Mitochondrial Transfer in *Saccharomyces cerevisiae*

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Abstract

This thesis investigated mitochondrial transfer in *Saccharomyces cerevisiae*, between respiratory compromised B18p⁰ recipient and respiratory competent donor cells.

The respiratory compromised strain had three red fluorescent proteins tagged to the membrane, nucleus and cytoplasm (triple RFP-B18p⁰) and is referred to as the B18p⁰ strain. B18p⁰ cells did not contain mitochondrial DNA, causing it to be respiratory compromised and required a fermentable carbon source, such as glucose/dextrose, for proliferation. The respiratory competent strain used had a green fluorescent protein tagged to the Tom70 mitochondrial protein (Tom70-GFP) and is referred to as the Tom70 strain. The Tom70 cells contained the nuclear encoded URA3 cassette, allowing for negative selectivity of this strain using 5-FOA.

S. cerevisiae strains were co-cultured together in media containing only non-fermentable carbon sources (YPGE), plated on YPGE plates containing 5-FOA and colonies grown were distinguished post-co-culture based on their distinct phenotypic and genotypic characteristics. Fluorescent analysis of co-culture colonies revealed the presence of 5-FOA resistant Tom70 cells and some red B18p⁰ cells that had acquired the ability to grow on non-fermentable carbon sources. Genotypic analysis revealed that the majority of these red colonies had acquired mtDNA as well as the nuclear encoded, Tom70 specific URA3 cassette.

Several permutations of co-cultures were performed, using different ratios of recipient and donor cells and single-gene deletion donor cells.

Purified mitochondria from Tom70 cells were tried to be transferred into B18p⁰ cells using centrifugation forces to induce a higher occurrence frequency of mitochondrial transfer. Metabolic support experiments were conducted to investigate if the Tom70 strain could provide metabolic support to the B18p⁰ strain without mitochondrial transfer.

Results indicate that no permutation induced potential mitochondrial transfer at a higher rate than others. However, results indicate that mitochondrial transfer did occur at low frequencies, potentially through the fusion of respiratory competent and respiratory compromised cells. Forced transfer did not increase the occurrence frequency of B18p⁰ cells to take up mitochondria and Tom70 cells did not provide metabolic support to B18p⁰ cells.

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Chapter 1

Introduction

In the last few years a new paradigm has emerged in the field of mammalian metabolism: the ability of mitochondria to move between cells. This phenomenon has been reported both *in vitro* (reviewed in [2]) and *in vivo* [3, 4] in both cancer and normal cells and has been suggested to be a response to injuries that result in compromised mitochondrial function, including respiration [5-8]. Not much is known about the mitochondrial donors *in vivo*, or the processes and signals involved in intercellular mitochondrial transport. This thesis investigates whether or not intercellular mitochondrial transport also occurs between respiratory competent strains and incompetent p^0 strains of *Saccharomyces cerevisiae*.

1.1 Mitochondria and Energy Metabolism

Mitochondria are a central component of cellular metabolism. Mitochondria play a crucial role in cellular bioenergetics (catabolism) and biosynthesis (anabolism) [9]. Through fission and fusion, mitophagy and mitogenesis, mitochondria meet the ever-changing metabolic demands of their host cells [10, 11]. Cross-talk between the nucleus and mitochondria allows each cell to effectively work in and respond to changes in their environment and maintain bioenergetic and biosynthetic homeostasis. Mitochondria also facilitate cellular stress responses [9]. Dysfunctional mitochondria have been implicated in many neurodegenerative and neuromuscular diseases [12, 13], cancer [14] and aging [15, 16]. Loss of functional mitochondria disturbs cellular metabolic homeostasis and leads to loss of crucial bioenergetic and biosynthetic mitochondrial function [2].

To understand the role of mitochondria in energy metabolism it is important to briefly describe how mammalian cells obtain energy. Energy generation occurs through a mixture of glycolysis and oxidative phosphorylation (OXPHOS), with the balance between these two pathways dependent on substrate availability, presence of oxygen as well as the specific genetic and epigenetic profiles of the cells (reviewed in [17]).

Figure 1.1 shows an overview of the mammalian glucose metabolism, aerobic respiration and lactic acid fermentation.

Figure 1. 3: Mammalian glucose metabolism: Glucose enters the cell (large green oval) via glucose transporters (Glut1, 2 and/or 3) and is oxidized to pyruvate through glycolysis in the cytoplasm, producing 2 ATP molecules per glucose and two reducing agents in the form of NADH. Pyruvate is transported into the mitochondrion (yellow oval) through the mitochondrial pyruvate carriers (MPC1/2) where it is oxidized to acetyl CoA, generating 2 NADH. Acetyl CoA enters the tricarboxylic acid cycle (TCA) and is oxidized to CO₂, generating additional reducing agents (6 NADH/ glucose, 2 FADH₂/ glucose). NADH is shuttled between the cytoplasm and the mitochondria via the malate-aspartate shuttle or the glycerol-3-phosphate shuttle. The mitochondrial electron transport chain (blue oval, MET) re-oxidizes all reduced NADH and FADH₂ at the inner mitochondrial membrane. The final electron acceptor of MET is O₂, generating H₂O at respiratory complex IV. MET creates a proton gradient over the inner mitochondrial membrane which is used by ATP synthase (complex V) to produce ATP. When MET is compromised, for instance under anaerobic conditions or through oxidative damage, pyruvate is reduced to lactate, which is transported out of the cell through the monocarboxylate transporter, re-oxidizing the NADH generating through glycolysis (green arrows).

1.1.1 Glycolysis

Glycolysis is the first step in glucose metabolism and converts glucose into pyruvate, generating a small amount of energy in the form of two adenosine triphosphate (ATP) molecules per molecule of glucose through substrate phosphorylation (Figure 1.1). Glucose oxidation is accompanied by the reduction of nicotinamide adenine dinucleotide (NAD^+ to NADH) [17].

1.1.2 Tricarboxylic Acid Cycle

Complete oxidation of pyruvate occurs in the tricarboxylic acid (TCA) cycle located in the mitochondrial matrix (MM) (Figure 1.1). Under aerobic conditions, pyruvate is preferentially imported into the MM via the mitochondrial pyruvate carrier 1 and 2 where it is oxidized to acetyl coenzyme A (accompanied by the reduction of NAD^+ to NADH). Acetyl coenzyme A then enters the TCA cycle and combines with oxaloacetate to form citrate [18, 19].

The TCA cycle ultimately oxidizes each acetyl coenzyme A to two molecules of carbon dioxide, generating 3 NADH , 1 flavin adenine dinucleotides (FADH_2) and 1 GTP through substrate phosphorylation [20].

1.1.3 Mitochondrial Electron Transport

In the mammalian mitochondrial electron transport chain (MET), electrons are transferred from NADH to respiratory complex (RC) I (NADH :ubiquinone oxidoreductase) and from succinate (produced in the TCA cycle) to RC II (succinate dehydrogenase). Electrons from RC I and II are then transferred to ubiquinone (CoQ_{10}), RC III (cytochrome bc_1) and RC IV and finally to oxygen as the terminal electron acceptor [21].

1.1.4 Oxidative Phosphorylation

Movement of electrons through RCs I, III and IV is coupled to proton translocation from the MM to the intermembrane space. The resulting proton gradient forms the mitochondrial membrane potential and is used at RC V (F_1F_0 ATP synthase) to produce ATP via oxidative

phosphorylation (Figure 1.1). In theory, the maximum ATP yield resulting from complete oxidation of glucose through glycolysis, TCA cycle and OXPHOS was recently shown to be 33.45 ATP molecules per mol glucose [22].

1.1.5 Lactic Acid Fermentation

In the absence of oxygen, pyruvate is reduced to lactic acid by lactate dehydrogenase (LDH) which is transported out of the cell through the monocarboxylate transporter (Figure 1.1). Lactic acid fermentation does not generate ATP but does recycle NADH back to NAD⁺ to sustain continued glycolysis. Glycolysis and lactic acid fermentation in the presence of oxygen is called aerobic glycolysis, or the Warburg effect, first described by Otto Warburg in 1924 [23-26].

1.2 Energy Metabolism in Cancer Cells

A common feature of cancer cells is metabolic reprogramming, caused by changes in expression of oncogenes and tumour suppressor genes, as well as changes in the tumour microenvironment. Reprogramming of energy metabolism results in an increased dependence on glycolysis to generate energy. Increased aerobic glycolysis requires increased glucose uptake, glycolytic rates and increased lactate dehydrogenase (LDH) activity to support continued proliferation and meet bioenergetic and biosynthetic demands. Highly glycolytic cancers tend to be more aggressive and invasive [26-31]. Nonetheless, these cancer cells still use very low rates of OXPHOS in addition to high glycolytic rates to meet their energy requirements. The ability to easily adjust the balance between glycolysis and OXPHOS is a hallmark for many cancers [28, 32-36] and conveys a selective advantage as it supports uninterrupted growth in the presence of fluctuating oxygen levels due to compromised vascularity in solid tumours [2, 17, 23].

1.3 Mitochondrial Transfer in Mammalian Cells

Mitochondria are inherited through the maternal line and this process is known as vertical gene transmission and describes the movement of genetic material, such as mitochondrial DNA, from one organism to another (unicellular or multicellular).

The acquisition of mitochondria other than through uniparental inheritance has only recently been discovered. The detection of ultrafine inter-cellular structures in mammalian cells, so called tunnelling nanotubes (TNT's) in 2004 opened the door to a new area of research [37]. These TNT's are 50-200 nm in diameter and show a continuous connection of membranes and cytosol between host and donor cell. Via staining with LysoTracker dye, the movement of acidic organelles through TNT's between PC12 rat pheochromocytoma cells but also between rat kidney and human embryonic kidney cells was demonstrated [37].

Further studies have supported these findings. Researchers were able to demonstrate active mitochondrial transfer from human mesenchymal stem cells to A549 petite cells (human lung carcinoma cells, depleted of mitochondria), resulting in a rescue of aerobic respiration [5]. The number of studies that demonstrate intracellular mitochondrial trafficking has increased since the findings of Rustom et al. in 2004 and Spees et al. in 2006 (recently reviewed in [2]). A variety of cell culture systems and a variety of different cell types have been established to demonstrate that mitochondrial transfer may rescue cells with dysfunctional mitochondrial respiration [4, 5, 8, 17, 38].

Mammalian rhozero (ρ^0) cells deprived of mitochondrial DNA (mtDNA), which encodes key proteins of the electron transport chain. Without these proteins, respiratory complex I, III and IV do not assemble and cells are unable to conduct MET and thus OXPHOS and are characterized by loss of anchorage-independent growth, tumour formation and increased sensitivity to chemotherapy and radiation therapy [39-43]. Mammalian ρ^0 cells require supplementation with pyruvate and uridine in cell cultures for proliferation [43, 44]. Acquiring new mitochondria from donor cells through intercellular mitochondrial transfer has been shown to restore mitochondrial functions in mammalian cells *in vivo* [3, 4] and *in vitro* (reviewed in [2]). Tan *et al.* and Dong *et al.* showed that mitochondrial transfer resulted in restoration of respiration in 4T1 ρ^0 breast carcinoma cells [3] and in mouse melanoma B16 ρ^0 cells [4] which was required for tumour formation. In these experiments, B16 ρ^0 cells were injected subcutaneously into C57BL/6J mice and 4T1 ρ^0 cells were injected subcutaneously into Balb/c mice. The formation of a tumour was delayed by 2-3 weeks compared to tumour formation by 4T1wt and B16wt cells, and the resulting tumour cells contained host mouse mtDNA. In addition, co-culture of B16 ρ^0 cells with mesenchymal stem cells of the C57BL/6J mice strain also resulted in mitochondrial transfer via TNT's to the depleted cells [4].

Gap junctional channels have also been shown to be a possible mode of mitochondrial transfer. In a study conducted by Islam et al. [38], bone marrow-derived stromal cells (BMSCs)

were airway-instilled into mice after treatment with lipopolysaccharides (LPS), causing acute lung injury (ALI). Mitochondrial transfer from BMSCs to the alveolar epithelia cells occurred via micro-vesicles that were released into gap junctional channels. This led to an increase in ATP concentrations in the alveolar cells and protected against the LPS-induced ALI [38].

The discovery of intercellular mitochondrial transfer has opened the way for a wide range of exploratory studies. Besides genetic associations to phenotypes established in cancer, diseases, disorders and aging, mitochondrial dysfunctions are gaining importance as intercellular mitochondrial transfer may be a potential new therapeutic target. However, the underlying signals that induce and stimulate mitochondrial transfer, the actual process itself and mitochondrial donors *in vivo* are not well understood. Furthermore, questions such as whether or not mitochondrial transfer is a universal and conserved process, have yet not been answered. Using simple cell models to investigate this process in more detail may give new insights that may ultimately translate into new therapies for mitochondrial diseases, cancer and aging.

1.4 *Saccharomyces cerevisiae* as a Model Organism

S. cerevisiae has a number of attributes that makes it a good model organism for many metabolic studies.

1. Yeasts share many molecular properties with humans; despite their simplicity, many genes are conserved in higher eukaryotes, providing a framework for multicellular comparisons [45]. This gene conservation can be observed in cellular activities such as mitochondrial biogenesis, DNA replication, recombination, repair, metabolism and intracellular trafficking [46]. Since the whole genome of *S. cerevisiae* was sequenced in 1996, multiple studies have investigated the conserved functions between yeast and human genes [47]. Botstein et al. showed in 1997 that around 31% of protein-encoding genes in yeast have a mammalian homologs, supporting the model function of yeast for higher eukaryotes [48].
2. Yeasts have a very short multiplication rate of 90 minutes.

3. Gene mutation libraries of yeasts are available to test compounds and pathways. Each mutant has a single gene expression change, causing over- and under-expression, or gene deletion, resulting in no expression at all. Growth, or lack thereof, under these changed conditions, can give a very detailed insight about the affected pathway.
4. Yeasts are also experimentally tractable, are non-hazardous and have been used as a model organism for nearly a century [49]. They are not only used as a model for eukaryotic cell biology but are increasingly used as a model for studies of functional genomics and systems biology due to their ability to perform homologous recombination and it is easy to modify their gene expression.
5. Yeast cells can be haploid or diploid. The diploid cells possess the Mat a and Mat α mating type while the haploids are either Mat a or Mat α . Culturing yeast cells of opposite mating types allows them to fuse to create diploid cells; haploid cells of the same mating type cannot mate and undergo asexual reproduction [50].
6. *S. cerevisiae* laboratory strains do not have the homothallic (HO) locus. This locus precludes stable haploid strains in nature as an active HO locus allows mating type switching. Inactivation of this locus results in stable, heterothallic haploid strains that can be used to investigate genetic mechanisms and allow controlled crossing [50]. The *S. cerevisiae* strain that is predominantly used in laboratories is the strain S288C, a strain isolated from rotting Californian figs [51]. It is naturally heterothallic and extensive literature is available for characteristics of this strain.

1.4.1 Energy Metabolism in *S. cerevisiae*

S. cerevisiae has more options when it comes to generating energy compared with mammalian cells (Figure 1.2). Like mammalian cells, the oxidation of glucose starts with glycolysis in the cytoplasm, followed by the TCA cycle in the mitochondrial matrix. In the mitochondrial electron transport chain, RCI is replaced by two external NADH dehydrogenases that receive electrons from cytosolic NADH (Nde1 and Nde2) and the internal NADH dehydrogenase which receives electrons from the mitochondrial matrix (Ndi1) [52-54]. Nde1/2 and Ndi1 do not translocate protons and only transfer electrons to the ubiquinone pool [52].

The mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase (Gut2p) also transfers electrons to the ubiquinone pool cytosolic from glycerol – 3 – phosphate. Electrons are then transferred via ubiquinone (CoQ6), cytochrome bc1 complex, cytochrome C and cytochrome c oxidase to oxygen as the final electron acceptor [52]. ATP synthase utilizes the proton gradient to produce ATP [52].

In *S. cerevisiae*, excess glucose results in additional pyruvate that cannot be shuttled across the inner mitochondrial membrane (IMM) [55]. This pyruvate is converted into acetaldehyde in a process known as the Crabtree effect [56, 57]. Acetaldehyde can be shuttled across the inner mitochondrial membrane where it is converted to ethanol via the mitochondrial alcohol dehydrogenase (Adh3p) or converted into acetate which can be converted into acetyl coenzyme A to enter the TCA [58, 59]. Ethanol is shuttled across the inner mitochondrial membrane and can also be converted into acetaldehyde by the cytosolic alcohol dehydrogenase (Adh1p/2p) [58] (Figure 1.2). In the absence of glucose or under anaerobic conditions, ethanol can be used as a carbon source to fuel the TCA through the conversion to acetyl Coenzyme A via acetate and acetaldehyde (Figure 1.2). Another alternative carbon source in the absence of glucose and oxygen is glycerol. Glycerol-3 phosphatase converts glycerol to glycerol – 3 – phosphate in the cytosol [60] which is used in the glycerol – 3 – phosphate shuttle to produce dihydroxy – acetone – phosphate (DHAP) via glycerol – 3 – phosphate dehydrogenase (Gut2p) [61]. Gut2p donates electrons to the ubiquinone pool of OXPHOS with concurrent re-oxidation of FAD^+ to FADH_2 [61]. DHAP is supplied to the shuttle via glycolysis when glucose is present. DHAP can be converted to glycerol – 3 – phosphate via the cytosolic glycerol – 3 – Phosphate dehydrogenase (Gpd1/2) [62] (Figure 1.2).

1.4.1.1 NADH Recycling

The intermediate electron acceptor, NAD^+ plays a crucial role in glycolysis and re-oxidation of NADH is therefore necessary to sustain continued glycolysis [63]. In mammalian cells, NADH is recycled predominantly via mitochondrial electron transport and lactate dehydrogenase.

S. cerevisiae has five strategies to recycle NADH [56, 58, 59, 64, 65]:

- Cytosolic NADH is re-oxidized via complex I (Nde1/2)
- Mitochondrial NADH is re-oxidized via complex I (Ndi1)
- Conversion of acetaldehyde to ethanol in the mitochondrial matrix re-oxidizes NADH
- Conversion of DHAP to Glycerol – 3 – Phosphate re-oxidizes NADH in the cytosol
- Conversion of Glycerol – 3 – Phosphate to glycerol is a cytosolic net NADH – oxidizing process

1.4.2 *S. cerevisiae* Mitochondrial Genome

Mitochondria have highly specialized functions. Their involvement in respiration and biosynthesis is evident in many cellular functions, from pyrimidine and amino acid synthesis or the synthesis of fatty acids, cholesterol and phospholipids to the oxidation of glycolysis products in the oxidative phosphorylation electron transport chain [9]. The human mitochondrial genome is a double-stranded, closed-circular molecule of 16,569 base pairs and contains 2 origins of replication and 37 genes encoding 13 polypeptides that encode respiratory complex subunits, 22 transfer RNAs (tRNAs) and the 12S and 16S ribosomal RNA (rRNA) genes for mitochondrial protein synthesis [47, 48, 66, 67]. Nuclear DNA encodes about 1500 proteins which contribute to the mitochondrial proteome. These n-mitoproteins include the additional respiratory complex subunits and enzymes of the TCA cycle, amino acid, nucleic acid and lipid biosynthesis, mtDNA and RNA polymerases, transcription factors, ribosomal proteins in addition to all components of DNA repair pathways [29].

Figure 1. 5 Mitochondrial genome of *S. cerevisiae*: The yeast mitochondrial genome has over 85,000 base pairs, contains seven origins of replication and 34 genes. Eight of these genes code for proteins that make up the structures of the electron transport chain. Figure adapted from [1].

The yeast mitochondrial genome is also a circular double stranded molecule but is larger than the human mtDNA, comprising 85,800 base pairs, with seven origins of replication and 34 genes. These encode 8 structural genes: ATP synthase subunits 6, 8 and 9 (atp6, atp8, atp9), cytochrome C-oxidase subunits 1, 2 and 3 (cox1, cox2, cox3), apocytochrome b (cytb), as well as two ribosomal proteins, 24 transfer RNAs and some intron-related open reading frames (ORFs) [68] (Figure 1.3).

Although the genes encoded by the mtDNA in both organisms are for similar subunits of the mitochondrial electron transport chain, the scaffold of their mtDNA is very different, emphasizing the complexity of single cellular organisms that is often understated. However, the similarities between the yeast and human mtDNA and nuclear DNA can be used to help identify human genes involved in or contributing to mitochondrial disease and disorder phenotypes [69]. If *S. cerevisiae* can be shown to undergo mitochondrial transfer, then genome-wide genetic approaches could be used to identify genes involved in mitochondrial transfer, and to understand the underlying regulatory mechanisms in *S. cerevisiae*. This in turn may give us a better insight into this process in cancer, increasing our knowledge of cancer genetics and chemotherapy resistance.

1.4.3 *Saccharomyces cerevisiae* rho zeros

Respiratory competent *S. cerevisiae* cells are often referred to as “grandes”. These cells form large colonies when grown on complete yeast medium (containing yeast extract, peptone and glucose) as well as on non-fermentable carbon sources such as ethanol or glycerol.

S. cerevisiae petites are respiratory compromised mutants. They retain some of their mtDNA; even though they still contain mitochondria that are biosynthetically functional, the mitochondria are morphologically deformed and swollen, and not part of networks [70, 71].

S. cerevisiae cells that are completely devoid of mitochondrial DNA are respiratory deficient and are called “rho zeros” (p^0). These p^0 cells form small colonies when grown on complete yeast medium and cannot grow at all on non-fermentable carbon sources such as glycerol or ethanol. This phenotype can be caused by mutation in, or loss of, mitochondrial DNA or nuclear genes involved in mitochondrial respiration [72, 73].

Respiratory deficient *S. cerevisiae* cells arise spontaneously in nature. As with mammalian p^0 cells [43], yeast p^0 cells can also be created through exposure to ethidium bromide (EtBr).

This phenanthridine dye preferentially binds to mitochondrial DNA, where it inhibits mtDNA replication and disrupts mitochondrial protein expression, without having much effect on nuclear DNA. The preference for mtDNA results from a more permeable mitochondrial membrane and less complex DNA structures compared to nuclear access and complexity [70, 74, 75]. As cells continue to divide during treatment with this dye (25 µg/ mL for 3 days), the remaining mtDNA is diluted out, and respiratory competent *S. cerevisiae* cultures will convert into respiratory deficient cultures.

1.5 Research Aim and Objectives

Intercellular mitochondrial transfer has never been investigated in *S. cerevisiae*. If mitochondria do travel between yeast cells, the mechanism behind it may be very different to mammalian cells, because the cell wall of yeast may prevent formation of tunnelling nanotubes or vesicles. It is also possible that this is a phenomenon only found in multicellular organisms. However, any information about this process in a simple unicellular eukaryotic model may be very helpful in answering questions pertinent to mammalian mitochondrial transfer, particularly in dissecting the signals and processes involved. This thesis therefore aims to determine the feasibility of using *S. cerevisiae* as a model to study intercellular mitochondrial transfer.

1.5.1 Aim

The research conducted for this thesis aims to investigate whether or not intercellular mitochondrial transport occurs between respiratory competent strains and incompetent ρ^0 strains of *Saccharomyces cerevisiae*.

The ρ^0 model represents an extreme form of respiratory injury and I hypothesize that this is the most likely scenario to provoke intercellular mitochondrial transfer.

1.5.2 Objectives

The aim is addressed through three main objectives:

Objective 1: Validation of mitochondrial donor and recipient strains and establishment of a system that allows for the detection of mitochondrial transfer. The mitochondrial acceptor strain will need to have its mitochondrial DNA removed (ρ^0) and the mitochondrial donor strain will need to have functional mitochondria and be able to be negatively selected against without harming the acceptor strain.

Objective 2: Determine whether or not mitochondrial transfer takes place between mitochondrial donor and recipient strains, by co-culturing both strains under different conditions.

Objective 3: Investigating whether or not mitochondrial transfer can be forced between mitochondrial donor and recipient strains, using purified mitochondria and investigating whether or not a mitochondrial donor strains can provide metabolic support to a respiratory compromised strain without mitochondrial transfer.

Chapter 2

Materials and Methods

2.1 Materials

All materials, including reagents, consumables and machines, are listed in Table 2.1 in accordance with their sourcing information.

Table 2. 10 Materials, reagents, consumables and machines

Material	Sourcing
1 kb+ DNA Ladder	Life Technologies, Auckland, NZ
2,3,5 – Triphenyltetrazolium chloride	Sigma-Aldrich, Auckland, NZ
96-well tissue culture plates	Interlab, Wellington, NZ
Acid-washed glass beads (0.45-0.52mm)	DNAture Ltd, Gisborne, NZ
Agar	Life Technologies, Auckland, NZ or Formedium, Norfolk, UK
Agarose (low electroendosmosis)	Medi'Ray, Auckland, NZ
Alpha Imager mini transilluminator	Alphatech Systems, Auckland, NZ
Amino acid powder stocks	Sigma Aldrich, Auckland, NZ or Formedium, Norfolk, UK
Amino acids	Sigma Aldrich, Auckland, NZ or Formedium, Norfolk, UK
BioReady rTaq 10x reaction buffer	Bulldog Bio, New Hampshire, UK
BioReady rTaq Polymerase	Bulldog Bio, New Hampshire, UK
Boric acid	Thermo Fisher Scientific, Auckland, NZ
Bromophenol blue	Sigma Aldrich, Auckland, NZ
Bovine Serum Albumin (BSA)	Thermo Fischer Scientific, Auckland, NZ
Canon Eos 600D camera	Canon NZ Ltd., Wellington, NZ
Carbonyl cyanide <i>m</i> -chlorophenyl hydrazone (CCCP)	Sigma-Aldrich, Auckland, NZ
Chloroform	Sigma Aldrich, Auckland, NZ
Deoxynucleotide (dNTP) mix	Thermo Fisher Scientific, Auckland, NZ
Dithiothreitol (DTT)	Sigma Aldrich, Auckland, NZ
DMSO	Sigma Aldrich, Auckland, NZ
Electrophoresis gel tank	Gentaur, Paris, FR

Elmer Perkin OPERA high throughput microscope	Perkin Elmer, Waltham, MA, USA
EnVision 2102 Multilabel Plate Reader	Perkin Elmer, Waltham, MA, USA
Eppendorf Centrifuge 5415 R	Sigma Aldrich, Auckland, NZ
Eppendorf Centrifuge 5810	Sigma Aldrich, Auckland, NZ
Ethanol (absolute, >99%)	Pure Science Ltd., Porirua, NZ
Ethidium bromide	Thermo Fisher Scientific, Auckland, NZ
Ethylenediaminetetraacetic acid (EDTA)	AppliChem, Darmstadt, Germany
E.Z.N.A. Plasmid Mini Kit II	Omegabiotek, Queenstown, NZ
Falcon tube (15 mL)	Interlab, Wellington, NZ
Falcon tube (50 mL)	Interlab, Wellington, NZ
Glucose (powder, 100%)	Sigma Aldrich, Auckland, NZ
Glycerol	Thermo Fisher Scientific, Auckland, NZ
Hank's Buffered Salt Solution (HBSS)	Thermo Fisher Scientific, Auckland, NZ
Hydrochloric acid (HCl)	Sigma Aldrich, Auckland, NZ
IN Cell Analyzer 6500 high throughput microscope	GE Healthcare, Life Sciences, Germany
Kapa 2G Fast PCR Ready Mix (Hotstart)	Sigma Aldrich, Auckland, NZ
Lithium acetate	Sigma Aldrich, Auckland, NZ
Microcentrifuge tubes (1.5 mL)	Interlab, Wellington, NZ
Monosodium glutamate salt (MSG)	Sigma Aldrich, Auckland, NZ
Morpholinepropanesulfonic acid (MOPS)	Sigma Aldrich, Auckland, NZ
Nitrogen base	Formedium, Norfolk, UK
pAG60 plasmid (URA3 from <i>C. albicans</i>)	Addgene, Waltham, MA, USA
PCR tube (0.2 mL)	Interlab, Wellington, NZ
Peptone	Fort Richard, Auckland, NZ or Formedium, Norfolk, UK
Perkin Elmer CellCarrier-384 Ultra Microplates, tissue culture treated	Sigma Aldrich, Auckland, NZ
Polyethersulfone (PES) membrane filter (0.22 and 0.45µm)	Interlab, Wellington, NZ
Polyethylene glycol (PEG)	Sigma Aldrich, Auckland, NZ
Potassium acetate	Sigma Aldrich, Auckland, NZ
Potassium hydroxide (KOH)	Sigma Aldrich, Auckland, NZ
Primers	Integrated DNA Technologies, SG
Salmon sperm DNA	Sigma Aldrich, Auckland, NZ
Singer plates	Singer Instrument Co. Ltd., Somerset, UK
Singer RoToR HDA	Singer Instrument Co. Ltd., Somerset, UK
Sodium acetate (NaOAc)	Sigma Aldrich, Auckland, NZ

Sodium chloride (NaCl)	Sigma Aldrich, Auckland, NZ
Sodium dodecyl sulfate (SDS)	Life Technologies, Auckland, NZ
Sodium hydroxide (NaOH)	VWR Global Science, Auckland, NZ
Sulfuric acid (H ₂ SO ₄)	Sigma Aldrich, Auckland, NZ
Suspension Mixer/ Rotator	Digestion Laboratory Instruments Int., SanFran, USA
Talboys standard vortex mixer	Bio-strategy Ltd., Auckland, NZ
Transonic T460 Sonicating water bath	VWR Global Science, Auckland, NZ
Tris-(hydroxymethyl)-aminomethane base	Formedium, Norfolk, UK
Triton X-100	VWR Global Science, Auckland, NZ
Tryptone	Acumedia, Michigang, USA
UNICAM 8625 UV/VIS spectrophotometer	Thermo Fisher Scientific, Auckland, NZ
P Selecta Precistem 5L 100°C waterbath	Group Selecta, Barcelona, SP
WST-1	Sigma-Aldrich, Auckland, NZ
Yeast extract	Formedium, Norfolk, UK
Yeast Nitrogen bases without amino acids or ammonium sulphate	Fort Richard, Auckland, NZ or Formedium, Norfolk, UK
Zymolyase 100T	Zymo Research, Ngaio Diagnostics Ltd., Nelson, NZ

2.2 Yeast strains

All *Saccharomyces cerevisiae* strains used were derived from S288C and are listed in Table 2.2. Strains with a yCG number were generated by Dr. David Maass and the strain B18 wildtype was generated by Dr. Remy Schneider, Chemical Genetics Laboratory within the School of Biological Sciences at the Victoria University of Wellington. Strains were maintained in YPD glycerol stocks at -80°C. The BY4741 strain was obtained from the Boone library [76], the Tom70 strain and the Cox6 strain came from the Yeast-GFP Clone Collection from UCSF [77].

Table 2. 11 *S. cerevisiae* strains

Strain	Mating type	Genotype	Description
BY4741	<i>MAT a</i>	<i>kan leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 hoΔ0</i>	wildtype
yCG227	<i>MAT a</i>	<i>kan leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 hoΔ0</i>	BY4741 transformed with MRH1-egFP611, a red fluorescent protein (RFP) with a localisation to the cellular membrane.
yCG251	<i>MAT alpha</i>	<i>can1Δ::STE2pr-URA3 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hoΔ0</i>	Strain containing a NLS-RedStar2-HPH, a RFP linked with a nuclear localisation signal (NLS) and with the hygromycin dominant selectable marker.
yCG253	<i>MAT alpha</i>	<i>can1Δ::STE2pr-Sp_URA lyp1Δ::mCherry-Nat his3Δ1 leu2Δ0 ura3Δ0::NLS-RedStar2-HPH LYS2+ hoΔ0</i>	yCG251 transformed with NATMX4-TEFpr-mCherry; double positive RFP with localisation to the nucleus and cytosol.
B18 wildtype	<i>Mat a</i>	<i>kan leu2Δ0 his3Δ1 ura3Δ met15Δ0 can1Δ::STE2pr-Sp_URA lyp1Δ::mCherry-Nat leu2Δ0 ura3Δ0::NLS-RedStar2-HPH LYS2+ hoΔ0</i>	yCG253 mated with yCG251; triple positive RFP with localisation to the membrane, nucleus and cytosol.
Tom70	<i>Mat a</i>	<i>ura3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+ hoΔ0</i>	Tom70-GFP-HIS6; strain transformed with the pAG60 plasmid containing URA3 cassette. Strain contains a green fluorescent protein (GFP) with

			localisation to the mitochondrial membrane.
Cox6	<i>Mat a</i>	<i>ura3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i> <i>LYS2+ hoΔ0</i>	Cox6-GFP-HIA6 strain; Strain contains a green fluorescent protein (GFP) with localisation to the mitochondrial membrane.
Gem1	<i>Mat a</i>	<i>ura3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i> <i>LYS2+ hoΔ0</i> <i>gem1 Δ::URA3</i>	Cox6-GFP-HIS6, strain transformed with the pAG60 plasmid containing URA3 cassette. Strain contains a green fluorescent protein (GFP) with localisation to the mitochondrial membrane.
YPT11	<i>Mat a</i>	<i>ura3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i> <i>LYS2+ hoΔ0</i> <i>ypt11 Δ::URA3</i>	Cox6-GFP-HIS6, strain transformed with the pAG60 plasmid containing URA3 cassette. Strain contains a green fluorescent protein (GFP) with localisation to the mitochondrial membrane.
DDI1	<i>Mat a</i>	<i>ura3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i> <i>LYS2+ hoΔ0</i> <i>ddi1 Δ::URA3</i>	Cox6-GFP-HIS6, strain transformed with the pAG60 plasmid containing URA3 cassette. Strain contains a green fluorescent protein (GFP) with localisation to the mitochondrial membrane.

2.3 Yeast Media

Yeast cells were grown and cultured in YPD or SD under non-selective conditions, static at 30°C when grown on agar or shaking at 30°C when grown in liquid.

Media containing yeast (Extract or Nitrogen bases), peptone, amino acids and MSG were autoclaved before the addition of glycerol, ethanol, glucose or agar which were all autoclaved separately. Medium was additionally filter sterilised after addition of 5-Fluoroorotic Acid.

The different types of media used in this study are listed in Table 2.3. Percentages represent weight per volume.

Table 2.3 Media

Media	Components	
Yeast Peptone Dextrose (YPD) medium	Yeast extract	1%
	Peptone	2%
	Adenine	0.012%
	Glucose	2%
	Agar (if relevant)	2%
Synthetic Complete medium (SC)	Yeast Nitrogen Bases (without amino acids or ammonium sulphate)	0.17%
	Monosodium glutamate salt (MSG)	0.1%
	Complete amino acid mixture*	0.2%
	Glucose	2%
	Agar (if relevant)	2%
Yeast Peptone Glycerol Ethanol (YPGE) medium	Yeast extract	1%
	Peptone	2%
	Adenine	0.012%
	Glycerol	3%
	Ethanol	3%
	Agar (if relevant)	2%
Yeast Peptone Glycerol Ethanol (YPGE) supplemented with 5-Fluoroorotic Acid (5-FOA)	Yeast extract	1%
	Peptone	2%
	Adenine	0.012%
	Glycerol	3%
	Ethanol	3%
	5-FOA	0.2%
	Agar (if relevant)	2%
Lysogeny broth (LB) medium	Yeast extract	0.5%
	Tryptone	1%
	NaCL	0.5%
	Agar (if relevant)	2%

* Complete amino acid mixture: 3 g adenine, 2 g uracil, 2 g inositol, 0.2 g para-aminobenzoic acid, 2 g alanine, 2 g arginine, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g isoleucine, 10 g leucine, 2 g lysine, 2 g methionine, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tyrosine, 2 g tryptophan, and 2 g valine (55.2 g total).

2.4 Yeast Transformation

A single colony of the strain to be transformed was inoculated into 2mL liquid YPD medium overnight in a Lab suspension rotator (Digestion Laboratory Instruments Int., San Francisco, CA) at 30°C and 200 revolutions per minute (rpm) to an OD₆₆₀ of 0.4 to 0.6. Cultures were diluted to an OD₆₆₀ of 0.15 to 0.2 in 50mL YPD and grown shaking at 30°C to an OD₆₆₀ of 0.5 to 0.7. Cells were pelleted by centrifugation in an Eppendorf 5810 Centrifuge (Sigma Aldrich, Auckland, NZ) for 5min at 3000rpm and washed twice with ddH₂O. Then, the supernatant was removed and the pellet resuspended in 1mL lithium acetate (LiAc). The transformation mix was made consisting of 33.3% w/v polyethylene glycol (PEG), 100μM lithium acetate, 27.8 mg/mL denatured salmon sperm DNA, 50μL of the plasmid PCR product and 100μL of competent cells (cells that were resuspended in 1mL lithium acetate) and the final volume was made up to 1mL with ddH₂O. Transformant mixtures were vortexed for 30 sec, then incubated in a suspension rotator at 30°C for 30min, followed by an incubation at 42°C for 30min. Transformant cultures were spun down using an Eppendorf 5415R table top centrifuge (Sigma Aldrich, Auckland, NZ) for 5min at 7000rpm. Supernatant was pipetted off carefully and pellet was resuspended in 4mL YPD, incubated in a suspension rotator at 30°C for 2h, centrifuged at 3000g for 5min and resuspended in 100μL ddH₂O. Next, 100μL were plated on selective media (uracil deficient) and plates were incubated at 30°C for 2 days.

2.5 Plasmid Mini-Prep

E.coli containing the paG60 plasmid were streaked from frozen stock on LB agar plates and were grown overnight at 37°C. A single colony was suspended in 3mL liquid LB medium and grown overnight in a suspension rotator at 30°C and 200rpm. The plasmid was isolated using the E.Z.N.A. Plasmid Mini Kit II (cat. Number D6945-01) according to manufacturer's instructions. The presence of purified plasmid was confirmed by PCR.

2.6 Growth Rates

In order to determine growth rates, strains were grown in liquid SC overnight for 12 hours at 30°C with shaking in a Lab suspension rotator at 200 revolutions per minute (rpm). The OD₆₆₀ was determined by a 1:10 dilution of cell suspension in distilled water using the UNICAM 8625 UV/VIS spectrophotometer (Thermo Fischer, Auckland, NZ) to establish the concentration of the overnight cultures. All strains were then diluted with liquid SC to obtain a concentration of 5×10^5 cells (lab standard to minimise variation that occurs with lower cell concentrations). Then, 200µl of each of the diluted cultures were added in octuplets to a 96-well plate and then read in the PerkinElmer microplate plate reader (PerkinElmer Inc, Waltham, MA, USA) at 30°C for 48h.

2.7 Generation of the B18 ρ^0 Strain

The B18 wildtype strain was treated with Ethidium Bromide (25 µg EtBr/ mL SC media) and incubated shaking at 30°C for three days to remove mitochondrial DNA and create the ρ^0 phenotype like previously described [78]. Absence of aerobic respiration was verified by lack of growth on YPGE medium.

2.8 Viability

Yeast were grown in 3mL SC in the suspension rotator for 16h at 30°C. Cultures were spun down for 3min at 3000rpm and the supernatant discarded. The pellet was resuspended in 3mL PBS and the centrifugation was repeated. The cell pellets were the resuspended in 3mL of YPGE and the OD₆₆₀ was read to establish the concentration at time 0 before re-incubation at 30°C in the suspension rotator at 200rpm. At time intervals of 48h, samples were streaked on SC media, incubated at 30°C and grown colonies counted.

2.9 Fluorescent Imaging

Fluorescence was measured using either the Elmer Perkin OPERA high throughput light microscope (PerkinElmer Inc, Waltham, MA, USA) or the IN Cell Analyzer 6500 high throughput light microscope (GE Healthcare, Life Sciences, Germany) as follows.

Green fluorescence was excited with the 488nm laser at 1800uW, and emission at 509nm wavelength captured with an exposure of 200ms. Red fluorescence was excited using the 561nm laser at 1000uW and emission at 588nm captured with 200ms exposure.

Image J (<https://imagej.nih.gov/ij/download.html>) was used to create colour composite images with the green signal in channel one and the red signal in channel two.

2.10 5-FOA Selectivity

A stock of 150mg 5-Fluoroorotic acid (5-FOA) was created by dissolving 150 mg/mL 5-FOA in DMSO. To establish the lowest effective concentration of 5-FOA to selectively kill strains containing the URA3 cassette, 5-FOA was added to SC in the range of 0.5 mg/mL to 3.5 mg/mL in 0.5 mg/mL increments. All strains were grown in SC overnight to an OD₆₆₀ of 0.4-0.6. The cultures were spun down for 3min at 3000rpm using an Eppendorf 5415R table top centrifuge (Sigma Aldrich, Auckland, NZ). The supernatant was discarded, and the cells were resuspended in 3mL of distilled water (ddH₂O) and the washing step was repeated. The strains were resuspended in 3mL SC and were then diluted until a concentration of 5×10^7 cells were obtained. Then, 200µl of each of SC/5-FOA concentrations and 2µL of each of the cultures were added in quadruplets to a 96-well plate to obtain a starting concentration of 5×10^5 cells/well and the OD₆₆₀ was read in the microplate plate reader at 30°C every 60min for 27h.

2.11 Co-Culture Experiments

The Tom70 strain and B18p⁰ cells were grown in 3mL liquid SC at 30°C to an OD₆₆₀ of 0.4 to 0.6. Cultures were pelleted by centrifugation, supernatant was discarded and re-suspended in 3mL YPGE. This washing step was repeated twice, and the final pellets were re-suspended in YPGE to a concentration of 5.3×10^7 cells/mL.

For co-cultures, cells from both cultures were combined in at specified ratios, in a 15mL falcon tube to a volume of 3mL and incubated at 30°C in a Lab suspension mixer (Digestion Laboratory Instruments Int., San Francisco, USA) at 200rpm. Samples were streaked onto YPGE plates containing 5-FOA as described. As controls for selectivity, Tom70 and B18p⁰ cells were grown on their own under the same conditions as the co-cultures.

2.12 Genomic DNA extraction

Yeast cells were grown for 20 to 24h in liquid SC medium to an OD₆₆₀ of 0.4 to 0.6. An aliquot of 1.5mL was transferred into a microcentrifuge tube containing 0.3g glass beads (0.52 mm, DNAture, NZ), 200µL Yeast DNA breaking buffer (2% Triton X-100, 10% sodium dodecyl sulphate, 100mM NaCl, 10mM Tris-HCL (pH 8.0), 1mM Ethylenediaminetetraacetic acid (EDTA, pH 8.0)) and 200µL phenol/chloroform (ratio 1:1). The tube was then vortexed on a multi-Eppendorf rack attached to a Talboy standard vortex mixer (Biostrategy Ltd, Auckland, NZ) for 6min and centrifuged for 5min using a tabletop centrifuge at 13200rpm. The aqueous phase was transferred to a new tube containing 1mL 95% ethanol and 40µL NaOAc, was inverted by hand 5 times and centrifuged for 5min at 13,200rpm. The supernatant was removed, and the pellet was air dried before re-suspension in 40µL 1X TE buffer (10mM Tris-HCL (pH 8.0), 1mM EDTA (pH 8.0)). DNA was incubated in a water bath (P Selecta Precistem 5L, Group Selecta, Barcelona, SP) at 65°C for 10 minutes to aid dissolution before storing at -20°C.

2.13 Concentration, Yield and Purity of Extracted Genomic DNA

Absorbance readings of the extracted DNA were performed using a spectrophotometer at 260 nm (A_{260}), 280 nm (A_{280}) and 320 (A_{320}) nm. The DNA concentration was estimated using the following equation:

$$\text{Concentration } (\mu\text{g/mL}) = (A_{260} \text{ reading} - A_{320} \text{ reading}) \times \text{dilution factor} \times 50 \mu\text{g/mL}$$

The total yield was measured by multiplying the DNA concentration by the total volume of the purified sample, using the following equation:

$$\text{DNA yield } (\mu\text{g}) = \text{DNA concentration} \times \text{total sample volume (mL)}$$

DNA purity was evaluated using the following equation:

$$\text{DNA purity } (A_{260} / A_{280}) = (A_{260} \text{ reading} - A_{320} \text{ reading}) / (A_{280} \text{ reading} - A_{320} \text{ reading})$$

A A_{260} / A_{280} ratio of 1.7 to 2.0 is indicative of good quality DNA while ratios outside this range indicate the presence of contaminants.

2.14 Primers

Primers were designed based on sequence information obtained from the *Saccharomyces* Genome Database (SGD) and checked with BLAST primer search and Primer3. Primers were suspended in 1x TE to a stock concentration of 100µM and stored at -20°C. Primer sequences highlighted in grey represent paG60 specific sequences used to amplify the URA3 cassette of the plasmid/transformants. Information about the general primers used in this study are listed in Table 2.4 and gene-deletion and confirmation primers in Table 2.5.

Table 2. 4 General Primers

Primer name	Sequence (5' -> 3')	Predicted product size
General primers		
COX3 forward (sc_Cox3_F)	TAGGTGCATGTTGACCACCC	128 bp
COX3 reverse (sc_Cox3_R)	ACCTGCGATTAAGGCATGATGA	
URA3 forward (URA3_paG60)	ATGCCTCACCAGTAGCACAG	536bp
URA3 reverse (URA3_paG60)	CACCCATATCACGTTGGGCA	
Mating loci (sc_Mat_loci_F)	AGTCACACTAAGATCGTTTATGG	
Mat alpha (sc_Mat_alpha_R)	GCACGGAATATGGGACTACTTCG	404bp
Mat a (sc_Mat_a_R)	ACTCCACAAGTAAGAGTTTG	534bp

Table 2. 5 Transformation specific primers

Transformation specific primers		
Gene-deletion primers Gem1 forward	TAGAAATGCAACACTTCCCTAATATAGAAATTTGGGCATTAATTATG GAGGCCCCAGAATACCCT	
Gene-deletion primers Gem1 reverse	AAAAATAGCGGACTTCTAAATACTAATGTGTTGAACAACACAAAGTA TAGCGACCAGCATTAC	
Gene-deletion primers YPT11 forward	GTTTCATCATGGTGGCACGGTTTTGAATCCTGGGTTGGGCATAATGA TGGAGGCCCCAGAATACCCT	
Gene-deletion primers YPT11 reverse	ACAATGGCTGCCTGCGAATCTTGTTGTATAATTTGTGAAGACTAA GTATAGCGACCAGCATTAC	
Gene-deletion primers DDI1 forward	ACAAAGTACATACCAACATAACAGCAAAAATATACGTAAAGATGAT GGAGGCCCCAGAATACCCT	
Gene-deletion primers DDI1 reverse	TGGGCTACATACGTAGAGGCCGATCACAATATCAGTGGTTGCTCAA GTATAGCGACCAGCATTAC	
Gem1 transformation confirmation primer	A	ATCACCCCTTTGAAGGTTTCTTGC
	D	TCATACGTTTATAAGGGACCAAAAA

YPT11 transformation confirmation primer	A	GAAGAGAAAGAAGAAAGGAGCGTAT
	D	TCAATATAGCCGAAACCAAATAAA
DDI1 transformation confirmation primer	A	TAGAGGTCGAGACCCTAATTCTTTT
	D	ATCACCCCTTTGAAGGTTTCTTGC
Transformation confirmation UR3 internal forward primer B		AATTCAACGCGTCTGTGAGG
Transformation confirmation URA3 internal reverse primer C		GACACCTGGAGTTGGATT

Expected product sizes for the confirmation of gene-deletion transformations are found in Table 2.6.

Table 2. 6 Expected gene-deletion product sizes

Deleted gene	Confirmation primers	Expected product size
Gem1	Primers AB	343bp
	Primers CD	605bp
YPT11	Primers AB	609bp
	Primers CD	622bp
DDI1	Primers AB	578bp
	Primers CD	734bp

2.15 Polymerase Chain Reaction (PCR)

PCRs were performed in 0.2mL PCR tubes with a total volume of 25µL. The components of the PCR for Bioready rTaq polymerase are listed in Table 2.7. The components of the PCR for the Kapa 2G Fast Polymerase are listed in Table 2.8. The cycle profile for the BioReady rTaq Polymerase and Kapa 2G Fast are displayed in Table 2.9. Kapa 2G extension times varied, depending on the amplification length.

Table 2. 7 PCR Reaction Mix Components (BioReady)

PCR Reaction Mix Components	Volume (µL)
ddH ₂ O	17.25
dNTP mix	2
10 x BioReady rTaq buffer	2.5
Forward primer (10µM)	1
Reverse primer (10µM)	1
BioReady rTaq Polymerase	0.25
Template DNA	1

Table 2. 8 PCR Reaction Mix Components (Kapa 2G)

PCR Reaction Mix Components	Volume (µL)
ddH ₂ O	9
Kapa 2G Fast PCR ready Mix	12.5
Forward primer (10µM)	1.25
Reverse primer (10µM)	1.25
Template DNA	1

Table 2. 9 PCR cycle profile

Molecular process		Temperature (°C)	Time (Bioready rTaq Polymerase)	Time (Kapa 2G Fast polymerase)
Initial denaturation		94	5min	3min
36 Cycles	Denaturation	94	45sec	10-15sec
	Annealing	59	45sec	10-15sec
	Extension	68	2min	1-15sec
Final extension		72	10 minutes	1min

2.16 Gel Electrophoresis

Agarose gels were prepared with 2% w/v concentration using low electroendosmosis (LE) agarose and 1X TBE buffer (89mM Tris-(hydroxymethyl)-aminomethane, 89mM boric acid, 2mM ethylenediaminetetraacetic acid (EDTA; pH 8.0)), allowing optimal resolution for linear DNA of 50 to 2000 base pairs. Components were mixed in a microwavable flask, microwaved in pulses until the agarose was completely dissolved, then ethidium bromide was added to a concentration of 0.5 µg/mL to allow for nucleic acid visualisation. The agarose gel mixture was poured into appropriate casts and left to set. Loading buffer (1µL 5X loading dye (glycerol 30% v/v, bromophenol blue 0.25% w/v)) was added to the sample of interest (4µL) and added into the wells of the agarose gel. A 1 kb+ DNA Ladder mixture (10% v/v 1 kb+ DNA Ladder, 20% v/v loading dye, 70% v/v ddH₂O) was also added to one well for size comparison.

The gel was run submerged in TBE buffer containing ethidium bromide at 80V for 60 minutes, then visualized using an Alpha Imager mini transilluminator at 302 nm.

2.17 CCCP Treatment and WST-1 Reduction

A stock of 500µM Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was dissolved in DMSO in a dark bottle and stored at -20°C. Cells were grown in YPD in an overnight culture in the suspension rotator at 200rpm and 30°C for 16h. Cells were spun down, supernatant discarded and resuspended in fresh YPD and then diluted to a concentration of 1 x 10⁶ cells/mL. Cells were treated with CCCP at concentrations of 1.5 and 2.5µM for 24h, shaking at 200rpm and 30°C. Next, cells were collected, washed twice with HBSS buffer and resuspended in HBSS buffer. Then, 150µL of cells were added to a microcentrifuge tube at a concentration of 1 x 10⁷ cells/tube. 15µL of WST-1/PMS stock solution was added to each tube and tubes were incubated in a suspension rotator at 200rpm and 30°C for 2h. Cells were collected by centrifugation at 300g for 5min and 100µL of the supernatant was removed and transferred to a 96-well plate. Absorbance at 450nm was measured using the microplate reader.

2.18 TTC Overlay Technique

Cells were grown on YPD agar plates for 48 hours at 30°C. Varying concentrations of 2,3,5 – Triphenyltetrazolium chloride TTC (0.1, 0.25, 0.5, 0.75, 1%) dissolved in ddH₂O and filter sterilized (0.22µm filter) were mixed with a second agar at 1.5% (autoclaved) at 55°C. 15mL of this TTC containing agar was poured over the 48 hours old colonies. Plates were then incubated for 3 hours at 30°C.

2.19 Mitochondrial Isolation and Purification

The protocol for the isolation and purification of mitochondria from yeast cells was performed as previously described [79]. Briefly, 1L of cells were grown in YPD in a suspension rotator at 200rpm and 30°C for 48h. Cells were harvested by centrifugation for 5min at 3000g. Cells were washed twice with ddH₂O and collected by centrifugation for 5min at 3000g. Pellets were resuspended in DTT buffer (100mM Tris/H₂SO₄ (pH 9.4), 10mM dithiothreitol), incubated at 70rpm for 20min at 30°C and harvested by centrifugation for 5min at 3000g. Pellets were resuspended in Zymolyase buffer (20mM potassium phosphate (pH 7.4), 1.2M sorbitol) and Zymolyase 100T (1mg Zymolyase 100T/g (wet weight)) was added to the cell suspension. Suspension was incubated at 70rpm for 30min at 30°C. The spheroplasts formed by this process were collected by centrifugation for 8min at 2200g and resuspended in ice-cold homogenization buffer (10mM Tris/HCl (pH 7.4), 0.6M sorbitol, 1mM EDTA, 0.2% (w/v) BSA) before homogenization by making 15 strokes with a tight pestle. Unbroken cells, nuclei and large debris were removed by centrifugation for 5min at 1500g and supernatant transferred to a new tube. Supernatant was centrifuged for 5min at 3000g and resulting supernatant was centrifuged for 15min at 12000g. Supernatant was then discarded, and pellet suspended in ice -cold homogenization buffer before centrifugation for 5min at 3000g. Resulting supernatant was centrifuged for 15min at 12000g and resulting pellet was resuspended in 3mL of ice-cold SEM buffer (10mM MOPS/KOH (pH 7.2), 250mM sucrose, 1mM EDTA).

Next, 60% Sucrose in EM buffer (10mM MOPS/KOH (pH 7.2), 1mM EDTA) was overlaid with 32%, 23% and 15% sucrose in EM buffer and crude mitochondrial fraction was added to the top of the overlay. The crude mitochondrial fraction was purified by centrifugation for 1h at 134000g. Mitochondrial collection was removed and suspended in SEM buffer and centrifuged for 30min at 10000g. Supernatant was discarded, and pure mitochondria were re-suspended in 3mL SEM-buffer.

2.20 Forced Mitochondrial Transfer

B18p⁰ cultures were grown in a suspension rotator at 200rpm and 30°C for 16h. Cultures were washed twice with ddH₂O and re-suspended in YPGE to a concentration of 1×10^6 cells/500μL per treatment.

Cultures underwent one of three treatments:

1. Zymolyase treatment contained cells treated with Zymolyase T100 for 8min. Cells then received 50μL SEM buffer containing mitochondrial fraction or pure SEM buffer (negative control).
2. Centrifugation treatment contained cells that did not receive any pre-treatment but received either 50μL SEM buffer containing mitochondrial fraction or pure SEM buffer (negative control).
3. Incubation treatment contained cells that were incubated in a suspension rotator for 1h at 30°C at 200rpm in the presence of 50μL SEM buffer containing mitochondrial fraction or pure SEM buffer (negative control).

All treatments were then centrifuged at either one of three speeds (1000g, 6500g, 12000g) for either 1, 5 or 10 minutes.

Supernatant was discarded after centrifugation. Cell pellets were re-suspended in 50μL YPGE and plated onto YPGE plates containing 5-FOA. Plates were then incubated at 30°C.

2.21 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics software or Casio fx-9750GII calculator. Assumptions of homogeneity of variance (Leven's Test of Equality of Error Variances), normality (Normal Q-Q plot of standardized residuals), sphericity (Mauchly's Test of Sphericity) and homogeneity of co-variance (Box's Test of Equality of Covariance Matrices) were assessed before statistical analyses were performed. Greenhouse-Geisser corrections were performed when sphericity assumption was violated. Bonferroni correction was applied for repeated measures ANOVA.

One-way ANOVA was used for the comparison of means between groups. Tukey HSD Post-Hoc test was used to identify statistical differences between all individual treatment groups. Repeated measures ANOVA was used for the comparison of means between groups over time. Independent sample T-test was performed to test for difference between treatment independently for each timepoint. P-values of <0.05 were considered statistically significant.

Chapter 3

Saccharomyces cerevisiae strain validation & establishment of a system to detect mitochondrial transfer

3.1 Background

The literature regarding intercellular mitochondrial transfer in mammalian settings, *in vitro* [41] and *in vivo* [4], has increased steadily in the last few years. However, underlying molecular pathways and molecular signals of this phenomenon are still poorly understood.

Single cellular organisms, such as *S. cerevisiae*, represent an ideal model in which to study cell biological processes. These single cell organisms are easy to grow, experimentally tractable and amenable to both genetic analysis and molecular biological techniques and share many molecular properties with mammalian cells. They can be used to investigate the genetic basis of processes through the presence of a stable haploid locus, absence of a homothallic HO locus and availability of gene mutation libraries. In order to determine the feasibility of using *S. cerevisiae* to examine the underlying mechanisms of intercellular mitochondrial transfer, it was necessary to determine whether or not *S. cerevisiae* can transfer mitochondria.

This thesis used *S. cerevisiae* BY4741 B18p⁰ cells whose mtDNA had been removed through exposure to ethidium bromide to induce injured, respiratory deficient cells. *S. cerevisiae* BY4741 Tom70 cells were used as respiratory competent cells. Both strains have different fluorescent markers and PCR profiles to allow for identification of cells independent of the selective conditions used.

This chapter validated the phenotypic and genotypic characteristics of B18p⁰ cells, B18wt cells and Tom70 cells.

3.2 Results

3.2.1 Phenotypic Characteristics

3.2.1.1 Growth Characteristics in Selective Media

S. cerevisiae ρ^0 cells have no mitochondrial DNA and thus cannot perform mitochondrial electron transport (MET) and oxidative phosphorylation (OXPHOS). This means they require a fermentable/sugar carbon source for proliferation such as glucose or dextrose. In the absence of these, for instance in YPGE medium, ρ^0 cells cannot grow, whereas mitochondrial competent cells can utilise glycerol and ethanol for ATP production and subsequent proliferation. Validation of substrate and selective requirements for B18 ρ^0 cells, B18wt and Tom70 cells was done in both solid and liquid media.

To assess the ability to grow on solid media, cells were streaked from agar plate to agar plate, followed by incubation for at least 48h at 30°C. All cell types grown on solid YPD, containing fermentable glucose, formed the same number and size of colonies after two days of incubation (Figure 3.1 A).

As expected, B18 ρ^0 cells did not grow on YPGE medium, even after 48h incubation (Figure 3.1 B). The absence of a fermentable/sugar carbon source requires the cell to conduct OXPHOS, something the B18 ρ^0 cells cannot do. Incubation for B18 ρ^0 cells on solid YPGE medium, even for a prolonged period of 14 days, did not result in any colony formation. The ability of B18wt and Tom70 cells to form colonies on solid YPGE was not affected, consistent with functional MET and OXPHOS.

Growth in liquid YPD medium was also assessed over 48h. Growth curves show a prolonged lag phase for four independent B18 ρ^0 strains in comparison with B18wt and Tom70 (Figure 3.2 A). Overall, growth in liquid YPD was not affected in any of the B18 ρ^0 strain, however, these strains grew at a slower rate compared to respiratory competent cells. B18 ρ^0 cells reached optimum growth, log phase (OD_{660} ~0.4 – 0.5), approximately three hours after B18wt and Tom70 cells.

Respiratory deficiency was shown by lack of growth in liquid YPGE over 48h by the four B18 ρ^0 strains, further validating their metabolic impairment due to loss of mtDNA. B18wt and Tom70 cells grew equally well in liquid YPGE as in liquid YPD (Figure 3.2 B).

A YPD

B18p⁰

B18wt

Tom70

B YPGE

B18p⁰

B18wt

Tom70

Figure 3. 1 Growth of B18p⁰, B18wt and Tom70 on solid media: Growth on YPD (A) and YPGE (B)

Figure 3. 2 Growth of B18p⁰, B18wt and Tom70 in liquid media: Growth of all strains in liquid YPD (A). Growth of all strains in liquid YPGE (B)

3.2.1.2 Viability Assay

B18p⁰ cells cannot proliferate in YPGE due to absence of mtDNA and subsequent loss of MET and OXPHOS. In contrast to mammalian cells, *S. cerevisiae* p⁰ cells do not die in medium containing non-fermentable carbon sources, such as glycerol and ethanol. Here we determined how long B18p⁰ can survive in the absence of a fermentable carbon source.

B18p⁰ cells were grown overnight, washed twice then suspended in YPGE. Cultures were incubated at 200rpm and 30°C. The OD₆₆₀ was determined every 48h for 2 weeks and samples were plated on SC plates. SC plates were incubated at 30°C and colony forming units (CFU) were measured to determine the proportion of cells that were viable after prolonged exposure to medium without a fermentable/sugar source (YPGE).

Figure 3.3 shows the SC plates. All plates showed the growth of yeast carpets and colonies were indistinguishable from one another, indicating that viability was not affected by prolonged exposure to YPGE medium.

Figure 3. 3 Viability of B18p⁰ after prolonged exposure to YPGE

3.2.1.3 Fluorescent Phenotypes

B18p⁰ and Tom70 cells could be distinguished by fluorescent microscopy.

The B18p⁰ is a triple red fluorescent strain. B18p⁰ cells contain the MRH1-egFP611-KANMX4 protein that localises to the cellular membrane, the NLS-RedStar2-HPH protein that is linked to a nuclear localisation signal and the NATMX4-TEFpr-mCherry protein, a double positive red fluorescent protein with localisation to the nucleus and cytosol. Fluorescent microscopy, as shown in Figure 3.4, revealed a bright red signal for the yeast plasma membrane, nucleus and a fainter signal for the cytoplasm. Fluorescence was not affected by the carbon source in the medium (Figure 3.4) and did not differ between the B18p⁰ cells and B18wt cells, confirming expression is not reliant on mitochondrial function.

In Tom70 cells, the green fluorescent protein is linked to the Tom70 protein, an outer mitochondrial membrane transporter protein. The signal shows a distinct green localization close to the plasma membrane, characteristic of mitochondrial networks in *S. cerevisiae* [80]. This signal is also unaffected by the carbon source (Figure 3.4).

A SC

B18wt

B18p⁰

Tom70

B YPD

B18wt

B18p⁰

Tom70

Figure 3. 4 Fluorescent phenotypes: Fluorescent phenotype in SC (A) or YPD (B) medium.

3.2.1.4 5-Fluoroorotic Acid Selectivity

There was potential to select against Tom70 cells using 5-FOA. The Tom70 cells contain a URA3 cassette. The URA3 gene encodes orotidine-5'-phosphate decarboxylase (ODCase), allowing the Tom70 cells to grow on media not supplemented with uracil or uridine. While this confers a growth advantage to Tom70 cells, addition of 5-FOA allows these cells to convert 5-fluoroorotic acid (5-FOA) into the toxic compound 5-fluorouracil, causing cell death. This feature can be used to negatively select for cells containing this cassette.

To determine the minimum concentration of 5-FOA that reduced growth of Tom70 cells without affecting growth of B18p⁰ cells, all cells were grown in varying concentrations of 5-FOA (0 mg/mL to 3.5 mg/mL in 0.5 mg/mL increments) for 27h and the OD₆₆₀ of the culture was determined over time. Measurements were standardized to the 0 mg/mL 5-FOA control.

As expected, growth of B18wt cells and B18p⁰ cells was not affected by 5-FOA and showed consistent growth. There was a slight reduction in growth at concentrations of 3mg/mL 5-FOA and higher (Figure 3.5). However, the difference in growth at the different concentrations was not statistically significant (B18wt: P-value = 0.524; B18p⁰: P-value = 0.3223).

The Tom70 strain showed a drastic reduction in growth at 5-FOA concentrations of 1 mg/mL and slight to no growth at 1.5 and 2 mg/mL 5-FOA (Figure 3.5 A). The differences in growth at different concentrations of 5-FOA were statistically significant for the Tom70 cells (P-value < 0.001), confirming the URA3 activity.

The 5-FOA concentrations of 1.5 mg/mL and 2 mg/mL were the most effective working concentrations for selective killing of Tom70 without affecting the B18p⁰ strain. A working concentration of 2 mg/mL 5-FOA was chosen to ensure negative selection of the Tom70 cells.

To ensure that the effects seen were due to 5-FOA and not DMSO, the same experiment was conducted using SC media containing DMSO without 5-FOA as a control. The concentrations unit (mg/mL) refer to the amount of DMSO required for the stated concentrations of 5-FOA. The Tom70 strain showed relatively consistent growth at all control concentrations (Figure 3.5 B). A one-way ANOVA of the comparisons between the different concentrations was not significant (P-value = 0.361), indicating no statistically significant difference in growth rates and that the effects observed in presence of 5-FOA were due to 5-FOA and not DMSO.

To assess the timing of residual resistance of Tom70 cells to 5-FOA, Tom70 cultures, both liquid and agar, were plated on SC agar plates supplemented with 2 mg/mL 5-FOA. Plates were incubated at 30°C for 14 days and the time of colony appearance was recorded. Colonies appeared between 72 and 96 hours of incubation and increased in size but not in number until day 14 of incubation (Figure 3.6).

Tom70 cultures were also plated on YPGE agar plates, containing 2 mg/mL 5-FOA, to assess resistance frequency and to determine the efficacy of 5-FOA in media containing non-fermentable carbon sources. Plates were incubated at 30°C for 14 days and colony numbers were counted. On average for four repeats (range 12 to 21 colonies), 17 colonies grew on SC agar plates supplemented with 2mg/mL 5-FOA. Seventeen out of 1×10^7 cells plated represents less than 0.0002% ($\frac{17}{1 \times 10^7} \times 100 = 0.00017$) of cells showed resistance to 5-FOA, indicating the strong toxicity of 5-FOA and demonstrating its effective negative selectivity against Tom70 cells (Figure 3.7).

Figure 3. 5 Effects of 5-FOA on *S. cerevisiae* proliferation in liquid media: Effects of varying concentrations of 5-FOA on B18wt, B18p0 and Tom70 cell proliferation (A). Effects of vehicle control (DMSO) on Tom70 proliferation (B).

A

B

Figure 3. 6 Residual resistance of Tom70 cells to 5-FOA: Cells were streaked from agar to SC plates containing 2 mg/mL 5-FOA (A). Cells were plated from liquid to SC plates containing 2 mg/mL 5-FOA (B).

YPGE/5-FOA

Figure 3. 7 Residual resistance of Tom70 to YPGE containing 5-FOA: 1×10^7 cells were plated in triplicate on YPGE containing 2 mg/mL 5-FOA.

3.2.1.5 WST-1/PMS Reduction and CCCP Treatment

The colourless cell-impermeable dye, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium monosodium salt (WST-1), is reduced to its bright yellow coloured formazan in the presence of its obligate intermediate electron acceptor, 1-methoxy phenazine methosulphate (PMS) [81]. WST-1/PMS reduction was used to measure plasma membrane electron transport (PMET) in B18p⁰ cells, B18wt and Tom70 cells. In mammalian cells PMET has been shown to be an additional NADH recycling pathway in the plasma membrane, in addition to MET and lactate dehydrogenase activity [31]. However, in sharp contrast to mammalian cells, in *S. cerevisiae* PMET relies on the presence of components of the mitochondrial electron transport chain, located in the inner mitochondrial membrane which has been shown to be located in very close proximity the plasma membrane [80]. B18p⁰ cells that do not have components of the mitochondrial electron transport chain due to absence of mtDNA are expected not to have strong PMET activity.

Cells were grown to mid-exponential phase and washed twice with HBSS buffer. WST-1/PMS reduction was measured after incubating 1x10⁷ cells per 150µL HBSS, containing 10% WST-1 at 30° C for two hours. Control samples of HBSS and WST-1/PMS only were used to measure auto oxidation. Absorbance at 450nm was adjusted for auto oxidation.

B18wt and Tom70 cultures had 7 to 8-fold higher reduction rates than B18p⁰ cells (Figure 3.8). Differences in WST-1/PMS reduction were statistically significant between B18p⁰ on one hand and Tom70 and B18wt cells on the other hand (P-value < 0.0001). The loss of mitochondrial electron transport chain components in B18p⁰ cells greatly reduced their capacity to reduce the tetrazolium dye compared to the respiratory competent Tom70 and B18wt cells.

Next, the effect of carbonyl cyanide m-chlorophenyl hydrazine (CCCP) on WST-1/PMS reduction was established for all cell types. CCCP is an ionophore which uncouples OXPHOS from MET, which on short exposures (10-30min) destroys the mitochondrial membrane potential in mammalian cells and increases MET as a consequence. An increase in MET equates to a decrease in PMET because both processes compete for the intracellular NADH pool [31, 78, 81]. Exposures of several hours kills mammalian cells (PM Herst, personal communication, 16 January 2019). In *S. cerevisiae*, prolonged exposure (24h) affects mitochondrial membrane potential without affecting cell viability.

B18p⁰, Tom70- and B18wt cultures were grown overnight in YPD to mid-exponential phase, washed twice with YPD and re-suspended to a concentration of 1 x 10⁶ cells/mL and received

either 0 μ M (control), 1.5 μ M or 2.5 μ M CCCP treatment. Cultures were then incubated at 30°C for 24h, washed twice with HBSS, re-suspended to a concentration of 1 x 10⁷ cells/150 μ L and WST-1/PMS reduction was assessed.

B18p⁰ cultures showed no biologically significant difference in WST-1/PMS reduction between treatments and control (Figure 3.8). The difference in OD₆₆₀ between CCCP-treated and untreated B18p⁰ cells (1), (2) and (3) were statistically significant (P-value = 0.048; P-value = 0.008; P-value = 0.049 respectively) but the effect size was in the range of 0.003 to 0.018 which is biologically insignificant.

B18wt and Tom70 cultures both showed a reduced WST-1/PMS reduction that was CCCP concentration dependent (Figure 3.8). The difference between WST-1/PMS reduction of CCCP-treated and untreated Tom70 cells was statistically significant (P-value = 0.002). Tukey's HSD Post-Hoc test showed the most significant difference for Tom70 in dye reduction was for the 2.5 μ M CCCP versus untreated control (P-value = 0.0019) that coincided with a biological significant difference in A450 of around 0.2.

Figure 3. 8 WST-1/PMS reduction of CCCP treated and untreated cells

The difference between WST-1/PMS reduction of CCCP-treated and untreated B18wt cells was significantly different (P-value = 0.0001). Tukey's HSD Post-Hoc test identified the greatest difference between the 2.5 μ M CCCP treatment and the untreated control (P-value < 0.0001) with a significant biological effect size in A450 of 0.3.

These results indicate that Tom70 and B18wt cells have functional MET and OXPHOS that can be uncoupled and thus disrupt mitochondrial membrane potential. B18 ρ^0 cells were unaffected by the uncoupler because they do not have respiratory subunits and thus no MET or OXPHOS.

3.2.1.6 TTC Assay

Triphenyl tetrazolium chloride (TTC) is a cell permeable redox indicator. This white compound is reduced to a red compound (1,3,5 – triphenylformazan) in the mitochondrial electron transport chain of living cells. This compound was used to assess the MET activity of B18 ρ^0 , Tom70 and B18wt cultures grown on SC agar plates, different to the cell impermeable WST-1/PMS assessment of PMET activity in liquid cultures. Cultures were grown on agar at 30°C for 48h and then overlaid with agar containing varying concentrations of TTC.

Figure 3. 9 Reduction of varying TTC concentrations by B18 ρ^0 , B18wt and Tom70 cells

Figure 3.9 showed that B18p⁰ cultures remained white in the presence of 0.1% to 1% TTC, confirming that no TTC reduction occurred in these cells. Tom70 and B18p⁰ cells showed an increasing intensity of red colour with increasing concentrations of TTC. Increased TTC concentrations corresponded to more compound being available for reduction, indicating that Tom70 and B18wt cells have high MET function.

3.2.2 Genotypic Characteristics

Finally, the mitochondrial DNA status of the B18p⁰ cells was confirmed by PCR. Figure 3.10 A shows the absence of a PCR product for Cox3, a gene product encoded by mtDNA, in B18p⁰ cells. In contrast, Tom70 and B18wt cells showed clear bands of the Cox3 product. Absence of a product in B18p⁰ cells was not due to a technical problem or the complete absence of DNA (see Figure 3.10 B). A PCR for mating type, a nuclear encoded gene, showed all DNA samples could be amplified and all *S. cerevisiae* strains used were of the Mat a mating.

5-FOA selectivity is conferred by the presence of the URA3 cassette. Figure 3.10 C shows that Tom70 was the only strain that contained the URA3 cassette. PCR for URA3 was negative in B18p⁰ and B18wt cells.

Figure 3. 10 Genotypic characteristics: Assessment of Cox3 (mitochondrial DNA) – product size 128bp (A), mating type – product size 534bp (B) and presence of URA3 cassette – product size 536bp (C).

3.3 Discussion

The advantages of using unicellular yeasts in functional genomics and systems biology have led to an increasing amount of research being conducted with these organisms to study human diseases. However, mitochondrial transfer in *S. cerevisiae* or other unicellular organisms has not been part of active research to date.

Yeast offer a unique opportunity to study this phenomenon and can aid in obtaining more knowledge and understanding of this process. If yeast cells have the ability to transfer mitochondria between them, studying this process in yeasts could answer questions such as whether or not this is an evolutionary conserved process and elucidate the underlying mechanisms.

However, this type of research requires the *S. cerevisiae* populations of interest to be different in their phenotypic and genotypic profiles.

B18p⁰, Tom70 and B18wt cells demonstrated different growth characteristics on selective media as expected. Treatment with ethidium bromide resulted in B18p⁰ cells without mtDNA, MET or OXPHOS. Absence of mtDNA correlated with the absence of growth on or in media containing only glycerol and ethanol as carbon sources. Mitochondrial components such as the NADH oxidase, bc1 complex and cytochrome c oxidase are required for the complete utilization of glycerol and ethanol for subsequent ATP production (see Chapter 1). Absence of these complexes in B18p⁰ cells abolishes their ability to use non-fermentable carbon sources. Tom70 and B18wt cells are not affected in their growth as these strains have functional MET and OXPHOS.

Tom70 and B18wt cells demonstrated a slight delay in lag phase between growth in YPD and YPGE medium. Delayed lag phase and slower log phase are generally attributed to diauxic growth characteristics [82, 83]. Glycerol and ethanol are readily used in bioenergetic pathways; however, glucose is the preferred metabolite. In the absence of glucose, remodelling metabolism to utilize alternative carbon sources slows the lag phase compared to growth in YPD [82, 83]. B18wt and Tom70 cells reach saturation nonetheless and, therefore, do not have significant differences in growth in either medium.

B18p⁰ cells had a slower growth rate in YPD compared to Tom70 and B18wt cells. The reliance solely on glycolysis is less efficient than using OXPHOS for energy generation.

Metabolic parameters were assessed in different ways, by WST-1/PMS reduction, the effect of CCCP on WST-1/PMS reduction and TTC reduction.

B18p⁰ cells only had about 10% of the WST-1/PMS reduction seen in the respiratory competent strains. WST-1/PMS is a cell impermeable dye that, in mammalian cells, is reduced at the cell surface, in the presence of the obligate intermediate electron acceptor, PMS. In *S. cerevisiae* this process is independent of the ferric reductase system but dependent on cytochrome bc1 and cytochrome c oxidase [80]. It has previously been hypothesized that WST-1/PMS reduction on the cell surface can be facilitated in *S. cerevisiae* through the formation of restricted domains between the plasma membrane and the mitochondrial membrane [80, 84], allowing for the transfer of reducing agents between the plasma membrane and mitochondrial membrane in yeast mitochondria. This was supported by fluorescent images as mitochondrial networks were located close to the outer plasma membrane [80].

The effect of CCCP on WST-1/PMS reduction further supports these results. As an uncoupling agent, CCCP disrupts mitochondrial membrane potential, resulting in a sudden increase of MET and subsequent collapse of OXPHOS and MET. This collapse was shown by a decreased WST-1/PMS reduction, demonstrating that Tom70 and B18wt cells had functional MET to start with.

The WST-1/PMS reduction was not biologically or statistically significantly different between the CCCP treated and untreated B18p⁰ cells. The equal rates of WST-1/PMS reduction in the B18p⁰ cells under untreated and CCCP treated conditions further supports their lack of functional OXPHOS and MET.

Independently of cell surface WST-1/PMS reduction, intracellular TTC reduction followed the same pattern. TTC is a cell permeable dye and is reduced within the mitochondria. TTC dislocates O₂ as the final electron acceptor and is reduced to its red colour upon acceptance of electrons moving through MET [85, 86]. In B18p⁰ cells, TTC entered the cells but could not accept any electrons without functional electron transport. In Tom70 and B18wt cells, functional electron transport resulted in reduction of TTC. Increased intensity in red colour was TTC concentration dependent, correlating with functional MET.

Genotypically, the difference between the different strains were as expected. The absence of mtDNA in B18p⁰ cells was confirmed by the absence of Cox3 by PCR. The presence of the 5-FOA cassette, also confirmed by PCR, in the Tom70 strain conveyed negative selectivity on medium containing 2mg/mL 5-FOA. Functionality of this cassette was assessed by growth in

liquid and on agar containing 5-FOA. While growth in liquid was greatly reduced, growth on agar plates showed a slight resistance to the chemical. Previous studies have shown that phenotypic mutations at the URA3 locus occur at a rate of approximately 3×10^{-10} per base pair per generation [87, 88]. Tom70 cells showed a residual resistance to 5-FOA of below 0.00001%, falling into the expected range of resistance through loss of function of the URA3 enzyme. The number of colonies grown on 5-FOA containing agar would be manageable. Co-culture experiments will include sampling and plating of approximately 1×10^6 to 1×10^7 cells/sample. Growth of 100 to 1000 colonies will represent 0.00001 to 0.0001% of all cells plated, is likely to make growth of about 20 resistant Tom70 cells inconsequential in the actual experimental aim of identifying mitochondrial transfer.

In summary, this chapter has validated some useful phenotypic and genotypic characteristics of B18 ρ^0 cells, Tom70 and B18wt strains. Differences in their growth characteristics, fluorescent appearance, metabolic profile and genotype were validated and a procedure to distinguish between the experimental strains lacking or containing functional mitochondria was successfully established to identify presence of functional mitochondria by means of microscopy, PCR and 5-FOA sensitivity.

Chapter 4

Co-culture

4.1 Background

Chapter 3 validated phenotypic and genotypic characteristics of B18 ρ^0 cells, B18wt cells and Tom70 cells. Differences in their growth characteristics, fluorescent appearance, metabolic profile and genotype mean that these strains can be used to study intercellular mitochondrial transfer between B18 ρ^0 cells and Tom70 cells. A co-culture system was used, growing both cell types together in YPGE, which contains only glycerol and ethanol and no other fermentable carbon sources. These conditions were expected to induce metabolic stress in the B18 ρ^0 cells with the aim that subsequent communication with the respiratory competent Tom70 cells would induce intercellular mitochondrial transfer. Mitochondrial transfer from Tom70 cells with green fluorescent mitochondria to red fluorescent B18 ρ^0 cells would result in red B18 ρ^0 cells that are able to grow on YPGE and the 5-FOA will prevent growth of the Tom70 cells. Green mitochondria within red cells would only be visible for a few cell divisions as the green colour is nuclear-encoded and be diluted out over several generations.

4.2 Results

In this chapter multiple co-cultures of B18p⁰ cells and Tom70 cells were carried out at different ratios of B18p⁰ cells and Tom70 cells. Table 4.1 summarises the different conditions for co-cultures.

4.2.1 Preliminary Co-Culture Experiment

Duplicate co-cultures of B18p⁰ cells and Tom70 cells were set up in liquid YPGE/5-FOA medium at 2×10^7 cells/mL, then kept at optimal growth conditions (outlined in Figure 4.1.) The OD₆₆₀ of each co-culture was determined every 24h for 11 days to determine cell concentration and co-cultures were washed daily with YPGE then re-suspended to of 2×10^7 cells/mL. Additionally, on each day, samples were streaked on YPGE/5-FOA agar plates, with the exception of day 4, day 8 and day 10. YPGE/5-FOA agar plates were incubated for 10 days at 30°C.

The preliminary co-culture experiment was defined as CC1

Table 4.1: Summary of different co-culture conditions

Co-culture conditions	CC1	CC2	CC3 + CC3a	CC4 + CC4a	CC5	CC6
Ratio B18p ⁰ :Tom70	1:1				100:1	50:1
Negative control replicates	NA	Duplicate	Single	Duplicate	Single	single
Length of co-culture (days)	11	12	14			
Sampling frequency	24h				Day 2, 6, 14	
Daily washing and resuspension to initial cell numbers (2x10 ⁷ cells/mL)	yes	yes	CC3 only	CC4 only	No	no
Incubation of YPGE/5-FOA plates (days)	10		14			
Colonies suspended in 96 well plate	yes					
Samples streaked onto YPGE plate then into 96-well plates	no		yes			
Samples streaked onto YPGE plate then into 96-well plates w/ 5-FOA	no			yes		

4.2.1.1 Fluorescent Analysis of Duplicate Co-cultures

In order to determine the number of red and green colonies that had grown on the YPGE/5-FOA plates, all colonies were picked, re-suspended in liquid SC in 96-well plates and grown for 16h at 30°C and 200rpm. Five microlitres were taken from each well and combined with 50µL SC medium in a 384-well clear bottom microscopy plate. All samples were then screened for the presence of either a red and green fluorescent signal (“red” and “green”).

The fluorescent screen of the colonies grown on YPGE/5-FOA identified 179 green and 19 red colonies (Figure 4.2).

All 179 green colonies had the same fluorescent appearance (Figure 4.3A), characteristic of mitochondrial networks in *S. cerevisiae*. This was consistent with resistant Tom70 parental cells that had grown despite the presence of 5-FOA.

Of the 19 red colonies, 8 colonies showed a green haze at the gap in the red plasma membrane (Figure 4.3B). This was possibly due to autofluorescence as the red signal of these images also appears to be more orange than a deep red.

Cells of another 8 of the red colonies were red with a yellow grainy appearance within the cells (Figure 4.3C and D). This grainy yellow was caused by the overlay of a green and red fluorescent signal. The green signal co-localized with the red signal of the plasma membrane, a characteristic of mitochondrial networks in *S. cerevisiae*. Thus, the yellow grainy appearance may indicate mitochondrial transfer from Tom70 cells to B18p⁰ cells. Separating the green and red fluorescent channels from each other also confirmed that the yellow grainy appearance within cells was not due to auto-fluorescence or apoptotic cells (Figure 4.3E). Cells of the remaining 3 red colonies only showed a red signal.

Representative images of all 19 red colonies are in Appendix A.

Figure 4. 1 Graphic overview of co-culture set-up: B18p⁰ and Tom70 cultures were grown separately in liquid SC for 24h. Cultures were washed and combined in a 1:1 ratio in 3mL YPGE. Co-cultures were incubated at 30°C and 50µL were taken each every 24h to determine the OD₆₆₀ and for plating onto YPGE/5-FOA agar. Plates were incubated at 30°C and colonies grown were screened for fluorescence.

Figure 4. 2 Number of green and red colonies for CC1 duplicates over time

Figure 4. 3 Fluorescent images of CC1 colonies: Green fluorescence of Tom70 mitochondria (A) Green haze localized at membrane gap (B) Yellow grainy appearance within red membrane of B18p⁰ cells (C) Colour composite image of a red colony (D) Separate RFP and GFP channels of colour composite image showing B18p⁰ cells phenotypes and mitochondrial phenotype of Tom70 cells.

4.2.1.2 Genotypic Validation of Intercellular Mitochondrial Transfer

To determine whether or not intercellular mitochondrial transfer was responsible for the growth of B18p⁰ cells on YPGE, PCR analysis was performed. B18p⁰ cells, Tom70 cells and B18p⁰ cells that have acquired mitochondria from Tom70 cells should have a unique PCR profile as shown in Chapter 3 and outlined in Table 4.2.

1. Tom70 and B18p⁰ cells that have acquired mitochondria from Tom70 cells, but not B18p⁰ cells, should contain the mitochondrial encoded Cox3.
2. All strains should be mating type Mat a.
3. The Ura3 cassette is specific to the Tom70 cells.

Table 4. 2 Unique PCR profile for B18p⁰ cells, B18p⁰ with mtDNA and Tom70 cells.

	Cox3	Mat a	Ura3
B18 p0 that have not acquired mitochondria	-	+	-
B18 p0 that have acquired mitochondria	+	+	-
Tom70	+	+	+

DNA extractions were performed on BY4741, Tom70, B18wt and B18p⁰ cells, and on four red colonies as a representation of the 19 red colonies. Representatives of the red colonies were a red colony of CC2 – day9 (yellow grainy appearance), CC1 – day 11 (yellow grainy appearance), CC1 – day 9 (green haze) and CC1 – day 7 (yellow grainy appearance). BY4741 was used as a genetic background for all strains as all the strains for this thesis were derived from BY4741. Samples were grown in triplicate and three independent DNA extractions were performed for each strain and sample.

Figure 4.4 shows the presence or absence of the Cox3 gene, which is encoded by mitochondrial DNA. BY4741, Tom70 and B18wt triplicates were all positive for presence of the Cox3 gene with a band of the correct product size of 128bp (Figure 4.4A). The B18p⁰ cell

triplicates had no bands, confirming that the B18 ρ^0 cells do not contain the mitochondrial DNA. Results for the four red colonies were mixed. One colony tested, CC2-day 9, did not have the Cox3 band, but the other three showed the presence of mitochondrial DNA, despite a defect in the gel that impeded migration of some of the bands (Figure 4.4B).

To confirm that all samples were of the same mating type and to validate that the negative results obtained in the Cox3 PCR were not due to absence of any DNA in the extraction, mating type PCR was performed on the same gene extraction triplicates for BY4741, Tom70, B18wt, B18 ρ^0 cells and the 4 representative red colonies.

In order to completely validate these findings, a PCR demonstrating presence of nuclear DNA would be required. Mating type locus was selected as a nuclear DNA control and also to demonstrate that mating type switching had not occurred. Positive results for Mat a were expected for each triplicate for each sample. However, only bands for the Mat a product of 534bp were visible for the triplicates of BY4741 and the first sample of the Tom70 triplicates. All other samples and triplicates did not show any band for Mat a or Mat α product (data not shown). These results indicate that this PCR did not work properly and means that intercellular mitochondrial transfer could not be validated by PCR.

Unfortunately, not enough DNA or sample material was available to repeat the fluorescent screen or PCR analysis.

Figure 4. 4 PCR assessment of mitochondrial DNA: PCR for the mitochondrial encoded Cox3 gene from triplicate DNA extractions (1-3). (A). Control strains show band of the correct product size (128bp) (B). MtDNA in representative red colonies D9CC2G4 (D9 = day 9, CC2 = Co-culture 2, G4 = 384-well plate location), D11CC1A4, D9CC1E4 and D7CC1B5.

4.2.2 Repeat of the Co-culture Experiment

The main difference between the preliminary duplicate co-culture 1 set-up and these co-culture duplicates, defined as CC2, was the addition of duplicate negative controls for B18p⁰ and Tom70 cells.

4.2.2.1 Fluorescent Analysis of CC2 Duplicate Co-cultures and Controls

All colonies grown on the CC2 plates were picked, re-suspended and grown in liquid SC in 96-well plates and checked for fluorescent signal in a 384-well plate. A total of 118 green colonies and 4 red colonies were obtained this way (Figure 4.5). Cells from all 118 green colonies showed the same fluorescent appearance (Figure 4.6A), characteristic of mitochondrial networks in *S. cerevisiae*. Occurrence of green colonies was similar between CC1 and CC2, indicating a consistent basal level of resistant Tom70 cells between experiments.

None of the 4 red colonies showed a green haze or yellow grainy appearance (Figure 4.6B).

Again, green colonies grew from the negative control containing only Tom70 cells.

No red colonies grew from the B18p⁰ negative controls.

Figure 4. 5 Number of green and red colonies for CC2 over time

Figure 4. 6 Fluorescent images of CC2 colonies: Green fluorescent signal of mitochondria of resistant Tom70 cells (A). Red fluorescent signal of B18p⁰ cells of red colonies of CC2 (B).

4.2.3 Does increasing co-culture stress affect mitochondrial transfer?

Co-cultures in CC1 and CC2 had been washed and resuspended in fresh medium daily. Leaving the co-cultures to reach saturation could potentially have increased nutrient stress and subsequently might increase the rate of mitochondrial transfer. For this co-culture experiment, defined as CC3, duplicates were set up with two different conditions:

1. The first condition comprised CC3 duplicates that were washed/resuspended daily as with CC1 and CC2.
 2. For the second condition, CC3a duplicates were left undisturbed to reach saturation and beyond. Samples were taken daily for determining the OD₆₀₀ and for streaking on YPGE/5-FOA agar plates without washing and resuspending.
- Negative control cultures for B18p⁰ and Tom70 cells were set up for CC3 and CC3a.

Sampling for each culture was taken out to 14 days.

4.2.3.1 Proliferation of Co-cultures and Controls

Proliferation of the co-cultures in YPGE was measured and compared to the control strains, by taking 50μL of the cultures and 950μL YPGE, so that the OD₆₆₀ represents a 1:20 dilution of the original concentration of the cultures. Figure 4.7 shows the OD₆₆₀ of the B18p⁰ cell control, Tom70 control and CC3a duplicates.

The Tom70 and CC3a duplicate cultures all increased their OD₆₆₀ on day 1 compared to the B18p⁰ cell culture, indicating robust cell proliferation (Figure 4.7). OD₆₆₀ increased for all cultures over time to approximately 0.9. As expected, the B18p⁰ cell culture showed an OD₆₆₀ of 0.05 for day 1 to day 4 (Figure 4.7). However, on day 5, the B18p⁰ cell culture OD₆₆₀ increased to approximately 0.3 and to 0.6 on day 14, which could have been a sign of contamination.

Figure 4. 7 Proliferation for CC3a and controls over 14 days: The change in OD₆₆₀ over time for CC3a duplicates, B18p⁰ and Tom70 negative cultures over time.

Figure 4. 8 Number of green and red colonies of CC3 and CC3a over time

4.2.3.2 Fluorescent Analysis of CC3 Duplicate Co-cultures and Control Cultures

All colonies grown on all YPGE/5-FOA plates were picked and re-streaked on YPGE agar plates, then incubated at 30°C for 48h. This was done to create enough yeast material for fluorescent analyses, DNA extractions and to preserve the samples for future use.

Colonies grown when re-streaked on YPGE were resuspended in liquid SC in 96-well plates and incubated at 30°C and 200rpm for 24h. Samples from each well were screened for presence of red and green fluorescent colonies in a 384-well plate.

All of the 337 colonies from these YPGE plates were green and none of the colonies was red (Figure 4.8). No further analysis of mtDNA was performed.

Again, consistent with the lack of mtDNA, MET and OXPHOS activity, no colonies grew on the B18p⁰ YPGE/5-FOA plates.

4.2.4 Does fresh mitochondrial DNA depletion affect mitochondrial transfer?

The comparison of washed/resuspended and saturating cultures was repeated using fresh stocks of each strain.

Frozen stocks were streaked on SC agar plates and incubated at 30°C for 48h for the duplicate co-cultures CC4. Samples were re-suspended in liquid SC and grown at 30°C and 200rpm for 24h. B18p⁰ cells were also re-exposed to ethidium bromide (25 µg/mL) for 3 days. This was done to ensure true rho zero status of B18p⁰ cells. Duplicate co-cultures for both conditions were then set up as described in CC3.

Duplicate controls for B18p⁰ and Tom70 cells alone were set up in parallel.

4.2.4.1 Proliferation of Co-cultures and Controls

Figure 4.10 shows the OD₆₆₀ of the B18p⁰ cell control cultures, Tom70 control cultures and the CC4a duplicates.

The Tom70 and CC4a cultures all increased their OD₆₆₀ on day 1 compared to the B18p⁰ cell culture, indicating robust cell proliferation (Figure 4.9). OD₆₆₀ increased for all cultures over time to approximately 0.8. The B18p⁰ control cultures had an OD₆₆₀ of 0.05 for day 1 to day 3 or 4. On day 5 and 4 respectively, the OD₆₆₀ increased from 0.05 to approximately 0.8 day 14. (Figure 4.9). This increase in OD₆₆₀ can best be explained by contamination.

4.2.4.2 Fluorescent Analysis of Duplicate CC4 Co-cultures and Controls

All colonies grown on all YPGE/5-FOA plates were picked and re-streaked on YPGE agar plates. Colonies grown when re-streaked on YPGE were resuspended in liquid SC in 96-well plates, supplemented with 2mg/mL 5-FOA and incubated at 30°C and 200rpm for 24h. The addition of 5-FOA to the liquid SC medium was done to have an additional measure of identifying potentially false positives relating to the Tom70 cell type. While colonies grown on YPGE/5-FOA agar plates may have only survived through the slow degradation and reduced efficacy of 5-FOA, growing them in SC with fresh 5-FOA could show an increased effect on those cells compared to just growth in liquid SC.

The fluorescent screen identified 133 green colonies and one colony that was a mix of red and green cells (Figure 4.10).

Figure 4. 9 Proliferation of CC4a duplicates, Tom70 and B18p⁰ cultures over 14 days

Figure 4. 10 Number of green and red colonies for all co-cultures over 14 days: The mixed colony is represented as 1 green colony and 0 red colonies.

Figure 4.11 shows the colour composite image of the mixed colony. Green fluorescent signals showed characteristics of mitochondrial networks in *S. cerevisiae* as expected in Tom70 cells. Red fluorescent signals were characteristic of the B18p⁰ cells.

Figure 4. 11 Fluorescent composite images: The mixed colony shows presence of green and red cells.

4.2.4.3 Contaminant Characterisation

The increase in OD₆₆₀ for the negative B18p⁰ control cultures after day 3 and day 4 was the second time that a negative B18p⁰ control showed an increase in OD₆₆₀ (CC3). B18p⁰ cells cannot grow in or on liquid YPGE (see Chapter 3). This increase in OD₆₆₀ can best be explained by contamination with bacteria and/or yeasts other than B18p⁰.

After 14 days of co-culture, samples were taken from the negative B18p⁰ control cultures and screened for fluorescence. Figure 4.12 A shows RFP and GFP channels for the sample from the negative B18p⁰ control culture. The RFP channel shows red fluorescent cells, indicative of B18p⁰ cells, and non-fluorescent budding yeast cells. Absence of a red fluorescent signal in the contaminant suggest that B18p⁰ cells and the contaminant were not able to mate. To investigate whether or not these could be Tom70 cells, signals for green fluorescence were checked. The GFP channel showed no green fluorescent signal but outlines of non-fluorescent budding yeast.

Figure 4. 12 Characterisation of contaminant: (A). Fluorescent analysis shows presence of red cells and non-fluorescent budding yeasts. (B). PCR analysis for presence of mitochondrial DNA (Cox3, left) and mating type (right) (C). PCR profile to distinguish between cell types used and specific yeast contamination this research).

DNA extractions were performed on B18 ρ^0 colonies, Tom70 colonies and liquid samples of the negative B18 ρ^0 control cultures after the end of the 14-day co-culture experiment. Figure 4.12 B shows the assessment of Cox3 presence for B18 ρ^0 and Tom70 cells and the negative B18 ρ^0 control culture samples. Tom70 was positive for presence of the Cox3 gene with a band of the correct product size of 128bp as expected. B18 ρ^0 cells were negative for the presence of Cox3 as expected. The negative B18 ρ^0 control cultures show presence of a band. The bands do not match the expected product size and thus do not show presence of the *Saccharomyces cerevisiae* Cox3 mitochondrial gene.

To assess whether or not the correct mating type could be detected in the negative B18 ρ^0 control cultures, PCR for mating type was performed on the same DNA extractions. B18 ρ^0 cells are of mating type Mat a and show a band of the correct product size (534bp). The negative B18 ρ^0 control cultures show two bands. These are not of the correct size for Mat a or α , indicating that the contaminant over-grew B18 ρ^0 cells in these cultures as the B18 ρ^0 cells would not have proliferated in YPGE medium.

Figure 4.12 C shows the PCR profile that could distinguish the contaminant in the negative B18 ρ^0 control cultured from the other cell types.

The fluorescent images indicate that the contaminant is a budding yeast. Mating type and Cox3 assessment however show no indication of a *S. cerevisiae* mating type locus nor any *S. cerevisiae* mitochondrial Cox3. Absence of growth on any colonies on any of the negative B18 ρ^0 control YPGE/5-FOA agar plates suggest that the contaminant can grow in liquid YPGE but not on YPGE/5-FOA. It is likely that this yeast contaminant is a different species to the *S. cerevisiae* used in this research.

4.2.5 Does the ratio of cells in co-culture affect mitochondrial transfer?

Data from mammalian cell work in our lab suggested that induction of mitochondrial transfer was linked to the ratio of stressed donor to recipient cell. If more donor cells were present, there was less transfer, and the frequency of transfer increased as the proportion of stressed recipients increased relative to the potential donor (Rebecca Dawson, personal communication). To determine if the same could occur in a yeast model, two different ratios of B18p⁰ cells to Tom70 cells were chosen: 100:1 and 50:1.

The final concentration of B18p⁰ cells in co-culture 5 (100:1) was 30×10^7 cells to 3×10^6 Tom70 cells in a total of 3mL YPGE. Co-culture 6 (50:1) had 15×10^7 B18p⁰ cells with 3×10^6 Tom70 cells in 3mL YPGE.

Two negative control cultures of B18p⁰ and Tom70 cells were set up for each ratio with identical concentrations of each cell type.

To avoid contamination, cultures were not washed and resuspended every day, nor were they sampled every day. Instead, day 2, day 6 and day 14 were chosen as days for sampling, representing an early-, mid- and late-phase of the co-culture experiment.

4.2.5.1 Proliferation of CC5 and CC6 and Controls

Figure 4.13 A shows the OD₆₆₀ for the B18p⁰ control culture, Tom70 control, quadruplicates of CC5 and duplicates for CC6.

The 100:1 co-cultures (CC5) had a higher OD₆₆₀ than the 50:1 co-cultures (CC6) due to the larger number of B18p⁰ cells at the start of the experiment. Figure 4.13 B shows the average OD₆₆₀ of the quadruplets and duplicates, graphed with standard deviations. None of the standard deviations for either time point overlapped with the standard deviation of the other, indicating a clear difference in OD₆₆₀ for each timepoint between CC5 and CC6 cultures.

Figure 4. 13 Proliferation for CC5 and CC6 over time: OD_{660} graphed for all individual replicates and controls of CC5 and CC6 (A). Average OD_{660} for CC5 quadruplicates and CC6 duplicates, graphed with standard deviations (B)

A **B**

Figure 4. 14 Number of green and red colonies for CC5 and CC6 over time: Individual counts of green and red colonies for all co-culture replicates over time (A) Total number of green and red colonies for CC5 and CC6 co-cultures over time (B)

The OD₆₆₀ of the B18 ρ^0 (CC5) and B18 ρ^0 (CC6) negative controls were consistent over time, indicating lack of growth of the B18 ρ^0 negative control in YPGE, indicative of successful control of contamination (Figure 4.13 A).

The Tom70 control cultures showed an increase in OD₆₆₀ over time. The low starting concentration of these cultures could explain the smaller increase in growth compared to previous experiments (Figure 4.13 A).

4.2.5.2 Fluorescent Analysis of CC5 and CC6

All colonies were picked and re-streaked on YPGE and samples were suspended in liquid SC, supplemented with 2 mg/mL 5-FOA in 96-well plates.

A total of 119 and 68 green colonies and 6 and 8 red colonies were obtained for CC5 and CC6, respectively (Figure 4.14 A and B).

Out of all 14 red colonies, 13 colonies showed presence of a substantial amount of cellular debris in the 384-well plate (Figure 4.15). This was not seen with green colonies.

Neither control for B18 ρ^0 cultures or Tom70 cultures showed growth of any colonies. Representative images of all red colonies can be found in Appendix B.

Figure 4. 15 Representative fluorescent images of red colonies of CC5: Images show DIC (left) or no DIC (right). CC5 (4) = co-culture 5, replicate 4

4.2.5.3 Genotypic Validation of Intercellular Mitochondrial Transfer

DNA was extracted from B18wt, Tom70, B18 ρ^0 cells and all 14 red colonies. Figure 4.16 A shows the assessment of mitochondrial DNA (Cox3). B18wt and Tom70 were positive for the Cox3 gene as expected. The B18 ρ^0 sample had no band, indicating that the B18 ρ^0 cells did not contain mitochondrial DNA. The results of the red colonies were variable. Nine of the 14 red colonies show bands of the correct product size, indicative of mitochondrial DNA in B18 ρ^0 cells. These came from both the 100:1 and 50:1 ratio. The proportion of red, mtDNA-positive colonies (65%) was similar to that observed in CC1 (75%).

Figure 4.16 B shows presence of nuclear DNA and confirmation of the mating type. All DNA samples had nuclear DNA, regardless of the presence of mitochondrial DNA and both co-cultures and controls were positive for the Mat a mating type.

To exclude the presence of Tom70 cells, PCR for the presence of the Tom70 specific, nuclear encoded URA3 cassette was performed. Figure 16 C shows the assessment of the presence of the URA3 cassette. Tom70 was positive for presence of URA3 while B18wt and B18 ρ^0 were negative for URA3 as expected. Interestingly, all red colonies that were positive for Cox3 were also positive for URA3. The red colonies that did not show a band for Cox3 were also negative for URA3.

Figure 4. 16 PCR profile of red CC5 and CC6 colonies: 240-410 = experimental names for the red colonies. Assessment of mitochondrial DNA (128bp) (A), mating type (534bp) (B) and URA3 (536bp) (C).

4.3 Summary

The results present in this Chapter have given some new insight into intercellular mitochondrial transfer between Tom70 and B18p⁰ cells under conditions of metabolic stress. Table 4.3, Figure 4.17 and Figure 4.18 summarise the occurrence of green and red colonies for all co-cultures, respectively.

The level of 5FOA-resistant cells was consistent between experiments, as determined by the range of green cells that could grow. These had all the characteristics of Tom70 cells, including mtDNA, URA3 and the green fluorescent signal.

Intriguingly, there were a small number of red colonies that grew on a non-fermentable carbon source, implying they had re-gained the ability to perform MET and OXPHOS. While a minority did not have detectable levels of mitochondrial DNA, most red colonies had gained mitochondrial DNA. Given the total number of cells analysed over the course of these experiments, these very rare events suggest that if intercellular mitochondrial transfer occurs in yeast, it is at a very low frequency.

Table 4.3: Summary of different co-culture results

Co-culture conditions	CC1+CC2	CC3+CC4	CC5+ CC6+ CC5-s + CC6-s	CC7s+ CC8s+ CC7-s + CC8-s	CC9	CC10
# Total colonies on all CC YPGE/5-FOA plates (range per plate)	239 (7-50)	206 (0-20)	422 (0-39)	160 (0-11)	161(3-53)	126 (9-64)
#Total green colonies on all YPGE/5-FOA CC plates (range per plate)	187 (7-40)	128 (1-20)	329 (0-30)	154 (0-11)	119 (3-32)	68 (2-17)
# Total red colonies on all YPGE/5-FOA CC plates (range per plate)	19 (1-5)	4 (1-2)	0	0	6 (1-3)	8 (1-5)
PCR Cox3: Tom70, B18wt, B18p⁰	as expected	ND	ND	ND	as expected	as expected
PCR Cox3 for representative red colonies	variable	ND	ND	ND	variable	variable
PCR: Mat a	variable	ND	ND	ND	as expected	as expected
PCR: Ura3	inconclusive	ND	ND	ND	unexpected	unexpected

Figure 4. 17 Summary of all green colonies grown for all co-cultures on YPGE/5-FOA: Boxplots represent maximum and minimum colony numbers, interquartile ranges and outliers. X represents the median; middle line represents the average. Green highlighted background represents range of 5-FOA resistant Tom70 cells observed in co-culture Tom70 controls.

Figure 4. 18 Summary of all red colonies grown for all co-cultures on YPGE/5-FOA: Boxplots represent maximum and minimum colony numbers, interquartile ranges and outliers. X represents the median; middle line represents the average.

The presence of URA3 in the red colonies that had mtDNA was surprising and suggests that these red colonies carried nuclear cassettes from both Tom70 (URA3) and B18p⁰ cells (NLS-RedStar2-HPH (nucleus), MRH1-egFP611 (membrane) and NATMX4-TEFpr-mCherry (cytoplasm)).

A breakdown of each co-culture colony composition, including controls, false positives and contaminants, can be found in Appendix C to G.

4.4 Discussion

This chapter describes various permutations of co-cultures of B18p⁰ and Tom70 cells, with differences in sampling times (10-14 days), cell ratios (1:1, 50:1 and 100:1) and growth conditions. The focus in all instances was on the number of green fluorescent colonies (5-FOA-resistant Tom70 cells) and red fluorescent colonies (B18p⁰ cells with potentially acquired mtDNA) grown on YPGE/5-FOA plates.

Neither changing the ratio of B18p⁰ to Tom70 cells, the growth conditions or the time in co-culture altered the frequency of red colonies and putative mitochondrial transfer. This suggests that inter-cellular transfer does not increase over time nor that any given time represents a “peak” or “preferred” time for this phenomenon to occur.

The one apparent mixed colony seen in CC4 could be the result of picking a green colony and some of the surrounding cells on the YPGE/5-FOA plate that had not grown but were still alive. Only proliferating cells will form colonies, however, cells that do not proliferate, will still be present on the plate. It was shown in chapter 3 that B18p⁰ cells remained viable even after 14 days in YPGE culture. It is possible that the mixed colony was a resistant Tom70 cell colony but that B18p⁰ cells were picked up simultaneously when picking the colony and subsequently would proliferate in the liquid SC in the 96-well plate and fluoresce in the 384-well plate.

The number of green colonies growing on YPGE/5-FOA varied between 0 and 40 per plate and represent 5-FOA resistant Tom70 cells. This fits in well within the expected number of colonies on YPGE/5-FOA plates determined in the standardized sensitivity experiments in Chapter 3 (range 15-22) and the corresponding co-culture negative Tom70 control cultures (range 0-34) in CC2 – CC6.

There was a discrepancy between total number of colonies on YPGE/5-FOA and the total number of green and red fluorescent colonies. This was mainly due to a number of colonies not growing in SC in the 96-well plates (CC1 and CC2) or when re-streaked on YPGE (CC3 – CC6). However, several of the co-cultures suffered from contamination. A few colonies did not fluoresce (possibly a yeast contaminant) and very few colonies suffered from a bacterial/fungal contaminant.

All the experiments were conducted in a “yeast laboratory” on the bench with Bunsen burners to provide a “sterile” workplace. The “yeast laboratory” contains equipment that is shared with

microbiologists, ecologists and other students using different yeast strains, potentially introducing a variety of organisms into the laboratory environment.

Contamination was also seen in some co-cultures as an increase in OD₆₆₀ for B18p⁰ cells in liquid YPGE/5-FOA medium. The characterisation of the yeast contaminant confirmed that increases in OD₆₆₀ were not due to growth of B18p⁰ cells but through the presence of a foreign yeast strain. Moreover, presence of a bacterial contaminant in the liquid cultures could have led to an overgrowth of these cultures. However, YPGE/5-FOA plates of negative B18p⁰ control cultures stayed negative and colonies that did grow on these plates stayed in the range of 0-45 per plate, which is not characteristic of a bacterial contamination.

As shown in Table 4.3, 5 experiments consisting of 14 co-cultures and 168 plates only grew 38 (range 0-5) red fluorescent colonies and only 8 of these (CC1) contained a few cells with an intracellular yellow grainy appearance. This yellow grainy appearance was due to mixing of the green and red signals and was indicative of intracellular mitochondrial transfer of green mitochondria into red cells. The lack of yellow in cells of other red colonies is probably because the gene that encodes Tom70 GFP signal is nuclear-encoded and will not transfer with mitochondria. Thus, while mitochondria will replicate, the green protein will dilute out over several generations. Given the very short generation time of *Saccharomyces cerevisiae* (90 minutes) this signal will likely be lost within a day.

Although tempting to assume that all 38 red colonies of YPGE/5-FOA represent mitochondrial transfer, this needed to be verified by the restoration of mtDNA in B18p⁰ cells with the ability to grow on YPGE. This PCR was not done for all cultures for a number of reasons, but CC1, CC5 and CC6 PCRs showed the absence of Cox3 in B18p⁰ cells, and its presence in Tom70 as expected. A total of 4 (of 19) colonies (of CC1 and all 14 colonies of CC5 and CC6) were tested for mtDNA. Only 12 (of 18 total) contained mtDNA. This is interesting as it shows that it may be possible for B18p⁰ cells to grow on YPGE/5-FOA without needing OXPHOS to utilize glycerol or ethanol. This suggests some adaptive metabolic changes in B18p⁰ cells or the presence of something in the immediate environment of B18p⁰ cells that could also provide necessary metabolites required for B18p⁰ cells to grow.

The fact that 12 of 18 samples did have mtDNA does not necessarily mean that they have acquired mitochondria from Tom70 cells; it is possible that very low undetectable levels of mtDNA remained after ethidium bromide exposure that replenished once ethidium bromide was removed. However, none of the negative B18p⁰ control cultures showed any red colonies

which does not support this theory. Increase in OD₆₆₀ for the negative B18 ρ^0 control cultures was most likely due to the introduction of a contaminant as characterised for CC4. Thus, the results do seem to indicate that mitochondrial transfer from Tom70 cells to B18 ρ^0 cells is a possibility for an extremely small number of cells. High-resolution confocal microscopy of mitochondria might be able to see a difference in morphology between remnant mitochondria in ρ^0 cells and restored/rescued mitochondria from transfer.

To exclude presence of Tom70 cells in the red colonies, PCR for presence of URA3 was conducted on the 14 red colonies of CC5 and CC6. All red colonies that were positive for the presence of mtDNA were also positive for URA3. The red colonies did not show the bright yellow appearance as seen in CC1, however, upon closure examination, faint yellow hazes were visible in some of the red colonies. These URA3 and COX3 positive red colonies contain both Tom70 nuclear DNA and Tom70 mitochondrial DNA as well as B18 ρ^0 cell nuclear DNA (as seen by the triple red fluorescent appearance encoded by three nuclear genes). *S. cerevisiae* cells of opposite mating type easily fuse but B18 ρ^0 cells and Tom70 cells are of the same mating type. Fusion of ρ^0 yeasts with wildtype yeast of the same mating type has only been described for protoplasts, cells that do not comprise a cell wall [89].

In summary, none of the co-culture setups was superior to another with respect to producing red fluorescent colonies. A small number of the B18 ρ^0 cells acquired the ability to grow on YPGE, some of which contained mitochondrial DNA and some which had no detectable levels of mtDNA. However, colonies positive for mtDNA also carried the URA3 gene, suggesting these colonies were not formed exclusively by mitochondrial transfer.

Chapter 5

Gene-deletion transformants

5.1 Background

Intercellular mitochondrial transfer has been demonstrated *in vitro* (reviewed in [2]) and *in vivo* [3, 4]. However, the underlying signals and mechanisms are currently not well understood and further research is required to elucidate the pathways involved in intercellular mitochondrial transfer.

Miro1 was the first protein to demonstrate a regulatory function in intercellular mitochondrial transfer. Miro1 is a Rho GTPase that binds to the outer mitochondrial membrane and to motor proteins, such as kinesin and dynein, facilitating mitochondrial movement along microtubules [90]. Overexpression of Miro1 in mesenchymal stem cells led to enhanced intercellular mitochondrial transfer to epithelial cells [6] and neonatal mice cardiomyocytes [91], while Miro1 knockdown led to loss of transfer [6, 91].

The yeast homolog to Miro1 is Gem1p, encoded by the Gem1 gene, which has a role in mitochondrial inheritance [92]. Mitochondrial inheritance is decreased in yeast mutants with defective Gem1p while overexpression of wildtype Gem1p did not have an effect on mitochondrial inheritance, indicating that Gem1p is not rate limiting *in vivo* for the movement of mitochondria [93].

Ypt11p is Rab GTPase which binds the class V myosin motor protein Myo2p and this complex formation is essential to facilitate the function of Myo2p in mitochondrial distribution. One regulatory function of this complex is the mother-to-bud transport of mitochondria. Overexpression of YPT11 resulted in accumulation of mitochondria in the bud while YPT11 deletion resulted in a delay of the mitochondrial transmission toward the bud [94].

Deletion of Gem1p or Ypt11p abolishes mitochondrial trafficking between mother and daughter cells. However, overexpression of Ypt11p, but not Gem1p, resulted in accumulation of mitochondria in the daughter bud.

Another potential pathway for mitochondria to move between cells is in extracellular vesicles. Key to this is DDI1, a regulator involved with the yeast homologues of SNAREs. Deletion of

DDI1 showed increased protein secretion, and it is proposed to be a negative regulator of exocytosis.

Single-deletion mutants of Gem1, YPT11 and DDI1 were analysed to study their potential involvement in intercellular mitochondrial transfer. Their regulatory involvements and functions regarding mitochondrial inheritance, trafficking and exocytosis offer the potential to gain insight into their involvement of intercellular mitochondrial transfer in *S. cerevisiae*.

Deletion of Gem1p and Ypt11p were hypothesized to inhibit all intercellular mitochondrial transfer, whereas deletion of DDI1 was hypothesized to increase this process when compared with transfer in the co-cultures of B18p⁰ cells and Tom70 cells.

5.2 Strain Generation & Validation

5.2.1 Genotypic Characteristics

5.2.1.1 Confirmation of Gene Deletions

5-FOA selectivity is required and is conferred by the URA3 cassette. The gene-deletion strains were created by homologous recombination of the URA3 cassette into the gene to be deleted. In addition, in YPT11, DDI1 and Gem1 cells, the green fluorescent protein is linked to the Cox6 protein, an inner mitochondrial membrane transporter protein.

To transform the URA3 cassette into these strains, the URA3 open reading frame (ORF) needed to be amplified from the paG60 plasmid, flanked by regions of homology for each gene to be deleted (Figure 5.1).

E.coli containing the paG60 plasmid with the URA3 cassette, were grown in LB medium (see Chapter 2) and the plasmid DNA containing the URA3 gene cassette was extracted as previously described (see Chapter 2).

To amplify the URA3 ORF with gene specific regions of homology, a PCR was performed using gene-deletion specific primers, each for YPT11, DD1 and Gem1 (see Chapter 2). Figure 5.2 A shows the PCR product for the amplification of the URA3 DNA fragment of the pAG60 plasmid, each of which contained regions of homology specific to the gene to be deleted.

The Cox6-GFP strain, which was wildtype for each gene, was transformed with the URA3 PCR product as per protocol for each gene (see Chapter 2). Verification of the correct integration of the URA3 cassette was carried out by PCR on genomic DNA (gDNA) for each of the transformants and product size was analysed. The gDNA from putative transformants was used as the template in PCR utilizing the confirmation forward and reverse Primers (see Chapter 2) which flank each integration site with the corresponding internal URA3 cassette primer. Figure 5.1 shows a schematic overview of this process. Figure 5.2 B shows the confirmation of URA3 presence and gene-deletion confirmation.

The Cox6-GFP gene deletion strains, *ypt11Δ0::URA3*, *ddi1Δ0::URA3* and *gem1Δ0::URA3* will be referred to as YPT11, DDI1 and Gem1 from here onwards.

Figure 5. 1 Schematic overview of gene-deletion transformation: URA3 cassette is amplified by PCR from the paG60 plasmid using primers that contain gene-specific regions of homology. PCR product is then used in the transformation, enabling regions of homology to undergo homologous recombination. Gene-deletion was confirmed by using internal and external confirmation primers.

Figure 5. 2 PCR amplifications of transformation: URA3 cassette of paG60 was amplified using primers that contained gene-specific regions of homology (A). Gene-deletion confirmation using gene-deletion confirmation primers A + B and C + D. NC denotes negative control (B).

PCR for Cox3, a mitochondrial encoded gene (Chapter 3 and 4), showed a clear band of the correct product size for YPT11, DDI1 and Gem1, demonstrating presence of mtDNA (Figure 5.3 A).

PCR for mating type, a nuclear encoded gene, showed all deletion strains used were of the same Mat a mating type (Figure 5.3 B).

Figure 5. 3 PCR assessment of gene-deletion strains: PCR assessment of mating type (Mat a - 534bp) (A) Assessment of mitochondrial DNA (Cox3 - 128bp) (B).

5.2.2 Phenotypic Characteristics

5.2.2.1 Growth Characteristics in Selective Media

Validation of substrate requirements for YPT11, DDI1 and Gem1 cells was done in both solid and liquid media.

To assess the ability to grow on solid media, cells were streaked from agar plate to agar plate, followed by incubation for at least 48h at 30°C. All cell types grew equally well on solid YPD, containing fermentable glucose, and on solid YPGE, containing glycerol and ethanol (Figure 5.4).

Growth in liquid YPD medium was also assessed over 48h. YPT11, DDI1 and Gem1 cells grew equally well in liquid YPGE as in liquid YPD (Figure 5.5).

5.2.2.2 Fluorescent Phenotypes

When using fluorescent microscopy to distinguish between B18p⁰ cells and YPT11, DDI1 or Gem1 cells, these cell types need to possess distinct fluorescent phenotypes.

In YPT11, DDI1 and Gem1 cells, the green fluorescent protein is linked to the Cox6 protein, an inner mitochondrial membrane transporter protein. The signal showed a distinct green localization close to the plasma membrane, characteristic of mitochondrial networks in *S. cerevisiae* [80]. This signal was also unaffected by type of medium (Figure 5.6).

A YPD

B YPGE

Figure 5. 4 Growth of YPT11, DDI1 and Gem1 on solid media: Growth on YPD (A) and YPGE (B) media

Figure 5. 5 Growth in liquid media: Growth of YPT11, DDI1 and Gem1 in liquid YPD (A) and YPGE (B) over 48 h.

5.2.2.3 5-Fluoroorotic Acid Selectivity

5-FOA sensitivity can be used to negatively select for cells containing the URA3 cassette (Chapter 3).

The effective concentration of 5-FOA for Tom 70 cells was calculated to be 1.5-2.0mg/mL (Chapter 3). This sensitivity assay was repeated for all gene-deletion strains and is shown in Figure 5.7 A. The differences in growth at different concentrations of 5-FOA were statistically significant for all three mutants (P-value < 0.05). The working concentration of 2 mg/mL 5-FOA, as established for Tom70 cells (see Chapter 3) was suitable to induce negative selection of the gene-deletion strains.

Analysis of DMSO, the vehicle for 5-FOA, showed that DMSO exposure resulted in only a minimal effect on growth, showing growth inhibition was largely due to 5-FOA and not DMSO (Figure 5.7 B).

To assess any residual resistance of YPT11, DDI1 and Gem1 cells to 5-FOA, liquid cultures were plated on YPGE agar plates, supplemented with 2mg/mL 5-FOA. Plates were incubated at 30°C for 14 days and the time of colony appearance was recorded.

On average for four repeats, 54 (range 0-78), 0.5 (range 0-1) and 24.25 (range 0-63) colonies grew on YPGE agar plates supplemented with 2mg/mL 5-FOA for Gem1, YPT11 and DDI1 respectively. The largest number of colonies on any of the plates was out 78 of 1×10^7 cells plated and indicates less than 0.0008% ($\frac{78}{1 \times 10^7} \times 100 = 0.00078$), of cells were resistant to 5-FOA. This demonstrates the strong toxicity of 5-FOA and its effective negative selectivity against YPT11, DDI1 and Gem1 cells (Figure 5.8). This was consistent with 5-FOA sensitivity of the Tom70 strain (Chapter 3).

A SC

Gem1

YPT11

DDI1

B YPD

Gem1

YPT11

DDI1

Figure 5. 6 Fluorescence of gene-deletion strains: Fluorescence in SC (A) and YPD (B) media.

Figure 5. 7 5-FOA Sensitivity of gene-deletion strains: Effects on proliferation of Gem1, YPT11 and DDI1 at various concentrations of 5-FOA (A) and effects of vehicle control (DMSO) on proliferation (B). All measurements are standardized to 0 mg/mL 5-FOA control.

Figure 5. 8 Residual resistance of gene-deletion strains to 5-FOA: Plates are replicates to determine residual resistance.

5.2.2.4 WST-1/PMS Reduction and CCCP Treatment

WST-1/PMS reduction was carried out as described previously (Chapter 2 and 3) with the appropriate controls for auto-oxidation. YPT11, DDI1 and Gem1 cultures showed high capacity to reduce the tetrazolium dye (Figure 5.9).

Next, the effect of carbonyl cyanide m-chlorophenyl hydrazine (CCCP) on WST-1/PMS reduction was established for all cell types (Chapter 5.9).

All three gene-deletion strains showed a reduced WST-1/PMS reduction that was CCCP concentration dependent (Figure 5.9). The difference between WST-1/PMS reduction of CCCP-treated and untreated cells was statistically significant for all strains (Gem1: P-value = 0.0000; YPT11: P-value < 0.0001; DDI1: P-value < 0.0001). This confirmed that all gene-deletion strains had functional MET and OXPHOS and that CCCP was able to uncouple MET and OXPHOS, leading to reduced WST-1/PMS reduction.

Gene deletions did also not alter sensitivity to 5-FOA.

Figure 5. 9 WST-1/PMS reduction after CCCP treatment

5.2.2.5 TTC Assay

Different to the cell impermeable WST-1/PMS assessment of PMET activity in liquid cultures, Triphenyl tetrazolium chloride (TTC) was used to assess the MET activity of B18 ρ^0 , YPT11, DDI1 and Gem1 cultures grown on SC agar plates. This was done to further confirm that the gene-deletion strains, YPT11, DDI1 and Gem1, were respiratory competent, not only in liquid culture but also on solid medium and that deletion of YPT11, DDI1 or Gem1 did not alter respiration capacity.

Cultures were grown on agar at 30°C for 48h and then overlaid with agar containing 0.75% TTC.

Figure 10 shows that the B18 ρ^0 culture remained white in the presence of 0.75% TTC, indicating that no TTC reduction occurred in these cells. YPT11, DDI1 and Gem1 cells showed a deep red colour in the presence of 0.75% TTC, indicating that YPT11, DDI1 and Gem1 cells have high MET function.

Figure 5. 10 TTC reduction of gene-deletion strains: Respiratory competent cells reduce the colourless TTC to a bright red colour.

5.3 Co-culture Experiments with Gene-Deletion Mutants

The research for this chapter includes multiple co-cultures of B18p⁰ cells and gene-deletion transformant cells in a 1:1 ratio. Experimental definition for each co-culture can be found in Table 5.1.

Table 5. 1 Experimental definition of gene-deletion co-cultures

Co-culture	CC7	CC8	CC9
Gene-deletion transformant	YPT11 (ypt11Δ0::URA3)	DDI1 (ddi1Δ0::URA3)	Gem1 (gem1Δ0::URA3)

CC7, CC8 and CC9 were set up in triplicate as described in Chapter 2 and Chapter 4. The final concentration of B18p⁰ cells was 1 x 10⁷ cells to 1 x 10⁷ gene-deletion transformant cells in a total of 3mL YPGE.

Triplicate negative control cultures for YPT11, DDI1 and Gem1 were set up each for CC7, CC8 and CC9. Two negative B18p⁰ cultures were set up.

Cultures were not disturbed other than on day 2, day 6 and day 14 when samples were taken.

5.3.1 Proliferation of CC7, CC8 and CC9

Figure 5.11 A shows the OD₆₆₀ of each of the triplicate gene-deletion co-cultures and Figure 5.11 B shows the average OD₆₆₀ of the triplicates for each gene-deletion co-cultures, graphed with standard deviations. All standard deviations overlapped, indicating no difference in OD₆₆₀ for each of the timepoints for the average OD₆₆₀ for each set of triplicates.

The YPT11, DDI1 and Gem1 negative controls showed an increase in OD₆₆₀ over time, similar to that of CC7, CC8 and CC9 (Figure 5.11 C).

Figure 5.11 Proliferation of gene-deletion co-cultures and controls: Individual gene-deletion triplicate co-culture OD₆₆₀ over time (A) and average OD₆₆₀ for all gene-deletion co-cultures (B). OD₆₆₀ of Gem1, YPT11 and DDI1 control cultures and B18p⁰ controls over time (C)

All cultures showed a slight increase in OD₆₆₀ over time. However, an increase in OD₆₆₀ of about 0.2 suggests low cell proliferation compared with the co-cultures CC1 to CC6.

The OD₆₆₀ of the B18 ρ^0 (1) and B18 ρ^0 (2) negative controls were consistent over time, indicating lack of growth of the B18 ρ^0 negative control in YPGE (Figure 5.11 C).

5.3.2 Fluorescent Analysis of CC7, CC8 and CC9

All colonies were picked and re-streaked on YPGE and samples were suspended in liquid SC, supplemented with 2 mg/mL 5-FOA in 96-well plates.

For the DDI1 co-culture (CC8), 26 green colonies and 9 red colonies were obtained, and for the YPT11 co-culture (CC9), 49 green colonies and 8 red colonies.

Gem1 plates showed more than 1500 colonies per plate, the highest number of colonies for any co-culture. 38 representative colonies were picked, avoiding colonies that were part of a cluster or touching other colonies. Of 38 colonies, 36 were green 2 were red.

All green colonies showed the same fluorescent appearance, characteristic of mitochondrial networks in *S. cerevisiae*.

Of 19 red colonies, 18 colonies showed presence of a large amounts of cellular debris (Figure 5.12) that was not seen in the presence of green colonies.

Neither control for B18 ρ^0 cultures showed growth of any colonies, and all gene-deletion control cultures were green, as expected.

Figure 5. 12 Fluorescent imaged of red colonies

5.3.3 Genotypic Validation of Intercellular Mitochondrial Transfer

DNA was extracted from B18wt, Tom70, B18 ρ^0 cells and all 8 red colonies. Figure 5.13 A shows the assessment of nuclear DNA and confirmation of the mating type. B18wt, Tom70 and the B18 ρ^0 cells were positive for Mat a as expected. All DNA samples had nuclear DNA, however, two samples (475 and 490) showed very faint bands, indicating low levels of nuclear DNA.

Presence of mitochondrial DNA was assessed on the same DNA extractions. B18wt and Tom70 were positive for the Cox3 gene as expected. The B18 ρ^0 sample had no band, indicating that the B18 ρ^0 cells did not contain mitochondrial DNA, as expected.

The results of the 8 red colonies varied. Only sample 415 showed detectable levels of mitochondrial DNA. All other samples were negative for Cox3 (Figure 5.13 B), but showed high levels of cellular debris.

Assessment of the Tom70 specific, nuclear encoded URA3 cassette was also performed on the same DNA extractions. Tom70 was positive for presence of URA3 while B18wt and B18 ρ^0 were negative for URA3 as expected. Only sample 415 showed presence of the URA3 cassette (Figure 5.13 C), consistent with the results obtained for CC5 and CC6.

Figure 5. 13 PCR assessment of red colonies of gene-deletion co-cultures: Assessment of mating type locus (*Mat a* - 534bp) (A), mitochondrial DNA (*Cox3* - 128bp) (B) and nuclear encoded, *Tom70* specific *URA3* cassette (*URA3* - 536bp) (C). 415 to 498 = experimental number. NC denotes negative control.

5.3.4 Summary

The results present in this Chapter have given some insight into intercellular mitochondrial transfer between three gene-deletion mutants and B18p⁰ cells under conditions of metabolic stress. Table 4.2 and Figure 4.14 summarise the occurrence of green and red colonies for all co-cultures, respectively.

There was a consistent level of 5-FOA-resistant cells, as determined by the range of green cells that could grow. Presence of mtDNA and the green fluorescent signal confirmed that these were 5-FOA resistant gene-deletion mutants.

Table 5. 2 Results summary of gene-deletion co-cultures

Co-culture result	CC7			CC8			CC9		
Gene-deletion transformant	YPT11 (ypt11Δ0::URA3)			DDI1 (ddi1Δ0::URA3)			Gem1 (gem1Δ0::URA3)		
# Total YPGE/5-FOA plates	9			9			9		
# Total colonies on all YPGE/5-FOA plates (range per plate)	280 (1-98)			222 (2-91)			>13.500 (1500-1600)		
# total colonies per day of sampling (range per plate)	Day 2	Day 6	Day 14	Day 2	Day 6	Day 14	Day 2	Day 6	Day 14
	9 (1-5)	9 (2-5)	262 (17-98)	11 (2-5)	24 (5-13)	187 (10-91)	>1500	>1500	>1500
#Total green colonies on all YPGE/5-FOA plates (range per plate)	26			49			36		
# Total red colonies on all YPGE/5-FOA plates (range per plate)	9 (0-7)			8 (0-8)			2 (0-2)		
PCR Cox3: Tom70, B18wt, B18p⁰	as expected			as expected			as expected		
PCR Cox3 for representative red colonies	negative			negative			variable		
PCR: Mat a	positive			positive			positive		
PCR: Ura3	variable			negative			negative

Figure 5. 14 Summary of green and red colonies of gene-deletion co-cultures: Boxplots represents range of colonies, line represents the average number of colonies and x denotes the median.

A small number of red colonies observed to grow on a non-fermentable carbon source, implying they had re-gained the ability to perform MET and OXPHOS. However, only one red colony showed presence of mtDNA while the majority did not have detectable levels of mitochondrial DNA. Given the total number of cells analysed over the course of these experiments, these very rare events suggest that intercellular mitochondrial transfer can occur in yeast but only at a very low frequency.

A breakdown of each co-culture colony composition, including controls, false positives and contaminant, can be found in Appendix H.

5. 4 Discussion

This chapter describes the generation and validation of three gene-deletion strains, Gem1, YPT11 and DDI1 followed by co-cultures of each of these with B18p⁰ cells.

Validation of the genotypic and phenotypic characteristics of the three mutant strains was shown by growth on both SC and YPGE solid and liquid media, sensitivity to 5-FOA, the presence of Cox3, strong WST-1/PMS reduction, CCCP sensitivity and TCC reduction.

By deleting genes that are known to be involved in mitochondrial transport and exocytosis, the expectation was that intercellular mitochondrial transport between Gem1p and YPT11 and B18p⁰ cells might be inhibited but promoted in co-cultures between DDI1 and B18p⁰ cells. However, both YPT11 co-cultures and DDI1 co-cultures grew very few colonies (range 1-98 and 2-91, respectively) which is within the range of CC1 to CC6 (range 0-64), suggesting that neither gene-deletion has an impact on the occurrence of intercellular mitochondrial transfer.

If the results of the 38 picked colonies from the Gem1 plate were to be extrapolated, this plate would have a slightly higher number of red colonies than produced with the Tom70-GFP gene replete or the YPT11 and DDI1 strains.

However, this assumption must be viewed with caution as there was an extremely high number of colonies on all of the Gem1 plates including the negative controls. If this was due to a sudden increase in 5-FOA resistance, from 0.0002% (resistance assay) to 3.6% in this co-culture experiment, there is no obvious explanation for this.

Both YPT11 and DDI genes play a role in mitochondrial inheritance and vesicular trafficking, indicating that intercellular mitochondrial transfer may be distinct from these processes. However, it is possible that deletion of a single gene does not abolish or promote transfer through rescue effects of other genes. In order to demonstrate this, it would be necessary to conduct co-cultures between all single gene-deletion, double gene-deletion and triple gene-deletion strains and B18p⁰ cells.

Interestingly, like with CC1-CC8, the aggregating clustering seen in 18 of 19 red colonies, but not green colonies, suggests presence of large amounts of cellular debris. Given that these red colonies grew on YPGE without mtDNA, the debris could provide nutritional support. Round morphology and clustering could also indicate presence of a bacterial contaminant such as *Staphylococcus*, a common skin commensal. However, presence of a bacterial

contaminant would likely have resulted in carpet like growth on the YPGE/5-FOA and YPGE plates, which was never seen.

In summary, the results shown in this chapter indicate that neither gene-deletion mutant increased or decreased the level of intercellular mitochondrial transfer in these co-cultures.

Chapter 6

Metabolic support

6.1 Background

Mitochondria are essential to tissue homeostasis. Many of the hallmarks of metabolic disorders, cancer and aging can be attributed to mitochondrial dysfunction. Studies that have reported intercellular mitochondrial transfer *in vivo* [3, 4] and *in vitro* (reviewed in [2]) have also suggested that metabolic exchange and direct cell-to-cell contact can convey new properties to the recipient cells, leading to cellular reprogramming and to phenotypes that can protect against tissue injury and resistance to therapeutic agents.

Mammalian ρ^0 cells are unable to conduct MET and OXPHOS. In cell culture, mammalian ρ^0 cells require supplementation with pyruvate and uridine for proliferation; these cells cannot survive without presence of pyruvate and uridine [43, 44].

Data from mammalian cell work in our lab suggested that the presence of respiratory competent cells was able to prolong lifespan of respiratory deficient cells in the absence of pyruvate and uridine (Schneider et al., manuscript in preparation).

Bone-marrow derived stromal cells could maintain viability of respiration-deficient ρ^0 astrocytes in a co-culture setting with metabolic stress induced by pyruvate-uridine withdrawal. Close cell contact between cells was observed without occurrence of intercellular mitochondrial transfer.

Mitochondrial transfer has also been studied under forced/induced conditions to precisely characterize the effects of respiration competent mitochondria on recipient cells.

Artificial transfer of mitochondria from a donor to a recipient cell was performed in oocytes, where mitochondria were directly injected into mitochondrially devoid oocytes [95-97].

Trans-mitochondrial cytoplasmic hybrids (cybrids) were created through the fusion of enucleated cells with respiration deficient ρ^0 cells [98, 99]. Endogenous mitochondrial DNA in a mouse cell line that was poorly metastatic was replaced with mtDNA from a highly metastatic cell line and vice versa in these cybrids to study the effects of mtDNA on tumour cell behaviour [98].

The complex and challenging techniques used in these studies allowed for a better characterization of the effects of respiration-competent mitochondria on recipient cells; however, the frequency of cells that would take up mitochondria and would be viable was relatively small and thus, these methods are not feasible for large-scale frequency studies.

One large-scale approach to study these effects involves the extraction, purification and centrifugation of respiratory-competent mitochondria into mitochondrially deficient cells [100, 101]. The protocol called MitoCeption was designed to directly and quantitatively transfer isolated mitochondria from cell A into recipient cell B [7]. Hereby, isolated and purified human mesenchymal stem cell (hMSC) mitochondria were centrifuged into MDA-MB-231 cancer cells, allowing for a more quantitative approach to study the effects of mtDNA on cell behaviour. This showed that hMSC mitochondria transferred by MitoCeption had the capacity to enhance energetic metabolism and increase the endogenous pool of mitochondria within MDA-MB-231 cancer cells [7].

While isolated and purified mitochondrial fractions can still contain both intact and damaged mitochondria, another protocol to study the effects of exogenous mtDNA on recipient cells was established. The magnetomitotransfer protocol used magnetic beads attached to an anti-TOM22 antibody, a mitochondrial outer membrane protein [101]. These magnetic antibodies attached to mitochondria and were extracted from the cells using magnetic forces [101]. Isolated mitochondria were added to a MRC-5 fibroblast cell monolayer in a single chamber slide and pulled down into the cells by a magnetic field [101]. Magnetomitotransfer resulted in significant elevated oxygen consumption of MRC-5 fibroblasts [101], implying that active mitochondria were transferred into the recipient cells.

This Chapter investigates whether or not 1) the same metabolic support could be observed in *S. cerevisiae* p^0 cultures as seen with mammalian p^0 cultures and 2) whether mitochondrial transfer could be forced in *S. cerevisiae* cells.

6.2 Results

6.2.1 Metabolic Support

In section 3.2.1.3, a few B18 ρ^0 cells were shown to survive and proliferate on YPGE/5-FOA without mitochondrial transfer. It was feasible that Tom70 cells could secrete metabolites or molecules into the culture medium to induce ρ^0 cell proliferation.

To test this, Tom70 cells were grown overnight, washed twice with ddH₂O and re-suspended to a concentration of 1×10^6 cells/mL in 3mL YPGE. Triplicate cultures were grown for 72h at 200rpm and 30°C. Then, cultures were spun down (2000g for 5min) and supernatant was collected, and filter sterilized. These Tom70 conditioned media were then transferred to a new tube.

B18 ρ^0 cells were grown in SC overnight and OD₆₆₀ was measured to ensure that cells had grown overnight, indicating that healthy cells were used for this experiment. B18 ρ^0 cultures were then washed twice with ddH₂O and re-suspended in conditioned YPGE medium or control medium (YPGE only) to a concentration of 1×10^7 cells/200 μ L in 96-well plates. Conditioned medium only and control YPGE medium only were also added in triplicate to the 96-well plate as negative controls.

Cultures were then incubated at 200rpm and 30°C for 11 days. OD₆₆₀ was determined as a measure of proliferation on day 0, 1, 2, 6, 9 and 11.

Figure 6.1 shows the experimental set-up.

Figure 6. 1 Proliferative support experimental set-up

Figure 6.2 shows the average OD₆₆₀ for B18p⁰ proliferation in conditioned and control medium over time. Samples show high variability. However, no increase in OD₆₆₀ over time and subsequent proliferation were observed in B18p⁰ cells under either condition. Differences in OD₆₆₀ between conditioned and control medium over time were not statistically significant (P-value < 0.05), indicating that conditioned medium did not induce proliferation of B18p⁰ cells in medium containing only glycerol and ethanol as carbon sources.

This suggests that Tom70 cells did not secrete metabolites into the medium that could promote B18p⁰ proliferation in the absence of a fermentable/sugar carbon source.

Figure 6. 2 Proliferation of B18p0 in condition and control medium: Proliferation was measured for 6 time-points for B18p0 cells in conditioned and control medium. Conditioned and control medium alone were included as negative controls.

6.2.2 Forced Mitochondrial Transfer

Green mitochondria from Tom70 cells were isolated and purified as described in Chapter 2. The purified mitochondrial fraction was imaged to confirm that extracted mitochondria were still fluorescent post extraction (Figure 6.3).

B18p⁰ cultures were grown overnight, washed twice with ddH₂O and re-suspended in YPGE to a concentration of 1 x 10⁶ cells/500μL. Then, B18p⁰ cultures underwent one of 2 pre-treatments; all treatments and controls were performed in triplicate.

1. Zymolyase treatment: To test whether or not reduced cell wall integrity could lead to increased mitochondrial uptake, B18p⁰ cells were treated with Zymolyase T100 for 8min. Then, 50μL SEM buffer containing mitochondrial fraction or pure SEM buffer (negative control) were added and suspension was centrifuged.
2. Incubation treatment: To test whether or not prior incubation with mitochondria or SEM buffer (negative control) could induce signals to increase mitochondrial uptake, B18p⁰ cells were incubated in a suspension rotator for 1h at 30°C at 200rpm in the presence of 50μL SEM buffer containing mitochondrial fraction or pure SEM buffer (negative control) before centrifugation.
3. No pre-treatment: To test whether or not centrifugation only could induce desired uptake of mitochondrial transfer, B18p⁰ cells did not receive any pre-treatment but either 50μL SEM buffer containing mitochondrial fraction or pure SEM buffer (negative control) were added before centrifugation.

Centrifugation occurred at either one of three speeds and one of three durations. Pre-treatments and centrifugation conditions are shown in Table 6.1 and the experimental set-up is shown in Figure 6.4 A.

Supernatant was discarded after centrifugation and pellets were re-suspended in 50μL YPGE and plated onto YPGE (treatments) or SC (control) plates containing 5-FOA to account for any Tom70 not excluded in the purification. B18p⁰ cells that were able to take up and utilise isolated Tom70 mitochondria were selected by growth on YPGE/5-FOA. Plates were then incubated at 30°C for 14 days.

Table 6. 1 Forced mitochondrial transfer experimental conditions

Treatment		1500 g			6500g			12000g		
Zymolyase	speed	Low			Medium			High		
	Time (min)	1	5	10	1	5	10	1	5	10
Zymolyase (no mitochondria)	speed	Low			Medium			High		
	Time (min)	1	5	10	1	5	10	1	5	10
Incubation	speed	Low			Medium			High		
	Time (min)	1	5	10	1	5	10	1	5	10
Incubation (no mitochondria)	speed	Low			Medium			High		
	Time (min)	1	5	10	1	5	10	1	5	10
Centrifugation only	speed	Low			Medium			High		
	Time (min)	1	5	10	1	5	10	1	5	10
Centrifugation only (no mitochondria)	speed	Low			Medium			High		
	Time (min)	1	5	10	1	5	10	1	5	10

Figure 6. 3 Experimental set-up of forced mitochondrial transfer experiment: Tom70 mitochondria were isolated and purified, then added to B18p⁰ cells undergoing one of three treatments before centrifugation at different speeds and durations. Samples were then plated on YPGE/5-FOA plates and incubated.

Figure 6.4 B shows a representative YPGE/5-FOA plate. No treatment YPGE/5-FOA plate showed growth of any colonies, even after 14 days or 30 days incubation.

YPGE/5-FOA plates for each treatment control showed growth of a yeast carpet on SC plates, indicating that spinning lengths and speeds did not interfere with the ability of cells to grow. Control YPGE/5-FOA plates can be found in Appendix I.

These results suggest that the treatment conditions set out here were not sufficient to induce forced uptake of mitochondria in B18p⁰ cells.

Figure 6. 4 Forced transfer results: Purified mitochondria appear as green dots under fluorescent analysis (A). YPGE/5-FOA treatment plates did not show colonies after 14 days of incubation (B).

6.3 Discussion

The results shown in this chapter investigated if 1) metabolic support provided by Tom70 cell-conditioned YPGE medium would promote *S. cerevisiae* proliferation and 2) whether forced mitochondrial transfer was possible in *S. cerevisiae* p^0 .

While yeasts are a useful eukaryotic model organism, there are some significant differences between mammalian and yeast cells. First, as *S. cerevisiae* cells do not die under metabolite withdrawal, proliferation was assessed instead of viability. Tom70 conditioned medium could not induce proliferation of B18 p^0 cells. Under these conditions, Tom70 cells did not secrete metabolites into the medium that allowed B18 p^0 cells to proliferate.

Centrifugation of purified mitochondria into mitochondrial depleted cells presents a large-scale approach to obtain higher frequencies of mitochondrial transfer to study effects of mitochondria on recipient p^0 cells in mammalian cell cultures. In yeast research, this method had not been used previously.

The results shown here indicated that forced transfer of isolated mitochondria could not be induced in *S. cerevisiae* under the conditions used. This experiment does not rule out that MitoCeption into *S. cerevisiae* p^0 cells may be possible under different experimental conditions. The experimental set-up required high levels of mitochondrial activity immediately upon uptake as cells were centrifuged and plated onto YPGE. Maybe mitochondria were taken up but cross-communication between mitochondria and nucleus was not sufficient at this stage to perform all MET and OXPHOS required.

Confocal microscopy immediately after centrifugation could give an indication to whether or not green mitochondria could be seen within red cells.

Chapter 7

General Discussion and Future Directions

This thesis describes research investigating several permutations of co-cultures to facilitate intercellular mitochondrial transfer between respiratory competent strains and incompetent p^0 strains of *Saccharomyces cerevisiae*.

7.1 Does intercellular mitochondrial transfer occur between respiratory competent and incompetent p^0 strains of *Saccharomyces cerevisiae*?

Mitochondria are inherited through the maternal line in mammalian cells. It has been widely accepted that mitochondria are restricted to their cell of origin. However, intercellular mitochondrial transfer has been observed in mammalian cells *in vitro* (reviewed in [2]) and *in vivo* [3, 4]. Whether this is a conserved and universal process poses an interesting question regarding its evolutionary origin and purpose. If such a system does exist universally in all eukaryotes, multicellular and unicellular, intercellular mitochondrial transfer could represent many advantages; a possible rescue mechanism of cells with dysfunctional mitochondria; a mechanism to increase the diversity of the mtDNA pool between cells; or it could simply be a remnant of endosymbiotic movement of prokaryotic material between eukaryotic hosts.

Mammalian intercellular mitochondrial transfer studies focused on respiratory injury and have showed that intercellular mitochondrial transfer could restore respiration in p^0 cells.

The mechanisms of intercellular mitochondrial transfer included tunnelling nanotubes (TNTs), vesicular transport and cell fusion.

The p^0 model represent an extreme form of respiratory injury and was hypothesized to be the most likely model to induce intercellular mitochondrial transfer in *Saccharomyces cerevisiae*.

7.1.1 Intercellular Mitochondrial Transfer in *Saccharomyces cerevisiae*

The research for this thesis used *S. cerevisiae* BY4741 B18p⁰ cells whose mtDNA had been removed through exposure to ethidium bromide. These represented injured, respiratory deficient cells while *S. cerevisiae* BY4741 Tom70 cells represented healthy, respiratory competent cells with fluorescent mitochondria.

The use of a simple unicellular eukaryotic model may be very helpful in answering questions pertinent to mammalian mitochondrial transfer, such as defining the signals and processes involved. Intercellular mitochondrial transfer had never been investigated in *S. cerevisiae*. It was possible that this is a phenomenon only of mammalian cells, or of multicellular organisms. It was also possible that even if mitochondria do travel between yeast cells, the mechanism behind it may be very different because the cell wall of yeast cells may prevent formation of tunnelling nanotubes or vesicles. However, the potential benefits of the yeast genetics made it worth investigating.

Results shown in Chapter 4 indicate that intercellular mitochondrial transfer may indeed occur in *Saccharomyces cerevisiae* but that this is an extremely rare occurrence. Co-culture experiments with red fluorescent B18p⁰ cells and green fluorescent Tom70 cells showed that a very small number of red colonies grew on YPGE, which does not contain a fermentable carbon source. Some of these colonies had re-acquired mtDNA, suggesting mitochondrial transfer but some had not acquired mtDNA and should not have been able to grow on YPGE.

The morphology of aggregating clusters seen in the presence of most red colonies, but not green colonies, suggests presence of either large amounts of cellular debris or bacterial contamination, potentially of the genus *Staphylococcus*. However, the presence of so many bacterial cells amongst the yeast cells would have resulted in a carpet of bacterial colonies covering the plate. Bacterial growth would also have been seen in at least some of the 5-FOA-resistant Tom 70 cells. In addition, bacteria would have been seen clustering and covering yeast cells. Thus, the presence of aggregates can be most likely be explained by cellular debris rather than bacterial contamination. Dying cells and cellular debris would release all cellular contents and potentially provide enough nutritional support for B18p⁰ cells to proliferate, particularly in the absence of mitochondrial DNA.

B18p⁰ cells that had acquired mtDNA had also acquired the nuclear encoded, Tom70 specific URA3 cassette as well as retaining the original three red cassettes (NLS-RedStar2-HPH

(nucleus), MRH1-egFP611 (membrane) and NATMX4-TEFpr-mCherry (cytoplasm)). This suggests that cell fusion between Tom70 cells and B18p⁰ cells may be the potential mechanism behind intercellular mitochondrial transfer. These results fit in with the results observed in mammalian co-culture experiments conducted in our laboratory (Matthew Rower and Dr Remy Schneider – unpublished data) where cell fusion occurred between respiratory competent and respiratory compromised p⁰ cells.

It would be expected that if cell fusion were to have occurred here, that all cells would show a yellow grainy appearance, similar to that seen in CC1, due to the presence of the nuclear encoded Tom70 GFP tag. No red cell showed a predominant yellow appearance.

However, the research conducted in our lab has shown that fused, binucleated cells result in mononucleated cells over time; one nucleus establishes dominance over the other, resulting in the other nucleus shrinking. Similar could have occurred here; DNA of both nuclei may be present but only DNA of one nucleus being expressed. This would explain the absence of a green/yellow signal within the cells. To address this, mRNA levels of the URA3 or GFP gene could be measured to give insight into the expression levels of the Tom70 nuclear DNA.

B18p⁰ cells and Tom70 cells have the same mating type and cell fusion of cells of the same mating type has not been described in *S. cerevisiae* other than for protoplasts [102] as the cell wall prohibits fusion of cells of identical mating type, so this would be a highly unusual finding. Live-cell imaging of co-cultures would allow to capture any fusion-events and potentially the broader mechanisms of intercellular mitochondrial transfer.

An alternative explanation for the co-presence of Tom70 mitochondrial DNA and nuclear DNA within B18p⁰ cells would be horizontal gene transfer, induced by metabolic stress and selective pressure due to 5-FOA. It could be that Tom70 cells did not only transfer mitochondrial DNA but also the URA3 gene.

7.1.2 Can gene-deletion co-cultures give insight into underlying mechanisms?

Chapter 5 investigated intercellular mitochondrial transfer between B18p⁰ cells and the single-gene deletion strains, YPT11, DDI1 and Gem1, that had either shown involvement in mitochondrial transfer in mammalian cells or were implicated in a potential underlying mechanism of transfer.

7.1.2.1 Gem1p

S.cerevisiae Gem1p is the homologue of Miro1_h, which facilitates intercellular mitochondrial transfer in mammalian cells. Overexpression of Miro1 in mesenchymal stem cells led to enhanced intercellular mitochondrial transfer to epithelial cells [6] and neonatal mice cardiomyocytes [91], while Miro1 knockdown led to loss of transfer [6, 91]. Miro1 can bind the MFN2 protein mitofusin, another GTPase embedded in the outer mitochondrial membrane, promoting mitochondrial fusion [103]. Degradation of Miro1 and mitofusin in damaged mitochondria disrupts mitochondrial transport and prevents fusion, isolating damaged mitochondria from healthy ones and promoting mitophagy; this could explain why intercellular mitochondrial transfer is abolished in the absence of Miro1.

The human RHOT1 gene encodes one of the two mitochondrial Rho GTPase 1 (MIRO1) isoforms, a unique subgroup of the Ras superfamily involved in mitochondrial dynamics [90, 103-105]. The Miro1 enzyme facilitates mitochondrial transport by binding to motor and adaptor proteins, and is involved in mitochondrial homeostasis, apoptosis and cancer [90, 106-108]. Miro1, and subsequently mitochondrial transport, are regulated by calcium ion levels; high calcium ion levels cause dissociation of the motor/adaptor complex and thus no transport along microtubules.

7.1.2.2 Ypt11p

S. cerevisiae Ypt11p is the homologue of the mammalian Rab11 GTPase. Rab GTPases, like Rab11, have been implicated in the regulation of specificity in membrane trafficking and SNARE complex assembly through interactions with SNARE components at the level of

SNARE activation [109, 110]. Rab11 regulates the endosomal recycling pathway in mammalian cells [111]. Rab11 has also been shown to play a role in mitochondrial distribution and is involved in the division of mitochondrial networks into smaller organelles that can be transported by motor proteins [112, 113]. Thus, enzymes such as Rab11, offer an interesting potential for intercellular mitochondrial involvement.

In yeasts, mitochondrial organisation and transportation are reliant on actin filaments. Mitochondrial inheritance is decreased in yeast mutants with defective Gem1p while overexpression of wildtype Gem1p did not have an effect of mitochondrial inheritance, indicating that Gem1p activity is not rate-limiting (*in vivo*) for the movement of mitochondria [93]. Miro1 and subsequent mitochondrial transport are regulated by calcium ion levels in mammalian cells; however, Gem1p mutants without or with increased calcium ion binding motifs demonstrated that calcium ion binding was not necessary for its function in mitochondrial inheritance, suggesting that calcium binding does not stimulate or inhibit GTPase activity [114]. It is possible that mitochondrial Rho GTPase activity is regulated differently in different organisms. However, Miro1 protein function are conserved between human and yeasts, despite the differences of cytoskeletal tracks and motor proteins used for movement. In yeasts, organelles move mostly along actin filaments and require a myosin motor (Myo2p) and two motor association proteins, Mmr1p and Ypt11p in addition to Gem1p [94, 115].

Mmr1p and Ypt11p, contribute to myosin dependant mitochondrial movement and have been shown to contribute to mitochondrial inheritance. Mmr1p and Ypt11p promote mitochondrial inheritance in independent pathways; defects in either protein results in daughter buds without mitochondria [93, 114].

Ypt11p expression peaks during G1 phase of the cell cycle, corresponding to the timing of bud emergence [116, 117], and is regulated by phosphorylation and degradation [118].

Miro1 promotes intercellular mitochondrial transfer while Rab11 has a link to mitochondrial movements but has not been implicated in intercellular mitochondrial transfer in mammalian cells. Intercellular mitochondrial transfer has not been investigated in *S. cerevisiae* before. Based on mammalian studies, Gem1p and Ypt11p deletion mutants were used to investigate whether or not they would inhibit intercellular transfer in *S. cerevisiae*.

7.1.2.3 DDI1p

The possibility of increased intercellular mitochondrial transfer was addressed by using a DDI1p deletion mutant. In yeasts, Snc1 and Snc2 are SNARE homologues [119] and the *S. cerevisiae* DDI1p is a DNA damage-inducible v-SNARE binding protein. DDI1 has shown to act at the level of secretory vesicle docking and fusion and to play a role in the suppression of protein secretion [120]. Deletions of DDI1 in *S. cerevisiae* mutants displayed increased protein secretion, increased chronological lifespan and competitive fitness and decreased stress resistance. It is proposed that DDI1 is a negative regulator of exocytosis in *S. cerevisiae* [120].

Thus, Gem1, YPT11 and DDI1 were chosen as candidate genes for this research. Their regulatory involvements and functions regarding mitochondrial inheritance, trafficking and exocytosis offer the potential to gain insight into their involvement of intercellular mitochondrial transfer in *S. cerevisiae*.

7.1.2.4 Co-cultures with Single Gene-Deletion Strains

Co-cultures of each of the gene-deletion strains with B18p⁰ cells did not affect the number of red colonies on YPGE in *S. cerevisiae*.

It is possible that the single gene-deletions were not sufficient to induce an effect that could indicate their involvement. Co-cultures with the complete single gene-deletion, double gene-deletion and triple gene-deletion libraries would be better suited to identify the pathways of intercellular mitochondrial transfer. However, this would require a feasible, large-scale approach as co-cultures, as performed in this research, with approximately 4000 different yeasts strains was not feasible within the timeframe available.

Similar difficulties had been observed in mammalian studies, where frequency of mitochondrial transfer was not high enough to thoroughly study the effects of donor mitochondria on recipient cells. In response, methods have been developed for high frequency, large-scale mitochondrial transfer approaches.

7.2 Can mitochondrial transfer be forced in *Saccharomyces cerevisiae* p^0 cells?

Chapter 6 investigated a large-scale approach to induce mitochondrial transfer at higher frequencies with the aim to develop a methodology that could allow utilisation of whole gene-deletion libraries.

In mammalian research, two methods have been developed recently to study the effects of donor mitochondria on recipient cells. These methods used centrifugation (MitoCeption) [7] or magnetic forces (Magnetomitotransfer) [101] to force isolated mitochondria into recipient cells.

MitoCeption was used in this research to force mitochondria into B18 p^0 cells. Various treatment conditions and varying centrifugation speeds and durations were used. However, results shown in Chapter 6 demonstrated that forced mitochondrial transfer could not be established in *S. cerevisiae* B18 p^0 cells under set conditions.

7.2.1 Can respiratory competent cells provide metabolic support?

Research in our lab has shown that presence of respiratory competent cells could prolong survival of p^0 mammalian cells (Rebecca Dawson, Dr Remy Schneider, Matthew Rowe, personal communication). These observations were not replicated in *S. cerevisiae* as shown in Chapter 6. B18 p^0 cells were viable after 14 days of culture in YPGE but did not proliferate in Tom70 conditioned YPGE medium.

7.3 Why would *Saccharomyces cerevisiae* undergo intercellular mitochondrial transfer?

While these data indicated that mitochondrial transfer, accompanied by transfer of nuclear DNA, potentially through cell fusion, may indeed occur in *S. cerevisiae*, it is necessary to evaluate if the extremely low frequency events are of any significant biological value that would suggest further research in this model organism would be useful.

In mammalian cells, mitochondrial transfer could be a possible rescue mechanism for cells with dysfunctional respiration [4, 5, 8, 17, 38]. Replacing respiratory incompetent mitochondria with respiratory competent ones could be a way of dealing with mitochondrial injury, caused by reactive oxygen and nitrogen species (ROS and RNS) due to oxidative stress. In the case of cancer, obtaining healthy mitochondria to replace radiation- and chemotherapy-injured mitochondria, could potentially lead to a novel form of treatment resistance [4, 40, 41].

The requirement for mammalian p^0 cells to obtain mitochondria from their environment *in vivo* is based on their need for pyrimidine biosynthesis and not on mitochondrial ATP production. Respiratory capacity is required for the activity of dihydroorotate dehydrogenase (DHODH). DHODH, a flavoprotein found on the outer surface of the inner mitochondrial membrane, catalyses the fourth step in the pyrimidine biosynthesis. DHODH catalyses the oxidation of dihydroorotate to orotate, a ubiquinone-mediated process. Electrons from this oxidation are used to reduce coenzyme Q (CoQ) just prior to complex III in the mitochondrial electron transport chain [43]. In p^0 cancer cells, which are lacking functional mitochondrial electron transport, DHODH is unable to oxidize dihydroorotate to orotate and thus pyrimidine synthesis is blocked. Blocked pyrimidine synthesis means DNA cannot be synthesized and cells cannot proliferate.

In culture, uridine supplementation bypasses this block. However, *in vivo*, p^0 cells must acquire mtDNA to re-establish mitochondrial electron transport to allow pyrimidine biosynthesis. When DHODH was deleted in 4T1 cells and B16 cells, these respiratory competent cells did not form tumours. Restoring DHODH resulted in normal tumour growth in both models. However, disabling mitochondrial ATP production by deleting ATPb5 did not really affect tumour growth [121]. After mitochondrial transfer, or when cells were supplemented with an alternative oxidase, p^0 cells formed tumours, respired and contained mtDNA of the host genotype [3, 4, 121].

In contrast to mammalian DHODH, DHODH in *S. cerevisiae* is cytosolic and catalyses the oxidation of dihydroorotate to orotate and the reduction of fumarate to succinate; it can use fumarate as an electron acceptor and is independent of oxygen [122].

Two isoforms of the fumarate reductase exist; a cytosolic and pro-mitochondrial isoform. Both are essential for anaerobic growth [123] and are required for the re-oxidation of intracellular NADH during anaerobic growth [124]. While proliferation and cell growth in *S. cerevisiae* in the absence of oxygen lowers the available fumarate and subsequent DHODH function, mesaconate can drive oxidation of dihydroorotate as an alternative electron acceptor and can support pyrimidine *de novo* synthesis if the level of fumarate is low [125].

In summary, *S.cerevisiae* ρ^0 cells do not have the extreme need that mammalian ρ^0 cells have to acquire mitochondria to ensure pyrimidine biosynthesis.

7.3.1 Stress Responses in *Saccharomyces cerevisiae*

Changes in the environment such as temperature or nutrient availability, presence of toxic substances, represent stresses that can cause cells to stop growing or multiplying, cause cell damage or even cell death if organisms cannot adapt. In *S. cerevisiae*, the 'environmental stress response' changes the expression of more than 900 genes, emphasizing the importance for these single cellular organisms to respond appropriately to changes in their environment. The single celled yeasts are much less able to control certain environmental variables than mammalian cells which are to some extent sheltered from very large fluctuation within the homeostatic protection present for tissues, organs and organ systems [126, 127].

Diploid yeast cells respond to stress by inducing sporulation and tetrad formation. Haploids in comparison, as used in this research, will enter a stress tolerance state that offers a trade-off between growth and survival. While cells do not duplicate under stressed conditions, they focus on cellular robustness and often use anaerobic mechanisms to sustain life. Yeast can survive a variety of stresses and can switch to active growth and duplication upon return to appropriate environmental conditions [126, 128, 129]. This was seen in the ability of B18 ρ^0 cells to survive but not to proliferate on YPGE. In contrast to yeasts, mammalian cells die if severe stresses persist beyond their ability to adapt and leads to cell death [130].

The ability to survive and hibernate until return to appropriate environmental conditions offers yeasts an adaptation that mammalian cells lack. Thus, yeasts cells might simply not require mitochondria to an extend that mammalian cells do, explaining the extremely low frequency of intercellular mitochondrial transfer observed in Chapter 4 and Chapter 5. If this is the case, then their utility as model to explore the signals and processes that drive transfer will be limited.

7.4 Future Directions

The results presented in this thesis suggest that intercellular mitochondrial transfer may indeed occur in a very small number of cells. Interestingly, mitochondrial transfer is co-occurring with nuclear DNA transfer, potentially through cell fusion.

The small frequency of intercellular mitochondrial transfer events observed in this research suggest that this phenomenon is an extremely rare event. Through selective pressure, this rare event can still be of importance to the population as such. However, the low occurrence frequency of mitochondrial transfer established with the experimental parameters set out in this research makes *S. cerevisiae* a less suitable model to study and mimic mitochondrial transfer of mammalian cells.

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Appendences

Appendix A

Appendix A: Fluorescent Images of all red colonies of CC1

Appendix B

Appendix B: Fluorescent Images of all red colonies of CC5 and CC6

Appendix C

Appendix C: Summary of co-culture colony composition of CC1: Discrepancy between total number of colonies grown on YPGE/5-FOA and the green/red colonies can be attributed to physical contamination (dark grey) or false positives (black and white dots). False positives were defined as colonies that did not re-grow when suspended in liquid SC in the 96-well plate.

Appendix D

Appendix D: Summary of co-culture colony composition of CC2: Total colony composition of CC2 (A) and CC2 controls (B). False positives were defined as colonies that did not re-grow when suspended in liquid SC in the 96-well plate. Contamination in B was defined as presence of a bacterial/yeast contaminant in the fluorescent screen.

Appendix E

A

B

Appendix E: Summary of co-culture colony composition of CC3: Total colony composition of CC3 (A) and CC3 controls (B). False positives were defined as colonies that did not re-grow when re-streaked on YPGE.

Appendix F

A

B

Appendix F: Summary of co-culture colony composition of CC4: Total colony composition of CC4 (A) and CC4 controls (B). False positives were defined as colonies that did not re-grow when re-streaked on YPGE. 5-FOA sensitives were defined as colonies that did not re-grow when suspended in liquid SC, supplemented with 5-FOA, in the 96-well plates.

Appendix G

A

B

Appendix G: Summary of co-culture colony composition of CC5 and CC6: Total colony composition of CC5 (A) and CC6 (B). False positives were defined as colonies that did not re-grow when re-streaked on YPGE. 5-FOA sensitives were defined as colonies that did not re-grow when suspended in liquid SC, supplemented with 5-FOA, in the 96-well plates.

Appendix H

A

B

C

Total number of colonies
 Green colonies
 Red colonies
 False positives
 5-FOA sensitive
 Contamination
 Normal red colonies
 Normal green colonies

Appendix H: Summary of co-culture colony composition of gene-deletion co-cultures: Total colony composition of YPT11 (CC7) (A), DDI1 (CC8) (B) and Gem1 (CC9) (C). False positives were defined as colonies that did not re-grow when re-streaked on YPGE. %-FOA sensitives were defined as colonies that did not re-grow when suspended in liquid SC, supplemented with 5-FOA, in the 96-well plates.

Appendix I

Treatment speed		Zymolyase control	Just spinning control	Incubation control
1500g (low)	1 min
	5 min
	10 min
6500g (medium)	1 min
	5 min
	10 min
12000g (high)	1 min
	5 min
	10 min

Appendix I: Growth of B18p⁰ cultures after MitoCeption: B18p⁰ control cultures underwent three treatments (containing SEM buffer but no mitochondria) and underwent one of three spinning durations and speeds before plating on SC.

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