Elucidating the anti-cancer mechanisms for transition-state structure inhibitors of nucleoside phosphorylases methylthioadenosine-DADMe-immucillin A and forodesine

By

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Abstract

Transition-state structure analogues are among the most powerful chemical inhibitors discovered to date with picomolar efficacy for enzymes. The nucleoside analogue methylthioadenosine-DADMe-immucillin A (MTDIA) is an inhibitor of the enzyme methylthioadenosine phosphorylase (MTAP) in polyamine biosynthesis. The recently approved forodesine (Mundesine®) is an inhibitor of purine nucleoside phosphorylase (PNP) and purine synthesis. Although the targets of these drugs were known at the time of drug design, it is important to know the compendium of cellular perturbations resulting from use of these inhibitors. Several suspected mechanisms of MTDIA and forodesine in progression of apoptotic cell death have been identified but the underlying mechanisms initiating apoptosis remain elusive. We hypothesize that numerous cellular processes are affected in MTDIA and forodesine treatments given the importance of polyamine and purine synthesis in cancer cells. To elucidate the unsuspected mechanisms mediating anti-cancer activity, unbiased genomic analyses were employed using *Saccharomyces cerevisiae*. First, gene-gene interactions with *MEU1* (the MTAP orthologue in yeast) were determined using Synthetic Genetic Array methodology followed by assessment of drug-gene interactions with MTDIA treatment under a *MEU1* essential condition with MTA as the sole source of sulphur. Disruptions to suspected mechanisms of amino acid metabolism, carbohydrate metabolism, response to starvation, vesicle-mediated transport, vacuole fusion, lipid homeostasis, chromatin organisation, transcription, and translation were implicated well as unsuspected mechanisms of NAD⁺ dependent cellular processes, multi-vesicular body formation, endosomal transport, ion homeostasis, mitochondrion organisation, and cell cycle progression. Induction of autophagy was subsequently confirmed with MTDIA to validate the disruptions to vesicle-mediated transport, response to starvation, multi-vesicular body formation and vacuolar fusion. Reduction in ergosterol levels and disruptions to ergosterol biosynthetic proteins were confirmed with MTDIA and *meu1Δ* to validate disruptions to lipid homeostasis. To complement the genetic analyses, the abundance and localisation of proteins were evaluated in response to MTDIA or *MEU1*-deficiency. Disruptions to proteins

implicated in carbohydrate metabolism, methionine salvage, transcription, translation, transmembrane transport, lipid homeostasis, cell cycle and DNA repair were identified with *meu1Δ* and MTDIA. Key findings from the analysis of protein abundance and localization were the relocalisation of plasma membrane proteins and disruptions to vesicle mediated transport proteins consistent with the induction of autophagy and disruptions to proteins in homeostasis of all major lipid classes, further corroborating the findings of screening gene deletion mutants for elucidating drug mechanisms. To investigate the mechanisms of forodesine toxicity, genetic interactions with *PNP1* (the PNP orthologue in yeast) were determined using Synthetic Genetic Array methodology. Disruptions to amino acid metabolism, starvation responsive genes, vacuolar organisation and vesicle mediated transport, carbohydrate metabolism, lipid homeostasis, chromatin organisation, chromosome segregation, transcription, and translation were identified in response to *PNP1*-deficiency. Despite the introduction of several human genes and supplementation of metabolites required for forodesine bioactivity in humans, forodesine was not sufficiently bioactive in yeast to evaluate sensitivity of gene deletion mutants to forodesine. Overall, chemical genomic analyses in yeast with transition-state structure analogues MTDIA and forodesine effectively highlight the vast number of cellular processes affected by inhibition of a single target. Moreover, genome-wide pre-screening should be carried out in yeast to identify side-effects and secondary effects from drug target inhibition prior to assessing desired and undesired outcomes of highly specific drugs in human cells.

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List of Abbreviations

A549	Human alveolar basal epithelial cells	SGA	Synthetic genetic array
AraC	Cytarabine	SGD	Saccharomyces Genome Database
Atg8	Yeast orthologue of LC3	SMS	Spermine
CCL	Chronic lymphatic leukaemia	SRM	Spermidine
CM	Complete minimal	SSAT	Spermidine/spermine-N1-acetyltransferase
CNT2	Concentrative nucleoside transporter 2	TOR	Target of rapamycin
Ctv	Cytosol to vacuole transport	WT	Wild type
dCK	Deoxycytidine kinase	xxxΔ	Non-essential gene deletion collection
dCyt	Deoxycytidine	YPD	Yeast/peptone/dextrose
DFMO	L-α-difluoromethylornithine		
dGuo	Deoxyguanosine		
DMSO	Dimethyl sulfoxide		
ENT1	Equilibrative nucleoside transporter 1		
EV	Empty vector		
FIC	Fractional inhibitory concentration		
Forodesine	Immucillin-H		
G418	Geneticin		
GC-MS	Gas chromatography–mass spectrometry		
GFP	Green fluorescent protein		
GO	Gene ontology		
HSV-TK	Herpes simplex virus type-1 thymidine kinase		
IC20	20% growth inhibitory concentration		
IMP	Inosine monophosphate		
kanR	Kanamycin resistance		
Kd	Dissociation constant		
Ki	Equilibrium constant		
KIE	Kinetic isotope effect		
MAU	Met/Ade/Ura		
MAUC	Met/Ade/Ura/Cys		
Meu1	MTAP orthologue in yeast		
MIC	Minimum inhibitory concentration		
MTA	Methylthioadenosine		
MTAP	Methylthioadenosine phosphorylase		
MTDIA	Methylthioadenosine-DADMe-immucillin A		
NAT	ClonNAT		
natR	NAT resistance		
ODC	Ornithine decarboxylase		
ORF	Open reading frame		
PI	Phosphatidylinositol		
PI3K	Phosphoinositide 3-kinase		
PI3P	Phosphatidylinositol 3-phosphate		
PNP	Purine nucleoside phosphorylase		
SC	Synthetic complete		
SD	Synthetic dropout		
SD-N	Starvation media		

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Chapter 1: Literature review

1.1 Burden of cancer and current cancer therapies

1.1.1 Burden of cancer

Cancer is the leading cause of death in both economically developed and developing countries (Jemal et al., 2011). In 2012, there were 14.1 million new cases of cancer world-wide which contributed to 8.2 million estimated deaths with lung, liver, stomach and colorectal cancers being the major causes of cancer related deaths (Torre et al., 2015). The number of new cases of cancer is expected to rise to 21.6 million and 13 million deaths annually by 2030 due to growth and increased lifespans of the current population (Ferlay et al., 2015). On average \$5.6 billion is spent every year on cancer research by National Institutes of Health, which is greater than the NIH budget for cardiovascular disease, infectious disease and Alzheimer's disease combined ("NIH Categorical Spending -NIH Research Portfolio Online Reporting Tools (RePORT)," 2015). The current consensus is that a large fraction of these deaths can be prevented by better management of cancer risk factors, early detection, physical activity and healthy dietary practises.

Cancer is the collective name given to a group of related diseases leading to development of an abnormal cell mass by uncontrollable cell division, replicative immortality and resistance to cell death (Perez-Herrero & Fernandez-Medarde, 2015). It is a result of both genetic and environmental factors. The development of cancer is a multi-step process and is a result of gain of function mutations to proto-oncogenes that promote cell proliferation and differentiation as well as loss of function mutations in tumour suppressor genes that promote growth inhibition, arrest and cell death (Hanahan & Weinberg, 2000). These changes are further accentuated by changes to cell energy metabolism and immune evasion (Hanahan & Weinberg, 2011). Clonal expansion of these cells with unregulated cell division eventually leads to genetic instability and different evolutionary pressure from the tumour environments, which together result in multiple mutations and tumour heterogeneity (Marusyk et al., 2012). The access to vasculature spreads the cancerous cells throughout the body leading to development of new and diverse tumours away from the primary site (Hanahan & Weinberg, 2011).

The spread of cancer into vital organs leads to gradual loss of organ function and degeneration of health like other age-related pathologies resulting in death (Cooper, 2000).

1.1.2 Current cancer therapies, undesired effects and resistance

There are several approaches to manage and treat cancers. If diagnosed early, cancer is treatable either by surgical removal and radiotherapy that are often applied to localised tumours and are the most effective treatment for cancer (Perez-Herrero & Fernandez-Medarde, 2015). Upon metastasis, the treatment of cancers requires the use of small molecular inhibitors or monoclonal antibodies capable of reaching every organ via the circulatory system (Perez-Herrero & Fernandez-Medarde, 2015). The most readily used approach to treat metastatic cancers is chemotherapy and it often targets rapidly dividing cells. It uses cytotoxic compounds to inhibit processes in the cell division cycle such as DNA replication with either DNA alkylating agents (cisplatin, temozolomide), topoisomerase II with inhibitors (adriamycin, doxorubicin) and nucleoside analogues (gemcitabine, 5-FU), and cell division machinery with inhibitors of microtubule dynamic (paclitaxol, nocodazole) (Zhao et al., 2013). Targeting these key cellular processes essential to all cells with chemotherapeutics leads to numerous undesired side effects by its toxic action on normal cells. As a consequence, cancer sufferers have to endure unwanted side effects of chemotherapy that can range from minor discomforts such as fatigue, vomiting and nausea to more severe side effects such as myelosuppression and organ damage which can be life threatening and a hindrance to continuation of chemotherapy (Attar et al., 2000). Chemotherapy can also cause permanent damage to nerves, lead to sterility and in rare occasions lead to development of other types cancers (Park, 2014). Some of these unwarranted side-effects are managed by administering cytoprotectants such as dexrazoxane and amifostine (Hensley et al., 2009). To reduce these side effects, novel approaches are being developed to specifically target cancerous cells and are known collectively as targeted therapy.

Molecularly targeted therapy from here on is referred to as targeted therapy, which unlike chemotherapy, exploits the differences in cancer cell metabolism. There are several targeted therapies available for cancer treatment including hormone therapies, signal transduction inhibitors

(tyrosine kinase and mTOR inhibitors), gene expression modulators, apoptosis inducers, angiogenesis inhibitors, and toxin delivery molecules (<https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/targeted-therapies-fact-sheet>, 2016). Targeted therapies are deliberately designed based on interactions with a desired molecular target that is either differentially expressed or altered in the cancer cell. The fusion protein Bcr-Abl kinase or mutant allele BRAFV600E are examples of such targets that are expressed only in cancer cells (An et al., 2010; Cantwell-Dorris et al., 2011). Most targeted therapies modulate a cellular process that cancer cells depend on for cell proliferation such as polyamine and purine biosynthesis inhibitors investigated in this dissertation. Other promising alternatives to chemotherapy and targeted therapy include immunotherapy that uses engineered T-cell therapy, cancer vaccines (*e.g.* Sipuleucel-T (Anassi & Ndefo, 2011)), oncolytic viruses (*e.g.* talimogene laherparepvec (Rehman et al., 2016)) and small molecule immune check-point inhibitors (anti-CTLA-4 Ipilimumab, anti-PD-1 nivolumab (Boutros et al., 2016)) to improve cancer cell recognition and boost the immune system to eliminate the cancer.

Kinase inhibitors are one of the most pursued small molecules for targeted cancer therapy in the recent decade. There are over 900 human kinases that catalyse the transfer of γ -phosphate group of ATP to target proteins to initiate signal transduction required for an array of cellular processes including cell proliferation, survival and differentiation (Wu et al., 2015). BCR-Abl kinase inhibitors (imatinib and dasatinib), ErbB inhibitors against EGFR and HER2 kinases (lapatinib) and multiple kinase inhibitor (vandetanib) against EGFR, VEGFR and RET, lipid kinase inhibitor (idelalisib) and serine/threonine kinase B-Raf inhibitors (vemurafenib and dabrafenib) are some of the commonly used kinase inhibitors (Wu et al., 2015). In addition to treatment of tumours, kinase inhibitors are also effective against inflammatory diseases, central nervous system disorders, cardiovascular diseases (Wu et al., 2015). From the success of tyrosine kinase inhibitors, several other small molecular inhibitors that modulate the activity of proteins in cancer have become the focus of attention. One such class of small molecules that has gained recent attention is transition state structure inhibitors.

1.1.3 Transition state structure analogues as antitumor therapeutics

Transition state structure analogues are chemically stable structures that by design resemble an enzymatic transition state of a substrate that has a lifetime in femtoseconds (Schramm, 2013b). The enzymatic transition state structures are determined by kinetic isotope effects (KIEs) of labelled enzymes and reactants, combined with computational chemistry and the transition state structure analogues, are synthesised to closely match the Van der Waal's geometry and electrostatic features of transition state structures (Schramm, 2011; Schramm, 1999). By resembling a stable transition state structure, transition state analogues lower the activation barrier that is required for the reaction with the enzyme and bind more tightly than the substrate in the ground state (Wolfenden & Radzicka, 1991). For some transition state structures, a single substituent is responsible for reaction with an enzyme and changing that one substituent can cause a severe decrease in binding affinity (Grubmeyer et al., 1989). Others may involve interactions with many substituents and these can be modified in the transition state analogue to further match the environment of the binding pocket in improve the binding affinity and specificity for the enzyme (Janes & Schulz, 1990). Transition state analogues are among the most powerful chemical inhibitors of enzymes discovered to date (Schramm, 2011).

Formycin, an inhibitor of AMP nucleoside hydrolase, is a notable transition state structure analogue that was determined by analysis of KIEs (DeWolf et al., 1979). Several other transition state structure analogues have since been developed by analysis of KIEs. Notable of these are purine biosynthesis and polyamine biosynthesis inhibitors designed by the laboratory group of Professor Vern Schramm (Albert Einstein College of Medicine) and synthesised by members of the Ferrier Institute (Victoria University of Wellington) (Evans et al., 2004; Evans et al., 2003). Methylthio-DADMe-Immucillin A (MTDIA) is an inhibitor of polyamine biosynthesis and a promising preclinical anticancer agent that is evaluated for therapeutic mechanisms in chapters 2 and 3. Immucillin H (forodesine) is an inhibitor of purine nucleoside biosynthesis for which therapeutic investigations were investigated in chapter 4 and was approved for treatment of refractory leukaemia in Japan during the writing of this dissertation.

1.2 Polyamine biosynthesis and MTAP inhibition as target for cancer therapy

1.2.1 Polyamine biosynthesis in mammals

Putrescine, spermidine and spermine are polyamines that are nucleophilic polycationic molecules found in all eukaryotic cells, which cumulatively comprise 7-10% of all cellular content (Gugliucci, 2004). The structure and sheer volume of polyamines results in interactions with many negatively charged molecules including DNA, RNA and proteins (Minois et al., 2011), which associate polyamines with many important cellular processes. These include DNA replication, transcription and translation as well as affecting the activity of many kinases and phosphatases to modulate cell growth, proliferation and survival (Minois et al., 2011).

The levels of polyamines are tightly regulated through synthesis, catabolism and transport (Fig. 1.1) (Pegg, 2009). Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthesis pathway and it catalyses the conversion of ornithine to putrescine (Woster, 2006). ODC is rapidly degraded by ODC-antizyme and the 26S proteasome to modulate the levels of putrescine (Murakami et al., 1992; Kahana et al., 2005). Putrescine is then converted to spermidine by aminopropyltransferase spermidine synthase, which is subsequently converted to spermine by another closely related aminopropyltransferase spermine synthase (Woster, 2006). 5'-methylthioadenosine is produced as a by-product during spermine and spermidine synthesis from the co-substrate decarboxylated S-adenosyl methionine. 5'-methylthioadenosine is a potent inhibitor of the aminopropyl transfer process and must be rapidly hydrolysed by 5'-methylthioadenosine phosphorylase (MTAP) to maintain the forward reaction (Raina et al., 1984; Pajula et al., 1979b). Further control of polyamine levels are exerted through acetylation of spermidine and spermine by spermidine/spermine-N1-acetyltransferase (SSAT) and subsequent oxidative deamination by acetylpolyamine oxidase (PAO) (Woster, 2006). Spermine is also converted back to spermidine by spermine oxidase (SMO). SMO and SSAT/PAO provide interconversion of polyamines to maintain the appropriate levels of each of the polyamines (Pegg, 2009). Several polyamine transport mechanisms

Although polyamine biosynthesis is highly characterised, the cellular mechanisms by which different polyamines exert their function have not been completely elucidated. It is well established that they are required for normal cellular homeostasis (Woster, 2006). The depletion of polyamine levels are observed with ageing and impressively, supplementation with putrescine and spermidine increases cellular survival (Vivó et al., 2001; Minois et al., 2011). In yeast, chronological ageing leads to an accumulation of acetic acid that induces apoptosis like phenotype, which can be alleviated by exogenous spermidine (Kaeberlein, 2009). Likewise, the polyamine system is highly upregulated in

various human diseases such as cystic fibrosis, neurodegenerative disease including Parkinson's and Alzheimer's to cancers, carcinoma, melanoma and lymphoma, and depletion of polyamines induce quiescence and apoptosis (Wallace & Fraser, 2004; Minois et al., 2011). Thus, presents a target for cancer therapy.

Several steps in polyamine biosynthesis, transport and metabolism have been targeted by inhibitors with varying therapeutic anti-cancer effects from quiescence to cytotoxicity. The catalyst of the first and rate-limiting step in polyamine biosynthesis (ODC) was the first to be targeted and α -difluoromethylornithine (DFMO) has been the most effective and the only FDA approved inhibitor of ODC to date (Metcalf et al., 1978). The rapid turnover of ODC coupled with poor transport leads to a requirement of high DFMO concentrations to maintain ODC inhibition (Casero & Marton, 2007; O'Shaughnessy et al., 1999). Subsequent synthesis of other symmetrically and unsymmetrically substituted alkylpolyamines capable of inhibiting the ODC such as AdoMet-DC (5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine) as well as spermidine and spermine aminopropyltransferases (S-adenosyl-1,8-diamino-3-thiooctane and S-adenosyl-1,12-diamino-3-thio-9-azadodecane respectively) have been generated (Tang et al., 1981; Woster et al., 1989). Some but not all alkylpolyamines induce cell-type specific induction of SSAT expression, in some cases more than a 1000-fold (Woster, 2006). The subsequent generation of hydrogen peroxide by deamination activity of PAO is thought to be the mechanism by which these compounds induce cytotoxicity (Ha et al., 1997). The cause of toxicity in non-SSAT inducing alkylpolyamines is yet to be determined.

1.2.3 MTAP in methionine salvage and synthesis of S-adenosyl methionine

Methylthioadenosine phosphorylase (MTAP) encodes the initial enzyme in the methionine salvage pathway. It catalyses the conversion of 5'-methylthioadenosine (MTA) that is generated as a by-product of polyamine biosynthesis to adenine and methylthioribose-1-phosphate (Avila et al., 2004). 5'-methylthioribose-1-phosphate is further processed in subsequent reactions to salvage methionine (Backlund & Smith, 1981; Finkelstein, 1990). Methionine is also re-synthesised from homocysteine by betaine-homocysteine methyltransferase and 5-methyltetrahydrofolate homocysteine

synthesis (Stoner & Eisenberg, 1975; Hall & Dietrich, 2007), a ribosyl groups for post-translational modification of tRNA (Iwata-Reuyl, 2003), a methylene donor for synthesis of cyclopropane fatty acids (Taylor & Cronan Jr, 1979), a source for re-synthesis methionine and glutathione formation (Lu, 2000) and a source for generation of 5'-deoxyrybosyl radicals from reaction with iron sulphur clusters containing metalloenzymes (Cheek & Broderick, 2001). More importantly, 90% of S-adenosyl methionine (AdoMet) is used in methyltransferase reactions where AdoMet acts as a methyl donor for an array of molecules including nucleic acids, proteins and lipids (Tehlivets et al., 2013). S-adenosyl methionine is therefore an important molecule generated by polyamine biosynthesis. During methylation, S-Adenosyl homocysteine is generated as an inhibitor of methyltransferase reactions involving S-adenosyl methionine and is therefore rapidly metabolised to adenosine and homocysteine (Finkelstein, 1990).

1.2.4 MTAP as a target for cancer

Although numerous inhibitors of polyamine biosynthetic pathway have been generated, the recycling of the toxic by-product 5'-methylthioadenosine by 5'-methylthioadenosine phosphorylase (MTAP) has not been investigated as a target for therapeutic intervention until recently (Basu et al., 2007). MTAP is a tumour suppressor located in the chromosome 9p21-22 region (Carrera et al., 1984). Activity of MTAP is lost in numerous human cancers either through methylation of its promoter or is co-deleted with flanking tumour suppressors (*e.g.*, CDKN2A that encodes P15, p16 and p19) and interferon genes (Cairns et al., 1995; Chen et al., 1996). MTAP deficiency is common in numerous solid tumours including pancreatic, lung, endometrial, metastatic melanoma, oesophageal carcinoma, biliary tract cancer, gastrointestinal stromal tumours, soft tissue and osteosarcoma, gliomas, and in hematologic malignancies including adult T-cell leukemia, B-lineage ALL, T-cell acute leukemia, diffuse large cell lymphoma and mantle cell lymphoma (Bertino et al., 2011).

Ectopic MTAP expression in MTAP negative cancers led to inhibition of anchorage-independent growth as measured by colony formation on soft agar plates and suppression of tumour formation in SCID mice in both the fibrosarcoma cell line HT1080 and the breast adenocarcinoma cell

line MCF-7 (Christopher et al., 2002; Tang et al., 2015). Decreases in levels of all three major polyamines and the ratio of putrescine to total polyamines were observed following MTAP re-expression for MCF-7 cells but not HT1080 cells. The ODC inhibitor DFMO inhibited anchorage-independent growth of MTAP negative MCF-7 cells but not MTAP negative HT1080 cell (Christopher et al., 2002; Tang et al., 2015). In contrast, expression of catalytically inactive MTAP in HT1080 cells inhibited anchorage-independent growth and promoted wound healing to similar levels of catalytically active MTAP but not in MCF-7 cells (Christopher et al., 2002; Tang et al., 2015). These results suggest that MTAP exerts its tumour suppressor activity via at least two independent mechanisms: the enzymatic mechanism that involves processing of MTA and the non-enzymatic mechanism that was observed in HT1080 cells.

1.2.5 MTDIA: transition state analogue of MTA

Methylthio-DADMe-Immucillin A (MTDIA) is a transition state structure analogue of 5'-methylthio adenosine (MTA) bound by the human enzyme 5'-methylthio adenosine phosphorylase (MTAP). MTDIA is one of most potent inhibitors of MTAP generated by transition state structure analysis and is the most characterised (Singh et al., 2004). It is a 86 pM K_i^* inhibitor of human MTAP *in vitro* (Schramm, 2011). It is currently preclinical with proven *in vivo* efficacy in mouse xenografts models for human lung, prostate, colon, cervical, head and neck, and triple negative breast cancers, as well as *in vitro* efficacy in various human cancer cell lines (Basu et al., 2007; Basu et al., 2011). MTAP inhibition by MTDIA leads to an accumulation of MTA, which inhibits polyamine biosynthesis (specifically the syntheses of putrescine, spermidine and spermine) and consequently the recycling of S-adenosyl methionine (Basu et al., 2011) (Fig. 1.1). One or more of these processes are responsible for subsequent cellular processes such as G2/M arrest, loss of mitochondrial membrane integrity and cleavage of PARP and caspases resulting in apoptosis (Basu et al., 2011). The initiation of apoptosis however is cell type specific as normal fibroblast cells do not undergo apoptosis nor activate caspases, PARP or other cellular alterations observed with increased levels of MTA (Basu et al., 2007). The

genetic mechanisms underlying the initiation of these processes are not understood and are the subject of this dissertation.

1.3 Purine biosynthesis as a target for cancer therapy

1.3.1 Structure and function of purine nucleoside phosphorylase (PNP) in humans

Purine nucleoside phosphorylase (PNP) is a ubiquitous protein belonging to the nucleoside phosphorylase-I family and is an enzyme of the purine salvage pathway (Parks Jr & Agarwal, 1972). It is a 90 kDa enzyme that exists as homotrimer in the cytosol for which the crystal structure has been solved (Toro et al., 2006). Each subunit consists of a disoriented β structure flanked by seven α helices with the catalytic sites found between the subunit-subunit boundaries (Canduri et al., 2005). PNP in the β structure configuration is specific for purine nucleosides. PNP is differentially expressed in tissues and is most abundant in peripheral lymphocytes and granulocytes (Toro et al., 2006; Carson et al., 1977). It reversibly metabolises purine nucleosides deoxyguanosine, deoxyinosine, guanosine, and inosine to their nucleobases and 2'-ribose (or deoxyribose) -1 -phosphate (Kim et al., 1968). Guanine and hypoxanthine are phosphorylated to GMP and IMP by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and subsequently to GTP and incorporated into RNA. Alternatively, the purine nucleobases can be metabolised by xanthine oxidase and excreted as uric acid (Toro et al., 2006).

A genetic deficiency of PNP was first identified in 1975 in a five year old child presenting with T-cell lymphopenia and erythrocytes lacking the PNP enzyme activity (Giblett et al., 1975). The mutation was later characterised as a consequence of a homozygous 265G>A mutation in exon 3 of the PNP gene resulting in a Glu89Lys amino acid substitution (Williams et al., 1987). The genetic deficiencies in the purine salvage pathway is one of the common causes of autosomal recessive Severe Combined ImmunoDeficiency (SCID) (Hirschhorn et al., 2013). In addition to the characteristic loss of T-cells with age, some SCID cases also present with neurological defects, bone marrow development abnormalities, poor humoral immunity and autoimmunity (Hirschhorn et al., 2013).

Loss of PNP function leads to accumulation of the nucleoside substrates of PNP in all tissue types, body compartments, plasma and urine (Chantin et al., 1996; Hershfield & Mitchell, 1995). Of these deoxyguanosine has an alternative fate where it is phosphorylated to dGMP by deoxynucleoside kinases and becomes trapped within the cell. dGMP is subsequently phosphorylated to dGTP by two non-rate limiting nucleotide kinases leading to pathologically elevated levels dGTP (Cohen et al., 1978). It is the accumulation of dGTP that is toxic to T-cells. The accumulation of dGTP leads to the disruption of the dNTP pool and inhibition of DNA synthesis leading to an induction in apoptosis (Balakrishnan et al., 2006). The loss of PNP activity also leads to depletion of GTP and uric acid levels (Wortmann et al., 1979). Conversely, the accumulated dGMP can be catabolised back to deoxyguanosine by 5'-nucleotidase. These enzymes provide important balance to the dGTP levels.

1.3.2 PNP inhibitors and other deoxyguanosine analogues to treat T-cell activation

The importance of PNP and deoxyguanosine (dGuo) in T-cell development from the literature just reviewed on T-cell lymphopenia reveals opportunities for regulation of diseases characterised by T-cell proliferation. Several PNP inhibitors and deoxyguanosine analogues that elevate dGuo in plasma have been assessed *in vivo* in animal models and in patients in clinical trial (Ravandi & Gandhi, 2006). Well known PNP inhibitors allopurinol, 6-mercaptopurine, 6-methoxypurine and acyclovir showed poor affinity for human and bovine PNP and poor growth inhibition in cell lines (Krenitsky et al., 1968; Tuttle & Krenitsky, 1984). From these early discovered inhibitors it was observed that >95% continuous inhibition of PNP is required for sufficient dGTP accumulation to abrogate T-cell function and proliferation (Gandhi, 2009; Morris & Montgomery, 1998). The C-8 substituted purine analogues (8-aminoguanosine and 8-iodoguanosine) and the dGuo analogue 8-amino-9-(2-thienylmethyl) guanine showed potent activity against T-cells in primary cell cultures but not in animal models (Gilbertsen et al., 1987; Kazmers et al., 1981). The subsequent, N-7 substitutions of inosine and guanosine were then developed based on the crystal structure of PNP, which resulted in clinical trials of BCX-34 (peldesine) that demonstrated limited activity as it failed to achieve >95% continuous inhibition of PNP (Viegas et al., 2000; Ravandi & Gandhi, 2006).

Following the partial success of these inhibitors, a series of 9-deazanucleotide analogues (termed Immucillins) were designed to mimic the transition state structure of nucleosides inosine and guanosine bound to PNP (Schramm, 2002). Immucillin-H (forodesine) ($K_d \sim 56$ pM) and Immucillin-G ($K_d \sim 42$ pM) are potent inhibitors of human PNP with dissociation constants in the picomolar range (Evans et al., 2003). Forodesine has undergone 14 phase I and II clinical trials to evaluate efficacy, safety, and pharmacokinetics in patients with lymphatic leukaemia and lymphoma (<https://clinicaltrials.gov/ct2/results?term=bcx-1777>). On 30 March 2017, forodesine was approved for treatment of recurrent/refractory peripheral T-cell lymphoma in Japan (<http://www.mundipharma.com.sg/2017/03/30/mundipharma-wins-approval-antineoplastic-agent-mundesine-treatment-relapsedrefractory-peripheral-t-cell-lymphoma-japan/>, 2017). Nelarabine is another promising deoxyguanosine analogue that causes accumulation of dGTP in plasma. It is a PNP- and deoxycytidine kinase (dCK)-resistant prodrug of arabinosyl guanine (AraG) that is converted to the active form by adenine deaminase (Lambe et al., 1995). It is phosphorylated exclusively by deoxyguanosine kinase to generate toxic AraG-GTP and it inhibits the growth of B-cells and monocytes as well as T-cells (Lambe et al., 1995). It has undergone extensive clinical trials and has been approved by the FDA for treatment of patients with T-cell acute lymphoblastic leukaemia and T-cell lymphoblastic lymphoma (Reilly & Kisor, 2009; Ravandi & Gandhi, 2006).

1.3.3 High deoxyguanosine kinase, low 5' nucleosidase activity and nucleoside transport determines T-cell toxicity

Although the PNP activity is not dissimilar between the B and T lymphocytes, the activity of the PNP inhibitors differ drastically between these lymphocytes (Fridland & Verhoef, 1985). This is attributed to the 2-3-fold greater deoxyguanosine kinase activity and reduced 5'-nucleotidase activity in T-cells leading to pathologically elevated dGTP formation in the presence of inhibitors (Osborne & Scott, 1983; Madrid-Marina et al., 1993; Kazmers et al., 1981) (Fig. 1.4). The deoxyguanosine phosphorylation is conferred by the cytosolic/nuclear dCK and the mitochondrial deoxyguanosine kinase (dGK) leading to accumulation of dGTP in their respective organelles (Eriksson et al., 2002) (Fig.

1.3). The dCK can phosphorylate all four deoxyribonucleotides with varying catalytic efficiency, while dGK phosphorylates only deoxyadenosine and deoxyguanosine nucleosides (Arnér & Eriksson, 1995). dGK can also phosphorylate dCyt but only at 2% of its efficiency for dGuo. There is some contradiction as to whether it is the dGTP accumulation in the mitochondria or the cytosol that is responsible for the T-cell toxicity. Most nucleoside analogues phosphorylated by dGK are also phosphorylated by dCK

Figure 1.3 – **Transport and metabolism of nucleosides in humans.** CNTs, concentrative nucleoside transporters; ENTs, equilibrative nucleoside transporters; dA, deoxyadenosine; dG, deoxyguanosine; dC, deoxycytidine; dT, deoxythymidine; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; TK1, thymidine kinase 1; TK2, thymidine kinase 2; AK, adenylate kinase; AK3, adenylate Kinase 3; GMPK, deoxyguanosine monophosphate kinase; NDPK, nucleotide diphosphate kinase.

with greater efficiency except for AraG and 2-chloro-2'-deoxyadenosine (CdA). dCK is expressed at much higher levels than dGK in actively dividing cells and lymphocytes (Arnér & Eriksson, 1995) and the mechanism of resistance to these compounds including AraG and CdA have been through down-regulation of dCK and not dGK (Curbo et al., 2001). The resistance to deoxyguanosine in the absence of PNP where deoxyguanosine phosphorylase activity is concerned is due to down-regulation of dCK and not dGK (Ullman et al., 1979). In summary, dCK is predominantly responsible for the accumulation of deoxyguanosine monophosphate in T-cells in response to loss or inhibition of PNP activity. In addition to deoxyguanosine phosphorylation, deoxyguanosine transport and the transport of PNP inhibitors are equally important to T-cell toxicity and PNP deficient cells with defective nucleoside transport are resistant to deoxyguanosine (Ullman et al., 1979). In T lymphoblast (CCRF-CEM) cells, forodesine and deoxyguanosine entry into the cell is carrier-mediated. The equilibrative nucleoside transporter 1 (ENT1) is predominantly responsible for forodesine transport and ENT2 to a lesser degree (Huang et al., 2008). Deoxyguanosine transport is predominantly mediated by the concentrative nucleoside transporters 2 (CNT2) but is also transported by ENT1 and ENT2 (Huang et al., 2008). In addition, ribonucleotide reductase (RR) resistance to feedback inhibition by dGTP have also been identified and confers resistance to deoxyguanosine (Ullman et al., 1979).

1.3.4 Proposed mechanisms for forodesine activity in T-cells

Forodesine (Immucillin H or BCX-1777) is a non-cleavable, transition state structure analogue of inosine bound human PNP and binds human PNP $\sim 7 \times 10^5$ fold more strongly than its native substrate inosine with a 56 pM K_d (Schramm, 2004). Forodesine induces apoptosis in activated T-cells at low nanomolar concentrations and lacks activity against resting T-cells and other cell types (Kicska et al., 2001). The proposed mechanism for forodesine mimics the loss of PNP activity. Forodesine and deoxyguanosine treatment of T-acute lymphoblastic leukemia (CEM-SS) cells lead to 154-fold increase in the accumulation of dGTP and an 8-fold increase in dATP accumulation (Bantia et al., 2003). Lamivudine ((-)-β-L-2',3'-dideoxy-3'-thiacytidine) mediated depletion of dGTP was able to partially rescue the forodesine toxicity in CEM-SS cells, although it led to a further increase in dATP (8-30 fold)

(Bantia et al., 2003). This suggests that it is the accumulation of dGTP and not dATP that is responsible for the main mechanism of forodesine mediated toxicity. The proposed mechanisms for dGTP-mediated toxicity is that it binds and allosterically facilitates the ribonucleotide reductase binding to ADP and conversion to dADP (Ahmad & Dealwis, 2013). dADP is subsequently converted to dATP, which then binds and inhibits ribonucleotide reductase leading to the depletion of dCTD and dTTD pools and inhibition of DNA synthesis. However, no such disruptions were observed to pools of deoxycytidine and deoxythymidine following forodesine treatment, rather, it lead to an increase in deoxycytidine and deoxythymidine levels (Bantia et al., 2003). Marrow stromal cells treated with forodesine accumulate dGTP to levels similar to chronic lymphatic leukaemia (CCL) cells but without apoptosis (Balakrishnan et al., 2010b). Therefore, the accumulation of dGTP alone is also not sufficient to induce apoptosis and the exact mechanisms leading to apoptosis are not fully understood.

Following initiation of apoptosis, several mechanisms involved in the progression of mitochondria-dependent apoptotic cell death have been characterised in PNP-deficient human and mouse cells. Mitochondrial membrane dissipation and release of cytochrome C have been identified in CD4+ and CD8+ mouse thymocytes (Arpaia et al., 2000; Papinazath et al., 2011). Nuclear DNA fragmentation has also been identified in these cells. Several changes to proteins also involved in the intrinsic apoptotic pathway were identified such as stabilisation of p53 and activation of downstream p21, activation of caspases 3, 8 and 9, subsequent PARP cleavage and downregulation of antiapoptotic BCL-2 and MCL-1 and upregulation of proapoptotic BIM (Balakrishnan et al., 2006; Alonso et al., 2009). Forodesine-treated cells were also able to complete apoptosis when p53 function was compromised and this was via upregulation of p73 and expression of proapoptotic genes FOXO1 and FOXO3A (Alonso et al., 2009).

In addition to induction of apoptosis, a positive correlation was observed in cells undergoing apoptosis and the phosphorylation of dCK in forodesine-treated CLL cells (Alonso et al., 2009). In addition to the increases in dGTP and dATP, significant decreases in ribonucleotide levels of ATP (20%) and GTP (50%)

Figure 1.4 – **Proposed mechanisms for forodesine and loss of PNP.** dGuo, deoxyguanosine.

were observed for forodesine-treated CLL cells. Reduction in both RNA and protein synthesis was also observed with forodesine and dGuo in CCL cells (Balakrishnan et al., 2010b). The nuclear DNA fragmentation observed in PNP-deficient cells was also observed in treatment with a nucleoside analogue that accumulate dATP, deplete NAD⁺ and ATP pools, confer deficiency in DNA repair (Carson et al., 1986). However, the underlying genetic mechanisms and cellular processes that link dGTP accumulation and apoptosis are yet unknown.

1.4 Yeast as a model for cell metabolism-targeted cancer therapeutics

1.4.1 Modifications to energy metabolism in cancer

Modifications to energy metabolism are considered one of the hallmarks in development of cancer and are consequences of some of the same oncogenic changes driving tumour development (Hanahan & Weinberg, 2011). Under aerobic conditions, cells use glycolysis to convert glucose to pyruvate and then to CO₂ in the mitochondria by oxidative phosphorylation. In the majority of tumours, energy metabolism is reprogrammed to preferentially use lactate fermentation even in the presence of O₂ and unimpaired mitochondria, which is termed the Warburg effect or aerobic glycolysis (Warburg, 1956; Ferreira, 2010). Under aerobic glycolysis, ~85% of glucose in cancer cells is utilised for synthesis of lactate contributing to as high as 40 times the normal level of lactate in some cancers (Holm et al., 1995; Vander Heiden et al., 2009). Only 5% is transported to the mitochondria as pyruvate

for oxidative phosphorylation. The remaining 10% is diverted to biosynthetic pathways upstream of pyruvate production including glycolysis, nucleotide and fatty acid synthesis (DeBerardinis et al., 2007). To maintain a higher metabolic flux under hypoxic conditions, lactate is transported out of the cells along a concentration gradient. In oxygenated environments, lactate is recaptured by tumour cells via monocarboxylate transporter 1 and converted to pyruvate by lactate dehydrogenase B and is used as an energy source instead of glucose (Corbet & Feron, 2017). The heterogeneity in the tumour microenvironment leads to different metabolic preferences of cells within a tumour and coexistence of cancer cells with normal levels of mitochondrial oxidation and cancer cells that preferentially generate lactate as an end product (Hensley et al., 2016).

In addition to aerobic glycolysis, proliferating cells at least partly rely on endogenous pathways for synthesis of amino acids, fatty acids and cholesterol, although these can also be supplied by the bloodstream (Beloribi-Djefalia et al., 2016; Gaglio et al., 2011). The majority of metabolic flux in proliferating cells is dedicated to the synthesis of proteins and lipids with several enzymes in lipid synthesis crucial for proliferating cancer cells. Cholesterol is an essential sterol in the membranes of mammals that is required for membrane fluidity, localisation of membrane proteins and establishment of local asymmetry and membrane curvature. Cholesterol can be esterified and stored as triacylglycerol (TAG) in the lipid droplets to serve as a reserve for acetyl-CoA for use in energy production and synthesis of more complex lipids (Röhrig & Schulze, 2016). Acetyl-CoA liberated from fatty acids also constitutes another source of TCA cycle intermediates and reducing equivalents such as NADH and FADH₂. The uptake and conversion of fatty acids into acyl-CoA and subsequent conversion to acetyl-CoA by beta-oxidation in the mitochondria are upregulated in some cancers (Camarda et al., 2016).

Glutamine is another key molecule for cancers as it can provide both oxaloacetate and acetyl-CoA to maintain the TCA cycle independent of glucose and pyruvate as well as for synthesis of lipids (Corbet & Feron, 2017). Glutamine is the obligate amide donor for at least three independent enzymatic reactions in purine biosynthesis and two reactions in pyrimidine biosynthesis where it is

converted to glutamate (Wise & Thompson, 2010). Glutamate subsequently serves as an amine donor for α -ketoacids for synthesis of non-essential amino acids by transamination. When synthesis exceeds demand, glutamate can also be exchanged for essential amino acids leading to activation of mTOR to promote protein synthesis and cell growth (Chen & Cui, 2015). Both glutamine synthesis and transport of exogenous glutamine are required for survival of some cancers (Gross et al., 2014).

1.4.2 Yeast as a model for cancer cell metabolism and human disease

The exponentially growing Baker's yeast (*Saccharomyces cerevisiae*) share many metabolic similarities with proliferating cancer cells. These yeast cells possess the ability to adapt its metabolism to its environment and switch between respiratory and fermentative growth (the Crabtree effect). In the presence of high glucose, it preferentially uses fermentation as the main metabolic pathway and represses oxidative phosphorylation, similar to the aerobic glycolysis in tumour cells (Diaz-Ruiz et al., 2011). The central carbon metabolism in yeast is evolutionarily conserved and uses glucose and glutamate catabolism for growth and energy production (Natter & Kohlwein, 2013). Sterol synthesis in yeast is also essential for viability (Bard et al., 1993), and along with glycerolipids and sphingolipids, are synthesised from acetyl-CoA generated by the central carbon metabolism and oxidation of fatty acids by conserved mechanisms (Natter & Kohlwein, 2013).

Yeast has been used to study several other cancer relevant cellular processes including DNA replication (DiNardo et al., 1984), recombination and repair (Lisby et al., 2001), genome stability (Yuen et al., 2007; Stirling et al., 2011), cell division (Hartwell et al., 1974; Nurse, 1975), autophagy (Takeshige et al., 1992) and programmed cell death (Carmona-Gutierrez et al., 2010) as the cellular machinery for such basic processes are conserved from yeast to humans. Yeast has also been used to elucidate novel cancer biology where functional orthologs or entire pathways are absent. Heterologous expression of mammalian isoforms of PKC (Riedel et al., 1993), mammalian caspases (Lisa-Santamaría et al., 2009) and BCL2 (Khoury & Greenwood, 2008) family proteins in yeast were used to identify suppressors of such pathways. Employing translational yeast genomics, several human tumour suppressor genes that were involved in cell cycle checkpoints, DNA replication/ repair, sister chromatid segregation have

been identified (de Clare & Oliver, 2013). The conservation of many higher eukaryotic features involved in cancer makes *S. cerevisiae* a powerful tool to unveil molecular mechanisms underlying basic human biology and a good model system for screening cancer therapeutics that target cell metabolism.

1.4.3 Genome-wide tools in yeast for elucidating gene function

The simple unicellular eukaryote *S. cerevisiae* is a genetically tractable system, with a short 90-minute generation time (Guthrie & Fink, 2002) and has the most annotated genome of all organisms (Simon & Bedalov, 2004; Forsburg, 2001). It has a small genome of 16 chromosomes comprised of ~6000 open reading frames (ORFs), of which nearly 1,000 have orthologs that are implicated in human disease and can complement yeast loss of function mutations (Goffeau et al., 1996; Engel et al., 2014; Botstein & Fink, 2011; Kachroo et al., 2015). *S. cerevisiae* has both haploid and diploid life cycles (Forsburg, 2001). Of the ~6000 ORFs in *S. cerevisiae* only ~1000 are indispensable for viability and the remaining ~5000 are non-essential genes that have been systematically inactivated to create comprehensive non-essential gene deletion libraries in haploid and diploid organisms (Tong et al., 2001; Ho et al., 2011; Winzeler et al., 1999; Boone et al., 2007). The haploid deletion library is readily utilised in Synthetic Genetic Array (SGA) experiments to generate double mutants by mass mating against a query gene deletion to identify gene-gene (genetic) interactions (Tong et al., 2001). Several other comprehensive mutant libraries are also available to investigate essential genes including the essential heterozygous diploid gene deletion library, the decreased abundance by mRNA perturbation (DAmP) library (Schuldiner et al., 2005) and the TET-off library (Mnaimneh et al., 2004). Gene overexpression libraries are useful for complementing deletion mutant phenotypes and identifying new functions of genes (Douglas et al., 2012). GST- and HIS-tagged libraries are useful for investigating protein-protein interactions (Zhu et al., 2001). The green fluorescent protein (GFP) fusion library is used to visualise protein localisation and abundance (Tkach et al., 2012). The comprehensive genome-wide tools in yeast permit unbiased assessment of genes and small molecule function.

The genetic interaction data available for *S. cerevisiae* are unmatched by other models organisms (Ho et al., 2011). Approximately 89% of the protein coding genes in *S. cerevisiae* have been annotated with a biological function (Jouhten et al., 2016). Other resources on *S. cerevisiae* include the Saccharomyces Genome Database (SGD; <http://www.yeastgenome.org/>) which provides extensive literature-based information on gene function for every gene that has been studied (Botstein & Fink, 2011). In addition, a maintained three structured controlled vocabulary referred to as gene ontologies (GO categories) that describes gene/protein function in terms of biological processes, cellular components and molecular functions that are independent of organism, permits the transfer of findings from other organisms to yeast (Ashburner et al., 2000). The conservation of many higher eukaryotic features involved in cancer coupled with its genetically tractability, less complex, short life cycles and the most annotated genome of any organism makes *S. cerevisiae* a powerful tool to investigate cancer biology.

1.4.4 Nucleoside transport in humans and yeast

Nucleosides and their derivatives are hydrophobic molecules; hence the expression of membrane permeases determine their distribution in cells and presence in the extracellular environment. In humans, nucleoside transport is facilitated either by the Na⁺ coupled symporter SCL28 family concentrative nucleoside transporters (CNT1-3) or by the energy dependent SCL29 family equilibrative nucleoside transporters (ENT1-4) (Young et al., 2013). These nucleoside transporters differ in their substrate specificity, subcellular localisation and expression in specific tissues leading to regulation of the pools of specific permeants (Young et al., 2013). In addition to transport of nucleosides, nucleobases and monoamines, nucleoside transporters also control the transport of various anticancer and antiviral drugs (Mackey et al., 1998; Pastor-Anglada et al., 1998). In addition to the CNTs and ENTs, SCL15 family peptide transporters and SLC22 family organic anion and cation transporters have also been implicated in transport of nucleoside drugs in humans (Cano - Soldado & Pastor - Anglada, 2012; Ciarimboli, 2008).

Both nucleoside transporter families are evolutionary conserved in wide range of eubacteria and eukaryotes. ENTs are distantly related to the major facilitator superfamily transporters found in *S. cerevisiae*. Yeast encodes a single ENT related nucleoside transporter (FUN26) and an unrelated putative nucleoside transporter (FUI1). Uptake is such a fundamental process that with only 18% amino acid identity between FUN26 and ENT1/ENT2, heterologous expression of *FUN26* in *X. laevis* oocytes permits uptake of both purine and pyrimidine nucleosides and nucleobases (Vickers et al., 2000). *FUN26* in yeast localises to the vacuolar membrane and functions as a high affinity nucleoside/nucleobase transporter. In contrast, *FUI1* is a nucleobase:cation symporter of plasma membrane protein and it shares 69-70 % amino acid similarity with uracil and allantoin permeases *FUR4* and *DAL4*, respectively (Loewen et al., 2003; Vickers et al., 2000). *FUI1* transports uridine, uracil, 5-fluorouracil, and thymine nucleobases and analogues but not nucleosides. There are also dipeptide transporters (*PTR2* and *DAL5*) (Cai et al., 2007), oligo peptide transporters (*OPT1* and *OPT2*) (Hauser et al., 2001), and organic anion/cation transporters (André, 1995) that transport nucleoside-derived small molecules in yeast. However, *S. cerevisiae* lack plasma membrane transporters that are capable of permitting cell entry to purine and pyrimidine nucleosides as well as the majority of nucleoside analogues which is a major limitation for the investigating the mechanisms of MTDIA and forodesine.

1.4.5 Purine nucleotide biosynthesis is highly conserved in yeast

The yeast purine nucleoside phosphorylase is encoded by *PNP1*. The catalytic and substrate binding residues of PNP are conserved in yeast with closer similarity to the human enzyme (50% similarity) than to *E. coli* (<10% identity) (Lecoq et al., 2001b). It is also more similar in structure and function to the human enzyme as it only metabolises guanosine and inosine nucleosides, while *E. coli* encodes a hexameric enzyme that can also process adenosine (Jensen & Nygaard, 1975). The yeast *PNP1* deletion (*pnp1Δ*) does not have any obvious growth defect while these cells accumulate guanosine and inosine (Lecoq et al., 2001b) similar to PNP-deficient mice (Arpaia et al., 2000). Yeast do not encode either a cytosolic deoxycytidine kinase or a mitochondrial deoxyguanosine kinase required for the conversion of deoxyguanosine to deoxyguanosine monophosphate (dGMP), nor do

yeast express the 5'-nucleotidase responsible for catabolism of dGMP. However, the remainder of the nucleotide biosynthesis pathway is conserved in yeast (Fig. 1.5).

Figure 1.5 – **Nucleoside metabolism is conserved from *S. cerevisiae* to humans.** The dotted arrows indicate the pathways that are absent in yeast. The orthologue genes/complexes in humans are shown in parentheses. dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; 5'-NT, 5'-nucleotidase; GMPK, deoxyguanosine monophosphate kinase (GUK1); NDPK, nucleotide diphosphate kinase (NME1, NME2, NME3, NME4, NME6, NME7, NME1-NME2, AK9); HPT, hypoxanthine phosphoribosyltransferase (HPRT1); RR, ribonucleotides

1.4.6 Structural conservation of MTAP and methionine salvage pathways

The human methylthioadenosine phosphorylase (MTAP) is not conserved in bacteria and plants but is conserved in yeast. The MTAP activity in bacteria and plants are facilitated by a dual substrate specific enzyme 5'-methylthioadenosine/S-adenosylhomocysteine (MTA/AdoHcy) nucleosidase (MTAN) that reversibly cleaves MTA and AdoHcy to liberate adenine therefore shares little similarity with MTAP (Lee et al., 2004). MTAP in yeast is encoded by *MEU1*, an enzyme comprising 275 amino acids with 35% identities and 53% similarities with human MTAP (Subhi et al., 2003). The interactions involved in the active site for binding of MTA and the nucleoside analogue MTDIA are conserved in yeast (Lee et al., 2004; Singh et al., 2004)

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Get&RID=FD3YFTNJ113#alnHdr_Query_134717).

Moreover, the polyamine biosynthesis and methionine and adenine salvage pathways are conserved from yeast to humans (Pirkov et al., 2008).

Loss of *MEU1* in yeast has no obvious growth defect when cultured in synthetic complete media. However, following mutations that permit the uptake of nucleosides, *meu1Δ* cannot grow in the presence of MTA as the sole sulphur source and is required for salvage of methionine from MTA (Thomas et al., 2000). Loss of *MEU1* leads to upregulation of ODC, leading to accumulation of both putrescine and spermidine and a depletion of spermine (Subhi et al., 2003). This is contrary to MTAP inhibition in mouse xenografts of human cancers that reduced gene expression of polyamine biosynthesis, ODC (ornithine decarboxylase), SSAT1 (spermine/spermidine N1-acetyltransferase 1), SRM (spermidine synthase) and SMS (spermine synthase) and a depletion of both spermidine and spermine (Basu et al., 2007; Basu et al., 2011). The differences at the transcriptional level may reflect a difference between knock-down and complete loss of MTAP function or a difference between loss of MTAP function in tumour only as compared to the whole organism. MTAP negative cancers also

Figure 1.6 – Polyamine biosynthesis and methionine salvage pathways are conserved from yeast to humans.

show elevated ODC levels that are inhibited by MTAP re-expression but in cell type-specific manner (Christopher et al., 2002). This overexpression of ODC in the absence of MTAP/*MEU1* is a consequence of 4-methylthio-2-oxobutanoic acid, an inhibitor of ODC in both mammals and yeast and a downstream product of MTAP (Subhi et al., 2003; Tang et al., 2006). An ODC antizyme is also present in higher eukaryotes that is generated by a frameshifting event that is stimulated by elevated ODC levels, yet these mechanisms are absent in yeast (Coffino, 2001). The conservation of polyamine biosynthesis at the transcription level and similar post-translational regulation mechanisms further corroborates the use of yeast as a model system to elucidate mechanisms MTAP inhibition (Fig. 1.6).

1.4.7 Macroautophagy mechanisms are conserved in yeast

Macroautophagy (hereafter referred to as autophagy) is a membrane transport pathway where cytosolic content such as longed lived proteins and damaged or surplus organelles are transported to the vacuole (lysosome in mammalian cells) and recycled to maintain cell homeostasis (Ohsumi, 2014). Autophagy is conserved across eukaryotes and it is induced in cells under nutrient deprivation or inhibition of TORC1 by rapamycin (Baba et al., 1994; Kamada et al., 2000). Following induction of autophagy, a cup shaped structure known as the phagophore forms in the cytosol and the leading edge fuses with the other to form a double-membraned vesicle known as the autophagosome that traps the cytosolic content within it (Suzuki & Ohsumi, 2010). The autophagosomes subsequently fuse with the vacuolar membrane, and autophagic bodies are released within the vacuole/lysosome and degraded by the hydrolases within the vacuolar/lysosomal lumen. The resultant macromolecules are subsequently effluxed by vacuolar membrane permeases to the cytosol and reused in metabolic reactions. The process involves many genes that are collectively named autophagy related (ATG) genes (Klionsky et al., 2003). Although autophagy is non-selective, it shares a majority of molecular components with selective forms of autophagy such as cytosol to

vacuole transport pathway, autophagy of peroxisomes (pexophagy), mitochondria (mitophagy) and ribosome (ribophagy) where cytosolic content is selectively degraded under nutrient rich conditions.

The autophagy pathway in yeast has been extensively studied and provided the basis for what is known about autophagy in mammalian cells (*e.g.*, the 2016 Nobel Prize in Physiology/Medicine). Genes involved in each step of the autophagy pathway: autophagy induction, vesicle nucleation, expansion of the phagophore and autophagosome completion, docking and fusion of autophagosome with the vacuole, degradation of cargo and efflux of macromolecules have been thoroughly described (Yang & Klionsky, 2009; Wen & Klionsky, 2016). Briefly, nutrient deprivation or rapamycin inhibits TORC1 and phosphorylation of Atg13. Atg13 in turn promotes associations of Atg1 with Atg17-Atg31-Atg29 (assembly of Atg1 kinase complex) and leading to induction of autophagy. Atg11 also associates with Atg1 under nutrient replete conditions for induction of selective forms of autophagy. During nucleation, Atg14-containing class III PtdIns3K complex (Vps34, Vps30/Atg6, Vps15, Atg14 and Atg38) is recruited to the PAS (phagosome assembly site) that requires Atg23, Atg27 and Atg11. The expansion and completion of the autophagosome requires two ubiquitin-like conjugation systems: Atg8-PE (requires Atg4, Atg3 and Atg7) and Atg12-Atg5-Atg16 (requires Atg7 and Atg10), and Atg12-Atg5-Atg16 can subsequently act as a E3 like enzyme for lipidation of Atg8. Atg9, a membrane associated protein cycles between the peripheral site and the PAS carrying or directing membrane for autophagosome expansion. The trafficking of Atg9 from the peripheral site to the PAS requires Atg23, Atg27 and Atg11. Likewise, the trafficking from the PAS to the peripheral site requires Atg1-Atg13, Atg2-Atg18 and Atg14-containing class III PtdIns3K complex. Docking and fusion of the autophagosome with the vacuole requires SNARE proteins: Vam3, Vam7, Vti1, Ykt6, class C Vps/HOPS complex, Mon1-Ccz1 complex and the Rab GTPase, Ypt7. The degradation of vesicles within the vacuolar lumen requires hydrolases Pep4, Prb1 and Atg15 and the resulting macromolecules are effluxed by Atg22, Avt3 and Avt4 permeases. The evolutionary conservation of autophagy mechanisms permits evaluation of disruptions to autophagy in the well characterised yeast system to identify points of perturbation by mammalian active small molecules.

Chapter 2 - Elucidating the anticancer mechanisms of nucleoside analogue MTDIA via with assessment of fitness for non-essential gene deletions

2.1 Introduction

Polyamine biosynthesis has been long proposed as a candidate for cancer therapy as levels of polyamines are elevated in proliferating cells (Wallace, 1996). Previous attempts at inhibiting the polyamine biosynthesis pathway by ornithine decarboxylase (ODC) inhibitors had limited success as consequence of poor transport, poor target affinity and presence of compensatory mechanisms (Casero & Marton, 2007). 5'-methylthioadenosine phosphorylase (MTAP) is also an enzyme of the polyamine biosynthesis pathway and the only enzyme in humans that can catabolise 5'-methylthioadenosine (MTA) (Della Ragione et al., 1986). MTA is a potent inhibitor of the aminopropyl transferases spermidine and spermine synthases and must be rapidly hydrolysed to maintain the forward reaction (Pajula et al., 1979a; Raina et al., 1984). MTA is required for recycling of adenine, methionine and S-adenosyl methionine, the primary methyl donor for many methylation reactions (Backlund & Smith, 1981; Finkelstein, 1990; Lu, 2000). Due to close association of MTAP with the polyamine biosynthesis pathway and recycling of S-adenosyl methionine, it has recently been proposed as a target for cancer therapy (Basu et al., 2007).

MTDIA is a potent picomolar inhibitor of the human enzyme 5'-methylthioadenosine phosphorylase (MTAP) and mimics the enzymatic transition state structure of MTA bound by MTAP (Schramm, 2011). It has a 1.75 nM K_i and 86 pM K_i^* for MTAP *in vitro* (Singh et al., 2004). It is currently in preclinical stages, but has *in vivo* efficacy in mouse xenografts models for human lung, prostate, colon, cervical, head and neck, and triple negative breast cancers, as well as *in vitro* efficacy in various human cancer cell lines (Basu et al., 2007; Basu et al., 2011). MTDIA thus, mimics the loss of MTAP and leads to an accumulation of MTA and a reduction in AdoMet and polyamines (spermidine and spermine) levels (Basu et al., 2007; Basu et al., 2011). One or more of these processes are responsible for subsequent cellular processes mediating apoptosis such as G2/M arrest, loss of mitochondrial membrane integrity, and cleavage of PARP and caspases (Basu et al., 2011). The initiation of apoptosis,

however, is cell type specific as normal fibroblast cells do not undergo caspase or PARP-mediated apoptosis or other cellular alterations observed with increased levels of MTA (Basu et al., 2007). Therefore, genetic mechanisms underlying these processes are not fully understood and an additional level of complexity exists beyond MTA-initiated apoptosis.

To elucidate the genetic mechanisms of MTDIA that lead to initiation of cell death, a two-pronged approach was employed that utilises the *S. cerevisiae* genetic model. First, the genetic (gene-gene) interactions surrounding loss of the MTDIA drug target in yeast were identified as genetic interactions with *MEU1* (the yeast orthologue of MTAP). Second, the chemical genetic (drug-gene) interactions were investigated to provide a closer approximation of MTDIA action in humans. The mechanisms that were identified from these two approaches were investigated further to confirm these mechanisms as points of perturbation by MTDIA.

2.2 Experimental procedures

2.2.0 Yeast strains, media and compounds

2.2.0.1 Yeast strains

All *S. cerevisiae* strains used in this chapter were derived from the diploid strain BY4743 (Brachmann et al., 1998) and were constructed by PCR mediated disruption or heterologous expression of human equilibrative nucleoside transporter 1 (ENT1) gene from plasmid pYhENT1 ((Vickers et al., 1999); gift of Carol E. Cass) as follows:

Strain	Genotype	Reference
BY4743	<i>MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0</i>	(Brachmann et al., 1998)
BY4742	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0</i>	(Brachmann et al., 1998)
Y7092 (wild type)	<i>MATα can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	(Tong & Boone, 2007)
<i>meu1Δ</i> (Y7092 background)	<i>MATα meu1Δ::natR; can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
WT(Y7092) + hENT1	<i>MATα CEN3::hENT1::URA3; can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
WT(Y7092) + hENT1 + dCK	<i>MATα CEN3::hENT1::URA3 CEN3::dCK::LEU2 can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
<i>meu1ΔxxxΔ</i>	<i>MATa meu1Δ::natR; xxxΔ::kanR; can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study

xxxΔ	MATa xxxΔ::kanR his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2	(Tong et al., 2001)
xxx-GFP (GFP library, constructed in chapter 3)	MATa xxx-GFP::HIS5 can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::URA3	This study
meu1Δ xxx-GFP (GFP library, constructed in chapter 3)	MATa meu1Δ::kanR; xxx-GFP::HIS5; can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::URA3	This study
Y7092 + hENT1::natR (yCG640 background)	MATa can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::natR	This study
GFP-Atg8	MATa GFP-Atg8::URA3 can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::natR	This study
meu1Δ::kanR + hENT1::natR (yCG643 background)	MATa meu1Δ::natR; can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::natR	This study
meu1Δ GFP-Atg8	MATa GFP-Atg8::URA3; meu1Δ::natR; can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::natR	This study
xxxΔ GFP-Atg8 (pep4Δ, atg1Δ, atg4Δ; yCG640 background)	MATa xxxΔ::kanR; GFP-Atg8::URA3; can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::natR	This study

Table 2.2.1 – Yeast strains used in this chapter.

2.2.0.2 Media components

All *S. cerevisiae* strains were cultured in one of the following growth media supplemented with 100 µg/mL ClonNAT (Werner bioAgents) and/or 200 µg/mL geneticin (G418). The amino acid analogues L-canavanine (Sigma-Aldrich) and L-thialysine (Sigma-Aldrich) were used at 50 µg/mL in the absence of their amino acid analogues. All yeast growth media was autoclaved at 121°C and cooled to 65°C before adding 2% glucose and antibiotics unless otherwise stated. All *E. coli* strains were cultured in lysogeny broth (LB) supplemented with 100 µg/mL ampicillin (Sigma-Aldrich).

Yeast peptone dextrose (YPD) agar: 1% (w/v) yeast extract (Becton, Dickinson and Company), 2% (w/v) bacto-peptone (Becton, Dickinson and Company), 0.12% (w/v) adenine (Sigma-Aldrich), 2% (w/v) agar (Invitrogen) and 2% (w/v) glucose (Sigma-Aldrich). YPD broth was prepared as above without the addition of agar.

Synthetic complete (SC) and synthetic dropout (SD) agar: 0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulphate (Becton, Dickinson and Company), 0.1% (w/v) monosodium glutamate (Sigma-Aldrich), 0.2% (w/v) amino acid mixture to suit (Sigma-Aldrich), 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) glucose (Sigma-Aldrich). SC broth was prepared as

above without the addition of agar. SD media was made the same as SC, minus the appropriate amino acid supplement.

SD-Met/Ade/Ura/Cys (MAUC) + 2% raffinose + 2% galactose agar: 0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulphate (Becton, Dickinson and Company), 0.1% (w/v) monosodium glutamate (Sigma-Aldrich), 0.2% (w/v) amino acid mixture to suit (Sigma-Aldrich), 2% (w/v) agar (Becton, Dickinson and Company), 2% (w/v) raffinose (Sigma-Aldrich) and 2% (w/v) galactose (Sigma-Aldrich). Media was autoclaved at 121°C and cooled to 65°C prior to adding filter sterilised raffinose and galactose. SD broth was prepared as above without the addition of agar.

Complete minimal (CM)-Ura + 2% raffinose: 0.725 (w/v) % CM amino acids and ammonium sulphate mixture to suit, 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) raffinose (Cabosynth). Media was autoclaved at 121°C and cooled to 65°C prior to adding filter sterilised raffinose. SD broth was prepared as above without the addition of agar.

CM- Ura + 2% galactose agar: 0.725 (w/v) % CM amino acids and ammonium sulphate mixture to suit, 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) galactose (Cabosynth). Media was autoclaved at 121°C and cooled to 65°C prior to adding filter sterilised galactose. SD broth was prepared as above without the addition of agar.

CM-Met/Ade/Ura (MAU) + 2% galactose: 0.725 (w/v) % CM amino acids and ammonium sulphate mixture to suit, 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) galactose (Cabosynth). Media was autoclaved at 121°C and cooled to 65°C prior to adding filter sterilised raffinose and galactose. SD broth was prepared as above without the addition of agar.

SD-N + 2% galactose (starvation media) agar: 0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulphate (Becton, Dickinson and Company), 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) galactose (Cabosynth). Media was autoclaved at 121°C and cooled to 65°C prior to adding filter sterilised galactose. SD broth was prepared as above without the addition of agar.

Enriched sporulation (SPO) agar: 1% (w/v) potassium acetate (Scharlau Chemie S.A.), 0.1% (w/v) yeast extract (Becton, Dickinson and Company), 0.05% (w/v) glucose (Sigma-Aldrich), 0.2% (w/v) amino acid

mixture (Sigma-Aldrich), 2% (w/v) agar (Becton, Dickinson and Company). Enriched sporulation broth was prepared as above without agar.

SC/SD amino acid mixtures: Amino acid mixture of SD media is a mixture of the following amino acids minus the appropriate amino acid supplement: 3 g adenine, 2 g uracil, 2 g inositol, 0.2 g para-aminobenzoic acid, 2 g alanine, 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 tryptophan, 2 g tyrosine and 2 g valine. Amino acid mixture for enriched sporulation composed of 2 g histidine, 10 g leucine, 2 g lysine and 2 g uracil.

CM amino acid and ammonium sulphate mixture: 100 g yeast nitrogen base without amino acids or ammonium sulphate, 293.11 g of ammonium sulphate, 1.2 g adenine, 1.2g arginine, 1.2 g histidine, 1.8 isoleucine, 3.6 g leucine, 1.8 lysine, 1.2 methionine, 3 g phenylalanine, 1.2 g tryptophan, 1.8 g tyrosine, 1.2 g uracil, and 9 g valine.

Lysogeny broth (LB) Agar: 1% (w/v) bacto-tryptone (Becton, Dickinson and Company), 0.5% (w/v) yeast extract (Becton, Dickinson and Company), 1% (w/v) NaCl (Scharlau Chemie S.A.) and adjusted to pH 7.5 with 10 M NaOH. The media was autoclaved at 121°C and cooled to 65°C before adding antibiotics unless otherwise stated. LB broth was prepared as above without addition of agar.

2.2.0.3 Compounds and chemicals

MTDIA was kindly provided by Prof Gary Evans (The Ferrier institute, Victoria University of Wellington) was dissolved in DMSO at a concentration of 10 mM and stored at -20°C. MTA (AK Scientific) was dissolved in ddH₂O at a concentration of 5 mM and stored at -20°C.

2.2.1 Liquid phase growth assays

All liquid growth assays were performed in 96-well plates (TCP-000-096, JET BIOFIL) as previously described (Coorey et al., 2014). 5×10^5 cells/mL were treated with either compound or vehicle control, and were incubated at 30°C. The residual growth ($OD_{600} \text{Exp} / OD_{600} \text{vehicle control} \times 100$) was assessed in mid-log cultures (0.3-0.5 OD_{600} units in the vehicle control) using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer).

2.2.2 Agar phase growth assays

All agar growth assays were performed in 24 well plates (JET BIOFIL) as previously described (Coorey et al., 2014). 1×10^5 , 1×10^3 and 1×10^1 cells were plated for each condition of compound or vehicle control. Growth reduction was visually inspected at 24 h and 48 h in SC + glucose media and 48 h and 96 h in SD-MAUC + 2% raffinose + 2% galactose media.

2.2.3 Quantification of genetic interactions with *MEU1*

To investigate the genetic interactions with MTAP, the *meu1Δ::natR* strain was constructed in Y7092 background (Tong & Boone, 2007) by PCR mediated disruption of the *MEU1* gene with the *natMX4* selectable marker from plasmid p4339 (Goldstein & McCusker, 1999) (Appendix Fig. 7.2.1; Appendix Table 7.2.1). The non-essential haploid yeast gene deletion library (Boone et al., 2007) was maintained across 14 plates at 1536 colony format with each deletion strain in quadruplicate. This gene deletion library was mated *en masse* against the *meu1Δ::natR* deletion strain using the RoToR HDA replicator (Singer Instrument Co. Ltd, Somerset, UK) and SGA methodology (Tong et al., 2001), which generated haploid double gene deletion mutants that were assessed for epistasis (based on colony size) using Gitter tools (Wagih & Parts, 2014) on RStudio v0.99.896 (Racine, 2012) and ScreenMill (Dittmar et al., 2010). Double gene deletion strains that showed a growth defect (growth < 40%, $p < 0.05$) or growth improvement (growth > 40%, $p < 0.05$) compared to the parental haploid non-essential gene deletion library in at least two out of three SGAs were considered as putative epistatic interaction of *meu1* and were independently validated.

2.2.4 Validation of genetic interactions with *MEU1*

To validate the putative epistatic interactions, the *meu1ΔxxxΔ* double mutants were re-constructed by performing a mini SGA comprised of 154 deletion strains (31 putative genetic interactions and 123 functionally related strains). A control SGA was performed using *ura3Δ::natR* as a query strain to exclude mating- and sporulation-deficient deletion strains. The double mutants were assessed for growth defects in liquid 96-well plate growth assays using the parental single deletion and Y7092 (wild type) as control strains. The yeast strains were cultured in 96-well plates for 2 d at

room temperature and inoculated into fresh SC media in 96-well plates using the 96-long pin RePads® on the RoToR HDA replicator (Singer Instrument Co. Ltd). Plates were incubated at 30°C and growth was evaluated by measuring OD₆₀₀ from 14 -24 h at 2 h intervals as previously described (Section 2.2.1). The epistatic growth defect of the double mutants was calculated relative to the growth defect of the *meu1Δ* single mutant ($(meu1ΔxxxΔ/meu1Δ) / (xxxΔ/WT)$), modified from the calculation used for assessment of drug sensitivity. The relative growth of the double mutants to the single mutants was normalised against the *meu1Δhis3Δ* double gene deletion strain, which is phenotypically identical to *meu1Δ* single gene deletion strain that contains a partial deletion of the *HIS3* gene. Stringent cut-offs of growth reduction (<40% treated compared to untreated with $p < 0.05$) and growth improvement (>40% treated compared to untreated with $p < 0.05$) were used to determine significant changes in growth. The double gene deletion mutants with extreme growth defects (<5% treated compared to untreated with $p < 0.05$) were considered synthetic lethal with the *meu1Δ* strain.

The validated genes were assigned to the three major hierarchical gene ontology (GO) categories: biological process, molecular function and cellular component using the haploid non-essential gene deletion library as a reference set on YeastMine (Gene Ontology Consortium, 2004; Ashburner et al., 2000; Balakrishnan et al., 2012) with p -values corrected for multiple testing using the Benjamin and Hochberg false discovery rate of 0.05 (Benjamini & Hochberg, 1995). In addition, genes were assigned to functional categories according to the simplified GO category biological process on GO slim mapper on SGD tools and the gene descriptions on YeastMine (Cherry et al., 2012) (Balakrishnan et al., 2012).

2.2.5 Nucleoside uptake in *S. cerevisiae*

To assess the uptake of nucleosides in *S. cerevisiae*, yeast strains: Y7092 (wild type, methionine auxotroph) and *ade2Δade3Δ* (adenine and methionine auxotroph) were transformed with either the human equilibrative nucleoside transporter (hENT1) containing yeast expression plasmid pYhENT1(Vickers et al., 1999) or empty vector pYES2 as previously described (Gietz & Schiestl, 2007). The *ade2Δade3Δ* double mutant was cultured in media supplemented with MTA or adenosine in the

absence of methionine or adenine, respectively. Bioactivity of known toxic nucleoside analogues 3'-deoxyadenosine (cordycepin) and 7-deaza-7-cyanoadenosine (toyocamycin) was evaluated using growth assays in liquid as previously described (Section 2.2.1).

2.2.6 Assessment of toxicity of MTDIA

To assess if MTDIA was cytotoxic or cytostatic in *S. cerevisiae*, two approaches were employed. In the first approach, WT+hENT1 cells in 99 μ L volumes SD-Met/Ade/Ura/Cys + MTA + 2% raffinose + 2% galactose (SD-MAUC+MTA+RG) of liquid media were treated with inhibitory concentrations of MTDIA and incubated at 30°C as previously described (Section 2.2.1). At 0, 6, 12, 24 and 48 h intervals, 1 μ L of cells was removed and plated onto SD-MAUC+RG agar media or SD-Ura agar media. The plates were incubated at 30°C and photographed at 12h and 48h using a digital camera (EOS 600D, Canon). Alternatively, WT+hENT1 cells were treated with MTDIA in a liquid growth assay for 48 h and growth was assessed at OD₆₀₀ as previously described (Section 2.2.1). The cells were centrifuged at 2400 rpm for 5 min, washed twice with MAUC+MTA+RG media, and suspended in fresh MAUC+MTA+RG media. The cells were then incubated for 48 h at 30°C and assessed for growth at OD₆₀₀.

2.2.7 Construction of hENT1 expression yeast gene deletion library

To construct the haploid yeast gene deletion library expressing hENT1 (xxx Δ +hENT1), the WT+hENT1 strain was crossed with the non-essential haploid yeast gene deletion library (gift of Charles Boone) using SGA methodology (Tong et al., 2001).

2.2.8 Screening the hENT1 yeast gene deletion library for sensitivity to MTDIA

To assess deletion strains for sensitivity to MTDIA, the haploid xxx Δ +hENT1 gene deletion library was prepared on agar in 1536 format with each strain in quadruplicate and 384 strains per plate. The deletion strains were freshly pinned in 1536 format on SD-Ura + 4% raffinose agar plates and incubated at 30°C for 24 h. Colonies were then replicated on SD-MAUC+MTA+RG agar plates with and without 3 nM MTDIA, incubated for 48 h at 30°C, and photographed using a digital camera (EOS 600D, Cannon). All replications of treated and untreated strains were performed in triplicate, colony

sizes were measured using Gitter tools (Wagih & Parts, 2014) in RStudio v0.99.896 (Racine, 2012) and subsequently compared using ScreenMill (Dittmar et al., 2010).

2.2.9 Fluorescent microscopy: analysis of autophagy

To monitor autophagy, cells were transformed with GFP-Atg8 (Suzuki et al., 2001) using lithium acetate/single-stranded carrier DNA/PEG as previously described (Gietz & Schiestl, 2007). Transformed cells were cultured overnight ($OD_{660} = 0.2$) in SD-Ura + 2% raffinose, washed twice in dH_2O , and resuspended in fresh media (YPD + 2% glucose, SD-N + 2% galactose, SD-Ura + 2% galactose, SD-MAU+ 2% galactose + 250 μM MTA $\pm$ MTDIA), and cultured at 30°C for 3 h. Cells expressing GFP-Atg8 were visualised under 100x magnification using a fluorescent microscope (BX63, Olympus) equipped with camera (Dp70, Olympus) using DIC and filters suitable for FITC and Texas Red.

2.2.10 Automated fluorescent microscopy

Cells cultured for 20 h in SD-Ura+2% raffinose agar media were inoculated into black-walled, clear bottom 384 well plates (Perkin Elmer Cell Carrier) containing SD-MAU+ MTA + 2% galactose liquid media with and without MTDIA to an OD_{660} of 0.3, using the 384 long pin RePads© on the RoToR HDA replicator (Singer Instrument Co. Ltd) as previously described (Bircham et al., 2011). Plates were incubated at 30°C for 6 h and the fluorescent signal was detected at 488 nm (GFP) and 561 nm (nuclear localisation signal fused to RedStar2 and cytosolic mCherry) using the 60x water immersion lens (NA 1.2) in the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin Elmer). Three visual fields were imaged for each condition for two channels at a height of 0.4 μm as previously described (Bircham et al., 2011).

2.2.5.11 Phloxine B viability assay

To assay viability, cells ($OD_{660} = 0.2$ for WT, 0.6 for *meu1Δ* and 10 nM MTDIA) cultured overnight in SD-Ura + 2% raffinose were harvested, washed twice with 10 mL CM-MAUC + RG+ MTA, resuspended in 50 mL of CM-MAUC + RG + MTA, and cultured for 10 d at 30°C. At 0, 2, 4, 6, 8 and 10 d, 1 mL of culture was transferred into a microfuge tube, stained with 2 $\mu g/mL$ phloxine B (Sigma-

Aldrich) as previously described (Noda, 2008). Cells were visualised under 60x magnification using a fluorescent microscope (BX63, Olympus) equipped with camera (Dp70, Olympus). Percent viability (unlabelled cells/GFP labelled cells + unlabelled cells) x 100 was determined from a minimum of 1000 cells across five visual fields for each condition.

2.2.5.2 GC-MS analysis of sterols

To quantify sterols, yeast cells from an overnight culture were inoculated to an OD₆₆₀ of 0.05 in 15 mL of SD-MAUC + 250 µM MTA + 2% galactose media with and without MTDIA, and then incubated at 30 °C until an OD₆₆₀ of 1.0 was reached. For *meu1Δ* and 10 nM MTDIA, 0.2 OD units were inoculated into 15 mL volumes to account for the slow growth rate. Then 5.0 OD units of cells were harvested, washed twice with dH₂O, stored at -80°C, and the lipids were extracted as previously described (Munkacsı et al., 2011). In brief, 150 µL of glass beads and 900 µL of chloroform:methanol (1:2) were added to the cell pellet, vortexed for 10 min at 4°C, and incubated overnight at 4°C at 1100 rpm using a thermomixer (Eppendorf, Hamburg, Germany). Then 300 µL chloroform and 300 µL water were added, vortexed for 30 s, and incubated on ice for 2 min. The samples were centrifuged at 9000 rpm for 5 min and the lower organic phase (~850 µL) was collected. The extraction was repeated with 600 µL of chloroform and the two organic extracts were combined and dried using a Centrivap Concentrator (Labconco). The lipid film was stored at -80°C. To derivatise, 1.2 mg/ml methoxamine HCl in pyridine was added to the lipid film, vortexed for 15 min, and incubated at room temperature for 16 h. Samples were silylated with 30 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane, vortexed for 1 min, incubated at 70°C for 30 min, and vortexed for an additional 1 min. The sterols were diluted in hexane to a final volume of 200 µL and 100 µL was transferred into 2 mL glass vials with microvolume insert (Agilent). Gas chromatograph-mass spectrometer (GC-MS) data acquisition and post run analysis was performed using Shimadzu GCMS solution v2.72 (Shimadzu, Kyoto) as previously described (Alzeer et al., 2016; Wu et al., 2012). Briefly, the samples were analysed with a Shimadzu QP2010-Plus GC-MS (Shimadzu) with an RXI – 5SiIMS column (30 m x 0.25 mm ID with 0.25 µm film thickness, Restek) attached to an electron impact mass spectrometer operating at

70 eV in positive ion mode, scanning from m/z 42-600 every 0.3 s. 1 μ L of samples were introduced using a an AOC-20i auto-sampler with a split injection (20:1) at an injector temperature of 250°C using He as the carrier gas at a linear velocity of 43.4 cm/s (1.37 mL/min) at constant flow. Each sample was injected at an initial oven temperature of 60°C for 1 min, followed by a ramp at 10°C/min to 325°C with a final hold for 3.5 min. Samples were carried to the mass spectrometer at an interface temperature of 330°C. Sterols were identified by comparison of the detected fragmentation patterns and retention times with those of authentic standards for ergosterol, lanosterol and squalene (Avanti Polar Lipids).

2.2.5.3 Calculation of synergy and fractional inhibitory concentration (FIC) index

To investigate if MTDIA treatment induces autophagy, cells treated with rapamycin (RPM), a known inducer of autophagy, were co-treated with MTDIA and the growth inhibition was assessed in a liquid phase growth assay as previously described (Section 2.2.1). The growth defect by rapamycin and MTDIA in combination relative to either drug alone $(\text{MTDIA}+\text{RPM}/\text{MTDIA})/(\text{RPM}/\text{DMSO})\times 100$ was determined using a modified drug sensitivity calculation (Best et al., 2013). In addition, fractional inhibitory concentration FIC index was calculated to quantify drug-drug interaction as the following: synergistic ($\text{FIC} \leq 0.5$), additive ($0.5 < \text{FIC} < 1$), indifferent ($1 < \text{FIC} < 4$), and antagonistic ($\text{FIC} \geq 4$) where FIC is defined as $(\text{drug A in combination}/\text{drug A alone}) + (\text{drug B in combination}/\text{drug B alone})$ (Hall et al., 1983; Odds, 2003; McElhaney-Feser et al., 1998).

2.3 Results

2.3.1 SGA analysis identifies genetic interactions with the *MEU1* target of MTDIA

The assessment of gene-gene (genetic) interactions provides insight into the buffering mechanisms for a pathway or cellular process of interest and can be used to discern novel biology. To investigate the genetic interactions with the target of MTDIA, *MEU1* (the yeast orthologue of MTAP) was deleted in a query strain as previously described (Section 2.2.3). Haploid *meu1 Δ xxx Δ* double gene deletion mutants were generated by crossing the haploid non-essential gene deletion library *en masse* against the query strain by SGA methodology as previously described (Tong et al., 2001). A significant

reduction in growth of *meu1Δ* was conferred with the addition of 31 single gene deletions (Fig. 2.3.1; Appendix Table 7.2.2a). To validate the putative *MEU1* genetic interaction, double mutants were reconstructed in a mini SGA comprised of 123 gene deletion strains (31 putative genetic interactions and 123 functionally related strains). The resulting double mutants were evaluated for reduction in growth phenotype relative to the single gene deletion parents and wild type. Validations were performed in triplicate in liquid media as the growth can be accurately quantified by measuring turbidity at OD₆₀₀. Using a stringent cut-off of growth reduction > 40% growth inhibition in the double gene deletion mutant relative to the corresponding single gene deletion mutants (*i.e.*, *aah1Δpnp1Δ/pnp1 Δ* vs. *aah1Δ/WT*), 52 gene deletion strains (11/31 from putative genetic interactions and 41/123 selected based on shared function) were determined to have a growth defect when co-deleted for *meu1Δ* (Fig. 2.3.2; Appendix Table 7.2.2b). These genes were assigned to cellular processes using the GO slim mapping tool in SGD (Cherry et al., 2012) and were involved in amino acid metabolism, response to starvation, vacuole fusion and vesicle-mediated transport, carbohydrate metabolism, lipid homeostasis, chromatin organisation, chromosome segregation, transcription and translation.

Synthetic lethality of *meu1Δ* with gene deletions involved in amino acid metabolism and transport (*SPE2*, *TAT2*, *AMD1* and *AAH1*) are likely the consequence of *MEU1* function in amino acid salvage and NAD⁺ biosynthesis. *SPE2* catalyses the conversion of S-adenosyl-L-methionine (AdoMet) to S-adenosylmethioninamine and is required for the syntheses of spermidine and spermine as well as the recycling of AdoMet back to MTA. Loss of *SPE2* would lead to depletion of MTA and hence the growth defect. This is consistent with the inability of the *meu1Δspe2Δ* double deletion mutant to accumulate MTA (Chattopadhyay et al., 2006). *TAT2* encodes a high affinity tryptophan transporter. Tryptophan is used as a precursor for synthesis of nicotinic acid mononucleotide (NaMN) in NAD⁺ synthesis and it bypasses the enzymatic step catalysed by *MEU1* (nicotinamide ribose to nicotinamide) (Belenky et al., 2007). As *TAT2* is also mislocalised to the vacuole in response to ergosterol biosynthesis inhibition (Gaber et al., 1989), the exacerbated growth defect in *meu1Δtat2Δ* may reflect a further

reduction of NAD⁺ and a link between NAD⁺ and ergosterol. *AMD1* and *AAH1* are required for synthesis of inosine monophosphate (IMP) from adenine and deletion of *MEU1* prevents the recycling of adenine from MTA. The double deletions *meu1Δamd1Δ* and *meu1Δaah1Δ* must rely solely on adenine and inosine in the media and other functionally redundant enzymes (encoded by *APT1*, *PNP1* and *HPT1*) for synthesis of IMP. It is likely that reduced IMP in these double mutants is responsible for these growth defects. This is consistent with the poor growth of *meu1Δade2Δ* double mutant compared with the *ade2Δ* and *meu1Δ* single mutants in the absence of adenine (Subhi et al., 2003).

As MTA inhibits DNA methyl transferases in bacteria and humans (Woodcock et al., 1983; Lautenberger & Linn, 1972), the changes in chromatin organisation likely underlie growth defects in *meu1Δ* caused by deletions in YLR184W (adjacent to TOS4/YLR183C), *HIR2*, *EAF1*, *IES5*, *UME6*, *PAT1* and *HTZ1*. Likewise, changes in transcription also underlie growth defects in *meu1Δ* caused by deletions in *SIP3*, *MED1*, *DAL81*, *KNS1* and *YDR290W* (overlaps RTT103). Changes in DNA methylation patterns were also observed in human cell lines following MTAP inhibition with MTDIA (Basu et al., 2011). Interestingly, yeast do not encode DNA methyltransferases (Tehlivets et al., 2013). Therefore, the mechanisms by which *MEU1* affect chromatin organisation are independent of S-adenosylmethionine dependent reactions. The depletion of polyamine in yeast by deletion of *SPE1* (ornithine decarboxylase, the rate limiting step in polyamine biosynthesis) was previously shown to modulate chromatin remodelling and transcriptional regulation by bypassing a core histone deacetylase (*GCN5*) (Pollard et al., 1999). Furthermore, *SPE3* (spermidine synthase) gene deletion alleviated mitotic repression of sporulation specific genes (Friesen et al., 1998). Therefore, altered chromatin modulation in response to loss of *MEU1* is likely a result of polyamine depletion. The overexpression of ornithine decarboxylase in mice lead to upregulation of both histone acetyl transferases and histone deacetylase (Hobbs & Gilmour, 2000; Hobbs et al., 2002) , thus disruptions to chromatin remodelling and transcription in *meu1Δ* were expected results. Disruptions to translation such as deletions in *RPL9A*, *YPR126C* (overlaps YLH47), *RPL22A*, *RPS6A*, *RPS19A* and *RPS10A* were also expected. For example, L-α-difluoromethylornithine (the ODC inhibitor DFMO) or GC7 (spermidine

synthesis inhibitor) inhibited translation initiation by stimulating the phosphorylation of translation initiation factor eIF2 α and 4E-BP1 (eIF4E repressor) as well as translation elongation by inhibiting the hypusination of translation elongation factor eIF5A by spermidine (Landau et al., 2010). Disruptions of genes involved in lipid homeostasis (*OPI3*, *CSG2*, *ETR1*, *INO2*, *SHR5*, *CEM1*), vacuole fusion and vesicle-mediated transport (*VAM3*, *VPS41*, *ROM2*, *MTC5*, *GGA2*, *CCZ1*, *GOS1*, *SRO9*, *VAM6* and *RIM1*) and response to starvation (*RVS167*, *PHO5*, *IRS4* and *RVS161*) were observed with loss of *MEU1* and are novel findings in this thesis.

Figure 2.3.1 – **Growth of gene deletions with the *MEU1* deletion.** (a) Growth of exemplar double *xxx* Δ *meu1* Δ and single *xxx* Δ mutants in technical quadruplicate assessed on agar. (b) Percent growth of 4,286 *xxx* Δ *meu1* Δ deletion mutants relative to *xxx* Δ from biological triplicate of technical quadruplicate experiments. Three independent SGAs were performed using *meu1* Δ query strain agar using the RoToR HAD (Singer Instrument Co. Ltd) and mated against the haploid single gene deletion library *en masse* using SGA methodology (Tong et al., 2001). The haploid *meu1* Δ xxx Δ double mutant meiotic progeny were assessed for epistatic colony size relative to the haploid parental mutants using

Gitter tools (Wagih & Parts, 2014) in RStudio v0.99.896 (Racine, 2012) and ScreenMill (Dittmar et al., 2010).

Figure 2.3.2 – **52 negative and 2 positive genetic interactions with Meu1**. The growth of the double mutants relative to the single haploid parental strains (*meu1ΔxxxΔ /meu1Δ*)/ (*xxxΔ*/ WT) x 100 was determined in liquid media at OD600 when the single mutant control (*xxxΔ*) was 0.3-0.5. The growth of the double mutants was normalised to *meu1Δhis3Δ* control strain and the double deletion mutants with an average growth > ± 40%, *p* < 0.05 of the *meu1Δhis3Δ* strain were considered a growth defect or a growth improvement.

2.3.2 Heterologous expression of human gene results in nucleoside uptake in yeast

S. cerevisiae lack the orthologous plasma membrane transporters present in humans that permit the uptake of exogenous purines, pyrimidines and derivatives, including MTDIA and MTA (Young et al., 2013). The heterologous expression of recombinant human equilibrative nucleoside transporter 1 (hENT1) in *S. cerevisiae* (Vickers et al., 1999; Griffiths et al., 1997) permitted the uptake of pyrimidines, thymidine and uridine and derivatives 3'-deoxy-3'-fluorothymidine and 5-bromodeoxyuridine (Paproski et al., 2008; Vernis et al., 2003) and in humans also transports purine and purine derivatives (Young et al., 2013). To achieve the uptake MTDIA, hENT1 was expressed in *S. cerevisiae* and the uptake of purine nucleosides and derivatives were assessed. The adenine auxotroph *ade2Δade3Δ* double mutant was assessed for growth in the presence of nucleosides adenosine and 5'-methylthioadenosine (MTA) in the absence of exogenous adenine. The adenine auxotroph expressing the empty vector (*ade2Δade3Δ*+EV) exhibited normal growth in the absence of ENT1 when cultured in media supplemented with adenine and no growth was observed with adenosine or MTA to concentration as high as 2 mM (Fig. 2.3.3a). The growth in adenosine and MTA in the absence of adenine as expected required the expression of hENT1 (Fig. 2.3.3b-c). In addition to permitting transport dependent growth on nucleosides, the expression of hENT1 also improves growth on adenine compared to expression of EV, and is a consequence of hENT1 permitting the uptake of several nucleobases including adenine (Yao et al., 2011). Expression of hENT1 permitted growth of *ade2Δade3Δ* mutant following supplementation with adenosine and MTA with optimum growth with supplementation of 250 μM adenosine and 500 μM MTA. Less than optimal growth was observed for at 2 mM MTA, but not adenosine. This is consistent with MTA-mediated inhibition of cell proliferation in humans (Andreu-Pérez et al., 2010; Pajula et al., 1979b). As wild type yeast do not uptake MTA, this is the first demonstration of MTA mediating growth inhibition in yeast.

In addition, the WT strain was assessed for growth inhibition by toxic nucleosides toyocamycin and cordycepin. The treatment of WT with toyocamycin showed no growth inhibition at 100 μM, the

highest concentration tested (Fig. 2.3.3d). Upon expression of hENT1, toyocamycin was growth

Figure 2.3.3 – **hENT1 permits the uptake of adenosine nucleosides and analogues.** (a.i-ii) *ade2Δade3Δ*+EV and *ade2Δade3Δ*+hENT1 supplemented with adenine. (b.i-ii) adenosine. (c.i-ii) 5'-methylthioadenosine (MTA). (d) WT+EV and WT+hENT1 treated with toyocamycin. (e) Cordycepin. Cells cultured overnight in SD-U+R media were seeded in SD-U+RG (for WT, methionine auxotroph) or

SD-UA+RG (for *ade2Δade3Δ*, adenine and methionine auxotroph) to 5×10^5 cells/mL, treated with nucleosides or nucleobases and were incubated at 30°C. OD₆₀₀ was determined using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer). Where appropriate, percent growth (OD₆₀₀Exp/OD₆₀₀ vehicle control x 100) was determined in mid-log cultures (0.3-0.5 OD₆₀₀ units in the vehicle control). SD-U+R, SD-Uracil +2% raffinose; SD-U+RG, SD-Uracil +2% raffinose +2% galactose; SD-UA+RG, SD-Uracil/adenine +2% raffinose +2% galactose; EV, empty vector.

inhibitory at low nanomolar concentrations (IC₅₀ ~6.6 nM). Although, the drug target of toyocamycin is conserved in yeast (Kiburu & LaRonde-LeBlanc, 2012), this is the first instance where bioactivity of toyocamycin has been demonstrated *in vivo* due to the absence of plasma membrane nucleoside transporters in *S. cerevisiae*. In contrast, cordycepin, a natural product that has been previously analysed in chemogenomic analyses of yeast (Holbein et al., 2009), was bioactive in WT (IC₅₀ ~17 μM) and the toxicity was improved ~85 fold with the expression of hENT1 (IC₅₀ ~2 nM) (Fig. 2.3.3e). This suggests that hENT1 is not the sole nucleoside transporter of cordycepin and the permease(s) responsible for uptake have not been characterised.

2.3.3 Meu1 is essential when MTA is the only exogenous source of sulphur

The IC₁₀₋₂₀ of compounds in WT cells are required for screening of gene deletion mutants for hypersensitivity and growth inhibition requires a perturbation of an essential target or essential macromolecule in cells (Parsons et al., 2004; Cong et al., 2012). *MEU1* in yeast encodes a non-essential protein and, we sought to make a *MEU1* conditionally essential by screening the methionine auxotroph yeast strains WT+EV and WT+hENT1 with Meu1 inhibitors MT-DADMe-immucillin A (MTDIA) or DADMe-immucillin A (DIA) in different media conditions. The growth inhibition was assessed in cells cultured with or without the supplementation of MTA (native substrate of *MEU1*) in media with or without methionine and cysteine. WT expressing empty vector (WT+EV) showed slight growth inhibition with MTDIA at high micromolar concentration (IC₂₀ = 0.32-1 mM) in SC media and the growth inhibition was unchanged by the expression of hENT1 (Fig. 2.3.4a). WT+EV and WT+hENT1 strains also showed similar levels of growth inhibition to DIA at micromolar concentrations (IC₂₀ >320

μM) (Fig. 2.3.4b). This slight growth inhibition at high micromolar concentrations are likely a result of induction of a general stress mechanism (Petrenko et al., 2013) or nucleoside analogues interfering with the uptake of other nutrients (Yao et al., 2011).

In contrast, the treatment of WT+hENT1 in MTA with MTDIA lead to significant growth inhibition at low nanomolar concentrations ($IC_{20} \sim 15$ nM) (Fig. 2.3.4c). The growth inhibition plateaued at 42% and was not further inhibited at concentrations >1 μM MTDIA. In the absence of methionine/adenine/cysteine, the growth of WT+hENT1 treated MTDIA and 250 μM MTA was inhibited by 93% at the lowest concentration tested of 10 nM and the MIC was 31.6 nM. Similarly, DIA+ 250 μM MTA inhibited the growth of WT+hENT1 at nanomolar concentrations ($IC_{20} \sim 125$ nM) in SC but plateaued at 68% growth at 31.6 μM and in the absence of methionine/adenine/cysteine the growth the was inhibited by 33% at 10 nM. These results indicate an essential condition that include the heterologous expression of hENT1, MTA as the sole sulphur source in the absence of amino acids methionine/cysteine are required to render *MEU1* as conditionally essential.

In the absence of methionine and cysteine, MTA is processed by Meu1 to generate adenine and 5-methylthioribose-1-phosphate that are further processed in subsequent steps to salvage methionine (Pirkov et al., 2008). Inhibition of this salvage pathway by MTDIA/DIA-facilitated inhibition of *MEU1* was likely responsible for the observed growth inhibition. The absence of adenine enhanced growth inhibition but was not required to achieve complete inhibition (Appendix Fig. 7.2.2). This was likely a consequence of reduced adenine available for synthesis of s-adenosyl methionine from which methionine is salvaged (Thomas et al., 2000). From here forth, MTDIA was used as the *MEU1* inhibitor as it was more potent than DIA and media lacking amino acids methionine/adenine/uracil/cysteine supplemented with 250 μM MTA and 2% galactose as the carbon source (SD-MAUC+MTA+2% Gal) was used as the condition where *MEU1* is essential.

Following identification of the *MEU1* essential condition, the nature of growth inhibition by MTDIA (*i.e.*, cytostasis or cytotoxicity) was assessed. Two approaches were employed for this

assessment. In the first approach, WT+hENT1 cells were treated with MTDIA (as previously described in section 2.2.1), and at set intervals (0, 6, 12, 24 and 48 h), a subset of cells were removed and

Figure 2.3.4 – **MTDIA is a potent inhibitor of yeast MTAP and cytostatic when methionine salvage is essential.** (a) WT+EV and WT+hENT1 treated with MT-DADMe-immucillin A (MTDIA). (b) DADMe-immucillin A (DIA). (c) WT+hENT1 treated with MTDIA in SD-URA+RG, SD-URA+MTA+RG and SD-MAUC+MTA+RG. (d) DIA. (e.i) Recovery WT+hENT1 pre-treated with MTDIA for 0, 6, 12, 24, and 48 h

in SD-MAUC+MTA+RG agar (e.ii) Recovery of MTDIA pre-treated WT+hENT1 in SD-URA+RG agar. (f) Recovery WT+hENT1 cells pre-treated with MTDIA for 48 h, following 48 h in SD-MAUC+MTA+RG or SD-MAUC+MTA+RG+ MTDIA[μ M]. WT cells (expressing EV or hENT1) were cultured overnight in SD-U+R media were seeded in fresh SD-U+RG or SD-MAUC+RG media supplemented $\pm$ 250 μ M MTA to 5×10^5 cells/mL, treated with MTDIA or DIA and were incubated at 30°C. OD₆₀₀ was determined using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer) and where appropriate, % growth ($\text{OD}_{600}\text{Exp}/\text{OD}_{600}\text{ vehicle control} \times 100$) was determined in mid-log cultures (0.3-0.5 OD₆₀₀ units in the vehicle control). The MTDIA cells were recovered by either by plating on agar media or resuspending in media without MTDIA following MTDIA treatment. The recovery was measured either by visual inspection on agar or measuring OD₆₀₀ in liquid media. SD-U+R, SD-Uracil +2% raffinose; SD-U+RG, SD-Uracil +2% raffinose +2% galactose; SD-MAUC+MTA+RG, SD-Methionine/Adenine/Uracil/Cystine + 250 μ M 5'-methylthiodenosine +2% raffinose +2% galactose; EV, empty vector.

incubated for growth on SD-MAUC+MTA+RG agar media and SD-Ura agar media (Fig. 2.3.4e.i). The growth of cells immediately following MTDIA treatment was generally comparable to untreated cells for all MTDIA concentrations with only slight growth inhibition observed for the highest concentration (3.16 μ M MTDIA). In contrast, cells pre-treated with MTDIA for more than 6 h were unable to recover beyond MTDIA concentrations ≥ 316 nM. This was however, an order of magnitude greater than the MIC of MTDIA. When the cells were plated in SD-Ura media (without MTA), cells were able to recover independent of the time or concentration of the MTDIA treatment (Fig. 2.3.4e.ii). Together, these results suggest that MTDIA is cytostatic and not cytotoxic. MTDIA only inhibits growth as long as *MEU1* remains essential (*i.e.*, in the absence of methionine and cysteine with supplemented MTA). The growth inhibition is thus a consequence of the inability to salvage methionine.

In the second approach, WT+hENT1 cells were treated with MTDIA for 48 h, washed in fresh SD-MAUC+MTA+RG media, incubated at 30°C for 48 h, and growth was measured at OD₆₀₀ (Fig. 2.3.4f). After treatment with MTDIA, cells were able to recover at concentrations two orders of magnitude beyond the MIC of MTDIA. The recovery however was incomplete as cells pre-treated with higher concentrations (>10 μ M) of MTDIA were unable to recover. The recovery was absent in the presence of MTDIA even after 96 h from the initial MTDIA treatment. Together, this shows that following entry

into the cell, MTDIA is a potent inhibitor of yeast *MEU1*. This is consistent with the high degree of conservation of the MTAP active site from yeast to humans (Subhi et al., 2003; Lee et al., 2004)

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Get&RID=FD3YFTNJ113#alnHdr_Query_134717,

2017) and low nM IC_{50} of MTDIA (10-100 nM MTDIA with 20 μ M MTA) for human head and neck and lung cancer cell lines (Basu et al., 2007; Basu et al., 2011).

2.3.4 MTDIA inhibits growth by inhibiting *MEU1* function and is bioactive in agar

To confirm that MTDIA toxicity in *S. cerevisiae* is mediated through *MEU1*, WT and *meu1 Δ* deletion strains were screened for sensitivity to MTDIA or MTA alone. The growth of the *meu1 Δ* +hENT1 strain was mostly unaffected by MTDIA treatment alone and was similar to WT+hENT1 (Fig. 2.3.5a). Only a small reduction in growth (90% growth at 100 μ M) was observed in both strains at the highest concentration tested and was consistent with previous experiments. This is likely a result of MTDIA saturating the hENT1 transporter and interfering with the uptake of other nucleobases (Yao et al., 2011).

The growth of WT+hENT1 in the absence of methionine/adenine/cystine was dependent on MTA concentration as previously observed (Fig. 2.3.3). Maximum growth was achieved between 100-316 μ M and was slightly growth inhibitory at 1mM (Fig. 2.3.5b). In contrast, the growth of *meu1 Δ* +hENT1 was significantly impaired (15% of WT at 100 nM MTA) but not abolished in the presence of MTA in the absence of methionine/adenine/cysteine. These results and the near lethal combination of MTA and MTDIA (Fig. 2.3.4c) validates *MEU1* as the target of MTDIA in yeast. The slight growth observed for *meu1 Δ* (OD_{600} = 0.07 at 100nM) reflects the presence of other methionine salvage pathways in yeast (Thomas et al., 2000; Finkelstein, 1990).

Following the validation of *Meu1* as the target of MTDIA, the conditions for screening the *xxx Δ* +hENT1 deletion library were optimised in agar. The *his3 Δ* +hENT1 strain, that is phenotypically equivalent to WT that carries a partial deletion of the *HIS3* gene was screened for sensitivity to MTDIA in SD-MAUC + 250 μ M MTA + RG agar media by spotting 5×10^5 , 5×10^3 and 5×10^1 cells from an overnight culture. Plates were incubated at 30°C and were visually inspected for growth reduction in comparison

to the vehicle control at 24, 48, 72 and 96 h (Fig. 2.3.5c). A reduction in growth was observed at MTDIA concentrations ≥ 3.125 nM for all three cell densities dependent on the incubation time. The

Figure 2.3.5 – MTDIA reduces growth by inhibiting Meu1 dependent salvage of methionine and is growth inhibitory in agar. (a) WT+hENT1 and *meu1Δ*+hENT1 treated with MTDIA. (b) WT+hENT1 and *meu1Δ*+hENT1 treated with MTA. (c) 5×10^5 , 5×10^3 and 5×10^1 *his3Δ*+hENT1 (WT equivalent) cells treated with MTDIA and 250 μ M MTA in agar. Growth of WT+hENT1 and *meu1Δ*+hENT1 treated with MTDIA or MTA alone in SD-U+RG or SD-MAUC+RG were determined at OD₆₀₀ was using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer) and where appropriate, percent growth ($\text{OD}_{600}\text{Exp}/\text{OD}_{600}\text{vehicle control} \times 100$) was determined in mid-log cultures (0.3-0.5 OD₆₀₀ units in the vehicle control). In the agar screen, 5×10^5 , 5×10^3 and 5×10^1 cells from an overnight culture in SD-U+R was spotted onto SD-MAUC+250 μ M MTA+RG agar media, incubated at 30°C and imaged at 24 (ci), 48 (cii), 72 (ciii) and 96 h (civ). The arrow heads indicate the lowest concentration that shows a notable growth reduction for each cell density. SD-U+R, SD-Uracil +2% raffinose; SD-U+RG, SD-Uracil +2% raffinose +2% galactose; SD-MAUC+MTA+RG, SD-Methionine/Adenine/Uracil/Cysteine +250 μ M 5'-methylthiodenosine +2% raffinose +2% galactose.

his3Δ+hENT1 strain as previously shown was unable to grow in the absence of MTA. These results show that MTDIA was not inactivated by adding to 65°C agar media and therefore the chemogenomic analysis can be performed in agar. Further, MTDIA is growth inhibitory at MTDIA concentrations as low as 3.125 nM in agar media.

2.3.5 Genome-wide analysis identifies genetic interactions with MTDIA

The IC₁₀₋₂₀ of compounds in WT cells are used for screening of gene deletions mutants for hypersensitivity (Lee et al., 2014; Hoepfner et al., 2014). The IC₂₀ was used for screening yeast gene deletion libraries as it identifies sufficient number of drug hypersensitive strains and permits sufficient growth to distinguish sensitive from hypersensitive sensitive strains. To optimise screening conditions to evaluate sensitivity of the *xxxΔ*+hENT1 deletion library to MTDIA, we sought to determine the ~IC₂₀ in 1536 colony format, the format to be used for the genome-wide analysis. A single plate from the *xxxΔ*+hENT1 library was screened against semi-inhibitory concentrations of MTDIA previously determined in agar. Growth of plate 12 of the *xxxΔ*+hENT1 deletion library was quantified at 3 nM, 6 nM, and 12 nM MTDIA relative to a vehicle control. The median colony size of plate 12 was reduced 19, 26 and 33% compared to the vehicle control with treatments of 3 nM, 6 nM and 12 nM MTDIA,

respectively (Fig. 2.3.6a). In addition to reduction of the plate-wide median colony size, several gene deletion strains also showed a growth defect in comparison to the plate median, such as *hir2Δ* that encodes a subunit of the nucleosome assembly complex required for regulation of transcription (Prochasson et al., 2005). From the three semi-inhibitory MTDIA concentrations assessed in 1536 colony format, 3 nM was chosen as the screening for the *xxxΔ*+hENT1 deletion library as this concentration inhibited a panel of 384 strains by 18% compared to untreated cells (*i.e.*, within the desired IC₁₀₋₂₀ level of inhibition).

For assessment of MTDIA drug-gene interactions, the *xxxΔ*+hENT1 library comprising 4310 unique gene deletion strains was treated with 3 nM MTDIA or vehicle control in SD-MAUC+MTA+RG media in agar in three biological replicates and 12 technical replicates. Colony sizes of treated and untreated cells were quantified using Gitter (Wagih & Parts, 2014) and compared using ScreenMill (Dittmar et al., 2010). Relative to the vehicle control in at least two of the three biological replicates, 39 deletion strains exhibited a significant growth defect based on a growth ratio < 0.8 with a $p < 0.05$. In contrast, 13 deletion strains exhibited a significant growth improvement based on a growth ratio > 120% with a $p < 0.05$ (Fig. 2.3.7b; Appendix Table 7.2.3a). To validate the putative drug-gene interactions with MTDIA, growth of selected *xxxΔ*+hENT1 gene deletions strains (52 putative genetic interactions with MTDIA and 558 additional deletion strains that were either genetic interactors with *MEU1* or had shared function with the putative interactions with MTDIA) was quantified in the presence and absence of 2 nM MTDIA, the IC₂₀ concentration in liquid media. The validations were performed in triplicate and in liquid media as it is more quantitative than comparing colony size in agar. Stringent cut-offs were applied to determine MTDIA hypersensitivity (growth < 75%, $p < 0.05$) or resistance (growth > 125%, $p < 0.05$) and were assigned to GO categories using the haploid non-essential gene deletion library as a reference set on YeastMine (Balakrishnan et al., 2012).

The growth defect of 61 gene deletion strains (20/52 putative and 41/558 related) was significantly exacerbated (growth < 75%, $p < 0.05$) in response to MTDIA treatment and were

Figure 2.3.6 – **Gene deletion strains hypersensitive or resistant to 3 nM MTDIA.** (a) The plate-wide reduction in median colony size with 3, 6 and 12 nM MTDIA treatment and *hir2Δ+hENT1* showing dose dependent sensitivity to MTDIA. (b) *xxxΔ+hENT1* library screened against 3nM MTDIA in agar. A single *xxxΔ+hENT1* library plate was screened against semi-inhibitory concentrations of MTDIA in SD-MAUC+MTA+RG agar media. The plates were imaged and the median colony size was determined using Gitter. Percent reduction in plate-wide median colony size was determined by comparing against the vehicle control and are presented in parenthesis. For assessment of MTDIA drug-gene interactions, the 4310 *xxxΔ+hENT1* gene deletion strains were treated with 3 nM MTDIA or vehicle control in SD-MAUC+MTA+RG media in agar in triplicate experiments. The *xxxΔ+hENT1* strains with a growth defect or growth improvement (growth<80% or growth>120%, $p < 0.05$) in at least two out of three SGAs were considered as hypersensitive or resistant to MTDIA.

considered hypersensitive to MTDIA (Fig. 2.3.7a; Appendix Table 7.2.3b). These genes were significantly enriched for the major biological processes of regulation of gene expression ($p = 5.97E-04$) and 'de novo' IMP biosynthetic process ($p = 1.45E-04$) following Benjamin-Hochberg correction for

multiple testing ($p < 0.05$). Several sub-categories within these biological processes were also significantly enriched including negative regulation of gene expression ($p = 1.18\text{E-}04$), histone deacetylation ($p = 1.39\text{E-}02$) chromatin organization ($p = 2.80\text{E-}02$), transcription from RNA polymerase III promoter ($p = 3.81\text{E-}02$) and negative regulation of transcription during mitotic cell cycle ($p = 4.67\text{E-}02$) (Appendix Table 7.2.4). In contrast, 30 gene deletions (4/52 putative and 26/558) conferred a significant growth improvement (growth > 130%, $p < 0.05$) in response to MTDIA treatment and were considered resistant to MTDIA (Fig. 2.3.7b; Appendix Table 7.2.3c). These 30 genes did not show enrichment for GO categories when corrected for multiple testing.

In addition to MTDIA-sensitive and MTDIA-resistant strains, several gene deletion strains failed to grow when MTA was provided as the only sulphur source (Appendix Table 7.2.5). Several of these were noteworthy such as *MEU1*, *MRI1*, *MDE1*, *UTR4* and *ADI1* that encode enzymes involved in salvage of methionine from MTA (Pirkov et al., 2008). Genes required for endocytosis (*RSV167* and *RSV161*) (Youn et al., 2010), guanine nucleotide exchange factor (*CCZ1*), and iron transport (*FTR1* and *FET3*) were inviable in MTA and also showed an exacerbated growth defect with *meu1Δ*.

There is strong evidence that the MTDIA screen is targeting *MEU1* based on overlap with the *meu1Δ* SGA results. The MTDIA hypersensitive genes included gene deletion strains defective in adenine metabolism (*ADE1*, *ADE4*, *ADE5,7*, *ADE6*, *ADE8*) and involved in inosine monophosphate (IMP) biosynthesis. Interestingly, these genes sensitive to MTDIA were involved in *de novo* synthesis of IMP from PARP (Rébora et al., 2001; Jones & Fink, 1982), while the *meu1ΔxxxΔ* strains with exacerbated growth defects were involved in the salvage of IMP from adenine (Pirkov et al., 2008). This was perhaps a consequence of performing the *meu1Δ* screen in adenine-replete conditions and the MTDIA screen in adenine-deficient media supplemented with MTA. Loss of either pathway in conjunction with *MEU1* inhibition with MTDIA or with deletion of *MEU1* further reduces availability of adenine for purine nucleoside biosynthesis. This is consistent with inhibition of purine synthesis by MTA in MTAP^{+/+} human lymphoblasts and the increased sensitivity of MTAP negative cancers to purine synthesis inhibitors (Bertino et al., 2011; Gordon et al., 1987; Gordon & Emmerson, 1986). Gene deletions

sensitive to MTDIA that also were synthetic lethal with *meu1Δ* also included genes involved in gene expression at the levels of chromatin organisation (*BRE1*, *DPB4*, *HDA3*, *HIR2*, *HIR3*, *HOS4*, *HST1*, *HST3*, *IES1*, *NPT1*, *PAT1*, *RFM1*, *RXT2*, *SIR4*, *SNF5*, *SUM1*), transcription (*SOK2*, *YLR236C*, *YLR235C*, *TDA5*, *RPN4*, *PSP2*, *CKA2*, *DHH1*, *YGR054W*, *KNS1*, *CTH1*, *DPB4*, *GTR2*, *YOR024W*), and translation (*RPL9A*, *YPR126C* (overlaps *YLH47*), *RPL22A*, *RPS6A*, *RPS19A* and *RPS10A*). These were likely consequences of depletion in polyamine levels that resulted in negatively regulated gene repression (Hobbs & Gilmour, 2000; Hobbs et al., 2002) as well as inhibition of both translation initiation and elongation (Landau et al., 2010).

In addition, several novel cellular processes were identified in the MTDIA screen that were not previously identified with MTAP inhibition or the *meu1Δ* SGA. First, zinc homeostasis was suggested by hypersensitivity of the zinc ion homeostasis genes (*ICE2* and *ZRT1*) to MTDIA. In response to zinc depletion, CDP-DAG pathway (*CHO1*, *OPI3*, *CHO2*) is down regulated (Iwanyshyn et al., 2004) and phosphatidylinositol synthase is upregulated (Henry et al., 2012). The already depleted phospholipid biosynthesis pathway by MTAP inhibition may be further downregulated by low zinc conditions generated by *ZRT1* and *ICE2* loss. Second, pH homeostasis was implicated by genes involved in the RIM101 pathway (*RIM20*, *DFG16*, *RIM21*) that were sensitive to MTDIA treatment. These genes along with RIM9 forms a protein complex that regulate RIM101, a transcriptional repressor of alkaline pH responsive genes (Xu & Mitchell, 2001) and RIM101 pathway that acts in parallel to PKC pathway in responding to cell wall damage (Castrejon et al., 2006). This pathway is constitutively activated by a deletion of *VPS4* (Hayashi et al., 2005), a gene deletion that was resistant to MTDIA. The poor cell wall composition in cells cultured in galactose (Aguilar - Uscanga & Francois, 2003) and disruptions to sterol homeostasis by MTDIA would be exacerbated by the deletions in genes required for cell synthesis, thus, leading to the growth defect as these pathways are interconnected.

Figure 2.3.7a – **61 gene deletion strains were hypertensive to MTDIA.** The growth of the single gene deletion mutants treated with MTDIA relative to WT treated with MTDIA ($\frac{xxx\Delta + \text{MTDIA}}{xxx\Delta + \text{carrier}} / \frac{\text{WT} + \text{MTDIA}}{\text{WT} + \text{carrier}} \times 100$) was determined in liquid media at OD₆₀₀ when the carrier control was 0.3-0.5. The *his3Δ* deletion strain was used as the WT control. The single mutants with average growth <75%, $p < 0.05$ from three replicates compared to the WT control were considered hypertensive to MTDIA. These were assigned to simplified GO categories using GO slim mapping tool on SGD and were involved in amino acid biosynthesis, multivesicular body formation, Sterol biosynthesis, ion homeostasis, ribosome biogenesis, glucose metabolism, gene expression, NAD⁺ biosynthesis, and cell cycle progression.

Figure 2.3.7b – **29 gene deletion strains were resistant to MTDIA**. The growth of the single gene deletion mutants treated with MTDIA relative to WT treated with MTDIA ($xxx\Delta$ +MTDIA/ $xxx\Delta$ +carrier)/ (WT+MTDIA/ WT+carrier) x 100 was determined in liquid media at OD₆₀₀ when the carrier control was 0.3-0.5. The *his3Δ* deletion strain was used as the WT control. The single mutants with average growth >130%, $p < 0.05$ from three replicates compared to the WT control were considered resistant to MTDIA. These were assigned to simplified GO categories using GO slim mapping tool on SGD and were involved in endosomal transport, vacuole fusion, mitochondrion organization, translation, and chromatin organisation.

2.3.6 Further evaluation of MTDIA and *meu1Δ* mechanisms

2.3.6.1.0 Macroautophagy genes are sensitive to MTDIA treatment

A subset of the gene deletions that were resistant to MTDIA were involved in fusion of vesicles and autophagosomes with the vacuole (*ELO2*, *VPS17*, *VPS4*, *VPS5*, *VPS53*, *YDR455C*/overlaps *VPS44*, *VPS8*, *VAM10* and *MON1*) (Klionsky, 2005), pexophagy (*GPR1* (Budovskaya et al., 2004) and *PEX5*) and negative regulation of TORC1 (*NPR2*). Another subset of gene deletions hypersensitive to MTDIA were involved in negative regulation of autophagy (*TOR1* and *SOK2*). Interestingly, some of the same deletion that were resistant to MTDIA showed a growth defect in the *meu1Δ* SGA, including genes involved in vesicle fusion with the vacuole (*CCZ1*, *VAM3*, *VAM6*, *VPS41*, *ROM2*, *MTC5*, *GGA2* and *RIM1*) and response to starvation (*PHO5*, *IRS4*, *RVS161*, *RVS167*). This disparity between the two experiments might be a reflection of the condition in which the screens were performed where mild autophagy may promote survival while induction excessive autophagy may be detrimental to viability (Tsujimoto & Shimizu, 2005). To determine if MTDIA disrupts the autophagy process in yeast, several assays were employed to measure responses to MTDIA treatment and *meu1Δ* are investigated below. We thus hypothesize that autophagy is a novel mechanism of MTDIA toxicity.

2.3.6.1.1 MTDIA exacerbates the rapamycin induced growth arrest and induces autophagic cell death

To determine if MTDIA treatment disrupts the autophagic flux, cells were co-treated with MTDIA and rapamycin, an inhibitor of the TOR nutrient sensing pathway and a hallmark inducer of autophagy (Kamada et al., 2000). The growth of WT cells treated with semi-inhibitory concentrations of MTDIA and its genetic mimic *meu1Δ* were assessed following rapamycin treatment (Fig. 2.3.8.1a) and the fractional inhibitory concentration (FIC) index (Hall et al., 1983; Odds, 2003) was assessed to determine synergy (Appendix Table 7.2.6). A concentration dependent growth reduction of WT was observed following rapamycin treatment (6.5, 12.5 and 25 nM) that was exacerbated by 1, 3, and 5 nM MTDIA treatment. FIC <0.5 was observed for co-treatment of rapamycin and MTDIA at concentrations lower than IC₅₀ of each compound. This increased sensitivity to rapamycin was also observed for *meu1Δ* compared to WT (Fig. 2.3.8.1b). However, the *meu1Δ* strain was slow growing as

Figure 2.3.8.1 – MTDIA treatment and *meu1Δ* decreases chronological lifespan and exacerbates the growth defect induced by rapamycin. (a) Growth of Rapamycin and MTDIA co-treated cells relative to MTDIA treatment alone. (c) WT and *meu1Δ* treated with rapamycin. (c-d) Viability of WT cells treated MTDIA and *meu1Δ* assessed with phloxine B at 2 d intervals. MTDIA and rapamycin co-treatments were performed in liquid media 30°C for 48 h. Percent growth was determined in liquid from the growth in the MTDIA + rapamycin co-treatment relative to MTDIA and rapamycin alone $(RPM+MTDA/MTDIA)/(RPM/DMSO) \times 100$ at OD₆₀₀ from six biological replicates. Fractional inhibitory concentration (FIC) index was calculated to distinguish synergy from additive (Appendix Table 7.2.6). Viability assays was performed by culturing yeast strains in 50 mL volumes of SD-MAUC+ 250 μM MTA+2% raffinose and 2% galactose media at 30°C for 10 d. At 2 d intervals, 1mL of cells were strained with 2 μg/mL phloxine B and were visualised under 60x magnification under DIC and 488 nm using Upright Automated Fluorescence Microscope (BX63, Olympus) equipped with camera (Dp70, Olympus). Percent viability (unlabelled cells/GFP labelled cells + unlabelled cells) $\times 100$ was determined from 1000-1300 cells across five visual fields for each condition and the standard deviation from the five different visual fields are presented.

it is unable to utilise MTA and relied on other methionine salvage pathways for growth. These results are also consistent with the *TOR1* gene deletion showing increased sensitivity to MTDIA compared to WT (in the MTDIA chemical screen, Fig. 2.3.7). These results suggest that the exacerbated rapamycin-induced growth arrest by MTDIA and *meu1Δ* was either due to further induction of autophagy or exacerbation of some other mechanism of rapamycin. The synergistic interaction between rapamycin and MTDIA suggests a TORC1 independent pathway for induction of autophagy.

Defects in autophagy can result in loss of viability (Liu & Levine, 2015). Measure of cell survival is one approach to monitoring the impact of autophagy and this can be assayed with phloxine B, which stains the cytosol of dead cells with disrupted plasma membranes (Noda, 2008). To investigate autophagic cell death, the WT cells treated with MTDIA and *meu1Δ* were assessed for viability with phloxine B. The WT cells treated with MTDIA showed a reduction in viability starting at 4 d (82.9% for 3nM MTDIA ($\sim IC_{20}$) compared with 89.6% for WT untreated) (Fig. 2.3.8.1c-d). This defect was further enhanced at 10 d resulting in a concentration dependent reduction in viability with MTDIA (74.5% for WT untreated, 61.7% for 1nM MTDIA($\sim IC_{10}$), 28.2% for 3nM MTDIA). The decrease in cell viability was

further exacerbated in the *meu1Δ* gene deletion strain in the absence of MTDIA (66.2% viability at 4 d and 13.0% viability at 10 d). This shows that MTAP inhibition maintained over a period of days is cytotoxic and contrary to the cytostatic phenotype observed in section 2.3.3. Together, the exacerbation of rapamycin-mediated growth arrest by MTDIA and increase in autophagic cell death suggests that MTDIA treatment may induce toxic levels of autophagy in yeast. Given that some genes required for autophagy were dispensable in response to MTDIA treatment while others were not, suggesting that some autophagy is needed for survival following MTDIA treatment while too much autophagy is detrimental.

2.3.6.1.2 MTDIA regulates abundance and localisation of core autophagy proteins

The autophagy pathway in yeast have been extensively studied and genes involved in each step of autophagy: induction, recognition of cargo and packaging, vesicle nucleation, expansion of the phagophore and autophagosome completion, Atg protein cycling, autophagosome fusion with the vacuole, brake down of cargo and recycling of macromolecules have been identified (Yang & Klionsky, 2009). To further investigate the potential induction of autophagy in response to MTDIA treatment, the deletion of genes that are essential for both non-selective (macroautophagy) and selective forms of autophagy (cytosol to vacuole transport pathway, pexophagy, mitophagy and ribophagy) were assessed for sensitivity to MTDIA. The genes deletions of core-autophagy proteins did not present sensitive or resistant to MTDIA compared to WT (i.e. $\pm$ 25% growth difference) (Fig. 2.3.8.2a). It is possible that although these deletions do not show altered sensitivity, that their function may be altered at the protein level. To investigate if the functions of core-autophagy proteins were affected by MTDIA, the GFP fusion proteins of the core-autophagy genes (from the GFP/dual RFP+hENT1 libraries constructed in Chapter 4) were assessed for change in localisation and abundance in response to MTDIA treatment and loss of *meu1Δ* by Acapella automated image analysis software (Perkin Elmer). The autophagy proteins Atg1 (Atg1 kinase complex), Vps34 (phosphoinositide 3-kinase), Atg3 and Atg4 (Atg8 conjugation system), Atg2 and Atg18 (ATG9 recycling), and Atg20 (cytosol to vacuole targeting

pathway) showed changes in localisation or change in protein abundance or both either in response to MTDIA treatment or in response to loss of *MEU1*. Atg1-GFP relocated from endosomes to the vacuole, and similarly Atg2-GFP relocated from the cytosol to endosomes (Fig. 2.3.8.2b) following MTDIA treatment, results that were consistent with rapamycin-induced autophagy phenotypes (Nakatogawa et al., 2012; Suzuki et al., 2007). The *meu1Δ* in these strains showed phenotypes consistent with MTDIA treatment. The Atg18-GFP was excluded from the vacuole (Suzuki et al., 2007), Atg3-GFP increase in abundance (Huh et al., 2003) and Atg20-GFP relocated cytosol and endosomes to the mitochondria (Koh et al., 2015) showed nutrient starvation consistent phenotypes in the *meu1Δ* deletion but not with MTDIA treatment under the conditions investigated. The changes in distribution of core-autophagy protein with *meu1Δ* in the absence of changes in WT treated with MTDIA may reflect the difference in knock out and knock down of the drug target and a higher concentration of MTDIA or longer treatment regime may be needed to observe these phenotypes with MTDIA treatment.

In contrast, Vps34-GFP showed a change in localisation from the vacuole and vacuolar membrane to cytoplasmic foci and ER in *meu1Δ* but not with MTDIA treatment. Interestingly, the mislocalisation of Vps34 in *meu1Δ* was also observed in complete minimal (methionine replete) media compared to WT (Appendix Fig. 7.2.3). Therefore, it may present a likely point of perturbation for autophagy induction. All the other observed changes in localisation and abundance were also confirmed by assessment of GFP abundance and localisation in complete minimal (methionine replete) and starvation media. In addition, significant changes in GFP localisation or abundance within the cell were also detected for Atg11, Atg27 and Atg31 with MTDIA treatment in the *meu1Δ* by Acapella. However, these were indistinguishable from WT untreated upon visual inspection but may reflect actual changes and could be validated by western blot analysis. The changes in abundance and localisation of autophagy proteins to MTDIA treatment or loss of its drug target (with the exception of Vps34) are consistent with starvation media and rapamycin treatment in published literature and confirms an induction of starvation induced autophagy although the deletions of their genes did not show altered sensitivity from WT. The change in Vps34 in complete media may reflect some additional

Figure 2.3.8.2 – Protein levels and localisation of core autophagy proteins in response to MTDIA treatment although the gene deletions do not show altered sensitivity. (a) Deletions strains defective in core-autophagy genes treated with 3 nM MTDIA ($\sim IC_{30}$). (b) Abundance and localisation of core-autophagy proteins fused to GFP in WT, WT with 100 nM MTDIA and in *meu1Δ*. The gene deletion strains were treated with 2 nM MTDIA in SD-MAUC + 2% galactose +250 μM MTA at 30°C for 48 h. The growth of the mutants relative to WT in MTDIA and carrier (MTDIA *xxxΔ*/DMSO *xxxΔ*)/ (MTDIA WT /DMSO WT) x 100 were determined at OD₆₀₀. Mutants with a growth defect or growth improvement > ± 25%, *p* < 0.05 relative to WT were considered a growth defect or a growth improvement. The core-autophagy GFP strains were cultured in SD-MAU + 2% galactose +250 μM MTA media for 6 h at 30°C. Florescent signals were detected at 488 nm (GFP) and 561 (nuclear localisation signal fused to RS2 and cytosolic mCherry) using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin Elmer) using the 60x water immersion lens (NA 1.2). The GFP abundance and localisation was quantified by automated image analysis (Acapella, Perkin Elmer)(Bircham et al., 2011). Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated. Phosphoinositide 3-kinase (PtdIns3K); cytosol to vacuole transport (cvt).

inducer of autophagy besides amino acid starvation. Several potential inducers of autophagy that are amino acid starvation independent were identified in the *meu1Δ* and MTDIA screens notable of these were disruptions to ergosterol biosynthesis and phospholipid biosynthesis both of which can induce autophagy (Cheng et al., 2006).

2.3.6.1.3 MTDIA induces autophagic trafficking of GFP-Atg8 independent of starvation

To further confirm an induction of autophagy following MTDIA treatment, the localization of GFP-Atg8, the yeast orthologue of the mammalian autophagosome marker LC3, was evaluated. LC3/Atg8 is one of two proteins that remain associated with the completed vesicle throughout the autophagy pathway and is frequently used as a marker for monitoring autophagic activity in yeast and mammalian cells (Merkulova et al., 2014; Klionsky et al., 2007; Strømhaug et al., 2004). Following induction of autophagy in starvation media or following rapamycin treatment, Atg8 localises from cytosolic foci (autophagosomes) to the vacuole (Suzuki et al., 2001; Kim et al., 2001). The yeast strains YCG640 (WT) and YCG643 (*meu1Δ*) with hENT1::URA3 (constructed in Chapter 4) were transformed

with *natMX4* selectable marker from p4339 plasmid (Goldstein & McCusker, 1999) (to replace URA3 with *natR*) followed by GFP-ATG8::Cen::URA3 plasmid (Suzuki et al., 2001) to generate GFP-Atg8 expressing strains in the dual RFPs background (to observe GFP-Atg8 fluorescence relative to RFP fluorescence of cytosol and nucleus in each cell). The localisation of GFP-Atg8 in WT and *meu1Δ* were assessed by fluorescence microscopy after 3h incubation in SD-MAU+ 2% galactose + 250 μM MTA media with and without MTDIA. In the absence of MTDIA treatment, WT cells showed nearly equal amounts of vacuolar and cytosolic localisation of GFP-Atg8 (47.2% and 52.9% respectively) (Fig. 2.3.8.3a-b). Following treatment of 10 nM MTDIA, 72.8% of cells exhibited vacuolar localisation of GFP-Atg8. In the genetic mimic *meu1Δ*, this autophagy phenotype was also apparent with 79.43% of the cells showing vacuolar localisation of GFP-Atg8.

To reduce the background contribution of starvation-induced autophagy, cells were cultured in media supplemented with 1 mM MTA (Fig. 2.3.8.3c-d) and were treated with 100 nM MTDIA to account for higher levels of MTA and the short incubation time. Under these conditions ~88% of the WT cells showed cytosolic location for GFP-Atg8 in the absence of MTDIA. Following 100 nM MTDIA treatment, a direct inverse of this was observed where nearly 90% of the cells showed vacuolar localisation of GFP-Atg8. This phenotype was conserved in the *meu1Δ* strain in the absence of MTDIA wherein vacuolar localisation of GFP-Atg8 was observed.

To further investigate the induction of autophagy by MTDIA treatment, the autophagy pathway was disrupted with gene deletions that halted the autophagy process at various steps. The conjugation of Atg8 to the phosphatidylethanolamine (PE) on the autophagosomal membrane and deconjugation of ATG8 from the PE requires the cysteine protease, Atg4 (Yu et al., 2012). The dissociation of Atg proteins from the phagophore assembly site (PAS) requires Atg1 kinase (Cheong et al., 2008). Following delivery of autophagic bodies to the vacuolar lumen, intra vacuolar breakdown requires the vacuolar hydrolase, Pep4 (Xie et al., 2008). The deletions of genes *ATG1*, *ATG4* and *PEP4* were used for assessment of GFP-Atg8 localisation following induction of autophagy. As expected, WT cells showed mostly cytosolic localisation of GFP-Atg8 without treatment and mostly vacuolar

Figure 2.3.8.3 – **MTDIA treatment or *meu1Δ* induces autophagy that is Atg1, Atg4 and Pep4 dependent.** The localisation of GFP-ATG8 was visualised in cells treated with MTDIA (a-b) WT and *meu1Δ* treated with 250 μM MTA or 250 μM MTA with 3 and 10 nM MTDIA. (c-d) WT and *meu1Δ* treated with 1 mM MTA or 1mM MTA with 100 nM MTDIA. (e) *atg1Δ*, *atg4Δ*, *pep4Δ* and WT cells subcultured in SD-N, SD-Ura or SD-MAU media with 250 μM MTA or 250 μM MTA + 10 nM MTDIA. GFP-ATG8 expressing strains were subcultured with 250 or 1 mM MTA and appropriate concentrations of MTDIA in SD-MAU+2% galactose media for 3h (or SD-N +2% galactose, SD-Ura+2% galactose). The fluorescent signals were detected at 488 nm (GFP-ATG8) and 589 (nuclear localisation signal fused to RS2) using Upright Automated Fluorescence Microscope (BX63, Olympus) equipped with camera (Dp70, Olympus). Where appropriate, the localisation of GFP-ATG8 is presented as an average from five visual fields with 300-400 cells counted per condition.

localisation following MTDIA treatment. In contrast, the *atg1Δ* and *atg4Δ* deletions prevented the localisation of GFP-Atg8 to the vacuole and showed predominantly cytosolic location in both untreated and MTDIA treated cells (Fig. 2.3.8.3e). The *pep4Δ* prevented the degradation of GFP-Atg8 at the vacuolar lumen with localisation to vesicles in the vacuole in both MTDIA-treated and untreated cells. The GFP-Atg8 localisations observed for *atg1Δ*, *atg4Δ* and *pep4Δ* in MTDIA treated and untreated cells were unchanged from phenotypes observed in starvation-induced autophagy (Yeh et al., 2010; Kim et al., 2001), suggesting that MTDIA is inducing a stable and conserved form of autophagy. Overall, the increased localisation of GFP-Atg8 to the vacuole by MTDIA treatment that is dependent on a functional autophagic pathway confirms an induction of autophagy as a mechanism of MTDIA toxicity.

2.3.6.2.0 MTDIA inhibits ergosterol biosynthesis

Gene deletions involved in sterol homeostasis were sensitive (*ERF2*, *ETR1*, *YHL008C*) or resistant (*CGS2*) to MTDIA and showed an exacerbated growth phenotype with *meu1Δ* (*OPI3*, *CSG2*, *ETR1*, *INO2*, *SHR5*, *CEM1*). We hypothesize that sterol biosynthesis is not directly affected by loss of *MEU1* activity but rather by depletion of the downstream product S-adenosyl methionine, a co-enzyme required for the delta(24)-sterol C-methyltransferase activity facilitated by *ERG6* (Hardwick &

Pelham, 1994) and phospholipid methyl transferases activity by *CHO2* and *OPI3* (Kodaki & Yamashita, 1987). In contrast higher eukaryotes, the yeast plasma membrane consists of mainly ergosterol and minor amounts of zymosterol. The conversion of fecosterol to zymosterol is facilitated by Erg6 (Heidepriem et al., 1992; Lees et al., 1995). Although Erg6 function is non-essential for yeast cell division, it is required for normal membrane permeability and fluidity as reflected by altered sensitivity and resistance to xenobiotics and the permeability of nutrients (Gaber et al., 1989). Notable is Tat2, a high-affinity tryptophan transporter of the plasma membrane that is missorted to vacuole in response to loss of Erg6 function (Umebayashi & Nakano, 2003) showed an exacerbated growth defect with *meu1Δ*. Phospholipid composition of the membrane regulated by *OPI3* and *CHO2* are also required for membrane fluidity (de Kroon et al., 2013). The *de novo* biosynthesis of phosphatidylcholine from phosphatidylethanolamine by S-adenosyl methionine dependent phospholipid methyl transferases is the major consumer of S-adenosyl methionine in mice (Noga et al., 2003), in humans (Stead et al., 2006), and in yeast cultured in the absence of exogenous choline and ethanolamine (Tehlivets, 2011). It is possible that decreased S-adenosyl methionine is leading to a reduction in Erg6, Cho2 and Opi3 activity and consequently, a downregulation in ergosterol and phospholipid biosynthetic pathways.

To investigate disruptions to sterol synthesis, the abundance and localisation of four GFP fusion proteins in middle and late stages of the ergosterol biosynthesis pathway were assessed in response to MTDIA treatment of WT cells or in the *meu1Δ* strain by confocal microscopy. The localisation of three proteins was altered in response to MTDIA treatment as well as *MEU1* deletion. Erg24 was relocalised from the ER to cytosol and vacuole, Erg6 from ER and lipid droplets to lipid droplets, and Erg3 from ER to the vacuole (Fig. 2.3.8.4a). In addition, the levels of Erg1 was significantly decreased with MTDIA. These results suggest that MTDIA treatment leads to a disruption in the sterol biosynthesis pathway. To further characterise disruptions to sterol biosynthesis pathway, the levels of ergosterol and ergosterol precursors (squalene and lanosterol) were quantified by GC-MS (Fig. 2.3.8.4c). Reduced levels of ergosterol coincident with increased levels of squalene and lanosterol were observed in MTDIA-treated WT cells or untreated *meu1Δ* cells. These results are consistent with

a

c

d

Figure 2.3.8.4 – **MTDIA treatment and *meu1Δ* inhibits ergosterol biosynthesis.** (a) WT expressing ergosterol biosynthesis proteins fused to GFP treated with 100 nM MTDIA and *meu1Δ*. (b) Simplified ergosterol biosynthesis pathway showing sterol intermediates and genes of relevance. (c) Quantitation of sterol intermediates in WT and *meu1Δ* by GC-MS. GFP fusion strains within ergosterol biosynthesis were cultured in SD-MAU + 2% galactose +250 μM MTA media for 6 h at 30°C. Florescent signal was detected at 488 nm (GFP) and 561 (nuclear localisation signal fused to RS2 and cytosolic mCherry) using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin Elmer) using the 60x water immersion lens (NA 1.2). Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated. Sterols were extracted from OD600 5.0 units using chloroform-methanol. The lipid pellets were dried, derivatised, diluted in hexane and run on a GC-MS (QP2010-Plus, Shimadzu). The identity of the sterols were confirmed using authentic standards. The percent of each sterol relative to the total sterols (i.e., Ergosterol / (Squalene+Lanosterol+Ergosterol)x100) from six biological replicates experiments are presented.

reduced protein levels of Erg1 and Erg24 (Fig. 2.3.8.4a) and overall confirm that MTDIA and loss of *MEU1* inhibits the ergosterol biosynthesis in yeast.

2.4 Discussion

Screening of *S. cerevisiae* gene deletion mutants for gene-drug and gene-gene interactions permits an unbiased assessment of drug and drug target functions. In this chapter, we employed these methodologies for assessment of mechanisms in MT-DADMe-immucillin A (MTDIA), a transition state structure inhibitor of human MTAP. In this two-pronged approach, the non-essential nature of *MEU1* (yeast orthologue of MTAP) was exploited for assessment of gene-gene interactions with the drug target of MTDIA. However, the non-essentiality of *Meu1* also prevented the bioactivity of MTDIA coupled with the lack of nucleoside uptake in yeast. To achieve bioactivity, *MEU1* was made essential by providing 5'-methylthioadenosine (MTA) as the only sulphur source in the *met17Δ* background that requires the function of *MEU1* for synthesis of methionine from MTA, and the uptake of nucleoside MTA and MTDIA was permitted by expression of the human ENT1 nucleoside transporter. The drug-gene interaction screen that followed identified the same cellular processes as the gene-gene interaction screen, thus corroborating the use of the drug target deletion as a proxy for MTDIA. These

cellular processes included both expected and novel mechanisms for MTDIA. The cellular processes chromatin organisation, transcription and translation were expected and are consistent with findings in mammalian cell line and murine models (Pollard et al., 1999; Mandal et al., 2013). The novel findings included disruptions to amino acid metabolism, response to starvation, vacuole fusion and vesicle-mediated transport, carbohydrate metabolism, lipid homeostasis, and NAD⁺ biosynthesis. From these, disruptions to autophagy and ergosterol biosynthesis were further investigated in follow-up experiments.

Autophagy is an intracellular degradation pathway where cytosolic content is degraded via the vacuole and recycled to compensate for nutrient deprivation (Suzuki & Ohsumi, 2010). Autophagy was investigated as a possible mechanism of MTDIA mediated growth inhibition in yeast and was shown to induce TOR1C-independent autophagic cell death. MTDIA and *meu1Δ* exacerbated the rapamycin induced growth arrest and reduced replicative lifespan. The protein levels and localisation of several core-autophagy genes changed in response to MTDIA treatment and *meu1Δ* including Atg8 (yeast orthologue of LC3), a well characterised marker for autophagy. The change in localisation of Atg8 was prevented by deletion of essential autophagy pathway genes such as *ATG1*, *ATG4* and *PEP4*. These results confirmed induction of autophagy by MTAP inhibition or loss in yeast. Of these core autophagy proteins, Vps34 (PI3PK) distribution was altered from the vacuolar lumen/vacuolar membrane to cytoplasmic foci in *meu1Δ*. Interestingly, Vps34 inhibitors (e.g. alpelisib and buparlisib) are promising drugs currently in clinical trials (Massacesi et al., 2016).

Sterol biosynthesis is an essential process required for viability under both aerobic and anaerobic conditions (Bard et al., 1993). Ergosterol is the major sterol in yeast membranes and is synthesised from acetyl-CoA in a pathway that is highly conserved from yeast to humans (Nielsen, 2009; Nes, 2011). Following MTDIA treatment or in the *meu1Δ* strain, the localisation and abundance of several ergosterol biosynthetic proteins were altered. In addition, there was a reduction in ergosterol levels coincident with increases in two sterol intermediates (lanosterol and squalene), which was consistent with the downregulation of Erg1 and Erg24 proteins that process these sterol

intermediates. Interestingly, Erg1 and Erg6 were downregulated with MTDIA and *meu1Δ* which, based on the upregulation of these proteins in response to amino acid deprivation (Huh et al., 2003), suggests that the sterol changes with MTDIA and *meu1Δ* are independent of starvation. Therefore, inhibition of ergosterol biosynthesis is a novel mechanism of Meu1 inhibition. The inhibition of ergosterol biosynthesis is likely mediated through inhibition of Meu1 leading to an accumulation of decarboxylated S-adenosyl methionine, an inhibitor of S-adenosylmethionine dependent methyltransferase activity of Erg6 (McCammon et al., 1984; Frostesjo et al., 1997). This prediction would be consistent with our observation of reduced Erg6 levels with MTDIA treatment or loss of Meu1 and localisation. We also observed mislocalisation of Erg3 from the ER to the vacuole, which may be due to degradation of sterols and lipid droplets by autophagy (lipophagy) to compensate for the reduced ergosterol levels resulting from Meu1 inhibition (Liu & Czaja, 2013). Although the Erg6 mechanism suggested for MTDIA-mediated sterol biosynthesis is not conserved from yeast to humans (*i.e.*, the conversion of zymosterol to fecosterol catalysed by s-adenosyl methionine dependent Erg6 is not conserved (Umate, 2015)), polyamine depletion in mammals reduces cholesterol levels and may still present a viable mechanism of MTAP inhibition and an inducer of autophagy (Kee et al., 2004). Compellingly, MTDIA treatment of A549 tumours in mice lead to >3-fold change in expression of genes required for cholesterol homeostasis (Basu et al., 2011).

Lipids are critical in the regulation of many biochemical processes including the membrane remodelling that underlies autophagosome biogenesis and autophagy process (Jaishy & Abel, 2016). In humans, lipids and lipid metabolising enzymes mediate the autophagy process (Dall'Armi et al., 2013). For example, mammalian phosphoinositide 3-kinase class I (PI3K-I), when activated phosphorylates PI(4,5)P₂ to PI(3,4,5)P₃ on the plasma membrane that recruits and activates AKT, which in turn promotes mTORC1 signalling pathway and inhibits autophagy (Zoncu et al., 2011). Although, PI3K-I kinases are absent in yeast, the pathway downstream of PI3K-I kinase is conserved from yeast to humans including TOR and regulators of TOR (Vivanco & Sawyers, 2002). Phosphatidylinositol 3-phosphate (PI3P) synthesised by PI3Kinase class III (PI3K-III, yeast Vps34

orthologue) acts as a membrane-bound signal for assembly of protein scaffolds at the membrane-cytosol interface and is required for autophagosome formation, maturation and transport (Knævelsrud & Simonsen, 2012). Also related to autophagy, phosphatidylethanolamine (PE) is required for lipidation of LC3/Atg8 to LC3/Atg8-PE (Kabeya et al., 2004). Lipids also directly affect physiochemical properties of the lipid bilayer itself (*e.g.*, curvature and fluidity) (Haucke & Di Paolo, 2007). In addition, non-canonical mTOR independent forms of autophagy have been identified in mammals that are mediated by small molecules and unesterified fatty acids that can bypass the requirement of majority of Atg proteins (Codogno et al., 2012; Niso - Santano et al., 2015). The roles of Vps34/PI3K-III and lipids highlighted here reflects the importance of Vps34 and lipids in regulation of autophagy in mammals.

In yeast, there are two Vps34 (PI3K-III) complexes that differ in their subcellular localisation and function. Vps34 complex II (Vps34, Vps30/Atg6, Vps15, Vps38) is localised to endosomes and involved in carboxypeptidase Y (CPY) sorting to the vacuole. Vps34 complex II in humans also plays a role in autophagy mediated through BECN1 (Vps30/Atg6 orthologue) interactions with UVRAG (Vps38 orthologue) (Simonsen & Tooze, 2009). Vps34 complex I (Vps34, Vps30/Atg6, Vps15, Atg14) is localised to the PAS (phagosome assembly site) and required for induction of autophagy. In higher eukaryotes, vesicles containing Vps34 complex I are targeted to subdomains of ER during starvation where PI3P is synthesised and autophagy is induced (Axe et al., 2008). The recruitment of Vps34 complex I to the subdomains of ER in response to starvation is well characterised in mammals, but has not been observed in yeast previously. Based on the mislocalisation of Vps34 observed with Meu1 loss in yeast, we predict a similar role for Vps34 complex I to PI3K-III complex I. The increased levels of PI3P produced by mislocalised Vps34 at subdomains of the ER/sites of autophagosome formation is responsible for autophagy observed with Meu1 loss and presents novel mechanism for induction of autophagy by MTDIA in yeast. The depletion of cholesterol is also known to induce autophagy in mammalian cells (Cheng et al., 2006), thus it will be interesting to investigate any link between the ergosterol reduction by MTDIA and autophagy induction by MTDIA. Polyamine depletion by

upregulation of SSAT (spermidine/spermine N(1)-acetyltransferases) reduces the plasma cholesterol levels (Pirinen et al., 2010), therefore the cholesterol biosynthesis pathway might yet be modulated by MTAP inhibition. Moreover, changes in membrane curvature by depletion membrane sterol may be responsible for mislocalisation of Vps34, and thus would be interesting to investigate.

Overall, the results of this chapter provided a global survey of the cellular effects of MTAP inhibition, either via MTDIA treatment or *MEU1* deletion. Suspected pathways were identified that justified the use of yeast, and notable among the unsuspected pathways was the induction of autophagy via a rapamycin-independent and Vps34/PI3P-dependent mechanism. As phospholipid synthesis (methyltransferase dependent) and phosphoinositide-3-phosphate synthesis (Vps34) are conserved from yeast to humans, MTAP inhibition may also induce autophagy in humans and might be a mechanism for MTDIA mediated cell death in humans. Given that small molecules modulating autophagy in a myriad of human disorders including cancer, heart disease, obesity and neurodegeneration are currently the focus of intensive investigation (Jiang & Mizushima, 2014; Kovsky et al., 2010; Levine & Kroemer, 2008), the results of this chapter suggest that MTDIA can be considered among these autophagy modulators.

Chapter 3 - Elucidating the anticancer mechanisms of nucleoside analogue MTDIA by global analysis of protein localisation and abundance

3.1 Introduction

MTDIA is a potent inhibitor of the human enzyme 5'-methylthioadenosine phosphorylase (MTAP) *in vitro* ($K_d = 86$ pM) that mimics the enzymatic transition state structure of MTA bound by MTAP (Schramm, 2011). Its efficacy has been demonstrated *in vivo* in mouse xenograft models for human lung, prostate, colon, cervical, head and neck, and triple negative breast cancers, as well as various human cancer cell lines (Basu et al., 2007; Basu et al., 2011). MTDIA mimics the loss of MTAP, resulting in an accumulation of MTA, a reduction in S-adenosyl methionine, and a reduction in polyamine levels (spermidine and spermine). The mechanisms responsible for activation of apoptotic pathways leading to G2/M arrest, loss of mitochondrial membrane integrity, cleavage of PARP and caspases are yet to be characterised (Basu et al., 2011). Since the initiation of apoptosis is cell type specific (Basu et al., 2007), an additional level of complexity exists beyond the accumulation of MTA initiating apoptosis and the genetic mechanisms underlying these processes are not characterised.

In Chapter 2, several potential mechanisms for MTDIA mediated growth inhibition were identified including induction of autophagy and inhibition of ergosterol biosynthesis. To complement the gene-gene and gene-drug interactions that were identified with genomic libraries of gene deletion strains, this chapter investigated MTDIA mechanisms via measurements of the abundance and localisation of normal (not mutated) proteins. Specifically, experiments were conducted with a GFP library expressing human ENT1 (4,900 strains) where each strain contains a different protein fused at the C-terminus to GFP. Protein localisation and abundance were investigated in *meu1Δ* (genetic mimic of MTDIA treatment) and MTDIA-treated cells. This unbiased approach both complements the growth-specific phenotypes in deletion library screens as well as provides new insight into changes at the protein level that may not necessarily alter the growth phenotype. The GFP library also permits the assessment of proteins for essential genes that were absent in the gene deletion libraries screen with *meu1Δ* and MTDIA in Chapter 2.

3.2 Experimental procedures

3.2.0 Yeast strains, media and compounds

3.2.0.1 Yeast strains

All *S. cerevisiae* strains used in this chapter were derived from yeast strains BY4741 and BY4742 (Brachmann et al., 1998) and were constructed by heterologous expression of human equilibrative nucleoside transporter 1 (ENT1) gene from plasmid pYhENT1 ((Vickers et al., 1999); gift of Carol E. Cass) (Table 3.2.1).

Strain	Genotype	Reference
BY4741	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 MET15 ura3Δ0</i>	(Brachmann et al., 1998)
BY4742	<i>MATα his3Δ1 leu2Δ0 LYS2 met15Δ ura3Δ0</i>	(Brachmann et al., 1998)
YCG386	<i>MATα can1Δ::STE2pr-URA3 lyp1Δ::HPH::TEFpr-NLS-RedStar2-TEF2pr-mCherry his3Δ1 met15Δ0 ura3Δ0 leu2Δ0 SEC63-GFP-HISp</i>	(Low, 2013)
Y7039	<i>MATα can1Δ::STE2pr-Sp_LEU2; his3Δ1 leu2Δ0 LYS2 met15Δ ura3Δ0</i>	(Tong & Boone, 2007)
WT (diploid)	<i>MATα/α can1Δ::STE2pr-Sp_LEU2/CAN1; lyp1Δ::HPH::TEFpr-NLS-RS2::TEF2pr_mCherry/LYP1; his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2 CEN3::hENT1::URA3</i>	This study
WT (YCG640)	<i>MATα can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::TEFpr-NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::URA3</i>	This study
<i>meu1Δ</i> (YCG640)	<i>MATα meu1Δ::kanR; can1Δ::STE2pr-Sp_LEU2 lyp1Δ::HPH::TEFpr-NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::URA3</i>	This study
xxx-GFP (GFP library)	<i>MATα can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::TEFpr-NLS-RedStar2-TEF2pr-mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::URA3 XXX-GFP-sp_HIS5</i>	This study
<i>meu1Δ</i> xxx-GFP (GFP-library)	<i>MATα meu1Δ::kanR; can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::TEFpr-NLS-RedStar2-TEF2pr-mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::URA3 XXX-GFP-sp_HIS5</i>	This study

Table 3.2.1 – Yeast strains used in this chapter.

3.2.0.2 Media

All *S. cerevisiae* strains were cultured in one of the following growth media supplemented with the appropriate antibiotics: 100 µg/mL ClonNAT (NAT; Werner BioAgents), 200 µg/mL geneticin (G418; Carbosynth) and 300 µg/mL hygromycin B (HPH; Formedium). The amino acid analogues canavanine (Sigma-Aldrich) and thialysine (Sigma-Aldrich) were used at 50 µg/mL in the absence of

their amino acid analogues. All yeast growth media was autoclaved at 121°C and cooled to 65°C, before adding 2% glucose and antibiotics unless otherwise stated. All *E. coli* strains were cultured in lysogeny broth (LB) supplemented with 100 µg/mL ampicillin (Sigma-Aldrich).

Yeast peptone dextrose (YPD) agar: 1% (w/v) yeast extract (Becton, Dickinson and Company), 2% (w/v) bacto-peptone (Becton, Dickinson and Company), 0.12% (w/v) adenine (Sigma-Aldrich), 2% (w/v) agar (Invitrogen) and 2% (w/v) glucose (Sigma-Aldrich). YPD broth was prepared as above without the addition of agar.

Synthetic complete (SC) and synthetic dropout (SD) agar: 0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulphate (Becton, Dickinson and Company), 0.1% (w/v) monosodium glutamate (Sigma-Aldrich), 0.2% (w/v) amino acid mixture to suit (Sigma-Aldrich), 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) glucose (Sigma-Aldrich). SC broth was prepared as above without the addition of agar. SD media was made same as SC minus the appropriate amino acid supplement.

CM (complete minimum)-Ura + 2% galactose agar: 0.725% (w/v) CM amino acids/ammonium sulphate mixture to suit, 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) galactose (Carbosynth). Media was autoclaved at 121°C and cooled to 65°C prior to adding filter sterilised galactose. SD broth was prepared as above without the addition of agar.

CM-Ura + 2% raffinose agar: 0.725% (w/v) CM amino acids/ammonium sulphate mixture to suit, 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) raffinose (Carbosynth). Media was autoclaved at 121°C and cooled to 65°C prior to adding filter sterilised galactose. SD broth was prepared as above without the addition of agar.

CM-Met/Ade/Ura (MAU) + 2% galactose: 0.725% (w/v) CM amino acids/ammonium sulphate mixture to suit, 2% (w/v) agar (Becton, Dickinson and Company) and 2% (w/v) galactose (Carbosynth). Media was autoclaved at 121°C and cooled to 65°C prior to adding filter sterilised raffinose and galactose. SD broth was prepared as above without the addition of agar.

Enriched sporulation (SPO) agar: 1% (w/v) potassium acetate (Scharlau Chemie S.A.), 0.1% (w/v) yeast extract (Becton, Dickinson and Company), 0.05% (w/v) glucose (Sigma-Aldrich), 0.2% (w/v) amino acid mixture to suit (Sigma-Aldrich), 2% (w/v) agar (Becton, Dickinson and Company). Enriched sporulation broth was prepared as above without agar.

Amino acid mixture for SC media: 3 g adenine, 2 g uracil, 2 g inositol, 0.2 g para-aminobenzoic acid, 2 g alanine, 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 tryptophan, 2 g tyrosine, and 2 g valine. Amino acid mixture of SD media is a mixture of the amino acids (Sigma-Aldrich) above minus the appropriate amino acid supplement. Amino acid mixture for enriched sporulation composed of 2 g histidine, 10 g leucine, 2 g lysine, and 2 g uracil.

Amino acid/ammonium sulphate mixture for CM): 100 g yeast nitrogen base without amino acids or ammonium sulphate, 293.11 g of ammonium sulphate, 1.2 g adenine, 1.2 g arginine, 1.2 g histidine, 1.8 g isoleucine, 3.6 g leucine, 1.8 g lysine, 1.2 g methionine, 3 g phenylalanine, 1.2 g tryptophan, 1.8 g tyrosine, 1.2 g uracil, and 9 g valine.

3.2.0.3 Compounds and chemicals

MTDIA was kindly provided by Prof Gary Evans (Ferrier Institute, Victoria University of Wellington), dissolved in DMSO at a concentration of 10 mM, and stored at -20°C. 5'-methylthio adenosine (MTA) (W1114, AK Scientific) was dissolved in ddH₂O at a concentration of 5 mM and stored at -20°C.

3.2.1 GFP library construction

A significant proportion of the GFP library labelled with the dual RFP markers available in my laboratory showed a significant vacuolar morphology defect. This was inherent to the query strain (YCG256) used in construction of the GFP/dual RFP library. Therefore, the GFP library was reconstructed by crossing the GFP library (*MAT α*) with a *MAT α* query strain expressing hENT1. The

new query strain was constructed via a single selectable marker for the dual RFPs (*LEU2*) and another selectable marker for hENT1 expression (*URA3*). PCR-mediated disruption was used to engineer BY4741, specifically replace the *CAN1* and *LYP1* loci with *STE2pr-Sp_LEU2* from y7039 (gift from C. Boone)(Tong & Boone, 2007) and integrate the *HPH::TEFpr-NLS-RS2::TEF2pr_mCherry* construct from YCG383 (Low, 2013). BY4742 was transformed with pYhENT1 (*CEN3::hENT1::URA3*) (Vickers et al., 1999) using primers in Appendix Table 7.3.1 (Appendix Fig. 7.3.1) (Janke et al., 2004; Gietz & Schiestl, 2007). The resulting BY4741-based strain (*can1Δ lyp1Δ::HPH::TEFpr-NLS-RS2::TEF2pr_mCherry*) and the BY4742 + *hENT1::URA3* transformants were mated to generate a diploid strain. Briefly, the diploid strain was cultured overnight in SD-Ura + HPH + 2% glucose liquid media, washed with water, resuspended in sporulation media, incubated at 30°C for 7 d, plated on SD-Arg/Leu/Ura + Can/Thia + 2% glucose agar, and incubated for 2 d at 30°C. The resulting colonies were inoculated into SD-Ura media in a 96-well plate (one colony per well) and incubated at 30°C overnight. The random spore analysis was performed as previously described (Tong & Boone, 2006) in liquid media, where ~1 µL of spores in sporulation media were cultured in SD-Ura were transferred into SD-Leu, SD-Met/Cys, SD-His, SD-Ura, and SD-MAUC + 2% galactose liquid media in 96 well plates using the RoToR HDA replicator (Singer Instrument Co.) to assess for genotypes in Table 3.2.2. Concomitantly, *MATa* (*his3::kanR*) and *Mata* (*pdr1::natR*) mating type strains with selectable markers were cultured overnight, resuspended in YPD to an OD of 0.3 in 90 µL of either *MATa* or *MATa* were transferred into wells of a 96 well plate followed by 10 µL SD-Ura cultured spores and incubated at 30°C for 1 d. 10 µL of cells (mated with *MATa* or *MATa*) were inoculated into SD-Ura+G418 or SD-Ura+NAT were incubated at 30°C for 2 d. Eight spores were acquired by random spore analysis that showed phenotypes consistent with WT YCG640 genotype (*MATa can1Δ::STE2pr-Sp_LEU2; lyp1Δ::HPH::TEFpr-NLS-RS2::TEF2pr_mCherry; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 CEN3::hENT1::URA3*) and were assessed for normal (unfragmented) vacuolar morphology by light microscopy and fluorescence microscopy utilising the nuclear (NLS-RedStar2) and cytoplasmic (TEF2pr_mCherry) RFP markers. Cells derived from five spores showed normal morphology, three

were frozen down and one was used as the query strain in the construction of the GFP/dual RFP + hENT1 library by synthetic genetic array (SGA) methodology as previously described (Tong et al., 2001) in Chapter 2 (section 2.2.3). *MEU1* was replaced by PCR mediated disruption with pFA6-kanMX4 (Goldstein & McCusker, 1999) with primers (Appendix Table 7.3.1) to generate an isogenic *meu1Δ::kanR* (YCG643) strain, the genetic mimic of MTDIA employed in the construction of *meu1Δ* GFP/dual RFP + hENT1 library by SGA methodology as previously described (Tong et al., 2001).

Media	Expected viability	Construct confirmed
SD-Leu	-	<i>leu2Δ0</i>
SD-Ura	+	<i>CEN3::hENT1::URA3</i>
SD-Met/Cys	-	<i>met15Δ0</i>
SD-MAUC+MTA+2% galactose	+	<i>CEN3::hENT1::URA3</i>
SD-His	-	<i>his3Δ1</i>
SD-Arg/Lys +Can/Thia+HPH	+	<i>lyp1Δ::HPH-NLS-RedStar2-TEF2pr-mCherry</i> <i>can1Δ::STE2pr-Sp_LEU2</i>
SD-Ura+G418	+	<i>MATα</i> mating type
SD-Ura+NAT	-	<i>MATα</i> mating type

Table 3.2.2 – **Expected phenotypes for WT (YCG640) in random spore analysis.** Spores were inoculated into appropriate amino acid dropout media supplemented with 2% glucose and were incubated at 30°C overnight unless specified otherwise. Spores cultured in SD-MAUC+MTA+2% galactose media were grown for 2d to obtain sufficient growth. +/- indicates growth/no growth. MAUC, Met/Ade/Ura/Cys; MTA, 5'-methylthoadenosine.

3.2.2 Automated fluorescence microscopy

Cells expressing the GFP-tagged protein and the dual RFPs maintained on agar plates in 384 colony format were pinned on SD-Ura+2% raffinose agar using RoToR HDA replicator (Singer Instrument Co), cultured at 30°C for 20 h, inoculated into a black-walled clear bottom 384 well cell carrier plate (Perkin Elmer) to an OD₆₆₀ of 0.3 in 50 μL volumes of SD-MAU+ MTA+ 2% galactose liquid media with and without MTDIA and were cultured at 30°C for 6 h. Cells were visualised using a high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin Elmer) as previously described in Chapter 2 (section 2.2.10). The change in GFP intensity (>20% increase or decrease compared to WT untreated) was quantified using Acapella automated image analysis software (Perkin Elmer), and

change in localisation was confirmed by visual inspection and were validated by repeat evaluation and analysis. A cut-off >20% change in abundance was used as significant change, as changes in abundance >18% were consistently detectable by visual inspection.

3.3 Results

3.3.1 Construction of the GFP/dual RFPs + hENT1 libraries

A new GFP library comprised of ~4,900 unique GFP-tagged fusion proteins in the dual nuclear and cytosolic RFPs with hENT1 heterologous expression was constructed by mating the query strain YCG640 with the GFP library (Open Biosystems) as previously described (Tong et al., 2001). *MEU1* was deleted from YCG640 to create the genetic mimic of MTDIA (YCG643) and was mated against the GFP library to create an isogeneic *meu1Δ* GFP/dual RFP + hENT1 library.

3.3.2 Changes in protein abundance and localisation in response to MTDIA treatment and *meu1Δ*

To further investigate the cellular processes that were perturbed by MTDIA treatment (gene-drug interactions) or loss of *MEU1* (gene-gene interactions), the newly constructed genome-wide GFP libraries were assayed using high throughput confocal microscopy. The localisation and abundance of individual proteins were assessed *in vivo* in response to MTDIA treatment and *meu1Δ* using Acapella automated image analysis software (Perkin Elmer) and by visual inspection. Out of ~4,900 GFP strains grown in three conditions (untreated, MTDIA treated, and in the *meu1Δ* condition; Fig. 3.3.1a), 762 proteins were identified by Acapella to show a change in abundance and 124 strains to show a change in localisation with visual inspection with either *meu1Δ* or with MTDIA treatment. Of 886 that exhibited a change in localisation/abundance, 336 GFPs were altered with *meu1Δ* and 147 GFPs were altered with MTDIA (with 135 overlapping) based on reproducibility in an independent assay. Specifically, 242 showed a change only in abundance (median GFP >±20% of WT untreated control), 47 showed a change only in localisation, and 59 proteins showed changes in both localisation and abundance compared to WT untreated (Fig. 3.3.1b-c).

The 201 localisation/abundance changes for *meu1Δ* that were not observed with MTDIA mostly showed responses that were similar to untreated WT, indicating a genetic dosage effect. The

12 changes with MTDIA that were different from WT untreated and *meu1Δ* included only changes in abundance of mitochondrial, cytoplasmic and nuclear proteins with MTAP inhibition (Fig. 3.3.1b). An additional 17 proteins that relocalised *meu1Δ* showed intermediary responses with MTDIA. Fourteen proteins were downregulated and three proteins were upregulated with MTDIA without clear relocalisation observed in *meu1Δ*. Overall, there were 118 of 147 changes in abundance/localisation with MTDIA that were in agreement with *meu1Δ*.

The 348 proteins that showed a change in either localisation/abundance with MTDIA and *meu1Δ* were in various organelles within cells (Fig. 3.3.1d-e) (Appendix Fig. 7.3.2; Appendix Table 7.3.2). The majority of the 242 proteins that showed a change in abundance alone were localised to cytoplasm, nucleus and mitochondria. The majority of the 106 proteins that showed a change in localisation were relocalised from cytoplasm to nucleus, from nucleus to cytoplasm, from ER/plasma membrane to vacuole, and from ER/cytoplasmic foci to cytoplasmic foci with either MTDIA or *meu1Δ* (Fig. 3.3.1f-g). In addition, five bud neck proteins relocalised to the plasma membrane and three microtubule associated proteins relocalised to nuclear foci with *meu1Δ* but not with MTDIA with the exception of the bud neck protein Sec8 that relocalised to the plasma membrane.

These 348 proteins that changed in localization/abundance were assigned into the three major hierarchical categories. Most informative was the biological process based on enrichment. The proteins that changed in abundance/localisation with MTDIA were significantly enriched for transport ($p = 1.27E-05$) within subcategories of transmembrane transport ($p = 4.39E-07$), organic acid transport ($p = 5.67E-07$), carboxylic acid transmembrane transport ($p = 1.38E-06$) and establishment of

Figure 3.3.1 – Global analysis of protein relocalisation and abundance in response to MTDIA and its genetic mimic *meu1Δ* highlights mislocalisation of plasma membrane/ER proteins to vacuole. (a) Schematic representation of the screening and quantification approach used in analysis of ~4900 GFP fusion proteins. (b) Proteins that changed in localisation/abundance with MTDIA vs. *meu1Δ*. (c) Changes in localisation vs. abundance. (d) Localisation of proteins in WT vs. WT with MTDIA. (e) Localisation of proteins in WT vs. *meu1Δ*. (f) Relocalisations with MTDIA treatment. (g) Relocalisation with *meu1Δ*. ~4,900 GFP fusion proteins in the dual RFP (NLS-RS2 and mCherry) background were screened against MTDIA and *meu1Δ* using high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin Elmer) and change in abundance (>20% increase or decrease) was quantified with automated image analysis software (Acapella, Perkin Elmer) (Bircham et al., 2011) and relocalisation

by visual inspection. Shown here are changes in localisation and abundance reproducible from the high-throughput screen.

localization ($p = 3.63\text{E-}05$) (Appendix Table 7.3.3). The notable significant enrichments with *meu1Δ* were for pyruvate metabolic process ($p = 1.70\text{E-}10$), glycolytic process ($p = 5.62\text{E-}08$), generation of precursor metabolites and energy ($p = 2.96\text{E-}08$), acyl-CoA metabolic process ($p = 5.12\text{E-}06$), cytoplasmic translation ($p = 4.34\text{E-}09$), nicotinamide nucleotide metabolic process ($p = 2.02\text{E-}09$) and nucleotide phosphorylation ($p = 2.74\text{E-}08$) (Appendix Table 7.3.4). This shows that loss of MTAP/*MEU1* affects cellular processes involving synthesis of metabolites, while MTDIA treatment affects transport of metabolites. This is likely a difference between downregulation of the drug target (MTDIA treatment) and the complete loss of gene function (*meu1Δ*). Metabolite transport is relied upon under downregulation of MTAP to compensate for the reduced metabolites, while loss of MTAP requires upregulation of synthesis since upregulation of transport cannot compensate. Alternatively, such differences may be a reflection of recently determined non-enzymatic activities of MTAP that are still present during MTAP inhibition yet absent in *meu1Δ* (Tang et al., 2015).

3.3.2 MTDIA regulates proteins involved in glucose metabolism

In addition to GO enrichment, the proteins showing changes in localisation/abundance also showed enrichment for pathways of central carbon metabolism, such as glycolysis ($p = 1.83\text{E-}04$), glucose fermentation ($p = 1.71\text{E-}07$), gluconeogenesis ($p = 3.21\text{E-}04$) and pyruvate dehydrogenase complex ($p = 2.11\text{E-}02$) with *meu1Δ* (Appendix Table 7.3.5). These results were consistent with deletions of glucose metabolism genes (*e.g.*, pyruvate dehydrogenase complex E1 alpha subunit *PDA1* and methylglyoxalase *HSP31*) showed a growth defect with *meu1Δ* in chapter 2 and Pda1 and Hsp31 were downregulated and upregulated, respectively, in *meu1Δ*. A total of 50 proteins in central carbon metabolism were deregulated with MTDIA or *meu1Δ* and were assigned to respective pathways of glycolysis/gluconeogenesis, trehalose metabolism, pentose phosphate pathway, glycogen metabolism, acetoin and butanediol synthesis, glycerol biosynthesis, ethanol fermentation, TCA cycle,

glyoxylate cycle, and long chain fatty acid biosynthesis (Fig. 3.3.3). The enzymes that catalyse the rate limiting step in glycolysis (Hxk1, Hxk2, Glk1) were increased whilst Cdc19 that catalyses the final step in pyruvate synthesis was reduced with MTDIA and *meu1Δ*. The enzymes that catalyse the intervening steps of glycolysis/gluconeogenesis are bidirectional and showed either an increase (Pfk2, Thd1, Thd2, Pgi1, Gpm1, Gpm2, Eno1) or decrease (Pgi1, Thd3) in protein levels (Fig. 3.3.2a). There was a reduction in Pgm2, which catalyses the initial step glycogen metabolism and shunts glucose-6-phosphate away from glycolysis, whilst proteins that catalyse synthesis of the final products (*e.g.*, glycogen synthase Gsy2 and transketolase Tkl2 of pentose phosphate pathway) were increased compared to control (Fig. 3.3.2c). Similarly, MTDIA and *meu1Δ* resulted in upregulation of the enzyme Tps1 that salvages UDP-glucose from glycogen metabolism for subsequent synthesis of glucose by Nth1 (Fig. 3.3.2b) and as well as the enzymes Tps1 and Tkl2 that salvage glyceraldehyde-3-phosphate from pentose phosphate pathway and glycerol metabolism (Fig. 3.3.2b,d), respectively, for the utilisation in glycolysis/gluconeogenesis. The proteins Gpd1 and Gpd2 that synthesise glycerol-3-phosphate were also upregulated while Gut2 that catalyses the reverse reaction in glycerol metabolism pathway was downregulated with MTDIA and *meu1Δ*, which may also reflect the requirement for glycerol-3-phosphate in the synthesis of glycerophospholipids (Fig. 3.3.2e).

Pyruvate is synthesised by pyruvate kinase Cdc19 from glycolysis or by malate dehydrogenase Mae1 from the TCA cycle, both of which were downregulated with *meu1Δ*. Likewise, pyruvate decarboxylases that metabolise pyruvate for ethanol fermentation and acetoin/butanediol biosynthesis (Pdc1, Pdc6) and for the TCA cycle (Pyc1) were increased with both MTDIA and *meu1Δ* (Fig. 3.3.2g). In contrast, there was downregulation in pyruvate dehydrogenases Pda1 and Lat1 that synthesise acetyl CoA from pyruvate and acetyl-coA transferase Ach1 as well as acetyl-coA synthetases Acs1 and Acs2 that synthesise acetyl-CoA from acetate (Fig.3.3.10a). Citrate synthases (Cit1, Cit2) that catalyse the rate limiting step in the TCA cycle and acetyl-CoA carboxylase Acc1 from long chain fatty acid synthesis that utilises acetyl-CoA as a reactant were increased whilst the protein levels of Cit3 (a

a

dual specific citrate/methylcitrate synthase that utilises propionyl-CoA for synthesis of methylcitrate and acetyl-CoA for citrate synthesis) were reduced. Protein levels of three alcohol dehydrogenases (Adh3, Adh4, Adh6) that metabolise acetaldehyde to ethanol and the aldehyde dehydrogenase Ald6 that metabolises acetaldehyde to acetate were reduced (Fig.3.3.2f). Interestingly, the levels of alcohol

g

Figure 3.3.2 – **Proteins of central carbon metabolism that either increased or decreased in abundance or relocalised with MTDIA and *meu1Δ*.** (a) Glycolysis/gluconeogenesis. (b) Trehalose metabolism. (c) Glycogen metabolism. (d) Pentose phosphate pathway. (e) Glycerol biosynthesis. (f) Ethanol fermentation. (g) TCA cycle. GFP fusion proteins were visualised using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin) using the 60x water immersion lens (NA 1.2). The GFP abundance was quantified by automated image analysis (Acapella, Perkin Elmer)(Bircham et al., 2011) and relocalisation was determined by visual inspection. Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated.

dehydrogenases Adh2 that catalyse the reverse reaction and aldehyde dehydrogenase Ald3 that also synthesises β -alanine from spermine metabolism were increased. Protein levels of ketoglutarate dehydrogenase complex KGD2 were increased while levels of ketoglutarate dehydrogenase Lpd1 that also function in the glycine cleavage complex were increased with MTDIA but relocalised to mitochondrial foci with *meu1Δ*. Similarly, malate dehydrogenase Mdh2 levels increased with MTDIA

Figure 3.3.3 – **Central carbon metabolism and proteins levels altered with *meu1Δ* or MTDIA.** Pathway is compiled from sources KEGG, SGD and Tripodi et al. (Cherry et al., 2012; Kanehisa & Goto, 2000; Tripodi et al., 2015).

but decreased with *meu1Δ*. In addition, fumarate hydrolase Fum1 was increased with MTDIA treatment. The membrane anchor of succinate dehydrogenase complex (Sdh3) was also reduced with *meu1Δ*. Since glycolysis serves as a sink for acetyl-CoA synthesis, the regulation of central carbon metabolism processes may be a result of disruptions to lipid homeostasis. Several glycolysis genes were also upregulated or downregulated by >2-fold with MTDIA in A549 lung cancer cells but this was not further investigated (Basu et al., 2011). For example, the deletion of *Meu1* here resulted in upregulated levels of alcohol dehydrogenase *Adh2*, which was concordant with the 2.37-fold upregulation of the human orthologue of *Adh2* with MTDIA in A549 lung cancer cells (Basu et al., 2011).

3.3.3 Methionine salvage from glycine, cystine, choline and sulphate is regulated by MTDIA and *meu1Δ*

In the absence of methionine and cysteine, MTA is processed by *Meu1*/MTAP to generate adenine and 5-methylthioribose-1-phosphate that is subsequently processed to methionine (Pirkov et al., 2008). Deletion of genes required for salvage of methionine from MTA (*MRI1*, *MDE1*, *UTR4*, *ADI1* and *MEU1*) were inviable in MTA as the sole source of methionine in Chapter 2. Proteins required for salvage of methionine by pathways independent of MTA metabolism were increased in abundance with either MTDIA or *meu1Δ* (Fig. 3.3.5), which was consistent with increased requirement for methionine under the *meu1Δ* essential condition (Chapter 2). Glycine decarboxylase complex proteins (*Gcv1*, *Gcv2*, *Gcv3* and *Lpd1*) were enriched for the glycine cleavage complex pathway ($p = 2.11\text{E-}02$), which catalyses the cleavage of glycine to NH_3 and CO_2 and concomitant synthesis 5,10-methylene tetrahydrofolate from tetrahydrofolate for methionine salvage. The levels of *Gcv1*, *Gcv2* and *Gcv3* were increased at the mitochondria and the levels of *Lpd1* mitochondrial foci were increased with *meu1Δ* (Fig. 3.3.4a). Related, *MEU1*-deficiency resulted in the upregulation of the cytosolic serine

Figure 3.3.4 – **Methionine salvage was increased with MTDIA and *meu1Δ*.** (a) Tetrahydrofolate and 10-formyl- tetrahydrofolate assimilation. (b) Methionine salvage from ccysteine and polyamine biosynthesis (c) Sulphate assimilation. (d) methionine sulfoxide reduction. GFP fusion proteins were visualised using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin) using the 60x water immersion lens (NA 1.2). The GFP abundance was quantified by automated image analysis (Acapella, Perkin Elmer) (Bircham et al., 2011) and relocalisation was determined by visual inspection. Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated.

hydroxymethyltransferase Shm2 that catalyses the synthesis of glycine from serine and concomitant synthesis of 5,10-methylene tetrahydrofolate, which is subsequently converted to 5-methyl tetrahydrofolate and then methionine (Fig. 3.3.4a). Also related in *meu1Δ* was the upregulation of Ade17 that catalyses for synthesis of the tetrahydrofolate from 10-formyl-THF. Cystathionine gamma-synthase Str2 required for synthesis of cystathionine from cysteine, which is subsequently converted to homocysteine and then methionine was also upregulated in *meu1Δ* (Fig. 3.3.4b). In contrast, spermidine synthase Spe3 required for methionine salvage from decarboxylated adenosyl methionine in a pathway inhibited by *meu1Δ* and MTDIA was downregulated, possibly because of product inhibition by MTA (Pajula et al., 1979b). Met14 and Met5 are required for salvage of homocysteine and subsequently methionine from sulphate assimilation. Met14 catalyses the second step in sulphate assimilation and was increased in *meu1Δ*. Met5 catalyses the penultimate step in the synthesis of homocysteine and was decreased in *meu1Δ*. This is likely due to homocysteine serving as a reactant in the synthesis of S-adenosyl homocysteine, a potent inhibitor of methyltransferase reactions requiring S-adenosyl methionine. Related, Met22 catalyses metabolism of sulphate assimilation pathway product adenosine 3',5'-bisphosphate to AMP and phosphate to maintain the forward reaction and was increased in *meu1Δ* (Fig. 3.3.4c).

Thioredoxins are required for cellular oxidant detoxification reactions and the reduction of methionine sulfoxide to methionine. Levels of several thioredoxins (methionine-S-sulfoxide reductase Mrx1, sulfiredoxin Srx1, glutaredoxin Grx8 and alkyl hydroperoxide reductase Ahp1) were increased in *meu1Δ*, whilst levels of thioredoxin peroxidase Tsa1 were increased with MTDIA and accumulation increased at cytosolic foci in *meu1Δ* (Fig. 3.3.4d). In addition, permeases required for uptake of precursors for methionine salvage such as cystine (Bpa2, Bpa3, Yct1), cystine/methionine (Mup1), adenine (Fcy2), choline (Hnm1) and polyamines (Top4) relocalised from the plasma membrane to the vacuole with MTDIA treatment and in *meu1Δ*.

Figure 3.3.5 – **Methionine salvage pathway proteins changed in abundance with *meu1Δ***. Pathway is compiled from sources KEGG, SGD and Kanehisa and Goto (Tripodi et al., 2015; Cherry et al., 2012; Kanehisa & Goto, 2000).

3.3.4 MTDIA regulates proteins involved in chromatin remodelling and transcription

Gene deletions of transcription and chromatin remodelling genes (*HIR2*, *EAF1*, *IES5*, *UME6*, *PAT1*, *HTZ1*, *SIP3*, *MED1*, *DAL81*, *KNS1*, *YLR184W* (adjacent to *TOS4/YLR183C*), *YDR290W*) showed a growth defect in *meu1Δ*, while these deletions (*BRE1*, *DPB4*, *HDA3*, *HIR2*, *HIR3*, *HOS4*, *HST1*, *HST3*, *IES1*, *NPT1*, *PAT1*, *RFM1*, *RXT2*, *SIR4*, *SNF5*, *SUM1*, *SOK2*, *YLR236C*, *YLR235C*, *TDA5*, *RPN4*, *PSP2*, *CKA2*, *DHH1*, *YGR054W*, *KNS1*, *CTH1*, *DPB4*, *GTR2*, *YOR024W*) showed a growth defect with MTDIA treatment (Chapter 2). Likewise, several proteins involved in overlapping cellular processes of transcription, chromatin organisation and gene expression showed altered abundance with MTDIA

Figure 3.3.6 – **Proteins regulating gene expression, transcription and chromatin organisation changed in abundance with MTDIA and *meu1Δ*.** GFP fusion proteins in the dual RFP (NLS-RS2 and mCherry) background were screened against MTDIA and *meu1Δ* using a high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin Elmer). Change in abundance (>20% increase or

decrease) was quantified with automated image analysis software (Acapella, Perkin Elmer) as previously described (Bircham et al., 2011). Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated.

treatment and loss of Meu1. Transcription proteins downregulated with MTDIA or *meu1Δ* include RNA polymerase constituents (Rpa12, Rpa43, Rpa135 and Rpc10), RNA polymerase II mediator complex subunit Srb5, TFIID subunit Taf10, TFIIH subunit Tfb1, galactose responsive genes Gal3, superoxide dismutase Sod1, histone H2B Htb1, histone deacetylase of Rpd3S and Rpd3L complexes RPD3, DNA double strand break repair proteins Mre11 and Rad6, transcription elongation factor TFIIIS Dst1, and transcription termination factor Pcf11 (Fig. 3.3.6). In contrast, nucleosome remodelling protein required for genes transcription initiation of tRNA genes Nhp6b, TFIID subunit Taf7 and cytoplasmic peptidyl-prolyl cis-trans isomerase Cpr1 were upregulated with MTDIA and *meu1Δ* (Fig. 3.3.6). Disruptions to levels of proteins in chromatin organisation, transcription and gene expression observed were consistent with polyamine regulated changes in expression of both histone deacetylases and histone acetyltransferases (Pollard et al., 1999; Christopher et al., 2002). The histone modification in response to polyamine depletion would alter the expression of many of genes and are concordant with our observation of the deregulation of proteins involved in chromatin organisation, transcription and gene expression, as well as the altered expression of hundreds of genes in MTDIA-treated A549 lung cancer cells and with expression of MTAP in MTAP negative MCF-7 breast cancer cells (Basu et al., 2011; Christopher et al., 2002).

3.3.5 Proteins regulating translation are inhibited MTDIA and *meu1Δ*

Polyamine depletion by ornithine decarboxylase and spermidine synthase inhibition inhibits both translation initiation and elongation (Landau et al., 2010). Consistent with this proposed role of polyamines in translation, deletions of translation genes *RPL9A*, *YPR126C* (overlaps mitochondrial ribosomal associated protein *YLH47*), *RPL22A*, *RPS6A*, *RPS19A* and *RPS10A* showed a growth defect with *meu1Δ* and deletion of *RPS10A* *RPL23B* showed a growth defect with MTDIA treatment (Chapter 2). GFP fluorescence of 72 proteins required for translation changed in abundance in response to MTDIA treatment or *meu1Δ*. These proteins were involved in different stages of translation (Fig. 3.3.7a-e). Out of 72 proteins, 65 were down regulated with MTDIA and *meu1Δ* and were involved in pre-ribosomal RNA processing (C/D box snoRNA proteins Bcd1 and Bud21, C/D snoRNA interacting protein Slx9, rRNA and ribosome biosynthesis regulon protein Fcf2, DExD/H box RNA helicase Dbp3, H/ACA snoRNP proteins Gar1, Nhp2, RNA polymerase II C-terminal domain binding protein Hrr25, RNA recognition motif containing protein Nop6, RNA polymerase I subunits Rpa12, Rpa43, Rpa135, 27SA3 pre-rRNA processing proteins Rpl17a, Rpl7b, Rpl8a, Rpl8b, 20S pre-rRNA processing Rps14b, RNA metabolism protein of unknown function Rtc3) (Fig. 3.3.7a), constituents of the 60S ribosomal subunit (Rpl1a, Rpl1b, Rpl3, Rpl6b, Rpl9a, Rpl9b, Rpl11a, Rpl12b, Rpl15b, Rpl16a, Rpl21a, Rpl21b, Rpl22a, Rpl23a, Rpl24b, Rpl33b, Rpl34a, Rpl36a, Rpl36b, Rpl40a, Rpl40b), constituents of the 40S ribosomal subunit (Rps6a, Rps10a, Rps18b, Rps22b, Rps23b, Rps24a, Rps24b, Rps26b, Rps30a) (Fig. 3.3.7b), proteins requires for nuclear export of pre-60s ribosomal subunit (Alb1, Arx1, Bud20 Ecm1 Nog2 Rrp12 Rpp1a) (Fig. 3.3.7c), proteins that regulate translation (mRNA decapping protein Edc2, translational activator of mRNAs with internal ribosome entry sites Gis2, glutamine tRNA synthetase Gln4, P-body-dependent granule assembly protein Pbp1, and translation machinery associated protein Tma22) (Fig. 3.3.7d). Translation elongation related proteins (Eft1, Eft2, Yef3, Tma7, ribosomal stalk protein P1 alpha Rpp1a, and cytoplasmic RNA-binding protein Sro9) were also reduced in response to MTDIA and *meu1Δ* (Fig. 3.3.7e). These results are indicative of translation inhibition with MTDIA and *meu1Δ* and

a

Figure 3.3.7 – Proteins regulating translation were downregulated in response to MTDIA or *meu1Δ*.

(a) RNA processing and ribosome biogenesis. (b) Ribosomal constituents and assembly. (c) Ribosomal subunit and rRNA export from the nucleus. (d) Translation regulation. (e) Translation elongation. GFP fusion proteins were visualised using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin) using the 60x water immersion lens (NA 1.2). The GFP abundance was quantified by automated image analysis (Acapella, Perkin Elmer)(Bircham et al., 2011) and relocalisation was determined by visual inspection. Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated.

are consistent with growth defects in non-essential gene deletion of translation related genes in Chapter 2.

In contrast, two proteins, methionyl-tRNA synthetase Mes1 and mitochondrial ribosomal subunit Mrp10 were upregulated with MTDIA and *meu1Δ*. Three proteins changed localisation were the ATP-dependent RNA helicase Dbp2 and the pre-ribosomal particle component Nop7 that relocalised to the nucleus as well as the 20S pre-rRNA processing protein Nob1 that relocalised to nuclear foci (Fig. 3.3.7a-e). Growth defects in translation genes and mostly deregulation of translation proteins are concordant with the role of polyamines in other systems where polyamines stimulate ribosomal RNA and aminoacyl-tRNA synthesis as well as improve translation fidelity by stabilising ribosomal subunits and ribosomal RNA, improving discrimination of aminoacyl-tRNA binding to the ribosome and subsequent proof reading, and inhibiting of polypeptide chain termination (Thompson et al., 1981; Sperrazza & Spremulli, 1983; Igarashi et al., 1982; Tabor & Tabor, 1984).

3.3.6 Cellular transport mechanisms disrupted by MTDIA and *meu1Δ* are consistent with autophagy

Disruptions to vesicle-mediated transport was a novel mechanism of MTDIA and MTAP loss identified in this thesis represented by altered growth observed for deletions of vesicle-mediated transport genes (*ELO2*, *VPS17*, *VPS4*, *VPS5*, *VPS53*, *YDR455C*/overlaps *VPS44*, *VPS8*, *VAM10* and *MON1*) observed with MTDIA and deletions of *CCZ1*, *VAM3*, *VAM6*, *VPS41*, *ROM2*, *MTC5*, *GGA2* and *RIM1* observed with *meu1Δ* (Chapter 2). Consistent with these findings were changes in proteins involved in transmembrane transport either in intensity or localisation with MTDIA and *meu1Δ*. Amino

acid permeases of the plasma membrane such as the plasma membrane regulator of polyamine and carnitine transport Apg2, branched amino acid permeases Bap2 and Bap3, 7-keto 8-aminopelargonic acid permease Bio5, high affinity glutamine permease Gnp1 that also transports other amino acids (Leu, Ser, Thr, Cys, Met and Asn), high-affinity histidine permease Hip1, choline, ethanolamine, and carnitine permease Hnm1, high affinity methionine permease Mup1 relocalised from the plasma membrane to the vacuole in response to MTDIA or *meu1Δ* (Fig. 3.3.8a). Likewise, plasma membrane ion transport proteins (high-affinity copper transporter Ctr1, ferro-O₂-oxidoreductase Fet3, high affinity iron permease Ftr1, iron siderophore Arn1, negative regulator of Pma1 H⁺-ATPase HSP30, high-affinity cysteine permease Yct1, and acetic acid responsive plasma membrane protein Yro2 relocalised from the plasma membrane to the vacuole (Fig. 3.3.8b). Plasma membrane xenobiotic efflux transporters such as ATP-binding cassette (ABC) transporters (Pdr5, Snq2, Yor1 and Pdr12), and major facilitator superfamily proteins (Hol1 and Tpo4) also relocalised from the plasma membrane to the vacuole (Fig. 3.3.8c). Several other plasma membrane permeases (high-affinity H⁺/biotin (vitamin H) symporter Vht1, ammonium transporter Ato3, purine-cytosine permease Fcy2, high affinity uridine permease Fui1, and myo-inositol transporters Itr1 and Itr2) also relocalised from the plasma membrane to the vacuole (Fig. 3.3.8d). Several ER membrane transport proteins (the ER high affinity Ca²⁺/Mn²⁺ permease Pmr1 and the protein translocon complex subunit Ssh1) increased in abundance, while the low-affinity amino acid permease Apg1 decreased in abundance with *meu1Δ*. Vacuolar membrane proteins such as the zinc transporter Zrc1, vacuolar H⁽⁺⁾-ATPase subunit Vma1 were down regulated with *meu1Δ* (Fig. 3.3.8a-d).

Mitochondrial proteins were also regulated by MTDIA and *meu1Δ*. Mitochondrial F₁F₀ ATP synthase subunits Atp7 and Atp15, subunit IV of cytochrome c oxidase Cox4, ubiquinol cytochrome-c reductase complex subunit Qcr6, inner membrane citrate transporter Ctp1, phosphate carrier Mir1, citrate and oxoglutarate transporter Yhm2, as well as mitochondrial translocase of outer membrane (TOM) complex subunit Tom7 were downregulated with MTDIA or *meu1Δ* (Fig. 3.3.8e). Only eight mitochondrial proteins are encoded and synthesised in the mitochondria itself while the remainder

a

b

f

Figure. 3.3.8 – MTDIA and *meu1Δ* deregulates membrane proteins. (a) Amino acid permeases. (b) Ion transporters. (c) Xenobiotic efflux transporters. (d) Mitochondria targeted transport. (e) Other transport mechanisms. (f) Vesicle-mediated transport proteins. GFP fusion proteins were visualised using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin). The GFP abundance was quantified by automated image analysis (Acapella, Perkin Elmer)(Bircham et al., 2011) and relocalisation was determined by visual inspection. Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated.

are encoded by nuclear genes and synthesised by the cytosolic ribosomes and are translocated to the mitochondria with the aid of translocase of outer mitochondrial membrane (TOM) complex and complexes of the translocase of the inner membrane (TIM) (Lithgow, 2000; Koehler, 2000). Therefore, the localisation of nuclear genome encoded mitochondrial proteins are dependent on vesicle-

mediated transport and are sensitive to secretory pathway and vesicle-mediated transport disruptions.

Consistent with vacuolar localisation of plasma membrane proteins, several proteins required for vesicle-mediated transport were also localised to the vacuole (Fig. 3.3.8f). These included the ER to Golgi transport protein *Erp1*, the *Kar9* localisation determinant protein *Tvp38*, the endosomal-vacuolar trafficking pathway protein *Rcr2* and the plasma membrane t-SNARE *Sso2*. Whilst others such as casein kinase *HRR25* relocalised from cytoplasm to cytoplasmic foci and ADP-ribosylation factor

Figure. 3.3.9 – **Vesicle-mediated transport proteins that were deregulated with MTDIA and *meu1Δ*.** Proteins levels that were upregulated (red) downregulated (blue) and relocalised to the vacuole (*). The assignments of genes in the pathway are based on SGD (Cherry et al., 2012).

Arf1 increased in the number of cytoplasmic foci, levels of other proteins were reduced (the endocytosis protein Gvp36, ER/Golgi inheritance protein Sec8, guanyl-nucleotide exchange factor Sec2) or increased (high affinity $\text{Ca}^{2+}/\text{Mn}^{2+}$ P-type ATPase eisosome core component Pil1, calcineurin substrate Aly1) in response to MTDIA and *meu1* Δ (Fig. 3.3.8f; Fig.3.3.9). These disruptions to vesicle-mediated transport are likely responsible for the mislocalisation of plasma membrane proteins to the vacuole and would be consistent with the induction of autophagy as a mechanism of MTDIA described in chapter 2.

3.3.7 Proteins involved in synthesis of cell wall, acetyl-CoA and homeostasis of sterols, glycerolipids and sphingolipids are regulated by MTDIA and *meu1* Δ

Ergosterol and zymosterol are the major sterols in yeast, and with fatty acids, glycerophospholipids and sphingolipids, comprise the lipid constituents of membranes. The synthesis of ergosterol is co-regulated with the synthesis of other lipids. The inhibition of HMG-CoA of ergosterol synthesis also leads to downregulation of sphingolipid synthesis and synthetic lethality of *erg2* $\Delta*erg24* Δ is suppressed by deletion of fatty acid elongases *ELO2* or *ELO3* critical to sphingolipid synthesis (Storey et al., 1998; Bhuiyan et al., 2007). Deletion of *ERG26* also changes the phospholipid composition of membranes, and inhibition of Erg11 inhibits fatty acid synthesis by inhibiting the transcription of *ELO1*, *FAS1* and *OLE1* (Baudry et al., 2001; Bammert & Fostel, 2000). In chapter 2, deletions of genes involved in lipid homeostasis *OPI3*, *CSG2*, *ETR1*, *INO2*, *SHR5*, *CEM1* showed a growth defect with *meu1* Δ and deletion of *ERF2*, *ETR1*, *YHL008C* genes showed a growth defect with MTDIA, while deletion of fatty acid elongase *ELO2* was resistant to MTDIA. Likewise, deletions of genes required for cell wall synthesis and Rim101 pathway *RIM20*, *DFG16*, *RIM21* also showed a growth defect with MTDIA and *vps4* Δ that constitutively activate *RIM101* improved growth. Herein we show disruptions to proteins involved in acetyl-CoA metabolism, sphingolipid metabolism, phospholipid metabolism and cell wall synthesis following MTDIA treatment or with *meu1* Δ (Fig. 3.3.10a-e).$

Acetyl-CoA synthetases Acs1, Acs2 and Ccw14 as well as CoA transferase Ach1 were downregulated, fatty acid uptake protein Fat3 was upregulated, and acetyl-CoA carboxylase Acc1

mislocalised from the cytoplasm to cytoplasmic foci (Fig. 3.3.10a). Ergosterol biosynthetic proteins such as squalene epoxidase Erg1 were downregulated, while two others (C-5 sterol desaturase Erg3 and C-14 sterol reductase Erg24) relocalised from ER and cytoplasmic foci to the vacuole. Delta(24)-sterol C-methyltransferase Erg6 was relocalised from ER to the cytoplasmic foci as previously reported in Chapter 2 (section 2.3.6.2 and Fig. 3.3.10b). Proteins required for sphingolipid metabolism were also affected such as long chain fatty acyl-CoA synthetase Faa4 that relocalised from the ER to cytoplasmic foci, essential 3-hydroxyacyl-CoA dehydratase Phs1 and phosphatidylinositol phosphate (PtdInsP) phosphatase Sac1 that relocalised from the ER to vacuole, glycerol 3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferase Sct1 that decreased in expression levels at the ER, and levels of integral plasma membrane proteins required for sphingolipid homeostasis (Fmp45 and Ynl194c) were upregulated with MTDIA and *meu1Δ* (Fig. 3.3.10c). Phospholipid synthesis proteins were also affected by MTDIA and *meu1Δ* such as the downregulation of cholinephosphate cytidylyltransferase Pct1, major CTP synthase isozyme Ura7, Phosphatidylethanolamine methyltransferase Cho2, and phospholipid metabolism regulator Scs2. ER methylene-fatty-acyl-phospholipid synthase Opi3, plasma membrane transporter for choline/ethanolamine/carnitine Hnm1, and ER phospholipid translocase Neo1 relocalised to the vacuole. Inter-organellar calcium and phospholipid exchange protein Mmm1 localised to cytoplasmic foci that are likely peroxisomes (Fig. 3.3.10d). Glycerol-3-phosphate dehydrogenases (Gpd1 and Gpd2) required for synthesis of glycerol-3-phosphate (a precursor for glycerophospholipid synthesis) were also upregulated with MTDIA and in *meu1Δ* (Fig. 3.3.2e). These disruptions to phospholipid synthesis proteins were expected since Opi3 and Cho2 that catalyse the penultimate and final steps in phospholipid synthesis are s-adenosyl methionine dependent methyl transferases inhibited by accumulation of MTA as consequence of MTAP inhibition (Kodaki & Yamashita, 1987; Williams-Ashman et al., 1982).

a

b

c

d

Figure. 3.3.10 – **MTDIA and *meu1Δ* disrupts lipid homeostasis and cell wall synthesis.** (a) Acetyl-CoA metabolism. (b) Ergosterol biosynthesis. (c) Sphingolipid metabolism (d) Phospholipid metabolism. (e) Cell wall synthesis. GFP fusion proteins were visualised using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin). The GFP abundance was quantified by automated image analysis (Acapella, Perkin Elmer)(Bircham et al., 2011) and relocalisation was determined by visual inspection. Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated.

In addition to lipid homeostasis several cell wall synthesis and cell wall associated proteins were also perturbed by MTDIA and *meu1Δ*. The structure and biosynthesis of a fungal cell wall is unique to fungi being comprised of chitin, glucans, mannans and glycoproteins crosslinked together (Bowman & Free, 2006). The glycoproteins in particular are synthesised at the ER where a glycosylphosphatidylinositol (GPI) anchor is attached that consists of ceramides attached to phosphatidylinositol linked to a oligosaccharide chain ending with a phosphatidylethanolamine moiety to which the glycosylated protein is attached (Bowman & Free, 2006). The disruptions to vesicle-mediated transport or lipid homeostasis would therefore impact on glycoprotein composition of the cell wall. Reduction in levels of several cell wall synthesis and cell wall associated proteins were

observed with MTDIA and *meu1Δ* (e.g., the bifunctional triacylglycerol lipase and 1-acyl DHAP reductase Ayr1, covalently linked cell wall glycoproteins (Ccw14 and Cwp2) and mitochondrial beta-1,3-glucanotransferase Gas1 required for cell wall assembly) whilst others relocated to the vacuole (plasma membrane associated protein cell wall integrity protein Pun1, GPI-cell wall glycoprotein Sed1 and glycosylphosphatidylinositol-dependent cell wall protein Tod6) (Fig. 3.3.10e).

3.3.8 Cell cycle and DNA repair proteins are regulated by MTDIA and *meu1Δ*

Deletions of cell cycle progression genes *MBP1*, *WHI2*, *WHI4* and *CLB3* and the nucleotide excision repair gene *RAD4* showed a growth defect with MTDIA or *meu1Δ* (Chapter 2). Consistent with these findings, several cell cycle related proteins and DNA repair proteins were deregulated in response to MTDIA and *meu1Δ*. Bud neck associated septin ring components (Cdc10, Cdc11 and Shs1) and bud-site selection protein Bud4 showed reduced accumulation at the bud neck with *meu1Δ* (Fig. 3.3.12). Likewise, microtubule associated essential subunits of the Dam1/DASH complex (Ask1, Dad2, Duo1) also showed reduced spindle formation following *meu1Δ*. In addition, nuclear localised bud-site selection protein Bud20, cohesin complex subunit Irr1, and MIND kinetochore complex protein Mtw1 were downregulated. Cytosolic foci protein required for asymmetric localization of Kar9p during mitosis Tvp38 was localised to the vacuole with *meu1Δ*. DNA double-strand break repair proteins (Mre11, Rad6, Rdh54, Tfb1) were downregulated with *meu1Δ*. The dysregulation of cell cycle associated mRNAs were previously observed with MTDIA treatment of A549 lung cancer cell line but the dysregulation of DNA repair mRNAs was not detected. The depletion of polyamines by inhibition of ornithine decarboxylase however, impaired the repair of DNA double strand breaks generated during the repair of thymine dimers generated UV and was partially reversed by polyamine supplementation (Snyder & Sunkara, 1990). Therefore, the downregulation of DNA repair proteins is likely a result of polyamine depletion.

Figure. 3.3.11 – **MTDIA disrupts cell cycle and DNA repair mechanisms.** (a) cell cycle associated proteins. (b) DNA repair proteins. GFP fusion proteins were visualised using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin). The GFP abundance was quantified by automated image analysis (Acapella, Perkin Elmer)(Bircham et al., 2011) and relocalisation was determined by visual inspection. Numbers inset in each image are the percent changes in GFP abundance with either MTDIA or *meu1Δ* relative to WT untreated.

3.4 Discussion

The global analysis of proteins *in vivo* provides unparalleled information on changes to both abundance and localisation that is not discernible to transcriptomic and proteomic methods that measure only abundance. In this chapter, the *Saccharomyces cerevisiae* GFP fusion collection comprising ~4,900 strains was screened with the transition state structure inhibitor of 5'-methylthioadenosine phosphorylase (MTAP) MTDIA and the genetic mimic in yeast *meu1Δ*. These experiments complemented the mechanisms identified in chapter 2 using non-essential gene deletions. Of the 4,900 GFP-fusion proteins screened against MTDIA, 147 showed altered protein abundance/localisation compared to WT untreated and as expected nearly twice as many (336 proteins) showed altered abundance/localisation with complete loss of *MEU1* compared to WT, demonstrating dose-dependent response. As a consequence, there were 135 overlaps between MTDIA treatment and *meu1Δ*. Out of 348 total proteins, the majority (242/348) increased in abundance and under a third (106/348) showed change in localisation. Approximately half of the mislocalised proteins (59/106) exhibited a significant change in abundance. The remaining 47 proteins showed a change in localisation without a significant change in protein abundance and that would be undetectable to transcriptomic or proteomic methods. Proteins that increased in abundance showed mostly cytoplasmic, nuclear or cytoplasmic foci localisation and proteins that changed in localisation relocated from cytoplasm to nucleus or plasma membrane/ER to the vacuole for both MTDIA treatment and with *meu1Δ*. Vacuolar relocated proteins with MTDIA and in *meu1Δ* were mostly transmembrane proteins (amino acid permeases, ion transporters, drug efflux transporters and transporters of other solutes of the plasma membrane). These proteins are synthesised in the ER and are secreted to the plasma membrane via Golgi apparatus by vesicle-mediated transport (Van der Rest et al., 1995). The vesicle-mediated transport proteins themselves were either downregulated or vacuolar localised with MTDIA and *meu1Δ*.

Several cellular processes directly related the synthesis of polyamine were perturbed with MTDIA and *meu1Δ*. MTDIA is an inhibitor of polyamine synthesis and MTAP/*MEU1*, which processes

5'-methylthioadenosine (MTA) generated as a by-product and inhibitor of spermidine and spermine synthesis to ribose-1-phosphate and adenine to salvage methionine (Avila et al., 2004). Previous studies have shown that inhibition of *meu1Δ* induces ODC activity by ~8-fold leading to 20-fold elevation of putrescine and >2-fold elevation of spermidine levels that was attributed to a reduction in 4-methylthio-2-oxobutanoic acid, a downstream product of MTAP and inhibitor ODC (Subhi et al., 2003). These findings were consistent with the role of MTAP as a tumour suppressor where expression of MTAP in MTAP negative breast cancer cell line (MCF-7) lead to ~6-fold decrease in putrescine levels and ~2-fold reduction in spermidine and spermine levels (Christopher et al., 2002). However, a follow-up study into MTAP expression in MTAP negative fibrosarcoma cell line (HT1080) showed no change in ODC or polyamine levels (Tang et al., 2015). Other studies have shown that MTA accumulation in MTAP positive cells is pro-apoptotic in human hepatocytes and cytotoxic in melanocytes (Ansorena et al., 2002; Andreu-Pérez et al., 2010). MTAP inhibition by MTDIA and MTA co-treatment induced apoptosis of MTAP positive cell lines of lung, head and neck, and mouse xenografts models for both MTAP positive and negative lung, prostate, colon, cervical and triple negative breast cancers, and the polyamine levels were depleted in MTDIA treated cancers (Basu et al., 2007; Basu et al., 2011; Schramm, 2013a). In the current work, the upregulation of ornithine decarboxylase Spe1 and spermidine synthase Spe3 was not observed with either MTDIA or *meu1Δ* and the spermidine synthase was downregulated 84% and 69% of untreated WT with MTDIA and *meu1Δ*, respectively. These results were consistent with spermidine synthase inhibition by MTA observed in bovine brain tissue and reduction in spermidine levels with MTDIA dependent inhibition of MTAP in lung cancer and head and neck cancer cells (Pajula et al., 1979b; Basu et al., 2007; Basu et al., 2011). Furthermore, the processing of MTA by MTAP/*MEU1* is necessary for salvage of methionine (Pirkov et al., 2008), therefore, proteins required for salvage methionine from glycine, serine, cysteine, choline and sulphate and reduction of methionine sulfoxide were all upregulated with *meu1Δ* and MTDIA. These results functionally validate the MTAP inhibition by MTDIA in yeast.

Several mechanisms relating to polyamine depletion were detected in the screening of the GFP-fusion proteins with MTDIA and in *meu1Δ*. Proteins in overlapping cellular processes of transcription, chromatin organisation and gene expression were downregulated with MTDIA and *meu1Δ* and disruptions to these proteins were consistent with polyamine depletion (Pollard et al., 1999). The role of acetyl-CoA in histone modification is likely a contributor for these disruptions, given that yeast do not encode DNA methyl transferase present in mammals (Tehlivets et al., 2013). Notable was the upregulation in *meu1Δ* of the Nhp6 architectural transcription factor Nhp6b that functions in a parallel pathway to the histone acetyltransferase Gcn5 that is bypassed by polyamine depletion (Yu et al., 2000; Pollard et al., 1999). Many proteins required for translation were also downregulated with MTDIA and *meu1Δ*, consistent with the positive regulatory role of polyamines in translation and subsequent polyamine depletion with MTAP inhibition. Several proteins involved in resolving DNA interstrand cross-links were also downregulated with MTDIA and in *meu1Δ* and was a consequence of polyamine depletion (Snyder & Sunkara, 1990). These mechanisms were not detected with MTDIA treatment of A549 tumours (Basu et al., 2011). Given that some of the commonly used chemotherapeutics are used for the sole purpose of inducing DNA strand cross-links (Dronkert & Kanaar, 2001), MTDIA in combination with these chemotherapeutic agents may improve the effectiveness preventing the repair of DNA strand cross-linking.

A comparison of the proteins altered with MTDIA and *meu1Δ* in this study with the gene expression changes detected in the MTDIA treated A549 mouse xenograft tumours showed overlaps in perturbed cellular processes. GO analysis of >2-fold upregulated or downregulated gene transcripts from Basu et al. enriched for biological processes including processes regulating steroid biosynthesis ($p = 1.92\text{E-}04$), response to lipids ($p = 4.69\text{E-}03$) vesicle-mediated transport ($p = 1.68\text{E-}03$), and gene expression ($p = 9.99\text{E-}05$) (Appendix Table 7.3.6), albeit only regulation of cell cycle and apoptotic processes were reported (Basu et al., 2011). In our study, we observed disruptions to proteins that regulate all three lipids classes, vesicle-mediated transport, gene expression and cell cycle consistent

with these disruptions in A549 tumours (Basu et al., 2011). The overlaps in the genetic screens confirm the use of yeast as a valid model for elucidation of drug mechanisms affecting human diseases.

The vacuolar relocalisation of the plasma membrane/ER proteins and disruptions to vesicle-mediated transport are consistent with induction of autophagy mechanisms with MTDIA and *meu1Δ* (Chapter 2). Notable was aldehyde dehydrogenase Ald6 that is preferentially degraded by starvation induced autophagy and in response was downregulated (Onodera & Ohsumi, 2004). Another example is the multifunctional casein kinase Hrr25 that phosphorylates and promotes association of Atg19 with Atg11 a core autophagy protein required for cytosol to vacuole targeting pathway (Tanaka et al., 2014). Hrr25 also phosphorylates proteins Atg36 to promote pexophagy and Atg32 to promote mitophagy (Nakatogawa, 2015). In response to MTDIA treatment and *meu1Δ*, Hrr25 accumulated in cytoplasmic foci. Loss of Hrr25 does not affect nitrogen starvation induced non-selective autophagy (Tanaka et al., 2014); therefore, induction of Hrr25 with MTDIA and *meu1Δ* may reflect induction of other selective forms of autophagy such as lipid droplet autophagy (lipophagy) in response to disrupted lipid homeostasis. Lipid droplet autophagy was only recently described (Singh et al., 2009), thus the proteins involved in lipophagy are less well characterised than that of other forms of autophagy and the role of Hrr25 in lipophagy is yet to be investigated. Long chain fatty acyl-CoA synthetase Faa4, localises to the ER/lipid droplets and following induction of lipophagy increases in accumulation at lipid droplets with some localised to the vacuole (van Zutphen et al., 2014). Here we observed increased localisation of Faa4 at lipid droplets with MTDIA and further increase in accumulation at the lipid droplets as well as what appears to be vacuolar localisation of Faa4 with *meu1Δ*. A similar relocalisation was also observed for the ER-localised Erg6-GFP, which would also be consistent with induction of lipid droplet autophagy. Consistent with the degradation of lipid droplets for fatty acid synthesis, the bifunctional triacylglycerol lipase Ayr1 was also upregulated with *meu1Δ*.

Cellular processes affected by regulation of acetyl-CoA were also disrupted with MTDIA and *meu1Δ*. These included proteins involved in central carbon metabolism, cell wall synthesis and lipid homeostasis. Acetyl-CoA is an intermediate of central carbon metabolism and a precursor common to

the synthesis of all lipid classes (Carman et al., 2008; Schug et al., 2015). Acetyl-CoA levels are elevated in various cancers by upregulation of enzymes that metabolise acetate such as acetyl-coA synthetases (Comerford et al., 2014). The acetyl-CoA synthesis in our yeast model would be expected to be increased with the demand for synthesis of ergosterol and glycerophospholipids, given that MTDIA/*meu1Δ* inhibits methyl transferase activities of Erg6 in ergosterol synthesis (Chapter 2) and Opi3/Cho2 in glycerophospholipid synthesis (in this chapter). However, acetyl-CoA synthetases Acs1 and Acs2, and acetyl-coA transferase Ach1 levels were downregulated in *meu1Δ*, possibly due to inhibition by Erg6/Opi3/Cho2. Acetyl-CoA transferase ACAT1 was also downregulated in A549 tumours following MTDIA treatment (Basu et al., 2011). The downregulation of acetyl-CoA synthetases/transferases may play a role in the anti-tumour activity of MTDIA, given that loss of acetyl-CoA synthetase enzyme, ACSS2 reduce the tumour burden in models of hepatocellular carcinoma (Comerford et al., 2014).

In summary, previously suspected mechanisms of MTDIA detected in Chapter 2 in screening of non-essential gene deletion mutants with MTDIA and *meu1Δ* (disruption to amino acid metabolism, carbohydrate metabolism, transcription, translation, chromatin organisation, gene expression, vesicle-mediated transport, lipid homeostasis) were validated with perturbations to GFP-fusion proteins involved in these processes. Several additional cellular perturbations were also identified in disruptions to essential cell cycle and DNA repair proteins, yet were not evident in the results obtained with the non-essential deletion collection and were not detected previously. Furthermore, this chapter identified vacuolar localisation of numerous plasma membrane proteins, further suggesting induction of autophagy as a mechanism of MTDIA. The increased accumulation of lipid homeostasis proteins to lipid droplets suggests lipid droplet autophagy as an additional mechanism of MTDIA. Overall, the screening of the GFP library in this chapter both complemented and validated the screening of gene deletions in chapter 2. In addition, screening the GFP collection with MTDIA and the genetic mimic *meu1Δ* showed a dose response of MTAP inhibition, which may give insight into cellular mechanisms resulting from moderate and extended treatments with MTDIA.

Chapter 4 – Elucidating the therapeutic mechanisms of PNP inhibitor forodesine via assessment of fitness of non-essential gene deletions

4.1 Introduction

Purine nucleoside phosphorylase (PNP) is an enzyme of the purine salvage pathway that is required for T-cell proliferation (Parks Jr & Agarwal, 1972). Loss of PNP leads to T-cell selective suppression and reveals opportunities for regulation of diseases characterised by T-cell proliferation (Bantia & Kilpatrick, 2004). Previous attempts at inhibiting the purine salvage pathway had limited success due to lack of target affinity and the absence of 95% continuous inhibition of PNP required to abrogate T-cell function and proliferation (Gandhi, 2009; Morris & Montgomery, 1998). Forodesine is a potent inhibitor of the human PNP enzyme *in vitro* (*e.g.*, $IC_{50} = 56 \text{ pM}$ K_d *in vitro*) (Evans et al., 2003). Between 2001-2017, forodesine has undergone 14 phase I and II clinical studies to evaluate efficacy, safety and pharmacokinetics in patients with lymphatic leukaemia and lymphoma (<https://clinicaltrials.gov/ct2/results?term=bcx-1777>). In April 2017, forodesine was approved in Japan to treat recurrent and refractory peripheral T-cell Lymphoma (<http://www.mundipharma.com.sg/2017/03/30/mundipharma-wins-approval-antineoplastic-agent-mundesine-treatment-relapsedrefractory-peripheral-t-cell-lymphoma-japan/>, 2017). Forodesine mimics the transition state structure of the PNP substrate inosine bound to the enzyme PNP; therefore, $\sim 7 \times 10^5$ fold more strongly to PNP than inosine (Schramm, 2011). Thus, forodesine inhibits PNP with previously unparalleled efficacy.

The proposed mechanism for forodesine-mediated toxicity essentially mimics the genetic deficiency of PNP. The inhibition of *PNP1* causes an accumulation of its substrates (deoxyguanosine, deoxyinosine, guanosine, and inosine) within cells (Kim et al., 1968; Fox et al., 1980; Wortmann et al., 1979; Chantin et al., 1996). Of these, deoxyguanosine has an alternate fate where it is phosphorylated to dGTP by elevated dCK activity in T-cells (Osborne & Scott, 1983). As dGTP inhibits the ribonucleotide diphosphate reductase (RR) and disrupts the dNTP pool, forodesine inhibits DNA synthesis leading to apoptosis of T-cells (Tattersall et al., 1975; Arpaia et al., 2000). The mechanisms by which PNP

inhibition results in apoptosis have been investigated in T-cells wherein the progression of apoptosis is mediated by changes in mitochondrial membrane potential, P53 stabilisation, caspase activation and cleavage of PARP (Balakrishnan et al., 2006; Balakrishnan et al., 2010a). However, the underlying mechanisms that link dGTP accumulation to initiation of apoptosis have not been characterised. Furthermore, not all cells with elevated dGTP in response to forodesine treatment succumb to apoptosis (Balakrishnan et al., 2006; Balakrishnan et al., 2010a).

Purine nucleoside phosphorylase activity in *S. cerevisiae* is encoded by *PNP1*, which is a structural and functional homologue of human PNP (Lecoq et al., 2001b). Yeast *PNP1* shares 50% sequence identity with the human enzyme and the catalytic and substrate binding residues of PNP are conserved (Lecoq et al., 2001b). This chapter uses yeast genetic tools not available in human cell cultures to elucidate the mechanisms responsible for forodesine mediated toxicity to better understand its human cell toxicity. To elucidate the therapeutic mechanisms of forodesine, the genetic (gene-gene) interactions surrounding loss of the yeast drug target *PNP1* were identified as functional interactions underlying forodesine toxicity. In addition, conditions required to conduct chemical genetic (gene-drug) screening were also investigated.

4.2 Experimental procedures

4.2.1 Yeast strains, media components and compounds

All *S. cerevisiae* strains used in this chapter were derived either from the haploid strains BY4742 (Brachmann et al., 1998) or Y7092 (Tong & Boone, 2007) and were constructed by PCR mediated disruption or heterologous expression of human genes equilibrative nucleoside transporter 1 (ENT1) ((Vickers et al., 1999); gift of Carol E. Cass), deoxycytidine kinase (dCK) ((O’Konek et al., 2009); gift of Donna S. Shewach) and herpes simplex virus type-1 thymidine kinase (HSV-TK) ((O’Konek et al., 2009); gift of Donna S. Shewach) in plasmids. The haploid non-essential gene deletion mutant library ((Tong et al., 2001); gift of Charles Boone) was mated in *en masse* in the construction of genome-wide deletion libraries. All *S. cerevisiae* and *E. coli* strains were cultured in growth media as stated in Chapter

2 unless otherwise specified. The PNP inhibitors forodesine, DADMe-immucillin G and immucillin H were kindly provided by Prof Gary Evans (Ferrier Institute, Victoria University of Wellington) and Cytarabine (AraC) (Sigma-Aldrich) were dissolved in DMSO at a concentration of 10 mM and stored at -20°C.

Strain	Genotype	Reference
BY4743	<i>MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0</i>	(Brachmann et al., 1998)
Y7092 (wild-type)	<i>MATα can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	(Tong & Boone, 2007)
BY4742	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0</i>	(Brachmann et al., 1998)
<i>pnp1Δ</i> (Y7092 background)	<i>MATα pnp1Δ::natR; can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
Y7092 + hENT1 + dCK	<i>MATα CEN3::hENT1::URA3 CEN3::dCK::LEU2 can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
Y7092 + hENT1 + HSV-TK	<i>MATα CEN3::hENT1::URA3 CEN3::HSV-TK::LEU2 can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
<i>pnp1Δ</i> + hENT1 + dCK	<i>MATα CEN3::hENT1::URA3 CEN3::dCK::LEU2 pnp1Δ::natR can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
<i>ade2Δade2Δ</i> + hENT1 + dCK	<i>MATα CEN3::hENT1::URA3 CEN3::dCK::LEU2 ade2Δ::natR ade2Δ::kanR can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
<i>xxxΔ</i> (haploid nonessential gene deletion library)	<i>MATa xxxΔ::kanR his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0</i>	(Tong et al., 2001)
<i>pnp1ΔxxxΔ</i> (double mutants generated in the <i>pnp1Δ</i> SGA)	<i>MATa pnp1Δ::natR xxxΔ::kanR can1Δ::STE2pr-Sp_HIS5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
<i>ura3Δ</i> (Y7092 background SGA control strain)	<i>MATα pnp1Δ::natR; can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	(Bircham et al., 2011)
<i>ura3ΔxxxΔ</i> (double mutants generated in the control mini SGA)	<i>MATa ura3Δ::natR xxxΔ::kanR can1Δ::STE2pr-Sp_HIS5 lyp1Δ his3Δ1 leu2Δ0 met15Δ0 LYS2</i>	This study
Y7092 + pBY011 (<i>CEN::URA3</i> control strain)	<i>MATα CEN3::URA3 can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study

xxxΔ + pBY011 (control gene deletion library used in the minimal media experiment)	<i>MATα CEN3::URA3 can1Δ::STE2pr- Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2</i>	This study
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Table 4.2.1 – Yeast strains used in this chapter.

4.2.2 *PNP1* Synthetic Genetic Array (SGA)

To investigate the genetic interactions with PNP, the *pnp1Δ::natR* was constructed in yeast by PCR mediated disruption of the *PNP1* gene with *natMX4* selectable marker from p4339 plasmid (Goldstein & McCusker, 1999) (Appendix Fig. 7.4.1; Appendix Table 7.4.1). The haploid yeast gene deletion library (Boone et al., 2007) was maintained in 1536 colony format on agar with each deletion strain in quadruplicate with the entire non-essential gene deletion library occupying 14 plates. This gene deletion library was mated *en masse* against the *pnp1::natR* deletion strain using the RoToR HDA replicator (Singer Instrument Co. Ltd.) according to SGA methodology (Tong et al., 2001). The haploid resulting were analysed with Gitter (Wagih & Parts, 2014) and ScreenMill (Dittmar et al., 2010) previously described (section 2.2.3). Double gene deletion strains that showed a growth defect (growth < 30%, $p < 0.05$) compared to the parental haploid non-essential gene deletion library in at least two out of three SGAs were considered as putative epistatic interactions of *PNP1* and were independently validated.

4.2.3 Screening of the *pnp1ΔxxxΔ* deletion strains in minimal media

To identify a media condition where *pnp1Δ* is essential, the *pnp1ΔxxxΔ* double mutants were pinned onto minimal media supplemented with three essential amino acids (SD-N + Met/Ura/Leu + 2% glucose) and the ability to utilise *de novo* amino acid biosynthesis was assessed. The plates were incubated for 5 d at 30°C and colony sizes were assessed using Gitter tools (Wagih & Parts, 2014) on RStudio v0.99.896 (Racine, 2012) and ScreenMill (Dittmar et al., 2010) against the WT control (*Cen::URA3*) SGA screened under the same conditions. Genes responsible for uracil biosynthesis were excluded from this analysis as these were only present in the *Cen::URA3* control SGA.

4.2.4 Validation of *PNP1* interactions

To validate the putative epistatic interactions, the *pnp1ΔxxxΔ* double mutants were re-constructed by performing a mini SGA comprised of specific deletion strains. A control mini SGA was performed using *ura3Δ::natR* as a query strain to filter out mating and sporulation deficient deletion strains. The double mutants were assessed for growth defects in liquid 96-well plate growth assays as previously described in Chapter 2 (Section 2.2.4) using the parental haploid strains and Y7092 wildtype (WT) as controls. The epistatic growth defect of the double mutants was calculated relative to the growth defect of the *pnp1Δ* single mutant ($((pnp1ΔxxxΔ/pnp1Δ) / (xxxΔ/WT))$) modified from the calculation used for assessment of drug sensitivity (Best et al., 2013). The relative growth of the double mutants to the single mutants was normalised against the control *pnp1Δhis3Δ* double deletion strain, which is phenotypically identical to *pnp1Δ* single gene deletion strain that carries a partial deletion of the *HIS3* gene. Stringent cut-offs of growth reduction (>40% growth in double mutant compared to single mutants, $p < 0.05$) and growth improvement (>40% growth in double mutant compared to single mutants, $p < 0.05$) were used to determine significant changes in growth. The double gene deletion mutants with extreme growth defects (<5% growth in double mutant compared to single mutants, $p < 0.05$) were considered synthetic lethal interactions.

4.2.5 Optimisation for forodesine chemical genetic analysis

To achieve forodesine bioactivity (growth inhibition) in yeast, heterologous expression of human genes responsible for the uptake and metabolism of nucleosides was conducted. Plasmids expressing human genes hENT1 (pYhENT1), dCK (pTW382) and herpes simplex gene HSV-TK (pTW383) expression plasmids, and were introduced into the yeast strains using lithium acetate/single-stranded carrier DNA/PEG as previously described (Gietz & Schiestl, 2007). The activity of hENT1 was validated with uptake of adenosine and 5'-methylthioadenosine in Chapter 2 (Section 2.3.2). The dCK and HSV-TK activity was assessed in yeast liquid growth assays against cytarabine (AraC) in SD-Uracil + 2% raffinose(Raf) + 2% galactose(Gal) media. To identify a condition where *PNP1* is conditionally essential, yeast strains WT+hENT1+hdCK, *pnp1Δ*+hENT1+hdCK, and *ade2Δade3Δ*+hENT1+hdCK strains were

assessed for growth in a range of synthetic dropout media conditions. Unless otherwise stated, all growth assays were performed in liquid in appropriate media supplemented with 2% galactose and 2% raffinose to induce the expression of hENT1 under the Gal1-10 promoter.

4.3 Results

4.3.1 SGA analysis identifies genetic interactions with the yeast *PNP1* target of forodesine

Forodesine is an inhibitor of purine nucleoside phosphorylase (PNP) that was recently approved to treat recurrent and refractory peripheral T-cell lymphoma (<http://www.mundipharma.com.sg/2017/03/30/mundipharma-wins-approval-antineoplastic-agent-mundesine-treatment-relapsedrefractory-peripheral-t-cell-lymphoma-japan/>, 2017). However, the mechanisms of action are not fully understood. Some mechanisms have been identified and these are the suspected mechanisms already previously associated with apoptosis (Balakrishnan et al., 2006; Balakrishnan et al., 2010a). To identify unsuspected mechanisms of action of forodesine, *PNP1* (the yeast orthologue of human PNP) was used as a genetic mimic of forodesine treatment. Genome-wide representatives of *pnp1ΔxxxΔ* double deletion meiotic progeny generated using *pnp1Δ* as a query against the nonessential gene deletion library by SGA methodology and evaluated for epistatic growth defect. Based on (growth < 30%, $p < 0.05$), 43 gene deletions conferred a significant reduction in growth of *pnp1Δ* (Fig 4.3.1a; Appendix Table 7.4.2a). To validate the putative *PNP1* genetic interactions identified from the *PNP1* SGA, the double mutants were reconstructed in a mini SGA comprised of 42 putatively interacting gene deletion strains and 112 additional functionally related gene deletion strains. The resulting 154 double mutants were evaluated for reduction in growth phenotype relative to the single deletion mutant parents and wildtype. Validations were performed in triplicate and in liquid media as the growth of a specific number of cells can be accurately measured by measuring turbidity in a spectrophotometer.

Using a stringent cut-off of >40% growth reduction in double mutant compared to parental single mutants and WT ($p < 0.05$), 53 gene deletion strains (18/42 from putative genetic interactions and 35/112 functionally related genes) were considered to exacerbate the growth defect of *pnp1Δ*

(Fig. 4.3.2; Appendix Table 7.4.2b). These genes were assigned to cellular processes using the GO Slim mapping tool on SGD (Cherry et al., 2012) and were involved in amino acid metabolism, response to starvation, vacuole fusion, vesicle-mediated transport, carbohydrate metabolism, lipid homeostasis, chromatin organisation chromosome segregation, transcription, translation and other cellular processes. As proof-of-principle, the genes defective in amino acid metabolism (*AMD1*, *AAH1* and *ADE5,7*) were all involved in purine salvage, and *BAS1*, a transcriptional regulator of genes involved in purine metabolism, helps explain molecular mechanisms of purine deficiency caused by *PNP1* deletion (Daigian-Fornier & Fink, 1992). *PDC5*, although involved in pyruvate degradation, also catalyses the catabolism of several amino acids including tryptophan (Dickinson et al., 2003). *INO2* is required for growth in the absence of inosine and regulates the expression of genes in synthesis of phosphatidylcholine from phosphocholine including *OPI3* (Kersting et al., 2004). *VPS41/VAM2*, *VAM3*, *MON1* and *CCZ1* are GTPase activating enzymes involved in regulation of YPT/Rab GTPase *YPT7* that functions in endosome transport and autophagy (De Antoni et al., 2002; Plemel et al., 2011; Brett et al., 2008; Chen et al., 2014). Vps29 forms the cargo sorting complex with Vps26 and Vps35 which sorts cargo from endosome to trans-Golgi and is an effector of *YPT7* (Liu et al., 2012). Several other genes involved in autophagy (*YDR128W/MTC5*, *PHO5*, *IRS4* and *ATG13*) also showed a growth defect when *PNP1* was co-deleted and are consistent with reduced autophagic activity observed for *pnp1Δ* in nitrogen starvation and with rapamycin treatment (Dengjel et al., 2012).

Deletion of *PNP1* also exacerbated the growth defect of numerous genes that regulate gene expression in response to nutrient limitation at the levels of transcription and translation. *DAL81* positively regulates nitrogen utilisation and promotes the expression of genes involved in catabolism of poor nitrogen sources such as leucine, γ -aminobutyric acid and allantoin and also the expression genes involved in amino acid uptake in nitrogen limited conditions (Palavecino et al., 2015). *GCR2* (overlaps *YNL198C*) is required for transcriptional initiation of glycolytic genes (Uemura & Fraenkel, 1990). *KNS1* is a protein kinase that negatively regulates RNA Pol III and an effector of the TOR pathway (Lee et al., 2012). *RTT103* (overlaps *YDR290W*) regulates transcription termination from RNA Pol II

(Kim et al., 2004). *YKE2* is a subunit of the prefoldin complex that is recruited to chromatin and facilitates transcriptional elongation by RNA Pol II (Millan-Zambrano et al., 2013). In addition, translation elongation factor *TEF4* also showed an exacerbated growth defect with the *pnp1Δ* deletion. *SNF5* is subunit of the SWI/SNF chromatin remodelling complex that is involved in transcriptional regulation. Other genes were also involved in ribosomal function (*RPS19A*, *RPL22A*, *RPS6A*, *RPL9A*, *BUD21*), chromatin remodelling including histone acetyl transferases (*EAF1*, *UME6* and *TOS4* (adjacent *YLR184W*), nucleosome and histone assembly (*HIR2* and *HTZ1* and *SNF5*), DNA repair (*CST9*, *RAD4*), mitochondrial maintenance and inheritance (*RIM1* and *PTC1*) and ATPases (*VPH1*, *PMA2*).

Eight gene deletions (*med1Δ*, *ent2Δ*, *yke2Δ*, *pba1Δ/ylr199cΔ*, *vid21Δ*, *syg1Δ*, *opi3Δ* and *vam3Δ*) conferred extreme growth defects in *pnp1Δ* (< 5% growth in double mutant compared to parental single mutants, $p < 0.05$) and were identified as synthetic lethal interactions (Appendix Table 7.4.2b). In addition, 21 additional double deletion strains were inviable (11/53 putative genetic interactions) but were also inviable in the control SGA and were thus excluded as these were deficient in either the mating or sporulation steps of the SGA procedure. Five gene deletion strains (*ymr085wΔ*, *rim13Δ*, *ynl296wΔ*, *yor082cΔ* and *hst3Δ*) conferred a growth improvement in *pnp1Δ* (>40% growth in double mutant compared to parental single mutants, $p < 0.05$). Of these *MON2* (overlaps *YNL296W*) negatively regulates another GTPase *ARL1*, involved in membrane trafficking and trans-Golgi organisation. *HST3* encoded NAD⁺-dependent protein deacetylases involved in gene silencing. *WHI5* (overlaps *YOR082*) that encodes G1/S phase transcription repressor. *YMR084W-YMR085W* fused open reading frame encode a paralogue to *GFA1* that involved in the initial step of chitin synthesis. *RIM13* involved in proteolytic activation of *Rim101* in response to alkaline pH.

Figure 4.3.1a – **Growth of gene deletion strains impacted by *PNP1* deletion.** (a) Growth of double *xxxΔpnp1Δ* and single *xxxΔ* in technical quadruplicate assessed on agar. (b) Percent growth of 4,294 *xxxΔmeu1Δ* deletion mutants relative to *xxxΔ* from biological triplicate of technical quadruplicate experiments. Three independent SGAs were performed using *pnp1Δ* query strain agar using the RoToR HDA (Singer Instrument Co. Ltd) and mated against the haploid single gene deletion library *en masse* to according to SGA methodology (Tong et al., 2001). The haploid *pnp1ΔxxxΔ* double mutant meiotic progeny were assessed for epistatic colony size relative to the haploid parental mutants using Gitter tools (Wagih & Parts, 2014) in RStudio v0.99.896 (Racine, 2012) and ScreenMill (Dittmar et al., 2010).

Fig. 4.3.1b- **53 negative and 5 positive genetic interactions with *PNP1***. The growth of the double mutants relative to the single haploid parental strains (*pnp1ΔxxxΔ* / *pnp1Δ*) / (*xxxΔ* / WT) $\times 100$ was determined in liquid media at OD⁶⁰⁰ between 0.3-0.5 for single mutant (*xxxΔ*) control. The growth of the double mutants was normalised to *pnp1Δhis3Δ* control strain and the double deletion mutants with an average growth $> \pm 40\%$, $p < 0.05$ of the *pnp1Δhis3Δ* strain were considered a growth defect or a growth improvement.

4.3.3 Minimal media identifies conditions where *PNP1* is essential

The IC_{10-20} of compounds in WT cells are required for screening of gene deletions mutants for hypersensitivity and growth inhibition requires a perturbation of an essential target or essential macromolecule in cells (Parsons et al., 2004; Cong et al., 2012). *PNP1* in yeast encodes a non-essential protein and, we sought to make a *PNP1* conditionally essential by screening the ~4200 haploid *pnp1ΔxxxΔ* double deletion strains generated by SGA methodology on minimal media supplemented with the essential amino acids leucine, histidine, and uracil. Based on (growth $>\pm 30\%$ treated compared to untreated, $p < 0.05$) criteria, the deletion of 47 genes significantly altered the growth of *pnp1Δ* relative to the *xxxΔ* single gene deletions strains and included 24 growth defects and 23 improvements (Fig. 4.3.2a-b; Appendix Table 7.4.3). The deletion of genes involved in chorismite metabolism (*ARO7*, *PHA2*, *TRP1*, *TRP2*), inosine monophosphate metabolism (*ADE2*, *ADE4*, *ADE8*) and threonine biosynthesis (*THR1*) were affected by deletion of *pnp1Δ* and included growth defects and improvements. Chorismite is a precursor for the syntheses of aromatic amino acids and the branch point in the shikimate pathway that precedes synthesis of phenylalanine, tyrosine, and tryptophan (Braus, 1991). Likewise, the deletion of genes required for the synthesis of phenylalanine and tyrosine show a growth improvement in the *pnp1Δ* deletion strain while deletion of genes in tryptophan biosynthesis exacerbated the *pnp1Δ* growth defect (Fig. 4.3.2c). These results suggest a requirement of tryptophan in the absence of *PNP1*. In contrast, deletions of genes involved in inosine monophosphate metabolism were non-informative as their growth was severely impaired in the absence of adenine independent of *PNP1* while only *THR1* of the threonine biosynthesis pathway showed a growth defect with *pnp1Δ*.

Figure 4.3.2 – **Tryptophan biosynthesis from chorismate is required for growth in the absence of *PNP1* in minimal media.** (a) Growth of double *xxxΔpnp1Δ* and single *xxxΔ* in technical quadruplicate assessed on agar. (b) Percent growth of 4,286 *xxxΔpnp1Δ* deletion mutants relative to *xxxΔ* from technical quadruplicate experiments. (c) Simplified shikimate pathway. The *pnp1ΔxxxΔ* double mutants and the *xxxΔ* parental haploid mutants were pinned onto minimal media supplemented with the essential amino acids methionine, leucine and uracil using the RoToR HDA (Singer Instrument Co.

Ltd) and were incubated for 5 d at 30°C. Deletions of genes labelled red in the simplified shikimate pathway reduced growth of *pnp1Δ* and deletion of genes labelled in blue improved the growth *pnp1Δ*.

4.3.4 *PNP1* inhibitors are variably potent in yeast due to non-essential functionality of *PNP1*

To evaluate the bioactivity of *PNP1* inhibitors, forodesine (inosine analogue) and DADMe-Immucillin G (DIG) (guanosine analogue) were screened against the WT+hENT1 strain. Forodesine was inactive in yeast at concentrations up to 1 mM (the highest concentration tested) (Fig. 4.3.3a). DIG conferred 13% growth defect at 316 μM and a 68% growth defect at 1 mM, As these compounds are low nanomolar inhibitors of human T cell leukemia cell lines MOLT-4 and CCRF-CEM (forodesine IC₅₀ = 0.1-10 nM with 20 μM deoxyguanosine (Kicska et al., 2001), the potent bioactivity at 1mM only with DIG was likely due to secondary target effects. Under the same conditions, the loss of *PNP1* is non-essential (Fig. 4.3.3b), indicating that the activity of DIG is not mediated through *PNP1* inhibition. A minute but significant growth defect was observed for *pnp1Δ* at saturation (>36 hours) compared with WT. Therefore, DIG was excluded from consideration as a *PNP1* inhibitor in subsequent experiments.

To determine whether absence of growth inhibition by forodesine was due to lack of forodesine transport, the ability of forodesine to competitively inhibit the uptake of nucleosides adenosine and cordycepin via hENT1 was assessed. Both compounds are transported by hENT1 as previously shown in Chapter 2 section 2.3.2. The growth of *ade2Δade3Δ*+hENT1 with adenosine in the absence of exogenous adenine and the growth WT+hENT1 under cordycepin treatment was assessed following forodesine co-treatment. A forodesine concentration-dependent decrease in growth (IC₂₀ = 0.8 μM and IC₄₀=100 μM) was observed in the *ade2Δade3Δ*+hENT1 strain cultured in 250 μM adenosine supplemented media. In contrast, the *ade2Δade3Δ*+hENT1 strain in 250 μM adenine supplemented media showed no growth inhibition with forodesine (Fig. 4.3.3c) and was similar to the WT+hENT1 strain treated with forodesine in synthetic complete media (Fig. 4.3.3a). The WT+hENT1 strain treated with semi-inhibitory concentrations of cordycepin showed a growth improvement when co-treated with forodesine (Fig. 4.3.3d). As expected, growth improvement was more pronounced at

Figure 4.3.3 – Forodesine treatment or loss of *PNP1* does not cause a growth defect in yeast. (a) Bioactivity of DADMe-Immucillin G (DIG) and forodesine evaluated in the WT+hENT1 strain. Percent growth ($OD_{600}Exp/OD_{600}vehicle\ control \times 100$) was determined in mid log cultures (0.3-0.5 OD_{600} units in the vehicle control) using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer). (b) The growth of the WT+hENT1 and *pnp1Δ*+hENT1. (c) *ade2Δade3Δ*+hENT1 strain treated with forodesine and 250 μM adenine or 250 μM adenosine. (d) WT+hENT1 strain co-treated with toxic nucleoside analogue cordycepin and 100 or 1000 μM forodesine. The percent growth inhibition by cordycepin in the absence of forodesine is shown in blue. The percent growth improvement ($OD_{600}Cor+Foro/OD_{600}Cor\ control \times 100$) with forodesine treatment shown in orange (100 μM forodesine) and grey (1000 μM forodesine). (e) WT+hENT1 and *pdr1Δpdr3Δ*+hENT1 with forodesine.

higher concentrations of both cordycepin and forodesine due to saturation of the hENT1 transporter. This is also consistent with the ~85-fold increase in IC_{50} of cordycepin with expression of the hENT1 in WT (Chapter 2 section 2.3.2). Together, the results with adenosine and cordycepin confirm that forodesine enters the cells via the hENT1 transporter, although forodesine is not growth inhibitory.

To determine if the secondary effects observed with DIG are also present for forodesine but masked by the highly-regulated drug efflux mechanisms in yeast, a pleotropic drug response (PDR) attenuated strain (Coorey et al., 2015) expressing hENT1 (*pdr1Δpdr3Δ*+hENT1) was evaluated for growth inhibition by forodesine. No significant difference in bioactivity was observed with various forodesine concentrations in the *pdr1Δpdr3Δ*+hENT1 strain compared to the WT+hENT1 strain (Fig. 4.3.3e). This indicates that the lack of growth inhibition is not due to PDR-network mediated efflux of forodesine, and moreover that secondary target effects observed for DIG is absent for forodesine. The mechanisms of forodesine are likely mediated through the non-essential *PNP1* in yeast that needs to be made conditionally essential in order to investigate its therapeutic mechanism.

4.3.5 Forodesine sensitivity is increased with deoxycytidine and deoxyguanosine

Increased expression of dCK is necessary for PNP inhibition mediated T-cell toxicity and dCK is absent in lysates of forodesine-resistant human leukaemia cells (Ullman et al., 1979; Osborne & Scott, 1983; Huang et al., 2008). To achieve *PNP1* inhibition-facilitated growth inhibition in yeast, WT+hENT1 and *pnp1Δ*+hENT1 strains were transformed with the yeast expression plasmid pTW382 (O'Konek et al., 2009) expressing the human dCK gene. Cytarabine, a toxic analogue of 2'deoxyctidine, is converted to cytosine arabinoside triphosphate by dCK inducing cytotoxicity (Verschuur et al., 2001). The dCK activity was thus assessed by screening against cytarabine. Cytarabine conferred 19% growth inhibition in WT+hENT1+dCK at 100 nM (the lowest concentration tested), and completely inhibitory at concentrations $\geq 3.1 \mu\text{M}$ (Fig. 4.3.4a). In contrast, cytarabine was inactive in the absence of dCK at all concentrations between 100 nM - 1mM in both WT and WT+hENT1.

Following the confirmation of dCK function in yeast, the mechanism by which forodesine causes T-cell toxicity was investigated in yeast. The bioactivity of *PNP1* inhibitor forodesine and nucleosides deoxyguanosine and deoxyadenosine were assayed in WT, WT+hENT1 and WT+hENT1+dCK strains. As expected forodesine was inactive in the WT in the absence of the hENT1 required for forodesine transport (Fig. 4.3.4b). A small (7%) reduction in growth was observed with 10 μ M forodesine in the presence of the hENT1 transporter and it was further exacerbated (16%) with expression of human dCK. Similarly, deoxyguanosine was inactive in WT in the absence of hENT1 from 0.063-1 mM and active and inhibiting growth when hENT1 was expressed (IC_{20} ~656.25 μ M) (Fig. 4.3.4c). The growth inhibition by deoxyguanosine was significantly increased in the WT+hENT1+dCK strain (IC_{20} ~93.75 μ M) but only reached IC_{50} at 1 mM. The growth inhibition by both forodesine and deoxyguanosine observed in the absence of human dCK expression is therefore likely mediated through a yet uncharacterised deoxyguanosine kinase in *S. cerevisiae* with a lower catalytic efficiency than the human dCK. The closest fungal orthologs of human dCK is encoded by a hypothetical protein GLOINDRAFT_331615 in *Rhizophagus irregularis* (23% identities and 46% similarities) and CCR4-NOT transcription complex subunit 3 of *Cryptococcus neoformans* (26% identities and 47% similarities), likewise, the hypothetical protein GLOINDRAFT_331615 (28% identities and 46% similarities) is also the closest fungal orthologue of human dGK and are distant orthologue (Balakrishnan et al., 2005). GLOINDRAFT_331615 shares minor homology (similarities 21-23%) with 50 proteins in *S. cerevisiae* with unshared function that do not possess kinase activity.

The yeast strains WT+hENT1+dCK and *pnp1* Δ +hENT1+dCK were assessed for growth inhibition by deoxyguanosine and forodesine. Upon deletion of the drug target of forodesine, the *pnp1* Δ deletion mutant showed only slight increase in sensitivity to deoxyguanosine (IC_{20} ~93.75 μ M, IC_{50} ~593.75 μ M) compared to WT (IC_{20} ~93.75, IC_{50} >1000 μ M) (Fig. 4.3.4d). The growth defect of WT+hENT1+dCK in forodesine was exacerbated when co-treated with 250 μ M deoxyguanosine (IC_{20} ~5 μ M) and this growth defect was negated when *PNP1* was deleted (Fig. 4.3.4e). A slight improvement in growth was

Figure 4.3.4 – **dCK expression improves growth inhibition by forodesine, and *pnp1* Δ confers resistance.** Growth assays demonstrating the bioactivity of cytarabine, forodesine and 2'-deoxyguanosine (dGuo) in yeast strains WT and *pnp1* Δ , expressing the human equilibrative nucleoside transporter (hENT1) and the human deoxycytidine kinase (hdCK). (a) Cytarabine in WT, WT+hENT1, WT+hENT1+dCK. (b) Forodesine. (c) dGuo. (d) dGuo in WT+hENT1+hdCK and *pnp1* Δ hENT1+hdCK strains. (f) 250 μ M dGuo+ forodesine. % growth ($OD_{600}Exp/OD_{600}Vehical\ control \times 100$) was determined in mid-log cultures (0.3-0.5 OD_{600} units in the vehicle control) using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer). Standard deviation from triplicate experiments are presented.

observed at the higher doses of forodesine for *pnp1Δ*, albeit this is likely due to saturation of the hENT1 transporter by forodesine leading to a reduction in transport of deoxyguanosine. Overall, these results suggest that the growth inhibition by forodesine is mediated through *PNP1*.

4.3.6 Mitochondrial accumulation of dGTP is insufficient to inhibit yeast growth

To further improve on the toxicity induced by *PNP1* inhibition or deletion, the herpes simplex virus type-1 thymidine kinase (HSV-TK) was expressed in the WT+hENT1 strain in the absence of dCK. HSV-TK is a human TK-2 related kinase with broad substrate specificity and phosphorylates various guanosine analogues including ganciclovir (GCV) (O'Konek et al., 2009). Given that heterologous expression of HSV-TK in mice liver accumulates phosphorylated GCV in the mitochondria (van der Eb et al., 2003) (Herraiz et al., 2003) and that deoxyguanosine was previously shown to be a substrate HSV-TK (Fyfe et al., 1978), we sought to evaluate the contribution of the mitochondrial deoxyguanosine kinase activity in response to a treatment of forodesine and 250 μM deoxyguanosine (Fig. 4.3.5a&b). The expression of HSV-TK was confirmed by phosphorylation cytarabine to toxic AraC triphosphate by HSV-TK. WT expressing HSV-TK showed growth inhibition (7% at 100 μM and 53% at 1 mM) to cytarabine whilst, WT and WT+hENT1 strains showed on growth inhibition to cytarabine treatment from 100 nM- 1 mM. (Fig. 4.3.5a). The expression of HSV-TK in WT+hENT1 strain resulted in increased sensitivity to deoxyguanosine with 100 nM forodesine (21% growth inhibition) compared to basal inhibition in the WT+hENT1 strain (13% growth inhibition) (Fig. 4.3.5b). The treatment of WT+HSV-TK with deoxyguanosine alone showed similar growth inhibition to WT (10% in WT+hENT1+HSV-TK and 9% in WT+hENT1). However, dCK in WT showed more sensitivity to deoxyguanosine with 100 nM forodesine (37% inhibition) and deoxyguanosine alone (inhibition 28% than the expression of cytosolic dCK. (Fig. 4.3.5b). From these results, it cannot be excluded that mitochondrial accumulation of dGMP is not a significant contributor to the growth inhibition by forodesine.

Figure 4.3.5 – Expression of HSV-TK exacerbates the growth inhibition by deoxyguanosine and forodesine. (a) WT, WT+hENT1 and WT+hENT1+HSV-TK against cytarabine. (b) WT+hENT1, WT+hENT1+dCK and WT+hENT1+HSV-TK against 500 μM dGuo and forodesine relative to forodesine alone. Triplicate growth assays were performed in liquid media at 30°C for WT cells expressing human equilibrative nucleoside transporter (hENT1) and human deoxycytidine kinase (dCK) or herpes simplex viral thymidine kinase (HSV-TK). Percent Growth ($OD_{600}Exp/OD_{600}Vehical\ control \times 100$) was determined in mid-log cultures (0.3-0.5 OD_{600} units in the vehicle control) using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer).

4.3.7 MTA exacerbates the forodesine growth defect

Forodesine and deoxyguanosine treatment in T-cells lead to increased accumulation of dGTP (154-fold) and dATP (8-fold) (Bantia et al., 2003) although phosphorylation of adenosine and deoxyadenosine is catalysed by adenosine kinase an enzyme conserved in yeast (Lecoq et al., 2001a). As the growth inhibition by forodesine and deoxyguanosine was reversed by deoxycytidine without a reduction in dATP accumulation (Bantia et al., 2003), the disruptions to the adenine salvage pathways (*amd1Δ*, *aah1Δ*, *ade2Δ*, *ade5*, *ade7Δ*) leading to exacerbation of the *pnp1Δ* growth defect was unexpected. To investigate this further and attempt to make *PNP1* essential, the growth defect of WT and *pnp1Δ* was assessed against deoxyadenosine or 5'-methylthioadenosine (MTA) with deoxyguanosine and forodesine. Treatment of WT+hENT1+dCK with deoxyadenosine showed sensitivities similar to those previously observed for deoxyguanosine ($\sim IC_{50}$ 900 μM) and deletion of *PNP1* showed only slight exacerbation in sensitivity ($\sim IC_{50}$ 500 μM) to deoxyadenosine (Fig. 4.3.6a). In contrast, the *ade2Δade3Δ* double mutant showed resistance to deoxyadenosine (39% growth

inhibition at 1 mM), which is likely a consequence of adenosine and adenine being generated from deoxyadenosine catabolism.

The treatment of WT with deoxyguanosine plus forodesine with MTA, in the absence of exogenous adenine or methionine, showed 1%, 68% and 94% growth inhibition 1 μ M 10 μ M and 100 μ M, respectively (Fig. 4.3.6b). This, however, was at micromolar ($\sim$ IC₅₀ 6 μ M) concentrations of forodesine compared with MTDIA (in Chapter 2 section 2.3.3) which was inhibitory at sub pico/low nanomolar concentrations (Kicska et al., 2001). Likewise, the growth of *pnp1* Δ was similarly inhibited although it was less sensitive to forodesine with deoxyguanosine than WT (Fig. 4.3.6b), which was not expected as the deletion of *PNP1* was expected to reverse the growth defect induced by forodesine with deoxyguanosine and MTA. The sensitivity of *pnp1* Δ to forodesine suggests that the growth inhibition observed under these conditions was not solely mediated by *PNP1* inhibition. WT+hENT1 treated with forodesine with MTA in the absence of methionine and cystine resulted in growth inhibition (in the absence of dCK or deoxyguanosine) due to decreased the MTA uptake (Fig. 4.3.6c), and is consistent with the *pnp1* Δ inhibition observed for deoxyguanosine with forodesine with MTA. These observations suggest that forodesine-mediated *PNP1* inhibition achieved with MTA is a consequence of hENT1 saturation and the inability to transport MTA. Therefore, MTA was no longer used in subsequent experiments in combination with forodesine.

The growth inhibition by forodesine with deoxyguanosine was also assessed in adenosine supplemented media in the absence of exogenous adenine (Fig. 4.3.6c). WT and *pnp1* Δ were resistant to forodesine with deoxyguanosine when treated with adenosine (WT+hENT1+dCK, while *ade2* $\Delta*ade3* Δ exhibited minor sensitivity to forodesine with deoxyguanosine (18% inhibition with 100 μ M forodesine and 250 μ M deoxyguanosine) (Fig. 4.3.6d). The resistance to deoxyguanosine in WT and *pnp1* Δ was likely a result of adenosine competing with the deoxyguanosine kinase activity by dCK and the uptake of forodesine and deoxyguanosine by hENT1. The growth reduction in *ade2* $\Delta*ade3* Δ at$$

higher concentrations of forodesine is likely a result of hENT1 saturation, which results in reduced uptake of adenosine required by the *ade2Δade3Δ* (adenine auxotroph).

Figure 4.3.6 – MTA-mediated growth exacerbation is not mediated through inhibition of Pnp1p. Growth assays evaluating WT+hENT1+dCK, *ade2Δade3Δ* +hENT1+dCK and *pnp1Δ* +hENT1+dCK strains for growth inhibition by 250 μM dAdo and 250 μM dGuo plus forodesine in 250 μM MTA supplemented media in absence of exogenous adenine and methionine. (a) WT, *ade2Δade3Δ* and *pnp1Δ* with dAdo. (b) WT and *pnp1Δ* with 250 μM dGuo + forodesine in 250 μM MTA supplemented media. (c) WT + hENT1 with forodesine in 250 μM MTA supplemented media. (d) WT and *pnp1Δ* with 250 μM dGuo + forodesine in 250 μM adenosine supplemented media. Percent growth (OD600Exp/OD600Vehical control x 100) was determined in mid-log cultures using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer). dAdo, deoxyadenosine; dGuo, deoxyguanosine; Ado, adenosine; MTA, methylthioadenosine.

4.3.8 Nicotinic acid depletion does not exacerbate the growth defect of *pnp1Δ*

PNP1 is involved in NAD⁺ biosynthesis, wherein with *URH1* and *MEU1*, constitutes alternative pathways in salvage of nicotinic acid from nicotinamide riboside (via *PNP1*, *URH1*, *Meu1*) and nicotinate riboside (via *PNP1*, *URH1*) that is subsequently converted to nicotinamide mononucleotide

(Belenky et al., 2007; Belenky et al., 2009). Likewise, tryptophan is also used as a precursor for synthesis of nicotinamide mononucleotide (Bedalov et al., 2003). Since there is a requirement of tryptophan and nicotinic acid either through transport or synthesis to maintain appropriate NAD⁺ levels required for replicative lifespan and possibly general fitness, we sought to investigate the requirement of *PNP1* in nicotinamide mononucleotide and NAD⁺ biosynthesis. Growth inhibition by deoxyguanosine in the absence of tryptophan, nicotinic acid (NA) and other nutrients was evaluated in *pnp1Δ* and WT.

In the absence of tryptophan and nicotinic acid, *pnp1Δ* (28% with 1 mM deoxyguanosine) showed more growth inhibition to deoxyguanosine than WT (19% with 1mM deoxyguanosine) (Fig. 4.3.7a), but the inhibition of both strains occurred at higher concentrations than previously observed in synthetic complete media 47%, 57% with 1 mM deoxyguanosine in WT and *pnp1Δ* respectively (Fig. 4.3.4d). In the absence of non-essential amino acids, the growth defect was observed for WT and *pnp1Δ* with lower concentrations of deoxyguanosine (62.5-250 μM and 62.5 μM deoxyguanosine with nitrogen sources MSG or ammonium sulphate respectively). (Fig. 4.3.7b). However, no growth difference was observed between WT and *pnp1Δ* at higher concentrations of 0.5 mM and 1 mM deoxyguanosine whether the nitrogen source was MSG (Fig. 4.3.7b) or ammonium sulphate (Fig. 4.3.7c). The deoxyguanosine-facilitated growth defect in WT and *pnp1Δ* with minimal amino acids (cysteine and glutamine to permit the synthesis of methionine and tryptophan, respectively) in the absence of MSG, ammonium sulphate or nicotinic acid was assessed. Relative to poor growth in the absence of nitrogen in the form of MSG or ammonium sulphate (OD₆₀₀ =0.06 for WT at 5 d with 1.7g/L yeast nitrogen base), incremental increases in the concentration of yeast nitrogen base improved growth (e.g., OD₆₀₀ 0.16 and 0.23 for WT at 5 d with 3.4 and 6.8 g/L yeast nitrogen base) but also alleviated the growth inhibition of *pnp1Δ* and WT to deoxyguanosine (Fig. 4.3.7d-e). In summary, although the absence of nicotinic acid decreases replicative lifespan in the *pnp1Δ* strain, it does not impact viability.

Figure 4.3.7 – The growth defect of *pnp1* Δ to dGuo is not further exacerbated in the absence of tryptophan and nicotinic acid. (a) WT and *pnp1* Δ in minus Trp and Na. (b) WT and *pnp1* Δ in minus Na and all non-essential amino acids. (c) WT and *pnp1* Δ in minus Na, AA and non-essential amino acids. (d) WT and *pnp1* Δ in Cys and Gly and increasing concentration of yeast nitrogen base without Na. (e) Relative growth defect of WT and *pnp1* Δ to 250 μ M deoxyguanosine in Cys and Gly and increasing concentration of yeast nitrogen base without Na. Yeast strains WT+hENT1+dCK and *pnp1* Δ +hENT1+dCK were screened against deoxyguanosine in SD-Trp/Na, SD-MSG, SD-AA plus essential amino acids and SD-N+Cys/Glu. The liquid growth assays were performed in 2% raffinose and

2% galactose in triplicate with the exception of Fig. 4.3.7d-e for which six replicates were performed for each condition. % Growth ($OD_{600}^{Exp}/OD_{600}^{Vehical\ control} \times 100$) was determined in mid-log cultures (OD_{600} 0.3-0.5 unless otherwise stated) using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer). Na, nicotinic acid; AA, ammonium acetate, MSG, monosodium glutamate; Trp, tryptophan; Cys, cysteine; Glu, glutamine.

4.3.9 Forodesine does not sufficiently inhibit yeast *PNP1*

To assess if forodesine sufficiently inhibits *PNP1* function in yeast, the yeast strains WT, *nrk1Δ*, *urh1Δ*, *aah1Δ*, *amd1Δ* and *pnp1Δ* expressing hENT1 and dCK were assessed for sensitivity to forodesine and deoxyguanosine. The yeast strains *aah1Δ* and *amd1Δ* have growth defects with loss of *PNP1* (Section 4.3.1). *NRK1* and *URH1* are genes involved in NAD⁺ synthesis, and the deletions of each of these genes have previously been identified as inviable with *pnp1Δ* in the absence of tryptophan and nicotinic acid (Tempel et al., 2007). In the current studies, *nrk1Δ* and *urh1Δ* deletions did not have a growth defect with the deletion of *pnp1Δ* in synthetic complete media (Section 4.3.1), therefore these were assessed in synthetic complete media in the absence of nicotinic acid and tryptophan. The WT strain showed sensitivity to both deoxyguanosine and forodesine in both complete and Trp/Nicotinic acid drop out media and the growth defect in each media was slightly exacerbated (12% in SC and 15% in Trp/Na) with forodesine treatment. The *pnp1Δ* strain, as previously observed showed sensitivity to deoxyguanosine (Fig. 4.3.8c), and did not exacerbate growth inhibition with forodesine treatment in both synthetic complete and Trp/Nicotinic acid dropout media (Fig. 4.3.8a-b). The growth defects of *aah1Δ* and *amd1Δ* in synthetic complete media as well as *nrk1Δ* and *urh1Δ* in Trp/Nicotinic acid dropout media were exacerbated with co-treatment of forodesine and deoxyguanosine. However, the growth defect was not different from WT for these deletion strains (Fig. 4.3.8a-b). Interestingly, *urh1Δ* showed resistance to forodesine compared with WT. This was unexpected as these deletions had a defect in chronological lifespan when the *PNP1* drug target of forodesine was deleted in the background (Belenky et al., 2007). This is likely the difference between knockout of the *PNP1* gene in the double mutant compared to the knockdown *PNP1* in the single

mutant treated with forodesine. Interestingly, deoxyguanosine sensitivity in both WT and *pnp1Δ* was reduced in the absence of tryptophan and nicotinic acid compared to synthetic complete media. The lack of growth reduction in genes deletion strains that previously demonstrated a growth defect with complete abolition of *PNP1* function suggest that forodesine does not sufficiently inhibit *PNP1* in yeast.

Figure 4.3.8 – **Forodesine does not exacerbate the growth defect of gene deletions that are synthetic sick or synthetic lethal with *pnp1Δ*.** (a) WT, *aah1Δ*, *amd1Δ* and *pnp1Δ* in SC. (b) WT, *nrk1Δ*, *urh1Δ*, and *pnp1Δ* in SD-Trp/Na. (c) WT and *pnp1Δ* against 250 μM dGuo in SC and SD-Trp/Na. (d) WT and *pnp1Δ* against 10 μM forodesine in SC and SD-Trp/Na. Percent growth ($OD_{600}Exp/OD_{600}Vehical\ control \times 100$) was determined in mid-log cultures using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer).

4.4 Discussion

To investigate the therapeutic mechanism of PNP inhibition, we attempted a two-pronged approach in *S. cerevisiae*. The first identified the gene-gene interactions with the drug target. These

interactions were then used to optimise media conditions to make PNP essential in yeast so the effect of the PNP inhibitor forodesine could be better discerned. The second approach was to identify gene-drug interactions under the *PNP1* essential condition in order to follow up on these findings in biologically relevant experiments. To these ends, a null deletion of the *S. cerevisiae PNP1* was constructed and genetic interactions were investigated among 4400 pairwise deletions of *pnp1Δ* and non-essential genes. Of these, 53 negative genetic interactions (growth reduction) and five positive genetic interactions (growth improvement) were identified. These genes were classified into two main categories: 1) genes that are modulated in response to nutrient limitation such as amino acid metabolism, response to starvation, vacuole organisation, vesicle-mediated transport, carbohydrate metabolism, and lipid homeostasis; and 2) genes that modulate gene expression such as chromatin organisation, chromosome segregation and repair, transcription and translation.

Of these, the amino acid metabolism genes (*AMD1*, *AAH1* and *ADE5,7*) and *PNP1* were involved exclusively in the salvage of purine nucleotides via three different pathways each ending with synthesis of IMP. IMP is synthesised either from phosphorylation of inosine to hypoxanthine by *PNP1* and subsequent phosphorylation of hypoxanthine by *HPT1* or deamination of adenine to AMP by *AAH1* and subsequent deamination of AMP by *AMD1* or hydrolysis of 5'-phosphoribosyl-5-formamido-4-imidazole carboxamide by *ADE16* and *ADE17* (Walther et al., 2010; Tibbetts & Appling, 1997). The first of such pathways is compromised by deletion of *PNP1*. Second, by the deletion of *AMD1* and *AAH1* genes and third, by the deletion of *ADE5,7*. Loss of these otherwise buffering pathways for synthesis of IMP would lead to exacerbation of the growth defect of *pnp1Δ*. This degree of genetic and pathway redundancy is conserved from yeast to humans (Stepchenkova et al., 2005; Simoni et al., 2007) and demonstrates the importance of purine ribonucleotide salvage and the dispensability of *PNP1*.

Furthermore, the majority of the genes involved in vacuolar and vesical transport are GTPases (*VPS41/VAM2*, *VAM3*, *MON1*, *CCZ1* and *ARL1* (negatively regulated by *MON2*); and these cellular

processes may be compromised indirectly by the loss of *PNP1* as a result of GTP depletion (Balakrishnan et al., 2010b). The disruptions to transcription and translation (*DAL81*, *GCR2* (overlaps YNL198C), *KNS1*, *RTT103* (overlaps *YDR290W*), *YKE2*, *TEF4*, *SNF5*, *RPS19A*, *RPL22A*, *RPS6A*, *RPL9A*, *BUD21*) observed is also a reflection of the reduced ATP and GTP levels in the absence of *PNP1*. This is consistent with the reduction in incorporation of radioactively labelled uridine to mRNA and leucine to proteins observed in forodesine treated CCL cells (Balakrishnan et al., 2010b) as well as depletion of purine ribonucleotides ATP (20%) and GTP (50%) observed in forodesine-treated CLL cells (Balakrishnan et al., 2010b).

The *PNP1*-interacting genes that are involved in chromatin organisation and gene expression are also involved in mitigating the depletion of purines nucleotides. *EAF1/VID21* encodes NuA4 histone acetyltransferase complex specific subunit and directly interacts with *BAS1* and *PHO2* transcription factors (Cheng et al., 2015). Bas1 regulates the expression of genes involved in purine synthesis and is constitutively bound to the promoters of ADE genes. During adenine starvation, Bas1 undergoes a conformation change that recruits histone variant H2A.Z (encoded by *HTZ1*), SAGA histone acetyltransferase complex and SWI/SNF chromatin re-modelers leading to nucleosome disassembly and transcription initiation (Cheng et al., 2015). The loss of *EAF1* also leads to reduced ADE gene expression. The expression of *PHO5*, a nucleotide phosphatase, is also regulated by recruitment HTZ1 by NuA4 and is also impaired by the deletion of *EAF1* (Auger et al., 2008). Under phosphate starvation, *PHO5* is positively regulated by both Ino2/Ino4 transcription factors (He et al., 2012). The carbohydrate metabolism was also impaired in the absence of *PNP1*, but this is likely an indirect effect of reduced levels of both nicotinate riboside salvage in NAD⁺ biosynthesis. Ribose-1-phosphate molecules generated by *PNP1* during purine nucleoside phosphorylation are converted further to ribose-5-phosphate and used as precursors in the non-oxidative pentose phosphate pathway (Xu et al., 2013). *PNP1* with *URH1* and *MEU1* constitute alternative pathways synthesis of precursors for NAD⁺ biosynthesis that is independent of *NRK1* (Belenky et al., 2007; Belenky et al.,

2009); however, deletions of these genes did not exacerbate the growth *pnp1Δ* in synthetic complete media or synthetic complete minus Trp/nicotinic acid media.

From the cellular processes identified herein that buffer the loss of *PNP1* in *S. cerevisiae*, it is evident that *PNP1* is required for homeostasis of ribonucleotides. This however, differs from the main proposed mechanism of dGTP accumulation in CCL cells treated with PNP inhibitors (Bantia et al., 2003). This is due to the absence of deoxyguanosine kinase activity in yeast required for conversion of accumulated deoxyguanosine to deoxyguanosine monophosphate. Although the remainder of the pathway is conserved, *S. cerevisiae* cannot accumulate dGTP to the extent required to inhibit growth. Therefore, *PNP1* loss in *S. cerevisiae* does not completely mirror the PNP loss in mammals, at least with the situation in T-cells.

To replicate the mechanisms by which *PNP1* inhibitors induce T cell toxicity several modifications to the *S. cerevisiae* genome were required. The *S. cerevisiae* genome lacks both ENT and CNT families of plasma membrane nucleoside transporters required for transport of most nucleosides (Young et al., 2013) (Vickers et al., 2000) (including forodesine and deoxyguanosine (Huang et al., 2008)) as well as lacks deoxyguanosine kinase activity (Arner & Eriksson, 1995). Therefore, the human equilibrative nucleoside transporter (ENT1) and the human deoxycytidine kinase were expressed in *S. cerevisiae*. This modified strain was treated with forodesine in combination with 250 μ M deoxyguanosine to achieve growth inhibition. The forodesine-facilitated growth defect in yeast (IC_{20} $\sim$ 5.0 μ M, IC_{50} > 100 μ M) however paled in comparison to picomolar/low nanomolar activity in human cells (Kicska et al., 2001).

To improve the toxicity in yeast, media conditions were manipulated to target pathways in which *PNP1* functions. The nicotinamide mononucleotide salvage of NAD^+ biosynthesis was targeted with forodesine or *pnp1Δ* in the absence of precursors nicotinic acid and tryptophan (Belenky et al., 2009). Purine salvage (Walther et al., 2010; Tibbetts & Appling, 1997) was targeted with adenosine, natural analogues of adenosine (MTA and dAdo), and different variants of minimal and synthetic

media were also assessed but all were unsuccessful in improving the toxicity of forodesine plus deoxyguanosine. When exploring the minimal media conditions, supplementation with some amino acids was necessary as for viability of the genetic background of yeast strains, maintenance of plasmids, and for the SGA manipulations used for construction of the haploid hENT1 + dCK gene deletion library. It is possible these may have prevented toxicity of forodesine.

In addition to the aforementioned cytosolic dCK, deoxyguanosine phosphorylation at the mitochondria was assessed by expression of broad specificity nucleoside kinase HSV-TK. However, less inhibition was observed with HSV-TK expression than with dCK expression. It is likely that *PNP1* was not inhibited due to the genetic redundancies unique to *S. cerevisiae*. Examples of such redundancies include *URH1* that functions in the salvage of nicotinamide riboside to nicotinate (Belenky et al., 2007) and *PNC1* that catalyses the subsequent step in NAD⁺ synthesis (Ghislain et al., 2002) and do not have mammalian orthologues but are conserved across fungi (Wood, 2006; Byrne & Wolfe, 2005). In addition to presence of genetic redundancy in yeast, the lack of gene conservation required for further processing of metabolites may also be a reason for lack of forodesine toxicity, which was the case with deoxycytidine and deoxyguanosine kinases. The absence of other pathways that are yet to be characterised may prevent the mechanisms required for forodesine toxicity in yeast. It is also possible that forodesine does not achieve the 95% continuous inhibition of *PNP1* required to accumulate sufficient dGTP to get similar growth inhibition in yeast as observed for T-cells.

In summary, findings from yeast herein present novel targets to improve the efficacy of forodesine. The synthetic lethal and synthetic sick interactions with *pnp1Δ* in yeast show the requirement for inosine monophosphate (IMP) salvage, tryptophan, inosine and phospholipid biosynthesis for growth in the absence of *PNP1* activity. Immucillin-HP and Immucillin-GP are transition state structure inhibitors of hypoxanthine guanine phosphoribosyl transferase (HGPRT) (Li et al., 1999) and the AMP deaminase (AMD) inhibitor 3-[2-(3-carboxy-4-bromo-5,6,7,8-tetrahydronaphthyl)ethyl]-3,6,7,8-tetrahydroimidazo[4,5-d][1,3]diazepin-8-ol (24b) (Kasibhatla et al.,

2001), and these are two parallel pathways required for salvage of IMP (Walther et al., 2010; Tibbetts & Appling, 1997). Inhibitors of either pathway in conjunction with forodesine would improve the toxicity of forodesine. The inhibition of HPRT (or AMD) would deplete the cells of GMP, IMP, and AMP (also by AMD inhibition) as well as the allosteric regulation of ribonucleotide reductase by dGTP (Ahmad & Dealwis, 2013) that converts the already depleted ADP to dADP thereby inhibiting transcription. The elevated dADP would thus inhibit dCTP and dTTP synthesis by ribonucleotide reductase, leading to disruption of nucleotide pools and forodesine-mediated growth inhibition.

Chapter 5 – Implications and future directions

5.1 Summary

Herein elucidated are the mechanisms for transition state structure analogues methylthioadenosine-DADMe-immucillin A (MTDIA) and the recently approved forodesine (Mundesine®). It is necessary to know the compendium of cellular perturbations resulting from use of these inhibitors, given the importance of polyamine and purine synthesis in cancer. Perturbation to glucose metabolism, translation, transcription, vesicle-mediated transport, lipid homeostasis, cell wall synthesis and cell cycle progression were discerned with MTDIA and its genetic mimic *meu1Δ* in the evaluation of non-essential gene deletions. Disruptions to these cellular processes were also revealed in the global analysis of protein abundance and localisation, confirming disruptions to these processes as mechanisms of MTAP inhibition or MTAP loss. Many plasma membrane proteins were relocalised to the vacuole consistent with autophagy as a mechanism of MTDIA. The disruptions to lipid homeostasis were further evaluated by quantification of sterols and significant reduction in ergosterol levels coincident with accumulation of ergosterol precursors were confirmed. This dissertation highlights the importance of genome-wide studies in discerning drug mechanisms and understanding gene function, given that initiating apoptotic pathways (Basu et al., 2007) and changing DNA methylation patterns (Basu et al., 2011) were the only known mechanisms of MTDIA prior to this dissertation. The novel cellular processes perturbed by MTAP inhibition and MTDIA provide additional insight into possible combinatorial solutions for treating cancers such as in combination with DNA interstrand cross-links inducers (*e.g.*, cisplatin and melphalan). The assessment of the drug target of forodesine in yeast, Pnp1, for genetic interactions identified similar cellular processes to MTDIA and loss of MTAP. However, despite the introduction of several human genes and supplementation of metabolites required for bioactivity in humans, forodesine lacked efficacy in yeast, thus a condition where forodesine was sufficiently bioactive for genomic analyses was not determined. Findings with transition state structure analogues herein illustrate that even highly target-specific inhibitors affect

vast numbers of cellular processes. Thus, genome-wide pre-screening of drugs could efficiently be carried out in yeast prior to assessing mechanisms in human cells.

5.2 Implication for MTDIA as a cancer therapeutic

Remodelling of cell energy metabolism is one of the driving causes of cancer development. These changes include altered glycolysis, lipid metabolism, glutamine metabolism and autophagy to meet the demands for cell proliferation (Zhao et al., 2013). Targeting these changes are one such way to specially target the growth of cancer cells. MTDIA is an inhibitor of methylthioadenosine phosphorylase (MTAP), an enzyme that processes MTA, the toxic byproduct of polyamine synthesis, to maintain the forward reaction and salvage methionine and adenine (Schramm, 2011; Pajula et al., 1979b). Polyamines are important molecules in cancer that promote cell proliferation by facilitating transcription (Friesen et al., 1998), translation (Landau et al., 2010), DNA repair (Snyder & Sunkara, 1990) and maintaining basal levels of autophagy (Eisenberg et al., 2009). Exogenously added polyamines such as spermidine induce autophagy in yeast and mammalian cells, and likewise, depletion of spermidine has previously been shown to inhibit autophagy (Morselli et al., 2011; Vanrell et al., 2013). The depletion of polyamines leading to induction of autophagy has not been detected previously. The induction of excessive autophagy leading to cell death by MTAP inhibition identified herein in yeast would be a novel means to treat cancers if conserved in mammals. It is possible that the differences in programmed cell death between yeast and humans may lead to different outcomes from excessive induction of autophagy. For example, autophagy may not be a key mechanism in mammalian cells if apoptosis is efficient.

MTDIA may potentially regulate cell energy metabolism and immune evasion in cancer cells at several different stages. MTDIA treatment or loss of MTAP in yeast downregulated pyruvate synthesis, oxidation of acetaldehyde, and the synthesis of acetyl-CoA. Other changes to cell energy metabolism with MTDIA/*meu1Δ* included disrupted synthesis of all major lipids, as well as glutamine metabolism where glutamine synthases (Gln1) was mislocalised to cytoplasmic foci (Appendix Fig.

7.3.3). In addition, a product of polyamine synthesis, S-adenosyl methionine (downstream of MTAP) regulates innate immunity through modulation of methyltransferase reactions (Ding et al., 2015). Although the immune processes are beyond investigations carried out in yeast, the GO analysis of gene expression changes with MTDIA in A549 tumours was enriched for myeloid leukocyte mediated immunity ($p = 2.13E-02$) and neutrophil mediated immunity ($p = 4.93E-02$) (Appendix Table 7.3.6). These changes along with induction of autophagy may be responsible for the induction of pro-apoptotic responses observed in cancers.

5.3 Implication for MTDIA in treating lysosomal storage disorders and other disease with defective autophagy

Autophagy is an essential catabolic process that degrades cytoplasmic protein aggregates, lipids, and damaged organelles via the lysosome/vacuole to maintain cellular homeostasis. Large numbers of human diseases result from deregulated autophagy including cancer, heart disease, obesity and neurodegenerative diseases (Cheng et al., 2013; Choi et al., 2013). The findings from this dissertation add MTDIA to the existing arsenal of autophagy regulators (Li et al., 2015), suggesting MTDIA can be effective modulating diseases other than cancer. Particularly, yeast orthologues of proteins that drive development of neurodegenerative diseases such as late-onset Parkinson disease (GLUD2, ATXN2, PARK7, GSK3B), Alzheimer's disease (HSD11B1, DLST, SOD1, GSK3B) and Huntington's disease (CS, GAPDH, ATXN2, SGTA, UBB, ABCB1, ASL, HSPB1, GSK3B) (Hamosh et al., 2005) were regulated by MTDIA.

5.4 Implications of Vps34 in TORC1-dependent and independent autophagy

Several mechanisms responsible for induction of autophagy in yeast and mammals have been identified, the most characterised being the TOR1/mTOR-dependent autophagy in which 40 different genes are required (Sarkar, 2013; Feng et al., 2014). TOR1/mTOR is regulated by extracellular signals such as amino acids and growth factors, availability of ATP, and O₂ levels (Burman & Ktistakis, 2010). Phosphoinositide 3-kinase Vps34 converts phosphoinositide to phosphoinositide 3-kinase, is required

for autophagosome maturation, and is an upstream regulator of TOR1/mTOR (Dall'Armi et al., 2013). Vps34 activates TOR1/mTOR in the presence but not the absence of glutamine and therefore plays a role in amino acid starvation-induced autophagy (Tanigawa & Maeda, 2017). Given that Vps34 relocates from vacuole/lysosome to subdomains of the ER in response to starvation (Burman & Ktistakis, 2010) and that exogenously added phosphoinositide 3-kinase induces autophagy in human colon cancer cells (Petiot et al., 2000), our observation that Vps34 relocates from the vacuole to what appears to be subdomain of the ER with *meu1Δ* under methionine replete conditions suggests that Vps34 is likely the inducer of autophagy, and if so, it is not yet certain if it is TOR-dependent or TOR-independent. Therefore, the role of Vps34 should be further investigated in the induction of autophagy with MTDIA. Comparisons of MTDIA and the Vps34 inhibitor wortmannin (Blommaert et al., 1997) would provide valuable insight into the role of Vps34 in MTDIA mediated induction of autophagy. The mislocalisation of Vps34 as the cause of autophagy induction by MTDIA would be confirmed if wortmannin prevents the relocation of Vps34 to sub-domains of ER in *meu1Δ*. It is also yet to be determined if the autophagic vesicles accumulated within the vacuole are degraded following induction of autophagy with MTDIA. The GFP-Atg8 used herein for microscopy-based detection of autophagy induction is also used in a Western blot analysis to detect degradation of autophagic vesicles at the vacuole. Atg8 is rapidly degraded in the vacuolar lumen by vacuolar/lysosome proteases, resulting in free GFP. Thus the ratio of GFP-ATG8 to free GFP will give an indication of rate of degradation of autophagic vesicles (autophagic flux) (Klionsky, 2011).

5.5 Implications of yeast ergosterol synthesis inhibition on sterol synthesis in mammals

In addition to induction of autophagy, MTAP inhibition/loss in yeast also disrupted lipid homeostasis. Deletions of lipid homeostasis genes OPI3, SIP3, and SYG1 were synthetically lethal with *MEU1* deletion and localisation/abundance of these proteins required for homeostasis of all major classes of lipids were altered with *meu1Δ* or MTDIA. The synthesis of sterols in yeast involves methyltransferase reactions by delta(24)-sterol C-methyltransferase Erg6. Although the

methyltransferases are absent in sterol synthesis in humans, polyamine depletion via MTAP inhibition of A549 tumours in mice lead to >3-fold change in expression of cholesterol homeostasis genes (Kee et al., 2004; Basu et al., 2011), suggesting that non-methyltransferase reactions of sterol synthesis are affected by MTAP inhibition. The role of other methyltransferases in lipid homeostasis are also conserved and worthy of further investigation. The yeast glycerophospholipid methyltransferases Cho2 and Opi3 required for conversion of PE to PC are encoded by a single methyltransferase PEMT in humans, and therefore prone to product inhibition by MTA (Gaynor et al., 1991; Vance & Ridgway, 1988). The observed MTDIA-mediated disruptions to fatty acid synthesis and sphingolipid synthesis likely do not involve methyltransferases. The downregulation of acetyl-CoA synthetases and acetyl-CoA transferases by MTAP inhibition in yeast (in this study) and in humans (Basu et al., 2011) suggest that it maybe causal rather than consequence of lipid homeostasis disruption given that acetyl-CoA is used for as precursor for synthesis of all major classes of lipids (Natter & Kohlwein, 2013). The disruption of MTDIA on the synthesis of lipids other than sterols needs to be quantified in yeast and the translation of these findings to A459 or FaDu cells would give a better indication of the conservation of lipid homeostasis disruptions as a mechanism in head and neck and lung cancers treated with MTDIA.

MTDIA as a consequence of sterol synthesis inhibition may induce lipid droplet autophagy. Consistent with induction of lipid droplet autophagy were increased accumulation of Erg6 and Faa4 at the lipid droplets (Bouchez et al., 2015) with MTDIA and *meu1Δ*. Another protein of interest, the multi-functional casein kinase Hrr25, was induced by MTDIA and *meu1Δ*. Hrr25 has roles in several non-selective forms of autophagy but not in starvation induced autophagy, and its role in lipophagy has not been investigated. Therefore, the uncharacteristic mislocalisation of Hrr25 from cytoplasm to cytoplasmic foci with MTDIA and *meu1Δ* should be investigated to confirm the role in lipophagy with other lipophagy inducers in yeast such as oleate (Niso - Santano et al., 2015). If Hrr25 is not

mislocalised with induction of lipophagy, it may reflect activation of other selective forms of autophagy by MTDIA or yet uncharacterised role of Hrr25 in starvation induced autophagy.

Sterol synthesis inhibition is a promising anti-cancer mechanism, given the requirement of lipids for cell division and changes in cell energy metabolism in cancer cells. Sterol synthesis inhibitors HMG-CoA (Statins) are some of the most heavily prescribed drugs worldwide for cardiovascular diseases, and results from clinical trials show reduced incidence of cancer with statins as chemopreventatives (Boudreau et al., 2010; Demierre et al., 2005). Statins are currently undergoing or have recently completed nearly 150 clinical trials for its efficacy as a cancer therapeutic against various cancers including lung, liver, breast and prostate cancers (<https://clinicaltrials.gov/ct2/results?cond=cancer&term=statin&cntry1=&state1=&Search=Search#wrapper>). Interestingly, statins also induce autophagy and are at least in part responsible for the anti-metastatic effects of statins (Parikh et al., 2010). Given the mechanisms of MTDIA as an inhibitor of sterol synthesis and an inducer of autophagy, it is possible that anti-cancer mechanisms of MTDIA are mediated through disruptions to lipid homeostasis in a similar manner to statins. Thus, MTDIA in combination with statins may improve the potency of both compounds as chemotherapeutics.

5.6 Implications of toxicity in the inhibition of yeast Pnp1

Loss of Pnp1 in yeast illustrated the role of PNP in purine synthesis and the subsequent impacts on many cellular processes (*e.g.*, amino acid metabolism, response to starvation, vacuole fusion, vesicle-mediated transport, carbohydrate metabolism, lipid metabolic processes, chromatin organisation chromosome segregation, transcription, and translation) were similar to what was observed with MTAP inhibition and suggests a similar stress response. As PNP inhibition/loss mediated toxicity in mammals is a combination of dGTP accumulation (proposed mechanisms (Osborne & Scott, 1983)) and GTP depletion (Balakrishnan et al., 2010b), the assessment of the *pnp1ΔxxxΔ* double deletions for genetic interactions did not determine cellular process perturbed by dGTP accumulation but rather was a result of GTP depletion. Yeast lack deoxyguanosine kinase activity (dCK/dGK

orthologues) required for conversion of the Pnp1 product deoxyguanosine to dGMP, enzymes crucial for the proposed toxicity in humans. Yeast also lack the nucleoside transporters required for entry of deoxyguanosine (Arnér & Eriksson, 1995). Therefore, the genetic screen of *pnp1ΔxxxΔ*+hENT1+dCK library against 250 μM deoxyguanosine should be performed (following construction of the *pnp1ΔxxxΔ*+hENT1+dCK gene deletion library) to identify the complete Pnp1 interactome affected by both GTP depletion and dGTP accumulation. Under these conditions, *pnp1Δ* is growth inhibitory and non-lethal therefore permitting the assessment of genetic interactions with *PNP1*. This experiment was not performed in this dissertation due to time constraints and a concerted effort investigating the mechanisms of MTDIA; however, this dissertation provided insight into dGTP independent mechanism of forodesine via *PNP1* deficiency.

The poor efficacy of forodesine for yeast Pnp1 was also a contributor to lack of bioactivity, given that gene deletions sensitive to *pnp1Δ* were insensitive to forodesine. Therefore, better transport mechanisms should be investigated such as combinations of the expression of the CNT2 major transporter for deoxyguanosine (Huang et al., 2008), expression of the major forodesine transporter hENT1, and dGTP accumulation should be measured. Alternatively, the expression of human mitochondrial deoxyguanosine kinase (Arpaia et al., 2000) or *Drosophila melanogaster* multifunctional nuclear Dm-dNK (Solaroli et al., 2007; Munch-Petersen et al., 1998) with hENT1 may also improve the accumulation of dGTP. The lack of specificity for yeast Pnp1 by forodesine can possibly be overcome by replacing the yeast Pnp1 with the human PNP. Removing redundant pathways in yeast may increase the reliance on *PNP1* and present conditions where PNP is conditionally essential. To that end, *PNP1* loss herein rendered yeast sensitive to disruptions of inosine monophosphate salvage. The deletion of hypoxanthine guanine phosphoribosyl transferase (HPT1/HGPRT) or inhibition by transition state structure inhibitors of HGPRT by immucillin-HP and immucillin-GP (Li et al., 1999), would render yeast more sensitive to inhibition by forodesine. These and other inhibitors of IMP synthesis (*e.g.*, the AMP deaminase inhibitor 3-[2-(3-carboxy-4-bromo-

5,6,7,8-tetrahydronaphthyl)ethyl]-3,6,7,8-tetrahydroimidazo[4,5-d][1,3]diazepin-8-ol (Kasibhatla et al., 2001)) would also render lymphatic leukemia cells more sensitive to the action of forodesine and would improve the toxicity in treating lymphatic leukemia.

5.7 Implications on transition state structure analogues

Transition state structure analogues are structures chemically tailored to inhibit the function of a protein of interest and are some of the most potent inhibitors discovered to date (Schramm, 2011). Forodesine was the first of these transition state analogues designed base on the kinetic isotope effects and computational chemistry to be approved for human use (<http://www.mundipharma.com.sg/2017/03/30/mundipharma-wins-approval-antineoplastic-agent-mundesine-treatment-relapsedrefractory-peripheral-t-cell-lymphoma-japan/>, 2017) and is likely the first of many given the efficacy and potency of MTDIA as an anti-cancer inhibitor (Basu et al., 2007; Basu et al., 2011) and the efficacy of other immucillins against infectious pathogens (Ting et al., 2005; Singh et al., 2006). In the next few years, the genome-wide studies performed in this thesis can be carried out human cells with application of CRISPR/Cas9 based gene editing to reveal the complete genetic interactions resulting from inhibition of MTAP (MTDIA target) or PNP (forodesine target) as well as pairwise synthetic lethal interactions with specific cancer alleles. These types of analyses have recently been reported for subsets of the human genome (*e.g.*, 73 pairwise interactions of tumour suppressor genes with relevant drug targets (Shen et al., 2017)). With time, these studies could also be carried out *in situ* in cancer cells. The synthetic lethal (oncogene expression/loss of tumour suppressor-geneX) interactions detected by CRISPR/Cas9 gene editing would identify novel drug targets, that can be drugged with the application of kinetic isotope effects and computational chemistry in designing novel transition state structure inhibitors. Synthetic lethality based transition state structure inhibitor design would not be limited to treatment of cancers but also in the treatment of other human disorders.

6 - Bibliography

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7- Appendix

7.2.1 *meu1*Δ mutant construction

Figure 7.2.1 – ***meu1*Δ construction and validation PCRs**. PCR products were run on a 1% agarose gel with 1KB plus ladder at 100 mV. (a) *meu1*Δ::*natR* deletion construction PCR. (b) PCR validation of *MEU1* knockout.

Primer	Sequence
Fw primer (383)	AGATGAACGTGTGAATGAAAAAAAACTATTTGAGATGAGATGAGATTAAACATGGA GGCCCAGAATACCCT
Rev primer (384)	ATATATGTTTTCTCTCCTATTTATATTTACATATGATTAGCGGCAACCACAGTATAGCG ACCAGCATTCAC
Conformation primer A (3)	CATGTCTATACGCAAATG
Conformation primer B (388)	TACGAGACGACCACGAAGC
Conformation primer C (389)	CATGTCTATACGCAAATG
Conformation primer D (4)	TTATACGTTACTGTACAT

Table 7.2.1 – ***meu1*Δ construction and conformation primers**. Primers were designed according to Janke et al. 2006 (Janke et al., 2004).

7.2.2 Genetic interactions with *MEU1*

ORF name	Gene name	Function	p-value	Z-Score	% Growth
YCL025C	AGP1	Low-affinity amino acid permease with broad substrate range	3.04E-02	2.17	61.73
YOR141C	ARP8	Nuclear actin-related protein involved in chromatin remodelling	3.62E-02	2.09	62.50
YGR157W	CHO2	Phosphatidylethanolamine methyltransferase (PEMT)	7.03E-03	2.70	54.35
YBR036C	CSG2	Endoplasmic reticulum membrane protein	3.35E-02	2.13	60.98
YNL286W	CUS2	Putative checkpoint factor in transcription	3.87E-02	2.07	64.52
YIR023W	DAL81	Positive regulator of genes in multiple nitrogen degradation pathways	2.93E-03	2.98	51.02
YDL160C	DHH1	Cytoplasmic DEAD-box helicase, stimulates mRNA decapping	3.10E-29	11.22	7.52
YBL016W	FUS3	Mitogen-activated serine/threonine protein kinase involved in mating	4.66E-02	1.99	64.52
YNL281W	HCH1	Heat shock protein regulator	4.13E-02	2.04	64.52
YPR179C	HDA3	Subunit of the HDA1 histone deacetylase complex	1.30E-02	2.48	57.14
YIL090W	ICE2	Integral ER membrane protein with type-III transmembrane domains	3.99E-02	2.05	64.52
YDR123C	INO2	Transcription factor	1.37E-03	3.20	47.17
YJR073C	OPI3	Methylene-fatty-acyl-phospholipid synthase	9.09E-03	2.61	55.56
YBR093C	PHO5	Repressible acid phosphatase	1.42E-02	2.45	57.47
YER162C	RAD4	Protein that recognizes and binds damaged DNA (with Rad23p) during NER	6.56E-03	2.72	55.56
YMR154C	RIM13	Calpain-like cysteine protease	4.16E-02	2.04	64.94
YLR371W	ROM2	Guanine nucleotide exchange factor (GEF) for Rho1p and Rho2p	1.98E-02	2.33	59.17
YGL147C	RPL9A	Ribosomal 60S subunit protein L9A	9.05E-24	10.05	9.88
YLR357W	RSC2	Component of the RSC chromatin remodelling complex	4.83E-04	3.49	45.25
YPR069C	SPE3	Spermidine synthase	4.88E-02	1.97	64.52
YNL179C	SRF6	Dubious open reading frame next to RHO5	4.46E-02	2.01	65.36
YCL032W	STE50	Adaptor protein for various signalling pathways	1.33E-02	2.48	58.82
YDR146C	SWI5	Transcription factor that recruits Mediator and Swi/Snf complexes	4.99E-02	1.96	64.52
YKL081W	TEF4	Gamma subunit of translational elongation factor eEF1B	2.94E-02	2.18	61.35
YDR074W	TPS2	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex	1.15E-02	2.53	55.56
YDR080W	VPS41	Subunit of the HOPS endocytic tethering complex	2.23E-02	2.29	58.82
YDR128W	YDR128W	Subunit of SEACAT, a subcomplex of the SEA complex	1.08E-02	2.55	55.25
YLR184W	YLR184W	Dubious open reading frame next to TOS4	4.42E-02	2.01	62.89
YNL285W	YNL285W	Dubious open reading frame that overlaps tRNA-Gly and putative ASR1409	2.79E-02	2.20	60.98
YGL255W	ZRT1	High-affinity zinc transporter of the plasma membrane	2.33E-02	2.27	59.17
YNL241C	ZWF1	Glucose-6-phosphate dehydrogenase (G6PD)	1.39E-02	2.46	56.50

Table 7.2.2a – **31 putative genetic interactions with *MEU1***. The putative interactions with an epistatic growth ($(meu1\Delta xxx\Delta/xxx\Delta) < 70\%$, $p < 0.05$) in at least two out of the triplicate SGAs are presented.

Three independent SGAs were performed on agar using the RoToR HAD (Singer Instrument Co. Ltd). *meu1Δ* query strain was mated against the haploid single gene deletion library *en masse* to according to SGA methodology (Tong et al., 2001). The haploid *meu1ΔxxxΔ* double mutant meiotic progeny were assessed for epistatic colony size relative to the haploid parental mutants using Gitter tools (Wagih & Parts, 2014) in RStudio v0.99.896 (Racine, 2012) and ScreenMill (Dittmar et al., 2010).

ORF name	Gene name	Function	p-value	Standard deviation	Growth %
YNL141W	AAH1	Adenine deaminase (adenine aminohydrolase)	2.63E-15	5.09	19.84
YML035C	AMD1	AMP deaminase	0.000146	6.20	9.74
YNL259C	ATX1	Cytosolic copper metallochaperone	1.69E-08	8.34	29.43
YBR131W	CCZ1	Subunit of a heterodimeric guanine nucleotide exchange factor (GEF)	1.24E-10	2.66	51.81
YER061C	CEM1	Mitochondrial beta-keto-acyl synthase	6.74E-08	7.84	37.86
YBR036C	CSG2	Endoplasmic reticulum membrane protein	7.86E-09	8.89	12.23
YIR023W	DAL81	Positive regulator of genes in multiple nitrogen degradation pathways	6.15E-18	2.54	23.01
YDR359C	EAF1	Component of the NuA4 histone acetyltransferase complex	6.21E-07	9.97	25.51
YNL080C	EOS1	Protein involved in N-glycosylation	4.51E-13	6.47	11.17
YBR026C	ETR1	2-enoyl thioester reductase	2.15E-10	7.75	18.24
YBR020W	GAL1	Galactokinase	1.39E-05	11.45	21.91
YHR108W	GGA2	Protein that regulates Arf1p, Arf2p to facilitate Golgi trafficking	2.02E-05	9.89	45.94
YHL031C	GOS1	v-SNARE protein involved in Golgi transport	0.04846	39.77	175.05
YOR038C	HIR2	Subunit of HIR nucleosome assembly complex	1.48E-09	8.23	16.36
YOL012C	HTZ1	Histone variant H2AZ	5.7E-05	10.78	52.43
YER092W	IES5	Non-essential INO80 chromatin remodelling complex subunit	0.000237	13.49	26.97
YDR123C	INO2	Transcription factor	1.39E-05	11.71	29.72
YKR019C	IRS4	EH domain-containing protein	0.000404	10.59	55.22
YLL019C	KNS1	Protein kinase involved in negative regulation of PolIII transcription	0.000385	13.45	41.74
YPR070W	MED1	Subunit of the RNA polymerase II mediator complex	4.3E-08	4.32	3.46
YDR128W	MTC5	Subunit of SEACAT, a subcomplex of the SEA complex	4.24E-10	6.05	43.92
YJR073C	OPI3	Methylene-fatty-acyl-phospholipid synthase	3.58E-24	1.57	-1.61
YCR077C	PAT1	Deadenylation-dependent mRNA-decapping factor	3.49E-12	4.99	35.39
YLR134W	PDC5	Minor isoform of pyruvate decarboxylase	1.42E-13	6.05	20.75
YBR093C	PHO5	Repressible acid phosphatase	0.010172	20.69	48.93
YPL036W	PMA2	Plasma membrane H ⁺ -ATPase	4.43E-12	6.35	25.73
YER162C	RAD4	Protein that recognizes and binds damaged DNA (with Rad23p) during NER	4.23E-10	7.70	19.72
YCR028C-A	RIM1	ssDNA-binding protein essential for mitochondrial genome maintenance	4.42E-05	8.57	57.26

YLR371W	ROM2	Guanine nucleotide exchange factor (GEF) for Rho1p and Rho2p	0.025124	26.01	35.89
YLR061W	RPL22A	Ribosomal 60S subunit protein L22A	3.73E-07	8.88	34.92
YGL147C	RPL9A	Ribosomal 60S subunit protein L9A	3.09E-21	3.16	0.34
YOR293W	RPS10A	Protein component of the small (40S) ribosomal subunit	0.013664	9.38	55.11
YOL121C	RPS19A	Protein component of the small (40S) ribosomal subunit	0.021084	18.73	51.26
YPL090C	RPS6A	Protein component of the small (40S) ribosomal subunit	0.001387	16.24	39.66
YCR009C	RVS161	Amphiphysin-like lipid raft protein	8.19E-08	5.65	58.15
YDR388W	RVS167	Calmodulin-binding actin-associated protein	6.98E-18	3.05	22.29
YOL110W	SHR5	Palmitoyltransferase subunit	0.001225	15.63	36.34
YNL257C	SIP3	Putative sterol transfer protein	1.74E-12	2.03	0.00
YMR016C	SOK2	Nuclear protein that negatively regulates pseudohyphal differentiation	1.28E-05	10.86	35.27
YOL052C	SPE2	S-adenosylmethionine decarboxylase	6.11E-07	10.74	2.55
YCL037C	SRO9	Cytoplasmic RNA-binding protein	0.000207	18.38	268.71
YIL047C	SYG1	Plasma membrane protein of unknown function	3.96E-18	5.20	0.80
YOL020W	TAT2	High affinity tryptophan and tyrosine permease	5.6E-10	7.73	23.01
YDR207C	UME6	Rpd3L histone deacetylase complex subunit	4.09E-05	5.13	32.41
YOR106W	VAM3	Syntaxin-like vacuolar t-SNARE	2.27E-06	11.45	4.09
YDL077C	VAM6	Guanine nucleotide exchange factor for the GTPase Gtr1p	0.016927	18.40	54.79
YDR080W	VPS41	Subunit of the HOPS endocytic tethering complex	1.69E-10	8.13	5.05
YDR290W	YDR290W	Dubious open reading frame overlaps RTT103	0.007849	18.21	42.96
YLR184W	YLR184W	Dubious open reading frame adjacent to TOS4	4.29E-18	5.18	0.40
YNL198C	YNL198C	Dubious open reading frame overlaps GCR2	0.001642	16.59	26.43
YOL024W	YOL024W	Putative protein overlaps ARS1511	0.042438	30.98	29.10
YOR097C	YOR097C	Putative protein of unknown function	0.000405	13.97	41.22
YPR126C	YPR126C	Dubious open reading frame overlaps YLH47	5.23E-14	5.70	20.38
YNL241C	ZWF1	Glucose-6-phosphate dehydrogenase (G6PD)	1.96E-06	3.04	9.00

Table 7.2.2b – **52 negative and 2 positive genetic interaction with *Meu1***. The growth of the double mutants relative to the single haploid parental strains (*meu1ΔxxxΔ /meu1Δ*)/ (*xxxΔ*/ WT) x 100 was determined in liquid media at 16h and OD₆₀₀ for all cultures with the exception *xxxΔ* slow growers where the OD₆₀₀ was measured at later time points. The growth of the double mutants was normalised to *meu1Δhis3Δ* control strain and the double deletion mutants with an average growth > ± 40%, *p* <0.05 of the *meu1Δhis3Δ* strain were considered a growth defect or a growth improvement.

7.2.3 MTDIA screening of the gene deletion library

Figure 7.2.2 – **Absence of adenine improves sensitivity MTDIA.** Wt+hENT1 treated with MTDIA with and without adenine. The growth assays were performed as previously described in SD-MAUC+MTA+RG media.

7.2.4 Drug-gene interactions and GO enrichment MTDIA

ORF name	Gene name	Function	p-value	Z-Score	% Growth
YOR128C	ADE2	Phosphoribosylaminoimidazole carboxylase	4.22E-02	2.03	75.19
YMR300C	ADE4	Phosphoribosylpyrophosphate amidotransferase (PRPPAT)	5.64E-02	1.91	78.74
YGL234W	ADE5,7	Enzyme of the 'de novo' purine nucleotide biosynthetic pathway	9.46E-03	2.60	72.99
YGR061C	ADE6	Formylglycinamide-ribonucleotide (FGAM)-synthetase	3.68E-04	3.56	60.98
YDR408C	ADE8	Phosphoribosyl-glycinamide transformylase	4.04E-02	2.05	77.52
YCL025C	AGP1	Low-affinity amino acid permease with broad substrate range	6.08E-03	-2.44	128.21
YNR074C	AIF1	Mitochondrial cell death effector	7.04E-02	1.81	80.00
YKR099W	BAS1	Myb-related transcription factor	8.70E-03	2.62	77.52
YDL074C	BRE1	E3 ubiquitin ligase	9.45E-03	-2.60	129.87
YOR026W	BUB3	Kinetochore checkpoint WD40 repeat protein	7.25E-05	3.27	67.57
YBR131W	CCZ1	Subunit of a heterodimeric guanine nucleotide exchange factor (GEF)	1.70E-04	-1.83	128.21
YLR394W	CST9	SUMO E3 ligase	3.00E-02	1.86	79.37
YOR037W	CYC2	Mitochondrial peripheral inner membrane protein	2.55E-02	1.77	80.00
YBL101C	ECM21	Protein involved in regulating endocytosis of plasma membrane proteins	3.54E-03	2.92	75.76
YCR034W	ELO2	Fatty acid elongase, involved in sphingolipid biosynthesis	2.74E-03	-3.00	135.14
YBR026C	ETR1	2-enoyl thioester reductase	1.21E-02	2.51	70.42
YER145C	FTR1	High affinity iron permease	2.22E-03	-3.06	138.89
YER083C	GET2	Subunit of the GET complex	4.90E-02	-1.97	131.58
YJR090C	GRR1	F-box protein component of an SCF ubiquitin-ligase complex	2.55E-02	2.23	77.52
YOR038C	HIR2	Subunit of HIR nucleosome assembly complex	1.86E-04	3.74	64.10

YJR140C	HIR3	Subunit of the HIR complex	4.92E-04	3.48	69.44
YOL068C	HST1	NAD(+)-dependent histone deacetylase	7.97E-04	3.35	67.11
YOR025W	HST3	Member of the Sir2 family of NAD(+)-dependent protein deacetylases	1.87E-03	3.11	68.97
YOL012C	HTZ1	Histone variant H2AZ	5.99E-03	1.95	78.74
YKR019C	IRS4	EH domain-containing protein	9.80E-03	2.58	78.13
YLL007C	LMO1	Homolog of mammalian ELMO (Engulfment and cell Motility)	1.79E-02	2.37	80.00
YKR007W	MEH1	Component of the EGO and GSE complexes	1.93E-02	2.34	80.00
YPR118W	MRI1	5'-methylthioribose-1-phosphate isomerase	2.25E-02	2.28	75.76
YOR209C	NPT1	Nicotinate phosphoribosyltransferase	2.76E-02	2.20	76.34
YCR077C	PAT1	Deadenylation-dependent mRNA-decapping factor	6.78E-03	-2.71	131.58
YLR044C	PDC1	Major of three pyruvate decarboxylase isozymes	6.12E-02	1.87	79.37
YGL059W	PKP2	Mitochondrial protein kinase	4.72E-02	1.98	78.13
YML017W	PSP2	Asn rich cytoplasmic protein that contains RGG motifs	5.01E-02	1.96	78.74
YOR279C	RFM1	Component of the Sum1p-Rfm1p-Hst1p complex	9.87E-04	3.29	72.99
YCR028C-A	RIM1	ssDNA-binding protein essential for mitochondrial genome maintenance	3.08E-04	-3.61	144.93
YJL121C	RPE1	D-ribulose-5-phosphate 3-epimerase	4.25E-03	2.86	75.76
YDR388W	RVS167	Calmodulin-binding actin-associated protein	5.98E-05	2.20	76.34
YMR016C	SOK2	Nuclear protein that negatively regulates pseudohyphal differentiation	5.41E-03	2.78	68.03
YCL037C	SRO9	Cytoplasmic RNA-binding protein	4.68E-02	-1.99	123.46
YDR310C	SUM1	Transcriptional repressor that regulates middle-sporulation genes	2.77E-02	2.20	73.53
YLR426W	TDA5	Putative protein of unknown function	2.55E-03	3.02	74.63
YCR053W	THR4	Threonine synthase	1.65E-03	-3.15	138.89
YFR010W	UBP6	Ubiquitin-specific protease	4.13E-02	-2.04	131.58
YEL038W	UTR4	Protein with sequence similarity to acireductone synthases	1.33E-02	-2.48	129.87
YAL002W	VPS8	Membrane-binding component of the CORVET complex	4.42E-02	-2.01	123.46
YDR290W	YDR290W	Dubious open reading frame; overlaps RTT103	2.17E-04	3.70	64.52
YJL120W	YJL120W	Dubious open reading frame; overlaps the RPE1	2.55E-02	2.23	76.34
YLR236C	YLR236C	Dubious open reading frame; overlaps ORF YLR235C	2.56E-05	4.21	55.87
YNR025C	YNR025C	Dubious open reading frame; overlaps MPP6	4.03E-02	2.05	77.52
YOR024W	YOR024W	Dubious open reading frame; Overlaps 5'UTR of HST3	4.56E-02	2.00	78.13
YOR072W	YOR072W	Dubious open reading frame; overlaps the dubious gene YOR072W-A	1.59E-02	2.41	74.63
YPR195C	YPR195C	Dubious open reading frame; at the 5' end of OPT2 (Oligo peptide transporter 2)	5.19E-02	1.94	78.74

Table 7.2.3a – **52 putative drug-gene interactions with 3nM MTDIA.** 4310 gene deletion strains (xxxΔ+hENT1) were screened against 3 nM MTDIA or Vehicle control in SD-MAUC+MTA+RG media in agar in triplicate experiments. Deletion strains with a growth defect or growth improvement (80%>growth>120%, *P*-value <0.05) in at least two out of three SGAs were considered as hypersensitive or resistant to MTDIA and are shown.

ORF name	Gene name	Function	% Growth	Standard deviation	p-value
YAR015W	ADE1	N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase	64.78	6.55	1.07E-04
YMR300C	ADE4	Phosphoribosylpyrophosphate amidotransferase (PRPPAT)	69.80	3.05	2.16E-05
YGL234W	ADE5,7		62.03	2.45	2.05E-07
YGR061C	ADE6	Formylglycinamide-ribonucleotide (FGAM)-synthetase	72.67	3.73	5.62E-05
YDR408C	ADE8	Phosphoribosyl-glycinamide transformylase	69.67	1.45	2.14E-05
YDR127W	ARO1	Pentafunctional AROM protein	69.01	1.93	1.74E-05
YDL074C	BRE1	E3 ubiquitin ligase	68.16	4.25	2.39E-05
YOR061W	CKA2	Alpha' catalytic subunit of casein kinase 2 (CK2)	53.31	1.69	8.82E-07
YDR151C	CTH1	Member of the CCCH zinc finger family	72.27	10.13	7.53E-03
YOR030W	DFG16	Probable multiple transmembrane protein	69.09	11.02	1.12E-02
YDL160C	DHH1	Cytoplasmic DEAD-box helicase, stimulates mRNA decapping	65.57	9.96	2.09E-03
YDR121W	DPB4	Subunit of DNA pol epsilon and of ISW2 chromatin accessibility complex	72.37	0.73	4.77E-05
YGL043W	DST1	General transcription elongation factor TFIIIS	71.23	0.00	3.89E-05
YBL101C	ECM21	Protein involved in regulating endocytosis of plasma membrane proteins	19.33	2.28	7.26E-09
YBR076W	ECM8	Non-essential protein of unknown function next to SLM4	39.46	23.52	3.35E-02
YLR246W	ERF2	Subunit of a palmitoyltransferase	67.54	4.98	5.38E-05
YBR026C	ETR1	2-enoyl thioester reductase	69.53	7.42	1.37E-03
YDR221W	GTB1	Glucosidase II beta subunit, forms a complex with alpha subunit Rot2p	71.92	5.85	1.34E-04
YGR163W	GTR2	Subunit of a TORC1-stimulating GTPase complex	74.99	7.83	1.30E-03
YPR179C	HDA3	Subunit of the HDA1 histone deacetylase complex	40.89	16.43	1.15E-02
YOR038C	HIR2	Subunit of HIR nucleosome assembly complex	33.48	14.19	4.96E-03
YJR140C	HIR3	Subunit of the HIR nucleosome assembly complex	74.74	4.50	2.14E-04
YIL112W	HOS4	Subunit of the Set3 complex	72.26	12.21	2.91E-02
YDR533C	HSP31	Methylglyoxalase that converts methylglyoxal to D-lactate	60.20	9.66	1.69E-03
YOL068C	HST1	NAD(+)-dependent histone deacetylase	41.48	5.46	1.03E-06
YOR025W	HST3	Member of the Sir2 family of NAD(+)-dependent protein deacetylases	70.76	10.64	1.18E-02
YIL090W	ICE2	Integral ER membrane protein with type-III transmembrane domains	55.40	5.56	8.49E-06
YLL019C	KNS1	Protein kinase involved in negative regulation of PolIII transcription	72.25	1.25	3.98E-05
YDR532C	KRE28	Subunit of a kinetochore-microtubule binding complex	73.47	9.32	4.27E-03
YDL056W	MBP1	Transcription factor involved in regulation of cell cycle progression	72.75	9.77	1.26E-02
YDR128W	MTC5	Subunit of SEACAT, a subcomplex of the SEA complex	66.33	9.22	5.15E-03
YOR209C	NPT1	Nicotinate phosphoribosyltransferase	58.34	3.85	2.13E-06

YER178W	PDA1	E1 alpha subunit of the pyruvate dehydrogenase (PDH) complex	73.78	3.66	7.63E-05
YML017W	PSP2	Asn rich cytoplasmic protein that contains RGG motifs	48.04	10.95	2.21E-03
YOR279C	RFM1	Component of the Sum1p-Rfm1p-Hst1p complex	46.33	4.52	3.38E-07
YOR275C	RIM20	Protein involved in proteolytic activation of Rim101p	67.67	1.69	1.06E-05
YNL294C	RIM21	pH sensor molecule, component of the RIM101 pathway	74.40	15.10	4.64E-02
YER117W	RPL23B	Ribosomal 60S subunit protein L23B	67.52	11.52	1.29E-02
YDL020C	RPN4	Transcription factor that stimulates expression of proteasome genes	44.62	7.84	7.84E-05
YOR293W	RPS10A	Protein component of the small (40S) ribosomal subunit	58.46	6.53	8.32E-05
YDL194W	SNF3	Plasma membrane low glucose sensor,	74.44	7.57	1.46E-03
YBR289W	SNF5	Subunit of the SWI/SNF chromatin remodelling complex	72.20	4.79	4.92E-05
YMR016C	SOK2	Nuclear protein that negatively regulates pseudohyphal differentiation	4.26	1.36	5.07E-09
YCR081W	SRB8	Subunit of the RNA polymerase II mediator complex	32.35	6.67	5.00E-04
YDR310C	SUM1	Transcriptional repressor that regulates middle-sporulation genes	36.77	3.49	5.16E-08
YLR426W	TDA5	Putative protein of unknown function	43.22	4.64	2.95E-07
YGR260W	TNA1	High affinity nicotinic acid plasma membrane permease	67.56	3.67	1.56E-04
YJR066W	TOR1	PIK-related protein kinase and rapamycin target	74.48	7.11	2.00E-03
YLR425W	TUS1	Guanine nucleotide exchange factor (GEF) that modulates Rho1p activity	65.50	10.22	4.00E-03
YOR043W	WHI2	Protein required for full activation of the general stress response	48.58	5.44	2.86E-06
YDL224C	WHI4	Putative RNA binding protein	73.89	3.09	5.25E-05
YHR161C	YAP1801	Protein of the AP180 family, involved in clathrin cage assembly	74.44	7.81	1.28E-03
YCR099C	YCR099C	Putative protein overlaps another putative protein YCR100C	73.99	12.95	3.68E-02
YDL242W	YDL242W	Dubious open reading frame	74.56	6.07	8.06E-04
YGR054W	YGR054W	Eukaryotic initiation factor eIF2A	69.50	3.23	2.23E-05
YHL008C	YHL008C	Putative protein of unknown function	70.58	8.35	2.23E-03
YLR235C	YLR235C	Dubious open reading frame; overlaps gene TOP3 and YLR236C	30.87	9.11	1.08E-04
YLR236C	YLR236C	Dubious open reading frame; overlaps gene TOP3	7.77	3.16	1.54E-09
YNL179C	YNL179C	Dubious open reading frame; deletion in <i>cyr1</i> mutant results in loss of stress resistance	52.79	9.31	4.15E-04
YOR024W	YOR024W	Dubious open reading frame adjacent to HST3	64.34	6.03	1.04E-04
YOR072W	YOR072W	Dubious open reading frame; overlaps gene YOR072W-A and adjacent to NRT1	19.98	1.66	1.67E-08
YGL255W	ZRT1	High-affinity zinc transporter of the plasma membrane	62.82	2.56	3.60E-06

Table 7.2.3b – **62 validated gene deletions hypertensive to 3 nM MTDIA**. The growth of the single gene deletion mutants treated with MTDIA relative to WT treated with MTDIA ($xxx\Delta$ +MTDIA/ $xxx\Delta$ +carrier)/(WT+MTDIA/WT+carrier) x 100 was determined in liquid media at 24 h and OD₆₀₀. For slow growing deletion strains, OD₆₀₀ was measured at later time points where OD₆₀₀ units in the vehicle

control was between 0.3-0.5. The *his3Δ* deletion strain was used as the WT control. The single mutants with average growth <75%, P-value <0.05 from three replicates compared to the WT control were considered hypersensitive to MTDIA. These were assigned to simplified GO categories using GO slim mapping tool on SGD and were involved in amino acid biosynthesis, multivesicular body formation, Sterol biosynthesis, ion homeostasis, ribosome biogenesis, glucose metabolism, gene expression, NAD⁺ biosynthesis, cell cycle progression. (61 without SRB8)

ORF name	Gene name	Function	% Growth	Standard deviation	p-value
YBR036C	CSG2	Endoplasmic reticulum membrane protein	132.93	9.55	1.20E-02
YOR037W	CYC2	Mitochondrial peripheral inner membrane protein	131.28	8.49	4.83E-03
YIR023W	DAL81	Positive regulator of genes in multiple nitrogen degradation pathways	148.57	14.70	3.47E-02
YCR034W	ELO2	Fatty acid elongase, involved in sphingolipid biosynthesis	138.73	12.58	1.71E-02
YAL048C	GEM1	Outer mitochondrial membrane GTPase, subunit of the ERMES complex	131.29	9.08	2.91E-02
YDL035C	GPR1	Plasma membrane G protein coupled receptor (GPCR), <i>gpr1Δ</i> inhibits pexophagy	132.76	10.12	1.02E-02
YNL164C	IBD2	Component of the BUB2-dependent spindle checkpoint pathway	137.60	17.76	3.29E-02
YFL013C	IES1	Subunit of the INO80 chromatin remodelling complex	131.32	11.59	3.55E-02
YOR235W	IRC13	Dubious open reading frame; Overlaps SNR17A	134.45	2.45	1.52E-04
YGL124C	MON1	Subunit of a heterodimeric guanine nucleotide exchange factor (GEF)	136.65	6.91	1.63E-03
YEL062W	NPR2	Subunit of the Iml1p/SEACIT complex	135.99	5.92	4.26E-04
YCR077C	PAT1	Deadenylation-dependent mRNA-decapping factor	152.76	1.10	3.23E-06
YJR034W	PET191	Protein required for assembly of cytochrome c oxidase	135.98	8.40	6.18E-03
YDR244W	PEX5	Peroxisomal membrane signal receptor for peroxisomal matrix proteins	135.14	9.37	9.90E-03
YDL006W	PTC1	Type 2C protein phosphatase (PP2C), cell wall integrity	159.48	3.28	6.28E-07
YOR001W	RRP6	Nuclear exosome exonuclease component	158.32	11.63	5.37E-03
YBR095C	RXT2	Component of the histone deacetylase Rpd3L complex	154.26	3.38	9.62E-07
YDR227W	SIR4	SIR protein involved in assembly of silent chromatin domains	131.87	9.75	2.13E-02
YEL031W	SPF1	P-type ATPase, ion transporter of the ER membrane	179.63	9.00	1.23E-04
YKL081W	TEF4	Gamma subunit of translational elongation factor eEF1B	132.93	13.40	1.62E-02
YDR074W	TPS2	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex	161.10	12.45	3.67E-03
YOR068C	VAM10	Protein involved in vacuole morphogenesis	144.82	15.63	2.01E-02
YOR132W	VPS17	Subunit of the membrane-associated retromer complex	133.06	5.58	4.49E-04
YPR173C	VPS4	AAA-ATPase involved in multivesicular body (MVB) protein sorting	181.28	26.75	2.80E-02
YOR069W	VPS5	Nexin-1 homolog	156.87	13.40	7.05E-03

YJL029C	VPS53	Component of the GARP (Golgi-associated retrograde protein) complex	162.27	7.87	1.40E-04
YAL002W	VPS8	Membrane-binding component of the CORVET complex	142.09	7.35	7.80E-04
YBR016W	YBR016W	Tail-anchored plasma membrane protein with a conserved CYSTM module	144.15	7.08	6.21E-04
YBR209W	YBR209W	Dubious open reading frame	140.61	10.01	4.42E-03
YDR455C	YDR455C	Dubious open reading frame; overlaps gene YDR456W	164.06	21.16	4.51E-02

Table 7.2.3c – **30 validated gene deletion strains were resistant to MTDIA**. The growth of the single gene deletion mutants treated with MTDIA relative to WT treated with MTDIA ($xxx\Delta$ +MTDIA/ $xxx\Delta$ +carrier)/ (WT+MTDIA/ WT+carrier) x 100 was determined in liquid media at 24 h and OD₆₀₀. For slow growing deletion strains, OD₆₀₀ was measured at later time points where OD₆₀₀ units in the vehicle control was between 0.3-0.5. The *his3* Δ deletion strain was used as the WT control. The single mutants with average growth >130%, *P*-value <0.05 from three replicates compared to the WT control were considered resistant to MTDIA. These were assigned to simplified GO categories using GO slim mapping tool on SGD and were involved in endosomal transport, vacuole fusion, mitochondrion organization, translation, chromatin organisation (29 without *dal81*).

Gene ontology: Biological process	Corrected P-values
Negative regulation of gene expression	1.18E-04
Negative regulation of macromolecule biosynthetic process	1.31E-04
IMP biosynthetic process	1.46E-04
Regulation of gene expression	5.97E-04
Negative regulation of transcription, DNA-templated	6.15E-04
Gene silencing	1.98E-02
Chromatin organisation	2.80E-02
Ribose phosphate biosynthetic process	3.08E-02
Transcription from RNA polymerase III promoter	3.81E-02
Histone deacetylation	4.45E-02
Negative regulation of transcription from RNA polymerase II promoter during mitotic cell cycle	4.67E-02

Table 7.2.4 – **GO enrichment of validated MTDIA hypersensitive strains**. The validated genes were assigned to the three major hierarchical gene ontology (GO) categories: biological process, molecular function and cellular component using the haploid non-essential gene deletion library as a reference set on YeastMine (Gene Ontology Consortium, 2004; Ashburner et al., 2000; Balakrishnan et al., 2012) with *p*-values corrected for multiple testing using the Benjamin and Hochberg false discovery rate of 0.05 (Benjamini & Hochberg, 1995).

ORF	Gene	OD ₆₀₀ at 32 h	Function
YMR009W	ADI1	0.019	Acireductone dioxygenase involved in methionine salvage pathway
YBR131W	CCZ1	0.034	Subunit of a heterodimeric guanine nucleotide exchange factor (GEF)
YMR058W	FET3	0.012	Ferro-O ₂ -oxidoreductase
YER145C	FTR1	0.011	High affinity iron permease
YBR020W	GAL1	0.013	Galactokinase
YBR019C	GAL10	0.001	UDP-glucose-4-epimerase
YDR009W	GAL3	0.022	Transcriptional regulator
YJR024C	MDE1	0.011	5'-methylthioribulose-1-phosphate dehydratase
YLR017W	MEU1	0.03	Methylthioadenosine phosphorylase (MTAP)
YPR118W	MRI1	0.007	5'-methylthioribose-1-phosphate isomerase
YJL121C	RPE1	0.013	D-ribulose-5-phosphate 3-epimerase
YLR357W	RSC2	-0.003	Component of the RSC chromatin remodelling complex
YCR009C	RVS161	0.019	Amphiphysin-like lipid raft protein
YDR388W	RVS167	0.016	Calmodulin-binding actin-associated protein
YGL026C	TRP5	0.001	Tryptophan synthase
YEL038W	UTR4	0.005	Protein with sequence similarity to acireductone synthases
YDR290W	YDR290W/ overlaps RRT103	0.006	Protein involved in transcription termination by RNA polymerase II
YJL022W	YJL022W/ overlaps PET130	0.01	Protein required for respiratory growth

Table 7.2.5 – Genes that were unviable in MTA as the only sulphur source.

7.2.5 MTDIA treatment induces autophagy

		MTDIA [nM]			
		0.5 (IC ₅)	1 (IC ₁₀)	3 (IC ₂₀)	5 (IC ₃₀)
Rapamycin [nM]	6.25 (IC ₁₇)	0.89	0.96	1.14	1.12
	12.5 (IC ₂₀)	0.86	0.93	1.09	1.08
	25 (IC ₄₀)	0.63	0.68	0.81	0.79
	50 (IC ₅₇)	0.46	0.50	0.59	0.58
	100 (IC ₆₈)	0.35	0.38	0.45	0.45

FIC>1
 FIC 0.5-1.0
 FIC 0.4-0.5
 FIC 0.3-0.4

Table 7.2.6 – FIC index for rapamycin and MTDIA in combination. FIC index was calculated to confirm drug interactions where FIC is defined as (drug A in combination/drug A alone) + (drug B in

combination/ drug B alone) (Hall et al., 1983; Odds, 2003; McElhaney-Feser et al., 1998). Synergistic ($FIC \leq 0.5$), $0.5 < FIC < 1$ additive, $1 < FIC < 4$ indifferent and $FIC \geq 4$ is antagonistic.

Figure 7.2.3 – **Vps34-GFP** in WT and *meu1Δ* in methionine replete complete minimal media. Florescent signal was detected at 488 nm (GFP) and 561 (nuclear localisation signal fused to RS2 and cytosolic mCherry) using the high-throughput spinning disk confocal microscope (Evo Tec OPERA, Perkin Elmer, Massachusetts, United States) using the 60x water immersion lens (NA 1.2). The GFP abundance and localisation was quantified by automated image analysis (Acapella, Perkin Elmer, Massachusetts, United States)(Bircham et al., 2011). Numbers inset in each image are the percent changes in GFP abundance in *meu1Δ* relative to WT untreated.

7.3.1 yCG640 and yCG643 construction

Figure 7.3.1 – YCG640 and YCG643 construction. PCR products were run on a 1% agarose gel with 1KB plus ladder at 100 mV. (a) *lyp1Δ::HPH-NLS-RS2* construction PCR. (b) *can1Δ::STE2pr-Sp_LEU2* construction PCR. (c) *meu1Δ::kanR* construction PCR. (d) *Cen::hENT1::URA3*.

Primer	Sequence
<i>lyp1Δ::HPH-NLS-RS2-mCherry</i> Fw (#224)	AATTGCATTGTCTATAACGATAACAAAAGACATCGTATATATATATATATCGTAC GCTGCAGGTCGAC
<i>lyp1Δ::HPH-NLS-RS2-mCherry</i> Rev (#252)	TCTATTTTTTTATTTTTTCTATTTGAAGGCATGCAAGAGGTCTGTGAACTATAG GGAGACCGGCAGA
<i>meu1Δ::kanR</i> Fw (#831)	AGATGAACGTGTGAATGAAAAAACTATTTTCGAGATGAGATGAGATTAAACGC CGGGTCACCCGGCCAGC
<i>meu1Δ::kanR</i> Rev (#832)	ATATATGTTTTCTCTCCTATTTATATTTTACATATGATTAGCGGCACCCACGTTAG TATCGAATCGACAG
<i>can1Δ::STE2pr_LEU2</i> Fw (#749)	CGGCTTTTTTGAATTTCTTTT
<i>can1Δ::STE2pr_LEU2</i> Rev (#754)	CTTTTCGGTGTATGACTTATG

Table 7.3.1 – **yCG640 and yCG643 construction primers**. Primers were designed according to Janke et al. (Janke et al., 2004).

7.3.2 GFP proteins localised to subcellular structures.

Figure 7.3.2 – GFP proteins localised to subcellular structures.

7.3.3 Changes in either protein localisation or abundance with 100 nM MTDIA or in *meu1Δ* and Go enrichment

ORF name	Gene name	Function	Median WT GFP intensity (RFU)	% GFP intensity relative to WT (WT + MTDIA /WT)	Median WT + MTDIA GFP intensity (RFU)	% GFP intensity relative to WT (<i>meu1Δ</i> /WT)	Median <i>meu1Δ</i> GFP intensity (RFU)	Localisation with WT	Localisation with 100 nM MTDIA	Localisation with <i>meu1Δ</i>
YNR016C	ACC1	Acetyl-CoA carboxylase, biotin containing enzyme	176.04	87.39	153.85	123.70	217.76	Cytoplasm	Lipid droplets, cytoplasm	Lipid droplets, cytoplasm
YBL015W	ACH1	Protein with CoA transferase activity	548.31	117.41	643.78	52.57	288.27	Mitochondrion	Mitochondrion	Mitochondrion
YAL054C	ACS1	Acetyl-coA synthetase isoform	299.41	97.20	291.04	93.53	280.03	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm
YLR153C	ACS2	Acetyl-coA synthetase isoform	402.41	95.98	386.21	85.46	343.88	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YMR120C	ADE17	Enzyme of 'de novo' purine biosynthesis	79.72	107.58	85.77	121.27	96.68	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YMR303C	ADH2	Glucose-repressible alcohol dehydrogenase II	352.70	111.55	393.42	152.45	537.69	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YMR083W	ADH3	Mitochondrial alcohol dehydrogenase isozyme III	137.93	100.70	138.89	81.21	112.01	Mitochondrion	Mitochondrion	Mitochondrion
YGL256W	ADH4	Alcohol dehydrogenase isoenzyme type IV	43.70	97.02	42.40	79.64	34.80	Cytoplasm	Cytoplasm	Cytoplasm
YMR318C	ADH6	NADPH-dependent medium chain alcohol dehydrogenase	206.76	79.48	164.33	61.44	127.03	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDR226W	ADK1	Adenylate kinase, required for purine metabolism	481.02	105.44	507.21	52.80	253.96	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YCL025C	AGP1	Low-affinity amino acid permease with broad substrate range	180.39	82.05	148.00	69.10	124.64	ER	ER	ER

YBR132C	AGP2	Plasma membrane regulator of polyamine and carnitine transport	68.76	97.79	67.24	93.73	64.45	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YDR214W	AHA1	Co-chaperone that binds Hsp82p and activates its ATPase activity	118.59	148.01	175.53	100.29	118.94	Cytoplasm	Cytoplasm, nucleus	Cytoplasm, nucleus
YLR109W	AHP1	Thiol-specific peroxiredoxin	884.41	117.98	1043.43	205.54	1817.80	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YJL122W	ALB1	Shuttling pre-60S factor	65.06	66.48	43.26	76.31	49.65	Nucleus	Nuclear foci	Nuclear foci
YMR169C	ALD3	Cytoplasmic aldehyde dehydrogenase	32.62	108.90	35.52	256.40	83.64	Cytoplasm	Cytoplasm	Cytoplasm, nucleus
YPL061W	ALD6	Cytosolic aldehyde dehydrogenase	751.09	77.96	585.57	75.28	565.40	Cytoplasm	Cytoplasm	Cytoplasm
YNL219C	ALG9	Mannosyltransferase, involved in N-linked glycosylation	57.93	88.50	51.26	81.26	47.07	ER	ER	ER
YKR021W	ALY1	Alpha arrestin, substrate of calcineurin	36.89	101.87	37.59	709.88	261.91	Cytoplasm	Cytoplasm	Cytoplasm
YCL050C	APA1	AP4A phosphorylase	36.00	103.57	37.28	291.04	104.77	Mitochondrion	Mitochondrion	Cytoplasm
YKL103C	APE1	Vacuolar aminopeptidase yscI	75.27	105.80	79.64	89.19	67.14	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci
YML022W	APT1	Adenine phosphoribosyltransferase	71.32	75.92	54.15	79.60	71.32	Cytoplasm	Cytoplasm	Cytoplasm
YBR149W	ARA1	NADP+ dependent arabinose dehydrogenase	86.88	111.76	97.10	218.83	190.13	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDL192W	ARF1	ADP-ribosylation factor	304.19	93.83	285.43	120.87	367.69	Cytoplasmic foci	Cytoplasmic foci	Cytoplasmic foci
YHL040C	ARN1	ARN family transporter for siderophore-iron chelates	42.62	81.57	34.76	117.80	50.21	Plasma membrane, cytoplasmic foci	Vacuolar lumen	Vacuolar lumen
YHR137W	ARO9	Aromatic aminotransferase II	90.56	101.87	92.25	147.11	133.22	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDR101C	ARX1	Nuclear export factor for the ribosomal pre-60S subunit	75.58	78.37	59.23	82.94	62.69	Nucleus	Nucleus	Cytoplasm, nucleus
YOR058C	ASE1	Mitotic spindle midzone-localized microtubule bundling protein	28.62	106.95	30.61	117.85	33.73	Cytoplasm	Cytoplasm	Cytoplasm, vacuolar lumen

YKL052C	ASK1	Essential subunit of the Dam1 complex (aka DASH complex)	54.33	85.56	46.49	82.28	44.70	Nuclear foci, spindle	Nuclear foci, spindle	Nuclear foci
YGL180W	ATG1	Protein serine/threonine kinase	44.25	106.35	47.06	104.21	46.12	Cytoplasm, cytoplasmic foci	Cytoplasm, vacuolar lumen	Cytoplasm, vacuolar lumen
YJL178C	ATG27	Type I membrane protein involved in autophagy and the Cvt pathway	51.36	80.99	41.60	94.77	48.68	Cytoplasmic foci	Vacuolar lumen	Cytoplasmic foci
YDR384C	ATO3	Plasma membrane protein, putative ammonium transporter	62.26	93.37	58.13	80.48	50.11	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Vacuolar lumen
YPL271W	ATP15	Epsilon subunit of the F1 sector of mitochondrial F1F0 ATP synthase	263.26	77.41	203.80	63.45	167.04	Mitochondrion	Mitochondrion	Mitochondrion
YKL016C	ATP7	Subunit d of the stator stalk of mitochondrial F1F0 ATP synthase	196.97	105.36	207.53	55.95	110.21	Mitochondrion	Mitochondrion	Mitochondrion
YIL124W	AYR1	Bifunctional triacylglycerol lipase and 1-acyl DHAP reductase	146.17	114.67	167.61	78.49	114.73	ER, lipid droplet	ER, lipid droplet	ER
YBR068C	BAP2	High-affinity leucine permease	84.47	83.35	70.40	83.53	70.55	Plasma membrane, vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YDR046C	BAP3	Amino acid permease	72.86	75.95	55.34	87.62	63.83	Plasma membrane, vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YHR040W	BCD1	Essential protein required for the accumulation of box C/D snoRNA	38.77	102.86	39.88	92.53	35.88	Nucleus	Nucleus	Cytoplasm, nucleus
YAL061W	BDH2	Putative medium-chain alcohol dehydrogenase with similarity to BDH1	50.73	123.51	62.66	121.17	61.47	Cytoplasm	Cytoplasm	Cytoplasm
YOR304C-A	BIL1	Protein that binds Bud6p and has a role in actin cable assembly	42.33	96.00	40.64	115.92	49.07	Bud neck	Bud neck	Bud neck
YNR056C	BIO5	Putative transmembrane protein involved in the biotin biosynthesis	59.34	164.25	97.47	160.39	95.18	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen

YNL166C	BNI5	Linker protein responsible for recruitment of myosin to the bud neck	48.13	97.63	46.99	96.14	46.27	Bud neck	Bud neck	Bud neck
YLR074C	BUD20	C2H2-type zinc finger protein required for ribosome assembly	59.83	68.69	41.10	72.65	43.46	Nucleus	Nucleus	Nucleus
YOR078W	BUD21	Component of small ribosomal subunit (SSU) processosome	47.51	72.00	34.21	82.58	39.24	Nuclear foci	Nuclear foci	Nuclear foci
YJR092W	BUD4	Anillin-like protein involved in bud-site selection	34.33	99.25	34.07	101.08	34.70	Bud neck	Bud neck	Bud neck
YLR438W	CAR2	L-ornithine transaminase (OTase)	134.29	81.40	109.31	57.27	76.91	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YLR390W-A	CCW14	Covalently linked cell wall glycoprotein	121.94	75.29	91.81	71.08	86.68	ER	ER	ER
YCR002C	CDC10	Component of the septin ring, required for cytokinesis	66.39	96.21	63.87	107.80	71.57	Bud neck	Bud neck	Bud neck, plasma membrane
YJR076C	CDC11	Component of the septin ring that is required for cytokinesis	63.61	98.18	62.45	98.11	62.41	Bud neck	Bud neck	Bud neck, plasma membrane
YAL038W	CDC19	Pyruvate kinase	741.87	94.83	703.49	72.38	536.94	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGR157W	CHO2	Phosphatidylethanolamine methyltransferase (PEMT)	87.73	77.04	67.59	73.36	64.36	ER	Cytoplasmic foci	Cytoplasmic foci
YNR001C	CIT1	Citrate synthase	241.03	153.05	368.89	185.02	445.96	Mitochondrion	Mitochondrion	Mitochondrion
YCR005C	CIT2	Citrate synthase, peroxisomal isozyme involved in glyoxylate cycle	38.64	106.13	41.01	121.89	47.10	Cytoplasm	Cytoplasm	Cytoplasm
YPR001W	CIT3	Dual specificity mitochondrial citrate and methylcitrate synthase	56.66	82.36	46.66	71.22	40.35	Mitochondrion	Mitochondrion	Mitochondrion
YGL187C	COX4	Subunit IV of cytochrome c oxidase	464.40	100.83	468.24	54.10	251.23	Mitochondrion	Mitochondrion	Mitochondrion
YDR155C	CPR1	Cytoplasmic peptidyl-prolyl cis-trans isomerase (cyclophilin)	395.38	100.54	397.53	153.34	606.28	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YOL007C	CSI2	Protein of unknown function	37.49	93.11	34.91	97.83	36.68	Vacuolar lumen	Vacuolar lumen	Vacuolar lumen

YBR291C	CTP1	Mitochondrial inner membrane citrate transporter	49.06	69.65	34.17	77.09	37.82	Mitochondrion	Mitochondrion	Cytoplasmic foci
YPR124W	CTR1	High-affinity copper transporter of plasma membrane	47.73	96.08	45.86	130.79	62.42	Plasma membrane	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YGR088W	CTT1	Cytosolic catalase T	34.22	101.80	34.84	143.82	49.22	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YKL096W-A	CWP2	Covalently linked cell wall mannoprotein	786.37	85.31	670.85	57.16	449.46	ER	ER	ER
YKR083C	DAD2	Essential subunit of the Dam1 complex (aka DASH complex)	52.01	95.15	49.49	82.49	42.90	Spindle, nuclear foci	Spindle, nuclear foci	Nuclear foci
YNL112W	DBP2	ATP-dependent RNA helicase of the DEAD-box protein family	38.62	97.26	37.56	318.01	122.81	Bud neck	Bud neck	Nucleus
YGL078C	DBP3	RNA-Dependent ATPase, member of DExD/H-box family	73.43	85.40	62.71	76.32	56.04	Nuclear foci	Nuclear foci	Nuclear foci
YLR270W	DCS1	Non-essential hydrolase involved in mRNA decapping	62.07	144.21	89.51	132.58	82.29	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YOL052C-A	DDR2	Multi-stress response protein	87.57	121.09	106.04	63.96	56.00	ER	ER	Vacuolar lumen
YLR348C	DIC1	Mitochondrial dicarboxylate carrier	44.77	68.65	30.74	89.63	40.13	Mitochondrion	Mitochondrion	Mitochondrion
YEL071W	DLD3	2-hydroxyglutarate transhydrogenase, and minor D-lactate dehydrogenase	178.77	74.64	133.43	69.12	123.57	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGL043W	DST1	General transcription elongation factor TFIIS	73.49	100.14	73.59	66.28	48.70	Nucleus	Nucleus	Nucleus
YGL061C	DUO1	Essential subunit of the Dam1 complex (aka DASH complex)	41.43	99.71	41.31	85.42	35.39	Spindles, nuclear foci, cytoplasm	Spindles, nuclear foci, cytoplasm	Nuclear foci, cytoplasm
YAL059W	ECM1	Pre-ribosomal factor involved in 60S ribosomal protein subunit export	51.12	74.20	37.93	73.32	37.48	Nucleus	Nucleus	Cytoplasm, nucleus
YER035W	EDC2	RNA-binding protein that directly activates mRNA decapping	79.76	106.50	84.95	73.68	58.77	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus

YOR133W	EFT1	Elongation factor 2 (EF-2), also encoded by EFT2	106.44	53.00	56.41	39.15	41.67	Cytoplasm	Cytoplasm	Cytoplasm
YDR385W	EFT2	Elongation factor 2 (EF-2), also encoded by EFT1	256.47	63.31	162.38	24.35	62.44	Cytoplasm	Cytoplasm	Cytoplasm
YGR254W	ENO1	Enolase I, a phosphopyruvate hydratase	1063.48	166.33	1768.88	263.92	2806.70	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGR175C	ERG1	Squalene epoxidase	143.62	77.87	111.83	87.48	125.64	ER	ER	ER
YNL280C	ERG24	C-14 sterol reductase	58.14	77.56	45.10	78.47	45.63	Lipid droplets	Lipid droplets	Vacuolar lumen
YLR056W	ERG3	C-5 sterol desaturase	91.28	75.82	69.21	72.81	66.46	ER	ER	Vacuolar lumen
YML008C	ERG6	Delta(24)-sterol C-methyltransferase	122.33	86.79	106.18	92.65	113.35	ER, lipid droplet	ER, lipid droplet	Lipid droplet
YAR002C-A	ERP1	Member of the p24 family involved in ER to Golgi transport	108.83	66.69	72.58	63.25	68.84	Cytoplasmic foci	Cytoplasmic foci, vacuolar lumen	Vacuolar lumen
YMR246W	FAA4	Long chain fatty acyl-CoA synthetase	133.13	89.46	119.10	101.54	135.17	ER	Lipid droplets	Lipid droplets
YKL187C	FAT3	Protein required for fatty acid uptake	42.04	120.92	50.83	112.99	47.50	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Plasma membrane
YLR051C	FCF2	Nucleolar protein involved in the early steps of 35S rRNA processing	48.02	78.17	37.54	88.56	42.53	Nuclear foci	Nuclear foci	Nuclear foci
YER056C	FCY2	Purine-cytosine permease	82.39	70.00	57.67	78.76	64.88	Plasma membrane, vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YMR144W	FDO1	Protein involved in directionality of mating type switching	35.39	95.38	33.75	100.55	35.58	Nucleus	Nucleus	Cytoplasm
YMR058W	FET3	Ferro-O2-oxidoreductase	128.31	70.20	90.08	73.35	94.11	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Vacuolar lumen
YDR070C	FMP16	Protein of unknown function	206.80	223.70	462.60	241.06	498.51	Mitochondrion	Mitochondrion	Mitochondrion
YDL222C	FMP45	Integral membrane protein localized to mitochondria	50.47	150.37	75.89	174.55	88.10	Plasma membrane	Plasma membrane	Plasma membrane

YER145C	FTR1	High affinity iron permease	82.76	88.15	72.95	100.00	82.76	Plasma membrane	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YBL042C	FUI1	High affinity uridine permease, localizes to the plasma membrane	62.04	76.90	47.71	88.15	54.69	Plasma membrane	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YPL262W	FUM1	Fumarase	210.60	128.94	271.54	93.38	196.65	Mitochondrion	Mitochondrion	Mitochondrion
YDR009W	GAL3	Transcriptional regulator	91.65	82.19	75.33	71.61	65.64	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YHR089C	GAR1	Protein component of the H/ACA snoRNP pseudouridylase complex	42.47	73.59	31.25	88.66	37.65	Nuclear foci, cytoplasm	Nuclear foci, cytoplasm	Vacuolar lumen
YMR307W	GAS1	Beta-1,3-glucanosyltransferase	239.05	52.53	125.58	43.41	103.77	Mitochondrion	Mitochondrion	Mitochondrion
YDR019C	GCV1	T subunit of the mitochondrial glycine decarboxylase complex	55.77	140.37	78.29	165.09	92.07	Mitochondrion	Mitochondrion	Mitochondrion
YMR189W	GCV2	P subunit of the mitochondrial glycine decarboxylase complex	68.59	119.93	82.27	134.95	92.57	Mitochondrion	Mitochondrion	Mitochondrion
YAL044C	GCV3	H subunit of the mitochondrial glycine decarboxylase complex	71.08	115.34	81.98	194.01	137.90	Mitochondrion	Mitochondrion	Mitochondrion
YOR375C	GDH1	NADP(+)-dependent glutamate dehydrogenase	634.56	156.74	994.62	142.24	902.62	Cytoplasm, vacuolar membrane foci	Cytoplasm, vacuolar membrane foci	Cytoplasm, vacuolar membrane foci
YDL215C	GDH2	NAD(+)-dependent glutamate dehydrogenase	124.83	112.08	139.91	91.27	113.93	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Cytoplasm
YOR355W	GDS1	Protein of unknown function	37.44	88.23	33.03	96.68	36.19	Nuclear foci, cytoplasm	Cytoplasm	Cytoplasm, vacuolar lumen
YNL255C	GIS2	Translational activator for mRNAs with internal ribosome entry sites	226.78	77.84	176.52	78.64	178.33	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YCL040W	GLK1	Glucokinase	47.67	108.61	51.78	123.50	58.88	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci

YPR035W	GLN1	Glutamine synthetase (GS)	310.36	98.82	306.69	89.98	279.26	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YOR168W	GLN4	Glutamine tRNA synthetase	165.32	98.26	162.44	70.72	116.91	Cytoplasm	Cytoplasm	Cytoplasm
YDR508C	GNP1	High-affinity glutamine permease	164.99	75.23	124.12	78.93	130.23	ER	Vacuolar lumen	Vacuolar lumen
YNL274C	GOR1	Glyoxylate reductase	64.51	121.42	78.33	158.60	102.32	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDL022W	GPD1	NAD-dependent glycerol-3-phosphate dehydrogenase	200.86	114.78	230.55	126.11	253.31	Cytoplasmic foci	Cytoplasmic foci	Cytoplasmic foci
YOL059W	GPD2	NAD-dependent glycerol 3-phosphate dehydrogenase	111.04	122.00	135.48	99.13	110.08	Cytoplasm	Cytoplasm	Cytoplasm
YPR160W	GPH1	Glycogen phosphorylase required for the mobilization of glycogen	135.75	110.67	150.24	54.04	73.36	Cytoplasm	Cytoplasm	Cytoplasm
YKL152C	GPM1	Tetrameric phosphoglycerate mutase	851.45	116.33	990.49	138.16	1176.37	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDL021W	GPM2	Homolog of Gpm1p phosphoglycerate mutase	44.08	115.79	51.04	137.82	60.75	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YER062C	GPP2	DL-glycerol-3-phosphate phosphatase involved in glycerol biosynthesis	169.54	81.36	137.95	46.13	78.21	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YPL223C	GRE1	Hydrophilin essential in desiccation-rehydration process	36.19	77.03	27.87	233.70	84.57	Cytoplasm	Cytoplasm	Cytoplasm, nucleus
YOL151W	GRE2	3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase	51.12	106.53	54.46	132.87	67.92	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YLR364W	GRX8	Glutaredoxin that employs a dithiol mechanism of catalysis	83.31	113.01	94.15	165.89	138.20	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YLR258W	GSY2	Glycogen synthase	88.24	112.35	99.14	118.44	104.52	Cytoplasm	Cytoplasm	Cytoplasm
YIL155C	GUT2	Mitochondrial glycerol-3-phosphate dehydrogenase	40.28	107.56	43.33	68.20	27.47	Mitochondrion	Mitochondrion	Mitochondrion
YIL041W	GVP36	BAR domain protein that localizes to early and late Golgi vesicles	232.29	97.85	227.29	73.55	170.84	Cytoplasmic foci	Cytoplasmic foci	Cytoplasmic foci
YDL223C	HBT1	Shmoo tip protein, substrate of Hub1p ubiquitin-like protein	54.24	78.22	42.42	67.02	36.35	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus

YGR191W	HIP1	High-affinity histidine permease	54.48	74.12	40.39	88.68	48.32	Cytoplasm, vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YGL077C	HNM1	Plasma membrane transporter for choline, ethanolamine, and carnitine	90.23	76.91	69.40	92.63	83.59	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Vacuolar lumen
YNR055C	HOL1	Putative transporter in the major facilitator superfamily	38.70	94.54	36.58	97.28	37.64	Plasma membrane	Plasma membrane	Plasma membrane, vacuolar lumen
YMR251 W-A	HOR7	Protein of unknown function	297.99	92.39	275.32	119.76	356.88	ER	ER	ER
YPL204W	HRR25	Conserved casein kinase	57.45	95.01	54.58	90.09	51.75	Cytoplasm	Cytoplasmic foci	Cytoplasmic foci
YMR186 W	HSC82	Cytoplasmic chaperone of the Hsp90 family	1189.26	99.20	1179.76	127.46	1515.89	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YFL014W	HSP12	Plasma membrane protein involved in maintaining membrane organization	860.19	167.27	1438.82	138.52	1191.55	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YBR072W	HSP26	Small heat shock protein (sHSP) with chaperone activity	39.81	130.19	51.83	204.26	81.32	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci
YCR021C	HSP30	Negative regulator of the H(+)-ATPase Pma1p	43.20	81.48	35.20	85.82	37.08	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YDR533C	HSP31	Methylglyoxalase that converts methylglyoxal to D-lactate	77.91	91.76	71.49	155.19	120.91	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDR258C	HSP78	Oligomeric mitochondrial matrix chaperone	118.17	103.47	122.27	128.35	151.67	Mitochondrion	Mitochondrion	Mitochondrion
YDR224C	HTB1	Histone H2B	1862.32	103.93	1935.47	63.32	1179.22	Nucleus	Nucleus	Nucleus
YFR053C	HXK1	Hexokinase isoenzyme 1	1815.01	139.93	2539.72	84.02	1525.05	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGL253W	HXK2	Hexokinase isoenzyme 2	1304.57	119.75	1562.22	135.76	1771.07	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YOR136W	IDH2	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	189.73	105.68	200.51	159.27	302.17	Mitochondrion	Mitochondrion	Mitochondrion

YNL157W	IGO1	Protein required for initiation of G0 program	59.28	77.57	45.98	129.84	76.97	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YLR413W	INA1	Protein of unknown function	105.52	71.21	75.13	58.91	62.16	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Vacuolar lumen
YIR036C	IRC24	Putative benzil reductase	62.49	127.93	79.94	89.77	56.09	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YIL026C	IRR1	Subunit of the cohesin complex	41.79	99.52	41.59	97.11	40.58	Nucleus	Nucleus	Nucleus
YDR497C	ITR1	Myo-inositol transporter	217.37	99.87	217.09	102.45	222.70	Plasma membrane, vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YOL103W	ITR2	Myo-inositol transporter	42.93	88.58	38.03	92.31	39.63	Plasma membrane	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YHR158C	KEL1	Protein required for proper cell fusion and cell morphology	41.95	102.92	43.18	116.29	48.79	Bud neck	Bud neck	Plasma membrane
YDR148C	KGD2	Dihydrolipoyl transsuccinylase	77.51	114.16	88.48	122.74	95.14	Mitochondrion	Mitochondrion	Mitochondrion
YPR159W	KRE6	Type II integral membrane protein	71.67	90.61	64.95	84.13	60.30	ER	ER	ER
YNL071W	LAT1	Dihydrolipoamide acetyltransferase component (E2) of the PDC	84.39	107.24	90.51	75.01	63.30	Mitochondrion	Mitochondrion	Mitochondrion
YFL018C	LPD1	Dihydrolipoamide dehydrogenase	396.21	124.26	492.32	71.31	282.54	Mitochondrion	Mitochondrion	Mitochondrion
YKL029C	MAE1	Mitochondrial malic enzyme	47.94	89.20	42.77	82.07	39.35	Mitochondrion	Mitochondrion	Mitochondrion
YKL021C	MAK11	Protein involved in an early step of 60S ribosomal subunit biogenesis	52.21	101.61	53.05	108.59	56.69	Nuclear membrane	Nuclear membrane	Nuclear foci
YNL307C	MCK1	Dual-specificity ser/thr and tyrosine protein kinase	91.60	133.47	122.25	100.94	92.46	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YOL126C	MDH2	Cytoplasmic malate dehydrogenase	262.38	142.11	372.86	47.64	124.99	Cytoplasm, cytoplasmic foci, nucleus	Cytoplasm, cytoplasmic foci, nucleus	Cytoplasm, cytoplasmic foci, nucleus
YLR188W	MDL1	Mitochondrial inner membrane half-type ABC transporter	43.60	64.71	28.21	94.06	41.01	Mitochondrion	Mitochondrion	Mitochondrion

YGR264C	MES1	Methionyl-tRNA synthetase	199.31	87.68	174.77	119.02	237.22	Cytoplasm	Cytoplasm	Cytoplasm
YKL001C	MET14	Adenylylsulfate kinase	135.83	95.07	129.13	142.68	193.81	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YOL064C	MET22	Bisphosphate-3'-nucleotidase	128.80	133.73	172.25	103.03	132.70	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YJR137C	MET5	Sulfite reductase beta subunit	194.90	92.35	179.98	64.20	125.12	Cytoplasm	Cytoplasm	Cytoplasm
YJR077C	MIR1	Mitochondrial phosphate carrier	225.79	69.77	157.55	56.59	127.77	Mitochondrion	Mitochondrion	Mitochondrion
YLL006W	MMM1	ER integral membrane protein, ERMES complex subunit	140.48	105.76	148.57	28.06	39.42	Cytoplasm	Cytoplasm	Cytoplasmic foci
YMR224C	MRE11	Nuclease subunit of the MRX complex with Rad50p and Xrs2p	36.18	100.24	36.27	96.67	34.98	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDR033W	MRH1	Protein that localizes primarily to the plasma membrane	137.91	100.06	138.00	71.06	98.00	Plasma membrane	Plasma membrane	Plasma membrane
YDL045W-A	MRP10	Mitochondrial ribosomal protein of the small subunit	72.74	81.19	59.06	131.61	95.73	Mitochondrion	Mitochondrion	Mitochondrion
YAL034W-A	MTW1	Essential component of the MIND kinetochore complex	42.96	80.30	34.50	82.50	35.44	Nuclear foci	Nuclear foci	Nuclear foci
YGR055W	MUP1	High affinity methionine permease	384.45	111.90	430.20	88.99	342.12	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YER042W	MXR1	Methionine-S-sulfoxide reductase	67.42	89.17	60.12	160.45	108.18	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YPR149W	NCE102	Protein of unknown function	281.90	128.42	362.03	108.15	304.88	Plasma membrane	Plasma membrane	Plasma membrane
YIL048W	NEO1	Phospholipid translocase (flippase), role in phospholipid asymmetry o	48.68	80.23	39.05	102.02	49.66	Cytoplasm	Cytoplasm	Vacuolar lumen
YDL208W	NHP2	Protein related to mammalian high mobility group (HMG) proteins	214.32	91.32	195.73	70.31	150.69	Nucleus	Nucleus	Nucleus
YBR089C-A	NHP6B	High-mobility group (HMG) protein	128.17	97.07	124.42	145.47	186.45	Nucleus	Nucleus	Nucleus
YKL151C	NNR2	Widely-conserved NADHX dehydratase	39.82	118.96	47.37	131.86	52.51	Cytoplasm	Cytoplasm	Cytoplasm

YOR056C	NOB1	Protein involved in proteasomal and 40S ribosomal subunit biogenesis	55.61	111.94	62.25	101.18	56.27	Cytoplasm	Cytoplasm	Nuclear foci
YPL093W	NOG1	Putative GTPase	97.44	82.99	80.87	76.58	74.62	Nucleus	Nucleus	Nuclear foci
YNR053C	NOG2	Putative GTPase	46.19	85.63	39.55	90.44	41.77	Nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDL213C	NOP6	rRNA-binding protein required for 40S ribosomal subunit biogenesis	98.66	83.04	81.93	57.77	57.00	Nucleus	Nucleus	Nucleus
YGR103W	NOP7	Component of several different pre-ribosomal particles	31.98	102.10	32.65	153.25	49.01	Cytoplasm	Cytoplasm	Nucleus
YGR043C	NQM1	Transaldolase of unknown function	56.72	111.10	63.02	135.34	76.77	Nucleus	Nucleus	Nucleus
YDR001C	NTH1	Neutral trehalase, degrades trehalose	61.53	105.55	64.94	151.34	93.12	Cytoplasm	Cytoplasm	Cytoplasm
YJR073C	OPI3	Methylene-fatty-acyl-phospholipid synthase	163.76	71.96	117.85	112.21	183.75	ER	ER, vacuolar lumen	ER, vacuolar lumen
YJL002C	OST1	Alpha subunit of the oligosaccharyltransferase complex of the ER lumen	124.27	92.27	114.67	78.43	97.47	ER	ER	ER

YHR179W OYE2 Conserved observed in mon-2()n1 0cleof

YLR044C	PDC1	Major of three pyruvate decarboxylase isozymes	2120.15	111.25	2358.72	148.68	3152.25	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGR087C	PDC6	Minor isoform of pyruvate decarboxylase	95.33	151.71	144.62	273.38	260.61	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YPL058C	PDR12	Plasma membrane ATP-binding cassette (ABC) transporter	51.76	114.50	59.27	125.52	64.97	Vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YOR153W	PDR5	Plasma membrane ATP-binding cassette (ABC) transporter	66.46	85.81	57.02	98.88	65.71	Plasma membrane, vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YKR046C	PET10	Protein of unknown function that localizes to lipid particles	124.97	162.26	202.77	67.23	84.02	Lipid droplets	Lipid droplets	Lipid droplets
YDR244W	PEX5	Peroxisomal membrane signal receptor for peroxisomal matrix proteins	36.23	100.03	36.24	92.17	33.39	Peroxisomes	Peroxisomes	Peroxisomes
YMR205C	PFK2	Beta subunit of heterooctameric phosphofructokinase	505.42	103.38	522.49	137.87	696.81	Cytoplasm	Cytoplasm	Cytoplasm
YNL149C	PGA2	Essential protein required for maturation of Gas1p and Pho8p	56.16	93.26	52.37	96.88	54.40	ER, lipid droplets	ER, lipid droplets	ER
YBR196C	PGI1	Glycolytic enzyme phosphoglucose isomerase	635.08	109.81	697.40	78.80	500.44	Cytoplasm, nucleus	Nucleus	Nucleus
YCR012W	PGK1	3-phosphoglycerate kinase	2328.94	126.40	2943.72	139.66	3252.51	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YMR105C	PGM2	Phosphoglucomutase	465.15	107.24	498.80	62.43	290.39	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YJL097W	PHS1	Essential 3-hydroxyacyl-CoA dehydratase of the ER membrane	73.21	85.11	62.31	86.99	63.69	ER	ER, vacuolar lumen	Vacuolar lumen
YGR086C	PIL1	Eisosome core component	243.97	140.82	343.55	168.16	410.25	Plasma membrane	Plasma membrane	Plasma membrane
YLR016C	PML1	Subunit of the RES complex	39.01	77.92	30.40	98.12	38.28	Nuclear foci,	Nuclear foci	Cytoplasm
YEL017C-A	PMP2	Proteolipid associated with plasma membrane H(+)-ATPase (Pma1p)	403.09	99.44	400.84	67.10	270.46	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen

YGL167C	PMR1	High affinity Ca ²⁺ /Mn ²⁺ P-type ATPase	39.06	69.20	27.03	213.36	83.33	Cytoplasm	Cytoplasm	ER
YNR032W	PPG1	Putative serine/threonine protein phosphatase	32.61	99.02	32.29	132.90	43.34	Cytoplasm	Cytoplasm	Cytoplasm
YCL057W	PRD1	Zinc metalloendopeptidase	113.23	102.21	115.73	80.36	90.99	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YMR314W	PRE5	Alpha 6 subunit of the 20S proteasome	199.69	94.82	189.35	65.61	131.02	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGR135W	PRE9	Alpha 3 subunit of the 20S proteasome	183.90	98.98	182.02	69.06	127.00	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YJR059W	PTK2	Serine/threonine protein kinase	55.28	99.60	55.06	131.45	72.66	Cytoplasm	Cytoplasm	Cytoplasm
YLR414C	PUN1	Plasma membrane protein with a role in cell wall integrity	75.96	91.26	69.32	110.45	83.90	Plasma membrane	Plasma membrane	Plasma membrane, vacuolar lumen
YEL020C	PXP1	Peroxisomal matrix protein	62.18	124.07	77.14	138.98	86.41	Cytoplasm	Cytoplasm	Cytoplasm
YBR218C	PYC2	Pyruvate carboxylase isoform	129.60	98.64	127.85	95.87	124.25	Cytoplasm	Cytoplasm	Cytoplasm
YFR033C	QCR6	Subunit 6 of the ubiquinol cytochrome-c reductase complex	301.87	109.09	329.31	83.80	252.96	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGL058W	RAD6	Ubiquitin-conjugating enzyme (E2)	94.64	83.27	78.80	43.71	41.37	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDL090C	RAM1	Beta subunit of the CAAX farnesyltransferase (FTase)	42.62	102.66	43.75	93.98	40.05	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm
YDR003W	RCR2	Vacuolar protein	49.46	87.88	43.46	106.25	52.55	Cytoplasm	Cytoplasm	Cytoplasm, vacuolar lumen
YBR073W	RDH54	DNA-dependent ATPase	36.32	91.06	33.08	99.45	36.12	Nucleus	Nucleus	Cytoplasm
YOR285W	RDL1	Thiosulfate sulfurtransferase	236.78	102.10	241.76	61.05	144.55	Cytoplasmic foci	Cytoplasmic foci	Cytoplasmic foci
YIL057C	RGI2	Protein of unknown function	107.71	129.55	139.53	103.39	111.35	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YMR139W	RIM11	Protein kinase	49.09	106.88	52.47	131.28	64.44	Cytoplasm	Cytoplasm	Cytoplasm
YBR030W	RKM3	Ribosomal lysine methyltransferase	37.38	104.07	38.90	95.03	35.52	Nucleus	Nucleus	Cytoplasm, nucleus

YDL001W	RMD1	Cytoplasmic protein required for sporulation	49.07	97.87	48.02	79.36	38.95	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm
YER070W	RNR1	Major isoform of large subunit of ribonucleotide-diphosphate reductase	731.72	103.99	760.93	124.61	911.83	Septins	Septins	Septins, cytoplasm
YJR063W	RPA12	RNA Polymerase A	109.81	71.43	78.44	86.87	109.81	Nucleus	Nucleus	Nucleus
YPR010C	RPA135	RNA polymerase I second largest subunit A135	103.41	96.61	99.91	66.92	69.20	Nucleus	Nucleus	Nucleus
YOR340C	RPA43	RNA polymerase I subunit A43	72.93	87.81	64.04	71.38	52.06	Nucleus	Nucleus	Nucleus
YHR143W-A	RPC10	RNA polymerase subunit ABC10-alpha, found in RNA pol I, II, and III	112.31	80.64	90.57	56.79	63.78	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YNL330C	RPD3	Histone deacetylase, component of both the Rpd3S and Rpd3L complexes	49.33	100.13	49.39	90.68	44.73	Cytoplasm	Cytoplasm	Nucleus
YPR102C	RPL11A	Ribosomal 60S subunit protein L11A	90.50	83.28	75.37	61.66	55.80	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDR418W	RPL12B	Ribosomal 60S subunit protein L12B	484.89	94.51	458.25	67.95	329.49	Cytoplasm	Cytoplasm	Cytoplasm
YMR121C	RPL15B	Ribosomal 60S subunit protein L15B	41.92	69.59	29.17	93.88	39.35	Cytoplasm, nuclear foci	Cytoplasm, nuclear foci	Cytoplasm, nuclear foci
YIL133C	RPL16A	Ribosomal 60S subunit protein L16A	201.42	76.55	154.19	22.14	44.60	Cytoplasm	Cytoplasm	Cytoplasm
YKL180W	RPL17A	Ribosomal 60S subunit protein L17A	109.89	90.20	99.12	66.55	73.13	Plasma membrane	Plasma membrane	Plasma membrane
YPL220W	RPL1A	Ribosomal 60S subunit protein L1A	110.39	75.41	83.24	50.36	55.59	Cytoplasm	Cytoplasm	Cytoplasm
YGL135W	RPL1B	Ribosomal 60S subunit protein L1B	585.16	74.51	436.02	57.93	339.01	Cytoplasm	Cytoplasm	Cytoplasm
YBR191W	RPL21A	Ribosomal 60S subunit protein L21A	140.20	85.92	120.46	66.11	92.69	Cytoplasm	Cytoplasm	Cytoplasm
YPL079W	RPL21B	Ribosomal 60S subunit protein L21B	283.79	84.17	238.87	50.71	143.92	Cytoplasm	Cytoplasm	Cytoplasm
YLR061W	RPL22A	Ribosomal 60S subunit protein L22A	1141.26	104.89	1197.12	62.63	714.73	Cytoplasm	Cytoplasm	Cytoplasm
YBL087C	RPL23A	Ribosomal 60S subunit protein L23A	64.25	53.71	34.51	64.93	41.71	Nucleus	Nucleus	Nucleus
YGR148C	RPL24B	Ribosomal 60S subunit protein L24B	353.74	71.68	253.57	68.43	242.07	Cytoplasm	Cytoplasm	Cytoplasm
YOR063W	RPL3	Ribosomal 60S subunit protein L3	418.92	104.22	436.60	70.26	294.32	Cytoplasm, nuclear foci	Cytoplasm, nuclear foci	Cytoplasm, nuclear foci
YOR234C	RPL33B	Ribosomal 60S subunit protein L33B	175.82	59.48	104.58	73.07	128.48	Cytoplasm	Cytoplasm	Cytoplasm

YER056C-A	RPL34A	Ribosomal 60S subunit protein L34A	334.68	78.00	261.05	57.19	191.40	Cytoplasm	Cytoplasm	Cytoplasm
YMR194W	RPL36A	Ribosomal 60S subunit protein L36A	207.02	71.13	147.24	57.08	118.16	Cytoplasm	Cytoplasm	Cytoplasm
YPL249C-A	RPL36B	Ribosomal 60S subunit protein L36B	223.06	86.89	193.81	62.75	139.97	Cytoplasm	Cytoplasm	Cytoplasm
YIL148W	RPL40A	Ubiquitin-ribosomal 60S subunit protein L40A fusion protein	51.11	60.29	30.81	72.67	37.14	Cytoplasm, nucleus	Cytoplasm	Cytoplasm
YKR094C	RPL40B	Ubiquitin-ribosomal 60S subunit protein L40B fusion protein	163.57	38.61	63.15	22.21	36.32	Cytoplasm	Cytoplasm	Cytoplasm
YLR448W	RPL6B	Ribosomal 60S subunit protein L6B	303.44	79.41	240.98	46.61	141.42	Cytoplasm	Cytoplasm	Cytoplasm
YPL198W	RPL7B	Ribosomal 60S subunit protein L7B	148.57	94.15	139.88	70.31	104.47	Cytoplasm, nuclear foci	Cytoplasm, nuclear foci	Cytoplasm, nuclear foci
YHL033C	RPL8A	Ribosomal 60S subunit protein L8A	905.39	87.74	794.42	52.19	472.53	Cytoplasm	Cytoplasm	Cytoplasm
YLL045C	RPL8B	Ribosomal 60S subunit protein L8B	502.72	87.99	442.33	59.70	300.11	Cytoplasm	Cytoplasm	Cytoplasm
YGL147C	RPL9A	Ribosomal 60S subunit protein L9A	201.33	68.52	137.95	62.79	126.42	Cytoplasm	Cytoplasm	Cytoplasm
YNL067W	RPL9B	Ribosomal 60S subunit protein L9B	1137.98	98.87	1125.17	56.14	638.81	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YFR052W	RPN12	Subunit of the 19S regulatory particle of the 26S proteasome lid	154.66	66.88	103.44	76.58	118.45	Nucleus	Nucleus	Nucleus
YPR108W	RPN7	Essential non-ATPase regulatory subunit of the 26S proteasome	34.32	99.45	34.13	147.38	50.58	Vacuolar lumen	Vacuolar lumen	Nucleus
YDL081C	RPP1A	Ribosomal stalk protein P1 alpha	808.19	106.79	863.03	52.95	427.95	Cytoplasm	Cytoplasm	Cytoplasm
YOR293W	RPS10A	Protein component of the small (40S) ribosomal subunit	43.88	71.49	31.37	82.68	36.28	Nuclear foci	Nuclear foci	Nuclear foci
YJL191W	RPS14B	Protein component of the small (40S) ribosomal subunit	59.37	70.39	41.79	73.29	43.52	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YML026C	RPS18B	Protein component of the small (40S) ribosomal subunit	65.32	68.08	44.47	64.45	42.10	Cytoplasm	Cytoplasm	Cytoplasm
YLR367W	RPS22B	Protein component of the small (40S) ribosomal subunit	234.68	57.32	134.52	46.92	110.11	Nucleus	Nucleus	Nucleus

YPR132W	RPS23B	Ribosomal protein 28 (rp28) of the small (40S) ribosomal subunit	131.72	70.72	93.16	54.83	72.23	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YER074W	RPS24A	Protein component of the small (40S) ribosomal subunit	233.52	87.07	203.33	79.23	185.01	Cytoplasm	Cytoplasm	Cytoplasm
YIL069C	RPS24B	Protein component of the small (40S) ribosomal subunit	107.37	90.14	96.78	53.28	57.20	Cytoplasm	Cytoplasm	Cytoplasm
YER131W	RPS26B	Protein component of the small (40S) ribosomal subunit	383.18	90.13	345.38	58.52	224.22	Cytoplasm	Cytoplasm	Cytoplasm
YLR287C-A	RPS30A	Protein component of the small (40S) ribosomal subunit	294.68	82.18	242.17	68.97	203.23	Cytoplasm	Cytoplasm	Cytoplasm
YPL090C	RPS6A	Protein component of the small (40S) ribosomal subunit	525.86	104.67	550.42	67.50	354.98	Cytoplasm	Cytoplasm	Cytoplasm
YPL012W	RRP12	Protein required for export of the ribosomal subunits	64.49	83.96	54.15	79.66	51.38	Cytoplasm, nuclear foci	Cytoplasm, nuclear foci	Nuclear foci
YIL127C	RRT14	Putative protein of unknown function	50.59	75.57	38.23	82.18	41.57	Nuclear foci	Nuclear foci	Nuclear foci
YHR087W	RTC3	Protein of unknown function involved in RNA metabolism	390.72	217.41	849.46	39.76	155.34	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDL204W	RTN2	Reticulon protein	40.78	114.59	46.73	147.14	60.01	Plasma membrane, cytoplasm	Plasma membrane, cytoplasm	Plasma membrane, cytoplasm
YOR077W	RTS2	Basic zinc-finger protein	46.40	81.63	37.88	87.57	40.64	Nucleus	Nucleus	Nucleus
YKL212W	SAC1	Phosphatidylinositol phosphate (PtdInsP) phosphatase	90.23	81.71	73.72	76.03	68.60	ER	ER, vacuolar lumen	ER, vacuolar lumen
YKL117W	SBA1	Co-chaperone that binds and regulates Hsp90 family chaperones	295.67	99.60	294.50	74.14	219.22	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YER120W	SCS2	Integral ER membrane protein, regulates phospholipid metabolism	52.90	78.61	41.58	90.17	47.70	ER	Plasma membrane	Plasma membrane
YBL011W	SCT1	Glycerol 3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferase	50.90	102.87	52.36	82.72	42.10	ER	ER	ER
YDR178W	SDH4	Membrane anchor subunit of succinate dehydrogenase (SDH)	169.21	107.46	181.84	59.70	101.02	Mitochondrion	Mitochondrion	Mitochondrion

YNL272C	SEC2	Guanyl-nucleotide exchange factor for the small G-protein Sec4p	51.54	100.28	51.69	86.46	44.56	Cytoplasm, bud neck	Cytoplasm, bud neck	Cytoplasm
YPR055W	SEC8	Essential 121 kDa subunit of the exocyst complex	47.10	81.27	38.28	86.94	40.95	Bud neck, plasma membrane foci	Bud neck, plasma membrane foci	Bud neck, plasma membrane foci
YDR077W	SED1	Suppression of Exponential Defect	244.84	121.45	297.37	85.36	244.84	ER	ER	ER, vacuolar lumen
YOR007C	SGT2	Glutamine-rich cytoplasmic cochaperone	119.81	100.36	120.25	78.69	94.28	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci
YLR058C	SHM2	Cytosolic serine hydroxymethyltransferase	176.15	108.51	191.14	147.33	259.53	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci
YDL225W	SHS1	Component of the septin ring that is required for cytokinesis	50.52	97.33	49.17	115.85	58.53	Bud neck	Bud neck	Bud neck, plasma membrane
YGR081C	SLX9	Protein required for pre-rRNA processing	59.58	75.47	44.96	79.30	47.25	Nucleus	Nuclear foci	Nuclear foci
YML058W	SML1	Ribonucleotide reductase inhibitor	224.77	59.11	132.86	54.65	122.83	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDR011W	SNQ2	Plasma membrane ATP-binding cassette (ABC) transporter	68.22	96.57	65.88	98.65	67.30	Plasma membrane	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YJR104C	SOD1	Cytosolic copper-zinc superoxide dismutase	907.13	113.60	1030.47	57.42	520.85	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YNR034W	SOL1	Protein with a possible role in tRNA export	57.60	102.98	59.31	125.61	72.35	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YPR069C	SPE3	Spermidine synthase	105.40	84.22	88.77	68.59	72.30	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGR104C	SRB5	Subunit of the RNA polymerase II mediator complex	57.65	94.67	54.58	66.30	38.22	Mitochondrion	Mitochondrion	Mitochondrion
YCL037C	SRO9	Cytoplasmic RNA-binding protein	157.03	86.89	136.44	71.30	111.97	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus

YKR092C	SRP40	Nucleolar serine-rich protein	124.38	82.97	103.20	60.47	75.21	Nuclear foci	Nuclear foci	Nuclear foci
YKL086W	SRX1	Sulfiredoxin	42.11	98.86	41.63	131.68	55.45	Cytoplasm	Cytoplasm	Cytoplasm, nucleus
YPL106C	SSE1	ATPase component of heat shock protein Hsp90 chaperone complex	536.49	76.10	408.28	61.60	330.47	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YBR283C	SSH1	Subunit of the Ssh1 translocon complex	39.41	70.82	27.91	229.60	90.48	Cytoplasm	Cytoplasm	ER
YMR183C	SSO2	Plasma membrane t-SNARE	38.45	70.60	27.14	105.44	40.54	Cytoplasm	Cytoplasm	Cytoplasm, vacuolar lumen
YOR212W	STE4	G protein beta subunit	54.17	83.59	45.28	71.59	38.78	ER	ER	Cytoplasm
YDL130W-A	STF1	Protein involved in regulation of the mitochondrial F1F0-ATP synthase	174.55	123.68	215.89	139.60	243.67	Mitochondrion	Mitochondrion	Mitochondrion
YJR130C	STR2	Cystathionine gamma-synthase, converts cysteine into cystathionine	39.54	102.40	40.49	106.84	42.24	Cytoplasm	Cytoplasm	Cytoplasm, nucleus
YDR167W	TAF10	Subunit (145 kDa) of TFIID and SAGA complexes	70.30	75.75	53.26	61.09	42.95	Nucleus	Nucleus	Nucleus
YMR227C	TAF7	TFIID subunit (67 kDa)	32.47	101.23	32.87	116.60	37.86	Cytoplasm	Cytoplasm	Nucleus
YOL020W	TAT2	High affinity tryptophan and tyrosine permease	47.45	89.91	42.66	97.15	46.10	Vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YJL052W	TDH1	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 1	285.70	142.31	406.59	212.55	607.26	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YJR009C	TDH2	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 2	2051.60	103.20	2117.26	43.82	898.93	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGR192C	TDH3	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 3	2315.14	149.08	3451.40	175.44	4061.58	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDR311W	TFB1	Subunit of TFIIF and nucleotide excision repair factor 3 complexes	47.65	95.64	45.57	79.50	37.88	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YLR178C	TFS1	Inhibitor of carboxypeptidase Y (Prc1p), and Ras GAP (Ira2p)	54.47	80.55	43.88	151.95	82.77	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YBR117C	TKL2	Transketolase	33.72	103.23	34.81	142.48	48.04	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus

YLR327C	TMA10	Protein of unknown function that associates with ribosomes	767.19	106.31	815.58	6.86	52.62	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YJR014W	TMA22	Protein of unknown function	99.49	87.07	86.62	65.81	65.47	Cytoplasm	Cytoplasm	Cytoplasm
YLR262C-A	TMA7	Protein of unknown that associates with ribosomes	116.81	82.79	96.70	67.72	79.11	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YNL070W	TOM7	Component of the TOM (translocase of outer membrane) complex	46.79	65.38	30.59	75.93	35.53	Mitochondrion	Cytoplasmic foci	Cytoplasm
YNL300W	TOS6		70.37	75.43	53.08	85.45	70.37	ER	ER	ER, vacuolar lumen
YDR050C	TPI1	Triose phosphate isomerase, abundant glycolytic enzyme	354.11	98.25	347.90	165.00	584.30	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YOR273C	TPO4	Polyamine transporter of the major facilitator superfamily	74.99	112.90	84.67	108.33	81.24	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YBR126C	TPS1	Synthase subunit of trehalose-6-P synthase/phosphatase complex	219.30	132.70	291.01	162.86	357.15	Cytoplasm	Cytoplasm	Cytoplasm
YDR074W	TPS2	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex	306.90	96.14	295.04	47.87	146.92	Cytoplasm	Cytoplasm	Cytoplasm
YNL299W	TRF5	Non-canonical poly(A) polymerase	33.15	94.61	31.37	99.15	32.87	Nuclear foci	Nuclear foci	Cytoplasm
YML028W	TSA1	Thioredoxin peroxidase	1157.80	121.49	1406.58	90.44	1047.10	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci
YKR088C	TVP38	Integral membrane protein	60.82	99.14	60.30	98.31	59.79	Cytoplasmic foci	Vacuolar lumen	Vacuolar lumen
YBR082C	UBC4	Ubiquitin-conjugating enzyme (E2)	104.84	47.05	49.33	51.31	53.79	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YGR019W	UGA1	Gamma-aminobutyrate (GABA) transaminase	81.02	115.14	93.29	144.69	117.23	Cytoplasm	Cytoplasm	Cytoplasm
YKL035W	UGP1	UDP-glucose pyrophosphorylase (UGPase)	106.46	104.93	111.71	125.07	133.14	Cytoplasm	Cytoplasm	Cytoplasm, nucleus
YBL039C	URA7	Major CTP synthase isozyme (see also URA8)	167.86	98.34	165.08	55.56	93.26	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci

YDR400W	URH1	Uridine nucleosidase (uridine-cytidine N-ribohydrolase)	35.33	121.53	42.94	117.48	41.50	Cytoplasm	Cytoplasm	Cytoplasm
YGL212W	VAM7	Vacuolar SNARE protein	58.59	105.05	61.54	82.14	48.12	Vacuolar lumen	Vacuolar lumen	Cytoplasm, vacuolar lumen
YGR065C	VHT1	High-affinity plasma membrane H ⁺ -biotin (vitamin H) symporter	79.34	170.21	135.05	111.25	88.27	Plasma membrane, vacuolar lumen	Vacuolar lumen	Plasma membrane, vacuolar lumen
YDL185W	VMA1	Subunit A of the V1 peripheral membrane domain of V-ATPase	573.31	112.70	646.12	53.56	307.06	Vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YEL027W	VMA3	Proteolipid subunit c of the V0 domain of vacuolar H ⁽⁺⁾ -ATPase	37.06	92.95	34.45	1612.18	597.49	Cytoplasm, cytoplasmic foci	Cytoplasm, cytoplasmic foci	Nucleus
YER072W	VTC1	Regulatory subunit of the vacuolar transporter chaperone (VTC) complex	45.87	94.17	43.19	77.44	35.52	ER, vacuolar lumen	ER, vacuolar lumen	Vacuolar lumen
YOR230W	WTM1	Transcriptional modulator	181.54	100.79	182.97	117.07	212.53	Nucleus	Nucleus	Nucleus
YBL055C	YBL055C	3'→5' exonuclease and endonuclease with a possible role in apoptosis	47.22	98.15	46.35	81.97	38.71	Mitochondrion	Mitochondrion	Cytoplasm
YCR087C-A	YCR087C-A	Putative protein of unknown function	47.15	67.23	31.70	83.22	39.24	Nuclear foci	Nuclear foci	Nuclear foci
YLL055W	YCT1	High-affinity cysteine-specific transporter	55.86	140.31	78.37	266.87	149.07	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDL124W	YDL124W	NADPH-dependent alpha-keto amide reductase	155.31	135.27	210.10	173.29	269.15	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YDL129W	YDL129W	Protein of unknown function	36.47	77.80	28.38	101.88	37.16	Cytoplasm	Cytoplasm	Cytoplasm, nucleus
YDR262W	YDR262W	Putative protein of unknown function	36.43	98.96	36.05	131.88	48.04	Vacuolar lumen	Vacuolar lumen	Vacuolar lumen
YLR249W	YEF3	Translation elongation factor 3	1100.43	87.53	963.23	70.36	774.21	Cytoplasm	Cytoplasm	Cytoplasm
YER053C-A	YER053C-A	Protein of unknown function	43.33	96.47	41.81	169.94	73.64	ER	ER	ER
YMR241W	YHM2	Citrate and oxoglutarate carrier protein	149.58	80.29	120.10	64.13	95.93	Mitochondrion	Mitochondrion	Mitochondrion
YKL071W	YKL071W	Putative protein of unknown function	502.12	82.43	413.92	69.46	348.75	Cytoplasm	Cytoplasm	Cytoplasm

YKR018C	YKR018C	Protein of unknown function	74.16	94.41	70.02	133.67	99.13	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YLR297W	YLR297W	Protein of unknown function	37.96	79.31	30.11	124.11	47.12	Cytoplasm, vacuolar lumen	Cytoplasm, vacuolar lumen	Vacuolar lumen
YMR099C	YMR099C	Glucose-6-phosphate 1-epimerase (hexose-6-phosphate mutarotase)	95.47	106.19	101.38	70.73	67.53	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YMR178W	YMR178W	Protein of unknown function	51.29	86.80	44.52	127.40	65.34	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YNL194C	YNL194C	Integral membrane protein	44.31	105.64	46.82	144.02	63.82	Plasma membrane	Plasma membrane	Plasma membrane
YGR281W	YOR1	Plasma membrane ATP-binding cassette (ABC) transporter	39.66	99.25	39.37	106.73	42.33	Cytoplasm, vacuolar lumen	Cytoplasm, vacuolar lumen	Cytoplasm, vacuolar lumen
YOR292C	YOR292C	Putative protein of unknown function	39.01	74.61	29.11	102.27	39.90	Cytoplasm	Cytoplasm, vacuolar lumen	Cytoplasm, vacuolar lumen
YPR127W	YPR127W	Putative pyridoxine 4-dehydrogenase	33.20	105.76	35.11	118.29	39.28	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YPR172W	YPR172W	Protein of unknown function	64.03	92.97	59.53	67.50	43.22	Cytoplasm, nucleus	Cytoplasm, nucleus	Cytoplasm, nucleus
YBR054W	YRO2	Protein with a putative role in response to acid stress	54.76	158.37	86.73	178.73	97.88	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen	Plasma membrane, vacuolar lumen
YNL310C	ZIM17	Protein co-chaperone with a zinc finger motif	40.13	72.53	29.11	109.77	44.05	Nuclear foci	Nuclear foci	Nucleus
YMR243C	ZRC1	Vacuolar membrane zinc transporter	50.57	74.58	37.71	145.48	73.57	Cytoplasmic foci	Vacuolar membrane	Vacuolar membrane

Table 7.3.2 – Proteins with change in either localisation or abundance with 100 nM MTDIA or with *meu1Δ*.

Go: biological process	Corrected enrichment
Transmembrane transport	4.39E-07
Organic acid transport	5.67E-07
Carboxylic acid transport	1.38E-06
Establishment of localization	3.63E-05
Ion transport	1.48E-04
Amino acid transmembrane transport	3.06E-04

Table 7.3.3 – **GO Enrichment for proteins affected by MTDIA treatment.** Presented are the non-overlapping GO categories for biological processes following correction for multiple testing using Benjamini Hochberg false discovery rate of 0.05.

Go: biological process	Corrected enrichment
Pyruvate metabolic process	1.70E-10
Pyridine nucleotide metabolic process	2.02E-09
Nicotinamide nucleotide metabolic process	2.02E-09
Cytoplasmic translation	4.34E-09
Glycolytic process	5.62E-08
Acyl-CoA metabolic process	5.12E-06
Acetyl-CoA metabolic process	1.34E-05
Ribosomal large subunit biogenesis	2.05E-04
Transmembrane transport	7.07E-04
Glycine catabolic process	7.58E-04
Gluconeogenesis	9.58E-04
Glyceraldehyde-3-phosphate metabolic process	7.90E-03
Glutamate metabolic process	1.08E-02
Ion transport	1.45E-02
Acetate metabolic process	2.61E-02
Amino acid transport	4.31E-02
Tricarboxylic acid cycle	4.36E-02
Citrate metabolic process	4.36E-02

Table 7.3.4 – **GO enrichment for proteins affected by *meu1Δ*.** Presented are the non-overlapping GO categories for biological processes following correction for multiple testing using Benjamini Hochberg false discovery rate of 0.05.

Pathway	Corrected enrichment	Genes
Glucose fermentation	1.71E-07	<i>ADH2, ADH3, ADH4, ALD6, CDC19, ENO1, GLK1, GPM1, HXK2, PDC1, PDC6, PFK2, PGI1, PGK1, TDH1, TDH2, TDH3, TPI1</i>
Glycolysis	1.83E-04	<i>CDC19, ENO1, GPM1, PFK2, PGI1, PGK1, TDH1, TDH2, TDH3, TPI1</i>
Gluconeogenesis	3.21E-04	<i>ENO1, GPM1, MAE1, MDH2, PGI1, PGK1, PYC2, TDH1, TDH2, TDH3</i>

Glycine cleavage complex	2.11E-02	<i>GCV1, GCV2, GCV3, LPD1</i>
Pyruvate dehydrogenase complex	2.11E-02	<i>GCV1, GCV2, GCV3, LPD1</i>

Table 7.3.5 – **Pathway enrichment for *meu1Δ***. Presented are the pathways enriched on Yeastmine following correction for multiple testing using Benjamini Hochberg false discovery rate of 0.05.

Figure 7.3.3 – Relocalisation Gln1-GFP to cytoplasmic foci with MTDIA and in *meu1Δ*.

7.3.4 Go analysis of >2-fold upregulated or downregulated gene transcripts from Basu et al. 2011.

GO biological process complete	Homo sapiens (reference list of proteins = 20972)	Basu et al. 2011 (proteins with annotations = 690)	Fold enrichment (proteins in Basu et al. /reference list)	Go enrichment (p-value)
Negative regulation of wound healing (GO:0061045)	63	13	6.27	2.42E-03
Regulation of steroid biosynthetic process (GO:0050810)	81	16	6	1.92E-04
Negative regulation of response to wounding (GO:1903035)	74	13	5.34	1.41E-02
Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043154)	84	14	5.07	1.04E-02
Negative regulation of cysteine-type endopeptidase activity (GO:2000117)	89	14	4.78	2.02E-02
Regulation of steroid metabolic process (GO:0019218)	106	16	4.59	6.69E-03
Regulation of mitotic nuclear division (GO:0007088)	149	18	3.67	3.13E-02
Regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043281)	202	21	3.16	4.99E-02
Mitotic cell cycle phase transition (GO:0044772)	265	25	2.87	3.59E-02
Response to drug (GO:0042493)	411	37	2.74	6.01E-04
Negative regulation of hydrolase activity (GO:0051346)	407	36	2.69	1.37E-03
Small molecule biosynthetic process (GO:0044283)	436	38	2.65	8.96E-04
Positive regulation of transferase activity (GO:0051347)	657	57	2.64	6.41E-07
Positive regulation of protein kinase activity (GO:0045860)	481	41	2.59	5.06E-04
Positive regulation of kinase activity (GO:0033674)	519	43	2.52	5.22E-04

Regulation of protein serine/threonine kinase activity (GO:0071900)	475	38	2.43	7.07E-03
Organic hydroxy compound metabolic process (GO:1901615)	412	33	2.43	3.62E-02
Response to growth factor (GO:0070848)	495	39	2.39	7.32E-03
Mitotic cell cycle (GO:0000278)	693	54	2.37	8.00E-05
Response to wounding (GO:0009611)	554	43	2.36	2.97E-03
Wound healing (GO:0042060)	466	36	2.35	2.97E-02
Cellular response to growth factor stimulus (GO:0071363)	468	36	2.34	3.26E-02
Mitotic cell cycle process (GO:1903047)	635	48	2.3	1.32E-03
Myeloid leukocyte mediated immunity (GO:0002444)	518	39	2.29	2.13E-02
Negative regulation of cellular protein metabolic process (GO:0032269)	1023	77	2.29	2.22E-07
Regulation of protein kinase activity (GO:0045859)	750	56	2.27	1.77E-04
Regulation of mitotic cell cycle (GO:0007346)	523	39	2.27	2.66E-02
Neutrophil mediated immunity (GO:0002446)	497	37	2.26	4.93E-02
Positive regulation of protein phosphorylation (GO:0001934)	886	66	2.26	9.85E-06
Myeloid cell activation involved in immune response (GO:0002275)	514	38	2.25	4.32E-02
Symbiosis, encompassing mutualism through parasitism (GO:0044403)	789	58	2.23	1.67E-04
Interspecies interaction between organisms (GO:0044419)	791	58	2.23	1.82E-04
Response to organonitrogen compound (GO:0010243)	880	64	2.21	4.41E-05
Positive regulation of cell death (GO:0010942)	632	46	2.21	6.68E-03
Positive regulation of apoptotic process (GO:0043065)	591	43	2.21	1.55E-02
Negative regulation of programmed cell death (GO:0043069)	845	61	2.19	1.34E-04
Negative regulation of apoptotic process (GO:0043066)	832	60	2.19	1.83E-04
Positive regulation of programmed cell death (GO:0043068)	597	43	2.19	1.99E-02
Negative regulation of protein metabolic process (GO:0051248)	1087	78	2.18	1.47E-06
Regulation of kinase activity (GO:0043549)	810	58	2.18	4.04E-04
Regulated exocytosis (GO:0045055)	686	49	2.17	4.94E-03
Regulation of cellular response to stress (GO:0080135)	615	44	2.17	1.80E-02
Regulation of transferase activity (GO:0051338)	984	70	2.16	1.96E-05
Positive regulation of phosphorylation (GO:0042327)	929	66	2.16	6.11E-05
Response to organic cyclic compound (GO:0014070)	885	63	2.16	1.28E-04
Regulation of protein phosphorylation (GO:0001932)	1300	92	2.15	5.74E-08
Regulation of cell motility (GO:2000145)	748	53	2.15	2.16E-03
Response to nitrogen compound (GO:1901698)	981	69	2.14	4.02E-05
Positive regulation of phosphorus metabolic process (GO:0010562)	1055	74	2.13	1.19E-05
Positive regulation of phosphate metabolic process (GO:0045937)	1055	74	2.13	1.19E-05
Positive regulation of protein modification process (GO:0031401)	1146	80	2.12	2.98E-06
Positive regulation of intracellular signal transduction (GO:1902533)	905	63	2.12	2.84E-04
Regulation of phosphorylation (GO:0042325)	1395	97	2.11	3.77E-08

Regulation of cell migration (GO:0030334)	695	48	2.1	1.61E-02
Regulation of MAPK cascade (GO:0043408)	670	46	2.09	3.07E-02
Response to oxygen-containing compound (GO:1901700)	1410	97	2.09	6.81E-08
Negative regulation of cell death (GO:0060548)	917	63	2.09	4.53E-04
Exocytosis (GO:0006887)	774	53	2.08	5.97E-03
Regulation of proteolysis (GO:0030162)	803	55	2.08	3.63E-03
Response to hormone (GO:0009725)	777	53	2.07	6.69E-03
Regulation of cell cycle (GO:0051726)	1019	69	2.06	1.75E-04
Response to lipid (GO:0033993)	831	56	2.05	4.69E-03
Response to endogenous stimulus (GO:0009719)	1264	85	2.04	4.67E-06
Regulation of locomotion (GO:0040012)	818	55	2.04	6.36E-03
Regulation of cellular component movement (GO:0051270)	820	55	2.04	6.84E-03
Secretion by cell (GO:0032940)	963	64	2.02	1.14E-03
Cellular response to oxygen-containing compound (GO:1901701)	874	58	2.02	4.74E-03
Leukocyte mediated immunity (GO:0002443)	725	48	2.01	4.89E-02
Positive regulation of protein metabolic process (GO:0051247)	1543	102	2.01	1.76E-07
Positive regulation of cellular protein metabolic process (GO:0032270)	1449	96	2.01	6.69E-07
Positive regulation of cellular component organization (GO:0051130)	1180	78	2.01	5.39E-05
Negative regulation of molecular function (GO:0044092)	1136	75	2.01	1.14E-04
Immune effector process (GO:0002252)	1003	66	2	1.02E-03
Negative regulation of catalytic activity (GO:0043086)	866	57	2	7.67E-03
Carboxylic acid metabolic process (GO:0019752)	868	57	2	8.23E-03
Regulation of protein modification process (GO:0031399)	1680	110	1.99	4.39E-08
Regulation of phosphate metabolic process (GO:0019220)	1633	107	1.99	8.68E-08
Regulation of organelle organization (GO:0033043)	1190	78	1.99	7.70E-05
Cell cycle process (GO:0022402)	1006	66	1.99	1.13E-03
Apoptotic process (GO:0006915)	903	59	1.99	6.18E-03
Regulation of response to stress (GO:0080134)	1382	90	1.98	6.59E-06
Cell death (GO:0008219)	1061	69	1.98	7.96E-04
Programmed cell death (GO:0012501)	1026	67	1.98	1.07E-03
Regulation of phosphorus metabolic process (GO:0051174)	1647	107	1.97	1.43E-07
Regulation of programmed cell death (GO:0043067)	1437	93	1.97	4.51E-06
Regulation of apoptotic process (GO:0042981)	1423	92	1.97	5.95E-06
Regulation of intracellular signal transduction (GO:1902531)	1740	112	1.96	7.68E-08
Cellular response to endogenous stimulus (GO:0071495)	1053	68	1.96	1.28E-03
Positive regulation of cell proliferation (GO:0008284)	851	55	1.96	2.05E-02
Regulation of cellular component biogenesis (GO:0044087)	825	53	1.95	3.71E-02
Regulation of cell death (GO:0010941)	1537	98	1.94	3.14E-06
Cell cycle (GO:0007049)	1333	85	1.94	5.37E-05
Secretion (GO:0046903)	1068	68	1.94	2.12E-03
Regulation of cellular protein metabolic process (GO:0032268)	2436	155	1.93	5.95E-12

Regulation of anatomical structure morphogenesis (GO:0022603)	951	60	1.92	1.49E-02
Positive regulation of signal transduction (GO:0009967)	1443	90	1.9	5.15E-05
Oxoacid metabolic process (GO:0043436)	976	61	1.9	1.64E-02
Regulation of protein metabolic process (GO:0051246)	2684	167	1.89	2.32E-12
Negative regulation of signal transduction (GO:0009968)	1134	70	1.88	4.27E-03
Regulation of cellular component organization (GO:0051128)	2321	143	1.87	1.33E-09
Organic acid metabolic process (GO:0006082)	993	61	1.87	2.80E-02
Response to organic substance (GO:0010033)	2612	159	1.85	1.01E-10
Negative regulation of response to stimulus (GO:0048585)	1411	86	1.85	3.22E-04
Cellular response to organic substance (GO:0071310)	1967	119	1.84	7.33E-07
Regulation of cell differentiation (GO:0045595)	1544	93	1.83	1.45E-04
Positive regulation of molecular function (GO:0044093)	1916	115	1.82	2.59E-06
Negative regulation of signalling (GO:0023057)	1236	74	1.82	6.10E-03
Nitrogen compound transport (GO:0071705)	1692	100	1.8	9.90E-05
Regulation of hydrolase activity (GO:0051336)	1386	82	1.8	2.33E-03
Negative regulation of cell communication (GO:0010648)	1232	73	1.8	1.06E-02
Response to stress (GO:0006950)	3263	192	1.79	1.63E-12
Regulation of catalytic activity (GO:0050790)	2447	144	1.79	3.30E-08
Positive regulation of catalytic activity (GO:0043085)	1612	95	1.79	2.78E-04
Regulation of cell proliferation (GO:0042127)	1558	92	1.79	4.32E-04
Positive regulation of cell communication (GO:0010647)	1570	92	1.78	6.10E-04
Positive regulation of macromolecule metabolic process (GO:0010604)	2892	168	1.77	8.09E-10
Multi-organism process (GO:0051704)	2398	140	1.77	1.26E-07
Organic substance transport (GO:0071702)	2048	119	1.77	8.13E-06
Positive regulation of signalling (GO:0023056)	1578	92	1.77	7.65E-04
Positive regulation of nitrogen compound metabolic process (GO:0051173)	2814	163	1.76	2.76E-09
Positive regulation of developmental process (GO:0051094)	1207	70	1.76	3.66E-02
Regulation of molecular function (GO:0065009)	3032	174	1.74	7.14E-10
Cellular protein localization (GO:0034613)	1331	76	1.74	2.41E-02
Positive regulation of metabolic process (GO:0009893)	3129	178	1.73	7.17E-10
Positive regulation of cellular metabolic process (GO:0031325)	2917	165	1.72	1.32E-08
Cellular localization (GO:0051641)	2104	119	1.72	3.83E-05
Positive regulation of response to stimulus (GO:0048584)	2088	118	1.72	4.72E-05
Cellular macromolecule localization (GO:0070727)	1341	76	1.72	3.14E-02
Protein localization (GO:0008104)	1920	108	1.71	3.03E-04
Cellular response to chemical stimulus (GO:0070887)	2416	135	1.7	5.75E-06
Regulation of developmental process (GO:0050793)	2284	128	1.7	1.50E-05
Vesicle-mediated transport (GO:0016192)	1748	98	1.7	1.68E-03
Tissue development (GO:0009888)	1643	92	1.7	4.42E-03
Establishment of localization in cell (GO:0051649)	1647	92	1.7	4.90E-03
Establishment of protein localization (GO:0045184)	1498	84	1.7	1.39E-02

Positive regulation of multicellular organismal process (GO:0051240)	1464	82	1.7	1.95E-02
Cellular response to stress (GO:0033554)	1561	87	1.69	1.13E-02
Negative regulation of nitrogen compound metabolic process (GO:0051172)	2249	124	1.68	7.39E-05
Regulation of multicellular organismal development (GO:2000026)	1738	96	1.68	4.40E-03
Single-organism localization (GO:1902578)	2646	145	1.67	4.16E-06
Regulation of signal transduction (GO:0009966)	2783	152	1.66	1.74E-06
Macromolecule localization (GO:0033036)	2214	121	1.66	1.90E-04
Regulation of multicellular organismal process (GO:0051239)	2685	146	1.65	6.01E-06
Negative regulation of cellular metabolic process (GO:0031324)	2405	130	1.64	9.54E-05
Phosphorus metabolic process (GO:0006793)	2185	118	1.64	5.58E-04
Small molecule metabolic process (GO:0044281)	1801	97	1.64	1.14E-02
Positive regulation of macromolecule biosynthetic process (GO:0010557)	1628	88	1.64	3.26E-02
Positive regulation of biological process (GO:0048518)	5400	288	1.62	4.20E-16
Negative regulation of macromolecule metabolic process (GO:0010605)	2444	130	1.62	2.43E-04
Positive regulation of cellular process (GO:0048522)	4808	254	1.61	1.29E-12
Regulation of signalling (GO:0023051)	3139	166	1.61	2.18E-06
Regulation of cell communication (GO:0010646)	3086	163	1.61	3.70E-06
Response to external stimulus (GO:0009605)	1808	96	1.61	2.35E-02
Positive regulation of biosynthetic process (GO:0009891)	1791	95	1.61	2.79E-02
Immune system process (GO:0002376)	2512	132	1.6	3.74E-04
Localization (GO:0051179)	5375	283	1.6	7.77E-15
Single-organism transport (GO:0044765)	2521	133	1.6	2.60E-04
Cellular component assembly (GO:0022607)	2254	119	1.6	1.59E-03
Phosphate-containing compound metabolic process (GO:0006796)	2092	110	1.6	6.17E-03
Establishment of localization (GO:0051234)	4499	235	1.59	1.41E-10
Regulation of response to stimulus (GO:0048583)	3761	196	1.58	8.62E-08
Regulation of biological quality (GO:0065008)	3487	181	1.58	1.08E-06
Regulation of localization (GO:0032879)	2553	132	1.57	9.39E-04
Response to chemical (GO:0042221)	3897	199	1.55	3.41E-07
Negative regulation of metabolic process (GO:0009892)	2688	137	1.55	1.15E-03
Cell surface receptor signalling pathway (GO:0007166)	2217	113	1.55	1.85E-02
Cellular component biogenesis (GO:0044085)	2508	127	1.54	5.25E-03
Negative regulation of cellular process (GO:0048523)	4291	214	1.52	3.77E-07
Transport (GO:0006810)	4368	217	1.51	3.64E-07
Animal organ development (GO:0048513)	2961	145	1.49	4.69E-03
Regulation of macromolecule metabolic process (GO:0060255)	6010	293	1.48	6.25E-11
Negative regulation of biological process (GO:0048519)	4778	233	1.48	2.76E-07
Organelle organization (GO:0006996)	3097	151	1.48	3.27E-03
Regulation of metabolic process (GO:0019222)	6533	316	1.47	5.05E-12

Regulation of primary metabolic process (GO:0080090)	5976	289	1.47	3.34E-10
Single-multicellular organism process (GO:0044707)	5539	267	1.47	1.11E-08
Cellular component organization (GO:0016043)	5242	253	1.47	6.17E-08
Regulation of nitrogen compound metabolic process (GO:0051171)	5811	279	1.46	3.48E-09
Single-organism metabolic process (GO:0044710)	3572	171	1.46	1.38E-03
Cellular component organization or biogenesis (GO:0071840)	5457	258	1.44	3.09E-07
Regulation of gene expression (GO:0010468)	4544	213	1.42	9.99E-05
Response to stimulus (GO:0050896)	7844	367	1.42	2.37E-13
Regulation of cellular metabolic process (GO:0031323)	6029	282	1.42	5.59E-08
Multicellular organism development (GO:0007275)	4745	221	1.42	7.68E-05
Developmental process (GO:0032502)	5436	252	1.41	4.77E-06
Single-organism developmental process (GO:0044767)	5350	247	1.4	1.21E-05
Anatomical structure development (GO:0048856)	5073	234	1.4	4.94E-05
System development (GO:0048731)	4156	191	1.4	3.50E-03
Regulation of biosynthetic process (GO:0009889)	4432	202	1.39	2.48E-03
Organonitrogen compound metabolic process (GO:1901564)	5458	248	1.38	5.08E-05
Organic cyclic compound metabolic process (GO:1901360)	4945	224	1.38	6.02E-04
Cellular response to stimulus (GO:0051716)	6204	278	1.36	1.12E-05
Regulation of macromolecule biosynthetic process (GO:0010556)	4169	186	1.36	3.73E-02
Organic substance biosynthetic process (GO:1901576)	4742	210	1.35	1.07E-02
Cell communication (GO:0007154)	5341	236	1.34	1.74E-03
Biosynthetic process (GO:0009058)	4809	211	1.33	1.93E-02
Cellular metabolic process (GO:0044237)	8936	388	1.32	3.78E-09
Multicellular organismal process (GO:0032501)	6633	289	1.32	8.04E-05
Signalling (GO:0023052)	5254	229	1.32	8.82E-03
Single organism signalling (GO:0044700)	5250	228	1.32	1.25E-02
Single-organism cellular process (GO:0044763)	9841	424	1.31	1.12E-10
Organic substance metabolic process (GO:0071704)	9416	404	1.3	3.68E-09

Table 7.3.6 – **GO analysis of >2-fold upregulated or downregulated gene transcripts from Basu et al.**

2011. GO enrichment for biological processes were determined using Panter (Mi et al., 2017) with human genome as a reference list with Bonferroni (Simes, 1986) false discovery rate of 0.05. Highlighted in red are biological processes relevant to this decertation.

7.4.1 *pnp1Δ* mutant construction

Figure 7.4.1 – ***pnp1Δ* construction and validation PCRs**. PCR products were run on a 1% agarose gel with 1KB plus ladder at 100 mV. (a) *pnp1Δ::natR* deletion construction PCR. (b) PCR validation of *PNP1* knockout.

Primer	Sequence
Fw primer (#381)	ATCTTGCTAATCTTCTAGAACACATGAGAACTCCGAATAAACTTAAAAGACATGGAGGCCC AGAATACCCT
Rev primer (#382)	AGGTACATCTTCCCGCTATGTAATAAATAGAGGTATTTAAGTTATAACAACAGTATAGCGAC CAGCATTAC
Conformation primer A (#3)	TCAAGCATCATCTGTCAA
Conformation primer B (#385)	TACGAGACGACCACGAAGC
Conformation primer C (#386)	TGGAACCGCCGGCTGACC
Conformation primer D (#4)	TTATACGTTACTGTACAT

Table 7.4.1 - ***pnp1Δ* construction and conformation primers**. Primers were designed as previously described (Janke et al., 2004).

7.4.2 Genetic interactions with *PNP1* in complete and minimal media

ID Column	Gene	Function	p-value	Z-Score	% Growth
YNL141W	AAH1	Adenine deaminase (adenine aminohydrolase)	1.64E-02	2.40	53.76
YGL105W	ARC1	Protein that binds tRNA and methionyl- and glutamyl-tRNA synthetases	1.12E-11	6.79	16.67
YNL242W	ATG2	Peripheral membrane protein required for autophagic vesicle formation	1.37E-02	2.46	52.91
YGR157W	CHO2	Phosphatidylethanolamine methyltransferase (PEMT)	2.17E-02	2.29	54.95
YBR036C	CSG2	Endoplasmic reticulum membrane protein	1.38E-03	3.20	43.29
YIR023W	DAL81	Positive regulator of genes in multiple nitrogen degradation pathways	8.03E-03	2.65	50.51
YHL031C	GOS1	v-SNARE protein involved in Golgi transport	3.99E-02	2.06	59.17
YJR090C	GRR1	F-box protein component of an SCF ubiquitin-ligase complex	3.14E-18	8.71	9.99
YNL021W	HDA1	Putative catalytic subunit of a class II histone deacetylase complex	4.31E-02	2.02	59.17
YPR179C	HDA3	Subunit of the HDA1 histone deacetylase complex	5.09E-03	2.80	48.08
YIL090W	ICE2	Integral ER membrane protein with type-III transmembrane domains	1.49E-03	3.18	43.86
YDR123C	INO2	Transcription factor	1.69E-03	3.14	44.25
YIL125W	KGD1	Subunit of the mitochondrial alpha-ketoglutarate dehydrogenase complex	2.56E-15	8.09	11.81
YDR034C	LYS14	Transcriptional activator involved in regulating lysine biosynthesis	1.46E-12	7.08	15.55
YHR194W	MDM31	Mitochondrial protein that may have a role in phospholipid metabolism	1.43E-10	6.41	18.45
YGL124C	MON1	Subunit of a heterodimeric guanine nucleotide exchange factor (GEF)	3.72E-02	2.08	58.48
YJR073C	OPI3	Methylene-fatty-acyl-phospholipid synthase	3.77E-02	2.08	58.48
YBR093C	PHO5	Repressible acid phosphatase	1.75E-04	3.75	37.45
YBR106W	PHO88	Protein involved in SRP-independent targeting of substrates to the ER	5.21E-12	6.90	16.10
YER162C	RAD4	Protein that recognizes and binds damaged DNA (with Rad23p) during NER	2.80E-02	2.20	57.14
YJL204C	RCY1	F-box protein involved in recycling endocytosed proteins	1.37E-05	4.35	31.85
YLR371W	ROM2	Guanine nucleotide exchange factor (GEF) for Rho1p and Rho2p	4.08E-02	2.05	59.17
YLR357W	RSC2	Component of the RSC chromatin remodelling complex	1.44E-04	5.16	38.91
YBR289W	SNF5	Subunit of the SWI/SNF chromatin remodelling complex	4.22E-08	5.48	23.53
YCL032W	STE50	Adaptor protein for various signalling pathways	1.84E-02	2.36	54.35
YIL047C	SYG1	Plasma membrane protein of unknown function	3.14E-12	7.37	16.21
YKL081W	TEF4	Gamma subunit of translational elongation factor eEF1B	2.38E-05	4.23	32.89
YIL011W	TIR3	Cell wall mannoprotein	8.77E-16	8.04	11.93
YDR074W	TPS2	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex	2.24E-02	2.28	55.56
YGL026C	TRP5	Tryptophan synthase	2.59E-12	7.00	15.77

YDR359C	VID21	Component of the NuA4 histone acetyltransferase complex	1.67E-09	6.03	20.33
YOR270C	VPH1	Subunit a of vacuolar-ATPase V0 domain	1.73E-02	2.38	53.76
YHR012W	VPS29	Subunit of the membrane-associated retromer complex	2.19E-02	2.29	55.56
YDL041W	YDL041W	Dubious open reading frame overlaps SIR2	4.24E-18	8.67	10.09
YDR128W	YDR128W	Subunit of SEACAT, a subcomplex of the SEA complex	3.16E-02	2.15	57.47
YFL013W-A	YFL013W-A	Dubious open reading frame overlaps IES1	4.32E-02	2.02	59.52
YIL012W	YIL012W	Putative protein of unknown function	1.84E-10	6.37	57.14
YLR184W	YLR184W	Dubious open reading frame adjacent to TOP4	2.08E-03	3.08	44.84
YPR024W	YME1	Catalytic subunit of i-AAA protease complex	3.10E-05	4.17	33.56
YNL080C	YNL080C	Protein involved in N-glycosylation	1.04E-02	2.56	51.55
YGL255W	ZRT1	High-affinity zinc transporter of the plasma membrane	7.32E-03	2.68	49.75
YNL241C	ZWF1	Glucose-6-phosphate dehydrogenase (G6PD)	2.45E-02	2.25	56.18

Table 7.4.2a – **42 putative genetic interactions with *PNP1* gene in synthetic complete media.** The haploid single gene deletion library was mated *en masse* against the *pnp1Δ* query strain on agar according to SGA methodology using the Singer RoToR HAD (Singer Instrument Co) (Tong et al., 2001). The haploid *pnp1ΔxxxΔ* double mutant meiotic progeny were assessed for epistatic colony size relative to the haploid parental mutants using Gitter on RStudio and ScreenMill. The putative interactions with an epistatic growth defect (*pnp1ΔxxxΔ/pnp1Δ*) in colony size with a $p < 0.05$ in at least two out of three independent SGAs are presented.

ORF name	Gene name	Function	<i>p</i> -value	Standard deviation	% Growth
YNL141W	AAH1	Adenine deaminase (adenine aminohydrolase)	3.53E-26	1.59	25.99
YGL234W	ADE5,7	Enzyme of the 'de novo' purine nucleotide biosynthetic pathway	5.31E-05	7.28	46.48
YML035C	AMD1	AMP deaminase	1.50E-02	19.15	19.39
YPR185W	ATG13	Regulatory subunit of the Atg1p signalling complex	7.85E-04	8.78	48.89
YKR099W	BAS1	Myb-related transcription factor	4.75E-03	10.64	54.11
YOR078W	BUD21	Component of small ribosomal subunit (SSU) processosome	1.52E-24	1.41	30.86
YBR131W	CCZ1	Subunit of a heterodimeric guanine nucleotide exchange factor (GEF)	3.15E-02	20.39	38.69
YER061C	CEM1	Mitochondrial beta-keto-acyl synthase	2.59E-03	12.67	30.69
YLR394W	CST9	SUMO E3 ligase	4.16E-06	7.49	20.42
YIR023W	DAL81	Positive regulator of genes in multiple nitrogen degradation pathways	6.22E-08	5.91	30.18
YDR359C	EAF1	Component of the NuA4 histone acetyltransferase complex	1.45E-43	0.90	2.45
YLR206W	ENT2	Epsin-like protein required for endocytosis and actin patch assembly	1.38E-06	7.99	-1.00
YNL080C	EOS1	Protein involved in N-glycosylation	2.02E-13	4.41	22.58
YBR026C	ETR1	2-enoyl thioester reductase	1.06E-13	4.55	16.64

YOR038C	HIR2	Subunit of HIR nucleosome assembly complex	1.24E-07	6.48	23.39
YOR025W	HST3	Member of the Sir2 family of NAD(+)-dependent protein deacetylases	3.30E-02	51.91	239.38
YOL012C	HTZ1	Histone variant H2AZ	1.06E-17	3.13	33.41
YDR123C	INO2	Transcription factor	2.67E-07	5.97	13.90
YKR019C	IRS4	EH domain-containing protein	2.80E-09	4.78	41.32
YLL019C	KNS1	Protein kinase involved in negative regulation of PolIII transcription	2.84E-05	6.50	56.73
YPR070W	MED1	Subunit of the RNA polymerase II mediator complex	2.62E-04	11.62	-1.02
YGL124C	MON1	Subunit of a heterodimeric guanine nucleotide exchange factor (GEF)	5.44E-05	12.00	34.97
YDR128W	MTC5	Subunit of SEACAT, a subcomplex of the SEA complex	2.19E-02	15.59	43.05
YJR073C	OPI3	Methylene-fatty-acyl-phospholipid synthase	2.69E-05	7.98	3.65
YCR077C	PAT1	Deadenylation-dependent mRNA-decapping factor	1.84E-07	5.74	38.15
YLR199C	PBA1	Protein involved in 20S proteasome assembly	7.68E-21	3.66	2.34
YLR134W	PDC5	Minor isoform of pyruvate decarboxylase	7.91E-07	7.10	20.50
YBR093C	PHO5	Repressible acid phosphatase	1.78E-03	11.67	19.52
	PHO88				
YPL036W	PMA2	Plasma membrane H ⁺ -ATPase	1.46E-13	3.79	39.87
YDL006W	PTC1	Type 2C protein phosphatase (PP2C)	2.24E-30	1.03	36.91
YER162C	RAD4	Protein that recognizes and binds damaged DNA (with Rad23p) during NER	1.02E-19	3.17	25.75
YCR028C-A	RIM1	ssDNA-binding protein essential for mitochondrial genome maintenance	4.74E-03	11.00	58.95
YMR154C	RIM13	Calpain-like cysteine protease	2.75E-09	5.27	159.25
YLR061W	RPL22A	Ribosomal 60S subunit protein L22A	4.20E-10	5.41	17.62
YGL147C	RPL9A	Ribosomal 60S subunit protein L9A	3.60E-13	4.20	28.68
YOL121C	RPS19A	Protein component of the small (40S) ribosomal subunit	4.93E-10	5.52	14.68
YPL090C	RPS6A	Protein component of the small (40S) ribosomal subunit	8.60E-07	7.27	18.22
YNL257C	SIP3	Putative sterol transfer protein	5.75E-11	5.36	10.55
YBR289W	SNF5	Subunit of the SWI/SNF chromatin remodelling complex	4.99E-04	7.93	48.47
YMR016C	SOK2	Nuclear protein that negatively regulates pseudohyphal differentiation	6.56E-06	6.92	37.26
YOR190W	SPR1	Sporulation-specific exo-1,3-beta-glucanase	1.70E-06	6.73	31.69
YIL047C	SYG1	Plasma membrane protein of unknown function	1.57E-27	2.76	2.76
YKL081W	TEF4	Gamma subunit of translational elongation factor eEF1B	7.49E-04	9.14	50.20
YDR074W	TPS2	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex	8.87E-03	12.19	57.53
YDR207C	UME6	Rpd3L histone deacetylase complex subunit	2.61E-06	7.30	21.83
YOR106W	VAM3	Syntaxin-like vacuolar t-SNARE	4.68E-07	6.32	3.82
YOR270C	VPH1	Subunit a of vacuolar-ATPase V0 domain	2.00E-02	19.02	35.25
YHR012W	VPS29	Subunit of the membrane-associated retromer complex	5.45E-10	5.18	27.26
YDR080W	VPS41	Subunit of the HOPS endocytic tethering complex	4.43E-03	17.15	23.05
YDR290W	YDR290W	Dubious open reading frame overlaps RTT103	2.95E-03	11.27	47.45
YLR200W	YKE2	Subunit of the heterohexameric Gim/prefoldin protein complex	7.10E-21	3.66	-0.75

YLR184W	YLR184W	Dubious open reading frame adjacent to TOS4	4.05E-03	14.53	18.69
YMR085W	YMR085W	Putative protein adjacent YMR084W and are merged in related strains, and together forms a paralogous to GFA1	3.31E-04	9.27	152.12
YNL198C	YNL198C	Dubious open reading frame overlaps GCR2	3.17E-02	19.29	42.44
YNL296W	YNL296W	Dubious open reading frame overlaps MON2	1.07E-03	13.23	189.83
YOR082C	YOR082C	Dubious open reading frame overlaps WHI5	9.44E-04	13.85	193.95
YOR097C	YOR097C	Putative protein of unknown function	2.43E-03	11.78	33.27

Table 7.4.2b - **53 negative and 5 positive genetic interactions with *PNP1***. The growth of the double mutants relative to the single haploid parental strains (*pnp1ΔxxxΔ /pnp1Δ*)/ (*xxxΔ*/ WT) x 100 was determined in liquid media at 16h and OD⁶⁰⁰ for all cultures with the exception *xxxΔ* slow growers where the OD600 was measured at later time points. The growth of the double mutants was normalised to *pnp1Δhis3Δ* control strain and the double deletion mutants with an average growth > ± 40%, *p* <0.05 of the *pnp1Δhis3Δ* strain were considered a growth defect or a growth improvement.

ORF name	Gene name	Function	% Growth	<i>p</i> -value	Z-Score
YLR131C	ACE2	Transcription factor required for septum destruction after cytokinesis	53.76	2.99E-04	3.62
YOR128C	ADE2	Phosphoribosylaminoimidazole carboxylase	32.15	1.93E-11	6.71
YMR300C	ADE4	Phosphoribosylpyrophosphate amidotransferase (PRPPAT)	256.41	9.94E-09	-5.73
YGR061C	ADE6	Formylglycinamide-ribonucleotide (FGAM)-synthetase	153.85	7.70E-03	-2.66
YDR408C	ADE8	Phosphoribosyl-glycinamide transformylase	222.22	1.05E-06	-4.88
YMR282C	AEP2	Mitochondrial protein	60.61	3.42E-03	2.93
YPR060C	ARO7	Chorismate mutase	178.57	3.13E-04	-3.60
YNL315C	ATP11	Molecular chaperone	67.57	2.48E-02	2.24
YDR375C	BCS1	Protein translocase and chaperone required for Complex III assembly	61.35	4.42E-03	2.85
YDR254W	CHL4	Outer kinetochore protein required for chromosome stability	138.89	4.07E-02	-2.05
YGR157W	CHO2	Phosphatidylethanolamine methyltransferase (PEMT)	166.67	1.86E-03	-3.11
YOL008W	COQ10	Coenzyme Q (ubiquinone) binding protein	67.57	2.36E-02	2.26
YIR023W	DAL81	Positive regulator of genes in multiple nitrogen degradation pathways	250.00	3.81E-08	-5.50
YCR076C	FUB1	Proteasome-binding protein	69.93	4.14E-02	2.04
YJL165C	HAL5	Putative protein kinase	52.36	1.55E-04	3.78
YER057C	HMF1	Member of the p14.5 protein family	144.93	2.05E-02	-2.32
YGL168W	HUR1	Protein of unknown function	151.52	1.00E-02	-2.57
YOR135C	IRC14	Dubious open reading frame	65.36	1.45E-02	2.45
YDR475C	JIP4	Protein of unknown function	60.61	3.65E-03	2.91
YDR318W	MCM21	Component of the kinetochore sub-complex COMA	144.93	2.35E-02	-2.27
YOL126C	MDH2	Cytoplasmic malate dehydrogenase	69.93	3.83E-02	2.07

YDR336W	MRX8	Protein that associates with mitochondrial ribosome	136.99	4.89E-02	-1.97
YEL033W	MTC7	Protein of unknown function	136.99	4.96E-02	-1.96
YNL316C	PHA2	Prephenate dehydratase	196.08	3.28E-05	-4.15
YJL198W	PHO90	Low-affinity phosphate transporter	68.97	3.29E-02	2.13
YCR028C-A	RIM1	ssDNA-binding protein essential for mitochondrial genome maintenance	35.97	1.48E-09	6.05
YLR357W	RSC2	Component of the RSC chromatin remodelling complex	13.33	4.43E-33	11.98
YNL254C	RTC4	Protein of unknown function	149.25	1.20E-02	-2.51
YCR008W	SAT4	Ser/Thr protein kinase involved in salt tolerance	49.02	2.79E-05	4.19
YDR104C	SPO71	Meiosis-specific protein required for prospore membrane morphogenesis	138.89	4.33E-02	-2.02
YDR410C	STE14	Farnesyl cysteine-carboxyl methyltransferase	10.62	1.21E-40	13.35
YDR463W	STP1	Transcription factor	147.06	1.88E-02	-2.35
YKL081W	TEF4	Gamma subunit of translational elongation factor eEF1B	149.25	1.42E-02	-2.45
YHR025W	THR1	Homoserine kinase	12.14	4.41E-36	12.54
YDR074W	TPS2	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex	140.85	3.00E-02	-2.17
YDR007W	TRP1	Phosphoribosylanthranilate isomerase	37.74	8.76E-09	5.75
YER090W	TRP2	Anthranilate synthase	35.71	1.19E-09	6.08
YDR092W	UBC13	E2 ubiquitin-conjugating enzyme	138.89	3.96E-02	-2.06
YFR010W	UBP6	Ubiquitin-specific protease	43.10	6.92E-07	4.96
YOR106W	VAM3	Syntaxin-like vacuolar t-SNARE	45.66	3.79E-06	4.62
YDL077C	VAM6	Guanine nucleotide exchange factor for the GTPase Gtr1p	58.82	2.03E-03	3.09
YEL014C	YEL014C	Putative protein of unknown function	144.93	1.91E-02	-2.34
YER067C-A	YER067C-A	Dubious open reading frame overlaps RGI1	136.99	4.97E-02	-1.96
YGL218W	YGL218W	Dubious open reading frame	44.64	2.12E-06	4.74
YMR244W	YMR244W	Putative protein of unknown function	144.93	2.33E-02	-2.27
YOR139C	YOR139C	Dubious open reading frame overlaps IDH2, subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	144.93	2.24E-02	-2.28
YJL139C	YUR1	Mannosyltransferase involved in protein N-glycosylation	69.93	4.06E-02	2.05

Table 7.4.3 – **47 putative genetic interactions with *PNP1* gene in minimal media.** The *pnp1ΔxxxΔ* double mutants and the *xxxΔ* parental haploid mutants were pinned onto minimal media supplemented with the essential amino acids methionine, leucine and uracil using the RoToR HAD (Singer Instrument Co. Ltd) and were incubated for 5d at 30°C. The putative interactions with an epistatic growth defect ($(xxxΔpnp1Δ / xxxΔ \times 100) < 30\%$, $p < 0.05$) or improvement ($(xxxΔpnp1Δ / xxxΔ \times 100) > 130\%$, $p < 0.05$) in the *pnp1ΔxxxΔ* double mutants relative to the single mutants *xxxΔ* were determined using the Gitter package Gitter tools (Wagih & Parts, 2014) in RStudio v0.99.896 (Racine, 2012) and ScreenMill (Dittmar et al., 2010).