Investigation of the Glutaredoxin system with the biogenesis of mitochondrial intermembrane space proteins

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ABSTRACT

Mitochondrial protein biogenesis depends on the import of nucleus-encoded precursors from the cytosol. Import is highly regulated and specific for different subcompartments, with intermembrane space (IMS) import driven by an oxidative mechanism on conserved cysteine residues. Oxidative folding in the IMS is facilitated by the mitochondria import and assembly (MIA) pathway. Proteins can only be imported into the IMS in Cys-reduced unfolded forms, as oxidation prevents translocation into the IMS. How the import-competent forms are maintained in the cytoplasm is lesser characterised compared to the MIA pathway. Two recent studies suggest that the cytosolic Thioredoxin (Trx) and Glutaredoxin (Grx) reductase systems play a role in facilitating IMS protein import, with previous evidence identifying a role for yeast Trxs in small Tim protein biogenesis.

In this study, the redox properties of the yeast Trx and Grx systems were investigated, as well as whether the yeast Grx system play a role in the biogenesis of two typical types of IMS precursor proteins. First, *in vitro* studies were carried out to determine the standard redox potentials (E°) of the Trx and Grx enzymes. This was a quantifiable parameter of reducing activity and the results were described in Chapter 3. This study determined the E°<sub>Trx1</sub> value, which was shown to be a more effective reductant compared to other orthologs. Experimental limitations prevented the Grx system E° values being determined. Next, whether the Grx plays a role in the biogenesis of the CX<sub>3</sub>C motif-containing small Tim proteins were investigated using yeast genetic *in vivo* and biochemical analysis methods. The results were described in Chapter 4. There, Grxs were observed to not affect cell growth, but in using overexpressed Tim9 as an import model, significant differences were observed for the Grx system as *GRX* deletion significantly decreased overexpressed Tim9 levels. Study on the isolated mitochondria and cytosol with overexpressed Tim9 was unclear however. This study also observed a genetic interaction between *GRX* and *YME1* that recovered cell functioning under respiratory conditions. Finally, whether the Grx system plays a role in the biogenesis of CX<sub>3</sub>C motif-containing proteins (Mia40, Mia40C and Cox17) was studied in Chapter 5. Whilst Mia40C (C-domain of Mia40) and Cox17 are imported into mitochondria via the MIA pathway, the full-length Mia40 is a substrate of the presequence-targeted TIM23 pathway. The results indicated that import of the full-length Mia40 was unaffected by *GRX* deletion. However, studies of an overexpressed Mia40C as a substrate of the MIA pathway, showed strong mitochondrial protein level decreases caused by deletion of the Grx proteins. This decrease was also accompanied by an accumulation of unimported Mia40C in the cytosol. Cox17 as an alternative MIA pathway substrate also showed decreased mitochondrial levels in the *GRX* deletion mutants. These results altogether suggest that the cytosolic Grx system can function in the biogenesis of CX<sub>3</sub>C motif-containing IMS proteins imported through the MIA pathway, as well as the CX<sub>3</sub>C small Tim proteins. The topic of how IMS proteins are degraded in the cell was also raised by the study of Yme1.
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PREFACE ABOUT THE AUTHOR

I have studied at the University of Manchester since 2011 and before attending, I received a First Class BSc (Hons) from the University of Newcastle for Biomedical Sciences (2008-2011). With regards to research experience, in this study I have covered a wide field of techniques from \textit{in vitro} redox analysis to \textit{in organello} experimentation and the genetic modification of \textit{S. cerevisiae} strains. The focus of this study was on the \textit{in vivo} study of cell biology and protein expression. Previous research experience came from my undergraduate degree, where I performed western blot analyses in the neurosciences laboratories, as well as a summer placement focused on the \textit{in vitro} study of mammalian tissue.
LIST OF ABBREVIATIONS

$A_{260}$ Absorbance measured at 260nm wavelength
$A_{280}$ Absorbance measured at 280nm wavelength
$A_{600}$ Absorbance measured at 600nm wavelength
AAC ATP/ADP Carrier
ACN Acetonitrile
ADP Adenosine diphosphate
Amp Ampicillin
AMS 4-acetomido-4'-maleimidylstilbene-2,2-disulfonic acid
APS Ammonium persulfate
Arc1 Arcyl-RNA-Complex 1
ATP Adenosine trisphosphate
BB7.4 Breaking buffer pH 7.4
Ccs1 Copper chaperone superoxide dismutase 1
Cox17 Cytochrome c oxidase 17
Ct qPCR Cycle threshold
$CX_2C$ Dithiol cysteine motif with a 2 amino acid interval
$CX_3C$ Dithiol cysteine motif with a 3 amino acid interval
$CX_9C$ Dithiol cysteine motif with a 9 amino acid interval
Cys Cysteine
Cyt $b_2$ Cytochrome $b_2$
Cyt c Cytochrome c
DTT Dithiothreitol
$E^{**}$ Redox potential
EDTA Ethylenediaminetetraacetic acid
ER Endoplasmic reticulum
Erv1 Essential for respiration and vegetative growth 1
FAD Flavin adenine dinucleotide - oxidised
FADH$_2$ Flavin adenine dinucleotide - reduced
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Glr</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>Grx</td>
<td>Glutaredoxin</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione - reduced</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione – oxidised</td>
</tr>
<tr>
<td>HLH</td>
<td>Helix-Loop-Helix</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>i-AAA</td>
<td>Inner membrane ATPases associated with diverse cellular activities</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>Intermembrane space</td>
</tr>
<tr>
<td>ITS</td>
<td>Intermembrane space-targeting signal</td>
</tr>
<tr>
<td>K_{eqm}</td>
<td>Equilibrium constant</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy broth media</td>
</tr>
<tr>
<td>LM</td>
<td>Lactate media</td>
</tr>
<tr>
<td>MDM</td>
<td>Mitochondria distribution and morphology</td>
</tr>
<tr>
<td>MIA</td>
<td>Mitochondria import assembly</td>
</tr>
<tr>
<td>MISS</td>
<td>Mitochondrial intermembrane space sorting</td>
</tr>
<tr>
<td>MPP</td>
<td>Mitochondria processing peptidase</td>
</tr>
<tr>
<td>MQH2O</td>
<td>Milli-Q purified water</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide adenine dinucleotide phosphate - oxidised</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate – reduced</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OE</td>
<td>Overexpression</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>Oxa1</td>
<td>Oxidase assembly 1</td>
</tr>
<tr>
<td>O₂⁻•</td>
<td>Superoxide anion radical</td>
</tr>
<tr>
<td>PAM</td>
<td>Presequence translocase-associated motor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methane sulfonyl fluoride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
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</tr>
<tr>
<td>POTRA</td>
<td>Polypeptide transport-associated domain</td>
</tr>
<tr>
<td>PPE</td>
<td>Protein-protein equilibration</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative/real time polymerase chain reaction</td>
</tr>
<tr>
<td>RNR</td>
<td>Ribonucleotide reductase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase high pressure liquid chromatography</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S-URA</td>
<td>Synthetic minimal media, uracil selective media</td>
</tr>
<tr>
<td>SAM</td>
<td>Sorting and assembly machinery</td>
</tr>
<tr>
<td>SB±DTT</td>
<td>Sample buffer with/without DTT</td>
</tr>
<tr>
<td>SBTI</td>
<td>Soy bean trypsin inhibitor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SFH-PCR</td>
<td>Short frame homology polymerase chain reaction</td>
</tr>
<tr>
<td>Sod1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of the inner membrane</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the outer membrane</td>
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<td>Trx</td>
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<td>Trr</td>
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<td>Ub</td>
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<tr>
<td>WT</td>
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<tr>
<td>Yme1</td>
<td>Yeast mitochondria escape 1</td>
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<td>YP</td>
<td>Yeast peptone media</td>
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<tr>
<td>-LEU</td>
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<td>Δψ</td>
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<td>Overexpression from the pYES2 plasmid constructs</td>
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<td>-URA</td>
<td>Uracil deficiency</td>
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1. INTRODUCTION

1.1. Overview of mitochondria

The mitochondrion is a rod-shaped structure that controls and facilitates numerous functions within eukaryotic cells and is responsible for generating adenosine triphosphate (ATP) by aerobic respiration. ATP is the molecular unit of energy that is utilised for numerous functions including amongst others: metabolism, cell signalling and motility. As such, mitochondria levels vary depending on the cell behaviour and energy requirements, with a greater abundance observed in cytoskeletal and myocardial cells (~30% of the total volume) compared to epithelial cells for example (Ferrari et al., 1993). As well as ATP production, mitochondria are also involved in regulating and contributing to other cellular processes. These include calcium homeostasis, the control of regulated cell death as apoptosis and the detoxification of reactive oxygen species (ROS) produced by the mitochondria under oxidative stress conditions (Zhu et al., 1999, Murphy, 2009). With these roles, mitochondria dysfunction has several implications in disease pathologies that range from Parkinson’s disease to cardiovascular diseases (Giorgi et al., 2012, Ballinger, 2005). The role of mitochondria in oxidative stress has also made it a target in investigating the mechanisms involved in ageing.

Mitochondria are also notable for containing DNA separate to the nucleus. According to the endosymbiotic theory, this is a carryover from when the mitochondrion was a separate protobacterium that was taken into the cytosol of a proto-eukaryote cell (Margulis, 1970). Over time, the bacterium became integrated into the eukaryote cell with protobacterium genes being gradually introduced into the genomic nucleus DNA. Through this symbiotic relationship, the activity and biogenesis of mitochondria became strongly integrated into the functioning of the larger eukaryote (Kurland and Andersson, 2000). The mitochondrial biogenesis pathways for protein assembly are elaborated in further detail in the following sections.
1.2. Mitochondria structure and biogenesis in *S. cerevisiae*

The mitochondrion structure is highly conserved across eukaryotic organisms. The name ‘mitochondria’ itself is descended from the Greek words of ‘mitos’ and ‘chondrion’ for ‘thread’ and ‘granule’ (van der Giezen, 2011). This is reflected in the general mitochondria morphology of ‘granule’ structures that during cellular division, mitochondria elongate into thread-like tubular forms. The mitochondria structure and sub-compartments are observable using electron microscopy (EM) (Figure 1.1).

![Image of mitochondria structure](image.png)

**Figure 1.1. Electron microscope overview of the mitochondrion structure.**

Mitochondria observed through the midplane section. Overview of the compartments were detailed, with the double mitochondrial membranes being observable: the outer membrane (OM) and the inner membrane (IM). The internal mitochondria matrix is enclosed by the IM, while the area between the IM and OM compose the intermembrane space (IMS). The extended cristae branch across the mitochondria matrix to maximise the IM surface area. Mitochondrial DNA and ribosomes are present in the matrix. Adapted from (Quarton, 1967).
The individual mitochondrion contains two membranes for the separation and compartmentalisation of different sections, whilst also performing a number of necessary functions. The inner membrane (IM) encloses the mitochondrial matrix, whilst the outer membrane (OM) closes the mitochondria from the cytosol. Between the two membranes is the intermembrane space (IMS) that is the focus of interest in this study.

These four compartments regulate mitochondria functioning and biogenesis with specific import and assembly pathways. The OM is a semi-permeable phospholipid structure that facilitates and regulates the insertion of molecules into the mitochondria. Porin channels enable free-diffusion of small molecules and ions across the OM (typically <5 kDa in size) into the IMS, while proteins are inserted in through the use of translocase complexes (translocase of the outer membrane, TOM) (Ha et al., 1993, Schmidt et al., 2010). The TOM complexes also contain cytosolic receptors to recognise a number of mitochondrial proteins for import.

The IM in contrast, is impermeable to free diffusion as it entirely encloses the mitochondrial matrix. As such, proteins imported into or across the IM must utilise translocases of the inner membrane, TIM complexes. The IM is also responsible for the final stage of aerobic respiration; oxidative phosphorylation by the electron transport chain. As such, the IM forms numerous folds known as cristae to maximise the available surface and the amount of ATP that can be produced from aerobic respiration (Gilkerson et al., 2003). The IMS compartment between the two makes up less than 5% of the total mitochondria volume, but facilitates the organisation and import of IM and OM-targeted proteins as well as the assembly of the IM respiratory chain required for oxidative phosphorylation (Hu et al., 2008, Hüttemann et al., 2007).

The mitochondria matrix is an aqueous compartment within the IM and facilitates the citric acid cycle of aerobic respiration, while also functioning as stores in calcium homeostasis (Vandecasteele et al., 2001). The matrix contains ribosomes and the free-floating circular mitochondrial DNA (mtDNA), but while the ribosomes there enable RNA transcription, the mtDNA ultimately transcribes <1% of the total mitochondria proteins. From the endosymbiotic theory, as the protobacterium became integrated into the host protoeukaryotes, the majority of genes for mitochondrial proteins became transferred to the host nucleus. Thus, mitochondria
biogenesis is reliant on the import and assembly of nucleus-transcribed proteins (Kurland and Andersson, 2000). From nucleus transcription and translation in the cytosol, proteins are then sorted at the mitochondria and imported to their target destinations before folding and assembly into matured forms (Herrmann et al., 2012).

1.2.1. Nucleus transcription and targeting to the mitochondria

The majority of mitochondrial proteins are nucleus-transcribed and translated in the cytosol, with translocation across the cytosol to the mitochondria being directed by a number of factors. After gene transcription, the cytosolic ribosomes translate the precursor RNA which is directed to the mitochondria OM for import. Chaperones in the cytosol facilitate translocation for specific mitochondrial proteins featuring import targeting sequences and also direct the docking with mitochondrial OM receptors (Young et al., 2003).

Mitochondrial proteins utilise post-translational import for the transport of the ribosome products to the OM surface (chaperoned or by diffusion). This differs to the co-translational biogenesis observed in the endoplasmic reticulum (ER). There, proteins translated by the cytosolic ribosomes are detected by a signal recognition particle, which initiates translocation of the ribosome to the ER translocation channels. Synthesised proteins are then immediately translocated into the ER without exposure to the cytosolic environment (Nyathi et al., 2013). Co-translational import does occur in the biogenesis of a few mitochondrial proteins (e.g. Fumarase, Cox2), but the post-translational system is the prevalent import pathway (Yogev et al., 2007, Szyrach et al., 2003, Reid and Schatz, 1982).

Chaperones in the cytosol bind to select mitochondrial protein precursors, such as IM carriers and matrix-targeted substrates, which feature sequences that are sensitive to the redox environment of the cytosol. Cytosolic chaperones include the matrix stimulating factor (MSF) and heat shock protein (HSP; Hsp70) in S. cerevisiae (Bukau and Horwich, 1998). These chaperone proteins to dock with the Tom70 surface receptor of the TOM complex for import with ATP hydrolysis (Young et al., 2003). Matrix-targeted proteins exhibit an N-terminus associated presequence, between 20 and 50 amino acids. This cleavable presequence enables the protein
to assume an alpha-helical form and exhibits a net positive charge that produces specific interaction with negatively-charged receptors (Pfanner, 2000). Channel proteins in the IM do not feature an N-terminus presequence but instead contain hydrophobic internal targeting sequences that facilitate sorting (Pfanner and Geissler, 2001). Chaperones in the cytosol protect sensitive sequences in the precursors while facilitating docking to the Tom70 receptors for import. Other mitochondrial proteins can exhibit a combination of these targeting sequences or none, with import then being driven by an alternate mechanism, such as the IMS proteins.

Sorting of the mitochondrial proteins from the cytosol occurs at the OM-embedded TOM complex; identified with an overview of the import pathways in Figure 1.2. Each pathway has specificities in the substrates and receptor complexes for facilitating import; OM β-barrel proteins (A), IM carrier proteins (B), IM and matrix proteins with targeting presequences (C) and redox-sensitive IMS proteins (D). Each pathway is explored in further detail in each of the following subsections.
Figure 1.2. Overview of the mitochondria protein import pathways.

Import pathways through the TOM complex with sorting for mitochondrial protein biogenesis. Chaperones in the cytosol (Hsp70) direct IM and matrix precursor translocation to the TOM complex. A) After IMS translocation of OM β-barrel proteins, IMS chaperones (Tim9-Tim10) guide proteins for insertion at the SAM complex. B) Import of IM precursors across the IMS with chaperones. Precursors dock with TIM22 for insertion, dependent on the membrane potential (Δψ). C) Import of presequence containing proteins to the TIM23 complex. Proteins are imported into the matrix with cleavage of the presequence by the mitochondria processing peptidase (MPP). Precursors can be imported laterally into the IM, through sorting in TIM23. D) Import of IMS precursors by oxidative folding with the MIA pathway. Oxidation prevents diffusion back across the TOM pore. Adapted from (Harbauer et al., 2014).
1.2.2. Mitochondria entry via TOM complex and the import of OM proteins

Import for the different mitochondria proteins begins at the translocase of the outer membrane (TOM) complex. At 450 kDa in size, the complex comprises seven components with the OM pore of Tom40 as the central subunit and Tom20 and Tom70 as cytosol-facing receptors (Rapaport et al., 1998). These interact with precursors, before allowing the translocation across the membrane. While Tom 20 and Tom70 constitute the primary receptors of the TOM complex, the transmembrane units of Tom22, Tom5, Tom6 and Tom7 form the general import pore (GIP) with Tom40 (Ahting et al., 2001) (Figure 1.3).

![Diagram of mitochondrial protein import](image)

**Figure 1.3. Import of mitochondria precursors across the TOM complex.**

The TOM complex is composed of Tom40 as the general import pore (GIP) and the subunits of Tom5, Tom6, Tom7 and Tom22. The Tom70 and Tom20 subunits function as receptors to recognise IM and presequence-containing precursors. Matrix-targeted protein substrates express a positively charged N-terminal sequence to direct import to the TIM23 complex after translocation. IMS precursors do not express targeting sequences and feature redox-sensitive motifs for oxidative folding. Biogenesis of β-barrel OM proteins requires import through the SAM complex from the IMS. Translocation in the IMS for OM or IM-targeted proteins requires chaperone binding (e.g. Tim9-Tim10 complex). Adapted from (Wiedemann et al., 2004).
Tom20 and Tom70 as the TOM complex cytosolic receptors feature single N-terminal transmembrane domains anchored into the OM. Tom70 binds proteins exhibiting internal targeting signals (IM-associated proteins) and Tom20 bind precursors with positively-charged N-terminal sequences (Brix et al., 1997). Tom70 recognises and docks the precursor-bound cytosolic chaperone (Hsp70) through the tetra-tricopeptide repeat (TPR) motifs in the cytosol-exposed C-terminus recognising the precursor's internal targeting sequences (Brix et al., 2000). Tom22 differentiates from Tom20 and Tom70 by having the C-terminal and N-terminal domains exposed to the IMS and cytosol respectively. While Tom22 also binds N-terminal presequence proteins through the transmembrane domain, the cytosolic domain of the protein interacts with Tom20 and Tom70 as the central receptor for transfer into the Tom40 translocation pore (Pfanner and Geissler, 2001). After docking with the receptors, ATP hydrolysis by the ATPase drives translocation into the IMS (Young et al., 2003).

Tom40 is the central subunit of the GIP and is a transmembrane β-barrel protein, with the channel of the pore measuring ~20A in diameter (Ahting et al., 2001). The other subunits of Tom5, Tom6 and Tom7 contribute to the promotion and regulation of import across the GIP. Tom5 presents a cytosol-exposed N-terminal domain that is functionally involved in the transfer of pre-proteins into the Tom40 pore, whilst Tom6 and Tom7 modulate import from the receptors to the GIP (Dietmeier et al., 1997, Hönlinger et al., 1996).

Import through the TOM complex is not passive like with porins, with evidence suggesting it to function more as a co-chaperone in preventing the aggregation of the imported precursor proteins and driving membrane translocation (Rapaport et al., 1998). While the OM lacks a membrane potential or ATP motors like the IM, import is instead driven through a stepwise increase in binding site affinity for the positively charged mitochondrial targeting sequences in the protein, as part of the ‘acid-chain’ hypothesis (Komiya et al., 1998). The cytosol-facing cis binding site is composed of the acidic domains of Tom70, Tom20, Tom22 and Tom5 with import then being driven across the OM by the increasing affinity of the binding sites in the IMS-facing trans binding sites. These more acidic sites are present in the IMS-exposed Tom22 C-terminus and Tom7. This net transfer effect allows for translocation of the presequence-containing
proteins across the OM, for later sorting and import to the inner membrane (Komiya et al., 1997).

OM proteins can be subdivided based on their secondary structures: \(\alpha\)-helical or \(\beta\)-barrel. The \(\beta\)-barrel proteins, such as porin and Tom40, are inserted into the OM through the sorting and assembly machinery (SAM) complex after OM translocation via the TOM40 complex and chaperoning by Tim9-Tim10 across the IMS to the SAM complex (Taylor and Pfanner, 2004). The three subunits that compose the 200 kDa SAM core are Sam35, Sam37 and Sam50, with Mdm10 altogether forming the full holo-complex (Meisinger et al., 2006). After import into the IMS, \(\beta\)-barrel proteins are translocated to the trans IMS-facing Sam35 receptor for insertion. In the IMS, OM proteins are bound to chaperones to prevent aggregation; 70 kDa hexameric complexes of small Tim proteins (Tim9-Tim10 or Tim8-Tim13). The Sam50 channel forming subunit contains the N-terminal Polypeptide-transport-associated (POTRA) domain to bind and insert the \(\beta\)-barrel precursor into the OM in conjunction with Sam37 as a stabilising factor (Dukanovic et al., 2009, Stroud et al., 2011). The mitochondrial distribution and morphology protein 10 (Mdm10) binds to the SAM complex and is involved in the later steps of \(\beta\)-barrel protein assembly (Meisinger et al., 2004). The import pathway of \(\beta\)-barrel OM proteins is depicted in Figure 1.4.
Figure 1.4. Import and assembly of β-barrel OM proteins.

Import of β-barrel protein require import through the TOM complex into the IMS. Complexes of small Tim proteins (Tim9-Tim10) protect exposed hydrophobic regions of the OM proteins in the IMS, while chaperoning proteins to dock with the IMS-face of the sorting and assembly machinery (SAM) complex. The SAM complex is assembled from Sam50, Sam38 and Sam37 to insert β-barrel proteins into the OM. Mdm10 assists in protein assembly and interaction with the TOM complex. Figure adapted from (Mokranjac and Neupert, 2009).

The Tom7 subunit in the TOM complex mediates assembly of the β-barrel Tom40 subunit through Mdm10 in the SAM complex (Becker et al., 2011a). Tom7 interacts directly with Mdm10 for recruitment into the SAM complex and this enables release of Tom40 into the OM for further TOM complex assembly. This is part of a functional equilibrium between the import and assembly of OM channel proteins and shows the TOM complex to have further roles in mitochondria protein sorting (Meisinger et al., 2006, Yamano et al., 2010). While the import of OM β-barrel proteins is facilitated by TOM and SAM complexes, α-helical OM proteins (e.g. Tom20) are imported into the OM without IMS translocation, through an alternative pathway across the OM (Figure 1.5).
The Tom20 and Tom70 receptors along with additional TOM40 subunits of Tom5, Tom6 and Tom7 are imported using the Mim1-SAM complex pathway. Import differs from β-barrel proteins by insertion occurring across the cytosol-exposed face of the OM, without IMS translocation through Tom40. Substrate proteins can be differentiated with the presence of single or multiple α-helical transmembrane domains. Adapted from (Becker et al., 2009).

The α-helical targeting sequences range from single to multiple repeats and are typically located in the N-terminus transmembrane domains of proteins, with Tom5, Tom6 and Tom7 however featuring the domain in the C-terminus tail (Hammen and Weiner, 2000, Endo et al., 2003). This import is less characterised compared to β-barrel proteins, but a dedicated import pathway has been identified as the OM-bound 200 kDa MIM complex which is composed of mitochondria import proteins: Mim1 and Mim2. Mim1 was identified as being vital to the MIM complex formation and functioning in α-helical OM import, while the non-essential Mim2 interacts with Mim1 for assembly (Becker et al., 2011b, Dimmer et al., 2012). Mim1 has been identified to be necessary for the import of Tom20 and Tom70 precursors into the OM, without insertion through the SAM complex (Hulett et al., 2008). This contrasts to Tom22 which does not require import through the MIM complex, but is dependent on the SAM complex for import. Tom22 precursor import is then facilitated by the TOM complex with recognition of the precursor by the Tom20 and Tom70 receptors before insertion (Ahting et al., 2005). Biogenesis of the Tom5, Tom6 and Tom7 subunits is facilitated through the MIM complex interaction with Sam37 (Dukanovic et al., 2009, Stojanovski et al., 2007).
1.2.3. Insertion into the IM: TIM22 complex

Following import across the OM, proteins targeted to the inner membrane (IM) can be imported by three different mechanisms:

A. The IM-associated TIM22 pathway
B. The TIM23 stop-transfer pathway
C. The TIM23 conservative pathway

The stop-transfer and conservative pathways utilise the TIM23 complex and are detailed in the next section. The pathway focused here involves the direct insertion of carrier proteins into the IM through the TIM22 complex (Sirrenberg et al., 1996). TIM22 is about 300 kDa in size and is composed of the core proteins: Tim22, Tim18 and Tim54 as well as a peripheral Tim12 subunit (Kovermann et al., 2002). IM and OM precursor proteins imported across the TOM complex are chaperoned across the IMS by small Tim proteins to protect hydrophobic regions. TIM22 substrates contain positively-charged and hydrophobic internal targeting signals as opposed to TIM23 substrates that feature N-terminal presequences (Arnold et al., 1998, Peixoto et al., 2007). In the cytosolic translocation of TIM22 precursors, Hsp70 chaperones enable docking with TOM receptors for import into the IMS (Pfanner and Meijer, 1997). The TIM22 import pathway is outlined in Figure 1.6.
Figure 1.6. Import and assembly of IM carrier proteins through the TIM22 complex.

Following import across the OM, the IM precursor proteins are protected from aggregation in the aqueous IMS environment by the small Tim proteins before docking to the TIM22 translocase. The chaperone associates with the IM-bound Tim12 before the IM precursor is through the Tim22 pore into the IM. Import is driven by the membrane potential of the IM (Δψ). Adapted from (Wiedemann et al., 2004).

After crossing the IMS bound to the Tim9-Tim10 chaperone, the precursor protein is inserted into the Tim22 channel. The IM-bound Tim12 mediates the transfer by associating with the Tim9-Tim10 IMS chaperone and Tim22 for the transit of the IM precursor protein, but the molecular mechanisms for its interactions have not been elucidated (Lionaki et al., 2008). Tim22 exhibits a twin-pore translocase structure that serves as a gating mechanism in controlling IM protein import (Rehling et al., 2003). The roles for Tim18 and Tim54 have not been fully elucidated, but Tim18 has been identified as being involved in TIM22 biogenesis and stability, while Tim54 is thought to also have a similar role in assembly (Kerscher et al., 2000, Koehler et al., 2000). Import through the TIM22 and TIM23 complexes requires the membrane potential (Δψ) across the IM to drive the process. While not fully characterised, it has been determined
that the membrane potential works with the twin pore Tim22 structure to serve gate-wise import (Rehling et al., 2003).

1.2.4. Import into the matrix and IM: TIM23 complex

Import of N-terminal presequence-bearing proteins into the matrix, as well as certain IM proteins, is facilitated by the TIM23 complex (Bauer et al., 1996, Truscott et al., 2001). The TIM23 complex comprises several units to produce the transmembrane pore and receptors and can be subdivided into three categories that relate to their general function. These are the subunits that form the translocation pore (Tim17, Tim23 and Tim21), participate in ATP hydrolysis to drive import (Tim44, Pam16, Pam18 and mtHsp70) and facilitating import to the TIM23 complex as a receptor (Tim50) (Yamamoto et al., 2002). The structure of the TIM23 complex and the import pathways are outlined in Figure 1.7.
Figure 1.7. Import and assembly of IM and matrix proteins through the TIM23 complex.

TIM23 complex structure of the TIM23 (green) and PAM-associated subunits (yellow), with the Tim23 pore as the central channel (orange). IM proteins translocated into TIM23 are inserted laterally by the stop-transfer pathway. Otherwise, proteins are imported across the matrix with ATP hydrolysis and the membrane potential (Δψ) driving translocation. Matrix-targeting presequences are cleaved from proteins by the mitochondria processing peptidase (MPP). Adapted from (Mokranjac and Neupert, 2009).

The central IM core of TIM23 is a dimer of the Tim17, Tim23 and Tim44 subunits (Bauer et al., 1996). Tim23 is composed of three domains: the OM-associated N-terminus (residues 1-50), the IMS-situated domain (residues 51-100) and the IM-embedded C-terminus (residues 100-222). The OM-associated N-terminus is exposed to the cytosol-facing cis site and in close proximity to the TOM complex (but without interaction). This anchors Tim23 for the IMS domain.
to dimerise and form the presequence-binding receptor (Donzeau et al., 2000). This receptor domain is negatively charged to the positive charged presequence in precursors and mediates transfer in the IMS to the IM import channel. Tim50 is the primary receptor for the TIM23 complex; binding and releasing the protein into the Tim23 channel, whilst also modulating Tim23 activity (Schulz et al., 2011, Yamamoto et al., 2002). Tim17 forms the twin pore structure with Tim23 and facilitates sorting of precursors. Tim21 and Pam17 are non-essential subunits, with roles in sorting precursors for import.

Similar to TIM22, translocation across TIM23 is dependent on the membrane potential ($\Delta\psi$) as a driving force in the gating of the Tim23 twin pores. ATP hydrolysis is also required for the cleavage and release of precursor proteins into the matrix. This is driven by the presequence translocase-associated motor (PAM) complex (subunits of Pam16, Pam17 and Pam18) on the trans matrix-side of the IM (Wagner et al., 2009, van der Laan et al., 2005). Hydrolysis is catalysed by mtHsp70 (Ssc1 in yeast), which binds the molecule before the co-chaperone Mge1 dissociates ADP from mtHsp70 and the cycle continues for TIM23 import. Binding of the PAM complex to TIM23 is mediated by Tim44 (Schiller et al., 2008). The targeting presequence is cleaved from the protein with the mitochondria processing peptidase (MPP) as the final translocation step.

The aforementioned conservative and stop transfer pathways can be used in the import of IM-targeted proteins and occurs through the TIM23 complex. Substrates of the conservative pathway (e.g. Cox18) present the TIM23 targeting presequence as well as hydrophobic membrane-spanning domains (Park et al., 2013). The IM precursors are imported into the mitochondria matrix, where they are bound by mtHsp70 as the MPP enzyme cleaves off the presequence. The substrate is translocated into the IM through the trans matrix-exposed Oxa1 membrane protein (Neupert and Herrmann, 2007). Oxa1 is a 45 kDa translocase that contains a conserved domain with five transmembrane spans to mediate transfer and is itself a substrate of the conservative pathway. While Oxa1 has been characterised in the export of mitochondria-encoded precursors across the IM, the molecular mechanism of conservative translocation has not been fully elucidated (Kruger et al., 2012).
The stop-transfer pathway occurs with lateral translocation into the IM through the TIM23 complex (Figure 1.7) and substrates include Cox4, Cox5 and Yme1 as well as the *S. cerevisiae* Mia40 ortholog. Substrates feature the TIM23 targeting presequence with a strong hydrophobic transmembrane domain that arrests import across the TIM23 complex. Matrix precursor proteins also feature hydrophobic regions, but to a decreased strength that does not impede TIM23 translocation (Mokranjac and Neupert, 2010). Lateral insertion into the IM occurs in a Δψ-dependent manner, though not fully understood, where a hydrophobic interaction of the transmembrane domains with the lipid membrane anchors the protein in the IM (Bohnert et al., 2010, Park et al., 2013). IM-embedded proteins may also have matrix or IMS-exposed N-terminal and C-terminal domains as well to express functions (e.g. Mia40 has the active C-terminal core domain exposed to the IMS). Enzymatic cleavage of the IMS soluble domains can also release proteins into the IMS (e.g. Cytb2) as an alternate import pathway to the traditional MIA disulfide relay in IMS import (Schwarz et al., 1993). Additionally in the stop-transfer pathway, the absence of proline amino acids in precursors combined with a strong hydrophobic nature promotes sorting into the IM, while the presence of proline prevents the arrest of TIM23 translocation, so the IM protein is then imported in with the Oxa1-associated conservative pathway (Meier et al., 2005, Bohnert et al., 2010). The inclusion of proline residues destabilises the α-helical structures of the IM substrate, indicating that recognition and arrest is related to the secondary structure of the protein (Mokranjac and Neupert, 2009).

### 1.2.5. Import into the IMS: TIM23 and MIA pathway

Precursors of IMS proteins are translocated across the TOM complex and imported through three different pathways, linked to the IM or directly into the IMS:

A. Import of proteins with bipartite presequences

B. Import through affinity binding sites

C. Import through the redox-regulated MIA pathway

Previously mentioned, import of IM precursor proteins can occur laterally through the TIM23 complex. IMS proteins with bipartite sequences are imported into the IM in the same manner,
with proteolytic cleavage releasing the protein into the IMS (Chacinska et al., 2004, Glick et al., 1992). Substrates include proteins such as cytochrome \( b_2 \) (Cyt \( b_2 \)) and cytochrome \( c_1 \) (Cyt \( c_1 \)). Precursors contain the matrix-targeting sequence for translocation to the TIM23 complex with an adjacent hydrophobic sorting sequence to arrest import at the IM. After lateral insertion into the IM, proteolytic cleavage of the sorting sequence releases the protein into the IMS.

IMS protein import by affinity binding sites is less characterised in comparison to the other import pathways, in which import is not directed by targeting presequences, ATP hydrolysis or the inner membrane potential (\( \Delta \psi \)) (Herrmann and Hell, 2005). After translocation across the TOM complex, precursor proteins permanently associate with binding sites present on the IMS-exposed faces of the IM or OM. Substrates include the cytochrome \( c \) and mitochondrial heme lyases, Cox12 and porins (Pfaller and Neupert, 1987). For heme lyase, folding of the protein in the IMS was not fully elucidated in terms of occurring either during or immediately after import into the compartment. Import was identified as not being a driving force for the affinity site binding (Steiner et al., 1995).

Import of IMS proteins by the redox-regulated mitochondria import and assembly (MIA) pathway retains them in the IMS by the oxidative folding of protein structures. This then inhibits the diffusion of proteins back out into the cytosol through the TOM complexes. MIA substrates range between 8 to 24 kDa in an unfolded form that may diffuse freely across the TOM complex between the cytosol and IMS (Lutz et al., 2003). Like with affinity site binding, substrates lack targeting presequences but precursor import is instead driven by the presence of conserved redox-sensitive cysteine residues in the precursor. Through the MIA pathway, these residues are oxidised to form intramolecular disulfide bonds with other cysteine residues; folding the secondary protein structure into the appropriate matured form (Figure 1.8). A more comprehensive analysis of the MIA pathway and the disulfide-bond redox mechanism is discussed in the next section.
IMS precursors imported into the IMS compartment through the TOM complex. The IMS precursors feature conserved cysteine residues, with oxidation by the IM-embedded Mia40 to form disulfide bonds for folding IMS proteins. The matured proteins are then retained in the IMS compartment as Mia40 is reoxidised by Erv1 to restore its activity. Adapted from (Neupert and Herrmann, 2007)

1.3. The IMS disulfide relay in the MIA pathway

The MIA pathway was observed to catalyse oxidative folding of many IMS proteins and this disulfide relay system is comprised of two central components: the oxidoreductase Mia40 and the FAD-dependent sulfhydryl oxidase Erv1 (Mesecke et al., 2005, Bottinger et al., 2012). After translocation of the IMS precursor in its Cys-reduced form across the TOM complex, it is oxidised through the MIA pathway by transient disulfide bonds (S-S). To restore the oxidised Mia40 state for continued activity, electrons are removed and shuttled down the relay with removal by the reduction of an electron acceptor such as cytochrome c (Cyt c) or oxygen (O₂) molecules (Dabir et al., 2007). The disulfide relay system of the MIA pathway is presented in Figure 1.9.
Figure 1.9. MIA pathway disulfide relay and electron flow.

Outline of the electron transfer between Mia40, Erv1 and Cyt c for the oxidation of proteins and the removal of electrons from the IMS. **A)** Reduced IMS substrates oxidised by Mia40, which becomes reduced. **B)** Electron shuttling between the reduced Mia40 and the Erv1 sulfhydryl oxidase. **C)** Electron shuttling within the Erv1 homodimer. **D)** Reduced Erv1 shuttles electrons to the bound FAD co-factor, with removal by the reducing of Cyt c or O$_2$ molecules. Figure adapted from (Allen et al., 2005).

In the context of the MIA pathway with cell functioning, the mitochondrial disease of the neurodegenerative deafness dystonia (Mohr-Tranebjaerg) syndrome in humans is associated with defective MIA pathway activity from failed import of DDP1 (deafness dystonia polypeptide 1) protein (Koehler et al., 1999, Hell, 2008). Substitution of a cysteine residue was observed to impair protein folding and the biogenesis of necessary complexes (Hofmann et al., 2002). Thus, failure to IMS protein biogenesis can have implications in disease pathologies. The MIA pathway is analysed in further detail with the individual components in the proceeding sections.

**1.3.1. Mia40 oxidoreductase of the MIA pathway**

Mia40 functions as the IMS import receptor, with the oxidative-folding of precursors. This occurs through the covalent interactions of conserved dithiol motifs between Mia40 and the IMS substrate. The *S. cerevisiae* ortholog of Mia40 differs from other eukaryotes such as *H. sapiens* by being a ~40 kDa anchored into the IM by TIM23 lateral insertion with exposure of the C-terminal active domain into the IMS, whereas *H. sapiens* Mia40 (hMia40) is soluble in the IMS. The structures and domains of the two Mia40 orthologs are presented in Figure 1.10.
Figure 1. The structures and domains of different eukaryotic Mia40 orthologs.

Mia40 orthologs presented for *S. cerevisiae* Mia40 and *H. sapiens* hMia40 with the identification of the key sequences for the protein domains. In *S. cerevisiae*, TIM23-targeting presequences and the hydrophobic transmembrane domain are presented at the N-terminus of Mia40. The redox-active CPC-CX₉C-CX₉C motifs are conserved in both species. Adapted from (UniProtKB, 2015b, UniProtKB, 2015a).

In *H. sapiens*, hMia40 is free in the IMS with ALR (*H. sapiens* ortholog to Erv1) to make up the Mia40 pathway. A conserved structure across the different eukaryotes features three α-helices arranged as a lid (α1) and core (α2, α3). Mia40 features six conserved cysteine residues (C₁-C₆: CPC-CX₉C-CX₉C) that are arranged in each of the α-helices with the CPC motif in the α1 lid, while the two CX₉C motifs are each present on the α2 and α3 helices (Terziyska et al., 2008). Intramolecular disulfide bonds between the CX₉C motifs assemble a folded hairpin structure between the α2 and α3 helices, while the α1 lid is free of the core as the import receptor. The conserved Mia40 structures also contain a hydrophobic cleft into which IMS substrates bind to before docking with the catalytic CPC lid for oxidative folding (Chatzi et al., 2013) (Figure 1.11).
IMS precursors (in α-helical structures) are oxidised through covalent interactions with the Mia40 active site, which ultimately form hairpin structures in the precursor. The hydrophobic cleft in Mia40 adjacent to the CPC motif enables effective docking of the IMS precursor with mitochondria intermembrane space sorting (MISS)/intermembrane space targeting signal (ITS). The hydrophobic interaction enhances substrate binding to Mia40 and the cleft is composed of hydrophobic amino acids surrounding the CPC and CX₉C motifs (e.g. leucine, methionine, phenylalanine) (Chatzi et al., 2013). After oxidation of the precursor by the CPC motif, Mia40 is
left reduced from the redox reaction and to restore oxidative activity, Mia40 is then oxidised by Erv1 in the MIA pathway.

1.3.2. Erv1 FAD-dependent sulfhydryl oxidase

Erv1 (Essential for respiration and vegetative growth) is a 21 kDa FAD-dependent sulfhydryl oxidase present in the mitochondria, which is conserved across species such as the *H. sapiens* ortholog of *Alr1* (augmenter for liver regeneration) that was observed with a proliferation of hepatocytes in mammalian studies (Hagiya et al., 1994). The electron shuttling pathway in Erv1 downstream from reduced Mia40 to removal by the electron acceptors is presented in Figure 1.12.

![Figure 1.12. Electron shuttling in the Erv1 homodimer.](image)

Electrons shuttled downstream from Mia40 after the oxidation of IMS precursors. A) Electrons shuttled from reduced Mia40 to the N-domain of Erv1. Mia40 is reoxidised. B) Shuttling of electrons from the N-domain to the C-domain of the second Erv1 in the homodimer. C) Shuttling of electrons to the bound FAD co-factor in the Erv1 core. D) Electrons from the reduced FADH$_2$ co-factor are donated to Cyt c or O$_2$ molecules. Oxidised Erv1 is restored and recycled for continued function with Mia40. Figure adapted from (Riemer et al., 2011).
The individual Erv1 molecule is comprised of the C-domain (core) and the N-domain (N-terminal arm); both containing conserved CXXC motifs for transient redox interactions for electron shuttling. The N-domain is freely flexible from the core and presents the CXXC motif at amino acids residues 30-33. After reduction from the disulfide-relay reaction with Mia40, the N-domain interacts with the CXXC motif in the C-domain core (residues 130-133) in the second Erv1 of the homodimer in Erv1-Erv1 electron shuttling (step B, Figure 1.12) (Guo et al., 2012). The core domain contains the FAD-binding domain (residues 166-183) for FAD as a redox co-factor (1 FAD molecule per subunit) as well as a CX_16C structural disulfide (residues 159-176) (Ang et al., 2014). The FAD-binding domain is itself adjacent to the CX_2C motif and after shuttling within the Erv1 homodimer, the reduced FADH_2 co-factor in the C-domain transfers the electrons out and restores the oxidised Mia40-Erv1 disulfide relay (steps C and D) (Coppock and Thorpe, 2006). Oxygen molecules and Cyt c are reduced by FADH_2 and released into the IMS as water molecules and reduced Cyt c. The cytochrome c oxidase (Cox) in the IM electron transport chain can remove electrons from reduced Cyt c as a link between the MIA pathway and aerobic respiration in the IM (Dabir et al., 2007).

1.4. Mitochondria IMS substrates

1.4.1. Overview of IMS substrates

IMS substrates of the MIA pathway are oxidatively-folded to be retained in the IMS. As such, substrates feature conserved redox-sensitive cysteine residues, which are usually arranged in twin CX_2C or CX_9C motifs (Gabriel et al., 2007). These substrate proteins form intermolecular transient disulfide bonds with Mia40 upon protein import and result in the substrates forming the oxidised hairpin structures. Being redox sensitive, IMS precursors may go between being reduced and oxidised in either the cytosol or IMS (Figure 1.13).
Figure 1.13. IMS precursor disulfide bond formation and oxidative folding.

Intramolecular disulfide bonds between conserved CXnC motifs fold the protein structure when oxidised. Protein folding occurs to produce a hairpin structure of the protein. Reduction linearises the structure for OM pore translocation.

Aside from the conserved CXnC motifs, IMS precursors exhibit signals for recognition by the Mia40 import receptor: the mitochondria intermembrane space sorting (MISS) or the IMS-targeting signal (ITS) sequences (Milenkovic et al., 2009). These sequences comprise of specific hydrophobic (Hy) and aromatic (Ar) residues at specific sites upstream or downstream of the critical cysteine residue that primes it for interaction with the Mia40 oxidoreductase. Upon folding of the precursor, the MISS/ITS signal form an amphipathic helix that exhibits hydrophilic and hydrophobic sides. This is presented in Figure 1.14 for the CX3C small Tim proteins and CX9C Cox17.
Figure 1.14. MISS/ITS signals of Cox17 and small Tim proteins.

Helical wheel representation of the MISS/ITS signals: hydrophobic (blue) and hydrophilic side (green). A) CX₃C protein sequence prepared as a consensus sequence from different small Tim proteins (Tim9, Tim10 and Tim12). MISS signal upstream of the docking cysteine (the black circle in the helical wheel). B) CX₃C protein Cox17 sequence with the MISS signal downstream of the docking cysteine (the black circle in the helical wheel). In folding, the conserved amino acids form the MISS/ITS signal on the hydrophobic side of the protein substrate. Adapted from (Sideris et al., 2009).

From the helical structure, the docking cysteine and the other key amino acids of the MISS signal are located on the hydrophobic side, while charged amino acids are present on the hydrophilic face of the protein. For Tim9 in yeast, the C1 cysteine (residue 35) is the critical cysteine amino acid for the interaction with Mia40, with the MISS sequence located upstream at -3, -4 and -7 positions in the sequence, while in the CX₃C Cox17, the essential MISS residues are located downstream at +3, +4 and +7 of the critical cysteine at residue 47 (Milenkovic et al., 2009, Sideris et al., 2009). Protein folding aligns the MISS/ITS signal with the docking cysteine in the hydrophobic face of Mia40 (Figure 1.11A) to form the docking interface for the critical cysteine residue in the Mia40 CPC motif. In the oxidative folding steps for the precursor substrate, the outer intramolecular disulfide bond is dependent on Mia40 and after release into
the IMS, the inner bond is formed independently (Banci et al., 2010). For the oxidative folding of IMS precursors, two different conserved twin cysteine-motifs are present which proteins can be identified into: CX_3C and CX_9C (Longen et al., 2014). These are detailed in the following sections.

1.4.2. IMS CX_3C proteins – Small Tim proteins

The CX_3C-containing proteins are characterised as small Tim proteins: Tim9, Tim10, Tim8, Tim13 and Tim12 (Koehler, 2004). These are associated with chaperoning IM and OM-targeted proteins across the IMS as the Tim9-Tim10 and Tim8-Tim13 complexes. Tim12 is instead associated with the IM-bound TIM22 complex (Kovermann et al., 2002). Each protein is 9 to 13 kDa in size and adopts α-helical structural forms. Proteins are conserved across different orthologs, expressing the CX_3C motif for disulfide-bond oxidation (Figure 1.15).
Figure 1.15. Sequence allignment of conserved eukaryotic Tim9 and Tim10 orthologs.

Sequence alignments for the A) Tim9 and B) Tim10 orthologs in eukaryotic species. Green highlighting denotes identical ortholog sequences with blue for high conservation. Cysteine residues (marked in red) are presented as C1 and C2 (inner helix) and C3 and C4 (outer helix). Oxidative folding forms the α-helical hairpin structures with disulfide bonds between C1-C4 (outer) and C2-C3 (inner). Adapted from (Webb et al., 2006)
In small Tim proteins, disulfide bridges fold the α-helices into hairpin structures (inner bond C$_2$-C$_3$, outer bond C$_1$-C$_4$). Soluble hexameric complexes (~70 kDa) of Tim9-Tim10 and Tim8-Tim13 were formed at 1:1 ratios. For the Tim9-Tim10-Tim12 docking complex bound to the TIM22, it was suggested that one Tim10 subunit is substituted by Tim12 (Koehler et al., 1998). Crystal structures for both Tim9-Tim10 and Tim8-Tim13 complexes are solved and are similar (Webb et al., 2006, Beverly et al., 2008). The ribbon structures of oxidised Tim9 and Tim10 subunits are presented in Figure 1.16.

**Figure 1.16. Ribbon structures of the Tim9-Tim10 hexameric complex.**

*S. cerevisiae* hexameric complex displayed as the individual Tim9 and Tim10 subunits. **A)** Ribbon structures for *S. cerevisiae* Tim9 (red) and Tim10 (blue). The two α-helices form hairpin structures with disulfide bonds (yellow) between the conserved dithiol residues: C$_1$-C$_4$ (outer) and C$_2$-C$_3$ (inner). **B)** Overlaid ribbon structures of Tim9 and Tim10 from *S. cerevisiae* (red and blue) and *H. sapiens* (orange and green). **C)** Side and top views of the hexameric complex. Intertwining Tim9 and Tim10 molecules form the propeller-like structure of the complex. Adapted from (Baker et al., 2009).
Both Tim9 and Tim10 adopt α-helical hairpin structures after oxidation by Mia40 (Figure 1.16A). Numerous residues of the small Tim proteins are conserved across the different Tim9 and Tim10 orthologs, observed in the overlay of the S. cerevisiae and H. sapiens protein structures that show near-identical ribbon structures (Figure 1.16B). The Tim9-Tim10 complex is the hexameric chaperone complex of interlocking subunits (Figure 1.16C); where Tim9 has been identified to stabilise and protect Tim10 from degradation by forming the complex, while Tim10 serves as the functional moiety (Spiller et al., 2015, Baker et al., 2009). Tim8-Tim13 also forms the hexameric complex structure and fulfils a chaperone role in the IMS, but compared to the Tim9-Tim10 chaperone, was identified as non-essential for cell viability (Koehler et al., 1999).

### 1.4.3. IMS CX₉C proteins

IMS precursor proteins express twin CX₉C motifs, which are conserved sequences where the redox-sensitive cysteine residues are separated by nine amino residues between them. In contrast to the five CX₃C proteins readily detected and characterised, the CX₉C protein are a larger sub-group of IMS proteins, where genome-wide analysis identified 17 CX₉C proteins in S. cerevisiae (Cavallaro, 2010). As with the small Tim proteins, the CX₉C protein structures are generally observed in α-helical forms with the adoption of a coiled-helix-coiled helix (CHCH) folded structure after disulfide bond formation between the intramolecular dithiol residues. Each of the proteins can be subdivided based on their function in the cell; structural scaffolding in the mitochondria and assembly of the cytochrome c oxidases being the most generalised functions (Cavallaro, 2010, Horn et al., 2010). The CX₉C proteins most characterised for structure and function are Mia40 and Cox17.

The CX₉C motif Mia40 in S. cerevisiae is not imported into the mitochondria through the IMS MIA pathway. Import instead uses the presequence-targeted pathway through TIM23 to anchor it into the IM with the active domain exposed to the IMS (Chatzi et al., 2013). In other eukaryotes such as H. sapiens, hMia40 biogenesis is dependent on the MIA pathway (Chacinska et al., 2008). Cox17 is a 9 kDa cytochrome c oxidase (COX) in the IMS that binds copper ions as a metallochaperone, before donating two copper ions to the IM proteins Sco1 and Cox11 (Banci et al., 2008, Horng et al., 2004). Similar proteins in the IMS identified as part
of the COX family include Cox19, Cox23, Cmc1 and Cmc2 that participate in the biogenesis of oxidase complexes and the distribution of copper ions (Longen et al., 2009, Khalimonchuk and Winge, 2008).

1.4.4. Other IMS protein substrates of the MIA pathway

Other IMS proteins have been identified with proteins featuring twin dithiol motifs and one example is the Erv1 sulfhydryl oxidase, which was the first FAD-linked sulfhydryl oxidase identified in yeast and shuttles electrons downstream from Mia40 (Lee et al., 2000). Erv1 does not feature twin CX\_3C or CX\_9C motifs for disulfide bonds folding, instead featuring core and tail domains with conserved CX\_2C motifs. Import of Erv1 utilises the MIA pathway, but the Erv1 CX\_2C motifs associated with electron shuttling have been identified to not be involved in stabilising the protein structure (Terziyska et al., 2007). The molecular interactions of Erv1 import with Mia40 has not been fully characterised compared to other IMS proteins.

The superoxide dismutase 1 (Sod1) is ubiquitously expressed and localised to the cytosol, endoplasmic reticulum (ER) and the mitochondrial IMS (Kawamata and Manfredi, 2010). The protein is imported directly into the IMS and oxidative folding is facilitated by the Ccs1 copper chaperone. The MIA pathway can help facilitate import as Ccs1 is a MIA substrate (23 kDa) that features twin CX\_2C motifs. A mixed disulfide between Mia40 and Ccs1 traps it in the IMS for later IMS oxidation of Sod1 by Ccs1 (Reddehase et al., 2009, Field et al., 2003).

1.5. The cytosolic Thioredoxin and Glutaredoxin oxidoreductase systems

1.5.1. Overview of redox homeostasis in the cell

The redox-sensitivity of mitochondrial IMS proteins in post-translational import requires the redox homeostasis systems in the cytosol for import (Reid and Schatz, 1982). This is compared to the co-translational import of ER proteins from the cytosolic ribosomes at the ER surface. Only reduced mitochondrial proteins are competent for entry across the TOM complexes and need to be maintained in the cytosol against oxidising conditions.
Glutathione (GSH, reduced form) is a tripeptide synthesised in the cytosol, which is considered as a main reducing source. While transported to different locations within the cell, up to 10 mM GSH is retained in the cytosol, producing a general reducing environment (Chakravarthi et al., 2006). Homeostasis of GSH is by the reduction of oxidised glutathione (GSSG) by glutathione reductase (Glr1) using NADPH as a reducing source to donate electrons. Despite GSH in the cytosol, IMS proteins can still be oxidised with *in vitro* analysis of small Tim proteins showing them as being able to be oxidised by GSH at concentrations observed in cells (Morgan and Lu, 2008). Oxidative folding of IMS proteins was observed as being kinetically competitive to mitochondrial import with regards to rates of function. For the relationship between GSH and IMS precursors, *in vitro* analysis for the standard redox potential (E°', units of voltage) quantified the reducing ability of a species and was able to characterise the cytosolic redox environment.

Analysis of GSH compared to the IMS precursors identified them as being a greater reductants compared to GSH, as measured by a larger negative E°' value. IMS proteins (with small Tim proteins in particular) were measured between the range of E°'=-310 to -330 mV, whereas E°'\textsubscript{GSH}=-240 mV and E°'\textsubscript{NADPH}=-315 mV (Åslund et al., 1997). For *S. cerevisiae* small Tim proteins, E°'=-310 mV for both Tim9 and Tim13 (Morgan and Lu, 2008, Tienson et al., 2009). For Tim10, a similar value was determined as E°'=-320 mV (Lu and Woodburn, 2005), while for the CX\textsubscript{9}C proteins, E°'\textsubscript{Mia40}=-289 mV and *H. sapiens* E°'\textsubscript{Cox17}=-324 mV, similar to the general range observed with IMS proteins (Zovo and Palumaa, 2008, Tienson et al., 2009). Analysis of the cytosol environment calculated a redox potential of E°'=-289 mV, based on the glutathione GSH/GSSG redox status, which further illustrated the fact that IMS precursors would be stable in oxidised forms in the cytosol (Ostergaard et al., 2004). Mitochondria GSH levels in IMS were measured to determine the redox environment as E°'=-255 mV, with the more positive value showing the IMS as being more oxidising than the cytosol (Hu et al., 2008). From the quantitative values of redox stability measured, the IMS proteins were observed to be thermodynamically stable in the cytosol in their oxidised forms. The greater negative values indicate the IMS proteins to be more reducing to the GSH in the cytosol that would then lead to the proteins being oxidised. This consequently promotes the requirement of reducing factors in the cytosol to maintain IMS proteins in import-suitable reduced forms.
1.5.2. Thioredoxin and glutaredoxin systems

In this study, the cytosolic factors being analysed were the multi-functional oxidoreductases: Thioredoxins (Trx) and Glutaredoxins (Grx). Both protein classes feature ubiquitously expressed protein isoforms in the cell, with overlapping roles in several biological functions. These include the redox homeostasis of reactive oxygen species (ROS), repairing oxidative-damaged protein and downstream activation of ribonucleotide reductases (RNR) (Sengupta and Holmgren, 2014). Aside from these functions, there is the central mechanism of both redoxin systems in reducing protein disulfide-bonds and suggests the two redoxins to be possible factors in maintaining reduced IMS proteins in the cytosol, which has not been well characterised (Brandes et al., 2009).

Both Trxs and Grxs contain species-conserved cysteine (CX2C) residues, which reduce substrate proteins by a thiol-disulfide exchange with a disulfide bridge present in the oxidised substrate. The exchange is initiated by the N-terminal cysteine of the oxidoreductase CX2C motif to form a mixed disulfide with the substrate. Electrons are transferred from the C-terminal cysteine of the CX2C motif to the intermediate complex; reducing and releasing the substrate while oxidising and deactivating the oxidoreductase (Holmgren, 1989, Meyer et al., 2009). Individual redox homeostasis pathways are used by Trxs and Grxs to restore their reducing functions. The two redoxin systems are individually outlined in the following sections for the isoforms present in the cytosol, specific system functions and homeostasis to restore function.

1.5.2.1. Thioredoxin (Trx) system

The S. cerevisiae thioredoxin (Trx) system is present as three different isoforms with Trx1 and Trx2 (11 to 12 kDa) present in the cytosol, with Trx3 (14 kDa) in the mitochondria matrix. After reducing substrates and becoming oxidised in the process, Trxs are reduced through a direct interaction with NADPH and thioredoxin reductase (Trr) catalysis (Zhang et al., 2009). The two cytosolic isoforms are reduced by Trr1, while Trx3 is reduced by Trr2 which is also localised to the mitochondria matrix. The reducing pathway and electron flow for the cytosolic Trx system is presented in Figure 1.17.
Figure 1.7. Electron flow in the cytosolic Trx redox pathway.

Substrate proteins are reduced by Trx1 and Trx2 in the cytosolic Trx system, with oxidised Trxs being directly reduced by NADPH, catalysed by thioredoxin reductase Trr1. In the *S. cerevisiae* mitochondria matrix, the Trx system comprises Trx3 and Trr2. Adapted from (Karlenius and Tonissen, 2010).

Catalysis of Trx reduction by Trr1 also requires FAD as a co-factor in the transfer of electrons and bound as one FAD molecule per Trr1 subunit (Zhang et al., 2009). The cytosolic Trx2 differentiates from Trx1 as it can be induced after exposure to oxidative stress conditions with superoxide anion radicals (O$_2^\cdot$-) or hydrogen peroxide (H$_2$O$_2$). The Yap1 and Skn7 transcription factors are induced to express several proteins to restore the cellular redox homeostasis, including expression of the *TRX2* and *TRR1* genes (Lee et al., 1999, Mulford and Fassler, 2011, Morgan et al., 1997). Trx2 performs negative feedback by translocation to the nucleus from the cytosol to reduce Yap1 and inhibit further induction of the Trx system (Bao et al., 2007). Expression of the *TRX1* gene was observed in *H. sapiens* to be induced by DJ-1 transcriptional factor as part of the anti-oxidant defence mechanism (Im et al., 2012). Altogether, the cytosolic system has been identified as an effective contributor to the defence against oxidative stress.

1.5.2.2. Glutaredoxin (Grx) system

The glutaredoxin (Grx) oxidoreductases contrast to the Trxs with a greater complexity in function and diversity in the isoforms present in cells. The *S. cerevisiae* Grx system is the most diverse compared to other eukaryotes, with eight isoforms present ubiquitously throughout the different cell compartments (Allen and Mieyal, 2012) (Figure 1.18).
Figure 1.18. Distribution of *S. cerevisiae* Grx isoforms.

The *S. cerevisiae* Grx isoforms are localised to the cytosol, mitochondria, nucleus and golgi apparatus. The dithiol (CXXC) Grxs of Grx1 and Grx2 were identified to be involved with protein folding, with the monothiol Grxs (CXXS) being associated with redox homeostasis and the removal of oxidative species. Localisation of Grxs into the mitochondria matrix requires a targeting presequence (marked ‘M’). After translocation, the targeting presequence is cleaved by MPP. Grx2 is localised to the cytosol and mitochondria by alternate translation. Unprocessed Grx2 during translocation presents mitochondria OM-bound Grx2 exposed to the cytosol. Adapted from (Lillig et al., 2008).

The *S. cerevisiae* Grx system differs from *E. coli* which has Grx1-Grx4 isoforms free in the cytosol and the five Grx *H. sapiens* isoforms, where the dithiol Grx2 is also localised into the mitochondria matrix (Lillig et al., 2008). Similar to the Trxs, the cytosolic Grx system is associated with the defence against oxidative stress conditions, by the direct reduction of H$_2$O$_2$ in a peroxidase-like function (Collinson et al., 2002). While the Yap1 transcription factor induces expression of *GSH* and *GLR1* genes in oxidative stress, as well as *GLR1* in stationary-phase growth to prevent GSSG accumulation in cells, the two *GRX* genes are not induced (Grant et al., 1996b). Exposure to stress conditions (e.g. oxidative, heat) increases the activation and expression of the *GRX1* and *GRX2* genes by stress response elements (STRE); short DNA sequences to stimulate transcription of the *GRX* genes (Grant et al., 2000). The *GRX1* gene
contains one element in the promoter region, while \textit{GRX2} contains two STRE. The elements are mediated by activation with the Hog1 MAPK kinase pathway. Expression is negatively regulated by the Ras-protein kinase A pathway and cAMP levels. For the cytosolic Grx paralogs, functionality with oxidative stress and redox sensitivity was seen to vary for each oxidoreductase; observed with a \textit{Δgrx1} mutant sensitised to superoxide (O$_2^•$-)‐induced stress and \textit{Δgrx2} to H$_2$O$_2$ (Luikenhuis et al., 1998). Overexpression of the \textit{GRX1} and \textit{GRX2} genes was observed with increased resistance to oxidative stress.

Grx1 and Grx2 share 40-52% identity and 61-76% similarity in \textit{E. coli} and mammals, while in \textit{S. cerevisiae}, 64% sequence identity is shared (Luikenhuis et al., 1998, Li et al., 2010). Grx1 is a ~13 kDa identified free only in the cytosol, while localisation of Grx2 is more complex with the isoform present in the cytosol (12 kDa) and mitochondria (~15 kDa unprocessed and 12 kDa processed). This is from the alternate translation of the Grx2 mRNA as amino acid residues 1-35 (initiated by methionine) translate the mitochondria-targeting presequence to direct import to the TIM23 complex for translocation into the matrix. The mitochondria targeting sequence is then cleaved from the protein by MPP as it is retained in the compartment. Translation of Grx2 from the secondary methionine in the amino acid sequence (res. 36) forms the central core of Grx2 (res. 36-143) without the mitochondria targeting sequence and remains free in the cytosol like Grx1. Grx2 has also been identified on the cytosol-facing mitochondrial OM from mitochondrial import across the OM being observed to be relatively inefficient (Porras et al., 2006). The unprocessed mitochondria-targeted protein with the attached presequence remains at the TOM complex, but the specific functions of the OM-bound Grx2 has not been fully elucidated. To restore activity in the reduced Grx after oxidation from the IMS substrate, a redox system with GSH and NADPH are utilised (Figure 1.19).
Figure 1.19. Electron flow in the cytosolic Grx-glutathione redox pathway.

Substrate proteins are reduced by the Grxs: Grx1 and Grx2 in the cytosol, only Grx2 in the mitochondria matrix. Grxs directly reduced by GSH (reduced glutathione) which is then reduced by NADPH as the electron source for the redox pathway like Trxs. NADPH reduction of GSSG is catalysed by glutathione reductase (Glr1), which binds FAD as a redox co-factor. Adapted from (Salinas et al., 2004).

Reduction of Grxs differs from the Trxs as it is not a direct reaction with NADPH. Instead, the oxidised Grx is reduced by a glutathione molecule (GSH), which connects the Grx system to the GSH resources and supplies available in the cytosol and mitochondria. Reduction of the oxidised glutathione (GSSG) requires NADPH with catalysis by glutathione reductase (Glr1). Glr1 (53 kDa) is the only isoform of the protein present in the mitochondria and cytosol for the two localised redox systems. Like Trr1, the Glr1 also binds with an FAD co-factor as one molecule per subunit in shuttling electrons from NADPH to GSSG (Yu and Zhou, 2007).

1.5.3. Interaction between the Trx and Grx systems

For the two cytosolic systems, overlap in redox functioning was observed by the requirement of a single TRX or GRX gene for cell growth, with a quadruple deletion strain (Δgrx1grx2trx1trx2) being inviable (Draculic et al., 2000). Both systems depend on reducing pathways to restore functioning, with NADPH as the shared primary reducing source. However, functional overlaps between the two systems have been identified. The Yap1 transcription factor that induces expression for TRX2 and TRR1 also induces GLR1 and GSH to form the cytosolic Trx2 system as well as GSH from NADPH-reduction of oxidised GSSG in the cytosol (Lee et al., 1999).
Deletion of the *TRX1* and *TRX2* genes has been observed to increase GSSG levels, which indicates a functional overlap between the two systems through the glutathione (GSH) reducing source. While oxidised GSSG accumulated in the Δ*glr1*; forming 63% of the total glutathione levels (compared to 6% in the Wild Type), the Δ*trx1trx2* mutant showed a 22% GSSG ratio. Similarly, in combined Trx and Glr1 mutants (Δ*trx1glr1* or Δ*trx2glr1*), GSH:GSSG ratios were observed as ~70% oxidised for each mutant (Muller, 1996). Studies performed *in vitro* study also supported the cross-reactive function for the *S. cerevisiae* Trx system in a role similar to Glr1 in reducing GSSG (Tan et al., 2010). In contrast, combined deletion of *GRX1* or *GRX2* with *GLR1* was observed to not affect the GSH:GSSG ratio significantly like with the Trx system. Together, these results showed an effectiveness of the Trx system as an alternate reducing agent to Glr1 for GSSG present in the cytosol.

Individual redox function of the Trx and Grx oxidoreductases can be measured *in vitro* using standard redox potentials (E°) values, which measure the ability of a redox species to acquire electrons and become reduced (units in voltage, V). A larger negative value indicates stronger function as a reductant. The *S. cerevisiae* has not been fully characterised between the different oxidoreductase systems, with only cytosolic Trx2 previously determined (Gonzalez Porque et al., 1970). However in *E. coli*, E°*_{Trx1}*=−270 mV and E°*_{Grx1}*=−230 mV have been determined, which shows the Trx system to be stronger reductants in that species (Krause et al., 1991, Åslund et al., 1997). Collectively, the cytosolic Trx system has been characterised to exhibit stronger redox activity than the Grxs, which instead shows more complex functioning in the cell with gene expression and redox activity in stress and in disulfide-bond exchange.

### 1.6. Yme1 protease as an IMS folding assistant

The protease Yme1 (*yeast mitochondrial escape 1*) is an ATPase that hydrolyses ATP and exhibits a role in the quality control of IM and IMS proteins in the mitochondria. Yme1 is an 82 kDa protein imported into the IM through the TIM23 lateral insertion pathway, with three domains in the structure: the N-terminal, AAA and proteolytic domains (Graef et al., 2007). The Yme1 N-terminal domain contains a single transmembrane domain for anchoring into the IM with the N-terminus exposed into the matrix and the large soluble catalytic domain (~55 kDa) in
the C-terminal exposed in the IMS (Leonhard et al., 1996). The AAA domain (ATPase associated with diverse cellular activities) coordinates ATP hydrolysis for binding of substrates to Yme1. This is translocation of the substrate protein to the proteolytic domain at the C-terminal for degradation (Gerdes et al., 2012). The C-terminus is capped by a helical structure that has not been elucidated in terms of its function (Arnold and Langer, 2002).

Yme1 assembles into a large hexameric complex as the IMS-exposed i-AAA protease on the IM, which utilises the collective ATP hydrolysis for the translocation of substrate proteins to the proteolytic domains for degradation. The IM-embedded Mgr1 and Mgr3 proteins can associate with the i-AAA protease as part of an adaptor complex to help facilitate the protease activity (Dunn et al., 2008, Fiumera et al., 2009). Yme1 has been suggested to express chaperone-like activity to prevent aggregation, with later identification as a folding assistant in IMS protein biogenesis and homeostasis (Leonhard et al., 1999, Schreiner et al., 2012). Yme1 was identified in the targeting and clearance of misfolded or unassembled small Tim proteins in the IMS by degradation (Baker et al., 2012). Finally, Yme1 was recently identified as a negative regulator to Tim10 biogenesis in the IMS as substrates, which is protected from Yme1 degradation by assembly into the Tim9-Tim10 hexameric complex, where Tim10 is the functional moiety (Spiller et al., 2015).
1.7. Aims of study

1.7.1. Characterising the redox properties of Trx and Grx (Chapter 3)

The reductant functions of the two systems have only been partially-elucidated in yeast, so the aim was to characterise the individual cytosolic Trx and Grx isoforms and their reductant functions. This was to understand the reductant effectiveness of the two systems in relation to each other. This was achieved by the *in vitro* determination of the standard redox potential (E°') values, which quantifies the reductant properties of a species. The reducing functions were characterised with IMS protein import and *in organello* study of the OM-bound Grx2 isoform.

1.7.2. Investigating the cytosolic Grx reducing activity in the biogenesis of CX₃C IMS proteins (Chapter 4)

The biogenesis of IMS small Tim (CX₃C) proteins was investigated to determine if the cytosolic Grx system maintained protein reduction for import. To achieve this, *in vivo* studies were utilised to measure the mitochondrial levels of IMS proteins in GRX deletion mutant (∆grx) yeast strains. In addition, the degradation systems of the cytosolic 26S proteasome and IMS Yme1 protease were studied with the Grx system as negative regulators to IMS protein biogenesis.

1.7.3. Investigating the cytosolic Grx reducing activity in the biogenesis of CX₉C IMS proteins (Chapter 5)

The biogenesis of *S. cerevisiae* IMS CX₉C proteins are less characterised compared to the small Tim proteins. The Grx system was observed to be effective in facilitating *H. sapiens* CX₉C protein import, so this study looks to address the Grx reducing functions using *in vivo* approaches. This would further the compared Grx in IMS protein biogenesis compared to CX₃C IMS proteins. Mitochondrial protein levels were analysed in Δgrx yeast strains for CX₉C proteins in the IMS and IM as a control, where import is independent to the redox-sensitive MIA pathway. Full-length and C-terminal truncated forms for Mia40 (Mia40C representative of the *H. sapiens* hMia40 ortholog) were studied as CX₉C substrates of the different import pathways.
2. METHODS AND MATERIALS

2.1. Molecular methods

2.1.1. Molecular biology

In this study, a number of plasmid constructs were generated. Inserted genes were amplified by PCR using a genomic DNA template. PCR primers for the 5’- and 3’- sequence ends were used, with restriction sites for ligation into plasmids (appendix Table 6). If unavailable from laboratory stocks, oligonucleotides were designed manually and synthesized by Eurofins UK.

Phusion PCR for new DNA synthesis was performed with ~100 ng WT genomic DNA as a template, a DNA polymerase (New England BioLabs, NEB) and 0.5 μM primers with 0.2 μM deoxynucleotide (dNTP) mixtures. PCR was run for 35 cycles of: 10 seconds at 98°C, 15 seconds at 60°C and 90 seconds at 72°C. Efficiency of the PCR reaction was analysed with 0.8% agarose gel electrophoresis. Oligonucleotides and plasmids were treated with relevant restriction enzymes (NEB) and purified with the Qiaquick kit (Qiagen). The cleaved target gene was ligated into as expression vector using T4 DNA ligase (Fermentas) at RT for 2 hours. Intact plasmids were selected through transformation of E. coli competent cells (DH5α) on 100 μg/ml carbenicillin LB agar. A successful cell colony was picked with a sterile pipette tip and inoculated in liquid LB media (100 μg/ml carbenicillin) with growth overnight at 37°C. DNA was isolated with the QIAPrep miniprep kit (Qiagen). Plasmid constructs were identified by restriction digest analysis with the previous restriction enzymes and conditions. The cleaved plasmid and gene insert were verified using 0.8% agarose gel electrophoresis. Correct base sequence was determined externally (GATC Biotech). All plasmid constructs used in this study are listed in the appendix, Table 7.
2.1.2. Preparation of *E. coli* competent cells

DH5α and Top10 *E. coli* competent cells were prepared for this study (appendix Table 5). To prepare competent cells, 1 ml LB preculture was inoculated with a single colony and incubated overnight at 37°C. 200 ml fresh LB was inoculated 1:100 and incubated until mid-log phase growth (~4-7x10⁷ cells/ml, A₆₅₀= 0.45), followed by transfer onto ice (30 minutes). Cells were pelleted at 2,500 rpm, 4°C (5 minutes, Sigma 3-18K) and gently resuspended in 66 ml RF1 buffer (100 mM RbCl; 50 mM MnCl₂∙4H₂O; 30 mM CH₃COOK; 10 mM CaCl₂∙2H₂O; 15% glycerol; pH 5.8) and incubated on ice (1 hour). Centrifugation was repeated and cells were resuspended in 8 ml RF2 buffer (10 mM MOPS; 10 mM RbCl; 75 mM CaCl₂∙2H₂O; 15% glycerol; pH 6.8) on ice for 15 minutes. Sealed aliquots were then submerged in liquid nitrogen for immediate freezing. After removal, aliquots were transported on ice to the -81°C freezers for long-term storage.

2.1.3. Colony PCR

Selected colonies were removed from an agar plate and resuspended in 10μl sterile MQH₂O. PCR preparation and running conditions were performed as outlined in the previous section. For genomic DNA-transformed *S. cerevisiae* cells, additional steps were required for cell lysis. PCR mixture samples were pre-heated at 95°C for 5 minutes, before chilling on ice for 1 minute and 10 second centrifugation (14,800 rpm, Sigma 1-14K). 0.5 μl DNA polymerase was added afterwards. After colony PCR, products were analysed with 0.8% agarose gel electrophoresis.

2.1.4. Isolation of genomic DNA

Selected colonies were used to inoculate YPD media and grown overnight. Cells equivalent to A₆₀₀=20 were harvested and treated with 60U zymolyase (50 minutes, 30°C). Cells were then permeabilised with 1% SDS (65°C, 30 minutes). Proteins were precipitated with 1.3 M potassium acetate (on ice for 1 hour). The supernatant was centrifuged at 14,800 rpm (10 minutes, Sigma 1-14K) to isolate DNA for extraction. DNA was isolated using phenol:chloroform:isoamyl (25:24:1, Sigma-Aldrich) within the upper aqueous layer after brief vortexing and centrifugation (14,800 rpm Sigma 1-14K, 2 minutes). After repetition, the DNA
was centrifuged from the aqueous solution (15 minutes) and retained as a pellet. After washing in 70% ethanol, DNA was resuspended in TE buffer (10 mM TrisHCl pH 7.5; 1 mM EDTA) and 10 mg/ml RNAse (37°C, 30 minutes) to remove residual RNA.

### 2.2. Biochemical methods

#### 2.2.1. Protein expression and purification from *E. coli*

Genes for Grx1, Grx2, Trx1, Trx2 and Trr1 previously ligated into pBAD vector plasmid constructs (Collinson and Grant, 2003, Mukhopadhyay and Rosen, 2002) were transformed into DE3 *E. coli* cells. Proteins contain an N-terminal six-histidine residue tagged sequence for later Ni²⁺-NTA purification. Luria broth media (LB, Formedium) with 100 μg/ml Ampicillin was inoculated with a loop of the transformed *E. coli* glycerol stock. The pre-culture was incubated overnight at 37°C, 200 rpm and used to inoculate a fresh LB (100 μg/ml Ampicillin) media (20 ml in 1 litre). Cultures were further incubated at 37°C, 200 rpm until the A₆₀₀~0.5 where gene expression was induced with 0.002% w/v L (+) arabinose. Cells were incubated for a further 4 hours at which point the A₆₀₀~1.5-2.0. Cells were harvested at 5,000 rpm, 4°C in a JLA8.1 rotor for 15 minutes, followed by resuspension in buffer: 50 mM Tris-HCl pH 7.5; 500 mM NaCl; 1 mM imidazole. After repeated centrifugation, cells were resuspended in lysis solution containing a dissolved EDTA-free protease inhibitor cocktail tablet (1 tablet for 50ml lysis buffer, Roche). 5ml of solution used per gram of pellet weighed. After incubation on ice for 15 minutes, sonication was performed to break cell walls (10 cycles of 10 seconds off, 30 seconds on and amplitude of 30-35%). Solutions were retained at 4°C with ultracentrifugation at 17,000 rpm (20 minutes; JA17 rotor, Beckmann centrifuge). The supernatant was then filtered through a 0.45 μm filter membrane.

#### 2.2.2. Gravity-flow affinity chromatography

To isolate His-tagged proteins from the *E. coli* protein mixture, the lysate was passed through a gravity-flow chromatography column. Columns were prepared in 2 ml volumes packed with Ni²⁺-charged agarose resin for His-tagged proteins (Qiagen). Columns were maintained at 4°C and
cleaned and charged before use. Lysate was passed through the column, with 20x resin volume of the wash buffer applied (50 mM Tris-HCl pH 7.5; 500 mM NaCl; 20 mM imidazole). The His-tag bound proteins were eluted at a 3x volume of the elution buffer (50 mM Tris-HCl pH 7.5; 500 mM NaCl; 250 mM imidazole) compared to the resin volume. The efficiency of protein expression and purification was analysed on SDS-PAGE, with Coomassie Blue R250 staining to detect proteins and observe the efficiency of elution.

2.2.3. Size-exclusion chromatography

Proteins were further purified by size exclusion chromatography before study. Samples were applied to a Superdex 75 10/300 GL column (GE Healthcare) attached to the AKTA flow system (Amersham Pharmacia Biotech). BAE buffer (50 mM Tris; 150 mM NaCl; 1 mM EDTA; pH 7.0) was used for running at a 0.5 ml/min flow rate. Centrifugation was used to remove precipitate from the protein before loading onto the column by syringe injection (14,800 rpm Sigma 1-14K, 10 minutes). The 0.5 ml eluate fractions containing the target protein were collected and stored at -81°C. Reducing (DTT) or oxidising agents (oxidised glutathione (GSSG) or Cystine) were incubated with proteins for 30 minutes before injection to adjust redox states and were washed out of the column during the initial flow-through.

2.2.4. SDS-PAGE electrophoresis techniques

Electrophoresis conducted using SDS-PAGE gels (sodium dodecyl sulphate polyacrylamide gel electrophoresis) were run in the Bio-Rad mini-Protean III system. Protein samples were pre-heated at 95°C for 5 minutes and centrifuged at 14,800 rpm for 1 minute before loading the supernatant. Gels were run at a 26 mA constant per gel in each tank. Staining was performed with Coomassie Blue stain (0.25% Coomassie Brilliant Blue R-250 (Life Technologies); 7.5% acetic acid (Sigma); 50% methanol (Fisher)) was used to detect protein bands. SDS-PAGE gels were manually prepared in this study. Recipes for the SDS-PAGE compositions used are presented in the appendix (Chapter 8.2.1.1).
2.2.5. Gel blotting

Proteins were transferred to a nitrocellulose membrane (0.2 μm, Bio-Rad) in chilled conditions with the Hoefer TE22 mini tank gel electrophoresis unit. Membranes, sponges and transfer papers (Trans blot, Bio-Rad) were pre-soaked in the chilled transfer buffer (25 mM tris (Formedium); 150 mM glycine (Formedium); 20% methanol (Fisher)). Transfer performed at a 300 mA, 45 minutes. Transfer efficiency was monitored with Ponceau S staining (0.1% Ponceau S (Sigma-Aldrich); 5% acetic acid (Fisher)). Excess stain was removed by rinsing in Milli-Q purified water (MQH₂O).

2.2.6. Western blotting

Nitrocellulose membranes were treated with blocking buffer (5% w/v BSA or dried skimmed milk in MQH₂O) for 1 hour at room temperature (RT), with gentle agitation or continuous rotation. This was followed with three consecutive washes in PBS buffer (phosphate-buffered saline, 137 mM NaCl (Fisher); 2.68 mM KCl (VWR International); 7.5 mM Na₂HPO₄ (Fisher); 1.5 mM Na₂HPO₄·H₂O (Fisher)), before the application of antibody solutions.

Primary antibodies (rabbit) were applied overnight in PBST containing 5% w/v BSA (0.1% Tween-20; 1x PBS). Secondary antibodies (goat anti-rabbit) were applied for ~ 90 minutes in a dark box with 2% BSA in PBST. Both were used at RT, with gentle agitation or rotation. Between applications of the antibodies, the membranes were washed three times in PBS buffer. After additional PBS buffer washes, blots were scanned and visualised with the Odyssey Sa system (Li-Cor Biosciences).

2.2.7. Agarose gel electrophoresis

Agarose gels were prepared to 0.8% agarose in TAE (40 mM tris; 0.114% acetic acid; 1 mM EDTA; pH 8.5). Gels were run at 80 mV constant for 40 minutes in the Fisherbrand HU6 tank, with Generuler 1kb (Life Technologies) as a molecular weight marker. All samples were prepared in loading dye (Fermentas). Agarose gels were visualised under UV light (U:Genius³ Syngene).
2.2.8. UV/Vis spectrophotometry

NADPH and NADP⁺ (Sigma-Aldrich) were prepared fresh before spectrophotometry analysis. Different concentrations of Trx and NADPH were loaded in the quartz micro-cell cuvette (150 mm Ultra-micro cell 105.252-QS Suprasil, Hellma Analytics), with BAE buffer to make up the final volume to 150 μl. Spectrophotometry was performed at 25°C, using the Cary300Bio spectrophotometer (Varian).

Redox reactions were monitored by NADPH oxidation to NADP⁺, with NADPH absorption at 340 nm and NADP⁺ at 260 nm. After 5 minutes to measure background rate of aerobic oxidation, Trr1 was added to catalyse the redox reaction between NADPH and Trx1. A₃₄₀ was monitored continuously and excess NADP⁺ (>600 μM) was applied after equilibrium was reached, in order to reverse the reaction and produce NADPH. A control reaction for background NADPH oxidation was performed simultaneously without adding Trr1. The NADPH concentration was calculated by A₃₄₀ changes using the 6.22 mM⁻¹·cm⁻¹ molar extinction coefficient. Values were then used to determine the concentrations of NADP⁺ and Trx redox species.

2.2.9. Reverse Phase Chromatography (RP-HPLC)

Redox equilibrium between Trx and Grx was measured by detection of the different redox species separated by an increasing hydrophobicity gradient. RP-HPLC was performed using the Phenomenex Aeris Widepore column (3.6μ, XB-C18) as the stationary binding phase. Elution gradients were established using increasing concentrations of organic solvent acetonitrile (ACN, Sigma-Aldrich). Buffer A (5% ACN) was used as the initial baseline with increasing flow of buffer B (80% ACN) across the column to elute proteins in increasing hydrophobicity. ACN buffers were prepared with 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich) in Chromasolv HPLC-grade water (Sigma-Aldrich).

2.2.10. Oxygen consumption assay

Oxygen consumption as a maker of mitochondria function was measured using a Clark-type oxygen electrode (Hansatech Instruments). From an overnight cell preculture at 30°C, cells
were inoculated in fresh media and grown to late-exponential phase. Cells were applied to 2 ml of fresh media in the electrode; final concentration between 0.5-2.0 \( A_{600} \text{/ml} \). Media in the electrode was retained in an air-tight chamber, with continuous stirring by a magnetic bar. The chamber is connected to the electrode by a permeable filter paper membrane and the background oxygen consumption was measured before cells were added. Oxygen consumption was conducted at 30°C and monitored every second for 10 minutes. Data was recorded as nmol O\(_2\) min\(^{-1}\) and normalised for the cell density.

2.3. **In vivo and in organello techniques**

Unless stated otherwise, yeast strains were handled at 30°C with 200 rpm shaking in aerobic conditions for liquid cultures.

2.3.1. **Maintenance of yeast strains for study**

The yeast strains used in this study were prepared and maintained in YPD (1% bacto yeast extract (Scientific Laboratory Supplies Ltd); 2% peptone (Formedium); 2% glucose), and listed in Table 4 inside the appendix. Other YP media used were YPEG (3% ethanol (Fisher Scientific); 3% glycerol, (Fisher Scientific)) and YPGal (2% w galactose, (Fisher)). Agar media prepared to 2% (Formedium).

2.3.2. **Yeast cell spot testing**

A yeast colony was inoculated in liquid YPD and grown overnight into the late-exponential growth phase (20 hours). Based on the \( A_{600} \), cells were centrifuged (eppendorfs in Sigma 1-14K at 14,800 rpm for 1 minute), washed in MQH\(_2\)O and adjusted to \( A_{600}=1.0^0, 1.0^{-1}, 1.0^{-2}, 1.0^{-3} \). Cells spotted onto fresh agar plates and incubated at 30°C for up to 3 days.

2.3.3. **Yeast cell growth curves**

An initial YPD culture was inoculated with an agar plate colony and grown under aerobic conditions to the late-exponential phase (~20 hours). Based on the \( A_{600} \), fresh media was inoculated at \( A_{600}=0.2 \) for the growth curve. For transfer to different media (e.g. YPGal or
YPEG), cells were washed in MQH₂O before inoculation. Cell growth and density was monitored at \(A_{600}\).

2.3.4. Crude yeast protein preparation as whole cell extracts

Cells prepared in an overnight YPD preculture were shifted to fresh YPD or YPEG at a starting \(A_{600}=0.05\) with continued growth at 30°C until the late-exponential phase (~24 hours). The volume of cells equivalent to \(A_{600}=5.0\) were removed and centrifuged. The pelleted cells were resuspended on ice with 500 \(\mu\)l 0.2 M NaOH (sodium hydroxide, Formedium) to each sample for 10 minutes. 8% TCA (trichloroacetic acid, Fisher) was added and incubated on ice for an additional 10 minutes. After centrifugation (14,800 rpm Sigma 1-14K, 3 minutes), pellets were resuspended in 0.2 M Tris and sample buffer (11.7% 4x stock SDS-PAGE stacking gel buffer; 5% w/v glycerol (Fisher Scientific); 58 mM SDS (Fisher); 17 \(\mu\)M Bromophenol blue (Sigma-Aldrich)). 100 mM DTT (Sigma-Aldrich) was added to samples in reducing conditions.

For OE-transformed cells, an overnight SD-URA pre-culture was prepared with later MQH₂O washing and resuspension at \(A_{600}=0.05\) in fresh SGal-URA. Cultures were grown to the late-exponential phase (~24 hours) before measurement of the \(A_{600}\) value. Cells were collected for the preparation of whole cell extracts, as described before.

2.3.5. Isolation of mitochondria

2.3.5.1. Lactate media

0.3% yeast extract (Melford); 0.05% glucose (Formedium), 0.05% calcium chloride dihydrate (Sigma); 0.05% sodium chloride (Formedium); 0.06% magnesium chloride-6-hydrate (Fisher); 0.1% potassium phosphate (Fisher); 0.1% ammonium chloride (Fisher); 2% lactic acid (Sigma-Aldrich); 0.8% sodium hydroxide (Formedium). The pH was then adjusted to 5.5.

2.3.5.2. Mitochondria isolation procedure

50 ml LM was inoculated with an agar plate colony and grown to the stationary phase. Cells then used to inoculate fresh LM in litre cultures at \(A_{600}=0.1\). The new LM cultures were grown
until the late-exponential phase (~20 hours) at 30°C before harvesting. LM was centrifuged at 5,000 rpm; 10 minutes (JLA8.1000 rotor, J2-HS centrifuge) and pellets were resuspended and collected in MQH₂O. After centrifugation (2,850 rpm; Sigma 3-18K centrifuge, SciQuip Ltd), the pellet was weighed to measure the yield. It was then resuspended in 25 ml Tris-DTT buffer (100 mM Tris-SO₄ pH 9.4; 10 mM DTT) and incubated at 30°C with shaking at 80 rpm. After 30 minutes, cells were centrifuged and washed in 1.2 M Sorbitol buffer (1.2 M Sorbitol (Sigma); 20 mM KPi (Fisher) pH 7.4).

After repeated centrifugation, the pellet was resuspended in zymolyase buffer to permeabilise the cells. The constituents depended on the wet weight; 3 mg zymolyase 20T (Seikagaku Corporation) and 2 ml 1.2 M Sorbitol buffer per gram cell weight. The culture was then incubated further at 30°C, 80 rpm shaking for 40 minutes to spheroblast the cells. Spheroblasting efficiency was monitored by A₆₀₀ measurement; using a 1:500 dilution in MQH₂O against a control of 1.2 M Sorbitol buffer in MQH₂O. If successful, the A₆₀₀ drops to <10% of the original value after ~30 minutes. After centrifugation, cells were washed in chilled 1.2 M sorbitol buffer and stored on ice. Cells were resuspended in a chilled buffer for homogenisation: 0.6 M Sorbitol; 20 mM HEPES-KOH pH 7.4; 200 mM PMSF; together filtered through Whatman paper (GE Healthcare) before use.

Homogenisation was performed with a glass-teflon homogeniser and Dounce tissue grinder (Sigma-Aldrich). Twenty strokes were used before centrifugation (1,500 x g) to pellet unruptured spheroblasts, while the supernatant was retained. Homogenisation was repeated for the pellet and the resulting supernatant fractions were combined. After centrifugation (4,000 x g, 10 minutes) to pellet cell debris, the supernatant was taken for ultracentrifugation (10,000 rpm, 4°C for 10 minutes (J25.50 rotor, Beckmann). Mitochondria pelleted from the cytosol were then resuspended in 2 ml BB7.4 (0.6 M sorbitol; 20 mM HEPES-KOH pH 7.4). Protein concentration was measured from the A₂₈₀ reading (diluted 1:100 in 1 ml 0.6% SDS), with A₂₈₀=0.21~10 mg/ml crude mitochondrial proteins. The concentration was adjusted to 10mg/ml and decanted into 1mg aliquots with 10 mg/ml fatty acid-free BSA (bovine serum albumin, Sigma-Aldrich). Aliquots were snap-frozen by immersion in liquid nitrogen and after removal, were promptly stored in freezers at -81°C.
For mitochondria isolated from transformed OE strains, the methods used were as described before, but with modification to the media used. Colonies from agar plates were inoculated in a SD-URA culture that was grown to the stationary phase (~22 hours). This was used to inoculate a 50 ml SGal-URA culture at $A_{600}=0.05$ for overnight growth (~16 hours). Cells were used to inoculate 1 litre of YPGal (2% galactose) at $A_{600}=0.05$ and grown to the late-exponential phase at 30°C (~21 hours) when plasmid expression was induced. Isolation of mitochondria with permeabilisation and homogenisation was then used, as described earlier in this section. Preparation of isolated mitochondria after inducing protein OE is outlined in Figure 2.1.
OE plasmids containing genes for target proteins were transformed into *S. cerevisiae* cells (B1), with unmodified pYES2 as an endogenous control (A1). Gene OE was induced (AII, BII) following cell exposure to 2% galactose. BIII) OE protein import into the mitochondria. AI, BIV) Proteins recovered from the isolated mitochondria and cytosol after homogenisation and levels were measured by Western blotting. Figure is original to this study.
2.3.6. Solubilisation of isolated mitochondria proteins

From -81°C storage, aliquots were thawed on ice (~15 minutes) and divided into two 50 μl volumes (50 mg mitochondrial proteins). Cells were centrifuged in eppendorfs (14,800 rpm Sigma 1-14K, 1 minute) to pellet mitochondria. These were then resuspended in sample buffer (±DTT), with proteins loaded in SDS-PAGE gels as 25, 50, or 75μg.

2.3.7. Protein solubilisation from isolated cytosol

From the post-homogenisation supernatant after ultracentrifugation, 1 ml was treated with 5% TCA on ice (1 hour) to precipitate proteins. After centrifugation in eppendorfs (14,800 rpm Sigma 1-14K, 3 minutes) and decantation; the pellet was washed twice in acetone followed with repeated centrifugation. The pellet was dried in a heating block, before resuspension in 100 μl sample buffer (SB±DTT; 100 mM Tris).

2.3.8. AMS alkylation assay

AMS (4-acetamido-4'-maleimidylstilbene-2, 2'-disulfonic acid, Sigma Alrich) alkylates free cysteines in proteins; increasing the molecular weight by ~0.5 kDa (per cysteine) to identify the different reduced species. AMS incubated with samples including Tris and SB-DTT buffer at RT for 30 minutes.

2.3.9. Fluorescence microscopy

A yeast colony (transformed with the RFP fluorescent tag in a plasmid) was inoculated in a preculture and grown overnight into the late-exponential phase. Cells were measured by the A600 value, collected and washed in MQH2O. Fresh 50 ml SGal-URA media was inoculated (A600=0.003) and grown until early exponential phase between A600=0.6-1.0. From this, 1 ml was centrifuged (4,000 rpm Sigma 1-14K, 1 minute) and resuspended in 200 μl fresh media. Samples were then studied under fluorescence microscopy (Nikon Eclipse E600 UV-2A) with images then being captured.
2.3.10. Inhibition of cytosolic proteasome with MG132

Cells were prepared in a modified knockout minimum media: 0.17% Yeast nitrogen base without amino acids or ammonium sulphate (Amresco Biochemicals); 0.13% uracil-deficient (-URA) amino acid single drop-out mixture (Formedium); 0.1% proline (Formedium). 2% glucose and 2% galactose were added for SD\textsuperscript{MG}-URA and SGal\textsuperscript{MG}-URA. Plate colonies were used to inoculate SD\textsuperscript{MG}-URA media and grown until the late exponential phase (~19 hours). CY4-derived cells were permeabilised with 0.003% w/v SDS for 3 additional hours. Cells were subsequently washed in MQH\textsubscript{2}O and transferred to fresh SGal\textsuperscript{MG}-URA (0.003% SDS) at A\textsubscript{600}=2.0. For the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-al, Sigma-Aldrich), 75 μM was added in SGal\textsuperscript{MG}-URA at 15 hours into cell growth, while in control samples, the equivalent volume of DMSO was added instead. Cell growth was monitored at A\textsubscript{600} with whole cell extracts prepared to measure protein levels.

2.3.11. Isolation of plasmid and genomic DNA from transformed yeast

Using a manufacturer-developed protocol (Qiagen), the QIAprep Spin miniprep kit was used to isolate plasmid DNA from transformed yeast cells. The protocol includes 425-600 μm acid-washed glass beads (Sigma) being vortexed with cells for lysis. Cells were prepared from inoculated SD-URA precultures grown ~16 hours at 30°C. Genomic DNA was also isolated in the plasmid mixture.

2.3.12. Radiolabelled protein import into isolated mitochondria

2.3.12.1. Preparation of mitochondria for import

LM mitochondria aliquots were thawed from -81°C on ice (30 minutes), while reagents were prepared. Samples were resuspended in 900 μl ice-cold BB7.4 and centrifuged at 12,000 rpm (4°C, 5 minutes). The pellet was washed with 1ml ice-cold BB74 and centrifuged (13,000 rpm Sigma 1-14K, 1 minute). Mitochondria were resuspended with 100 μl BB7.4 (final concentration to 10 mg/ml) and kept on ice.
2.3.12.2. Preparation of radiolabelled precursor proteins/reticulocyte lysate

Using the Tim9-pGEM plasmid DNA as a template, precursor Tim9 radiolabelled with $^{35}$S-methionine (PerkinElmer) was prepared on ice through the SP6 TnT reticulocyte lysate system (Promega). For each import sample, 5 μl lysate was required in the following ratios: 0.2 μl TnT reaction buffer, 0.1 μl Methionine knockout (-Met) mixture (Amresco Biosciences), 0.1 μl SP6 TnT RNA polymerase (Promega), 0.1 μl RNasin ribonuclease inhibitor (Promega), 0.4 μl $^{35}$S-methionine and 2.5 μl TnT rabbit reticulocyte lysate. As a template, 1.0 μg minimum of plasmid DNA was used. The volume was made up to 5 μl with nuclease-free water (Fermentas).

Reagents were mixed and centrifuged for 5 seconds, with incubation at 30°C for 30 minutes. 5 mM DTT was added, with further 30°C incubation for 30 minutes. The lysate was then centrifuged at 38,000 rpm, 4°C for 15 minutes in 200 μl polypropylene thickwall ultracentrifuge tubes (TLA-100 rotor titanium 30° angle 20x0.2 ml, Beckman Coulter). The radiolabelled supernatant was removed and stored on ice until use with the isolated mitochondria. A total volume of 150 μl was used for each import assay with the mitochondria and lysate. In import studies with the presence of reduced glutathione (GSH, Sigma-Aldrich), different concentrations were added to the import assay solutions from a 250 mM stock. Sample volumes were prepared with 75 μl 2x Import buffer (IB), 7.5 μl 10 mg/ml mitochondria, 5 μl reticulolysate and 62.5 μl nuclease-free water (volume adjusted for GSH addition). For 1xIB: 0.6 M Sorbitol; 2 mM KH$_2$PO$_4$; 1.25 mM KCl; 50 mM HEPES; 10 mM MgCl$_2$; 2.5 mM Na$_2$EDTA; 5 mM L-methionine; 1 mg/ml fatty acid-free BSA. The lysate was added last and after mixing, the solution was incubated at 25°C for 20 minutes. A negative control was prepared from solubilised mitochondria with 0.1% w/v digitonin (Fisher Scientific) added 3 minutes prior to the end of incubation.

Trypsin (Sigma-Aldrich) was applied to digest unimported precursors from the import assay and the mitochondrial outer membrane (OM). 50 mg/ml Trypsin was applied to samples and were kept on ice for 30 minutes. Digestion was stopped with the soybean-derived trypsin inhibitor (SBTI, Sigma-Aldrich); added to ≥30x the trypsin concentration used. Samples were kept on ice for 10 minutes before centrifugation (13,000 rpm 4°C, AccuSpin MicroR (Fisher Scientific) for 5
minutes). The pellet was washed in BB7.4 and centrifuged as before. Samples were washed again in BB7.4 before being resuspended in ~8 μl 2xSB+DTT. Samples were loaded onto SDS-PAGE gels and after electrophoresis, were used with gel blotting. Detection of radiolabelled bands was performed by autoradiography using a phosphor plate (BAS-MP imaging plate screen, Fujifilm) and scanning (Typhoon Trio imager, GE Healthcare).

2.4. Yeast genetic methods

2.4.1. Short frame homology PCR transformation (SFH-PCR)

To knock out selected genes, SFH-PCR was used to replace genes with the KanMX4 cassette. PCR was used to amplify the cassette with matching 5’ upstream and 3’ downstream sticky ends corresponding to the gene. Cassettes were then transformed into yeast strains using the high efficiency yeast transformation method (Section 2.4.2.1). Transformed colonies were selected on YPD agar containing 400 μg/ml Geneticin (G418, ThermoFisher Scientific). Colony PCR analysis was performed with primers for the KanMX4 cassette and detection on 0.8% agarose gel.

2.4.2. Plasmid transformation of yeast strains

2.4.2.1. High efficiency yeast transformation

An overnight YPD culture (~18 hours at 30°C, 200 rpm) was inoculated with an agar plate colony. Cells were diluted to A600=0.3 in fresh YPD media and grown to A600=1 (~3 hours 30°C, 200 rpm). Cells were centrifuged at 3,000 g (Sigma 3-18K) and after MQH2O washing, were permeabilised with 100 mM lithium acetate (LiAc, 1.5 ml) before resuspension in 250 μl 100 mM LiAc. From this, 50 μl was used for transformation with 4 μl plasmid (or 40 μl linear DNA fragments), 5 μl single-strand carrier DNA (pre-heated at 95°C, 5 minutes then chilled on ice), 240 μl 50% PEG and 36 μl 1 M LiAc. Incubation at 30°C for 30 minutes was performed, with heat-shock for 15 minutes at 42°C. Following centrifugation (14,800 rpm Sigma 1-14K, 30 seconds), cells were resuspended in 400 μl YPD and incubated as before, with centrifugation and resuspension to 100 μl in MQH2O. Cells were plated onto selective agar for growth at 30°C
(≈2 days). The list of plasmid-transformed yeast strains prepared is presented in the appendix, Table 8.

2.4.2.2. Maintenance of pYES2-transformed strains

Colonies were maintained in uracil-deficient (–URA) media for selection of the pYES2-transformed yeast strains containing the URA3 gene in the plasmid. Media contains 0.5% Yeast nitrogen base without amino acids or ammonium sulphate (Amresco Biochemicals and Life Sciences); 0.13% -URA amino acid single drop-out mixture (Formedium) and 0.5% ammonium sulphate (Fisher). Solid –URA media for agar plates was prepared with the addition of 2% agar (Fisher). Expression in pYES2 is fully repressed in SD-URA (2% glucose) and fully induced in SGal-URA (2% galactose).

2.4.3. Quantitative PCR (real time polymerase chain reaction, qPCR)

2.4.3.1. DNA preparation for qPCR

To measure the level of plasmid DNA in transformed cells, precultures were prepared overnight, before centrifugation and washing in MQH₂O. Cells were inoculated in fresh media at A₆₀₀=0.02 and grown until the mid-exponential phase (14-18 hours, 30°C 200 rpm) when A₆₀₀>2.0. At this point, the plasmids products can begin to be observed; while the purity of DNA recovered is higher compared to the late exponential phase. DNA isolation was performed with A₆₀₀=20 total of cells using acid-washed glass beads for cell lysis and the modified QIAprep Spin miniprep protocol (User-developed protocol PR04, Qiagen). The concentration of isolated DNA (ng/μl) was measured according to the A₂₆₀ absorbance (Nanodrop 1000, ThermoScientific). Results were measured in triplicate for accuracy. DNA purity was verified by measuring the 260/280 and 260/230 ratios, with ~1.8 and ~2.0-2.2 respectively being equivalent for high DNA purity.

2.4.3.2. Run conditions for qPCR

Samples were prepared in 96-well plates (MicroAmp Fast Optical reaction plates (0.1 ml), Applied Biosystems by Life Technologies) to working volumes of 10 μl. SYBR Green mixture + ROX passive reference dye (Bio-Rad) were used to detect synthesised DNA. DNA samples
were loaded to 0.2 nM with 0.5 μM of each primer. All samples were prepared in RT-PCR-grade nuclease-free water (Ambion Life Technologies) to prevent contamination. One pair of forward and reverse primers was used for a pYES2 plasmid region, while another was used for the genomic β-tubulin gene (appendix, Table 6). Serially-diluted (1:10) standards of the purified plasmid and β-tubulin were used against the samples, as well as negative qPCR controls without DNA. Samples were run in triplicate, while the standards and negatives were run in duplicate. The qPCR runs were performed in the StepOne Plus real-time PCR system. Prior to running, plates were sealed (Microseal B adhesive seals, Bio-Rad) before brief vortexing and centrifugation (max speed, 15 seconds) to ensure mixing and collection at the base of the wells.

For the experimental run, an initial holding stage (95°C, 20 seconds) was used before 40 cycles of 95°C (3 seconds) and 60°C (30 seconds) were used. Samples were then prepared as a melting curve with initial heating at 95°C (15 seconds) before a continual 0.3°C increase per minute as a gradient from 60°C up to 95°C and then holding for 15 seconds. All data was collected in the StepOne Real-Time PCR system software for quantification and analysis (Applied Biosciences).
3. RESULTS AND DISCUSSION I – REDOX POTENTIAL MEASUREMENT OF CYTOSOLIC REDOXINS AND IN ORGANELLO IMPORT

3.1. Introduction

To understand whether the cytosolic thioredoxin (Trx1, Trx2) and glutaredoxin (Grx1, Grx2) systems facilitate the maintenance of IMS precursor proteins in import-competent forms, the redox properties of the different oxidoreductases need to be characterised. Though the standard redox potential ($E^\text{ox}$) value of some bacterial and human homologues have been reported, only the S. cerevisiae $E^\text{ox}\text{Trx2}$ value has been determined out of the different isoforms (Gonzalez Porque et al., 1970). The standard redox potential ($E^\text{ox}$) provides a quantitative measurement of the redox ability of a chemical species, specifically in terms of their tendency to acquire electrons. A species measured with a large negative $E^\text{ox}$ value is described to be a strong reductant, while a positive value denotes it as being an oxidant with the ready-acceptance of electrons from other reductants. The $E^\text{ox}$ values can also be related to IMS proteins and other redox sources and elements (GSH, NADPH) present in the environment to develop a map of electron flow between redox pathways. Studies in E. coli comparing the Trx and Grx systems determined Trxs to be stronger reductants with $E^\text{ox}\text{Trx1}=-270$ mV compared to $E^\text{ox}\text{Grx1}=-230$ mV (Åslund et al., 1997). For the S. cerevisiae Trx and Grx orthologs, $E^\text{ox}$ values have not been fully determined and were the focus of this chapter.

The results presented in this chapter were carried out during the first year of my PhD study; focused on in vitro characterisation of the redox properties of the yeast Trx and Grx enzymes, using purified proteins. Firstly, the Trx1 $E^\text{ox}$ was determined based on its redox reaction with NADPH catalysed by Trr1. The NADPH concentration change was monitored by following its $A_{340}$ intensity change as it was oxidised and from this, the different redox species concentrations were calculated. The Nernst equation was used to calculate the $E^\text{ox}\text{Trx1}$ based on the known $E^\text{ox}\text{NADP}$ value. Secondly, I attempted to determine the $E^\text{ox}$ for Grxs by developing a method of protein-protein equilibration (PPE) between Trxs and Grxs. This is because it is GSH, not
NADPH that reduces Grx directly (Figure 1.19), and a direct equilibrium between Grx and GSH would produce mixed-disulfides that interfere with the detection of the separate redox species.

Furthermore, mitochondrial protein import analyses were used to investigate whether the OM-located Grx2 plays a role during the import of Tim9. To address this, in organello import of radiolabelled Tim9 precursors was developed for the mitochondria isolated from WT and Δgrx2 yeast strains. Imported precursors in the mitochondria were then analysed by measurement of radioactive signals.

3.2. Determination of the Trx1 redox potential

3.2.1. Expression and purification of recombinant yeast Trx1 and Trr1 from E. coli

In order to determine the $E^{\circ}_{\text{Trx1}}$ from the equilibrium reaction, recombinant Trx1 and Trr1 in pBAD/His vectors (Invitrogen) were expressed and purified from BL21 (DE3) E. coli cells, as described in (Terpe, 2006, Guzman et al., 1995). Protein expression was induced with the addition of L (+) arabinose to a 0.002% final concentration, before cells were lysed by sonication. After centrifugation, the N-terminal His-tagged recombinant proteins were isolated by passage of the supernatant through a charged Ni-NTA column. The bound recombinant proteins were then eluted from the column using an increased concentration of imidazole (250 mM from 20 mM in the wash). Analysis of the efficiency of Trx1 purification was performed by SDS-PAGE examination (Figure 3.1). It showed detection of the 12 kDa poly His-tagged Trx1 with ~90% purity as higher kDa proteins were also detected in eluates E₁ to E₃ with Trx1. These impurities were then removed using size exclusion chromatography (SEC, Superdex 75 HPLC columns). The E₁-E₃ eluates were pooled together beforehand to improve purification efficiency, initially by centrifugation to remove precipitated proteins. Purification by SEC was performed using fast protein liquid chromatography (FPLC, Figure 3.1B).
Figure 3.1. Recombinant Trx1 expression and purification.

A) 16% Tris-tricine SDS-PAGE analysis and Coomassie staining of samples from each step of Trx1 purification from E. coli. Keys: U (uninduced cells), I (L (+) arabinose-induced cells), P (pellet of centrifugation after sonication), S (supernatant of sonication), FT (flow through of the supernatant through the Ni-NTA column), W (washing of the column, 20 mM imidazole), E₁-E₅ (eluate fractions with 250 mM imidazole), M (protein markers). B) FPLC size-exclusion profile of Trx1 purification. Further purification of pooled E₁-E₅ eluates from (A) by size-exclusion chromatography (Superdex75) in BAE buffer (50 mM Tris; 150 mM NaCl; 1 mM EDTA) pH 7.0.

After performing SEC, eluate fractions (500 µl in size) were collected for the lowest molecular weight peak that corresponded to the Trx1 size (the primary peak at 11.5-13.5 ml flow, Figure 3.1B) and were pooled together for further study. Protein concentration was determined using the A₂₈₀ absorbance with the extinction coefficient of 10.095 mM⁻¹ cm⁻¹ (Durigon et al., 2012). Aliquots of Trx1 were then stored at -20°C for short-term storage before use, while other samples were snap-frozen in liquid nitrogen and stored at -81°C.

Similarly, the His-tagged recombinant Trr1 (35 kDa) was purified from the pBAD plasmid vector in BL21 (DE3) E. coli cells, as described for Trx1. Induction of expression required 0.002% L (+) arabinose and SDS-PAGE was used to analyse Trr1 purification efficiency (Figure 3.2A). Due to Trr1 requiring FAD as a bound co-factor (1:1 ratio) in transferring electrons, 100 µM FAD was
added to affinity-purified Trr1 for 30 minutes before further purification with SEC, with the profile observed being shown in Figure 3.2B.

Figure 3.2. Recombinant Trr1 expression and purification.

A) SDS-PAGE analysis (16% Tris-Tricine gel) and Coomassie staining of samples from each step of Trr1 purification from E. coli. Keys: U (uninduced cells), I (L (+) arabinose-induced cells), P (pellet of centrifugation after sonication), S (supernatant of sonication), FT (flow through of the supernatant through the Ni-NTA column), W (washing of the column with 20 mM imidazole), E1-E5 (eluate fractions with 250 mM imidazole). M (protein markers). B) FPLC size-exclusion profile of Trr1 purification. Further purification of pooled E1-E3 eluates from (A) by size-exclusion chromatography (Superdex75) in BAE buffer (50 mM Tris; 150 mM NaCl; 1 mM EDTA) pH 7.0.

Eluate fractions (500 μl volume size) were collected from FPLC, corresponding to the primary peak in the profile that corresponded to Trr1 (35 kDa, 7-10 ml column flow). Trr1 concentration was measured based on the absorption intensity at 450 nm for the bound FAD co-factor, with an extinction coefficient of 11.3 mM−1 cm−1 (Durigon et al., 2012). Trr1 was snap-frozen and stored at -81°C before use.

3.2.2. Determination of the Trx1 E°′ value

To determine the Trx1 redox potential (E°′Trx1), a redox equilibrium reaction was performed using oxidised Trx1 (Trx1Ox) and the reduced form of NADP+ (NADPHRed). Prior to the reaction, Trx1 was fully oxidised through prolonged exposure to an aerobic environment. Trr1 catalysed
the thiol-disulfide exchange leading to a shift in the redox equilibrium to form reduced Trx1 (Trx1_{Red}) and oxidised NADP⁺ (NADP⁺_{Ox}) (Eq. 1). NADPH oxidation was monitored by its absorbance at 340nm and was used to measure the concentration differences using the extinction coefficient of 6.22 mM⁻¹cm⁻¹ (Fruscione et al., 2008). Differences in the NADPH concentration, after factoring in the background rate of oxidation, were equivalent to the amount of Trx1 reduced in the equilibrium. Equilibrium was performed in aerobic conditions at 25°C, pH 7.0 in BAE buffer and normalisation against an uncatalysed equilibrium mixture, to measure aerobic oxidation of NADPH. The equilibrium constant (K_{eqn}) for the performed thiol-disulfide exchange reaction was then determined for the different concentrations of the Trx1 and NADPH redox species observed (Eq. 2).

\[
\text{Trx1}_{Ox} + \text{NADPH}_{Red} \rightleftharpoons _{Trr1} \text{Trx1}_{Red} + \text{NADP}^+_{Ox} \quad (\text{Eq. 1})
\]

\[
K_{eqn} = \frac{[\text{Trx1}_{Red}][\text{NADP}^+_{Ox}]}{[\text{Trx1}_{Ox}][\text{NADPH}_{Red}]} \quad (\text{Eq. 2})
\]

\[
E^{\circ\prime}_{(\text{Trx1})} = E^{\circ\prime}_{(\text{NADP})} + \frac{RT}{nF} \ln K_{eqn} \quad (\text{Eq. 3})
\]

The calculated K_{eqn} value was used with the Nernst equation (Eq. 3) to determine the redox potential of Trx1 (E^{\circ\prime}_{Trx1}) against E^{\circ\prime}_{NADP}=-315 mV (Åslund et al., 1997) and where \( R= \) Universal gas constant (8.314 J K⁻¹ mol⁻¹), \( T= \) Absolute temperature (298°K), \( n= \) number of electrons transferred (here \( n=2 \)) and \( F= \) Faraday’s constant (9.6485x10⁴ J mol⁻¹) (Åslund et al., 1997).

Trx1 and NADPH were mixed (BAE, pH 7.0) in a cuvette inside the spectrophotometer and were equilibrated at 25°C before measurement. Enzyme Trr1 was added to the Trx1-NADPH mixture after 5 minutes of baseline measurement to initiate the redox reaction. In each study, a parallel control experiment was performed for the same concentrations of Trx1 and NADPH, without the addition of Trr1. For each experiment, a set of UV visibility spectra were recorded at intervals of every 4 minutes over 250-440 nm (Figure 3.3). The peak at 340 nm for NADPH was monitored for the shift of redox equilibrium (Eq. 1). In Figure 3.3, 20 μM Trx1 was incubated with 20 μM NADPH and catalysed by 100 nM Trr1.
Figure 3.3. Spectra of the Trx1-NADPH redox reaction catalysed by Trr1.

In a spectrophotometer cuvette, 20 μM oxidised Trx1 and 20 μM NADPH were incubated (total volume of 150 μl). 100 nM Trr1 was added after 5 minutes of baseline measurement to catalyse the redox reaction of Trx1 and NADPH. Spectras were recorded at 4 minute intervals, with 9 measurements shown to demonstrate overall changes in the A₃₄₀ peaks. Equilibrium was reached with consistent A₃₄₀ readings being observed comparable to the baseline control samples. Redox reactions were performed at 25°C in BAE pH 7.0 buffer.
The $A_{340}$ values measured were normalised against the baseline control that was performed simultaneously in a separate cuvette. Spectra readings were also recorded at each 4 minute interval, which was done to accurately determine the concentration of NADPH oxidised over time in the redox reaction with Trx1. Relative $A_{340}$ differences were presented in Figure 3.4.

![Figure 3.4. Timecourse for the Trx1-NADPH reaction.](image)

Timecourse of the Trx1-NADPH redox equilibrium reaction, previously presented in Figure 3.3. Changes in 340 nm absorbance in each 4 minute interval correspond to changes in NADPH concentration. Values were measured relative to the Trx1-NADPH control reaction performed simultaneously in aerobic conditions, without Trr1. Trr1 was added after 5 minutes to initiate the redox reaction. Excess NADP$^+$ (140 μM) was added at 50 minutes to reverse the reaction; $A_{340}$ intensity increased as NADPH was reformed from the reversal of the redox equilibrium (Eq. 1).
From consistent A$_{340}$ readings (40, 44, 48 minutes) that established the new redox equilibrium, excess NADP$^+$ (140 μM) was applied to the cuvette to reoxidise Trx1. It confirms the reaction is reversible as NADPH was reform (Eq. 1). The concentrations of the Trx1 and NADPH redox species were calculated and used to determine the K$_{eqm}$ for the reaction (Eq. 2). The value was then applied to the Nernst equation to determine the E$_{Trx1}^{°'}$ value against E$_{NADP}^{°'}$ (Eq. 3). For the Trx1-NADPH experiment presented in Figure 3.3 and Figure 3.4, K$_{eqm}$=12.90 and E$_{Trx1}^{°'}$=-282 mV were calculated. To verify the result, the same experiment was carried out using different Trx1 and Trr1 concentrations; the obtained results were similar and presented in Table 1. The averaged S. cerevisiae E$_{Trx1}^{°'}$ was determined as -280±1.53 mV.

**Table 1. The calculated K$_{eqm}$ and E$_{eqm}$ values of S. cerevisiae Trx1.**

Substrate concentrations used to determine the K$_{eqm}$ values presented in the table. All values calculated at pH 7.0 and 25°C in BAE buffer. Standard E$_{eqm}$ error=±1.53 mV with n=3.

<table>
<thead>
<tr>
<th>NADPH (μM)</th>
<th>Trx1 (μM)</th>
<th>Trr1 (nM)</th>
<th>K$_{eqm}$</th>
<th>E$_{Trx1}^{°'}$ (mV)</th>
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<td>20</td>
<td>20</td>
<td>100</td>
<td>13.92</td>
<td>-281</td>
</tr>
</tbody>
</table>

**3.3. Determination of the Grx1 redox potential**

To determine the standard redox potential of Grx1 (E$_{Grx1}^{°'}$), a redox equilibrium reaction with NADPH is not suitable, as Grx reduction does not occur directly with NADPH like Trxs. Grxs are reduced by glutathione (GSH) which is then itself reduced by NADPH, catalysed by the glutathione reductase (Glr) (Figure 1.19). Thus, a Grx-GSH redox equilibrium reaction was unsuitable for study due to the propensity of Grxs to form mixed-disulfides with glutathione, since the change in GSH concentration would not translate fully to redox changes in Grx as being properly reduced. An attempt was made based on the Trx1-Grx1 redox reaction to
determine $E^{''}_{\text{Grx}}$; a method previously used to determine $E^{''}$ of *E. coli* Grx1 and Grx3 against Trx (Åslund et al., 1997). Detection of redox species can be performed with the AMS assay or alternatively, the more accurate method of reverse-phase HPLC (RP-HPLC) to isolate proteins based on their hydrophobicity.

### 3.3.1. Recombinant Grx1 expression and purification from *E. coli*

Using the method from Trx1 purification, His-tagged recombinant Grx1 was expressed and purified from *E. coli* with His-tag affinity purification followed by SEC (Figure 3.5). Eluate fractions (500 μl) were collected corresponding to the lower molecular weight peak, between 11-13 ml flow for the 13 kDa Grx1 and polyhistidine (6xHis) tag. Samples were pooled together and the concentration was measured based on $A_{280}$ using the extinction coefficient of 6.085 mM$^{-1}$ cm$^{-1}$ (Yu et al., 2008).

![Figure 3.5. Recombinant Grx1 expression and purification.](image)

**A)** SDS-PAGE analysis of samples taken during the expression and purification of Grx1 from *E. coli*. Keys: U (uninduced cells), I (L (+) arabinose-induced cells), P (pellet of centrifugation after sonication), S (supernatant of sonication), FT (flow through of the supernatant through the Ni-NTA column), W (washing of the column), E$_1$-E$_6$ (eluate fractions with 250 mM imidazole). M (protein markers). **B** FPLC size-exclusion profile of Grx1 purification. Further purification of pooled E$_3$-E$_4$ eluates from (A) by size-exclusion chromatography (Superdex75) in BAE buffer (50 mM Tris; 150 mM NaCl; 1 mM EDTA; pH 7.0).
3.3.2. Redox state analysis of Trxs and Grxs by AMS assay

After purification of Grx1 and Trx1, AMS assays were performed for each protein to establish their cysteine redox state as purified. AMS assays determine the redox state by AMS covalently interacting with free thiols in a reduced protein; increasing the mass by ~0.5 kDa for each AMS bound. In SDS-PAGE analysis, reduced proteins are then detected at higher molecular weights compared to oxidised proteins. AMS assays can be performed to measure redox species of proteins in a reaction mixture using western blotting and densitometry analysis of bands. The assay was performed with untreated and GSSG or DTT-treated Trx1, as shown in Figure 3.6.

AMS assays with 20 μM Trx1 samples to produce and detect Trx1 redox species. Reduced proteins after AMS application, had a mass that was increased by ~2 kDa. Treatment of Trx1 with redox agents: 1 mM oxidising GSSG or 1mM reducing DTT. AMS treatment confirmed the formation of the redox species. Samples were analysed by SDS-PAGE with Coomassie Blue staining.

AMS analysis of Trx1 showed the protein to be stable in its oxidised state (lane 2). As controls, redox species were examined after incubation with redox agents and AMS application. Reduction by DTT was confirmed with the AMS assay (lane 4); while oxidation with GSSG was also confirmed after AMS was applied (lane 6). These control samples treated with redox agents confirmed the ability of Trx1 to fully produce the different redox species. Similarly, the redox state of the purified Grx1 was analysed and shown in Figure 3.7.
Figure 3.7. Redox AMS analysis of Grx1 redox species.

A) SDS-PAGE analysis of the redox state of Grx1 ±1 mM DTT or GSSG pre-treatment. B) Redox analysis as in (A) but Grx1 is treated with cystine, diamide and GSSG. In (A) and (B), 20 μM Grx1 was used for each sample and AMS assays were used to detect Grx1 redox species after treatment conditions. SDS-PAGE analysis performed with Coomassie Blue staining.

AMS assays for Grx1 showed GSSG and DTT to be effective as redox agents though unlike Trx1, Grx1 was observed in a mixture of reduced and oxidised forms, with greater amounts of reduced Grxs (lane 2, Figure 3.7A). Due to the possibility of mixed-disulfide formation between Grx1-GSSG, other oxidative agents (0.5 mM cystine and diamide) were used. Figure 3.7B shows that the reagents can oxidise Grx1 better than GSSG (lanes 3-5), as whilst a fraction of reduced Grx1 was still present after GSSG-treatment (lane 5), Grx1 was fully oxidised by cystine and diamide (lanes 3 and 4). Untreated Grx1 with AMS (lane 2) was observed as a mixture of reduced and oxidised species, more as a 1:1 ratio than in Figure 3.7A. Treatment of Trx1 and Grx1 samples with DTT (lane 3 in both Figure 3.6 and Figure 3.7A) formed two bands with the a small amount of sample being detected as the lower band. This can be explained as the fully-reduced protein being able to migrate faster through the polyacrylamide; enabling detection at what would be equivalent to a lower molecular weight. In summary ahead of the redox-equilibrium reaction, Trx1 was observed to be readily-oxidised from aerobic oxidation (Figure 3.6), whereas Grx1 was observed as a mixture of redox species that could be fully reduced by DTT pre-treatment (Figure 3.7).
3.3.3. Redox state analysis by Reverse phase chromatography (RP-HPLC)

RP-HPLC analysis was performed to examine if the reduced and oxidised forms of a protein can be separated from each other for quantification. Reduced and oxidised Trx1 and Grx1 were prepared based on the results of the AMS assays and analysed by RP-HPLC at various conditions; aiming to separate the different species into different peaks. Samples for Trx1 redox species prepared under the conditions, as detailed in Figure 3.6, were studied by RP-HPLC and presented in Figure 3.8.

![Graph showing RP-HPLC analysis of Trx1 redox species](image)

**Figure 3.8.** RP-HPLC analysis of the Trx1 redox species.

RP-HPLC profiles of the untreated, GSSG- and DTT-treated Trx1, prepared with the conditions used in Figure 3.6. RP-HPLC was performed with the Aeris Widepore 3.6μ XB-C18 column (Phenomenex) and a gradient of 40-60% buffer B (80% ACN) over a 20 ml flow through the column, in order to elute the different Trx1 species. Secondary y-axis denotes the ACN %-gradient used to elute Trx1.
Trx1 was eluted in two peaks at 13-15 ml in the column gradient flow. The identical overlap of oxidised (GSSG-treated) Trx1 and the untreated sample was consistent with the AMS assay (Figure 3.6) and confirms that Trx1 was purified in an oxidised form. The DTT-treated reduced Trx1 was eluted at a different position upstream from the oxidised form. Thus, the different Trx1 redox species could be detected separate from one another, with the reduced Trx1 being more polar to the oxidised form, resulting in earlier elution from the column. Additionally, the chromatogram profile for Trx1 was consistently seen as a twin-peak form that was still maintained after treatment with the different redox agents. The peaks did not coalesce into a single peak in the profiles after treatment with the redox agents. With the previous Trx1 AMS assays confirming the production of the different redox species under identical conditions, indicated that these chromatogram profiles were representative of Trx1 in this study. After this, the reduced and oxidised forms of Grx1 were analysed by RP-HPLC and the resulting data is presented in Figure 3.9.
Figure 3.9. RP-HPLC analysis of the Grx1 redox species.

RP-HPLC analysis of the Grx1 redox species, corresponding to the samples prepared as stated in Figure 3.7A and B; each with 20 μM Grx1. A) Chromatogram profiles of GSSG and DTT-treated Grx1 overlaid with the profile of untreated Grx1. B) Chromatogram profiles of cystine and DTT-treated Grx1 overlaid with the profile of untreated Grx1. Grx1 was prepared from a fresh purification of *E. coli* expression. The secondary y-axis denotes the acetonitrile (ACN) flow gradient (%) used to elute Grx1.
Chromatogram profiles from Grx1 RP-HPLC showed an inability to separate the different redox species from each other (Figure 3.9A). This was despite samples being prepared to the same conditions as in Figure 3.7A, which produced the different redox species. With repeated RP-HPLC analysis from a different sample preparation, untreated samples were observed as an even mix of the two redox species in an AMS assay (Figure 3.7B) and as a twin-peak chromatogram profile (Figure 3.9B). Unlike Trx1, redox treatment with cystine and DTT produced single, but broad peaks for the different Grx1 forms. However, despite clear separation of the redox species in the AMS assay (Figure 3.7); strong overlap of the redox RP-HPLC peaks was still observed. This complicates specific RP-HPLC detection of the Grx1 redox species in the PPE mixture with Trx1. RP-HPLC was then focused on measuring concentration changes of the Trx1 redox species.

3.4. Determination of $E^{o'}_{Grx1}$ by protein-protein equilibration

PPE was performed by mixing 20 μM oxidised Trx1 and 20 μM reduced Grx1 under anoxic conditions in an anaerobic chamber. Trx1 was oxidised by exposure to an aerobic atmosphere, reduced Grx1 was prepared by incubation with 1mM DTT and buffer exchanged using SEC to remove excess DTT in an anaerobic chamber. Individual samples of oxidised Trx1 and reduced Grx1 were analysed by RP-HPLC as well as the mixture after 6 hours of incubation (Figure 3.10A). Unfortunately, no changes were observed in the Trx1 or Grx1 redox species as shown by no shifts in the Trx1 and Grx1 chromatogram peaks compared to the control samples. Next, the AMS assay coupled with western blotting analysis was employed to detect the redox states of Trx1 and Grx1 after incubation under anaerobic conditions (Figure 3.10B).
**Figure 3.10.** RP-HPLC and AMS assay analysis of the Trx1-Grx1 PPE reaction.

**A)** RP-HPLC analysis from 20 μM oxidised Trx1 and reduced Grx1. Absorbance was monitored at 215 nm (secondary y-axis). The primary y-axis denotes the ACN %-gradient used for elution.

**B)** Redox AMS assay for the PPE as in (A), with samples for analysis prepared at 2 and 4 hours into incubation. The sample was incubated with AMS for 30 minutes at RT. Samples were loaded in two separate 16% Tris-Tricine SDS-PAGE gels, followed by western blotting with anti-Grx and anti-Trx antibodies (each membrane strip labelled with the antibody used on the right).
Redox AMS assay detected the oxidised Trx1 and reduced Grx1 (lanes 1-4), with an additional small fraction of oxidised Grx1 (lane 4). However, the Grx antibody displayed cross-reactivity with Trx, so overlap between Trx1 (11.2 kDa) and Grx1 (12.4 kDa) was observed. PPE performed over 4 hours at RT did not present a change in the redox equilibrium between Trx1 and Grx1, based on AMS assays. Thus, the $E^{\text{red}}_{Grx1}$ was not successfully determined.

### 3.5. The role of OM-bound Grx2 in the import of radiolabelled IMS proteins

Grx2 is not only localised to the cytosol, but also in the mitochondrial OM and matrix (Porras et al., 2006). To understand whether the OM Grx2 plays a role during the import of IMS proteins, in organello analysis was performed. Mitochondria were isolated from the WT and Δgrx2 mutant yeast strains. Preliminary studies were performed with a western blot for Grx2 in isolated mitochondria from WT and Δgrx2 strains (Figure 3.11). As expected, two Grx bands at 15 and 12 kDa corresponding to the OM and matrix forms of Grx2 were detected in the WT, but not in the mutant mitochondria.

![Western blotting of Grx2 in mitochondria isolated from WT and Δgrx2 yeast.](image)

**Figure 3.11.** Western blotting of Grx2 in mitochondria isolated from WT and Δgrx2 yeast.

Mitochondria isolated from lactate media (LM) and samples were loaded with 25, 50 and 75 μg of proteins. Western blotting was performed with antibodies against Grx2 and mitochondrial marker proteins AAC (IM) and mtHsp70 (matrix).
In Grx2 import into the mitochondria matrix, the N-terminal sequence is cleaved in the matrix to free Grx2. The OM-bound Grx2 has a higher molecular weight due to the attached presequence. The two differently-sized bands appear in an equal ratio in the WT. Mitochondrial marker proteins AAC and mtHsp70 confirmed equal loading in the WT and Δgrx2 mutant.

An import assay was performed using $^{35}$S-Methionine-labelled Tim9 and the WT mitochondria, $^{35}$S-Tim9 was synthesised in reticulocyte lysate and incubated with the mitochondria. Concentrations of GSH were applied to reduce Grx2 for Tim9 import (Figure 3.12).

Figure 3.12. Tim9 mitochondrial import with reduced glutathione (GSH).

Preliminary import studies with $^{35}$S-Tim9 in WT mitochondria in different GSH concentrations. GSH was added to the lysate to determine the optimal concentrations for import with the OM-bound Grx2. Samples extracted after 30 minutes incubation on ice with the mitochondria and lysate. After performing a gel transfer, phosphor-plate radiography was used for observation. Dig. = Digitonin-treated samples (negative control). 10% and 30% (lanes 1 and 9) indicates 10% and 30% of the total $^{35}$S-Tim9 amount used in each import reaction.

The results of the import experiment showed that 5 mM GSH increased the levels of imported Tim9. The total GSH concentration observed in S. cerevisiae cells was described previously to range from 0.5 to 10 mM (Maher, 2005). Excess GSH concentrations (15 and 30 mM) decreased Tim9 import while no import was observed without added GSH. This preliminary result indicated successful in organello Tim9 precursor import in the WT mitochondria containing OM-Grx2, but further studies to develop the time course mitochondria import did not work due to problems in replicating the experiment with a suitable plasmid template. Further investigation needs to be carried out to elaborate on these experiments.
3.6. Discussion

3.6.1. Determination of the \textit{S. cerevisiae} E'_{\text{Trx1}} value

The redox property of a biomolecule can be quantified and measured using the standard redox potential (E'') values. By determining the E'' of Trx and Grx, these could then be related to IMS proteins and cytosolic reducing sources (NADPH, GSH) to determine thermodynamic gradients for the individual redox pathways. Combining \textit{in vitro} experimentation with \textit{in vivo} studies provides further information on the Trx and Grx systems with IMS protein biogenesis. The \textit{S. cerevisiae} cytosolic Trx system has only been previously studied with Trx2 in an \textit{in vitro} redox equilibrium against NADPH and determined as -240 mV (Gonzalez Porque et al., 1970). This study using a similar methodology, determined E''_{\text{Trx1}} to be -280 mV that would show Trx1 to be a stronger reductant compared to the yeast Trx2 and more close to that of \textit{E. coli} Trx (-270 mV). A comparison between the yeast Trx to other orthologs in different species is shown in Table 2.

\textbf{Table 2. Redox potential values (E'') for Trx and Grx species paralogs.}

<table>
<thead>
<tr>
<th>Species</th>
<th>Paralog</th>
<th>Standard Redox potential E'' (mV)</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Escherichia coli}</td>
<td>Trx</td>
<td>-270</td>
<td>(Krause et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>Grx1</td>
<td>-233</td>
<td>(Åslund et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Grx3</td>
<td>-198</td>
<td></td>
</tr>
<tr>
<td>Bacteriophage T4</td>
<td>Trx1</td>
<td>-230</td>
<td>(Joelson et al., 1990)</td>
</tr>
<tr>
<td>\textit{Saccharomyces cerevisiae}</td>
<td>Trx1</td>
<td>-280</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Trx2</td>
<td>-240</td>
<td>(Gonzalez Porque et al., 1970)</td>
</tr>
<tr>
<td>\textit{Homo sapiens}</td>
<td>Trx1</td>
<td>-230</td>
<td>(Watson et al., 2003)</td>
</tr>
</tbody>
</table>
With *E. coli* $E^{\prime\prime}_{\text{Trx1}}=-270$ mV, it serves as the closest ortholog in terms of quantified reducing function (Krause et al., 1991). In addition to the redox AMS assays, Trx1 was observed in its steady state as oxidised after reducing a substrate. This agrees with the lower *in vitro* $E^{\prime\prime}_{\text{Trx1}}$ result observed as being more readily-reducing compared to other established paralogs and thus, more able to be oxidised.

### 3.6.2. Limitations of determining the $E^{\prime\prime}_{\text{Grx1}}$ redox potential

The $E^{\prime\prime}$ values for the Grx-GSH system has been analysed in *E. coli* with Grx1 and Grx3 measured by PPE against TrxPDI as $E^{\prime\prime}=-198$ mV and -233 mV respectively (Table 2) (Åslund et al., 1997). With $E^{\prime\prime}_{\text{GSH}}=-260$ mV, the *E. coli* Grx system was supported in the reducing electron flow downstream to GSH and NADPH ($-315$ mV); the increasing $E^{\prime\prime}$ values down from NADPH reflecting a decrease in reductant functioning.

The AMS assay confirmed the detection of Grx1 redox species in advance of RP-HPLC analysis (Figure 3.7). RP-HPLC is an *in vitro* method and has been used successfully in the determination of *E. coli* Grx, but chromatograms of the yeast Grx1 redox species could not be effectively separated (Figure 3.9, Figure 3.10A). The semi-quantitative method of redox AMS assay was therefore considered to be more practical for this study (Figure 3.10B). Redox AMS assays are less accurate due to the requirement of quantifying Grx1 redox species by the measurement of protein band intensities after western blotting. Successful measurement of the bands could be affected by the quality of the antibodies used and any inefficiency in the western blot process. The Grx antibody used displayed cross-reactivity with Trx1 (Figure 3.10B), that presented interference with the AMS-treated samples. An extended PPE incubation time in anaerobic conditions would also be required, such as an overnight period (~18 hours) when 6 hours was observed as being inadequate, as part of determining the $E^{\prime\prime}_{\text{Grx1}}$ value (Morgan and Lu, 2008).

Differences between Trx1 and Grx1 redox states were observed in purified proteins, with Grx1 in aerobic-exposed samples as a mixture of reduced and oxidised forms (Figure 3.7), whereas Trx1 was readily detected as being oxidised (Figure 3.6). In relation to the known $E^{\prime\prime}$ of the
proteins, such as $E_{\text{Trx}1}^0$ in yeast (-280 mV); the low negative value shows it is a strong reductant and makes Trx1 likely to become oxidised from aerobic exposure as its reductant behaviour results in it donating electrons to other species. With purified Grx1 measured in equal mixtures of redox species only from exposure to the aerobic environment (Figure 3.6, Figure 3.9), shows a difference to Trx1 by being more stable in reduced forms. This could be translated to a greater $E^0$ value as it would mean that the protein has a greater tendency to acquire electrons and be reduced than Trx1 for instance. Similarly, for the *E. coli* orthologs of Trx1 and Grx1 (Table 2), Grx1 along with Grx3 were observed to have exhibited higher $E^0$ values compared to Trx1 and this difference shows Trx1 to be a stronger reductant. The Grx and Trx systems have not been fully quantified for eukaryotic species, so whether this difference would be observable cannot be stated at present.

With $E^0$ as quantitative measures of reducing abilities, the Grx system could be compared *in vitro* to Trxs, as well as to the other Grx-GSH components: GSH (-260 mV) and NADPH (-315 mV). These *in vitro* studies provide a better understanding of the cytosolic systems’ molecular functioning compared to each other and in the biogenesis of IMS precursors.

### 3.6.3. Limitations in investigating the role of OM Grx2 with in Tim9 import

In investigating cytosolic Trx and Grx systems in IMS precursor biogenesis, an *in organello* study was used specifically for Grx2 and its OM-associated isoform. This was to determine if it could facilitate Tim9 import under *in vitro* conditions. Grx2 is localised to several intracellular compartments from alternate translation, with the matrix-targeted precursor directed by a 35 amino acid-length presequence. Import into the mitochondria was identified to be too inefficient to produce immature Grx2 bound to the OM surface before translocation (Porras et al., 2006).

Whether this form facilitated reduction of IMS precursors for import was a topic suitable for investigation in this study. Previously, mitochondrial import with isolated mitochondria and a reconstituted cytosolic Trx system (Trx1, Trr1, NADPH) provided evidence of Trx functioning in IMS protein biogenesis (Durigon et al., 2012).
Preliminary data in this study identified 5 mM GSH requirement for optimal import of the Tim9 precursor into the mitochondria, whereas incubation of the precursor with the WT mitochondria and without GSH, resulted in no import (Figure 3.12). This would indicate the partial system of just the OM-associated Grx2 was not efficient for import, instead requiring GSH to facilitate protein reduction into an import-competent. Radiolabelled import studied with GSH applied to Δgrx2 isolated mitochondria was used to determine if GSH was essential for Grx2 activity to facilitate IMS precursor reduction for import, or if GSH was capable in reducing IMS substrate proteins. Limitations were observed in this experiment in acquiring a suitable Tim9 plasmid template for the radiolabelled precursor after the initial experiment as well as difficulties in setting up RP-HPLC analysis creating difficulties in performing the import experiment in a suitable timespan. The import of radiolabelled IMS precursors would need to be measured in isolated WT mitochondria compared to the Δgrx2 mutant for this investigation. This would also be elaborated into investigating import with reconstituted cytosolic Grx1 and Grx2 systems with GSH and isolated mitochondria, as well as the redox states of unimported IMS precursors in the lysate over a time course import study.
4. RESULTS AND DISCUSSION II: EFFECTS OF THE CYTOSOLIC GLUTAREDOXINS IN THE BIOGENESIS OF MITOCHONDRIAL SMALL TIM (CX$_3$C-MOTIF CONTAINING) PROTEINS

4.1. Introduction

In mitochondrial IMS protein biogenesis, the thiol-disulfide redox regulation plays a key role for the biogenesis of small Tim proteins; all of which contain two CX$_3$C motifs. The precursors used in this study were Tim8, Tim9, Tim10 and Tim13. In the IMS, they are oxidised to then form hexameric complexes of Tim9-Tim10 and Tim8-Tim13. These proteins act as chaperones; facilitating the translocation of hydrophobic membrane proteins from the TOM complex and across the IMS to the SAM or TIM22 complex for their insertion into the membranes (Milenkovic et al., 2007). However, the oxidised small Tim proteins would not be import-competent across TOM40 from the cytosol, with only Cys-reduced proteins being able to traverse the OM pores (Morgan et al., 2009). How the precursors are maintained in import-competent Cys-reduced forms has not been fully elucidated. Only recently, a study showed that the Trx oxidoreductases can act as cytosolic factors for maintaining small Tim precursors in a reduced and import-competent form (Durigon et al., 2012). This chapter focuses on Glutaredoxins (Grx) and determining whether these oxidoreductases can also serve as cytosolic factors for maintaining the small Tim precursors in competent forms for mitochondrial import.

Grxs are like Trxs in term of being ubiquitous, with roles in several important biological processes, but differ by being glutathione-dependent to restore reductive functions, whereas Trxs are directly reduced by NADPH. The cytosolic Grx system features an isoform (Grx2) that can be detected in the cytosol, mitochondria matrix and the mitochondria OM surface (Porras et al., 2006). In this chapter, the effects of single and double GRX gene (GRX1 and GRX2) deletions on the biogenesis of twin CX$_3$C motif-containing small Tim proteins are investigated. First, the effects of the single (Δgrx1 and Δgrx2) and double deletion (Δgrx1grx2) mutants on
yeast growth and mitochondrial protein levels were examined. Growth studies identified no clear
defect in growth from GRX deletion in either fermentable or non-fermentable (respiratory)
media. Then, protein levels were measured both in whole cell extracts prepared in the two
different growth conditions as well as in isolated mitochondria; to observe if GRX1 and GRX2
deletion had in vivo effects on small Tim biogenesis in the mitochondria.

Secondly, genetic techniques were used to generate strains that can be induced to overexpress
(OE) the CX_3C proteins in the cytosol, which was aimed to saturate mitochondrial import and be
able to examine unimported precursor proteins in the cytosol. This technique of protein OE has
been previously used with success in investigating hMia40 biogenesis in situ (Banci et al.,
2013). In my study, yeast strains were transformed with inducible plasmid constructs containing
genes for small Tim proteins. Protein expression was regulated by the fermentable sugars used
in the growth media; 2% glucose-containing media repressing plasmid activity, whilst 2%
galactose-containing media induced it. Mitochondrial protein levels were studied in whole cell
extracts and in isolated mitochondria; between the overexpressing strains (e.g. ↑Tim9) against
empty plasmid-transformed strains (↑pYES2), which were used to reflect endogenous protein
levels. Protein levels were investigated at different stages of growth to monitor the progress and
extent of expression from the plasmid.

Thirdly, possible effects of the cytosolic ubiquitin proteasomes on the degradation and removal
of unimported precursors was examined. By inhibiting cytosolic proteasomes, otherwise-
degraded mitochondrial proteins in excess within the cytosol could be detected. Being able to
examine the unimported proteins provides further details into the possible roles of the cytosolic
Grx system in mitochondrial protein biogenesis.

Finally, the effects of the mitochondrial i-AAA-protease Yme1 (yeast mitochondria escape 1) on
stability and import of IMS precursors were investigated. Yme1 forms an IM-anchored complex,
with the i-AAA proteolytic domains exposed to the IMS and performs several functions
(Schreiner et al., 2012). The focus here was its protease function in degrading misfolded or
unfolded protein precursors, previously observed to degrade misfolded small Tim proteins
Therefore in conjunction with the study of the cytosolic proteasomes, Yme1 was examined in its interaction with IMS precursor biogenesis and the Grx systems.

4.2. Basic characterisation of the WT and GRX deletion mutants

4.2.1. Growth phenotypes of yeast strains

In investigating the Grx system, cell growth was examined for the necessity of the oxidoreductases in mitochondria function. Single and double GRX deletion mutants were compared to the WT in fermentative and respiratory growth conditions. Spot tests were performed on agar media, followed by incubation at 30°C for up to 3 days (Figure 4.1).
Figure 4.1. Effects of single and double GRX deletion on yeast cell growth.

A) Spot-test analysis of WT and Δgrx strains on YPD (fermentative) and YPEG (respiratory) conditions respectively. YPD cultures were grown to the late-exponential phase (18 hours), before serial dilution to $A_{600}=10^0$, $10^{-1}$, $10^{-2}$ and $10^{-3}$. Cells were spotted on YPD and YPEG agar media with incubation at 30°C for 3 days. B) WT and mutant strains grown in fresh YPD liquid media at 30°C for 24 hours, starting at $A_{600}=0.2$ (after inoculation of an initial YPD culture with individual plate colony). C) Cell growth study as in (B) but the cells from the initial culture were washed and inoculated in YPEG at $A_{600}=0.2$. The YPEG growth study was performed in parallel to the YPD cultures in (B). Growth curves were performed once each.
Spot test analysis identified no obvious effects on cell growth in either growth conditions (Figure 4.1A). Growth in respiratory conditions was performed with the metabolism of a non-fermentative carbon source, where no effects in the Δgrx mutant strains were observed, as growth was comparable to the WT yeast. With consistent results, liquid media studies over time were used to better monitor Δgrx yeast cell growth compared to the WT; better observing if there were lags or delays in cell growth. In fermentative conditions (Figure 4.1B), similar growth rates were observed for the GRX deletion mutants to WT strains. In respiratory conditions (Figure 4.1C), yeast growth was slower, with slower exponential growth rates and decreased final A_{600} values measured in yeast strains (at 24 hours) compared to fermentative conditions. Collectively, results showed Grx1 and Grx2 not to be immediately necessary for cell growth, though they may still play a role in IMS protein import.

4.2.2. **Mitochondrial protein levels between WT and Δgrx mutant strains**

To determine whether Grx1 and Grx2 served as factors in the biogenesis of the small Tim proteins; protein levels were analysed and compared between the WT and double GRX deletion mutant (Δgrx1grx2). Mitochondria protein levels in yeast strains were analysed by western blotting using both whole cell extracts (Figure 4.2) and isolated mitochondria (Figure 4.3).
Figure 4.2. Effects of double GRX deletion on whole cell protein levels in yeast.

A) WT and Δgrx1grx2 strains grown in fermentative YPD media to the late exponential phase (~22 hours, 30°C 200 rpm), before collection of whole cell extracts (equivalent to A600=5.0 of cells). B) As outlined in (A), but with cells grown in respiratory YPEG growth media as a medium shift from the initial YPD culture. Western blot analysis performed with antibodies for mitochondrial proteins; Tim9 (IMS), Tim23 and Mia40 (IM). Cytosolic G6PDH was used as a loading control. The Grx antibody used to confirm the Δgrx1grx2 identity. Samples loaded in increasing three-fold amounts (1x, 3x, 9x).

Tim9 levels were similar between the two strains on both fermentative (Figure 4.2A) and respiratory media (Figure 4.2B). Other mitochondrial proteins were analysed with the IM-embedded Mia40 and Tim23: both proteins imported into the mitochondria independent to the thiol-oxidising IMS pathway. Between the WT and double GRX deletion mutant (Δgrx1grx2), no clear differences in Tim23 and Mia40 levels were observed. Cytosolic protein G6PDH was used as a loading control, with matching levels observed between the WT and mutant in both growth conditions.

Mitochondrial protein levels were then analysed in isolated mitochondria, as they should provide more accurate information on whether mitochondria protein levels are affected by GRX deletion. Mitochondria were isolated from the WT and Δgrx yeast cells (grown in respiratory lactate
and after normalisation based on protein concentrations, levels were measured using western blotting (Figure 4.3). In the isolated mitochondria, similar levels of the IMS proteins (Tim9, Tim10 and Tim13) were observed between the WT and Δgrx1grx2 yeast, as were the other mitochondrial proteins (e.g. Mia40). These results support those observed in the whole cell extracts, showing no evidence for the Grxs to facilitate IMS protein biogenesis.

![Western Blot Image](image)

**Figure 4.3. Effects of double GRX deletion on mitochondrial protein levels.**

Cells were grown to the late-exponential phase in respiratory lactate media (LM), before harvesting mitochondria. Protein samples for the western blot were loaded to 25, 50 and 75μg from each mitochondria sample. Immunoblotting was used for a number of IMS small Tim proteins; Tim9, Tim10 and Tim13. Mitochondrial proteins for the IM; Mia40 and Tim23, OM; Tom22 and the matrix; mtHsp70 were used for comparison. The Grx antibody confirmed the identity of the Δgrx1grx2 mutant.
4.3. Mitochondria levels of OE CX₃C small Tim proteins

4.3.1. Generation of *S. cerevisiae* strains to OE small Tim proteins

Overexpression (OE) of human Mia40 (hMia40) was used in mammalian cells to show Grx1 as being involved in facilitating hMia40 biogenesis (Banci et al., 2013). This investigation therefore utilised the OE of IMS precursor proteins with *in vivo* study, in order to examine the cytosolic Grx system with IMS protein biogenesis. It was hoped that usage of OE proteins in the cytosol would provide better analysis of mitochondrial protein levels, from saturating the mitochondria import pathways. This would allow for study of Grx1 and Grx2 as potential cytosolic factors in import as well as better analysing the behaviour of IMS precursors in the cytosol; from accumulation after OE and how they are regulated or responded to (e.g. Proteasome degradation). The technique required transformation of *S. cerevisiae* strains with plasmid constructs containing genes for IMS precursors. The pYES2 overexpression plasmid (Invitrogen) was used with genes for IMS precursors being ligated into the multi-copy insert region. Genes with matching sticky-ends for the plasmid were generated by PCR using template DNA and oligonucleotide primers for the genes being investigated (appendix Table 6). Plasmid constructs were cloned in *E. coli* competent cells before transformation into the appropriate *S. cerevisiae* strains. Transformed OE yeast were selected and maintained in uracil-deficient media (-URA), sustained by the pYES2 *URA3* gene. The pYES2 plasmids are stable due to being 2μ-based and expression was regulated by the strongly inducible *GAL1* promoter (*P*<sub>GAL1</sub>). An overview of the pYES2 plasmid map and the process developed to induce pYES2 expression in this study were presented in Figure 4.4.
Figure 4.4. Overview of generating plasmid strains to overexpress proteins (↑).

A) Schematic plasmid map of the pYES2 expression vector (Cat. no. V825-20, Invitrogen) used as the vector the insertion of mitochondria precursor genes. Transformed plasmids identified by ampicillin selection and plasmid-transformed yeast cells were selected on uracil deficient media (-URA). The GAL1 promoter in pYES2 induces expression after exposure to galactose, with repression in glucose. B) Overview for inducing plasmid expression in transformed cells. Cells from agar media were prepared in an initial 2% glucose-containing culture (SD-URA) and grown at 30°C. Cells were then washed and inoculated into fresh 2% galactose media (SGal-URA) to induce expression.
Protein OE was regulated and induced by specific fermentable monosaccharide sources in growth media: glucose for repression and galactose for the induction of the \textit{GAL1} promoter (Figure 4.4B). Until OE is induced, all strains were maintained in SD-URA to prevent possible selection bias as glucose fully-represses the promoter. Cell growth was measured after plasmid induction in SGal-URA to analyse if there were any growth defects from stressing cells with excess IMS proteins. Protein levels were then measured in whole cell extracts and isolated mitochondria samples using western blots; with levels also compared to unmodified pYES2-transformed strains (\textit{\textsuperscript{↑}pYES2}), to reflect endogenous protein levels.

4.3.1.1. Preparation of \textit{\textsuperscript{↑}pYES2}-transformed control yeast strains

In developing the OE plasmids, endogenous basal levels of proteins would be measured using strains transformed with unmodified pYES2 plasmids (\textit{\textsuperscript{↑}pYES2}), for comparison of OE levels to. Initially, untransformed yeast strains were grown in 2% galactose YP media (YPGal) to check that fermentative growth with galactose would match that with glucose in YPD and that equal growth rates were observed in the mutants and WT, presented in Figure 4.5.

![Figure 4.5. Effects of \textit{GRX} deletion in fermentative yeast cell growth with galactose.](image)

The WT and different \textit{Δgrx} mutant yeast strains were grown in YP media containing 2% galactose (YPGal). Cells initially prepared in an initial YPD culture inoculated from a plate colony. This culture was used with resuspension in YPGal at \textit{A}_{600}\textsuperscript{0}\textsuperscript{0}=0.2 and cell cultures were incubated at 30°C, 200 rpm for up to 26 hours.
Using galactose as a fermentable source, no defects were observed with growth rates comparable to YPD (Figure 4.1B). The different yeast strains were then transformed with unmodified ↑pYES2 plasmids and maintained on SD-URA media. Cell growth was measured initially using spot tests on SD-URA and SGal-URA, followed by study in growth curves, presented in Figure 4.6.

![Figure 4.6](image)

**Figure 4.6. Effects of GRX deletion on cell growth of ↑pYES2-transformed yeast.**

**A)** Cell growth of the empty pYES2-transformed (↑pYES2) strains measured using spot testing on the different fermentable sources in SD-URA (glucose) and SGal-URA (galactose). Cells prepared in an initial SD-URA culture before adjustment based on $A_{600}$ and spotting on agar media. **B)** WT and Δgrx mutants from an SD-URA culture were inoculated in SGal-URA media at $A_{600}=0.2$ and incubated at 30°C, 200 rpm, with monitoring of cell growth and density by $A_{600}$ measurement.
Cell growth analysed by the spot tests observed slower growth for the ↑pYES2-transformed Δgrx mutants in SGal-URA media. Analysis in the growth curves showed a slower rate of initial cell growth for the Δgrx1grx2 yeast strains compared to the Wild Type. Later into the exponential phase (15 hours), growth rates of the mutant would match the WT. Examination of the protein levels was performed for the different transformed yeast strains, with whole cell extracts prepared from the cultures in SD-URA and SGal-URA media, presented in Figure 4.7.

![Figure 4.7](image_url)

**Figure 4.7.** Effects of GRX deletion on protein levels in ↑pYES2-transformed yeast.

**A)** Whole cell extracts prepared from SD-URA media (2% glucose). **B)** Whole cell extracts prepared from SGal-URA media (2% galactose) that induces plasmid expression. Western blotting performed with detection for IMS proteins (Tim9, Tim10), mitochondrial matrix/cytosol (Hsp70), IM (Mia40) and OM (Tom22). The loading marker of G6PDH (cytosol) was used, while deletion of the GRX2 gene was confirmed with the use of the Grx2 antibody.
In the different conditions for ↑pYES2-transformed strains, endogenous levels of the IMS proteins (Tim9, Tim10) were comparable between the Δgrx mutants and WT. Other mitochondrial proteins showed similar levels, with Δgrx mutant growth in SGal-URA not having effects on protein expression. However, decreased WT:↑pYES2 loading in SD-URA samples were observed with Hsp70 and G6PDH. This would have been from handling or pipetting issues rather than protein expression in the WT strain, as this was limited only to Figure 4.7A.

For each of the IMS small Tim proteins studied (Tim9, Tim10, Tim8 and Tim13), genes were ligated into the pYES2 plasmid to form OE plasmid constructs (appendix, Table 7). These were transformed into the WT and different Δgrx mutant strains and maintained in SD-URA. The effects of small Tim OE are detailed further in the proceeding sections with analysis for cell growth and protein levels relative to the ↑pYES2 endogenous levels.

4.3.1.2. Generation and analysis of ↑Tim9-transformed yeast strains

WT and Δgrx yeast strains were transformed with the pYES2-Tim9 plasmid (↑Tim9), while cell growth (after inducing Tim9 OE) was studied using spot tests on SGal-URA agar, as done previously with the ↑pYES2-transformed yeast (Figure 4.8A). This was also followed up with liquid media study using growth curves (Figure 4.8B). Protein levels were finally studied, comparing OE levels between the WT and GRX deletion mutant strains, as well as to endogenous ↑pYES2 levels (Figure 4.8C).
Figure 4.8. Effects of *GRX* deletion with the cell growth and protein levels in ↑Tim9 yeast.

**A)** Spot test analysis of ↑Tim9 strains in suppressing (SD-URA) and inducing (SGal-URA) media. Cell densities modified to $A_{600}$ before spotting on plates. **B)** Cell growth in the WT and Δgrx1grx2·↑Tim9 transformed strains inoculated in fresh SGal-URA at $A_{600}$=0.4 and incubation at 30°C, 200 rpm. **C)** Western blotting for ↑Tim9 in the WT and Δgrx mutants measured against endogenous Tim9 levels in ↑pYES2 strains. Cytosolic protein G6PDH was used as a loading control.
Cell growth was observed as being slowed in the GRX deletion mutants in both the untransformed (\textit{pYES2}) and overexpressing (\textit{Tim9}) yeast strains. However, relative OE Tim9 levels in a western blot analysis (Figure 4.8C) showed decreases in Tim9 levels in the different GRX deletion mutants; particularly in the \textit{Δgrx2} and \textit{Δgrx1grx2} mutant strains. This OE study shows observable differences compared to the initial results in this chapter (Figure 4.2), while supporting previous studies where differences in OE proteins were readily detectable in \textit{Δgrx} mutants (Banci et al., 2013).

4.3.1.3. Analysis of \textit{Tim10}, \textit{Tim8} and \textit{Tim13}-transformed yeast strains

As described with \textit{Tim9}, OE plasmid constructs for other small Tim proteins were prepared and transformed into the WT and GRX deletion strains to generate different yeast strains: \textit{Tim10}, \textit{Tim8} and \textit{Tim13}. For each transformed yeast strain, cell growth and protein levels after induced expression were studied. It was hoped that these studies would together provide \textit{in vivo} data for the OE precursor proteins with the GRX deletion mutants, as well as observing the differences in protein levels after induction.

The \textit{Tim10} strains were analysed in Figure 4.9. The expression of Tim10 similarly did not incur defects in cell growth, in either the spot tests or growth curves. Protein studies into the whole cell levels did not present observable differences in OE strains. Later study identified inefficiencies in Tim10 antibody functioning.
Figure 4.9. Effects of GRX deletion with cell growth and protein levels of ↑Tim10 yeast.

A) The growth of ↑Tim10-transformed WT and Δgrx yeast strains in plasmid-repressing (SD-URA) or inducing (SGal-URA) conditions were measured using spot testing. Cells prepared from an initial SD-URA culture (grown to the late-exponential phase) before washing and adjusting based on the A_600 cell density. Plates then spotted and incubated for up to 72 hours at 30°C. B) The ↑Tim10-transformed WT and GRX deletion yeast strains were inoculated in SGal-URA at A_600=0.4 from an initial SD-URA culture. Growth curves performed at 30°C, 200 rpm. C) Whole cell extracts prepared for western blotting and samples were compared to ↑pYES2 samples. The cytosolic protein G6PDH was used as a loading control.
For the ↑Tim8-transformed yeast, cell growth and protein levels were studied in Figure 4.10. Tim8 OE did not show observable effects on cell growth and similarly to ↑Tim10 samples; the protein levels observed in western blotting were not clearly detectable from the ↑pYES2-transformed samples (Figure 4.10B).

**Figure 4.10. Effects of GRX deletion with cell growth and protein levels of ↑Tim8 yeast.**

**A)** Cell growth of strains measured on SD-URA (repressing) and SGal-URA media (inducing plasmid expression) plates. Cells were prepared in an initial SD-URA culture from plate colonies. Cells were washed and adjusted to set $A_{600}$ measurements before spotting onto plates. Agar plates incubated at 30°C for 72 hours. **B)** Protein levels measured by the western blotting of whole cell extracts prepared from the transformed WT and Δgrx mutants. Protein levels compared to ↑pYES2 samples as representative of endogenous protein levels. Mitochondria proteins analysed were Tim8 (IMS), Mia40 (IM), Tom22 and Tom40 (OM) with G6PDH from the cytosol as the loading control.
Finally, for ↑Tim13-transformed yeast, cell growth and protein levels after inducing OE of Tim13 were studied (Figure 4.11). Cell growth of the transformed GRX deletion mutants was similar to the WT after inducing Tim13 expression. Overexpressed Tim13 protein levels, which were detectable compared to Tim8 or Tim10; were not clearly overexpressed compared to the ↑pYES2 endogenous levels. Thus, the ↑Tim9 OE strains were used for further study in examining mitochondrial protein levels in the Δgrx mutant yeast strains.

Figure 4.11. Effects of GRX deletion with cell growth and protein levels in ↑Tim13 yeast.

A) Spot testing of ↑Tim13 strains on SD-URA (repressing) and SGal-URA (plasmid-inducing) media. Plate colonies were inoculated in an initial SD-URA culture, before cells were washed and prepared to set A₆₀₀ densities. Agar plates were spotted with incubation at 30°C for 3 days.

B) Whole cell extracts from the ↑Tim13 strains compared against protein levels in ↑pYES2 samples. Western blot analysis for Tim13 (IMS), Mia40 (IM), Tom22 and Tom40 (OM). G6PDH from the cytosol was used as the loading control.
4.3.2. Effects of GRX deletion on an OE cytosolic protein – Arc1

In the investigation of the cytosolic Grx system in IMS protein biogenesis and protein OE, a control with a cytosolic protein was developed. This was to understand if the differences observed with OE Tim9 levels were from GRX deletion and the decreased ability to maintain reduced Tim9 forms for import into the IMS. As such, expression of a cytosolic protein in the pYES2 plasmid vector was induced, to show that the effects of the thiol-reducing Grxs were specific for IMS proteins. Protein levels should therefore be unaffected in the different GRX deletion mutants.

A tagged form of cytosolic protein Arc1 was prepared in a pYES2 plasmid using PCR to add in a polyhistidine tagged sequence (↑Arc1Tag), as this allowed for specific detection of the plasmid-expressed protein separate to the endogenous Arc1 levels in a western blot. The expressed proteins would be observed at a higher molecular weight (~1 kDa) to Arc1. Through this, the expression of the Arc1Tag product could be observed relative to endogenous levels (in ↑pYES2 yeast). The ↑Arc1Tag yeast strains were analysed in terms of cell growth, with protein levels measured by western blotting (Figure 4.12).
Figure 4.12. Effects of GRX deletion on cell growth and protein levels in ↑Arc1\(_{\text{tag}}\)-yeast.

A) Cell growth analysed by spot testing on repressing (SD-URA) and inducing (SGal-URA) media. Cells prepared to set \(A_{600}\) densities before spotting. Plates incubated at 30°C for 3 days.

B) Growth in liquid SGal-URA media between transformed WT and \(\Delta\text{grx}\) strains. Media inoculated with SD-URA preculture cells at \(A_{600}=2.0\) (30°C, 200 rpm) with monitoring of cell density.

C) Protein levels analysed by western blotting. The expressed \(\text{Arc1}_{\text{tag}}\) proteins were detected by the Arc1 antibody. Cytosolic protein G6PDH was used as a loading control.
Cell growth was about identical in the Δgrx mutants in the spot tests, with a slight increase in the growth curve with the Δgrx1grx2↑Arc1Tag yeast compared to the WT. However, this may have been an effect from the higher starting A600 density used in this study. With protein expression (Figure 4.12C), Arc1Tag levels were consistent across the ↑Arc1Tag–transformed stains, with endogenous levels remaining similar between to the ↑pYES2 strains. Levels of the OE Arc1Tag were about to the same levels as Arc1, but with the overlap of both expressed and endogenous Tim9 being detected in a blot due to being the same size, densitometry analysis was later used to examine the ratio of overexpression observed (chapter 4.3.4.1). Collectively, deletion of the GRX genes did not affect levels of the OE cytosolic protein levels, which supports the previous results observed of OE Tim9 precursors decreasing in GRX deletion as being a specific observation.

4.3.3. Visualising imported IM and IMS precursors in the mitochondria

For the biogenesis of IMS precursor proteins, import was visualised under fluorescence microscopy with RFP-tagged proteins. This was to demonstrate that non-redox sensitive proteins were being imported into the organelle and not accumulating in the cytosol after plasmid expression. Two RFP-tagged plasmid constructs were transformed into the different WT and Δgrx mutants: matrix-targeted Cox4 and IMS-targeted Cyt b2. After inducing plasmid expression in the cytosol, cells were grown to the early exponential phase and imaged. Usage of mitochondria from young growing cells improves the detection of the fluorescent-tagged proteins in healthy mitochondria (Figure 4.13).
Figure 4.13. Imaging of expressed mitochondrial Cox4 and Cyt b2 in GRX deletion yeast.

Plasmid constructs to OE matrix protein Cox4 and IMS protein Cyt b2 with the RFP fluorescent tagged-presequences, were transformed into WT and Δgrx mutants. Cultures were inoculated in SGal media from an initial SD culture and grown to the early exponential phase (A₆₀₀=0.6-1.0) before observation under a fluorescent microscope. A) ↑Cox4_RFP transformed in the WT and Δgrx mutants strains and maintained in –URA media. B) ↑Cyt b2_RFP plasmids transformed into WT and Δgrx2 strains and maintained in –LEU media. Images prepared in triplicate.
Mitochondria were observed as long and narrow rod structures around the edges of the cytosol, from detection of the RFP-tagged mitochondrial proteins imported inside. For the matrix-targeted Cox4_RFP substrate (independent of the IMS import pathway), imported precursors were clearly observed inside the mitochondria, for the GRX deletion mutant strains as well as the WT. This was an in vivo observation of OE precursor import, with matrix-targeted import being unaffected in the absence of the cytosolic Grx system.

Due to the –LEU transformation marker being interfered with by the grx1::LEU2 knockout mutation (appendix, Table 4), visualisation of the Cyt b2_RFP was only performed with the WT and Δgrx2-transformed strains, where interference would not occur. Fluorescence imaging for the IMS precursor studies was performed in triplicate. Together, these results demonstrated that overexpressed mitochondrial proteins in the cytosol could be successfully imported into the mitochondria, as observed by fluorescence imaging. Additionally with the different Δgrx mutants and the WT, cells were of a matching size (3-4 μm in diameter) without any swelling in cells that could affect the measurement of cell densities.

4.3.4. Effects of GRX deletion on the overexpression of Tim9

4.3.4.1. Absolute levels of Tim9 in induced overexpression strains

From the initial western blot for OE Tim9, the readily detectable OE protein levels were observed with differences between the WT and GRX-deleted yeast strains. Western blot analysis was repeated for protein levels in ↑Tim9-transformed yeast strains. Densitometry analysis was used to quantify the absolute ratio of Tim9 OE, and if there were significant differences between the WT and assorted Δgrx mutants (Figure 4.14).
Figure 4.14. Absolute ratios of induced Tim9 OE (↑Tim9) in GRX deletion mutants.

Absolute ratio levels of overexpressed (OE) Tim9 in ↑Tim9-transformed WT and Δgrx yeast strains. Absolute ratios compared the induced-OE of proteins from ↑pYES2-transformed strains in relation to the loading control of G6PDH. Results were quantitated from western blots (an example shown in Figure 4.8C) with densitometry using ImageJ processing software (National Institute of Health, USA). Error bars represent the Standard Error (SE) with n=3. Data analysis was performed with the Student's t-test, assuming equal variance (p<0.05).
The absolute ratios of Tim9 levels in the ↑Tim9-transformed yeast strains were observed to be significantly decreased in the different Δgrx strains compared to the WT. These also showed that deletion of a single GRX gene was sufficient to affect the protein levels of the OE Tim9; contrasting to the equal levels observed in the unmodified and ↑pYES2-transformed yeast strains. The significant decreases in ↑Tim9 levels therefore suggest that the cytosolic Grx system may play a role in Tim9 biogenesis.

4.3.4.2. Time course of Tim9 overexpression in yeast

Above studies were using samples prepared from cells in late-exponential growth. To help verify the results, protein levels were analysed over the initial growth and later exponential growth phases, with cell growth first being monitored (Figure 4.15).

![Figure 4.15. Timecourse of WT-transformed cell growth in galactose (SGal-URA).](image)

Induced OE of the WT-transformed ↑pYES2 and ↑Tim9 strains. Cultures inoculated in the plasmid-inducing SGal-URA (2% galactose) media at $A_{600}=0.4$ and incubated with at 30°C, 200 rpm with $A_{600}$ monitoring. Error bars represent the Standard Error (SE) for $n=2$ (WT∙↑pYES2) and $n=3$ (WT∙↑Tim9).
The growth of the WT·↑Tim9 transformed strain was similar to the empty vector strain (WT·↑pYES2), as observed by the overlap of standard error bars. Thus, OE of Tim9 did not have effects on cell growth. At 3 hour intervals, whole cell extracts were prepared to examine protein levels after inducing plasmid expression. Western blotting for Tim9 in the ↑pYES2 and ↑Tim9 was performed with analysis for other mitochondrial (Tim23, Mia40) and cytosolic marker G6PDH (Figure 4.16).

**Figure 4.16. Tim9 levels during exponential growth of ↑pYES2 and ↑Tim9 strains.**

A & C; Western blotting of whole cell extracts prepared from cultures in Figure 4.15 at set time intervals. A) WT·↑pYES2 Tim9 protein levels over exponential growth. B) Densitometry analysis of Tim9 and G6PDH bands (performed by ImageJ) from (A) relative to the samples at 0 hours. C) WT·↑Tim9 protein levels over exponential growth. D) Densitometry analysis of Tim9 and G6PDH from (C), relative to samples at 0 hours. For the western blots, antibodies for Tim9 (IMS), Tim23 and Mia40 (IM) were used with G6PDH (cytosol) as a loading control.
The endogenous Tim9 levels in the ↑pYES2 strain showed the same pattern increase (up to 3x the initial level observed at time 0 from transfer to SGal-URA inducing media) as the cytosolic G6PDH and mitochondrial proteins Tim23 and Mia40 in exponential growth (Figure 4.16A and B). Towards the plateau at the end of exponential growth (18 hours), mitochondria protein expression levels showed a small decline. For Tim9 levels in ↑Tim9 yeast (Figure 4.16C and D), OE was observed at the later-exponential and early-stationary growth phases (15 hours, 18 hours to ∼9x the initial amount), while other mitochondrial proteins and G6PDH showed similar patterns to ↑pYES2 strains with a ∼3x increase (Figure 4.16A and B). However, a biphasic trend was observed with Tim9 in the WT∙↑Tim9 whole cell extracts (Figure 4.16C and D), where the levels temporarily increased at 3 hours, before decreasing and increasing once again after 12 hours of growth in SGal-URA. This required repetition to examine if this was reproducible (presented in Figure 4.17). However, induced plasmid OE of IMS precursors would only be readily detectable during late-exponential growth, when the previous western blots were prepared at. Together, these results provide further details about the pYES2 plasmid activity and its galactose-induced expression. These results also demonstrated the optimal amount of incubation time required, before cell extracts can be collected or cells can be harvested for isolated mitochondria.

Next, levels of protein expression in the ↑Tim9-transformed WT and Δgrx1grx2 yeast going into stationary growth were examined. This was to observe if the OE Tim9 levels, after being expressed in the late-exponential phase of growth (Figure 4.15, Figure 4.16), would eventually decrease from proteasome degradation. For this, cell cultures inoculated in plasmid-inducing galactose media were incubated for up to 48 hours, when yeast cell growth would have fully advanced from being into the stationary phase for an extended period of time. During this analysis, cell growth was monitored as whole cell extracts were prepared for western blot analysis (Figure 4.17).
Figure 4.17. Tim9 protein levels in the WT and the Δgrx yeast in late exponential growth.

A) Cells inoculated in the inducing SGal-URA media at $A_{600}=2.0$ from an initial SD-URA culture that was inoculated with a plate colony. Cells were incubated at 30°C, 200 rpm with regular monitoring by the monitoring of the $A_{600}$ values. B) Western blotting for whole cell extracts prepared from the time points marked in (A) during the initial lag, late-exponential growth and stationary phase. Proteins analysed were the IMS protein Tim9, mitochondrial OM Porin and IM proteins, Mia40 and Tim23. All were detected by immunoblotting.
Whole cell extracts were prepared during the initial lag phase, late exponential phase (21 hours) and late into the stationary phase (30 and 48 hours) between the transformed WT and Δgrx mutant strains. Western blotting showed greater levels of OE Tim9 observed in the WT than in the Δgrx1grx2 mutant, visible with the time points at 9 hours and 21 hours: representative of the initial and late exponential growth phases respectively. Comparing protein levels in the early stages of growth (0 to 9 hours) to the previous western blot (where a biphasic trend of Tim9 was observed, Figure 4.16B), showed levels here to gradually increase after inoculation in SGal-URA media, as well as for the Δgrx1grx2 yeast strain. In both conditions where cells were incubated into the stationary phase (30 and 48 hours), the Tim9 levels showed a continuing decline, which could indicate that the OE proteins were unstable and being degraded; either following import into the mitochondria or in any unimported proteins present in the cytosol.

4.3.5. Measurement of plasmid loading in transformed WT and Δgrx yeast

Tim9 was repeatedly observed at a decreased level in the Δgrx1grx2 mutant to the WT in the above western blots for OE proteins. While the multi-copy pYES2 vector is highly stable, the high 2µ copy number can vary between different plasmids, which can then affect the level of expression from it (Futcher and Cox, 1984). To determine whether these results observed were due to GRX deletion or from variances with the plasmid loading, qPCR analysis was used to detect and quantify pYES2 plasmid and genomic DNA in the transformed cells. Real time PCR (quantitative, qPCR) was performed with extracted DNA from the different WT and Δgrx1grx2-transformed strains.

Genomic and plasmid DNA were both prepared from yeast by a modified manufacturer’s protocol (Qiagen, Chapter 2.4.3.1), after cell growth reaching mid-exponential growth (15 hours), when plasmid activity was induced. After measuring the total DNA concentration (at \( A_{260} \)), qPCR was used to detect and quantify the amount of the pYES2 plasmid in a transformed cell. Primers unique to the pYES2 ampicillin gene were used along with primers for the genomic β-tubulin used in the same DNA samples in a separate reaction mixture as a control. The qPCR analytical technique was performed to detect newly-synthesised DNA and was measured as being significant after passing the cycle threshold (Ct) point compared to any background.
signals. Lower Ct values marked earlier detection significant levels of DNA before PCR continued DNA synthesis to the maximum levels. The recorded Ct points in the different transformed strains used (↑pYES2, ↑Tim9, ↑Tim10 and ↑Arc1Tag) were presented in Figure 4.18.

Figure 4.18. DNA levels in transformed WT and Δgrx1grx2 yeast strains by qPCR.

qPCR analysis was performed using DNA samples isolated from yeast cells. DNA isolated was a mixture of plasmid and genomic DNA. Cells prepared into the mid-exponential phase of cell growth in SGal-URA media (collected to a total of 20 A_600 cell volume 15 hours into growth). DNA sample concentrations were measured at A_260 before normalisation and the preparation of qPCR samples for analysis. Primers specific for the pYES2 plasmid (the Ampicillin region) were used to measure the plasmid DNA concentration (A). The same sample concentrations were used with primers for a genomic DNA marker (the β-Tubulin gene) in (B). Samples performed in triplicate for each qPCR run and overexpression strain and results were analysed with the StepOne Plus PCR software. Error bars presented for the standard error (SE) with n=3. Statistical analysis (One way ANOVA) showed that for between the WT and Δgrx1grx2 transformed yeast, no significant differences were observed in either (A) or (B) as p> 0.05.
Analysis by qPCR showed similar levels of plasmid DNA load in the transformed WT and Δgrx1grx2 strains. Variability was observed with the wide standard error bars with the WT∙↑pYES2 and WT∙↑Tim9 samples, while levels of the genomic β-tubulin marker were closer in range. Difficulties in developing the plasmid and genomic DNA standard curves prevented the determination of the corresponding DNA copy number from the cycle threshold (Ct) values recorded. This would have also enabled the determination of the copy number ratio between the pYES2 plasmids and genomic DNA, which would better examine the plasmid loading in the different yeast strains. However, from the data available within the results, any variation was not statistically significant as p> 0.05 for both the plasmid and genomic DNA levels investigated within each transformed WT and Δgrx1grx2 yeast strain.

4.3.6. Effects of GRX deletion on the mitochondrial import of Tim9

To further examine the decreased levels of OE Tim9 in the GRX deletion mutants, observed in whole cell extract samples, mitochondrial protein levels were studied after isolation of both the mitochondria and cytosol. The protein levels for the ↑Tim9-transformed WT and Δgrx yeast strains (compared to the endogenous ↑pYES2 levels), were studied by western blotting (Figure 4.19).
Figure 4.19. Effects of GRX deletion on OE Tim9 levels in isolated mitochondria.

Isolated mitochondria from ↑Tim9-transformed yeast compared to ↑pYES2 samples, with analysis by western blotting. A) Proteins equivalent to 75 μg were loaded from each ↑Tim9 and ↑pYES2 mitochondria samples for comparison. B) Tim9 OE in WT and Δgrx1grx2 mitochondria directly compared, loaded as 25, 50 and 75 μg. C) Single GRX gene deletion mutants (Δgrx1, Δgrx2) and WT isolated mitochondria directly compared to each other to the same amounts as in (B). Western blotting was performed for IMS proteins Tim9 and Tim10 along with IM (Tim22, Tim23, Mia40), OM (Porin) and matrix (mtHsp70) proteins. Grx2 antibody confirmed GRX2 deletion in mutants.
Compared to the ↑pYES2 mitochondrial levels, the general amounts of mitochondrial proteins were higher in the ↑Tim9 strains, observed with the mitochondrial protein controls of Porin, Mia40, Tim23 and mtHsp70. The IMS Tim9 and Tim10 proteins showed stronger differences in intensity relative to the endogenous ↑pYES2 levels. For the ↑Tim9 isolated mitochondria, protein levels between the WT and the Δgrx1grx2-transformed strains (Figure 4.19B and C) were observed with minor differences, but near-identical and contrasts to what was observed in the whole cell extract samples. Interestingly, immunoblotting of Tim10 (which forms the hexameric complex with Tim9) also shows similar level increase of expression in the ↑Tim9 mitochondria; greater to the endogenous ↑pYES2 mitochondrial levels. This observation could indicate that the level of Tim10 is closely tied to Tim9 expression or the levels that are present in either the cytosol or mitochondrial IMS.

The levels of the OE Tim9 in the WT and Δgrx1grx2 mitochondria were compared to the isolated cytosol, both subcompartments isolated after homogenisation of cells during cell growth. Levels for the OE Tim9 as well as markers to examine the efficiency of isolation for the two cellular subcompartments were analysed by western blotting, presented in Figure 4.20.

![Image](image-url)

**Figure 4.20. Effects of GRX deletion in the subcellular protein levels of ↑Tim9 yeast.**

Whole cell extracts from SGal-URA culture during mitochondria isolation, with cytosol samples precipitated after homogenisation. Cytosolic G6PDH used as a loading control in whole cell extracts and in analysing the efficiency of cytosol isolation. 75 μg mitochondria was loaded and an antibody for Hsp70 (mtHsp70) was used to check the efficiency of mitochondria isolation.
Mitochondrial mtHsp70 and cytosolic G6PDH were analysed as markers for the respective compartments, which showed clear separation of the two fractions in the isolation process. As in previous western blots, Tim9 levels in whole cell extracts were decreased in the Δgrx1grx2 mutant, while for the isolated mitochondria (lanes 7 and 8), differences in Tim9 levels between the WT and Δgrx mutant were less strong, but still observable compared to in Figure 4.19. In the cytosol, no Tim9 was detectable in either strain and indicates that it was not retained, either being imported into the mitochondria or degraded by cytosolic proteasomes.

4.4. Effects of the cytosolic proteasome on Tim9 protein levels

A discrepancy in the Tim9 levels between the whole cell extracts and the isolated mitochondria between the WT and Δgrx strains was observed without any detection in the cytosol either, the cytosolic degradation of unimported OE Tim9 was studied. This was to determine if the stability of OE Tim9 in the cytosol was responsible for the differences observed in whole cell extracts. This was working on the hypothesis that in both the WT and Δgrx mutant strains, mitochondrial import of the OE precursors was similar, while the absence of the cytosolic Grx system may lead to more rapid degradation of OE Tim9 due to oxidation or aggregation. This is compared to in the WT yeast, where the Grx system is still present. This would present decreased levels in the whole cell, but not in the isolated mitochondria or be detected in the cytosol.

4.4.1. Effects of proteasome inhibition on OE protein levels

MG132 (Z-Leu-leu-Al (Sigma-Aldrich)) inhibits the cytosolic 26S proteasome, which tags substrates with ubiquitin (Ub) molecules before degradation. To address whether OE Tim9 could be recovered from cytosolic degradation and if a greater ratio would be recovered from the Δgrx mutants, cells were treated with MG132 after necessary permeabilisation of cell walls with SDS (0.003%) and follows previous studies in yeast (Liu et al., 2007, Bragoszewski et al., 2013b). The effectiveness of inhibition was analysed using western blotting for accumulated Ub-tagged proteins. Cell growth was initially studied before the analysis of protein levels in treated samples. This was performed for protein levels in whole cell extracts due to costs and the experimental amounts required; preventing usage of the inhibitor during mitochondria isolation.
MG132 was added to ↑Tim9-transformed cell cultures during exponential growth, where OE of the precursor would start occurring (~15 hours, Figure 4.16). Cultures grown in the inducing galactose media and permeabilised with 0.003% SDS, were then treated with 75 μM MG132 (or DMSO in untreated samples) and the A$_{600}$ cell density was measured into the late-exponential and stationary phases (Figure 4.21A). The density was monitored in the event of growth being arrested by the inhibitor as the ubiquitin proteasome pathway is also associated with degrading regulatory molecules that control the cell cycle (Tu et al., 2012). The initial A$_{600}$ values were also regularly measured to track when exponential growth occurred in the cultures (~10 hours). The effectiveness of proteasome inhibition was confirmed using western blotting for Ub-tagged proteins, presented in Figure 4.21B.

Figure 4.21. Cell growth of ↑Tim9 yeast strains after proteasome inhibition during the exponential phase.

75 μM MG132 added to cultures during exponential growth at 15 hours. A) Duplicate WT and Δgrx1grx2↑Tim9 cultures were inoculated in SGal-URA at A$_{600}$=2.0 with incubation at 30°C, 200 rpm. MG132 was added to one culture and DMSO to the other as the control. Cell growth was monitored by A$_{600}$. B) Western blot analysis for MG132 effectiveness. Samples were taken at 18 hours, when MG132 was applied for 3 hours. FK2 Ub antibody detected Ub-tagged proteins. Tom40 was used to confirm loading of the different treated samples.
MG132 treatment showed no effect on cell growth as the measured A₆₅₀ values were near-identical to the untreated yeast cultures. Furthermore, the successful MG132 inhibition was confirmed with western blotting for Ub-tagged proteins (Figure 4.21B). From the extracts prepared during late-exponential growth, protein levels with the proteasome inhibition were analysed by western blotting and presented in Figure 4.22.

![Western Blot Images](image)

**Figure 4.22. Effects on Tim9 levels after proteasome inhibition at mid-exponential growth.**

SGal-URA cultures treated during incubation after 15 hours with 75 μM MG132 (+). In untreated samples (-), the equivalent volume of DMSO was applied. At specific timepoints during cell growth and during the late-exponential phase (Figure 4.21A), whole cell extracts were prepared. **A)** Western blotting for WT·↑Tim9 cultures with Δgrx1grx2·↑Tim9 samples in **B**). Tim9 levels analysed with cytosolic G6PDH as a loading control.

No differences with OE Tim9 protein levels in the WT and Δgrx1grx2·↑Tim9 strains were observed after MG132 treatment over the time course, despite the effectiveness of proteasome inhibition being confirmed by western blotting (Figure 4.21B). Initial experiments with proteasome inhibition at the start of growth in OE-inducing media stunted cell growth, which was reflected in the prepared protein samples. In contrast, later treatment of cultures during
mid-exponential growth did not seem to affect the cell density or Tim9 levels. The induced OE of Tim9 was also observed to not have been strongly effective, between the levels observed in the initial protein samples (time at “0 hours”) to the later samples from exponential growth. Together, mitochondrial protein levels were not observed to have been affected by inhibition of the cytosolic proteasome in this study; prompting study of whether the IMS-proteasome degrades the OE Tim9 precursor during mitochondrial import.

4.5. Effects of YME1 deletion on small Tim proteins in the WT and Δgrx yeast strains

The IM-associated Yeast mitochondrial escape 1 (Yme1) forms the i-AAA protease complex for the degradation of IMS and IM proteins, as well as functioning as a folding assistant to IMS unfolded proteins (Schreiner et al., 2012). The Yme1 protease has been previously identified to be involved in the degradation of Tim9 and Tim10 in the IMS (Baker et al., 2012). Thus, whether Yme1 was a factor in the inability of IMS proteins to be detected in the isolated mitochondria was investigated, using the overexpressed Tim9 in the different Δgrx mutant strains. In this study, YME1 deletion mutants (Δyme1) were developed by SFH-PCR modification of the Wild Type and Δgrx1grx2 yeast strains by substitution with the KanMX4 cassette. Colony PCR analysis confirmed deletion of the YME1 genomic sequence.

4.5.1. Effects of YME1 and GRX deletion mutants on cell growth and function

Spot testing was performed to examine effects of the Δyme1 and Δgrx1grx2yme1 triple-deletion on cell growth, compared to the WT and Δgrx1grx2 yeast. Cell growth was analysed in fermentative and respiratory growth conditions and presented in Figure 4.23.
Plates colonies were inoculated in YPD and grown to the late exponential phase (~20 hours at 30°C, 200 rpm). Cells were collected, washed and based on the $A_{600}$ value, were adjusted to $A_{600}=1 \times 10^0$, followed by serial dilution. Cells were spotted onto fermentative (YPD) and respiratory (YPEG) agar media and incubated at 30°C for up to 3 days.

On fermentative media (YPD), cell growth of the different mutants and the WT was identical. However, in respiratory media (YPEG), $\Delta yme1$ mutant cell growth was observed with a strong decline compared to other strains. This indicated a strong negative effect from $YME1$ deletion while the $\Delta grx1grx2$ mutant showed growth identical to the WT, previously observed in Figure 4.1. The mutant $\Delta grx1grx2yme1$ triple deletion strain showed WT-like growth under respiratory conditions. The results suggest there is a genetic interaction between the $GRX$ and $YME1$ genes.

Next, the mitochondria function of the four yeast strains were studied by oxygen consumption analysis in fermentative and respiratory conditions. After baseline measurements initially with the YPD or YPEG media, cells were inoculated into the oxygen electrode chamber. Oxygen consumption was monitored continuously and the oxygen consumption rates (OCR) were calculated and normalised for the cell densities used, presented in Figure 4.24.
Figure 4.24. Effects on oxygen consumption in *YME1* and *GRX* deletion mutants.

Oxygen consumption was analysed in a sealed chamber attached to a Clark-type electrode. Baseline oxygen consumption with the media without yeast cells was initially performed. After baseline, cells were injected and oxygen consumption of the cells at 4 OD units/ml were recorded. Samples were isolated from the fermentative YPD-prepared cells (A) and respiratory YPEG-prepared cells (B) after growth for 24 hours at 30°C. Oxygen consumption measured as μM/min and normalised against the cell density used (OD$_{600}$) to calculate the normalised rate (μM/min/OD$_{600}$). Error bars presented for the standard error (SE) with n=3. All experiments were done at 30°C.
A number of observations were obtained from studying the OCR of the GRX and YME1 deletion strains. In respiratory YPEG media, all cells showed increased oxygen consumption rates compared with that in YPD, the exception being the Δyme1 mutant. The Δyme1 mutant strain in both the fermentative and respiratory growth conditions, showed minimal OCR that was comparable to the baseline measurements performed for YPD and YPEG media. The Δgrx1grx2yme1 mutant strain was observed to exhibit comparable rates to the WT and Δgrx1grx2 strains in both of the growth conditions used. These OCR results are consistent with those of the spot tests (Figure 4.23), showing that deletion of the two GRX genes lead to a recovery of cell growth and mitochondrial functioning that declined in the YME1 deletion strain. Further study was performed for the mitochondrial protein levels in the different YME1 deletion mutants.

4.5.2. Effects of YME1 and GRX deletion on mitochondrial protein levels

Whole cell extracts of the GRX and YME1 deletion yeast strains were prepared from cells in both YPD and YPEG media for western blotting (Figure 4.25). Aside from IMS Tim9, levels of other mitochondrial marker proteins were analysed. Proteins such as Hsp70, Tim23 and Tom22 were measured as being the same in the different mutant strains. The levels of Tim9 were observed as being similar between the different mutants and the WT (fermentative conditions). In respiratory conditions (YPEG), Tim9 levels were also similar between the different mutants, with minor decreases compared to the WT. To further investigate protein levels with the YME1 deletion strains, mitochondrial proteins were studied in isolated mitochondria prepared from the four yeast strains.
Figure 4.25. Effects of $GRX$ and $YME1$ deletion on mitochondrial protein levels.

**A)** Protein samples from whole cell extracts prepared in both fermentative (YPD) and respiratory (YPEG) growth media after growth at 30°C for 24 hours. **B)** Proteins in isolated mitochondria, prepared in respiratory lactate media (LM). For each sample, loading equivalent to 25, 50 and 75 μg were used for western blotting. Mitochondrial proteins analysed in western blots ranged from different compartments: IMS (Tim9), IM (Mia40, Tim23), OM (Porin, Tom22) and the mitochondrial matrix (mtHsp70). Deletion of the $GRX$ and $YME1$ genes was confirmed using antibodies.
Mitochondrial Tim9 levels seem to be decreased in the \( \Delta yme1 \) and \( \Delta grx1grx2yme1 \) yeast strains. However the decreased protein levels were also observed in other mitochondrial proteins such as Porin, Mia40 and mtHsp70, which indicated that loading was not accurate with decreased loading of the mitochondria samples, likely based on the \( A_{280} \) measurement. Consequently, despite the differences observed in cell growth and mitochondria functioning, the endogenous levels of Tim9 were thought to not be affected in \( \Delta yme1 \), with results matching previous western blots in which the two \( GRX \) genes are deleted (Figure 4.2, Figure 4.3). After the results observed for OE Tim9, protein levels in the above yeast strains were further investigated after transformation with the ↑Tim9 plasmids.

4.5.3. **Effects of YME1 and GRX deletion on mitochondrial ↑Tim9 protein levels**

4.5.3.1. **Cell growth with ↑Tim9-transformed strains**

Following earlier studies with the OE Tim9 in this investigation (Figure 4.14), the different \( \Delta yme1 \)-associated mutant yeast strains were transformed with the ↑pYES2 and ↑Tim9 plasmids to further examine possible differences in protein levels. Cell growth was initially studied with the induced OE of Tim9 and presented in Figure 4.26.
Spot tests performed on promoter-respressing SD-URA and inducing SGal-URA. Cell cultures were initially prepared in SD-URA cultures from individual plate colonies before serial dilution to set $A_{600}$ values and spotting on agar media. **A)** Yeast strains transformed with the unmodified pYES2 ($\uparrow$pYES2) plasmid and the Tim9 OE ($\uparrow$Tim9) plasmid construct **(B).** After spoting, agar plates were incubated at 30°C for 3 days.

In the $\uparrow$pYES2-transformed yeast, cell growth between the different mutants was similar to the WT in the fermentative and respiratory conditions, while growth was weaker for the $\Delta$yme1 and $\Delta$grx1grx2yme1 yeast strains on YPEG media. The $\uparrow$Tim9-transformed strains showed similar growth to the $\uparrow$pYES2 strains in both conditions. However, the $\Delta$grx1grx2$\uparrow$Tim9 strain showed defects in YPEG media, which contrasts to previous spot test results (Figure 4.8A) and may have been the result of handling issues that would necessitate a repeat. Thus, mitochondria functioning with the OE of Tim9 in the $\Delta$yme1 mutants were further investigated with oxygen consumption analysis.
4.5.3.2. Mitochondrial functioning in ↑Tim9-transformed GRX and YME1 deletion yeast

Mitochondria functioning in the different GRX and YME1 deletion mutants were analysed by measurement of the oxygen consumption rates (normalised as O₂ μM/min/OD₆₀₀), with the OE of Tim9 in ↑Tim9-transformed yeast. This was compared to the ↑pYES2-transformed strains as controls and presented in Figure 4.27.

**Figure 4.27. Effects on oxygen consumption in ↑Tim9-transformed GRX and YME1 deletion mutants.**

Oxygen consumption assays performed for A) WT and Δyme1 B) Δgrx1grx2 and Δgrx1grx2yme1 yeast strains: each transformed with the ↑pYES2 and ↑Tim9 plasmid constructs. Cell cultures were prepared in an initial SD-URA culture from individual plate colonies, before inoculation in SGal-URA and incubation at 30°C for 24 hours. Oxygen consumption was performed with an initial baseline with SGal-URA in the electrode. After 1 minute of baseline measurement, cells were injected into the sealed electrode chamber. Error bars are presented for the standard error (SE) with n=3. All experiments were performed at 30°C.
The OE of Tim9 was observed as not affecting the OCR in transformed YME1 deletion mutants (Δyme1, Δgrx1grx2yme1) as rates remained similar to the ↑pYES2-transformed strains. The ↑Tim9-transformed WT and Δgrx1grx2 strains showed a small decrease in the OCR rates from the ↑pYES2 strains. Compared to the untransformed strains (Figure 4.24), the normalised OCR rates in the plasmid-transformed strains showed an overall decrease, which may have extended from the maintenance of cells in synthetic minimum media. However, the Δyme1-transformed strains showed equivalent OCR to the Δyme1 mutant at strongly-declined rates. As the OCR rates between the ↑pYES2 and ↑Tim9-transformed Δyme1 mutant strains were equal after inducing Tim9 OE, these results show OE of Tim9 in the YME1 deletion mutants to not affect mitochondria functioning.

4.5.3.3. Protein levels in ↑Tim9-transformed GRX and YME1 deletion mutant strains

Endogenous mitochondrial proteins levels in the different YME1 deletion mutant strains did not present observable differences (Figure 4.25). Since differences in protein levels were previously observed with OE Tim9 in whole cell extracts (Figure 4.14), proteins in the ↑Tim9-transformed GRX and YME1 deletion mutant strains were analysed using western blots and presented in Figure 4.28.
Figure 4.28. Effects on ↑Tim9 protein levels in GRX and YME1 deletion mutant yeast.

Whole cell extracts prepared from SGal-URA to induce OE in ↑pYES2 and ↑Tim9-transformed YME1 and GRX deletion yeast strains. Western blotting was used to analyse proteins for different mitochondrial marker proteins: IMS (Tim9), IM (Tim23, Mia40), OM (Porin, Tom40) and the matrix (Hsp70). Cytosolic protein marker G6PDH was used as a loading control.

The OE Tim9 levels were observed in the WT and Δyme1∙↑Tim9 samples compared to in the ↑pYES2-transformed strains. As with previous ↑Tim9 western blots (Figure 4.14), decreased levels were observed in the Δgrx1grx2 mutant strain. While deletion of YME1 did not have an effect on OE Tim9 levels, the Δgrx1grx2yme1∙↑Tim9 protein sample (lane 8, Figure 4.28) was notable for a recovery in OE Tim9 levels. This was observed as an increase in band intensity greater than with Δgrx1grx2 (lane 6, Figure 4.28), but still lower compared to the WT and Δyme1 protein samples (lanes 2 and 4, Figure 4.28). The other mitochondrial and cytosolic proteins analysed showed identical levels across the different yeast strains and conditions.
4.6. Discussion

4.6.1. The Grx system has an effect on the levels of OE Tim9 proteins

In the initial part of this study, the effect of GRX deletion mutants on cell growth was studied. Results showed no differences in cell growth between the WT and the GRX deletion mutants and are in agreement to previous analysis comparing the Trx and Grx systems with cell viability in respiratory growth (Durigon et al., 2012). Comparisons between the Trx and Grx systems identified inhibited respiratory growth from yeast strains with TRX deletions, rather than with GRX deletions; where growth remained comparable to the WT.

Furthermore, no differences in the mitochondria functioning were observed based on the oxygen consumption rates between the WT and Δgrx1grx2 yeast strains, in either fermentative or respiratory conditions (Figure 4.24). Consistently, western blots showed mitochondrial protein levels in GRX deletion mutants to be equivalent to the WT. The unaffected mitochondrial functioning and protein levels observed are in agreement with research that identified a single GRX or TRX gene to be essential for yeast viability (Draculic et al., 2000). As the TRX genes were still expressed, their identified roles in facilitating small Tim biogenesis continued mitochondrial functioning without the cytosolic Grx system (Durigon et al., 2012).

Overexpression (OE) of CX3C IMS precursor proteins in vivo was performed with the hope to stress the mitochondria IMS import pathways and investigate if the cytosolic Grx system had a role in facilitating small protein Tim biogenesis. Initially, possible effects of OE proteins on cell growth and mitochondrial respiration were analysed, with no toxic effects observed from OE Tim9 in the WT or Δgrx yeast strains.

To examine for possible variances in plasmid loading in the WT and GRX deletion yeast that could affect the level of OE protein observed, qPCR was used to quantify the DNA in the different transformed strains. Measurement of the expressed pYES2 mRNA product was not feasible in this study as the necessary primers would not have distinguished between the plasmid-expressed and the endogenous mRNA. A possible solution would be to develop plasmid constructs for IMS proteins with an additional sequence (e.g. Polyhistine tag) for
specific selection by primers. For this study with the available plasmids, a simpler method was utilised in each transformed WT and GRX deletion yeast strain, with primers selective for the pYES2 plasmid and a genomic DNA marker. This would detect the plasmid load in a lysed sample of both purified genomic and plasmid DNA. Between transformed WT and GRX deletion yeast strains, similar-to-equal levels were observed for the plasmids, as well as equal levels for the genomic DNA markers (Figure 4.18). The variability observed for ↑pYES2 and ↑Tim9 plasmids could have been from handling issues as similar effects were not observed with the other samples or with the genomic DNA, where Ct values were separated by ≤1 qPCR cycle. Overall, results showed that transformation with the ↑Tim9 plasmid did not affect cell functioning and that there was an even plasmid load in WT and GRX deletion yeast strains.

The cytosolic Grx system was studied to test whether or not it facilitated CX3C IMS protein biogenesis, using in vivo study of OE proteins in GRX deletion mutants. Using OE IMS proteins in protein import had been used previously, where the Grx system was identified as being able to maintain OE human CX3C Mia40 in unfolded reduced forms in the cytosol for import (Banci et al., 2013). However, for CX3C proteins like Tim9, import had not been investigated up to this point with the cytosolic Grx system. Thus, protein levels were investigated in Δgrx yeast strains transformed with the ↑Tim9 OE plasmid, before the study of levels in isolated mitochondria.

In whole cell extracts using OE Tim9, levels were observed to be significantly decreased in both partial and complete deletions of the Grx system (Figure 4.14). However in the isolated mitochondria, OE Tim9 was observed in a slight decrease in the Δgrx mutant to the WT. The increased Tim10 levels in the ↑Tim9 mitochondria fits with the model of theTim9-Tim10 hexameric complex preventing possible aggregation occurring in the IMS. Additionally, Tim9 was described as a protective factor to Tim10, so the increased amounts present in the IMS enabled greater accumulation of Tim10 by stabilisation into the complex (Spiller et al., 2015). In the time course study of ↑Tim9 yeast, the declined OE Tim9 levels in cells grown to the stationary phase indicated protein degradation and a decrease in stability (Figure 4.17). Overall under the experimental conditions, whilst decreased Tim9 levels in the whole cell extracts were clearly observed in the GRX double deletion mutant (Δgrx1grx2), similar mitochondrial Tim9 levels were detected in the isolated mitochondria and no detection of unimported Tim9 in the
cytosol. These results indicated that Tim9 precursors may be partially degraded in the cytosol before import into the mitochondria in the mutant yeast strains. This explanation is consistent with a very recent study that showed an over-accumulation of mitochondrial precursors in the cytosol to induce stress, which then activates a proteasome degradation response to clear proteins (Wrobel et al., 2015).

4.6.2. Inhibition of cytosolic proteasomes did not recover mitochondrial proteins

The cytosolic proteasome system has been identified to clear mitochondrial proteins that have accumulated in the cytosol (Wrobel et al., 2015). This was inclusive of MIA and TIM23 pathway substrates from inefficiencies in mitochondrial import, where the over-accumulation of MIA precursors in the cytosol stressed the environment. This activated the protective UPRam response that increases proteasome activity to clear proteins. This mechanism would help understand the results of this study where no unimported Tim9 was detected in the cytosol, while mitochondrial levels remained similar between the WT and Δgrx yeast strains, as the overexpressed Tim9 in the cytosol, which could become toxic with accumulation, were degraded in a rapid response. While Tim9 import into the IMS occurred at similar rates between the different yeast strains, there was a greater accumulation of oxidised Tim9 in the Δgrx cytosol, leading to earlier degradation by the 26S proteasome (Wrobel et al., 2015). This would present the decreased Tim9 levels observed in the whole cell extracts of Δgrx yeast.

To examine the proteasome system with IMS protein levels in this study, previously established methods with the MG132 proteasome inhibitor were used (Liu et al., 2007, Bragoszewski et al., 2013a, Bragoszewski et al., 2013b). In this study, MG132 was applied during incubation in pYES2-inducing media at mid-exponential growth (15 hours, Figure 4.21), while western blots confirmed 26S proteasome inhibition. Treatment during the mid-exponential cell growth did not increase the levels of Tim9 detected.

The results of my study are not consistent with the previous findings of the cytosolic ubiquitin-proteasome system in the cytosolic clearance of IMS proteins (Bragoszewski et al., 2013b,
Wrobel et al., 2015). This may have been possible from the experimental conditions used here, compared to the previous studies. Yeast cells were prepared as to previously-outlined studies, with SDS permeabilisation prior to 75 μM MG132 being added and incubation at 30°C (Liu et al., 2007). Western blots in this study appropriately detected Ub-tagged proteins from treated cell cultures. In this study, higher optical densities were used for inoculation in the inducing SGal-URA media, due to the simultaneous preparation of additional samples for AMS-modified Immunoprecipitation. This was to examine the redox states of proteins after proteasome inhibition. Limitations in the method did not present clear results for the Tim9 protein. However, the mechanisms outlined in those previous studies would be applicable to this investigation for the OE Tim9 differences observed between the WT and GRX deletion mutant yeast (Wrobel et al., 2015). Additionally, IMS precursors import has been observed with proteins diffusing back into the cytosol. This retro-translocation of reduced IMS precursors could function as part of the homeostasis mechanisms between the two compartments for excess amounts of proteins in the IMS protein biogenesis (Bragoszewski et al., 2015).

4.6.3. There is a genetic interaction between GRX and YME1

The IMS-exposed Yme1 protease was studied to understand whether the OE precursors translocated into the IMS can be degraded by it. A recent study in our lab identified Yme1 to preferably degrade unassembled IMS Tim10 over Tim9 (Spiller et al., 2015). In this study, YME1 deletion mutant strains were prepared with a Δgrx1grx2yme1 triple mutant strain to examine if there was a possible interaction between it and the cytosolic Grx system.

This study demonstrates a new genetic interaction between the two Grx paralogs with the Yme1 protease that was previously not studied. The Δgrx1grx2yme1 triple deletion mutant displayed growth equivalent to the WT in respiratory conditions whereas growth of the Δyme1 single deletion mutant was greatly-diminished (Figure 4.23). Similarly with mitochondrial functioning, OCR rates were recovered in the triple mutant compared to the Δyme1 (Figure 4.24). The slow Δyme1 respiratory growth agrees with previous research that identified IM integrity in Δyme1 yeast to be compromised with a decline in cell viability, as well as a failure to assemble subunits of the IM respiratory chain (Weber et al., 1996, Kominsky and Thorsness, 2000). In terms of
protein levels, the overexpressed Tim9 in \( \Delta grx1grx2yme1 \) yeast showed increased Tim9 levels compared to \( \Delta grx1grx2 \), while \( YME1 \) deletion did not decrease Tim9 levels. Taken together, this study shows a functional interaction between the Grx system and Yme1 protease, but at a genetic level. The molecular mechanisms for this interaction would need to be elucidated in the future.

Genetic interactions between the IM-anchored Yme1 and the cytosolic environment has been previously identified for the 26S proteasome, where mutation of the \( YNT1 \) gene (a regulatory subunit for the proteasome) compensated for the \( \Delta yme1 \)-associated defects (Campbell et al., 1994). This interaction with the 26S proteasome agreed with this study for the Grx system and suggests that the IMS substrates of Yme1 bridged a connection to cytosolic systems. The presence of Grx activity in the IMS of \( S. \ cerevisiae \) has also been recently identified, from diffusion of Grx1 and Grx2 into the IMS along with reduced glutathione (GSH) (Kojer et al., 2015). This raises the possibility of an interaction with Yme1 occurring within the IMS. The presence of GSH in the IMS was described to negatively regulate Mia40 activity by reduction (Kojer et al., 2012). In low IMS concentrations, Grxs act as a kinetic barrier to GSH and maintains oxidised Mia40 for continued activity. Similarly, activity in the IMS of the yeast Trx system (Trx1, Trr1) has also been identified (Vögtle et al., 2012). Cytosolic Grx1 has also been identified in the IMS for de-glutathionylation activity with \( H. \ sapiens \) mitochondria, while GSH reduces Mia40 in mammals as a negative regulator (Pai et al., 2007, Fischer et al., 2013).

**4.6.4. Conclusions**

Regarding the aim of this chapter, the results showed evidence for the Grx systems to be a factor in the import of small Tim proteins, by the significant decreases of overexpressed Tim9 levels in \( \Delta grx \) mutant yeast. Further study in isolated mitochondria did not show clear differences like in whole cell extracts. Study of the protein stability with proteasome degradation did not detect Tim9 that could possibly be degraded in the cytosol. Interestingly, a genetic interaction between \( GRX \) and \( YME1 \) was observed in this study. It would be interesting to investigate the mechanisms involved in the mitochondrial functioning and how protein levels (with overexpression for detection) can be recovered.
5. RESULTS AND DISCUSSION III – EFFECTS OF THE CYTOSOLIC GLUTAREDOXINS IN THE BIOGENESIS OF MITOCHONDRIAL CX₉C-MOTIF CONTAINING PROTEINS

5.1. Introduction

Substrates of the MIA pathway can be classed on the basis of the conserved twin dithiol motifs present. The CX₃C proteins were studied in the previous chapter, while IMS proteins that contain CX₉C motifs are the focus here. These motifs are used to mature the protein structure in by oxidative folding, as the formed disulfide bonds stabilise the coiled-coil-helix-coiled-helix (CHCH) substrate fold. More twin CX₉C motif-containing proteins are present in the IMS compared to the just five twin CX₃C motif-containing proteins. The most studied CX₉C proteins are Mia40 and Cox17: both contributing to the biogenesis of mitochondrial proteins. A screening analysis has identified a number of IMS CX₉C proteins to also have roles in assembling the mitochondrial morphology, such as Mdm35 (Cavallaro, 2010). In this study, the effects of GRX deletion on the biogenesis of CX₉C motif-containing proteins Mia40 and Cox17 were investigated.

Cox17 is a cytochrome c oxidase that functions as a metallochaperone in the IMS. The protein is imported into the IMS through the MIA pathway, where it functions as a donor to Cox11 and Sco1 in chaperoning Cu²⁺ ions to the Cytochrome c Oxidase (CcO) in the IM respiratory chain (Koch and Schmid, 2014, Horng et al., 2004). Thus, Cox17 has a role in aerobic respiration and has also been recently identified to have a role in maintaining the IM architecture (Chojnacka et al., 2015). Mia40 is an essential component of the MIA pathway (mitochondria import and assembly), where it functions with the FAD-dependent sulfhydryl oxidase Erv1 (yeast ortholog to the H. sapiens ALR; Augmenter of liver regeneration) to facilitate the oxidative folding of IMS substrates. This includes Cox17 and the small Tim proteins, as discussed in Chapter 1. In S. cerevisiae, Mia40 is anchored to the IM with the functional domain exposed to the IMS. Yeast Mia40 has a targeting presequence for import into the mitochondria through the TIM23 pathway,
followed by insertion into the IM (Figure 1.7). However, the human Mia40 (hMia40) is much smaller than the yeast ortholog, only having the conserved C-terminal oxidoreductase domain. Import of hMia40 is through the redox sensitive MIA pathway (Sztolsztener et al., 2013). Previous investigations identified that the human cytosolic Grx1 is more effective than Trx1 in maintaining overexpressed hMia40 in reduced states for mitochondrial import (Banci et al., 2013).

In order to understand whether the Grx systems play a role in the biogenesis of the CX$_9$C motif-containing proteins in yeast, effects of the double deletion of the GRX genes ($\Delta$grx1grx2) were investigated using the full-length yeast Mia40, the C-terminal domain of Mia40 (Mia40C) and Cox17 as models. Analyses for both the endogenous proteins and plasmid overexpression (OE) proteins were utilised in examining the biogenesis of these proteins. Both the full length Mia40 and Mia40C were studied as they use different mitochondrial import pathways. Mia40C contains the necessary CX$_9$C motifs of Mia40, but behaves like other substrates of the MIA oxidative-folding pathway, as observed with hMia40 (Figure 1.10) (Chacinska et al., 2008). The full-length yeast Mia40 contains an N-terminal domain with the TIM23-targeting sequence and a transmembrane domain for insertion into the IM; independent to the MIA pathway (Chatzi et al., 2013). Studying both provides a better understanding into the specific biogenesis of CX$_9$C MIA pathway substrates compared to a pathway independent of substrate redox state. Furthermore, Cox17 was studied as an alternative model to verify the results obtained based on Mia40C, to see if the Mia40C results were due to being a non-native protein or as an IMS-soluble CX$_9$C protein.

From using naturally-occurring and novel model proteins, the results suggested that the cytosolic Grx oxidoreductases play a role in facilitating the biogenesis of CX$_9$C motif-containing IMS proteins that use the MIA pathway for import. However, it does not seem to affect the full-length Mia40 in yeast; which uses the TIM23 pathway for import.
5.2. Effects of GRX deletion on the endogenous CX₃C proteins levels

First, western blots of Cox17 and IM-targeted full-length Mia40 were performed with mitochondria isolated from the WT and Δgrx1grx2 yeast strains (Figure 5.1). In the ↑pYES2-transformed yeast strains to represent endogenous protein levels in the synthetic minimum media (↑pYES2 yeast being controls to OE conditions), levels for Mia40 and Cox17 were also analysed.

Figure 5.1. Effects of GRX deletion on endogenous Mia40 and Cox17 levels.

A) Mitochondria isolated from cells prepared in respiratory lactate media (LM). Western blotting for Cox17 (IMS), mtHsp70 (matrix), Mia40 and Tim23 (IM). A Grx antibody was used to confirm GRX deletion. Mitochondria loaded to 25, 50 and 75 μg protein amounts. B) Western blot of whole cell extracts prepared from SGal-URA incubation (24 hours, 30°C). Mitochondrial proteins analysed were Cox17 (IMS), Mia40 (IM) and Hsp70 (matrix, cytosol). The cytosolic protein G6PDH was used as a loading control.
The same levels of Cox17 as well as Mia40 were observed in both WT and GRX deletion mutants. Since previous studies for CX$_3$C proteins identified decreased OE Tim9 in Δgrx mutants (whole cell extracts, Figure 4.14) and OE hMia40 being measured in GRX deletion strains (Banci et al., 2013), the next step was to test overexpressed CX$_3$C proteins in the yeast strains and see if similar patterns would be observed. Plasmid constructs with pYES2 (↑Mia40, ↑Mia40C and ↑Cox17) were transformed into yeast strains for investigation (listed in appendix, Table 8). Transformed strains were maintained in GAL1 promoter-repressing SD-URA before inducing plasmid expression in SGal-URA (2% galactose).

5.3. Effects of GRX deletion on TIM23-targeted full-length Mia40

5.3.1. Cell growth of Δgrx mutant yeast strains with overexpressed Mia40

The cell growth of ↑Mia40-transformed WT and GRX deletion yeast strains were studied to test whether overexpression (OE) had any effects on cell growth (Figure 5.2). Cell growth of the GRX deletion mutants with OE of Mia40 was observed as not being affected, as growth remained equal to the WT strains.
Figure 5.2. Cell growth of ↑Mia40-transformed WT and Δgrx mutant yeast strains.

Cell growth studied with the induced OE of Mia40. **A)** Spot test analysis on repressing SD-URA and inducing SGal-URA media for the ↑Mia40 yeast. From an intial culture (inoculated with individual plate colonies), cells were serially-diluted before spotting on agar plates. Plates were incubated at 30°C for 3 days. **B)** SGal-URA cultures were inoculated at $A_{600}=0.2$ from an initial SD-URA culture and incubated at 30°C, 200 rpm. Cell growth was monitored by $A_{600}$ measurement.
5.3.2. Effects of Δgrx mutant yeast strains on OE Mia40 protein levels

From induced OE of the ↑Mia40 yeast, whole cell extracts were prepared to examine protein levels (Figure 5.3). Protein samples were compared to ↑pYES2-transformed strains for the endogenous protein levels. Mia40 OE was strong, with a mild decrease observed in the GRX deletion yeast strains. For the G6PDH loading control, increased levels were observed for the WT↑Mia40 sample (lane 2). From the ↑Mia40:↑pYES2 ratios for G6PDH and Mia40, the absolute ratios for Mia40 OE in the Δgrx mutants are presented in Figure 5.3B.

![Western blot and densitometry analysis](image)

**Figure 5.3. Effects of Δgrx mutants with ↑Mia40 protein levels.**

A) Whole cell extract samples of ↑Mia40-transformed strains compared to the ↑pYES2 endogenous levels. Western blotting for Mia40, with cytosolic G6PDH as a loading control. B) Densitometry analysis of repeated western blots prepared as in (A), for Mia40 in ↑Mia40 strains. Absolute ratios presented, compared to the G6PDH loading control. Error bars for the standard error (SE), n=3. Student’s t-test analysis showed no significant differences with p> 0.05.
In the ↑Mia40-transformed yeast, no significant differences were observed in the whole cell extract levels of Mia40 with the different Δgrx mutants compared to the WT strain (p>0.05, Student’s t-test). The large standard error (SE) observed in Δgrx1grx2 ↑Mia40 samples showed greater variability compared to other samples. Further study of whether the cytosolic Grx system functioned in the biogenesis of the TIM23-imported Mia40, protein levels in the isolated mitochondria were examined by western blotting (Figure 5.4).

**Figure 5.4. Isolated mitochondria protein levels in ↑Mia40-transformed Δgrx strains.**

Mitochondrial protein levels from different ↑Mia40-transformed yeast analysed by western blotting. Protein samples from the isolated mitochondria were loaded as 25, 50 and 75 μg for analysis. Antibodies used were Mia40 (IM), Tom40 and Porin (OM) and mtHsp70 (matrix).

Overexpressed Mia40 was detected in the isolated mitochondria with similar levels observed between the WT and different GRX deletion yeast strains, while other mitochondrial proteins were at identical levels. Further study was performed with the subcompartments of transformed yeast, using the isolated cytosol and mitochondria from the WT and Δgrx1grx2 strains, presented in Figure 5.5. The full-length Mia40 was not detectable in the isolated cytosol, with similar levels observed between the WT and Δgrx1grx2 for the whole cell extracts and isolated mitochondria. Minor amounts of cytosolic G6PDH were detected in the Δgrx1grx2 isolated...
mitochondria (lane 6), which was otherwise detected in the cytosol as a marker of efficiency of mitochondrial isolation. In conclusion for these results, protein levels of the TIM23-targeted Mia40 were not affected by the cytosolic system, as measured under in vivo conditions.

Figure 5.5. Protein levels in subcompartments from ↑Mia40 yeast strains.

Isolation of mitochondria and cytosol in the WT·↑Mia40 and Δgrx1grx2·↑Mia40 strains was analysed by western blotting. Whole cell extracts were from the incubation of cells in SGal-URA during isolation. Proteins in the cytosol were precipitated for use while 75 μg of isolated mitochondria were used. Western blotting was performed with the Mia40 and G6PDH antibodies to examine the OE effectiveness and the efficiency of isolation.

5.4. Effects of GRX deletion on MIA-pathway targeted Mia40C

5.4.1. Effects on cell growth in ↑Mia40C-transformed strains

For the examination of the MIA pathway with overexpressed protein levels, the cell growth of ↑Mia40C-transformed yeast were initially studied. As it was not the endogenous yeast isoform, this was to check that OE did not present side effects to cell growth. Spot tests and growth curves were performed, with presentation in Figure 5.6. The OE of Mia40C did not present effects in the spot tests, with a slight decrease for the Δgrx1grx2 being observed. However, growth curves for the WT and Δgrx1grx2·↑Mia40C yeast presented identical growth rates after some initial variation was observed for the readings at 4 and 6 hours (Figure 5.6B). Collectively, these results show that the OE of Mia40C did not affect cell growth.
**Figure 5.6. Cell growth of GRX deletion mutants with ↑Mia40C.**

**A)** Spot test analysis on repressing SD-URA and inducing SGal-URA. From inoculation of a SD-URA culture with individual agar plate colonies, cells were grown for 24 hours, before being collected. After washing, cell cultures were set to specific cell densities, based on $A_{600}$ measurements and serial dilution. Plates were incubated at 30°C for 3 days. **B)** Growth curve analysis with induced overexpression. Cells were prepared in an initial SD-URA culture before washing and inoculation to $A_{600}=0.2$ in fresh SGal-URA media. Cultures were incubated at 30°C, 200 rpm and cell growth was monitored by $A_{600}$ measurement.
5.4.2. Effects of GRX deletion on Mia40C whole cell extract levels

To examine the effects of the Grx system on the protein levels of Mia40C, western blotting was performed with the whole cell extracts of ↑Mia40C and ↑pYES2-transformed yeast, presented in Figure 5.7. Because Mia40C was a non-native protein being expressed, ratios were measured to the native full-length Mia40 expressed in the ↑Mia40C-transformed yeast (Figure 5.7B).

Figure 5.7. Effects of Δgrx mutants on ↑Mia40C protein levels in whole cell extracts.

A) Western blotting analysis for proteins in ↑Mia40C and ↑pYES2-transformed WT and Δgrx mutants. Mitochondrial proteins analysed were IMS (Mia40C), IM (Tim23, Mia40), OM (Tom40) and the matrix/cytosol (Hsp70). Cytosolic G6PDH was used as a loading control. B) Absolute ratios of Mia40C to Mia40 in the WT and ↑Mia40C-transformed yeast strains. Error bars are presented for the standard error (SE), with n=3. Differences between the WT and different Δgrx mutant strains were examined by Student’s t-test analysis (p< 0.05).
The expressed Mia40C, as the isolated C-terminal domain, has a molecular weight of ~15 kDa compared to the full-length yeast Mia40 bound to the IM (~70 kDa). Much higher protein levels of Mia40C were observed in the WT, compared to in the single and double GRX deletion mutants. Equal amounts of the endogenous Mia40 levels were observed between the different ↑pYES2 and ↑Mia40C strains, so Mia40C expression did not affect levels. Identical levels were measured for other mitochondrial proteins and the cytosolic G6PDH in the different yeast strains. From densitometry analysis, the absolute ratios of Mia40C levels in the different Δgrx mutant strains were observed with a significant decrease from the OE levels observed in the WT (p < 0.05 with the Student’s t-test, n=3).

Study of Mia40C expression was then performed over the course of exponential and stationary phases of growth to further investigate the WT and Δgrx1grx2 yeast, presented in Figure 5.8. Due to Mia40C having a different molecular weight to the full-length Mia40 (at 15 kDa), the specific point of plasmid expression during cell growth could be measured without overlap from the endogenous protein levels. In examining the exponential growth of cells, Mia40C was first detected in the WT·↑Mia40C yeast strain at 12 hours (lane 4, Figure 5.8A), which is when cells were observed to enter exponential growth (Figure 5.6B).
Figure 5.8. Effects of GRX deletion on ↑Mia40C expression during exponential growth.

A) WT and Δgrx1grx2·↑Mia40C individual plate colonies were prepared in an initial SD-URA culture. Cells were washed and inoculated in SGal-URA at $A_{600}=0.5$, with incubation at 30°C, 200 rpm. During exponential growth, whole cell extracts were removed and prepared for western blotting. Mitochondrial proteins analysed were Mia40C (IMS), Mia40 and Tim23 (IM) and Porin (OM). The Grx antibody was used to confirm GRX deletion, while the cytosolic protein G6PDH was used as a loading control. B) Yeast strains prepared as in (A), but were incubated for an extended period of time (48 hours). Whole cell extracts were prepared from cell cultures during the late-exponential and stationary growth phases. Western blotting was performed for Mia40C (IMS), Tim23 and Mia40 (IM) and Arc1 as a cytosolic loading control.
Greater levels of Mia40C in the WT yeast compared to the Δgrx1grx2 mutant were continually detected after expression, including much later in stationary cell growth (48 hours, Figure 5.8B). Additionally, no decline of Mia40C levels was observed in either yeast strain up to the late-exponential growth phase (21 hours). However, later into the stationary phase (48 hours, Figure 5.8B), minimal decreases were observed in the WT compared to in the Δgrx1grx2 yeast.

Mitochondrial proteins Tim23 and Porin as well as cytosolic G6PDH levels were not affected in either strain over exponential growth, while Mia40 was unaffected in the WT∙↑Mia40C, momentarily increasing in the Δgrx1grx2 mutants (lane 12, Figure 5.8A). This was observed only in this one sample, while levels remained comparable between the WT and GRX deletion yeast strains. In the stationary phase of growth, Tim23 and Mia40 were observed to decrease in both strains after 27 hours, while levels of Arc1 remained consistent up to the end of the time course. Together, these blots indicate a strong stability of Mia40C expressed in the cytosol, as well as GRX deletion strongly decreasing the Mia40C levels observed in the whole cell extracts.

5.4.3. Effects of GRX deletion on mitochondrial Mia40C levels

Further study of the Mia40C protein levels was performed in isolated mitochondria from the different yeast strains. Analysis of the mitochondrial levels between the WT and GRX deletion mutants were by western blotting, as presented in Figure 5.9. Specific comparison of mitochondrial Mia40C levels showed a strong decrease in the Δgrx1grx2 (Figure 5.9A), while in single GRX deletion mutants, a slight decrease was observed for Mia40C (Figure 5.9B). Densitometry analysis of the mitochondrial Mia40C levels was performed and presented in Figure 5.9C.
Figure 5.9. Effects of GRX deletion on mitochondrial Mia40C levels.

A) Western blotting of isolated mitochondria from the WT·↑Mia40C and Δgrx1grx2·↑Mia40C-transformed yeast. (B) The mitochondrial protein levels analysed in the ↑Mia40C-transformed WT yeast to the single GRX deletion Δgrx1 and Δgrx2 mutants. For (A) and (B), proteins analysed were Mia40C and Tim9 (IMS), Mia40 and Tim23 (IM) as well as Tom40 (OM). Each samples was loaded as 25, 50 and 75 μg. C) Densitometry analysis of the preceeding Mia40C bands, with the relative Mia40C levels between the the WT and different Δgrx mutant yeast strains. Values were compared to the WT where the intensity measured with 75 μg protein was taken as 100% and what the other samples were relative to. Error bars presented for the WT·↑Mia40C and Δgrx1grx2·↑Mia40C samples with the standard error (SE) for n=2. No error bars were included for the Δgrx1·↑Mia40C and Δgrx2·↑Mia40C samples as n=1.
The MIA pathway substrate of Tim9 was observed in the Δgrx mutants as being similar to the WT yeast. The other mitochondrial proteins analysed, including Mia40, were identical in the WT and GRX deletion yeast strains. In the densitometry analysis for Mia40C, levels of the protein in the Δgrx1grx2 yeast showed declines to less than 10% of the proteins analysed in the WT (where the intensity observed with 75 μg represented the standard). Single deletion of a GRX gene decreased Mia40C levels to ~60% (in a 75 μg load) compared to the WT. Together, the results agreed with the previous western blots performed in whole cell extracts for Mia40C (Figure 5.7, Figure 5.8). Decreases of mitochondrial levels were observed with a singular deletion of a GRX gene, while deletion of both GRX1 and GRX2 greatly diminished Mia40C levels. This contrasts to the similar levels observed for the full-length yeast Mia40 and the MIA substrate of CX3C Tim9, which showed minimal changes in mitochondrial levels of the protein in OE conditions.

As Mia40C was observed to be a stable protein after expression (Figure 5.8), the WT and Δgrx1grx2 isolated cytosol were examined for Mia40C levels. As decreased mitochondrial levels of Mia40C was observed in the Δgrx1grx2, there was the matter of whether there would be an accumulation of the unimported proteins in the cytosol and if they would be degraded. Protein samples prepared after cell homogenisation were analysed by western blotting, compared to whole cell extracts (from the SGal-URA culture during isolation) and isolated mitochondria samples. Together, the results were presented in Figure 5.10A. In the western blot, Mia40C was detected in the cytosolic fractions for each yeast strain and was followed up with an AMS assay to examine the redox states of the unimported proteins (Figure 5.10B).
Figure 5.10. Sub-cellular localisation and redox state analysis of Mia40C in Δgrx yeast.

A) Western blotting of samples from the sub-cellular compartments of the WT·↑Mia40C and Δgrx1grx2·↑Mia40C yeast. Whole cell extracts prepared from SGal-URA (before inoculation in YPGal), while proteins were precipitated from 1 ml each of isolated cytosol. Mitochondrial proteins were loaded to 75 μg for study. Antibodies for the cytosolic G6PDH and mitochondrial Mia40 measured the efficiency of isolation as well as the detection of Mia40C.

B) AMS assay for Mia40C in the isolated cytosol. Cytosolic samples were treated with redox agents; 5 mM cystine, 5 mM DTT (1 hour, RT), before treatment with 12.5 mM AMS (30 minutes, RT). AMS was used to detect reduced thiols in proteins (4x –SH, Mia40C). Reduced (red.) and oxidised (ox.) proteins were indicated.
Following previous western blots, the levels of Mia40C (both whole cells and isolated mitochondria) were observed with strong decreases in the Δgrx1grx2 yeast. Notable as well, was that Mia40C was detected in the cytosol for each yeast strain and a greater amount was observed for the Δgrx1grx2 (lane 7) than in the WT (lane 2). This supported the hypothesis of the Grx systems maintaining reduced IMS protein for import, with unimported precursors accumulating in the cytosol for later degradation. To investigate whether the unimported proteins were oxidised, in particular for the proteins detected from the Δgrx1grx2 yeast, an AMS assay was utilised (Figure 5.10B). Results reproduced the greater cytosolic levels of Mia40C in the Δgrx1grx2 mutant; however AMS treatment (lanes 2 and 8) identified unimported Mia40C in both yeast strains in reduced forms. Whether this was the thermodynamically-favoured form of Mia40C in the cytosol or from the incubation of yeast with DTT prior to zymolyase degradation of cell walls, would require further investigation.

Collectively, results showed strong differences in overexpressed Mia40C levels with GRX deletion. This was observed in both the whole cell extracts (like Tim9 in chapter 4) and in the isolated mitochondria, where unimported Mia40C also accumulated in the Δgrx1grx2 cytosol. Compared to the full-length yeast Mia40 (imported through the TIM23 pathway), where levels still remained similar with GRX deletion, strong effects were observed specifically for Mia40C as a CX9C MIA pathway substrate. Because Mia40C was not a native IMS protein, Cox17 was also examined while the plasmid loading was checked between the WT and GRX deletion mutant yeast strains.

5.4.4. Plasmid loading of ↑Mia40 and ↑Mia40C in Δgrx mutant yeast

To confirm the different protein overexpression levels observed in the WT and Δgrx1grx2 mitochondria, loading of both the ↑Mia40 and ↑Mia40C plasmid constructs were examined. As outlined previously for the small Tim proteins (Chapter 4.3.5), plasmid and genomic DNA was isolated from the different plasmid-transformed yeast cells during exponential growth in plasmid-inducing media (15 hours, SGal-URA). PCR analysis was used to measure loads of the ↑Mia40 and ↑Mia40C plasmid constructs as well as for the genomic β-tubulin levels. The results were presented in Figure 5.11.
DNA mixtures from plasmid-transformed cells were used with primers specific for the ampicillin resistance regions of the pYES2 gene or the genomic β-tublin gene. Newly-synthesised DNA was detected by binding to the passive SYBR-Green dye. PCR analysis performed for the ↑pYES2, ↑Mia40 and ↑Mia40C plasmid constructs in the WT and Δgrx1grx2 yeast. A) Measurement of plasmid DNA in transformed yeast, while the genomic DNA levels in the same samples were measured in (B). The qPCR study was performed in parallel to the study of small Tim plasmid-transformed yeast (Figure 4.18). DNA was detected after passing the cycle threshold (Ct). Each Ct cycle denotes a doubling of DNA. Error bars represent the standard error (SE) with n=3. Statistical analysis (One way ANOVA) identified no significant differences between the WT and Δgrx1grx2 yeast in (A) or (B) with p> 0.05.
Expression analysis by qPCR for the plasmid and genomic DNA confirms the equal amounts of the plasmid DNA and genomic DNA in the three respective yeast strains. For the ↑Mia40 plasmid construct between the WT and Δgrx1grx2 strains, the lower cycle threshold (Ct) value in the WT-↑Mia40 is equivalent to earlier detection (significant compared to background signals), with a single Ct cycle representing a doubling in DNA. For levels of the genomic DNA, equal levels were observed in the different strains, with greater variability noted for the Δgrx1grx2-↑Mia40 yeast. The earlier threshold detection of plasmid DNA in the WT to the GRX deletion mutant was consistent to the results for the genomic DNA, where a greater variability was also observed in the results. However, no statistical differences were analysed between the different transformed WT and Δgrx1grx2 yeast strains as p> 0.05. As outlined with the qPCR data presented in Figure 4.18, standard curves for plasmid and genomic DNA were not developed due to difficulties. Consequently, the Ct values could not be utilised to determine the copy numbers of the pYES2 plasmid and genomic DNA from absolute quantification. These in turn, would have been used to provide copy number ratios for the plasmids transformed into yeast, as a quantification of the plasmid load. However, from the results obtained, equal ↑Mia40C plasmid loads in the transformed yeast strains were observed.

5.5. Effects of GRX deletion on the import of OE Cox17

Decreased mitochondrial levels of Mia40C were observed in GRX deletion mutants, with the strongest effects in the Δgrx1grx2 mutant. This suggested a decreased efficiency of import without the cytosolic Grx system. However, Mia40 is an essential component of the MIA pathway, which may have contributed to the defect. To verify the results, another CX3C-motif containing protein that was native to the IMS, Cox17 was examined using the same overexpression (OE) method. Western blotting analysis showed that in the endogenous proteins levels, equal amounts were observed (Figure 5.1), so overexpression in yeast was examined using an inducible plasmid construct with the pYES2 vector (↑Cox17).
Cell growth was studied with the OE of Cox17 through spot tests (Figure 5.12A). Identical rates of growth in the different transformed yeast strains were observed after expression was induced. Protein levels of Cox17 between the WT and different GRX deletion mutant yeast strains were then analysed by western blotting, with comparisons to the endogenous protein levels in ↑pYES2-transformed yeast (Figure 5.12B).

**Figure 5.12. Effects of GRX deletion with ↑Cox17 expression on cell growth and protein levels.**

**A)** Cell growth of ↑Cox17-transformed yeast on repressing SD-URA and inducing SGal-URA media. From an SD-URA culture inoculated with individual plate colonies, the A_{600} of cells were adjusted with serial dilution before spotting on agar plates. Incubation was then performed at 30°C for 3 days. **B)** Western blotting of Cox17 OE compared to ↑pYES2-transformed yeast in the different WT and GRX deletion yeast strains. Proteins analysed in the whole cell extracts were Cox17 (IMS), Tim23 (IM), Tom40 (OM) and Hsp70 (matrix, cytosol). A loading control was performed with the cytosolic protein G6PDH.
High levels of Cox17 in the ↑Cox17 OE conditions were observed in the WT yeast, compared to ↑pYES2 endogenous levels. This followed the previous results observed with Mia40C and Mia40. Whole cell Cox17 levels were observed with decreases in each GRX deletion mutant, with single deletion strains showing strong decreases of Cox17 levels, as seen before with Mia40C (Figure 5.7). Other mitochondrial proteins analysed were observed to identical levels in the different Δgrx mutant strains, as well as the G6PDH loading control. Further study would be performed to measure levels of OE Cox17 in GRX deletion mutants during exponential growth.

Mitochondrial protein levels were examined for each yeast strain in isolated mitochondria, with the Δgrx1grx2 samples not presented due to contamination in the preparation. This would be examined with repetition of the isolation process for study of OE Cox17 in a double GRX deletion mutant. Mitochondrial Cox17 levels were analysed between the WT and single GRX deletion mutants with western blotting, presented in Figure 5.13.

![Western Blot](image)

**Figure 5.13. Effects of GRX deletion on the mitochondrial Cox17 levels.**

Mitochondria isolated from the ↑Cox17-transformed WT and the GRX deletion mutant (Δgrx1 and Δgrx2) yeast strains. Mitochondrial proteins analysed by western blotting were Cox17 (IMS), Mia40 and Tim23 (IM), Tom22, Tom40 and Porin (OM) as well as mtHsp70 (matrix). The Grx2 antibody confirmed deletion of GRX2. Samples loaded to 25, 50 and 75 μg.
Depletion of Cox17 in the Δgrx1 and Δgrx2 mutants was observed in comparison to the WT-transformed yeast. Western blotting for other mitochondrial proteins identified identical levels across the Δgrx mutants and the WT yeast. These results for the mitochondrial levels support those observed in the whole cell extracts, for a decrease in OE Cox17 levels with GRX deletion. From the differences observed in whole cell extracts and isolated mitochondria, the subcompartments of the different yeast strains were analysed. Protein levels in the isolated cytosol and mitochondria were detected by western blotting, presented in Figure 5.14.

![Figure 5.14. Compartmentalisation of ↑Cox17-transformed WT and Δgrx yeast.](image)

Cytosol and mitochondria isolated from ↑Cox17-transformed cells after induced OE of the plasmid in galactose-containing media. Proteins were precipitated from the cytosol for study, while 75 μg of mitochondrial proteins were loaded for each sample. Proteins Mia40 and G6PDH were used as loading controls and to examine the efficiency of the isolation process.

Efficient isolation of the cytosol and mitochondria fractions was observed with specific detection of Mia40 in the mitochondria and G6PDH in the cytosol. A small fraction of unimported Cox17 was detectable in the WT·↑Cox17 cytosol. For the Δgrx1 and Δgrx2 yeast, minimal amounts of Cox17 were detected in the mitochondria, with none detected in the cytosol. These results follow the previous whole cell and mitochondrial protein levels for Cox17 (Figure 5.12, Figure 5.13). Altogether, these results for Cox17 support those observed with Mia40C, for the mitochondrial levels of CX2C MIA pathway substrates to be strongly decreased in the absence of the cytosolic Grx system.
5.6. Discussion

The biogenesis of yeast CX₃C IMS proteins is less well characterised compared to small Tim CX₃C proteins. This was particularly the case for the maintenance of proteins in the cytosol, where the thioredoxin (Trx) system was identified to facilitate small Tim protein biogenesis (Durigon et al., 2012). The decision to examine CX₃C proteins with induced-overexpression (OE) was based on a recent study that showed Grx1 being more effective than Trx1 in maintaining reduced human Mia40 (hMia40) for mitochondrial import (Banci et al., 2013).

In this study, the C-terminal domain of yeast Mia40 (Mia40C), an analogue to hMia40, was used to serve as a model substrate featuring the twin CX₃C motifs for oxidative folding via the MIA pathway. Overexpression in yeast (↑Mia40C) was compared to the full-length yeast Mia40 (↑Mia40) to investigate import of proteins between the MIA and TIM23 pathways. The native IMS protein Cox17 was also examined as a MIA pathway substrate (↑Cox17). A summary of the results observed for overexpressed protein with GRX deletion is presented in Table 3.

Table 3. Levels of overexpressed (OE) proteins observed in GRX deletion yeast.

<table>
<thead>
<tr>
<th>OE of precursor protein</th>
<th>Dithiol motifs contained</th>
<th>Import pathway</th>
<th>Effect on growth</th>
<th>Effects of Δgrx1grx2 on OE protein levels</th>
<th>Detection in Δgrx1grx2 cytosol</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Whole cell</td>
<td>Mitochondrial</td>
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<tr>
<td>Tim9</td>
<td>CX₃C</td>
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<td>~WT</td>
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<td>Slight decrease</td>
</tr>
<tr>
<td>Mia40</td>
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<td>TIM23</td>
<td>~WT</td>
<td>&lt;50% decrease</td>
<td>Slight decrease</td>
</tr>
<tr>
<td>Mia40C</td>
<td>CX₃C</td>
<td>MIA</td>
<td>~WT</td>
<td>Strong ~5x decrease</td>
<td>Strong ~10x decrease</td>
</tr>
<tr>
<td>Cox17</td>
<td>CX₃C</td>
<td>MIA</td>
<td>~WT</td>
<td>Strong 5x decrease</td>
<td>Strong decrease</td>
</tr>
</tbody>
</table>
5.6.1. The cytosolic Grx system does not play a role in TIM23-targeted Mia40 biogenesis.

Mia40 in *S. cerevisiae* differs to higher eukaryotes with import being facilitated by the TIM23 pathway for insertion into the IM (Figure 1.7). This contrasts to hMia40 which is assembled by the MIA pathway without any targeting sequences (Hofmann et al., 2005). To determine if TIM23 import of the redox-active yeast Mia40 required reduction by the cytosolic Grx system during import, the †Mia40-transformed GRX deletion yeast strains were analysed. Compared to the other MIA-pathway substrates examined with strong differences being observed between different GRX deletion yeast strains, Mia40 levels remained unaffected or similar in the different Δgrx mutant yeast. This means that import of Mia40 in yeast did not require reduction by the cytosolic Grx system. This study agrees with the hypothesis of TIM23 pathway import being more efficient than the diffusion of IMS proteins across the OM, where oxidative folding in the cytosol kinetically competes with import (Morgan and Lu, 2008, Bragoszewski et al., 2013).

5.6.2. Effects of GRX deletion on the MIA pathway proteins of Mia40C and Cox17

After results identified the TIM23 pathway to not be affected by GRX deletion, the MIA pathway was examined with CX₉C proteins, with the investigation of the Mia40C isoform and Cox17. Strong differences were observed with a ~10x decrease in levels of Mia40C in the Δgrx1Δgrx2 yeast strain compared to the WT, offset by a greater detection of unimported Mia40C in the cytosol (Table 3). Similarly, strong effects were observed for Cox17, which was studied a native IMS protein. Altogether, the strongly-decreased levels in GRX deletion mutant yeast, compared to study of full-length Mia40 showed import of CX₉C MIA pathway proteins to be affected and not TIM23 substrates. This study concludes that the cytosolic Grx system is able to play a role in facilitating IMS protein import like the Trx system, but was specific here for CX₉C proteins and not CX₃C small Tim proteins (as studied with Tim9).

This study agrees with previous *in situ* research for overexpressed hMia40, where cytosolic Grx1 was identified as being effective in maintaining reduced proteins for import (Banci et al., 2013). Unimported hMia40 in the cytosol without the Grx system, was detected as being
oxidised while in this study, the unimported Mia40C was identified to be in reduced states from an AMS assay (Figure 5.10B). Whether this was the thermodynamically favoured form in the yeast cytosol was uncertain due to the required DTT treatment of cells prior to homogenisation. This reduces surface proteins and there was the possibility that cytosolic proteins were also being reduced. Mitochondrial isolation in the absence of DTT resulted in a greatly-diminished yield and quality, without Mia40C detection in the cytosol. Further study would require trapping reduced cytosolic proteins with a reverse AMS assay during mitochondria isolation.

Due to unimported Cox17 not being detected in the cytosol of GRX deletion mutant yeast, whether degradation occurred with accumulated proteins was raised. Clearance of mistargeted MIA pathway substrates in the cytosol was identified to be performed by the 26S proteasome as a negative regulator to IMS protein import (Bragoszewski et al., 2013b, Wrobel et al., 2015). Overexpressed proteins accumulating in the cytosol unable to cross the OM would then be degraded. Greater amounts of unimported Mia40C were detected compared to Cox17, which with the time course studies performed (Figure 5.8); show Mia40C to be a more stable protein under the experimental conditions.

5.6.3. Conclusions

This in vivo study of CX9C protein suggests the cytosolic Grx oxidoreductases to play a role in the protein biogenesis of CX9C proteins of the MIA pathway. This was observed with overexpressed proteins in the cytosol to saturate import pathways as differences were not initially observed in the endogenous protein levels. Deletion of the GRX genes presented clear decreases for mitochondrial Cox17 and Mia40C levels, while greater amounts of unimported Mia40C being detected in the cytosol. This was compared to the full-length yeast Mia40, which was much less affected by the cytosolic Grx system. The results here for Mia40C and Cox17 with the cytosolic Grx system are consistent with what was shown for the H. sapiens cytosolic Grx1, where it was effective in maintaining reduced hMia40 for mitochondrial import (Banci et al., 2013).
6. CONCLUSIONS AND FUTURE RESEARCH

6.1. Conclusions from this study

The aims were to investigate the biogenesis of redox-sensitive IMS proteins with how reduced protein states were maintained in the cytosol for mitochondrial import. The mechanisms studied were the reducing Trx and Grx oxidoreductase systems present in the cytosol. Recent studies identified in vivo reduction of the yeast small Tim CX₃C proteins by the Trx system (Durigon et al., 2012). An investigation into H. sapiens CX₃C proteins identified stronger effectiveness of cytosolic Grx1 than Trx1 in reducing proteins for import (Banci et al., 2013).

The individual Trx and Grx paralogs were studied in vitro to determine the standard redox potential (E°') values as a quantifiable marker of reductant function (Chapter 3). The Grx system was studied in vivo for different MIA pathway substrates in GRX deletion mutant yeast strains, including the induced overexpression (OE) to stress import pathways. Small Tim CX₃C proteins were studied with a focus on Tim9 in Chapter 4, while Cox17 and Mia40 were studied as model CX₃C proteins for the TIM23 and MIA import pathways in Chapter 5.

In this study, the previously-unmeasured S. cerevisiae E°'ₜᵣₓ₁ value was determined as -280 mV. This marked it as the strongest reductant of the different species paralogs, as the stronger negative value marked a greater tendency to reduce a chemical species (Table 2). The E°'ₜᵣₓ₂ of -240 mV was previously determined, which in the context of this study, is interpreted as Trx2 being a weaker reductant of the two cytosolic Trx isoforms (Gonzalez Porque et al., 1970). However, studies for Grx1 were met with limitations in the requirement of an alternate methodology than what was used with Trx1 to determine the E°' value. Further limitations were observed in clearly isolating the different protein redox species from the experimental mixtures. Additional modifications to use a semi-quantitative redox AMS assay method was better suited and in future research, could be performed to determine the E°' values for the Grx system to compare their reductant function to the Trx system in yeast. Import assays for the OM-bound Grx2 to characterise its reductant function in organello, were limited in this study, but could be further investigation to determine if the specific isoform has a role in IMS protein biogenesis.
In chapter 4, overexpression of the small Tim proteins precursors was utilised after previous success was observed in examining mitochondrial import of hMia40 (Banci et al., 2013). This study found overexpressed Tim9 to have been significantly decreased in Δgrx yeast, with single deletion of a GRX gene being effective to affect levels from the WT. In contrast, the mitochondrial levels of overexpressed Tim9 in Δgrx yeast strain samples were similar compared to the WT. Since unimported Tim9 was not detected in the isolated cytosol prepared with the mitochondria, the stability of the expressed Tim9 was with the cytosolic proteasome systems. Despite successful inhibition being observed, Tim9 levels were not recovered in this study, which differed to research that identified the cytosolic ubiquitin-proteasome system as being a negative regulator in IMS protein biogenesis (Bragoszewski et al., 2013b, Wrobel et al., 2015). This mechanism would explain the differences observed between the Tim9 mitochondrial and whole cell levels, where Tim9 in the cytosol was more rapidly degraded in the GRX deletion mutants before import. However for the studies utilised, evidence is presented for the cytosolic Grx system to play a possible role in CX9C protein biogenesis that can be further investigated.

In studying the CX9C IMS proteins in chapter 5, studies for the TIM23-targeted yeast Mia40 did not identify differences in mitochondrial levels between the different GRX deletion yeast mutants. Thus, for the CX9C protein import independent to the MIA pathway, the Grx system did not affect import. The isolated C-terminal core of yeast Mia40 (as Mia40C) was used as a MIA pathway substrate, which renders the protein suitable for oxidative folding, without the TIM23-targeting for import. The protein also functioned as a direct analogue to hMia40, which is a MIA pathway substrate itself. Compared to the full-length Mia40, mitochondrial levels of Mia40C were diminished in the Δgrx1grx2 to a tenth of the overexpressed levels observed in the WT yeast. A singular GRX deletion was also observed to be effective in decreasing the Mia40C levels detected. Greater amounts of unimported Mia40C were also detected in Δgrx1grx2 cytosol compared to the WT, which showed that the expressed precursor remained in the cytosol after mitochondrial import did not occur. The unimported Mia40C was identified to be reduced, which differed from other studies identifying the oxidised protein states to be thermodynamically favoured in the cytosol (Banci et al., 2013). Further study with the MIA pathway substrate Cox17 showed single GRX deletion to be sufficient in drastically decreasing
the mitochondrial levels observed. Combined with the results for Mia40C, these in vivo studies presented the cytosolic Grx system as being effective in IMS CX₉C protein biogenesis compared to other small Tim proteins. The mechanisms for this would need to be elucidated in a further study, as well as investigating the cytosolic Trx system with the import of CX₉C proteins. Together, this would elucidate upon the findings observed in higher eukaryotes and determine the effectiveness of each system (Banci et al., 2013). This could be combined with the in vitro standard redox potential studies to further understand the roles of the two oxidoreductase systems.

Finally for this study, a genetic interaction was identified between the GRX and YME1 genes that was observed with strong recoveries in cell growth and functioning. The Yme1 protease has been identified to degrade misfolded IMS proteins as a regulator (Baker et al., 2012). Further study of overexpressed Tim9 levels in the Δgrx1grx2yme1 triple mutant compared to Δgrx1grx2, showed a recovery in whole cell protein levels as well. The results indicated a regulatory relationship between the different genes, though whether this was direct or mediated through a shared factor would need to be further investigated. Recent studies have also identified fractions of the Trx and Grx systems to be translocated from the cytosol to the IMS, also raising the topic of whether the interactions occurred solely within the IMS to recover cell function (Kojer et al., 2015). Future investigations would elaborate upon the observed relationship with consideration to the intracellular distribution of the Grx system, with what the mechanisms and effects are for the interaction with the YME1 gene.

6.2. Future research topics

6.2.1. In vitro analysis of the individual Trx and Grx paralogs

The S. cerevisiae E°⁻Trx1 was calculated in this study and it would be of interest to determine the E°⁻ for the cytosolic Grx system (Grx1, Grx2) as an in vitro measure of the reductant properties between the Trx and Grx systems. From the limitations observed in RP-HPLC, redox AMS assay with selective antibodies would examine the redox species in a Grx1-Trx1 equilibration mixture. Equilibrium of the two proteins (in opposite redox states) would be performed in an
anaerobic degassed environment over an extended period of time (e.g. 18 hours). AMS treatment followed by western blotting and densitometry analysis, would quantify the redox species concentrations to calculate the equilibrium constant (K_{eqm}) and redox potential (E^\circ). The Trx-NADPH redox equilibrium reaction could also be performed for Trx2 in yeast, to check the previous result (E^\circ=-240 mV (Gonzalez Porque et al., 1970)), while investigating the other individual oxidoreductases.

The protein-protein equilibration (PPE) technique can be later used with purified MIA pathway substrates to further investigate the redox mechanisms with the cytosolic oxidoreductases. Analysis by RP-HPLC or AMS assays would determine the E^\circ values and establish an in vitro thermodynamic gradient for the different yeast oxidoreductase systems in IMS protein biogenesis.

6.2.2. Study of the effects of OM-bound Grx2 in mitochondrial protein import

Initial studies were developed for studying the OM-bound Grx2 isoform with the import of radiolabelled IMS proteins (^{35}S-Tim9) in isolated mitochondria. Import studies would be performed in the WT and Δgrx2 mitochondria with 5 mM GSH for the initial Grx2 reduction. Imported radiolabelled proteins in the mitochondria would be visualised by autoradiography. AMS assays can also be performed to detect the ratios of redox species in the unimported precursors remaining in the lysate, as well as within the imported mitochondrial proteins. The investigation can also be furthered by in vitro reconstitution of the individual cytosolic Grx1 and Grx2 systems with the radiolabelled precursors to obtain in vitro evidence for the Grx systems in IMS protein biogenesis. MIA pathway substrates such as Cox17 can also be examined with the Grx system to compare import for the CX_{9}C and CX_{3}C IMS proteins. This would further develop the in vivo results performed in this study.

6.2.3. Examination of the Grx and Trx systems with CX_{9}C protein biogenesis

From the results of this study with Mia40C and Cox17, as well as prior research in humans for CX_{9}C IMS proteins (Banci et al., 2013), further study could be conducted to expand the focus to the cytosolic Trx system. This would determine if the effectiveness observed for the human Grxs
over Trxs in CX$_2$C IMS proteins were similar in yeast. This would further the understanding of the Trx system in IMS biogenesis after it was identified with import of the CX$_2$C Tim9 (Durigon et al., 2012). For overexpression of proteins with the pYES2 vector used in this study, a compatible $\Delta$trx1trx2 yeast mutant would need to be used without interference in the selection of transformed strains on uracil-deficient (-URA) media. Otherwise, a suitable mutant would have to be developed using PCR-transformation and the insertion of the kanMX4 cassette, as previously used to develop the YME1 deletion mutants (Table 4).

The induced overexpression of proteins in a respiratory cell growth could also be performed to better investigate mitochondrial functioning and proteins levels with the Trx and Grx systems. Concentrations of 1.5% glycerol for respiration and 0.5% galactose would be required to induce the pYES2 $P_{GAL1}$ promoter for expression, based on recent literature (Wrobel et al., 2015). From the conditions observed there, overexpression could still induced without interfering with mitochondrial respiration, as galactose was a fermentable carbon source. Induced overexpression in respiratory conditions was not investigated in this study due to the optimisation required to determine effective concentrations and the lack of appropriate studies to act as examples for developing a method from. By inducing respiratory stress, mitochondrial function is increased as well as the mitochondrial mass that can be prepared from cell cultures (Madeo et al., 2009). This would provide greater in vivo details into the molecular mechanisms of IMS protein import from the cytosol and how the different oxidoreductases present there can contribute to it.

6.2.4. Investigating the GRX and YME1 genetic interaction

This study identified a genetic interaction between GRX and YME1, observed as a recovery in cell functioning for the $\Delta$grx1grx2yme1 triple-deletion mutant yeast from the deficiencies observed in the $\Delta$yme1 mutant. Overexpressed Tim9 levels were also recovered in comparison to the $\Delta$grx1grx2 yeast. Further study would examine the mitochondrial levels to investigate the GRX-YME1 interaction in more detail with IMS protein biogenesis.
Additional study into the individual *GRX* gene deletion can be analysed to better understand the interaction observed with *YME1*. Double deletion mutants for individual *GRX* genes and the removed *YME1* would determine if the recovery observed in this study was specific for *GRX1* or *GRX2*, or only with the deletion of both genes. Whether the interaction was direct or through an intermediate such as Mia40 or a MIA substrate would also be a necessary step in the investigation. As the central step to examine gene interactions, a genome-wide association study can be utilised to investigate the *YME1* and *GRX* genes. Additionally, with a cytosolic Trx system also detectable in the IMS and identified in the cytosol as a redox factor in import, studying for an interaction between *TRX1* and *TRX2* with *YME1* would elaborate on what was observed in this study for the *GRX* genes (Vögtle et al., 2012, Durigon et al., 2012).

Finally, Tim9 was suggested to be a protective factor for Tim10 from degradation by the Yme1 protease (Spiller et al., 2015). Thus, Tim10 levels (with overexpression) can also be studied in the existing *GRX* and *YME1* deletion yeast strain to examine their effects on protein biogenesis. In this study, the overexpressed Tim10 (↑Tim10) was not investigated like Tim9 due to limitations in detection with the Tim10 antibodies available, though a functional antibody became available much later in the studies. By investigating Tim10 in *YME1* deletion mutants with the Trx and Grx oxidoreductase systems, greater detail can be elucidated in examining the mechanisms of IMS protein biogenesis.
7. REFERENCES


KAWAMATA, H. & MANFREDI, G. 2010. Import, Maturation, and Function of SOD1 and Its Copper Chaperone CCS in the Mitochondrial Intermembrane Space. Antioxid Redox Signal, 13, 1375-84.


8. APPENDIX

8.1. Appendix tables

Table 4. List of *S. cerevisiae* strains used in this study.

<table>
<thead>
<tr>
<th>Code (PTY)</th>
<th>Strain</th>
<th>Details</th>
<th>Genotype</th>
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<tr>
<td>04 CY100</td>
<td>grx2</td>
<td>As in CY4 but grx2::HIS3</td>
<td>This study</td>
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</table>
| 05 CY117   | grx1grx2 | As in CY4 but grx1::LEU2
grx2::HIS3 | This study |
| 06 CY117ΔY | grx1grx2yme1 | As in CY4 but grx1::LEU2
grx2::HIS3 YPR024w::kanMX4 | This study |
Table 5. List of competent *E. coli* strains used in this study.

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<th>Genotype</th>
<th>Reference</th>
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Table 8. List of plasmid-transformed *S. cerevisiae* strains used in this study.

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</table>

Chris Grant's laboratory
8.2. Appendix recipes

8.2.1. SDS-PAGE electrophoresis gels and buffers

8.2.1.1. 10% Tris SDS-PAGE gel

A) 10% Separating gel: 1.25 ml 4x stock SDS-PAGE separating gel buffer (1.5 M tris; 10 mM EDTA; 13.9 mM SDS; pH 8.8); 1.665 ml 30% (w/v) acrylamide (National Diagnostics); 2.085 ml MQH₂O; 25 μl 10% w/v APS (ammonium persulfate, Sigma); 5 μl TEMED

B) Stacking gel: 0.3 ml 30% (w/v) acrylamide, (National Diagnostics); 0.625 ml 4x stock SDS-PAGE stacking gel buffer (0.5 M tris; 10 mM EDTA; 13.9 mM SDS; pH 6.8); 1.625 ml MQH₂O; 25 μl 10% APS (Sigma); 5 μl TEMED

C) 5x Running buffer (cathode and anode): 124 mM tris; 946 mM glycine; 6 mM EDTA; 8.7 mM SDS.

8.2.1.2. 16% Tris-Tricine SDS-PAGE gel

A) 16% Tris Tricine SDS-PAGE separating gel: 2.4 ml 30% acrylamide; 150 μl 2.2% bis-acrylamide; 1.5 ml 3x tricine buffer (3 M tris; 10 mM SDS; pH 8.45.); 450 μl 87% v/v glycerol; 20 μl 10% APS; 2 μl TEMED

B) Tris Tricine stacking gel: 1.2 ml MQH₂O; 225 μl 30% acrylamide; 10 μl 2.2% bis-acrylamide; 460 μl 3x tricine gel buffer; 20 μl 10% APS; 2 μl TEMED

C) 1x Running buffer (anode): 0.2 M tris; pH 8.9.

D) 1x Running buffer (cathode): 0.1 M tris; 0.1M tricine; 3.47 mM SDS; pH 8.24.