink, 1992). Possibly as a result of feedback from this outcome, elevated cellular Ca^{2+} uptake is observed in *pmr1Δ* strain. This is mediated by Cch1p and Mid1p and recalls the mammalian capacitative Ca^{2+} entry (CCE) response introduced in **section 3.3**. *CCH1* encodes a high-affinity Ca^{2+} channel with homology to mammalian voltage-gated Ca^{2+} channels (Diriong, 1995). Both *cch1Δ* and *mid1Δ* mutants display reduced tolerance to monovalent cations such as lithium, suggesting a role for Ca^{2+} uptake in the calcineurin-dependent ion stress response, providing further support for the hypothesis that Mid1p and Cch1p function in the same Ca^{2+} uptake process (Paidhungat & Garrett, 1997). Cch1p functions together with the stretch-activated cation channel Mid1p (identified in the primary screen but not confirmed by the spot dilution assays) to form a channel for Ca^{2+} influx at the plasma membrane level (Locke, 2000). Along with the transporters Pmr1p (discussed above), Pmc1p, and the vacuolar ion exchanger Vcx1p, Cch1p and Mid1p are involved in the regulation of cellular Ca^{2+} levels and mediate Ca^{2+} signalling. Strains lacking either *CCH1* or *MID1* show hypersensitivity to ER stress including accumulation of misfolded proteins in the ER (Bonilla, 2002), alkaline (Viladevall, 2004), and iron stress (Peiter, 2005); upon lithium/galactose treatment, however, the loss of Cch1p seems to be more

debilitating than Mid1p. In a similar fashion, the hit *ECM7* encodes for a non-essential putative integral membrane protein and its transcription is induced under conditions of zinc deficiency. Ecm7p has a role in Ca^{2+} uptake and *ecm7Δ* mutants have cell wall defects and Ca^{2+} uptake deficiencies which could explain the sensitivity observed for this deletion strain.

When relating the results of this study to mammals, a mention should be made of the higher number of hits identified in this study for Ca^{2+} related processes happening in the Golgi compared to ER. This finding might relate to the fact that yeast Golgi plays a role functionally similar to that of mammalian ER, in its potential to mediate a temporary increase in cytosolic Ca^{2+} , which will then be amplified by a CCE-like mechanism (Aiello, 2002). Endorsing this explanation is the knowledge that the yeast ER appears to play a lesser role in cellular Ca^{2+} storage supported by the finding that the free Ca^{2+} concentration in this compartment has been reported to be only about 10 μM (Strayle, 1999) compared to the mM concentration reported for the Golgi apparatus (Fasolato, 1991).

Studies have demonstrated that some misfolded luminal proteins are exported from the ER into the cytosol for ubiquitination and subsequent degradation by the proteasomal structure (reviewed in Brodsky & McCracken, 1997). If this process is impaired by the action of lithium in competing with Ca^{2+} interactions, it would be expected that overexpression of a translational encoding gene with the intent of enhancing protein synthesis could overcome the toxicity the effect of a poor recycling could be overcome, as shown for *TIF2* (Montero-Lomeli, 2002). Adjunctive evidence that points to a loss of glycosylation is also suggested by observations reported in **section 3.30.3**.

3.32. The TOR pathway

S. cerevisiae adapts to changing nutrient conditions by regulating its transcription profile and complementing cellular protein requirements in correlation with the nutrient conditions. The TOR signalling pathway is one of the major control mechanisms within the cell that facilitates these changes, which actuates, among others, the transcription, intracellular trafficking, and protein turnover of nutrient transporters which are regulated in response to nutrient conditions, as highlighted in **section 3.17** for the “generation of precursor metabolites and energy” GO term.

The relevance of TOR as an important signalling component in the regulation of cellular responses is reflected in the wealth and variety of cellular processes indicated by this screen in assisting the cell to tolerate and overcome the lithium/galactose toxicity, many of which share TOR-mediated regulation. Indeed, a link between TOR signalling and lithium sensitivity has already been established by studies on both baker's yeast and *C. albicans*, where deletion of one of the components of the TOR complex TORC1, Tco89p, induces sensitivity to both lithium and rapamycin (Zheng, 2007).

Under nutrient-rich conditions, the TOR complex 1 (TORC1) prevents general autophagy and promotes ribosome biosynthesis and protein translation, however, nutrient limitation or the addition of rapamycin leads to TORC1 inhibition and consequent autophagy. Ubiquitin-mediated degradation could be involved in the mechanisms of regulation of autophagy and ribophagy (**section 3.30.1**). It is still unclear whether and to what extent TOR regulates autophagy and ribophagy. Similarly, it is not clear whether TOR signalling regulates a connection between the targeted disposal of stalling ribosomes and the

translation of specific mRNAs. TOR regulation extends to the CWI pathway (**section 3.21**), where the signal is mediated via Rho1p; this latter interacts with Pkc1p activating the MAP kinase pathway cascade, which is essential for regulating cell wall integrity and actin cytoskeleton (Helliwell, 1998) (**section 3.22**). The inter-communication which allows the adjustment of the two important processes of glycosylation and protein synthesis could be, in some measure, TOR-mediated (**section 3.30.3**).

3.33. Targets of lithium

This study recapitulated the majority of the long-time agreed targets of lithium. These include glycogen synthase kinase 3β (GSK- 3β), phosphoglucomutase, and FBPase as discussed below.

i. Glycogen synthase kinase 3β

The adverse effects observed upon lithium treatment in the developmental processes of many different organisms are thought to have a common cause. One possibility is that this occurs through inhibition of the GSK- 3β (Klein, 1996), since loss of function of this enzyme produces the same phenotypes in organisms as different as the social amoeba *Dictyostelium discoideum* (Harwood, 1995) and the frog *Xenopus laevis* (He, 1995). Some of lithium effects could be easily explained by a negative regulation of GSK- 3β proteins and other proteins by competitively displacing Mg^{2+} from its binding site.

Four homologs of GSK- 3β are present in yeast: *RIM11*, *MCK1*, *MRK1*, and *YGK3*. Among them, *RIM11* and *MCK1* are homologs with a highly probability that

they arose from a recent genome duplication followed by a rapid divergence, as shown by the ability of the two homologs to phosphorylate different specific substrates (Puziss, 1994). Rim11p is essential for entry into meiosis, whereas Mck1p is required for growth at elevated and low temperatures. While both kinases transmit nutrient signals, Mck1p transmits additional signals including stress signals such those related to temperature and osmotic shock. The other two paralogs (*MRK1* and *YGK3*) show redundant roles, though believed to be less important. Among the four GSK-3 β yeast homologs, only one was reported in this study: *MCK1*; the fact that *MCK1* deletion leads to lithium sensitivity indicates that the protein product of this gene is not a prime candidate as a target. However, it is notable that *MCK1* shows both a synthetic growth defect (Hirata, 2003) and negative genetic interaction (Fiedler, 2009; Costanzo, 2010) with *RIM11*, amongst other genes. It would be plausible, therefore, that lithium leads to inhibition of Rim11p, which in combination with *MCK1* deletion leads to a growth defect analogous to the growth defect observed in the dual deletion.

ii. Phosphoglucomutase (PGM).

Lithium has been previously shown to inhibit the PGM enzymes as extensively discussed in **section 3.2**. The contribution of the different PGM genes to Pgmp activity is reflected in the sensitivity observed in the relative deletions under lithium/galactose treatment. Abolition of Pgm2p, the major isoform lead to a more severe growth defect than loss of Pgm1p; concordantly, *pgm3 Δ* strain is only associated to a mild sensitivity upon lithium/galactose treatment, as expected from its relatively minor contribution. Lithium-induced inhibition of Pgmp has been noted to impact on Ca²⁺ regulation, as discussed in **section 3.3**.

iii. FBPase

The FBPase enzyme is also a verified target of lithium; the mechanisms through which this drug inhibits enzymatic activity has been previously established and extensively discussed in **section 3.29**. Under the lithium/galactose dual treatment, a depletion of the F-6P reserves is observed leading to severe cellular consequences, likely triggered by the unavailability of carbohydrate necessary for the synthesis of fundamental building blocks of nucleic acids (e. g., ribose).

iv. Inositol Monophosphate (IMP).

According to the “inositol depletion hypothesis” (Berridge & Irvine, 1989), lithium inhibits the inositol monophosphatases (IMPases) (Hallcher & Sherman, 1980) and inositol polyphosphate 1-phosphatases (IPPases) enzymes (Inhorn & Majerus, 1988) ultimately leading to the impairment of the inositol 1,4,5-triphosphate-dependent signal transduction pathway (Berridge & Irvine, 1989). Despite literature reports of inhibition of inositol monophosphatases, deletions of IMPase encoding yeast genes *INM1* and *INM2*, did not appear to be sensitive in this study, possibly masked by other more highly sensitive deletions under the dual lithium/galactose treatment.

3.34. Implications for lithium’s mode of action and neuroprotective agents

Several features associated with neurodegenerative diseases, such as cellular toxicity mediated by misfolded proteins and oxidative stress have been partially recapitulated by findings highlighted by this study, providing

information on conserved mechanisms that may be further utilized to gain mechanistic and genetic insights into candidate drugs.

Impairment of mitochondrial energy metabolism has been clearly recognized as one of the mechanisms crucial in many neurodegenerative disorders. A growing set of evidence relates reduced energy production and mitochondrial dysregulation to bipolar disorder and indications of mitochondrial dysfunction in other neurodegenerative conditions are supported by studies on the molecular pharmacology of mood stabilizers pointing at the respiratory organelles as one of the drugs targets (Zarate, 2006). Results presented in this thesis could help to better define the current model, in which the mitochondrial ETC emerges as a major source for the generation of reactive oxygen species; findings of alterations in the expression patterns of genes associated with mitochondrial activity in the respiratory chain have been reported, for example in brain samples from patients treated for bipolar disorder (Konradi, 2004). This study indicates several mitochondria-localized processes (e.g., TCA, ETC, and partially the beta oxidation of fatty acids) as critical in supporting the cell under the tested treatment and altogether reinforces the current awareness that the etiology of neurodegenerative diseases has to be sought in the abnormality of the aforementioned related biological processes (Calabrese, 2001 for review; Schon & Manfredi, 2003).

The number of pathways reported in this study with ubiquitin-related links demonstrates convincingly the importance of regulated proteolysis for cellular metabolism and how relevant and ubiquitous this process is, with a highly connected net of interactions that impinge on fundamental and far off pathways. The selective degradation of ribosomes by autophagy (ribophagy) is

mediated by hits from this screen - ubiquitin-specific protease 3 (Ubp3p) and Ubp3p-associated cofactor, Bre5p. This result strengthens the connections between the autophagy-like degradation mechanisms and neurodegenerative disorders, given the established role of ubiquitin as a key regulator of neuronal biology and the notion that dysfunction in ubiquitin metabolism may contribute to their pathogenesis (Layfield, 2005).

Furthermore, the proposal of this study that FBPase is inhibited by lithium may be useful for other investigations. FBPase has a structural fold similar to that of inositol-1-phosphatase (I-1-Pase) (Zhang, 1993), and both are members of the sugar phosphatase superfamily which has been shown to be subjected to lithium action (**section 3.29**). Inhibition of mammalian I-1-Pase by lithium is of particular interest, since this enzyme is expressed in brain tissue and forms the main target in medical treatment of manic-depression (Pollack, 1994). The mechanisms of the lithium inhibition of FBPases and IMPases are believed to be essentially the same: lithium binds at one of the metal binding sites, thereby retarding turnover or phosphate release (Johnson, 2001). The residues that constitute this metal binding site are conserved in lithium-sensitive I-1-Pase and in FBPase (Verhees, 2002).

The discrete number of deletion strains corresponding to genes encoding for a functional protein of unknown function reported in this study is challenging and encouraging. It emphasizes the concept that several targets exist for this drug and it may mean that there is more investigation to be undertaken into lithium toxicity and mechanism(s), and that alternative pathways could be targeted. Current research is directing its efforts towards identification of alternative medications that could be used in combination with lithium or alone

as a replacement for it, as already established for medications such as carbamazepine and valproate. The existence of compensatory pathways is indeed endorsed by this study which found the MAP kinase cascade, HOG and Ca^{2+} /calcineurin-mediated signalling processes over-represented amongst the screen hits. Phosphorylation-mediated signalling dominates the cluster of related processes described for genes associated to signal transmission and transduction achieved via phosphorylation of proteins by kinases. As anticipated, the extensiveness of its targets makes the therapeutic path of lithium difficult to disentangle, and while more insights are sought for the neurobiology of mood disorders and antidepressants' mechanisms of action, a shift in emphasis has been registered. Research earlier orientated towards investigations of the changes in neurotransmitters' release and metabolism has been overtaken by the study of the regulation of gene expression and neuroprotection. This current approach is supported by evidence from animal studies suggesting that drug therapy may act on specific transcription factors and target genes that regulate processes such as neuroprotection and neuronal survival.

3.35. This study screen versus Zhao's and Hillenmeyer's

Analogous lithium genome-scale genetic screens were conducted by independent groups (Hillenmeyer, 2008; Zhao, 2010) utilizing rich medium (YP) and glucose as a carbon source. Those two studies can be compared to this investigation, as the same diploid BY4743 deletion sets were employed; the concentrations of lithium chloride tested in the two aforementioned studies were, however, higher than the one used in this study.

This latter study identified 114 lithium-sensitive and 6 lithium-tolerant gene mutations in *S. cerevisiae*, with the majority of the sensitive mutations being genes annotated to a function in the VPS pathway (discussed in **section 2.36**), sporulation and meiosis, mainly operating in membrane docking and fusion. This led the authors to conclude that the integrity of the VPS pathway is critical for the ion homeostasis, at least in yeast cells (Zhao, 2010). Results presented here do not mirror Zhao's in terms of most represented functional categories, although sharing 14 common lithium sensitive strains and two suppressors. This incongruity between the generated data sets reflects the effect of different carbon sources on cell metabolism, with different mechanisms characterizing glucose versus galactose-related hits. A higher drug working concentration (0.1 M, 0.2 M, and 0.4 M) could also have played a role, as adaptation to low-to-mid molarities can engage different mechanisms. This lack of overlapping between sensitive and suppressor hits under lithium treatment reinforces the notion that many targets exist for this drug.

Data from the lithium/galactose screen presented in this study can be compared to those generated from Hillenmeyer's study (2008). The working concentrations utilized in the two assays were different: LiCl treatment was done at 150 mM in the cited study, while 30 mM was the highest concentration tested in this study, reflecting the differences in growth inhibition in the galactose and YPD contexts. All the ORFs identified in this screen at 30 mM are also found in Hillenmeyer's study, with the ultra-sensitive gene deletions identified in this screen (e.g., *CNB1*, *CRZ1*, and *RCN1*) being among Hillenmeyer's screens top hits. As the suppressor phenotypes associated with certain deletion strains are specific for the carbon source utilized (galactose in -this study- vs glucose in -

Hillenmeyer's-), deletions showing enhanced growth in this screen may be found annotated as sensitive in Hillenmeyer's study. It should not pass unnoticed that, all the sensitive deletions reported in this screen are also annotated in screens undertaken for different inorganic salts, for instance NaCl (Hillenmeyer, 2008). This suggests that either screens need to be performed at sensible concentrations to be able to appreciate discrete drug specific effects, otherwise osmotic stress effect may overbear the specific processes involved in drug's mechanisms and toxicity in a genome-wide assay. For this reason, although useful preliminary tools, chemical screens have to be complemented with different approaches, to further validate the results.

3.36. Summary

Under lithium/galactose stress, cell communication, the complex system that governs and synchronizes almost all basic cellular activities was considerably over-represented amongst the screen hits. Particularly convincing evidence is gained from finding of the key regulators as being protein kinases. Additionally, the finding of genes involved in chromatin remodelling resonates with the model of a complex and intimate role for the regulation of transcription initiation by signalling kinases. Transcriptional repressors that bind to promoter sequences of the cyclin genes were also reported, defining a subset of genes related to repression of cell cycle progression.

The maintenance of the cell wall under stress conditions was brought to attention, along with its regulation delivered by calcineurin signalling, the HOG, and TOR pathways. The involvement of this latter pathway, also acting as an

important component by linking external signals to the actin/cytoskeletal machinery, is supported by the activation of chitin synthase which ultimately leads to an increase in chitin synthesis rate and content.

Defects in growth were observed in mitochondrial gene deletions, testifying to the interplay between the TCA and the glyoxylate cycle, particularly in a non- or weakly-fermentable environment. In this context, the extraordinary extent of duplicate genes' contribution to metabolic pathways could also be appreciated.

An interpretation of the result on the loss of *FBP26* strongly suggests that this condition induces cell death as a result of the organism's inability to proceed with the synthesis of fundamental bio-precursors such purines, pyrimidines, and histidine.

The deletion strains leading to suppression of lithium/galactose toxicity appear to coalesce on the single process of galactose metabolism. Inhibition of the lithium target protein Pgmp leads to a reduced flux through the Leloir pathway and a build up of metabolites that impair growth. Reasonably, loss of galactose import or processing prevents the toxic metabolites from being formed, resulting in a satisfactory cellular growth, by making use of other available carbon sources. A model where a reduction in glycosylation leads to a corresponding loss of active cell surface glycosylated transporters is also proposed as possible reason for the eased toxic effects observed with some suppressor strains.

Finally, although an extended Ca^{2+} regulation in several of the processes highlighted in this chapter discussion is strongly suspected, the degree of this contribution is not fully addressable from the results presented in this study.

Broadly agreed upon targets of lithium have been recapitulated (FBPase, PGM, GSK-3) with exception of the IMPase/IPPase system. The yeast *S. cerevisiae* has proven once again that, despite being evolutionary distant from humans, it can be used as a model to study conserved cellular processes in higher eukaryotes.

3.37. Different environmental challenges

To determine the extent of similarity and divergence between the *TIF1* and *TIF2* paralogs, growth of their individual deletion strains was assessed under different environmental conditions with the intent to explore the relative contributions of each paralog to response to environmental conditions, as reported in **method 3.14**. Inducing stresses used included high salt, alkaline pH, osmotic stress, carbon source switch, glucose starvation, and high temperature, etc..., however the pair of duplicates appeared convincingly robust in coping with these adversities in both rich and minimal media, as this study reported no differences for the majority of the experimental conditions tested (**section 3.18**). This result suggests a highly conserved, backed-up genetic network, strikingly even in the case of carbon source switch, where the appreciable growth delay expected was not observed.

The paralogous genes' network of interactions appears to confer robustness in almost any perturbed environment but still breaks down in response to a specific perturbation, as seen for the drug rapamycin. The basis for

this strain specificity in response to rapamycin requires further investigation, as discussed in chapter 4.

4. Chapter 4

4.1. Summary

Being one of the key steps in protein synthesis, the process of translation initiation is subjected to a multi-level regulation which is achieved via diverse mechanisms (Sonnenberg & Dever, 2003). The first part of this thesis focuses on identifying mechanisms of regulation in a translationally deficient system imparted by the loss of one or the other of the *TIF1/2* genes. The cell is understood to select from several processes with the final purpose to adjust protein synthesis accordingly to the cellular status and environment. These include control of: mRNA abundance, translational rate through regulation of ribosome density/occupancy, and protein specific degradation. Results presented in this study faithfully reflect these well agreed arrangements, including sensitivity induced from loss of genes for proteins that function directly in, or regulation of mRNA concentration and export, ribosome assembly, degradation of selective proteins, and levels of transcription. These therefore all contribute to the regulation of translation initiation. The definition of the different degrees of contribution for any of the aforementioned routes can be inferred from the data presented in this study.

Despite a quite neat compartmentalisation, coupling of the cellular processes of translation and transcription has been acknowledged (Hinnebusch, 2005; Topisirovic, 2011); in fact, the existence of a tied mechanism in eukaryotes has recently been demonstrated in *S. cerevisiae* (Harel-Sharvit, 2010). The interpretation of the results presented in this thesis indicates a sophisticated inter-communication between these two fundamental processes, which is

ultimately represented in the genetic networks of yeast translation initiation factor 4a encoding genes *TIF1* and *TIF2*. Furthermore, such dense arrangement of connections is endorsed by the wealth of physical interactions annotated in the existent literature (Keene, 2001; Halbeisen, 2008). A major finding of this research is related to the functional competences associated to the two duplicate members of the gene pair, expertises that appear somewhat separate. The *tif1Δ* genetic profile highlights a connection with transcription mediated via the direct formation of the TFIIA transcription subunit, a nuclear protein required for all RNA polymerase II-dependent transcription events. The majority of transcription-translation inter-talk, however, is allocated to *TIF2* duplicate. By virtue of the interactions held by this paralog, the translational initiation process has connections to control gene expression via RNA polymerase II assembly and functionality along with a dense network of genetic interactions surrounding the SAGA complex. *TIF2* is also described unequivocally as the only paralog devoted to interactions with a substantial group of functionally related genes involved in early meiotic gene expression. Altogether, the genetic portrait of *TIF1* and *TIF2* genes speaks for a mutual control between transcription and translation aimed to reinforce the control of the two processes singularly.

The correlation between mRNA and protein abundance is profoundly affected by protein turnover (Pratt, 2002): degradation of proteins in the global control of protein synthesis represents a fundamental process and accounts for diverse points of potential control, as also confirmed by this study's SGA data. Ubiquitin appears as a common denominator in degradative processes taking place in both the proteasome and MVBs vesicles; both degradative pathways are represented in this study's genetic investigation of translation. The interplay

between a functional vesicular system and the rate of protein synthesis for appropriate cellular up-keep during changes in cellular metabolism has also been reiterated.

Interestingly, events like NMD which leads to stalling of the ribosome on mRNA, and thereby induce mRNA cleavage and degradation (Doma & Parker, 2006), do not appear to play a major role in addressing cellular issues concerning the translational initiation process. Given the established link between the mRNA surveillance machinery and other stages of translation, there is the possibility that the NMD contribution may be concealed behind other connections, for instance those with reference to the ubiquitin pathway.

Finally, it is critically important to comment on the relative paucity of interactions reported for genes involved in ribosome biosynthesis/biogenesis in the *tif1Δ* and *tif2Δ* SGAs. This result seems to fall in with existent work which showed how repression of ribosome biosynthesis does not rule the rapid repression of translation, at least in secretory-deficient pathways (Deloche, 2004). For this condition, the same group also report no TOR-mediated regulation in the decrease of translation, as echoed by the data in the current study, where no immediate link to TOR was reported.

This research testifies to the critical importance of translation-transcription inter-talk among the several mechanisms put in place to amend protein synthesis according to cellular needs. This contribution is mastered through the intervention of the DUB enzymes via their counteracting ubiquitinating enzymatic activity on specific targets. These DUBs are crucial regulators of chromatin dynamics and gene expression, and assist with the recruitment of modules that involve selected components of the SAGA complex.

Functional turnover of proteins is, to a certain extent, regulated by the balance between post-translational modifications, in particular ubiquitination/deubiquitination. In particular H2B histone ubiquitination appears to be of particular relevance: a scenario is proposed in which changes in the ubiquitin pools could be sensed by the nucleus by affecting the histone-ubiquitin status. Following this rationale, the proposed mechanism may lead to either a signal to recycle/degrade ubiquitin or to up/downregulate ubiquitin *de novo* synthesis.

This research concludes that functional turnover of proteins and the translation/transcription inter-talk emerges as the most significant contributors to the sophisticatedly regulated translational regulation, whilst the participation of NMD machinery or ribosome biogenesis to this process appear minor in this work. This study offers an insight on the dynamics of translation regulation, which emerges as highly interconnected and amendable process, which is to be expected given the importance of protein synthesis as a cellular process. Over evolutionary timescales, the challenge of regulating translation may be overcome by allocating new competences to genes to achieve this remodelling. This seems to be reflected in the variety of genes identified in these SGA screens. Supported by a previous hypothesis which suggested that eIF4Ap function is necessary even when no melting activity of secondary structures is required (Linder, 2003), this thesis suggests that RNA helicase activity may not be the only mechanism in which this protein participates, some of which still not clearly resolved in the literature.

The second part of this thesis describes the use of chemical and environmental perturbations, to complement the *TIF1* and *TIF2* genome-wide screen results. This is achieved by accommodating a variety of potential growth environment in which the robustness of the duplicate pair is challenged under disparate cellular contexts. One of the primary treatments made use of lithium, condition which was hoped to help, along with furthering our understanding of the *TIF1* and *TIF2* networks, in formulating an explanation on how augmented translation initiation overcomes lithium toxicity.

Although this approach did not return enlightening results that could be used to address this unresolved topic, it was highly instructive of mechanisms of response triggered upon lithium/galactose stress. Regulation and synchronization of basic cellular processes were affected: emphasis brought on aspects of cell communication highlighted mechanisms articulated by kinases enzymes as key regulators. This was also endorsed by the finding of genes involved in chromatin remodelling. In fact, this latter result was interpreted in line with the model of a complex and intimate role for the regulation of transcription initiation by signalling kinases. Additionally, transcriptional repressors able to bind to promoter sequences of the cyclin genes were also reported, emphasizing the importance of repression of cell cycle progression in control of protein synthesis. The maintenance of the cell wall under stress conditions was also underscored, along with its regulation delivered by calcineurin signalling, the HOG, and TOR pathways. The involvement of this latter pathway, also acting as an important component by linking external signals to the actin/cytoskeletal machinery, is supported by the activation of chitin synthase enzymes. These hits from the screen also indicated the stress that

combined lithium/galactose treatment places on central metabolic pathways, for instance those between the Leloir route and the gluconeogenesis and trehalose pathways. Interpretation of the results concluded growth defects are induced as a result of three major factors: 1) Impairment of gluconeogenesis and the TCA 2) the build-up of growth inhibitory intermediate metabolites, and 3) the organism's inability to proceed with the synthesis of fundamental bio-precursors such purines, pyrimidines, and histidine, triggered by the inadequate amount of ribose available. Furthermore, the extraordinary extent of duplicate genes' contribution to metabolic pathways could be appreciated, in the context of mitochondrial localized cycles. In regard to this, the interplay between the TCA and the glyoxylate routes in a weakly-fermentable carbon source environment was discussed in detail.

Mechanisms of genetic suppression were observed for deletion strains in response to lithium/galactose toxicity: although they may have initially appeared related to the ubiquitination process and the metabolism of the carbon source galactose, they all ultimately coalesce on the single process of galactose metabolism. Inhibition of the lithium target protein Pgmp leads to a reduced flux through the Leloir pathway and a build up of metabolites that impair growth. Reasonably, loss of galactose import or processing prevents the toxic metabolites from being formed, resulting in a satisfactory cellular growth making use of other carbon sources. A model where a reduction in glycosylation leads to a corresponding loss of active cell surface glycosylated transporters is also proposed as possible reason for the eased toxic effect. Finally, although an extended Ca^{2+} regulation is strongly suspected in several of the processes

highlighted in this study, the underlying mechanisms of this cation's contribution cannot be inferred from the results obtained.

Finally, the lithium/galactose study recapitulated almost fully the broadly agreed upon targets of lithium, as suggested for its therapeutic action. Despite being quite far away from humans, the yeast *S. cerevisiae* has proven once again, to be a valuable model for the study of conserved cellular processes in higher eukaryotes.

To determine the extent of similarity and divergence between the *TIF1* and *TIF2* paralogs, growth of their individual deletion strains was challenged under different chemical and environmental conditions with the intent to explore the relative contributions of each paralog in response to an extend range of stresses. The pair of duplicates appeared convincingly robust in coping with these adversities, thus suggesting a highly conserved and backed-up genetic network. Although the paralogous genes' network of interactions appears to confer robustness in almost any perturbed environment, it still breaks down in response to a specific perturbation, as observed for the drug rapamycin. The basis for this strain specificity in response to such chemical treatment requires further investigation, as discussed later in this chapter.

4.2. Future directions

Although several interesting discoveries have been achieved over the course of this research, there are still a number of areas that warrant future investigation and/or confirmation. Moreover, some results suggested findings that fell outside the remit of the current study but that would be particularly appealing for future investigations.

The interpretation of results obtained in the chapter 2 of this thesis provides a captivating visualization and a rather detailed understanding of the similarities and differences between *TIF1* and *TIF2* duplicate genes. Underlying the glimpse offered by this study of the mechanisms of robustness supporting the functions of *TIF1* and *TIF2* gene pair is a wealth of information that could ultimately assist in extrapolating from the laboratory to broader time scales. Evolutionary-based approaches are required to fully elucidate whether and through which modalities robustness may facilitate evolutionary innovation, not only for this pair of duplicates, but also with the attempt to augment our comprehension of WGD-derived paralogs. The type of approach utilized in this research encountered a limiting weakness, as only the study of evolutionary novelty arising from modest laboratory time scales could be uncovered. To what extent robustness promotes evolutionary innovations could be possibly achieved through comparative genomics studies. Some further investigations, although intended, were not achieved mainly as time did not permit and will be addressed shortly with the aid of Green Fluorescent Protein (GFP) microscopy, utilizing resources available in our laboratory. This is particularly applicable to cases in which the contribution of one or both duplicates to a particular process might be assessed.

It also became apparent that limitations in the techniques used meant that this research was not able to prove some of the newly proposed and unconventional interpretations beyond reasonable doubt and auxiliary validations are needed to turn them in unambiguous assertions. This is the case of some findings highlighted in the second part of this thesis (chapter 3) that complemented and deepened the discoveries made in the previous chapter.

Potential links to the process of translation initiation and ultimately protein synthesis have been hinted at, linked to highly connected mechanisms of control at the cellular level (e.g., TOR, ubiquitination/deubiquitination, and Ca^{2+} -mediated regulation); for this reason further investigations are required to ascertain the level of their involvement with the translational initiation process. TOR, inactivation of initiation induces a well-established inhibitory effect on protein synthesis, was brought to attention over the course of this study analysis. It is hard to state with certainty whether TOR involvement is directly linked to its regulation on proteins synthesis rather than acting through its extended range of interactions. Furthermore, since the functional turnover of proteins is regulated to a certain extent by the balance between post-translational modifications, such ubiquitination/deubiquitination, further experiments should address whether this process represents the predominant mechanism to regulate Tif1/2p proteins' stability. Cellular implications of Ca^{2+} -mediated regulation have also been considered; however, given the complexity of this regulatory mechanism, specific experiments should be designed to indisputably address this topic.

5. Appendix

5.1. Negative genetic interactions identified in *tif1* SGA

ORF	Gene	Description
YBR093C	PHO5	Repressible acid phosphatase (1 of 3) that also mediates extracellular nucleotide-derived phosphate hydrolysis; secretory pathway derived cell surface glycoprotein; induced by phosphate starvation and coordinately regulated by PHO4 and PHO2
YBR106W	PHO88	Probable membrane protein, involved in phosphate transport; pho88 pho86 double null mutant exhibits enhanced synthesis of repressible acid phosphatase at high inorganic phosphate concentrations
YCR063W	BUD31	Protein involved in bud-site selection; analysis of integrated high-throughput datasets predicts an involvement in RNA splicing; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern
YCR081W	SRB8	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for transcriptional regulation; involved in glucose repression
YDL006W	PTC1	Type 2C protein phosphatase (PP2C); inactivates the osmosensing MAPK cascade by dephosphorylating Hog1p; mutation delays mitochondrial inheritance; deletion reveals defects in precursor tRNA splicing, sporulation and cell separation
YDL002C	NHP10	Protein related to mammalian high mobility group proteins; likely component of the INO80 complex, which is an ATP-dependent chromatin-remodeling complex
YBR289W	SNF5	Subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; functions interdependently in transcriptional activation with Snf2p and Snf6p
YCL010C	SGF29	SaGa associated Factor 29kDa; Probable 29kDa Subunit of SAGA histone acetyltransferase complex
YDR080W	VPS41	Vacuolar membrane protein that is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex; essential for membrane docking and fusion at the Golgi-to-

ORF	Gene	Description
		endosome and endosome-to-vacuole stages of protein transport
YDL232W	OST4	Subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes protein asparagine-linked glycosylation; type I membrane protein required for incorporation of Ost3p or Ost6p into the OST complex
YDR173C	ARG82	Inositol polyphosphate multikinase (IPMK), sequentially phosphorylates Ins(1,4,5)P3 to form Ins(1,3,4,5,6)P5; also has diphosphoinositol polyphosphate synthase activity; regulates arginine-, phosphate-, and nitrogen-responsive genes
YER061C	CEM1	Mitochondrial beta-keto-acyl synthase with possible role in fatty acid synthesis; required for mitochondrial respiration
YER162C	RAD4	Protein that recognizes and binds damaged DNA (with Rad23p) during nucleotide excision repair; subunit of Nuclear Excision Repair Factor 2 (NEF2); homolog of human XPC protein
YDR455C		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene YDR456W
YER092W	IES5	Protein that associates with the INO80 chromatin remodeling complex under low-salt conditions
YFL013W-A		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; overlaps the verified gene IES1/YFL013C
YFL036W	RPO41	Mitochondrial RNA polymerase; single subunit enzyme similar to those of T3 and T7 bacteriophages; requires a specificity subunit encoded by MTF1 for promoter recognition
YGL212W	VAM7	Component of the vacuole SNARE complex involved in vacuolar morphogenesis; SNAP-25 homolog; functions with a syntaxin homolog Vam3p in vacuolar protein trafficking
YGL219C	MDM34	Mitochondrial outer membrane protein, required for normal mitochondrial morphology and inheritance; localizes to dots on the mitochondrial surface near mtDNA nucleoids; interacts genetically with MDM31 and MDM32
YGL218W		Dubious open reading frame, unlikely to encode a protein; not conserved in closely

ORF	Gene	Description
		related <i>Saccharomyces</i> species; 93% of ORF overlaps the verified gene MDM34; deletion in <i>cyr1</i> mutant results in loss of stress resistance
YFR025C	HIS2	Histidinolphosphatase, catalyzes the eighth step in histidine biosynthesis; mutations cause histidine auxotrophy and sensitivity to Cu, Co, and Ni salts; transcription is regulated by general amino acid control
YGR092W	DBF2	Ser/Thr kinase involved in transcription and stress response; functions as part of a network of genes in exit from mitosis; localization is cell cycle regulated; activated by Cdc15p during the exit from mitosis
YGL253W	HXK2	Hexokinase isoenzyme 2 that catalyzes phosphorylation of glucose in the cytosol; predominant hexokinase during growth on glucose; functions in the nucleus to repress expression of HXK1 and GLK1 and to induce expression of its own gene
YGR105W	VMA21	Integral membrane protein that is required for vacuolar H ⁺ -ATPase (V-ATPase) function, although not an actual component of the V-ATPase complex; functions in the assembly of the V-ATPase; localized to the yeast endoplasmic reticulum (ER)
YGL147C	RPL9A	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl9Bp and has similarity to <i>E. coli</i> L6 and rat L9 ribosomal proteins
YGR118W	RPS23A	Ribosomal protein 28 (rp28) of the small (40S) ribosomal subunit, required for translational accuracy; nearly identical to Rps23Bp and similar to <i>E. coli</i> S12 and rat S23 ribosomal proteins; deletion of both RPS23A and RPS23B is lethal
YGL016W	KAP122	Karyopherin beta, responsible for import of the Toa1p-Toa2p complex into the nucleus; binds to nucleoporins Nup1p and Nup2p; may play a role in regulation of pleiotropic drug resistance
YGL154C	LYS5	Phosphopantetheinyl transferase involved in lysine biosynthesis; converts inactive apo-form of Lys2p (alpha-amino adipate reductase) into catalytically active holo-form by posttranslational addition of phosphopantetheine
YGL168W	HUR1	Protein required for hydroxyurea resistance; has possible roles in DNA replication and maintenance of proper telomere length

ORF	Gene	Description
YGL026C	TRP5	Tryptophan synthase involved in tryptophan biosynthesis, regulated by the general control system of amino acid biosynthesis
YGL046W		Merged open reading frame, does not encode a discrete protein; YGL046W was originally annotated as an independent ORF, but as a result of a sequence change, it was merged with an adjacent ORF into a single reading frame, designated YGL045W
YGL045W	RIM8	Protein of unknown function, involved in the proteolytic activation of Rim101p in response to alkaline pH; has similarity to <i>A. nidulans</i> PalF
YHR067W	HTD2	Mitochondrial 3-hydroxyacyl-thioester dehydratase involved in fatty acid biosynthesis, required for respiratory growth and for normal mitochondrial morphology
YGR188C	BUB1	Protein kinase that forms a complex with Mad1p and Bub3p that is crucial in the checkpoint mechanism required to prevent cell cycle progression into anaphase in the presence of spindle damage, associates with centromere DNA via Skp1p
YHL007C	STE20	Signal transducing kinase of the PAK (p21-activated kinase) family, involved in pheromone response and pseudohyphal/invasive growth pathways, activated by Cdc42p; binds Ste4p at a GBB motif present in noncatalytic domains of PAK kinases
YHL025W	SNF6	Subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; functions interdependently in transcriptional activation with Snf2p and Snf5p
YHR025W	THR1	Homoserine kinase, conserved protein required for threonine biosynthesis; expression is regulated by the GCN4-mediated general amino acid control pathway
YJL204C	RCY1	F-box protein involved in recycling plasma membrane proteins internalized by endocytosis; localized to sites of polarized growth
YIR023W	DAL81	Positive regulator of genes in multiple nitrogen degradation pathways; contains DNA binding domain but does not appear to bind the dodecanucleotide sequence present in the promoter region of many genes involved in allantoin catabolism

ORF	Gene	Description
YJL138C	TIF2	Translation initiation factor eIF4A, identical to Tif1p; DEA(D/H)-box RNA helicase that couples ATPase activity to RNA binding and unwinding; forms a dumbbell structure of two compact domains connected by a linker; interacts with eIF4G
YJL046W	AIM22	Putative lipoate-protein ligase, required along with Lip2 and Lip5 for lipoylation of Lat1p and Kgd2p; similar to E. coli LplA; null mutant displays reduced frequency of mitochondrial genome loss
YJR075W	HOC1	Alpha-1,6-mannosyltransferase involved in cell wall mannan biosynthesis; subunit of a Golgi-localized complex that also contains Anp1p, Mnn9p, Mnn11p, and Mnn10p; identified as a suppressor of a cell lysis sensitive pkc1-371 allele
YKR059W	TIF1	Translation initiation factor eIF4A, identical to Tif2p; DEA(D/H)-box RNA helicase that couples ATPase activity to RNA binding and unwinding; forms a dumbbell structure of two compact domains connected by a linker; interacts with eIF4G
YKR058W	GLG1	Self-glucosylating initiator of glycogen synthesis, also glucosylates n-dodecyl-beta-D-maltoside; similar to mammalian glycogenin
YKR057W	RPS21A	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps21Bp and has similarity to rat S21 ribosomal protein
YKR056W	TRM2	tRNA methyltransferase, 5-methylates the uridine residue at position 54 of tRNAs and may also have a role in tRNA stabilization or maturation; endo-exonuclease with a role in DNA repair
YKR055W	RHO4	Non-essential small GTPase of the Rho/Rac subfamily of Ras-like proteins, likely to be involved in the establishment of cell polarity
YKR054C	DYN1	Cytoplasmic heavy chain dynein, microtubule motor protein, required for anaphase spindle elongation; involved in spindle assembly, chromosome movement, and spindle orientation during cell division, targeted to microtubule tips by Pac1p
YKR053C	YSR3	Dihydrosphingosine 1-phosphate phosphatase, membrane protein involved in sphingolipid metabolism; has similarity to Lcb3p
YKR052C	MRS4	Mitochondrial iron transporter of the mitochondrial carrier family (MCF), very

ORF	Gene	Description
		similar to and functionally redundant with Mrs3p; functions under low-iron conditions; may transport other cations in addition to iron
YKR051W		Putative protein of unknown function
YKR050W	TRK2	Component of the Trk1p-Trk2p potassium transport system
YJR109C	CPA2	Large subunit of carbamoyl phosphate synthetase, which catalyzes a step in the synthesis of citrulline, an arginine precursor
YKR070W		Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YKR069W	MET1	S-adenosyl-L-methionine uroporphyrinogen III transmethylase, involved in the biosynthesis of siroheme, a prosthetic group used by sulfite reductase; required for sulfate assimilation and methionine biosynthesis
YKR067W	GPT2	Glycerol-3-phosphate/dihydroxyacetone phosphate dual substrate-specific sn-1 acyltransferase located in lipid particles and the ER; involved in the stepwise acylation of glycerol-3-phosphate and dihydroxyacetone in lipid biosynthesis
YKR066C	CCP1	Mitochondrial cytochrome-c peroxidase; degrades reactive oxygen species in mitochondria, involved in the response to oxidative stress
YKR065C	FMP18	Presequence translocase-associated motor subunit, required for stable complex formation between cochaperones Pam16p and Pam18p, promotes association of Pam16p-Pam18p with the presequence translocase
YKR064W		Zinc cluster protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YKR061W	KTR2	Mannosyltransferase involved in N-linked protein glycosylation; member of the KRE2/MNT1 mannosyltransferase family
YKR060W	UTP30	Possible U3 snoRNP protein involved in maturation of pre-18S rRNA, based on computational analysis of large-scale protein-protein interaction data
YKL081W	TEF4	Translation elongation factor EF-1 gamma
YKL212W	SAC1	Lipid phosphoinositide phosphatase of the ER and Golgi, involved in protein trafficking and secretion

ORF	Gene	Description
YKR084C	HBS1	GTP binding protein with sequence similarity to the elongation factor class of G proteins, EF-1alpha and Sup35p; associates with Dom34p, and shares a similar genetic relationship with genes that encode ribosomal protein components
YKR082W	NUP133	Subunit of the Nup84p subcomplex of the nuclear pore complex (NPC), localizes to both sides of the NPC, required to establish a normal nucleocytoplasmic concentration gradient of the GTPase Gsp1p
YKR078W		Cytoplasmic protein of unknown function, has similarity to Vps5p; potential Cdc28p substrate; contains a Phox homology (PX) domain and specifically binds phosphatidylinositol 3-phosphate (PtdIns-3-P)
YKR096W		Protein of unknown function that may interact with ribosomes, based on co-purification experiments; green fluorescent protein (GFP)-fusion protein localizes to the nucleus and cytoplasm; predicted to contain a PINc domain
YKR023W		Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YLL006W	MMM1	Mitochondrial outer membrane protein, component of the Mdm10-Mdm12-Mmm1 complex involved in import and assembly of outer membrane beta-barrel proteins
YKR034W	DAL80	Negative regulator of genes in multiple nitrogen degradation pathways; expression is regulated by nitrogen levels and by Gln3p; member of the GATA-binding family, forms homodimers and heterodimers with Deh1p
YKR033C		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene DAL80
YKR028W	SAP190	Protein that forms a complex with the Sit4p protein phosphatase and is required for its function; member of a family of similar proteins including Sap4p, Sap155p, and Sap185p
YKR047W		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene NAP1
YKR046C	PET10	Protein of unknown function that co-purifies

ORF	Gene	Description
		with lipid particles; expression pattern suggests a role in respiratory growth; computational analysis of large-scale protein-protein interaction data suggests a role in ATP/ADP exchange
YKR043C	SHB17	Sedoheptulose biphosphatase involved in riboneogenesis; dephosphorylates sedoheptulose 1,7-bisphosphate, which is converted via the nonoxidative pentose phosphate pathway to ribose-5-phosphate; facilitates the conversion of glycolytic intermediates to pentose phosphate units; also has fructose 1,6-bisphosphatase activity but this is probably not biologically relevant, since deletion does not affect FBP levels; GFP-fusion protein localizes to the cytoplasm and nucleus
YKR042W	UTH1	Mitochondrial outer membrane and cell wall localized SUN family member required for mitochondrial autophagy; involved in the oxidative stress response, life span during starvation, mitochondrial biogenesis, and cell death
YLL039C	UBI4	Ubiquitin, becomes conjugated to proteins, marking them for selective degradation via the ubiquitin-26S proteasome system; essential for the cellular stress response; encoded as a polyubiquitin precursor comprised of 5 head-to-tail repeats
YLR337C	VRP1	Proline-rich actin-associated protein involved in cytoskeletal organization and cytokinesis; related to mammalian Wiskott-Aldrich syndrome protein (WASP)-interacting protein (WIP)
YLR039C	RIC1	Protein involved in retrograde transport to the cis-Golgi network; forms heterodimer with Rgp1p that acts as a GTP exchange factor for Ypt6p; involved in transcription of rRNA and ribosomal protein genes
YLR048W	RPS0B	Protein component of the small (40S) ribosomal subunit, nearly identical to Rps0Ap; required for maturation of 18S rRNA along with Rps0Ap; deletion of either RPS0 gene reduces growth rate, deletion of both genes is lethal
YLR287C-A	RPS30A	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps30Bp and has similarity to rat S30 ribosomal protein
YLR182W	SWI6	Transcription cofactor, forms complexes with

ORF	Gene	Description
		DNA-binding proteins Swi4p and Mbp1p to regulate transcription at the G1/S transition; involved in meiotic gene expression; localization regulated by phosphorylation; potential Cdc28p substrate
YLR062C	BUD28	Dubious open reading frame, unlikely to encode a protein; not conserved in closely related <i>Saccharomyces</i> species; 98% of ORF overlaps the verified gene RPL22A; diploid mutant displays a weak budding pattern phenotype in a systematic assay
YLR056W	ERG3	C-5 sterol desaturase, catalyzes the introduction of a C-5(6) double bond into episterol, a precursor in ergosterol biosynthesis; mutants are viable, but cannot grow on non-fermentable carbon sources
YMR154C	RIM13	Calpain-like cysteine protease involved in proteolytic activation of Rim101p in response to alkaline pH; has similarity to <i>A. nidulans</i> palB
YMR080C	NAM7	ATP-dependent RNA helicase of the SFI superfamily, required for nonsense mediated mRNA decay and for efficient translation termination at nonsense codons; involved in telomere maintenance
YLR448W	RPL6B	Protein component of the large (60S) ribosomal subunit, has similarity to Rpl6Ap and to rat L6 ribosomal protein; binds to 5.8S rRNA
YNL294C	RIM21	Component of the RIM101 pathway, has a role in cell wall construction and alkaline pH response; has similarity to <i>A. nidulans</i> PalH
YNL106C	INP52	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase, synaptojanin-like protein with an N-terminal Sac1 domain, plays a role in endocytosis; hyperosmotic stress causes translocation to actin patches
YNR010W	CSE2	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; component of the Med9/10 module; required for regulation of RNA polymerase II activity
YNL236W	SIN4	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; contributes to both positive and negative transcriptional regulation; dispensable for basal transcription

ORF	Gene	Description
YNR006W	VPS27	Endosomal protein that forms a complex with Hse1p; required for recycling Golgi proteins, forming lumenal membranes and sorting ubiquitinated proteins destined for degradation; has Ubiquitin Interaction Motifs which bind ubiquitin (Ubi4p)
YOL052C	SPE2	S-adenosylmethionine decarboxylase, required for the biosynthesis of spermidine and spermine; cells lacking Spe2p require spermine or spermidine for growth in the presence of oxygen but not when grown anaerobically
YOR026W	BUB3	Kinetochore checkpoint WD40 repeat protein that localizes to kinetochores during prophase and metaphase, delays anaphase in the presence of unattached kinetochores; forms complexes with Mad1p-Bub1p and with Cdc20p, binds Mad2p and Mad3p
YOR141C	ARP8	Nuclear actin-related protein involved in chromatin remodeling, component of chromatin-remodeling enzyme complexes
YOR196C	LIP5	Protein involved in biosynthesis of the coenzyme lipoic acid, has similarity to E. coli lipoic acid synthase
YOR221C	MCT1	Predicted malonyl-CoA:ACP transferase, putative component of a type-II mitochondrial fatty acid synthase that produces intermediates for phospholipid remodeling
YPL069C	BTS1	Geranylgeranyl diphosphate synthase, increases the intracellular pool of geranylgeranyl diphosphate, suppressor of bet2 mutation that causes defective geranylgeranylation of small GTP-binding proteins that mediate vesicular traffic
YPL090C	RPS6A	Protein component of the small (40S) ribosomal subunit; identical to Rps6Bp and has similarity to rat S6 ribosomal protein
YOR355W	GDS1	Protein of unknown function, required for growth on glycerol as a carbon source; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YPL106C	SSE1	ATPase that is a component of the heat shock protein Hsp90 chaperone complex; binds unfolded proteins; member of the heat shock protein 70 (HSP70) family; localized to the cytoplasm
YOR275C	RIM20	Protein involved in proteolytic activation of Rim101p in response to alkaline pH;

ORF	Gene	Description
		PalA/AIP1/Alix family member; interaction with the ESCRT-III subunit Snf7p suggests a relationship between pH response and multivesicular body formation
YPL239W	YAR1	Cytoplasmic ankyrin-repeat containing protein of unknown function, proposed to link the processes of 40S ribosomal subunit biogenesis and adaptation to osmotic and oxidative stress; expression repressed by heat shock
YOR293W	RPS10A	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps10Bp and has similarity to rat ribosomal protein S10

5.2. Positive genetic interactions identified in *tif1* SGA

ORF	Gene	Description
YGL029W	CGR1	Protein involved in nucleolar integrity and processing of the pre-rRNA for the 60S ribosome subunit; transcript is induced in response to cytotoxic stress but not genotoxic stress
YDL074C	BRE1	E3 ubiquitin ligase for Rad6p, required for the ubiquitination of histone H2B, recruitment of Rad6p to promoter chromatin and subsequent methylation of histone H3 (on K4 and K79), contains RING finger domain
YEL042W	GDA1	Guanosine diphosphatase located in the Golgi, involved in the transport of GDP-mannose into the Golgi lumen by converting GDP to GMP after mannose is transferred its substrate
YEL020C		Hypothetical protein with low sequence identity to Pdc1p
YEL016C	NPP2	Nucleotide pyrophosphatase/phosphodiesterase family member; mediates extracellular nucleotide phosphate hydrolysis along with Npp1p and Pho5p; activity and expression enhanced during conditions of phosphate starvation
YEL028W		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YEL023C		Putative protein of unknown function; expression is increased greatly during sporulation; YEL023C is not an essential gene
YEL014C		Dubious open reading frame unlikely to encode

ORF	Gene	Description
		a functional protein, based on available experimental and comparative sequence data

5.3. Negative genetic interactions identified in *tif2* SGA

ORF	Gene	Description
YBL027W	RPL19B	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl19Ap and has similarity to rat L19 ribosomal protein; rpl19a and rpl19b single null mutations result in slow growth, while the double null mutation is lethal
YBR106W	PHO88	Probable membrane protein, involved in phosphate transport; pho88 pho86 double null mutant exhibits enhanced synthesis of repressible acid phosphatase at high inorganic phosphate concentrations
YCR063W	BUD31	Protein involved in bud-site selection; analysis of integrated high-throughput datasets predicts an involvement in RNA splicing; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern
YCR081W	SRB8	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for transcriptional regulation; involved in glucose repression
YDL006W	PTC1	Type 2C protein phosphatase (PP2C); inactivates the osmosensing MAPK cascade by dephosphorylating Hog1p; mutation delays mitochondrial inheritance; deletion reveals defects in precursor tRNA splicing, sporulation and cell separation
YDL002C	NHP10	Protein related to mammalian high mobility group proteins; likely component of the INO80 complex, which is an ATP-dependent chromatin-remodeling complex
YBR289W	SNF5	Subunit of the SWI/SNF chromatin

ORF	Gene	Description
		remodeling complex involved in transcriptional regulation; functions interdependently in transcriptional activation with Snf2p and Snf6p
YCL010C	SGF29	SaGa associated Factor 29kDa; Probable 29kKDa Subunit of SAGA histone acetyltransferase complex
YDR173C	ARG82	Inositol polyphosphate multikinase (IPMK), sequentially phosphorylates Ins(1,4,5)P3 to form Ins(1,3,4,5,6)P5; also has diphosphoinositol polyphosphate synthase activity; regulates arginine-, phosphate-, and nitrogen-responsive genes
YER061C	CEM1	Mitochondrial beta-keto-acyl synthase with possible role in fatty acid synthesis; required for mitochondrial respiration
YER164W	CHD1	Nucleosome remodeling factor that functions in regulation of transcription elongation; contains a chromo domain, a helicase domain and a DNA-binding domain; component of both the SAGA and SILK complexes
YDR455C		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene YDR456W
YER092W	IES5	Protein that associates with the INO80 chromatin remodeling complex under low-salt conditions
YEL057C		Protein of unknown function involved in telomere maintenance; target of UME6 regulation
YEL068C		Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YGL212W	VAM7	Component of the vacuole SNARE complex involved in vacuolar morphogenesis; SNAP-25 homolog; functions with a syntaxin homolog Vam3p in vacuolar protein trafficking
YGL219C	MDM34	Mitochondrial outer membrane protein, required for normal mitochondrial morphology and inheritance; localizes to dots on the

ORF	Gene	Description
		mitochondrial surface near mtDNA nucleoids; interacts genetically with MDM31 and MDM32
YGL218W		Dubious open reading frame, unlikely to encode a protein; not conserved in closely related <i>Saccharomyces</i> species; 93% of ORF overlaps the verified gene MDM34; deletion in <i>cyr1</i> mutant results in loss of stress resistance
YFR025C	HIS2	Histidinolphosphatase, catalyzes the eighth step in histidine biosynthesis; mutations cause histidine auxotrophy and sensitivity to Cu, Co, and Ni salts; transcription is regulated by general amino acid control
YGR097W	ASK10	Component of the RNA polymerase II holoenzyme, phosphorylated in response to oxidative stress; has a role in destruction of Ssn8p, which relieves repression of stress-response genes
YGL244W	RTF1	Subunit of the RNA polymerase II-associated Paf1 complex; directly or indirectly regulates DNA-binding properties of Spt15p and relative activities of different TATA elements; involved in telomere maintenance
YGL147C	RPL9A	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl9Bp and has similarity to <i>E. coli</i> L6 and rat L9 ribosomal proteins
YGL154C	LYS5	Phosphopantetheinyl transferase involved in lysine biosynthesis; converts inactive apo-form of Lys2p (alpha-amino adipate reductase) into catalytically active holo-form by posttranslational addition of phosphopantetheine
YGL026C	TRP5	Tryptophan synthase involved in tryptophan biosynthesis, regulated by the general control system of amino acid biosynthesis
YGL024W		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially/completely overlaps the verified ORF PGD1/YGL025C
YGL046W		Merged open reading frame, does not

ORF	Gene	Description
		encode a discrete protein; YGL046W was originally annotated as an independent ORF, but as a result of a sequence change, it was merged with an adjacent ORF into a single reading frame, designated YGL045W
YGL045W	RIM8	Protein of unknown function, involved in the proteolytic activation of Rim101p in response to alkaline pH; has similarity to <i>A. nidulans</i> PalF
YHR067W	HTD2	Mitochondrial 3-hydroxyacyl-thioester dehydratase involved in fatty acid biosynthesis, required for respiratory growth and for normal mitochondrial morphology
YGR188C	BUB1	Protein kinase that forms a complex with Mad1p and Bub3p that is crucial in the checkpoint mechanism required to prevent cell cycle progression into anaphase in the presence of spindle damage, associates with centromere DNA via Skp1p
YHL007C	STE20	Signal transducing kinase of the PAK (p21-activated kinase) family, involved in pheromone response and pseudohyphal/invasive growth pathways, activated by Cdc42p; binds Ste4p at a GBB motif present in noncatalytic domains of PAK kinases
YHL027W	RIM101	Transcriptional repressor involved in response to pH and in cell wall construction; required for alkaline pH-stimulated haploid invasive growth and sporulation; activated by proteolytic processing; similar to <i>A. nidulans</i> PacC
YHR025W	THR1	Homoserine kinase, conserved protein required for threonine biosynthesis; expression is regulated by the GCN4-mediated general amino acid control pathway
YJL204C	RCY1	F-box protein involved in recycling plasma membrane proteins internalized by endocytosis; localized to sites of polarized growth
YJL130C	URA2	Bifunctional carbamoylphosphate synthetase (CPSase)-aspartate transcarbamylase (ATCase), catalyzes

ORF	Gene	Description
		the first two enzymatic steps in the de novo biosynthesis of pyrimidines; both activities are subject to feedback inhibition by UTP
YJL128C	PBS2	MAP kinase kinase that plays a pivotal role in the osmosensing signal-transduction pathway, activated under severe osmotic stress
YJL126W	NIT2	Nit protein, one of two proteins in <i>S. cerevisiae</i> with similarity to the Nit domain of NitFhit from fly and worm and to the mouse and human Nit protein which interacts with the Fhit tumor suppressor; nitrilase superfamily member
YJL124C	LSM1	Lsm (Like Sm) protein; forms heteroheptameric complex (with Lsm2p, Lsm3p, Lsm4p, Lsm5p, Lsm6p, and Lsm7p) involved in degradation of cytoplasmic mRNAs
YJL123C	MCT1	Protein of unknown function that may interact with ribosomes; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and to COPI-coated vesicles (early Golgi); mtc1 is synthetically lethal with cdc13-1
YJL122W	ALB1	Shuttling pre-60S factor; involved in the biogenesis of ribosomal large subunit; interacts directly with Arx1p; responsible for Tif6p recycling defects in absence of Rei1p
YJL116C	NCA3	Protein that functions with Nca2p to regulate mitochondrial expression of subunits 6 (Atp6p) and 8 (Atp8p) of the Fo-F1 ATP synthase; member of the SUN family
YJL115W	ASF1	Nucleosome assembly factor, involved in chromatin assembly and disassembly, anti-silencing protein that causes derepression of silent loci when overexpressed
YIR023W	DAL81	Positive regulator of genes in multiple nitrogen degradation pathways; contains DNA binding domain but does not appear to bind the dodecanucleotide sequence present in the promoter region of many genes

ORF	Gene	Description
		involved in allantoin catabolism
YJL144W		Cytoplasmic hydrophilin with a role in dessication resistance; expression induced by osmotic stress, starvation and during stationary phase; GFP-fusion protein is induced by the DNA-damaging agent MMS
YJL142C	IRC9	Hypothetical protein; null mutant displays increased levels of spontaneous Rad52 foci
YJL141C	YAK1	Serine-threonine protein kinase that is part of a glucose-sensing system involved in growth control in response to glucose availability; translocates from the cytoplasm to the nucleus and phosphorylates Pop2p in response to a glucose signal
YJL139C	YUR1	Mannosyltransferase of the KTR1 family, involved in protein N-glycosylation; located in the Golgi apparatus
YJL138C	TIF2	Translation initiation factor eIF4A, identical to Tif1p; DEA(D/H)-box RNA helicase that couples ATPase activity to RNA binding and unwinding; forms a dumbbell structure of two compact domains connected by a linker; interacts with eIF4G
YJL137C	GLG2	Self-glucosylating initiator of glycogen synthesis, also glucosylates n-dodecyl-beta-D-maltoside; similar to mammalian glycogenin
YJL136C	RPS21B	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps21Ap and has similarity to rat S21 ribosomal protein
YJL135W		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified genes YJL134W/LCB3
YJL134W	LCB3	Long-chain base-1-phosphate phosphatase with specificity for dihydrosphingosine-1-phosphate, regulates ceramide and long-chain base phosphates levels, involved in incorporation of exogenous long chain bases in sphingolipids

ORF	Gene	Description
YJL133W	MRS3	Mitochondrial iron transporter of the mitochondrial carrier family (MCF), very similar to and functionally redundant with Mrs4p; functions under low-iron conditions; may transport other cations in addition to iron
YJL132W		Putative protein of unknown function; localizes to the membrane fraction; possible Zap1p-regulated target gene induced by zinc deficiency
YJL154C	VPS35	Endosomal subunit of membrane-associated retromer complex required for retrograde transport; receptor that recognizes retrieval signals on cargo proteins, forms subcomplex with Vps26p and Vps29p that selects cargo proteins for retrieval
YJL153C	INO1	Inositol 1-phosphate synthase, involved in synthesis of inositol phosphates and inositol-containing phospholipids; transcription is coregulated with other phospholipid biosynthetic genes by Ino2p and Ino4p, which bind the UASINO DNA element
YJL152W		Dubious ORF unlikely to encode a functional protein, based on available experimental and comparative sequence data
YJL151C	SNA3	Integral membrane protein localized to vacuolar intraluminal vesicles, computational analysis of large-scale protein-protein interaction data suggests a possible role in either cell wall synthesis or protein-vacuolar targeting
YJL150W		Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YJL149W	DAS1	Putative SCF ubiquitin ligase F-box protein; interacts physically with both Cdc53p and Skp1 and genetically with CDC34; similar to putative F-box protein YDR131C; null mutant suppresses dst1delta sensitivity for 6-azauracil
YJL148W	RPA34	RNA polymerase I subunit A34.5
YJL147C		Mitochondrial protein of unknown

ORF	Gene	Description
		function; homozygous diploid deletion strain has a sporulation defect characterized by elevated dityrosine in the soluble fraction; expression induced by Ca ²⁺ shortage; YJL147W is a non-essential gene
YJL146W	IDS2	Protein involved in modulation of Ime2p activity during meiosis, appears to act indirectly to promote Ime2p-mediated late meiotic functions; found in growing cells and degraded during sporulation
YJL145W	SFH5	Putative phosphatidylinositol transfer protein (PITP), exhibits phosphatidylinositol- but not phosphatidylcholine-transfer activity, localized to cytosol and microsomes, similar to Sec14p; may be PITP regulator rather than actual PITP
YJL164C	TPK1	cAMP-dependent protein kinase catalytic subunit; promotes vegetative growth in response to nutrients via the Ras-cAMP signaling pathway; inhibited by regulatory subunit Bcy1p in the absence of cAMP; partially redundant with Tpk2p and Tpk3p
YJL162C	JJJ2	Protein of unknown function, contains a J-domain, which is a region with homology to the E. coli DnaJ protein
YJL160C		Putative protein of unknown function; member of the PIR (proteins with internal repeats) family of cell wall proteins; non-essential gene that is required for sporulation; mRNA is weakly cell cycle regulated, peaking in mitosis
YJL159W	HSP150	O-mannosylated heat shock protein that is secreted and covalently attached to the cell wall via beta-1,3-glucan and disulfide bridges; required for cell wall stability; induced by heat shock, oxidative stress, and nitrogen limitation
YJL158C	CIS3	Mannose-containing glycoprotein constituent of the cell wall; member of the PIR (proteins with internal repeats) family
YJL157C	FAR1	Cyclin-dependent kinase inhibitor that

ORF	Gene	Description
		mediates cell cycle arrest in response to pheromone; also forms a complex with Cdc24p, Ste4p, and Ste18p that may specify the direction of polarized growth during mating; potential Cdc28p substrate
YJL175W		Dubious open reading frame unlikely to encode a functional protein; deletion confers resistance to cisplatin, hypersensitivity to 5-fluorouracil, and growth defect at high pH with high Ca ²⁺ ; overlaps gene for SWI3 transcription factor
YJL172W	CPS1	Vacuolar carboxypeptidase yscS; expression is induced under low-nitrogen conditions
YJL170C	ASG7	Protein that regulates signaling from a G protein beta subunit Ste4p and its relocalization within the cell; specific to a-cells and induced by alpha-factor
YKR059W	TIF1	Translation initiation factor eIF4A, identical to Tif2p; DEA(D/H)-box RNA helicase that couples ATPase activity to RNA binding and unwinding; forms a dumbbell structure of two compact domains connected by a linker; interacts with eIF4G
YJR109C	CPA2	Large subunit of carbamoyl phosphate synthetase, which catalyzes a step in the synthesis of citrulline, an arginine precursor
YKL081W	TEF4	Translation elongation factor EF-1 gamma
YKL212W	SAC1	Lipid phosphoinositide phosphatase of the ER and Golgi, involved in protein trafficking and secretion
YKL041W	VPS24	One of four subunits of the endosomal sorting complex required for transport III (ESCRT-III); forms an ESCRT-III subcomplex with Did4p; involved in the sorting of transmembrane proteins into the multivesicular body (MVB) pathway
YLR337C	VRP1	Proline-rich actin-associated protein involved in cytoskeletal organization and cytokinesis; related to mammalian Wiskott-Aldrich syndrome protein (WASP)-interacting protein (WIP)

ORF	Gene	Description
YLR039C	RIC1	Protein involved in retrograde transport to the cis-Golgi network; forms heterodimer with Rgp1p that acts as a GTP exchange factor for Ypt6p; involved in transcription of rRNA and ribosomal protein genes
YLR182W	SWI6	Transcription cofactor, forms complexes with DNA-binding proteins Swi4p and Mbp1p to regulate transcription at the G1/S transition; involved in meiotic gene expression; localization regulated by phosphorylation; potential Cdc28p substrate
YMR154C	RIM13	Calpain-like cysteine protease involved in proteolytic activation of Rim101p in response to alkaline pH; has similarity to <i>A. nidulans</i> palB
YMR201C	RAD14	Protein that recognizes and binds damaged DNA during nucleotide excision repair; subunit of Nucleotide Excision Repair Factor 1 (NEF1); contains zinc finger motif; homolog of human XPA protein
YMR223W	UBP8	Ubiquitin-specific protease that is a component of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) acetylation complex; required for SAGA-mediated deubiquitination of histone H2B
YNL257C	SIP3	Protein that activates transcription through interaction with DNA-bound Snf1p, C-terminal region has a putative leucine zipper motif; potential Cdc28p substrate
YNL255C	GIS2	Protein with seven cysteine-rich CCHC zinc-finger motifs, similar to human CNBP, proposed to be involved in the RAS/cAMP signaling pathway
YNL294C	RIM21	Component of the RIM101 pathway, has a role in cell wall construction and alkaline pH response; has similarity to <i>A. nidulans</i> PalH
YOL052C	SPE2	S-adenosylmethionine decarboxylase, required for the biosynthesis of spermidine and spermine; cells lacking Spe2p require spermine or spermidine for growth in the presence of oxygen but not when grown anaerobically

ORF	Gene	Description
YOR026W	BUB3	Kinetochore checkpoint WD40 repeat protein that localizes to kinetochores during prophase and metaphase, delays anaphase in the presence of unattached kinetochores; forms complexes with Mad1p-Bub1p and with Cdc20p, binds Mad2p and Mad3p
YOR141C	ARP8	Nuclear actin-related protein involved in chromatin remodeling, component of chromatin-remodeling enzyme complexes
YOR030W	DFG16	Probable multiple transmembrane protein, involved in diploid invasive and pseudohyphal growth upon nitrogen starvation; required for accumulation of processed Rim101p
YOR196C	LIP5	Protein involved in biosynthesis of the coenzyme lipoic acid, has similarity to E. coli lipoic acid synthase
YPL047W	SGF11	Integral subunit of SAGA histone acetyltransferase complex, regulates transcription of a subset of SAGA-regulated genes, required for the Ubp8p association with SAGA and for H2B deubiquitylation
YPL069C	BTS1	Geranylgeranyl diphosphate synthase, increases the intracellular pool of geranylgeranyl diphosphate, suppressor of bet2 mutation that causes defective geranylgeranylation of small GTP-binding proteins that mediate vesicular traffic
YPL178W	CBC2	Small subunit of the heterodimeric cap binding complex that also contains Sto1p, component of the spliceosomal commitment complex; interacts with Npl3p, possibly to package mRNA for export from the nucleus; contains an RNA-binding motif
YPL090C	RPS6A	Protein component of the small (40S) ribosomal subunit; identical to Rps6Bp and has similarity to rat S6 ribosomal protein
YOR358W	HAP5	Subunit of the heme-activated, glucose-repressed Hap2/3/4/5 CCAAT-binding complex, a transcriptional activator and global regulator of respiratory gene expression; required for assembly and

ORF	Gene	Description
		DNA binding activity of the complex
YOR275C	RIM20	Protein involved in proteolytic activation of Rim101p in response to alkaline pH; PalA/AIP1/Alix family member; interaction with the ESCRT-III subunit Snf7p suggests a relationship between pH response and multivesicular body formation
YGR122W		Probable ortholog of <i>A. nidulans</i> PalC, which is involved in pH regulation and binds to the ESCRT-III complex; null mutant does not properly process Rim101p and has decreased resistance to rapamycin; GFP-fusion protein is cytoplasmic
YGL029W	CGR1	Protein involved in nucleolar integrity and processing of the pre-rRNA for the 60S ribosome subunit; transcript is induced in response to cytotoxic stress but not genotoxic stress
YDL074C	BRE1	E3 ubiquitin ligase for Rad6p, required for the ubiquitination of histone H2B, recruitment of Rad6p to promoter chromatin and subsequent methylation of histone H3 (on K4 and K79), contains RING finger domain

5.4. Positive genetic interactions identified in *tif2* SGA

ORF	Gene	Description
YGL029W	CGR1	Protein involved in nucleolar integrity and processing of the pre-rRNA for the 60S ribosome subunit; transcript is induced in response to cytotoxic stress but not genotoxic stress
YDL074C	BRE1	E3 ubiquitin ligase for Rad6p, required for the ubiquitination of histone H2B, recruitment of Rad6p to promoter chromatin and subsequent methylation of histone H3 (on K4 and K79), contains RING finger domain

ORF	Gene	Description
YEL020C		Hypothetical protein with low sequence identity to Pdc1p
YEL016C	NPP2	Nucleotide pyrophosphatase/phosphodiesterase family member; mediates extracellular nucleotide phosphate hydrolysis along with Npp1p and Pho5p; activity and expression enhanced during conditions of phosphate starvation
YEL028W		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YEL023C		Putative protein of unknown function; expression is increased greatly during sporulation; YEL023C is not an essential gene
YEL014C		Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YPR044C	OPI11	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps verified gene RPL43A/YPR043W; deletion confers sensitivity to GSAO
YEL065W	SIT1	Ferrioxamine B transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; transcription is induced during iron deprivation and diauxic shift; potentially phosphorylated by Cdc28p
YLR200W	YKE2	Subunit of the heterohexameric Gim/prefoldin protein complex involved in the folding of alpha-tubulin, beta-tubulin, and actin

5.5. Primers

Table A-1

Lab identification number	Primer	Sequence
#26	<i>TIF1</i> 1.5 confirmation forward	GAATATACGCAGCCCTGACATTTGT
#27	<i>TIF1</i> 1.3 confirmation reverse	CCAATCTCTATTAGTAGGGCATCG
#28	<i>TIF2</i> 1.5 confirmation forward	TCATCTCAAGGAGAAGGAAACAGC
#29	<i>TIF2</i> 1.3 confirmation reverse	GAAGGGGAAAGTGGTTATGTGGCAG
#4	<i>nat</i> confirmation forward	TACGAGACGACCACGAAGC
#3	<i>nat</i> confirmation reverse	TGGAACCGCCGGCTGACC

Table A-2

Laboratory identification number	Primer	Sequence
#81	<i>Tet-tif2</i> forward	ATTCAACTAAACTACTACATAGT ATACATTAGCTGTACAGTCGTCA CATCAGACGGCCAGCTGAAGCTT CGTACGC
#82	<i>Tet-tif2</i> reverse	TGTCATAGTTGGTTTGAATTTGG GATTCTTCAATATCAGTAATACC TTCACAGACATATCCCCGAATT GATCCGGTAA
#79	<i>Tet-tif1</i> forward	AAGGTTTATCAGCAACAACATCC GATGCTTGCTGTTTATCTTTCTC ACCTCAGAGCCAGCTGAAGCTTC GTACGC
#80	<i>Tet-tif1</i> reverse	TGTCATAGTTGGTTTGAATTTGG GATTCTTCAATATCAGTAATACC TTCAGACATAT

Table A-3

Laboratory identification number	Primer	Sequence
#40	<i>TIF1 GAL</i> forward	CTTATTTTATAACAAATTCGTTA AACGGCCTCCTTCTAATTATATAC AAGATGCGTACGCTGCAGGTCGA C
#41	<i>TIF1 GAL</i> reverse	CCTTGTCATAGTTGGTTTGAATT TGGGATTCTTCAATATCAGTAAT ACCTTCAGACATCGATGAATTCT CTGTCTG
#42	<i>TIF2 GAL</i> forward	AGGACTGGTGTGTACAAGAACT AATAAATAGTAATTGCAATATGC GTACGCTGCAGGTCGAC
#43	<i>TIF2 GAL</i> reverse	AGTTGGTTTGAATTTGGGATTCT TCAATATCAGTAAATACCTTCAG ACATCGATGAATTCTCTGTCTG

Table A-4

Lab identification number	Primer	Sequence
#83	<i>MX4</i> forward	ACATGGAGGCCAGATACCC
#84	<i>MX4</i> reverse	CAGTATAGCGACCAGCATTAC

5.6. Lithium/galactose sensitive deletion strains

ORF	Gene	Description
YKL190W	<i>CNB1</i>	Calcineurin B; the regulatory subunit of calcineurin, a Ca ⁺⁺ /calmodulin-regulated type 2B protein phosphatase which regulates Crz1p (a stress-response transcription factor), the other calcineurin subunit is encoded by CNA1 and/or CMP1
YKL159C	<i>RCN1</i>	Protein involved in calcineurin regulation during calcium signaling; has similarity to H. sapiens DSCR1 which is found in the Down Syndrome candidate region
YML057W	<i>CMP2</i>	Calcineurin A; one isoform (the other is CNA1) of the catalytic subunit of calcineurin, a Ca ⁺⁺ /calmodulin-regulated protein phosphatase which regulates Crz1p (a stress-response transcription factor), the other calcineurin subunit is CNB1
YGR217W	<i>CCH1</i>	Voltage-gated high-affinity calcium channel involved in calcium influx in response to some environmental stresses as well as exposure to mating pheromones; interacts and co-localizes with Mid1p, suggesting Cch1p and Mid1p function together

ORF	Gene	Description
YLR443W	<i>ECM7</i>	Non-essential putative integral membrane protein with a role in calcium uptake; mutant has cell wall defects and Ca ⁺ uptake deficiencies; transcription is induced under conditions of zinc deficiency
YLL006W	<i>MMM1</i>	ER integral membrane protein, component of the ERMES complex that links the ER to mitochondria and may promote inter-organellar calcium and phospholipid exchange as well as coordinating mitochondrial DNA replication and growth
YJL187C	<i>SWE1</i>	Protein kinase that regulates the G2/M transition by inhibition of Cdc28p kinase activity; localizes to the nucleus and to the daughter side of the mother-bud neck; homolog of <i>S. pombe</i> Wee1p; potential Cdc28p substrate
YNL197C	<i>WHI3</i>	RNA binding protein that sequesters CLN3 mRNA in cytoplasmic foci; cytoplasmic retention factor for Cdc28p and associated cyclins; regulates cell fate and dose-dependently regulates the critical cell size required for passage through Start
YLR332W	<i>MID2</i>	O-glycosylated plasma membrane protein that acts as a sensor for cell wall integrity signaling and activates the pathway; interacts with Rom2p, a guanine nucleotide exchange factor for Rho1p, and with cell integrity pathway protein Zeo1p

ORF	Gene	Description
YIL011W	<i>TIR3</i>	Cell wall mannoprotein of the Srp1p/Tip1p family of serine-alanine-rich proteins; expressed under anaerobic conditions and required for anaerobic growth
YOL109W	<i>ZEO1</i>	Peripheral membrane protein of the plasma membrane that interacts with Mid2p; regulates the cell integrity pathway mediated by Pkc1p and Slt2p; the authentic protein is detected in a phosphorylated state in highly purified mitochondria
YOR231W	<i>MKK1</i>	Mitogen-activated kinase kinase involved in protein kinase C signaling pathway that controls cell integrity; upon activation by Bck1p phosphorylates downstream target, Slt2p; functionally redundant with Mkk2p
YHL027W	<i>RIM101</i>	Transcriptional repressor involved in response to pH and in cell wall construction; required for alkaline pH-stimulated haploid invasive growth and sporulation; activated by proteolytic processing; similar to <i>A. nidulans</i> PacC
YPL140C	<i>MKK2</i>	Mitogen-activated kinase kinase involved in protein kinase C signaling pathway that controls cell integrity; upon activation by Bck1p phosphorylates downstream target, Slt2p; functionally redundant with Mkk1p

ORF	Gene	Description
YNL233W	<i>BNI4</i>	Targeting subunit for Glc7p protein phosphatase, localized to the bud neck, required for localization of chitin synthase III to the bud neck via interaction with the chitin synthase III regulatory subunit Skt5p
YBR023C	<i>CHS3</i>	Chitin synthase III, catalyzes the transfer of N-acetylglucosamine (GlcNAc) to chitin; required for synthesis of the majority of cell wall chitin, the chitin ring during bud emergence, and spore wall chitosan
YLR330W	<i>CHS5</i>	Component of the exomer complex, which also contains Csh6p, Bch1p, Bch2p, and Bud7p and is involved in export of selected proteins, such as chitin synthase Chs3p, from the Golgi to the plasma membrane
YOR141C	<i>ARP8</i>	Nuclear actin-related protein involved in chromatin remodeling, component of chromatin-remodeling enzyme complexes; has mRNA binding activity
YER092W	<i>IES5</i>	Protein that associates with the INO80 chromatin remodeling complex under low-salt conditions
YFL049W	<i>SWP82</i>	Member of the SWI/SNF chromatin remodeling complex in which it plays an as yet unidentified role; has identifiable counterparts in closely related yeast species; abundantly expressed in many growth conditions; paralog of Npl6p

ORF	Gene	Description
YEL043W	<i>YEL043W</i>	Predicted cytoskeleton protein involved in intracellular signalling based on quantitative analysis of protein-protein interaction maps; may interact with ribosomes, based on co-purification studies; contains fibronectin type III domain fold
YDR388W	<i>RVS167</i>	Actin-associated protein with roles in endocytosis and exocytosis; interacts with Rvs161p to regulate actin cytoskeleton, endocytosis, and viability following starvation or osmotic stress; recruited to bud tips by Gyl1p and Gyp5p during polarized growth; homolog of mammalian amphiphysin
YIL017C	<i>VID28</i>	Protein involved in proteasome-dependent catabolite degradation of fructose-1,6-bisphosphatase (FBPase); localized to the nucleus and the cytoplasm
YLR373C	<i>VID22</i>	Glycosylated integral membrane protein localized to the plasma membrane; plays a role in fructose-1,6-bisphosphatase (FBPase) degradation; involved in FBPase transport from the cytosol to Vid (vacuole import and degradation) vesicles
YJL155C	<i>FBP26</i>	Fructose-2,6-bisphosphatase, required for glucose metabolism

ORF	Gene	Description
YLL043W	<i>FPS1</i>	Plasma membrane channel, member of major intrinsic protein (MIP) family; involved in efflux of glycerol and in uptake of acetic acid and the trivalent metalloids arsenite and antimonite; phosphorylated by Hog1p MAPK under acetate stress
YNL080C	<i>EOS1</i>	Protein involved in N-glycosylation; deletion mutation confers sensitivity to oxidative stress and shows synthetic lethality with mutations in the spindle checkpoint genes BUB3 and MAD1; YNL080C is not an essential gene
YJL101C	<i>GSH1</i>	Gamma glutamylcysteine synthetase catalyzes the first step in glutathione (GSH) biosynthesis; expression induced by oxidants, cadmium, and mercury
YNL307C	<i>MCK1</i>	Protein serine/threonine/tyrosine (dual-specificity) kinase involved in control of chromosome segregation and in regulating entry into meiosis; related to mammalian glycogen synthase kinases of the GSK-3 family
YKL127W	<i>PGM1</i>	Phosphoglucomutase, minor isoform; catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate, which is a key step in hexose metabolism

ORF	Gene	Description
YMR105C	<i>PGM2</i>	Phosphoglucomutase, catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate, which is a key step in hexose metabolism; functions as the acceptor for a Glc-phosphotransferase
YMR278W	<i>PGM3</i>	Phosphoglucomutase, catalyzes interconversion of glucose-1-phosphate and glucose-6-phosphate; transcription induced in response to stress; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus; non-essential
YDR359C	<i>EAF1/VID21</i>	Component of the NuA4 histone acetyltransferase complex; acts as a platform for assembly of NuA4 subunits into the native complex; required for initiation of pre-meiotic DNA replication, likely due to its requirement for expression of IME1
YJL168C	<i>SET2</i>	Histone methyltransferase with a role in transcriptional elongation, methylates a lysine residue of histone H3; associates with the C-terminal domain of Rpo21p; histone methylation activity is regulated by phosphorylation status of Rpo21p
YNL136W	<i>EAF7</i>	Subunit of the NuA4 histone acetyltransferase complex, which acetylates the N-terminal tails of histones H4 and H2A
YMR075W	<i>RCO1</i>	Essential subunit of the histone deacetylase Rpd3S complex; interacts with Eaf3p

ORF	Gene	Description
YJR040W	<i>GEF1</i>	Voltage-gated chloride channel localized to the golgi, the endosomal system, and plasma membrane, and involved in cation homeostasis; highly homologous to vertebrate voltage-gated chloride channels
YHL007C	<i>STE20</i>	Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family; involved in pheromone response, pseudohyphal/invasive growth, vacuole inheritance, down-regulation of sterol uptake; GBB motif binds Ste4p
YIL035C	<i>CKA1</i>	Alpha catalytic subunit of casein kinase 2 (CK2), a Ser/Thr protein kinase with roles in cell growth and proliferation; CK2, comprised of CKA1, CKA2, CKB1 and CKB2, has many substrates including transcription factors and all RNA polymerases
YJR049C	<i>UTR1</i>	ATP-NADH kinase; phosphorylates both NAD and NADH; active as a hexamer; enhances the activity of ferric reductase (Fre1p)
YHR030C	<i>SLT2</i>	Serine/threonine MAP kinase; involved in regulating maintenance of cell wall integrity, progression through the cell cycle, and nuclear mRNA retention in heat shock; required for mitophagy and pexophagy; affects recruitment of mitochondria to the phagophore assembly site (PAS); regulated by the PKC1-mediated signaling pathway

ORF	Gene	Description
YJL128C	<i>PBS2</i>	MAP kinase kinase of the HOG signaling pathway; activated under severe osmotic stress; mitophagy-specific regulator; plays a role in regulating Ty1 transposition
YJL095W	<i>BCK1</i>	Mitogen-activated protein (MAP) kinase kinase kinase acting in the protein kinase C signaling pathway, which controls cell integrity; upon activation by Pkc1p phosphorylates downstream kinases Mkk1p and Mkk2p
YER177W	<i>BMH1</i>	14-3-3 protein, major isoform; controls proteome at post-transcriptional level, binds proteins and DNA, involved in regulation of many processes including exocytosis, vesicle transport, Ras/MAPK signaling, and rapamycin-sensitive signaling
YDR532C	<i>KRE28\YDR532C</i>	Subunit of a kinetochore-microtubule binding complex with Spc105p that bridges centromeric heterochromatin and kinetochore MAPs and motors, and is also required for sister chromatid bi-orientation and kinetochore binding of SAC components
YML129C	<i>COX14</i>	Mitochondrial membrane protein, involved in translational regulation of Cox1p and assembly of cytochrome c oxidase (complex IV); associates with complex IV assembly intermediates and complex III/complex IV supercomplexes

ORF	Gene	Description
YJR077C	<i>MIR1</i>	Mitochondrial phosphate carrier, imports inorganic phosphate into mitochondria; functionally redundant with Pic2p but more abundant than Pic2p under normal conditions; phosphorylated
YCR028C-A	<i>RIM1</i>	Single-stranded DNA-binding protein essential for mitochondrial genome maintenance; involved in mitochondrial DNA replication
YNL052W	<i>COX5A</i>	Subunit Va of cytochrome c oxidase, which is the terminal member of the mitochondrial inner membrane electron transport chain; predominantly expressed during aerobic growth while its isoform Vb (Cox5Bp) is expressed during anaerobic growth
YIL111W	<i>COX5B</i>	Subunit Vb of cytochrome c oxidase, which is the terminal member of the mitochondrial inner membrane electron transport chain; predominantly expressed during anaerobic growth while its isoform Va (Cox5Ap) is expressed during aerobic growth
YOL095C	<i>HMI1</i>	Mitochondrial inner membrane localized ATP-dependent DNA helicase, required for the maintenance of the mitochondrial genome; not required for mitochondrial transcription; has homology to E. coli helicase uvrD
YOR136W	<i>IDH2</i>	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase, which catalyzes the oxidation of isocitrate to alpha-ketoglutarate in the TCA cycle; phosphorylated

ORF	Gene	Description
YIL125W	<i>KGD1</i>	Component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the tricarboxylic acid (TCA) cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA
YIL160C	<i>POT1</i>	3-ketoacyl-CoA thiolase with broad chain length specificity, cleaves 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA during beta-oxidation of fatty acids
YJL023C	<i>PET130</i>	Protein required for respiratory growth; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YMR189W	<i>GCV2</i>	P subunit of the mitochondrial glycine decarboxylase complex, required for the catabolism of glycine to 5,10-methylene-THF; expression is regulated by levels of 5,10-methylene-THF in the cytoplasm
YGR238C	<i>KEL2</i>	Protein that functions in a complex with Kel1p to negatively regulate mitotic exit, interacts with Tem1p and Lte1p; localizes to regions of polarized growth; potential Cdc28p substrate
YGR092W	<i>DBF2</i>	Ser/Thr kinase involved in transcription and stress response; functions as part of a network of genes in exit from mitosis; localization is cell cycle regulated; activated by Cdc15p during the exit from mitosis

ORF	Gene	Description
YEL061C	<i>CIN8</i>	Kinesin motor protein involved in mitotic spindle assembly and chromosome segregation
YBL031W	<i>SHE1</i>	Mitotic spindle protein that interacts with components of the Dam1 (DASH) complex, its effector Sli15p, and microtubule-associated protein Bim1p; also localizes to nuclear microtubules and to the bud neck in a ring-shaped structure
YDR436W	<i>PPZ2</i>	Serine/threonine protein phosphatase Z, isoform of Ppz1p; involved in regulation of potassium transport, which affects osmotic stability, cell cycle progression, and halotolerance
YLR113W	<i>HOG1</i>	Mitogen-activated protein kinase involved in osmoregulation; acts via three independent osmosensors; mitophagy-specific regulator; mediates the recruitment and activation of RNA Pol II at Hot1p-dependent promoters; localization regulated by Ptp2p and Ptp3p
YER118C	<i>SHO1</i>	Transmembrane osmosensor involved in activation of the Cdc42p- and MAP kinase-dependent filamentous growth pathway and the high-osmolarity glycerol response pathway; phosphorylated by Hog1p; interacts with Pbs2p, Msb2p, Hkr1p, and Ste11p
YCL032W	<i>STE50</i>	Protein involved in mating response, invasive/filamentous growth, and osmotolerance, acts as an adaptor that links G protein-associated Cdc42p-Ste20p complex to the effector Ste11p to modulate signal transduction

ORF	Gene	Description
YGL105W	<i>ARC1</i>	Protein that binds tRNA and methionyl- and glutamyl-tRNA synthetases (Mes1p and Gus1p), delivering tRNA to them, stimulating catalysis, and ensuring their localization to the cytoplasm; also binds quadruplex nucleic acids
YIL043C	<i>CBR1</i>	Microsomal cytochrome b reductase, not essential for viability; also detected in mitochondria; mutation in conserved NADH binding domain of the human ortholog results in type I methemoglobinemia
YIL123W	<i>SIM1</i>	Protein of the SUN family (Sim1p, Uth1p, Nca3p, Sun4p) that may participate in DNA replication, promoter contains SCB regulation box at -300 bp indicating that expression may be cell cycle-regulated
YPL157W	<i>TGS1</i>	Trimethyl guanosine synthase, conserved nucleolar methyl transferase that converts the m(7)G cap structure of snRNAs, snoRNAs, and telomerase TLC1 RNA to m(2,2,7)G; also required for nucleolar assembly and splicing of meiotic pre-mRNAs
YPL213W	<i>LEA1</i>	Component of U2 snRNP; disruption causes reduced U2 snRNP levels; physically interacts with Msl1p; putative homolog of human U2A' snRNP protein

ORF	Gene	Description
YOL081W	<i>IRA2</i>	GTPase-activating protein that negatively regulates RAS by converting it from the GTP- to the GDP-bound inactive form, required for reducing cAMP levels under nutrient limiting conditions, has similarity to Ira1p and human neurofibromin
YPL089C	<i>RLM1</i>	MADS-box transcription factor, component of the protein kinase C-mediated MAP kinase pathway involved in the maintenance of cell integrity; phosphorylated and activated by the MAP-kinase Slt2p
YDR363W-A	<i>SEM1</i>	Component of the lid subcomplex of the regulatory subunit of the 26S proteasome; involved in mRNA export mediated by the TREX-2 complex (Sac3p-Thp1p); ortholog of human DSS1
YER117W	<i>RPL23B</i>	Protein component of the large (60S) ribosomal subunit, identical to Rpl23Ap and has similarity to E. coli L14 and rat L23 ribosomal proteins
YBL027W	<i>RPL19B</i>	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl19Ap and has similarity to rat L19 ribosomal protein; rpl19a and rpl19b single null mutations result in slow growth, while the double null mutation is lethal
YKR057W	<i>RPS21A</i>	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps21Bp and has similarity to rat S21 ribosomal protein

ORF	Gene	Description
YGR089W	<i>NNF2</i>	Protein that exhibits physical and genetic interactions with Rpb8p, which is a subunit of RNA polymerases I, II, and III; computational analysis of large-scale protein-protein interaction data suggests a role in chromosome segregation
YMR037C	<i>MSN2</i>	Transcriptional activator related to Msn4p; activated in stress conditions, which results in translocation from the cytoplasm to the nucleus; binds DNA at stress response elements of responsive genes, inducing gene expression
YDR074W	<i>TPS2</i>	Phosphatase subunit of the trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose; expression is induced by stress conditions and repressed by the Ras-cAMP pathway
YDR001C	<i>NTH1</i>	Neutral trehalase, degrades trehalose; required for thermotolerance and may mediate resistance to other cellular stresses; may be phosphorylated by Cdc28p
YPR040W	<i>TIP41</i>	Protein that interacts physically and genetically with Tap42p, which regulates protein phosphatase 2A; component of the TOR (target of rapamycin) signaling pathway
YHR025W	<i>THR1</i>	Homoserine kinase, conserved protein required for threonine biosynthesis; expression is regulated by the GCN4-mediated general amino acid control pathway

ORF	Gene	Description
YHR104W	<i>GRE3</i>	Aldose reductase involved in methylglyoxal, d-xylose, arabinose, and galactose metabolism; stress induced (osmotic, ionic, oxidative, heat shock, starvation and heavy metals); regulated by the HOG pathway
YBL066C	<i>SEF1</i>	Putative transcription factor, has homolog in <i>Kluyveromyces lactis</i>
YOL089C	<i>HAL9</i>	Putative transcription factor containing a zinc finger; overexpression increases salt tolerance through increased expression of the ENA1 (Na ⁺ /Li ⁺ extrusion pump) gene while gene disruption decreases both salt tolerance and ENA1 expression
YCR009C	<i>RVS161</i>	Amphiphysin-like lipid raft protein; interacts with Rvs167p and regulates polarization of the actin cytoskeleton, endocytosis, cell polarity, cell fusion and viability following starvation or osmotic stress
YCL010C	<i>SGF29</i>	Component of the HAT/Core module of the SAGA, SLIK, and ADA complexes; HAT/Core module also contains Gcn5p, Ngg1p, and Ada2p; binds methylated histone H3K4; involved in transcriptional regulation through SAGA recruitment to target promoters and H3 acetylation

ORF	Gene	Description
YIL101C	<i>XBP1</i>	Transcriptional repressor that binds to promoter sequences of the cyclin genes, CYS3, and SMF2; expression is induced by stress or starvation during mitosis, and late in meiosis; member of the Swi4p/Mbp1p family; potential Cdc28p substrate
YMR275C	<i>BUL1</i>	Ubiquitin-binding component of the Rsp5p E3-ubiquitin ligase complex, functional homolog of Bul2p, disruption causes temperature-sensitive growth, overexpression causes missorting of amino acid permeases
YBL047C	<i>EDE1</i>	Key endocytic protein involved in a network of interactions with other endocytic proteins, binds membranes in a ubiquitin-dependent manner, may also bind ubiquitinated membrane-associated proteins
YGL141W	<i>HUL5</i>	Multiubiquitin chain assembly factor (E4); proteasome processivity factor that elongates polyUb chains on substrates, opposing Ubp6p, a branched polyubiquitin protease; required for retrograde transport of misfolded proteins during ERAD
YLR262C-A	<i>TMA7/RBF7</i>	Protein of unknown that associates with ribosomes; null mutant exhibits translation defects, altered polyribosome profiles, and resistance to the translation inhibitor anisomycin
YBL059W	<i>YBL059W</i>	Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies

ORF	Gene	Description
YGL057C	<i>GEP7</i>	Protein of unknown function; null mutant exhibits a respiratory growth defect and synthetic interactions with prohibitin (phb1) and gem1; authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YIL161W	<i>YIL161W</i>	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; mRNA is enriched in Scp160p-associated mRNPs; YIL161W is a non-essential gene
YJR054W	<i>YJR054W</i>	Vacuolar protein of unknown function; potential Cdc28p substrate
YMR003W	<i>AIM34</i>	Protein of unknown function; GFP-fusion protein localizes to the mitochondria; null mutant is viable and displays reduced frequency of mitochondrial genome loss
YMR196W	<i>YMR196W</i>	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YMR196W is not an essential gene
YNL011C	<i>YNL011C</i>	Putative protein of unknown function; YNL011C is not an essential gene

ORF	Gene	Description
YOR052C	<i>YOR052C</i>	Nuclear protein of unknown function; expression induced by nitrogen limitation in a GLN3, GAT1-independent manner and by weak acid; transcriptionally regulated by Rpn4p along with proteasome subunit genes; putative ortholog of human AIRAP, which stimulates proteasome activity in response to arsenic
YOR238W	<i>YOR238W</i>	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm
YJL201W	<i>ECM25</i>	Non-essential protein of unknown function; promoter contains a consensus binding sequence for factor Abf1p
YGL226W	<i>YGL226W</i>	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the mitochondrion; mtc3 is synthetically sick with cdc13-1
YJR120W	<i>YJR120W</i>	Protein of unknown function; essential for growth under anaerobic conditions; mutation causes decreased expression of ATP2, impaired respiration, defective sterol uptake, and altered levels/localization of ABC transporters Aus1p and Pdr11p
YHR079C	<i>IRE1</i>	Serine-threonine kinase and endoribonuclease; transmembrane protein that mediates the unfolded protein response (UPR) by regulating Hac1p synthesis through HAC1 mRNA splicing; Kar2p binds inactive Ire1p and releases from it upon ER stress

ORF	Gene	Description
YFL031W	<i>HAC1</i>	Basic leucine zipper (bZIP) transcription factor (ATF/CREB1 homolog) that regulates the unfolded protein response, via UPRE binding, and membrane biogenesis; ER stress-induced splicing pathway facilitates efficient Hac1p synthesis
YGL212W	<i>VAM7</i>	Vacuolar SNARE protein that functions with Vam3p in vacuolar protein trafficking; has an N-terminal PX domain (phosphoinositide-binding module) that binds PtdIns-3-P and mediates membrane binding; SNAP-25 homolog
YDR080W	<i>VPS41</i>	Vacuolar membrane protein that is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex; essential for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport
YDL077C	<i>VAM6</i>	Vacuolar protein that plays a critical role in the tethering steps of vacuolar membrane fusion by facilitating guanine nucleotide exchange on small guanosine triphosphatase Ypt7p

5.7. Lithium/galactose suppressor deletion strains

ORF	Gene	Description
YGL168W	<i>HUR1</i>	Protein of unknown function; reported null mutant phenotype of hydroxyurea sensitivity may be due to effects on overlapping PMR1 gene. The PMR1 gene encodes for High affinity Ca ²⁺ /Mn ²⁺ P-type ATPase required for Ca ²⁺ and Mn ²⁺ transport into Golgi; involved in Ca ²⁺ dependent protein sorting and processing; mutations in human homolog ATP2C1 cause acantholytic skin condition Hailey-Hailey disease
YGL167C	<i>PMR1</i>	High affinity Ca ²⁺ /Mn ²⁺ P-type ATPase required for Ca ²⁺ and Mn ²⁺ transport into Golgi; involved in Ca ²⁺ dependent protein sorting and processing; mutations in human homolog ATP2C1 cause acantholytic skin condition Hailey-Hailey disease
YBR020W	<i>GAL1</i>	Galactokinase, phosphorylates alpha-D-galactose to alpha-D-galactose-1-phosphate in the first step of galactose catabolism; expression regulated by Gal4p
YLR081W	<i>GAL2</i>	Galactose permease, required for utilization of galactose; also able to transport glucose
YPL248C	<i>GAL4</i>	DNA-binding transcription factor required for the activation of the GAL genes in response to galactose; repressed by Gal80p and activated by Gal3p
YML048W	<i>GSF2</i>	ER localized integral membrane protein that may promote secretion of certain hexose transporters, including Gal2p; involved in glucose-dependent repression

ORF	Gene	Description
YOR002W	<i>ALG6</i>	Alpha 1,3 glucosyltransferase, involved in transfer of oligosaccharides from dolichyl pyrophosphate to asparagine residues of proteins during N-linked protein glycosylation; mutations in human ortholog are associated with disease
YOR067C	<i>ALG8</i>	Glucosyl transferase, involved in N-linked glycosylation; adds glucose to the dolichol-linked oligosaccharide precursor prior to transfer to protein during lipid-linked oligosaccharide biosynthesis; similar to Alg6p
YGR078C	<i>PAC10</i>	Part of the heteromeric co-chaperone GimC/prefoldin complex, which promotes efficient protein folding. Protein required in the Absence of Cin8p
YNR051C	<i>BRE5</i>	Ubiquitin protease cofactor, forms deubiquitination complex with Ubp3p that coregulates anterograde and retrograde transport between the endoplasmic reticulum and Golgi compartments; null is sensitive to brefeldin A
YER151C	<i>UBP3</i>	Ubiquitin-specific protease that interacts with Bre5p to co-regulate anterograde and retrograde transport between the ER and Golgi; inhibitor of gene silencing; cleaves ubiquitin fusions but not polyubiquitin; also has mRNA binding activity
YDR092W	<i>UBC13</i>	Ubiquitin-conjugating enzyme involved in the error-free DNA postreplication repair pathway; interacts with Mms2p to assemble ubiquitin chains at the Ub Lys-63 residue; DNA damage triggers redistribution from the cytoplasm to the nucleus
YNL323W	<i>LEM3</i>	Membrane protein of the plasma membrane and ER, interacts specifically in vivo with the phospholipid translocase (flippase) Dnf1p; involved in translocation of phospholipids and alkylphosphocholine drugs across the plasma membrane

ORF	Gene	Description
YOL042W	<i>NGL1</i>	Putative endonuclease, has a domain similar to a magnesium-dependent endonuclease motif in mRNA deadenylase Ccr4p; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YNL123W	<i>NMA111</i>	Serine protease and general molecular chaperone; involved in response to heat stress and promotion of apoptosis; may contribute to lipid homeostasis; sequence similarity to the mammalian Omi/HtrA2 family of serine proteases
YAL023C	<i>PMT2</i>	Protein O-mannosyltransferase, transfers mannose residues from dolichyl phosphate-D-mannose to protein Ser/Thr residues; involved in ER quality control; acts in a complex with Pmt1p, can instead interact with Pmt5; target for new antifungal
YMR263W	<i>SAP30</i>	Subunit of a histone deacetylase complex, along with Rpd3p and Sin3p, that is involved in silencing at telomeres, rDNA, and silent mating-type loci; involved in telomere maintenance
YHR041C	<i>SRB2</i>	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; general transcription factor involved in telomere maintenance
YMR039C	<i>SUB1</i>	Transcriptional coactivator, facilitates elongation through factors that modify RNAP II; role in peroxide resistance involving Rad2p; role in the hyperosmotic stress response through polymerase recruitment at RNAP II and RNAP III genes
YPL247C	<i>YPL247C</i>	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus; similar to the petunia WD repeat protein an11; overexpression causes a cell cycle delay or arrest

ORF	Gene	Description
YBR162W-A	<i>YSY6</i>	Protein whose expression suppresses a secretory pathway mutation in <i>E. coli</i> ; has similarity to the mammalian RAMP4 protein involved in secretion
YOR304W	<i>ISW2</i>	ATP-dependent DNA translocase involved in chromatin remodeling; ATPase component that, with Itc1p, forms a complex required for repression of a-specific genes, INO1, and early meiotic genes during mitotic growth
YGL226C-A	<i>OST5</i>	Zeta subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes asparagine-linked glycosylation of newly synthesized proteins
YLR372W	<i>SUR4</i>	Elongase, involved in fatty acid and sphingolipid biosynthesis; synthesizes very long chain 20-26-carbon fatty acids from C18-CoA primers; involved in regulation of sphingolipid biosynthesis. Suppressor of Rvs161 and rvs167 mutations
YER111C	<i>SWI4</i>	DNA binding component of the SBF complex (Swi4p-Swi6p), a transcriptional activator that in concert with MBF (Mbp1-Swi6p) regulates late G1-specific transcription of targets including cyclins and genes required for DNA synthesis and repair

6. References

- (2009). Trans fatty acids are not significant in yeast. COFALEC.
- (2011). Global Yeast Industry Industrial Applications and Market Forecast (2010-2015). Markets and Markets (M&M).
- Abramson, R. D., T. E. Dever, et al. (1987). "The ATP-dependent interaction of eukaryotic initiation factors with mRNA." The Journal of Biological Chemistry **262**(8): 3826-3832.
- Achleitner, G., B. Gaigg, et al. (1999). "Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact." European Journal of Biochemistry / FEBS **264**(2): 545-553.
- Aguilar, P. S., F. Frohlich, et al. (2010). "A plasma-membrane E-MAP reveals links of the eisosome with sphingolipid metabolism and endosomal trafficking." Nature Structure Molecular Biology **17**(7): 901-908.
- Aiello, D. P., L. Fu, et al. (2002). "Intracellular glucose 1-phosphate and glucose 6-phosphate levels modulate Ca²⁺ homeostasis in *Saccharomyces cerevisiae*." The Journal of Biological Chemistry **277**(48): 45751-45758.
- Alepuz, P. M., E. de Nadal, et al. (2003). "Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II." The EMBO Journal **22**(10): 2433-2442.
- Alepuz, P. M., A. Jovanovic, et al. (2001). "Stress-induced map kinase Hog1 is part of transcription activation complexes." Molecular Cell **7**(4): 767-777.
- Alizadeh, P. and D. J. Klionsky (1996). "Purification and biochemical characterization of the ATH1 gene product, vacuolar acid trehalase, from *Saccharomyces cerevisiae*." FEBS Letters **391**(3): 273-278.
- Alper, H., J. Moxley, et al. (2006). "Engineering yeast transcription machinery for improved ethanol tolerance and production." Science **314**(5805): 1565-1568.
- Altmann, M., P. P. Muller, et al. (1993). "A *Saccharomyces cerevisiae* homologue of mammalian translation initiation factor 4B contributes to RNA helicase activity." The EMBO Journal **12**(10): 3997-4003.
- Altmann, M., N. Sonenberg, et al. (1989). "Translation in *Saccharomyces cerevisiae*: initiation factor 4E-dependent cell-free system." Molecular and Cellular Biology **9**(10): 4467-4472.
- Altmann, M., B. Wittmer, et al. (1995). "The *Saccharomyces cerevisiae* translation initiation factor Tif3 and its mammalian homologue, eIF-4B, have RNA annealing activity." The EMBO Journal **14**(15): 3820-3827.

- Alvarez, G., J. R. Munoz-Montano, et al. (1999). "Lithium protects cultured neurons against beta-amyloid-induced neurodegeneration." FEBS Letters **453**(3): 260-264.
- Amberg, D. C., D. J. Burke, et al. (2005). Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. United States, CSHL Press.
- Amerik, A. Y., J. Nowak, et al. (2000). "The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways." Molecular Biology of the Cell **11**(10): 3365-3380.
- Antebi, A. and G. R. Fink (1992). "The yeast Ca(2+)-ATPase homologue, PMR1, is required for normal Golgi function and localizes in a novel Golgi-like distribution." Molecular Biology of the Cell **3**(6): 633-654.
- Archer, C. T., A. Delahodde, et al. (2008). "Activation domain-dependent monoubiquitylation of Gal4 protein is essential for promoter binding in vivo." The Journal of Biological Chemistry **283**(18): 12614-12623.
- Ashburner, M., C. A. Ball, et al. (2000). "Gene ontology: tool for the unification of biology. The Gene Ontology Consortium." Nature genetics **25**(1): 25-29.
- Ashe, M. P., S. K. De Long, et al. (2000). "Glucose depletion rapidly inhibits translation initiation in yeast." Molecular Biology of the Cell **11**(3): 833-848.
- Atkin, A. L., L. R. Schenkman, et al. (1997). "Relationship between yeast polyribosomes and Upf proteins required for nonsense mRNA decay." The Journal of Biological Chemistry **272**(35): 22163-22172.
- Aury, J. M., O. Jaillon, et al. (2006). "Global trends of whole-genome duplications revealed by the ciliate *Paramecium tetraurelia*." Nature **444**(7116): 171-178.
- Ausubel, F. M., R. Brent, et al. (1987). Electrophoretic Separation of Proteins, Immunoblotting and Immunodetection. Current Protocols in Molecular Biology. New York, Greene Publishing Associates/Wiley Interscience.
- Babst, M. (2005). "A protein's final ESCRT." Traffic **6**(1): 2-9.
- Baggett, J. J. and B. Wendland (2001). "Clathrin function in yeast endocytosis." Traffic **2**(5): 297-302.
- Baim, S. B. and F. Sherman (1988). "mRNA structures influencing translation in the yeast *Saccharomyces cerevisiae*." Molecular and Cellular Biology **8**(4): 1591-1601.
- Baker, S. P. and P. A. Grant (2007). "The SAGA continues: expanding the cellular role of a transcriptional co-activator complex." Oncogene **26**(37): 5329-5340.

- Baudot, A., B. Jacq, et al. (2004). "A scale of functional divergence for yeast duplicated genes revealed from analysis of the protein-protein interaction network." Genome Biology **5**(10): R76.
- Beau, I., A. Esclatine, et al. (2008). "Lost to translation: when autophagy targets mature ribosomes." Trends in Cell Biology **18**(7): 311-314.
- Beck, T. and M. N. Hall (1999). "The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors." Nature **402**(6762): 689-692.
- Beese, S. E., T. Negishi, et al. (2009). "Identification of positive regulators of the yeast *fps1* glycerol channel." PLoS genetics **5**(11): e1000738.
- Beggs, J. D. (1978). "Transformation of yeast by a replicating hybrid plasmid." Nature **275**(5676): 104-109.
- Belanger, K. D., A. Gupta, et al. (2005). "Nuclear pore complex function in *Saccharomyces cerevisiae* is influenced by glycosylation of the transmembrane nucleoporin Pom152p." Genetics **171**(3): 935-947.
- Bell, W., P. Klaassen, et al. (1992). "Characterization of the 56-kDa subunit of yeast trehalose-6-phosphate synthase and cloning of its gene reveal its identity with the product of CIF1, a regulator of carbon catabolite inactivation." European Journal of Biochemistry / FEBS **209**(3): 951-959.
- Belli, G., E. Gari, et al. (1998). "An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast." Nucleic Acids Research **26**(4): 942-947.
- Berridge, M. J. and R. F. Irvine (1989). "Inositol phosphates and cell signalling." Nature **341**(6239): 197-205.
- Bertram, L. and R. E. Tanzi (2005). "The genetic epidemiology of neurodegenerative disease." The Journal of Clinical Investigation **115**(6): 1449-1457.
- Bertram, P. G., C. Zeng, et al. (1998). "The 14-3-3 proteins positively regulate rapamycin-sensitive signaling." Current Biology : CB **8**(23): 1259-1267.
- Beyer, A., J. Hollunder, et al. (2004). "Post-transcriptional expression regulation in the yeast *Saccharomyces cerevisiae* on a genomic scale." Molecular & Cellular Proteomics **3**(11): 1083-1092.
- Bhat, P. J., D. Oh, et al. (1990). "Analysis of the GAL3 signal transduction pathway activating GAL4 protein-dependent transcription in *Saccharomyces cerevisiae*." Genetics **125**(2): 281-291.
- Bian, C., C. Xu, et al. (2011). "Sgf29 binds histone H3K4me2/3 and is required for SAGA complex recruitment and histone H3 acetylation." The EMBO Journal **30**(14): 2829-2842.

- Bilodeau, P. S., S. C. Winistorfer, et al. (2003). "Vps27-Hse1 and ESCRT-I complexes cooperate to increase efficiency of sorting ubiquitinated proteins at the endosome." The Journal of Cell Biology **163**(2): 237-243.
- Bilsland, E., M. Hult, et al. (2007). "The Bre5/Ubp3 ubiquitin protease complex from budding yeast contributes to the cellular response to DNA damage." DNA Repair (Amst) **6**(10): 1471-1484.
- Bisson, L. F. and D. G. Fraenkel (1983). "Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae*." Proceedings of the National Academy of Sciences of the United States of America **80**(6): 1730-1734.
- Biswas, D., S. Takahata, et al. (2008). "A role for Chd1 and Set2 in negatively regulating DNA replication in *Saccharomyces cerevisiae*." Genetics **178**(2): 649-659.
- Biswas, D., Y. Yu, et al. (2005). "The yeast FACT complex has a role in transcriptional initiation." Molecular and Cellular Biology **25**(14): 5812-5822.
- Bjorklund, S. and C. M. Gustafsson (2005). "The yeast Mediator complex and its regulation." Trends in Biochemical Sciences **30**(5): 240-244.
- Blum, S., M. Mueller, et al. (1989). "Translation in *Saccharomyces cerevisiae*: initiation factor 4A-dependent cell-free system." Proceedings of the National Academy of Sciences of the United States of America **86**(16): 6043-6046.
- Blum, S., S. R. Schmid, et al. (1992). "ATP hydrolysis by initiation factor 4A is required for translation initiation in *Saccharomyces cerevisiae*." Proceedings of the National Academy of Sciences of the United States of America **89**(16): 7664-7668.
- Boles, E., W. Liebetrau, et al. (1994). "A family of hexosephosphate mutases in *Saccharomyces cerevisiae*." European Journal of Biochemistry / FEBS **220**(1): 83-96.
- Bon, E., P. Recordon-Navarro, et al. (2000). "A network of proteins around Rvs167p and Rvs161p, two proteins related to the yeast actin cytoskeleton." Yeast **16**(13): 1229-1241.
- Bonilla, M., K. K. Nastase, et al. (2002). "Essential role of calcineurin in response to endoplasmic reticulum stress." The EMBO Journal **21**(10): 2343-2353.
- Boone, C., H. Bussey, et al. (2007). "Exploring genetic interactions and networks with yeast." Nature Reviews. Genetics **8**(6): 437-449.
- Bowers, K., J. Lottridge, et al. (2004). "Protein-protein interactions of ESCRT complexes in the yeast *Saccharomyces cerevisiae*." Traffic **5**(3): 194-210.

- Boy-Marcotte, E., M. Perrot, et al. (1998). "Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*." Journal of Bacteriology **180**(5): 1044-1052.
- Brachmann, C. B., A. Davies, et al. (1998). "Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications." Yeast **14**(2): 115-132.
- Brewster, J. L., T. de Valoir, et al. (1993). "An osmosensing signal transduction pathway in yeast." Science **259**(5102): 1760-1763.
- Briand, J. F., F. Navarro, et al. (2001). "Partners of Rpb8p, a small subunit shared by yeast RNA polymerases I, II and III." Molecular and Cellular Biology **21**(17): 6056-6065.
- Bridges, C. B. (1922). "The origin of variation." American Naturalist **56**: 51-63.
- Briggs, S. D., T. Xiao, et al. (2002). "Gene silencing: trans-histone regulatory pathway in chromatin." Nature **418**(6897): 498.
- Brockmann, R., A. Beyer, et al. (2007). "Posttranscriptional expression regulation: what determines translation rates?" PLoS Computational Biology **3**(3): e57.
- Brodsky, J. L. and A. A. McCracken (1997). "ER-associated and proteasomemediated protein degradation: how two topologically restricted events came together." Trends in Cell Biology **7**(4): 151-156.
- Broгна, S. and J. Wen (2009). "Nonsense-mediated mRNA decay (NMD) mechanisms." Nature Structural and Molecular Biology **16**(2): 107-113.
- Brown, C. R., J. A. McCann, et al. (2002). "Vid22p, a novel plasma membrane protein, is required for the fructose-1,6-bisphosphatase degradation pathway." Journal of Cell Science **115**(Pt 3): 655-666.
- Burda, P. and M. Aebi (1998). "The ALG10 locus of *Saccharomyces cerevisiae* encodes the alpha-1,2 glucosyltransferase of the endoplasmic reticulum: the terminal glucose of the lipid-linked oligosaccharide is required for efficient N-linked glycosylation." Glycobiology **8**(5): 455-462.
- Burda, P., C. A. Jakob, et al. (1999). "Ordered assembly of the asymmetrically branched lipid-linked oligosaccharide in the endoplasmic reticulum is ensured by the substrate specificity of the individual glycosyltransferases." Glycobiology **9**(6): 617-625.
- Burke, D. J. and D. Church (1991). "Protein synthesis requirements for nuclear division, cytokinesis, and cell separation in *Saccharomyces cerevisiae*." Molecular and Cellular Biology **11**(7): 3691-3698.

- Bushell, M., M. Stoneley, et al. (2006). "Polypyrimidine tract binding protein regulates IRES-mediated gene expression during apoptosis." Molecular Cell **23**(3): 401-412.
- Cade, J. F. (1949). "Lithium salts in the treatment of psychotic excitement." Medical Journal of Australia **2**(10): 349-352.
- Calabrese, V., G. Scapagnini, et al. (2001). "Mitochondrial involvement in brain function and dysfunction: relevance to aging, neurodegenerative disorders and longevity." Neurochemical Research **26**(6): 739-764.
- Campanella, M., P. Pinton, et al. (2004). "Mitochondrial Ca²⁺ homeostasis in health and disease." Biological Research **37**(4): 653-660.
- Cavalieri, D., C. Barberio, et al. (1998). "Genetic and Molecular Diversity in *Saccharomyces cerevisiae* Natural Populations." Food Technology and Biotechnology **36**(1).
- Chalmers, S. and D. G. Nicholls (2003). "The relationship between free and total calcium concentrations in the matrix of liver and brain mitochondria." The Journal of Biological Chemistry **278**(21): 19062-19070.
- Chan, R. K. and C. A. Otte (1982). "Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a factor and alpha factor pheromones." Molecular and Cellular Biology **2**(1): 11-20.
- Chang, Y. F., J. S. Imam, et al. (2007). "The nonsense-mediated decay RNA surveillance pathway." Annual Review of Biochemistry **76**: 51-74.
- Chavan, M., A. Yan, et al. (2005). "Subunits of the translocon interact with components of the oligosaccharyl transferase complex." The Journal of Biological Chemistry **280**(24): 22917-22924.
- Chen, H. Z. and G. Zubay (1983). "Prokaryotic coupled transcription-translation." Methods in Enzymology **101**: 674-690.
- Chen, Q., Q. Ding, et al. (2005). "The stationary phase model of aging in yeast for the study of oxidative stress and age-related neurodegeneration." Biogerontology **6**(1): 1-13.
- Cherry, J. M., C. Adler, et al. (1998). "SGD: *Saccharomyces* Genome Database." Nucleic Acids Research **26**(1): 73-79.
- Chew, B. S., W. L. Siew, et al. (2010). "Transcriptional activation requires protection of the TATA-binding protein Tbp1 by the ubiquitin-specific protease Ubp3." The Biochemical Journal **431**(3): 391-399.
- Chow, C. W. and R. J. Davis (2006). "Protein kinases: chromatin-associated enzymes?" Cell **127**(5): 887-890.

- Christensen, K. A., J. T. Myers, et al. (2002). "pH-dependent regulation of lysosomal calcium in macrophages." Journal of Cell Science **115**(Pt 3): 599-607.
- Chua, G., Q. D. Morris, et al. (2006). "Identifying transcription factor functions and targets by phenotypic activation." Proceedings of the National Academy of Sciences of the United States of America **103**(32): 12045-12050.
- Chung, D. M., H. Wei, et al. (1999). "Lithium inhibits striatal damage in an animal model of Huntington's disease." Society of Neuroscience Abstracts **25**: 600.
- Cigan, A. M., L. Feng, et al. (1988). "tRNAi(met) functions in directing the scanning ribosome to the start site of translation." Science **242**(4875): 93-97.
- Cigan, A. M., E. K. Pabich, et al. (1989). "Yeast translation initiation suppressor sui2 encodes the alpha subunit of eukaryotic initiation factor 2 and shares sequence identity with the human alpha subunit." Proceedings of the National Academy of Sciences of the United States of America **86**(8): 2784-2788.
- Ciliberti, S., O. C. Martin, et al. (2007). "Robustness Can Evolve Gradually in Complex Regulatory Gene Networks with Varying Topology." PLoS Computational Biology **3**(2): e15.
- Cline, M. S., M. Smoot, et al. (2007). "Integration of biological networks and gene expression data using Cytoscape." Nature Protocols **2**(10): 2366-2382.
- Cline, M. S., M. Smoot, et al. (2007). "Integration of biological networks and gene expression data using Cytoscape." Nature Protocols **2**(10): 2366-2382.
- Cohen, M., F. Stutz, et al. (2003). "Ubp3 requires a cofactor, Bre5, to specifically de-ubiquitinate the COPII protein, Sec23." Nature Cell Biology **5**(7): 661-667.
- Collins, S. R., M. Schuldiner, et al. (2006). "A strategy for extracting and analyzing large-scale quantitative epistatic interaction data." Genome Biology **7**(7): R63.
- Colomina, N., F. Ferrezuelo, et al. (2008). "Whi3, a developmental regulator of budding yeast, binds a large set of mRNAs functionally related to the endoplasmic reticulum." The Journal of Biological Chemistry **283**(42): 28670-28679.
- Conant, G. C. and A. Wagner (2003). "Asymmetric sequence divergence of duplicate genes." Genome Research **13**(9): 2052-2058.
- Conant, G. C. and K. H. Wolfe (2006). "Functional partitioning of yeast co-expression networks after genome duplication." PLoS Biology **4**(4): e109.

- Conaway, J. W., A. Dvir, et al. (1998). "Mechanism of promoter escape by RNA polymerase II." Cold Spring Harbor Symposia on Quantitative Biology **63**: 357-364.
- Conaway, R. C. and J. W. Conaway (2009). "The INO80 chromatin remodeling complex in transcription, replication and repair." Trends in Biochemical Sciences **34**(2): 71-77.
- Coppolecchia, R., P. Buser, et al. (1993). "A new yeast translation initiation factor suppresses a mutation in the eIF-4A RNA helicase." The EMBO Journal **12**(10): 4005-4011.
- Correia, I., R. Alonso-Monge, et al. (2010). "MAPK cell-cycle regulation in *Saccharomyces cerevisiae* and *Candida albicans*." Future Microbiology **5**(7): 1125-1141.
- Costanzo, M., A. Baryshnikova, et al. (2010). "The genetic landscape of a cell." Science **327**(5964): 425-431.
- Costanzo, M. C., M. S. Skrzypek, et al. (2009). "New mutant phenotype data curation system in the *Saccharomyces* Genome Database." Database **2009**.
- Costigan, C. and M. Snyder (1994). "SLK1, a yeast homolog of MAP kinase activators, has a RAS/cAMP-independent role in nutrient sensing." Molecular & General Genetics : MGG **243**(3): 286-296.
- Cozzone, A. J. (1998). "Regulation of acetate metabolism by protein phosphorylation in enteric bacteria." Annual Review of Microbiology **52**: 127-164.
- Crespo, J. L. and M. N. Hall (2002). "Elucidating TOR signaling and rapamycin action: lessons from *Saccharomyces cerevisiae*." Microbiology and Molecular Biology Reviews **66**(4): 579-591, table of contents.
- Cruz, E., C. Whittington, et al. (2008). "A new 500 kb haplotype associated with high CD8+ T-lymphocyte numbers predicts a less severe expression of hereditary hemochromatosis." BMC Medical Genetics **9**: 97.
- Csutora, P., A. Strasz, et al. (2005). "Inhibition of phosphoglucomutase activity by lithium alters cellular calcium homeostasis and signaling in *Saccharomyces cerevisiae*." American Journal of Physiology and Cell Physiology **289**(1): C58-67.
- Cuenca-Bono, B., V. Garcia-Molinero, et al. (2010). "A novel link between Sus1 and the cytoplasmic mRNA decay machinery suggests a broad role in mRNA metabolism." BMC Cell Biology **11**: 19.
- Culotta, V. C., M. Yang, et al. (2005). "Manganese transport and trafficking: lessons learned from *Saccharomyces cerevisiae*." Eukaryotic Cell **4**(7): 1159-1165.

- Cunningham, K. W. and G. R. Fink (1994). "Ca²⁺ transport in *Saccharomyces cerevisiae*." The Journal of Experimental Biology **196**: 157-166.
- Cunningham, K. W. and G. R. Fink (1994). "Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca²⁺ ATPases." The Journal of Cell Biology **124**(3): 351-363.
- Cunningham, K. W. and G. R. Fink (1996). "Calcineurin inhibits VCX1-dependent H⁺/Ca²⁺ exchange and induces Ca²⁺ ATPases in *Saccharomyces cerevisiae*." Molecular and Cellular Biology **16**(5): 2226-2237.
- Czaplinski, K., Y. Weng, et al. (1995). "Purification and characterization of the Upf1 protein: a factor involved in translation and mRNA degradation." RNA **1**(6): 610-623.
- Dantuma, N. P., T. A. Groothuis, et al. (2006). "A dynamic ubiquitin equilibrium couples proteasomal activity to chromatin remodeling." The Journal of Cell Biology **173**(1): 19-26.
- Davis, J. C. and D. A. Petrov (2004). "Preferential duplication of conserved proteins in eukaryotic genomes." PLoS Biology **2**(3): E55.
- De Freitas, J., H. Wintz, et al. (2003). "Yeast, a model organism for iron and copper metabolism studies." BioMetals **16**(1): 185-197.
- de Jongh, W. A., C. Bro, et al. (2008). "The roles of galactitol, galactose-1-phosphate, and phosphoglucosyltransferase in galactose-induced toxicity in *Saccharomyces cerevisiae*." Biotechnology and Bioengineering **101**(2): 317-326.
- de la Cruz, J., I. Iost, et al. (1997). "The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in *Saccharomyces cerevisiae*." Proceedings of the National Academy of Sciences **94**(10): 5201-5206.
- de la Cruz, J., D. Kressler, et al. (1999). "Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families." Trends in Biochemical Sciences **24**(5): 192-198.
- De Nadal, E., M. Zapater, et al. (2004). "The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes." Nature **427**(6972): 370-374.
- De Virgilio, C., N. Burckert, et al. (1993). "Disruption of TPS2, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae*, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity." European Journal of Biochemistry / FEBS **212**(2): 315-323.
- de Vries, H. (1901). Die Mutationstheorie. Leipzig, Germany, Veit & Co.

- Deloche, O., J. de la Cruz, et al. (2004). "A membrane transport defect leads to a rapid attenuation of translation initiation in *Saccharomyces cerevisiae*." Molecular Cell **13**(3): 357-366.
- DeLuna, A., K. Vetsigian, et al. (2008). "Exposing the fitness contribution of duplicated genes." Nature Genetics **40**(5): 676-681.
- Dempski, R. E., Jr. and B. Imperiali (2002). "Oligosaccharyl transferase: gatekeeper to the secretory pathway." Current Opinion in Chemical Biology **6**(6): 844-850.
- Deng, J., H. P. Harding, et al. (2002). "Activation of GCN2 in UV-irradiated cells inhibits translation." Current Biology : CB **12**(15): 1279-1286.
- Densmore, L., W. E. Payne, et al. (1991). "In vivo genomic footprint of a yeast centromere." Molecular and Cellular Biology **11**(1): 154-165.
- Desfarges, L., P. Durrens, et al. (1993). "Yeast mutants affected in viability upon starvation have a modified phospholipid composition." Yeast **9**(3): 267-277.
- Dinant, C., A. B. Houtsmuller, et al. (2008). "Chromatin structure and DNA damage repair." Epigenetics Chromatin **1**(1): 9.
- Diriong, S., P. Lory, et al. (1995). "Chromosomal localization of the human genes for alpha 1A, alpha 1B, and alpha 1E voltage-dependent Ca²⁺ channel subunits." Genomics **30**(3): 605-609.
- Dittmar, K. and D. A. Liberles (2010). Evolution after Gene Duplication. Hoboken, NJ, Wiley-Blackwell.
- Dixon, S. J., Y. Fedyshyn, et al. (2008). "Significant conservation of synthetic lethal genetic interaction networks between distantly related eukaryotes." Proceedings of the National Academy of Sciences of the United States of America **105**(43): 16653-16658.
- Dobzhansky, T. (1946). "Genetics of natural populations; recombination and variability in populations of *Drosophila pseudoobscura*." Genetics **31**: 269-290.
- Doma, M. K. and R. Parker (2006). "Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation." Nature **440**(7083): 561-564.
- Donahue, T. F., A. M. Cigan, et al. (1988). "Mutations at a Zn(II) finger motif in the yeast eIF-2 beta gene alter ribosomal start-site selection during the scanning process." Cell **54**(5): 621-632.
- Donnelly, S. F., M. J. Pocklington, et al. (1993). "A proline-rich protein, verprolin, involved in cytoskeletal organization and cellular growth in the yeast *Saccharomyces cerevisiae*." Molecular Microbiology **10**(3): 585-596.

- Douglas, H. C. and F. Condie (1954). "The genetic control of galactose utilization in *Saccharomyces*." Journal of Bacteriology **68**(6): 662-670.
- Drubin, D. G., K. G. Miller, et al. (1988). "Yeast actin-binding proteins: evidence for a role in morphogenesis." The Journal of Cell Biology **107**(6 Pt 2): 2551-2561.
- Ernst, J. F. and L. H. Richman (1989). "Screening of Muteins Secreted by Yeast: Random Mutagenesis of Human Interleukin-2." Nature Biotechnology **7**: 716-720.
- Erpapazoglou, Z., M. Froissard, et al. (2008). "Substrate- and ubiquitin-dependent trafficking of the yeast siderophore transporter Sit1." Traffic **9**(8): 1372-1391.
- Estruch, F. (2000). "Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast." FEMS Microbiology Reviews **24**(4): 469-486.
- Estruch, F. and M. Carlson (1993). "Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*." Molecular and Cellular Biology **13**(7): 3872-3881.
- Fang, M., Z. Shen, et al. (2010). "The ER UDPase ENTPD5 promotes protein N-glycosylation, the Warburg effect, and proliferation in the PTEN pathway." Cell **143**(5): 711-724.
- Fasolato, C., M. Zottini, et al. (1991). "Intracellular Ca²⁺ pools in PC12 cells. Three intracellular pools are distinguished by their turnover and mechanisms of Ca²⁺ accumulation, storage, and release." The Journal of Biological Chemistry **266**(30): 20159-20167.
- Fendt, S. M. and U. Sauer (2010). "Transcriptional regulation of respiration in yeast metabolizing differently repressive carbon substrates." BMC Systems Biology **4**: 12.
- Fernandez-Sarabia, M. J., A. Sutton, et al. (1992). "SIT4 protein phosphatase is required for the normal accumulation of SWI4, CLN1, CLN2, and HCS26 RNAs during late G1." Genes & Development **6**(12A): 2417-2428.
- Fernandez, J., I. Yaman, et al. (2001). "Internal ribosome entry site-mediated translation of a mammalian mRNA is regulated by amino acid availability." The Journal of Biological Chemistry **276**(15): 12285-12291.
- Ferraiuolo, M. A., C. S. Lee, et al. (2004). "A nuclear translation-like factor eIF4AIII is recruited to the mRNA during splicing and functions in nonsense-mediated decay." Proceedings of the National Academy of Sciences of the United States of America **101**(12): 4118-4123.

- Fiedler, D., H. Braberg, et al. (2009). "Functional organization of the *S. cerevisiae* phosphorylation network." Cell **136**(5): 952-963.
- Fischer, E. and U. Sauer (2003). "Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS." European Journal of Biochemistry / FEBS **270**(5): 880-891.
- Fisher, R. A. (1935). "The sheltering of lethals." The American Naturalist **69**: 445-446.
- Fisk, D. G., C. A. Ball, et al. (2006). "Saccharomyces cerevisiae S288C genome annotation: a working hypothesis." Yeast **23**(12): 857-865.
- Fitzpatrick, D. A., M. E. Logue, et al. (2006). "A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis." BMC Evolutionary Biology **6**: 99.
- Flintoft, L. (2007). "Duplicates provide only limited cover." Nature Reviews. Genetics **8**(5): 328-328.
- Force, A., M. Lynch, et al. (1999). "Preservation of duplicate genes by complementary, degenerative mutations." Genetics **151**(4): 1531-1545.
- Forsburg, S. L. (2001). "The art and design of genetic screens: yeast." Nature Reviews. Genetics **2**(9): 659-668.
- Francois, J. and J. L. Parrou (2001). "Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*." FEMS Microbiology Reviews **25**(1): 125-145.
- Friedman, R. and A. L. Hughes (2003). "The temporal distribution of gene duplication events in a set of highly conserved human gene families." Molecular Biology and Evolution **20**(1): 154-161.
- Fu, L., A. Miseta, et al. (2000). "Loss of the major isoform of phosphoglucomutase results in altered calcium homeostasis in *Saccharomyces cerevisiae*." The Journal of Biological Chemistry **275**(8): 5431-5440.
- Fuchs, B. B. and E. Mylonakis (2009). "Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways." Eukaryotic Cell **8**(11): 1616-1625.
- Galione, A. and G. C. Churchill (2002). "Interactions between calcium release pathways: multiple messengers and multiple stores." Cell Calcium **32**(5-6): 343-354.
- Gancedo, C. and C. L. Flores (2004). "The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi." FEMS Yeast Research **4**(4-5): 351-359.

- Gasch, A. P., P. T. Spellman, et al. (2000). "Genomic expression programs in the response of yeast cells to environmental changes." Molecular Biology of the Cell **11**(12): 4241-4257.
- Gavin, A. C., P. Aloy, et al. (2006). "Proteome survey reveals modularity of the yeast cell machinery." Nature **440**(7084): 631-636.
- Gavin, A. C., M. Bosche, et al. (2002). "Functional organization of the yeast proteome by systematic analysis of protein complexes." Nature **415**(6868): 141-147.
- Giachetti, E., G. Pinzauti, et al. (1988). "Isocitrate lyase from Pinus pinea. Characterization of its true substrate and the action of magnesium ions." European Journal of Biochemistry / FEBS **172**(1): 85-91.
- Giaever, G., A. M. Chu, et al. (2002). "Functional profiling of the Saccharomyces cerevisiae genome." Nature **418**(6896): 387-391.
- Gietz, R. D. and R. H. Schiestl (2007). "High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method." Nature Protocols **2**(1): 31-34.
- Gingras, A. C., S. P. Gygi, et al. (1999). "Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism." Genes & Development **13**(11): 1422-1437.
- Gingras, A. C., B. Raught, et al. (1999). "eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation." Annual Review of Biochemistry **68**: 913-963.
- Glickman, M. H. and A. Ciechanover (2002). "The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction." Physical Review **82**(2): 373-428.
- Glinsky, G. V. (2005). "Death-from-cancer signatures and stem cell contribution to metastatic cancer." Cell Cycle **4**(9): 1171-1175.
- Goffeau, A., B. G. Barrell, et al. (1996). "Life with 6000 genes." Science **274**(5287): 546, 563-547.
- Goldstein, A. L., X. Pan, et al. (1999). "Heterologous URA3MX cassettes for gene replacement in Saccharomyces cerevisiae." Yeast **15**(6): 507-511.
- Gorner, W., E. Durchschlag, et al. (1998). "Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity." Genes & Development **12**(4): 586-597.
- Green, R., G. Lesage, et al. (2003). "A synthetic analysis of the Saccharomyces cerevisiae stress sensor Mid2p, and identification of a Mid2p-interacting protein, Zeo1p, that modulates the PKC1-MPK1 cell integrity pathway." Microbiology **149**(Pt 9): 2487-2499.

- Gregory, T. R. (2005). The Evolution of the Genome. Burlington, MA, Elsevier Academic.
- Grifo, J. A., S. M. Tahara, et al. (1982). "Characterization of eukaryotic initiation factor 4A, a protein involved in ATP-dependent binding of globin mRNA." The Journal of Biological Chemistry **257**(9): 5246-5252.
- Griggs, D. W. and M. Johnston (1991). "Regulated expression of the GAL4 activator gene in yeast provides a sensitive genetic switch for glucose repression." Proceedings of the National Academy of Sciences of the United States of America **88**(19): 8597-8601.
- Groothuis, T. A., N. P. Dantuma, et al. (2006). "Ubiquitin crosstalk connecting cellular processes." Cell Division **1**: 21.
- Gross, S. R. and T. G. Kinzy (2007). "Improper organization of the actin cytoskeleton affects protein synthesis at initiation." Molecular and Cellular Biology **27**(5): 1974-1989.
- Gruenberg, J. and H. Stenmark (2004). "The biogenesis of multivesicular endosomes." Nature Reviews. Molecular Cell Biology **5**(4): 317-323.
- Gu, X. (2003). "Evolution of duplicate genes versus genetic robustness against null mutations." Trends in Genetics : TIG **19**(7): 354-356.
- Gu, X., Z. Zhang, et al. (2005). "Rapid evolution of expression and regulatory divergences after yeast gene duplication." Proceedings of the National Academy of Sciences of the United States of America **102**(3): 707-712.
- Guerini, D. and C. B. Klee (1989). "Cloning of human calcineurin A: evidence for two isozymes and identification of a polyproline structural domain." Proceedings of the National Academy of Sciences of the United States of America **86**(23): 9183-9187.
- Guglielmi, B., N. L. van Berkum, et al. (2004). "A high resolution protein interaction map of the yeast Mediator complex." Nucleic Acids Research **32**(18): 5379-5391.
- Gustin, M. C., J. Albertyn, et al. (1998). "MAP kinase pathways in the yeast *Saccharomyces cerevisiae*." Microbiology and Molecular Biology Reviews **62**(4): 1264-1300.
- Gwizdek, C., N. Iglesias, et al. (2006). "Ubiquitin-associated domain of Mex67 synchronizes recruitment of the mRNA export machinery with transcription." Proceedings of the National Academy of Sciences of the United States of America **103**(44): 16376-16381.
- Ha, S. J., Q. Wei, et al. (2011). "Cofermentation of cellobiose and galactose by an engineered *Saccharomyces cerevisiae* strain." Applied and Environmental Microbiology **77**(16): 5822-5825.

- Haglund, K. and H. Stenmark (2006). "Working out coupled monoubiquitination." Nature Cell Biology **8**(11): 1218-1219.
- Halachmi, D. and Y. Eilam (1989). "Cytosolic and vacuolar Ca²⁺ concentrations in yeast cells measured with the Ca²⁺-sensitive fluorescence dye indo-1." FEBS Letters **256**(1-2): 55-61.
- Halbeisen, R. E., A. Galgano, et al. (2008). "Post-transcriptional gene regulation: from genome-wide studies to principles." Cellular and Molecular Life Sciences **65**(5): 798-813.
- Haldane, J. B. S. (1932). The Causes of Evolution. London, New York [etc.], Longmans, Green and co.
- Haldane, J. B. S. (1957). "The cost of natural selection." Journal of Genetics **55**: 511-524.
- Hallcher, L. M. and W. R. Sherman (1980). "The effects of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain." The Journal of Biological Chemistry **255**(22): 10896-10901.
- Hamel, P., Y. Saint-Georges, et al. (2004). "Redundancy in the function of mitochondrial phosphate transport in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*." Molecular Microbiology **51**(2): 307-317.
- Hanawalt, P. C. (2003). "Four decades of DNA repair: from early insights to current perspectives." Biochimie **85**(11): 1043-1052.
- Harbison, C. T., D. B. Gordon, et al. (2004). "Transcriptional regulatory code of a eukaryotic genome." Nature **431**(7004): 99-104.
- Hardy, C. F., O. Dryga, et al. (1997). "mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p." Proceedings of the National Academy of Sciences of the United States of America **94**(7): 3151-3155.
- Harel-Sharvit, L., N. Eldad, et al. (2010). "RNA polymerase II subunits link transcription and mRNA decay to translation." Cell **143**(4): 552-563.
- Harris, M. A., J. Clark, et al. (2004). "The Gene Ontology (GO) database and informatics resource." Nucleic Acids Research **32**(Database issue): D258-261.
- Harrison, J. C. and J. E. Haber (2006). "Surviving the breakup: the DNA damage checkpoint." Annual Review of Genetics **40**: 209-235.
- Harrison, R., B. Papp, et al. (2007). "Plasticity of genetic interactions in metabolic networks of yeast." Proceedings of the National Academy of Sciences of the United States of America **104**(7): 2307-2312.
- Hartman, J. L. t., B. Garvik, et al. (2001). "Principles for the buffering of genetic variation." Science **291**(5506): 1001-1004.

- Hartwell, L. H. (2002). "Nobel Lecture. Yeast and cancer." Bioscience Reports **22**(3-4): 373-394.
- Hartwell, L. H. (2004). "Yeast and cancer." Bioscience Reports **24**(4-5): 523-544.
- Hartwell, L. H., J. Culotti, et al. (1970). "Genetic control of the cell-division cycle in yeast. I. Detection of mutants." Proceedings of the National Academy of Sciences of the United States of America **66**(2): 352-359.
- Harwood, A. J., S. E. Plyte, et al. (1995). "Glycogen synthase kinase 3 regulates cell fate in Dictyostelium." Cell **80**(1): 139-148.
- He, X., J. P. Saint-Jeannet, et al. (1995). "Glycogen synthase kinase-3 and dorsoventral patterning in Xenopus embryos." Nature **374**(6523): 617-622.
- He, X. and J. Zhang (2005). "Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution." Genetics **169**(2): 1157-1164.
- He, X. and J. Zhang (2006). "Higher duplicability of less important genes in yeast genomes." Molecular Biology and Evolution **23**(1): 144-151.
- Hedges, S. B. (2002). "The origin and evolution of model organisms." Nature Reviews. Genetics **3**(11): 838-849.
- Helliwell, S. B., A. Schmidt, et al. (1998). "The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton." Current Biology : **CB** **8**(22): 1211-1214.
- Hellsten, U., M. K. Khokha, et al. (2007). "Accelerated gene evolution and subfunctionalization in the pseudotetraploid frog *Xenopus laevis*." BMC Biology **5**: 31.
- Henar Valdivieso, M., A. Duran, et al. (1999). "Chitin synthases in yeast and fungi." International Journal of Exercise Science **87**: 55-69.
- Henry, K. W., A. Wyce, et al. (2003). "Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8." Genes & Development **17**(21): 2648-2663.
- Hernandez, G., M. Altmann, et al. (2005). "Functional analysis of seven genes encoding eight translation initiation factor 4E (eIF4E) isoforms in *Drosophila*." Mechanisms of Development **122**(4): 529-543.
- Hernandez, G. and P. Vazquez-Pianzola (2005). "Functional diversity of the eukaryotic translation initiation factors belonging to eIF4 families." Mechanisms of Development **122**(7-8): 865-876.

- Herrero, P., R. Fernandez, et al. (1985). "Differential sensitivities to glucose and galactose repression of gluconeogenic and respiratory enzymes from *Saccharomyces cerevisiae*." Archives of Microbiology **143**(3): 216-219.
- Herscovics, A. and P. Orlean (1993). "Glycoprotein biosynthesis in yeast." Journal of the Federation of American Societies for Experimental Biology **7**(6): 540-550.
- Hershko, A., E. Eytan, et al. (1982). "Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. Relationship to the breakdown of abnormal proteins." The Journal of Biological Chemistry **257**(23): 13964-13970.
- Herz, H. M. and A. Bergmann (2009). "Genetic analysis of ESCRT function in *Drosophila*: a tumour model for human Tsg101." Biochemical Society Transaction **37**(Pt 1): 204-207.
- Herz, H. M., S. E. Woodfield, et al. (2009). "Common and distinct genetic properties of ESCRT-II components in *Drosophila*." PLoS One **4**(1): e4165.
- Hicke, L. and R. Dunn (2003). "Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins." Annual Review of Cell and Developmental Biology **19**: 141-172.
- Hilbert, M., F. Kebbel, et al. (2011). "eIF4G stimulates the activity of the DEAD box protein eIF4A by a conformational guidance mechanism." Nucleic Acids Research **39**(6): 2260-2270.
- Hillenmeyer, M. E., E. Fung, et al. (2008). "The chemical genomic portrait of yeast: uncovering a phenotype for all genes." Science **320**(5874): 362-365.
- Hinnebusch, A. G. (1997). "Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome." The Journal of Biological Chemistry **272**(35): 21661-21664.
- Hinnebusch, A. G. (2005). "Translational regulation of GCN4 and the general amino acid control of yeast." Annual Review Microbiology **59**: 407-450.
- Hinnebusch, A. G. and S. W. Liebman (1991). 11 Protein Synthesis and Translational Control in *Saccharomyces cerevisiae*.
- Hinnebusch, A. G. and K. Natarajan (2002). "Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress." Eukaryotic Cell **1**(1): 22-32.
- Hirata, Y., T. Andoh, et al. (2003). "Yeast glycogen synthase kinase-3 activates Msn2p-dependent transcription of stress responsive genes." Molecular Biology of the Cell **14**(1): 302-312.
- Hittinger, C. T. and S. B. Carroll (2007). "Gene duplication and the adaptive evolution of a classic genetic switch." Nature **449**(7163): 677-681.

- Hofmann, K. and L. Falquet (2001). "A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems." Trends in Biochemical Sciences **26**(6): 347-350.
- Hohmann, S., W. Bell, et al. (1996). "Evidence for trehalose-6-phosphate-dependent and -independent mechanisms in the control of sugar influx into yeast glycolysis." Molecular Microbiology **20**(5): 981-991.
- Holden, H. M., J. B. Thoden, et al. (2004). "Galactokinase: structure, function and role in type II galactosemia." Cellular and Molecular Life Sciences **61**(19-20): 2471-2484.
- Horak, J. and D. H. Wolf (1997). "Catabolite inactivation of the galactose transporter in the yeast *Saccharomyces cerevisiae*: ubiquitination, endocytosis, and degradation in the vacuole." Journal of Bacteriology **179**(5): 1541-1549.
- Howe, J. G. and J. W. Hershey (1984). "Translational initiation factor and ribosome association with the cytoskeletal framework fraction from HeLa cells." Cell **37**(1): 85-93.
- Hroudova, J. and Z. Fisar (2011). "Connectivity between mitochondrial functions and psychiatric disorders." Psychiatry Clinical Neuroscience **65**(2): 130-141.
- Hu, Z., J. M. Bonifas, et al. (2000). "Mutations in ATP2C1, encoding a calcium pump, cause Hailey-Hailey disease." Nature Genetics **24**(1): 61-65.
- Huang, P. H. and H. L. Chiang (1997). "Identification of novel vesicles in the cytosol to vacuole protein degradation pathway." The Journal of Cell Biology **136**(4): 803-810.
- Hughes, A. L. (1994). "The evolution of functionally novel proteins after gene duplication." Proceedings. Biological Sciences / The Royal Society **256**(1346): 119-124.
- Hughes, A. L. and R. Friedman (2007). "Sharing of transcription factors after gene duplication in the yeast *Saccharomyces cerevisiae*." Genetica **129**(3): 301-308.
- Huh, W. K., J. V. Falvo, et al. (2003). "Global analysis of protein localization in budding yeast." Nature **425**(6959): 686-691.
- Hung, G. C., C. R. Brown, et al. (2004). "Degradation of the gluconeogenic enzymes fructose-1,6-bisphosphatase and malate dehydrogenase is mediated by distinct proteolytic pathways and signaling events." The Journal of Biological Chemistry **279**(47): 49138-49150.
- Hurley, J. H. (2010). "The ESCRT complexes." Critical Reviews in Biochemistry and Molecular Biology **45**(6): 463-487.

- Hutzler, F., R. Gerstl, et al. (2008). "Protein N-glycosylation determines functionality of the *Saccharomyces cerevisiae* cell wall integrity sensor Mid2p." Molecular Microbiology **68**(6): 1438-1449.
- Ihmels, J., S. R. Collins, et al. (2007). "Backup without redundancy: genetic interactions reveal the cost of duplicate gene loss." Molecular Systems Biology **3**: 86.
- Iida, H., Y. Yagawa, et al. (1990). "Essential role for induced Ca²⁺ influx followed by [Ca²⁺]_i rise in maintaining viability of yeast cells late in the mating pheromone response pathway. A study of [Ca²⁺]_i in single *Saccharomyces cerevisiae* cells with imaging of fura-2." The Journal of Biological Chemistry **265**(22): 13391-13399.
- Ingvarsdottir, K., N. J. Krogan, et al. (2005). "H2B ubiquitin protease Ubp8 and Sgf11 constitute a discrete functional module within the *Saccharomyces cerevisiae* SAGA complex." Molecular and Cellular Biology **25**(3): 1162-1172.
- Inhorn, R. C. and P. W. Majerus (1988). "Properties of inositol polyphosphate 1-phosphatase." The Journal of Biological Chemistry **263**(28): 14559-14565.
- Ito, H., Y. Fukuda, et al. (1983). "Transformation of intact yeast cells treated with alkali cations." Journal of Bacteriology **153**(1): 163-168.
- Jackson, R. J., C. U. Hellen, et al. (2010). "The mechanism of eukaryotic translation initiation and principles of its regulation." Nature Reviews. Molecular Cell Biology **11**(2): 113-127.
- Jacq, C., J. R. Miller, et al. (1977). "A pseudogene structure in 5S DNA of *Xenopus laevis*." Cell **12**(1): 109-120.
- Jacquet, M., G. Renault, et al. (2003). "Oscillatory nucleocytoplasmic shuttling of the general stress response transcriptional activators Msn2 and Msn4 in *Saccharomyces cerevisiae*." The Journal of Cell Biology **161**(3): 497-505.
- James, T. Y., F. Kauff, et al. (2006). "Reconstructing the early evolution of Fungi using a six-gene phylogeny." Nature **443**(7113): 818-822.
- Janke, C., M. M. Magiera, et al. (2004). "A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes." Yeast **21**(11): 947-962.
- Jasnos, L. and R. Korona (2007). "Epistatic buffering of fitness loss in yeast double deletion strains." Nature Genetics **39**(4): 550-554.
- Jensen, R. A. (1976). "Enzyme recruitment in evolution of new function." Annual Review of Microbiology **30**: 409-425.

- Jensen, R. A. and G. S. Byng (1981). "The partitioning of biochemical pathways with isozyme systems." Isozymes Current Topics in Biological Medical Research **5**: 143-174.
- Jeong, H., S. P. Mason, et al. (2001). "Lethality and centrality in protein networks." Nature **411**(6833): 41-42.
- Jianzhi, Z. (2003). "Evolution by gene duplication: an update." Trends in Ecology & Evolution **18**(6): 292-298.
- Job, C., J. M. Soulie, et al. (1988). "Kinetic co-operativity of wheat-germ RNA polymerase II with adenosine 5'-[beta gamma-imido]triphosphate as substrate." The Biochemical Journal **252**(1): 55-63.
- Johansson, M. J., F. He, et al. (2007). "Association of yeast Upf1p with direct substrates of the NMD pathway." Proceedings of the National Academy of Sciences of the United States of America **104**(52): 20872-20877.
- Johnson, K. A., L. Chen, et al. (2001). "Crystal structure and catalytic mechanism of the MJ0109 gene product: a bifunctional enzyme with inositol monophosphatase and fructose 1,6-bisphosphatase activities." Biochemistry **40**(3): 618-630.
- Johnston, M. (1987). "A model fungal gene regulatory mechanism: the GAL genes of *Saccharomyces cerevisiae*." Microbiological Reviews **51**(4): 458-476.
- Jordan, I. K., Y. I. Wolf, et al. (2004). "Duplicated genes evolve slower than singletons despite the initial rate increase." BMC Evolutionary Biology **4**: 22.
- Jouaville, L. S., P. Pinton, et al. (1999). "Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming." Proceedings of the National Academy of Sciences of the United States of America **96**(24): 13807-13812.
- Kaessmann, H., N. Vinckenbosch, et al. (2009). "RNA-based gene duplication: mechanistic and evolutionary insights." Nature Reviews. Genetics **10**(1): 19-31.
- Kafri, R., A. Bar-Even, et al. (2005). "Transcription control reprogramming in genetic backup circuits." Nature Genetics **37**(3): 295-299.
- Kamath, R. S., A. G. Fraser, et al. (2003). "Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi." Nature **421**(6920): 231-237.
- Kao, C. F., C. Hillyer, et al. (2004). "Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B." Genes & Development **18**(2): 184-195.
- Karaoglu, D., D. J. Kelleher, et al. (1995). "Functional characterization of Ost3p. Loss of the 34-kD subunit of the *Saccharomyces cerevisiae*

- oligosaccharyltransferase results in biased underglycosylation of acceptor substrates." The Journal of Cell Biology **130**(3): 567-577.
- Katzmann, D. J., G. Odorizzi, et al. (2002). "Receptor downregulation and multivesicular-body sorting." Nature Reviews. Molecular Cell Biology **3**(12): 893-905.
- Keene, J. D. (2001). "Ribonucleoprotein infrastructure regulating the flow of genetic information between the genome and the proteome." Proceedings of the National Academy of Sciences of the United States of America **98**(13): 7018-7024.
- Kellermayer, R., D. P. Aiello, et al. (2003). "Extracellular Ca(2+) sensing contributes to excess Ca(2+) accumulation and vacuolar fragmentation in a pmr1Delta mutant of *S. cerevisiae*." Journal of Cell Science **116**(Pt 8): 1637-1646.
- Kelley, R. and T. Ideker (2005). "Systematic interpretation of genetic interactions using protein networks." Nature Biotechnology **23**(5): 561-566.
- Kellis, M., B. W. Birren, et al. (2004). "Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*." Nature **428**(6983): 617-624.
- Kimura, M. (1983). The Neutral Theory of Molecular Evolution. Cambridge Cambridgeshire ; New York, Cambridge University Press.
- Kimura, M. (1986). "DNA and the neutral theory." Philosophical transactions of the Royal Society of London. Series B, Biological sciences **312**(1154): 343-354.
- Kingsbury, T. J. and K. W. Cunningham (2000). "A conserved family of calcineurin regulators." Genes & Development **14**(13): 1595-1604.
- Kirschner, M. and J. Gerhart (1998). "Evolvability." Proceedings of the National Academy of Sciences of the United States of America **95**(15): 8420-8427.
- Kitano, H. (2004). "Biological robustness." Nature Reviews. Genetics **5**(11): 826-837.
- Klein, P. S. and D. A. Melton (1996). "A molecular mechanism for the effect of lithium on development." Proceedings of the National Academy of Sciences of the United States of America **93**(16): 8455-8459.
- Kline, N. S. (1969). Depression: its diagnosis and treatment. Lithium: the history of its use in psychiatry. Basel, New York,, S. Karger.
- Knauer, R. and L. Lehle (1999). "The oligosaccharyltransferase complex from *Saccharomyces cerevisiae*. Isolation of the OST6 gene, its synthetic interaction with OST3, and analysis of the native complex." The Journal of Biological Chemistry **274**(24): 17249-17256.

- Kobayashi, K., S. D. Ehrlich, et al. (2003). "Essential *Bacillus subtilis* genes." Proceedings of the National Academy of Sciences of the United States of America **100**(8): 4678-4683.
- Kolkman, A., M. M. Olsthoorn, et al. (2005). "Comparative proteome analysis of *Saccharomyces cerevisiae* grown in chemostat cultures limited for glucose or ethanol." Molecular & Cellular Proteomics **4**(1): 1-11.
- Kolling, R. and C. P. Hollenberg (1994). "The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquitinated form in endocytosis mutants." The EMBO Journal **13**(14): 3261-3271.
- Kondziela, J. R., M. W. Kaufmann, et al. (1985). "Diabetic ketoacidosis associated with lithium: case report." Journal of Clinical Psychiatry **46**(11): 492-493.
- Konradi, C., M. Eaton, et al. (2004). "Molecular evidence for mitochondrial dysfunction in bipolar disorder." Archives of General Psychiatry **61**(3): 300-308.
- Kopeck, K. O., V. Alva, et al. (2010). "Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria." Bioinformatics **26**(16): 1927-1931.
- Kopp, M., H. Muller, et al. (1993). "Molecular analysis of the neutral trehalase gene from *Saccharomyces cerevisiae*." The Journal of Biological Chemistry **268**(7): 4766-4774.
- Korneeva, N. L., B. J. Lamphear, et al. (2001). "Characterization of the two eIF4A-binding sites on human eIF4G-1." The Journal of Biological Chemistry **276**(4): 2872-2879.
- Kornmann, B., E. Currie, et al. (2009). "An ER-mitochondria tethering complex revealed by a synthetic biology screen." Science **325**(5939): 477-481.
- Kosman, D. J. (2003). "Molecular mechanisms of iron uptake in fungi." Molecular Microbiology **47**(5): 1185-1197.
- Koutelou, E., C. L. Hirsch, et al. (2010). "Multiple faces of the SAGA complex." Current Opinion in Cell Biology **22**(3): 374-382.
- Kozubowski, L., J. R. Larson, et al. (2005). "Role of the septin ring in the asymmetric localization of proteins at the mother-bud neck in *Saccharomyces cerevisiae*." Molecular Biology of the Cell **16**(8): 3455-3466.
- Kraus, P. R. and J. Heitman (2003). "Coping with stress: calmodulin and calcineurin in model and pathogenic fungi." Biochemical and Biophysical Research Communications **311**(4): 1151-1157.

- Kressler, D., J. de la Cruz, et al. (1997). "Fal1p is an essential DEAD-box protein involved in 40S-ribosomal-subunit biogenesis in *Saccharomyces cerevisiae*." Molecular and Cellular Biology **17**(12): 7283-7294.
- Krogan, N. J., M. Kim, et al. (2002). "RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: a targeted proteomics approach." Molecular and Cellular Biology **22**(20): 6979-6992.
- Kuepfer, L., U. Sauer, et al. (2005). "Metabolic functions of duplicate genes in *Saccharomyces cerevisiae*." Genome Research **15**(10): 1421-1430.
- Kuhn, K. M., J. L. DeRisi, et al. (2001). "Global and specific translational regulation in the genomic response of *Saccharomyces cerevisiae* to a rapid transfer from a fermentable to a nonfermentable carbon source." Molecular and Cellular Biology **21**(3): 916-927.
- Kurabe, N., K. Katagiri, et al. (2007). "Deregulated expression of a novel component of TFTC/STAGA histone acetyltransferase complexes, rat SGF29, in hepatocellular carcinoma: possible implication for the oncogenic potential of c-Myc." Oncogene **26**(38): 5626-5634.
- Kurtzman, C. P. and C. J. Robnett (2003). "Phylogenetic relationships among yeasts of the 'Saccharomyces complex' determined from multigene sequence analyses." FEMS Yeast Research **3**(4): 417-432.
- Kuwada, Y. (1911). "Meiosis in the pollen mother cells of *Zea Mays L.*" The Botanical Magazine **25**: 163.
- Lagunas, R., C. DeJuan, et al. (1986). "Inhibition of biosynthesis of *Saccharomyces cerevisiae* sugar transport system by tunicamycin." Journal of Bacteriology **168**(3): 1484-1486.
- Lamb, T. M., W. Xu, et al. (2001). "Alkaline response genes of *Saccharomyces cerevisiae* and their relationship to the RIM101 pathway." The Journal of Biological Chemistry **276**(3): 1850-1856.
- Lau, K. S., E. A. Partridge, et al. (2007). "Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation." Cell **129**(1): 123-134.
- Layfield, R., J. Lowe, et al. (2005). "The ubiquitin-proteasome system and neurodegenerative disorders." Essays Biochem **41**: 157-171.
- Lee, C. M., J. Sedman, et al. (1999). "The DNA helicase, Hmi1p, is transported into mitochondria by a C-terminal cleavable targeting signal." The Journal of Biological Chemistry **274**(30): 20937-20942.
- Lee, K. K., L. Florens, et al. (2005). "The deubiquitylation activity of Ubp8 is dependent upon Sgf11 and its association with the SAGA complex." Molecular and Cellular Biology **25**(3): 1173-1182.

- Lee, K. K., M. E. Sardi, et al. (2011). "Combinatorial depletion analysis to assemble the network architecture of the SAGA and ADA chromatin remodeling complexes." Molecular Systems Biology **7**: 503.
- Lee, M., S. Chatterjee, et al. (2000). "Genetic analysis of the role of Pol II holoenzyme components in repression by the Cyc8-Tup1 corepressor in yeast." Genetics **155**(4): 1535-1542.
- Lee, T. I., N. J. Rinaldi, et al. (2002). "Transcriptional regulatory networks in *Saccharomyces cerevisiae*." Science **298**(5594): 799-804.
- Lehner, B., J. Tischler, et al. (2006). "RNAi screens in *Caenorhabditis elegans* in a 96-well liquid format and their application to the systematic identification of genetic interactions." Nature Protocols **1**(3): 1617-1620.
- Lesne, A. (2008). "Robustness: confronting lessons from physics and biology." Biological Reviews of the Cambridge Philosophical Society **83**(4): 509-532.
- Lesuisse, E., M. Simon-Casteras, et al. (1998). "Siderophore-mediated iron uptake in *Saccharomyces cerevisiae*: the SIT1 gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily." Microbiology **144** (Pt 12): 3455-3462.
- Levin, D. E. (2005). "Cell wall integrity signaling in *Saccharomyces cerevisiae*." Microbiology and Molecular Biology Reviews **69**(2): 262-291.
- Lew, D. J. and S. I. Reed (1995). "A cell cycle checkpoint monitors cell morphogenesis in budding yeast." The Journal of Cell Biology **129**(3): 739-749.
- Li, J., Z. Yuan, et al. (2010). "The cellular robustness by genetic redundancy in budding yeast." PLoS Genetics **6**(11): e1001187.
- Li, K., H. Ouyang, et al. (2011). "Repression of N-glycosylation triggers the unfolded protein response (UPR) and overexpression of cell wall protein and chitin in *Aspergillus fumigatus*." Microbiology **157**(Pt 7): 1968-1979.
- Li, W., G. J. Belsham, et al. (2001). "Eukaryotic initiation factors 4A (eIF4A) and 4G (eIF4G) mutually interact in a 1:1 ratio in vivo." The Journal of Biological Chemistry **276**(31): 29111-29115.
- Li, W. and A. P. Mitchell (1997). "Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth." Genetics **145**(1): 63-73.
- Li, Y., J. Yan, et al. (2010). "Rad4 regulates protein turnover at a postubiquitylation step." Molecular Biology of the Cell **21**(1): 177-185.
- Lin, T. A., X. Kong, et al. (1994). "PHAS-I as a link between mitogen-activated protein kinase and translation initiation." Science **266**(5185): 653-656.

- Lin, Y. S., J. K. Hwang, et al. (2007). "Protein complexity, gene duplicability and gene dispensability in the yeast genome." Gene **387**(1-2): 109-117.
- Lindhahl, T. and B. Nyberg (1972). "Rate of depurination of native deoxyribonucleic acid." Biochemistry **11**(19): 3610-3618.
- Linder, P. (2003). "Yeast RNA helicases of the DEAD-box family involved in translation initiation." Biology of the Cell **95**(3-4): 157-167.
- Linder, P. and P. Lasko (2006). "Bent out of shape: RNA unwinding by the DEAD-box helicase Vasa." Cell **125**(2): 219-221.
- Linder, P., P. F. Lasko, et al. (1989). "Birth of the D-E-A-D box." Nature **337**(6203): 121-122.
- Linder, P. and P. P. Slonimski (1988). "Sequence of the genes TIF1 and TIF2 from *Saccharomyces cerevisiae* coding for a translation initiation factor." Nucleic Acids Research **16**(21): 10359.
- Linder, P. and P. P. Slonimski (1989). "An essential yeast protein, encoded by duplicated genes TIF1 and TIF2 and homologous to the mammalian translation initiation factor eIF-4A, can suppress a mitochondrial missense mutation." Proceedings of the National Academy of Sciences of the United States of America **86**(7): 2286-2290.
- Lindqvist, L. and J. Pelletier (2009). "Inhibitors of translation initiation as cancer therapeutics." Future Medicinal Chemistry **1**(9): 1709-1722.
- Liu, X., J. Tesfai, et al. (2003). "c-Myc transformation domain recruits the human STAGA complex and requires TRRAP and GCN5 acetylase activity for transcription activation." The Journal of Biological Chemistry **278**(22): 20405-20412.
- Locke, E. G., M. Bonilla, et al. (2000). "A homolog of voltage-gated Ca(2+) channels stimulated by depletion of secretory Ca(2+) in yeast." Molecular and Cellular Biology **20**(18): 6686-6694.
- Lohr, D., P. Venkov, et al. (1995). "Transcriptional regulation in the yeast GAL gene family: a complex genetic network." The Journal of the Federation of American Societies for Experimental Biology **9**(9): 777-787.
- Lommel, M., M. Bagnat, et al. (2004). "Aberrant processing of the WSC family and Mid2p cell surface sensors results in cell death of *Saccharomyces cerevisiae* O-mannosylation mutants." Molecular and Cellular Biology **24**(1): 46-57.
- Loper, J. C. (1988). "Gene engineering of yeasts for the degradation of hazardous waste." Journal of Hazardous Materials **19**(3): 323-326.
- Louis, E. J. (2007). "Evolutionary genetics: making the most of redundancy." Nature **449**(7163): 673-674.

- Lundblad, V. and K. Struhl (2010). Yeast. Current Protocols in Molecular Biology, John Wiley & Sons, Inc.
- Lynch, M. and J. S. Conery (2000). "The evolutionary fate and consequences of duplicate genes." Science **290**(5494): 1151-1155.
- Lynch, M. and A. Force (2000). "The probability of duplicate gene preservation by subfunctionalization." Genetics **154**(1): 459-473.
- Lynch, M. and V. Katju (2004). "The altered evolutionary trajectories of gene duplicates." Trends in Genetics : TIG **20**(11): 544-549.
- Ma, J. and M. Ptashne (1987). "The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80." Cell **50**(1): 137-142.
- Maechler, P. and C. B. Wollheim (2000). "Mitochondrial signals in glucose-stimulated insulin secretion in the beta cell." The Journal of Physiology **529 Pt 1**: 49-56.
- Maere, S., K. Heymans, et al. (2005). "BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks." Bioinformatics **21**(16): 3448-3449.
- Magdolen, V., D. G. Drubin, et al. (1993). "High levels of profilin suppress the lethality caused by overproduction of actin in yeast cells." FEBS Letters **316**(1): 41-47.
- Magni, G. E. (1963). "The Origin of Spontaneous Mutations during Meiosis." Proceedings of the National Academy of Sciences of the United States of America **50**: 975-980.
- Maltsev, N., E. M. Glass, et al. (2005). "Molecular mechanisms involved in robustness of yeast central metabolism against null mutations." The Journal of Biochemistry **137**(2): 177-187.
- Mamane, Y., E. Petroulakis, et al. (2006). "mTOR, translation initiation and cancer." Oncogene **25**(48): 6416-6422.
- Martin, D. E. and M. N. Hall (2005). "The expanding TOR signaling network." Current Opinion in Cell Biology **17**(2): 158-166.
- Martinez-Pastor, M. T., G. Marchler, et al. (1996). "The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE)." The EMBO Journal **15**(9): 2227-2235.
- Masuda, C. A., J. O. Previato, et al. (2008). "Overexpression of the aldose reductase GRE3 suppresses lithium-induced galactose toxicity in *Saccharomyces cerevisiae*." FEMS Yeast Research **8**(8): 1245-1253.

- Masuda, C. A., M. A. Xavier, et al. (2001). "Phosphoglucosyltransferase is an in vivo lithium target in yeast." The Journal of Biological Chemistry **276**(41): 37794-37801.
- Matsumoto, S., H. Friberg, et al. (1999). "Blockade of the mitochondrial permeability transition pore diminishes infarct size in the rat after transient middle cerebral artery occlusion." Journal of Cerebral Blood Flow and Metabolism **19**(7): 736-741.
- McCormack, J. G., A. P. Halestrap, et al. (1990). "Role of calcium ions in regulation of mammalian intramitochondrial metabolism." Physiological Reviews **70**(2): 391-425.
- McMillan, J. N., M. S. Longtine, et al. (1999). "The morphogenesis checkpoint in *Saccharomyces cerevisiae*: cell cycle control of Swe1p degradation by Hsl1p and Hsl7p." Molecular and Cellular Biology **19**(10): 6929-6939.
- McNabb, D. S., K. A. Tseng, et al. (1997). "The *Saccharomyces cerevisiae* Hap5p homolog from fission yeast reveals two conserved domains that are essential for assembly of heterotetrameric CCAAT-binding factor." Molecular and Cellular Biology **17**(12): 7008-7018.
- McNabb, D. S., Y. Xing, et al. (1995). "Cloning of yeast HAP5: a novel subunit of a heterotrimeric complex required for CCAAT binding." Genes & development **9**(1): 47-58.
- Mehta, D. V., A. Kabir, et al. (1999). "Expression of human inositol monophosphatase suppresses galactose toxicity in *Saccharomyces cerevisiae*: possible implications in galactosemia." Biochimica et biophysica acta **1454**(3): 217-226.
- Mendell, J. T., N. A. Sharifi, et al. (2004). "Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise." Nature genetics **36**(10): 1073-1078.
- Merico, D., D. Gfeller, et al. (2009). "How to visually interpret biological data using networks." Nature biotechnology **27**(10): 921-924.
- Metz, C. W. (1947). "Duplication of chromosome parts as a factor in evolution." The American naturalist **81**(797): 81-103.
- Meyer, A. (2003). "Molecular evolution: Duplication, duplication." Nature **421**(6918): 31-32.
- Meyer, J., A. Walker-Jonah, et al. (1991). "Galactokinase encoded by GAL1 is a bifunctional protein required for induction of the GAL genes in *Kluyveromyces lactis* and is able to suppress the gal3 phenotype in *Saccharomyces cerevisiae*." Molecular and Cellular Biology **11**(11): 5454-5461.

- Michelet, X., A. Djeddi, et al. (2010). "Developmental and cellular functions of the ESCRT machinery in pluricellular organisms." Biology of the Cell **102**(3): 191-202.
- Miseta, A., L. Fu, et al. (1999). "The Golgi apparatus plays a significant role in the maintenance of Ca²⁺ homeostasis in the vps33Delta vacuolar biogenesis mutant of *Saccharomyces cerevisiae*." The Journal of Biological Chemistry **274**(9): 5939-5947.
- Miyawaki, A., J. Llopis, et al. (1997). "Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin." Nature **388**(6645): 882-887.
- Mizunuma, M., D. Hirata, et al. (1998). "Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast." Nature **392**(6673): 303-306.
- Mizunuma, M., K. Miyamura, et al. (2004). "Involvement of S-adenosylmethionine in G1 cell-cycle regulation in *Saccharomyces cerevisiae*." Proceedings of the National Academy of Sciences of the United States of America **101**(16): 6086-6091.
- Montero-Lomeli, M., B. L. Morais, et al. (2002). "The initiation factor eIF4A is involved in the response to lithium stress in *Saccharomyces cerevisiae*." The Journal of Biological Chemistry **277**(24): 21542-21548.
- Mueller, C. L., S. E. Porter, et al. (2004). "The Paf1 complex has functions independent of actively transcribing RNA polymerase II." Molecular Cell **14**(4): 447-456.
- Muller, P. P., H. Trachsel, et al. (1989). "Genetic localization of the *Saccharomyces cerevisiae* genes *tif1* and *tif2*." Current Genetics **16**(2): 127-128.
- Munro, C. A., S. Selvaggini, et al. (2007). "The PKC, HOG and Ca²⁺ signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*." Molecular Microbiology **63**(5): 1399-1413.
- Musso, G., Z. Zhang, et al. (2007). "Retention of protein complex membership by ancient duplicated gene products in budding yeast." Trends in Genetics : TIG **23**(6): 266-269.
- Nehlin, J. O., M. Carlberg, et al. (1989). "Yeast galactose permease is related to yeast and mammalian glucose transporters." Gene **85**(2): 313-319.
- Nelson, K. K. and S. K. Lemmon (1993). "Suppressors of clathrin deficiency: overexpression of ubiquitin rescues lethal strains of clathrin-deficient *Saccharomyces cerevisiae*." Molecular and Cellular Biology **13**(1): 521-532.
- Ng, H. H., F. Robert, et al. (2003). "Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity." Molecular Cell **11**(3): 709-719.

- Nijtmans, L. G., L. de Jong, et al. (2000). "Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins." The EMBO Journal **19**(11): 2444-2451.
- Ning, C. and S. Segal (2000). "Plasma galactose and galactitol concentration in patients with galactose-1-phosphate uridylyltransferase deficiency galactosemia: determination by gas chromatography/mass spectrometry." Metabolism **49**(11): 1460-1466.
- Nonaka, H., K. Tanaka, et al. (1995). "A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*." The EMBO Journal **14**(23): 5931-5938.
- Nussenzveig, R. H., H. B. Lingam, et al. (2006). "A novel mutation of the cytochrome-b5 reductase gene in an Indian patient: the molecular basis of type I methemoglobinemia." Haematologica **91**(11): 1542-1545.
- O'Rourke, S. M. and I. Herskowitz (2002). "A third osmosensing branch in *Saccharomyces cerevisiae* requires the Msb2 protein and functions in parallel with the Sho1 branch." Molecular and Cell Biology **22**(13): 4739-4749.
- O'Rourke, S. M., I. Herskowitz, et al. (2002). "Yeast go the whole HOG for the hyperosmotic response." Trends in Genetics : TIG **18**(8): 405-412.
- Oh, D. and J. E. Hopper (1990). "Transcription of a yeast phosphoglucomutase isozyme gene is galactose inducible and glucose repressible." Molecular and Cell Biology **10**(4): 1415-1422.
- Ohlmeier, S., A. J. Kastaniotis, et al. (2004). "The yeast mitochondrial proteome, a study of fermentative and respiratory growth." The Journal of Biological Chemistry **279**(6): 3956-3979.
- Ohno, S. (1970). Evolution by Gene Duplication. London, Springer-Verlag.
- Ohno, S. (1973). Evolutional reason for having so much junk DNA. Modern Aspects of Cytogenetics: Constitutive Heterochromatin in Man. R. A. Pfeiffer. Stuttgart, Germany, F.K. Schattauer Verlag: 169-173.
- Oliveira, S. J., J. P. Pinto, et al. (2009). "ER stress-inducible factor CHOP affects the expression of hepcidin by modulating C/EBPalpha activity." PLoS One **4**(8): e6618.
- Olynyk, J. K., D. J. Cullen, et al. (1999). "A population-based study of the clinical expression of the hemochromatosis gene." The New England Journal of Medicine **341**(10): 718-724.
- Onishi, J., M. Mainz, et al. (2000). "Discovery of novel antifungal (1,3)-beta-D-glucan synthase inhibitors." Antimicrobial Agents and Chemotherapy **44**(2): 368-377.

- Ooi, S. L., X. Pan, et al. (2006). "Global synthetic-lethality analysis and yeast functional profiling." Trends in Genetics : TIG **22**(1): 56-63.
- Orgel, L. E. (1977). "Gene-duplication and the origin of proteins with novel functions." Journal of Theoretical Biology **67**(4): 773.
- Orphanides, G., W. H. Wu, et al. (1999). "The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins." Nature **400**(6741): 284-288.
- Ossareh-Nazari, B., M. Bonizec, et al. (2010). "Cdc48 and Ufd3, new partners of the ubiquitin protease Ubp3, are required for ribophagy." EMBO Reports **11**(7): 548-554.
- Ossareh-Nazari, B., M. Cohen, et al. (2010). "The Rsp5 ubiquitin ligase and the AAA-ATPase Cdc48 control the ubiquitin-mediated degradation of the COPII component Sec23." Experimental Cell Research **316**(20): 3351-3357.
- Ozier, O., N. Amin, et al. (2003). "Global architecture of genetic interactions on the protein network." Nature Biotechnology **21**(5): 490-491.
- Page, N., J. Sheraton, et al. (1996). "Identification of ASK10 as a multicopy activator of Skn7p-dependent transcription of a HIS3 reporter gene." Yeast **12**(3): 267-272.
- Paidhungat, M. and S. Garrett (1997). "A homolog of mammalian, voltage-gated calcium channels mediates yeast pheromone-stimulated Ca²⁺ uptake and exacerbates the cdc1(Ts) growth defect." Molecular and Cell Biology **17**(11): 6339-6347.
- Paiva, S., N. Vieira, et al. (2009). "Glucose-induced ubiquitylation and endocytosis of the yeast Jen1 transporter: role of lysine 63-linked ubiquitin chains." The Journal of Biological Chemistry **284**(29): 19228-19236.
- Papamichos-Chronakis, M., R. S. Conlan, et al. (2000). "Hrs1/Med3 is a Cyc8-Tup1 corepressor target in the RNA polymerase II holoenzyme." The Journal of Biological Chemistry **275**(12): 8397-8403.
- Papp, B., C. Pal, et al. (2003). "Dosage sensitivity and the evolution of gene families in yeast." Nature **424**(6945): 194-197.
- Papp, B., C. Pal, et al. (2004). "Metabolic network analysis of the causes and evolution of enzyme dispensability in yeast." Nature **429**(6992): 661-664.
- Parekh, A. B. and R. Penner (1997). "Store depletion and calcium influx." Physiological Reviews **77**(4): 901-930.
- Parsons, A. B., R. L. Brost, et al. (2004). "Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways." Nature Biotechnology **22**(1): 62-69.

- Parsons, A. B., A. Lopez, et al. (2006). "Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast." Cell **126**(3): 611-625.
- Peiter, E., M. Fischer, et al. (2005). "The *Saccharomyces cerevisiae* Ca²⁺ channel Cch1pMid1p is essential for tolerance to cold stress and iron toxicity." FEBS Letters **579**(25): 5697-5703.
- Pelletier, J. and N. Sonenberg (1985). "Insertion mutagenesis to increase secondary structure within the 5' noncoding region of a eukaryotic mRNA reduces translational efficiency." Cell **40**(3): 515-526.
- Peng, G. and J. E. Hopper (2002). "Gene activation by interaction of an inhibitor with a cytoplasmic signaling protein." Proceedings of the National Academy of Sciences of the United States of America **99**(13): 8548-8553.
- Peng, J., D. Schwartz, et al. (2003). "A proteomics approach to understanding protein ubiquitination." Nature Biotechnology **21**(8): 921-926.
- Pereira, M. D., E. C. Eleutherio, et al. (2001). "Acquisition of tolerance against oxidative damage in *Saccharomyces cerevisiae*." BMC Microbiology **1**: 11.
- Pfeffer, S. R. (1999). "Transport-vesicle targeting: tethers before SNAREs." Nature Cell Biology **1**(1): E17-22.
- Philip, B. and D. E. Levin (2001). "Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1." Molecular and Cellular Biology **21**(1): 271-280.
- Philpott, C. C. (2006). "Iron uptake in fungi: a system for every source." Biochimica et Biophysica Acta **1763**(7): 636-645.
- Piatigorsky, J. and G. Wistow (1991). "The recruitment of crystallins: new functions precede gene duplication." Science **252**(5009): 1078-1079.
- Piatigorsky, J. and G. J. Wistow (1989). "Enzyme/crystallins: gene sharing as an evolutionary strategy." Cell **57**(2): 197-199.
- Pickart, C. M. and D. Fushman (2004). "Polyubiquitin chains: polymeric protein signals." Current Opinion in Chemical Biology **8**(6): 610-616.
- Pietrangelo, A. (2004). "Hereditary hemochromatosis--a new look at an old disease." The New England Journal of Medicine **350**(23): 2383-2397.
- Pinton, P., C. Giorgi, et al. (2008). "Calcium and apoptosis: ER-mitochondria Ca²⁺ transfer in the control of apoptosis." Oncogene **27**(50): 6407-6418.
- Piskur, J., K. D. Schnackerz, et al. (2007). "Comparative genomics reveals novel biochemical pathways." Trends in Genetics : TIG **23**(8): 369-372.

- Platt, A. and R. J. Reece (1998). "The yeast galactose genetic switch is mediated by the formation of a Gal4p-Gal80p-Gal3p complex." The EMBO Journal **17**(14): 4086-4091.
- Platt, A., H. C. Ross, et al. (2000). "The insertion of two amino acids into a transcriptional inducer converts it into a galactokinase." Proceedings of the National Academy of Sciences of the United States of America **97**(7): 3154-3159.
- Pollack, S. J., J. R. Atack, et al. (1994). "Mechanism of inositol monophosphatase, the putative target of lithium therapy." Proceedings of the National Academy of Sciences of the United States of America **91**(13): 5766-5770.
- Popolo, L., D. Gilardelli, et al. (1997). "Increase in chitin as an essential response to defects in assembly of cell wall polymers in the ggp1delta mutant of *Saccharomyces cerevisiae*." Journal of Bacteriology **179**(2): 463-469.
- Popolo, L., T. Gualtieri, et al. (2001). "The yeast cell-wall salvage pathway." Medical Mycology **39 Suppl 1**: 111-121.
- Porter, S. E., T. M. Washburn, et al. (2002). "The yeast pafl-rNA polymerase II complex is required for full expression of a subset of cell cycle-regulated genes." Eukaryotic Cell **1**(5): 830-842.
- Poulin, F., A. C. Gingras, et al. (1998). "4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family." The Journal of Biological Chemistry **273**(22): 14002-14007.
- Powell, D. W., C. M. Weaver, et al. (2004). "Cluster analysis of mass spectrometry data reveals a novel component of SAGA." Molecular and Cell Biology **24**(16): 7249-7259.
- Prat, A., S. R. Schmid, et al. (1990). "Expression of translation initiation factor 4A from yeast and mouse in *Saccharomyces cerevisiae*." Biochimica et Biophysica Acta **1050**(1-3): 140-145.
- Pratt, J. M., J. Petty, et al. (2002). "Dynamics of protein turnover, a missing dimension in proteomics." Molecular & Cellular Proteomics **1**(8): 579-591.
- Pray-Grant, M. G., J. A. Daniel, et al. (2005). "Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation." Nature **433**(7024): 434-438.
- Prelich, G. (1999). "Suppression mechanisms: themes from variations." Trends in Genetics : TIG **15**(7): 261-266.
- Presser, A., M. B. Elowitz, et al. (2008). "The evolutionary dynamics of the *Saccharomyces cerevisiae* protein interaction network after duplication." Proceedings of the National Academy of Sciences of the United States of America **105**(3): 950-954.

- Proft, M., G. Mas, et al. (2006). "The stress-activated Hog1 kinase is a selective transcriptional elongation factor for genes responding to osmotic stress." Molecular Cell **23**(2): 241-250.
- Proud, C. G. (2002). "Regulation of mammalian translation factors by nutrients." European Journal of Biochemistry / FEBS **269**(22): 5338-5349.
- Puig, O., F. Caspary, et al. (2001). "The tandem affinity purification (TAP) method: a general procedure of protein complex purification." Methods **24**(3): 218-229.
- Putney, J. W., Jr. (1992). "Inositol phosphates and calcium entry." Advances in Second Messenger and Phosphoprotein Research **26**: 143-160.
- Puziss, J. W., T. A. Hardy, et al. (1994). "MDS1, a dosage suppressor of an mck1 mutant, encodes a putative yeast homolog of glycogen synthase kinase 3." Molecular and Cellular Biology **14**(1): 831-839.
- Raghavulu, S. V., R. K. Goud, et al. (2011). "Saccharomyces cerevisiae as anodic biocatalyst for power generation in biofuel cell: influence of redox condition and substrate load." Bioresource Technology **102**(3): 2751-2757.
- Raiborg, C., T. E. Rusten, et al. (2003). "Protein sorting into multivesicular endosomes." Current Opinion in Cell Biology **15**(4): 446-455.
- Raingeaud, J., S. Gupta, et al. (1995). "Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine." The Journal of Biological Chemistry **270**(13): 7420-7426.
- Ramos, J., K. Szkutnicka, et al. (1989). "Characteristics of galactose transport in Saccharomyces cerevisiae cells and reconstituted lipid vesicles." Journal of Bacteriology **171**(6): 3539-3544.
- Ray, W. J., Jr., E. S. Szymanski, et al. (1978). "The binding of lithium and of anionic metabolites to phosphoglucomutase." Biochimica et Biophysica Acta **522**(2): 434-442.
- Raymond, C. K., I. Howald-Stevenson, et al. (1992). "Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants." Molecular Biology of the Cell **3**(12): 1389-1402.
- Reinders, J., R. P. Zahedi, et al. (2006). "Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics." Journal of Proteome Research **5**(7): 1543-1554.
- Reiss, G., S. te Heesen, et al. (1997). "A specific screen for oligosaccharyltransferase mutations identifies the 9 kDa OST5 protein

- required for optimal activity in vivo and in vitro." The EMBO Journal **16**(6): 1164-1172.
- Ren, J., Y. Kee, et al. (2007). "Hse1, a component of the yeast Hrs-STAM ubiquitin-sorting complex, associates with ubiquitin peptidases and a ligase to control sorting efficiency into multivesicular bodies." Molecular Biology of the Cell **18**(1): 324-335.
- Riehman, K., C. Crews, et al. (2001). "Relationship between genotype, activity, and galactose sensitivity in yeast expressing patient alleles of human galactose-1-phosphate uridylyltransferase." The Journal of Biological Chemistry **276**(14): 10634-10640.
- Rizet, G. and C. Engelmann (1949). "Contribution à l'étude génétique d'un ascomycète tétrasporé: *Podospira anserina*." Rev Cytol **11**: 201-304.
- Robinson, K. M. and M. C. Schultz (2003). "Replication-independent assembly of nucleosome arrays in a novel yeast chromatin reconstitution system involves antisilencing factor Asf1p and chromodomain protein Chd1p." Molecular and Cellular Biology **23**(22): 7937-7946.
- Rodicio, R. and J. J. Heinisch (2010). "Together we are strong—cell wall integrity sensors in yeasts." Yeast **27**(8): 531-540.
- Rodriguez-Navarro, S. (2009). "Insights into SAGA function during gene expression." EMBO Rep **10**(8): 843-850.
- Rodriguez, A., T. De La Cera, et al. (2001). "The hexokinase 2 protein regulates the expression of the GLK1, HXK1 and HXK2 genes of *Saccharomyces cerevisiae*." The Biochemical Journal **355**(Pt 3): 625-631.
- Rogers, G. W., Jr., N. J. Richter, et al. (2001). "Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F." The Journal of Biological Chemistry **276**(33): 30914-30922.
- Rohde, J. R., S. Campbell, et al. (2004). "TOR controls transcriptional and translational programs via Sap-Sit4 protein phosphatase signaling effectors." Molecular and Cellular Biology **24**(19): 8332-8341.
- Rother, S., C. Burkert, et al. (2010). "Nucleocytoplasmic shuttling of the La motif-containing protein Sro9 might link its nuclear and cytoplasmic functions." RNA **16**(7): 1393-1401.
- Rothfels, K., J. C. Tanny, et al. (2005). "Components of the ESCRT pathway, DFG16, and YGR122w are required for Rim101 to act as a corepressor with Nrg1 at the negative regulatory element of the DIT1 gene of *Saccharomyces cerevisiae*." Molecular and Cellular Biology **25**(15): 6772-6788.
- Rothman, J. E. and L. Orci (1992). "Molecular dissection of the secretory pathway." Nature **355**(6359): 409-415.

- Sammons, M. A., P. Samir, et al. (2011). "Saccharomyces cerevisiae Gis2 interacts with the translation machinery and is orthogonal to myotonic dystrophy type 2 protein ZNF9." Biochemical and Biophysical Research Communications **406**(1): 13-19.
- Sarkar, S., R. Kiely, et al. (2010). "The Ino80 chromatin-remodeling complex restores chromatin structure during UV DNA damage repair." The Journal of Cell Biology **191**(6): 1061-1068.
- Schadt, E. E., S. A. Monks, et al. (2003). "Genetics of gene expression surveyed in maize, mouse and man." Nature **422**(6929): 297-302.
- Schafer, D. A. (2002). "Coupling actin dynamics and membrane dynamics during endocytosis." Current Opinion in Cell Biology **14**(1): 76-81.
- Schell, M. A. and D. B. Wilson (1977). "Purification and properties of galactokinase from Saccharomyces cerevisiae." The Journal of Biological Chemistry **252**(4): 1162-1166.
- Schenker, N. and J. F. Gentleman (2001). "On Judging the Significance of Differences by Examining the Overlap Between Confidence Intervals." The American Statistician **55**(3): 182-186.
- Scheper, G. C. and C. G. Proud (2002). "Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation?" European Journal of Biochemistry / FEBS **269**(22): 5350-5359.
- Schneider, R. (1999). "Brave little yeast, please guide us to thebes: sphingolipid function in S. cerevisiae." Bioessays **21**(12): 1004-1010.
- Schon, E. A. and G. Manfredi (2003). "Neuronal degeneration and mitochondrial dysfunction." Journal of Clinical Investigation **111**(3): 303-312.
- Schroder, M. and R. J. Kaufman (2005). "The mammalian unfolded protein response." Annual Review of Biochemistry **74**: 739-789.
- Schuldiner, M., S. R. Collins, et al. (2005). "Exploration of the function and organization of the yeast early secretory pathway through an epistatic miniarray profile." Cell **123**(3): 507-519.
- Schulze, A. and J. Downward (2001). "Navigating gene expression using microarrays--a technology review." Nature Cell Biology **3**(8): E190-195.
- Schulze, J. M., T. Hentrich, et al. (2011). "Splitting the task: Ubp8 and Ubp10 deubiquitinate different cellular pools of H2BK123." Genes & Development **25**(21): 2242-2247.
- Schwartzman, J. B. and J. Van't Hof (1982). "In the higher plant Pisum sativum maturation of nascent DNA is blocked by cycloheximide, but only after 4-8 replicons are joined." Nucleic Acids Research **10**(19): 6191-6205.

- Seal, S. N., A. Schmidt, et al. (1985). "Initiation factors eIF4A and C1 from wheat germ and the formation of mRNA X ribosome complexes." Archives of Biochemistry and Biophysics **238**(1): 146-153.
- Serebrovsky, A. S. (1938). "Genes scute and achaete in *Drosophila melanogaster* and a hypothesis of gene divergency." C.R. Acad. Sci. URSS **19**: 77-81.
- Shenkman, M., S. Tolchinsky, et al. (2007). "ER stress induces alternative nonproteasomal degradation of ER proteins but not of cytosolic ones." Cell Stress Chaperones **12**(4): 373-383.
- Sherman, F., J. W. Stewart, et al. (1980). "Mutants of yeast initiating translation of Iso-1-cytochrome c within a region spanning 37 nucleotides." Cell **20**(1): 215-222.
- Sheth, U. and R. Parker (2006). "Targeting of aberrant mRNAs to cytoplasmic processing bodies." Cell **125**(6): 1095-1109.
- Shi, G. and J. S. Trimmer (1999). "Differential asparagine-linked glycosylation of voltage-gated K⁺ channels in mammalian brain and in transfected cells." Journal of Membrane Biology **168**(3): 265-273.
- Shih, S. C., D. J. Katzmann, et al. (2002). "Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis." Nature Cell Biology **4**(5): 389-393.
- Silverman, B. W. (1986). Density Estimation for Statistics and Data Analysis. London ; New York, Chapman and Hall.
- Simic, R., D. L. Lindstrom, et al. (2003). "Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes." The EMBO Journal **22**(8): 1846-1856.
- Sinclair, D. A. and I. W. Dawes (1995). "Genetics of the synthesis of serine from glycine and the utilization of glycine as sole nitrogen source by *Saccharomyces cerevisiae*." Genetics **140**(4): 1213-1222.
- Sinclair, D. A., S. P. Hong, et al. (1996). "Specific induction by glycine of the gene for the P-subunit of glycine decarboxylase from *Saccharomyces cerevisiae*." Molecular Microbiology **19**(3): 611-623.
- Smith, M. M. (1987). "Molecular evolution of the *Saccharomyces cerevisiae* histone gene loci." Journal of Molecular Evolution **24**(3): 252-259.
- Smythe, E. and K. R. Ayscough (2003). "The Ark1/Prk1 family of protein kinases. Regulators of endocytosis and the actin skeleton." EMBO Reports **4**(3): 246-251.
- Sobel, S. G. and S. L. Wolin (1999). "Two yeast La motif-containing proteins are RNA-binding proteins that associate with polyribosomes." Molecular Biology of the Cell **10**(11): 3849-3862.

- Solomon, M., R. Booher, et al. (1988). "Cyclin in fission yeast." Cell **54**(6): 738-740.
- Sonenberg, N. and T. E. Dever (2003). "Eukaryotic translation initiation factors and regulators." Current Opinion in Structural Biology **13**(1): 56-63.
- Sonenberg, N. and A. G. Hinnebusch (2009). "Regulation of translation initiation in eukaryotes: mechanisms and biological targets." Cell **136**(4): 731-745.
- Sopko, R., D. Huang, et al. (2006). "Mapping pathways and phenotypes by systematic gene overexpression." Molecular Cell **21**(3): 319-330.
- Sprague, G. F., Jr. and I. Herskowitz (1981). "Control of yeast cell type by the mating type locus. I. Identification and control of expression of the a-specific gene BAR1." Journal of Molecular Biology **153**(2): 305-321.
- Spriggs, K. A., M. Stoneley, et al. (2008). "Re-programming of translation following cell stress allows IRES-mediated translation to predominate." Biology of the Cell **100**(1): 27-38.
- St Onge, R. P., R. Mani, et al. (2007). "Systematic pathway analysis using high-resolution fitness profiling of combinatorial gene deletions." Nature Genetics **39**(2): 199-206.
- Stadler, L. J. (1929). "Chromosome Number and the Mutation Rate in Avena and Triticum." Proceedings of the National Academy of Sciences of the United States of America **15**(12): 876-881.
- Stagljar, I., S. te Heesen, et al. (1994). "New phenotype of mutations deficient in glucosylation of the lipid-linked oligosaccharide: cloning of the ALG8 locus." Proceedings of the National Academy of Sciences of the United States of America **91**(13): 5977-5981.
- Stathopoulos, A. M. and M. S. Cyert (1997). "Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast." Genes & Development **11**(24): 3432-3444.
- Steinmetz, L. M., H. Sinha, et al. (2002). "Dissecting the architecture of a quantitative trait locus in yeast." Nature **416**(6878): 326-330.
- Stephens, S. G. (1951). "Possible significances of duplication in evolution." Advances in Genetics **4**: 247-265.
- Stevens, S. W., D. E. Ryan, et al. (2002). "Composition and functional characterization of the yeast spliceosomal penta-snRNP." Molecular Cell **9**(1): 31-44.
- Stiburek, L., H. Hansikova, et al. (2006). "Biogenesis of eukaryotic cytochrome c oxidase." Physiological Research **55 Suppl 2**: S27-41.

- Strayle, J., T. Pozzan, et al. (1999). "Steady-state free Ca(2+) in the yeast endoplasmic reticulum reaches only 10 microM and is mainly controlled by the secretory pathway pump pmr1." The EMBO Journal **18**(17): 4733-4743.
- Stringer, D. K. and R. C. Piper (2011). "Terminating protein ubiquitination: Hasta la vista, ubiquitin." Cell Cycle **10**(18): 3067-3071.
- Su, S. S. and A. P. Mitchell (1993). "Identification of functionally related genes that stimulate early meiotic gene expression in yeast." Genetics **133**(1): 67-77.
- Sumegi, A. (2008). "Mood stabilizers--past, present and future." Journal of the Hungarian Association of Neuropsychopharmacology **10**(1): 31-42.
- Svitkin, Y. V., A. Pause, et al. (2001). "The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure." RNA **7**(3): 382-394.
- Sychrova, H. and M. R. Chevallier (1993). "Cloning and sequencing of the *Saccharomyces cerevisiae* gene LYP1 coding for a lysine-specific permease." Yeast **9**(7): 771-782.
- Synetos, D., C. P. Frantziou, et al. (1996). "Mutations in yeast ribosomal proteins S28 and S4 affect the accuracy of translation and alter the sensitivity of the ribosomes to paromomycin." Biochimica et Biophysica Acta **1309**(1-2): 156-166.
- Szappanos, B., K. Kovacs, et al. (2011). "An integrated approach to characterize genetic interaction networks in yeast metabolism." Nature Genetics **43**(7): 656-662.
- Tagwerker, C., K. Flick, et al. (2006). "A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with in vivocross-linking." Molecular & Cellular Proteomics **5**(4): 737-748.
- Takahashi, S., Y. Araki, et al. (2008). "Upf1 potentially serves as a RING-related E3 ubiquitin ligase via its association with Upf3 in yeast." RNA **14**(9): 1950-1958.
- Tani, K. (2000). "Review of cancer gene therapy." Nihon Geka Gakkai Zasshi **101**(9): 618-624.
- Tarassov, K., V. Messier, et al. (2008). "An in vivo map of the yeast protein interactome." Science **320**(5882): 1465-1470.
- Teem, J. L., N. Abovich, et al. (1984). "A comparison of yeast ribosomal protein gene DNA sequences." Nucleic Acids Research **12**(22): 8295-8312.

- Teleman, A. A., Y. W. Chen, et al. (2005). "4E-BP functions as a metabolic brake used under stress conditions but not during normal growth." Genes & Development **19**(16): 1844-1848.
- Thanabalu, T. and A. L. Munn (2001). "Functions of Vrp1p in cytokinesis and actin patches are distinct and neither requires a WH2/V domain." The EMBO journal **20**(24): 6979-6989.
- Tiedje, C., D. G. Holland, et al. (2007). "Proteins involved in sterol synthesis interact with Ste20 and regulate cell polarity." Journal of Cell Science **120**(Pt 20): 3613-3624.
- Titov, A. A. and G. Blobel (1999). "The karyopherin Kap122p/Pdr6p imports both subunits of the transcription factor IIA into the nucleus." The Journal of Cell Biology **147**(2): 235-246.
- Tiwari, A. and J. P. Bhat (2008). "Molecular characterization reveals that YMR278w encoded protein is environmental stress response homologue of *Saccharomyces cerevisiae* PGM2." Biochemical and Biophysical Research Communications **366**(2): 340-345.
- Tkacz, J. S. and J. O. Lampen (1972). "Wall replication in *saccharomyces* species: use of fluorescein-conjugated concanavalin A to reveal the site of mannan insertion." Journal of General Microbiology **72**(2): 243-247.
- Tong, A. and C. Boone (2005). Synthetic Genetic Array (SGA) Analysis in *Saccharomyces cerevisiae*. Yeast Protocols. Totowa, NJ, U.S.A., The Humana Press Inc. **313**: 171-192.
- Tong, A. H. and C. Boone (2006). "Synthetic genetic array analysis in *Saccharomyces cerevisiae*." Methods in Molecular Biology **313**: 171-192.
- Tong, A. H., M. Evangelista, et al. (2001). "Systematic genetic analysis with ordered arrays of yeast deletion mutants." Science **294**(5550): 2364-2368.
- Tong, A. H., G. Lesage, et al. (2004). "Global mapping of the yeast genetic interaction network." Science **303**(5659): 808-813.
- Topisirovic, I., Y. V. Svitkin, et al. (2011). "Cap and cap-binding proteins in the control of gene expression." Wiley Interdisciplinary Reviews RNA **2**(2): 277-298.
- Torkko, J. M., K. T. Koivuranta, et al. (2001). "*Candida tropicalis* Etr1p and *Saccharomyces cerevisiae* Ybr026p (Mrf1'p), 2-enoyl thioester reductases essential for mitochondrial respiratory competence." Molecular and Cellular Biology **21**(18): 6243-6253.
- Torres, L., H. Martin, et al. (1991). "A protein kinase gene complements the lytic phenotype of *Saccharomyces cerevisiae* *lyt2* mutants." Molecular Microbiology **5**(11): 2845-2854.

- Traven, A., B. Jelicic, et al. (2006). "Yeast Gal4: a transcriptional paradigm revisited." EMBO Reports **7**(5): 496-499.
- Tschopp, J. F., S. D. Emr, et al. (1986). "GAL2 codes for a membrane-bound subunit of the galactose permease in *Saccharomyces cerevisiae*." Journal of Bacteriology **166**(1): 313-318.
- Tsvetanova, N. G., D. M. Klass, et al. (2010). "Proteome-wide search reveals unexpected RNA-binding proteins in *Saccharomyces cerevisiae*." PLoS One **5**(9).
- Uesono, Y., M. P. Ashe, et al. (2004). "Simultaneous yet independent regulation of actin cytoskeletal organization and translation initiation by glucose in *Saccharomyces cerevisiae*." Molecular Biology of the Cell **15**(4): 1544-1556.
- Valdivieso, M. H., L. Ferrario, et al. (2000). "Chitin synthesis in a *gas1* mutant of *Saccharomyces cerevisiae*." Journal of Bacteriology **182**(17): 4752-4757.
- van der Rest, M. E., A. H. Kamminga, et al. (1995). "The plasma membrane of *Saccharomyces cerevisiae*: structure, function, and biogenesis." Microbiology Reviews **59**(2): 304-322.
- Van Dyck, E., F. Foury, et al. (1992). "A single-stranded DNA binding protein required for mitochondrial DNA replication in *S. cerevisiae* is homologous to *E. coli* SSB." The EMBO Journal **11**(9): 3421-3430.
- Van Valen, L. (1963). "Haldane's Dilemma, Evolutionary Rates, and Heterosis." The American Naturalist **97**(894): 185-190.
- VanderSluis, B., J. Bellay, et al. (2010). "Genetic interactions reveal the evolutionary trajectories of duplicate genes." Molecular Systems Biology **6**: 429.
- Vazquez de Aldana, C. R. and A. G. Hinnebusch (1994). "Mutations in the GCD7 subunit of yeast guanine nucleotide exchange factor eIF-2B overcome the inhibitory effects of phosphorylated eIF-2 on translation initiation." Molecular and Cellular Biology **14**(5): 3208-3222.
- Velculescu, V. E., L. Zhang, et al. (1997). "Characterization of the yeast transcriptome." Cell **88**(2): 243-251.
- Verhees, C. H., J. Akerboom, et al. (2002). "Molecular and biochemical characterization of a distinct type of fructose-1,6-bisphosphatase from *Pyrococcus furiosus*." Journal of Bacteriology **184**(12): 3401-3405.
- Verna, J., A. Lodder, et al. (1997). "A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*." Proceedings of the National Academy of Sciences of the United States of America **94**(25): 13804-13809.

- Viladevall, L., R. Serrano, et al. (2004). "Characterization of the calcium-mediated response to alkaline stress in *Saccharomyces cerevisiae*." The Journal of Biological Chemistry **279**(42): 43614-43624.
- von der Haar, T. and J. E. McCarthy (2002). "Intracellular translation initiation factor levels in *Saccharomyces cerevisiae* and their role in cap-complex function." Molecular Microbiology **46**(2): 531-544.
- Wagner, A. (2000). "Robustness against mutations in genetic networks of yeast." Nature Genetics **24**(4): 355-361.
- Wagner, A. (2001). "Birth and death of duplicated genes in completely sequenced eukaryotes." Trends in Genetics : TIG **17**(5): 237-239.
- Wagner, A. (2008). "Robustness and evolvability: a paradox resolved." Proceedings. Biological Sciences / The Royal Society **275**(1630): 91-100.
- Wales, D. S., T. G. Cartledge, et al. (1980). "Effects of Glucose Repression and Anaerobiosis on the Activities and Subcellular Distribution of Tricarboxylic Acid Cycle and Associated Enzymes in *Saccharomyces carlsbergensis*." Journal of General Microbiology **116**(1): 93-98.
- Walker, L. A., C. A. Munro, et al. (2008). "Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins." PLoS Pathogens **4**(4): e1000040.
- Walsh, R. B., G. Kawasaki, et al. (1983). "Cloning of genes that complement yeast hexokinase and glucokinase mutants." Journal of Bacteriology **154**(2): 1002-1004.
- Weake, V. M., K. K. Lee, et al. (2008). "SAGA-mediated H2B deubiquitination controls the development of neuronal connectivity in the *Drosophila* visual system." The EMBO Journal **27**(2): 394-405.
- Wiesenberger, G., K. Steinleitner, et al. (2007). "Mg²⁺ deprivation elicits rapid Ca²⁺ uptake and activates Ca²⁺/calcineurin signaling in *Saccharomyces cerevisiae*." Eukaryotic Cell **6**(4): 592-599.
- Wightman, R., R. Bell, et al. (2008). "Localization and interaction of the proteins constituting the GAL genetic switch in *Saccharomyces cerevisiae*." Eukaryotic Cell **7**(12): 2061-2068.
- Williams, R. L. and S. Urbe (2007). "The emerging shape of the ESCRT machinery." Nature Reviews. Molecular Cell Biology **8**(5): 355-368.
- Williams, R. M., M. Primig, et al. (2002). "The Ume6 regulon coordinates metabolic and meiotic gene expression in yeast." Proceedings of the National Academy of Sciences of the United States of America **99**(21): 13431-13436.

- Williamson, D. H. (1965). "The timing of deoxyribonucleic acid synthesis in the cell cycle of *Saccharomyces cerevisiae*." The Journal of Cell Biology **25**(3): 517-528.
- Wilmes, G. M., M. Bergkessel, et al. (2008). "A genetic interaction map of RNA-processing factors reveals links between Sem1/Dss1-containing complexes and mRNA export and splicing." Molecular Cell **32**(5): 735-746.
- Winderickx, J., J. H. de Winde, et al. (1996). "Regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*: novel variations of STRE-mediated transcription control?" Molecular & General Genetics : MGG **252**(4): 470-482.
- Winzeler, E. A., D. D. Shoemaker, et al. (1999). "Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis." Science **285**(5429): 901-906.
- Wishart, J. A., A. Hayes, et al. (2005). "Doxycycline, the drug used to control the tet-regulatable promoter system, has no effect on global gene expression in *Saccharomyces cerevisiae*." Yeast **22**(7): 565-569.
- Wittmeyer, J., L. Joss, et al. (1999). "Spt16 and Pob3 of *Saccharomyces cerevisiae* form an essential, abundant heterodimer that is nuclear, chromatin-associated, and copurifies with DNA polymerase alpha." Biochemistry **38**(28): 8961-8971.
- Wolfe, K. (2000). "Robustness--it's not where you think it is." Nature Genetics **25**(1): 3-4.
- Wolfe, K. H. and D. C. Shields (1997). "Molecular evidence for an ancient duplication of the entire yeast genome." Nature **387**(6634): 708-713.
- Wong, J., Y. Nakajima, et al. (2007). "A protein interaction map of the mitotic spindle." Molecular and Cellular Biology **18**(10): 3800-3809.
- Wu, S., Y. Hu, et al. (2002). "Ultraviolet light inhibits translation through activation of the unfolded protein response kinase PERK in the lumen of the endoplasmic reticulum." The Journal of Biological Chemistry **277**(20): 18077-18083.
- Xiao, T., C. F. Kao, et al. (2005). "Histone H2B ubiquitylation is associated with elongating RNA polymerase II." Molecular and Cellular Biology **25**(2): 637-651.
- Xu, W. and A. P. Mitchell (2001). "Yeast PalA/AIP1/Alix homolog Rim20p associates with a PEST-like region and is required for its proteolytic cleavage." Journal of Bacteriology **183**(23): 6917-6923.
- Yan, A. and W. J. Lennarz (2005). "Unraveling the mechanism of protein N-glycosylation." The Journal of Biological Chemistry **280**(5): 3121-3124.

- Yen, K., P. Gitsham, et al. (2003). "An improved tetO promoter replacement system for regulating the expression of yeast genes." Yeast **20**(15): 1255-1262.
- Yoon, H. J. and T. F. Donahue (1992). "The suil suppressor locus in *Saccharomyces cerevisiae* encodes a translation factor that functions during tRNA(iMet) recognition of the start codon." Molecular and Cellular Biology **12**(1): 248-260.
- York, J. D., J. W. Ponder, et al. (1995). "Definition of a metal-dependent/Li(+)-inhibited phosphomonoesterase protein family based upon a conserved three-dimensional core structure." Proceedings of the National Academy of Sciences of the United States of America **92**(11): 5149-5153.
- Young, A. H. and J. I. Newham (2006). "Lithium in maintenance therapy for bipolar disorder." Journal of Psychopharmacology **20**(2 Suppl): 17-22.
- Young, K. H. (1998). "Yeast two-hybrid: so many interactions, (in) so little time." Biology of Reproduction **58**(2): 302-311.
- Young, M. E., T. S. Karpova, et al. (2002). "The Sur7p family defines novel cortical domains in *Saccharomyces cerevisiae*, affects sphingolipid metabolism, and is involved in sporulation." Molecular and Cellular Biology **22**(3): 927-934.
- Yu, F., Z. Wang, et al. (2012). "Lithium Ameliorates Neurodegeneration, Suppresses Neuroinflammation, and Improves Behavioral Performance in a Mouse Model of Traumatic Brain Injury." Journal of Neurotrauma **29**(2): 362-374.
- Yuen, K. W., C. D. Warren, et al. (2007). "Systematic genome instability screens in yeast and their potential relevance to cancer." Proceedings of the National Academy of Sciences of the United States of America **104**(10): 3925-3930.
- Zahringer, H., M. Burgert, et al. (1997). "Neutral trehalase Nth1p of *Saccharomyces cerevisiae* encoded by the NTH1 gene is a multiple stress responsive protein." FEBS Letters **412**(3): 615-620.
- Zarate, C. A., Jr., J. Singh, et al. (2006). "Cellular plasticity cascades: targets for the development of novel therapeutics for bipolar disorder." Biological Psychiatry **59**(11): 1006-1020.
- Zetoune, A. B., S. Fontaniere, et al. (2008). "Comparison of nonsense-mediated mRNA decay efficiency in various murine tissues." BMC Genet **9**: 83.
- Zetterberg, A., O. Larsson, et al. (1995). "What is the restriction point?" Current Opinion in Cell Biology **7**(6): 835-842.
- Zhang, B., O. Georgiev, et al. (2003). "Activity of metal-responsive transcription factor 1 by toxic heavy metals and H₂O₂ in vitro is modulated by metallothionein." Molecular and Cellular Biology **23**(23): 8471-8485.

- Zhang, B. and A. C. Zelhof (2002). "Amphiphysins: raising the BAR for synaptic vesicle recycling and membrane dynamics. Bin-Amphiphysin-Rvsp." Traffic **3**(7): 452-460.
- Zhang, X. Y., M. Varthi, et al. (2008). "The putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression." Molecular Cell **29**(1): 102-111.
- Zhang, Y. (2003). "Transcriptional regulation by histone ubiquitination and deubiquitination." Genes & Development **17**(22): 2733-2740.
- Zhang, Y., J. Y. Liang, et al. (1993). "Structural similarities between fructose-1,6-bisphosphatase and inositol monophosphatase." Biochemical and Biophysical Research Communications **190**(3): 1080-1083.
- Zhang, Z., J. Gu, et al. (2004). "How much expression divergence after yeast gene duplication could be explained by regulatory motif evolution?" Trends in Genetics : TIG **20**(9): 403-407.
- Zhang, Z., L. Zhou, et al. (2010). "Nonsense-mediated decay targets have multiple sequence-related features that can inhibit translation." Molecular Systems Biology **6**: 442.
- Zhao, J., W. Lin, et al. (2010). "The protein kinase Hal5p is the high-copy suppressor of lithium-sensitive mutations of genes involved in the sporulation and meiosis as well as the ergosterol biosynthesis in *Saccharomyces cerevisiae*." Genomics **95**(5): 290-298.
- Zheng, C., Z. Yan, et al. (2007). "Identification and characterization of a functional *Candida albicans* homolog of the *Saccharomyces cerevisiae* TCO89 gene." FEMS Yeast Research **7**(4): 558-568.
- Zimmer, S. G., A. DeBenedetti, et al. (2000). "Translational control of malignancy: the mRNA cap-binding protein, eIF-4E, as a central regulator of tumor formation, growth, invasion and metastasis." Anticancer Research **20**(3A): 1343-1351.