Split-DHFR as a Protein Complementation Assay for Localisation and Interactions of Yeast Mitochondrial Proteins

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Abstract

Protein complementation assays (PCAs) utilising two fragments of a reporter protein – fused to two potentially interacting proteins of interest – are a common method of analysing protein-protein interactions (PPIs). This approach, using split dihydrofolate reductase (DHFR) as a reporter protein, has been previously carried out for cytosolic *Saccharomyces cerevisiae* proteins. The focus of this study was to establish a split-DHFR assay specifically for use in analysing yeast mitochondrial PPIs in the intermembrane space (IMS), which has not been done before. A strategy to overcome the problem endogenous DHFR activity had to be developed using a modified strain of *S. cerevisiae* for the specific application here. Further, plasmids containing two positive control proteins, Tim9 and Tim10 (two well-known interacting proteins of the IMS) were cloned for transformation into yeast strain BY4741. Several other plasmids bearing various control proteins were designed and some of them cloned, although we required more time to have the full set of tools to establish the assay.

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The research reported in this thesis is my own work unless it is otherwise stated and it has not been submitted for any other degree.

Lisa Frances MacPherson

Abbreviations

AmpR	Ampicillin Resistance
AP/MS	Affinity Purification/Mass Spectrometry
BiFC	Bimolecular Fluorescence Complementation
CHO	Chinese Hamster Ovary
Cytb2	Cytochrome b2
CytC	Cytochrome C
DFR1	*S. cerevisiae* Dihydrofolate Reductase homologue
DHF	Dihydrofolic Acid
DHFR	Dihydrofolate Reductase
ER	Endoplasmic Reticulum
GFP	Green Fluorescent Protein
Glr	Glutathione Reductase
Gpx	Glutathione Peroxidase
Grx	Glutaredoxin
GSH	Glutathione
GSSG	Oxidised Glutathione
GST	Glutathione S-Transferase
H_2O_2	Hydrogen Peroxide
His	Hexahistidine
Hsp	Heat Shock Protein
IM	Inner Membrane
IMB	Inner Boundary Membrane
IMP	Inner Membrane Protease
IMS	Intermembrane Space
LC	Liquid Chromatography
LEU2	Leucine Marker
mDHFR	Murine Dihydrofolate Reductase
Mgr2	Mitochondrial Genome Required 2
MIA	Mitochondrial Intermembrane Space Assembly
MTS	Mitochondrial Targeting Sequence
MPP	Mitochondrial Processing Peptidase
MTX	Methotrexate
NES	Nuclear Export Signal

O_2^-	Superoxide Anion
OD	Optical Density
OM	Outer Membrane
ORF	Open Reading Frame
PAM	Presequence Translocase-Associated Motor
PCA	Protein Complementation Assay
PPI	Protein-Protein Interaction
Prx	Peroxiredoxin
ROS	Reactive Oxygen Species
SAM	Sorting and Assembly Machinery
TCA	Tricarboxylic Acid
THF	Tetrahydrofolic Acid
TIM	Translocase of the Inner Membrane
TOM	Translocase of the Outer Membrane
Trr	Thioredoxin Reductase
Trx	Thioredoxin
URA3	Uracil Marker
Y2H	Yeast Two-Hybrid

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1. Introduction

1.1 Protein Complementation Assays (PCAs)

1.1.1 PCAs and the Study of Protein-Protein Interactions (PPIs)

The study of protein-protein interactions (PPIs) is vital, not only for dissecting the function of new genes discovered during genome sequencing, but also for the study and treatment of diseases. Protein Complementation Assays (PCAs) are of great importance in studying PPIs, functioning through the fusion of two, complementary fragments of a reporter protein to two proteins of interest (Figure 1). If these two proteins interact, the reporter fragments are brought together so that they can now fold into their native structure and reconstitute their function (Remy et al., 2007). Many different PCAs approaches exist, and so the functioning reporter protein can give a detectable signal as colour (β-lactamase (Galarneau et al., 2002)), fluorescence (green fluorescent protein (GFP), bimolecular fluorescence complementation (BiFC)) (Hu & Kerppola, 2003; Kerppola, 2006), bioluminescence (Luciferase (Kaihara et al., 2003; Villalobos et al., 2007)) or even cell survival (dihydrofolate reductase (DHFR) (Pelletier et al., 1998)). Unlike other techniques such as Yeast Two-Hybrid (Y2H) screens, which require fusion proteins to be imported into the cell nucleus (Fields & Song, 1989), PCAs are advantageous as they can be used in any cellular compartment. They can also be used to study the formation of complexes containing three proteins, whereas Y2H screens are limited to binary protein complexes (Morell et al., 2009).

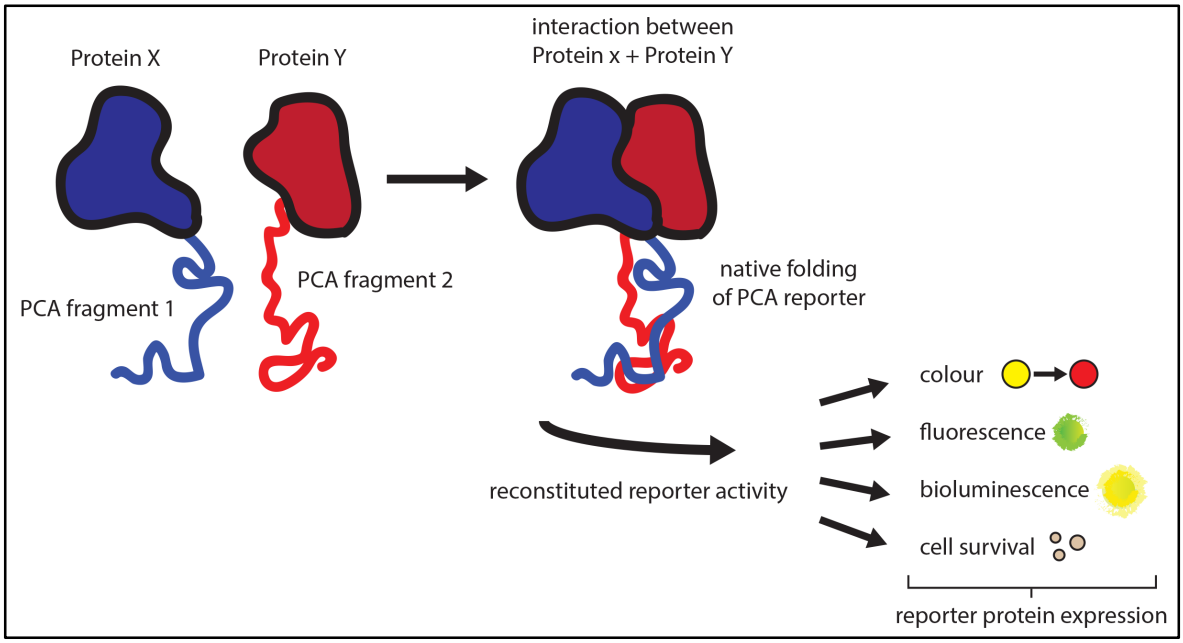


Figure 1 - General Protein Complementation Assay (PCA) approach (adapted from Remy et al., 2007). When Protein X and Protein Y interact, PCA fragments 1 and 2 are also brought together and native folding of the reporter protein occurs. This results in reconstituted reporter activity, which may be colour (β-lactamase), fluorescence (green fluorescent protein (GFP)), bioluminescence (Luciferase) or cell survival (dihydrofolate reductase (DHFR)).

As these PPIs can be directly detected *in vivo*, unlike with *in vitro* techniques such as affinity purification and mass spectrometry (AP/MS), they can be studied in the native environment of the proteins, which can influence their interactions with one another. The general approach of AP/MS involves using a cell lysate containing a protein of interest ('bait') bound to a tag, which is passed through an affinity column with a resin that will specifically bind to the tag. Multiple such tags exist, including TAP (as used in tandem affinity purification (Rigaut et al., 1999)), FLAG (Ho et al., 2002), hexahistidine (His) (Lichty et al., 2005), glutathione S-transferase (GST) (Smith & Johnson, 1988), human influenza haemaglutinin (HA) (Moon et al., 2012) and c-Myc (Hillman et al., 2001). The column is then washed to remove unbound proteins and the bait protein can be eluted along with any interacting proteins ('preys') (Gingras et al., 2007). The eluted protein complex is digested into smaller peptide fragments with trypsin and separated using either liquid chromatography (LC) (Wu & MacCoss, 2002) or gel-purified via SDS-PAGE (Nesvizhskii, 2012). These fragments are then identified via a mass spectrometer to generate MS spectra that can be compared to an online database of known peptide sequences. The results are generated as a list of proteins (the bait and interacting preys), i.e. potential PPIs. AP/MS is known to give false positives due to incorrect identification of interacting proteins (Nesvizhskii, 2010) and non-specific binding partners (such as heat shock proteins, ribosomal proteins, etc.) (Nesvizhskii, 2012), and true interactors with the bait can be less than 10% of those identified via MS in single-step AP (Trinkle-Mulcahy et al., 2008). Techniques such as Y2H and AP/MS, however, can often be used to complement the PCA approach and provide the initial data for potential PPIs (Remy et al., 2007). PCAs can then be used to study the localisation of these proteins, or even competition for binding to a specific protein (Morell et al., 2009). Full-length proteins of interest can be fused to the reporter fragments, although care must be taken to ensure the fragments do not interfere with protein targeting or post-translational modifications through their fusion to the N- or C-terminal domain (Remy et al., 2007). Linker sequences – of around 10-15 amino acids – are often added between the reporter and protein of interest. This is done to ensure that the reporter fragments are able to efficiently fold back into their native structure together, without being hindered by the size of the interacting proteins (Michnick et al., 2010).

Of all PCAs, fluorescent proteins – split-GFP (and its derivatives, such as BiFC) – are among the most widely used, with fluorescent signal strength indicating the strength of the PPI. High expression of the fluorescent reporter fragments can, however, result in association of the two fragments independent of the PPI (Morell et al., 2009). Split-GFP also interacts irreversibly, which can cause trapping of non-specific complexes and the disruption of endogenous PPIs (Tarassov et al., 2008). Another PCA, Luciferase, is reversible, but requires the addition of a substrate to provide its bioluminescent signal (Morell et al., 2009). Split-DHFR is also reversible, and useful in large-scale studies of PPIs – where it can be used to screen a cDNA library for potential interacting partners of a particular protein (Tarassov et al., 2008). Split-DHFR also has a distinct advantage in that is does not require any specialist equipment to visualise the results, as PPI is indicated by cell growth and not, for example, by fluorescence (which requires fluorescent microscopes to analyse) (Remy et al., 2007). These three PCAs – GFP, Luciferase and DHFR – are compared in Table 1.

Reporter Protein	SIgnal	Mass (kDa)	Reversible?	Advantage(s)	Disadvantage(s)
DHFR	cell growth	21.6	Yes	no specialist equipment required for visualisation	endogenous DHFR present in many cell types
GFP	fluorescence	26.9	No	stability of fragment interaction allows detection of transient PPIs	signal detection inhibited by background fluorescence of cell
Luciferase	bioluminescence	36.0 (Rluc) 19.9 (Gluc)	Yes	can be measured on the timescale of seconds	substrates required

Table 1 – Comparison of Protein Complementation Assays (PCAs) (adapted from Michnick et al., 2010). Protein mass for dihydrofolate reductase (DHFR), green fluorescent protein (GFP), Renilla reniformis *Luciferase (Rluc) and* Gaussia princeps *Luciferase (Gluc) were taken from UniProt* (Consortium, 2017).

1.1.2 Dihydrofolate Reductase (DHFR) as a PCA

DHFR (Figure 2A) is an enzyme that catalyses the synthesis of hydrofolate to allow for nucleotide biogenesis, via the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF) (Michnick et al., 2010) (Figure 2B). DHFR uses NADPH as a co-factor in this reaction, wherein it acts as an electron donor and is converted to NADP (Remy et al., 2007). DHFR has 3 domains, the discontinuous F1 and F3 domains, and the adenine-binding domain F2 (Pelletier et al., 1998). Both the substrate-binding pocket and NADPH-binding groove of DHFR are formed mainly by residues in the F2 and N-terminal portion of F1. Residues 101-108 form a disordered loop which can be disrupted with no significant effect on DHFR activity (Pelletier et al., 1998). This was first shown In 1992, when the loop was removed from murine DHFR (mDHFR) through circular permutation and the new variant was found to differ very little in terms of functionality (Buchwalder et al., 1992).

The split-DHFR PCA has been established not only in *Escherichia coli* (Pelletier et al., 1999; Pelletier et al., 1998) and *Saccharomyces cerevisiae* (yeast) cells – which will be discussed in detail later – but also mammalian cell lines (Michnick & Remy, 1999) and plant protoplasts (Subramaniam et al., 2001). The first of these studies was carried out in 1998, when Pelletier et al. showed that DHFR can be reassembled from complementary fragments (F1/2 and F3) when fused to interacting proteins. Due to its role in hydrofolate synthesis, DHFR activity is required for growth on minimal media lacking complex nutrients. *E. coli* DHFR is more sensitive to inhibition via trimethoprim than mDHFR. Trimethoprim is an anti-folate drug which binds to DHFR and inhibits the reduction of DHF to THF (Brogden et al., 1982). In Pelletier et al.'s study, mDHFR fragments were fused to interacting proteins in *E. coli* cells, which were then grown on minimal media in the presence of trimethoprim. The trimethoprim levels were high enough to inhibit the endogenous DHFR but not mDHFR, allowing only for the growth of cells where interacting proteins allowed for the reconstituted, mDHFR to assemble in a functional form. This approach allowed for the development of this technique as a general method for detecting protein-protein interactions *in vivo* via an enzyme-based detection system.

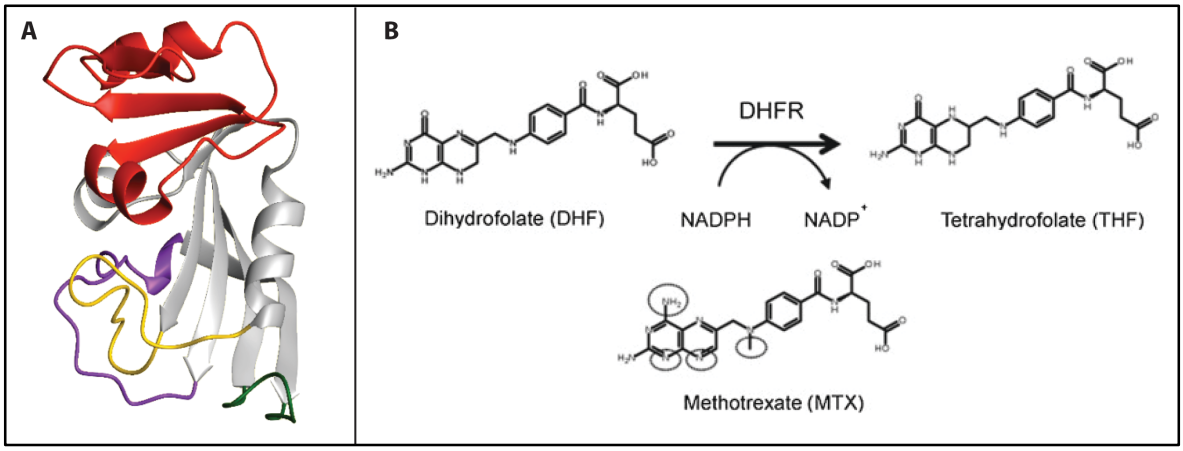


Figure 2 – Dihydrofolate reductase (DHFR) structure and function. A: Structure of DHFR (Ulrich et al., 2007). *The adenosine-binding domain is highlighted in red. B: Comparison of Dihydrofolate (DHF) and Methotrexate (MTX)* (Zheng & Kwon, 2013). *DHFR reduces its substrate, DHF, to Tetrahydrofolate (THF) via NADPH. MTX is able to act as a competitive inhibitor of DHF due to its similar structure. Differences in the chemical structure of MTX compared to DHF are circled.*

A modified approach to this assay was undertaken for use in mammalian cells. In 1980, a cell line derived from Chinese hamster ovaries (CHO) with no endogenous DHFR activity was generated (CHO-DUKX-B11) (G Urlaub & Chasin, 1980). This DHFR-negative cell line was used alongside fusion proteins with mDHFR fragments (Israel & Kaufman, 1993; Michnick & Remy, 1999). In this approach, it was shown that only 25 protein complexes per cell are needed for the split-DHFR assay to work (Michnick et al., 2010). The CHO-DUKX-B11 cell line, however, is prone to reverting to functional DHFR activity when mutagenised (G Urlaub & Chasin, 1980), and so a completely DHFR-deficient strain known as CHO-DG44 has also been developed via deletion of both DHFR alleles (Gail Urlaub et al., 1983). Mammalian cells with DHFR activity can also be used as an alternative approach to the split-DHFR PCA, via the use of mutated mDHFR fragments with a resistance to methotrexate (MTX) (Thillet et al., 1988). MTX, an anti-folate drug, can act as a competitive inhibitor of DHFR binding in its active site (i.e. as a DHF analog) (Figure 2B). The native DHFR is therefore inhibited by the MTX, but complemented by the activity of the reconstituted, mutant mDHFR fragments (Remy et al., 2007).

1.1.3 Split-DHFR in Yeast

Saccharomyces cerevisiae has a DHFR homologous gene, DFR1, which is localised to the cytoplasm and mitochondria (Huh et al., 2003) and is essential for viability. This presents an issue for the use of DHFR as a PCA in *S. cerevisiae*, as the endogenous DFR1 is necessary for cell growth but will interfere with the assay. With this in mind, in 2008 two groups took different approaches to adjust the split-DHFR assay for use in *S. cerevisiae* cells (Shibasaki et al., 2008; Tarassov et al., 2008). The first of these approaches, carried out by Shibasaki et al., used a combination of trimethoprim and sulphanilamide to inhibit the endogenous DHFR precursor. Sulphanilamide enhances the sensitivity of *S. cerevisiae* cells to trimethoprim, as the DHFR homolog present is not as sensitive to the drug as *E. coli* DHFR. mDHFR, which, as noted previously, has a lower affinity for trimethoprim, was used as the reporter protein fragments fused to the proteins of interest and was therefore able to complement for the lack of DFR1 activity.

Tarassov et al., 2008, however, undertook a different approach based on a MTX-resistant mutant of mDHFR fragments. They carried out site-directed mutagenesis to insert a L22F mutation into the mutant F31S mDHFR F1/2 fragment originally used in a mammalian cell split-DHFR assay (Michnick & Remy, 1999). By mutating two amino acids in the F1/2 fragment (L22F and F31S) (Figure 3), the reconstituted mDHFR remains active but becomes 10,000 times less sensitive to MTX than DFR1 (Ercikan-Abali et al., 1996). Therefore, when grown in the presence of MTX, the endogenous DFR1 is inhibited by the drug, whereas the MTX-resistant mDHFR fragments are not able to complement its function when protein complementation of the fragments occurs. Tarassov et al. used this approach to set up a large-scale genome-wide screen in *S. cerevisiae* cells. They achieved this by creating universal, DHFR fragment cassettes which were then used to create homologous recombination cassettes for 5756 genes in *S. cerevisiae* haploid strains. Open reading frames (ORFs) fused to the mutant F1/2 fragment of mDHFR were inserted into MAT**a** strains and then mated to MATα strains containing ORFs fused to the F3 fragment. Diploid strains containing reconstituted mDHFR were selected for on minimal media with MTX present, with PPIs analysed via the size of any colony growth.

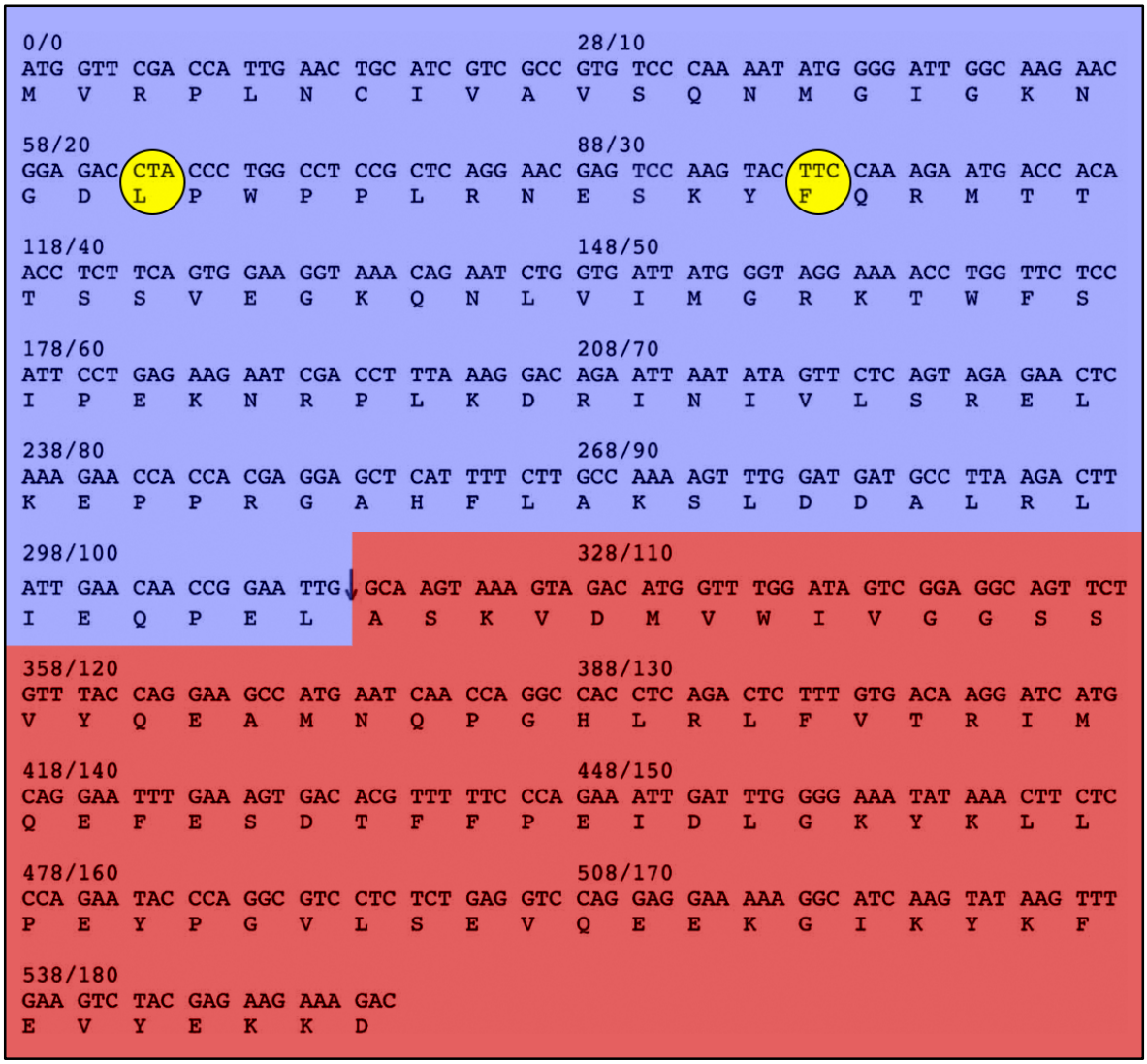


Figure 3 - Murine Dihydrofolate Reductase (DHFR) sequence (adapted from Remy et al., 2007; Tarassov et al., 2008). Fragment 1 (F1/2) of the split-DHFR approach is highlighted in blue and fragment 2 (F3) in red. The corresponding residues which were mutated in Tarassov et al.'s approach to create a Methotrexate (MTX) resistant strain of DHFR are indicated in yellow.

1.2 Protein Import into Yeast Mitochondria

1.2.1 Mitochondria and Protein Import

Mitochondria (Figure 4) are double-membraned organelles with an outer membrane (OM) acting as a barrier between mitochondria and the cytosol, and an inner membrane (IM) (Riemer et al., 2011). The surface area of the IM can be more than four times greater than that of the OM, due to invaginations in its structure known as cristae (Ikon & Ryan, 2017). The IM separates the two, main compartments of mitochondria known as the intermembrane space (IMS), where oxidative protein folding occurs, and the matrix. The IMS has been shown to be further organised into two, distinct regions – the 'bulk' of the IMS defined by the inner boundary membrane (IBM), and the cristae lumen (Ikon & Ryan, 2017). Cytochrome C (CytC) and the oxidative phosphorylation complexes, involved in the electron transfer reactions of respiration, are sequestered from the rest of the IMS in these cristae junctions (Perotti et al., 1983; Scorrano et al., 2002).

95% of mitochondrial protein precursors are encoded by the nucleus and synthesised in the cytosol before their import into mitochondria (Fraga & Ventura, 2013). In humans, mitochondrial DNA only encodes 13 mitochondrial polypeptides (Sickmann et al., 2003). Mitochondrial proteins can be destined for any compartment of mitochondria, including not only the IMS and matrix but also the OM and IM. It is therefore important to understand the various import pathways that these mitochondrial proteins undertake (Figure 6).

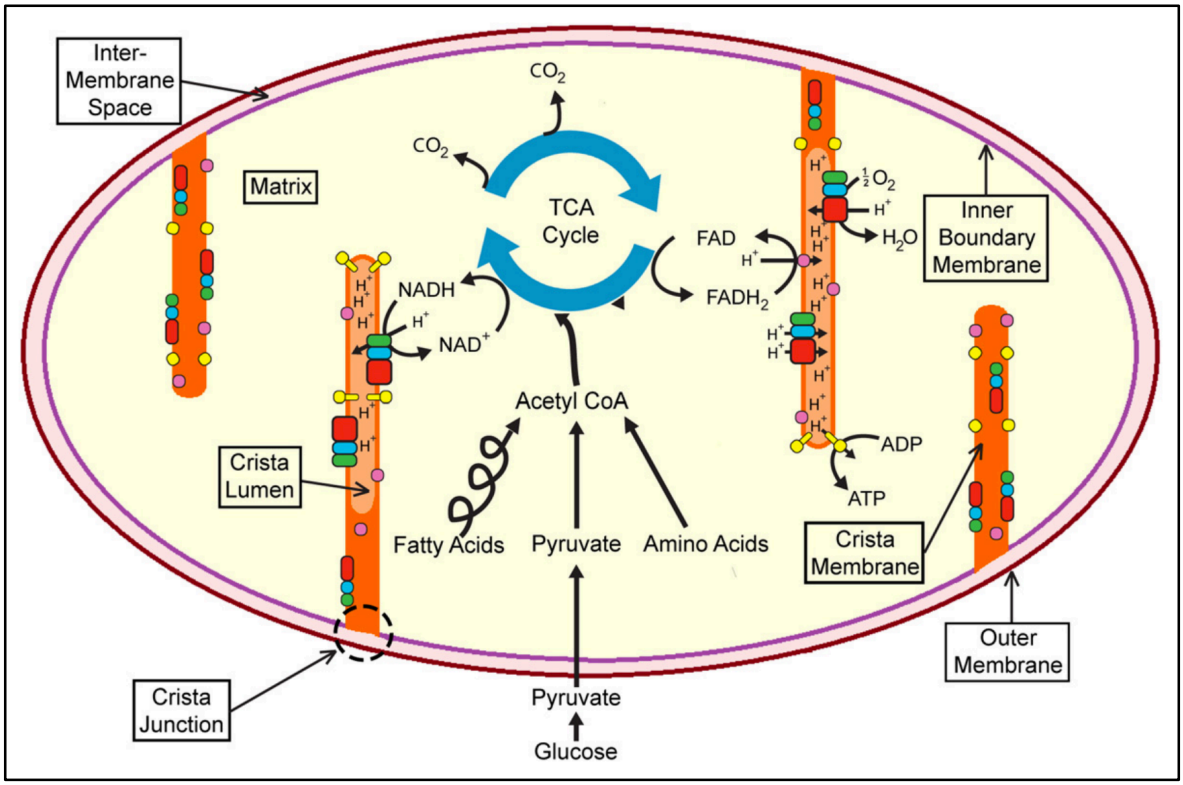


Figure 4 - Structure of mitochondria and metabolism organisation (Ikon & Ryan, 2017). *Glucose, fatty acids and amino acids provide the acetyl CoA which allows for the tricarboxylic acid (TCA) cycle to occur in the mitochondrial matrix. Electron transfer from Oxidised NADH and $FADH_2$ from the TCA cycle is then used to create an electron transfer gradient across the cristae membrane (via H^+ ions) which drives the production of ATP.*

1.2.2 Protein Import Pathways

1.2.2.1 Targeting Signals and Chaperones

Due to being synthesised in the cytosol, mitochondrial proteins require both targeting signals and chaperones to ensure that they are imported to the correct subcompartment of mitochondria. Specific targeting of mitochondrial preproteins to different subcompartments is determined based on targeting sequences found within them (Chatzi et al., 2016). For example, an N-terminal presequence known as the mitochondrial targeting sequence (MTS) will target preproteins to the matrix unless they contain a further internal targeting sequence (Manganas et al., 2017). The MTS is an amphipathic α-helix that is normally cleaved following preprotein import by the mitochondrial processing peptidase (MPP) (Braun & Schmitz, 1997). Cytosolic chaperones such as heat shock protein (Hsp) 70 and 90 prevent the aggregation of mitochondrial protein precursors and aid in their translocation to the general import pore of mitochondria – the TOM (translocase of the OM) complex (MacPherson & Tokatlidis, 2017; Neupert & Herrmann, 2007). The IMS also contains ATP-independent mitochondrial chaperones known as the small Tim family which aid in the translocation of polytopic IM and OM proteins that do not contain a targeting presequence (Chan et al., 2006). In *S. cerevisiae* two such small Tim complexes function alongside the TIM22 (translocase of the inner membrane 22) and SAM (sorting and assembly machinery) complexes to chaperone mitochondrial proteins (MacPherson & Tokatlidis, 2017). These are known as the TIM9/10 and TIM8/13 complexes, which form hexameric structures with 3 protomers of Tim9-Tim10 or Tim8-Tim13, respectively (Beverly et al., 2008; Webb et al., 2006).

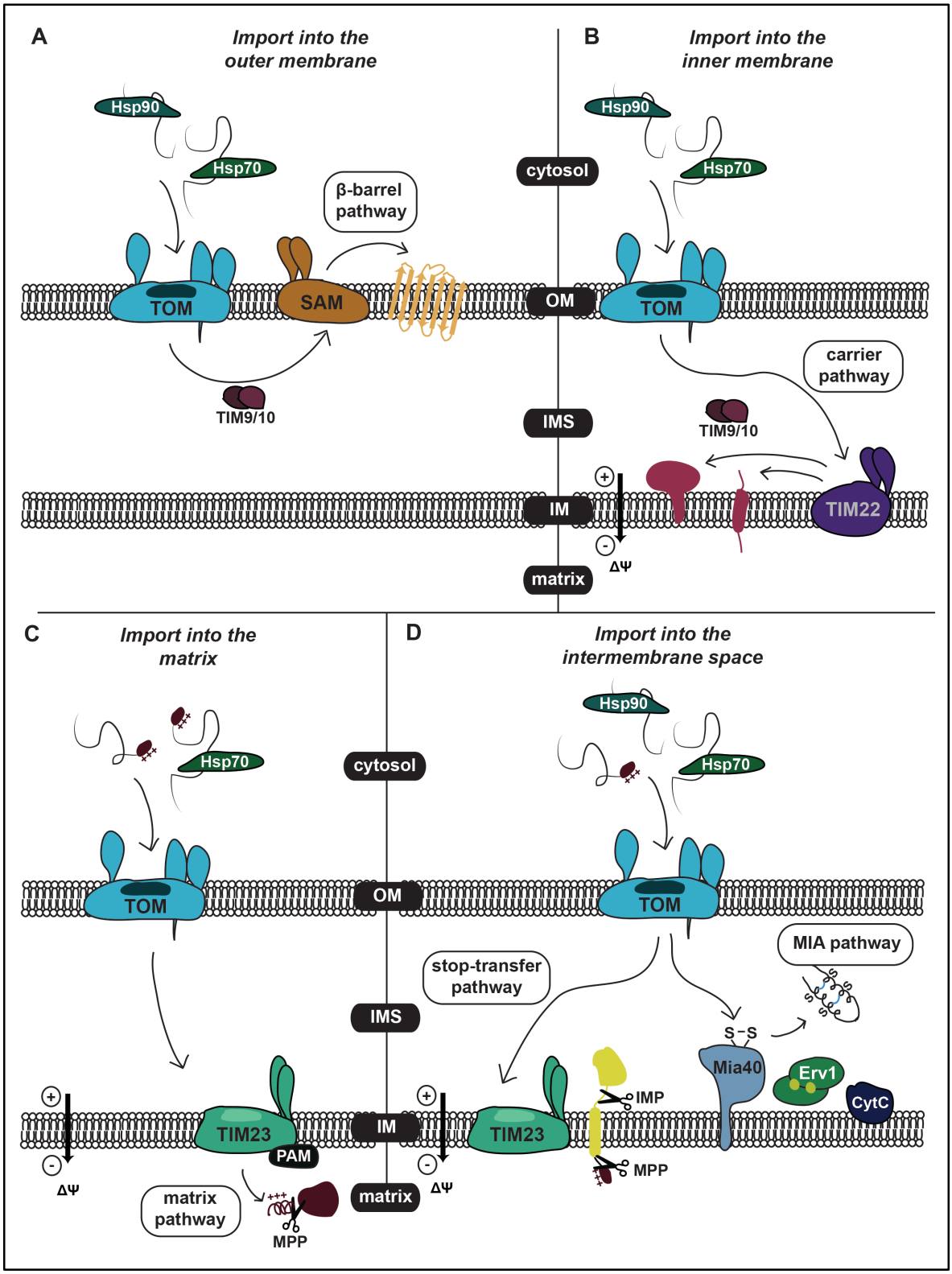


Figure 5 - Routes of protein import into mitochondria (adapted from Manganas et al., 2017). Precursor proteins are chaperoned through the cytosol, by heat shock protein (hsp) 70 and 90, to the general import pore of mitochondria, the translocase of the outer membrane (TOM) complex. A: Import into the outer membrane (OM). After their translocation through the TOM complex, precursor proteins are chaperoned through the intermembrane space (IMS) by the TIM9/10 complex. They are targeted to to the sorting and assembly machinery (SAM)

complex for insertion into the OM via the β-barrel pathway. B: Import into the inner membrane (IM). Precursor proteins are chaperoned by the TIM9/10 complex to the TIM22 (translocase of the inner membrane 22) complex for insertion into the IM via the carrier pathway. C: Import into the matrix. Precursor proteins are inserted into the matrix by TIM23 with translocation driven by the presequence translocase-associated motor (PAM) complex. The protein is then cleaved by the mitochondrial processing peptidase (MPP). D: Import into the IMS. Import into the IMS is carried out via the stop-transfer pathway and TIM23, with cleavage of precursor proteins by the inner membrane protease (IMP) and MMP. Proteins may also follow the Mitochondrial IMS Assembly (MIA) pathway and become trapped in the IMS via oxidative folding carried out by electron transfer between Mia40, Erv1 and Cytochrome C (CytC).

1.2.2.2 Outer Membrane (OM)

The general import pore of the OM, the TOM complex, has two receptors – Tom20 and Tom70 – which have hydrophilic domains exposed to the cytosol that interact with mitochondrial substrate proteins (MacPherson & Tokatlidis, 2017). Tom20 and Tom70 can compensate for one another's functions (Neupert & Herrmann, 2007), though Tom20 interacts with the hydrophobic residues of N-terminal presequences (i.e. the MTS) of incoming precursor proteins via its binding groove (Abe et al., 2000) and Tom70 recognises hydrophobic precursors with internal targeting sequences (Chan et al., 2006). Tom71 is a paralogue of Tom70 and can also partially compensate for its function (P. Rehling, 2003; Webb et al., 2006). Unlike Tom20 and Tom70 which are imbedded in the OM by their N-terminal domains, Tom22 exposes its N-terminus to the cytosol and its C-terminus to the IMS (van Wilpe et al., 2000). Tom22 connects Tom20 to the pore of TOM and has a large IMS domain that aids later translocation stages from the OM to the IM (MacPherson & Tokatlidis, 2017; Wagner et al., 2008). The central channel, Tom40, acts as the binding regions for precursor proteins (Neupert & Herrmann, 2007; Shiota et al., 2015) and Tom5, Tom6 and Tom7 modulate interactions with the channel (with it being lethal when all 3 channel modulating genes are deleted) (Dekker et al., 1998; Dietmeier et al., 1997; Sherman et al., 2005).

The TOM complex, along with the SAM complex, is also involved in the insertion of proteins to the OM (MacPherson & Tokatlidis, 2017) (Figure 5A). Some OM proteins need other complexes for their import into the OM, whereas β-barrel proteins are guided by a conserved β-signal (as they lack a MTS) as well as a conserved β-hairpin structure which is recognised by Tom20 (Bohnert et al., 2010; Hildenbeutel et al., 2012). The SAM complex also inserts proteins in the OM, and has a main component – Sam50 – which is highly conserved as well as 2 hydrophilic subunits exposed at its cytosolic side, Sam35 and Sam37 (MacPherson & Tokatlidis, 2017). Sam50 and Sam35 are essential, and Sam35 recognises the β-signal in β-barrel protein insertion and opens the Sam50 channel (Bohnert et al., 2010). Sam37, however, has been shown to be essential for the formation of the TOM-SAM complex during the insertion of OM proteins (Neupert & Herrmann, 2007).

1.2.2.3 Inner Membrane (IM) and Matrix

Polytopic proteins (with multiple transmembrane domains) follow the carrier pathway and are inserted into the IM via TIM22 (Hasson et al., 2010) (Figure 5B). The TIM22 pathway requires itself, the small Tims (specifically, the TIM9/10 complex) and the TOM complex in order to function. The Tim22 subunit makes up the main insertion channel, with conserved cysteine residues that stabilise Tim22 and are vital to its function (Davey et al., 2006). TIM22 also has accessory subunits Tim18 and Tim54, with Tim54 being non-essential (Kerscher et al., 1997; Kovermann et al., 2002).

Import into the mitochondrial matrix via TIM23 (Figure 5C) is similar to the carrier protein import pathway, but uses the TIM8/13 complex to chaperone preproteins instead of TIM9/10 (Paschen et al., 2000). The Tim23 subunit is the main channel and has an IMS domain receptor for presequences of incoming proteins (Kozany et al., 2004; Li et al., 2004). Tim50, on the other hand, interacts with mitochondrial precursor proteins via the IMS C-terminal domain of TIM23 (Geissler et al., 2002; Mokranjac et al., 2003; Yamamoto et al., 2002). Tim17 is also essential and although its function was originally unknown, it has been shown to interact with Pam17 of PAM (Presequence translocase-Associated Motor) complex (Jensen & Johnson, 2001; Peter et al., 2004; Ting et al., 2014). The PAM complex acts as the secondary driving force of protein import to the matrix and is powered by ATP hydrolysis (Bauer et al., 2000).

1.2.2.4 Intermembrane Space (IMS)

IMS-targeted proteins have sequences with conserved motifs necessary for their import. Some contain N-terminal bipartite sequences (a MTS domain followed by a hydrophobic domain) and follow a variation of the TIM23 import pathway (Glick et al., 1992). This is known as the ‘Stop Transfer’ Pathway (Figure 5D), wherein precursors are stopped during translocation through the TIM23 pore due to the presence of a hydrophobic targeting sequence (Glick et al., 1992). The MTS is then

cleaved by MPP and further proteolysis of the hydrophobic domain is carried out in an ATP-independent manner before the protein is released into the IMS (Glick et al., 1992). Mgr2 (Mitochondrial genome required 2) acts as a gatekeeper in this process by recognising positive residues found in the matrix-targeting signal of incoming peptides, therefore preventing incorrect import of preproteins into the IMS (Ieva et al., 2014). Proteins which lack a targeting presequence follow the Mitochondrial IMS Assembly (MIA) pathway (Figure 5D), utilising oxidative folding to trap precursor proteins in the IMS (Sideris & Tokatlidis, 2010), which will be discussed in more detail in the next section.

1.2.3 Redox Regulation in Mitochondria

1.2.3.1 Oxidation and Protein Folding

Mitochondria are organelles that function not only in energy production, but also apoptosis and iron-sulphur cluster assembly (Manganas et al., 2017). Due to their role within cells, mitochondria are a major source of reactive oxygen species (ROS) – for example when the mitochondrial respiratory chain produces hydrogen peroxide (H_2O_2) from the dismutation of the superoxide anion (O_2^-). ROS are involved in redox signalling when in low amounts but their production can also lead to mitochondrial dysfunction when in excess, resulting in the development of disease (Murphy, 2009). One such effect of ROS production is protein oxidation through the generation of disulphide bonds between cysteine residues (Morano et al., 2012). Oxidation allows cysteines to create intramolecular bonds to induce protein folding, or intermolecular bonds between different proteins as part of an interaction. Cysteines are therefore classified as either functional cysteines involved in the active sites of proteins, or essential structural cysteines maintaining the correct 3D shape of a protein (Chung et al., 2013) without a direct role in their function. Cysteines are less common within protein sequences than other amino acids. Together with other rare amino acids – tryptophan and methionine – they make up 5% of amino acids present in proteins, compared to more common amino acids, such as leucine, serine, lysine and glutamic acid, which together make up approximately 32% of all amino acids (Gaur, 2014; Lodish

et al., 2000). Cysteine residues themselves make up approximately only 2% of proteins (Hansen et al., 2013). Many cysteines are conserved across different eukaryotes, inferring that they have an important function within proteins (Riemer et al., 2011). This is because uncontrolled oxidation of cysteines can cause aberrant folding of native proteins, or indeed inactivation or modification of their function or regulation. It is therefore important for cells to have defence mechanisms against oxidation and the random formation of disulphide bonds.

Prevention or promotion of oxidative protein folding is ensured through compartmentalisation within cells, not only in mitochondria – specifically, the IMS – but also in the endoplasmic reticulum (ER) of eukaryotes and the periplasm of bacteria (Riemer et al., 2011). This comparative similarity between bacterial periplasm and the mitochondrial IMS is likely explained by endosymbiotic theory and the origins of mitochondria from prokaryotes (Sideris & Tokatlidis, 2010). Within the IMS, there are several examples of small proteins containing cysteine residues that can be affected by oxidation. These include substrates of the MIA pathway which contain cysteine motifs (CXnC), such as the small Tims, and oxidoreductases such as thioredoxin (Trx). The small Tims and Trx differ in that small Tims must be oxidised in the IMS in order to fold and function correctly, whereas the opposite is true for Trx (i.e. it must stay reduced for functionality). In this way, they represent a varied spectrum of proteins in the IMS which differ in their respective redox states.

1.2.3.2 Oxidative Folding in Mitochondria

The oxidative folding (or MIA) pathway of the mitochondrial IMS is centred round the oxidoreductase Mia40, which acts as a chaperone and a disulphide donor protein for imported precursors (Figure 6). The introduction of disulphide bonds is the catalytic event that induces their folding and traps them in the IMS (Sideris & Tokatlidis, 2010). Within *S. cerevisiae*, Mia40 is imported and inserted into the IM via TIM23 and bound to the membrane by its N-terminus, whereas the IMS-exposed C-terminus of Mia40 catalyses its reaction with protein precursors (Chatzi et al., 2013). MIA pathway substrates contain twin CXnC (n = 3 or 9) motifs that associate with the hydrophobic substrate binding cleft of Mia40. Following this association, the substrate interacts with a conserved CPC motif, the second cysteine of which forms the mixed disulphide intermediate with the substrate protein (Chatzi & Tokatlidis, 2013). Preproteins with CX3C motifs include small Tims such as Tim8, 9, 10, 12 and 13, which function as chaperone protein complexes that aid the movement of membrane proteins through the IMS (Sideris & Tokatlidis, 2010). CX9C motifs, on the other hand, made up around fifty-nine proteins in the *S. cerevisiae* genome, fourteen of which are shown to localise to mitochondria (Gabriel et al., 2007; Longen et al., 2009). Many of these proteins are involved in the assembly or stability of the mitochondrial respiratory chain (Chatzi & Tokatlidis, 2013).

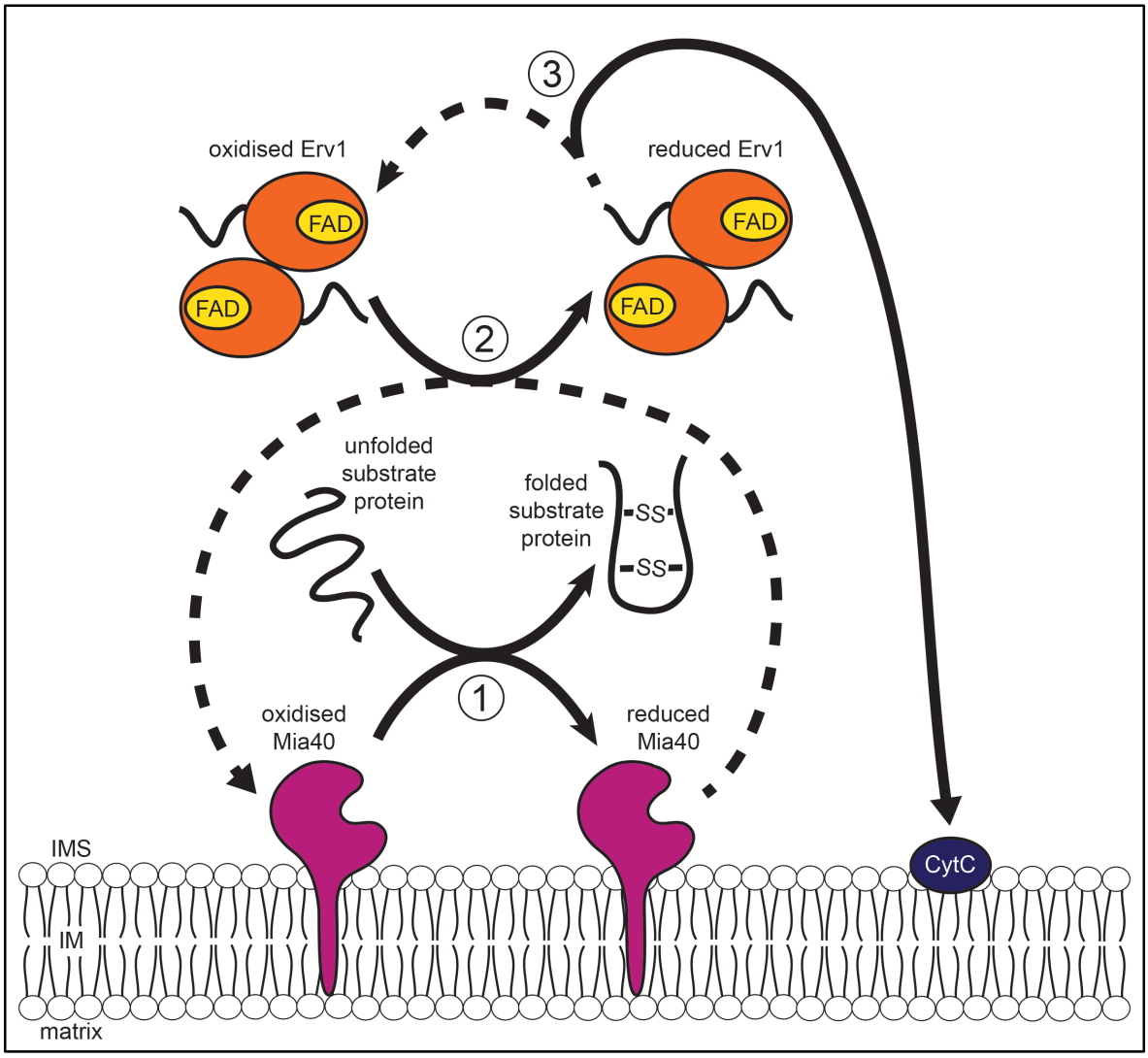


Figure 6 – The oxidative folding pathway in the mitochondrial intermembrane space (IMS) (adapted from MacPherson & Tokatlidis, 2017). Unfolded substrate proteins are folded by oxidised Mia40 (1), which becomes reduced as a result and is reoxidised through recycling by Erv1 (2). Erv1, which is reduced by this process, is then reoxidised by electron transfer to Cytochrome C (CytC) (3).

The incoming, reduced precursors interact with Mia40 by forming a mixed disulphide intermediate. Their release in the oxidised state results in the reduction of Mia40, the active site of which is recycled by Erv1, an essential, flavin adenine dinucleotide (FAD)-linked sulphydryl oxidase with no structural similarity or sequence homology to other Mia40 substrates (Chatzi & Tokatlidis, 2013). Erv1 has three conserved cysteine pairs (C30/C33, C130/C133 and C159/C176), the first of which acts as the shuttle disulphide interacting with Mia40 (Chatzi et al., 2013). The C-terminal cysteine pair is a structural disulphide, whereas the middle pair is involved in an electron transfer chain. Electrons flow from Erv1 to CytC and finally to molecular oxygen – both of which make up the final electron acceptors of the MIA pathway (Riemer et al., 2011; Sideris & Tokatlidis, 2010).

1.2.3.3 Redox Regulation in the IMS

Eukaryotic cells have developed further defence mechanisms against aberrant oxidative folding via the presence of peroxidase enzymes, redox proteins – such as glutaredoxins (Grxs) and Trxs – and the antioxidant glutathione (GSH) (Riemer et al., 2011). The mitochondrial IMS is unique in that oxidative folding of imported proteins occurs within it, as the IMS is a more oxidising environment than the cytosol (Sideris & Tokatlidis, 2010). It is not well understood how the maintenance of different redox states between mitochondrial subcompartments occurs. Studies by Kojer et al., however, have shown that GSH regulation is important in this process. Yeast strains lacking functional GSH are more susceptible to oxidative stress from O_2^- and peroxides (Grant, 2001). Kojer et al. noted that GSH diffuses freely between the IMS and cytosol through porin channels, thereby influencing the redox state of the IMS. This diffusion of GSH does not, however, occur between the IMS and the mitochondrial matrix, which maintains its own independent GSH levels and a more reducing environment than the IMS (Kojer et al., 2012). Similarly, by controlling the levels of Grxs – a reducing family of enzymes that use GSH as a cofactor – in the IMS, oxidative folding can occur in a reducing environment (Kojer et al., 2015).

Sulphydryl groups have been noted to play an important role in the oxidative stress response of cells through the Grx and Trx systems (Grant, 2001). Grxs and Trxs are small oxidoreductases which have structural similarity, both with active sites containing two, conserved cysteine residues. These cysteines are vital to the function of both Grx and Trx, which, although they share functional similarities, differ in their regulation. Grx, however, is recycled by GSH and oxidised GSH (GSSG) is then reduced again by electron transfer via GSH reductases (Glr) and NADPH. Grx reduction is therefore carried out indirectly by NADPH, unlike the Trx recycling system where oxidised Trx is reduced by Trx reductase (Trr) and NADPH directly (Holmgren, 1989; Trotter & Grant, 2002; Wheeler & Grant, 2004).

S. cerevisiae have 8 Grxs (Grx1-8) and one GSH reductase (Glr1). Grx1 and Grx2 are cytosolic and have a role in the cellular response to oxidative stress (Luikenhuis et al., 1998). Double deletion mutant strains in these genes are viable, though a single deletion in either Grx1 or Grx2 leaves yeast cells susceptible to particular ROS (O_2^- and H_2O_2 respectively) (Grant, 2001). Grx3, Grx4 and Grx5 are conserved in bacterial to mammalian species, and differ from most Grx in that they only have one cysteine residue at their active sites (Grant, 2001). Grx3 and Grx4 are involved in intracellular iron transport and localise in the nucleus, whereas Grx5 is involved in iron metabolism and mitochondrial iron cluster assembly (Mühlenhoff et al., 2010; Rodrıguez-Manzaneque et al., 2002). Grx6 and Grx7 are not well characterised by known to be present in both the ER and Golgi, and thought to be involved in the regulation of sulphydryl oxidation in these compartments (Izquierdo et al., 2008; Mesecke et al., 2008). Grx8 was identified as a Grx-like protein by Mesecke et al. in 2008, but a later study showed that is is likely not involved in defence against oxidative stress (Eckers et al., 2009).

There are 3 Trxs (Trx1, Trx2 and Trx3) in *S. cerevisiae* and 2 Trx reductases (Trr1 and Trr2). Two Trx pathways have been noted in yeast – the cytosolic pathway involving Trx1, Trx2 and Trr1, and the mitochondrial matrix Trx pathway with Trx3 and Trr2 (which may be involved in oxidative stress protection during respiration) (Miranda-Vizuete et al., 2000; Pedrajas et al., 1999; Trotter & Grant, 2005). The cytosolic Trx pathway is involved in the maintenance of proteins in a reduced state, whereas the matrix Trx pathway may be involved in oxidative stress

protection during respiration (Greetham et al., 2013). The redox states of these pathways are maintained independently (Trotter & Grant, 2005), likely due to their separate compartmentalisation. In 2012, the possibility of a third Trx pathway in the IMS arose due to the discovery of the presence of Trx1 and Trr1 in this compartment (Vögtle et al., 2012). Trx1 and Trr1 may therefore be involved in maintaining correct oxidative folding of proteins in the IMS alongside MIA pathway components.

Peroxidases include peroxiredoxins (Prxs) and glutathione peroxidases (Gpxs), of which Prxs – first discovered as peroxide recycling enzymes by Chae et al. – are better understood (H Z Chae & Rhee, 1994; H Z Chae et al., 1994). *S. cerevisiae* have three Gpxs – Gpx1, 2 and 3 – all of which are found in the cytosol, but are also associated with different mitochondrial subcompartments. Only the inactivation of Gpx3 – also associated with the IMS – leads to defective H_2O_2 tolerance (Inoue et al., 1999; Kritsiligkou et al., 2017; Vögtle et al., 2012). In the cytosol, Gpx3 acts as a redox sensor that interacts with the transcription factor Yap1 to activate oxidative stress response genes, such as Trx2 (Wood et al., 2004). The mechanism for this interaction relies on two of the three cysteine residues (C36, C64 and C82) of Gpx3. C36 of Gpx3 becomes sulphenylated by H_2O_2 and can then either form a mixed disulphide bond with C598 of Yap1, or an intramolecular bond with the resolving cysteine of Gpx3 (C82) (Delaunay et al., 2002). The intermolecular disulphide bond with Yap1 induces another Yap1 intramolecular disulphide bond. This results in a conformational change in protein that blocks its nuclear export signal (NES), leading to its accumulation in the nucleus and activation of stress response genes (Wood et al., 2004). Unlike its known function in the cytosol, there is currently no established role for Gpx3 within the mitochondrial IMS. It is also unknown how Gpx3 is targeted to the IMS, as the Tokatlidis lab has unpublished data showing that it does not require the MIA pathway (Tokatlidis, 2016). Gpx3 has been shown to be alternatively translated with an eighteen amino acid N-terminal extension under oxidative (H_2O_2) stress conditions (Gerashenko et al. 2012). Work from the Tokatlidis group has shown that both of these forms of Gpx3 are found in the IMS, and mitochondrial Gpx3 levels increased following treatment with H_2O_2. Gpx3 has also been shown to reoxidise Mia40, a protein involved in the oxidative folding of proteins in the IMS

(Kritsiligkou et al., 2017). As such, a possible hypothesis is that the N-terminal extension of Gpx3 improves its targeting into the IMS, where it is involved in an oxidative stress response to prevent H_2O_2 damage to proteins and aid in the correct functioning of the MIA pathway (Tokatlidis, 2016).

1.3 Focus of this Study

1.3.1 Study of PPI in Mitochondria

PPIs studying the different import pathways of mitochondrial proteins have traditionally been shown through pull down assays. The different subcompartments of mitochondria, however, mean that this approach is not always ideal. The study of IMS proteins is considered difficult as it is a constricted space with few proteins, and therefore much more material would be required to successfully carry out a successful pull down assay. IMS protein localisation has also been suggested by confocal GFP fluorescence in many studies, but this is not a reliable approach due to the large size of GFP (Table 1). The constrictive nature of the IMS also makes the GFP approach difficult, as an OM-localised protein in the cytosol or IMS could appear to co-localise based on GFP confocal microscopy. It is difficult to show that a given protein is indeed localised to the IMS and interacts with other proteins in the IMS without the creation of a stable intermediate for further study. This approach has been utilised in the Tokatlidis lab for Mia40 and its substrates by mutagenising the resolving cysteine and leaving the docking cysteine intact (Sideris & Tokatlidis, 2007). If a protein is imported into the IMS independently of Mia40, however, its localisation *in vivo* becomes more difficult to ascertain. One possible method of study would be to use a robust and reliable *in vivo* assay such as the split-DHFR PCA discussed previously.

1.3.2 Aims of the Project

Although the split-DHFR PCA approach (Figure 7) has been used in yeast before, it has only been used to examine the interactions of cytosolic proteins, and not mitochondrial proteins (Tarassov et al., 2008). Therefore, the aims of the project are to:

1. First, establish DHFR as a PCA technique (Figure 8) for use in proteins targeted to mitochondria, using control PPIs to verify that the assay can be used successfully. This approach could be used not only for IMS-targeted proteins, but also matrix proteins and even membrane proteins.
2. Then, use the split-DHFR approach to confirm any putative interactions of mitochondrial proteins, that have been investigated by other PPI assays (and functional protein interactions).
3. Further set up split-DHFR as a method of determining the route of import of mitochondrial proteins to the IMS (Figure 9).

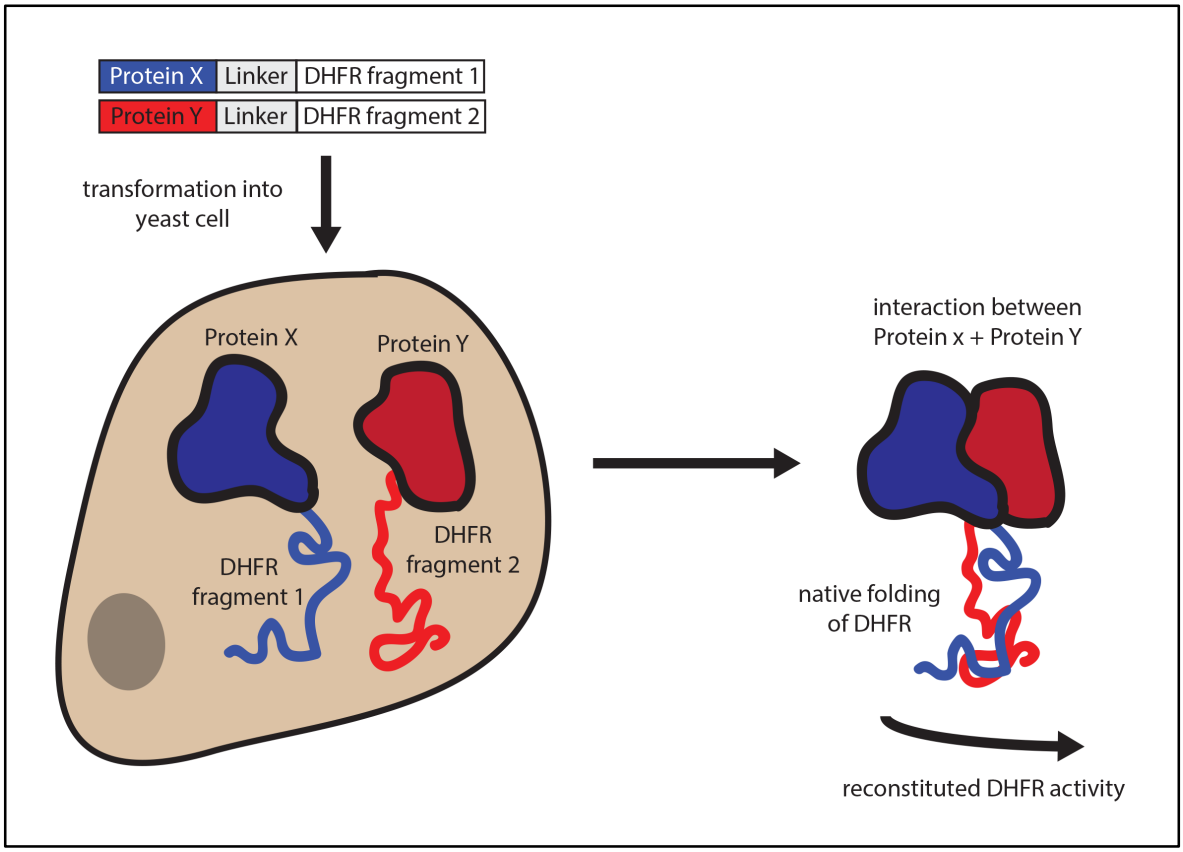


Figure 7 - A simplified version of the split-dihydrofolate reductase (DHFR) approach in yeast. Protein X and Protein Y are fused to the two split-DHFR fragments, respectively, and transformed into yeast cells. When Protein X and Protein Y interact, DHFR is reconstituted and the activity of the protein is recovered to allow for cell growth (adapted from Remy et al., 2007).

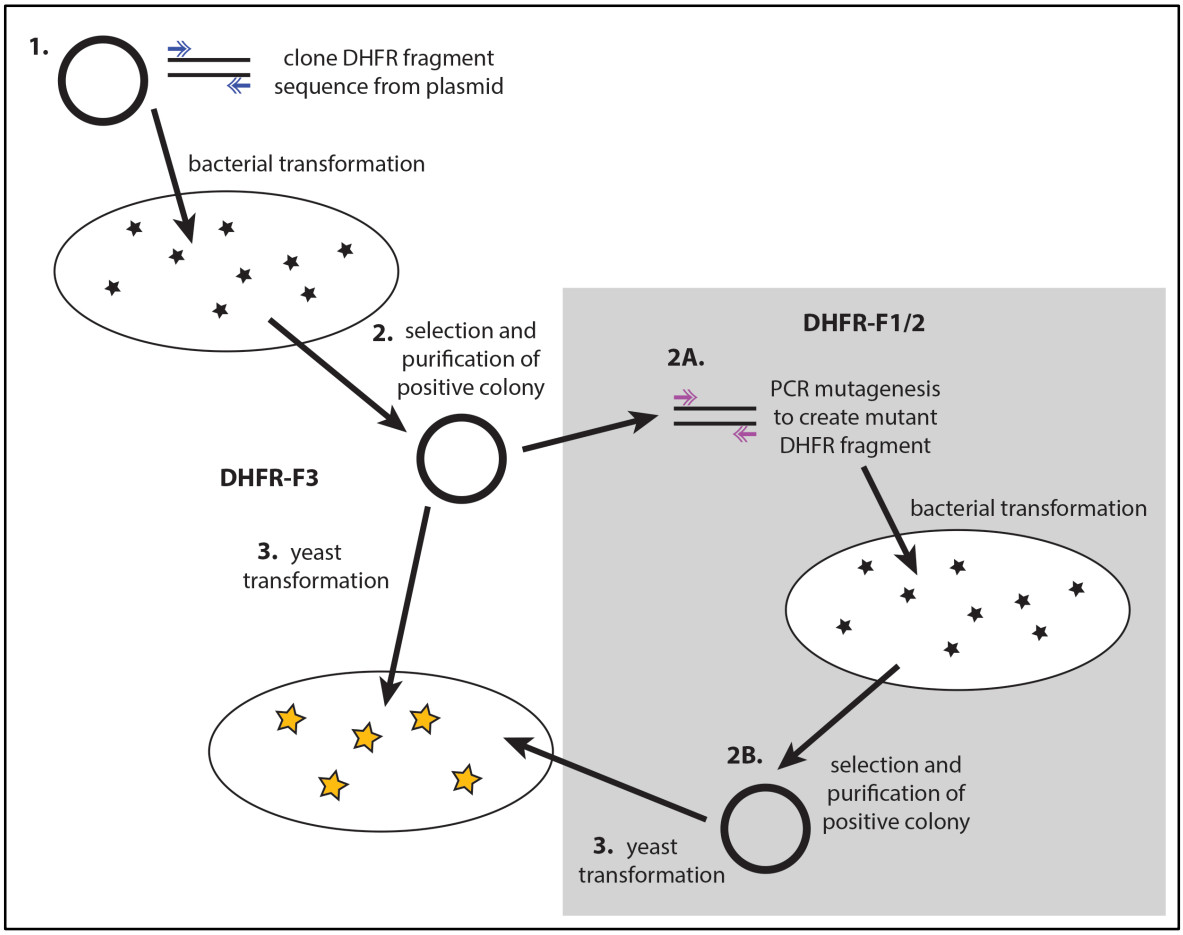


Figure 8 - The general approach for generating the split-dihydrofolate reductase (DHFR) fragments for use in this study. The DHFR fragment (either F1/2 or F3) is cloned from a plasmid containing the full murine DHFR sequence and transformed into bacterial cells (1). A positive colony containing the DHFR fragment are selected and purified (2). For DHFR-F1/2, PCR mutagenesis is carried out to generate a mutated form of DHFR-F1/2 (DHFR-F1/2mut) resistant to inhibition by methotrexate (2A). A positive colony containing DHFR-F1/2mut is then selected and purified (2B). Both DHFR fragments are then transformed into yeast for expression (3).

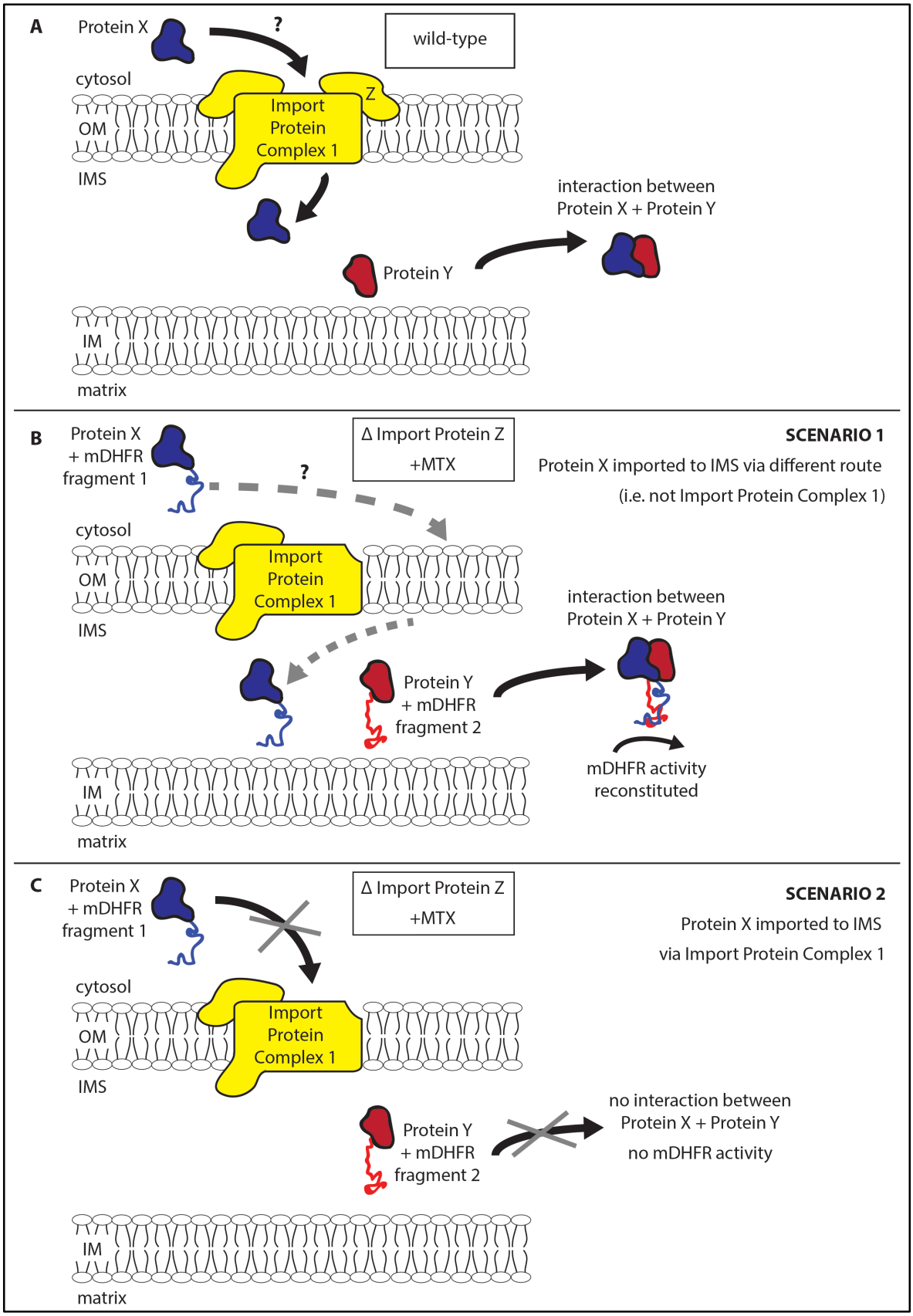


Figure 9 - Generalised example of using split-dihydrofolate reductase (DHFR) to determine the route of import of a protein into the intermembrane space (IMS). A: In wild-type yeast, Protein X is imported into the IMS (either by 'Import Protein Complex 1' or another route). In the IMS, Protein X and Protein Y interact. Two

potential scenarios can occur when this knowledge is applied to the split-DHFR assay. In this example, this is carried out using a deletion strain for a component of Import Protein Complex 1, Δ Import Protein Z, which prevents import of Protein X into the IMS. Protein X is fused to mutated murine DHFR (mDHFR) fragment 1, and Protein Y to mDHFR fragment 2 – able to form mDHFR resistant to inhibition by methotrexate (MTX). When these two fragments are able to form native mDHFR via the interaction of Protein X and Protein Y, mDHFR activity is reconstituted. This is carried out in the presence of MTX to inhibit endogenous DHFR. B: Protein X is not imported into the IMS by Protein Complex 1 but by another, unknown route. Its import is therefore not inhibited by the lack of functional Import Protein Complex 1, due to the deletion of its component, Protein Z. Protein X and Protein Y interact in the IMS and mDHFR activity occurs, allowing for cell survival in the presence of MTX. C: Protein X is imported via Import Protein Complex 1 and therefore is unable to be imported into the IMS. Protein X and Protein Y therefore do not interact and no mDHFR activity occurs as a result.

2. Materials and Methods

2.1 Transformation of Plasmids into *E. coli*

2.1.1 PCR Amplification

The primers designed were used for polymerase chain reactions (PCR) in the Biometra® T3 Thermocycler. The PCR reaction mixture of a single sample contained 2µl 10x buffer (New England BioLabs), 0.4µl dNTPs (Invitrogen), 0.5µl of each primer (10µM stock, Sigma), 11.4µl dH_2O, 5µl (10ng/µl) template DNA and 0.2µl Taq DNA polymerase (5000 units/ml, New England BioLabs) to make up a total volume of 20µl. One unit of Taq DNA polymerase is defined by New England BioLabs as 'the amount of enzyme that will incorporate 15 nmol of dNTP into acid-insoluble material in 30 minutes at 75°C'. All reaction mixtures were made up to 20µl excluding blanks with no Taq (10µl) and large-scale reactions (300µl). Conditions for the PCR thermocycle varied depending on the PCR reaction being carried out (Table 2).

Gene Amplified	Initial Denaturation	Denaturation	Primer Annealing	Extension	Cycle Length (trial/ large-scale)	Final Extension
DHFR-F1/2, DHFR-F3	1 minute 95°C	1 minute 95°C	1 minute 50°C	3 minutes 72°C	25 / 35	10 minutes 72°C
Tim9, Tim10	1 minute 95°C	1 minute 95°C	1 minute 58°C	30 seconds 72°C	25 / 35	10 minutes 72°C
Gpx3	1 minute 95°C	1 minute 95°C	1 minute 50°C	1 minute 72°C	25 / 35	10 minutes 72°C
Mia40, Yap1	1 minute 95°C	1 minute 95°C	1 minute 50°C	2 minutes 72°C	25 / 35	10 minutes 72°C

Table 2 - PCR thermocycle conditions. DHFR refers to dihydrofolate reductase.

2.1.2 DNA Gel Electrophoresis

The results of the PCR reactions were visualised using gel electrophoresis of 1% agarose made from 1x TAE buffer (40mM Tris pH 8.0, 20mM acetic acid, 1mM EDTA) and 0.5x SYBR®Safe DNA gel stain (Invitrogen). Each gel was ran at 60V for 50 minutes in 1x TAE buffer. A 1kb DNA ladder (Promega) was used to examine the relative sizes of the samples. The PCR products were cleaned using the Macherey-Nagel NucleoSpin® Gel and PCR Clean-up according to the manufacturer's instructions.

2.1.3 Cloning of Plasmids into *E. coli*

Digestion was carried out using 1x CutSmart™ buffer (New England Biolabs), 0.4u/µl of each restriction enzyme, approximately 40µl of PCR product (gene insert) or 3000ng of vector, 1x Bovine Serum Albumin (BSA; ThermoFisher Scientific) and dH_2O to make up to a total volume of 100µl for inserts and 50µl for vectors. Digestions were left at 37°C for 4 hours. Following digestion, the resulting samples were cleaned using NucleoSpin® Gel and PCR Clean-up according to the manufacturer's instructions.

Ligation of the digested products was carried out using 1x T4 DNA ligase buffer and T4 DNA ligase (400000 units/ml, New England Biolabs), 1mM ATP, plasmid vector, gene insert and dH_2O to give a total reaction volume of 10µl. One unit of T4 DNA ligase is defined by New England BioLabs as 'the amount of enzyme required to give 50% ligation of HindIII fragments of λ DNA (5´ DNA termini concentration of 0.12 µM, 300- µg/ml) in a total reaction volume of 20 µl in 30 minutes at 16°C in 1X T4 DNA Ligase Reaction Buffer'. Approximately 60ng of the vector was used for each reaction, but the concentration of insert used depended on the vector:insert ratio (with 60ng vector:22ng insert for 1:1). Ligation reactions were left for 2 hours RT, 1 hour 30 minutes at 25°C, 1 hour RT or 16°C overnight as specified.

The ligation mixtures were added to 100µl *E. coli* DH5α competent cells with transformation efficiency of 1 x 10^9cfu/µg pUC19 vector DNA (New England BioLabs) and left for 30 minutes on ice. The samples were then heat shocked for 45 seconds at 42°C and then put back on ice for a further 2 minutes. 900µl Luria-Bertani (LB) medium was added to each and left to incubate at 37°C for 1 hour. The cells were pelleted by 5 minutes of centrifugation at 15000g at room temperature (RT) and 700µl was removed. The pellet was resuspended in the remaining volume and plated on LB + antibiotic selection plates left to grow overnight at 37°C.

2.1.4 Colony PCR

Selected colonies were added to 50µl dH_2O and this was used as the DNA template for the PCR reaction, carried out for 20 cycles. Conditions for the PCR thermocycle varied depending on the PCR reaction being carried out as in Table 2.

2.1.5 *E. coli* DNA Extraction/Purification

E. coli DNA was extracted and purified using the QIAGEN QIAprep® Spin Miniprep Kit (250) according to the manufacturer's instructions. The DNA concentration of the samples was measured using NanoDrop® Spectrophotometer ND-1000 (Thermo).

2.1.6 Site-Directed Mutagenesis via PCR

The mutagenesis primers were designed for use in the Biometra® T3 Thermocycler. The PCR reaction mixture of a single sample contained 5µl 10x Accuzyme Buffer (Bioline), 1µl $MgCl_2$ (50mM), 2µl dNTPs (Invitrogen), 2µl of each primer (10µM stock, Sigma), 38µl dH_2O and 5µl (10ng/µl) DNA to make up a total volume of 50µl. 1µl Accuzyme Enzyme (250 units/100µl) Bioline) was then added, or omitted

altogether from control reactions. Conditions for the mutagenesis PCR thermocycle are shown in Table 3.

Gene Mutagenised	Initial Denaturation	Denaturation	Primer Annealing	Extension	Cycle Length	Final Extension
DHFR-F1/2	2 minutes 95°C	1 minute 95°C	1 minute 60°C	8 minutes 72°C	25	10 minutes 72°C

Table 3 - Mutagenesis PCR thermocycle conditions. DHFR-F1/2 refers to dihydrofolate reductase fragment 1.

The mutagenesis PCR products were digested by the addition of 1µl DpnI restriction enzyme (10 units/µl) and 5µl Buffer B (Promega) to the reaction mix. This was followed by incubation at 37°C for an hour and a half. Following digestion, this reaction mixture was transformed into *E. coli* DH5α competent cells as described previously.

2.2 Transformation of Plasmids into *S. cerevisiae*

2.2.1 *S. cerevisiae* Genomic DNA Extraction/Purification

3ml from an overnight culture of *S. cerevisiae* cells was pelleted by centrifugation at 15000g (RT) for five minutes, followed by washing with 500µl dH_2O and centrifugation at 15000g (RT) for a further 5 minutes. The pellet was vortexed for 4 minutes with 200µl lysis buffer (2% Triton X-100 (v/v), 1% SDS (v/v), 100mM NaCl, 10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0), 200µl glass beads and 200µl phenol-chloroform. 200µl dH_2O was added and the mixture was pelleted again by centrifugation at 15000g (RT) for 5 minutes. The supernatant was then transferred to a new tube and 1ml of ice-cold ethanol was added to it and mixed by inversion. The mixture was pelleted by centrifugation at 15000g (RT) for 2 minutes and the resulting pellet left to dry at RT. 400µl dH_2O and 60µg RNase A was added and the mixture incubated at 37°C for 10 minutes, followed by the addition of 400mM

ammonium acetate. 1ml ethanol was added and mixed by inversion, and left to incubate at -20°C for 20 minutes. The mixture was then pelleted by centrifugation at 15000g, 4°C for 15 minutes. The pellet was left to dry and then resuspended in 50µl dH_2O. The DNA concentration of the samples was measured using NanoDrop® Spectrophotometer ND-1000 (Thermo).

2.2.2 *S. cerevisiae* Transformation

Transformation of plasmids into *S. cerevisiae* cells was carried out as in the LiAc/SS-DNA/PEG Transformation method (Gietz & Schiestl, 1995), using carrier DNA instead of SS-DNA.

2.2.3 Growth Curves

S. cerevisiae cells were grown in 5ml overnight cultures of SD –uracil/-leucine. OD (Optical Density) was measured at 0 hours and then diluted to OD 0.8 in 5ml of the same media with the addition of 200µg/ml MTX. OD was measured after 1 hour and 6 hours before being further supplemented with 100µg/ml MTX and left overnight (approximately 21 hours). The OD of each culture was then measured at 24 hour intervals and either diluted into OD 0.2 in 5ml SD-ura-leu, or left to grow as indicated.

2.2.4 Spot Tests

OD 0.5 (10^7 cells/ml) *S. cerevisiae* cells were pelleted by centrifugation at 15000g (RT) for five minutes and then resuspended in 500µl dH_2O. Four serial dilutions of 50µl into 450µl dH_20 were carried out to give 10^6 cells/ml, 10^5 cells/ml, 10^4 cells/ml and 10^3 cells/ml. 5µl from each of these was then spotted onto a SD-ura-leu plate with 200µg/ml MTX added, to give 10^4 cells/ml, 10^3 cells/ml, 10^2 cells/ml and 10 cells/ml, respectively.

Results

3.1 Cloning of DHFR Fragments

To create the initial DHFR fragments, murine DHFR (Appendix Figure 1) from the pSP65-Su9-DHFR vector (created by E. Kallergi) was amplified via PCR (Figure 10). Primers for the first fragment (F1/2) were designed for its insertion into the pRS316 vector, and the second fragment (F3) for insertion into pRS415 (Table 4). These vectors, pRS316 and pRS415 (Appendix Figures 2 and 3), contain ampicillin resistance genes and also genes expressing either uracil (URA3) or leucine (LEU2), respectively. As seen in Figure x, each of the DHFR fragments gave expected band sizes of approximately 318bp (F1/2) and 243bp (F3) respectively.

Restriction sites were chosen to leave multiple, upstream cut sites for later insertion of proteins of interest to be linked to the DHFR fragments, with a C-terminal orientation of the fragments (Figure 11) as this has been shown to be the most efficient orientation for interaction (Remy et al., 2007).

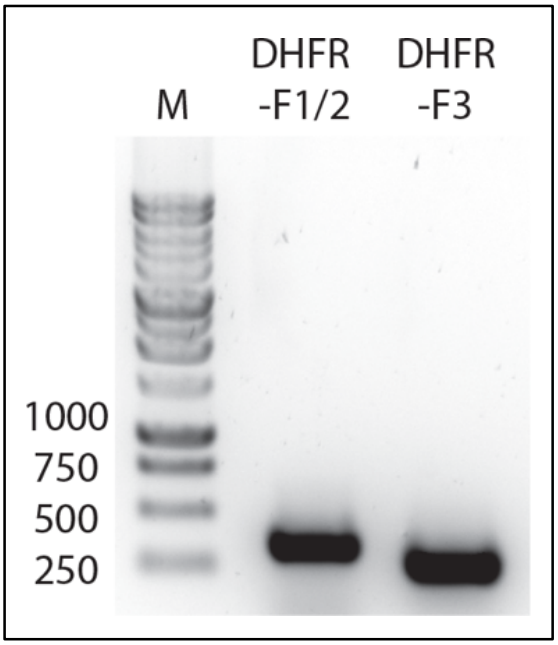


Figure 10 - PCR amplification of the dihydrofolate reductase (DHFR) gene fragments. DHFR-F1/2 (fragment 1) and DHFR-F3 (fragment 2) sequences compared to a 1kb DNA ladder (M) displaying bp size.

Gene	Vector	Insert Size (bp)	Restriction Sites
DHFR-F1/2	pRS316	318	XhoI / KpnI
DHFR-F3	pRS415	243	BamHI / XbaI

Table 4 - Vector, insert size and restriction sites used for the dihydrofolate reductase (DHFR) fragments. F1/2 refers to DHFR fragment 1 and F3 to DHFR fragment 2.

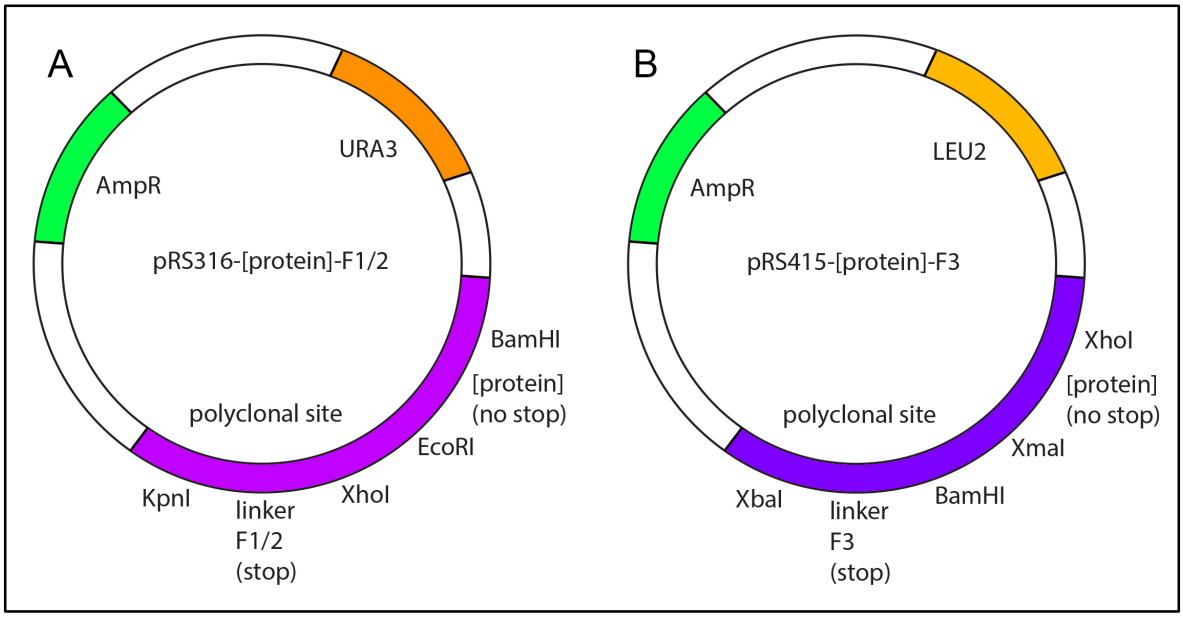


Figure 11 - Design of the dihydrofolate reductase (DHFR)-fragment containing plasmids. A: The first half of DHFR (F1/2) was inserted into the polyclonal site of pRS316 (purple), with an upstream protein of interest inserted for C-terminal expression of F1/2. pRS316 also contains ampicillin resistance (AmpR, green) and an uracil marker (URA3; orange) for growth on selective media. B: The second half of DHFR (F3) was inserted into the polyclonal site of pRS415 (purple) as in (A). pRS415 also contains AmpR (green) and a leucine marker (LEU2; orange) for growth on selective media. Different selection markers for growth on yeast media (URA3 and LEU2) were selected to allow the transformation of both plasmids into a single yeast strain for simultaneous expression of the two DHFR fragments.

The site for the division of full-length DHFR into two fragments was selected based on the work of Pelletier et al., who showed that residues 101-108 form a disordered loop which can be disrupted with little effect on the activity of the reconstituted DHFR (Pelletier et al., 1998). As well as cut sites for the relevant restriction enzymes, a linker amino acid sequence was also inserted to allow the DHFR fragments the most flexibility in both finding one another and folding correctly (Tarassov et al., 2008) when transformed into cells (Figure 7).

These DHFR fragment containing vectors were cloned into *E. coli* cells and positives were selected for via ampicillin plates, colony PCR and sequencing of purified DNA. Positive clones were then transformed into wild-type BY4741 *S. cerevisiae* cells – a yeast deletion strain lacking genes for histidine (HIS3), leucine (LEU2), methionine (MET15) and uracil (URA3) (Brachmann et al., 1998). Positive colonies were selected for using SD minimal media and auxotrophic selection for either uracil (pRS316) or leucine (pRS415). Three resulting yeast strains were created:

1. BY4741 pRS316-F1/2
2. BY4741 pRS415-F3
3. BY4741 pRS316-F1/2 + pRS415-F3

3.2 Generation of MTX-resistant DHFR Fragments

DHFR-F1/2 was mutated via sequential, site-directed PCR mutagenesis of the pRS316-F1/2 plasmid in order to create a MTX-resistant form of DHFR when the two fragments are reconstituted. The two sites mutated – L22F and F31S – were chosen based on the work of Tarassov et al., where they have been shown to create DHFR 10,000 times less sensitive to MTX than the wild-type (Tarassov et al., 2008). This mutant F1/2 (F1/2mut) was cloned into *E. coli* and transformed into *S. cerevisiae* cells as with the original F1/2. Two more yeast strains were created as a result:

1. BY4741 pRS316-F1/2mut
2. BY4741 pRS316-F1/2mut + pRS415-F3

3.3 Cloning of Control Proteins

In order to test the functionality of the split-DHFR assay created, a series of control proteins were selected to be fused N-terminally to the respective DHFR fragments (Table 5). Mia40 and its substrate Tim10 were selected as positive controls to show a transient interaction between two proteins known to interact in the IMS. Tim9 and Tim10, which form the TIM9/10 complex, were also chosen as another set of positive controls, as they demonstrate a more stable interaction. Mia40 and Yap1, which do not interact directly, were chosen as negative controls. Gpx3, being dually localised in the cytosol and IMS, was selected as a model protein to show that the split-DHFR assay could potentially be used to identify the localisation of proteins difficult to determine through other assays. The genes of these proteins were amplified via PCR (Figure 12), giving expected band sizes (as described in Table 6) for each of the genes. They were then cut with restriction enzymes to allow them to be inserted into either the pRS316-F1/2, pRS316-F1/2mut or pRS415-F3 plasmid (Table 6).

1st Interactor	2nd Interactor	Control Description
Mia40-F1/2mut	Tim10-F3	Positive *(transient interaction)*
Tim9-F1/2mut	Tim10-F3	Positive *(stable interaction)*
Mia40-F1/2mut	Yap1-F3	Negative *(non-interactors)*
Mia40-F1/2mut	-	Interaction specificity *(fragment does not interact with itself)*
Mia40-F1/2mut	F3	Interaction specificity *(DHFR reconstitution due to protein interaction)*
Mia40-F1/2	Tim10-F3	MTX selection *(mutation necessary for MTX resistance)*
-	-	Empty vector *(DHFR fragments necessary for MTX resistance)*
Mia40-F1/2mut	Gpx3-F3	Test interaction *(putative interacting proteins)*

Table 5 - Control interactions to determine the functionality of the split-DHFR assay. Mitochondrial proteins (Mia40, Tim9, Tim10, Yap1, Gpx3) are fused to either the first half of dihydrofolate reductase (DHFR; F1/2), a mutated form of F1/2 which is resistant to inhibition by methotrexate (MTX; F1/2mut) or the second half of DHFR (F3). A plasmid containing one fusion protein linked to either F1/2 or F1/2mut can be expressed alongside another plasmid with a fusion protein linked to F3, allowing for expression of both halves of DHFR in the same system.

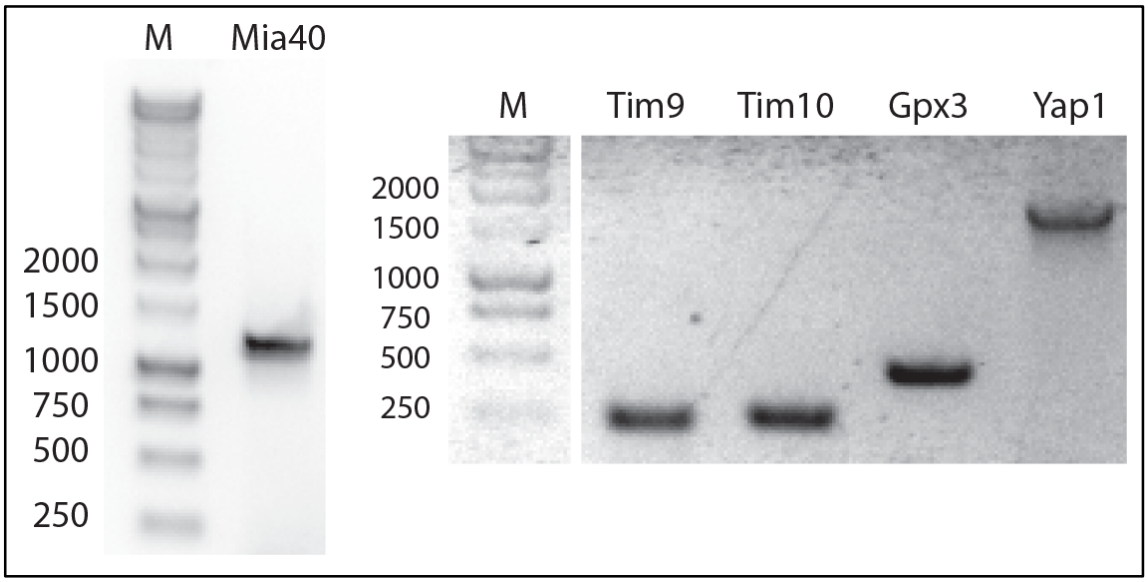


Figure 12 - PCR amplification of genes to be inserted N-terminally to the dihydrofolate reductase (DHFR) fragment plasmids. Sequences for Mia40, Tim9, Tim10, Gpx3 and Yap1 fragments compared to a 1kb DNA ladder (M) displaying bp size.

Gene	Vector	Insert Size (bp)	Restriction Sites
Mia40	pRS316-F1/2mut	1212	BamHI / EcoRI
Tim9	pRS316-F1/2mut	264	BamHI / EcoRI
Tim10	pRS415-F3	282	XhoI / XmaI
Gpx3	pRS415-F3	492	XhoI / XmaI
Yap1	pRS415-F3	1953	XhoI / XmaI

Table 6 - Vector, insert size and restriction sites used for the genes to be inserted N-terminally to the dihydrofolate reductase (DHFR) fragments. F1/2 refers to DHFR fragment 1 and F3 to DHFR fragment 2.

These control proteins were also cloned into *E. coli* cells and selected for via ampicillin plates, colony PCR and sequencing of purified DNA. Positive clones were obtained for both Tim9 (Figure 13) and Tim 10 (Figure 14), giving expected band sizes of 264bp and 282bp respectively. These positive clones were then transformed into wild-type BY4741 *S. cerevisiae* cells, with positive colonies selected for using SD minimal media and auxotrophic selection for either uracil (pRS316-F1/2mut) or leucine (pRS415-F3). This resulted in the creation of three more yeast strains:

1. BY4741 pRS316-Tim9-F1/2mut
2. BY4741 pRS415-Tim10-F3
3. BY4741 pRS316-Tim9-F1/2mut + pRS415-Tim10-F3

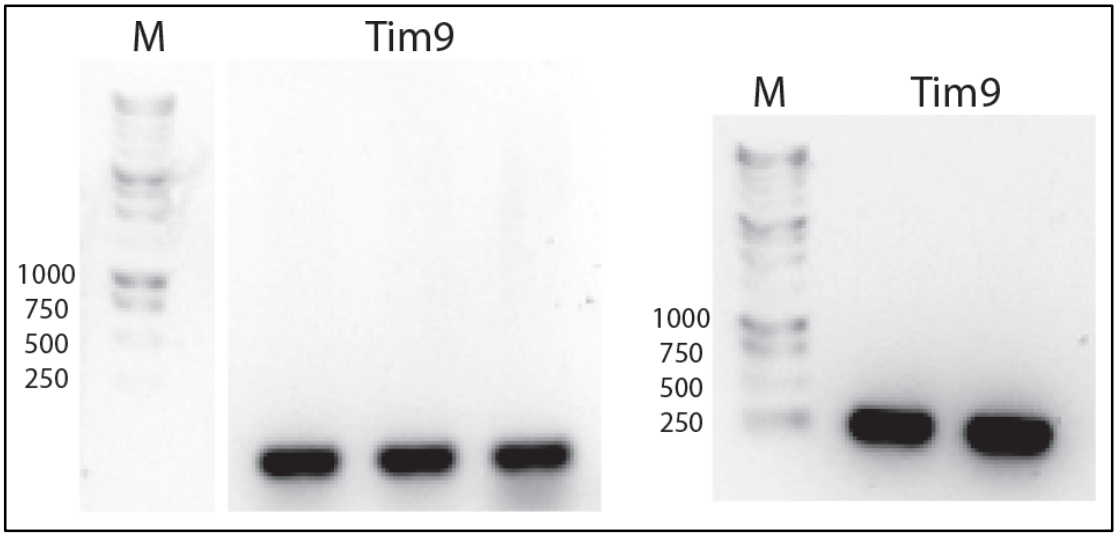


Figure 13 - PCR amplification of the Tim9 gene. Tim9 sequences compared to a 1kb DNA ladder (M) displaying bp size.

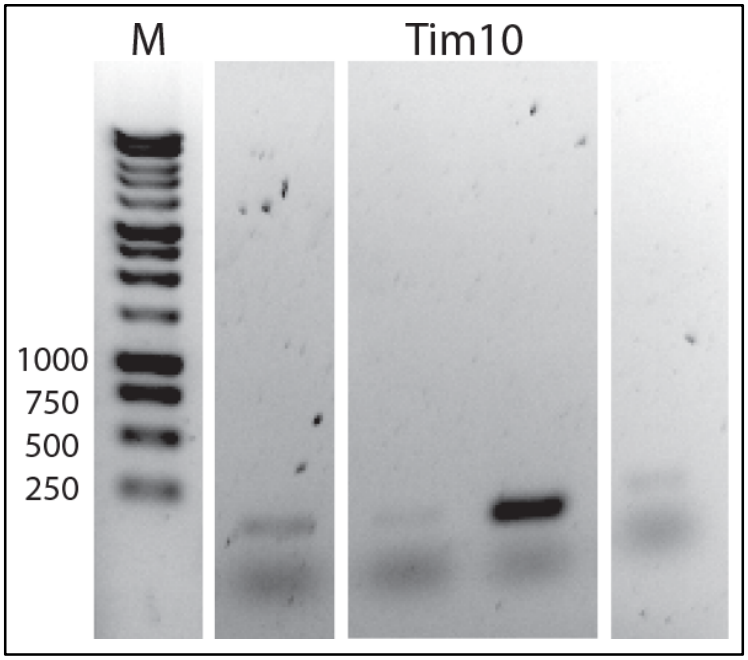


Figure 14 - PCR amplification of the Tim10 gene. Tim10 fragment sequences compared to a 1kb DNA ladder (M) displaying bp size.

3.4 Growth in the Presence of MTX

The BY4741 strain containing both Tim9-F1/2mut and Tim10-F3 was grown for several days in the presence of MTX, in order to assess if a positive interaction of the F1/2mut and F3 fragments would occur and reconstitute the DHFR protein. No significant difference in growth was observed between this strain and the negative control strains (Tim9-F1/2mut and F3, F1/2mut and F3, F1/2 and F3) (Appendix Table 1). The same strain was also used for spot tests on MTX plates alongside the negative controls strains, but no significant difference in growth was observed (data not shown).

Discussion

4.1 Approach

PPIs can be studied via PCAs utilising two, complementary fragments of reporter proteins fused to potentially interacting proteins, which give an indicative signal if PPI occurs. Many such PCAs approaches exist (such as those in Table 1), and have therefore been used to study a wide variety of PPIs. This approach, however, has not previously been used to study PPIs within mitochondrial subcompartments, such as the IMS – which was the focus of this study. Using GFP fusions to localise proteins to the IMS and confocal microscopy to analyse these interactions is not always straightforward, due to potentially poor folding of GFP. DHFR is known to fold in the IMS when an entire DHFR protein is fused to a protein of interest, but has not been shown to fold when split and linked to two different proteins.

To generate a split-DHFR assay for use in analysing mitochondrial protein-protein interactions in *S. cerevisiae*, the approach was similar to that of Tarassov et al., in that a MTX-resistant version of the split-DHFR protein was generated for use in yeast cells. Using MAT strains and homologous integration of the DHFR fragments was more advantageous in Tarassov et al.'s approach as it was a high throughput assay to screen for generic protein interactions (Tarassov et al., 2008). Constructs integrated directly into the genome are more stable, however, it would have been difficult to carry out the successful homologous recombination of multiple mitochondrial proteins fused to DHFR fragments into the yeast genome during the time available. Unlike Tarassov et al.'s approach, plasmids were generated containing multiple restriction sites before the DHFR fragments, so that the split-DHFR assay could be prepared for any protein. This was done using yeast expression vectors (pRS316 and pRS415, shown in Appendix Figures 2 and 3). The plasmid constructs were assured to be in-frame by DNA sequencing using a T3 promoter for pRS316 (Appendix Figure 2) and a T7 promoter for pRS415 (Appendix Figure 3), which was used to drive expression of the split-DHFR fusion protein for each of the control interactor proteins (Table 5).

The approach taken in this study was unique in that split-DHFR has never been used to assess the interaction of proteins imported into the yeast mitochondrial IMS before. Although this approach allowed for greater flexibility in application of the split-DHFR approach in the time given, as plasmid constructs are less reliable, integration into the genome could be considered later after the split-DHFR assay has been established and proteins have been shown to be properly targeted to the IMS. For this reason, specific mitochondrial proteins known to either interact with one another or not were used as positive and negative controls respectively (Table 5) to assess the validity of the approach for use in further experiments. A test set of mitochondrial pairs could have also been tested in one of the established systems for the split-DHFR assay in yeast, as working with a previously established assay would have given 'true' positive controls. As the approach by Tarassov et al. used MAT strains, however, this would have been difficult to test in the given timeframe as this assay was not set up for mitochondrial proteins (Tarassov et al., 2008).

Currently, eight *S. cerevisiae* strains containing one or more of the DHFR fragments have been produced (Table 7). As mentioned previously, the pRS316-F1/2, pRS316-F1/2mut and pRS415-F3 vectors contain multiple cut sites prior to the C-terminal DHFR fragment, and so can be used for N-terminal insertion of various proteins. Of the five mitochondrial proteins chosen to initially set up the split-DHFR assay – Mia40, Tim9, Tim10, Gpx3 and Yap1 – only vectors containing Tim9 and Tim10 were successfully cloned (Appendix Figures 7 and 8).

F1/2 Fragment (pRS316)	F3 Fragment (pRS415)
F/2	-
-	F3
F1/2	F3
F1/2mut	-
F1/2mut	F3
Tim9-F1/2mut	-
-	Tim10-F3
Tim9-F1/2mut	Tim10-F3

Table 7 – BY4741 S. cerevisiae *strains produced containing either the F/1 (or F1/2mut) dihydrofolate reductase (DHFR) fragment, the F3 DHFR fragment or both. F1/2 refers to DHFR fragment 1 and F3 to DHFR fragment 2.*

As Tim9 and Tim10 are known to interact and localise to the IMS, the *S. cerevisiae* strain containing both vectors was used as a positive control for the assay. Specific targeting sequences – such as Cytochrome b2 (Cytb2) – that localise to the IMS could also have been used to further ensure correction localisation of the fusion proteins, though this will be discussed later. The Tim9-F1/2mut+Tim10-F3 strain was grown both as yeast cell cultures containing MTX and as spot-tests on MTX-containing plates, though neither of these experiments gave the expected results in terms of cell growth. The growth of the Tim9-F1/2mut+Tim10-F3 strain was expected to be better in the presence of MTX than that of the F1/2+F3 strain it was compared to, but this was not observed (Appendix Table 1). The concentration of MTX used for these growth experiments was 200μg/ml, as was used by Tarassov et al., and yeast cell cultures were grown for at least 4 days in the presence of MTX as in their approach (Tarassov et al., 2008). As the approach taken in this study was different to that of Tarassov et al. and used yeast expression vectors, it is possible that the concentration of MTX must be adjusted for the split-DHFR assay to be successful. A range of different concentrations of MTX could be used to establish an upper limit at which neither the Tim9-F1/2mut+Tim10-F3 strain or the F1/2+F3 strain are able to grow in the presence of MTX, as well as a lower limit at which both strains are able to grow. Further yeast culture growth experiments carried out in this way would allow optimisation of the concentration of MTX required for growth of strains containing the MTX-

resistant split-DHFR plasmids. Similarly, as the focus was on the use of the split-DHFR assay for IMS proteins, it is possible that the assay may not be useable for such proteins due to incorrect localisation of, for example, Tim9 or Tim10, due to the addition of the C-terminal DHFR fragment.

4.2 Problems

Several setbacks were faced when setting up the split-DHFR assay, and not all of the work could be completed due to time constraints. Mutation of the DHFR-F1/2 fragment to produce the F1/2mut plasmid was delayed due to a random, single-point mutation in the F1/2 fragment that inserted a stop codon (TAG) into the sequence (Appendix Figure 4) by mutation of thymine to adenine at position 108. This was corrected using mutagenesis PCR primers, as was initially used to generate the mutated form of F1/2 (Appendix Figure 5). Similarly, a problem was found in that the pRS415 vector used could not be sequenced correctly, and so a new, empty pRS415 vector (Appendix Figure 3) had to be ordered. This caused a severe delay in experimental work, as the pRS415 plasmid had to be cloned again from the initial stages and also re-transformed into BY4741 strains for use in later experiments. Although Tim9 and Tim10 were the only two mitochondrial proteins which were successfully cloned into DHFR fragment vectors, Gpx3 (which has a dual localisation in the cytosol and the IMS) was also cloned and sequenced (Appendix Figure 9). Gpx3, however, had three single-point mutations at the beginning of its sequence (positions 35, 50 and 124 in Appendix Figure 9) and so was deemed unusable for further experimentation at this stage. If more time had been available, it is likely that Gpx3 would have been either successfully re-cloned or that these mutations would have been corrected by mutagenesis PCR. The initial PCR to create the initial Mia40 insert proved difficult and took several attempts. It was thought that the lack of success in creating the Mia40 and Yap1 clones may have been due to the size of the insert in comparison with Tim9, Tim10 and Gpx3, and that further work would be needed to clone these two proteins successfully.

4.3 Future Experimental Work

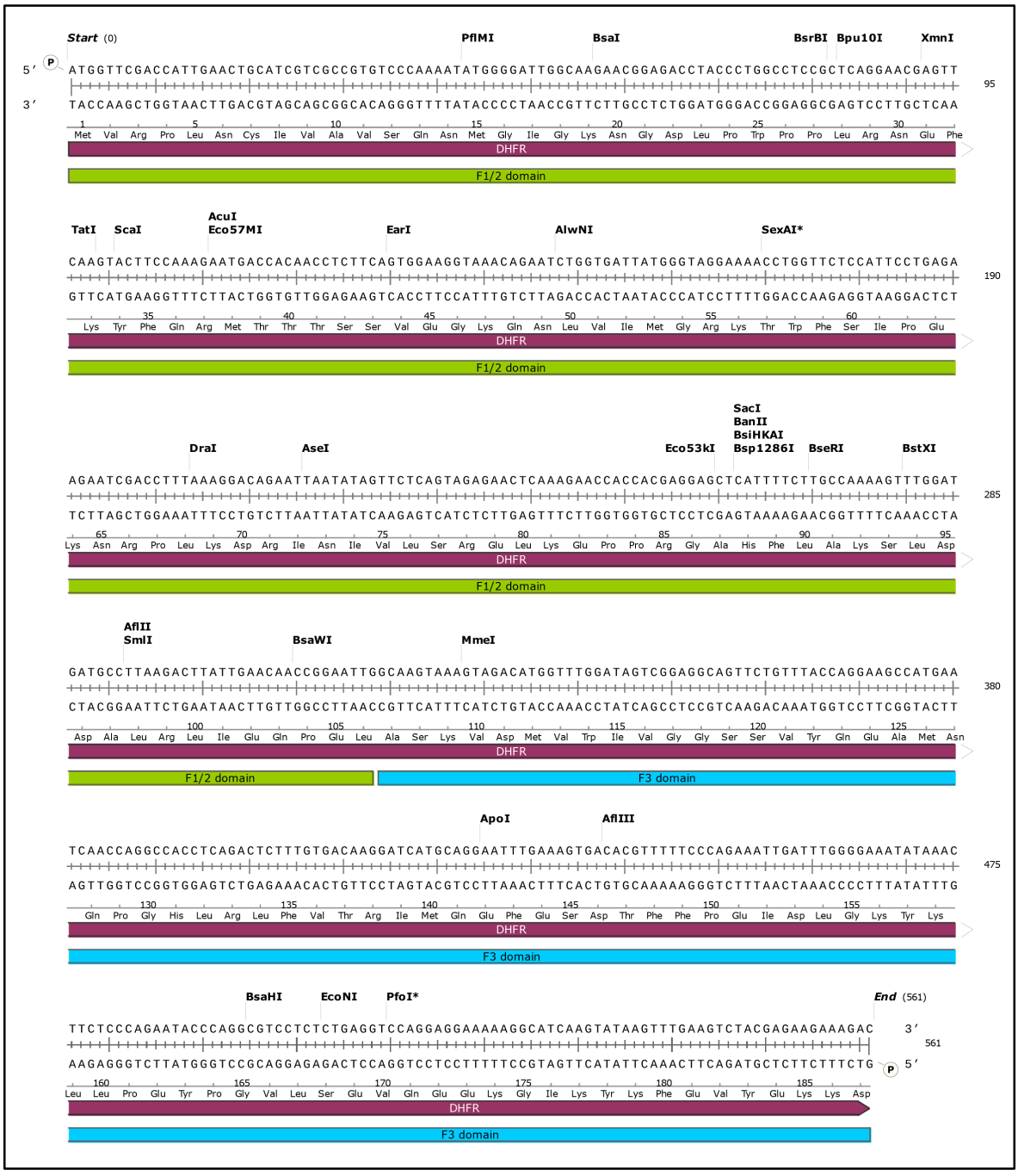
Ideally, the next step in experimental work would be to finish cloning Mia40, Gpx3 and Yap1 into DHFR fragment yeast expression vectors, and then transform these into BY4741 *S. cerevisiae* strains as with Tim9 and Tim10. This would allow for the split-DHFR assay to be set up fully with known interacting and non-interacting proteins before its use in the study of putative PPIs. The exact MTX conditions required for the growth of yeast cells containing split-DHFR assay must also be set up, as mentioned previously. The DHFR fragments could then be used alongside other, *in vitro* techniques such as AP/MS to study the PPIs of mitochondrial proteins in a native cellular context.

In the case of Tim9-F1/2mut+Tim10-F3, mitochondrial protein extracts could be taken from yeast cells expressing both plasmids and ran on a denaturing protein gel. Using antibodies for DHFR and/or Tim9 or Tim10 (which are available in the lab) would allow observation of whether the correct DHFR+Tim9Tim10 complex is present, as each TIM9/10 complex contains a heterohexamer of Tim9 and Tim10 (Webb et al., 2006), and therefore 3 complete DHFR proteins.

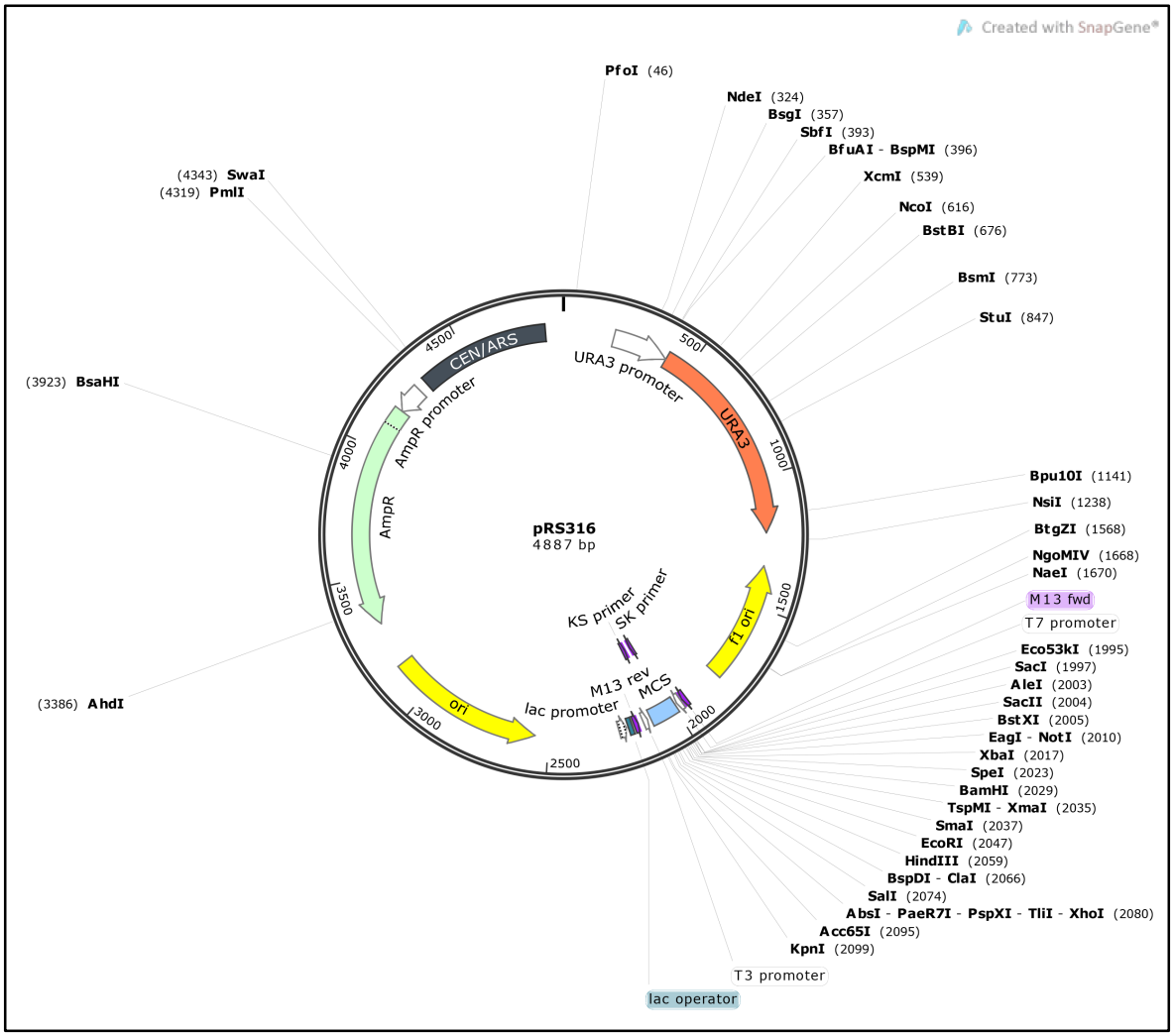
If the split-DHFR assay is shown to be functional, the same approach could then be used to determine the route of entry for proteins that interact in the IMS (Figure 9). This would be carried out by fusing two, interacting proteins of interest to each of the constructed DHFR fragments, and expressing these fragments in deletion yeast strains. Each knockout (K.O.) yeast strain would have a deletion of a gene expressing a protein involved in mitochondrial protein import (e.g. Tom70 of the TOM complex). If the two DHFR-fused proteins are able to enter the IMS and interact, the yeast cells will grow. If they are unable to grow in a particular deletion strain, this means that the deleted gene was somehow involved in the import of the protein. This would allow for protein import components or chaperones involved in this process to be discovered. This could be carried out, for example, with Trr1-F1/2mut and Trx1-Cytb2-F3 in a strain with no native Trx1. The Cytb2 targeting sequence would anchor the Trx1 to the IM, ensuring that Trx1 would definitely be present in the IMS. This would allow the import of Trr1 to be

assessed, as reconstitution of the DHFR fragments would depend on its import to the IMS and interaction with Trx.

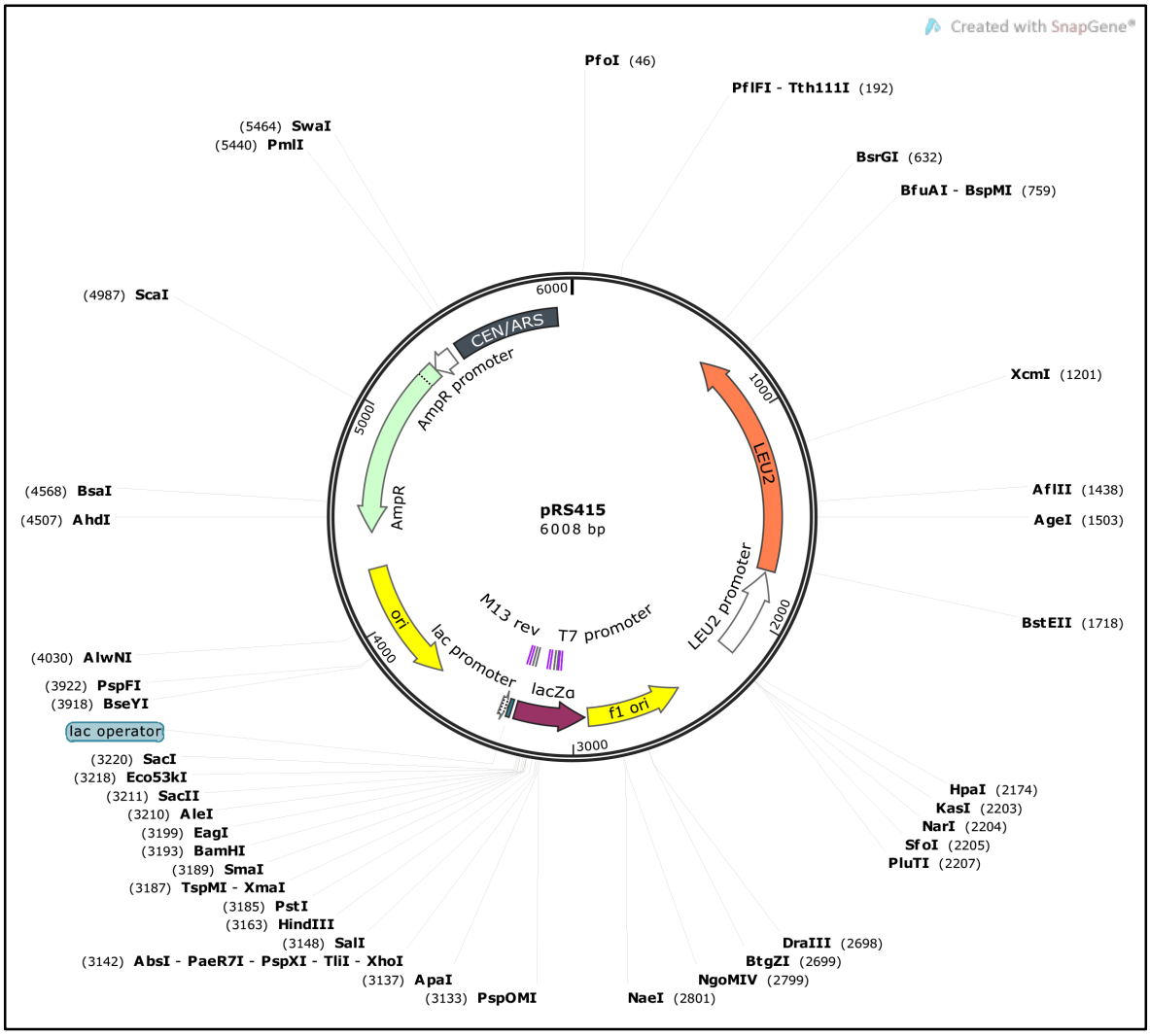
Appendix



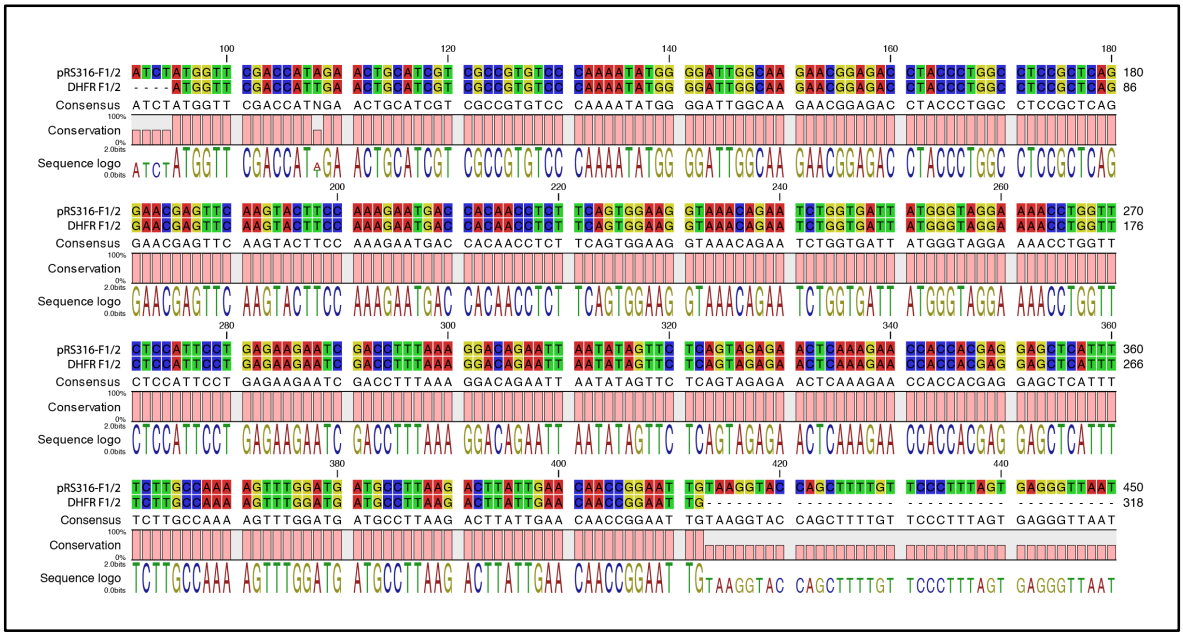
Appendix Figure 1 – Sequence of murine dihydrofolate reductase (mDHFR) used, showing the F1/2 (green) and F3 (blue) domains, created using SnapGene.



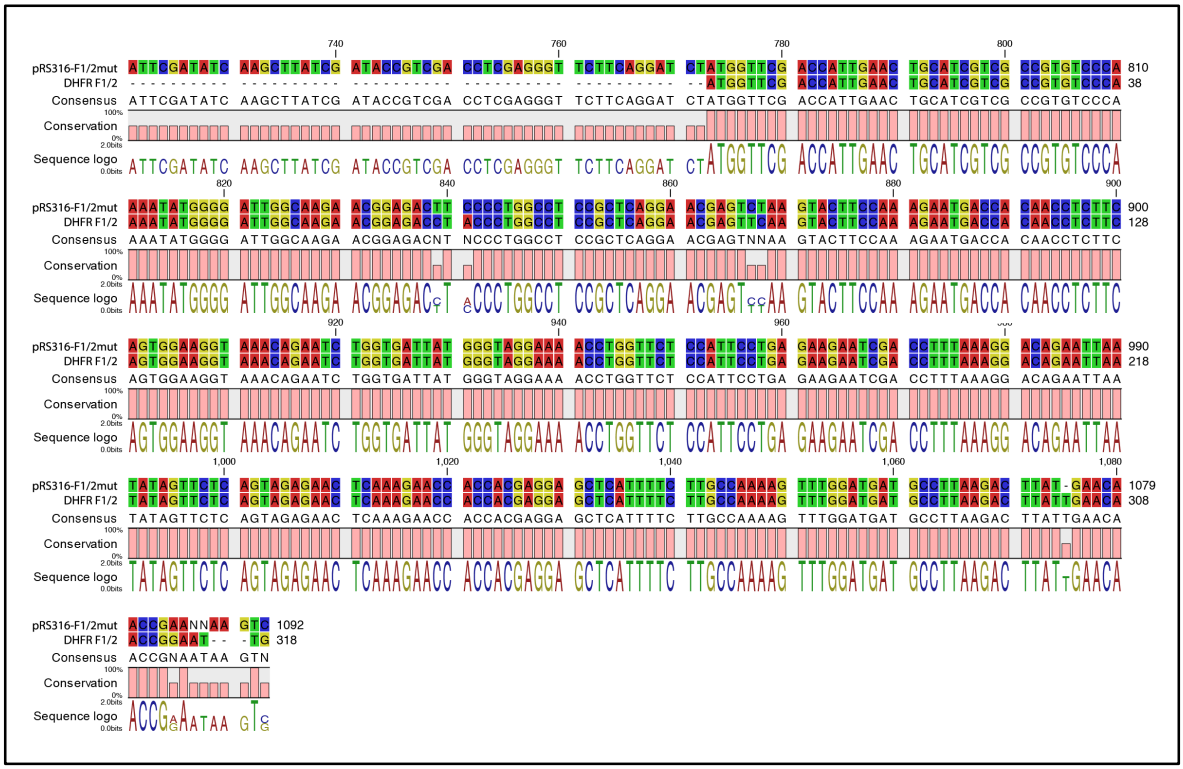
Appendix Figure 2 – pRS316 plasmid, created using SnapGene. Contains ampicillin resistance (AmpR; green) and an uracil marker (URA3; orange) for growth on selective media.



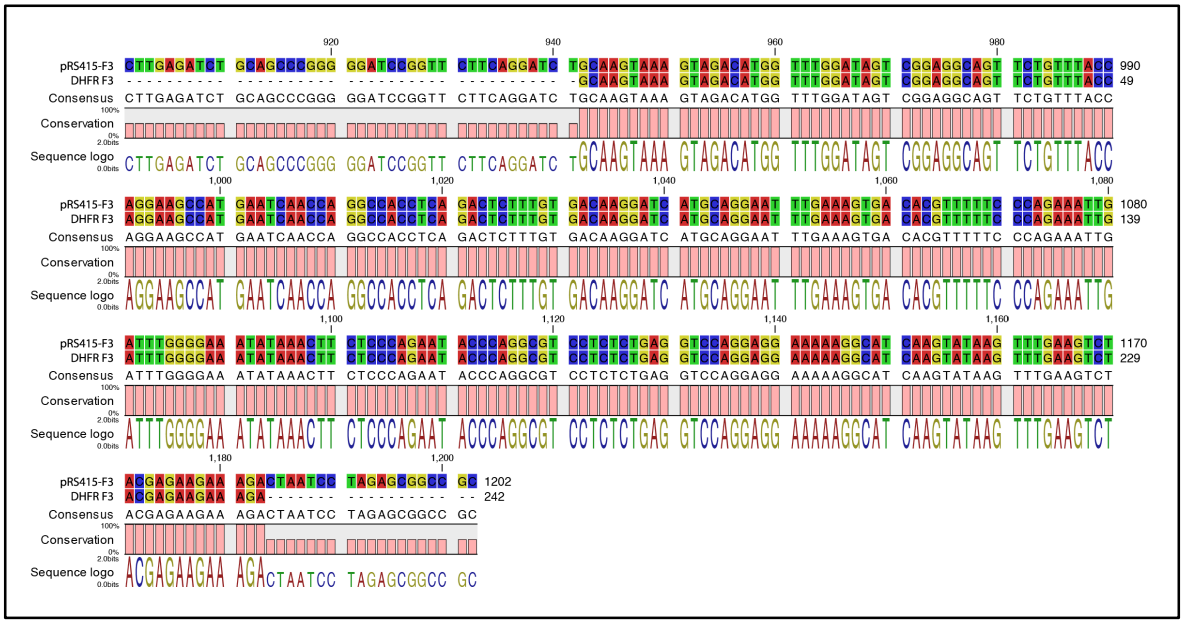
Appendix Figure 3 - pRS415 plasmid, created using SnapGene. Contains ampicillin resistance (AmpR; green) and a leucine marker (LEU2; orange) for growth on selective media.



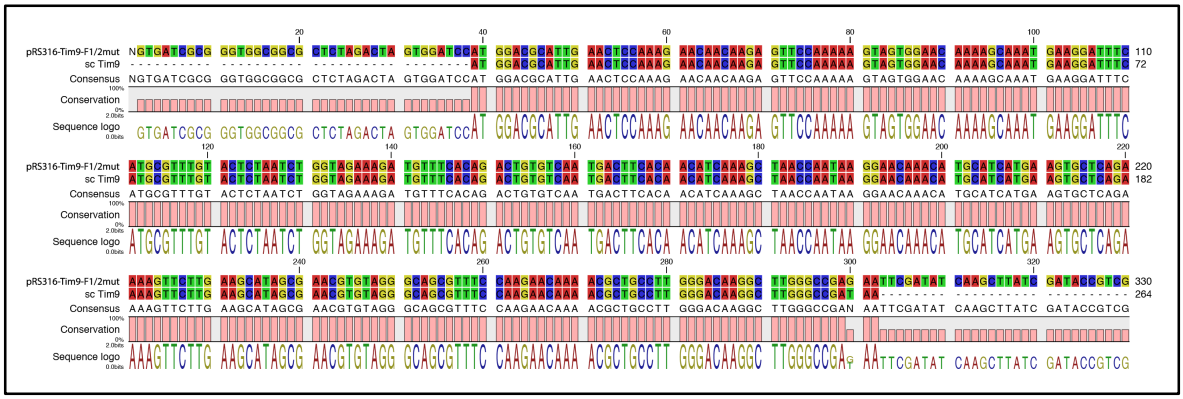
Appendix Figure 4 – pRS316-F1/2 aligned with murine dihydrofolate reductase fragment 1 (DHFR-F1/2).



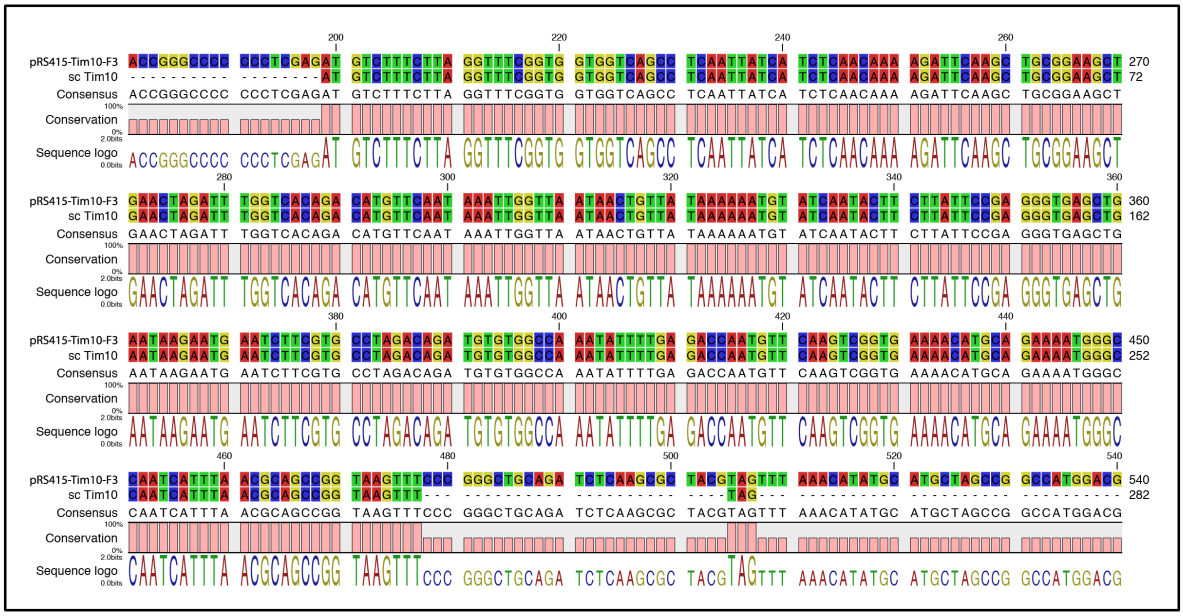
Appendix Figure 5 – pRS316-F1/2mut aligned with murine dihydrofolate reductase fragment 1 (DHFR-F1/2). F1/2mut refers to the mutated methotrexate (MTX) resistant DHFR-F1/2.



Appendix Figure 6 – pRS416-F3 aligned with murine dihydrofolate reductase fragment 2 (DHFR-F3).



Appendix Figure 7 - pRS316-Tim9-F1/2mut aligned with S. cerevisiae *Tim9. F1/2mut refers to the mutated methotrexate (MTX) resistant fragment 1 of dihydrofolate reductase (DHFR).*



Appendix Figure 8 – pRS415-Tim10-F3 aligned with S. cerevisiae *Tim10. F3 refers to fragment 2 of dihydrofolate reductase (DHFR).*

Appendix Figure 9 – pRS415-Gpx3-F3 aligned with S. cerevisiae *Gpx3. F3 refers to fragment 2 of dihydrofolate reductase (DHFR).*

DHFR Fragments		OD at Specific Time Intervals (Hours)									
F1/2	**F3**	*0*	*+1*	*+6*	*+21*	*+24*	*+24*	*+24*	*+24*	*+24*	*+24*
Tim9-F1/2mut	*Tim10-F3*	2.8	0.66	1.35	2.14	0.52	0.63	0.61	0.9	1.24	1.5
Tim9-F1/2mut	*F3*	2.9	0.69	1.88	1.78	0.94	0.64	0.66	1.06	1.36	1.39
F1/2mut	*F3*	2.8	0.87	1.62	2.44	0.69	0.58	0.73	1.19	1.31	1.48
F1/2	*F3*	2.8	0.6	1.37	2.38	0.56	0.82	0.73	1.32	1.26	1.36

Appendix Table 1 - Raw data of growth of S. cerevisiae *cells containing split-dihydrofolate reductase (DHFR) plasmids. These plasmids contained DHFR either the first (F1/2), second (F3) or mutated first (F1/2mut) fragment of DHFR. pRS316 was used as a vector for F1/2 or F1/2mut and pRS415 as a vector for F3.*

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180.

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