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Oxidative Regulation Mechanisms in the Mitochondrial Intermembrane Space

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy (PhD) in Biochemistry and Molecular Biology

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Abstract

Oxidative stress occurs when cells are unable to cope with the levels of various reactive oxygen species (ROS) that arise as part of regular cellular metabolism or in response to ionising radiation (H_2O_2, O_2^-, OH^-). The most well studied ROS is H_2O_2, due to its dual role as a mediator of oxidative stress and a signalling molecule for many cellular pathways. Cells possess a number of different mechanisms to combat ROS, in order to prevent their levels from becoming toxic.

In this thesis, we studied three different aspects of the antioxidant defence in *Saccharomyces cerevisiae*. In the first part, we explored the role of erythroascorbic acid – the yeast analogue of ascorbic acid (vitamin C) – and attempted to determine its role as an antioxidant in yeast. Our results were inconclusive, though there were indications that the presence of erythroascorbic acid may have a protective effect on the mitochondrial inner membrane potential ($\Delta\Psi$), protecting it from depolarisation.

The second part focused on elucidating the mitochondrial targeting of the main H_2O_2 sensor Gpx3 and, more specifically, whether the Yap1-binding proteins, Ybp1 and Ybp2, have an effect on the import of Gpx3 in yeast mitochondria. Our results show a slight effect of Ybp1 (but not Ybp2) on the import of Gpx3, indicating that Ybp1 may act as a chaperone for the more efficient targeting of Gpx3 from the cytosol to the outer mitochondrial membrane and, as a result, its eventual translocation into the IMS.

The final part of this thesis focused on elucidating the import of Trx1 and Trr1 in the mitochondrial IMS, as well as their function in this particular subcompartment. The discovery of two members of the thioredoxin system in the IMS is important, due to the absence of a known reducing mechanism in this oxidising compartment. Our results determined that several well-known import factors are dispensable for the import of either Trx1 or Trr1, indicating that they follow a yet unknown pathway for their translocation into the IMS. Importantly, we showed that Trx1 is reduced (and thus, active) in the IMS and that it can interact *in vitro* with both components of the MIA machinery (Mia40 and Erv1), while *in organello* experiments showed that Trx1 most probably interacts with a large number of Mia40 substrates.

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Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

List of abbreviations

ADP: adenosine diphosphate

AMP: adenosine monophosphate

AMS: 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid

ATP: adenosine triphosphate

Bam: β-barrel assembly

BSA: bovine serum albumin

bZIP: basic leucine zipper

CCCP: Carbonyl cyanide *m*-chlorophenyl hydrazone

CCHL: cytochrome c heme lyase

CC_1HL: cytochrome c_1 heme lyase

CE: carbonate extraction

CRD: cysteine-rich domain

DDM: n-dodecyl β-D-maltoside

DHFR: dehydrofolate reductase

D-IAA: D-(-)-isoascorbic acid

$DiSC_3(5)$: 3,3′ -dipropylthiadicarbocyanine iodide

DNP: 2,4-dinitrophenol

Dsb: disulfide bond formation

DTT: 1,4-dithiothreitol

EDTA: ethylenediaminetetraacetic acid

e**l:** elution

ER: endoplasmic reticulum

FAD: flavin adenine dinucleotide

FCCP: carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

FPLC: fast protein liquid chromatography

fth: flow-through

GSH: reduced glutathione

GSSG: oxidised glutathione

HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

IM: inner membrane

IMP: inner membrane protease

IMS: intermembrane space

IPTG: isopropyl β-D-1-thiogalactopyranoside

ITS/MISS: intermembrane space targeting signal/mitochondrial intermembrane space sorting signal

MBP: maltose-binding protein

MIA: mitochondrial import and assembly

MIM: mitochondrial import

MOPS: 3-(N-morpholino)-propanesulfonic acid

MP: mitoplasting

MPP: mitochondrial processing peptidase

MTS: mitochondrial targeting signal

NADH: reduced nicotinamide adenine dinucleotide

NADPH: reduced nicotinamide adenine dinucleotide phosphate

NEM: N-ethylmaleimide

NES: nuclear export signal

Ni-NTA: nickel-charged nitrilotriacetic acid resin beads

NLS: nuclear localisation signal

OM: outer membrane

o/n: overnight

p: pellet

PAGE: polyacrylamide gel electrophoresis

PAM: presequnece translocase-associated motor

PDI: protein disulfide isomerase

PK: proteinase K

PMSF: phenylmethylsulfonyl fluoride

ROS: reactive oxygen species

s: supernatant

SAM: sorting and assembly machinery

SDS: sodium dodecyl sulfate

SEC: secretory

TCA: trichloroacetic acid

TIM: translocase of the inner membrane

TNT: coupled transcription and translation (always used in reference to the TnT® SP6 Coupled Reticulocyte Lysate System

TOM: translocase of the outer membrane

Tris: 2-amino-2-(hydroxymethyl)-1,3-propanediol

w: wash

wt: wild type

ΔΨ: inner mitochondrial membrane potential

Chapter 1

Introduction

1. Introduction

1.1. Mitochondria

Mitochondria are double-membrane encircled intracellular organelles that are essential for the production of energy within cells. The first description of mitochondria came at the end of the 19th century by R. Altmann. He named these organelles "bioblasts" and characterised them as basic organisms that live within cells and perform important functions (Altmann 1890). In 1898, they were renamed "mitochondria" (Benda 1898).

There are two theories that have tried to explain the origin of mitochondria: the autonomous theory and the endosymbiotic theory. The autonomous theory suggests that the mitochondria came into existence when part of the nuclear DNA of the eukaryotic cell became detached and was enclosed by membranes that were impermeable to proteins. On the other hand, the endosymbiotic theory suggests that mitochondria are the remnants of an ancient event where a proteobacterium was internalised by a host cell. This proteobacterium is believed to have possessed the ability to catalyse a series of oxidative procedures that the eukaryotic host was unable to perform. As a result, the proteobacterium was retained within the eukaryotic cell as an endosymbiont (Martin and Müller 2007).

Figure 1 depicts a brief overview of all the important discoveries concerning the field of mitochondrial biology (Ernster and Schatz 1981; Schatz 2013). Some of the most notable points on this timeline are their discovery as the intracellular organelles that are responsible for cellular respiration, the discovery of the citric acid cycle (also known as the tricarboxylic acid (TCA) cycle or the Krebs cycle) (Krebs and Johnson 1937), the description of the unique structure of the mitochondria that are surrounded by two lipid bilayers (Sjöstrand 1953; Palay and Palade 1955), as well as the discovery of the mitochondrial DNA (mtDNA) (Nass and Nass 1963). The field of mitochondrial biology is under extensive research and new fragments of knowledge are constantly being added, as mitochondria are being linked to an increasing number of functions and phenotypes. Some of these include iron-sulfur cluster biogenesis, oxidative folding, and ageing, as well as a number of neurodegenerative diseases (Lill 2009; Fraga and Ventura 2013; Jefferies 2013; Scorrano 2013).

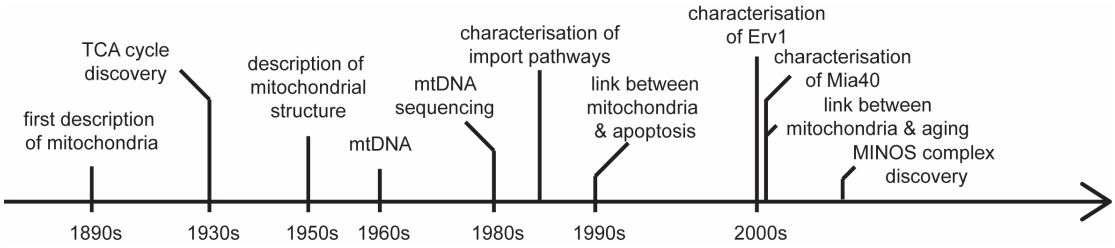

Figure 1: Schematic representation of some of the most important discoveries relating to mitochondria and their function.

The genetic map of the human mitochondrial DNA was published nearly 20 years after the discovery of the mtDNA (Anderson et al. 1981). This map showed that the mtDNA sequence contains coding regions for 13 mRNAs, 22 tRNAs and 2 rRNAs. These 13 mRNAs code for a set of extremely hydrophobic proteins that are core components of the respiratory chain complexes. As expected, these genes only code for a very small fraction of mitochondrial proteins. Human mitochondria have been found to contain approximately 1500 proteins responsible for their correct structure and function, while for the yeast *Saccharomyces cerevisiae* (hereby referred to by either name interchangeably) this number is closer to 1000 (Sickmann et al. 2003; Sztolsztener et al. 2013; Alexeyev et al. 2013). The remainder of the mitochondrial proteins are actually encoded by the nuclear genome and are synthesised by the ribosomes in the cytoplasm. This makes it necessary for protein import mechanisms to exist. Such mechanisms do indeed exist, have been studied extensively in the last 3 decades and will be detailed in chapter 1.3.

1.2. Mitochondrial structure

Mitochondria have a unique architecture, as they comprise of two lipid bilayers, which allow for the allocation of four distinct subcompartments (fig. 2). The two bilayers are distinctly different, both in lipid and protein content and, as such, are responsible for different cellular functions. One of the main differences between the two membranes is the fact that the inner mitochondrial membrane contains three times the amount of cardiolipin (a specific type of phospholipid) than the outer mitochondrial membrane. This difference in the composition of the two membranes contributes to the fact that the inner membrane is

impermeable even to small molecules, as opposed to the outer membrane. Cardiolipin is synthesised in mitochondria and seems to be especially enriched in the areas of contact between the outer and inner mitochondrial membranes (Houtkooper and Vaz 2008). Alterations in the phospholipid (and especially cardiolipin) content have been associated with a series of diseases, such as Barth syndrome and Tangier disease (Puntoni et al. 2012; Jefferies 2013).

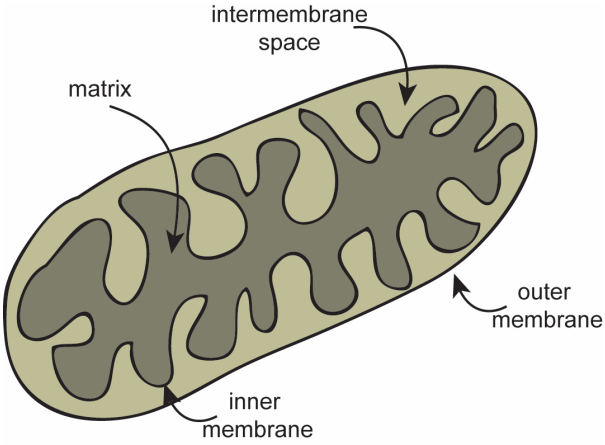

Figure 2: Depiction of the mitochondrial structure. This subcellular organelle is made up of four distinct subcompartments: the outer membrane (OM), the inner membrane (IM), the intermembrane space (IMS) and the matrix.

As mentioned before, the presence of the two membranes leads to the formation of four distinct subcompartments, each one with its own set of proteins and its own unique functions:

- the outer membrane (OM)

  The outer membrane acts as a barrier and separates the mitochondrion from the cytoplasm. It allows the free diffusion of small molecules of a size up to 5,000 Daltons between the two compartments through small channels called porins. The outer mitochondrial membrane also contains the subunits of the transloacase of the outer membrane (TOM) complex, which acts as the general entry gate for the import of proteins into the mitochondria.

- the intermembrane space (IMS)

  The intermembrane space is the subcompartment in which oxidative folding takes place, while also containing a number of protein factors that are responsible for the apoptotic response.

- the inner membrane (IM)

  The inner membrane contains proteins that are involved in a series of different and very important mitochondrial functions, such as certain components of the oxidative folding pathway, as well as the majority of the components of the respiratory chain complexes. It also contains the components of the translocase of the inner membrane (TIM) complexes that are essential for the translocation of proteins into the innermost compartment of mitochondria, the matrix.

- the matrix

  The matrix contains the vast majority of mitochondrial proteins that are involved in processes such as iron-sulfur cluster assembly (Lill 2009; Lill et al. 2014), apoptosis (Joza et al. 2001) and cellular respiration (McBride et al. 2006).

1.3. Mitochondrial import pathways

The entry of proteins into mitochondria is a rather complex process, with multiple control steps. As already mentioned, the presence of these import machineries is of vital importance due to the fact that the vast majority (more than 99%) of mitochondrial proteins are encoded in the nucleus and synthesised in the cytoplasm (Dudek et al. 2013). The protein precursors that are produced in the cytosol are targeted to the mitochondria and become completely folded once they are completely localised in the correct mitochondrial subcompartment (fig. 3).

The way in which each protein is targeted to the mitochondrion differs and is dependent on the compartment in which each protein is active. In most cases, the targeting signal is a small stretch of 15-20 residues at the N-terminus of each protein, which create a characteristic amphipathic α-helix, with all the positively charged amino acids on one side of the helix. This signal is usually cleaved after entry into the mitochondrion (Mossmann et al. 2012). In some cases, these targeting signals can be internal, with these usually being responsible for the translocation of the protein to the correct subcompartment of the mitochondrion after they have been translocated through the outer mitochondrial membrane.

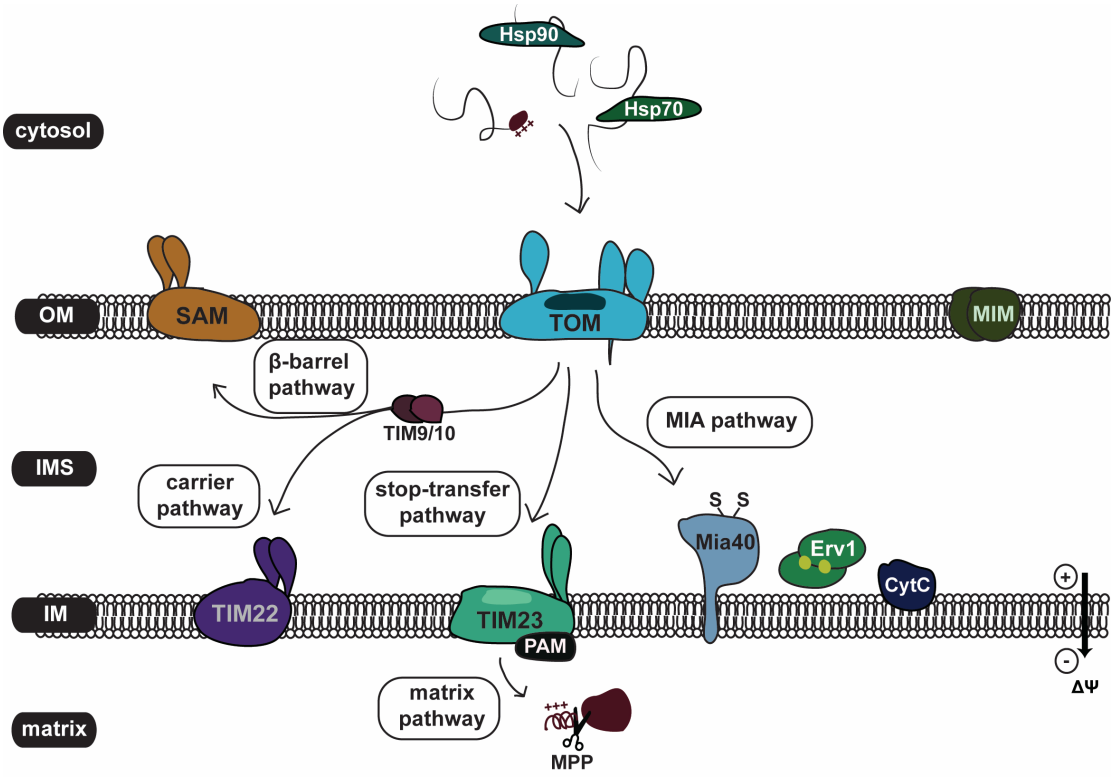

Figure 3: General overview of the protein import pathways in mitochondria. Depending on their final destination within the mitochondria, the incoming precursors make use of different import pathways, such as the MIA pathway for oxidative folding, the SAM pathway for β-barrel integration into the outer membrane, or the TIM23 pathway for inner membrane-targeted proteins.

Once the precursor proteins have been synthesised in the cytosol, a specific set of proteins called chaperones are recruited to the hydrophobic areas of the precursors and protect them from folding incorrectly and/or aggregating (Chacinska and Rehling 2004). The chaperones are able to bring the precursor proteins in close proximity to the outer mitochondrial membrane, making it possible for them to be recognised by the receptors of the translocase of the outer membrane (TOM) complex.

1.3.1. Import through and into the outer membrane

The TOM complex is the general route of entry for all proteins targeted to mitochondria. It is responsible for recognising the incoming precursor proteins through interactions with the cytosolic chaperones, while also playing an important role in the correct folding of the

proteins once they enter the IMS. The TOM complex is made up of seven subunits, which can be split into two main groups:

a) the receptor components

   Tom20, Tom22 and Tom70 are the three subunits that act as receptors and interact with the substrate proteins in the cytosol. Tom20 and Tom70 are the main receptors, with each one having a preference for a distinct set of substrate proteins, though they are also capable of compensating for one another to a certain extent (Neupert and Herrmann 2007). Both Tom70 and Tom20 are anchored to the outer mitochondrial membrane through their N-termini and expose their hydrophilic domains that are responsible for substrate interaction to the cytosol. On the other hand, Tom22 has a negatively charged N-terminal domain that is exposed to the cytosol, while its C-terminus (which is also negatively charged) is exposed in the IMS (van Wilpe et al. 1999). Tom20 is able to recognise proteins through a binding groove that recognises the hydrophobic residues of the mitochondrial targeting signal (MTS) (Abe et al. 2000), while Tom70 is responsible for the recognition of internal targeting signals (Chan et al. 2006).

b) the pore-forming components

   The main component of the pore is the Tom40 protein and contains binding regions for the mitochondrial preproteins (Neupert and Herrmann 2007; Shiota et al. 2015). Tom5, Tom6 and Tom7 are also part of the pore, but they are not essential for the correct function of the TOM complex unless all three proteins are deleted from the yeast genome (Dietmeier et al. 1997; Dekker et al. 1998; Sherman et al. 2005).

Proteins that are destined to become embedded in the outer mitochondrial membrane utilise the TOM complex in order to enter the mitochondria, but require the presence and function of an additional machinery known as the sorting and assembly machinery (SAM) complex (fig. 4). The SAM complex is responsible for the insertion of β-barrel proteins into the outer membrane. β-barrel proteins, prime examples of which are Tom40 and porin, are a class of proteins that can only be found in mitochondria and chloroplasts, a fact that is likely due to their prokaryotic origin (Neupert and Herrmann 2007). In prokaryotes, β-barrel proteins can be found in the outer membrane of Gram-negative bacteria. These proteins are synthesised in the cytosol and contain N-terminal targeting sequences that facilitate their translocation across the inner membrane via the Sec (secretory) system (Bos

et al. 2007). The insertion of these proteins into the outer membrane is carried out by the Bam (β-barrel assembly machinery) complex (Jores et al. 2016).

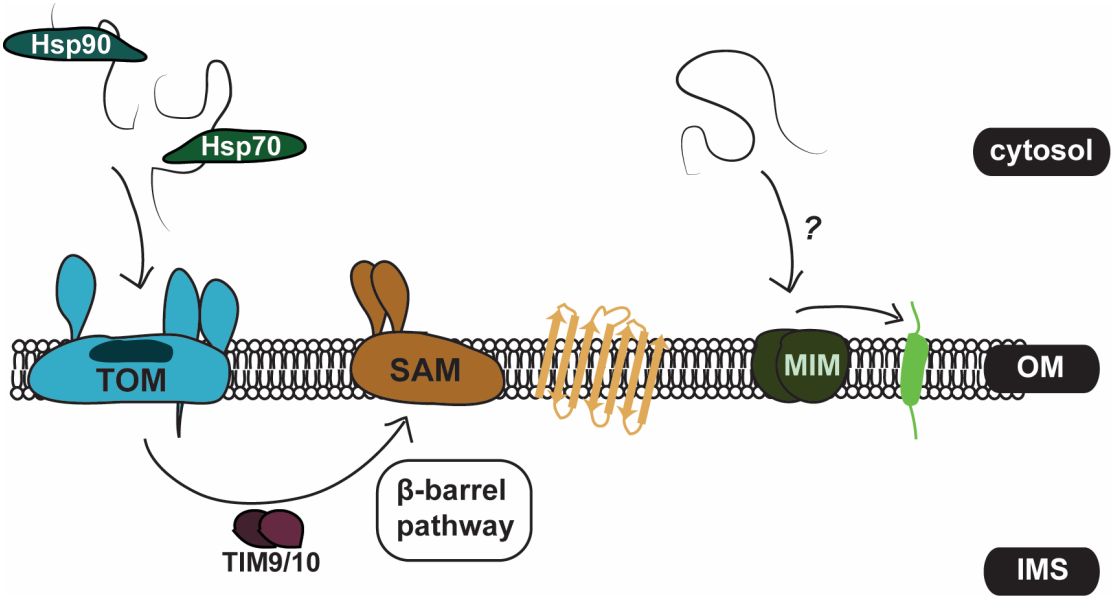

Figure 4: Protein import pathways of the outer mitochondrial membrane. β-barrel precursors enter the mitochondria through the general entry gate, the TOM complex. Once they are localised in the IMS, they are targeted to the SAM complex for sorting into the outer membrane, in a process that requires the interaction of the precursor with the Tim9/Tim10 chaperone complex. The outer membrane contains a second, less-well studied pathway called MIM, which may be responsible for the insertion of single- or multi-spanning α-helical proteins into the outer membrane (figure adapted from Manganas et al. 2017).

The main component of the SAM complex is Sam50, which is a highly conserved protein with two domains: an N-terminal hydrophilic domain exposed to the IMS and a C-terminal domain, which has a β-barrel structure of its own and is embedded in the outer membrane (Neupert and Herrmann 2007). Sam35 and Sam37 are also part of the SAM complex. They are both hydrophilic and located at the cytosolic surface of the complex. Of the three components, only Sam50 and Sam35 are essential for viability (Wiedemann et al. 2003; Chan and Lithgow 2008).

The way in which insertion into the outer membrane works is the following: β-barrel protein precursors interact with the TOM complex receptors and enter the mitochondrial IMS through the Tom40 pore. Studies were able to show that, despite β-barrel proteins

lacking an N-terminal presequence that facilitates the delivery of some mitochondrial proteins to the mitochondrial surface, they do contain a highly hydrophobic β-hairpin motif that can function as a targeting signal. This β-hairpin motif is located in the most C-terminal β-hairpin structure in the majority of β-barrel proteins that were tested and is recognised by the Tom20 receptor (Jores et al. 2016). After entering the mitochondria through the TOM complex, the β-barrel proteins are recognised by the Tim9/Tim10 chaperone complex in the IMS, which are able to bind to them and guide them to the SAM complex in order to become inserted into the outer membrane (Höhr et al. 2015).

An alternative pathway for the insertion of proteins into the outer membrane is the recently discovered mitochondrial import (MIM) pathway (fig. 4). This pathway consists of two proteins, Mim1 and Mim2, and was found to be important for the import of single- and multi-spanning α-helical proteins into the outer membrane, making their integration into the outer membrane a more efficient process (Becker et al. 2008; Popov-Celeketić et al. 2008; Becker et al. 2011; Papic et al. 2011). More specifically, research on Mim2 has shown that absence of this particular protein leads to problems with the import of mitochondrial proteins and morphological defects in mitochondria, while also affecting the correct assembly of the TOM complex (Dimmer et al. 2012; Neupert 2015).

1.3.2. Import into the inner membrane

The import of proteins that reside in the inner mitochondrial membrane is dependent on the TIM22 (translocase of the inner membrane) pathway. Such examples are the proteins of the solute carrier family as well as the membrane embedded Tim proteins (Tim17, Tim22 and Tim23) (Neupert and Herrmann 2007). The TIM22 pathway requires three protein complexes in order to function correctly: the TOM complex for the import of the precursors into the intermembrane space, the small Tim complexes for the recognition of the incoming precursors and efficient targeting to the TIM22 complex, as well as the TIM22 translocase itself, for the final integration into the inner mitochondrial membrane. The TIM22 complex consists of three subunits, the core component Tim22, as well as the accessory proteins Tim54 and Tim18. Tim22 is homologous to both Tim23 and Tim17 of the TIM23 complex (Sirrenberg et al. 1996). Of the three proteins, Tim54 is not necessarily required for the correct function of the complex (Kerscher et al. 1997;

Kovermann et al. 2002). Additonal components of this pathway are the small Tim complexes, which are hetero-oligomeric protein complexes of the proteins Tim8, Tim9, Tim10, Tim12 and Tim13. All these proteins are folded through their CX3C motifs, associate with each other in pairs (the most common being Tim9 with Tim10 and Tim8 with Tim13), are soluble in the IMS and associate with the TIM22 complex on the surface of the inner membrane (Kovermann et al. 2002; Vergnolle et al. 2005).

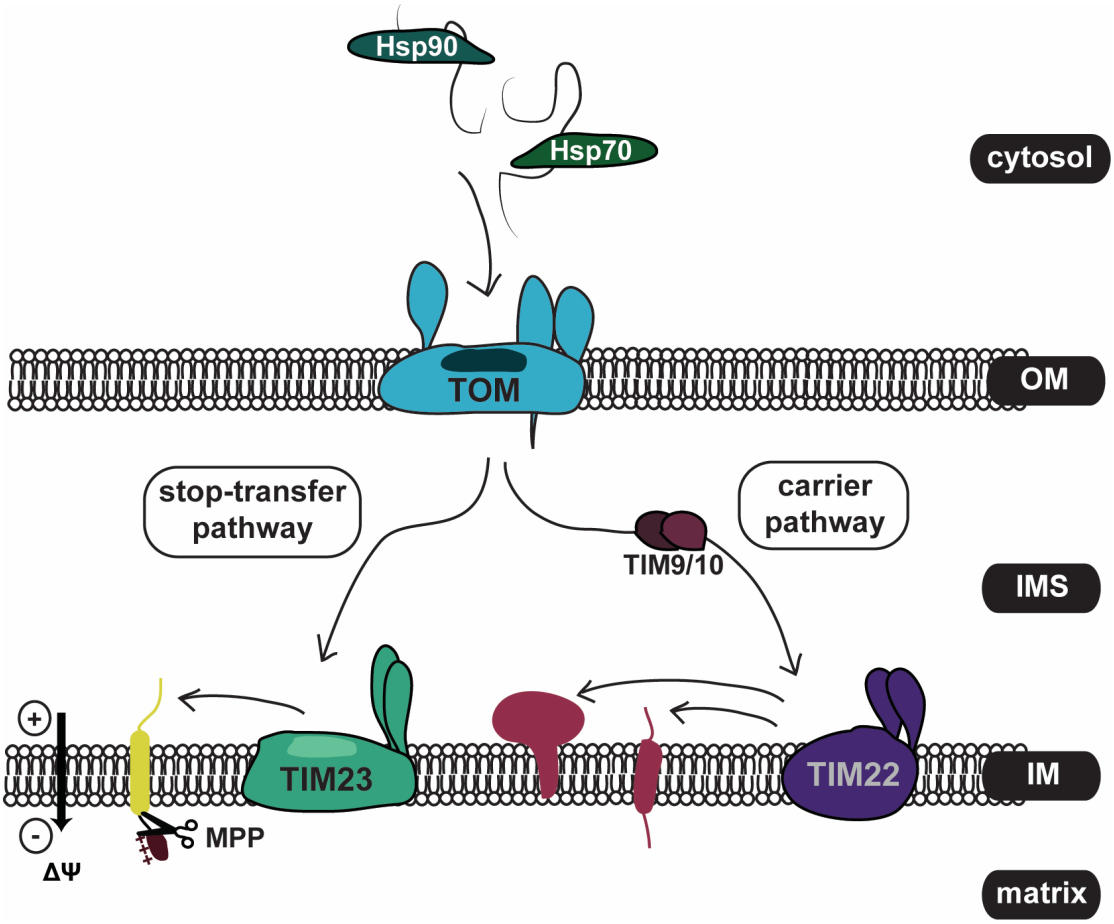

Figure 5: Protein import pathway of the inner mitochondrial membrane. Once the precursors have entered the IMS, they interact with the Tim9/Tim10 complex and subsequently with the TIM22 complex, in order to become inserted into the inner membrane. Inner membrane-targeted proteins can also make use of the TIM23 pathway (figure adapted from Manganas et al. 2017).

The first step for the import of precursors that utilise the TIM22 pathway is their recognition by the cytosolic chaperone Hsp70, which will lead them to the TOM complex of the outer mitochondrial membrane (Komiya et al. 1997). Once they have traversed the outer membrane, they are recognised by the Tim9/Tim10 complex in the IMS and form transient translocation intermediates that allow them to negotiate the aqueous environment

of the IMS. The small Tim complexes recognise the hydrophobic regions of the incoming precursors and bind to them, thus preventing their aggregation in the IMS (Truscott et al. 2002; Koehler 2004; Webb et al. 2006). These complexes deliver the precursors to the TIM22 complex, where they are inserted into the inner membrane in a membrane potential-dependent manner (fig. 5) (Dyall et al. 2003). One of the core components of the TIM23 complex, Tim23, utilises the TIM22 import pathway; however, it requires the non-essential Tim8/Tim13 chaperone complex in the IMS (Paschen et al. 2000).

1.3.3. Import into the matrix

Proteins that are destined to the matrix rely on the TIM23 (translocase of the inner membrane) pathway in order to reach their final destination (fig. 6). This pathway is also responsible for the sorting of certain inner membrane and IMS proteins. TIM23-dependent import also requires the presence of inner membrane potential and ATP hydrolysis as power sources for the translocation process (Neupert and Herrmann 2007). In a similar fashion to the TOM complex, the TIM23 complex also has two main groups of components:

a) the membrane channel

The membrane channel is made up by four different protein components: Tim23, Tim17, Tim50 and Tim21. Tim23 and Tim17 are the core components of the channel and expose their N-termini to the IMS (Donzeau et al. 2000). The N-terminal domain of Tim23 has a coiled-coil structure, which is important for its dimerisation and substrate binding properties (Bauer et al. 1996), while it also stretches to the outer membrane (Donzeau et al. 2000). On the other hand, the N-terminal domain of Tim17 is a lot shorter, though, due to the fact that it contains highly conserved negative residues, it is believed to be involved in the gating of the channel (Meier et al. 2005). Tim50 is anchored to the inner membrane by its N-terminus and exposes a domain to the IMS, which is responsible for substrate interaction (Geissler et al. 2002). Tim21, the only non-essential component of this channel, is responsible for the interaction with the IMS exposed domain of Tom22 (Chacinska et al. 2005; Mokranjac et al. 2005).

b) the import motor

The import motor, also known as the presequence translocase-associated motor (PAM) complex, is located in the matrix and is required for the final translocation of the preproteins into this subcompartment. The main component of the motor is the hydrophilic matrix protein Tim44, which is attached to the inner membrane and contains a hydrophobic pocket for substrate binding (Josyula et al. 2006). The incoming precursors are guided to this hydrophobic pocket by the mitochondrial chaperone Hsp70. mtHsp70 contains an N-terminal ATPase domain and a C-terminal substrate-binding domain, and has the ability to cycle between an ATP- and an ADP-bound state through the function of the nucleotide exchange factor Mge1. When mtHsp70 is in its ATP-bound form, it can be recruited by Tim44 into the import motor structure and complete the translocation process (Young et al. 2004; Bukau et al. 2006). The two final components of the import motor are the DnaJ-like proteins Tim14 and Tim16, which regulate the binding of the substrate proteins to mtHsp70.

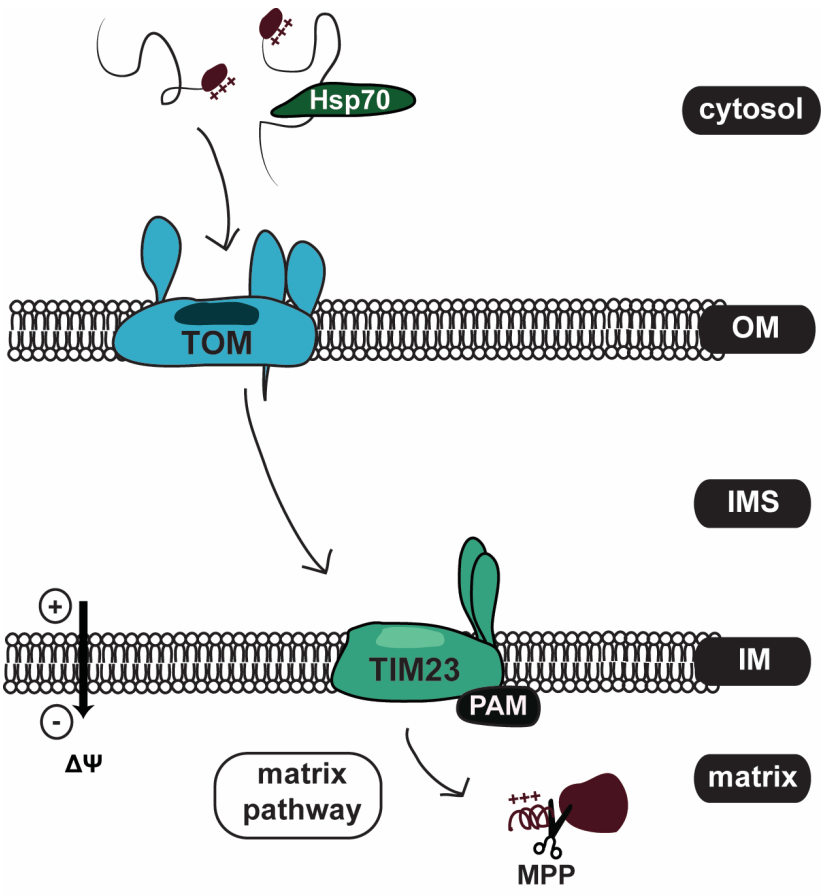

Figure 6: Protein import into the matrix. Precursors that are targeted to the matrix utilise the TIM23 pathway in order to reach their final destination. The presence of a positively charged N-terminal mitochondrial targeting signal (MTS) is essential for targeting to the TIM23 complex, with the translocation

requiring the assistance of the PAM complex. Once the precursor has been imported into the matrix, the MPP acts in order to cleave the MTS, thus leading to the maturation of the protein. Figure adapted from Manganas et al. 2017).

Once the protein has been translocated through to the matrix, it is usually subject to an additional processing step, which removes its matrix-targeting signal. This process is typically carried out by the mitochondrial processing peptidase (MPP). MPP is a heterodimeric zinc-binding metalloprotease that is located in the mitochondrial matrix, made up of two subunits designated as α-MPP and β-MPP (Braun and Schmitz 1997; Gakh et al. 2002). Its function is essential, as, once the targeting signal has sorted the protein into the correct subcompartment, it is not only no longer required, but can also interfere with the folding and further sorting within the compartment. MPP is also able to recognise and cleave sorting signals in proteins that are in transit to the inner mitochondrial membrane or the intermembrane space (Gakh et al. 2002). Sequence analysis of proteins that are processed by MPP were able to show that there is are three main features that characterise MPP-cleaved presequences: an overall positive charge, the ability to form an amphiphilic α-helix and the presence of an arginine residue at the -2 position from the position of cleavage (known as the "R-2 rule") (von Heijne et al. 1989; Gakh et al. 2002). However, this was found to not be as limiting, with the subsequent discovery of other cleavage consensus sequences being identified (R-3, R-10 and R-none) (Gakh et al. 2002).

1.3.4. Import into the intermembrane space

Proteins that reside in the IMS follow one of following pathways, depending on their characteristics:

a) they can utilise a variation of the TIM23 pathway, known as the "stop-transfer" pathway,
b) they can become folded through an interaction with the oxidative folding pathway
c) they can be interact with the TOM complex and become imported into the IMS without having to interact with the inner mitochondrial membrane, or
d) they can be retained in the IMS through the interaction with and binding of co-factors.

Proteins that utilise the first method of import into the IMS are characterised by the fact that they contain bipartite presequences (fig. 7). These are N-terminal targeting signals that contain two parts: an N-terminal mitochondrial targeting signal (MTS), directly followed by a hydrophobic region that has a high similarity to other transmembrane regions. As mentioned above, this pathway is also known as the "stop-transfer" pathway, due to the way in which the hydrophobic region of the bipartite presequence is able to arrest the translocation of the protein through the TIM23 pore (Glick et al. 1992). The MTS is cleaved off the protein through the function of the mitochondrial processing peptidase (MPP) in the same way as with the regular TIM23 pathway. However, due to the presence of the extra hydrophobic sorting signal, there is an additional processing step that is responsible for the cleavage of the hydrophobic region. This process is catalysed by an intermembrane space protease (such as IMP or Pcp1), which removes the hydrophobic sorting signal and releases the protein into the IMS (Nunnari et al. 1993; Esser et al. 2002; McQuibban et al. 2003). Due to the fact that there is no translocation towards the matrix, ATP hydrolysis is not necessary to power this import pathway; the inner membrane potential is the only driving force required to engage the precursor with the TIM23 channel.

Another pathway that is utilised mainly by cysteine-containing proteins is the oxidative folding pathway. This pathway is also known as the mitochondrial import and assembly (MIA) pathway and its two key components are the oxidoreductase Mia40 and the sulfhydryl oxidase Erv1 (essential for respiration and viability) (fig. 7) (Chacinska et al. 2004; Naoé et al. 2004; Hell 2008). The presence of such a pathway in the mitochondrial intermembrane space had been proposed based on *in vivo* studies where the small Tim proteins had been shown to contain internal disulfide bonds (Curran et al. 2002; Lu et al. 2004).

The first component of this pathway is the oxidoreductase Mia40, which is a 40kDa protein responsible for the insertion of disulfide bonds into the incoming precursor proteins. Mia40 (also known as Tim40) itself utilises the TIM23 pathway for its sorting in mitochondria. Through this pathway, Mia40 becomes anchored to the inner mitochondrial membrane by its N-terminus, with its C-terminal domain facing the IMS, which allows for the interaction of Mia40 with its substrates (Chatzi et al. 2013). Mia40 is a highly conserved protein within eukaryotes. However, it is important to note that although the *S. cerevisiae* Mia40 is anchored to the inner membrane, the human homologue

CHCHD4/MIA40 is soluble in the IMS, as it lacks the N-terminal 261 aminoacids, including the cleavable presequence and the transmembrane segment that anchors the yeast protein into the inner membrane (Hofmann et al. 2005).

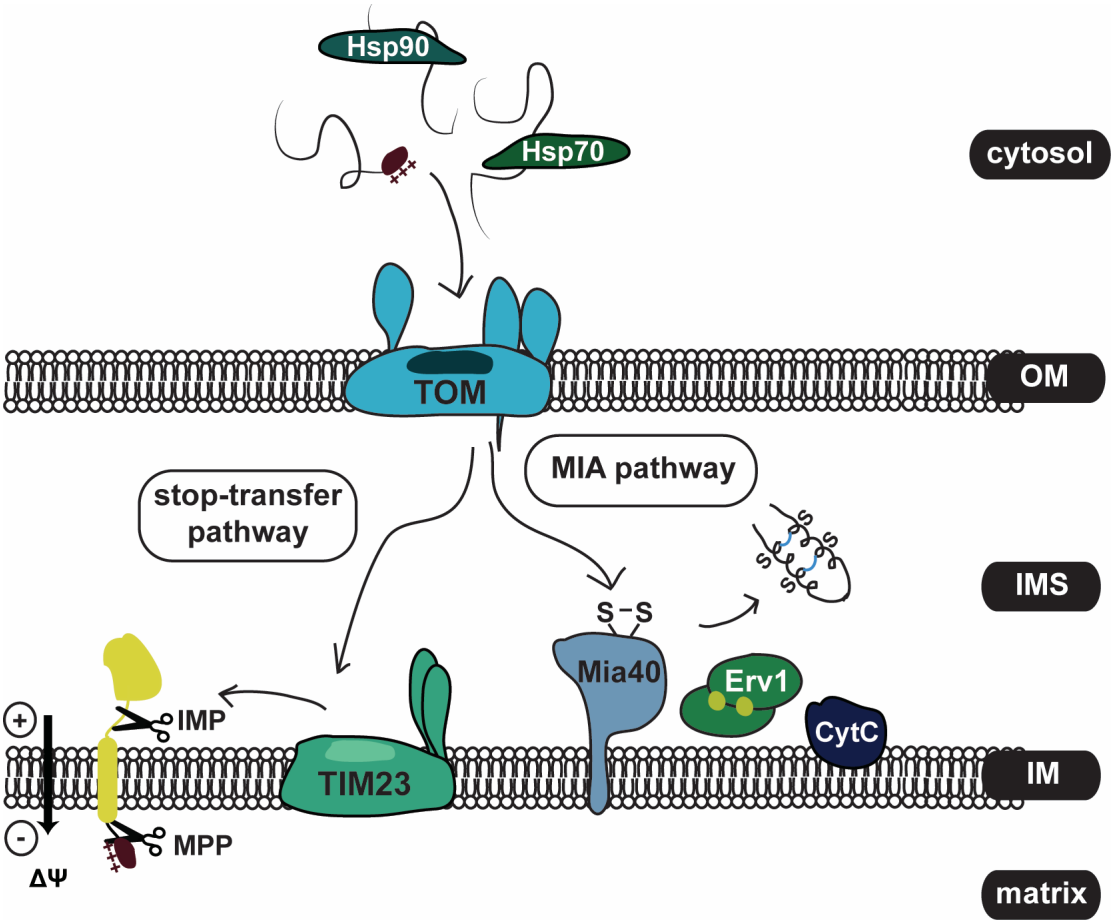

Figure 7: Protein import in the intermembrane space. IMS-targeted proteins typically utilise either a variation of the TIM23 pathway (known as "stop-transfer"), where the precursors become arrested at the TIM23 pore, due to the presence of a hydrophobic region. Through a two-step cleavage process involving MPP and IMP, the mature protein is released into the IMS. Alternatively, incoming precursors that contain cysteine residues interact with Mia40 and are retained in the IMS through the formation of disulfide bonds (figure adapted from Manganas et al. 2017).

Mia40 has the ability to recognise and bind to specific cysteine residues within the incoming precursors. These residues – hereby referred to as "docking" cysteines – are usually found upstream or downstream of certain intermembrane space-specific targeting signals that can be found in the imported substrates (Milenkovic et al. 2009; Sideris et al. 2009). The recognition of the substrates by Mia40 depends on the presence of these intermembrane space targeting signals (ITS), which are small, internal signals that have the

ability to form amphipathic α-helices, with all the hydrophobic residues on one side of the helix (Sideris et al. 2009). These signals have also been referred to as mitochondrial intermembrane space sorting signals (MISS) by another group, who identified such signals in Tim9 and Tim10 (Milenkovic et al. 2009). The amphipathic α-helix is extremely important as it is essential for the interaction with the hydrophobic cleft of Mia40, which is the first point of contact between the incoming protein and the disulfide donor.

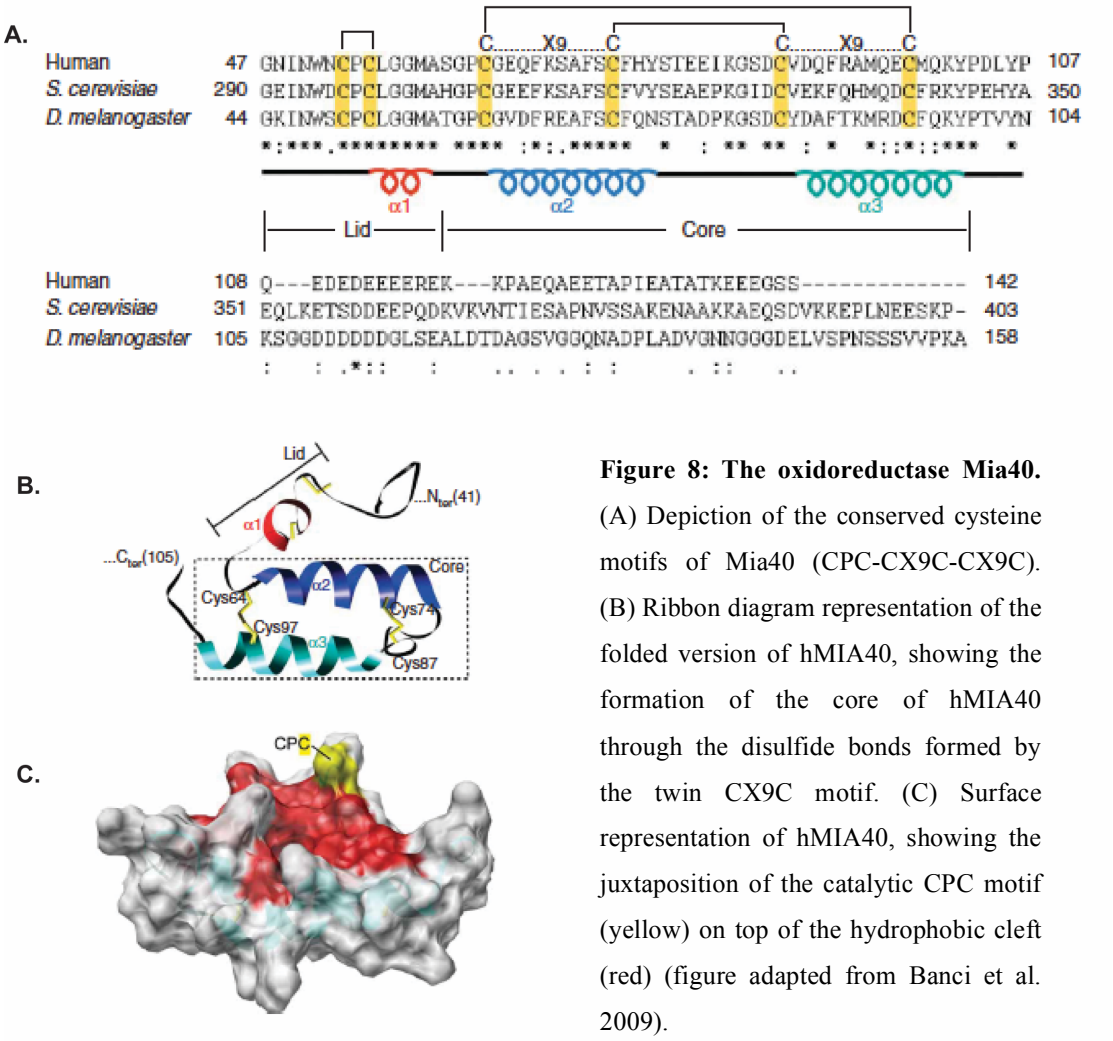

Figure 8: The oxidoreductase Mia40. (A) Depiction of the conserved cysteine motifs of Mia40 (CPC-CX9C-CX9C). (B) Ribbon diagram representation of the folded version of hMIA40, showing the formation of the core of hMIA40 through the disulfide bonds formed by the twin CX9C motif. (C) Surface representation of hMIA40, showing the juxtaposition of the catalytic CPC motif (yellow) on top of the hydrophobic cleft (red) (figure adapted from Banci et al. 2009).

The structure of Mia40 is characterised by two extremely important and functionally distinct elements: the hydrophobic cleft, which is important for protein binding, and the catalytic CPC motif, which is a redox active cysteine pair that can readily switch between an oxidised and a reduced state and which is positioned directly on top of the hydrophobic cleft (Banci et al. 2010). The hydrophobic cleft of Mia40 is a structure that is stabilised due

to the presence of two intramolecular disulfide bonds between the two CX9C motifs within the Mia40 protein (fig. 8) (Banci et al. 2009).

The introduction of disulfide bonds into the substrates has been proposed to occur in a two-step "sliding and docking" mechanism which requires the presence of both of the Mia40 structural elements. The first step ("sliding") requires the formation of non-covalent, primarily hydrophobic interactions between the hydrophobic targeting signal of the substrate and the binding cleft of Mia40 (Milenkovic et al. 2009; Longen et al. 2009; Sideris et al. 2009; Sideris and Tokatlidis 2010). Through this process, the cysteine residues of the substrate protein are placed in an ideal position directly juxtaposed to the oxidised cysteines of the catalytic CPC motif of Mia40. The second step ("docking") consists of the formation of a mixed disulfide intermediate between the second cysteine residue of the CPC motif and the docking cysteine of the substrate protein. In order for the protein to be released from Mia40, a nucleophilic attack by the partner cysteine of the substrate protein occurs and leads to the formation of an intramolecular disulfide bond in the substrate. Through this process, Mia40 itself becomes reduced and requires re-oxidation in order to regain its functionality (fig. 9). Essentially, what happens during this two-step process is the transfer of the disulfide bond of Mia40 to the substrate protein, an occurrence that assists with the folding and retention of the substrate in the IMS (Sideris and Tokatlidis 2010; Banci et al. 2010).

The second component of this pathway is Erv1, the protein responsible for the recycling of Mia40. Erv1 is a flavin adenine dinucleotide (FAD)-binding sulfhydryl oxidase. It contains three conserved cysteine pairs: C30/C33, C130/C133 and C159/C176. The C30/C33 pair is the one responsible for the interaction with Mia40 and is also called the shuttle disulfide (Lionaki et al. 2010). The third pair (C159/C176), which is also known as the structural disulfide, is important during the import process of Erv1 itself. Erv1 is a substrate of Mia40, even though it shares no structural similarities with any of the other Mia40 substrates, and the third cysteine pair is the one recognised by Mia40 during the import of Erv1 (Terziyska et al. 2007). The transfer of the disulfide bond from Mia40 to Erv1 during this process is important for the correct folding of Erv1, as is the binding of the FAD moiety (Kallergi et al. 2012).

Once it has been imported and properly folded, Erv1 plays an essential role in the electron transfer process. The Erv1 dimer has the ability of interacting with Mia40 through thiol-

dislufide exchange reactions. This process occurs through the shuttle cysteine motif of Erv1, which recognises and interacts with the reduced CPC motif of Mia40 and leads to the re-oxidation of the latter. The electrons that were removed from Mia40 to the shuttle motif during this process are transferred on to the FAD-proximal cysteine pair (C130/C133). From there, they are further transferred to the FAD moiety itself, which is responsible for the shuttling of the electrons to molecular oxygen either directly, in a process that leads to the production of hydrogen peroxide (H_2O_2), or indirectly through cytochrome c and the respiratory chain, which leads to the formation of H_2O as a by-product (fig. 9) (Farrell and Thorpe 2005; Ang and Lu 2009; Bien et al. 2010; Banci et al. 2011). Alternatively, other proteins, such as the cytochrome c peroxidase Ccp1, also have the ability to act as final electron acceptors (Dabir et al. 2007).

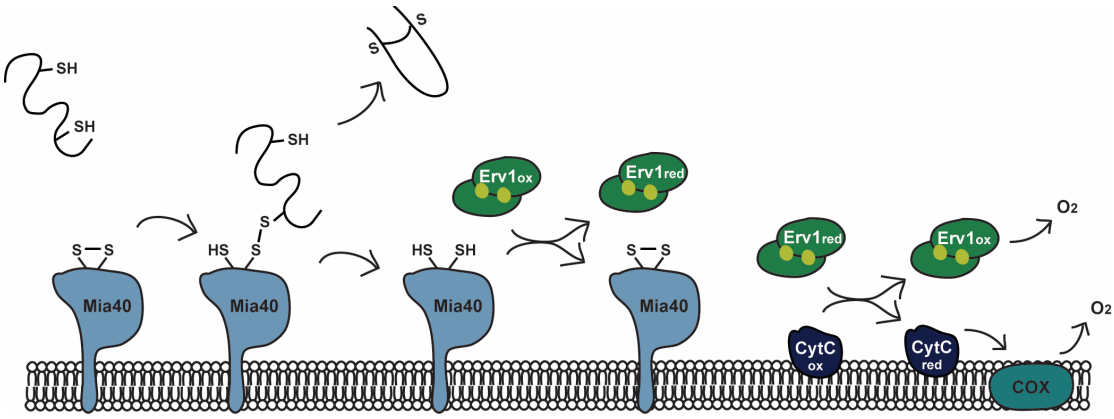

Figure 9: The oxidative folding pathway. The MIA pathway substrates, upon entering the IMS, interact with the oxidoreductase Mia40 through its catalytic CPC motif, thus creating mixed disulfide intermediates. Through this process, Mia40 transfers its disulfide bond to the substrate, leading to its release as a folded protein. Mia40 can be recycled back to its oxidised state through the function of the sulfhydryl oxidase Erv1. The electrons are transferred from Mia40, via Erv1, to molecular oxygen, either directly or indirectly (through cytochrome c and the respiratory chain).

The MIA pathway is also able to function under anaerobic conditions, but this particular matter has not been thoroughly investigated. It is unknown whether under these different conditions there are additional proteins (other than Mia40 and Erv1) that are involved in the process of disulfide bond formation. It is also worth mentioning that, even though Erv1 has not been found to have the capacity to directly interact with substrates and lead to their oxidation independently of Mia40, it has been proposed to form a so-called "ternary complex" *in organello*, together with Mia40 and the substrate (Stojanovski et al. 2008).

This particular hypothesis clashes with the proposed mechanism of "substrate mimicry", where Erv1 has been thought to interact with the CPC motif of Mia40 and re-oxidise it in a mechanism that is reminiscent of the interaction between Mia40 and its substrates (Banci et al, 2011). Further studies are required in order to elucidate the mechanism of interaction between the proteins involved in this pathway.

The third pathway that can be utilised by proteins destined to the IMS is a direct, "non-conservative" pathway, where certain proteins were found to become imported into the mitochondrial IMS in a one-step process that does not involve the inner mitochondrial membrane. Examples of such proteins are heme lyases, of which there are two identified in *S. cerevisiae* – the cytochrome c heme lyase (CCHL) and the cytochrome c_1 heme lyase (CC_1HL). Heme lyases are proteins that are involved in c-type cytochrome biogenesis and are localised in the intermembrane space, peripherally associated with the inner membrane (Steiner et al, 1995). These proteins traverse the outer mitochondrial membrane through the general import motor (TOM complex), with the receptor components Tom20 and Tom22 required for the recognition of the precursors (Mayer et al. 1995; Künkele et al. 1998; Diekert et al. 1999). The import and folding of the two proteins occcur independently of both the inner membrane potential and the presence of ATP, with the folding of the protein seeming to occur either during or immediately after the import, though without it being the driving force that provides the energy for the import to occur (Steiner et al. 1995). Additionally, the use of peptidylprolyl *cis-trans* isomerases was unable to inhibit the folding process, indicating that it occurs independently of any homologues of the known folding chaperones (Hsp60, Hsp70) (Steiner et al. 1995). Work that was done with the *Neurospora crassa* CCHL protein was able to identify an internal targeting signal within the protein that is necessary and sufficient for the import of non-mitochondrial proteins to the IMS (Diekert et al. 1999). This was shown to be a conserved feature, as the corresponding area of the *S. cerevisiae* CC_1HL was also sufficient for targeting (Diekert et al. 1999).

The import of the proteins mentioned above is an essential component of the final import pathway that has been found to apply for the translocation of apocytochrome c. Similarly to CCHL and CC_1HL, the import of apocytochrome c is not driven by the $\Delta\Psi$ or ATP. However, contrary to the heme lyases, apocytochrome c does not contain any targeting signals, nor does it depend on the receptor components of the TOM complex. Research was able to show that the import of this particular protein requires the interaction of the

apoprotein with the protease-resistant part of the TOM complex (Tom40), as well as the subsequent interaction with the heme lyase in the IMS. The interaction between the apocytochrome c and the CCHL results in the covalent attachment of a heme group to the apoprotein, a fact which leads to the final folding of the protein as cytochrome c and its irreversible retention in the IMS (Diekert et al. 2001). This discovery showed that there is a fourth pathway that can be followed for the import and retention of proteins in the IMS, which depends on the interaction of proteins with co-factors in order to be folded and become sorted into the correct compartment.

1.4. Oxidative stress in Saccharomyces cerevisiae

Reactive oxygen species (ROS) are molecules that arise from molecular oxygen and have been extensively studied concerning their role and involvement in oxidative stress. Examples of such molecules are hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and the hydroxyl radical (OH^-). Mitochondria themselves are major sources of ROS, alongside the endoplasmic reticulum and peroxisomes. ROS can be produced as a result of physiological cellular processes (such as the formation of disulfide bonds, as was discussed earlier in the text) or as a cellular response to ionising radiation (Margittai et al. 2012).

Hydrogen peroxide is one of the most studied ROS, both for the damaging effect it can have on proteins and DNA, as well as its more recently described role as a signalling molecule. The latter has been a source of much experimentation, in order to elucidate the role it plays in essential cellular processes such as the oxidation of cysteines and cellular proliferation, while also trying to determine the regulating elements responsible for the control of this toxic chemical that can eventually lead to apoptosis and cell death (Gough and Cotter 2011).

Research has shown that the majority of cellular ROS (up to 85%) is produced from the mitochondria (Aon et al. 2012). The production of ROS generally begins with the generation of the superoxide anion, which is rapidly converted to hydrogen peroxide and molecular oxygen, either spontaneously or through the function of the superoxide dismutase (Bray et al. 1974). The mitochondrial respiratory chain is thought to be the main source of reactive oxygen species, due to the process of oxidative phosphorylation

(Murphy 2009). The leakage of electrons during this process can lead to the reduction of oxygen, thus generating the ROS. Additionally, the oxidative folding process in the mitochondrial IMS, with oxygen acting as the final electron acceptor in a direct manner, can lead to the production of H_2O_2 (Ang and Lu 2009; Bien et al. 2010). The same is also true in the case of oxidative protein folding occuring in the endoplasmic reticulum (Tu and Weissman 2004).

It has been stated that oxidative stress is such a multi-faceted concept, that it cannot be explained through the extensive study of any one patricular oxidant (Temple et al. 2005). As such, each individual oxidant must be studied seperately in order to determine the molecular mechanisms it activates, as well as the ways in which it affects cellular function as a whole. Nonetheless, H_2O_2 remains the most widely used oxidant molecule, due to both its ease of use and its solubility in water. The removal of H_2O_2 from cells is essential due to its ability to participate in Fenton and Haber-Weiss reactions, which lead to the generation of highly reactive hydroxyl radicals (OH^-) (Morano et al. 2012).

As expected, all molecules within a cell are subject to the effects of oxidative stress. DNA can be affected by a variety of ROS and resulting damage can lead to an increased risk of cancer, along with other diseases. As such, it is vital for the oxidative damage to be repaired. Of the four base pairs, the most easily oxidised is guanine, with 8-oxo-7,8-dihydroguanine (8-oxoG) being the most common of all DNA lesions arising during highly oxidative conditions (Kino et al. 2017). Oxidative DNA damage repair is essential in order to preserve the genetic information and ensure that it is passed on from each generation to the next without changes. To this date, there have been more than 100 different DNA repair enzymes that have been discovered (Cadet and Davies 2017), including the ones involved in the widely known base excision repair (BER) and nucleotide excision repair (NER) pathways.

Lipids – especially polyunsaturated fatty acids (PUFAs) and cholesterol – are also susceptible to the effects of oxidative stress within the cell (Ademowo et al. 2017). The maintenance of lipids in a non-oxidised state is vital to cellular survival, as the lipids are responsible for maintaining the integrity of the cellular membranes. As such, it is of utmost importance for the cell to avoid lipid peroxidation, as this can alter the composition and structure of the membranes, as well as their functionality (Gaschler and Stockwell 2017). Lipid peroxides are especially problematic, due to their highly reactive nature that can

either lead to lipid peroxidation directly, or can result in the formation of higher quantities of ROS and other degradation products that can go on to affect DNA and proteins (Gaschler and Stockwell 2017). As is also the case with DNA, the severe effects oxidative stress can have on lipids necessitate the presence of repair mechanisms that can either prevent the lipid peroxides from being formed (such as the 5-lipoxygenase inhibitor Zileuton) (Bell et al. 1992; Steinhilber and Hofmann 2014) or eliminate the ones that have already been synthesised (such as the glutathione peroxidase enzymes) (Avery and Avery 2001).

Finally, proteins are also major targets for a number of different types of oxidation, as they contain 20 amino acid side chains, as well as the backbone, all of which can be oxidatively damaged (Davies 2016). Though all amino acids can become oxidised, our main focus tends to fall on the oxidation of cysteine residues, due to the fact that it leads to the formation of disulfide bonds.

1.5. Enzymatic and non-enzymatic defences for cell detoxification

It is important for normal cellular function that the cell maintains its redox homeostasis. As such, cells have developed a series of mechanisms in order to protect themselves from the adverse effects of redox imbalance. These mechanisms can either be non-enzymatic and involve molecules such as glutathione and ascorbic acid, or enzymatic and depend on proteins such as superoxide dismutases and catalases.

1.5.1. Non-enzymatic

1.5.1.1. Glutathione

Glutathione itself is a very important molecule for cellular function and has been proposed to be involved in a series of different cellular functions ranging from nucleic acid and protein synthesis to regulation of the activity of certain enzymes. It has also been studied quite extensively regarding its role in the metabolism of ROS (Schafer and Buettner 2001).

It is a metabolite that has been found to be essential for yeast viability and has an active role in the reduction of proteins even during normal growth conditions (Grant et al. 1996). Glutathione (γ-glutamylcysteinylglycine, GSH) is a molecule synthesised in the cystosol through two consecutive reactions in an ATP-independent manner. In the first step, glutamate and cysteine are linked to form a dipeptide through the function of γ-glutamylcysteine synthase (Gsh1), while the second step involves the addition of a glycine to the γ-Glu-Cys dipeptide and is a process mediated by glutathione synthase (Gsh2) (Lisowsky 1993; Grant et al. 1997). After it is synthesised, it is distributed to all cellular compartments, where it fulfills its role of the main cellular redox regulator.

However, despite the majority of published work indicating that GSH is a major cellular regulator, one group has proposed an alternative hypothesis whereby GSH is crucial for iron metabolism, with its role in redox control being only a secondary feature (Kumar et al. 2011). They proposed that despite redox control requiring a significant amount of GSH, it only serves as a backup for the thioredoxin system. On the other hand, iron metabolism only requires trace amounts of GSH, with these trace amounts being vital to the process. They further postulated that the reason why there are such high amounts of GSH in the cytosol, when its only vital function is in iron metabolism, is the safeguarding of its essential function in iron metabolism under stress conditions, where the cells are utilizing the majority of the cellular glutathione pool to combat the stress (Kumar et al. 2011).

Glutathione can be found in two pools within the cell: reduced glutathione (GSH) and oxidised glutathione (GSSG). Many studies have focused on measuring the levels of each one and figuring out the ratio between the two pools, as this can be indicative of the different functions carried out in the different cellular compartments. This measurement can also elucidate the different requirements for glutathione depending on the compartment/organelle, which can be associated with the differing levels of redox-sensitive proteins (Chatzi et al. 2016). Such measurements have shown that the cytosol is primarily a reducing environment with a GSH:GSSG ratio of 3000:1, while the endoplasmic reticulum is a much more oxidising environment with a GSH:GSSG ratio between 1:1 and 3:1 (Østergaard et al. 2004). There have also been attempts to measure the GSH:GSSG ratio in the mitochondria, a process that is quite challenging due to the presence of two compartments with very different characteristics: the IMS and the matrix. A study performed in 2008 was able to show that the IMS is much more oxidising than the matrix (IMS GSH:GSSG ratio → 250:1, matrix GSH:GSSG ratio → 900:1), a discovery

which the authors attributed to the fact that the IMS is host to a large number of proteins that are involved in redox pathways (Hu et al. 2008).

The most powerful tools that are used in order to measure the glutathione redox potential *in vivo* are the redox-sensitive fluorescent proteins, such as rxYFP and the roGFPs (Kojer et al. 2012). Their ability to detect the GSH:GSSG ratio depends on their catalysis by the dithiol glutaredoxins that are present in each compartment tested (Østergaard et al. 2004). As such, it became necessary to fine-tune the probes and create a detection system that is more dynamic and corresponds to the real-time conditions of each local pool of glutathione. This was achieved through the fusion of the human glutaredoxin 1 to a roGFP (Grx1-roGFP2), thus creating a probe that is able to equilibrate with the local glutathione pool in a rapid manner and detect small changes in the glutathione redox potential (Meyer and Dick 2010; Kojer et al. 2012).

A 2012 study found that the glutathione pools of the cytosol and the IMS are connected through the porins in the outer mitochondrial membrane and that the two compartments are closely connected, with the cytosolic Glr1 playing an important role in the regulation of the glutathione equilibrium in the IMS (Kojer et al. 2012). This is contrary to previous results that suggested that the two glutathione pools are independently regulated (Hu et al. 2008). This particular finding led to a rather puzzling question: How do the IMS proteins become (and remain) oxidised when the glutathione pool in this compartment is so reducing? Work by Kojer and coworkers was able to answer this question by identifying the presence of low amounts of cytosolic Grx2 in the IMS (Kojer et al. 2015). The formation of disulfide bonds in the IMS is the result of an equilibrium between the reducing glutathione pool and the limited amounts of Grx2 in the IMS, which prevents the rapid reduction of proteins. This comes in stark contrast with the cytosol, where the equally reducing glutathione pool, combined with the high glutaredoxin activity, are able to maintain all cysteine containing proteins in a reduced state (Kojer et al. 2015). This study utilised the roGFP2 probe fused to a b_2 presequence for IMS targeting, in order to assess the redox state of the intermembrane space in a more specific manner. Through the parallel use of single and multiple glutaredoxin mutants, they were able to detect that the IMS possesses glutaredoxin activity, which is exerted by the very limited amounts of Grx2 that are present within the compartment (Kojer et al. 2015). Through this particular study, the researchers were able to assess the differences between and the limitations of each type of redox-sensitive probe. More specifically, they concluded that the unfused probes are able to sense

how oxidising or reducing the environment is in the same way that the thiol residues in proteins do, while the fused Grx1-roGFP2 probe is the only one that truly measures the glutathione redox potential in any given compartment (Kojer et al. 2015), thus explaining why the two previous publications had measured a different glutathione redox potential in the IMS (Hu et al. 2008; Kojer et al. 2012).

Δgsh1 mutants have been a good model for the study of glutathione in yeast cells. These particular cells are unable to grow without the addition of external GSH to their growth medium, and one of the only ways to study them is to grow the cells for a certain period of time with GSH provided and then transfer them to a growth medium that is lacking GSH. This way, the *Δgsh1* cells are able to go through a small number of cell divisions, due to the amounts of GSH that have accumulated within the cells during the time they grew in glutathione-containing media (Spector et al. 2001; Lee et al. 2001). The results from experiments involving this particular strain were able to show that these mutants are sensitive to the majority of cellular oxidants (including hydrogen peroxide and the superoxide anion) as well as heavy metals (Grant et al. 1996; Thorsen et al. 2009).

1.5.1.2. Ascorbic acid

Ascorbic acid is the other non-enzymatic defense mechanism utilised by yeast, as well as other eukaryotic cells. It is water-soluble and plays the role of an antioxidant within the cell. L-ascorbic acid (also known as vitamin C) has been quite well characterised in mammalian cells for its role as an antioxidant molecule, with experiments showing that it can act as quite a powerful antioxidant. It has been shown that vitamin C protects the cells against oxidative injury by reducing oxidative cell death, inhibiting FAS-induced apoptosis and protecting the genome through the quenching of ROS (KC et al. 2005). Ascorbic acid can be transported into cells in its oxidised form, dehydroascorbic acid, through facilitative glucose transporters (Vera et al. 1993). The same method is utilised for the transport of ascorbic acid into the mitochondria, with the facilitative glucose transporter Glut1 being responsible for the mitochondrial uptake of vitamin C (KC et al. 2005). Once inside mitochondria, vitamin C can act in a protective capacity, by preventing (a) the oxidative damage that the free radicals produced by the respiratory chain can cause the mtDNA molecules and (b) the loss of the inner mitochondrial membrane potential (KC et al. 2005).

S. cerevisiae itself does not contain ascorbic acid, but rather a 5-carbon analogue known as erythroascorbate or D-erythroascorbic acid. It is synthesised from arabinose in a two-step process as shown in fig. 10. Deletion of either of the two enzymes involved in this biosynthetic pathway leads to an inability of the cell to produce erythroascorbic acid, a fact that signifies that these particular proteins are exclusively involved in erythroascorbate biosynthesis (Amako et al. 2006a; Amako et al. 2006b). The deletion of *ALO1* – the gene encoding for D-arabinono-1,4-lactone oxidase, the last step in erythroascorbate biosynthesis – does lead to an oxidative stress sensitive phenotype, specifically in the cases where the stress arises from increased levels of hydrogen peroxide and the superoxide anion (Huh et al. 1998).

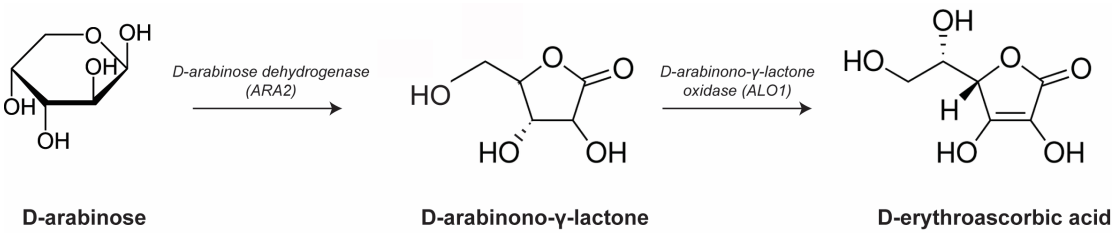

Figure 10: Chemical reaction showing the synthesis of D-erythroascorbic acid from D-arabinose in *S. cerevisiae*.

The main reason why erythroascorbate may not have as robust a role as an antioxidant when compared to its mammalian analogue is its limited availability within yeast cells (Spickett et al. 2000). Quantitative studies have shown that the levels of erythroascorbate in yeast cells are extremely low. It has been postulated that erythroascorbate could play a role as the main physiological reductant for single cysteine containing peroxiredoxins (Monteiro et al. 2007), though *in vivo* experiments showed no such requirement to support this claim (Greetham and Grant 2009).

1.5.2. Enzymatic

1.5.2.1. Superoxide dismutases

Saccharomyces cerevisiae contains two genes that encode for superoxide dismutases: *SOD1* and *SOD2*. These two proteins are localised in different cellular compartments, with Sod1 being localised mainly in the cytosol and Sod2 in the mitochondrial matrix (Fridovich 1995; Sturtz et al. 2001). The main role of these two proteins is to break down superoxide anions that can accumulate during normal cell function. The break down reaction performed by the superoxide dismutases leads to the formation of hydrogen peroxide, which is a substrate for other detoxifying mechanisms (fig. 11). Superoxide dismutases require the binding of specific metal cofactors in order to function properly: copper (Cu) and zinc (Zn) ions are required for Sod1 function, while Sod2 requires the binding of manganese (Mn).

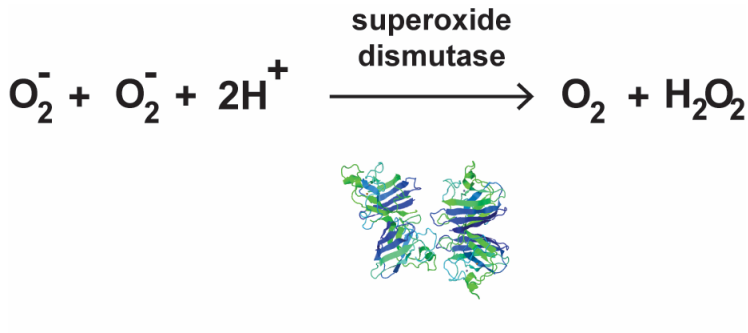

Figure 11: The reaction catalysed by the superoxide dismutases. Sod1 and Sod2 are responsible for breaking down superoxide anions in a reaction that leads to the production of hydrogen peroxide. [Sod1 structure was obtained from PDB (PDB code: 1SDY)]

1.5.2.2. Catalases

Catalases are enzymes that are able to break down hydrogen peroxide into water and molecular oxygen (fig. 12). The *S. cerevisiae* genome contains two genes that encode for two separate catalases: Ctt1, a catalase that can be found in the cytosol and Cta1, an enzyme that is dually localised in the peroxisomes and the mitochondrial matrix (Petrova

et al. 2004; Martins and English 2014). There have been a number of studies performed in order to assess the exact role of catalases within the cell, a task which has proven to be quite challenging due to the fact that the deletion of these two genes from the yeast genome does not lead to a strong enough phenotype to enable their complete characterisation (Grant et al. 1998). One study has suggested that, though catalases may not be particularly important in the scavenging of hydrogen peroxide during the exponential growth phase, they do seem to play the role of an emergency response mechanism during stress conditions, when the cells are in the stationary phase. Their results pointed out that catalase is essential for the cells to obtain maximum tolerance to H_2O_2 during the stationary phase, with the localisation of the catalase not affecting cellular tolerance, as Cta1 and Ctt1 were able to compensate for one another (Izawa et al. 1996). A more recent study showed that the cytoplasmic catalase, Ctt1, is an essential component of the H_2O_2 response when the cells are grown in nutrient-rich YPD media. More specifically, they showed that challenging cells growing in YPD with H_2O_2 led to an increase in the activity of Ctt1, while the same challenge when the cells were starved using KPi resulted in Ctt1 not being upregulated as part of the oxidative response (Martins and English 2014). The researchers attributed this phenomenon to the fact that catalases are synthesised *de novo* when the H_2O_2 challenge arises, a process which requires the presence of nutrients and the cells to be grown on rich media (Martins and English 2014).

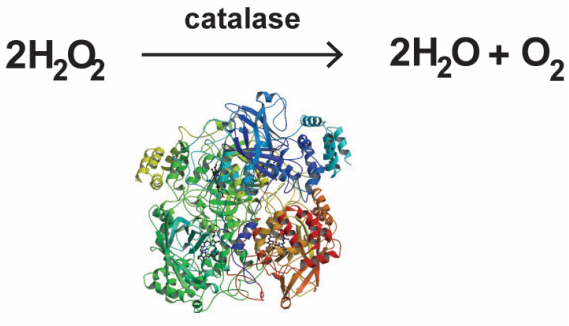

Figure 12: The reaction catalysed by catalases. Catalases are able to recognise hydrogen peroxide and break it down to water and molecular oxygen. [Cta1 structure was obtained from PDB (PDB code: 1A4E)]

1.5.2.3. The glutaredoxin system

The thioredoxin and glutaredoxin systems work together within the cell in order to maintain a reduced environment and, as such, also play a significant role in detoxifying the

cell and protecting it against oxidative stress. The function of these enzymes relies on the formation of disulfide bonds within their active sites. The glutaredoxin system consists of glutathione peroxidases, glutathione transferases and glutaredoxins, which will be discussed below.

In *S. cerevisiae*, there are three members in the glutathione peroxidase family: Gpx1, Gpx2 and Gpx3. These three proteins are not classical Gpxs (cGpx), but rather phospholipid hydroperoxide Gpxs (PHGpx) (Morano et al. 2012), with the ability to reduce phospholipid and non-phospholipid hydroperoxides and protect the cell from lipid peroxidation. Studies have shown that all three yeast PHGpxs behave more as atypical 2-Cys peroxiredoxins as opposed to Gpxs, as they form an intramolecular disulfide bond that is cleaved by thioredoxin as part of their catalytic cycle, as opposed to Gpxs, that utilise the reducing power of GSH (Delaunay et al. 2002; Tanaka et al. 2005; Ohdate et al. 2010). Of the three genes, only *GPX3* is constitutively expressed, with *GPX1* being induced under glucose starvation and *GPX2* becoming induced upon conditions of oxidative stress, where it is activated by Yap1 (Inoue et al. 1999). Gpx1 is a phospholipid-hydroperoxide glutathione peroxidase and can be found associated with the outer surface of the outer mitochondrial membrane (Inoue et al. 1999; Avery and Avery 2001). This localisation is important as it protects the cell from lipid peroxidation by ROS on the outer membrane of the mitochondria and helps maintain its permeability and function. Gpx1 is the only one out of the three yeast Gpxs that utilises glutathione and the thioredoxin system equally in order to become reduced (Ohdate et al. 2010). Gpx2 performs a very similar function to that of Gpx1, but is localised on the inner surface of the inner mitochondrial membrane and protects from lipid peroxidation by ROS that are produced within the mitochondrial matrix (Inoue et al. 1999; Ukai et al. 2011). Gpx2 was shown to depend on the thioredoxin system *in vivo* for its reduction, with glutathione being able to provide the reducing power only in an *in vitro* experimental setup (Tanaka et al. 2005). The third member of this family, Gpx3, is a paralog of Gpx1 and has been shown to function in a very different way to the other two members, as it acts as a hydrogen peroxide sensor and scavenger in the cytosol (Delaunay et al. 2002; Paulsen and Carroll 2009). Due to the fact that Gpx3 has recently been found to be present in the mitochondrial intermembrane space proteome (Vögtle et al. 2012; Kritsiligkou et al. 2017), the mechanism through which Gpx3 is able to sense and scavenge H_2O_2 will be analysed in detail in a separate section (see section 1.6).

The yeast genome contains two genes that encode for functional glutathione transferases (GSTs), *GTT1* and *GTT2* (Choi et al. 1998). These proteins are responsible for the conjugation of substrates to GSH before the cells remove these substrates via glutathione conjugate pumps (Morano et al. 2012). Both genes are non-essential and both single null mutant,, as well as the double null mutant yeast strains, are viable. This could possibly be attributed to the fact that two of the yeast glutaredoxins (Grx1 and Grx2) are able to perform as GSTs with certain substrates, such as 1-chloro-2,4-dinitrobenzene (CDNB). Yeast cells also contain three omega class glutathione transferases, known as Gto1, Gto2 and Gto3 (fig. 13) (Garcerá et al. 2006; Barreto et al. 2006). Cells that lack these three genes are sensitive to hydroperoxides.

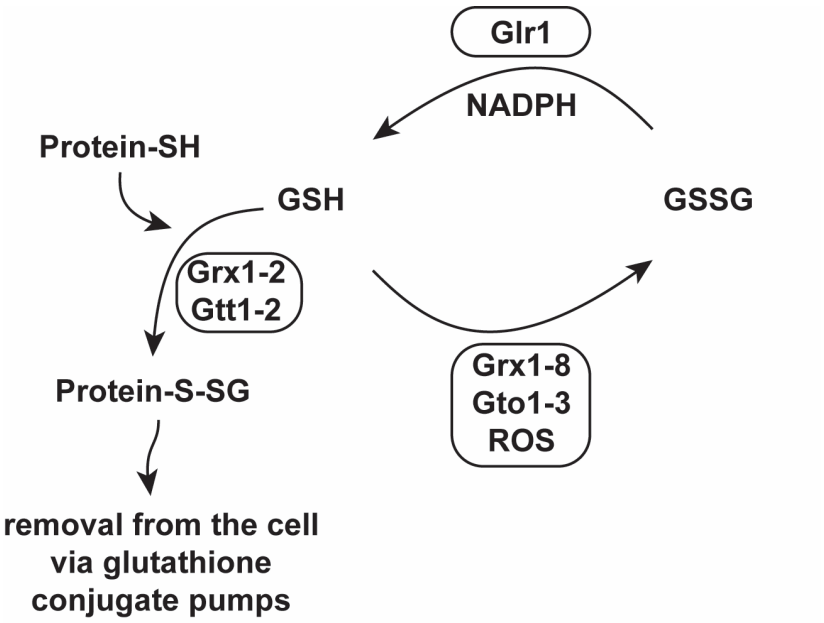

Figure 13: Overview of the glutaredoxin system. Glutathione can be oxidised through the function carried out by glutaredoxins and the omega glass glutathione transferases, as well as directly by ROS. The reverse reaction is catalysed by Glr1 in a NADPH-dependent reaction. GSTs can conjugate glutathione to proteins that need to be removed from the cell (figure modified from Morano et al. 2012)).

S. cerevisiae also contains 8 glutaredoxins, designated as Grx1-Grx8. As enzymes, glutaredoxins are oxidoreductases that seem to play important roles in many aspects of cellular function, such as protein regulation and sulfur metabolism (Holmgren 1989). Glutaredoxins can be classified as monothiol or dithiol, depending on the number of cysteine residues that are found within their active site (fig. 14) (Wheeler and Grant 2004). They are a focal part of the glutaredoxin system, as they are the acceptors of electrons

derived from NADPH (nicotinamide adenine dinucleotide phosphate), through the mediation of glutathione reductase (Glr1) and GSH (fig. 13).

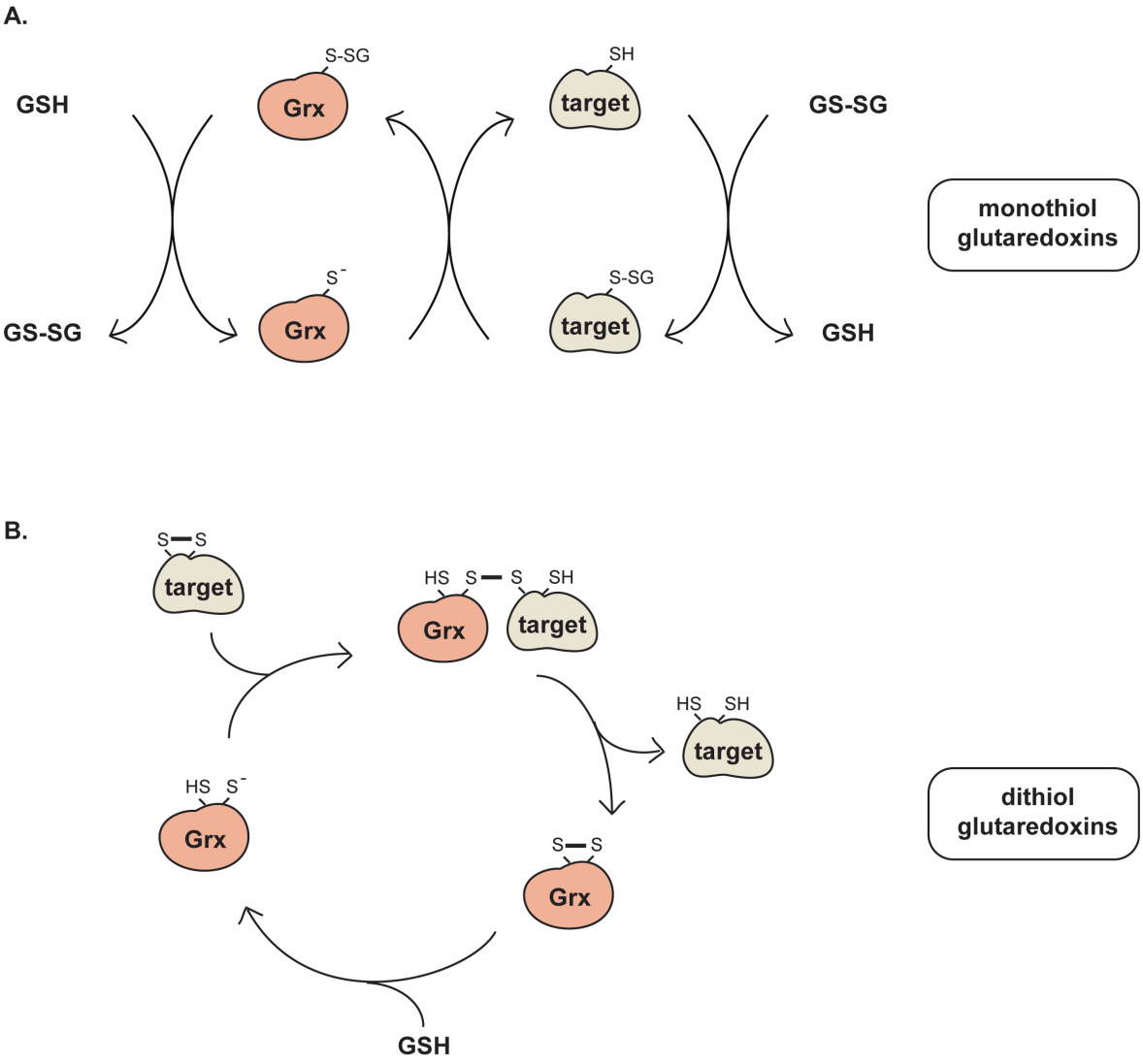

Figure 14: Mechanisms of monothiol and dithiol glutaredoxin function. (A) Monothiol glutaredoxins require only one active-site cysteine residue to reduce the mixed disulfide between the target protein and glutathione. (B) Dithiol glutaredoxins require two active-site cysteines, in order to form an intramolecular disulfide bond. This disulfide is subsequently reduced by GSH.

The dithiol glutaredoxins Grx1 and Grx2 are the two most studied members of the glutaredoxin family (Luikenhuis et al. 1998). Both are oxidoreductases that depend on GSH for their function, but differ in the cellular response they are involved in. *Δgrx1* cells are sensitive to oxidative stress that involves high levels of the superoxide anion, while *Δgrx2* cells are sensitive to hydrogen peroxide stress, indicating that the two proteins are

probably responsible for the regulation of different substrate proteins involved in the two distinct oxidative stress responses (Luikenhuis et al. 1998). Despite the fact that the two proteins share a high degree of homology, Grx2 seems to be responsible for the majority of the glutaredoxin activity in yeast cells (Grant 2001). The only other dithiol glutaredoxin is Grx8, however it is not believed to play a role in the response against oxidative stress (Eckers et al. 2009).

Grx3-7 are all monothiol glutaredoxins and play a variety of different roles within the cell. Grx3 and Grx4 are both localised in the nucleus, where they are involved in the trafficking of iron within the cell (Mühlenhoff et al. 2010). Grx5 is located in the mitochondrial matrix and is one of the proteins required for the assembly of [4Fe-4S] clusters in mitochondria (Rodríguez-Manzaneque et al. 2002). Grx6 and Grx7 are believed to play a part in the regulation of the oxidative state of sulfhydyl groups in proteins destined to be secreted (Mesecke et al. 2008; Izquierdo et al. 2008). Grx6 is localised in the ER and the Golgi, while Grx7 is localised mostly in the Golgi lumen. Both the *GRX6* and *GRX7* genes are upregulated during stress conditions induced by various stressors such as calcium (for Grx6), peroxides and sodium (for both Grx6 and Grx7), with different regulatory elements being responsible for their induction (Izquierdo et al. 2008).

1.5.2.4. The thioredoxin system

The other main system that, alongside the glutaredoxin system, is responsible for the maintenance of the redox balance of the cell is the thioredoxin system. This system is made up of two different types of enzymes: thioredoxins and thioredoxin reductases. The *S. cerevisiae* proteome contains three thioredoxins (Trx1, Trx2, Trx3) and two thioredoxin reductases (Trr1, Trr2). These enzymes actually make up two complete thioredoxin systems: the cytoplasmic system, which comprises of Trx1, Trx2 and Trr1, and the mitochondrial matrix thioredoxin system, with Trx3 and Trr2 working to detoxify this particular subcompartment.

Trxs, just like Grxs, are able to reduce disulfide bonds through a thiol-disulfide exchange reaction catalysed by the characteristic CX2C motif within their active site. These two cysteine residues form an intramolecular disulfide bond during the reduction of the

substrate protein. Oxidised Trx1 is recycled back to its active form through an interaction with the thioredoxin reductase, with the electrons that fuel this process coming from NADPH (fig. 15) (Toledano et al. 2013).

As is the case with most organisms, yeast thioredoxins have central roles in the detoxification process and the protection of the cell against ROS-induced oxidative stress (Kuge and Jones 1994; Izawa et al. 1999). Okazaki and coworkers were able to show that Trx1 and Trx2 become rapidly oxidised upon exposure of the cells to H_2O_2 and are detected for longer than 1 hr before becoming reduced (Okazaki et al. 2007)

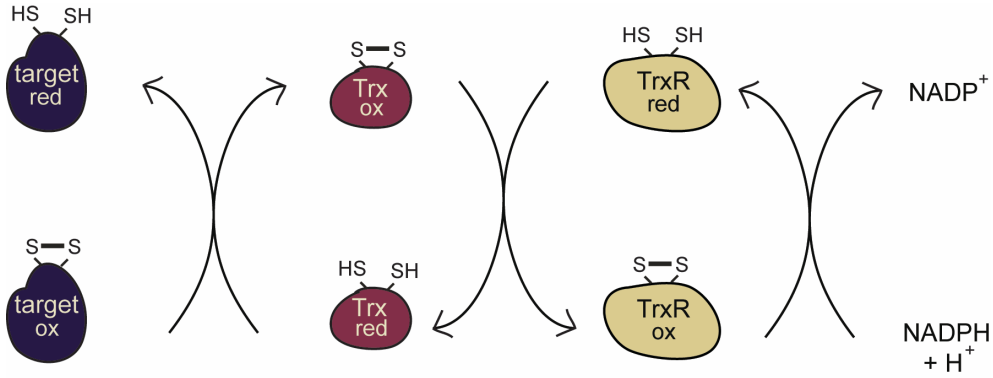

Figure 15: The thioredoxin pathway. Thioredoxins (Trx) – when in their reduced form – can interact with oxidised proteins in order to reduce them. In turn, Trx proteins can become reduced through interactions with thioredoxin reductases (TrxR). TrxR proteins are recycled by obtaining electrons from NADPH, in order to restore them to their active, reduced form.

Of the two cytoplasmic thioredoxins, Trx2 seems to be the one that possesses the main antioxidant activity, a fact which can be associated with the different ways in which the two genes are regulated and expressed. More specifically, Trx2 expression is significantly upregulated during oxidative stress conditions, as the *TRX2* gene is one of the ~100 genes regulated by the transcription factor Yap1 (the way in which this protein is activated as part of the oxidative stress response will be described in more detail below) (Paulsen and Carroll 2009). Additionally, *Δtrx2* cells show a hypersensitive to hydrogen peroxide phenotype, while *Δtrx1* mutants behave very similarly to the wild type (wt) cells when exposed to oxidative stress inducing conditions (Garrido and Grant 2002).

The last component of the cytosolic thioredoxin system is Trr1, a protein that was originally isolated and characterised because of its ability to reduce thioredoxin peroxidase

(Tsa1) (Chae et al. 1994). *Δtrr1* yeast strains also display hypersensitivity to H_2O_2 (Machado et al. 1997).

The mitochondrial thioredoxin system, which is resident in the mitochondrial matrix, consists of Trx3 and Trr2, which are responsible for the protection of the mitochondria from the oxidative stress-causing agents that are generated during respiration (Pedrajas et al. 1999). Surprisingly, the loss of Trr2 does not affect the oxidative state of Trx3, with experimental evidence showing that Trx3 accumulates in an oxidised state only in cells that lack both Trr2 and Glr1 (Trotter and Grant 2005). Further studies on the mitochondrial thioredoxin system revealed that although *Δtrx3* mutants do not seem to be affected by oxidative stress, *Δtrr2* mutants display a sensitive phenotype (Pedrajas et al. 2000; Trotter and Grant 2005). As such, it has been postulated that Trr2 plays an antioxidant role in the matrix that is independent of Trx3, which could be the maintenance of the antioxidant activity of Prx1, in a process that also includes GSH and Glr1 (Greetham and Grant 2009).

There have been a number of studies that have proposed a functional overlap between the thioredoxin and the glutaredoxin systems, with very interesting results. Cells that lack the complete cytosolic thioredoxin system (*Δtrx1/trx2/trr1*) or the genes responsible for the synthesis of glutathione (*Δgsh1/glr1*) are both completely viable, though mutants that have certain components of both systems deleted (eg. *Δtrx1/trx2/gsh1*) are not able to survive (Trotter and Grant 2002; Trotter and Grant 2003). The same question was postulated regarding whether there may be a functional overlap between the two thioredoxin systems. Work performed by Trotter & Grant showed that cells that lack either all the thioredoxins (*Δtrx1/trx2/trx3*) or all the thioredoxin reductases (*Δtrr1/trr2*) are able to survive, indicating that neither of the two systems is essential for cell viability (Trotter and Grant 2005). Furthermore, the lethal phenotype that is observed when both the thioredoxin and the glutaredoxin systems in the cytosol are compromised (*Δtrx1/trx2/grx1/grx2*) is indicative of the fact that the mitochondrial thioredoxin system can not compensate for the loss of either of the two cytosolic redox control systems (Draculic et al. 2000).

Another protein family that is part of the thioredoxin superfamily is the peroxiredoxin family. Peroxiredoxins (Prx) act as antioxidants, chaperones and signal transducers (Wood et al. 2003). Just like thioredoxins, Prxs utilise cysteine residues in their active sites that are redox-active in order to reduce peroxides and, much like the glutaredoxins, are

classified as 1-Cys or 2-Cys peroxiredoxins, according to the number of redox active cysteine residues (fig. 16) (Morano et al. 2012).

2-Cys peroxiredoxins are active when forming a dimer and their function is carried out through the oxidation of a cysteine on one of the monomers into a sulfenic acid group and the subsequent formation of a disulfide bond with the resolving cysteine of the second monomer (Morano et al. 2012). S-sulfenylation is a reversible post-translational modification, where the free thiol of a cysteine residue (Cys-SH) is oxidised to sulfenic acid (Cys-SOH) through the action of an oxidising agent (Gupta et al. 2016). This is a transient state of the cysteine residue and has an important role in the eventual formation of a disulfide bond. However, conditions of excessive and/or chronic stress, can convert the sulfenic acid group into the non-reversible forms of sulfinic (Cys-SO_2H) and sulfonic (Cys-SO_3H) acid (Gupta et al. 2016).

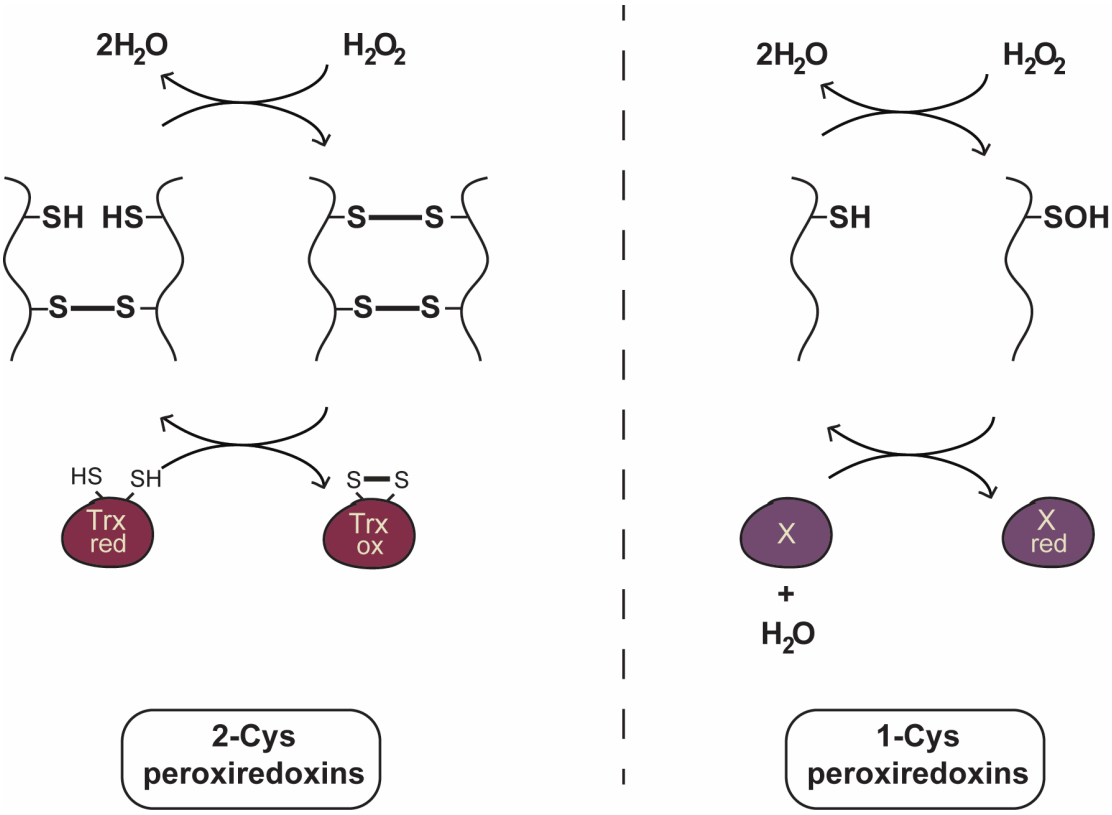

Figure 16: 1-Cys versus 2-Cys peroxiredoxins. 1-Cys peroxiredoxins have one redox active cysteine residue, while 2-Cys ones have two. Shown above is the proposed mechanism of peroxiredoxin function.

There are three cystoplasmic 2-Cys peroxiredoxins that display thioredoxin peroxidase activity that have been described in yeast: Tsa1, Tsa2 and Ahp1 (Morgan and Veal 2007).

All three have been characterised as antioxidants, with Tsa1 and Tsa2 having greater catalytic efficiency against H_2O_2 and Ahp1 being more efficient against alkyl hydroperoxides (Park et al. 2000; Garrido and Grant 2002; Jang et al. 2004). Tsa1 and Tsa2 are highly homologous and are capable of performing similar activities, though Tsa2 is expressed at much lower levels. Dot5 is another 2-Cys peroxiredoxin that can be found in the nucleus of yeast cells and is efficient at targeting alkyl hydroperoxides (Cha et al. 2003). Dot5 was originally found as one of the *DOT* (disruptor of telomeric silencing) genes (Singer et al. 1998). Though it was later re-discovered as a nuclear thioredoxin peroxidase (TPx) (Park et al. 2000), Dot5 was found to not have a very significant role in the antioxidant response against peroxide stress, as it cannot compensate for the lack of the cytosolic peroxiredoxins (when expressed ectopically in the cytosol), nor can it regulate the activity of Yap1 (Izawa et al. 2004). Additionally, it was found that deletion of the cysteine residues that are responsible for thioredoxin peroxidase activity (and are conserved throughout other TPxs) are not required for the telomeric silencing function of Dot5 (Izawa et al. 2004).

On the other hand, there is a single 1-Cys Prx that has been found and that is the mitochondrial Prx1 (Pedrajas et al. 2000). Prx1, as a 1-Cys peroxiredoxin, contains one cysteine residue, which means that it has no way of forming a disulfide bond. As such, the sulfenic acid intermediate that is formed in the case of 1-Cys peroxiredoxins is resolved through a reaction either with thioredoxins or with reduced glutathione, which creates a glutathionylated Prx1 (Morano et al. 2012). This form of Prx1 is recycled through its interaction with Trr2 (Greetham and Grant 2009) or Grx2 (Pedrajas et al. 2010). This finding is very important, as it further backs up the claims that the two main reductive systems, the glutaredoxin and the thioredoxin systems, are functionally overlapping.

1.6. The Yap1 activation pathway

Glutathione peroxidase 3 (Gpx3) is an 18kDa thiol peroxidase that acts as a receptor of H_2O_2 in the yeast cytoplasm. The role of this protein is to sense the levels of H_2O_2 within the cell and utilise it in order to activate a series of genes essential for the oxidative stress response (Delaunay et al. 2002). This particular protein is also known as Hyr1 (hydroperoxide response 1) or Orp1 (oxidant receptor peroxidase 1), names that have both

been proposed due to the fact that Gpx3 does not actually utilise glutathione in order to function, despite its similarities to the other two yeast glutathione peroxidases (Delaunay et al. 2002). As mentioned previously, Gpx3, along with Gpx1 and Gpx2, have been proposed to be classified as atypical 2-Cys peroxiredoxins (Ohdate et al. 2010).

In *S. cerevisiae*, one of the main regulators of the oxidative stress response is the transcription factor Yap1 (yeast AP-1). Yap1 is a 72kDa protein that belongs to the bZIP (basic leucine zipper) family of transcription factors. This particular protein is activated as part of the initial cellular response to rising levels of H_2O_2 or to the presence of other oxidising factors (eg. diamide) within the cell. This activation occurs through the formation of intramolecular disulfide bonds within Yap1, and, as a result, leads to its accumulation in the nucleus, where it can perform its function as a transcription factor. Yap1 is responsible for the transcriptional activation of approximately 100 genes, including *TRX2*, *TRR1*, and *TSA1* (Kuge and Jones 1994; Delaunay et al. 2002; Paulsen and Carroll 2009).

The Yap1 transcription factor has a rather characteristic primary structure that can be seen in fig. 17 . It contains the bZIP DNA binding region at its N-terminus, as well as two separate cysteine rich domains (CRDs), the N-terminal CRD (n-CRD) and the C-terminal CRD (c-CRD). Yap1 also contains a nuclear localisation signal (NLS) and a non-typical leucine-rich nuclear export signal (NES), that is in the same protein region as the c-CRD.

Under physiological cell conditions, Yap1 enters the nucleus, due to the presence of the NLS in its N-terminal region (Kuge et al. 1997). However, the protein is recognised by the nuclear exportin Crm1 through its NES region, a process that leads to the export of Yap1 from the nucleus and the lack of transcriptional activation. When the cell is undergoing oxidative stress, it has been shown that the formation of disulfide bonds between the n- and c-CRD regions or within the c-CRD region alone lead to the masking of the NES of Yap1. This process leads to the inability of the nuclear exportin to recognise this particular protein and thus, Yap1 is retained in the nucleus and can bind to the correct promoters in order to activate the transcription of the stress response genes (Yan et al. 1998; Mulford and Fassler 2011).

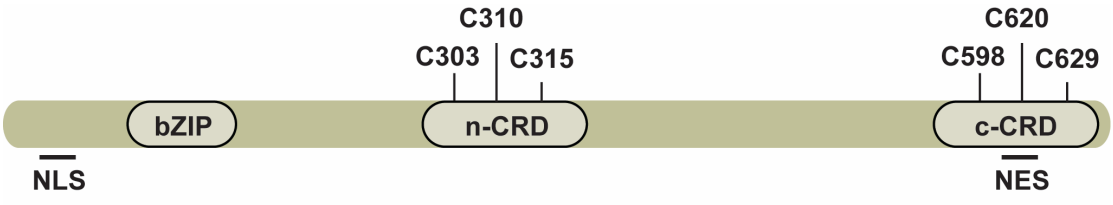

Figure 17: Primary structure of Yap1. Yap1 contains both a nuclear localisation signal (NLS), as well as a nuclear export signal (NES). The bZIP domain enables DNA binding. Yap1 also contains two cysteine-rich domains (CRDs).

It has been postulated that there are specific circumstances that lead to the formation of different disulfide bonds within Yap1. When the yeast cells are stressed with chemical oxidising agents such as diamide, a disulfide bond is formed between two of the cysteines within the c-CRD that leads to the masking of the export signal. The process through which this disulfide bond is formed has not yet been described in detail (Kuge et al. 1997; Gulshan et al. 2011). However, in the cases where the cells are stressed due to increasing concentrations of H_2O_2 in the cytosol, a different mechanism appears to be at work. The disulfide bond that is formed in this case requires cysteine residues from both CRDs and the presence of an additional protein, Gpx3.

The model that was proposed by Delaunay and coworkers in 2002 suggests that Gpx3 plays a dual role as a sensor and as a scavenger of hydrogen peroxide within the cytosol (fig. 18) (Delaunay et al. 2002). The first step in both cases is the same and entails the sulfenylation of the active cysteine residue of Gpx3 (C36) from a free thiol group (-SH) to a sulfenic acid group (-SOH). Once this has occured, the reaction can proceed in one of the two following ways:

a) Scavenger pathway: The sulfenylated C36 can react with the resolving C82 of Gpx3 itself, which leads to the formation of an intramolecular disulfide bond and thus, Gpx3 oxidation. Once Gpx3 is in its oxidised state, it can be recycled back to its reduced and fully functional state through the function of Trx1.

b) Sensor pathway: The sulfenylated C36 of Gpx3 can react with the C598 of the transcription factor Yap1, thus creating an intermolecular disulfide bond between the two proteins. Through a thiol-disulfide exchange reaction, this disulfide bond is transferred to Yap1, thus linking cysteine residues 598 and 303 with an

intramolecular disulfide bond. As discussed above, the formation of this disulfide bond masks the NES that is located at the C-terminus of Yap1 and blocks the release into the cytosol, allowing instead the retention of Yap1 in the nucleus (Delaunay et al. 2002; Paulsen and Carroll 2009).

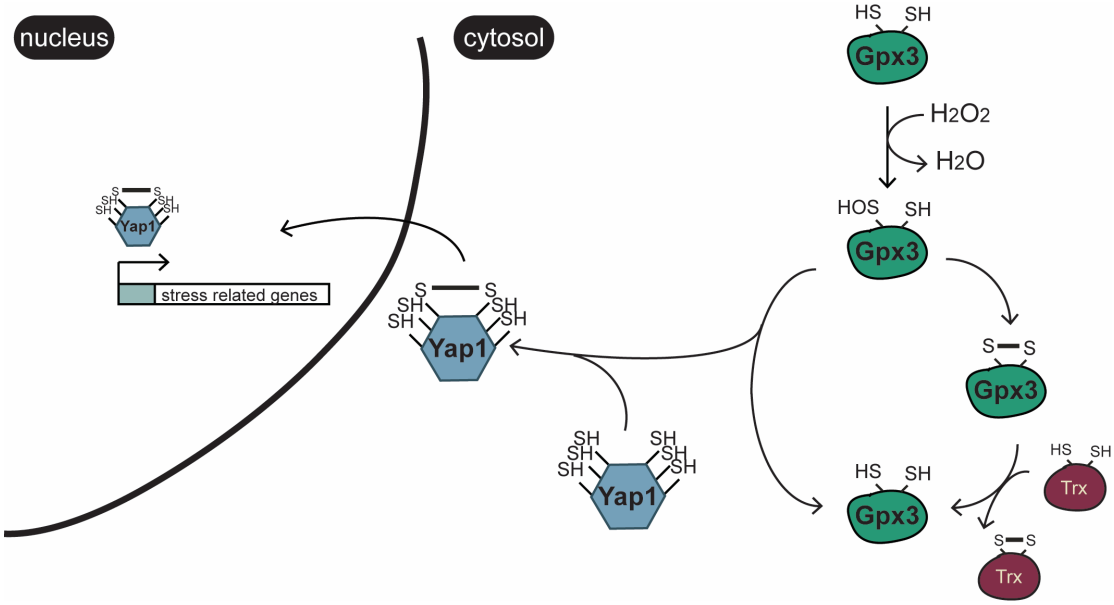

Figure 18: The Yap1 activation pathway. Gpx3 is able to function either as a sensor or as a scavenger of H_2O_2. In the scavenger pathway, Gpx3 can use H_2O_2 in order to become oxidised, with the oxidised form of Gpx3 being recycled through the function of the thioredoxin system. In the sensor pathway, the sulfenylated form of Gpx3 is able to form a mixed disulfide intermediate with Yap1, leading to the introduction of an intramolecular disulfide bond within Yap1. The formation of this bond is able to mask the NES of Yap1 and lead to the transcriptional activation of a number of genes involved in the oxidative stress response.

In 2003, Veal and coworkers were able to identify a new redox regulator protein, which they named Ybp1 (Yap1-binding protein) (Veal et al. 2003). In this study, they were able to find that Ybp1 is able to form a complex with Yap1 *in vivo* as part of the response to H_2O_2-induced stress. In *Δybp1* cells, where the complex cannot be formed, Yap1 was unable to become oxidised and, as such, activated. When the cells were stressed with diamide, another oxidising agent, this Yap1-Ybp1 complex was not formed, leading to the conclusion that Ybp1 is only involved in the response against high levels of hydrogen peroxide and acts in the same pathway as Gpx3 (Veal et al. 2003). Diamide is a thiol-oxidising agent that was identified in 1969, where it was shown to oxidise GSH to GSSG

in human red blood cells, in a reaction that did not form free radicals as by-products (Kosower et al. 1969; Kosower and Kosower 1995). This chemical compound acts as an oxidant that directly modifies the cysteine residues in proteins and leads to the formation of disulfide bonds. This comes into contrast with the oxidation of Yap1 by the elevated levels of H_2O_2, as the latter process depends on the presence of Gpx3 to mediate the oxidation process (fig. 19) (Gulshan et al. 2011)

The formation of the Yap1-Ybp1 complex was studied further, in an effort to understand the circumstances under which it is formed and how that can influence the oxidative stress response. Gulshan and coworkers were able to show that Yap1 exists in two distinct pools within the cell, depending on its association with Ybp1: unbound (or Ybp1-free) Yap1 and Ybp1-bound Yap1. The former was show to be associated mainly with the response against diamide – and to a lesser extent H_2O_2 – , while the latter was responsible for the H_2O_2-induced stress response (fig. 19) (Gulshan et al. 2011). In the cell, Yap1 exists predominantly in its free form, that is able to become imported into and exported from the nucleus according to the mechanism described above. Under normal circumstances, only a small fraction of the Ybp1-bound pool exists within the cell. Overexpression of Ybp1 leads to a sharp shift towards the H_2O_2-folded form of Yap1 and leads to a diamide-sensitive phenotype, as there is more Yap1 associated with Ybp1 than there is in free form (Gulshan et al. 2011).

Ybp1 is required for the successful folding of Yap1 during H_2O_2-induced stress conditions, a reaction which was shown to occur in the cytoplasm, prior to the localisation of Yap1 to the nucleus (Gulshan et al. 2011). As the cytoplasm is one of the first compartments to become oxidised upon exposure to oxidising agents, this makes it possible for the cell to detect the change at an early stage. This is thought to be particularly important for a timely cellular response, especially given the fact that Yap1 is not a direct antioxidant, but rather has a focal role in activating a series of genes that do encode for antioxidant enzymes (Gulshan et al. 2011). A recent study proposed that Ybp1 may be acting as a scaffold protein responsible for the transfer of the sulfenic acid modification of Orp1/Gpx3 to Yap1, by facilitating the transfer of the sulfenic acid group onto a specific cysteine residue of Yap1 and simultaneously blocking the formation of an intramolecular disulfide bond within Orp1/Gpx3 (Bersweiler et al. 2017).

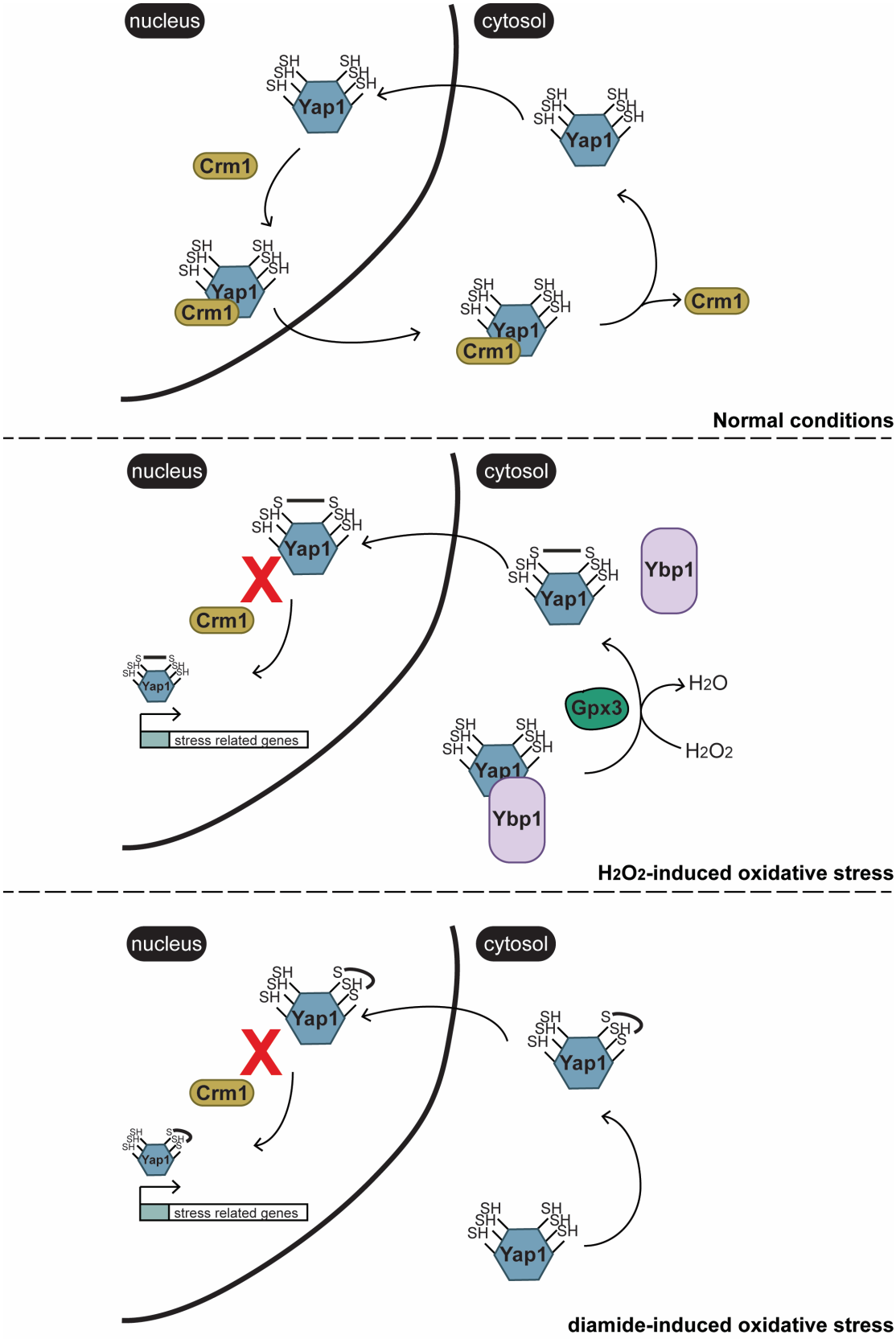

Figure 19: The activation of the Yap1 transcription factor under different stress conditions. Under normal conditions, Yap1 enters the nucleus due to its NLS, but Crm1 recognises its NES and transports it back to the cytosol. Gpx3-mediated activation of Yap1 in response to elevated levels of H_2O_2 requires the presence of the Yap1-Ybp1 complex. This leads to the formation of a disulfide bond between cysteines in the n-CRD and c-CRD domains, which mask the NES and allow for the retention of Yap1 in the nucleus and the

transcriptional activation of stress-related genes. When the cells are stressed with diamide, a disulfide bond is formed between cysteine residues of the c-CRD, which mask the NES and lead to the same result as in the case of H_2O_2-induced stress.

Genome analysis was also able to identify a protein that is homologous to Ybp1 in *S. cerevisiae* that was designated Ybp2 (Veal et al. 2003). Experiments performed in a 2004 study showed that, despite the fact that Ybp2 also seems to play a role in H_2O_2 tolerance, it is actually Ybp1 that has the most prominent role in the resistance phenotype (Gulshan et al. 2004). They were also able to show that Ybp2 does not seem to interact directly with Yap1, but might rather have a role in a parallel pathway to Ybp1. In order to show the different ways in which these two proteins play a role in the oxidative stress response, they proposed that Ybp2 should be changed to Ybh1 (Ybp1 homologue) (Gulshan et al. 2004). A hypothesis that was made regarding the actual role of Ybp2/Ybh1 in yeast takes into account the presence of several Yap1 homologues and states that Ybp2/Ybh1 may control the activity of one of these homologues (Gulshan et al. 2004). What makes this hypothesis quite interesting is the fact that Ybp2/Ybh1 has been shown to play a role in osmotic stress resistance, as have the two Yap1 homologues, Yap4 and Yap6 (Mendizabal et al. 1998; de Jesus Ferreira et al. 2001; Gulshan et al. 2004). Another possibility is that Ybp2/Ybh1 is responsible for the regulation of Yap1, but not in the same manner as Ybp1, which also requires the presence of Gpx3 in order to regulate Yap1 (Gulshan et al. 2004).

Recent research has been able to provide us with an abundance of new information regarding Gpx3, further describing its role outside of the Yap1 activation pathway. Gerashchenko and coworkers utilised a novel method known as ribosome profiling (RiboSeq), which was able to show that Gpx3 is among a series of possible protein candidates that can be alternatively translated from a non-AUG codon during H_2O_2-induced stress (Gerashchenko et al. 2012). Due to the fact that Gpx3 was found to localise in the mitochondrial IMS (Vögtle et al. 2012), as well as that it does not contain a mitochondrial localisation sequence, it was thought possible that this N-terminal extension could play a part in the dual localisation of Gpx3.

This was the focus of a study performed by in our lab in collaboration with the lab of Chris Grant at the University of Manchester (Kritsiligkou, Chatzi, Charalambous, Mironov, Grant & Tokatlidis), who were able to show that Gpx3 is targeted to the mitochondrial IMS through alternative translation that leads to the formation of an 18 amino-acid N-

terminal extension capable of targeting even non-mitochondrial proteins to the IMS (Kritsiligkou et al. 2017). The reason behind the localisation of Gpx3 in this mitochondrial compartment has not been fully elucidated, however, evidence suggests that it may be involved in a detoxification and redox control pathway in the IMS. Loss of Gpx3 leads to reduced matrix-targeted protein import, especially when cells are under oxidative stress (Kritsiligkou et al. 2017). The thioredoxin system has also been implicated in the regulation of protein import, as the absence of thioredoxins from the cytosol was found to lead to reduced protein import and cytosolic accumulation of oxidised mitochondrial precursors. More specifically, the cytosolic thioredoxin system was found to maintain the small Tim proteins in a reduced and import-competent state in the cytosol, prior to their translocation into the IMS. Trx1 was able to recognise the unfolded and partially folded Tim proteins and catalyse the reduction of the folding intermediates, thus preventing them from becoming fully folded in the wrong cellular location (Durigon et al. 2012).

Alongside Gpx3, two of the components of the cytosolic thioredoxin system were also found to reside in the IMS: Trx1 and Trr1 (Vögtle et al. 2012). Surprisingly, Trx2 was not on the list of proteins localised to the mitochondrial IMS under H_2O_2-induced stress conditions, a fact which is rather puzzling given the fact that Trx2 is the one whose expression is induced under such conditions. The presence of these two proteins means that the IMS contains a reducing mechanism to allow Gpx3 to remain functional in its antioxidant scavenger capacity, as well as that Trx1 could play an important role in the maintenance of the redox environment within this compartment (Kritsiligkou et al. 2017).

Chapter 2

Aims

2. Aims

The aims for this thesis can be split up into three separate categories, to reflect the three different sets of experiments that were performed.

A) The role of vitamin C as an antioxidant in yeast

With erythroascorbic acid (the yeast equivalent of ascorbic acid/vitamin C) not having as well described a role in the antioxidant defence of yeast cells, we decided to attempt to assess its effect in yeast mitochondria. More specifically, our aims were:

- To test whether arabinose – the substrate for the production of erythroascorbic acid in yeast – can be used as an efficient carbon source for the growth of yeast cells
- To assess the effect of erythroascorbic acid on the import of mitochondrial proteins and the mitochondrial membrane potential

B) The import of Gpx3 in the yeast mitochondrial IMS

The discovery of Gpx3 in the mitochondrial IMS (Vögtle et al. 2012) raised the questions of how it becomes imported into this subcompartment, as well as what function it carries out there. Work that was published through our lab's collaboration with Prof Chris Grant's lab at the University of Manchester was able to show that Gpx3 is targeted more efficiently to yeast mitochondria when in possession of an 18-aminoacid N-terminal extension (Kritsiligkou et al. 2017). In parallel to this work (prior to its publication), we decided to test whether there are any protein factors involved in the import of Gpx3. To this end, our aims were:

- To assess the import of the other proteins involved in the Yap1 activation pathway
- To determine if the additional presence of Ybp1 and/or Ybp2 during the import process has any effect on the import efficiency of Gpx3, as well as other proteins

C) *The import and function of Trx1 and Trr1 in the yeast mitochondrial IMS*

The discovery of Trx1 and Trr1 in the IMS at the same time as the discovery of Gpx3 also opened up questions concerning both the import and the function of a complete thioredoxin system in the IMS. Contrary to the work involving Gpx3, neither of these two proteins were identified as possibly being alternatively translated to include a targeting signal to mitochondria (Gerashchenko et al. 2012). The study of this particular system in the IMS was rather interesting, as, until then, the IMS was known to be home to a system for the oxidative folding of proteins, as well as being a generally more oxidising environment that allows the formation of disulfide bonds. The discovery of a reductive system in this subcompartment could possibly fill in a gap that does not exist in other such environments (ie the endoplasmic reticulum and the bacterial periplasm), both of which are known to possess ways of reducing wrongly-formed disulfide bonds. For this particular project, our aims were:

- To verify the import and localisation of Trx1 and Trr1
- To identify the mechanism of import of Trx1 and Trr1 into the mitochondrial IMS
- To determine the redox state of Trx1 and verify if it is active in the IMS
- To test the capacity of Trx1 and Trr1 to interact with components of the mitochondrial IMS using in *vitro* approaches
- To identify interactors of Trx1 and Trr1 in the IMS utilising *in organello* methods

Chapter 3

Materials and methods

3. Materials and Methods

3.1. Materials

3.1.1. Enzymes

All restriction enzymes used in the course of this thesis were obtained from New England Biolabs (NEB). T4 DNA ligase was also obtained from NEB. Taq DNA polymerase was obtained from NEB and Accuzyme DNA polymerase was obtained from Bioline.

3.1.2. Membranes

Western blotting was performed using Amersham™ Protran 0.45μm nitrocellulose (Fisher).

3.1.3. Antibodies

The antibodies used in this thesis, as well as the conditions of use, can be seen in table 1. All antibodies (except for the αHis antibody which was obtained from Bio-Rad) were produced by Davids Biotechnologie. Small quantities of each purified, recombinant protein were sent to the company to be injected into rabbits, in order for the antibody to be produced. The sera that were sent to us by the company were used without any further purification, to detect our proteins of interest. The antibodies that were gifted to our lab from Prof. Dr. N. Pfanner were also produced by Davids Biotechnologie, in the same manner described above.

Antibody	Company	Origin	Conditions of use	Source
αTrx1	Davids Biotechnologie	rabbit polyclonal	1/1000 in 1% milk	This study
αTrr1	Davids Biotechnologie	rabbit polyclonal	1/1000 in 5% milk	This study
αMia40	Davids Biotechnologie	rabbit polyclonal	1/1000 in 5% milk	(Sideris et al. 2009)
αErv1	Davids Biotechnologie	rabbit polyclonal	1/1000 in 5% milk	(Lionaki et al. 2010)
αmtHsp70	Davids Biotechnologie	rabbit polyclonal	1/20000 in 5% milk	Gifted by Prof. Dr. N. Pfanner
αporin	Davids Biotechnologie	rabbit polyclonal	1/20000 in 5% milk	Gifted by Prof. Dr. N. Pfanner
αcytc	Davids Biotechnologie	rabbit polyclonal	1/1000 in 5% milk	Gifted by Prof. Dr. N. Pfanner
αTim23	Davids Biotechnologie	rabbit polyclonal	1/1000 in 1% milk	Gifted by Prof. Dr. N. Pfanner
αHis	AbS Serotec (Bio-Rad)	mouse monoclonal	1/2000 in 5% milk	Obtained from Biorad

Table 1: List of antibodies used. The origin of each antibody (the company it was produced by and the study it was produced for or the lab it was obtained from), as well as the conditions of use, are shown in this table.

3.1.4. Plasmids

The list of all plasmids created for and used in this thesis can be seen in table 2. The process followed for the creation of the plasmids constructed during this study can be found in section 3.2.1.1.

Plasmids		Source
Ybp1	pSP64 – Ybp1 wt	This study
	pET24 – Ybp1His wt	This study
Ybp2	pSP64 – Ybp2 wt	This study
	pET24 – Ybp2His wt	This study
Yap1	pSP64 – Yap1 wt	This study
Su9DHFR	pSP65 – Su9DHFR	(Pfanner et al. 1987)
Gpx3	pSP64 – Gpx3 wt	(Kritsiligkou et al. 2017)
	pSP64 – N18Gpx3 wt	(Kritsiligkou et al. 2017)
	pET24 – Gpx3His	(Kritsiligkou et al. 2017)
Mia40	pET22 – ΔN290Mia40His	(Sideris et al. 2009)
Erv1	pET24 – Erv1His	Gifted by Dr. T. Lisowsky
Trx1	pSP64 - Trx1His wt	This study
	pSP64 - Trx1His C30S	This study
	pSP64 - Trx1His C33S	This study
	pSP64 - Trx1His C30/33S	This study
	pSP64 - Trx1His M35/39I	This study
	pSP65 - Trx1 wt	This study
	pET24 - Trx1His wt	This study
	pET24 - Trx1His C30S	This study
	pET24 - Trx1His C33S	This study
	pET24 - Trx1His C30/33S	This study
Trx2	pSP64 – Trx2 wt	This study
	pET24 – Trx2His wt	This study
Trr1	pSP64 - Trr1His wt	This study
	pSP64 - Trr1His C142S	This study
	pSP64 - Trr1His C145S	This study
	pSP64 - Trr1His C142/145S	This study

pET24 - Trr1His wt	This study
pET24 - Trr1His C142S	This study
pET24 - Trr1His C145S	This study
pET24 - Trr1His C142/145S	This study

Table 2: List of plasmids constructed and used. The genes of interest were cloned into pSP vectors for radioactive labelling and pET vectors for expression and purification.

3.1.5. Primers

All primers used for the creation of the constructs used in this study were obtained from Sigma. Table 3 shows the list of primers used for the cloning of our genes of interest into the appropriate vectors for radioactive labeling and/or protein overexpression, as well as the primers designed to mutagenise specific residues within each protein. The protocols followed for the clonings and mutagenesis can be found in section 3.2.1.

Plasmid	Primer name	Primer sequence (5'→3')
Clonings		
pSP64-Ybp1 wt	F_HindIII Ybp1	GCGCGCAAGCTTATGGAACCAATTGATGAC
	R_BamHI Ybp1	CGCGCGGATCCTCATTTTATACCAGTAAAATAG
pET24-Ybp1His wt	F_BamHI Ybp1	CGCGCGGATCCGATGGAACCAATTGATGAC
	R_nostop_HindIII Ybp1	GCGCGCAAGCTTTTTTATACCAGTAAAATAGTC
pSP64-Ybp2 wt	F_HindIII Ybp2	GCGCGCAAGCTTATGTACAACGAGCAGGTG
	R_BamHI Ybp2	CGCGCGGATCCTTACGAGCGTATGTTCAT
pET24-Ybp2His wt	F_BamHI Ybp2	CGCGCGGATCCGATGTACAACGAGCAGGTG

	R_nostop_HindIII Ybp2	GCGCGCAAGCTTCGAGCGTATGTTCATC TC
pSP64-Yap1 wt	F_HindIII Yap1	GCGCGCAAGCTTATGAGTGTGTCTACCG CC
	R_PstI Yap1	AACTGCAGTTAGTTCATATGCTTATTCA AAGC
pSP65-Trx1 wt	F_BamHI Trx1	CGGGATCCATGGTTACTCAATTCAAAAC TGCC
	R_HindIII Trx1	CGCAAGCTTTTAAGCATTAGCAGCAATG GCTTGC
pSP64-Trx2 wt	F_BamHI Trx2	CGGGATCCATGGTCACTCAATTAAAATC CGC
	R_EcoRI Trx1	GCGGAATTCCTATACGTTGGAAGCAATA GCTTGC
pET24-Trx2His wt	F_NdeI Trx2	CGCATATGATGGTCACTCAATTAAAATC CGC
	R_nostop_XhoI Trx2	GCCGCTCGAGTACGTTGGAAGCAATAGC TTGC

Mutagenesis

pSP64-Trx1His wt and pET24-Trx1His wt	F_Y11F Trx1	ACTGCCAGCGAATTCGACTCTGCAATT
	R_Y11F Trx1	AATTGCAGAGTCGAATTCGCTGGCAGT
	F_P70S Trx1	AAGAATGAAGTTTCCGCTATGCCAACT
	R_P70S Trx1	AGTTGGCATAGCGGAAACTTCATTCTT
pSP64-Trr1His wt and pET24-Trr1His wt	F_R196Q Trr1	TCTACCATTATGCAAAAGCGTGCTGAG
	R_R196Q Trr1	CTCAGCACGCTTTTGCATAATGGTAGA
pSP64-Trx1His C30S and pET24-Trx1His C30S	F_C30S Trx1	GTAGATTTCTACGCCACTTGGTCCGGTC CATGTAAAATGATTGCTCC
	R_C30S Trx1	GGAGCAATCATTTTACATGGACCGGACC AAGTGGCGTAGAAATCTACG
pSP64-Trx1His C30/33S and pET24-Trx1His C30/33S	F_C30/33S Trx1	CTTGGTCCGGTCCAAGTAAAATGATTGC TCC
	R_C30/33S Trx1	GGAGCAATCATTTTACTTGGACCGGACC AAG
pSP64-Trx1His	F_S30C Trx1	CGTAGATTTCTACGCCACTTGGTGCGGT

C33S and pET24-Trx1His C33S		CCATGTAAAATGATTGCTCC
	R_S30C Trx1	GGAGCAATCATTTTACATGGACCGCACC AAGTGGCGTAGAAATCTACG
pSP64-Trx1His M35/39I	F_M35/39I Trx1	CCATGTAAAATCATTGCTCCAATCATTG AAAAA
	R_M35/39I Trx1	TTTTTCAATGATTGGAGCAATGATTTTAC ATGG
pSP64-Trr1His C142S and pET24-Trr1His C142S	F_C142S Trr1	GGTATTTCTGCCTCTGCCGTGTGTGAT
	R_C142S Trr1	ATCACACACGGCAGAGGCAGAAATACC
pSP64-Trr1His C145S and pET24-Trr1His C145S	F_C145S Trr1	GCCTGTGCCGTGTCTGATGGTGCCGTC
	R_C145S Trr1	GACGGCACCATCAGACACGGCACAGGC
pSP64-Trr1His C142/145S and pET24-Trr1His C142/145S	F_C142/145S Trr1	GCCTCTGCCGTGTCTGATGGTGCCGTC
	R_C142/145S Trr1	GACGGCACCATCAGACACGGCAGAGGC

Table 3: List of primer sequences used. This table lists all the primer sequences that were used in order to clone our genes of interest into the corresponding vectors. The first column refers to the plasmid that was created with each set of primers. All plasmids shown in the "Clonings" section were created using the empty vectors (pSP64 or pET24-6xHis empty vectors) into which our genes of interest were cloned. Both plasmids and inserts were digested using the restriction enzymes that can be found in the primer name (column 2). In the "Mutagenesis" section, the first two rows refer to the correction of the mutagenised plasmids, as described in section 6.2.3, where the mutagenised plasmids were used as templates. The remainder of the "Mutagenesis" section refers to all the plasmids that were created specifically to contain point mutations for the purpose of this study. In these cases, the corrected Trx1 and Trr1 plasmids were used.

3.1.6. Bacterial strains and media for bacterial growth

The *Escherichia coli* strains that were used during the cloning process, as well as for plasmid propagation, were DH5α cells. For the expression of recombinant proteins, three different types of strains were used:

- BL21 (DE3) → no antibiotic selection

- BL21 (DE3) pLysS → chloramphenicol selection
- Origami™ 2 (DE3) → tetracycline selection

Cells were grown at 37°C in LB (Luria-Bertani) medium, containing 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. Solid media was prepared by also adding 2% agar. All bacterial media were autoclaved for 15min at 121°C before use.

During selection of the BL21(DE3) pLysS and the Origami™ 2 (DE3) cells, chloramphenicol was added at a final concentration of 25μg/ml, while tetracycline was added at a final concentration of 12.5μg/ml, respectively. Both antibiotics were added in addition to the antibiotic required for the plasmid selection, with final concentrations of 100μg/ml ampicillin being required for pSP vectors and 30μg/ml kanamycin being required for pET vectors..

3.1.7. Yeast strains

The *Saccharomyces cerevisiae* strains that were used in the course of this thesis, either for whole cell studies or for mitochondrial preparation can be seen in table 4. All yeast strains were grown on rich yeast media, as will be described in section 3.2.4.1.

Yeast strain	Genotype	Brief description	Source
FT5	*MATα, ura3-52, trp I-Δ63, his3-Δ200, leu2::PET56*	Wild type strain	(Tzamarias and Struhl 1994)
galMia40 (FT5) **(Mia40↓)**	*MATα, ura3-52, trp I-Δ63, his3-Δ200, leu2::PET56, kanMX-GAL1-10-MIA40*	Conditional yeast knockout strain, where the *MIA40* gene is placed under the control of the Gal1-10 promoter	(Banci et al. 2009)

galErv1 (FT5) **(Erv1↓)**	*MATα, ura3-52, trp I-Δ63, his3-Δ200, leu2::PET56, kanMX-GAL1-10-ERV1*	Conditional yeast knockout strain, where the *ERV1* gene is placed under the control of the Gal1-10 promoter	(Lionaki et al. 2010)
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Wild type strain	(Brachmann et al. 1998)
***Δtrx1* (BY4741)**	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, trx1Δ0::kanMX*	Strain deleted for the endogenous TRX1 gene	Dharmacon – GE Healthcare
***Δtrr1* (BY4741)**	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, trr1Δ0::kanMX*	Strain deleted for the endogenous TRR1 gene	Dharmacon – GE Healthcare
***Δtrr2* (BY4741)**	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, trr2Δ0::kanMX*	Strain deleted for the endogenous TRR2 gene	Dharmacon – GE Healthcare
D273-10B	*MATα mal*	Strain used for mitochondrial studies, constructed in the Sherman lab	(Sherman 1964)

Table 4: Yeast strains used. A short description of each yeast strain, as well as its genotype, are also shown. The source refers either to the first publication in which a particular strain was described or the company from which it was obtained.

3.2. Methods

3.2.1. Molecular biology assays

3.2.1.1. Cloning

All clonings for the projects discussed in this PhD thesis were prepared through PCR amplification of the inserts using yeast genomic DNA as a template. The process was followed by digestion of the PCR products and vectors with the desired restriction enzymes and ligation of the two digested components at different ratios (plasmid:insert – 1:3, 1:5, 1:7) depending on the size of the insert relative to the size of the vector. The quantities used in every case were calculated using the following equation:

$$\frac{(ng\ vector) \times (bp\ insert)}{bp\ vector} = ng\ insert$$

The ligation product was then transformed into DH5α bacterial cells and plated onto LB agar plates with the corresponding antibiotic selection, which were left to grow at 37^{o}C overnight. pSP64 and pSP65 vectors were selected for ampicillin (using a final concentration of 100μg/ml), while pET24 vectors were selected for kanamycin (using a final concentration of 30μg/ml). The positive colonies were analysed through colony PCR, and confirmed through sequencing analysis (GATC Biotech).

3.2.1.2. Mutagenesis

In order to mutate specific amino acid residues within proteins, the high fidelity Accuzyme DNA polymerase (Bioline) was used. The plasmid containing the sequence into which we wanted to make the mutation was used as the template for the PCR reaction, with the pair of primers required to introduce the point mutation being added to the reaction at a final concentration of 0.5μM, according to the Accuzyme DNA polymerase instructions. Once the PCR amplification of the plasmid had been completed, the reaction was treated with

1µl of DpnI (10U/µl) at 37°C for 1h. This enzyme is able to digest the non-mutated, supercoiled, double stranded parental plasmid DNA, but not the one produced during the PCR reaction, which also contains the point mutation. The DpnI-treated PCR mix was transformed into DH5α bacterial cells and the cells containing the plasmid were selected using the appropriate LB+antibiotic plates. Colonies were sent for sequencing analysis and those that were positive and contained the mutated residues were used.

3.2.2. In organello assays

3.2.2.1. Isolation and purification of yeast mitochondria

Mitochondria were isolated and purified from the wild type yeast strain D273-10B. The cells were grown at the optimal temperature of 30°C, using lactic acid, a non-fermentable carbon source that promotes respiration and requires functional mitochondria. According to the literature, this particular carbon source cannot be used for the production of energy within the cytoplasm (through glycolysis) (Turcotte et al. 2010). Essentially, this means that the only cells that survive and proliferate in this carbon source are the ones with fully functional mitochondria.

The isolation of the mitochondria is done according to the protocol described by Glick 1991. This protocol contains the use of the polysaccharide Nycodenz for the separation of the mitochondria from the rest of the subcellular organelles.

Following the isolation and purification processes, the mitochondria can be stored long term at -80°C, after the addition of 10mg/ml final concentration of fatty acid free BSA that maintains the membrane components in a stable condition.

3.2.2.2. Radioactive labelling of proteins using the TNT SP6-coupled transcription/translation system

For the production of radiolabelled proteins, the TnT® SP6 Coupled Reticulocyte Lysate System (Promega) was used, according to the instructions provided. The labelling of the proteins requires the presence of a plasmid, which contains the gene that will be expressed under the control of the SP6 polymerase promoter, as well as the presence of radiolabelled ^{35}S methionine during the reaction. The reaction itself is dependent on the components of the rabbit reticulocyte lysate provided with the kit and takes place at 30^{o}C for 90min. At the end of the incubation time the protein is separated from the ribosomes by centrifuging the samples in a TLA100 rotor at 55000rpm, at 4^{o}C for 15min.

3.2.2.3. Preparation and denaturation of radioactive and purified proteins

Ammonium sulfate precipitation was used in order to precipitate recombinant proteins for import assays. The desired amount of protein was calculated and precipitated by adding 3 volumes of saturated ammonium sulfate solution. After a 30min incubation on ice and a 30min spin at 25000g at 4^{o}C, the pellet was resuspended in the buffer required for the treatment of the protein at a concentration of 10mg/ml, as described below:

Native treatment: 50mM HEPES pH 7.4
Denaturing and reducing treatment: 8M urea, 50mM HEPES pH 7.4, 20mM DTT

The same method of precipitation was also used for the radiolabeled proteins that had to be denatured and reduced in order to be imported into yeast mitochondria. Ammonium sulfate was added to the supernatant from the protocol described in 3.2.2.2 and the process described above was followed. It is important to note that, in this case of the denaturing and reducing treatment, the pellet was resuspended in the appropriate buffer at half the volume of the initial TNT reaction, so as to minimise the concentration of urea in the final import reaction (always kept at less than 0.4mM final). The resuspended product was then incubated at 30^{o}C for 30min – 1h, in order for the denaturation to take place. After the incubation, another 1/2V of 50mM HEPES pH 7.4 was added to the mix and quickly vortexed, so as to bring it up to the initial volume of the TNT reaction.

3.2.2.4. Import of proteins into isolated yeast mitochondria

Mitochondria were resuspended in import buffer at a final concentration of 0.5mg/ml, in the presence of 2mM ATP and 2.5mM NADH and were equilibrated at 30^{o}C. Next, the radioactive precursor protein was added to the mix and the incubation at 30^{o}C continued for the required amount of time, following each experimental set up. At the end of the incubation period, the samples were placed on ice, so as to terminate the reaction ("freeze") at the desired moment.

After the import reaction, the mitochondria were isolated by centrifuging at 16000g, at 4^{o}C for 5min. The unimported material was removed by resuspending the mitochondrial pellet in an isotonic buffer solution (Breaking Buffer) in the presence of proteinase K (0.15mg/ml) at 4^{o}C for 20min or trypsin (0.1mg/ml) at 4^{o}C for 30min. The inhibition of the protease can be achieved by adding PMSF (2mM) (for proteinase K) or SBTI (1mg/ml) (for trypsin) for 10min at 4^{o}C. The mitochondria were re-isolated under the same centrifuging conditions as mentioned above. The supernatant was removed and the mitochondrial pellet was resuspended in 20µl of 2x Laemmli sample buffer (+/- β-mercaptoethanol). As a final step, the samples were boiled at 95^{o}C for 5min and were analysed using SDS gel electrophoresis.

For the import of proteins into mitochondria with disrupted membrane potential, NADH was omitted from the import mix. Instead, a membrane potential disrupting chemical, such as CCCP (final concentration 25µM) or valinomycin (final concentration 1mM), was added. After the disruption of the membrane, the same import procedure described above was followed.

For the import of proteins into mitochondria depleted of ATP, ATP was omitted from the import mix. Instead, 10µM oligomycin and 10mU/µl apyrase were added to the import mix and were incubated with the mitochondria for 10min prior to the addition of the precursor.

3.2.2.5. Trapping of mixed disulfide intermediates after import of proteins into isolated yeast mitochondria

For the trapping of mixed disulfide intermediates during the import process, the import protocol described in section 3.2.2.4 was performed with an additional step: before the first centrifugation of the samples, the redox state of the proteins in the reaction was arrested by adding 20mM of N-ethylmaleimide (NEM) for 5min on ice. Furthermore, all samples were analysed on SDS PAGE in the absence of a reducing agent.

3.2.2.6. Pulldown of 6xHis-tagged proteins after import into isolated yeast mitochondria

For these experiments, the import protocol described in the section 3.2.2.4 was also performed with some alterations. Instead of performing the protease treatment, samples were washed twice with Breaking Buffer to remove any non-imported material. This is done to avoid the addition of a protease to the solubilised material, as the protease may not become 100% deactivated, thus leading to proteolytic degradation of our solubilised proteins. The pellets were resuspended in solubilisation buffer, with the final concentration of mitochondrial proteins being 2.5mg/ml. The samples were solubilised with incubation on ice (20 min), followed by a 20min spin at 16000g at 4^{o}C for 5min. The supernatant, which contained all the proteins that were extracted from the mitochondria, was then added to Ni-NTA beads that have been pre-equlibrated with solubilisation buffer without detergent. The proteins were allowed to bind to the Ni-NTA beads for 1h at room temperature with gentle shaking. After the end of the binding period, the beads were washed twice: first with solubilisation buffer plus 10mM imidazole and then with the buffer plus 20mM imidazole. The protein material that was bound to the beads was eluted by increasing the concentration of imidazole present within the buffer to 300mM. Any residual amount of the bound protein was eluted by resuspending the beads in 2x Laemmli sample buffer. All samples were analysed on SDS PAGE according to the setup for each experiment.

When using this experimental setup to determine the disulfide-bonded partners of the bound proteins, the elution can also be done by adding 10mM dithiothreitol (DTT) to the

elution buffer. This will lead to the breakage of the disulfide bond and the release of the partner proteins, but not the His-tagged bound protein into the eluent.

3.2.2.7. Fractionation of mitochondria

Mitoplasting

For the creation of mitoplasts (mitochondria that lack their external membranes), mitochondria were incubated in a hypotonic solution. More specifically, the mitochondria were pelleted and resuspended in import buffer at a concentration of 5mg/ml. The rupturing of the outer membrane was achieved by diluting the mitochondria with 9 volumes of mitoplasting buffer, with the final concentration of mitochondrial proteins being 0.5mg/ml. Samples were treated with 0.1mg/ml proteinase K for 20min on ice (followed by a 10min inactivation using 2mM PMSF) where required. The samples were then centrifuged at 16000g at 4^{o}C for 5min. The supernatant fraction contained all the soluble proteins of the intermembrane space, while the pellet contained the mitoplasts, as well as outer membrane fragments.

Carbonate extraction

For the separation of the soluble from the membrane bound mitochondrial proteins, carbonate extraction was used. For this process, the mitochondria were resuspended in freshly prepared 0.1M ice-cold sodium carbonate buffer at a final concentration of mitochondrial proteins of 0.5mg/ml. The sample was incubated on ice for 30min and then centrifuged in a TLA100 rotor at 55000rpm at 4^{o}C for 30min. The resulting supernatant fraction contained all the soluble mitochondrial proteins, while the pellet contained all the membrane-anchored proteins.

3.2.2.8. Solutions

Import buffer: 600mM sorbitol, 2mM KH_2PO_4, 50mM KCl, 50mM HEPES, 10mM $MgCl_2$, 2.5mM Na_2EDTA pH7, 5mM L-methionine, 1mg/ml fatty acid free BSA
Isotonic buffer solution (Breaking buffer): 0.6M sorbitol, 20mM HEPES-KOH pH7.4
Solubilisation buffer: 150mM NaCl, 50mM Tris-HCl pH7.4, 2mM PMSF and 0.5% DDM
Mitoplasting buffer: 20mM HEPES-KOH pH 7.4, 1mM DTT

6x Laemmli sample buffer: 0.35M Tris-HCl pH6.8, 30% glycerol, 10% (w/v) SDS, 0.03% (w/v) bromophenol blue, +/- 1.43M β-mercaptoethanol

3.2.3. Biochemical assays

3.2.3.1. Tricine SDS-PAGE and Western Blotting

Samples for SDS-PAGE analysis were resuspended in 2x Laemmli sample buffer in the presence or absence of β-mercaptoethanol and were boiled for 5min at 95^{o}C prior to loading on the gels.

The gels used in this study were Tris-Tricine SDS gels prepared according to the protocol by Schägger 2006. For the preparation of these gels, the recipes in table 5 were followed.

The gels were either stained with Coomassie Blue Staining buffer or transferred onto nitrocellulose membrane using the BioRad Semi-Dry Transfer System at 25V constant for 25min. Membranes were stained with Ponceau buffer to determine the outcome of the transfer process. Membranes were blocked with 5% skimmed milk powder in TBST buffer for 1h at room temperature. Primary antibodies were diluted to working concentrations of 1:500 to 1:5000 in 1-5% skimmed milk powder in TBST buffer and were incubated with the membrane for 1-3h at room temperature or overnight at 4^{o}C. The conditions were optimised for each antibody used and are shown in table 1. After the end of the incubation time, the membranes were washed three times (3x5min) with TBST, before the addition of the secondary antibody. Secondary antibodies were either HRP-labelled or fluorescence-labelled and were diluted to a working concentration of 1:10000 in TBST with 1% skimmed milk powder (for the HRP-labelled antibodies) or in TBST (for the fluorescence-labelled antibodies). Blots were then washed twice (2x5min) with TBST and once (1x10min) with dH_2O. For the membranes incubated with the HRP antibodies, the signals were visualised through the use of X-ray films, while for the fluorescently-labelled antibodies, the LI-COR Odyssey CLx quantitative fluorescence imaging system was used.

	Stacking gel	Separating gel		
	5%	**10%**	**12%**	**14%**
Acryl/Bis-acryl (40%)	0.375ml	2.5ml	3ml	3.5ml
Tricine Gel buffer	0.75ml	3.3ml	3.3ml	3.3ml
87% glycerol	-	1.3ml	1.3ml	1.3ml
dH_2O	1.82ml	2.29ml	1.79ml	1.29ml
10% APS	30µl	100µl	100µl	100µl
TEMED	3µl	10µl	10µl	10µl
Total volume	3ml	10ml	10ml	10ml

Table 5: Recipes for the preparation of Tris-Tricine SDS gels. The percentage of the separating gel was chosen depending on the molecular weights of the proteins analysed in each experiment.

3.2.3.2. Large scale expression of recombinant proteins

All recombinant proteins were cloned into pET24-6xHis tagging vectors (except for ΔN290Mia40, which was cloned in a pET22-6xHis tagging vector) and were expressed in *Escherichia coli* BL21 (DE3) cells. Single colonies of transformed bacterial cells were used to inoculate small liquid LB cultures supplemented with the appropriate antibiotic selection. The cells were grown for 14h at 37°C and were diluted at a 1:50 ratio into fresh LB cultures. The new cultures were incubated at 37°C until they reached their logarithmic growth phase (OD_{600}=0.4-0.7). At this stage, the correct amount of IPTG was added and the cells were incubated for different times at different temperatures (depending on the appropriate expression conditions for each individual protein). The details of all the different recombinant protein expressions are shown in the table 6. After the induction, the cell pellets were collected by centrifugation at 5000g at 4°C for 15min and were stored at -20°C until purified.

Protein	IPTG/FAD	Induction temperature	Induction time
Trx1 (and cys mutants)	0.4mM IPTG	37°C	4h
Trx2	0.4mM IPTG	37°C	4h
Trr1	1mM IPTG, 10µM FAD	37°C	4h
ΔN290Mia40	0.4mM IPTG	37°C	4h
Erv1	0.1mM IPTG, 10µM FAD	18°C	o/n
Gpx3	0.4mM IPTG	18°C	o/n

Table 6: Induction conditions for all proteins expressed and purified during this study.

3.2.3.3. Purification of 6xHis-tagged recombinant proteins

The cell pellets obtained from protocol 3.2.3.2 were resuspended in buffer A by adding 5ml buffer per gram of cell pellet. 1mg/ml of lysozyme and 10µg/ml DNAse were added and the solution was incubated at 4°C for 15min. The cells were ruptured by passing them through a French Press twice at a stable pressure of 1,000psi. The broken cells were centrifuged at 21000g at 4°C for 30min. The supernatant was separated from the pellet and loaded onto Ni-NTA beads (Qiagen). The beads were washed once with buffer A supplemented with 10mM imidazole and once with buffer A supplemented with 20mM imidazole. The bound protein was eluted from the Ni-NTA beads by adding buffer A supplemented with 300mM imidazole.

3.2.3.4. Gel filtration analysis of purified recombinant proteins

Samples of the recombinant proteins that were purified as part of this thesis were analysed on gel filtration columns, in order to assess the level of purification and their state (monomeric-dimeric).

The purified, recombinant proteins were analysed using an ÄKTA Pure FPLC system (GE Healthcare), which had previously been equilibrated with degassed buffer A. The protein samples that were used in this assay were precipitated using the ammonium sulfate precipitation method (as described in 3.2.2.3) and resuspended in buffer A. Prior to loading, each sample was spun at 16000g for 5min at 4^{o}C, so as to ensure that no aggregates enter the gel filtration system. ~100μg of each protein were loaded onto a Superdex 200 10/300 GL (GE Healthcare) gel filtration column, which separates proteins and complexes based on their molecular weight.

Additionally, a mix of control proteins of known molecular weights was also analysed, so as to enable the determination of the molecular weights of our proteins of interest. Table 7 shows the data generated by the analysis of the protein standards, which allowed for the determination of the standard curve and equation shown in fig. 20. This equation was used to calculate (in approximation) the molecular weights of our proteins of interest.

Protein	Mr (Da)	Retention volume (ml) (V_E)	V_E/V_O
Ribonuclease A	13,700	17.62	2.0114
Carbonic anhydrase	29,000	16.25	1.855
Conalbumin	75,000	13.94	1.5913
Ferritin	440,000	10.4	1.187
Blue Dextran	2,000,000	8.76 (=V_O)	0

Table 7: Analysis of protein standards on the Superdex 200 10/300 GL gel filtration column. The retention volume (V_E) is the volume at which each protein is eluted from the ÄKTA Pure machine. The void volume (V_O) is the volume at which Blue Dextran is eluted and is used as the reference point for the

calculation of the molecular weights of other proteins analysed under the same conditions. For each protein, the V_E/V_O ratio must be calculated. This value is then added into the equation shown in fig. 20, in order to estimate the molecular weight of our proteins of interest.

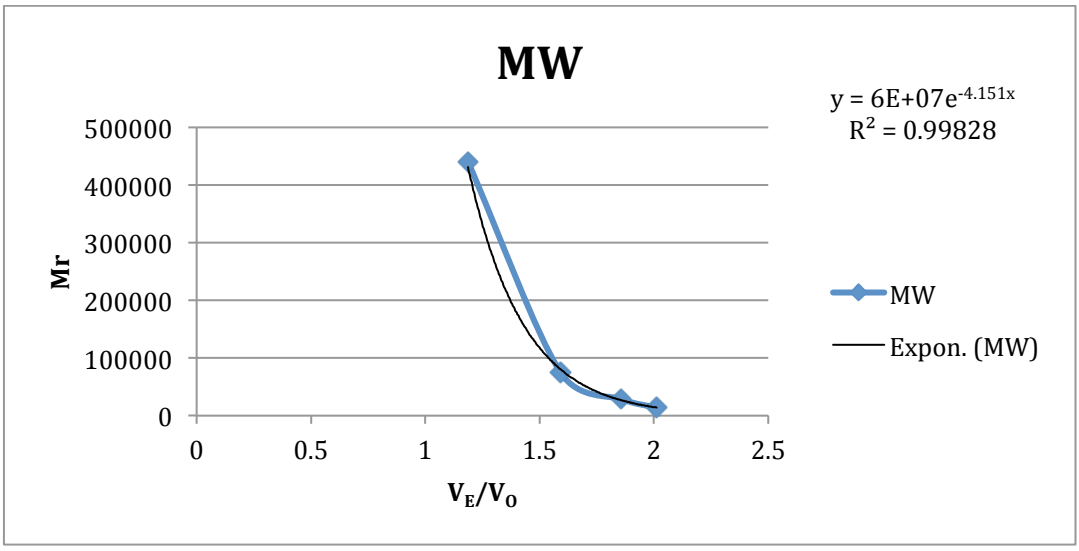

Figure 20: Standard curve generated by analysis of a mix of protein standards on a Superdex 200 10/300 GL column. The equation describing the standard curve can be used for the approximate determination of the molecular weight of other proteins analysed on the same column.

3.2.3.5. In vitro interaction assay

Before performing the *in vitro* interaction assay, all protein samples required preparation. The amounts of protein that needed to be precipitated for each experiment were calculated based on the use of 50µg of each recombinant protein per interaction. Proteins were precipitated using saturated ammonium sulfate, as described in 3.2.2.3. Samples were treated in order to change their oxidative state, depending on what required testing in each particular experiment. Proteins that required to be oxidised were treated with 200µM of freshly prepared H_2O_2 for 30min. Proteins that required to be reduced were treated with 20mM DTT for 30min. Trr1 was treated with 5mM NADPH for 30min (where required).

For the interactions, the following process was followed:

1) Each reaction was performed in 1ml of 50mM Tris-HCl pH8 buffer. When performing timecourse experiments, all reaction were placed in the same tube to ensure uniform conditions.

2) Addition of the proteins in the following order: Trr1, Trx1 and lastly, the substrate protein
3) Incubation at 30^{o}C for the desired amount of time
4) Collection of 1ml samples at each desired time point and transfer to eppendorf tubes containing 100µl TCA (10% final concentration)
5) Incubation on ice for 20min
6) Centrifugation at 16000g for 20min at 4^{o}C
7) Removal of supernatant and acetone wash with 1/2V of acetone
8) Centrifugation at 16000g for 20min at 4^{o}C
9) Removal of supernatant, air dry and addition of equal amounts of ETS buffer and 30mM AMS stock (15mM final concentration).
10) Incubation at 30^{o}C for 30min and 37^{o}C for 30min.
11) Addition of sample buffer. Sample buffer was added at the desired amount, depending on the final amount of protein that was to be loaded on to SDS PAGE for visualisation. In these experiments, the equivalent of 5µg of each protein was loaded on gels (1/10 of the total amount prepared).

3.2.3.6. Fluorimetry – Measurement of the inner mitochondrial membrane potential using fluorescence spectrophotometry

The effect of certain chemical substances on the inner mitochondrial membrane potential ($\Delta\Psi$) of isolated yeast mitochondria was measured by the quenching of the fluorescent signal emitted by the $DiSC_3(5)$ dye. This method was previously described by Sims et al. 1974 and Gärtner et al. 1995. All measurements were performed using a Horiba JobinYvonFL-1039/40 Fluorimeter at 25^{o}C at a 622nm excitation wavelength and a 670nm emission wavelength.

The reaction was carried out in $\Delta\Psi$ buffer and the reagents were added in the following order at certain intervals to allow for the fluorescent signal to stabilise:

1) Addition of 1ml $\Delta\Psi$ buffer in the cuvette
2) Addition of the $DiSC_3(5)$ dye (diluted in ethanol) at a final concentration of 2mM
3) Addition of D-isoascorbic acid (diluted in $\Delta\Psi$ buffer) at the desired final concentration (20mM or 30mM). In the case where no D-IAA was added, a small amount of $\Delta\Psi$ buffer was added to the reaction.

4) Addition of 50mg of mitochondria (before adding to the reaction, the mitochondria had been washed once and resuspended in SEM buffer, in order to get rid of the fatty acid free BSA that is added during the mitochondrial preparation).
5) Addition of valinomycin (diluted in acetone) at a final concentration of 1mM.

The assessment of the disruption of the inner mitochondrial membrane potential was made by comparing the difference in the fluorescence measurements before and after the addition of the uncoupler (valinomycin). All measurements were performed three times and the averages were calculated and presented as percentages of the total amount of fluorescence detected immediately before the addition of the mitochondria.

3.2.3.7. Solutions

Coomassie Staining Buffer: 30% (v/v) methanol, 10% (v/v) acetic acid, 0.2% (w/v) Coomassie Brilliant Blue R-250
Destaining Buffer: 15% (v/v) methanol, 10% (v/v) acetic acid
Ponceau buffer: 10% (v/v) acetic acid, 0.1% (w/v) Ponceau S
Tris-buffered saline with Tween 20 (TBST): 150mM NaCl, 100mM Tris-HCl pH7.4, 0.01% (w/v) Tween 20
Tricine Gel buffer: 3M Tris, 0.3% SDS, adjust pH to 8.45 with HCl
Buffer A: 150mM NaCl, 50mM Tris-HCl pH 7.4
ETS buffer: 3mM EDTA, 50mM Tris-HCl pH8, 3% (w/v) SDS
ΔΨ buffer: 0.6M sorbitol, 0.1% (w/v) BSA, 10mM MgCl2, 0.5mM EDTA, 20mM KPi pH 7.4
SEM buffer: 250mM sucrose, 1mM EDTA, 10mM MOPS-KOH pH 7.2

3.2.4. In vivo assays

3.2.4.1. Yeast growth media

Yeast cells were grown in rich (YPD/YPAra/YPGal/YPL) media at 30^{o}C.

This rich yeast medium contained 2% (w/v) carbon source (glucose for YPD, arabinose for YPAra, galactose for YPGal and lactic acid for YPL), 1% (w/v) yeast extract and 2% (w/v) peptone (+ 2% (w/v) agar, for solid media).

All media were autoclaved at 121^{o}C for 15min and were left to cool down to room temperature before use. Media with agar were poured into Petri dishes (20ml per dish) and left to solidify.

3.2.4.2. Spot growth assay

Solid growth media were prepared according to the recipe described in section 3.2.4.1. H_2O_2 was added to the plates at a 2mM final concentration where required.

The yeast cultures that were spotted onto the plates were prepared by growing the cells overnight at 30^{o}C. After measuring the OD_{600} of the culture, the appropriate volume corresponding to 10^7 cells (OD_{600}=1 → $2x10^7$ cells) was pelleted and resuspended in 500µl of autoclaved water. 1:10 serial dilutions were prepared and 5µl of each dilution were spotted onto each plate. Once the spots were dry, the plates were incubated at 30^{o}C for 2-4 days.

Chapter 4

The role of vitamin C as an antioxidant in yeast

4. The role of vitamin C as an antioxidant in yeast

4.1. Introduction

Ascorbic acid (vitamin C) is one of the most studied antioxidant molecules, due to the fact that was initially implicated in the treatment of scurvy, a disease that caused the gums of people unable to consume fresh fruit and vegetables to bleed. In 1937, Albert Szent-Györgyi – the person responsible for this discovery – was awarded the Nobel Prize in Physiology or Medicine, with Norman Haworth being awarded the Nobel Prize in Chemistry for his investigations on carbohydrates and vitamin C.. Since then, as vitamin C is an integral part of any healthy diet, researchers have worked in order to elucidate the precise reasons that cause this molecule to be so beneficial to human health.

Studies have shown that ascorbic acid is a rather effective antioxidant due to a series of properties that it possesses: a) it is able to interact with a variety of free radicals in an efficient manner, b) it is present in adequate amounts within the cell and c) it has the capability of being efficiently regenerated in the majority of compartments it is needed (Rose and Bode 1993; Arrigoni and De Tullio 2002). Details of the mechanism through which vitamin C acts as an antioxidant are difficult to be studied due to the challenge of quantifying the amounts of the antioxidant in the various cell compartments, as well as the fact that, due to it being an antioxidant that acts without the mediation of an enzyme, it is hard to predict any reactions that it may be part of (Arrigoni and De Tullio 2002).

As mentioned in the introduction, the yeast *S. cerevisiae* is not able to produce ascorbic acid, but instead produces a 5-carbon analogue known as erythroascorbic acid (Nick et al. 1986; Kim et al. 1998). The only known biosynthetic pathway for erythroascorbic acid is a two-step process, with D-arabinose as the initial substrate (fig. 10).

Vitamin C was also discussed in a publication by KC and coworkers, concerning its role in the import process (KC et al. 2005). Here, it was shown that vitamin C inhibits protonophore-induced loss of mitochondrial membrane potential ($\Delta\Psi$) in mammalian cells. They specifically looked into the loss of membrane potential caused by carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), a chemical that creates an uncoupling of the proton

gradient established during the normal activity of electron carriers in the electron transport chain. By using HL-60 cells and flow cytometry, they proved that the addition of ascorbic acid was able to restore the mitochondrial membrane potential that was chemically disrupted in these cells. More specifically, they found that ascorbic acid restored the mitochondrial potential in an exponential way and thus, significantly inhibited the increase in the percentage of cells with disrupted $\Delta\Psi$ caused by the addition of CCCP. Essentially, they suggested a protective role for vitamin C in cells under chemical disruption.

As such, our aims were a) to attempt to assess the antioxidant effect of erythroascorbic acid in yeast by utilising this biosynthetic pathway and b) to elucidate whether the yeast analogue of ascorbic acid has the same protective effect on the mitochondrial inner membrane potential as ascorbic acid.

4.2. Results

4.2.1. Growth of yeast cells in arabinose-containing media

As a first step, it was essential to test the growth conditions of wild type (wt) yeast cells in media containing arabinose. Two different wt yeast strains were used: FT5 and BY4741. The cells were grown in complete media (YP) containing either glucose or arabinose as a carbon source. Furthermore, cell growth was tested using the drop test method on solid media containing 2mM hydrogen peroxide. These experiments showed that both types of wild type cells exhibit slower growth when cultured on arabinose, as opposed to glucose, as well as that the presence of hydrogen peroxide seems to have a more severe effect when the cells are grown on arabinose as a sole carbon source (fig. 21).

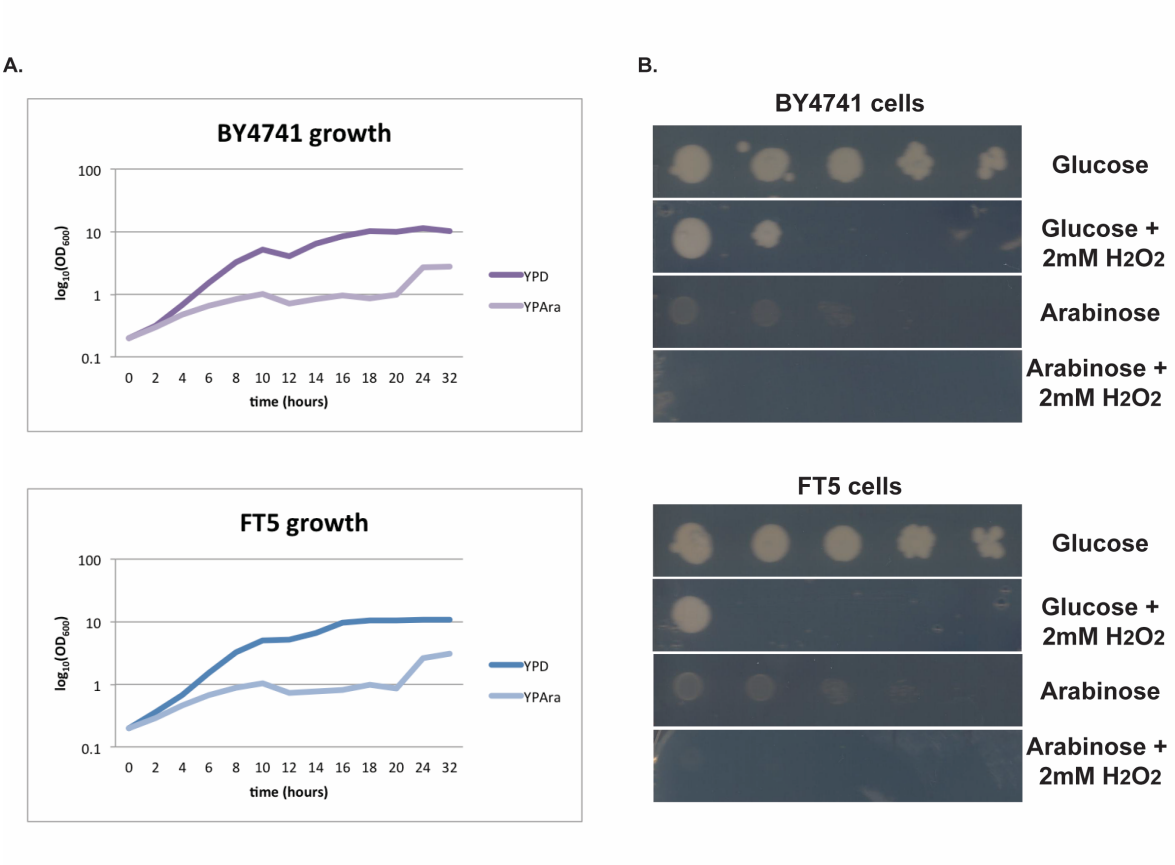

Figure 21: Growth of yeast cells in glucose versus arabinose. Liquid culture measurements (A) and drop tests (B) of BY4741 and FT5 cell growth in media containing glucose vs. media containing arabinose. All cells were grown in glucose (YPD) until the cultures were saturated and were subsequently transferred to fresh glucose or arabinose-containing media.

4.2.2. The effect of D-isoascorbic acid on protein import

Firstly, an indirect approach was used to check for the putative effect of vitamin C on the mitochondrial membrane potential. This approach was based on the matrix-targeted protein Su9-DHFR, which requires the membrane potential in order for it to become imported into mitochondria. When the membrane potential is abolished with the use of an ionophore such as CCCP or valinomycin, the import of Su9-DHFR is impaired. If vitamin C has the same protective effect in yeast as it does in mammalian cells, then, by incubating the purified mitochondria with the compound, while also chemically disrupting the membrane potential, the import mechanisms should remain active and Su9-DHFR should be translocated through the inner mitochondrial membrane.

The first step was to verify that the import of Su9-DHFR is indeed dependent on membrane potential. As presented in fig. 22, CCCP (25µM) alone did not seem to have a particular effect on the import of the precursor protein, especially when compared to the samples where the membrane potential had been disrupted using valinomycin (1mM) or a mix of the two.

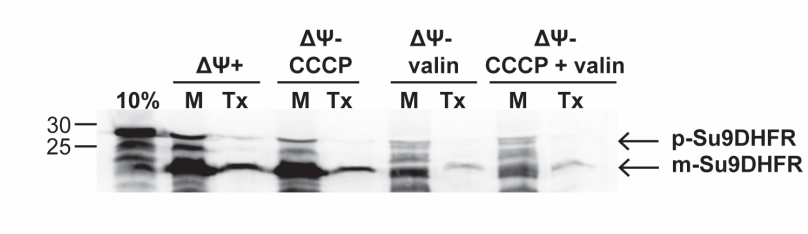

Figure 22: Trial import of Su9DHFR to assess ΔΨ disruption with CCCP and valinomycin. Import of radiolabelled Su9-DHFR into wild type (wt) mitochondria for 15min under normal conditions (ΔΨ+) or after the disruption of the membrane potential (ΔΨ-). The membrane potential was disrupted using either CCCP (25µM), valinomycin (1mM) or a mix of the two. [(10%)-corresponds to 10% of the precursor that was added for the import reaction – acts as a control lane, (M)-import into mitochondria and treatment with protease, (Tx)-import into mitochondria and treatment with Triton X-100 detergent and protease – this step is performed to verify the specificity of the import]

From the previous experiment, it was obvious that the concentration of CCCP that was used (25µM) was not sufficient for the disruption of the membrane potential, despite this being the optimal concentration already described in literature (Glick et al. 1992). This could be due to our particular stock preparation of CCCP, so the next step was to test the concentration of our CCCP solution that was required to impair the import of the Su9-DHFR precursor into the mitochondria. As shown in fig. 23, 50µM CCCP was sufficient to cause a noticeable difference in the percentage of precursor protein that is imported. The results presented in figures 22 and 23 established that the optimal concentration of CCCP for the disruption of the membrane potential is 50µM. Mitochondria were incubated with this particular concentration of CCCP in the presence or absence of ascorbic acid.

As mentioned above, the yeast equivalent of ascorbic acid is D-erythroascorbic acid. For this particular project, an analogue known as D-(-)-isoascorbic acid (hereby referred to as D-IAA) was used, due to this being the only commercially available analogue of erythroascorbic acid. In this experimental setup, the mitochondria were incubated with D-IAA at the same time as the radioactive precursor. A series of different concentrations

were used, to check whether any potential effect that might be seen was dosage-dependent. Due to the fact that there had been various problems with abolishing the membrane potential (as seen in the previous experiments), an additional reagent, 2,4-dinitrophenol (DNP), was also used. Both DNP and CCCP are protonophores and abolish the inner membrane potential of the mitochondria by transferring protons (H^+) down an electrochemical potential gradient.

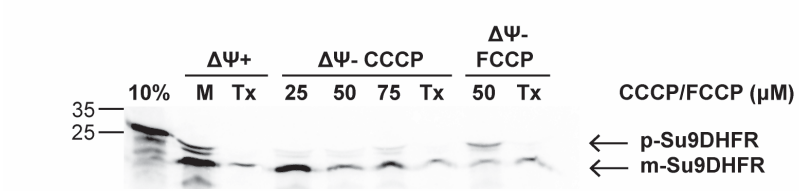

Figure 23: Trial import of Su9DHFR to assess Δ Ψ disruption through titration of CCCP. Su9-DHFR import is significantly reduced when mitochondrial membrane potential is abolished with 50 μ m of CCCP. The use of 50 μ M of FCCP also has the same effect. (The numbers 25, 50 and 75 indicate the μ M of CCCP/FCCP used for each treatment.)

As can be seen in fig. 24A, the inner mitochondrial membrane potential was completely abolished using CCCP and Su9-DHFR import was blocked. The addition of D-IAA to the import mix did not seem to rescue the phenotype, as the levels of imported Su9-DHFR were completely unaltered at all concentrations of D-IAA. On the other hand, as can be seen in figure 24B, DNP was not as efficient at abolishing the inner membrane potential, with the additional presence of D-IAA in the import mix leading to a further reduction in the levels of imported Su9-DHFR. The more prominent signal that can be detected in the two lanes with 5mM and 10mM of D-IAA added during import is most probably the result of insufficient cleavage by the protease, which is indicated by the fact that the precursor of Su9DHFR (ie. the non-cleaved version of the protein – p-Su9DHFR) is also present in the samples.

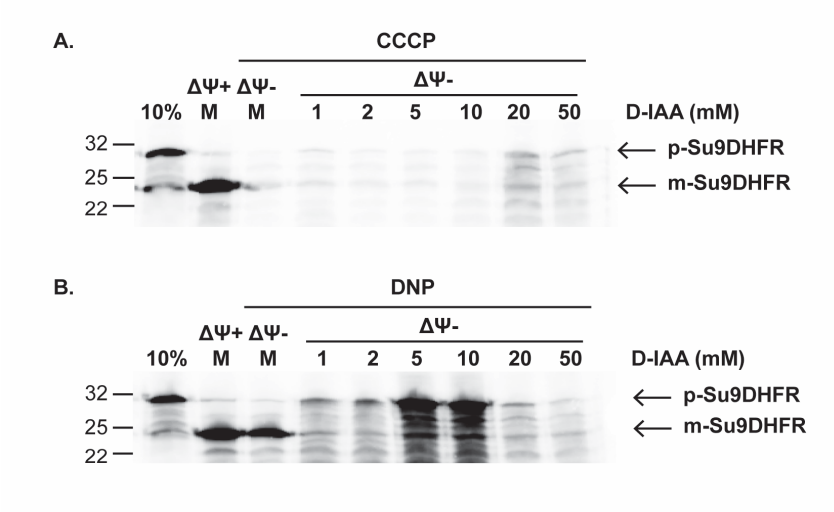

Figure 24: Assessing the effect of D-IAA on the import of Su9DHFR in mitochondria with disrupted ΔΨ. (A) Import of radiolabelled Su9-DHFR into wt mitochondria in the presence of various concentrations of D-IAA and 50μM of CCCP. (B) Import of radiolabelled Su9-DHFR into wt mitochondria in the presence of various concentrations of D-IAA and 25μM DNP. D-IAA was unable to rescue the effect that CCCP has on the import of Su9-DHFR.

4.2.3. Effect of D-isoascorbic acid on the mitochondrial membrane potential

As the indirect approach used in the above section failed to produce any results, a different experimental setup was designed in order to study the potential ability of D-IAA to rescue the inner mitochondrial membrane potential from being affected by the addition of uncouplers such as CCCP, FCCP, valinomycin and DNP. In this case, the mitochondrial membrane potential was measured through the ability of the fluorescent dye $DiSC_3(5)$ (3,3'-dipropylthiadicarbocyanine iodide) to be uptaken into mitochondria when the membrane potential is present and released when the membrane potential is abolished through the use of an uncoupler.

As can be seen in fig. 25, two time-points are important for this experiment: t=0sec, where the mitochondria were added to the reaction and t=120sec, where valinomycin, the membrane potential uncoupler, was added. The baseline for the $DiSC_3(5)$ fluorescent dye was always normalised as 100% of the fluorescent signal. Upon the addition of the mitochondria, the dye was taken up by the mitochondria and began to fluoresce at a different wavelength compared to when in solution without mitochondria, thus becoming undetectable to the fluorimeter. The lower the percentage of emission that can be recorded by the fluorimeter, the more dye has been internalised by the mitochondria. Upon the addition of the uncoupler, the fluorescent dye was released from the mitochondria and

thus, became accessible to the fluorimeter for detection. In the cases where D-IAA was required, it was added to the reaction before the addition of the mitochondria.

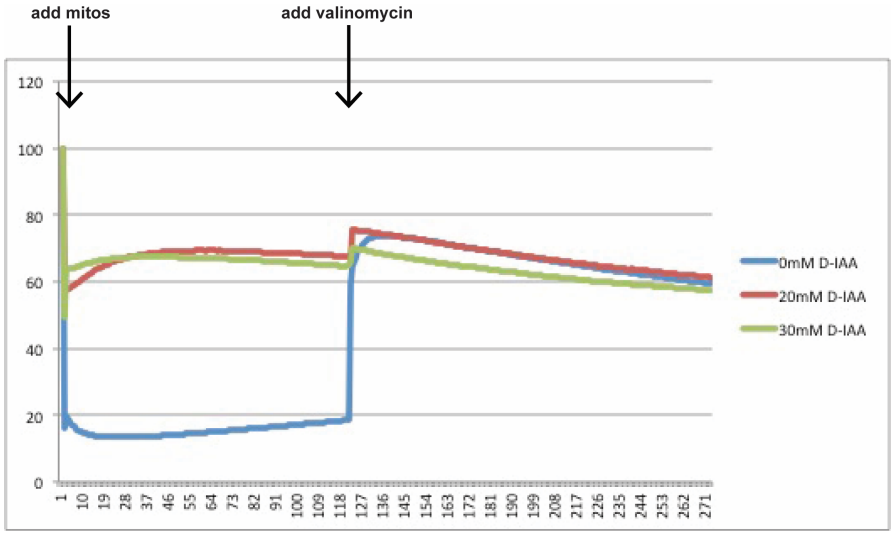

Figure 25: Measurement of $DiSC_3(5)$ emission (670nm) after treatment of mitochondrial samples with 1mM valinomycin. The D-IAA was added to the reaction buffer prior to the addition of the mitochondria.

In this particular experiment, we can see that the presence of D-IAA in the reaction leads to lower levels of internalisation of the $DiSC_3(5)$ dye by the mitochondria, while the addition of valinomycin also leads to a lower percentage of dye release.

4.3. Discussion

Arabinose can be used as a growth medium for yeast cells in order to initiate the pathway for the production of D-erythroascorbic acid, the equivalent of L-ascorbic acid (vitamin C) in mammals. Wild type yeast cells grow much slower when arabinose is used as a sole carbon source. As a result, it is very challenging to assess the antioxidant effect erythroascorbic acid may have in yeast, due to the smaller number of duplications in the same amount of time when comparing to the corresponding growth in glucose.

A possible way to circumvent this issue would be to supplement the glucose-containing growth medium with a small amount of D-arabinose, so as to induce the production of erythroascorbic acid, while at the same time maintaining the growth rate and optimal carbon uptake for the yeast cells. Even under such circumstances, it would be extremely

difficult to assess both the quantity of erythroascorbic acid produced under these conditions, as well as the effect that particular level of production might have against oxidative stress.

The import experiments that were performed in the presence of the erythroascorbic acid analogue, D-isoascorbic acid, were unable to lead to a conclusion concerning a protective effect that this particular chemical may have on the mitochondrial membrane potential, in a similar manner to what occurs in the mammalian cells used by KC and coworkers (KC et al. 2005). This particular experimental setup utilised an indirect method of detecting the mitochondrial inner membrane potential, through the abolishment of import of a matrix-targeted precursor. The addition of D-isoascorbic acid was unable to rescue the import defect caused by the addition of CCCP, a chemical uncoupler that depolarises the inner membrane. This could be due to either the D-isoascorbic acid not becoming internalised into the mitochondria in order to protect from the depolarisation or the D-isoascorbic acid not having the same effect on the yeast mitochondrial membrane potential.

The result from the fluorimetry assay was also quite inconclusive, as we seemingly have a lower percentage of depolarisation upon the addition of valinomycin. However, the presence of D-isoascorbic acid led to lower levels of $DiSC_3(5)$ internalisation into the mitochondria. More specifically, when D-isoascorbic acid was omitted, 80% of $DiSC_3(5)$ was internalised, with the addition of valinomycin leading to a ~50% release of the dye. In the two cases where D-isoascorbic acid was added, ~40% of the dye was internalized at the beginning of the experiment, with the subsequent addition of valinomycin causing a slight initial release of the dye. Interestingly, the amount of $DiSC_3(5)$ dye within mitochondria at the end of the experiment was greater than the point just before the addition of valinomycin.

As such, this particular result suggests that D-isoascorbic acid may have some protective effect on the mitochondrial membrane potential as, even though its addition to the reaction leads to lower levels of internalisation of the $DiSC_3(5)$ dye, it also causes less of this internalised quantity to be released upon the addition of valinomycin.

Chapter 5

The import of Gpx3 into the mitochondrial IMS

5. The import of Gpx3 into the mitochondrial IMS

5.1. Introduction

In 2012, the Meisinger group published a study wherein they identified 20 novel proteins that are localised in the mitochondrial IMS (Vögtle et al. 2012). Of these 20 proteins, three were the ones that caused the highest interest, due to their well described roles in pathways responsible for the regulation and combating of oxidative stress: Gpx3, Trx1 and Trr1.

A 2012 publication was able to show that Gpx3 is one of a number of different proteins that may be alternatively translated when the yeast cells are grown under hydrogen peroxide-induced stress (Gerashchenko et al. 2012). Work that was done in a collaboration between our lab and Chris Grant's lab at the University of Manchester was able to show that Gpx3 is more efficiently targeted to the IMS when it is alternatively translated and an 18 amino-acid N-terminal extension is added to the protein (Kritsiligkou et al. 2017). Gpx3 was proposed to play a different role to the one extensively characterised in the cytosol, with the loss of Gpx3 leading to abnormal mitochondrial morphology and defects in the import process, both of which can be rescued through the addition of the mitochondrially-targeted version of Gpx3 (Kritsiligkou et al. 2017). This particular publication was able to shine a light on the import of Gpx3 into the mitochondrial IMS, as well as its function in this subcompartment. Further work is required in order to fully understand the way in which Gpx3 becomes imported, as well the proteins it has the capability of interacting with in the IMS.

In this chapter, our aim was to look into the way in which Gpx3 becomes imported into the mitochondrial intermembrane space. Specifically, we wanted to address whether there is a possibility of the two Yap1-interacting proteins, Ybp1 and Ybp2/Ybh1 (throughout this experimental section, Ybp2/Ybh1 will be referred to as Ybp2), playing a role in the targeting of Gpx3 to the mitochondria. Despite the fact that no interaction partners of Ybp2 have been characterised, nor does Ybp1 seem to interact with Gpx3 directly as part of the Yap1 activation pathway (Gulshan et al. 2011), we posed the question whether either or both of the two factors could facilitate the import of Gpx3 into the mitochondria.

5.2. Results

5.2.1. Import of Ybp1 and Ybp2 into wild type mitochondria

We first wanted to test whether any of the components involved in the Yap1 activation pathway are imported into mitochondria (and more specifically into the IMS). This was done to check whether the imported Gpx3 in the mitochondrial IMS lacks the interactors it has in the cytosol, which in turn may suggest that it might serve a different function in the IMS compared to its known function in the cytosol. If all of the protein components of the Yap1 activation system have the ability to become imported, that would essentially suggest that the pathway is also active within mitochondria.

However, as can be seen from the image below (fig. 26), out of the four proteins involved in this pathway – which are Yap1, Gpx3, Ybp1 and Ybp2 – only Gpx3 (fig. 26C) becomes specifically imported in mitochondria. This suggests that Gpx3 could either play a different role in mitochondria or maintain a role similar to the cytosolic one, but engaging with a completely different set of interactors.

It is important to note the difference between the complete lack of import of Yap1 (fig. 26D) and the behaviour of Ybp1 (fig. 26A) and Ybp2 (fig. 26B). In the case of the latter two proteins, the import lane (M) and the Triton X-100-treated control lane (Tx) look exactly the same. The fact that the Tx lanes do not completely clear up could potentially mean that these two proteins strongly associate with the outer membrane of the mitochondria and cannot be completely cleaved by the addition of proteinase K, due to an association with the lipids. This could also account for the large amount of degradation visible, as partial cleavage by the protease could cause all of these smaller fragments of the radiolabelled proteins.

Indeed, when the radioactive precursors were cleaved using varying concentrations of proteinase K, they produced a reproducible cleavage pattern, which did not completely overlap with the cleavage pattern after import (fig. 27). As can be seen from the comparison between figures 26 and 27, the addition of proteinase K to the precursors alone, versus its addition to the samples after import into mitochondria have given different cleavage results. This could indicate that our two proteins associate with the outer

mitochondrial membrane in certain areas, thus masking cleavage sites and making them unavailable to the protease.

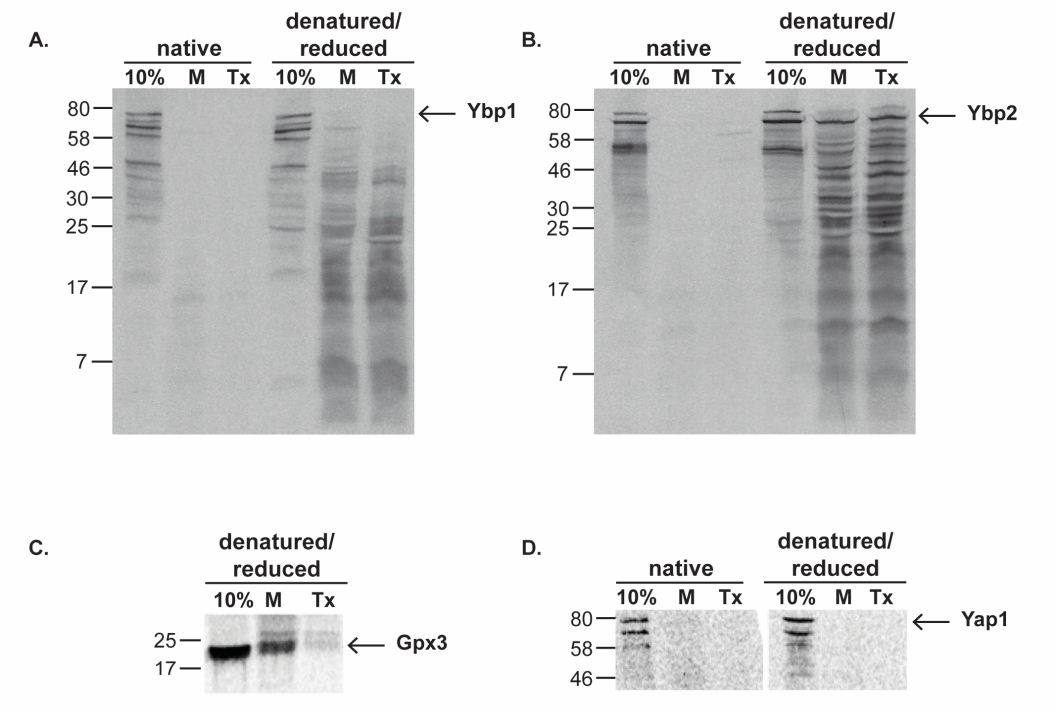

Figure 26: Import of radiolabelled precursors of the Yap1 activation pathway. Ybp1, Ybp2, Gpx3 and Yap1 were produced as radiolabelled precursors and presented to mitochondria using a standard import protocol. After the import, samples were treated with protease alone (M samples) or solubilized with 10% Triton X-100 in the presence of the protease (Tx samples) in order to verify whether the signal is indeed mitochondrial. Out of these proteins, only Gpx3 (C) becomes specifically imported into mitochondria under denaturing conditions. Ybp1 (A), Ybp2 (B) and Yap1 (D) do not become imported neither under native not under denaturing and reducing conditions, but the result for Ybp1 and Ybp2 suggests an association with the outer mitochondrial membrane.

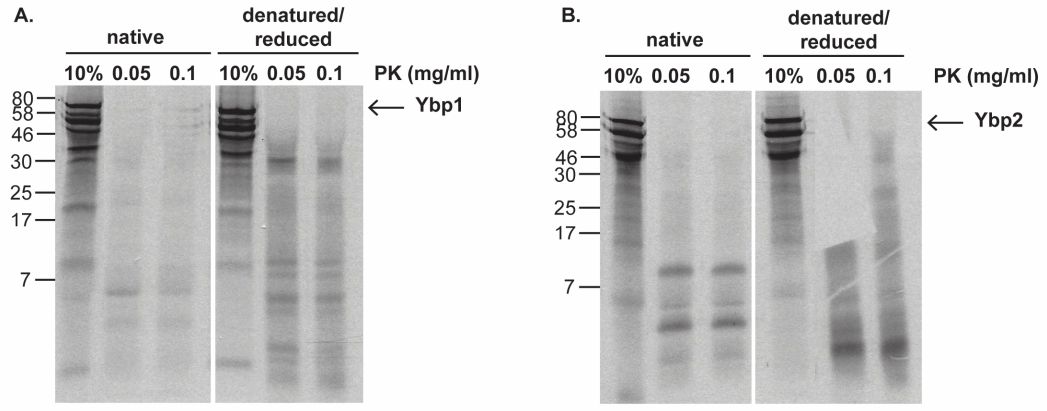

Figure 27: Cleavage of Ybp1 and Ybp2 precursors with different concentrations of proteinase K. Treatment of the two precursors with the protease produced distinct cleavage patterns, which differ between the native and denatured/reduced precursors. When comparing these results to the results obtained after

import (fig. 26), we can see that the cleavage patterns do not overlap, possibly indicating an association of Ybp1 and Ybp2 with the outer mitochondrial membrane.

5.2.2. Import of Gpx3 in the presence of Ybp1 and/or Ybp2

Due to the possible association of Ybp1 and Ybp2 with the outer mitochondrial membrane that was shown in the previous experiment, it was thought possible that either or both of the Ybp1/Ybp2 proteins could play a role in facilitating Gpx3 import to mitochondria.

To test this hypothesis, Ybp1 and Ybp2 were cloned into pET vectors, in order to produce them as purified proteins. These purified proteins could then be incubated together with the radioactive Gpx3 precursor and intact, import-competent mitochondria. If there was an increase in the amount of Gpx3 imported into the mitochondria in the presence of either or both of these proteins, that would mean that their presence has an effect on the mitochondrial import of Gpx3.

However, even though the clonings of these proteins were successful, their expression was not, despite testing various types of *E.coli* expression cells under different conditions (fig. 28).

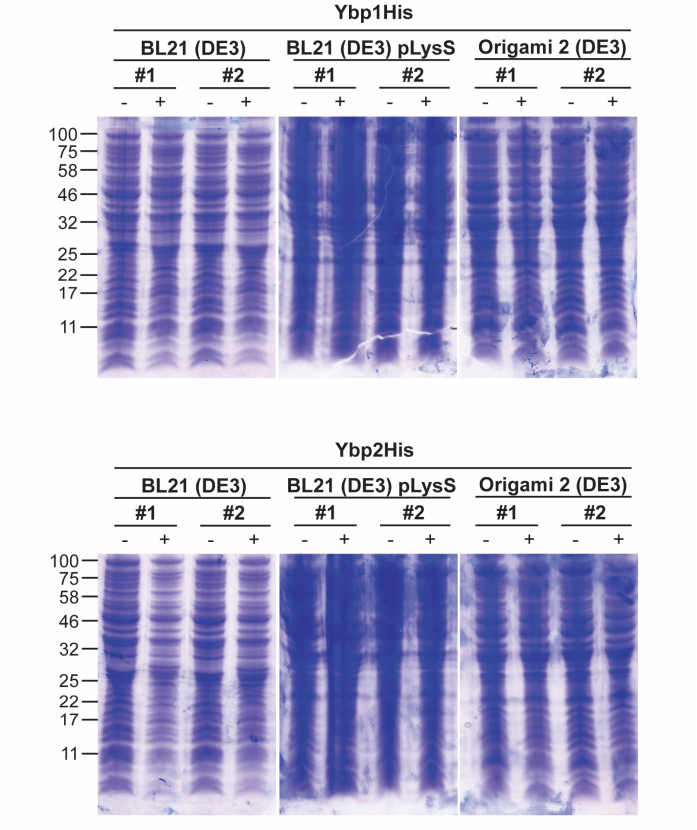

Figure 28: Induction of Ybp1 and Ybp2 expression. Three types of expression cells were used to test the induction of Ybp1 and Ybp2 expression from pET24 vectors. Various conditions were tested, but none were able to induce the production of either protein (here, the induction with 0.4mM IPTG (+) at 18°C overnight is shown). The absence of expression for both proteins was verified by performing Western blot analysis of the samples using an αHis antibody.

Due to the issues described above concerning the production of purified proteins, we decided to test the theory, by expressing all the proteins as radioactively labelled precursors. After the proteins were expressed using the TNT coupled transcription/translation kit, Gpx3 was treated with urea and DTT (for it to become denatured and reduced), while Ybp1 and Ybp2 were kept in their "native" state, with no additional treatment. The proteins were then incubated with wild type mitochondria, in order to determine whether the import of Gpx3 is affected in any way by the presence of either of the two proteins in the import mix.

As can be seen from the image below (fig. 29), there seems to be a small, but significant increase in the amount of Gpx3 imported into mitochondria, when Ybp1 is also present during the import process, while the imported levels of Gpx3 appear to be unaffected when incubated with Ybp2. This suggests that there is a relatively small, but noticeable effect on Gpx3 import that is specific for Ybp1. It is important to note here that the import of Gpx3 is very low, when compared to the result that was obtained in fig. 26, as well as the published work on Gpx3 import (Kritsiligkou et al. 2017). This is something that will require optimising in order to get a clearer image and a more definite idea of what is happening. This experiment has only given us a small indication that Ybp1 may play a role in the import of Gpx3 and a lot of further investigating is required in order to verify if this is actually true and occurs *in vivo*.

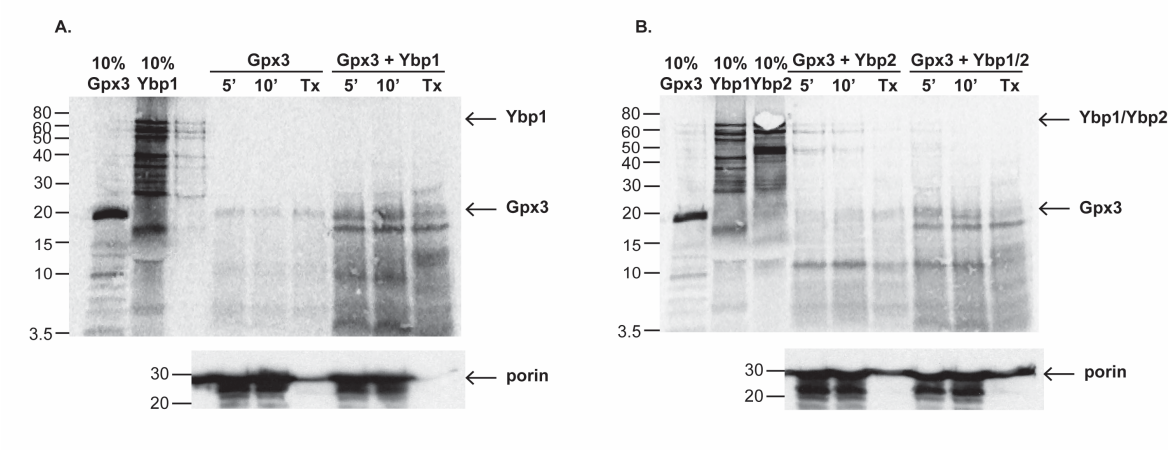

Figure 29: Import of Gpx3 in the presence or absence of Ybp1 and Ybp2. Gpx3 was denatured and reduced and imported into wt mitochondria alone or in the presence of Ybp1 (A) and in the presence of Ybp2 or the presence of both Ybp1 and Ybp2 simultaneously (B). Ybp1 and Ybp2 were added to the import mix as native. This experiment showed a higher amount of Gpx3 becoming imported into wild type yeast mitochondria, only in the cases where Ybp1 is present within the import mix.

5.2.3. Import of N18Gpx3 in the presence of Ybp1

Due to the results of the experiment above, where Gpx3 seems to be imported at a higher rate when Ybp1 is present during the import process, we thought to test whether Ybp1 might have a similar effect on the elongated version of Gpx3, N18Gpx3. As described by Kritsiligkou and coworkers, Gpx3 can be produced as an elongated version containing an 18-aminoacid N-terminal extension (Kritsiligkou et al. 2017). This particular version of the protein is produced upon oxidative stress and is believed to facilitate the import of Gpx3 into the mitochondrial IMS under these stress conditions. The N18Gpx3 version was reported to become imported more rapidly when compared to Gpx3, though both versions were found to be imported at similar levels at later time points (ie. the 10min used in this particular experiment) As Ybp2 did not seem to have an effect on the import of Gpx3, it was not tested any further. Following the same setup as before, where N18Gpx3 was treated with urea and DTT with Ybp1 being left untreated, we obtained the following result (fig. 30):

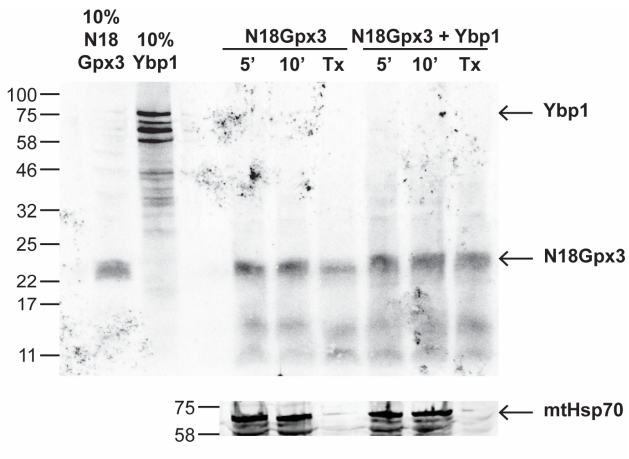

Figure 30: Import of N18Gpx3 in the presence or absence of Ybp1. N18Gpx3 was denatured and reduced and imported into wt mitochondria in the presence or absence of native Ybp1. The import of N18Gpx3 alone is stronger than that of Gpx3 alone (fig. 29). The presence of Ybp1 in the import mix did not have a particularly strong effect on the import of N18Gpx3.

From this image we can see that the import of the N18Gpx3 is stronger than that of Gpx3, a fact which agrees with the recently published data regarding the characterisation of Gpx3 in the mitochondrial IMS (Kritsiligkou et al. 2017). There does seem to be a slight increase in the import of the N18Gpx3 when Ybp1 is also added into the mix, however the difference between the two cases (N18Gpx3 alone versus N18Gpx3+Ybp1) is not as clear as it was in the case of Gpx3.

This result could possibly indicate that the N18 version of Gpx3 – whose 18 amino acid N-terminal extension seems to play a significant role in the import efficiency of Gpx3 – does not require the additional presence of Ybp1 in order to become imported more efficiently. Preliminary experiments that were performed with other mitochondrial proteins, such as Mia40, Tim10 and Su9DHFR, were unsuccessful due to issues with their translation from the TNT system. However, this is something that must be explored further and the import of the above mentioned (as well as additional) proteins must be compared in the presence and absence of Ybp1. This will allow us to explore the possibility that Ybp1 may play a role in the import of a number of different proteins and, as such, have a dual role as a cytosolic chaperone for proteins that are targeted to the mitochondria.

5.3. Discussion

Out of all the proteins involved in the Yap1 signalling pathway, only Gpx3 can be imported into yeast mitochondria. However, the fact that the signals for Ybp1 and Ybp2 did not clear up upon the addition of the detergent Triton X-100 and proteinase K led us to speculate that they could interact with the outer surface of the mitochondrial membrane. As a result, it was though possible that if this was indeed the case, one or both of these proteins could play a role in Gpx3 import, by interacting with Gpx3 for more efficient import into the mitochondria.

An experimental setup where Gpx3 was incubated with import-competent mitochondria at the same time as Ybp1, Ybp2 or both suggested that the import of Gpx3 is affected only when Ybp1 is present during import. A similar result was obtained for the case of the elongated version of Gpx3, N18Gpx3, with the effect not being as strong as in the case of Gpx3.

A working hypothesis that can be generated from the above results is that the import of Gpx3 into the mitochondrial IMS may be facilitated by the presence of Ybp1 in the import mix. This would further expand the role of Ybp1 to include it being a chaperone responsible for the correct targeting of Gpx3.

This particular hypothesis requires a lot of further testing in order to verify if this is something that can occur *in vivo*. Despite the fact that the effect of Ybp1 on Gpx3 import seems to be reproducible (observed from two individual experimental attempts), the result obtained for N18Gpx3 was only observed once (due to time limitations). As mentioned previously, preliminary experiments with other mitochondrial proteins (Mia40, Tim10 and Su9DHFR) were unsuccessful and must be repeated in order to test if the presence of Ybp1 during import affects the import of other proteins, or if the observed effect is specific for Gpx3.

Chapter 6

Characterisation of Trx1 and Trr1 import

6. Characterisation of Trx1 and Trr1 import

6.1. Introduction

As already mentioned in section 5.1, a 2012 study identified a pool of 20 novel proteins that are localised in the mitochondrial intermembrane space (Vögtle et al. 2012). From this list of proteins, three that caught our interest were Gpx3, Trx1 and Trr1, with Gpx3 having been the focus of chapter 5. The other two proteins are part of the cytosolic thioredoxin system with very well described roles in the detoxification of cellular stress. The presence of a complete thioredoxin system in the mitochondrial IMS is very interesting, as it adds the capability of reduction to this subcompartment, that up until now had only been described as an oxidising environment.

Up until this proteomic analysis was published in 2012, the IMS was known as an oxidising environment that allows the formation of disulfide bonds, just like the endoplasmic reticulum (ER) and the bacterial periplasm. What is interesting to note is that the proteins that are resident within these cellular compartments are typically translated in a different compartment than the one they end up in: the cytosol. One of the main characteristics of the cytosol is the fact that it is reducing and contains a series of proteins (such as thioredoxins and glutaredoxins) responsible for maintaining mitochondrial- or ER-targeted proteins in a reduced state (Herrmann and Riemer 2014).

As such, the discovery of a reducing system in the IMS of mitochondria opens up a wide field of research into further understanding the way in which this subcompartment functions, which must indicate a balancing act between the two systems: the MIA pathway and the thioredoxin pathway.

The presence of a reductive system in an otherwise oxidising environment is not unheard of. The Dsb (disulfide bond formation) system in the bacterial periplasm has been very well described and is made up of two separate branches: the oxidising branch – where the oxidoreductase DsbA and the sulfhydryl oxidase DsbB are responsible for the introduction of disulfide bonds – and the reductive/isomerisation branch – where DsbC and DsbD are

able to recognise and rectify incorrectly-formed disulfide bonds (Bardwell et al. 1991; Bardwell et al. 1993; Bader et al. 1999; Kadokura and Beckwith 2010).

Additionally, in the case of the ER, the presence of Pdi1 enables the oxidation and reduction/isomerisation functions to be carried out by a single protein. Pdi1, as well as 4 other yeast proteins and 20 mammalian proteins, belong to a superfamily of proteins, collectively known as PDIs (protein disulfide isomerases). These proteins are oxidoreductases that contain varying numbers of thioredoxin-like domains, with the most extensively studied member of the supefamily being the mammalian PDI (Hatahet and Ruddock 2009; Benham 2012). The isomerisation function is not common in all members of the PDI family, as there are certain members that are efficient at oxidation and isomerisation (such as PDI) (Oka and Bulleid 2013), while others are more efficient at reducing the incorrect disulfide bonds than at catalysing the formation of the correct ones (Bulleid and van Lith 2014).

In contrast to PDI, studies have not been able to show whether Mia40 has the ability to catalyse isomerisation reactions *in vivo*. Mia40 was shown to be able to interact with glutathione in a reaction that results in Mia40 being semi-oxidised. As this particular state of Mia40 was reminiscent of PDIs in the ER, it was though possible that Mia40 might also be able to reduce incorrectly formed disulfides (Riemer et al. 2009). The only supporting evidence of Mia40 having the ability to act as a reductase is an *in vitro* reconstitution assay, where reduced Mia40 was able to reduce the substrate protein Cox17. The reduction of the same substrate protein was also performed with PDI and DsbA, with Mia40 displaying the slowest kinetics (Koch and Schmid 2014a; Koch and Schmid 2014b).

The discovery of the presence of a thioredoxin system in the IMS could indicate that the two processes (oxidation and reduction) are uncoupled and more reminiscent of the Dsb system in the bacterial periplasm. Additionally, the putative presence of Grx2, a component of the cytosolic glutaredoxin system, in the IMS (Kojer et al. 2015), also points towards a separation between the two processes, with Mia40 introducing the disulfide bonds and the reductive systems correcting the non-native disulfides.

This chapter will focus on the characterisation of the import of Trx1 and Trr1 in the mitochondrial intermembrane space, in an attempt to understand how two proteins known

to reside in the cytosol can also become translocated across the outer mitochondrial membrane.

6.2. Results

6.2.1. Bioinformatics analysis of Trx1 and Trr1

Since the recent discovery of the fact that Trx1 and Trr1 also localise to the mitochondrial intermembrane space in addition to the cytosol, it became interesting to research the manner in which they become imported into this particular subcompartment of the mitochondria, as well as the reason for their existence in this oxidising environment.

The first step in this process was to perform bibliographical research and bioinformatics analysis, which could help determine some characteristics of the two proteins that could explain their presence in the mitochondria and, more specifically, the IMS.

Analysis of their amino acid sequences using the bioinformatics tool MITOPROT was able to determine their probability of become imported into mitochondria. This particular tool analyses the N-terminal regions of any sequence and is able to recognise specific patterns that could support a mitochondrial targeting sequence and are reminiscent of patterns found in known mitochondria-targeted proteins, as well as putative cleavage sites that can be recognised by mitochondrial proteases, such as MPP and IMP (Claros and Vincens 1996). Due to the fact that mitochondrial targeting prediction tools are not 100% reliable, we also used a number of other tools that are widely available online: Predotar – which also recognises N-terminal targeting sequences within proteins and which is quite reliable for the separation of mitochondrial and chloroplast targeting sequences (Small et al. 2004) –, TargetP – which predicts the subcellular localisation of proteins based on the recognition of mitochondrial targeting peptides, chloroplast transit peptides and secretory pathway signal peptides at the N-terminal region of the inputted sequence (Emanuelsson et al. 2000) – and MitoFates – an improved prediction tool for cleavable N-terminal presequences that detects positively charged amphiphilicity and presequence motifs, while

also making use of position weight matrices to map out the potential cleavage sites (Fukasawa et al. 2015).

By inputting our sequences of interest into these tools, we were able to see if they contain any possible mitochondrial targeting sequences that could explain their newly found presence in the mitochondrial IMS. However, the results from the different prediction tools were not always in agreement with one another and, as such, not very reliable. It is interesting to see how the different algorithms used by each tool are able to pick up different parts of the sequence and provide us with different outputs concerning the probability of mitochondrial localisation for the same protein. The results that were yielded from this attempt can be seen in Table 8.

Analysis of their amino acid sequences did not reveal the presence of a mitochondrial matrix-targeting signal (table 8), nor did it identify any classical cysteine motifs, which have been shown to play an important role in Mia40-dependent import (one of the main pathways of import followed by IMS proteins) (fig. 31). This is a consistent problem with bioinformatics tools, as matrix-targeting signals are the ones that are most readily recognised by the widely available prediction programmes. Other sorting signals that are responsible for targeting proteins to the other submitochondrial compartments are not very easily picked up. As can be seen from the data in table 8, the tools used for prediction analysis are not 100% reliable, as they all produce very different results due to the fact that they are unable to recognise proteins that have more unusual targeting motifs or depend on their cysteine residues and the MIA pathway in order to become imported (Erv1, Tim10).

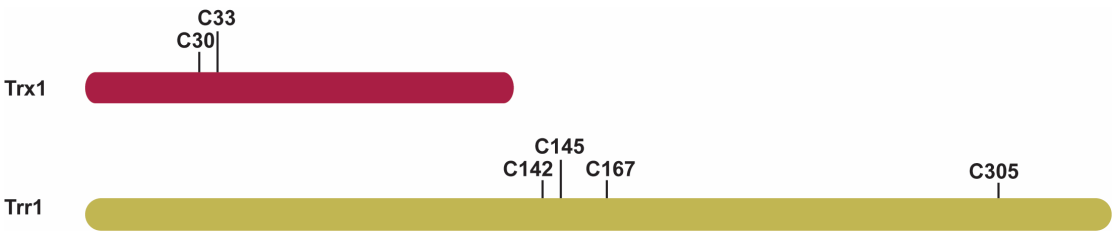

Figure 31: Cysteine residues in Trx1 and Trr1. Neither of the two proteins contains a typical cysteine motif that can be recognised by Mia40. Trx1 contains two cysteine residues arranged in a CX2C motif that is characteristic for all thioredoxins. Trr1 contains a CX2C motif, as well as two single cysteine residues.

	Prediction tool				
	MITOPROT	PREDOTAR	TargetP	MitoFates	
	Probabilty of mitochondrial targeting		Probability of mitochondrial localisation	Probability of mitochondrial presequence	Possible site of mitochondrial peptidase cleavage
Protein					
Trx1	0.065	0.00	0.054	0.00	F11 (MPP)
Trr1	0.111	0.01	0.236	0.008	A25 (MPP)
Trx2	0.053	0.00	0.055	0.00	V88 (MPP)
Trx3	0.895	0.93	0.865	0.996	F20 (MPP)
Trr2	0.973	0.66	0.771	0.996	M24 (MPP)
Mia40	0.987	0.84	0.932	0.996	L33 (MPP)
Erv1	0.154	0.00	0.074	0.00	S32 (MPP)
Tim10	0.019	0.02	0.207	0.00	C65 (MPP)

Table 8: Prediction of probability of mitochondrial localisation using online bioinformatics tools. Each number represents the probability of mitochondrial localisation with a number between 0 and 1 (0 → no mitochondrial localisation, 1 → 100% mitochondrial localisation), with the MitoFates tool also providing information concerning putative cleavage sites (shown here are the aminoacid predicted to be the cleavage site and its position within the protein, as well as the protease predicted to cleave at each position).

6.2.2. Expression of Trx1 and Trr1 as radiolabelled – First attempts

In parallel to the bioinformatics analysis that was performed, the two proteins in question were produced as radiolabelled using the Promega TNT kit. This could be done without doing any clonings, as the plasmids had already been constructed by a previous researcher in our lab, where both proteins had been cloned as His-tagged into pSP64 vectors. The proteins were produced as radiolabelled versions using ^{35}S-methionine. These radioactive precursors were presented to wild type mitochondria using a standard import protocol. It is important to note that term "precursor" is usually used for proteins that become cleaved after import into mitochondria. However, in this thesis, we have extended the term "precursor" to mean the version of the protein before import, despite the fact that the proteins tested are not likely to become cleaved or otherwise modified after they enter the mitochondria. This is something that would require verification and would necessitate the analysis of the state of the protein before and after import using mass spectrometry.

As can be seen in fig. 32, the import of Trx1 and Trr1 is rather difficult to track. First of all, there seems to be an issue with the translation of the two proteins (something that can be seen from the lanes designated as "10%"). In this gel lane, we have loaded 10% of the total amount that has been presented to the mitochondria during the import process. This is used as a way to both check the production of the protein from the TNT process, as well as to be able to assess and quantify the amounts of protein that actually do end up becoming imported into the mitochondria. In the case of Trx1 (fig. 32A), the TNT translation kit produces two translated proteins: one that migrates just above the 11kDa marker and another that migrates just below 11kDa, with the latter being the most prominently expressed one. The higher molecular weight band corresponds to the full-length Trx1 protein, with the lower one possibly being the product of alternative translation or truncation of the full-length protein. It is not clear why the second band is much more prominent than the one corresponding to the full-length protein, however it was consistently produced in every TNT reaction using this particular plasmid, irrespectively of the final concentration of the plasmid added to the TNT reaction.

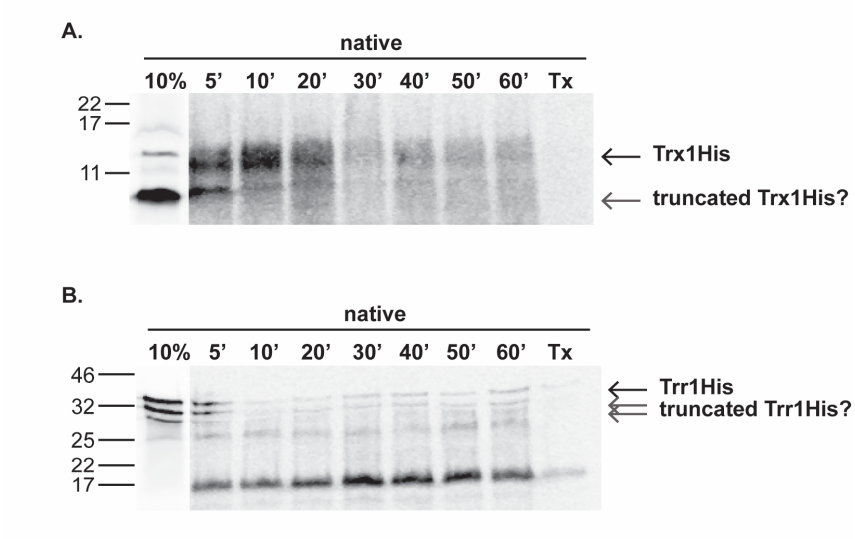

Figure 32: Import of native, radiolabelled Trx1His and Trr1His. Both Trx1His (A) and Trr1His (B) were produced as radiolabelled using the Promega SP6 TNT-coupled system and presented to wt mitochondria. The translation process lead to the production of two translated proteins in the case of Trx1 and three in the case of Trr1, with the higher band corresponding to the correctly synthesised protein in each case.

On the other hand, there seems to be a similar situation happening with Trr1 (fig. 32B). In this case, we have the production of three different precursor forms, all around the 32kDa marker (with a difference of a few kDa between each one of them), with the two highest molecular weight bands being of similar intensity. Similarly to Trx1, this issue arose consistently in every TNT reaction and could be due either to alternative translation initiation sites or to a small amount of degradation at the N- or C-termini of the protein.

Another issue that can be seen from these particular import figures is the fact that, even though we have followed the import process for various timepoints (from 5min to 1 hour), there doesn't seem to be any clear import and time-dependency for either of the two proteins. This is something that was not expected, as the conditions used in these experiments were the same as the ones used in previous work that has published by our lab as well as others in the same field of research. As such, this particular result cannot really be explained and further work is required in order to understand the import of Trx1 and Trr1.

In the previous experiment, the precursors were presented to the mitochondria in the state that they were produced during the TNT process, with no urea or DTT treatment in order to denature and unfold our proteins of interest. Due to the presence of a series of different Hsp chaperones, such as Hsp70 and Hsp90, in the rabbit reticulocyte lysate (one of the main components of the Promega TNT kit that we use for these types of experiments), we operate on the assumption that the proteins produced through this process are folded, even

if only partially (despite the fact that we have not assessed this experimentally). As such, it is common practice, when we have unclear results regarding the import of the "native" precursors, to try to denature them first (usually with urea), before presenting them to the mitochondria. This mimics the cellular conditions, as most proteins that are destined to the mitochondria are kept in an unfolded state in the cytosol (by different chaperones and cytosolic factors), until they can be imported into mitochondria, sorted into the correct subcompartment and folded into their native state in the compartment where they carry out their function. Due to the fact that import experiments utilise isolated mitochondria and do not contain any of the cytosolic components that might be required for protein import (other than the ones that are endogenously present in the reticulocyte lysate), the unfolding of the radioactive precursor prior to its presentation to the isolated mitochondria frequently facilitates the import process.

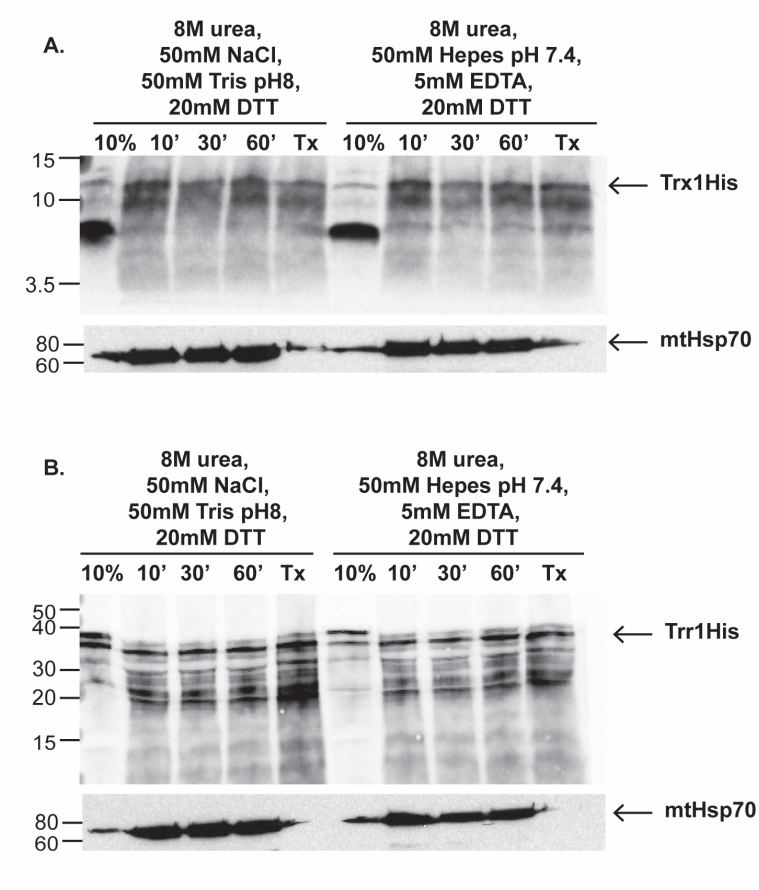

Figure 33: Import of denatured and reduced, radiolabelled Trx1His and Trr1His. Both Trx1His (A) and Trr1His (B) were produced as radiolabelled using the Promega SP6 TNT-coupled system, treated with the two different denaturing and reducing buffers and presented to wt mitochondria. The import experiment was inconclusive, as the presence of radioactive signals in the Triton X-100 treated lane (Tx) did not allow us to say whether the proteins that were presented to the mitochondria were actually imported.

As can be seen in fig. 33, we have used two different ways to completely denature and unfold the precursor proteins. The two common components of both buffers are urea and DTT (used at 8M and 20mM respectively) that are used in order to completely open up the protein molecules. Urea is a chaotropic agent that has the ability to interfere with the noncovalent bonds within proteins, thus leading to their denaturation. DTT is a reducing agent that can cleave disulfide bonds, while also preventing the formation of intra- and intermolecular disulfide bonds between the cysteine residues of proteins. Tris-HCl pH8 and HEPES-KOH pH7.4 are buffers responsible for keeping the pH of the reaction stable. The addition of NaCl to the first buffer can help with solubilising the proteins and preventing the formation of aggregates, while EDTA in the second buffer acts as a chelator for divalent ions and can facilitate the denaturation of proteins that require ions in order to fold and function. The reason why two different types of buffers were used is because experiments with other proteins have shown that certain proteins work better when in the Tris and NaCl environment, while others when in the HEPES and EDTA environment. Unfortunately, denaturing the precursors did not improve the previous result with the native precursors. An additional problem in these import experiments was the fact that the solubilisation of the mitochondria with the detergent Triton X-100 after import – a process which normally allows access of all mitochondrial components to the externally added protease and therefore results in complete digestion of the imported precursor – did not work. With such a result, we cannot even say that this particular import experiment was successful, as we cannot be sure that the signals we are seeing in the import lanes (lanes designated 10', 30' and 60' in fig. 33) are truly imported. This particular issue most likely has its roots in the quality of Triton X-100 used in the experiments, as this was a recurring issue for most such experiments performed in the lab, especially those that required treatment of the precursor with urea and DTT prior to import.

Due to the fact that in the case of the denatured import attempts, the Triton X-100-treated control samples did not become cleaved, we questioned whether the effectiveness of proteinase K cleavage of these particular proteins was affected by the presence of Triton X-100 in the mix. As is clear from fig. 34, this is not the case, as Trx1 is cleaved by PK regardless of the presence of Triton X-100 in the cleavage mix and regardless of its state.

As this particular issue could not be explained by the previous experiment, we went back to the original publication in which Trx1 and Trr1 had both been identified as novel intermembrane space localised proteins and looked for differences in the treatments that

were being used. Indeed, there was a significant difference in both the composition of the buffers used during the import process, as well as the buffers that were used during the protease cleavage step. The differences can be seen in detail in table 9.

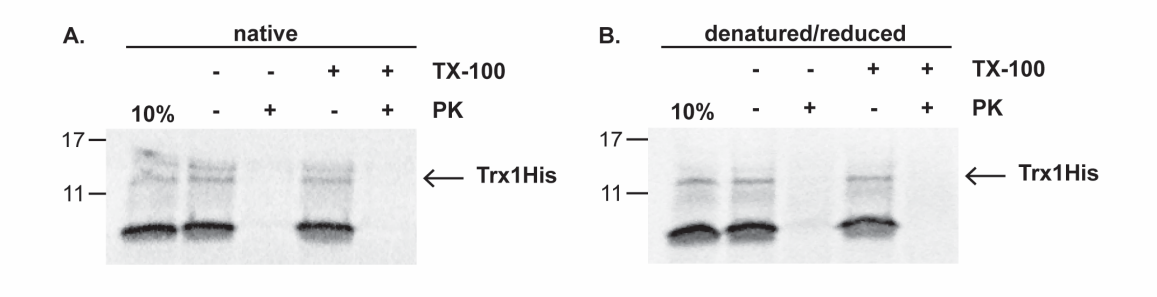

Figure 34: Cleavage of Trx1His precursor by PK in the presence or absence of the detergent Triton X-100. Both native (A), as well as denatured and reduced (B) Trx1 can be cleaved by PK, regardless of the presence of Triton X-100 during the cleavage process.

Our lab	Vögtle et al. 2012
Import buffer	
600mM sorbitol	250mM sucrose
2mM KH_2PO_4	10mM MOPS-KOH pH7.2
50mM KCl	80mM KCl
50mM HEPES	5mM KPi
10mM $MgCl_2$	5mM $MgCl_2$
2.5mM Na_2EDTA pH7	3% w/v BSA (omitted for Trx1)
5mM L-methionine	
1mg/ml fatty acid free BSA	
Resuspension/cleavage buffer	
600mM sorbitol	250mM sucrose
20mM HEPES-KOH pH7.4	0.5mM EDTA
	500mM MOPS-KOH pH7.2

Table 9: Comparison between the buffers used in our lab versus the ones used in the published work by Vögtle et al, 2012. Both the import and the resuspension/cleavage buffers have different components, as well as a different pH.

In order to test if the different import and cleavage buffers that were used were the cause the unclear results, we performed the imports using the buffers described in Vögtle et al. (2012) for both Trx1 and Trr1. As can be seen in fig. 35, the results were not particularly conclusive, neither for the native nor the denatured radioactive precursor import. More specifically, the change of buffers did not seem to help with clearing up the Triton X-100 (Tx) samples in the cases of the denatured protein imports, nor did the omission of BSA from the import buffer seem to have a significant effect on the import of Trx1.

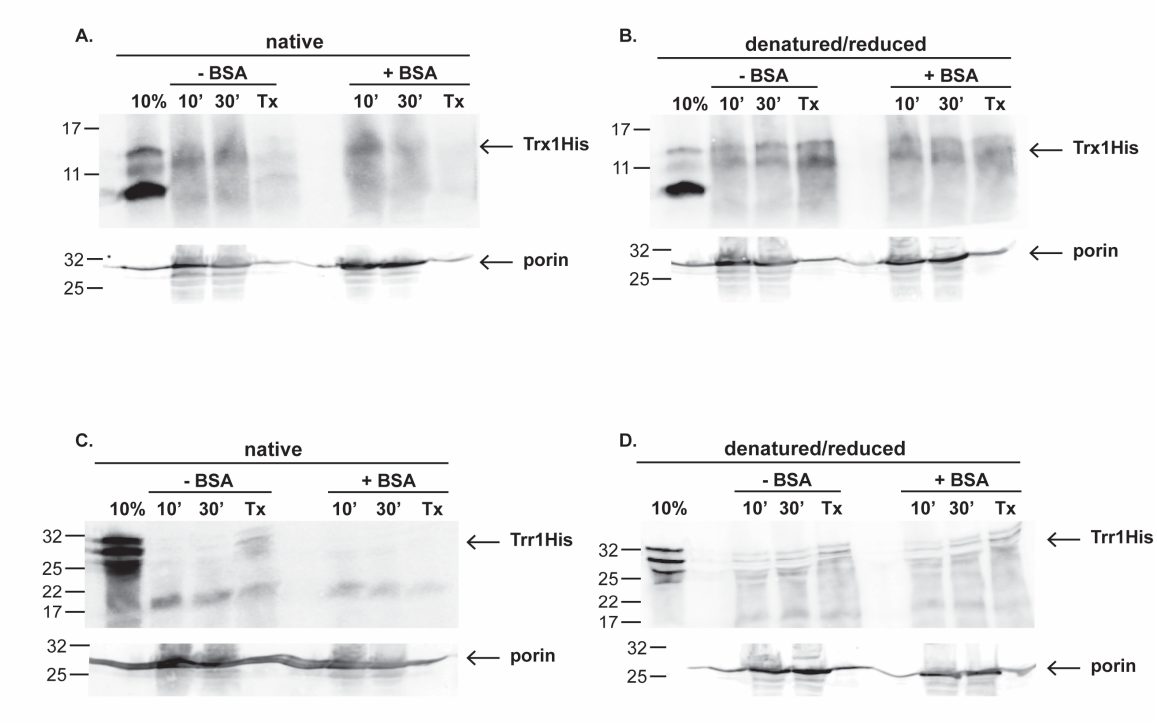

Figure 35: Import of Trx1 and Trr1 using the buffers described in Vogtle et al, 2012. The omission of BSA from the import buffer did not have a significant effect on the import of native (A) or denatured and reduced (B) Trx1. The use of different buffers did not help with the import of Trr1 (C+D).

At this stage, we thought it would be a good idea to re-sequence all of our plasmids and make sure that the materials we were working with were exactly what they should be and, interestingly, found that the particular constructs we had been working with contained mutations. More specifically, our Trx1 plasmids contained two point mutations (F11Y and S70P), while our Trr1 plasmids contained only a Q196R mutation. It was unknown whether these particular mutations may have affected the folding and function of our two proteins of interest and, as such, we decided that the correction of these mutations was essential during these early stages of experiments on this particular project.

6.2.3. Expression of Trx1 and Trr1 as radiolabelled – Test imports using the corrected plasmids

After correcting all the point mutations and re-sequencing all our constructs in order to make sure that this time we were working with 100% correct starting materials, we used the new plasmids to produce the radiolabelled proteins and imported the precursors into wt mitochondria. Unfortunately, the results were not substantially different (as can be seen in fig. 36), a fact that led us to start working on this particular project using two alternative approaches.

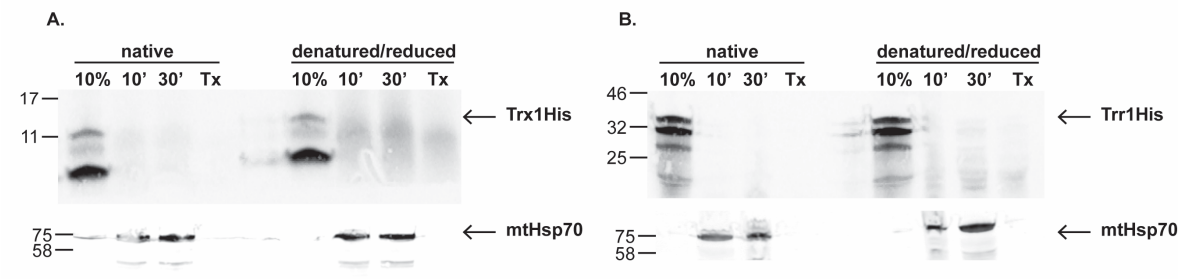

Figure 36: Import of Trx1 and Trr1 into wt mitochondria using the corrected plasmids as templates for the TNT reaction. Despite the plasmids containing the 100% correct sequences for our two genes of interest, the issue with the translation of multiple bands for both Trx1 (A) and Trr1 (B) remained unsolved.

6.2.4. Improving the translation of Trx1

When looking at the sequences of Trx1 and Trr1, it is clear that they contain more than one methionine residue. This could possibly account for the smaller precursors that are translated by the SP6 polymerase during the TNT reaction. In order to see if this is actually the case, we decided to mutagenise two of the methionine residues of Trx1. This new, mutagenised version of Trx1, where M35 and M39 were exchanged to isoleucine, was produced and imported into mitochondria (fig. 37).

When comparing the 10% samples of Trx1 produced by the wild type pSP64-Trx1His plasmid and the mutagenised pSP64-Trx1His-M35/39I plasmid, there is quite a clear difference. Even though both plasmids produce two protein species, the ratio between the

full-length Trx1 and the cleaved Trx1 bands is much better in the case of the mutagenised version of Trx1. When both translation products are imported into mitochondria, the import of the mutagenised Trx1 is elevated in comparison to the wild type version. Unfortunately, this particular result was not reliably reproducible, as in some cases the translation of Trx1 from this particular plasmid did not produce enough radioactive protein in order to be detected by the scanner.

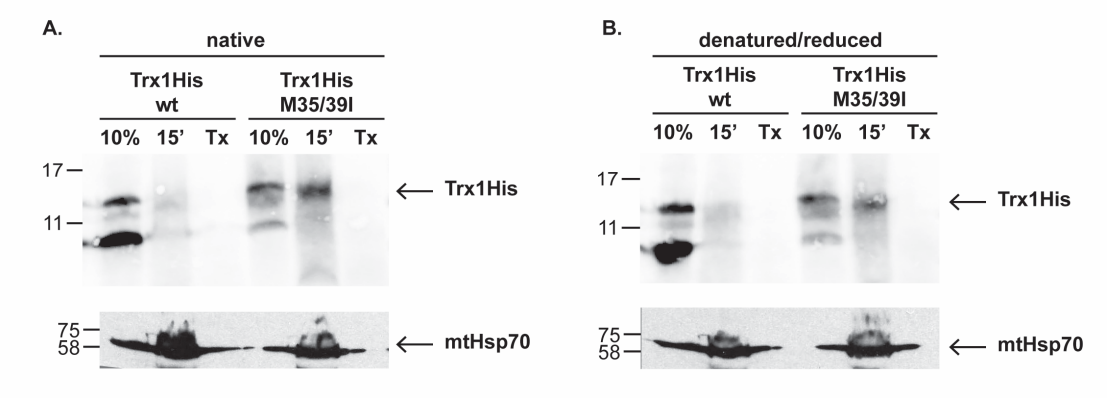

Figure 37: Import of wt and M35/39I Trx1 in wt mitochondria. Trx1His wt (A) and Trx1His M35/39I (B) were produced as radiolabelled precursors and imported into wt mitochondria either as native or after being treated with urea and DTT. The exchange of the two methionine residues at positions 35 and 39 to isoleucine led to the enrichment of the higher molecular weight band (which corresponds to the full-length Trx1), while also improving the level of import.

Therefore, despite the fact that the problem of translation could be due to the presence of the second and third methionine residues in the protein sequence, if we were to use this plasmid in our further studies, we would still be working with a mutated protein. Additionally, since the protein import assays were not reproducible even when using the methionine-mutated version of the protein, we decided not to pursue this approach with the *in vitro* translated radioactive precursors any further.

We also considered using this particular approach to determine if it would also help with the translation of Trr1. The sequence of Trr1 does indeed contain methionine residues near the N-terminal end of the sequence, which could possibly lead to the production of the shorter translation products (M35, M36, M68, M71). However, given the limited success this particular approach had for Trx1, we decided not to repeat the process of Trr1. Instead,

we decided to explore alternative ways of deciphering the import of our proteins of interest.

The two basic approaches that we wanted to use were a) to make use of the endogenous levels of Trx1 and Trr1 in mitochondria, an approach which could be limited by the levels and ease of detection of the two proteins and b) to attempt to assess the import of our two proteins of interest using recombinant purified proteins, as opposed to the radioactive counterparts with which so many issues arose.

6.2.5. Working with the endogenous Trx1 and Trr1 in mitochondria

As stated above, the first alternative approach that we followed was to attempt to work with the endogenous levels of the two proteins. In this particular case, our aim was to work with the system as is, with no external additions of proteins, in order to assess the effect that each protein has on mitochondrial function. Up until now, the proteins of the thioredoxin family had only been studied in relation to their effect on the whole cell, as they had only been known to localise in the cytosol. Consequently, the discovery of their dual localisation in the mitochondrial IMS made it interesting to investigate if there is a mitochondrial-specific phenotype that the lack of either of these two proteins causes, in a similar fashion to the abnormal mitochondrial morphology and dysfunction that the loss of Gpx3 can cause (Kritsiligkou et al. 2017).

One of the most significant limitations of this particular approach was the ability to detect the proteins with antibodies, something that would be dependent on the amounts of each protein within mitochondria. Given that these two particular proteins have a clear and well established role in the oxidative stress response in the cytosol, we expected that the amounts of Trx1 and Trr1 in the mitochondrial IMS would be very low and their detection may be challenging.

In order to detect the two proteins in mitochondria, we used rabbit polyclonal antibodies that we raised against the purified recombinant proteins and performed western blots against specific quantities (100μg) of purified mitochondria. The concentrations of the mitochondrial samples were determined prior to the experiment, a step that is particularly

important when comparing mitochondria from different yeast strains. We successfully detected Trr1 in mitochondria (the presence of the band that can be detected in the *Δtrr1* mitochondrial sample using the αTrr1 antibody will be addessed below), but, unfortunately, not Trx1 (fig. 38). This could be due to the fact that the quantities of Trx1 within mitochondria are below the antibody detection threshold.

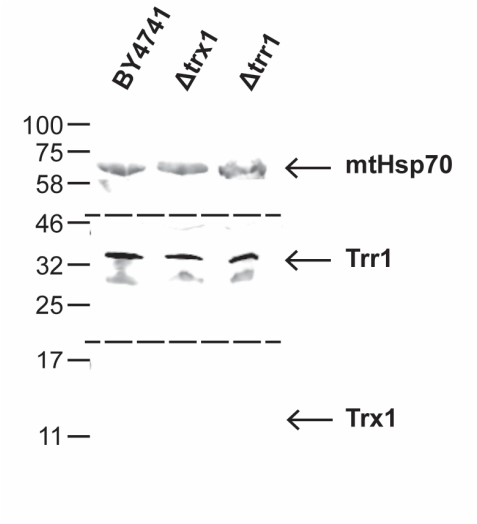

Figure 38: Detection of Trx1 and Trr1 in yeast mitochondria. 100µg of three different types of mitochondria were run on SDS PAGE and transferred onto nitrocellulose membrane, which was then cut along the dotted lines and blotted with three different antibodies. Trx1 was not detected in any sample, while Trr1 was detected in all samples, including the *Δtrr1* mitochondria.

In order to make sure that it was indeed the levels of Trx1 within mitochondria that led to the inability to detect it in our mitochondrial samples, we used a series of different antibody dilutions ranging from 1:100-1:1000, as well as tested the detection using two different detection systems: the HRP-luminescence detection using films and the LI-COR Odyssey CLx fluorescence imaging system. We further verified the capability of our antibody to detect Trx1 as a purified recombinant protein, as well as in whole cell extracts, which further backs up the claim that the levels of Trx1 within mitochondria are below the detection threshold of the antibody (fig. 39). The concentration of both types of samples was determined prior to the loading of the acrylamide gels: the recombinant protein was measured using the absorbance of the solution at 280nm and the extinction coefficient of Trx1 (10,095, when assuming the protein is oxidised), while the whole cell extract was measured using the Bradford assay (Biorad).

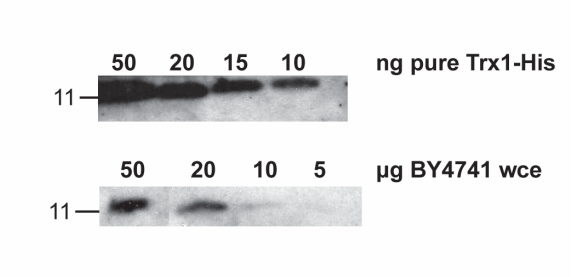

Figure 39: Detection of Trx1 using the αTrx1 antibody. Trx1 was detected in samples containing varying amounts of pure, recombinant Trx1His, as well as various quantities of whole cell extract (wce) prepared from a OD_{600}=1 BY4741 culture.

Due to the fact that we were able to detect endogenous Trr1 using our antibodies, we went on to verify its localisation within the mitochondria, using the mitoplasting and carbonate extraction methods that were described in section 3.2.2.7. From the results of this experiment, we were able to determine that Trr1 is indeed localised within mitochondria and seems to show an affinity for the pellet fraction, even though it still remains a soluble protein and does not become embedded in to the membrane. Unfortunately, due to the fact that it was not cleaved upon addition of proteinase K during the mitoplasting procedure, we were not able to definitively say that it is localised in the mitochondrial IMS (fig. 40).

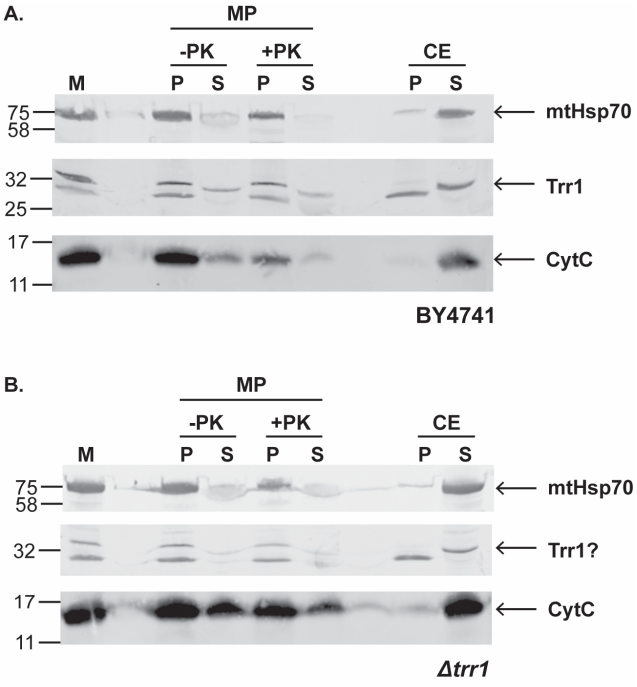

Figure 40: Localisation of endogenous Trr1. Analysis of the localisation of Trr1 in wt (BY4741) (A) and *Δ trr1* (B) mitochondria using mitoplasting (MP) and carbonate extraction (CE). Mitoplasting is the process of creating mitochondria that lack their outer membrane (mitoplasts) using osmotic shock. The presence or absence of protease (-/+PK) allows us to assess the localization of our protein of interest. After centrifugation of the treated samples, the mitoplasts can be found in the pellet fraction (P), while all the soluble IMS components can be found in the supernatant (S). Carbonate extraction is the process through which the soluble and membrane-integrated components of the mitochondrion can be separated, with the former remaining in the supernatant fraction (S) and the latter in the pellet fraction (P). Trr1 was shown to be localised in mitochondria, but the mitoplasting and carbonate extraction were unable to provide us with the information needed to pinpoint its submitochondiral localisation. This experiment also showed the detection of a band at ~35kDa in the *Δtrr1* mitochondria (see also fig. 38).

An interesting observation that was made during this experiment came about when we tested the detection and localisation of Trr1 in mitochondria derived from a *Δtrr1* yeast strain. This particular sample was used as a control and we were not expecting to see a signal when using the αTrr1 antibody against these mitochondria. However, when we used this particular antibody, we were able to detect a distinct – though fainter – signal. Additionally, it is important to note here that the αTrr1 antibody, both in this experiment,

as well as the initial detection experiment shown in fig. 38, detected two bands around the 32kDa molecular weight marker.

These two observations led us to look into the sequence similarity between the mitochondrial matrix-localised Trr2 and Trr1, in order to assess whether our antibody could also be detecting Trr2 in our samples. Trr1 and Trr2 are share an 86% identity (93% similarity) in their primary structure (calculated using blastp), except for the 23 amino-acid matrix targeting signal that is present only in Trr2 (fig. 41). According to the online mitochondrial targeting prediction tool MitoFates (Fukasawa et al. 2015), Trr2 is predicted to have a cleavage site at position 24, which is processed by MPP, thus leading both proteins to having the exact same number of residues. As such, with such high percentages of similarity and identity, as well as the fact that, once Trr2 is processed, it ends up being the same length as Trr1, our antibody is most likely detecting both proteins at the same molecular weight, with the second, lower molecular weight band that is detected being non-specific.

```
CLUSTAL 2.1 multiple sequence alignment


yeastTrr1       ----------------------MVHNKVTIIGSGPAAHTAAIYLARAEIKPILYEGMMA 37
yeastTrr2       MIKHIVSPFRTNFVGISKSVLSRMIHHKVTIIGSGPAAHTAAIYLARAEMKPTLYEGMMA 60
                                     *:*:**********************:** *******

yeastTrr1       NGIAAGGQLTTTTEIENFPGFPDGLTGSELMDRMREQSTKFGTEIITETVSKVDLSSKPF 97
yeastTrr2       NGIAAGGQLTTTTDIENFPGFPESLSGSELMERMRKQSAKFGTNIITETVSKVDLSSKPF 120
                ************:********:.*:*****:***:**:****:****************

yeastTrr1       KLWTEFNEDAEPVTTDAIILATGASAKRMHLPGEETYWQKGISACAVCDGAVPIFRNKPL 157
yeastTrr2       RLWTEFNEDAEPVTTDAIILATGASAKRMHLPGEETYWQQGISACAVCDGAVPIFRNKPL 180
                :************************************:*******************

yeastTrr1       AVIGGGDSACEEAQFLTKYGSKVFMLVRKDHLRASTIMQKRAEKNEKIEILYNTVALEAK 217
yeastTrr2       AVIGGGDSACEEAEFLTKYASKVYILVRKDHFRASVIMQRRIEKNPNIIVLFNTVALEAK 240
                ************:*****.***::******:***.***:* *** :* :*:********

yeastTrr1       GDGKLLNALRIKNTKKNEETDLPVSGLFYAIGHTPATKIVAGQVDTDEAGYIKTVPGSSL 277
yeastTrr2       GDGKLLNMLRIKNTKSNVENDLEVNGLFYAIGHSPATDIVKGQVDEEETGYIKTVPGSSL 300
                ******* *******.* *.** *.********:***.** **** :*:***********

yeastTrr1       TSVPGFFAAGDVQDSKYRQAITSAGSGCMAALDAEKYLTSLE 319
yeastTrr2       TSVPGFFAAGDVQDSRYRQAVTSAGSGCIAALDAERYLSAQE 342
                ***************:****:*******:******:**:: *
```

Figure 41: Alignment of Trr1 and Trr2 sequences using the Clustal O online multiple sequence alignment tool. The alignment clearly shows the high identity and similarity between the two proteins, as well as the N-terminal mitochondrial targeting signal of Trr2.

In order to test that this was indeed the case, we prepared mitochondria from a *Δtrr2* yeast strain background and tested the αTrr1 antibody against equal amounts of all three types of mitochondria. As can be seen in fig. 42, the signals detected in the two deletion strains (*Δtrr1* and *Δtrr2*) are fainter than the signal detected in the wt strain and the lower molecular weight signal is present in both types of mitochondria. This means that the signal we can detect in the wild type mitochondria is actually coming from the presence of the two thioredoxin reductases in the mitochondria.

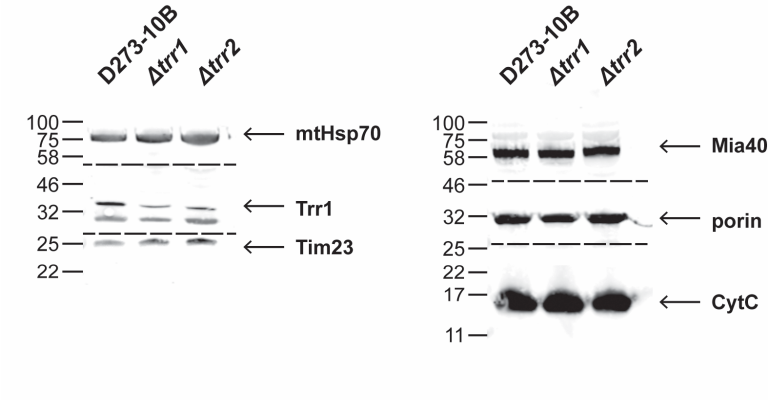

Figure 42: Testing the detection of Trr1 in *Δtrr2* mitochondria. Use of the αTrr1 antibody against 100μg of purified mitochondria from three different yeast strains (D273-10B, *Δtrr1* and *Δtrr2*) was able to show us that the signal detected in the wild type strain (D273-10B) is split between the two deletion strains (*Δtrr1* and *Δtrr2*), though further Western blot analysis using the double deletion mutant *Δtrr1/2* is required for us to definitively say that the signal corresponds to both IMS- and matrix-localised Trrs. Additionally, as the lower molecular weight band is detected in all three types of mitochondria, we believe this signal to be non-specific.

We then repeated the localisation experiment in the *Δtrr2* mitochondria and used our αTrr1 antibody in order to detect the distribution of Trr1 in the mitochondrial subcompartments. What we observed was the exact same pattern we had observed for the wild type yeast mitochondria, but with fainter signals – which was expected given the fact that the antibody is no longer also detecting Trr2 (fig. 43).

As can be determined from the experiments performed in this section, our attempt to utilise the endogenous mitochondrial pools of our two proteins of interest was only partially successful. Our inability to detect Trx1 in the mitochondria using our rabbit polyclonal antibody that was raised against purified Trx1 meant that this approach could only end up being used for Trr1. However, the αTrr1 antibody was also able to detect a signal in the *Δtrr1* strain, which most probably corresponds to the matrix-localised thioredoxin reductase Trr2. In order to definitively verify that the signal is coming from Trr1 and Trr2

we would have to test our antibody in the double deletion *Δtrr1/trr2* mutant, where, if our hypothesis is true, we would expect to be unable to detect the ~35kDa signal.

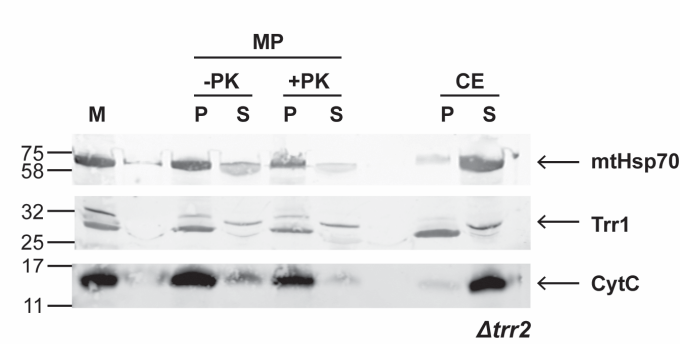

Figure 43: Localisation of endogenous Trr1 in *Δtrr2* mitochondria. This experiment had the same result as the one shown in fig. 40, although in this particular case, we are only detecting Trr1 in yeast mitochondria. As with the previous experiment, mitoplasting and carbonate extraction were unable to give us information concerning the submitochondrial localisation of Trr1.

One way to eliminate the possibility of detecting Trr2 in our experiments would be to use the mitochondria isolated from the *Δtrr2* background. As working with knockout strains is not optimum, unless the deleted protein is the one of interest, we decided against using *Δtrr2* mitochondria, as Trr2 is an important – if non-essential – component of the oxidative stress response in the mitochondrial matrix. Additionally, to further circumvent the issue of the αTrr1 antibody recognising both Trr1 and Trr2, as well as a second, non-specific band, we decided to attempt to assess the import and function of Trx1 and Trr1 using purified recombinant His-tagged proteins, which would enable us to specifically detect them with an αHis antibody, while also eliminating any detection of non-specific signals.

6.2.6. Purification of Trx1 and Trr1

The second approach that was used to investigate the two proteins of interest was to purify Trx1 and Trr1 as recombinant proteins from *E. coli* and use them in import assays with isolated mitochondria. We cloned the genes of interest into vectors that allow overexpression of the proteins in bacterial cells as His-tagged versions. The vector that was

used was the pET24 vector (Novagen) that adds a 6xHis tag at the C-terminus of any protein that is cloned into its multicloning site.

The vectors were sequenced in order to make sure that the genes were 100% correct and no mutations had arisen from the cloning process. They were then transformed into BL21 (DE3) bacterial expression cells. The expression of the proteins was induced using IPTG (FAD was also added in the case of Trr1, due to it being a FAD-binding protein) at the concentrations noted in table 6.

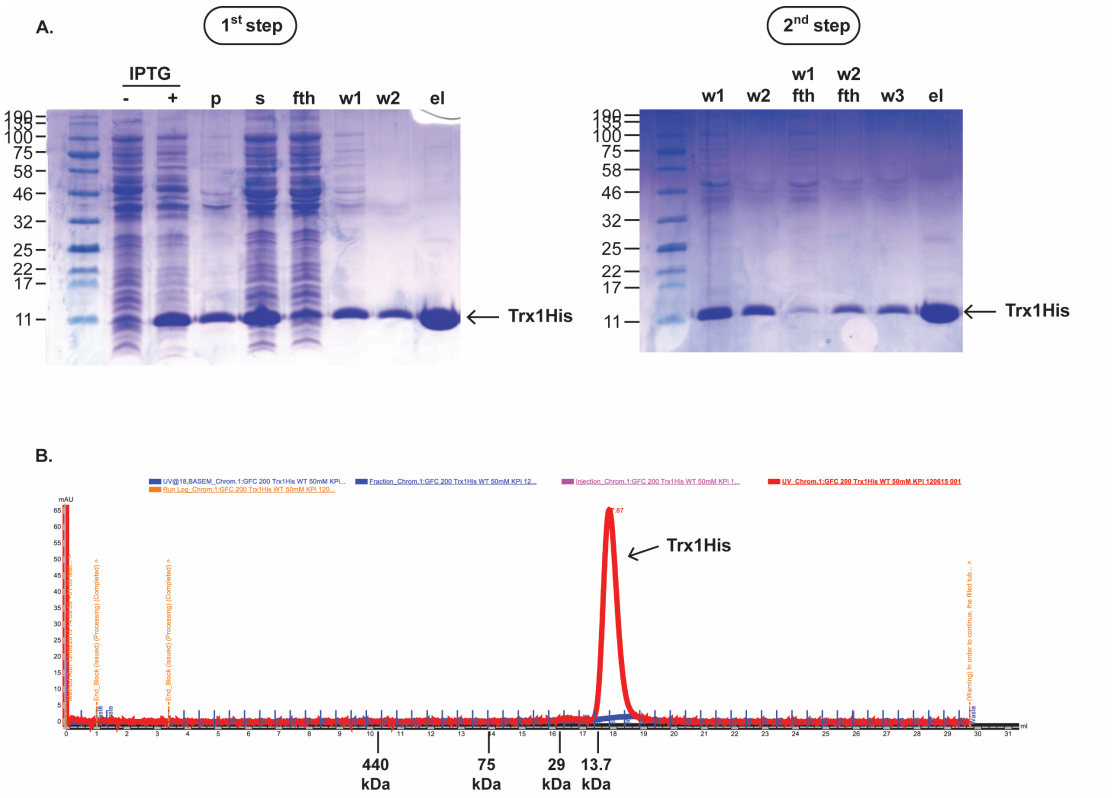

Figure 44: Purification of Trx1His wt. Trx1His wt was purified in two steps, due to the amount of material lost in the wash (w1 & w2) fractions during the first round of purification (A). The elution fractions from both rounds of purification were mixed and the protein was measured at 280nm in order to assess the final yield (total purified protein → 700mg). Trx1His wt was analysed on the ÄKTA Pure FPLC system (B) to assess the level of purification and its state, where it was assessed to be monomeric with an estimated molecular weight of 12.6kDa (this was calculated using the retention volume of 17.87ml, the void volume calculated with the elution of Blue Dextran for our standard curve and the equation calculated from our standard curve – see fig. 20).

Once the cells had been harvested, the proteins were purified in one-step using affinity purification chromatography with Ni-NTA affinity columns, as described in section 3.2.3.3 (fig. 44 & 45). Due to the fact that quite a significant amount of protein was lost in the flow-through and wash fractions during the purification process, these fractions were re-loaded onto the same Ni-NTA beads that had been used in the first round of purification. Through this approach, we were able to isolate and purify much larger quantities of both proteins than with just the first round of purification.

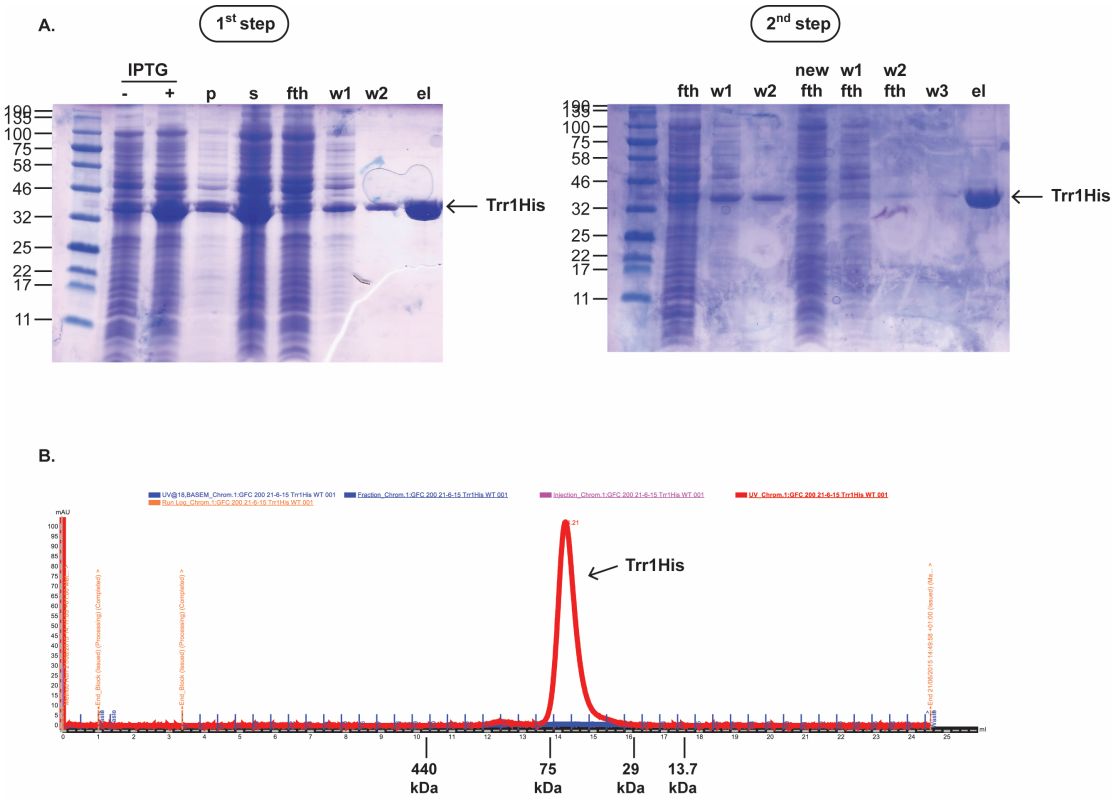

Figure 45: Purification of Trr1His wt. Trr1His wt was purified in two steps, due to the amount of material lost in the flow-through (fth) and wash (w1 & w2) fractions during the first round of purification (A). The elution fractions from both rounds of purification were mixed and the protein was measured at 280nm in order to assess the final yield (total purified protein → 1230mg). Trr1His wt was analysed on the ÄKTA Pure FPLC system (B) to assess the level of purification and its state, where it was assessed to be dimeric with an estimated molecular weight of 71.5Da (this was calculated using the retention volume of 14.21ml, the void volume calculated with the elution of Blue Dextran for our standard curve and the equation calculated from our standard curve – see fig. 20). This agrees with the published literature, which states that Trr1 is functional as a homodimer (Zhang et al. 2009).

Subsequently, the purified proteins were analysed by gel filtration using an ÄKTA Pure system, where Trx1 was found to be in a monomeric state (fig. 44) and Trr1 was found to be dimeric (fig. 45). This is an assumption that was made due to the relative molecular weight at which it was eluted (when compared to a standard mix of proteins run on the same gel filtration column under the same conditions), as well as the fact that their structure has been solved and are known to be globular proteins (Pinheiro et al. 2007; Zhang et al. 2009). A 2014 study showed that Trx1 is monomeric, both in its oxidised and its reduced states (Cruzeiro-Silva et al. 2014), and our gel filtration analysis was able to show that the purification process did not cause the formation of any aggregates. The result for Trr1 also agrees with the published literature, as Trr1 is known to be functional as a homodimer (Zhang et al. 2009).

6.2.7. Import and localisation of purified Trx1 and Trr1

In order to study these two proteins, the first step was to present them to wild type yeast mitochondria and test if they become imported. As with the radioactive precursors, the purified proteins were presented to the mitochondria both in their "native" folded state, as well as in a completely denatured and reduced state. Both proteins were found to become imported, with Trx1 becoming imported in both states, while Trr1 was imported only when denatured and reduced (fig. 46).

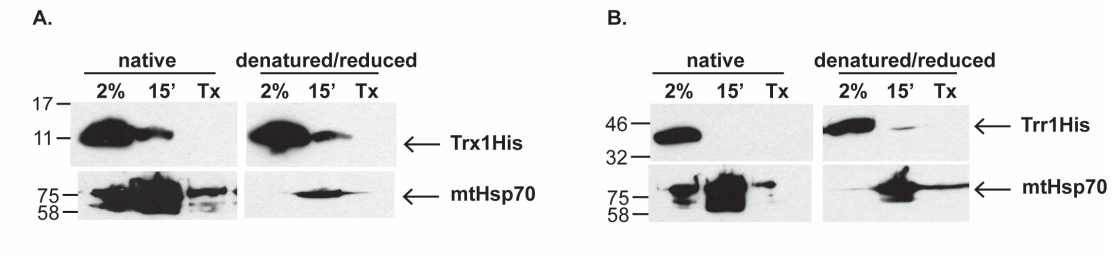

Figure 46: Import of purified, recombinant Trx1His and Trr1His in wt mitochondria. Trx1His was imported under both conditions (A), while Trr1His was imported only when denatured and reduced (B).

In order to visualise the localisation of these proteins, we used the same method that was used when we were testing the localisation of the endogenous Trr1 in fig. 40. Mitoplasting

(creation of mitochondria lacking the outer membrane) was able to show us that both proteins are localised in the mitochondrial IMS, as they can be found predominantly in the supernatant fraction (lanes designated MP-S) and can both be digested in the presence of the protease proteinase K. Carbonate extraction (separation of the soluble and membrane-anchored proteins of mitochondria) verified that our proteins are indeed soluble in the IMS (fig. 47).

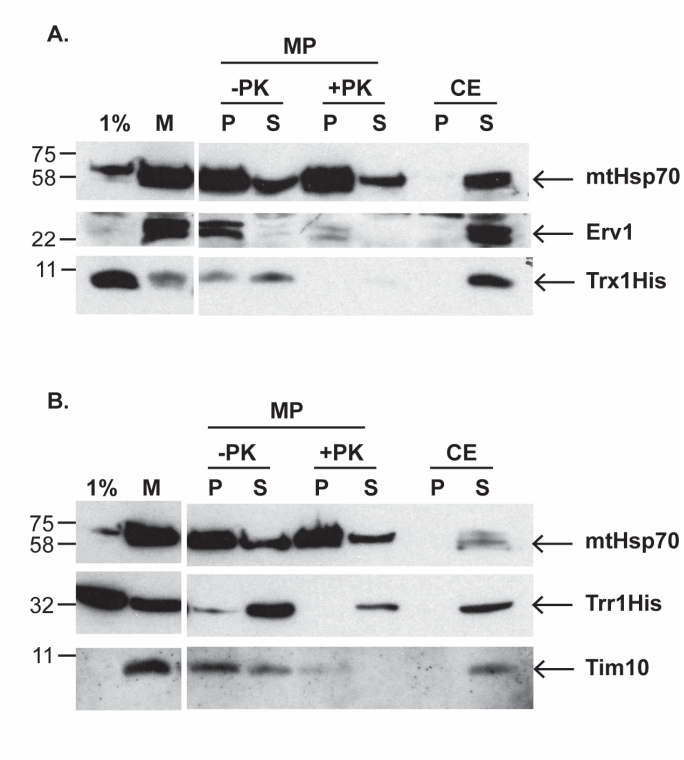

Figure 47: Localisation of Trx1His and Trr1His after import into wt mitochondria. Analysis of the localisation of Trx1His (A) and Trr1His (B) in wt mitochondria using mitoplasting (MP) and carbonate extraction (CE). Both Trx1His and Trr1His were shown to be soluble proteins localised in mitochondrial IMS, as indicated by their presence in the soluble fraction (S) after carbonate extraction, as well as their digestion by proteinase K during mitoplasting (–PK and +PK samples).

6.2.8. Effect of the depletion of the inner membrane potential or the mitochondrial ATP on the import of Trx1 and Trr1

The next important question was whether the inner mitochondrial membrane potential was required for the import process to be completed. The majority of mitochondrial precursors are targeted to the matrix and these proteins require an N-terminal positively charged targeting signal, in addition to the presence of the inner membrane potential. As both proteins lack an N-terminal targeting signal, as well as the fact that most IMS proteins do not depend on the membrane potential for their import, testing the import dependence on $\Delta\Psi$ might seem out of place. However, it was important to unequivocally assess the effect

of the membrane potential, since the latter is a critical factor for mitochondrial import. As can be seen from fig. 48, both proteins are indeed still imported into wild type mitochondria in the absence of membrane potential, further strengthening the claim that they are localised in the intermembrane space.

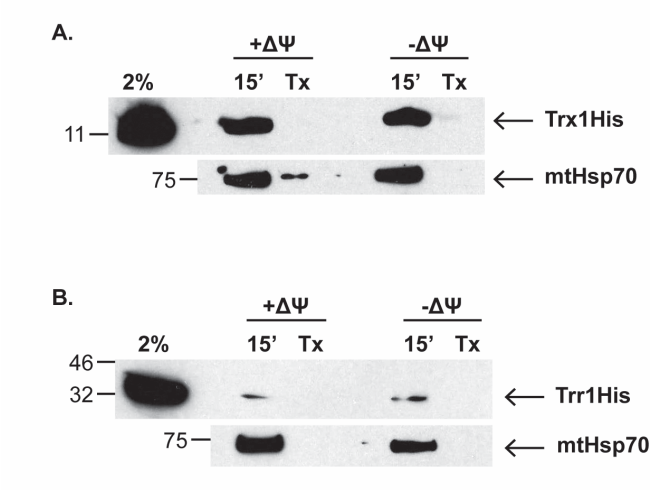

Figure 48: Import of Trx1His and Trr1His in the presence or absence of inner membrane potential. Trx1His (A) and Trr1His (B) were imported into wt mitochondria that were untreated (+ΔΨ) or treated with 1mM valinomycin (-ΔΨ). Both proteins become imported, regardless of the presence of inner membrane potential.

Another important energy source for the import of proteins into mitochondria is ATP hydrolysis. Proteins that are targeted to the matrix require ATP hydrolysis both in the matrix and the cytosol, whilst proteins that only cross the outer mitochondrial membrane but do not completely cross the inner membrane only require ATP in the cytosol to fuel the action of the cytosolic chaperones. In this particular case, ATP, which is added externally to all import assays, was eliminated, which, alongside the addition of apyrase, an ATP-diphosphohydrolase that converts ATP to AMP and inorganic phosphate, led to the complete depletion of ATP from the mitochondria. As can be seen from fig. 49, ATP is not required during the import process of Trx1. Unfortunately, no result was obtained for Trr1 and thus, the experiment must be repeated in order to assess whether ATP is required for its import.

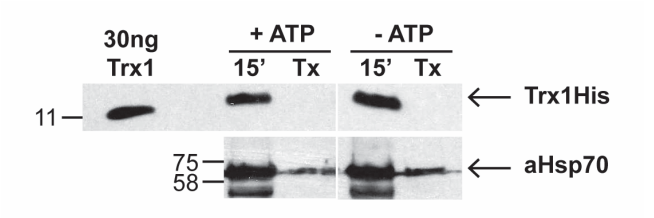

Figure 49: Import of Trx1His in the presence or absence of ATP. Trx1His was imported into mitochondria that were untreated using ATP-containing import buffer (+ATP) or mitochondria treated with 10μM oligomycin and 10mU/μl apyrase for 10min using import buffer without ATP (-ATP). The experiment showed that Trx1His does not depend on the presence of ATP in mitochondria to become imported. (ATP depletion protocol obtained from Terziyska et al. 2007).

6.2.9. Is the MIA pathway responsible for the import of Trx1 and Trr1?

As we have now verified that the two proteins are indeed localised in the mitochondrial IMS, the next step was to elucidate the pathway and mechanism they follow to get imported into the mitochondria and sorted into this particular subcompartment. Due to the fact that the majority of the proteins resident in the intermembrane space utilise the MIA pathway, we started looking into whether the two proteins in question also followed this same pathway.

The first step in this process was to closely look at the primary structure of the two proteins and look for any targeting signals that could make them possible substrates of the MIA pathway. As has already been mentioned in section 1.3.4 of the Introduction, the MIA pathway is a distinct import pathway responsible for the translocation of proteins that contain cysteine residues. More specifically, Mia40 substrates typically contain twin CX3C or CX9C motifs that are able to associate with the hydrophobic binding cleft of Mia40 and come into close proximity to its catalytic CPC motif. Through this interaction, a disulfide bond becomes transferred from the CPC motif to the cysteine residues of the substrate protein, leading to the folding of the latter and its retention in the intermembrane space. Additionally, Mia40 substrates typically contain a 9-aminoacid peptide known as the MISS/ITS, which facilitates the docking of the cysteine residue to the CPC motif of Mia40. This small stretch of sequence is characterised by the fact that it forms a short helix with the aromatic and hydrophobic residues on the side of the helix where the docking cysteine is also localised (Sideris et al. 2009).

When looking through the amino acid sequence of both Trx1 and Trr1, we found that both proteins lack any distinct cysteine motifs that would indicate that they may depend on Mia40 in order to become imported. More specifically, Trx1 contains two cysteine residues arranged in a WCGPC motif (which is typical of proteins in the thioredoxin family) (Pinheiro et al. 2007; Cruzeiro-Silva et al. 2014), while Trr1 contains a single CXXC motif, as well as two additional single cysteine residues (Zhang et al. 2009) (fig. 31).

Despite the lack of a clear motif that is known to be recognised by Mia40, we decided to test whether Trx1 and Trr1 were dependent on Mia40 for their import, as there are other known substrates of the MIA pathway that do not depend on their cysteine residues, but

still require Mia40 for their import. In order to test this hypothesis, we utilised mitochondria derived from yeast strains with depleted levels of the two main MIA pathway components, Mia40 and Erv1. Both of these proteins are essential for cell viability and their genes cannot be deleted from the yeast genome. In order to create Mia40- and Erv1-depleted mitochondria, we placed both genes under the control of a galactose-inducible promoter, to ensure that the genes are only transcribed when the yeast growth medium contains galactose. By growing the yeast cells in lactic acid, we are able to shut down the production of the galactose-induced gene, while at the same time promote the production of mitochondria. More specifically, starter yeast cultures were grown in the presence of galactose, in order to be able to produce Mia40 and Erv1 normally. 24 hours before the collection of the mitochondria, the yeast cells were transferred into fresh media containing lactic acid, as well a small amount of glucose (0.2%), to completely shut down the transcription of the MIA40 and ERV1 genes. A growth period of 24 hours allowed for the complete shutdown of the production of Mia40 and Erv1, as well as for their severe depletion from the mitochondria (due to the half-lives of the protein molecules).

Once the mitochondria were isolated from the Mia40- and Erv1-depleted cells, they were used in import assays for our two proteins of interest. Both types of mitochondria – Mia40-depleted (Mia40↓) and Erv1-depleted (Erv1↓) – were treated in exactly the same way as the wild type mitochondria and the results of the import experiments can be seen in fig. 50. From this experiment, we can clearly see that the import of Trx1 and Trr1 is independent of Mia40. Interestingly, in the case of Trx1, the import levels are elevated in the Mia40-depleted mitochondria compared to the wild type mitochondria, whilst in the case of Trr1, the import levels remain unaffected by the presence or absence of Mia40. As a control for these experiments, we imported Erv1 – a known Mia40 substrate – into the same types of mitochondria and indeed found that its import was completely abolished in the Mia40-depleted mitochondria.

The same can be observed in the case of the Erv1-depleted mitochondria, with the import of Trx1 being elevated compared to the wild type mitochondria and the import of Trr1 remaining unaffected. Erv1 import into Erv1-depleted mitochondria is only slightly affected, due to the lack of endogenous Erv1 present within mitochondria to recycle Mia40, which leads to the accumulation of reduced, and thus inactive, Mia40 (fig. 50).

This particular result concerning the import of Trx1 was unexpected and rather intriguing, as it suggests that the MIA pathway is not only not responsible for Trx1 import, but rather, its presence seems to affect the ability of Trx1 to become imported. This was a result that was reliably reproduced (with this experiment having been performed three separate times) and, as such, most probably has biological significance and a clear reason as to why it occurs. Consequently, this is a phenomenon that must be studied further, as it is the only protein that has been found to have such an import pattern, making it interesting to figure out the reason as to why it happens.

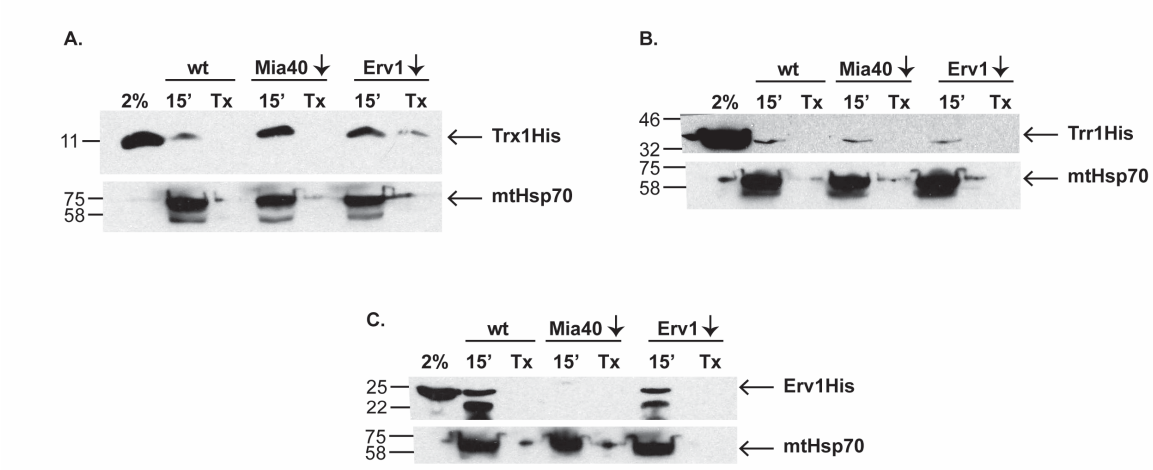

Figure 50: Import of Trx1His and Trr1His into wt, Mia40-depleted and Erv1-depleted mitochondria. Trx1His (A) and Trr1His (B) were imported into three different types of mitochondria – wild type, Mia40-depleted (Mia40↓) and Erv1-depleted (Erv1↓). The import of Trr1His was unaffected by the depletion of Mia40 and Erv1, while Trx1His import was elevated in the two depleted mitochondria. The import of Erv1His (C), a known substrate of Mia40, is shown as a control.

6.2.10. Are the cysteine residues of Trx1 and Trr1 required for import?

Given that neither of these two proteins seems to depend on the MIA pathway for its import, it would be logical to assume that they do not require their cysteine residues in order to be imported. For this reason, as well as to use in other types of experiments, the cysteine mutant versions of these proteins were created. In this particular thesis, only the Trx1 cysteine mutants were analysed, as the Trr1 mutants could not be purified in time for the experiments to be performed.

For Trx1, we generated two single cysteine and one double cysteine mutants (the single C30S and C33S mutants, as well as the double C30/33S mutant). All of these were produced by mutagenising the sequences in the pET24-Trx1-His vectors that carried the Trx1 gene. The mutant proteins were then overexpressed and purified via His-tag affinity purification from *E. coli* cells, using the exact same conditions that were used for the recombinant wild type Trx1 protein. The purified proteins were loaded onto the ÄKTA Pure Superdex 200 gel filtration column in order to assess the level of purification. The results of this process can be seen in figures 51, 52 & 53.

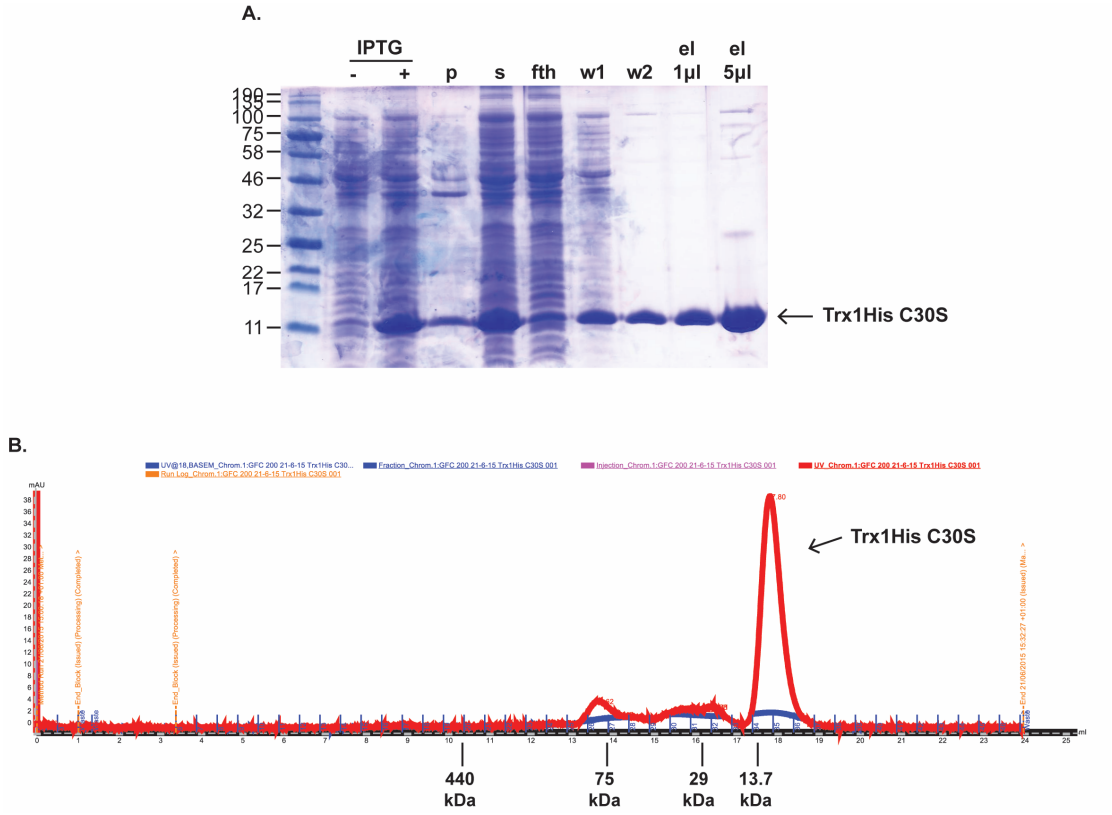

Figure 51: Purification of Trx1His C30S. Trx1His C30S was purified in one step, despite the loss of material in the wash (w1 & w2) fractions (A). The elution fraction was measured at 280nm in order to assess the final yield (total purified protein → 310mg). Trx1His C30S was analysed on the ÄKTA Pure FPLC system (B) to assess the level of purification and its state, where it was assessed to be monomeric with an estimated molecular weight of 13Da (this was calculated using the retention volume of 17.8ml, the void volume calculated with the elution of Blue Dextran for our standard curve and the equation calculated from our standard curve – see fig. 20).

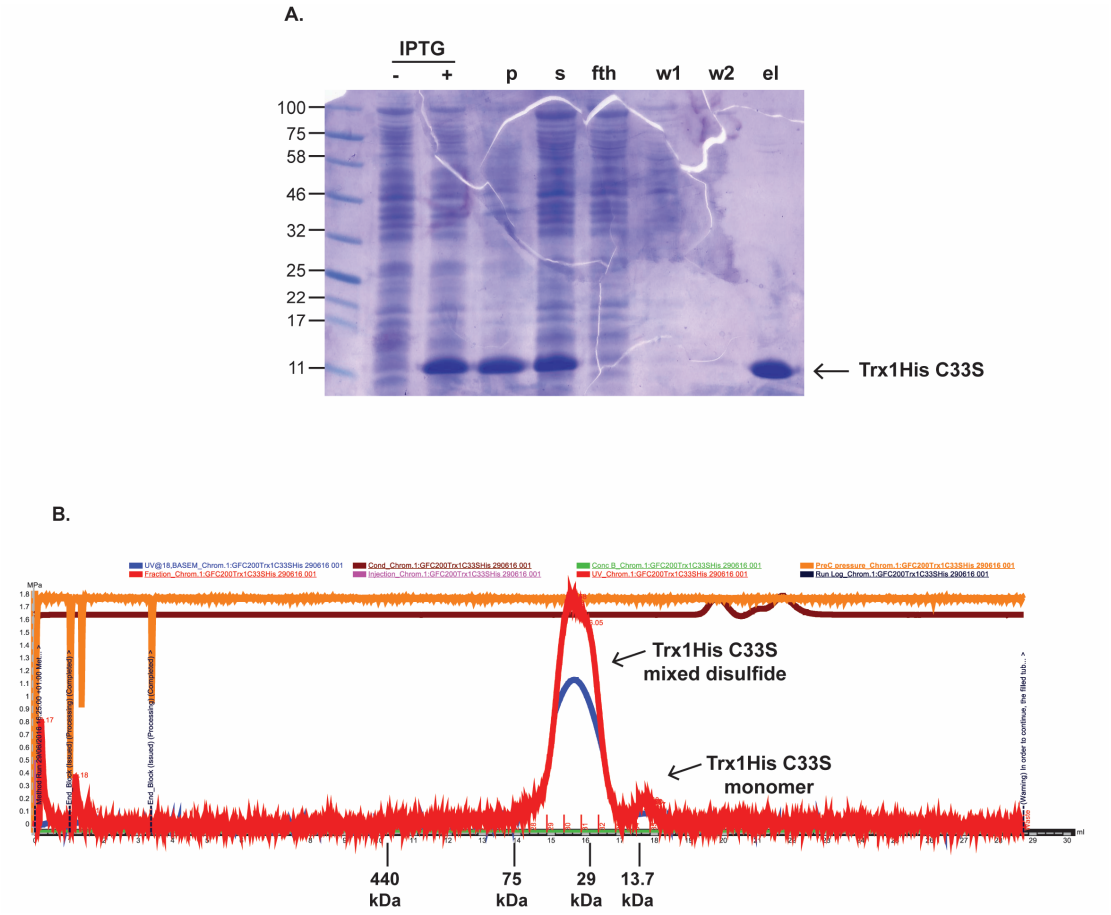

Figure 52: Purification of Trx1His C33S. Trx1His C33S was purified in one step (A). The elution fraction was measured at 280nm in order to assess the final yield (total purified protein → 120mg). Trx1His C33S was analysed on the ÄKTA Pure FPLC system (B) to assess the level of purification and its state, where it was assessed to be primarily dimeric with an estimated molecular weight of 29kDa, with a smaller fraction being monomeric with an estimated molecular weight of 13.9Da (this was calculated using the retention volume of 16.05ml and 17.66ml respectively, the void volume calculated with the elution of Blue Dextran for our standard curve and the equation calculated from our standard curve – see fig. 20).

Once the proteins had been purified, we used them in import assays, in order to assess the necessity of their cysteine residues when the proteins are presented to fully functional, wild type mitochondria. As can be seen in fig. 54, the absence of cysteine residues plays no role in the import process, as all three cysteine mutants were imported. This further supports the claim that Trx1 does not depend on the MIA pathway in order to become imported.

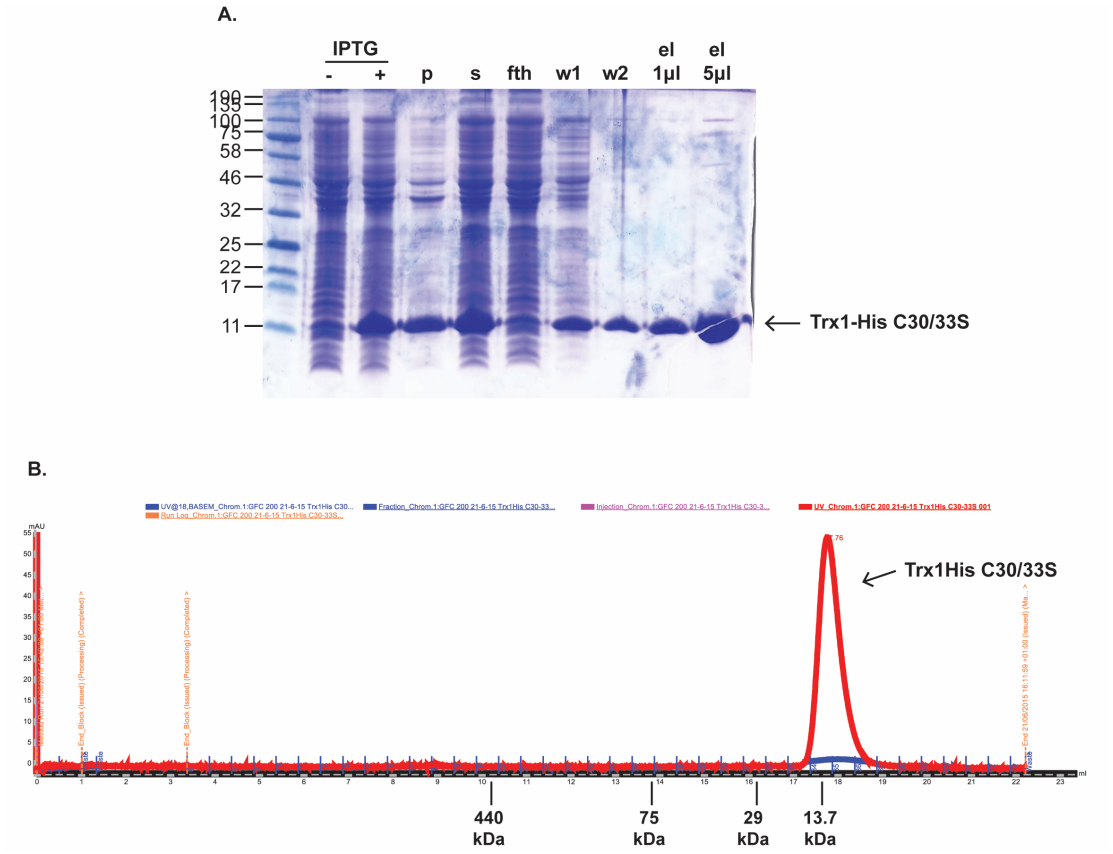

Figure 53: Purification of Trx1His C30/33S. Trx1His C30/33S was purified in one step (A). The elution fraction was measured at 280nm in order to assess the final yield (total purified protein → 330mg). Trx1His C30/33S was analysed on the ÄKTA Pure FPLC system (B) to assess the level of purification and its state, where it was assessed to be monomeric with an estimated molecular weight of 13.2Da (this was calculated using the retention volume of 17.76ml, the void volume calculated with the elution of Blue Dextran for our standard curve and the equation calculated from our standard curve – see fig. 20).

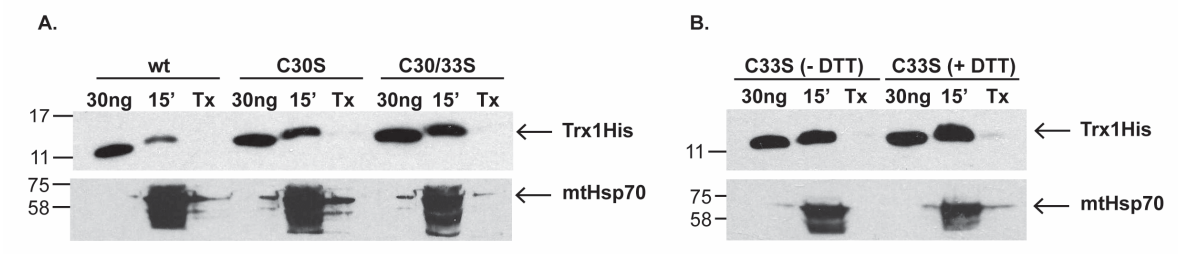

Figure 54: Import of Trx1His wt and all cysteine mutants into wt mitochondria. Trx1His wt, Trx1His C30S and Trx1His C30/33S (A), as well as Trx1His C33S that had been treated or not with 20mM DTT (B) were imported into wt yeast mitochondria. All versions of Trx1His were imported, indicating that the cysteine residues do not play a role during the import process.

6.2.11. Is the thioredoxin system itself required for the import of Trx1 and Trr1?

We have now established that Trx1 and Trr1 do not require the MIA pathway in order to become imported, however, the question of how the two proteins actually enter the mitochondria and become trapped in the IMS remains. An example of a protein that is dually localised and follows an unconventional import pathway is the case of Sod1 (superoxide dismutase 1), a major superoxide-scavenging yeast enzyme that is dually localised in the cytosol and the mitochondrial IMS. Sod1 requires the presence of Ccs1 (copper chaperone for superoxide dismutase 1) in order to become imported and is subsequently trapped in the IMS through the formation of a disulfide bond. Ccs1 itself is one of the few unconventional MIA pathway substrates that have been identified, with Mia40 promoting the import of Ccs1 through the introduction of a structural disulfide bond between Cys27 and Cys64 of Ccs1. When Ccs1 is absent from mitochondria, Sod1 becomes imported, but cannot be oxidised. As a result, Sod1 remains inactive (Varabyova et al. 2013). This particular example shows the dependence of import of a specific protein (Sod1) on the presence of another (Ccs1).

Whether this dependence is something that occurs in the cases of Trx1 and Trr1 can be easily tested. The experimental setup involved the preparation of highly purified mitochondria from the deletion strains for the two proteins we are interested in: *Δtrx1* and *Δtrr1*. Once the mitochondria had been obtained, they were used in import assays in order to assess the import of Trx1 and Trr1 purified proteins. The results show quite clearly that there is no dependence of one protein on the other, as Trx1 is imported normally into *Δtrr1* mitochondria and Trr1 in *Δtrx1* mitochondria (fig. 55).

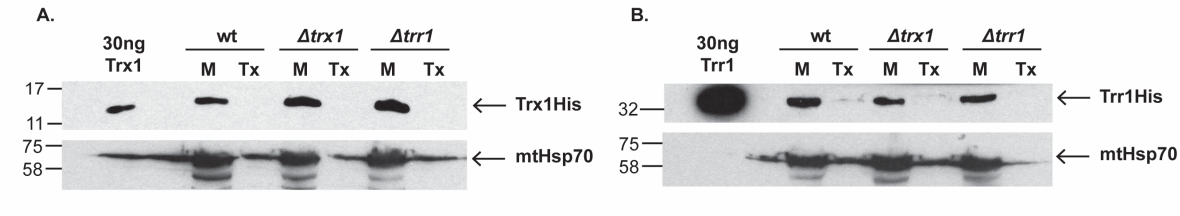

Figure 55: Import of Trx1His and Trr1His into wt, *Δtrx1* and *Δtrr1* mitochondria. Trx1His (A) and Trr1His (B) were imported into three different types of mitochondria – wild type, *Δtrx1* and *Δtrr1*. The import of Trx1His was unaffected by the absence of Trr1, while the import of Trr1His was unaffected by the absence of Trx1.

6.2.12. Purification and import of Trx2

As stated in the introduction, Trx1 and Trx2 share a very high degree of similarity (79% identity and 89% similarity, calculated using blastp). However, Trx2 was not in the list of novel proteins localised in the IMS (Vögtle et al. 2012). The authors of this publication assessed the peptides that were derived from their IMS extracts and were able to identify Trx1 as one of the novel IMS proteins. Due to the similarity between Trx1 and Trx2, we wondered whether some of the peptides that were attributed to the presence of Trx1 in the IMS could possibly be peptides that are also present in Trx2. This would mean that both proteins have the capacity to become imported into the IMS.

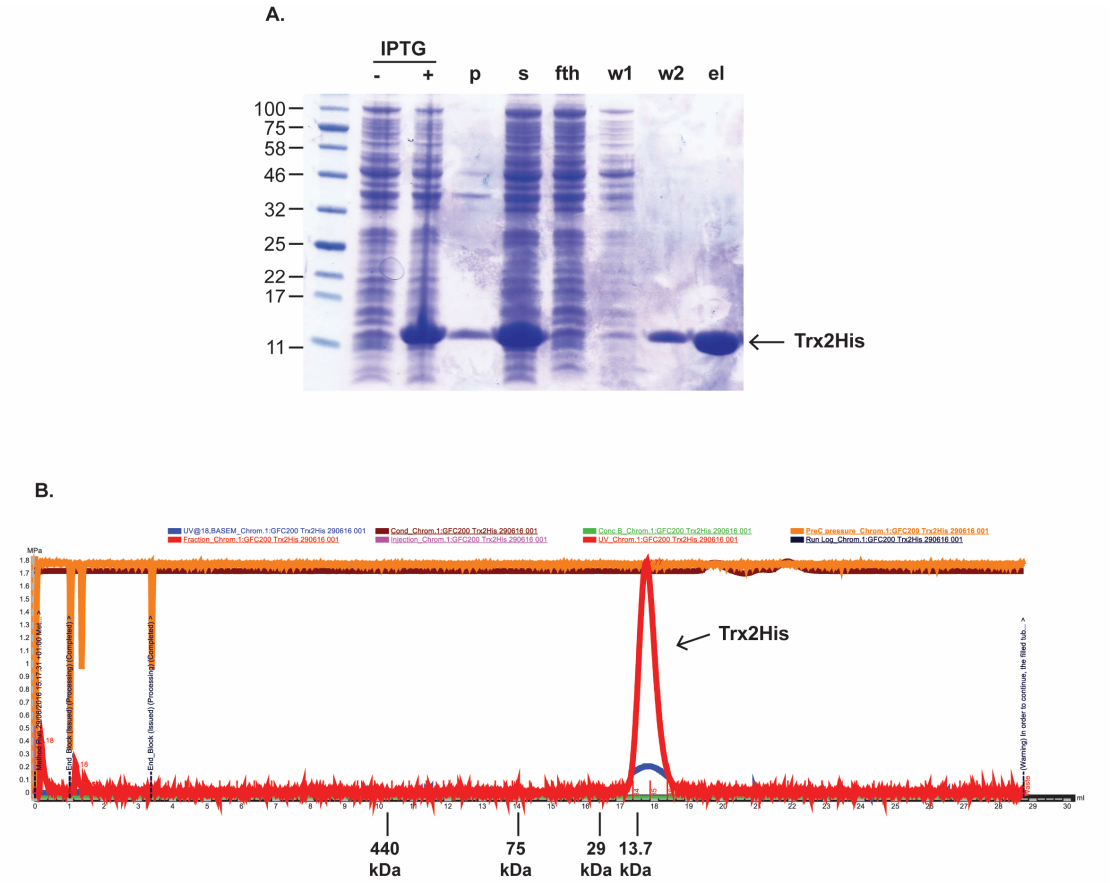

Figure 56: Purification of Trx2His wt. Trx2His was purified in one step (A). The elution fraction was measured at 280nm in order to assess the final yield (total purified protein → 110mg). Trx2His was analysed on the ÄKTA Pure FPLC system (B) to assess the level of purification and its state, where it was assessed to be monomeric with an estimated molecular weight of 13.2Da (this was calculated using the retention volume of 17.77ml, the void volume calculated with the elution of Blue Dextran for our standard curve and the equation calculated from our standard curve – see fig. 20).

Just like all the other proteins used in this study, Trx2 was cloned into a pET24 vector in order to be produced as His-tagged. The plasmid was then transformed into bacterial expression cells and protein production was induced. Trx2 was purified from the cell extract as described in section 3.2.3.3, and the result of this purification can be seen in fig. 56.

Once the protein had been purified, it was used in a standard import assay, in order to assess whether it is possible for Trx2 to become imported into the mitochondria. As can be seen in fig. 57, Trx2 can be imported into wild type mitochondria. This result opens up the possibility to assess whether it is also localised in the IMS. Future studies could focus on elucidating the mechanism of import for Trx2 and comparing it to the one used by Trx1. It is important to note that although Trx1 and Trx2 are highly homologous, Trx2 is expressed in higher levels upon oxidative stress, which could lead to an increase in the amount of Trx2 that is translocated into the mitochondria. This is an intriguing possibily and could be rationalised as a possible response to overcome putative hyperoxidation of critical proteins in the IMS during oxidative stress.

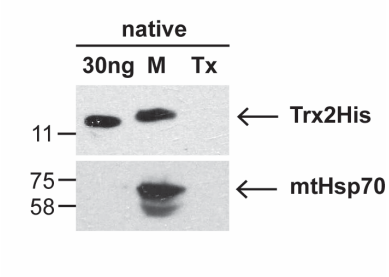

Figure 57: Import of Trx2His in wt mitochondria. This experiment showed that Trx2His is also capable of becoming imported into yeast mitochondria.

6.3. Discussion

The discovery of Trx1 and Trr1 in the mitochondrial IMS (Vögtle et al. 2012) led to the need to answer two main questions: a) how do the proteins become imported and b) what function do they carry out in the IMS. The aim of this chapter was to work on the first question and try to elucidate the mechanism of Trx1 and Trr1 import.

Our experimental process was filled with many challenges, from the mutations that had been generated in the DNA sequences, the various issues surrounding the production of the proteins as radiolabelled precursors and the extensive mutations required to make the translation process more specific, to the detection of both thioredoxin reductases in the mitochondria when using the αTrr1 antibody that was generated from the purified form of Trr1. In order to circumvent all the above issues, we decided to work with the purified His-tagged versions of our proteins of interest, which we would be able to detect specifically through the use of an αHis antibody.

The first step in the process of characterisation of Trx1 and Trr1 import was to determine if there are any putative targeting signals located within their sequences, through bioinformatics analysis. This process was done just to get a first indication of any patterns that might be recognised by the bioinformatics tools that are widely available (Claros and Vincens 1996; Emanuelsson et al. 2000; Small et al. 2004; Fukasawa et al. 2015). This was never going to be the basis of our experiments, as these tools are known to be more reliable in the prediction of matrix-targeting proteins, as opposed to proteins targeted to the other mitochondrial subcompartments. This particular fact was shown in table 8, where the same prediction tools were used to calculate the probability of mitochondrial translocation for known mitochondrial proteins, with varying degrees of success depending on the type of targeting sequence.

The second step was to verify the import and localisation of our two proteins, which indeed showed that both proteins are localised in the IMS. This meant that our first aim was to check whether Trx1 and Trr1 follow any of the known pathways for import of IMS proteins. The depletion of the inner mitochondrial membrane potential did not affect the import of either of the two proteins and the depletion of ATP allowed Trx1 to be imported as normal, thus minimising the probability of either of the two proteins utilising the TIM23 pathway for its import.

The dependence of the import of both proteins on the MIA pathway was also tested and showed a rather interesting result. Not only was the import not dependent on the presence of Mia40 in mitochondria, but also the depletion of Mia40 (and to a lesser extent, Erv1) resulted in higher levels of imported Trx1. In the case of Trr1, the levels of imported protein were the same, regardless of the type of mitochondria used.

The next step was to test whether the cysteine residues are dispensable during the import process. Taking into account the previous finding about neither protein depending on the MIA pathway for its import, it was thought to be unlikely that they would require their cysteine residues. Only Trx1 was tested concerning this particular hypothesis (due to time constraints with the purification of the cysteine mutants of Trr1), with the import experiments showing that neither of the two cysteine residues was required for import.

As there is already a case of a specific protein (Sod1) requiring the presence of another (Ccs1) in the IMS in order to become imported (Varabyova et al. 2013), we decided to test this hypothesis for Trx1 and Trr1. We thus wanted to elucidate whether either of these proteins was required for the import of the other. Import experiments in mitochondria derived from the corresponding deletion strains showed that there is no such dependence between our two proteins.

With the experiments performed above, we have managed to shine a light on the import of Trx1 and Trr1 in an indirect manner, by determining factors that are not involved in their import. As mentioned in the introduction (section 1.3.4), import into the IMS can also occur in a direct, "non-conventional' way, a pathway that until now is only known to be followed by heme lyases (Mayer et al. 1995; Künkele et al. 1998; Diekert et al. 1999). This is something that needs to be tested at the level of the outer mitochondrial membrane and involves the general entry gate of the outer membrane and its cytosolic receptors (TOM comlex). If this were found to indeed be the case, we would then have to assess the circumstances of interaction that would lead to the import of our proteins of interest under physiological or stress conditions.

One last but rather important finding was the fact that Trx2 can also become imported into mitochondria. This is interesting, as it was not included in the list of 20 novel IMS-localised proteins found by Vögtle and coworkers (Vögtle et al. 2012). However, when looking at the mass spectrometry data that was amassed during this study, we were able to see that despite Trx1-specific peptides being identified during the process, there were also peptides corresponding to Trx2. Additionally, when taking into account the high level of identity between the two proteins, it is highly possible that certain peptides that were allocated as belonging to Trx1 could also potentially belong to Trx2. The additional import of Trx2 into the mitochondrial IMS (even though this was not experimentally tested during the course of this thesis, we fully expect Trx2 to be sorted in the same subcompartment as

Trx1) is rather interesting, as Trx2 is one of the many genes upregulated during H_2O_2-induced oxidative stress. This could signify that the mitochondrial IMS also contains a detoxifying system for the alleviation of the effects of hydrogen peroxide stress, in addition to a constitutively active thrioredoxin system.

It must be noted here that for both the radioactive as well as the purified proteins, the His-tagged versions were used. The inclusion of the His-tag at the C-terminus of the two proteins should not affect their function, however this is something that would need to be verified by assessing whether the His-tagged versions of our two proteins of interest are able to rescue the null yeast mutant phenotypes *in vivo*.

Chapter 7

The function of Trx1 and Trr1 in the mitochondrial IMS

7. The function of Trx1 and Trr1 in the mitochondrial IMS

7.1. Introduction

In the previous chapter, we attempted to address the issue of how Trx1 and Trr1 become imported into the mitochondrial IMS. This is something that is very interesting to elucidate, as none of our data supports any of the known import pathways for the intermembrane space.

In addition to the import process, it is important to also determine the function of these proteins in this subcompartment. The thioredoxin system has been very well characterised concerning the way in which it works to reduce disulfide bonds in the cytosol, as well as being implicated as a system that is essential for the import of certain mitochondrial proteins, ie. the small Tims (Durigon et al. 2012).

As mentioned in section 6.1, the presence of Trx1 and Trr1 in the IMS could possibly be attributed to the requirement for a reductive system in this subcompartment. With Mia40 known only to be responsible for the introduction of disulfide bonds into cysteine-containing IMS-resident proteins and the only evidence of Mia40 possibly having the ability to reduce proteins coming from an *in vitro* experimental setup (Koch and Schmid 2014a; Koch and Schmid 2014b), a certain question arises: How are non-native disulfides resolved?

The formation of non-native disulfides is something that is expected, especially in the cases of proteins that contain more than two cysteine residues (Poet et al. 2017). As such, this creates a need for a reducing system that will "reset" the system, so that Mia40 can then introduce the correct disulfide into the protein, which, in turn, will lead to its correct folding and functionality.

Given the fact that we know the way in which the thioredoxin system works, the aim of this chapter is to find out whether Trx1 is able to function as a reductase in this oxidising environment, as well as discover proteins that can interact with the system. The characterisation of the interaction partners of the thioredoxin system in the IMS will be

able to tell us whether there is a specificity to the interactions or if Trx1 is able to interact with any disulfide-bonded protein in order to correct any wrongly formed disulfides.

7.2. Results

7.2.1. Redox state of Trx1 in the IMS

Trx1 must be in a reduced state in order to be functional. As stated in the Introduction, thioredoxins work by reducing disulfide bonds that may have been formed incorrectly or as part of a response to oxidative stress conditions and act in order to detoxify the cell from the adverse effects caused by elevated levels of oxidative agents within the cellular environment.

It was therefore important to ascertain the redox state of the imported Trx1 in the mitochondrial IMS. To this end, we checked the redox state of Trx1 after its import, using the AMS alkylation assay (as described in section 3.2.3.5). The AMS assay works as shown in fig. 58, where all reduced proteins with the SH group in their cysteines in a free thiol state are able to become modified by the addition of AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid), causing a distinct gel shift in SDS PAGE of 0.5kDa for every SH group modified when compared to the oxidised, non-modified counterparts.

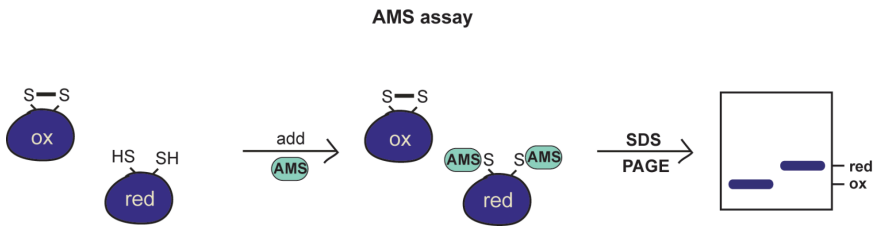

Figure 58: The AMS modification/alkylation assay. The free thiols of reduced proteins are able to bind AMS, while their oxidised counterparts are not. When the samples are analysed on non-reducing SDS PAGE, the two species can be distinguished due to a shift of the reduced species with each bound AMS moiety adding 0.5kDa of molecular weight to the protein.

The result of this assay for imported Trx1 is shown in fig. 59. SDS PAGE analysis showed quite clearly that Trx1 is maintained in its reduced state even 45min after import. This particular duration of time is calculated as 15min for the import process and 30min for the protease treatment. It is important to note that after the 30min of digestion, TCA was added in order to freeze all reactions, including the thiol-disulfide exchange ones. This is also quite a good indirect indicator that Trr1 must also be present in the IMS, as Trr1 is the only protein known to have the ability to reduce Trx1.

To further back this up, we performed the same experiment in mitochondria lacking Trr1 (from a *Δtrr1* strain). The result from this experiment can be seen in fig. 59 and clearly shows that in mitochondria lacking Trr1, the redox state of Trx1 remains oxidised, compared to the wild type mitochondria, where it is fully reduced. This can be taken as indirect proof of the presence of Trr1 in wild type mitochondria, as – as mentioned previously – Trr1 is the only protein known to be able to reduce Trx1. A more direct approach that could be used to verify the above result would be to supplement *Δtrr1* mitochondria with Trr1 through import before following the same procedure used in the experiment described above. Checking the redox state of Trx1 in these Trr1-supplemented mitochondria would show that it is indeed the presence of this particular protein that keeps Trx1 active in the IMS.

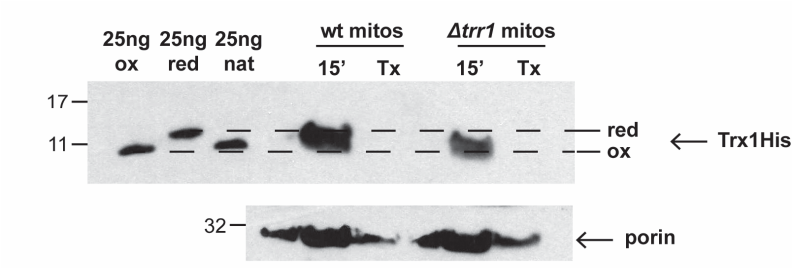

Figure 59: Redox state of Trx1His in wt and *Δtrr1* mitochondria. The first three lanes correspond to the standards on which we can interpret the results of the experiment. 25ng of Trx1 that had been treated with 200µM H_2O_2 (ox – oxidised), 20mM DTT (red – reduced) or left untreated (nat – native) were incubated with AMS so as to establish the way in which each form of the protein behaves in the presence of AMS. In the remainder of the lanes, we can see the experiment, where native (untreated) Trx1 was imported into wt and *Δtrr1* mitochondria. The samples were treated with proteinase K (-/+ Triton X-100) in the same way as all import samples, with the difference being that they were further treated with AMS, in order to assess the oxidative state of Trx1. The AMS modification analysis showed that Trx1His is found in a reduced state in wt mitochondria (upper dashed line). This observation seems to be dependent on the presence of Trr1, as in *Δtrr1* mitochondria Trx1His is oxidised (lower dashed line).

7.2.2. In vitro interactions between Trx1 and the MIA pathway components

In the previous chapter, we were able to show that neither Trx1 nor Trr1 interact with Mia40 during their import, as neither of the two main components of the MIA pathway is required in order for our two proteins of interest to become imported.

However, due to the fact that they both are cysteine-containing proteins, it is possible that they could interact with components of the MIA pathway in a process that occurs after import has been completed and involves the fully imported Trx1 and the MIA pathway. As described in more detail in the Introduction, we know that thioredoxins have the ability to interact with proteins that contain cysteines in order to reduce them. This acts as a defence mechanism for the cell, as thioredoxins are essential in maintaining the cellular homeostasis in situations of oxidative stress, where high levels of oxidising agents are present within the cell and lead to the disruption of physiological cellular activity through the modification of certain proteins.

We reasoned that if reduced Trx1 and/or Trr1 interact with Mia40 and Erv1, it would be important to be able to show this interaction both *in vitro* using purified components, as well as *in organello* using intact mitochondria. Therefore, to show the interaction *in vitro*, we reconstituted the thioredoxin system with the two purified components (Trx1 and Trr1) and NADPH, which is the substrate used by Trr1 to reduce Trx1 and keep it in its active state. It was important to have confidence that the *in vitro* system faithfully represents the interactions as they are known to occur *in vivo*. Changes in the redox state of the purified proteins were detected using the AMS alkylation assay, as described in the Materials and Methods and seen in fig. 58. As can be seen in fig. 60, the reconstitution of the system was successful and the result was in complete agreement with what is already known from the literature. More specifically, Trx1 was treated with 200μM of H_2O_2 to ensure that it is completely oxidised, while Trr1 was treated with 5mM NADPH to ensure that it is in a reduced, and thus active, state. Once both proteins were added to the reaction tube, we kept samples at the timepoints shown in fig. 60. It is very clear from this experiment that Trr1 rapidly reduces Trx1 and that the latter is maintained in a reduced state for the full duration of the assay. Control experiments, where NADPH was omitted and the system was reconstituted with oxidised Trr1, were also performed and verified the need for the presence of NAPDH in the reaction. Additional controls where NAPDH was added to Trx1 alone were also performed and showed that Trx1 remains oxidised under these conditions

(this was also used as an additional control in our interaction experiments – see last lanes in fig. 61A & B). This whole setup allowed us to completely exclude DTT from our experimental setup, as DTT is a powerful reductant and can, even at very small concentrations, lead to the reduction of proteins. These results gave us confidence that we can use this reliable reconstitution system to analyse putative interactions between Trx1/Trr1 and the MIA pathway components, Mia40 and Erv1.

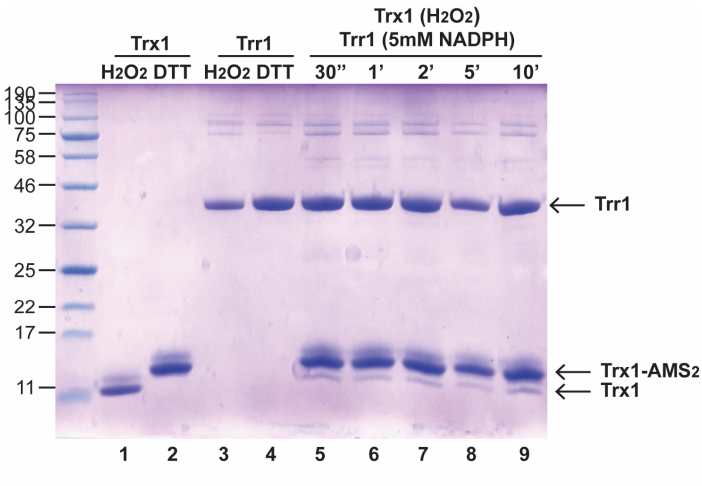

Figure 60: *In vitro* reconstitution of the thioredoxin system. Lanes 1-4 show the control reactions where Trx1 and Trr1 were treated with 200µM H_2O_2 or 20mM DTT, in order to establish how the oxidised and the reduced species migrate during electrophoresis. Lanes 5-9 show the reactions for the reconstitution of the thioredoxin system. Trx1His was treated with 200µM H_2O_2, while Trr1His was incubated with 5mM NADPH for 30min. Both proteins were then added to a single reaction tube, where the presence of reduced Trr1 led to the rapid reduction of Trx1 (lanes 5-9). This indicated that this experimental setup is able to activate Trx1 by leading to its reduction.

We assumed that if any interaction were to occur, this would be between the active (reduced) form of Trx1 and the oxidised forms of Mia40 and Erv1. As a consequence of such an interaction, we would expect to see a reduction in the components of the MIA pathway by the thioredoxin system, with a simultaneous oxidation of the latter.

Using this *in vitro* experimental approach, we found significant differences in the pairwise interactions of the different proteins. In the case of Trx1, the reduced form of the protein (which was generated through the reconstitution of the entire thioredoxin system) interacted with oxidised Mia40, resulting in an almost complete shift towards the reduced form of Mia40 (fig. 61A – lane 6). Oxidised Trx1 did not have any effect on the redox state of Mia40 (fig. 61A – lanes 8 and 9). On the other hand, Trr1 did not interact with Mia40 in any situation, as the addition of either oxidised or reduced Trr1 did not affect the redox state of Mia40 (fig. 61A – lanes 7 and 8).

When Erv1 was used in this assay instead of Mia40, the data followed the same pattern, but the magnitude of the effect seemed to be substantially decreased. Specifically, Erv1 also interacted with reduced Trx1, which resulted in the subsequent reduction of the former, albeit at a slower rate than the rate of the Mia40-Trx1 interaction (fig. 61B – lane 6).

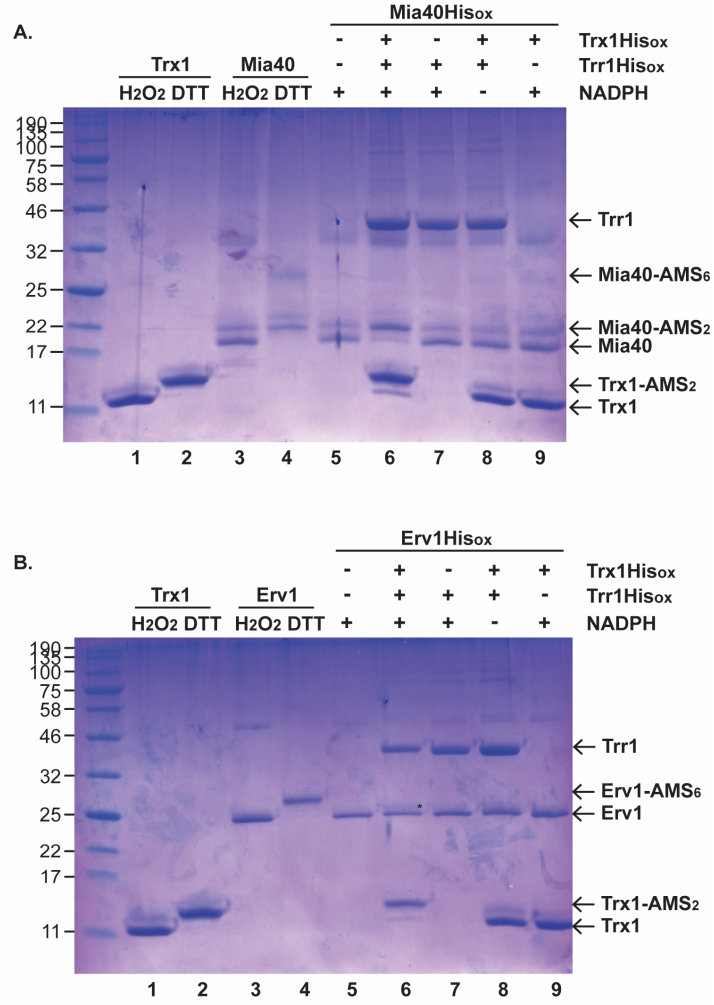

Figure 61: *In vitro* interaction between the thioredoxin system and the MIA pathway components. The interaction of the thioredoxin system with Mia40 (A) and Erv1 (B) was tested, in order to assess if the two systems have the capacity to interact with each other. Lanes 1-4 in both panels A + B show the control reactions where Trx1, Mia40 and Erv1 were treated with 200μM H_2O_2 or 20mM DTT, in order to establish how the oxidised and the reduced species migrate during electrophoresis. Panel A shows that oxidised Mia40 (the version used in this experiment is the ΔN290Mia40 and is referred to as Mia40 for simplicity) is able to interact with and become reduced by reduced Trx1 (lane 6), but cannot interact with reduced Trr1 (lane 7), oxidised Trr1 (lane 8), or oxidised Trx1 (lanes 8 and 9). NADPH is also unable to act directly upon Mia40 and alter its oxidative state (lane 5). Panel B shows that Erv1 is also able to interact with reduced Trx1 (lane 6), though to a lesser extent than Mia40. Erv1 is also unable to interact with reduced Trr1 (lane 7), oxidised Trr1 (lane 8), oxidised Trx1 (lanes 8 and 9) or NADPH directly (lane 5).

To gain a better understanding of these interactions, we then used a mutant version of Mia40, which is mutated in its hydrophobic cleft. The underlying concept in using the hydrophobic Mia40 mutant was that this hydrophobic cleft is key to all known interactions of Mia40 with substrate proteins and with Erv1 (Banci et al. 2009; Banci et al. 2011; Chatzi et al. 2013) and we wanted to test whether this was also the case for the interaction with Trx1. The mutation of 6 or 8 of the hydrophobic residues in cleft region of Mia40 was

shown to cause a similar effect as with the case of the SPS mutant (which is unable to form disulfide bonds with the incoming substrates). The presence of the hydrophobic cleft is essential, as cells that contained any of the mutations in the residues of the hydrophobic cleft were unable to survive (Banci et al. 2009).

We found that the thioredoxin system could still interact with the hydrophobic mutant of Mia40 (LMFFFM/A Mia40), as the latter becomes reduced over time, with Trx1 simultaneously becoming oxidised, thus following the behavior of the wild type version of Mia40 (fig. 62 – lanes 7-9). The image in this particular case is not very clear and contains a large number of non-specific bands, a problem that can be attributed to the purified version LMFFFM/A Mia40, which is not very stable and forms various aggregates. The bands that are of interest in this experiment are shown with arrows (fig. 62).

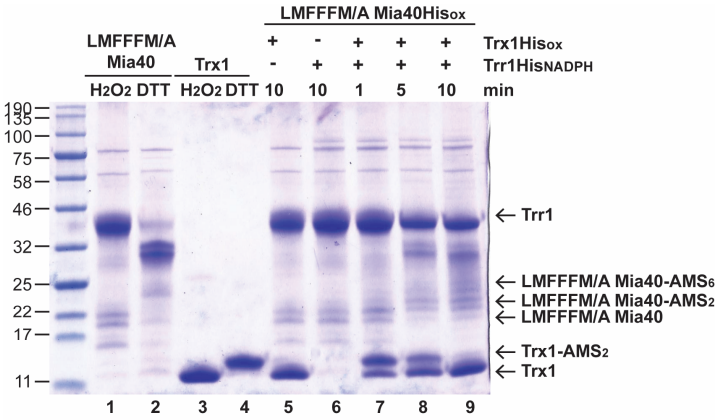

Figure 62: *In vitro* interaction between the thioredoxin system and the hydrophobic mutant of Mia40. Lanes 1-4 show the control reactions where Trx1 and LMFFFM/A Mia40 were treated with 200μM H_2O_2 or 20mM DTT, in order to establish how the oxidised and the reduced species migrate during electrophoresis. Oxidised LMFFFM/A Mia40 (the version used in this experiment is the LMFFFM/A ΔN290Mia40 and is referred to as LMFFFM/A Mia40 for simplicity) is able to interact with reduced Trx1 (lanes 7-9), but not with Trr1 (lane 6) or oxidised Trx1 (lane 5). This suggests that the interaction between Trx1 and Mia40 is not dependent on the hydrophobic cleft of Mia40. It must be noted that in this particular experiment, Trr1 cannot be seen clearly on the gel, due to the fact that the prominent aggregate from the LMFFFM/A Mia40 purification is at the same molecular weight as Trr1. The arrow indicates where we would expect to find Trr1, had the LMFFFM/A Mia40 aggregate signal been missing.

This is an intriguing result as it suggests that the observed interaction between Mia40 and Trx1 diverges from the "canonical" interactions of Mia40 with other proteins, which are known to be governed primarily by hydrophobic forces. Instead, it indicates that their interaction may be based on a different mechanism.

In addition to Mia40, we wanted to test this reconstitution system with another protein that is known to interact with Trx1, namely Gpx3. In this case, Gpx3 acted as a control sample, due to the fact that it is a well-described interactor of the thioredoxin system, with Trx1 being responsible for the recycling of Gpx3 when the latter becomes oxidised (D'Autréaux and Toledano 2007). However, it also is an interaction that is of interest to us, given the fact that Gpx3 was also one of the proteins dually localised in the cytosol and the IMS (Vögtle et al. 2012), as well as the fact that it seems to play an important role in mitochondrial morphology and the response to oxidative stress (Kritsiligkou et al. 2017).

As can be seen in fig. 63, Trx1 rapidly reduced Gpx3 in the presence of NADPH-treated Trr1, as after 1min Gpx3 was completely reduced (fig. 63 – lane 7). An observation that can be made at the 5min and 10min timepoints is that the oxidised form of Gpx3 began to re-appear (fig. 63 – lanes 8 and 9). However, the reason for this is unknown. Based on the findings and suggestions of Kritsiligkou and coworkers that Gpx3 may be able to act as an oxidoreductase in the IMS (Kritsiligkou et al. 2017), we could possibly explain the re-appearance of the oxidised version of Trx1 as a result of another interaction between the oxidised Trx1 and the reduced Gpx3. In this particular case, we could attribute the fact that no band reappears for the reduced Trx1 (at the 5min and 10min timepoints) to the fact that Trr1 is present in the reaction and can recycle Trx1 back to its reduced state.

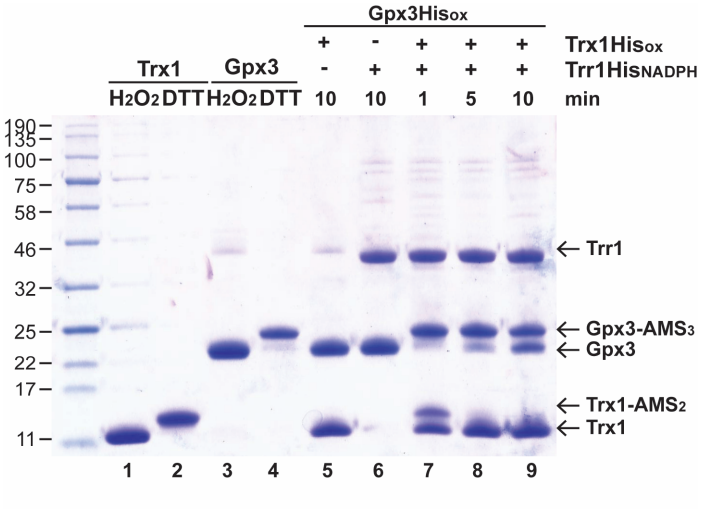

Figure 63: *In vitro* interaction between the thioredoxin system and Gpx3. Lanes 1-4 show the control reactions where Trx1 and Gpx3 were treated with 200µM H_2O_2 or 20mM DTT, in order to establish how the oxidised and the reduced species migrate during electrophoresis. Oxidised Gpx3 is able to interact with reduced Trx1 in a rapid manner (lanes 7-9), but not with Trr1 (lane 6) or oxidised Trx1 (lane 5). This verifies the literature, as Gpx3 is a known interactor of Trx1.

7.2.3. In organello interactions with the components of the MIA pathway

In addition to the *in vitro* interactions using purified proteins, it was obviously important to also test these in the physiological environment of intact mitochondria using *in organello* approaches. In this particular case, we tried to identify interactors by importing our proteins of interest into yeast mitochondria and then solubilising the whole organelles in order to pick out if our proteins were part of any complexes and if they were able to interact with other proteins *in vivo*. Clearly, the identification of protein interactors is ideally done with intact cells, but, in the case of Trx1 and Trr1, this is quite challenging because the majority of both proteins resides in the cytosol. It was therefore necessary to use *in organello* approaches, as this was the only way of ensuring minimal contamination from the large cytosolic pool of these two proteins.

Our approach focused on trying to find the possible interactors for Trx1, as Trr1 (a) is only known as a reductase for the thioredoxins and does not have any other known substrates and (b) did not seem to be able to interact with Mia40 and Erv1 in our *in vitro* experiments. As a continuation of this project, similar approaches can also be applied for the mitochondrial pool of Trr1, in order to address if it is indeed only able to interact with Trx1 in the IMS.

An approach that has been widely used to identify potential interactors of cysteine-containing proteins is the trapping of mixed disulfide intermediates. The concept behind this approach is simple: when we keep the cysteine residue responsible for the "docking" of our protein of interest onto its interactor, but mutagenise the residue required for the formation of the disulfide bond (resolving cysteine), the protein remains covalently bound to its interactor through a intermolecular disulfide bond, creating a mixed disulfide intermediate. This method has been extensively utilised for the identification of protein partners whose interactions are mediated by the formation of disulfide bonds (Sideris and Tokatlidis 2007; Sideris et al. 2009; Chatzi et al. 2013).

The first experiment that was performed was to try and trap any mixed disulfide intermediates formed after Trx1 was imported into mitochondria. In order to be able to visualise this, we used the alkylating agent NEM after importing Trx1 or the double cysteine mutant Trx1 C30/33S. The latter was used as a control in order to eliminate any signals that showed up in the case of the cysteine mutant as non-specific for interactions

mediated by the cysteine residues of Trx1. The results, shown in fig. 64, suggest that there are indeed a number of higher molecular weight species formed that are cysteine dependent and, as such, putative disulfide-bonded interactors of the thioredoxin system in the mitochondrial IMS. It is obvious that the Trx1 signal in these images is overexposed. This is something that typically is done when looking for interacting partners, as they tend to bind to our protein of interest (in this case, Trx1) in relatively low amounts, with the majority of the protein remaining unbound. As such, if we were to present the same image at a lower exposure, the higher molecular weight signals that could indicate the presence of Trx1 interactors would not be detectable.

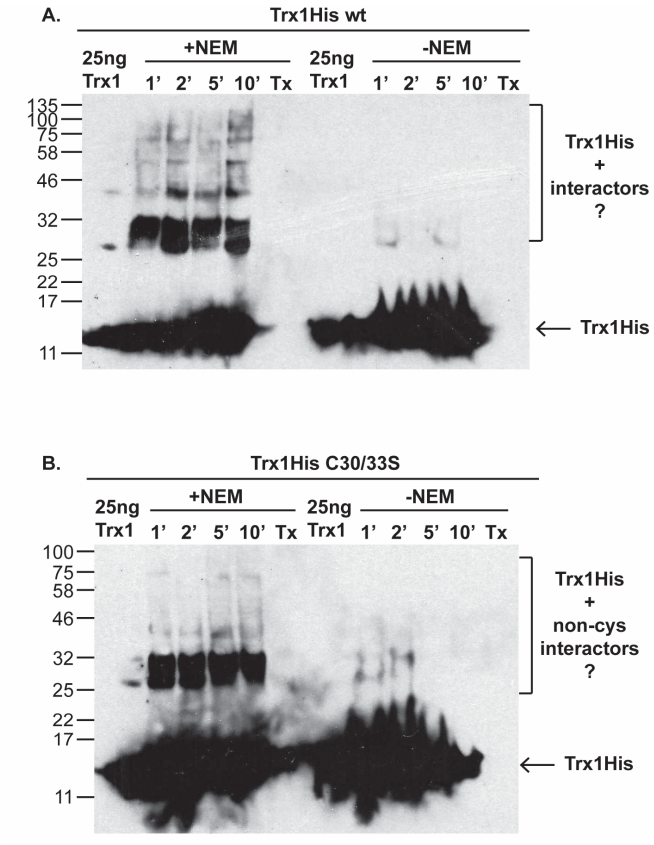

Figure 64: Import of Trx1His wt and Trx1His C30/33S in wt mitochondria and trapping of mixed disulfide intermediates with NEM. Trx1His wt (A) and Trx1His C30/33S (B) were imported into wt mitochondria. After incubating precursors and mitochondria for the specified timepoints, 25mM NEM (final concentration) was added to the import buffer to trap any mixed disulfide intermediates. Overexposure of the antibody signal for Trx1His wt was able to show the presence of a number of higher molecular weight species, which indicate putative interactors of Trx1His in mitochondria. The double cysteine mutant Trx1His C30/33S was used as a control for the detection of the background signal of interacting proteins that are not cysteine-mediated.

The same two proteins were also imported into wild type and Mia40-depleted mitochondria side by side, in order to test whether any specific bands disappeared, indicating an *in organello* interaction between Trx1 and Mia40. In this particular case, the results were quite puzzling, as the quite prominent thick band between 25kDa and 32kDa almost entirely disappeared when Mia40-depleted mitochondria were used (fig. 65). At this

particular molecular weight, this cannot be a complex between Trx1 and Mia40, but it is highly possible that it could be an interactor that depends on the MIA pathway in order to become imported, but does not depend on its cysteine residues in order to interact with Trx1.

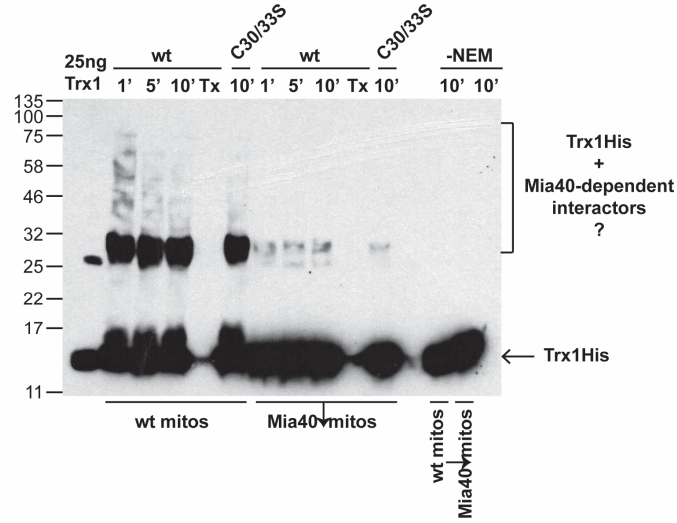

Figure 65: Import of Trx1His wt and Trx1His C30/33S in wt and Mia40-depleted mitochondria and trapping of mixed disulfide intermediates with NEM. Trx1His wt and Trx1His C30/33S were imported into wt and Mia40-depleted mitochondria. After incubating precursors and mitochondria for the specified timepoints, 25mM NEM (final concentration) was added to the import buffer to trap any mixed disulfide intermediates. Overexposure of the antibody signal was able to show the presence of a prominent band between 25kDa and 32kDa in the wt mitochondria that completely disappeared in the Mia40-depleted mitochondria. This could indicate an interactor that depends on the MIA pathway in order to become imported. The double cysteine mutant Trx1His C30/33S was used as a control for the detection of the background signal of interacting proteins that are not cysteine-mediated.

The final experiment that was performed was a pulldown of Trx1 and its cysteine mutants after being imported into wild type mitochondria. The aim of this experiment at this stage was to see if we could pick up any interactions between Trx1 and the two main components of the MIA pathway, Mia40 and Erv1, *in organello*. The results from this pulldown can be seen in fig. 66.

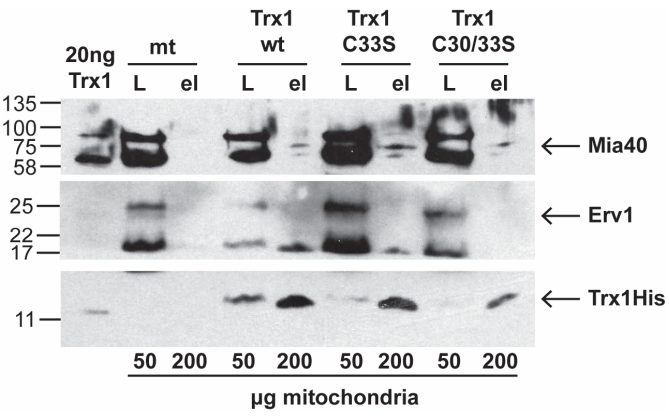

Figure 66: Pulldown of Trx1His wt, Trx1His C33S and Trx1His C30/33S after import into wt mitochondria. Trx1His wt, Trx1His C33S and Trx1His C30/33S were imported into wt mitochondria, which were then solubilised with solubilisation buffer containing 0.5% DDM. The solubilisation supernatant was incubated with equilibrated

Ni-NTA beads. The trap cysteine mutant (Trx1His C33S) was able to pull down higher amounts of Mia40 than the Trx1His wt. Additionally, neither of the two proteins was able to pull down Erv1. The non-imported mitochondria (mt) and the Trx1His C30/33S-imported mitochondria were used as controls for the detection of any background signals. (L) → loading control, (el) → elution.

As can be seen from fig. 66, the Trx1 cysteine trapping mutant (C33S) was able to pull down higher amounts of Mia40 than the wild type protein. There was no detectable signal in the case of Erv1, which could indicate that the interaction between Trx1 and Erv1 is not as favoured. This supposition would also agree with the results of the *in vitro* experiment between the two proteins.

7.3. Discussion

Equally important to the elucidation of the import pathway that Trx1 and Trr1 follow in order to reach the mitochondrial intermembrane space is the study of the function they perform in this particular subcompartment and how it ties in with the already known functions carried out in the IMS.

The first experiment was performed to test whether Trx1 is a redox-active state in the IMS. The result showed that Trx1 is indeed in a reduced state in the IMS, with the same experiment in the mitochondria lacking Trr1 (*Δtrr1*) showing that this is directly dependent on the presence of Trr1 in the mitochondria. From this result, we can safely assume that Trx1 is able to interact with IMS proteins in the same manner as it interacts with cytosolic ones, whereby it recognises oxidised and semi-oxidised proteins and reduces them.

In order to work on figuring out the function of the thioredoxin system in the IMS, we decided to first follow an *in vitro* approach and test the affinity of interaction between our two proteins of interest and the components of the MIA pathway. Our experimental setup allowed us to determine that Trx1 is the protein capable of interacting with the tested substrates, with Trr1 only being responsible for fuelling the reduction of Trx1. Trx1 showed a higher affinity for the reduction of oxidised Mia40 compared to oxidised Erv1.

Additionally, when we tested the interaction of the hydrophobic mutant of Mia40 (LMFFFM/A Mia40) with the thioredoxin system, we were able to detect the reduction of the hydrophobic mutant, indicating that the interaction between the two proteins does not involve the Mia40 hydrophobic cleft which mediates the majority of other Mia40 interactions.

The second approach used to assess the function of Trx1 in the IMS was the utilisation of *in organello* methods. Import of Trx1 into wild type mitochondria and blocking of the mixed-disulfide intermediates with the alkylating agent NEM showed the formation of a number of higher molecular weight species. All these species indicate interactions formed between Trx1 and IMS-localised proteins that are mediated by the formation of disulfide bonds between each pair of proteins.

Interestingly, when the same experiment was repeated in Mia40-depleted mitochondria, most of the higher molecular weight signals disappeared, a fact which could possibly indicate that the proteins able to interact with Trx1 are substrates of the MIA pathway and depend on the presence of Mia40 in order to become imported. This is a hypothesis that makes sense, if we consider the fact that the vast majority of Mia40 substrates are cysteine-containing proteins.

The final approach that was used was a pulldown assay, where three forms of Trx1 (wild type, C33S and C30/33S) were imported into wild type yeast mitochondria that were subsequently solubilised and incubated with Ni-NTA beads. This experiment was able to show us that Trx1 does interact with Mia40 *in organello*, with the highest amounts of Mi40 being found in the C33S Trx1 mutant. This is due to the fact that this mutant acts as a trap mutant, which can interact with the substrate proteins, but is unable to finalise the reduction process due to the lack of the resolving cysteine residue (C30). Additionally, no Erv1 was detected, a result that strengthens the one obtained from the *in vitro* experiment, which showed a higher affinity of Trx1 for Mia40 than for Erv1. Obviously, there are a significant number of other proteins resident within the IMS that were not tested in the samples from the pulldown, but the aim of this particular experiment was to find the conditions that could be used in order to be able to detect interaction partners of Trx1, in order to repeat it on a larger scale and analyse the samples with mass spectrometry.

Chapter 8

General discussion and future perspectives

8. General discussion and future perspectives

The exposure of cells to reactive oxygen species is something that occurs naturally as part of normal aerobic metabolism, but that can also happen when they are exposed to harmful chemicals and radiation that lead to the generation of radical compounds (Halliwell 2006). When the cells are unable to cope with the amounts of ROS or the damages caused by them, they face a condition called oxidative stress. This is a state where the cellular defence mechanisms and antioxidants are not able to efficiently detoxify the ROS and maintain a reduced environment within the cell. Oxidative stress can cause a number of adverse effects in cells, including protein oxidation, lipid peroxidation and DNA modification.

The study of oxidative stress and the cellular defence mechanisms responsible for the detoxification of ROS is important due to the implications of oxidative damage in a number of diseases (cancer, cardiovascular diseases and aging), as well as the food industry (Morano et al. 2012).

In this thesis, we attempted to address some of the mechanisms of oxidative regulation that could be at work in the mitochondrial intermembrane space of the yeast *Saccharomyces cerevisiae*.

8.1. The role of vitamin C as an antioxidant in yeast

Our aim in chapter 4 was to attempt to assess the effect that vitamin C – a powerful antioxidant in higher eukaryotes – might have in *S. cerevisiae* and, more specifically, its mitochondria.

Our attempt to utilise the endogenous pathway for the production of erythroascorbic acid was not successful, due to arabinose being a poor carbon source for yeast growth. As such, the addition of arabinose in a yeast growth medium containing a good carbon source (such as glucose) might be able to circumvent the growth issue, while simultaneously allow the

production of erythroascorbic acid within the cell. As mentioned in section 4.3, even this particular setup would have its pitfalls, as the measurement of the quantities of erythroascorbic acid produced under such circumstances would be quite difficult to assess. Additionally, as is generally the case when working with subcellular organelles, it would be challenging to assess the specific effect erythroascorbic acid may have on mitochondria. Mitochondria make up a small fraction of the cell and, with the cytosol being the centre of antioxidant defence, the study of the effect of erythroascorbic acid in yeast cells would most probably reflect its antioxidant activity in the cytosol.

The second approach involved the direct study of the effect of D-isoascorbic acid (a commercially available erythroascorbic acid analogue) on mitochondria. Addition of this chemical to mitochondria whose inner membrane potential was disrupted using chemical uncouplers showed mixed results, depending on the method of measurement. The indirect measurement using the import of the ΔΨ-dependent, matrix-localised precursor Su9DHFR did not show any evidence of D-isoascorbic acid being able to reverse/prevent the effect caused by CCCP. On the other hand, the more direct measurement involving the internalisation of the $DiSC_3(5)$ fluorescent dye into mitochondria and subsequent release upon addition of the uncoupler was not able to provide us with a definitive answer. The addition of D-isoascorbic acid showed a lower percentage of depolarisation, however, it also showed lower levels of $DiSC_3(5)$ internalisation.

From the above results, we were unable to determine whether this yeast equivalent of vitamin C has a similar protective effect as vitamin C does in mammalian cells (KC et al. 2005). Given the fact that erythroascorbic acid has been previously reported to not be as robust an antioxidant in yeast as ascorbic acid is in mammalian cells, it might be worth looking into other actions that could be carried out by this molecule. Such an example is the case of erythroascorbic acid playing a role in the induction of the alternative oxidase (AOX) gene in *Candida albicans*, which leads to the activation of cyanide-resistant respiration (Huh et al. 2008).

8.2. The import of Gpx3 into the mitochondrial IMS

Our aim in chapter 5 was to investigate the import process followed by Gpx3 and, more specifically, the initial step of how it can be targeted from the cytosol to the mitochondria.

As the import experiments performed for all components of the Yap1 activation pathway showed that only Gpx3 can become imported into mitochondria, we thought to look into whether any of the other proteins may play a role in facilitating Gpx3 import. Due to the import results for Ybp1 and Ybp2, which did not show any import, but did display a seemingly high affinity for the mitochondria (due to their resistance to cleavage by proteinase K after mitochondrial solubilisation with Triton X-100), we decided to test whether either of these two proteins might play a role in Gpx3 import.

After importing Gpx3 in the presence of Ybp1 and/or Ybp2, a small increase in the amount of internalised Gpx3 was seen, signifying that Ybp1 (but not Ybp2) could possibly play a role in facilitating Gpx3 import into mitochondria. There was also a slight increase in the amount of imported N18Gpx3, when the import was performed in the presence of Ybp1.

Despite these indications that Ybp1 could play a role in facilitating Gpx3 import into the mitochondrial IMS, they are by no means conclusive and require fine-tuning in order for us to definitely be sure that there is such an effect. Ybp1 was recently proposed to act as a scaffold protein that acts as a sulfenic acid chaperone, whereby it is able to transfer the H_2O_2 signal from Orp1/Gpx3 to Yap1 (Bersweiler et al. 2017). It may be possible that a second role of this chaperone function would be to promote an interaction of Gpx3 with the OM of the mitochondria. The hypothesis that Ybp1 could act as a chaperone for the more efficient targeting of Gpx3 to the outer mitochondrial membrane and, as a result, its translocation into mitochondria, would need to be tested for other mitochondrial proteins, so as to assess if this is a general mechanism for mitochondrially-targeted proteins or a more specific one for Gpx3 import.

Additionally, the hypothesis would need to be tested using different experimental setups. One such setup would be to supplement the import reaction with cytosolic extract from a yeast strain that contains Ybp1 (either at normal levels or at overexpressed levels, with a *Δybp1* cytosolic extract as a control). This would enable us to by-pass the step of the

production of Ybp1 as a radiolabelled precursor and ensure that it is correctly folded and fully functional.

Another approach we could use would be to produce Ybp1 as a purified, recombinant protein and use this in our experiments. Despite the fact that our efforts were unsuccessful and we were unable to induce the production of Ybp1 and Ybp2 in *E. coli* expression cells, these were all tested using the same vector into which our two genes of interest had been cloned. One common solution to such expression problems is to change the type of vector used to produce the protein. A setup that has been used extensively in our lab with great success is the cloning of the genomic sequences of difficult to express proteins downstream of a His-MBP-Tev coding sequence. In these cases, the translation of our protein of interest is driven by the translation of the maltose-binding protein (MBP), which increases the efficiency of the induction when compared to our sequence of interest alone. The addition of the Tev protease cleavage site enables us to isolate our protein without the bulky addition of MBP at the N-terminus.

Another setup that could be used to determine the effect of Ybp1 on the import of Gpx3 (and/or other mitochondrial proteins) would be to assess and quantify the amounts of certain mitochondrial proteins in highly purified mitochondria from *Δybp1* yeast cells. By comparing the levels of certain proteins of interest in the deletion strain with the corresponding wild type strain, we would be able to determine the effect of Ybp1 on the import of our proteins of interest.

8.3. Characterisation of Trx1 and Trr1 import

Our aim in chapter 6 was to elucidate the manner in which Trx1 and Trr1 become imported into the intermembrane space.

Our first attempts at elucidating the import of Trx1 and Trr1 were met with a number of different hurdles. The production of our proteins of interest as radioactive also led to the production of truncated versions and mutagenesis attempts to reduce the production of the additional proteins, though successful, were not consistent. Additionally, they created

another issue, which was the fact that we would not be working with the wild type proteins, as the mutations might lead to folding or functional defects.

One method that could be used to circumvent this issue would be to utilise alternative coupled and uncoupled transcription and translation systems. One such system is the *E. coli* S30 bacterial *in vitro* translation system (Robinson and Woolhead 2013) that is primarily used for the translation of bacterial proteins. On the other hand, there is also the eukaryotic wheat-germ extract, which is a plant cell-free expression system (Ogawa et al. 2017). If any of these alternative systems leads to the correct translation of our proteins of interest, then it could be used to repeat all the experiments that were done with the purified proteins, so as to verify all the results that have been obtained with the latter.

The second approach that involved attempting to work with the endogenous levels of Trx1 and Trr1 within our highly purified, isolated mitochondria was also not successful due to two major issues: a) the inability to detect Trx1 and b) the simultaneous detection of Trr1 and Trr2 with our αTrr1 antibody. A method that could be used to avoid these issues would be to add an epitope-tag our proteins of interest using homologous recombination, such as the FLAG-tag, Myc-Tag or His-tag. This will enable us to add one of the widely available tags to the genomic DNA encoding for Trx1 and Trr1, which will result in the cell producing only the tagged version of each protein. By using commercially available, highly specific monoclonal antibodies that recognise the epitope-tag sequences, we will be able to specifically detect our proteins of interest. The use of the above method will definitely solve the issue with the double detection of Trr1 and Trr2, as the tag-specific antibody will only recognise the tag that has been added to Trr1. It might also be helpful in the case of Trx1, as one of the reasons we might not have been able to detect it in our mitochondrial samples is the antibody detection threshold.

By using the purified, recombinant His-tagged versions of Trx1 and Trr1, we were able to work on elucidating their import into yeast mitochondria. Through the use of a number of deletion and conditional knockout strains, as well as various treatments, we were able to determine that the import of Trx1 and Trr1 does not depend on the MIA pathway, the inner membrane potential or the presence of the thioredoxin system within the IMS. Additionally, in the case of Trx1, we were also able to determine that its import does not depend on the presence of ATP during the import process or its cysteine residues.

An interesting result that was obtained during the course of these experiments was the fact that the import of Trx1 was elevated in Mia40-depleted mitochondria, a result that cannot really be explained at this point. Further studies regarding the import of Trx1 are required in order to assess the reason behind this phenomenon. As our results have shown that neither Trx1 nor Trr1 follow the MIA pathway, nor do they require the inner membrane potential (and consequently, the TIM23 pathway), future experiments will need to focus on alternative import pathways.

One such import pathway was described in section 1.3.4, where the two yeast heme lyases were shown to become imported into mitochondria using a direct, "non-conventional" pathway. To explore such a scenario for Trx1 and Trr1, we will have to look into the way in which they interact with the general entry gate in the outer mitochondrial membrane, the TOM complex. In order to assess whether the receptor components of the TOM complex are important, we could perform proteolytic digestion of the mitochondria prior to incubation with the precursor proteins. This particular treatment of the mitochondria leads to the cleavage of the receptors of the outer membrane and, thus, can eliminate the putative interactions of the incoming precursors with the TOM complex and inhibit the import process. If this experimental setup gives us any indication that there is a dependence on the outer mitochondrial membrane components, we could further investigate the issue by performing selective "blocking" of each separate receptor protein with specific antibodies and subsequently import our proteins of interest.

Another method that could be utilised to further investigate the components that interact with the incoming Trx1 and Trr1 precursors both at the level of the Tom40 pore, as well as in the IMS, is the creation of translocation arrested intermediates. The non-mitochondrial protein dehydrofolate reductase (DHFR) has been used extensively in the past for such studies, due to its ability to become tightly folded in the presence of methotrexate. In order to trap Trx1 and Trr1 during translocation, we would have to create the chimeric proteins Trx1-DHFR and Trr1-DHFR, which, upon the addition of methotrexate, will become arrested in the outer membrane channel. The folding of the DHFR domain on the outer surface of the outer membrane will allow for the Trx1 and Trr1 proteins to be partially stuck in the channel, with the majority of their length in the IMS. By performing an additional step of crosslinking, we can work towards identifying any proteins that interact with Trx1 and Trr1 during translocation, either by directly identifying specific components through immunoblotting, or by analysing the samples with mass spectrometry.

The experimental setup proposed above will be able to identify proteins involved in the translocation of Trx1 and Trr1. An additional strategy that can be used to identify IMS-localised interactors of the two proteins would be to add a 16G-polyglycine linker between the Trx1/Trr1 and the DHFR sequences. This will enable the full translocation of Trx1 and Trr1 across the outer membrane, with the polyglycine linker spanning the entirety of the OM. Essential controls in both experiments described above will be to verify that the addition of DHFR to Trx1 and Trr1 does not affect the import process, nor their correct localisation.

Even though bioinformatics analysis was performed as part of this thesis using a number of online prediction tools for mitochondrial localisation, these tools are generally regarded as not particularly efficient at recognising IMS-localisation patterns. Therefore, it is possible that Trx1 and Trr1 may contain unconventional targeting signals within their sequences that enable their import into the IMS. In order to test if this is true, we could create N- and C-terminal truncation mutants and test their capacity to become imported into isolated mitochondria. This will allow us to assess the presence of targeting signals within these regions.

The discovery that Trx2 is also able to become imported into mitochondria, despite not being identified as part of the updated IMS proteome (Vögtle et al. 2012), was very interesting as it is one of the genes upregulated as part of the response to oxidative stress. This could indicate that the IMS contains both a constitutuvely active thioredoxin system (Trx1 and Trr1), as well as stress-inducible one, through the translocation of Trx2 into the mitochondria as part of the oxidative stress response. Our experiment was only able to show that Trx2 has the capacity to become imported into isolated mitochondria, however, a number of follow-up experiments are required in order to check its submitochondrial localisation and the import pathway it utilises. As such, the study of the import of Trx2 would also follow along the lines of the methods described above for the determination of the import pathway utilised by Trx1 and Trr1.

8.4. The function of Trx1 and Trr1 the mitochondrial IMS

Our aim in chapter 7 was to study the function Trx1 and Trr1 carry out in the mitochondrial IMS. This is something highly interesting, as the discovery of these two proteins signified the discovery of a reductive pathway in this oxidising environment.

Our experiments were able to show that Trx1 is functional in the IMS of wild type mitochondria, as redox analysis revealed that it is capable of remaining reduced (and thus active), in a manner that depends on the presence of Trr1 within the IMS. Additionally, *in vitro* interaction experiments revealed the ability of Trx1 to interact with Mia40 independently of its hydrophobic cleft, as well as Erv1, though to a lesser extent. Trr1 was shown to be unable to directly interact with either of these proteins, with its presence being necessary only for the reduction of Trx1.

Our *in organello* approach was able to show that the trap cysteine mutant of Trx1 is able to pull down detectable amounts of Mia40, but not Erv1, further strengthening the case that Mia40 is a better interactor for Trx1 than Erv1. Another important finding was the disappearance of possible interactors of Trx1 in Mia40-depleted mitochondria. This could indicate that the majority of Trx1 interactors in the IMS are proteins that depend on the MIA pathway to become imported. This hypothesis makes sense if we take into account the fact that the majority of Mia40 substrates are cysteine-containing proteins and, as such, are possible interaction candidates for Trx1.

In order to understand the role Trx1 and Trr1 play in the IMS, the first step would be to identify the proteins that are capable of interacting with our proteins of interest. The interactors can be identified through the use of mass spectrometry after the import of the trap cysteine mutant versions of the two proteins. After the initial identification of possible interaction candidates, they can then be directly assessed through the use of deletion mutants, Blue Native PAGE analysis and SDS PAGE analysis, in order to verify the proteins that are true interactors of our two proteins of interest. This approach will allow us to assess whether the thioredoxin system is functional as a general reductive mechanism in the IMS or if either of the two proteins participates in other interactions within this compartment.

Chapter 9

Appendix

9. Appendix

9.1. List of publications

A. Chatzi, A., **Manganas, P.** & Tokatlidis, K., Oxidative folding in the mitochondrial intermembrane space: A regulated process important for cell physiology and disease. Biochim Biophys Acta. 1863(6 Pt A):1298-306. doi: 10.1016/j.bbamcr.2016.03.023.

B. Nuebel, E., **Manganas, P.** & Tokatlidis K., Orphan proteins of unknown function in the mitochondrial intermembrane space proteome: new pathways and metabolic cross-talk. Biochim Biophys Acta. (2016); 1863(11):2613-2623. doi: 10.1016/j.bbamcr.2016.07.004.

C. **Manganas P.**, MacPherson, L. & Tokatlidis, K., Oxidative protein biogenesis and redox regulation in the mitochondrial intermembrane space. Cell Tissue Res. (2017); 367(1):43-57. doi: 10.1007/s00441-016-2488-5.

Chapter 10

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