Structural and functional studies of mitochondrial small Tim proteins

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# LIST OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>5</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>7</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>8</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>9</td>
</tr>
<tr>
<td>COPYRIGHT STATEMENT</td>
<td>10</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>11</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>12</td>
</tr>
<tr>
<td><strong>1</strong> INTRODUCTION</td>
<td>14</td>
</tr>
<tr>
<td>1.1 <em>The Overview of mitochondria</em></td>
<td></td>
</tr>
<tr>
<td>1.2 <em>Biogenesis of mitochondrial proteins</em></td>
<td>18</td>
</tr>
<tr>
<td>1.2.1 Targeting signals of mitochondrial precursors</td>
<td>20</td>
</tr>
<tr>
<td>1.2.2 The mitochondrial entrance: TOM complex</td>
<td>23</td>
</tr>
<tr>
<td>1.2.3 The TIM23 complex and biogenesis of matrix proteins</td>
<td>24</td>
</tr>
<tr>
<td>1.2.4 The TIM22 complex and biogenesis of IM proteins</td>
<td>27</td>
</tr>
<tr>
<td>1.2.5 The SAM complex and biogenesis of β-barrel proteins</td>
<td>29</td>
</tr>
<tr>
<td>1.2.6 Biogenesis of IMS proteins</td>
<td>31</td>
</tr>
<tr>
<td>1.2.7 The MIA pathway</td>
<td>34</td>
</tr>
<tr>
<td>1.3 <em>Small Tim proteins</em></td>
<td>42</td>
</tr>
<tr>
<td>1.3.1 Twin CX$_3$C zinc-finger motif</td>
<td>43</td>
</tr>
<tr>
<td>1.3.2 Biogenesis of the small Tim proteins</td>
<td>46</td>
</tr>
<tr>
<td>1.3.3 Structures of small Tim complexes</td>
<td>52</td>
</tr>
<tr>
<td>1.3.4 Roles of small Tim complexes in the TIM22 pathway</td>
<td>54</td>
</tr>
<tr>
<td>1.3.5 Roles of small Tim complexes in other biogenesis pathways</td>
<td>57</td>
</tr>
<tr>
<td>1.4 <em>Aims and objectives</em></td>
<td>59</td>
</tr>
<tr>
<td><strong>2</strong> MATERIALS AND METHODS</td>
<td>61</td>
</tr>
<tr>
<td>2.1 <em>Molecular biology techniques</em></td>
<td>61</td>
</tr>
<tr>
<td>2.1.1 DNA cloning</td>
<td>61</td>
</tr>
<tr>
<td>2.1.2 Site-directed mutagenesis</td>
<td>62</td>
</tr>
<tr>
<td>2.1.3 Colony PCR of constructed plasmids</td>
<td>63</td>
</tr>
<tr>
<td>2.2 <em>Biochemical methods</em></td>
<td>63</td>
</tr>
<tr>
<td>2.2.1 Protein expression and purification from <em>E.coli</em></td>
<td>63</td>
</tr>
</tbody>
</table>
1.3.1.1 Expression and purification of GST-tagged protein
1.3.1.2 Expression and purification of His-tagged protein
1.3.2 Size exclusion chromatography
1.3.3 AMS thiol-alkylation assay
1.3.4 Micro scale thermophoresis (MST) analysis
1.3.5 Cross-linking
1.3.6 Agarose gel electrophoresis
1.3.7 Tris-Tricine SDS-PAGE
1.3.8 BN-PAGE
1.3.9 Gel transfer and western-blots

2.3 Yeast genetic techniques
2.3.1 High efficiency yeast transformation
2.3.2 Yeast colony PCR screening

2.4 In organelle and In vivo analysis
2.4.1 Crude protein preparation from yeast
2.4.2 Radioactive $^{35}$S pulse-labelling
2.4.3 Immunoprecipitation (IP)
2.4.4 Yeast mitochondria isolation
2.4.5 Solubilisation of yeast mitochondria

3 RESULTS AND DISCUSSION I:
Purification and characterisation of Tim12

3.1 Introduction
3.2 Purification of GST-tagged Tim12
3.3 Purification of N-terminal His-tagged Tim12
3.4 Purification of C-terminal His-tagged Tim12
3.5 Cysteine redox state of purified Tim12
3.6 Discussion

4 RESULTS AND DISCUSSION II:
In vitro study of the role of Tim9 disulphide bonds on complex formation

4.1 Introduction
4.2 Preparation of double cysteine mutants of Tim9
4.2.1 Expressions of recombinant Tim9 double cysteine mutants 102
4.2.2 Purifications of Tim9 double cysteine mutants 107
4.3 Characterisations of Tim9 double cysteine mutants 111
4.4 The effect of mutation on interaction between Tim9 and Tim10 112
4.5 The effect of mutation on Tim9-Tim10 complex formation 114
4.6 Discussion 125

5 RESULTS AND DISCUSSION III:
In vivo study of the role of cysteines in the biogenesis of small Tim proteins 127

5.1 Introduction 127
5.2 Phenotypes of small Tim cysteine mutants 128
  5.2.1 Phenotypes of KanMX4-tetO2 regulating strains 129
  5.2.2 Phenotypes of KanMX4-pMet3 regulating strains 140
5.3 Temperature sensitivities of Tim9 and Tim10 cysteine mutants 147
5.4 Redox stress sensitivities of Tim9 cysteine mutants 150
5.5 Steady-state levels of Tim9 and Tim10 in Tim9 cysteine mutants 153
5.6 Studies of disulphide bond mutants in TIM9 KO strains 158
  5.6.1 Phenotypes of disulphide bond mutants in TIM9 KO strains 158
  5.6.2 The protein stabilities of single disulphide bond mutants 160
5.7 Discussion 164
  5.7.1 Phenotypes of cysteine mutants of small Tim proteins 164
  5.7.2 Effects of temperature and redox on the phenotypes of mutants 166
  5.7.3 Protein steady-state levels in cysteine mutants of Tim9 and Tim10 167

6 CONCLUSIONS AND MODEL 168
7 REFERENCES 172
8 APPENDICES 186

  8.1 List of used yeast strains 186
  8.2 List of used E.coli host strains 187
  8.3 List of used plasmids 187
  8.4 List of used oligonucleotides 188
  8.5 List of used buffer solutions 190
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structure of a mitochondrion</td>
<td>15</td>
</tr>
<tr>
<td>1.2</td>
<td>Overview of the pathways used for import and assembly of mitochondrial proteins</td>
<td>19</td>
</tr>
<tr>
<td>1.3</td>
<td>Composition of the TOM complex</td>
<td>23</td>
</tr>
<tr>
<td>1.4</td>
<td>Composition of TIM23 complex and biogenesis of matrix proteins</td>
<td>25</td>
</tr>
<tr>
<td>1.5</td>
<td>Composition of the TIM22 complex and biogenesis of iM proteins</td>
<td>28</td>
</tr>
<tr>
<td>1.6</td>
<td>Composition of SAM complex and biogenesis of β-barrel proteins</td>
<td>30</td>
</tr>
<tr>
<td>1.7</td>
<td>TIM23 complex–dependent pathway for biogenesis of IMS proteins</td>
<td>33</td>
</tr>
<tr>
<td>1.8</td>
<td>Pathways of disulphide bond formation in cells</td>
<td>35</td>
</tr>
<tr>
<td>1.9</td>
<td>The model of disulphide relay system</td>
<td>39</td>
</tr>
<tr>
<td>1.10</td>
<td>The model of ternary complex formation</td>
<td>40</td>
</tr>
<tr>
<td>1.11</td>
<td>Sequence alignment of yeast small Tim proteins</td>
<td>44</td>
</tr>
<tr>
<td>1.12</td>
<td>Possible states of twin CX3C zinc-finger motif</td>
<td>45</td>
</tr>
<tr>
<td>1.13</td>
<td>The model for the biogenesis of Tim9-Tim10 complex</td>
<td>46</td>
</tr>
<tr>
<td>1.14</td>
<td>Model of the oxidative folding of Tim10 mediated by Mia40</td>
<td>49</td>
</tr>
<tr>
<td>1.15</td>
<td>The assembly process of Tim9-Tim10 complex</td>
<td>51</td>
</tr>
<tr>
<td>1.16</td>
<td>Crystal structure of the yeast Tim9–Tim10 hexameric complex</td>
<td>53</td>
</tr>
<tr>
<td>1.17</td>
<td>Tim9-Tim10 complex mediated the biogenesis of AAC</td>
<td>56</td>
</tr>
<tr>
<td>2.1</td>
<td>The scheme of MST assay</td>
<td>67</td>
</tr>
<tr>
<td>3.1</td>
<td>The construct of GST-Tim12 plasmid and purification process</td>
<td>79</td>
</tr>
<tr>
<td>3.2</td>
<td>Expression and solubility tests of GST-Tim12 in R.gami and BL21</td>
<td>81</td>
</tr>
<tr>
<td>3.3</td>
<td>Purification of Tim12 from the inclusion bodies of GST-Tim12</td>
<td>82</td>
</tr>
<tr>
<td>3.4</td>
<td>Optimisation of the dilution method for re-naturing proteins</td>
<td>83</td>
</tr>
<tr>
<td>3.5</td>
<td>Purification of Tim12 after the dialysis for re-naturing</td>
<td>84</td>
</tr>
<tr>
<td>3.6</td>
<td>The construct of His-Tim12 plasmid and purification process</td>
<td>86</td>
</tr>
<tr>
<td>3.7</td>
<td>Expressions of His-Tim12 in R.gami and BL21 at various temperatures</td>
<td>88</td>
</tr>
<tr>
<td>3.8</td>
<td>Purification of Tim12 from the inclusion bodies of His-Tim12</td>
<td>90</td>
</tr>
<tr>
<td>3.9</td>
<td>Gel filtration of Tim12 and SDS-PAGE analysis of the profile</td>
<td>92</td>
</tr>
</tbody>
</table>
Figure 3. 10 The construct of Tim12-His plasmid and purification process

Figure 3. 11 Expressions of Tim12-His in BL21 and R.gami

Figure 3. 12 Purification of Tim12-His from the inclusion body under denatured condition

Figure 3. 13 Dialysis of the denatured Tim12-His

Figure 3. 14 AMS assay of the redox states of Tim9, Tim10 and Tim12

Figure 4. 1 The scheme of double cysteine mutants of Tim9

Figure 4. 2 The expression tests of GST-Tim9C1,4S in BL21 and R.gami

Figure 4. 3 The expression tests of GST-Tim9C2,3S in BL21 and R.gami

Figure 4. 4 Purifications of WT and double cysteine mutants of Tim9

Figure 4. 5 Oligomerisation states of the Tim9wt and double cysteine mutants

Figure 4. 6 Far-UV CD spectra of Tim9wt and double cysteine mutants

Figure 4. 7 MST analysis of the interactions of Tim9wt and mutants with Tim10

Figure 4. 8 The complex formation of Tim9wt and Tim10

Figure 4. 9 The complex formation of Tim9C1,4S and Tim10

Figure 4. 10 The complex formation of Tim9C2,3S and Tim10

Figure 4. 11 The comparison for formed complexes by Tim9wt and mutants

Figure 4. 12 BN-PAGE analysis of complexes formed by Tim9wt and mutants

Figure 4. 13 SDS-PAGE analyses of hetero cross-link of Tim9wt and mutants with Tim10wt

Figure 5. 1 The construction scheme and regulation mechanism of KanMX4-tetO2 yeast strain

Figure 5. 2 Yeast colony PCR screening of KanMX4-tetO2 TIM9, TIM10 and TIM12 strains

Figure 5. 3 Phenotypes of Tim9, Tim10 and Tim12 regulated by KanMX4-tetO2 in yeast

Figure 5. 4 Spotting test of Tim9 cysteine mutation in the KanMX4-tetO2 yeast strains

Figure 5. 5 Spotting test of Tim10 cysteine mutation in the KanMX4-tetO2 yeast strains

Figure 5. 6 Spotting test of Tim12 cysteine mutation in the KanMX4-tetO2 yeast strains

Figure 5. 7 Phenotype of KanMX4-tetO2 regulated yeast strains on non-fermentable medium

Figure 5. 8 The scheme of constructing KanMX4-pMET3 yeast strains

Figure 5. 9 The process of constructing TIM9 KanMX4-pMET3 yeast strain

Figure 5. 10 Spotting test of KanMX4-pMET3 regulated Tim9 cysteine mutant strains
Figure 5.11 Spotting test of KanMX4-pMET3 regulated Tim10 cysteine mutant strains

Figure 5.12 Temperature sensitivities of TIM9 KanMX4-tetO strains

Figure 5.13 Temperature sensitivities of TIM9 KanMX4-pMET3 strains

Figure 5.14 Temperature sensitivities of TIM10 KanMX4-pMET3 strains

Figure 5.15 Redox stress sensitivities of TIM9 KanMX4-tetO strains

Figure 5.16 Oxidative stress sensitivities of TIM9 KanMX4-pMET3 strains

Figure 5.17 Protein steady-state levels of TIM9 KanMX4-pMET3 strains

Figure 5.18 Protein steady-state levels of TIM10 KanMX4-pMET3 strains

Figure 5.19 IP of 35S pulse labeled Tim9 in TIM9 KanMX4-pMET3 WT and mutants

Figure 5.20 Phenotypes of TIM9 KO disulphide bond mutants

Figure 5.21 Protein steady-state levels of the isolated mitochondria of TIM9 KO strains

Figure 5.22 Temperature stability of Tim9 in mitochondria of WT and mutants

Figure 6.1 Model for the biogenesis and complex formation of small Tim protein

LIST OF TABLES

Table 4.1 Protein interactions by ITC and MST

Table 5.1 Phenotypes of cysteine mutations of Tim9, Tim10 and Tim12
ABSTRACT

Most mitochondrial proteins are encoded by nuclear DNA, and synthesised in the cytosol, then imported into the different mitochondrial subcompartments. To reach their destination, mitochondrial inner membrane proteins require import across the outer mitochondrial membrane, and through the intermembrane space. This passage through the IMS is assisted by the small Tim proteins. This family is characterised by conserved cysteine residues arranged in a twin CX$_3$C motif. They can form Tim9-Tim10 and Tim8-Tim13 complexes, while Tim12 appears to form part of a Tim9-Tim10-Tim12 complex that is associated with the inner membrane translocase TIM22 complex. Current models suggest that the biogenesis of small Tim proteins and their assembly into complexes is dependent on the redox states of the proteins. However, the role of the conserved cysteine residues, and the disulphide bonds formed by them, in small Tim biogenesis and complex formation is not clear. As there is no research about the structural characterisation of Tim12 and double cysteine mutants of Tim9, purification of these proteins was attempted using different methods. To investigate how cysteine mutants affect complex formation, the purified double cysteine mutants of Tim9 were studied using *in vitro* methods. It showed that the double cysteine mutants were partially folded, and they can form complexes with Tim10 with low affinities, suggesting disulphide bonds are important for the structures and complex formation of small Tim proteins. The effect of cysteine mutants on mitochondrial function was addressed using *in vivo* methods. It showed that cysteines of small Tim proteins were not equally essential for cell viability, and growth defect of the lethal cysteine mutant was caused by low level of protein. Thus, the conclusion of this study is that disulphide bond formation is highly important for correct Tim9-Tim10 complex formation, and yeast can survive with low levels of complex, but it results in instability of the individual proteins.
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LIST OF ABBREVIATIONS

AMS: 4-acetamido-4′-maleimidylstilbene-2,2′-disulphonic acid
ALR: Augmenter of liver regeneration
ATP: Adenosine 5′-triphosphate
Ccs1: Copper chaperone of Sod1
CD: Circular dichroism
Cyt c: Cytochrome c
DNA: Deoxyribonucleic Acid
DOX: Doxycycline
Dsb: Disulphide bond
DTT: Dithiothreitol
EDTA: Ethylenediaminetetraacetic acid
ER: Endoplasmic reticulum
FAD: Flavin adenine dinucleotide
GSH: Reduced glutathione
GSSG: Oxidised Glutathione
GST: Glutathione-S-Transferase
IM: Inner membrane
IMS: Intermembrane space
IPTG: Isopropyl-β-D-thiogalactopyranoside
LB: Luria broth
MIA: Mitochondrial import and assembly
MISS/ITS: Mitochondrial IMS sorting signals/ IMS-targeting signals
**MPP**: Mitochondrial processing peptidase

**MST**: Micro scale thermophoresis

**OD**: Optical density

**OM**: Outer membrane

**PAM**: Presequence translocase-associated motor

**PMSF**: Phenyl methane sulphonyl fluoride

**PDI**: Protein disulphide isomerase

**SAM**: Sorting and assembly machinery

**SDS-PAGE**: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

**Sod1**: Super oxide dimutase

**TCEP**: Tris- (2-carboxyethyl) phosphine

**TIM**: Translocase complex of the inner membrane

**TOB**: Topogenesis of β-barrel proteins complex

**TOM**: Translocase complex of the outer membrane

**VT**: Vector

**WT**: Wild type
1 INTRODUCTION

1.1 The Overview of mitochondria

Mitochondria are essential organelles in nearly all eukaryotic cells excluding the protist *Chaos (Pelomyxa) carolinensis* (Alcock et al., 2008). They generate ATP that is used as a source of chemical energy for viability of cells, so they are described as the "energy factory" of cells. Apart from that, they are involved in many other biological activities, e.g. signaling, cellular differentiation, apoptosis, as well as the control of the cell cycle and cell growth (McBride et al., 2006). Therefore, it is not surprising that several human diseases are caused by mitochondrial dysfunction, including diabetes, cancer, cardiovascular diseases and process of ageing (de Ferranti and Mozaffarian, 2008).

Mitochondria have a similar size as bacteria ranging from 0.5µm to 1µm in diameter, and their number in one cell widely varies from one to over 1000 by organism and tissue types (Alberts, 1994, Voet, 2006). This unique feature of mitochondria was not only made by their characterised structure that carried out specialised functions, but also was related to their evolution.

Mitochondria can be separated into four compartments, from outside to inside. They are outer membrane (OM), inner membrane (IM), intermembrane space (IMS) and matrix (Figure 1. 1). The OM is fairly smooth, but the IM is highly convoluted, forming folds called cristae. The matrix is enclosed by the IM.
Figure 1. Structure of a mitochondrion. A mitochondrion is composed by two membranes: the outer membrane (OM) and inner membrane (IM). On the OM, there are porins for the transport of small molecules. The crista is generated by folded IM. Between these two membranes, the inter membrane space (IMS) is an aqueous compartment. Besides IMS, another aqueous compartment, the matrix, is enclosed by the IM.
The OM is composed of a relatively simple phospholipid bilayer, whose protein-to-phospholipid ratio is similar to that of eukaryotic plasma membrane, roughly 1:1 by weight. It contains many integral proteins. These proteins are usually called porins and render the OM permeable to small molecules (<5kDa). It is believed that ions, nutrient molecules, ATP, ADP, etc. can easily pass through the OM. Because porins of the OM allow free movement of small molecules into the IMS, the concentrations of these small molecules are believed to be the same as those in the cytosol (Alberts, 1994). For the import of larger molecules like mitochondrial proteins, a large dynamic complex, also called translocase of the outer membrane (TOM) complex, represents the entry gate of mitochondria (Mokranjac and Neupert, 2005). However, large molecules have to be transported via translocase of the OM, so the content of protein in the IMS is different to that of cytosol. If the OM is disrupted, proteins in the IMS would be released to cytosol resulting in death of cells (Chacinska et al., 2009). The mitochondrial OM can physically interact with the endoplasmic reticulum (ER) membrane, which is known as the mitochondria-associated ER membrane (MAM). It plays important “housekeeping” functions including the transfer of lipids between the ER and mitochondria and regulation of ER-mitochondria calcium signaling (Hayashi et al., 2009). Additionally, enzymes located at the OM catalyse several biochemical reactions involving fatty acid degradation (Carnitine palmitoyltransferase I, Long fatty acyl CoA synthetase), tryptophan metabolism (Kynureninase), and monoamine neurotransmitter metabolism (Monoamine oxidase) (Szalardy et al., 2012, Kerner et al., 2011).

The IMS is a compartment between the OM and IM and also named as perimitochondrial space. There are about 5% mitochondrial proteins located in this compartment. These proteins are essential for the biogenesis of matrix, IM and OM destined proteins, and participate in the redox-dependent activities which
are similar to mechanisms in the endoplasmic reticulum (ER) and the bacterial periplasm (Riemer et al., 2010). However, it is different to that in cytosol, since the presences of thioredoxins and glutaredoxins contribute to the high value of $[\text{GSH}]:[\text{GSSG}]$ that ranges from 30:1 to 100:1, and even to 3300:1 resulting in reducing condition that does not favour disulphide bond formation (Ostergaard et al., 2004). The IMS also serves the transfer of various metabolites between mitochondria and other cellular compartments. Furthermore, mitochondria regulated apoptosis is triggered by the release of the IMS proteins (e.g. cyt c) into the cytoplasm that promotes caspase activation in cytosol (Martinou and Youle, 2011, Scorrano, 2009). More importantly, the biogenesis of the respiratory chain complexes occurs in the IMS, which is regulated by the IMS proteins e.g. cytochrome bc(1) complex (Smith et al., 2012, Diaz et al., 2011).

The IM is also a phospholipid bilayer like the OM, but has higher protein-to-phospholipids ratio (more than 3:1 by weight). About 20% of mitochondrial proteins are located in the IM. Some of these proteins perform the redox reactions of oxidative phosphorylation. Some transmembrane proteins are used as transporters to regulate metabolite passages into and out of the matrix, or as a translocase of inner membrane (TIM) to facilitate the delivery of matrix proteins (Stojanovski et al., 2007). Unlike the OM, the IM does not allow free passage of small molecules. In fact, the permeability of ions such as potassium is highly regulated through ion conducting channels. Other small molecules, such as oxygen, carbon dioxide and water can passage freely across the IM.

The wrinkled IM forms an internal compartment, crista. There are many proteins including ATP synthase and a variety of cytochromes stubbed on the cristae. In other words, cristae increase the surface of IM for chemical reactions to produce more ATP, allowing the mitochondria to exert functions efficiently. However, cristae do not simply acquire random folds. They have a specialised topological
shape that is suggested to maintain the balance between membrane fusion and fission processes (Mannella, 2006).

The matrix of mitochondria houses about 2/3 of the mitochondrial proteins, as well as their own DNA, tRNA and ribosomes. Although aquaporins in the IM can regulate the flow of water, it’s still not clear how the balance of osmosis across the IM is maintained (Agre, 2006). The citric acid cycle (or Krebs cycle or TCA cycle), anaplerotic reactions, urea cycle and alcohol metabolism occur within the mitochondrial matrix. The NADH and FADH\(_2\) produced by the citric acid cycle in the matrix provide electrons for the electron transport chain, where oxygen ultimately accepts two electrons to generate water. Meanwhile, the protons released are pulled out into the IMS from the matrix by the energy produced from the electron transport chain. The protons return to the matrix through chemiosmosis that is carried out by ATP synthase at the IM. For the biogenesis of mitochondrial proteins, the details will be discussed in the following section.

### 1.2 Biogenesis of mitochondrial proteins

About 99% mitochondrial proteins are synthesized in the cytosol in the form of precursor that is also named as preprotein. Therefore, the correct import and sorting of precursor to their destinations is essential for biogenesis of mitochondria. So far, four major mitochondrial import translocase complexes have been identified in *Saccharomyces cerevisiae* (Figure 1. 2). All precursor proteins seem to use the translocase of the outer membrane (TOM) complex to enter mitochondria, then according to targeting information, different precursors use different pathways for their further import into the different sub-compartments of mitochondria (OM, IMS, IM and matrix respectively). Precursor proteins with a typical N-terminal targeting sequence use the translocase of the inner membrane (TIM) TIM23 pathway, which is the classical pathway and the
mainly used by matrix destined proteins. Precursor proteins with internal targeting signals utilise one of three following pathways: TIM22 translocation system for proteins destined for the IM like members of the carrier protein family; the sorting and assembly machinery (SAM) complex for β-barrel proteins on the OM; and the mitochondrial import and assembly (MIA) machinery for many IMS-localised proteins. Details of mitochondrial targeting signal and each pathway will be discussed in the following sections.

**Figure 1. Overview of the pathways used for import and assembly of mitochondrial proteins.** The precursors synthesised in the cytosol are imported into mitochondrion with different pathways. After crossing the TOM complex, the matrix precursors pass through the channel of the TIM23 complex to be imported into the matrix. The translocation of IMS precursors is mainly accomplished by the MIA machinery. The IM precursors are embedded in the IM via the TIM22 complex. The translocation of OM precursors in the OM employs the SAM complex. Small Tim proteins play chaperone-like functions for the delivery of incoming membrane precursors to translocase complexes in the OM and IM. Figure adapted from (Mokranjac and Neupert, 2009).
1.2.1 Targeting signals of mitochondrial precursors

Mitochondrial precursor proteins are guided to mitochondria by specific mitochondrial targeting signals, which contain cleavable or noncleavable targeting signals. The cleavable N-terminal sequences are usually called presequences, and are typically considered as a classical mitochondrial targeting signal. These presequences have several variations including some special subcompartment insertion information. Precursor proteins with no cleavable presequences depend on noncleavable targeting signals for their translocations, reflecting that various characterised patterns of precursor proteins determine different sorting pathways and ultimate destinations (Chacinska et al., 2009).

1.2.1.1 Cleavable targeting signals

All precursor proteins with cleavable presequences are imported into the mitochondrial matrix. Thus, presequence targeting signals are also called matrix targeting signals (MTS) (Ishihara et al., 2006). MTS contain about 10-80 amino acid residues that have propensity to form an amphipathic α-helix. It has a hydrophobic surface on one side of the helix and is positively charged in the other side of the surface. This kind of structure is necessary and sufficient to direct proteins into the mitochondrial matrix (van Loon and Schatz, 1987). MTS are not conserved among the primary sequences of different proteins. Even the same proteins in different species have considerably different MTS. However, MTS are normally conserved among fungi, mammals and some plants (Lister et al., 2003).

After precursor translocation into the matrix, the N-terminal targeting signals are proteolytically removed by the matrix processing peptidase (MPP). Some IM precursors have N-terminal presequences that can be cleaved by MPP as well, but the presequences are followed by noncleavable hydrophobic anchors which function as sorting signals to direct precursors into the IM. A group of IMS
precursors also contain cleavable presequences followed by hydrophobic sorting signals (bipartite presequence). Following MPP cleavage for their presequences removed, the bipartite presequences are cleaved off by the inner membrane peptidase (IMP) to release the protein into the IMS (Nunnari et al., 1993).

1.2.1.2 Noncleavable targeting signals

Compared with precursor proteins containing cleavable targeting signals, precursor proteins with noncleavable targeting signals distribute at mitochondrial OM, IM and IMS. For OM precursor proteins, there are two types of membrane proteins: β-barrel and α-helical. The β-barrel region is located at the C-terminus of precursor, which is composed of a TOM targeting domain and a series of anti parallel β-strands (Wimley, 2003). Every strand consists of 9~11 amino acids and is tilted about 45° to the membrane plane (Schulz, 2000). Until now, only five yeast OM proteins with β-barrel structure have been identified, such as Tom40, Sam50, Sam35, Sam37, and Mdm10.

Many OM precursor proteins contain α-helical domains, used as a signal for translocation into mitochondria. A type of α-helical signal is called signal-anchored sequence, which locates at the N-terminus of precursor. The precursor is anchored into the OM with using a small single transmembrane segment of signal-anchored sequence (Ahting et al., 2005). In contrast, another type of α-helical signal locates at the C-terminus of precursor is integrated into the OM using the single transmembrane segment, meanwhile the large N-terminal domain of precursor is exposed to the cytosol like a big tail. So this type of α-helical signal is called as C-tail anchor (Kemper et al., 2008). In addition, α-helical signal can also be found in the middle of some precursors, but that contain multiple transmembrane segments. The multiple transmembrane segments
makes the precursor span across the OM several times, resulting in formation of small loops of exposing to IMS or cytosol (Fritz et al., 2001).

The IM precursor proteins including the metabolite carrier proteins (e.g. the ADP/ATP carrier and phosphate carrier proteins) have multiple internal targeting signals. These internal targeting signals form several information elements, each with about 10 amino acid residues, distributed discontinuously within the entire precursor (Wiedemann et al., 2001). The residues at the C-terminus are generally important for insertion into the IM (Brandner et al., 2005). Some IM precursor proteins have an internal positively charged targeting signal like that of the cleavable presequence (Neupert and Herrmann, 2007). These targeting signals are recognised by several import receptors that facilitate their translocation into the IM. Details of the import mechanism of IM proteins will be discussed in the section 1.2.4.

The translocation signals of cysteine-containing IMS proteins (e.g. small Tim proteins and Cox17) are located at the inside of peptides, and are identified as mitochondrial intermembrane space sorting signals (MISS) or IMS-targeting signals (ITS). It has been confirmed that cysteine-containing IMS proteins are substrates of Mia40 (Stojanovski et al., 2012). The consensus sequence of MISS/ITS is composed of hydrophobic residues in positions ±3, ±4 and ±7 from the cysteine residue that first interacts with Mia40. For example, the MISS/ITS of yeast Tim9, Tim10 and Tim12 consists one aromatic residue and two other hydrophobic residues at positions -7, -4 and -3 upstream from the most N-terminal conserved cysteine. For the human Cox17 with twin CX_9C motifs, its MISS/ITS is located at downstream of the third cysteine, one aromatic residue and two other hydrophobic residues are located at positions +7, +4 and +3 from the docking cysteine. These amino acids are located on the same side as the docking cysteine in the α-helix and are essential for interaction with the Mia40
hydrophobic cleft (Sideris et al., 2009, Milenkovic et al., 2009). The further information about how Mia40 interacts with cysteine-containing substrates is provided in the section 1.2.7.

1.2.2 The mitochondrial entrance: TOM complex

Nearly all mitochondrial proteins are imported into mitochondria through the TOM complex, which works as the main protein entry gate. The TOM complex recognises mitochondrial precursor proteins synthesised in the cytosol and dissociates bound cytosolic chaperones to facilitate their channeling across the OM and ultimately releases precursor proteins into the IMS to follow their respective pathways. The TOM complex consists of the receptor subunits Tom70, Tom22, and Tom20, the membrane-embedded central subunit Tom40, and three small subunits Tom7, Tom6, and Tom5 (Figure 1.3) (Chacinska et al., 2009).

**Figure 1.3 Composition of the TOM complex.** The TOM complex is composed of seven subunits. Tom40 is the central pore component of the TOM complex with three receptor components (Tom70, Tom20 and Tom22) and three small Tom subunits (Tom7, Tom6, and Tom5).
Tom40 as a β-barrel protein is the central and pore-forming component of the TOM complex. It is essential for the viability of yeast. Tom40 and other three associated small subunits (Tom5, Tom6 and Tom7) form the general import pore (GIP), a translocation channel of the TOM complex, Tom40 is organised as an oligomer that forms two to three channels per TOM complex (Ryan et al., 2000). For functions of three small Tom proteins, Tom5 assists incoming precursors to dock on the GIP, while Tom6 and Tom7 are involved in the stabilisation and assembly of the TOM complex (Model et al., 2008).

The TOM complex consists of three receptors: Tom70, Tom20 and Tom22. Tom20 and Tom70 are the major receptors for recruiting precursors with and without N-terminal MTS respectively. They are anchored in the OM via N-terminal transmembrane segments, and their receptor domains are exposed to the cytosol. Tom70 recognises the hydrophobic precursors with multiple internal targeting signals, such as AAC (Young et al., 2003). While Tom20 is the major receptor for N-terminal cleavable presequences (Abe et al., 2000). Tom20 and Tom70 can also interact with cytosolic chaperones, e.g. Hsp70 of yeast, Hsp70 and Hsp90 of mammal, that keep hydrophobic proteins from aggregating in an aqueous environment. This interaction is essential for delivery of a set of mitochondrial membrane precursors (e.g. AAC) to the receptor for subsequent mitochondrial import (Young et al., 2003). Tom22 is not only the central receptor of the TOM complex, but also works as the docking point of the GIP complex where peripheral receptors Tom20 and Tom70 pass the recognised precursors to Tom22 for transport into the Tom40 channel (Pfanner and Geissler, 2001).

1.2.3 The TIM23 complex and biogenesis of matrix proteins

After translocation across the OM via the TOM complex, precursors of matrix proteins interact with components of the TIM23 complex of the IM (Figure 1. 4).
There are two energy sources for energetically driving the translocation of matrix proteins: the electrical membrane potential across the inner membrane ($\Delta \Psi$) and an ATP-dependent import motor. The components of TIM23 complex can be classified into two groups: membrane-embedded components and mtHsp70-associated import motor (also called presequence translocase-associated motor, PAM complex) (Pfanner and Geissler, 2001).

**Figure 1. 4 Composition of TIM23 complex and biogenesis of matrix proteins.** The TIM23 complex mainly consists of 9 subunits. Tim 21, Tim50, and Tim23 recruit incoming precursor, with exposing domains to the IMS. Tim17 and Tim23 form the translocation channel across the IM, which is driven by electrical membrane potential ($\Delta \psi$). Tim44, Tim16, Tim14 are exposed to the matrix, and function to deliver imported precursors to mtHsp70, and Mge1 that form an ATP-dependent import motor. The presequence of precursor is cleaved by the import motor leading to the maturation of matrix protein (Schmidt et al., 2010).
The membrane-embedded part of the TIM23 complex is composed of the three essential subunits, Tim17, Tim23, and Tim50. Tim17 and Tim23 build up the core translocation channel, while Tim50 functions as a receptor for recognising precursors. At the C-terminus of Tim23 and Tim17, there is a common transmembrane domain, where four transmembrane segments form the translocation channel (Truscott et al., 2001). The exposed N-terminus of Tim23 has a hydrophobic domain interacting with Tim50 that only recognises matrix-targeting proteins (Donzeau et al., 2000). As the major receptor of TIM23 complex, Tim50 anchors into the IM by a single transmembrane domain, exposing a large C-terminal domain to the IMS. This large domain directly associates with precursors that channel across the TOM complex (Mokranjac et al., 2003a).

The other part of the TIM23 complex is the ATP-dependent import motor (or PAM complex). It contains Tim14 (Pam18), Tim16 (Pam16), Tim44, Mge1 and mtHsp70. Tim44 is a hydrophilic matrix protein that tightly associates with the IM (Josyula et al., 2006). The assembly of Tim44 and mtHsp70 guides the translocation of precursors into the matrix driven by the hydrolysis of ATP. As a member of the hsp family, mtHsp70 contains a N-terminal ATPase domain and a C-terminal substrate-binding domain. If ATP binds to N-terminus of mtHsp70, the incoming precursor can be associated with the C-terminus of mtHsp70. However, if ADP occupies the N-terminus of mtHsp70, the C-terminus of mtHsp70 can not be open for recruitment of incoming precursor. Therefore, the association or dissociation of precursor with mtHsp70 is determined by the hydrolysis of ATP (Liu et al., 2003). Tim14 has been shown to act as a J-protein of the import motor, which stimulates the exchange of ADP to ATP (Mokranjac et al., 2003b). Tim14, Mge1 and Tim16 assist mtHsp70 to bind the incoming precursors. More than that, Tim16 can form a stable subcomplex with Tim14 to perform J-protein functions (Kozany et al., 2004).
At present, there are two models about how mtHsp70 drives protein translocation into the matrix. In the first model (trapping model), mtHsp70 associates with Tim44 dimer, and then traps the new incoming and unfolded precursors. The mtHsp70 acts as a Brownian ratchet to prevent precursors from sliding back into the import channel (Liu et al., 2003). In the second model (pulling model), mtHsp70 works as an active import motor in cooperation with Tim44 by interacting with Tim44 and precursors simultaneously. As the assembly of Tim44 and mtHsp70 undergoes an ATP-dependent conformational change, a pulling force is generated on precursors for translocation (Hutu et al., 2008). The trapping model is more suitable to explain the import of loosely folded precursors, while the pulling model is better suited for tightly folded precursors. However, both of these models may cooperate together in the import of matrix precursors (Neupert and Herrmann, 2007).

1.2.4 The TIM22 complex and biogenesis of IM proteins

The TIM22 complex plays a crucial role for the import of IM proteins including metabolic carrier proteins (e.g. AAC) and components of the TIM23 complex such as Tim23, Tim17 and Tim22. Although precursors of the IM proteins are synthesised in the cytosol without a cleavable presequence, they have internal targeting signals that guide them to the IM (Vergnolle et al., 2005). The TIM22 complex consists of three membrane-integrated components, Tim22 Tim54 and Tim18 (Figure 1.5). Tim22 is the central component of the complex and homologous to Tim23 and Tim17 (Sirrenberg et al., 1996). There are four transmembrane helices in Tim22 forming a channel for insertion of polypeptide substrates (Kovermann et al., 2002). Tim54 anchors in the IM using the N-terminus, leaving the C-terminus exposed towards the IMS. Tim18 is inserted in the IM by three transmembrane segments with its C-terminal domain faces to the IMS-like Tim54. Both Tim54 and Tim18 do not directly participate in the IM
protein insertion into the IM but function as assembly and stabilisation of TIM22 complex (Koehler et al., 2000, Hwang et al., 2007).

Figure 1. 5 Composition of the TIM22 complex and biogenesis of IM proteins. The TIM22 complex contains three subunits: Tim22, Tim18 and Tim54. Precursors traverse the OM through the TOM complex, and then are directed by the small Tim complex in the IMS to the TIM22 complex. In an electrical membrane potential ($\Delta\psi$)-dependent manner, precursors are integrated in the IM by the membrane-embedded component of this complex, Tim22, with the help of Tim8 and Tim54 (Bolender et al., 2008).
After channeling across the OM via the TOM complex, precursors of IM proteins are chaperoned by small Tim proteins in order to prevent protein aggregation in the aqueous environment of the IMS before reaching the IM. Then, Tim54 recruits incoming precursors to Tim22 that forms a signal-selective channel and specifically responds to internal targeting signals of carrier proteins. The membrane-insertion of mature proteins is driven by the force of membrane potential ($\Delta\psi$), but the exact mechanism is still unclear (Kovermann et al., 2002).

In addition to the TIM22 complex, the import of some IM proteins is TIM23-dependent. Those are imported into matrix by TIM23 complex, and then exported to the IM owing to the hydrophobicity of the transmembrane domains followed by the presence of charged residues (Meier et al., 2005). In the other TIM23-dependent pathway known as the “stop-transfer” pathway, precursors with one transmembrane domain are arrested at the level of IM during its import (Glick et al., 1992).

### 1.2.5 The SAM complex and biogenesis of β-barrel proteins

The SAM complex (sorting and assembly machinery complex), also known as the TOB complex (topogenesis of β-barrel proteins complex) has been identified to mediate the translocation of β-barrel proteins (e.g. Tom40 and Sam50) into the OM. All the OM β-barrel proteins are synthesised in the cytosol, and translocated through the OM by the TOM complex. After released from the TOM complex, these precursors interact with small Tim complexes (Tim8-Tim13 complex or Tim9-Tim10 complex) in the IMS to prevent them from aggregation. The SAM complex recruits the precursors from the small Tim complex-bound precursors and completes the insertion of β-barrel precursors (Paschen et al., 2005).
Figure 1. Composition of SAM complex and biogenesis of β-barrel proteins. The SAM complex contains Sam50, Sam35 and Sam37. Sam50 contributes to the major role in the SAM complex, while Sam35 and Sam37 function in assisting substrate binding and releasing from the SAM complex. Precursor of β-barrel protein passages through the OM via the TOM complex. The small Tim complexes bind to incoming precursor in the IMS and deliver to the SAM complex. The insertion of β-barrel proteins into the OM is accomplished by the SAM complex (Mokranjac and Neupert, 2009).
So far, it is known that the SAM complex is composed of three subunits: Sam50 (Tob55), Sam35 (Tob38 or Tom38) and Sam37 (Tob37 or Mas37) (Figure 1. 6). Sam50 exposes the N-terminal hydrophilic region in the IMS forming a polypeptide translocation associated domain as the major functional region. Precursors of β-barrel proteins are delivered by the small Tim complex to SAM complex and recognised by the N-terminal domain of Sam50 (Sanchez-Pulido et al., 2003). Sam35 is not embedded into the membrane but rather associated with Sam50 on the cytosolic side of the OM. Sam50 is required to bind substrates, because destabilisation of Sam35 inhibits substrate binding by the SAM complex (Chan and Lithgow, 2008). Sam37 is located at the cytosolic-side surface of OM (Gratzer et al., 1995). Sam37 and Sam35 form a codependent relationship, since the deletion of Sam37 leads to low levels of Sam35 (Chan and Lithgow, 2008). The function of Sam37 is suggested to assist the release of substrates from the SAM complex (Chan and Lithgow, 2008).

1.2.6 Biogenesis of IMS proteins

Although the IMS is the smallest compartment of the mitochondria, as described in the section 1.1.1, it contains proteins playing many important functions, such as the transfer of metabolites, the biogenesis and regulation of the respiratory chain and the regulation of apoptosis. According to the proteome study of the yeast mitochondrial IMS, there are at least 51 proteins located in the IMS, most of which are soluble proteins, but some are peripherally attached with the IM or membrane proteins (Voegtle et al., 2012). All the IMS proteins are encoded by the nuclear DNA and synthesised in the cytosol, followed by the import into mitochondria. So far, the biogenesis of IMS proteins mainly employs two pathways: TIM23 complex-dependent pathway and MIA pathway (Neupert and Herrmann, 2007, Schmidt et al., 2010). The first pathway is more closely related
to my PhD project and is described for more details in this section, while the second pathway is described in detail in this section.

The TIM23 complex-dependent pathway is employed by a group of IMS proteins that contain bipartite presequences, which consist of a typical N-terminal matrix targeting signal and a hydrophobic sorting domain (Mossmann et al., 2012). After passaging through the OM via the TOM complex, the precursor is directed by the N-terminal presequences to the TIM23 complex which arrests the hydrophobic sorting domain at the surface of the inner membrane. Depending on the TIM23 complex, the precursor is inserted into the IM, leading to the N-terminus towards the matrix and leaving the C-terminus in the IMS. The N-terminal targeting signal is cleaved off by the MPP in the matrix. Meanwhile, the inner membrane peptidase (IMP) complex located at the IM cleaves off the inserted precursor at the site of hydrophobic sequence and releases soluble and mature protein into the IMS (Mokranjac and Neupert, 2010, Mossmann et al., 2012).

The IMP complex consists of two catalytic subunits, Imp1 and Imp2, and the auxiliary protein Som1 (Jan et al., 2000, Nunnari et al., 1993). Imp1 and Imp2 are integral IM proteins, although their catalytic active is mainly contributed by the C-terminuses exposing to the IMS (Jan et al., 2000). The catalytic active is not found in Som1, but its interaction with Imp1 promotes cleavage efficiency of substrates (Bauerfeind et al., 1998). So far, only 4 IMS proteins (Gut2, Cyb2, Mcr1 and Ptc5) have been found to be the substrates of IMP complex (Voegtle et al., 2012, Mossmann et al., 2012).
The TIM23 complex–dependent pathway for biogenesis of IMS proteins. Precursors of IMS proteins with bipartite presequences utilise TIM23 complex–dependent pathway for translocation. Precursors are synthesised in the cytosol like most of mitochondrial proteins, followed by passage across the OM via the TOM complex. The imported precursors are arrested by the TIM23 complex and inserted into the IM. The matrix targeting signal of the presequence is cleaved off by the MPP complex. The IMP complex cleaves the hydrophobic sorting signal domain of presequence off precursor and releases the soluble and mature protein into the IMS. The IMP complex is formed by three subunits: Imp1, Imp2 and Som1 (Gakh et al., 2002).
1.2.7 The MIA pathway

Apart from the TIM23 complex-dependent pathway for IMS proteins with bipartite presequences, the MIA pathway is the major biogenesis route of many IMS proteins, especially for proteins containing cysteine residues (Naoe et al., 2004, Lee et al., 2000). Substrates of MIA pathway undergo oxidative folding for protein biogenesis through disulphide bond formation (Gabriel and Pfanner, 2007).

Like the MIA pathway in mitochondrial IMS proteins, disulphide bonds formation can occur in various organelles and is catalysed by different protein systems, such as DsbA/DsbB pathway in periplasmic space of prokaryotic cell, PDI/Ero1/Erv2 pathway in endoplasmic reticulum (ER) and G4L/A2.5L-E10R eukaryotic cytosol (Figure 1. 8) (Mesecke et al., 2005, Sevier and Kaiser, 2006, Kadokura et al., 2004). Although thiol-disulphide exchange reactions in these pathways are catalysed by respective redox enzymes, the common rule is that the formation of disulphide bonds in reduced substrates are exchanged from disulphide carriers, which are kept in the oxidised state by a sulfhydryl oxidase. Oxygen or another electron acceptor provides oxidising equivalents to sulfhydryl oxidase. During thiol-disulphide exchange reactions, the generated electrons move in the reverse direction form reduced substrate, disulphide carrier in next, then sulfhydryl oxidase, finally to oxidants (Sevier and Kaiser, 2002).
**Figure 1. 8 Pathways of disulphide bond formation in cells.** The disulphide bonds can be formed in different organelles. The substrates, key redox enzymes and electron acceptors are listed for various oxidation pathways. In each pathway, substrates are oxidised by disulphide carriers, which are re-oxidised by sulfhydryl oxidases. Meanwhile, the generated electrons in thiol-disulphide exchange reactions flow in the reverse directions, and finally are accepted by electron acceptors. Figure adapted from Chacinska et al., 2009.
1.2.7.1 Composition of the MIA pathway

The MIA pathway is mainly composed of two components: Mia40 and Erv1. Mia40 is conserved in all eukaryotic cells, and is soluble in the mitochondrial IMS of plant and animal cells, but is anchored in the mitochondrial IM in yeast cell (Chacinska et al., 2004, Daithankar et al., 2009). As the central and essential component, mutated Mia40 can specifically inhibit the import of cysteine rich IMS proteins without any affects on the import of other mitochondrial proteins in vitro. It has been found that Mia40 works not only as the import receptor that recruits substrates on the trans-side of the OM via formation of intermediate disulphide bond, but also as the oxidoreductase that catalyses oxidative folding of substrates (Sideris and Tokatlidis, 2007, Banci et al., 2009). Mia40 possesses 6 cysteine residues that are distributed into a CPC motif and twin CX9C motifs. The CPC motif is the redox-active site that performs thiol-disulphide exchange reactions with substrates, while the twin CX9C motifs form disulphide bonds to stabilise the structure of Mia40 (Grumbt et al., 2007).

The second essential component of the MIA path is Erv1 in yeast, which belongs to the family of FAD-dependent sulfhydryl oxidases sharing the FAD binding domain with other members. Homologues of Erv1 include augmenter of liver regeneration (ALR) in human and Erv2 in the ER lumen of yeast (Fass, 2008). Erv1 is important for the biogenesis of mitochondrial IMS proteins, but also can affect the morphology of yeast mitochondria, reactions of cellular respiration and biogenesis of cytosolic iron-sulfur (Fe/S) proteins (Becher et al., 1999, Lisowsky, 1992, Lange et al., 2001). Erv1 contains a conserved C-terminal catalytic domain and an N-terminal non-conserved domain. The conserved catalytic domain is functional for disulphide bond formation of substrate. It consists of a CXXC active motif, a CX16C motif and residues required for FAD binding (Gross et al., 2002). The CXXC active motif is closely located to the isoalloxazine ring of FAD, while
CX_{16}C motif forms a long range disulphide bond that may play a structural role in stabilising the FAD binding or substrate binding. The non-conserved domain containing a CX_nC motif is located at either N-terminus or C-terminus depending on different ERV/ALR proteins (Daithankar et al., 2009). Furthermore, the number of amino acids between cysteines is not conserved. The CXXC of Erv1 and ALR are located at the N-terminus of protein and closed to the catalytic core. However, the Erv2 CXC and AtErv1 CX_4C are located at the C-terminus. Based on the crystal structure of Erv2, the disulphide bond formed within the non-conserved CX_nC motif can be transferred to Mia40. Therefore, the role of Erv1 requires both catalytic disulphide domain and shuttle disulphide domain (Banci et al., 2011a, Hofhaus et al., 2003).

In the MIA pathway, the reduced Mia40 can be re-oxidised by Erv1, resulting in the reduced Erv1. For the re-oxidising of Erv1, apart from the direct electron acceptor molecular oxygen, the cyt c also serve as the oxidising agent for Erv1 (Allen et al., 2005, Tienson et al., 2009). The in vitro study showed that the reduced ALR can be re-oxidised by cyt c more efficiently than by molecular oxygen, suggesting cyt c is a better electron acceptor comparing to molecular oxygen (Farrell and Thorpe, 2005). Additionally, the cyt c and respiratory chain complexes (e.g. complex III and complex IV) were found relevant to the redox states of Mia40 (Bihlmaier et al., 2007). Thus, the cyt c makes the MIA pathway linked to the respiratory chain. Since The Hot13 (helper of Tim of 13kDa) can chelate zinc in the IMS, it was suggested to maintain Mia40 in the oxidised form. However, there were no more evidences to support this hypothesis (Morgan et al., 2009, Mesecke et al., 2008).
1.2.7.2 Two mechanism models of the MIA pathway

So far, there are two models for the molecular mechanism of MIA pathway, disulphide bond relay system and the ternary complex model (Figure 1. 9 and Figure 1. 10). In the first model, the disulphide bonds are introduced to substrates by the consecutive thiol-disulphide exchange reactions occurring between substrates and Mia40, and then Mia40 and Erv1 (Figure 1. 9) (Mesecke et al., 2005). However, some results support the second model suggesting that a Sub-Mia40-Erv1 ternary complex is formed in order to promote disulphide transfer to substrates, and then the oxidised substrates and Erv1 are dissociated from Mia40 (Stojanovski et al., 2008a).

In the case of a disulphide bond relay system, once a precursor of substrate is imported into mitochondria, it is recognised by the redox active CPC motif of Mia40 to form a mixed disulphide bond. This was supported by the observation that only oxidised Mia40 is able to participate in the formation of mixed disulphide bond (Muller et al., 2008, Chacinska et al., 2004, Mesecke et al., 2005). Then, a disulphide bond is transferred from oxidised Mia40 to reduced substrate through the thiol-disulphide exchange action. Subsequently, the substrate containing a disulphide bond is released in the oxidised form, meanwhile Mia40 CPC is reduced. Because substrates of Mia40 own more than one disulphide bonds, another oxidised Mia40 molecular may be required to continually donate disulphide bonds to the substrate. The sulfhydryl oxidase Erv1 is used to maintain Mia40 in the oxidised form. In turn, the reduced Erv1 is reoxidised by oxidised cytochrome c (cyt c) through FAD. Electrons derived from cyt c are coupled with respiratory chain complexes of the IM, which are ultimately delivered to O2 becoming H2O (Mesecke et al., 2005, Banci et al., 2009, Stojanovski et al., 2008b) (Figure 1. 9).
Figure 1. The model of disulphide relay system. The substrate is translocated into the IMS by the TOM complex, and subsequently recognised by Mia40 forming a mixed disulphide bond. While a disulphide bond is transferred to the substrate, Mia40 becomes reduced. Then the reduced Mia40 is reoxidised by the oxidised Erv1 which can be kept in the oxidised form by cyt c and O₂. In this way, the oxidised Mia40 is continually generated for another redox reaction.
Alternatively, the model of ternary complex formation suggests the substrate forms a ternary complex with Mia40 and Erv1 after emerging in the IMS (Figure 1. 10). In this model, Erv1 also participates in the donation of disulphide bonds to substrates, rather than in the reoxidation of Mia40 (Rissler et al., 2005, Stojanovski et al., 2008a). Therefore, the formation of a ternary complex increases the efficiency of thiol-disulphide exchange, leading to the formation of multiple disulphide bonds in one reaction. When the substrate is fully oxidised, the ternary complex is dissociated resulting in release of reduced Mia40 and Erv1. To oxidise the next incoming substrate, Erv1 is reoxidised by O₂ or cyt c, while the reduced Mia40 is reoxidised by oxidised Erv1 (Figure 1. 10).

Figure 1. 10 The model of ternary complex formation. After passaging through the OM via TOM complex, the reduced substrate forms a ternary complex with oxidised Mia40 and Erv1. Within the ternary complex, the oxidative folding of substrate is implemented by multiple thiol-disulphide exchange reactions, followed by the release of fully oxidised substrate. The dissociated Mia40 and Erv1 turn into the reduced form respectively, and can be individually reoxidised by the oxidised Erv1 and cyt c/O₂ (Stojanovski et al., 2008a).
The first model was proposed on the basis of \textit{in vitro} studies, while the second model was from the \textit{in organelle} studies. Thus, there are some questions for both models. Due to substrates of the MIA pathway having at least two disulphide bonds, according to the disulphide relay model, the MIA substrates have to enter a reaction cycle two times to obtain two disulphide bonds. According to the \textit{in vitro} capability of Mia40 to oxidise a substrate (Bien et al., 2010), it is possible that the two functional Mia40 can form a dimer in theory, so that the oxidation of substrate becomes more efficiently. However, this functional Mia40 dimer has not been found yet. For the ternary complex model, although it is known that Erv1 promotes the transfer of multiple disulphide bonds \textit{in vitro}, there is no evidence to show what delivers the oxidising equivalents to a substrate, a Mia40 monomer or dimer or Erv1 only.

\subsection*{1.2.7.3 Substrates of the MIA pathway}

All current known substrates of the MIA pathway are located in the mitochondrial IMS, which are low in molecular mass and mostly have characteristically conserved cysteine motifs such as twin CX$_3$C or CX$_9$C motifs (Herrmann and Kohl, 2007). In these motifs, cysteine residues are separated by 3 or 9 various residues. In the oxidised states, these proteins are folded in a helix-loop-helix structure, which is stabilised by two juxtaposed intramolecular disulphide bonds (Beverly et al., 2008, Webb et al., 2006). All members of the small Tim family, consisting of Tim8, Tim9, Tim10, Tim12 and Tim13, contain a twin CX$_3$C motif. These proteins form two complexes (Tim8-Tim13 and Tim9-Tim10) that play crucial roles in the transport of hydrophobic mitochondrial proteins across the aqueous IMS, and they are studied as model substrates of the MIA pathway. (Koehler, 2004). Since my project is focused on structure and function of the small Tim proteins, more information will be provided in the section 1.3.
For substrates with the twin CX\textsubscript{9}C motif, 14 proteins have been identified in the yeast mitochondrion, e.g. Mia40, Cox17, Cmc1, Cox19, Cox23, and Mdm35, which contribute more diverse functions than members of the twin CX\textsubscript{3}C motif group (Longen et al., 2009). Mia40 itself as the substrate of MIA pathway utilises the twin CX\textsubscript{9}C motif for import and to stabilise folding of the protein (Hofmann et al., 2005). Cox17 is one of the best-studied members of the twin CX\textsubscript{9}C motif group, which has a copper binding site and is involved in the biogenesis of cytochrome c oxidase (COX) (Banci et al., 2008). Additionally, Cmc1, Cox19, and Cox23 are also required for the biogenesis of COX, whereas they are not able to bind copper (Longen et al., 2009). Mdm35 plays an important role in the regulation of mitochondrial phospholipid metabolism by binding to the key regulation factor Ups1/PRELI-like protein (Potting et al., 2010).

In addition to substrates containing twin CX\textsubscript{9}C and twin CX\textsubscript{9}C motifs, some IMS proteins without these two motifs still use the MIA pathway for biogenesis. For instance, Erv1 has two CXXC and a CX\textsubscript{16}C motifs, and is a substrate of the MIA pathway (Gabriel et al., 2007). Ccs1, a copper chaperone in the IMS, is also a substrate of MIA pathway but contains a CXXC and a CXC motifs. It is involved in the import of Sod1 (Cu,Zn-superoxide dismutase) into mitochondrial IMS, after translation of Sod1 in cytosol (Field et al., 2003). Moreover, human anamorsin is the first identified cytosolic iron-sulfur (Fe/S) protein imported into the IMS, which has a twin CXXC motif at the C-terminal domain (Banci et al., 2011b).

1.3 Small Tim proteins

In the mitochondrial IMS, there is an evolutionarily conserved family of approximately 10kDa proteins, called the small Tim proteins. There are five small Tim proteins in Saccharomyces cerevisiae: Tim8, Tim9, Tim10, Tim12 and Tim13. Orthologs of the small Tim proteins can be found in humans and other eukaryotes,
although some organisms have other examples of related genes in their evolution (Gentle et al., 2007). Tim9, Tim10 and Tim12 are essential for viability of yeast, but Tim8 and Tim13 are dispensable (Koehler et al., 1999, Bauer et al., 1999). TIMM8A (or DDP1), the human ortholog of Tim8 is implicated in the neurodegenerative disorder Mohr-Tranebjaerg syndrome, also known as dystonia-deafness syndrome.

1.3.1 Twin CX₃C zinc-finger motif

According to the alignment of 5 yeast small Tim proteins, each protein of this family has 4 conserved cysteine residues forming a signature twin CX₃C zinc-finger motif (Figure 1.11). However, as well as 4 conserved cysteine residues within the twin CX₃C motif, the yeast Tim12 has two additional cysteine residues which seem not to be essential for function (Jarosch et al., 1996). In each CX₃C region, two cysteine residues are separated by three amino acids. Mass spectrometry and X-ray crystal structure studies show that the 4 cysteine residues of the twin CX₃C motif form two juxtaposed intramolecular disulphide bonds (Baker et al., 2009). As a whole, the secondary structure of the small Tim proteins is composed of two α-helices, each of which contain a CX₃C region. Between the two CX₃C regions, there are 11~16 amino acids, which form a central loop. Therefore, small Tim proteins adopt the structure of a helix-turn-helix α-hairpin-fold, which is stabilised by intramolecular disulphide bonds arising from cysteines of the twin CX₃C motif (Figure 1.12) (Webb et al., 2006, Baker et al., 2009).

So far, most studies about cysteines of small Tim proteins use single cysteine mutants of Tim9 and Tim10. The in vitro study of Tim10 found that mutation of single cysteine residue affected the folding of Tim10 and increased the tendency to form intermolecular covalent disulphide bonded dimers (Allen et al., 2003). For
the effects of the single cysteine mutants of Tim10 on function, the formation of Tim9-Tim10 complex is severely affected, the import and chaperone activity are reduced. Furthermore, the two intramolecular disulphide bonds of Tim10 are not equally important for the protein folding and complex formation, since it is found that the inner disulphide bond is more important for complex formation than the outer disulphide bond (Allen et al., 2003). On the other hand, the *in vivo* study using the single cysteine mutants of Tim9 and Tim10, showed that the first cysteine was essential for the interaction with Mia40, the last cysteine had a role for the release of fully oxidised Tim10 from Mia40 (Milenkovic et al., 2007, Sideris and Tokatlidis, 2007). In addition, it also has been shown that the imported first cysteine mutant of Tim10 can not form the Tim9-Tim10 complex *in vivo* (Lu et al., 2004a). Based on the *in vivo* studies, a model for the oxidative folding of Tim10 by Mia40 was proposed. It is presumed that the formation of inner disulphide bond is prior to the outer disulphide bond. However, the detailed mechanism of how each disulphide bond is formed is still unknown.

![Sequence alignment of yeast small Tim proteins](attachment:sequence_alignment.png)

**Figure 1. 11 Sequence alignment of yeast small Tim proteins.** The amino acid sequences of yeast small Tim proteins were retrieved from the Saccharomyces Genome Database (SGD, [http://www.yeastgenome.org](http://www.yeastgenome.org)). The alignment of 5 protein sequences is produced by using Cobalt Constraint-based Multiple Protein Alignment Tool from NCBI. The conserved residues including methionine and cysteine are highlight in red.
It has been shown that the cysteine thiol is one of the most prominent groups for binding to Zn$^{2+}$, so it was suggested that small Tim proteins could use four cysteine residues as a zinc-finger to hold Zn$^{2+}$ (Figure 1. 12)(Koehler, 2004, Morgan et al., 2009, Lu et al., 2004b). Trypsin treatments of mitoplasts that were incubated with EDTA or Zn$^{2+}$ showed that Tim10 and Tim12 could be stabilised in the presence of Zn$^{2+}$ but not in the present of EDTA(Murphy et al., 2001). Furthermore, ITC measurements of the Zn$^{2+}$ binding to the reduced and oxidised Tim9 or Tim10 showed that only the reduced proteins can bind Zn$^{2+}$ at a molar ratio of 1:1, but the oxidised proteins can not (Lu et al., 2004b). Subsequently, it has been shown that Tim9, Tim10 and Tim12 in functional states are not Zn$^{2+}$ bound, but instead form disulphide bonds (Gentle et al., 2007).

**Figure 1. 12 Possible states of twin CX$_3$C zinc-finger motif.** The mitochondrial small Tim proteins contain characteristic twin CX$_3$C zinc-finger motif. *In vitro*, there are three possible states for individual small Tim protein: (1) all four cysteine residues in the motif are reduced, (2) cysteine residues are reduced with coordination of a zinc ion (Zn$^{2+}$), (3) cysteine residues are fully oxidised and form two intramolecular disulphide bonds.
1.3.2 Biogenesis of the small Tim proteins

Like most of the mitochondrial proteins, the small Tim proteins are also encoded by nuclear DNA, and synthesised in the cytosol. Thus, the small Tim proteins have to be imported individually into mitochondrial IMS and form the Tim9-Tim10 or Tim8-Tim13 complex for their functions. According to in vitro studies, the mechanism of import and assembly has been developed by using Tim9 and Tim10 as models, and is regulated by the redox state of twin CX₃C motif (Figure 1. 13) (Lu et al., 2004a). Generally, this model can be divided into three major steps: (1) the import of cysteine-reduced small Tim proteins into mitochondria, (2) the oxidative folding of translocated small Tim proteins in the IMS, (3) formation of functional complexes. The details of each step are described in the following sections.

**Figure 1. 13 The model for the biogenesis of Tim9-Tim10 complex.** There are three major steps involved in this model. (1) Only the unfolded, cysteine reduced (not oxidised) proteins can be imported into mitochondria. (2) After passage across the outer membrane, the twin CX₃C motifs of Tim9 and Tim10 are oxidised to form two pairs of intra-molecular disulphide bonds. The oxidative folding is catalysed by the Mia40/Erv1 system. (3) The oxidised proteins interact with each other and assemble into a functional hexameric Tim9-Tim10 complex. Figure adapted from Lu, H., Allen, S., 2004.
1.3.2.1 Import of small Tim proteins

Although the small Tim proteins are small in size (~10 kDa), oxidatively folded proteins can not be imported (Morgan and Lu, 2008). The newly synthesised precursor proteins have to be maintained in an import-competent state by factors in the cytosol, because in vitro studies showed that the redox-sensitive twin CX\textsubscript{3}C motif can be oxidised by glutathione in the cytosol. Studies have shown that oxidized precursor proteins cannot be imported into mitochondria and the import-competent proteins are only in reduced forms (Lu et al., 2004a, Morgan and Lu, 2008).

One of the stabilisation factor for the small Tim proteins is zinc. As described in the section 1.3.1, the reduced twin CX\textsubscript{3}C motif is able to coordinate a molecular of Zn\textsuperscript{2+}, leading to stabilisation of the proteins in cysteine-reduced state (Lu and Woodburn, 2005). However, precursor in Zn\textsuperscript{2+} bound form can not be efficiently imported into mitochondria (Morgan et al., 2009). Furthermore, the affinity of Zn\textsuperscript{2+} binding is relatively low, compared with free Zn\textsuperscript{2+} concentration in cell. These suggest that Zn\textsuperscript{2+} binding may be not the only factor in the cytosol for stabilisation of reduced small proteins in the import-competent state.

In addition to the role of Zn\textsuperscript{2+} in stabilising the small Tim proteins in their reduced forms, recently it has been shown that the cytosolic thioredoxin (Trx) system is required for maintaining precursor proteins in the reduced and import-competent form before import into mitochondria (Durigon et al., 2012). The Trx system and the glutaredoxin (Grx) system play important roles in cellular thiol regulation and oxidative stress defence, and can reduce disulphide bonds of substrates while receiving electrons from nicotinamide adenine dinucleotide phosphate (NADPH) (Meyer et al., 2009). Although the enzymes of the Trx system, Trx1, Trx2 and thioredoxin reductase (Trr), are located in the cytosol, they can facilitate the
import of IMS redox-sensitive proteins, especially preferring small Tim proteins as substrates. In particular, the Trx enzymes favour partially oxidised over fully oxidised substrates, which may prevent the early stages of small Tim folding from happening in the cytosol (Durigon et al., 2012). Thus, the import efficiency or level of small Tim proteins is affected by oxidation of the proteins, and is relevant to Trx enzymes in the cytosol.

1.3.2.2 Oxidative folding of small Tim proteins

The oxidative folding of imported small Tim proteins is accomplished by the MIA pathway in the IMS. The in vitro studies of Tim9 and Tim10 showed their most N-terminal cysteines specifically interact with Mia40 (Milenkovic et al., 2007, Sideris and Tokatlidis, 2007). Additionally, it also showed that the most C-terminal cysteine of Tim10 was essential for release of oxidised substrates from Mia40 (Sideris and Tokatlidis, 2007). Based on above evidence, there is a model for oxidative folding of Tim10 by Mia40 (Figure 1. 14) (Sideris and Tokatlidis, 2007).

The N-terminal cysteine (C1) of Tim10 is recognised by oxidised Mia40 in the site-specific manner, meanwhile the intermolecular disulphide bond is formed between Tim10 C1 and Mia40. Following an unknown mechanism, the C2 and C3 form the inner disulphide bond. The formation of inner disulphide presumably creates a folded conformation that brings the C4 in proximity to C1. The C4 attacks the intermolecular Tim10-Mia40 disulphide bond, resulting in the formation of an outer disulphide bond. With release of folded Tim10, reduced Mia40 is re-oxidised through the MIA pathway as detailed in section 1.2.7. According to the folding trap hypothesis, the folded state prevents back-translocation out of the mitochondria, Tim10 is irreversibly folded (Neupert and Herrmann, 2007).
Figure 1. Model of the oxidative folding of Tim10 mediated by Mia40. The reduced Tim10 docks on the oxidised Mia40, and is recognised via the most N-terminal cysteine (C1). An intermolecular disulphide bond is formed between C1 and Mia40. Then, the inner disulphide bond of Tim10 is formed through an unknown mechanism, leading to the folded conformation. The intermolecular disulphide bond is attacked by the free C4, followed by the formation of outer disulphide bond of Tim10. At last, the folded Tim10 is released, which is coupled with the re-oxidation of Mia40 in the MIA pathway. Figure adapted from Sideris and Tokatlidis, 2007.
**1.3.2.3 Formation of small Tim complexes**

While maintaining the reduced and unfolded form is essential for import, it has been found that only oxidised small Tim proteins containing disulphide bonds can form the Tim9-Tim10 or Tim8-Tim13 complexes (Lu et al., 2004a). Using biophysical techniques coupled with protein mutagenesis, it has been demonstrated that the assembly of Tim9-Tim10 complexes process can be divided into 4 kinetically distinguishable steps (Figure 1. 15)(Ivanova et al., 2008). For the individual proteins, Tim9 forms a homo-dimer while Tim10 stays as a monomer. Firstly, upon interaction with Tim10, the Tim9 dimer dissociates, enabling the formation of a Tim9-Tim10 hetero dimer (Tim9*Tim10). Secondly, the hetero-dimers probably form into a tetramer intermediate whose inner layer consists of each subunit’s N-terminal helices and C-loops, while the outer layer is composed of unassembled C-terminal helices. The conformation of the tetramer intermediate results in exposed hydrophobic patches between helices, which might interact with substrates for chaperone-like functions. Subsequently, the outer layer of the tetramer intermediate accomplishes assembly and the hydrophobic patches become hidden. At the same time or in the following step, the hexamer Tim9-Tim10 complex is formed and packs into the final conformation. Before the formation of tetramer intermediate, the process of Tim9-Tim10 complex formation is fast, due to protein–protein interaction driven by the electrostatic force. However, the process form tetramer intermediate assembling into hexamer complex becomes slower, because protein-protein interaction is driven by the hydrophobic force that is less efficient than the electrostatic force.
Figure 1. The assembly process of the Tim9-Tim10 complex. The assembly requires 4 steps. The first step is the formation of hetero dimer coupled with the dissociation of dimeric Tim9. The second step is the formation of tetramer intermediate. The third and fourth steps are formation of the hexamer complex and final conformation packing. This figure is taken from Ivanova, E., Jowitt, T. A. & Lu, H. 2008.
1.3.3 Structures of small Tim complexes

Tim9 exclusively partners with Tim10, and Tim8 partners with Tim13 to form the hexameric Tim9-Tim10 and Tim8-Tim13 complex respectively. The crystal structures of both Tim9-Tim10 and Tim8-Tim13 complexes have been solved (Webb et al., 2006, Beverly et al., 2008, Baker et al., 2009). The 2.5Å X-ray crystal structure of the yeast Tim9-Tim10 hexameric complex revealed that it forms a six bladed α-propeller structure with alternating subunits (Figure 1. 16 A). The topology of the yeast Tim9–Tim10 complex is similar to that of its human counterparts and the yeast Tim8-Tim13 Complex (Beverly et al., 2008, Webb et al., 2006). Each subunit folds into a helix-loop-helix conformation; the two antiparallel N- and C-terminal helices are separated by a central loop (C-loop) (Figure 1. 16 B). This structure confirms that two intramolecular disulphide bonds are formed in the small Tim proteins by the juxtaposed cysteines of the twin CX3C motif. The C-loops of six subunits are linked into a circle by salt bridges, and arranged into a relatively flat doughnut-shaped core. The residues participating in the salt bridges are strictly conserved (Figure 1. 11). In the hexamer of the Tim9-Tim10 complex, twelve antiparallel helices extend from one face of the core to the other, and contact one another in coiled coil type interactions. These helices are divided into inner layer and outer layer, and each layer contains six helices. The six shorter N-terminal helices from each subunit form an inner layer, and are aligned approximately 15° to the molecular axis. Although there are few intermolecular contacts within the core region of the inner helices, hydrophobic residues in the N-terminal inner helices contact adjacent subunits. In contrast, six longer C-terminal helices form an outer layer and are inclined about 60° to the molecular axis, creating the blades of the propeller. The outer helices only make contacts in the core region. Almost all hydrophobic amino acids of the complex are hidden inside two layers of helices, and hydrophilic amino acids are
distributed at the inner and outer surfaces. This makes the complex soluble in the aqueous environment of the IMS (Baker et al., 2009, Webb et al., 2006).

**Figure 1. 16 Crystal structure of the yeast Tim9–Tim10 hexameric complex.** (A) Ribbon diagram depicting the hexameric Tim9-Tim10 complex with a horizontal view (left) and a vertical view (right). (B) Ribbon diagrams of subunits, Tim9 and Tim10, which are formed in hairpin-like structures by two intramolecular disulphide bonds (in yellow). The crystal structure is taken from BAKER, M. J., 2009.
The Tim9-Tim10 complex is essential for the viability of yeast, but the Tim8-Tim13 complex can be deleted without an obvious effect on the growth of yeast cells. The Tim9-Tim10 complex is 10-fold more abundant than the Tim8-Tim13 complex (Murphy et al., 2001).

The crystal structure of the yeast Tim9–Tim10 complex was found to be similar to that of the *Methanobacterium Thermoautotrophicum* prefoldin and *E. Coli* Skp chaperone (Walton and Sousa, 2004, Siegert et al., 2000). The function of these two proteins is to protect soluble and membrane proteins from aggregation, similar to that of the small Tim complex. The structural similarity suggested that the small Tim complex might employ a similar mode for its function. The crystal structures of prefoldin and Skp reveal that the there is a substrate-binding cavity formed by the tentacles, and the termini of the tentacles are used to grip substrate proteins. In the Tim9-Tim10 complex, twelve antiparallel helices also form a clamp-like structure similar to prefoldin and Skp. The tips of helices mediate all substantial lattice contacts, and evidence suggests that truncations at the N-terminus of Tim10 disable substrate binding (Webb et al., 2006, Vergnolle et al., 2005).

Recently, it was found that Tim12 also forms a soluble complex with the Tim9-Tim10 complex during its mitochondrial import process (Gebert et al., 2008). However, the structure and function of the ternary Tim9-Tim10-Tim12 complex is not clear.

### 1.3.4 Roles of small Tim complexes in the TIM22 pathway

The role of small Tim complexes was firstly found in the biogenesis of mitochondrial metabolite carrier proteins, which facilitates the imported proteins passaging the IMS for insertion into the IM by the TIM22 complex. Although there are more than 30 carrier proteins in the IM, the ADP/ATP carrier protein (AAC) is
one of the most studied examples. The AAC consists of six transmembrane helices forming three paired helix modules, which is similar to the hexameric Tim9-Tim10 complex (Pebay-Peyroula et al., 2003). The precursor of AAC is synthesised in the cytosol without presequences. Since these inner membrane proteins are highly hydrophobic, most of them require cytosolic chaperones (e.g. Hsp70 in yeast and Hsp70 and Hsp90 in mammal) to remain soluble in the cytosol, and the substrate-chaperone complexes dock onto Tom70 of the TOM40 complex (Young et al., 2003). After crossing the OM via the TOM40 complex in a loop conformation, they are bound by the Tim9–Tim10 complex in the IMS for delivery to the TIM22 complex (Figure 1. 2). The insertion of substrate proteins into the IM requires the membrane potential ($\Delta \psi$) across the IM.

Although little is known about how Tim9-Tim10 complex or subunits bind to AAC, there are two suggested models, based on the crystal structures of human Tim9-Tim10 complex and AAC (Figure 1. 17)(Webb et al., 2006). The first model suggested that two pairs of tentacles of Tim9 and/or Tim10 compact into a four-helix bundle to hold a molecule of AAC in the form of “bisecting U”. This model is similar to that of tyrosine kinase and JAK/STAT signaling molecules associating with their receptors APS, SH2-B and LNK proteins (Dhe-Paganon et al., 2004). In the second model, a module of AAC is suggested to interact with Tim9-Tim10 complex by replacing a Tim9 subunit from complex. This process is repeated, resulting in three Tim9 subunits being substituted by a single AAC. Thus, a recombined complex contains three Tim10 subunits and one AAC. This model is consistent with a previous study, in which radioactive AAC was used and it was found that the major crosslink arrested translocation products are AAC bound with one and two molecules of Tim10. This result is consistent with replacement of one or more Tim9 subunits by AAC (Endres et al., 1999). Both models suggested that some form of Tim9-Tim10 complex but not individual Tim9 or Tim10 could interact with AAC precursor.
Figure 1. 17 Tim9-Tim10 complex mediated the biogenesis of AAC. The AAC precursor is passaged through the OM by the TOM complex. In the IMS, there are two models of interaction with Tim9-Tim10 complex for transport to the TIM22 complex. In the bisecting U model, four tentacles of Tim9 and/or Tim10 insert into the helix bundle of AAC, by which the AAC is hold to the TIM22 complex. In the replacing model, the AAC replaces one Tim9 of hexamer Tim9-Tim10 complex with one double helix module, which is repeated until three Tim9 are replaced by the three repeat modules of AAC. Figure adapted from Webb et al., 2006.
Other than biogenesis of metabolite carrier proteins, TIM22 pathway also participates in the translocation of two integral subunits of the TIM23 complex, Tim17 and Tim23. The precursor proteins are chaperoned by the small Tim complexes across the IMS before insertion into the IM through the TIM22 pathway. Mitochondrial import and cross-linking assays indicated that the import of Tim17 is only mediated by the Tim9-Tim10 complex, while both Tim9-Tim10 and Tim8-Tim13 complexes share an equal role in the import of Tim23 though selective interaction with different regions of precursor Tim23 during its import (Leuenberger et al., 1999, Paschen et al., 2000). The Tim8-Tim13 complex prefers to bind the central region of Tim23 (amino acids 77–103), whereas Tim9-Tim10 complex binds to the C-terminal half of Tim23 (Beverly et al., 2008, Davis et al., 2007).

1.3.5 Roles of small Tim complexes in other biogenesis pathways

The mitochondrial OM has similarity to the outer membranes of bacterium and chloroplast, which contain β-barrel integral membrane proteins. Tom40, the central component and channel forming protein of the Tom complex, is one of them. After protein synthesis in the cytosol, Tom40 precursor is translocated into the IMS, and chaperoned by small Tim complexes to the SAM complex for insertion into the OM. Mitochondrial import assays show that defects of either Tim9-Tim10 complex or Tim8-Tim13 complex inhibits the biogenesis pathway of Tom40 at an early stage during formation of a Tom40-SAM intermediate (Wiedemann et al., 2004, Baker et al., 2009).

Apart from the roles of small Tim proteins in the TIM22 and SAM pathways, the small Tim proteins are also utilised in other pathways by some proteins containing only one transmembrane region. For example, Phb1 is a homologue of prohibitins located at the mitochondrial IM. The Phb1 precursor is chaperoned by
the Tim8-Tim13 complex in the IMS before insertion into the IM through the TIM23 complex (Tatsuta et al., 2005). Another example is the mammalian subunit ε which is essential for self-association of mammalian F₀F₁ ATP synthase (Bisetto et al., 2008). It was previously identified as a homologue to yeast Tim11, and uses both Tim9-Tim10 complex and Tim8-Tim13 complex for its translocation in the IMS to the matrix (Leuenberger et al., 1999, Devenish et al., 2000).
1.4 Aims and objectives

Although the small Tim proteins of the mitochondrial IMS have been known for many years, studies of their structures and functions in cells are still ongoing. Using in vitro methods, structural characterisation of Tim9 and Tim10 have been carried out. Moreover, using in vitro or in organelle methods, it was demonstrated that Cys-redox regulation plays an important role in the biogenesis of small Tim proteins and formation of the functional complex (Lu et al., 2004). In the case of Tim9 and Tim10, they import into mitochondria in the reduced form. In the IMS, their oxidative folding and formation of disulphide bonds are mediated by the MIA pathway. Finally, the oxidised proteins assemble into Tim9-Tim10 complex. Tim12 is found important for biogenesis of inner membrane proteins, and functions together with Tim9 and Tim10. The common feature of all three proteins is the presence of a pair of disulphide bonds. However, it has not been clear about the structural characterisations of Tim12 and roles of disulphide bonds of small Tim proteins in cell. Thus, overall aim of this study is to understand the role of individual cysteines on protein conformation and how Tim9, Tim10 and Tim12 form a functional complex. The following objectives need to be accomplished:

1. Purification of Tim12. Tim9 and Tim10 have both been well characterised, but much less is known about the biogenesis of the other essential Tim protein Tim12. So far, Tim12 was mainly studied using in vivo methods, as in vitro studies seem to be hindered by the lack of abundant purified Tim12. In order to overcome difficulties with purification, this study will test the optimal methods for expression and purification, using GST-tagged or His-tagged recombinant proteins. The purified protein will be studied using biophysical methods to in order to enable structural characterisation of Tim12.
2. Investigation of complex formation between purified proteins. Previously, Hui had studied about the complex formation of small Tim proteins used mutants of Tim10. In this study, using the purified proteins, the conformation will firstly be addressed. Importantly, the interaction and complex formation with Tim10 will be studied using *in vitro* methods.

3. Investigation of relevance between functions and formed complexes. Due most *in vivo* studies of small Tim proteins have concentrated on their interactions with other substrates, the questions about how the cysteines of small Tim proteins are involved in the biogenesis of themselves and assembly of complexes remain without answers. In this study, cysteine mutants of small Tim proteins will be studied for their effects on cell viability and functional complex formation, using yeast genetic methods coupled with in organelle methods.
2 MATERIALS AND METHODS

Most of general chemical materials and reagents used in this study were purchased from Sigma, unless otherwise stated.

2.1 Molecular biology techniques

All the primers (Eurofins MWG Operon, German), cloning vectors, *E.Coli* host strains and DNA templates used in this study are listed in Appendix.

2.1.1 DNA cloning

To construct a plasmid with the target gene, DNA fragment was amplified by Polymerase Chain Reaction (PCR). The primers for PCR were designed manually based on DNA template with desired restriction sites added on the 5’- and 3’-ends. Typically, each 20µl PCR mix contained 1U of Taq-polymerase (Fermentas, UK), 2µl of 10×PCR-buffer, 0.6µl of 10mM dNTPs, 1µl of 20 pmol/µl primer each and 100ng of plasmid DNA template. The rest of the volume was made up by sterile ddH₂O. PCR was performed at 94°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 45~65°C (depending on the Tₘ of synthesised primers) for 1 minute and 72°C for 1~3.5min (depending on the length of expected amplicon). A final cycle of 72°C for 10 minutes completed the reaction. The amplicon was analysed by 1% Agarose gel electrophoresis and then extracted from gel by using QIAGEN Gel Extraction Kit (Qiagen, UK).

To generate the insert for DNA cloning, purified PCR product was digested with selected restriction enzymes (New England Biolab, UK). Reaction conditions such as incubation time, template concentration and buffer for the different restriction enzymes varied and were adjusted according to manufacturer’s recommendations. Meanwhile, circularised vector was linearised by digestion with the same
restriction enzymes as the insert. Digested DNA was purified from 1% Agarose gel before ligation. The ligation reaction was performed by mixing the vector (50-200ng) and insert at 1:5 ratio in the presence of 1×ligation buffer, and 2U of T4-DNA ligase (Fermentas, UK) in a total volume of 10µl and incubated overnight at 16°C.

Bacterial transformation was achieved by incubating 5µl ligation mixture and 50µl of E.coli competent cells for 1 hour on ice before a heat-shock step at 42°C for 50 sec followed by an immediate incubation on ice for 5 minutes. The transformed cells were recovered in 250µl of SOC medium at 37°C for 1 hour with moderate shaking condition. The cell culture was centrifuged at low speed to remove 250µl of supernatant. The cell pellet was resuspended by pipetting and then spreaded on LB plates containing either 50 µg/ml of kanamycin or 100 µg/ml of ampicillin depending on the vector used. Plates were incubated overnight at 37°C. The bacterial colonies were screened by PCR or DNA sequencing (GATC Biotech, Germany).

### 2.1.2 Site-directed mutagenesis

The cysteine mutations of small Tim proteins to serine or alanine were generated by PCR site-directed mutagenesis. The primers were designed manually with the desired mutations located at the centre of primers. Each PCR reaction contained 5µl of 10×Pfu polymerase buffer (Fermentas, UK), 1µl of 10mM dNTP, 1µl of DNA template, 1µl of 20 pmol/µl primer each, 5U of Pfu polymerase (Fermentas, UK) and 40µl sterile ddH₂O. PCR was performed at 95°C for 30sec followed by 16 cycles of 95°C for 30sec, 55°C for 30sec and 68°C for 7 minutes. The parental DNA template was digested by adding 6µl 10×buffer 4 (NEB, UK), 2µl of Dpn1 and 2µl of sterile ddH₂O. The digestion mixture was incubated at 37°C for 3 hours, followed by transformation of 5µl digested plasmid into E.coli TOP1 cells. Colonies
containing plasmid with desired mutagenesis were screened by DNA sequencing (GATC Biotech, Germany).

2.1.3 Colony PCR of constructed plasmids

Selected colonies were picked up by a sterile toothpick and resuspended in 10µl sterile ddH$_2$O as a working template. Typically, each PCR contained 2µl of 10×PCR buffer, 1.2µl of 25mM MgCl$_2$, 0.6µl of 10mM dNTP, 1µl of each 20 pmol/µl primer, 0.5µl of Taq polymerase, 1µl of template and 12.7µl of ddH$_2$O. The reaction was run for 1 cycle of 95°C for 7 minutes, 35 cycles of 95°C for 1 minutes, 54°C for 1.5 minutes, 72°C for 1 minutes, and 1 cycle of 72°C for 5 minutes. PCR products were checked by 1% Agarose gel electrophoresis.

2.2 Biochemical methods

2.2.1 Protein expression and purification from E.coli

2.2.1.1 Expression and purification of GST-tagged protein

A pre-culture was prepared by inoculating a few colonies of E.coli carrying desired plasmid DNA constructs into sterile LB media containing 100 µg/ml Ampicillin and incubating overnight at 37°C with shaking at 200rpm. The cell culture was scaled up by addition of 20ml of the pre-culture into 1L of fresh LB media containing 100 µg/ml Ampicillin and the growth was continued at 30°C with shaking at 200rpm until the A$_{600}$ reached at 0.4~0.5. IPTG (0.1 g/L) was added into culture for induction, which took 3~4 hours at 30°C with shaking at 200rpm until the A$_{600}$ reached at 1.5~2.

Cultured cells were harvested by spinning at 4°C, 6500g for 15 minutes. The pellet was resuspended in ddH$_2$O and transferred into a 50ml falcon tube. Cells
were washed by Buffer A (50mM Tri-HCl, 150mM NaCl, pH 7.4) and then stored at -20°C for protein purification later.

In order to break the cell wall, the harvested cells were resuspended in pre-chilled 20ml Buffer A containing 0.1ml of 10 mg/ml lysozyme were incubated on ice for 15 minutes before sonication (30% amplitude power-on for each 10 sec with an interval of 30 sec in a total time of 20 minutes on ice). The soluble fraction was separated from inclusion bodies by centrifugation at 4°C, 27,000g for 15 minutes.

In the case of protein purification of insoluble protein, the pellets were resuspended in 10ml of Buffer B (50mM Tri-HCl, 150mM NaCl, pH 7.4, 6M urea, 10mM DTT). The solubilisation of inclusion bodies was achieved by incubation at room temperature for 1 hour. The denatured proteins were separated from the cell debris by centrifugation at 20°C, 48,000g for 30 minutes. The supernatant diluted 10-fold using 90ml Buffer A in the presence of 10mM DTT by gentle mixing for 2 hours to allow protein refolding. After centrifugation at 4°C 27,000g for 15 minutes, the supernatant containing refolded proteins was kept for protein purification.

Supernatants of either soluble or insoluble fractions were filtered by 0.45µm filter, and then incubated with about 2ml regenerated GST-beads at 4°C for overnight with gently rotating to allow protein binding. The protein bound beads were washed for at least three times using Buffer A before resuspending in 2 bed volumes of Buffer A containing 5 units/ml of thrombin for GST-tag cleavage from fusion proteins. The mixture was incubated at 4°C for overnight with gently rotating. The elution of target protein was collected and stored at -80°C for use later.
2.2.1.2 Expression and purification of His-tagged protein

The method of protein expression for His-tagged protein was similar to that of GST-tagged protein, except that the cells were grown in LB media containing 50 µg/ml kanamycin. Sonication was carried out as described in GST-tagged protein method except the cells were resuspended in Binding buffer (Buffer A + 5mM imidazole). The inclusion bodies were solubilised by 20ml of Binding buffer containing 6M urea.

Supernatants from soluble or insoluble fractions were filtered with 0.45µm filter unit before applying onto a column with 2ml packed volume of Ni\textsuperscript{2+} charged His-binding Resin (Novagen, Madison, WI, USA) that was equilibrated with Binding buffer at 4°C. Beads bound with target proteins were washed by 20ml of Washing Buffer 1 (Buffer A + 20mM imidazole) followed by Washing Buffer 2 (Buffer A + 40mM imidazole). The target protein was eluted with about 6ml of Elution Buffer (Buffer A + 250mM Imidazole). The imidazole was removed from the eluted fraction by dialysis against 2-3 successive changes of 1L of Buffer AE (Buffer A + 1mM EDTA) at 4°C with gently stirring. The proteins were stored at -80°C for further purification.

2.2.2 Size exclusion chromatography

The affinity purified proteins were further purified by size exclusion chromatography. In this study, Superdex 75 10/30 column (Amersham Pharmacia) was used for protein separation, which was fixed on an ÄKTA Purifier FPLC system (Amersham Pharmacia) operated by Unicorn software. Before loading, proteins were centrifuged at 12,500g for 5 minutes to remove any protein precipitates. 0.55ml of protein was injected into FPLC by syringe. Degased Buffer A was used as running buffer at a flow rate of 0.5 ml/ minute.
2.2.3 AMS thiol-alkylation assay

The redox states of protein were tested using thiol alkylation agent AMS (Invitrogen). AMS specifically reacts with reduced sulphydryl groups but not disulphide bond. Each reacted AMS molecule increases the molecular weight by ∼0.5kDa per thiol. Thus, increased molecular weight led to lower mobility of the protein on 16% Tris-Tricine SDS-PAGE. AMS was diluted in 2× non-reducing (−DTT) SDS-PAGE sample buffer. Proteins were incubated with equal volume of diluted AMS solution in dark for 15 minutes, before analysed by 16% Tris-Tricine SDS-PAGE. For reduced sample, 1mM TCEP was added to reduce proteins before AMS assay.

2.2.4 Micro scale thermophoresis (MST) analysis

The micro scale thermophoresis (MST) is a new developed technique for the investigation of molecular interaction, which is based on the directed movement of molecular along temperature gradients. The thermophoretic movement of the fluorescently labeled molecule is measured by monitoring the fluorescence distribution F inside a capillary with the NanoTemper Monolith instrument (Figure 2. 1, left panel). The microscopic temperature gradient is generated by an IR-Laser, which is focused into the capillary and is strongly absorbed by water. The temperature of the aqueous solution inside the laser spot is raised by 2K~8K compared to the periphery and is thus creating a strong temperature gradient. Before the IR-Laser is switched on a homogenous molecule distribution is observed inside the capillary. When the IR-Laser is switched on, two effects, separated by their time-scales, are observed. First, determined by the temperature relaxation time of approximately 50ms, the fluorescence of the dye changes because of its intrinsic temperature dependence. Second the molecules move from the locally heated region to the outer cold regions and thus the
concentration of molecules in the locally heated region decreases until it reaches a steady state on a time scale of about 10s~30s which is determined by mass diffusion (Figure 2. 1, right panel). The thermophoretic depletion can be changed by size, charge or solvation entropy of molecules. Due to the binding between a target molecule and a labeled molecule can be changed by at least one of above properties, it can be quantified by measuring the thermophoretic depletion (Schermer et al., 2011).

In this study, 2µM stock protein was labeled by fluorescence dye dissolved in Buffer A containing 0.1% Tween-20 for 1.5 hours, while 20µM target protein was unlabeled and serially 2-fold diluted for 15 dilutions using Buffer A containing 0.05% Tween-20. 15µl labeled stock protein was mixed with serial dilutions of unlabeled target protein. All measurements were performed by the Nano Temp Technologies during their demo in Manchester.

![Figure 2. 1 The scheme of MST assay.](image)

Left: The solution inside the capillary is locally heated with a focused IR-laser, which is coupled into the path of light using a hot mirror. Right: the normalized fluorescence in the heated spot is plotted against time. When the IR-laser was switched on at t=5s, the fluorescence would decrease. Because the temperature was increased, the labeled molecules or complexes moved away from the heated spot due to thermophoresis.
2.2.5 Cross-linking

The purified protein was incubated with 0.1% (v/v) of Gluteraldehyde (GA) for various time points (5, 10 and 20 minutes) at room temperature in 50mM Na\textsubscript{2}HPO\textsubscript{4}, pH7.2, 150mM NaCl. At designated time points, the reactions were quenched by addition of 100mM Tris with gently mixing at room temperature for 5 minutes before mixing with SDS PAGE sample buffer (+DTT). The samples were heated at 100°C for 5 minutes before centrifugation for 5 minutes with top speed. The supernatant was loaded on 16% Tris-Tricine SDS-PAGE for analysis and visualised by Coomassie staining.

2.2.6 Agarose gel electrophoresis

Agarose was weighted out as 1g/100ml (1% gel) and dissolved in TAE buffer by heating. When the melted gel is cool enough for handle, approximately 1-5\textmu l of a 10mg/ml Ethidium bromide was added into per 100 ml of agarose. Gel is run at 100V until DNA ladders can be distinguished clearly.

2.2.7 Tris-Tricine SDS-PAGE

16% Tris-Tricine SDS-PAGE was used for analyse the small Tim proteins. To make one 16% Tris-Tricine SDS-PAGE separating gel, the recipe was used as following: 2.4ml of 30% (w/v) Acrylamide and 0.8% (w/v) Bis-Acrylamide stock solution (ProtoGel, National diagnostics), 0.15ml of 2.2% (w/v) Bis-acrylamide, 1.5ml of 1.5M Tis/0.3% (w/v) SDS pH8.45, 0.9ml of 87% (v/v) Glycerol, 20\textmu l of 10% APS and 2\textmu l TEMED. To make one Tris-Tricine SDS-PAGE stacking gel, the recipe was used as following: 0.23ml of ProtoGel, 10\textmu l of 2.2% (w/v) Bis-acrylamide, 0.46ml of 1.5M Tis: 0.3% (w/v) SDS pH8.45, 1.2ml of MQH\textsubscript{2}O, 20\textmu l of 10% APS and 2\textmu l TEMED.
The Tricine SDS-PAGE running upper buffer (Cathode buffer) was prepared as 0.1M Tris pH8.25, 0.1M Tris-Tricine, and 0.05% (w/v) SDS. The Tricine SDS-PAGE running lower buffer (Anode buffer) was composed by 0.2M Tris pH8.9. The 2×SDS-PAGE sample buffer was prepared using 4% (w/v) SDS, 8% (v/v) Glycerol, 0.02% (w/v) bromophenol blue, 80mM Tris-HCl, pH 6.8, and stored in freezer. For reducing samples, 100mM DTT was added into 2×SDS-PAGE sample buffer. Samples were mixed with an equal volume of 2×SDS-PAGE sample buffer. Before loading on gel, samples were heated at 95°C for 5 minutes, followed by centrifugation at 12,500g for 5 minutes. All gels were run using Bio-Rad mini PSII system at 30mA per gel for about 4 hours.

2.2.8 Blue Native PAGE

BN-PAGE of 6%～16% gradient mini gel was used for the study of complex formation under the native condition. The separating gel was composed of 6% and 16% separating gel solutions, and then casted by the gradient mixer. The concentration of Acrylamide in separating gel was increased from the bottom to top. For each BN-PAGE, one portion of 6% separating gel solution was prepared following the recipe: 0.81ml of ProtoGel (the commercial product including 30% (w/v) Acrylamide and 0.8% (w/v) Bis-Acrylamide), 1ml of 2M 6-Aminocaproic acid pH7.0, 0.2ml of 1M Bis-Tris pH7.0, 2ml of MQH₂O, 30µl of 10% APS, 3µl of TEMED. For each BN-PAGE, one portion of 16% separating gel solution was prepared following the recipe: 1.83ml of ProtoGel, 0.86ml of 2M 6-Aminocaproic acid pH7.0, 172µl of 1M Bis-Tris pH7.0, 0.6ml of MQH₂O, 30µl of 10% APS, 3µl of TEMED. The BN-PAGE stacking gel for each separating gel was prepared using the following recipe: 0.35ml of ProtoGel, 625µl of 2M 6-Aminocaproic acid pH7.0, 125µl of 1M Bis-Tris pH7.0, 1.37ml of MQH₂O, 25µl of 10% APS, 6µl of TEMED.
The BN-PAGE Cathode buffer that would be filled in the running cassette was prepared including: 50mM Tricine, 15mM Bis-Tris, 0.02% (w/v) Coomassie blue, pH7.0. The BN-PAGE Anode buffer that would be filled in the tank contained 50mM Bis-Tris, pH7.0. The 10×BN-PAGE sample buffer consisted of 0.5M 6-Aminocaproic acid pH 7.0, 100mM Bis-Tris pH7.0, 20mM DTT and 5% Coomassie blue. Prepared samples were mixed 1/10 with 10×BN-PAGE sample buffer before loading on the gel. The BN-PAGE gel was run at 4°C, at 150V, until the dye front went into the separating gel, followed by 200V for about 2 hours.

2.2.9 Gel transfer and western-blot

In order to analyse by western-blot, gels were transferred onto nitrocellulose membranes (for Tricine SDS-PAGE) or onto PVDF membranes (for BN-PAGE). Gels and nitrocellulose membranes were soaked in transfer buffer (25mM Tris, 192mM Glycine, 20% (v/v) methanol) for 5 minutes. However, PVDF membranes were soaked in 100% methanol for 1 minute for activation. Gels and membranes were laid onto two pieces of filter paper as a sandwich and air bubbles were removed. This was dropped into a cold transfer buffer filled tank with the gel in the cathode (-) side, before. Normally, transfers were carried out at 300mA for 1 hour, but BN-PAGE transfer was performed at 150mM for 90 minutes. It is available for overnight transferring at 20mA locating in cold room.

To confirm transfer, membranes were stained with Ponceau S solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid). The stain can be completely removed from the protein bands by continued MQH₂O rinsing. For general proposes, transferred membranes were blocked for 1 hour at room temperature in Phosphate Buffer Saline Tween (PBS, 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 2mM NaH₂PO₄, pH 7.4, 0.1% Tween-20) containing 5% dried skimmed milk (or 0.3% (w/v) BAS for the western-blot of αHis-tag), and incubated with gentle
agitation. However, for PVDF membrane from BN-PAGE imaging by the Li-COR system, the blue on the membrane was faded by 90% methanol before blocking. At the end of the blocking incubation, membranes were washed twice for 5 minutes with fresh changes of PBST at room temperature. Primary antibody was diluted in PBST at 1:1000, and incubated for 1 hour at room temperature or overnight at 4°C. The blot was washed with PBST for 10 minutes, up to 3 times before incubation with diluted secondary antibody (Li-COR antibody) for 2 hours at room temperature in dark box. The blot was visualized using the Li-COR system at 680nm.

2.3 Yeast genetic techniques

2.3.1 High efficiency yeast transformation

The yeast stain used for transformation was inoculated into 5ml of liquid YPD (1% Yeast extract, 2% Peptone, 2% Glucose) medium from a fresh colony on an agar plate or glycerol stock, and incubated at 30°C for 16 hours with shaking at 180rpm. In order to get cells to log phase, the pre-culture was diluted into 50ml of pre-warmed YPD which adjusted the A_{600} to 0.2~0.3. The culture was incubated at 30°C until A_{600} reaches 0.6~0.8 which took about 4 hours.

Cells were harvest yeast by centrifuge at 4°C, 3000g for 5 minutes with low speed deceleration. The pellet was washed by ddH_{2}O washing followed by washing with 1ml of 100mM LiAc (Lithium Acetate). Cells were pipetted again with 100mM LiAc and made the final volume to 500µl. 50µl of cell suspension was aliquoted and pelleted. At the same time, 1ml of 2 mg/ml carrier ss-DNA was boiled for 5 minutes, then chilled on ice.

Transformation Mix was made by combining, in order, 240µl of 50% (W/V) PEG, 36µl of 1M LiAc, 25µl of boiled carried ss-DNA and 50µl of DNA fragment (5-15ug)
or plasmid DNA (1-10ug). The mixture was resuspended by vortex mixing vigorously and incubated at 30°C for 30 minutes. After 22 minutes of 42°C heat-shock, cells were centrifuged at 4700g for 30sec, and then resuspended with 200µl ddH₂O before plating on selective medium plates. For transformation of long DNA fragments, cells were cultured at 30°C for another 2 hours in corresponding liquid medium depending on yeast genotype.

2.3.2 Yeast colony PCR screening

Yeast colonies were resuspended in 10µl of fresh 200mM NaOH followed by heating for 10 minutes. A PCR working solution was made with 2µl of 5× Q-solution (QIAGEN), 10× PCR buffer, 0.2µl of 10mM dNTP, 0.2µl of 100 pmol/µl each primer, 0.1µl of Q-Taq (QIAGEN) and 5.3µl ddH₂O. 1µl of heated colony solution working as template was added into PCR tube last. The PCR was run as the following program: 1 cycle of 95°C for 5 minutes, 30 cycles of 95°C for 10 seconds, 50°C for 10 seconds, 72°C for 2 minutes and 1 cycle of 72°C for 10 minutes. All of PCR products were screened with 1% Agarose gel electrophoresis for screening.

2.4 In organelle and In vivo analysis

2.4.1 Crude protein preparation from yeast

One fresh colony was inoculated into 5ml appropriate medium for culture at 30°C until log or stationary phase. Approximately 10au of yeast cells were harvested by centrifuge at 1800g for 5 minutes. To extract total protein, pellet was resuspended in 500µl of fresh 0.2M Sodium hydroxide, and incubated on ice for 10 minutes. 50µl of 50% (w/v) TCA was added to neutralize Sodium hydroxide, which was incubated on ice for 10 minutes. The lysis solution was sedimented at 14,500g for 5 minutes. The pellet was resuspended in 35µl 2× reducing SDS
sample buffer and 15µl of 1M Tris, followed by heating and centrifugation. 20µl of sample was loaded per lane on 16% Tricine SDS-PAGE and analysed by western-blot.

2.4.2 Radioactive $^{35}$S pulse-labelling

Target yeast strain was incubated in appropriate medium at 30°C for 16 hours. Pre-culture was inoculated in fresh selective medium and incubated at 30°C for 6~8 hours until about $A_{600}=0.4$. Approximate 20au of cells were sedimented and washed twice with ddH$_2$O. To label protein with radioactive staff, the pellet was resuspended in 0.5ml medium containing 8µl of $^{35}$S-Mix and incubated at 30°C for 10 minutes with shaking. To chase, labeled cells were pelleted and resuspended in 2ml fresh selective medium. 0.5ml of culture was taken out at different time points. 0.5ml of 100mM chilled NaN$_3$ was then mixed with culture samples to kill the cells. This was left ice for 10 minutes before harvesting.

2.4.3 Immunoprecipitation (IP)

Cells were resuspended in 0.5ml of spheroplast buffer (1.4M sorbitol, 50mM Tris-HCl pH7.4, 2mM MgCl$_2$) and $A_{600}$ measured by using 5µl diluted in 995µl buffer. 6U/A600 of zymolyase (5U/µl stock solution in spheroplast buffer) was used to digest cell wall by incubation at 30°C for 30 minutes. The harvested pellet is spheroplasts, which were resuspended in 100µl of IP lysis buffer (1% SDS, 50mM Tris-HCl pH7.4, 5mM EDTA) and heated immediately at 95°C for 5 minutes followed by 2 minutes on ice. To remove non-specific protein background, 50µl of insoluble protein A and 0.9ml of IP buffer (187.5mM NaCl, 62.5mM Tris-HCl pH7.4, 6.25mM EDTA, 1.25% Triton X100) were mixed with solubilized spheroplasts and rotated at 4°C for 30 minutes before sedimentation at 12,500g for 10 minutes. The supernatant containing target proteins was moved to another tube for IP, and incubated with 1 µl/A600 of appropriate antibody at room
temperature for 1 hour. To precipitate the target protein, 25µl of protein A-sepharose (50% (w/v) stock solution in IP buffer) was added into the incubation at room temperature for 1 hour. The beads were spun down at 12,500g for 5 minutes and washed with 1ml of IP buffer three times. Target proteins were dissociated from beads by using 20µl of 2× reducing SDS sample buffer, followed by heating at 95°C for 5 minutes. After 5 minutes of centrifugation at top speed, 10µl per lane of sample was loaded on 16% SDS Tricine gel, then transferred to a membrane for visual analysis with the Typhoon system.

2.4.4 Yeast mitochondria isolation

Yeast strain was streaked on YPEG (1% Yeast extract, 2% Peptone, 3% Glycerol, 3% Ethanol) plate and grown at 30°C for 2 days for wt (ts-type) yeast, or at 25°C for 3 days for ts (temperature sensitive) yeast. A single colony was inoculated in 50ml of Lactate media (0.3 (w/v) Yeast extract, 0.05% (w/v) Glucose, 0.05% (w/v) CaCl₂•2H₂O, 0.05 % (w/v) NaCl, 0.06% (w/v) MgCl₂•6H₂O, 0.1% (w/v)KH₂PO₄, 0.1% (w/v) NH₄Cl, 0.8% (w/v) NaOH, 2.2% (v/v) 90% DL-lactic acid, in ddH₂O, pH 5.5 with NaOH) for pre-culture until grown to late exponential phase. This took 24 hours at 30°C for wt strain and 48 hours at 24°C for ts strain. 4×1L fresh lactate medium was inoculated with 10ml of each pre-culture and grown at 30°C for 16 hours for wt strain, while ts strain was grown at 24°C for 32 hours then shifted to 37°C for 8 hours.

Cells were harvested by spinning at 1800g for 10 minutes at 4°C, and washed once with chilled ddH₂O. Cells were sedimented by centrifugation at 4000g for 5 minutes. The pellet was weighed and resuspended in 25ml of Tris-DTT buffer (100mM Tris-SO₄, pH 9.4, 10mM DTT) followed by incubation in a 30°C shaking waterbath for 20 minutes. Cells were centrifuged at 3000g for 5 minutes and washed with 40ml of 1.2M Sorbitol buffer (1.2M Sorbitol, 20mM K₂HPO₄) followed
by centrifugation again. To digest cell wall, cells were resuspended in digestion buffer (1.2M Sorbitol, 20mM K$_2$HPO$_4$, 1.5 mg/ml Zymolase 20T (Seikagaku, Japan), 2ml/g cells), and incubated for 20~30 minutes in a 30°C shaking waterbath. To test the efficiency of the spheroplasting, 2µl of cell suspension was diluted in 998µl ddH$_2$O. When the $A_{600}$ approached 0.05, the spheroplasts were harvested by centrifugation at 3000g for 5 minutes at 4°C. The pellet was washed with 50ml of 1.2M Sorbitol buffer followed by spinning at 4000g for 5 minutes at 4°C.

The pellet of spheroplasts was resuspended in 40ml Homogenisation buffer (0.6M Sorbitol, 20mM HEPES, 1mM PMSF) and homogenized using a glass-teflon dounce with 15~20 strokes. The homogenised mixture was centrifuged at 1500g for 5 minutes to pellet cell debris and nuclei, and the supernatant was saved and kept on ice. The pellet was resuspended in 40ml Homogenisation buffer and homogenised as before, then spun again. The supernatants were combined before centrifugation at 7300g for 10 minutes at 4°C. The crude mitochondrial pellet was resuspended by gently pipetting up-down in 1~2ml of BB7.4 (0.6M Sorbitol, 20mM HEPES). The concentration of mitochondrial protein was determined by diluting 10µl mitochondria in 990µl of 0.6% (w/v) SDS and measuring $A_{280}$. An $A_{280}$ of a 0.2, equals a crude mitochondrial concentration of 10 mg/ml. The concentration of mitochondria was adjusted to 10 mg/ml using 10 mg/ml fatty acid free BSA (Bovine Serum Albumin) and the mitochondria were separated into 1mg aliquots which were snap frozen in liquid nitrogen and stored at -80°C until use.

### 2.4.5 Solubilisation of yeast mitochondria

40µg of isolated yeast mitochondria were centrifuged at 12,500g for 3 minutes, and then washed with BB7.4 to remove BSA. Solubilisation was carried out by
suspension in 30µl of S-buffer (20mM Bis-Tris pH 7.0, 50mM NaCl, 10% glycerol, 1mM PMSF, 1% Digitonin), incubated on ice for 30 minutes. The solubilized mitochondria were taken as supernatant from the centrifugation of the suspension at 63,000g for 30 minutes.
3 RESULTS AND DISCUSSION I:

Purification and characterisation of Tim12

3.1 Introduction

Tim12 is a unique member of the small Tim family. Apart from the conserved twin CX3C zinc-finger motif, Tim12 has two extra non-conserved cysteines and a longer amino acid sequence compared to other small Tim proteins (Figure 1. 11). It not only associates with the small Tim to form complexes, but also has an intrinsic affinity to the TIM22 complex on the IM (Lionaki et al., 2008). According to in organelle studies using in vitro translated Tim12, imported Tim12 is oxidised by Mia40-Erv1 disulphide relay system in the IMS, and then assembled into a complex with Tim9 and Tim10. This complex presents transiently and is recruited to dock onto the TIM22 complex of the IM. This recruitment process is guided by the C-terminus of Tim12 (Lionaki et al., 2008, Gebert et al., 2008). However, how the Tim9-Tim10-Tim12 ternary complex is formed is not clear. The ultimate aim of this study was to investigate the mechanism of Tim12 assembly into Tim9-Tim10-Tim12 complex by biophysical means using purified Tim12 proteins. Firstly, the ORF of Tim12 was cloned into either GST or His tag expression vector systems. Both systems allow overexpression of recombinant Tim12 proteins in the presence of IPTG and protein purification using high affinity beads against the corresponding tags. In this chapter, the protein expression and purification of Tim12, using both systems will be presented. Various parameters such as expression host cells, inducing agent concentration, expression temperature and time were optimised to obtain soluble and functional form of Tim12. Finally, purified Tim12 was briefly characterised.
3.2 Purification of GST-tagged Tim12

Since Tim9 and Tim10 have been successfully purified as GST-tagged recombinant proteins from either BL21 or Rosetta Gami (R.gami) E.coli strains (Ivanova et al., 2008, Lu et al., 2004), the GST-Tim12 plasmid was constructed using pGEX 4T-1 vector which expresses Tim12 as a N-terminal GST fusion protein (Figure 3. 1 A). The DNA construct was transformed into BL21 and R.gami E.coli strains for protein expression. The recombinant fusion protein is expected to be about 35kDa, and to bind to GST beads for affinity purification. Tim12 would be then released from the GST tag following thrombin treatment. Details of the construct restriction sites and methods for protein expression and purification were described in the chapter of methods and materials.
Figure 3.1 The construct of GST-Tim12 plasmid and purification process.

(A) The construct of GST-Tim12 plasmid. The TIM12 gene was cloned into pGEX 4T-1 vector using restriction sites BamHI and EcoRI. The vector contains an N-terminal GST tag and a cleavage site for thrombin. (B) The purification procedure of Tim12 using GST-tag. The 35kDa sized recombinant GST-Tim12 protein will be expressed and bound to GST beads. The Tim12 protein will be cleaved off from the recombinant protein by thrombin.
To ensure high protein yield, the GST-Tim12 protein expression level from BL21 and R.gami cells induced under the same conditions was compared in small-scale cultures (Figure 3. 2). GST-Tim12 recombinant protein was expressed in both R.gami and BL21 at a very similar level (Figure 3. 2 lane I). Meanwhile, the solubility of expressed GST-Tim12 was tested from each strain. It was anticipated that the recombinant protein would be expressed in a soluble form, as GST is a soluble protein that constitutes a major proportion in the fusion protein. However, more than ~90% of recombinant protein was found located in the pellet fraction in the cell lysate (Figure 3. 2 lanes S and P), meaning that the GST-Tim12 was expressed in the form of inclusion bodies. As there was no significant difference between the levels of protein expression in the strains used, R.gami strain was chosen for Tim12 expression because it offers the advantage of facilitating disulphide bond formation, as seen in the successful recombinant expression of Tim9 and Tim10.
Figure 3. 2 Expression and solubility tests of GST-Tim12 in R.gami and BL21. The un-induced cells (UI) were grown to log phase, and then induced by IPTG at 30°C for 16 hours (I). After harvesting cells, the total proteins (T) were released by lysis of sonication. Following the centrifugation, the soluble proteins (S) and the pellets (P) were separated. All samples collected from above process were analysed by 16% Tricine SDS-PAGE, and visualised by Coomassie Blue staining. (A) The SDS-PAGE analysis of the expression in R.gami; (B) The SDS-PAGE analysis of the expression in BL21.
After lysis of cells, 6M urea was used to solubilise the pellet of GST-Tim12 at room temperature for 1 hour, but there were still some pellet insoluble (Figure 3. 3 lane DP). Since the binding of GST-Tim12 to the affinity beads requires the GST tag to be in its active, correctly folded conformation, there is a requirement of re-naturing the fusion protein before binding. The concentration of urea was decreased to make the protein refold at 4°C by 10 times dilution with Tris buffer, resulting in a final concentration of 0.6M. A fraction of about 30% was precipitated as white aggregates in the refolding process, whilst the remaining 70% still remained soluble (Figure 3. 3 compare lane RS and RP). The soluble fraction was used for binding with GST beads. However, it was found that GST-Tim12 in 0.6M urea failed to or weakly bound to GST-beads, even with prolonged incubation overnight, with almost around 50% of the material found in the non-binding fraction (Figure 3. 3 lane NBF). Almost all the bound materials were eluted upon washing (Figure 3. 3 lane W), therefore resulting in an extremely low amount of Tim12 obtained after the cleavage of GST-tag (Figure 3. 3 lane E).

**Figure 3.** 3 Purification of Tim12 from the inclusion bodies of GST-Tim12.

DT: the total denatured protein; DS: the supernatant of denatured protein; DP: the pellet of denatured protein; RT: the total re-natured protein; RS: the supernatant of re-natured protein; RP: the pellet of re-natured protein; NBF: the non-binding fraction; W: the washing of beads; E: the elution after cleavage. Samples collected from every steps were analysed by the SDS-PAGE under reducing conditions, and visualised by Coomassie Blue staining.
To improve the affinity of renatured GST-Tim12 to beads, the dilution method for re-naturing was optimised by four approaches: (A) 10 times dilution with Tris buffer; (B) 20 times dilution with Tris buffer; (C) 10 times dilution with Tris buffer plus 1mM DTT; (D) doubling dilution with Tris buffer in every 40 minutes interval to a final dilution of 10 times (Figure 3. 4). Following that, all the re-natured proteins were applied to GST-beads, and the non-bound proteins were collected for comparing the effects of binding. Unfortunately, regardless of whichever dilution method used, the binding was still weak. Almost all re-natured materials were unable to bind to GST beads, as they were eluted into the non-bound fractions. This suggested that these four optimised dilution approaches had no significant effects to improvement protein binding to GST beads.

**Figure 3. 4 Optimisation of the dilution method for re-naturing proteins.** The dilution method for re-naturing proteins was optimised by the following four ways. A, 10 times dilution with Tris buffer and stirring at 4°C for 2 hours; B, 20 times dilution with Tris buffer and stirring at 4°C for 2 hours; C, 10 times dilution with Tris buffer plus 1mM DTT and stirring at 4°C for 2 hours; D, doubling dilution with Tris buffer in every 40min at 4°C with stirring until 10 times of dilution. Samples were collected from the re-natured and non-bound fractions, followed by SDS-PAGE analysis with Coomassie Blue staining.
Therefore, dialysis method was employed to re-nature the protein recombinant (Figure 3. 5). The 6M denatured pellet of GST-Tim12 was loaded into the 3kMW cut-off dialysis tubing, followed by two steps of dialysis at 4°C with gently stirring. Firstly, the dialysis was carried out in 1L Tris buffer for 3 hours. Next, the buffer was exchanged with 1L fresh Tris buffer for overnight dialysis. Similar with that of dilution method, almost all re-natured materials failed to bind to beads after dialysis (Figure 3. 5 lane RD and NBF). Some loosely bound proteins were washed away (Figure 3. 5 lane W), suggesting the beads affinity to re-natured dialysed protein is weak. However, some bound proteins were obtained, and successfully cleaved by thrombin. After the cleavage, the elution contained three major contents: GST-Tim12, GST tag and Tim12 (Figure 3. 5 lane E). Although some Tim12 can be detected in the SDS-PAGE, its concentration was too low to be further purified by gel filtration chromatography.

**Figure 3. 5 Purification of Tim12 after the dialysis for re-naturing.** The dialysis was carried out as described in the text for re-naturing GST-Tim12 (RD), followed by the binding of GST-beads. The non-bound fraction (NBF) and beads-washing (W) were flown out. After the cleavage using thrombin, the Tim12 was purified in the elution (E). Samples were collected from above steps and analysed by SDS-PAGE with Coomassie Blue staining.
3.3 Purification of N-terminal His-tagged Tim12

Due to the failure of refolding Tim12 in the form of GST-tagged recombinant protein, His-tag was considered as an alternative choice for purification of Tim12. Unlike GST-tagged fusion proteins, His-tagged proteins allow strong binding to nickel-charged beads even under denaturing conditions. The TIM12 gene was cloned into the pET-28b vector using NdeI and BamHI restriction sites (Figure 3.6 A). Because the constructed His-Tim12 plasmid can attach a His-tag and a thrombin cleavage site at the N-terminus of expressed protein, after the fusion protein binding to the His beads, the His-tag can be removed from Tim12 by the cleavage of thrombin (Figure 3.6 B). However, there will be three extra amino acids (Gly-Ser-His) at the N-terminus of Tim12.
Figure 3.6 The construct of His-Tim12 plasmid and purification process. (A) The construct of His-Tim12 plasmid. The TIM12 gene was cloned into pET-28b vector using restriction sites NdeI and BamHI. The vector contains an N-terminal His tag and a site for thrombin cleavage. (B) The purification procedure of Tim12 using N-terminal His-tag. The recombinant His-Tim12 protein bound to His beads can be eluted by thrombin cleavage.
The constructed His-Tim12 plasmids were transformed into R.gami and BL21 respectively. In order to get the high-yield expression condition, the expressions of two systems were tested at 16°C and 30°C overnight after 0.1g/L IPTG induction (Figure 3. 7). In the R.gami expression system, after induction, the His-Tim12 can be expressed with molecular weight of ~15kDa as expected, at either 16°C or 30°C (Figure 3. 7 A). There was no significant difference of expression level observed at the different temperatures tested, suggesting temperature had little effect on the expression of His-Tim12 using R.gami. On the other hand, no expression of His-Tim12 was observed in the BL21 system at either temperature (Figure 3. 7 B). Since low temperature is generally good for proper folding of expressed protein, His-Tim12 in R.gami was induced by 0.1g/L IPTG, and then expressed at 16°C for 16 hours in a large scale 4-litre culture.
**Figure 3.** *7 Expressions of His-Tim12 in R.gami and BL21 at two temperatures.* The R.gami (A) and BL21 (B) with His-Tim12 plasmids were grown to log phase. After induction by 0.1g/L IPTG, two expressions were tested at 16°C and 30°C for 16 hours. Samples were taken from the uninduced cells (UI) and induced cells (I), followed by SDS-PAGE analysis with Coomassie Blue staining.
After lysis of induced cells by sonication, most of the recombinant protein was located in the pellet (Figure 3. 8 compare lane P and S), meaning that the His-Tim12 was as insoluble as the GST-Tim12. Thus, the protein was solubilised by dissolving the pellet in 6M urea for 1 hour at room temperature (Figure 3. 8 lane DS and DP). The denatured proteins were re-natured using the one-step dilution method before affinity binding to His beads. The re-natured proteins were soluble (Figure 3. 8 lane RS), while a small proportion of protein remained in the insoluble pellet (Figure 3. 8 lane RP). A majority of the soluble His-Tim12 was successfully bound to the beads, as there was only about 10% of the recombinant protein found in the non-binding fraction (Figure 3. 8 lane NBF) with some loosely bound proteins washed out from the column (Figure 3. 8 lane W). After the thrombin treatment, the eluted product contained two major species: one was about 13kDa with the same size as Tim12, and the other one was less than 10kDa (Figure 3. 8 lane E). Since this smaller product did not exist before the cleavage of fusion protein, it was presumed that Tim12 was truncated after thrombin cleavage. Moreover, the amount of these two species of Tim12 was nearly equal, suggesting that the purified Tim12 was not stable, following His-tag removal. In order to get the pure full-length Tim12, gel filtration chromatography was used for further purification.
Figure 3. 8 Purification of Tim12 from the inclusion bodies of His-Tim12.
UI: the uninduced cell culture; I: the induced cell culture by IPTG; T: the total extracted protein; S: the soluble proteins; P: the pellet of insoluble proteins; DS: the supernatant of denatured protein; DP: the pellet of denatured protein; RT: the total re-natured protein; RS: the supernatant of re-natured protein; RP: the pellet of re-natured protein; NBF: the non-binding fraction; W: the washing of beads; E: the elution after cleavage. Samples collected from every steps were analysed by the reduced SDS-PAGE, and visualised by Coomassie Blue staining.
On gel filtration using Superdex 200 column equilibrated with Tris buffer, there were three peaks distributed from 13 to 20ml. Based on the site of standard marker on the same column, the molecular weight of P1 was 44kDa, P2 was 25kDa, and P3 was less 17kDa.

To verify contents of the peaks shown on the profile of gel filtration, the eluted fractions were analysed by SDS-PAGE under reducing and non-reducing conditions (Figure 3. 9 B and C). P1 contained mainly high oligomers of >44kDa, probably due to protein aggregation. P2 contained a mixture of 2 species of 25kDa and 13kDa whilst P3 only had a single species at 13kDa. The 25kDa species from P2 dissociated to 13kDa under reducing conditions (Figure 3. 9 C), indicating a reduction of disulphide bonded dimer to monomer. Although some monomeric form of Tim12 was successfully purified by gel filtration, the yield was too low for further experiments, such as CD and ITC analyses. Thus, a better method for the purification of Tim12 is required.
Figure 3. 9 Gel filtration of Tim12 and SDS-PAGE analysis of the profile.
(A) Gel filtration chromatography of Tim12. The elution of affinity purification was loaded on the Superdex 200 column for gel filtration. The protein was detected at 280nm. (B and C) SDS-PAGE analysis of the profile in A. Samples were taken from the corresponding peaks shown in (A), and analysed by SDS-PAGE under non-reducing (B) and reducing (C) conditions, followed by the visualisation of Coomassie Blue staining.
3.4 Purification of C-terminal His-tagged Tim12

The purification of Tim12 by means of a cleavable N-terminal His-tag was not ideal, since a large proportion of ~50% of the purified Tim12 was prone to protein truncation after cleavage using thrombin. In order to ensure Tim12 can be purified in the form of full-length, the plasmid of C-terminal His-tagged Tim12 was constructed by the insertion of TIM12 gene into the pET-24a vector using NdeI and XhoI (Figure 3. 10 A). Comparing to pET-28b, pET-24a does not encode any cleavage sites for His-tag removal, which might be ideal to maximise the yield of full-length Tim12. The expressed protein can be eluted from nickel-charged beads using imidazole, giving rise to a full-length recombinant Tim12-His (Figure 3. 10 B).
Figure 3. 10 The construct of Tim12-His plasmid and purification process. (A) The construct of Tim12-His plasmid. The TIM12 gene was cloned into pET-24a vector using restriction sites NdeI and XhoI. The vector expresses a C-terminal His-tag without any cleavage sites. (B) The purification procedure of Tim12-His. The recombinant Tim12-His protein bound to His beads can be eluted using 0.5M imidazole.
In order to choose the right expression host, expression of Tim12-His were compared between BL21 and R.gami cells (Figure 3. 11), under the same conditions as shown previously in Figure 3. 7. In contrast to His-Tim12, Tim12-His can be expressed in BL21 but not in R.gami. Thus, the BL21 was chosen for the expression of Tim12-His.

**Figure 3. 11 Expressions of Tim12-His in BL21 and R.gami.** The R.gami and BL21 with Tim12-His plasmids were grown to log phase. After induction by 0.1g/L IPTG, two expressions were performed at 30°C for 16 hours. Samples were taken from the uninduced cells (UI) and induced cells (I), followed by SDS-PAGE analysis with Coomassie Blue staining.

After cell lysis, the expressed Tim12-His was insoluble like GST-Tim12 and His-Tim12 (Figure 3. 12 lane P). Since His-tag can bind with nickel-charged beads under denaturing conditions, the urea-solubilised Tim12-His was directly loaded onto the column for binding without a pre-renaturing step as done previously to minimize protein loss. After a washing step to remove unbound material, Tim12-His was eluted using 0.5M imidazole containing 6M urea. The eluate contained a mixture of monomer and dimer with molecular weights of ~13kDa and ~26kDa.
respectively. The dimer was dissociated under reducing conditions, indicating the presence of intermolecular disulphide bond formation.

**Figure 3.** Purification of Tim12-His from inclusion bodies under denaturing conditions. T: total protein after cell lysis; S: soluble protein; P: pellet of expressed protein; D: denatured pellet by 6M urea; N: non-binding fraction; W: washing of beads; E: eluate in 1ml fractions. Samples collected from every steps were analysed under non-reducing and reducing conditioned SDS-PAGE, followed by the Coomassie Blue staining.
To re-nature the eluted Tim12-His, dialysis was immediately performed with decreasing urea concentration from 4M, 2M to no urea in Tris buffer (Figure 3. 13). The affinity purified Tim12-His was prone to dimerise due to prolonged handling in the presences of 4M, 2M or no urea and even before dialysis (Figure 3. 13). This indicated that the purified Tim12-His contained free, unpaired cysteines that can undergo oxidation to form intermolecular disulphide bonds. In addition, the re-natured Tim12-His was not stable under native conditions as white aggregations formed very quickly when urea was completely removed. Thus, there was no enough material for gel filtration analysis and further experiments.

**Figure 3. 13 Dialysis of the denatured Tim12-His.** The denatured Tim12-His (D) was dialysed against decreasing concentrations of urea (4M, 2M and 0M). Samples were analysed by SDS-PAGE under non-reducing and reducing conditions, and visualised by Coomassie Blue staining.
3.5 Cysteine redox state of purified Tim12

Results so far could suggest that Tim12 aggregation/insolubility was caused by the failure of the protein to acquire proper folding through disulphide bond formation, and that the freshly purified protein was not fully oxidised. To verify this, the redox state of Tim12 monomer that was successfully purified from cleaved His-Tim12 was analysed by SDS-PAGE-based assay in the presence or absence of the thiol modification reagent AMS (Figure 3. 14). Any free thiol in the protein will be alkylated by AMS, resulting in a molecular weight shift by ~0.5kDa per thiol modified. As a control, the protein was treated with the reducing agent TCEP to reduce any intramolecular disulphide bonds before AMS modification. In parallel, the experiment was repeated for Tim9 and Tim10 monomer for comparison. SDS-PAGE analysis showed that Tim9 and Tim10 had no change in molecular weight in the presence or absence of AMS (Figure 3. 14, compare lane 1 and 3; and lane 4 and 6) while the TCEP-reduced were shifted by ~2kDa by AMS (Figure 3. 14 lane 2 and 5), indicating that all four thiols present in purified Tim9 and Tim10 were in the oxidised forms. All 6 thiols of Tim12 monomer were fully oxidised as there was no clear shift in molecular weight with or without AMS (Figure 3. 14 lane 7 and 9), as opposed to a shift of ~3kDa upon TCEP treatment (Figure 3. 14 lane 8). Therefore, it seems like Tim12 has to be fully oxidised into the monomeric, but not the dimeric form in order to acquire proper protein conformation in a soluble form.
Figure 3. 14 AMS assay of the redox states of Tim9, Tim10 and Tim12. Samples were treated with or without (2mM) AMS and/or 1mM TCEP as indicated, before analysis by non-reducing 16% Tricine SDS-PAGE, and Coomassie Blue staining for visualisation.
3.6 Discussion

Expression and purification of Tim12 have been tested using three different expression systems that express the protein in the form of GST-tagged, N-terminal His-tagged or C-terminal His-tagged form from either R.gami or BL21 cells. However, none of those conditions used were ideal for Tim12 purification due to the insolubility of the expressed recombinant proteins. Although the urea-solubilised protein was treated with different methods of renaturation before or after protein purification, a vast majority or all the protein was lost due to aggregation. In addition, recovered protein did not seem to be able to acquire correct structural conformation, causing the inability of re-natured GST-Tim12 to efficiently bind to GST-beads and the massive aggregation of His-Tim12 and Tim12-His before or after purification. The lack of proper folding may be in part the result of covalent interactions between cysteine residues, since the purified Tim12-His could easily air-oxidise to form an intermolecular disulphide bonded dimer which then aggregated upon urea removal. However, the successfully purified Tim12 monomer from cleaved His-Tim12, although in a small amount, was fully oxidised by intra-molecular disulphide bonds between the 6 cysteines of Tim12. In addition to the conserved twin CX$_3$C motif that forms two disulphide bonds between Cys40 and Cys66, and Cys44 and Cys62, there seems to be a requirement for the two non-conserved cysteines (Cys30 and Cys61) to form a third disulphide bond in order for the protein to acquire the correct structural conformation in a soluble form.
4 RESULTS AND DISCUSSION II:

In vitro study of the role of Tim9 disulphide bonds on complex formation

4.1 Introduction

Precursors of small Tim proteins are encoded by nucleus DNA and synthesised in cytosol. Like all the other mitochondrial proteins, they have to be imported into mitochondrial IMS for their biogenesis. Only reduced precursors of the small Tim proteins can passage across the mitochondrial OM (Morgan and Lu, 2008). In the IMS, reduced precursors are oxidised by the Mia40-Erv1 system resulting in fully oxidised protein molecules containing two intramolecular disulphide bonds, formed by four conserved cysteines of the twin CX₃C motif e.g. Cys35-Cys59 and Cys39-Cys55 in Tim9. Functions of oxidised small Tim proteins are exerted by the formation of hexameric complexes, such as Tim9-Tim10 and Tim8-Tim13. The in vitro studies of biogenesis of small Tim proteins have revealed that the cysteine residues of Tim9 and Tim10 play an important role in the import and subsequent assembly of complex (Lu et al., 2004a, Ivanova et al., 2008). However, we have limited understanding in the functional mechanism of the Tim9-Tim10 complex, and do not know whether all four cysteines are required for their functions. In addition, the inner disulphide bond of Tim10 was found to be more important for complex formation than the outer disulphide bond (Allen et al., 2003). An in vivo study of single cysteine mutants of Tim9 and Tim10 found that the first conserved cysteine was crucial in the interaction with Mia40 (Sideris and Tokatlidis, 2007, Milenkovic et al., 2007). However, there are few in vitro and in vivo studies using double cysteine mutants of Tim9 to uncover the role of disulphide bonds of small Tim protein on biogenesis and function of mitochondria. In this study, two double cysteine mutants of Tim9, with inner or outer disulphide bond mutated to serine residues, were used to study the role of disulphide bonds of Tim9 in its folding
and interaction with Tim10wt in vitro. The WT and mutant Tim9 were purified by GST-tagged affinity and gel filtration chromatography. The expression of these two mutant proteins were studied in BL21 and R.gami cells with different incubation temperatures and concentrations of IPTG induction. Their folding was analysed by CD (Circular Dichroism). The microscale-thermophoresis, gel filtration chromatography, hetero cross-link and BN-PAGE were utilised to study the interactions between Tim9 mutants and the WT Tim10.

4.2 Preparation of double cysteine mutants of Tim9

4.2.1 Expressions of recombinant Tim9 double cysteine mutants

There are four cysteine residues in Tim9. Cysteines in pair forming disulphide bonds were mutated into serines by site-directed mutagenesis, such as Tim9C35,59S (Tim9C1,4S) and Tim9C39,55S (Tim9C2,3S) (Figure 4. 1). The plasmids of GST-Tim9C1,4S and GST-Tim9C2,3S were generated from GST-Tim9wt. Two rounds of single cysteine mutated PCR were used for each dual cysteine mutated plasmids methods. The mutated plasmids were confirmed by DNA sequencing before transformation into expression system. In order to get more recombinant proteins, the expression levels were compared between BL21 and R.gami cells, and were optimised for concentrations of IPTG induction and expression temperatures.
Figure 4. 1 The scheme of double cysteine mutants of Tim9. In this study, using the mutagenesis methods, the cysteines of Tim9 were mutated into serines generating two species: Tim9C35,59S and Tim9C39,55S, and were named as Tim9C1,4S and Tim9C2,3S for short. The inner disulphide bond was maintained in Tim9C1,4S, while the outer disulphide bond was kept in Tim9C2,3S.

The GST-Tim9C1,4S can be expressed in both BL21 and R.gami after induction, but it is obvious that more protein was produced in BL21 than in R.gami (Figure 4.2). The expression level in BL21 cells depends on the expression temperature and concentration of IPTG induction. In particular, after 1mg/ml IPTG of induction and 16 hours of 37°C incubation, the protein can be maximally expressed (Figure 4.2 upper panel). In contrast to the expression level in BL21, the expression level of R.gami is independent of temperature and concentration of IPTG, and seems to be inhibited at 37°C and 1mg/ml of IPTG (Figure 4.2 lower panel). Thus, the best expression condition for GST-Tim9C1,4S is to use BL21 at 37°C and 1mg/ml of IPTG.
Figure 4. The expression tests of GST-Tim9C1,4S in BL21 and R.gami.

The plasmids of GST-Tim9C1,4S were transformed into BL21 and R.gami respectively. The expressions of the GST-Tim9C1,4S indicated as single star, were tested under conditions shown in the panel below. The samples were harvested after 16 hours incubation, then analysed by Tricine SDS-PAGE. The upper panel is the expression in BL21; the lower panel is the expression in R.gami. The optimised expression condition was underlined in red.
For the expression of GST-Tim9C2,3S, BL21 and R.gami gave different results (Figure 4.3). BL21 can express more protein at high temperatures (30°C and 37°C) than low temperatures (16°C and 25°C). And increasing concentration of IPTG can improve the expression level at high temperatures (30°C and 37°C) (Figure 4.3 lane 8~13 in upper panel). In the case of R.gami, the expression of GST-Tim9C2,3S prefer to low temperatures (16°C and 25°C), rather than high temperatures (30°C and 37°C). The various concentrations of IPTG had little effect on the expression. Overall, the expression of GST-Tim9C2,3S in BL21 cells is better than that in R.gami cells (Figure 4.3 lane 2~7 in lower panel). Therefore, BL21, 37°C, and 1mg/ml of IPTG were also chosen as the condition for both mutants expression in the rest of this study.
Figure 4.3 The expression tests of GST-Tim9C2,3S in BL21 and R.gami.
The plasmids of GST-Tim9C2,3S were transformed into BL21 and R.gami respectively. The expressions of the GST-Tim9C2,3S indicated as double stars, were tested under conditions shown in the panel below. The samples were harvested after 16 hours incubation, then analysed by Tricine SDS-PAGE. The upper panel is the expression in BL21; the lower panel is the expression in R.gami. The optimised expression condition was underlined in red.
4.2.2 Purifications of Tim9 double cysteine mutants

The detailed procedure for the purification of GST tagged recombinant protein has been described in the chapter of Materials and Methods. The lysed cell solution is mixed with GST-beads for affinity binding. After the removal of non-binding fractions, the bound GST-tagged recombinant protein is cleaved by thrombin, followed by the elution of full-length target protein. Here, following the above procedure, the purifications of GST-tagged Tim9wt and two double cysteine mutants were analysed using SDS-PAGE (Figure 4.4).

The WT and both mutant recombinant proteins were highly expressed in the cells (Figure 4.4 lane I). However, a large proportion of both mutant proteins were insoluble, as they were mostly found in the pellet after cell lysis (Figure 4.4 lane P in B and C), whilst the WT protein was highly soluble (Figure 4.4 lane S in A). Providing that there was still a small fraction of soluble GST-Tim9 mutants in the supernatant, the soluble fractions was used as the crude material for purification of Tim9 mutants as for the WT protein. The recombinant proteins were successfully bound to the GST-beads, as they were not found in the non-bound (Figure 4.4 lane N) and wash (Figure 4.4 lane W) fractions after incubation with the beads. Following protease cleavage by thrombin to release Tim9 from GST-tag, a band of about 11kDa protein was observed, which was the same as the expected size of Tim9 protein (Figure 4.4 lane E) Meanwhile, a small proportion of purified Tim9 mutants appeared at about 20kDa observed under non-reducing condition (Figure 4.4 lane E-DTT in B and C), indicating that dimeric Tim9 mutants were formed by intermolecular disulphide bonds under native condition.
**Figure 4.** Purifications of WT and double cysteine mutants of Tim9. (A) The purification of Tim9WT. (B) The purification of Tim9C1,4S. (C) The purification of Tim9C2,3S. UI: the uninduced cell culture, I: the induced cell culture by IPTG, T: the total extracted proteins, S: the soluble proteins, P: the pellet of insoluble proteins, N: the non-bound fraction, W: the washed fraction, E: the elution was analysed under reducing condition, E-DTT: the elution was analysed under non-reducing condition. All samples were analysed by 16% Tricine SDS-PAGE and visualised by Coomassie Blue staining.
The affinity purified Tim9wt and two mutants were loaded onto Superdex 200 column for further purification by gel filtration chromatography (Figure 4. 5 A). The gel filtration profile of Tim9wt showed a main peak at 15-17ml (Figure 4. 5 Square in A). However, the major peaks of both mutants are located at 16-18ml (Figure 4. 5 Star in A), due to low concentrations. Comparing the value of peaks, it also suggested that the concentrations of two mutants were much lower than that of Tim9wt. Additionally, Tim9wt and two mutants have small peaks at 19-20ml (Figure 4. 5 Dot in A). Experiences for the gel filtration of Tim9wt suggested that this small peak was not generated by some small sized proteins, instead by the salt exchange in system indicating the separation of loaded sample was over on the column.

To know the content of corresponding elution at major peak, the SDS-PAGE analysis was performed under reducing (+DTT) and non-reducing (-DTT) conditions (Figure 4. 5 B). There were only ~11kDa bands existing on both reducing and non-reducing PAGE, suggesting that the peaks indicated by square and star on the profile of gel filtration were generated by monomeric proteins. Thus, the mutant proteins were successfully purified and most likely in a monomer form.
Figure 4. 5 Oligomerisation states of the Tim9wt and double cysteine mutants. (A) Gel filtration chromatography of Tim9wt and double cysteine mutants. The purified proteins were loaded in the same volume on Superdex 200 column. (B) 16% Tricine SDS-PAGE analysis of the peaks in (A) under reduced (+DTT) and non-reduced (-DTT) conditions. Based on the locations of peaks on the gel filtration profile, the elution of Tim9wt was collected at 15-17ml, while elution of two mutants were collected at 16-18ml for analysis.
4.3 Characterisations of Tim9 double cysteine mutants

To know whether the double cysteine mutants were folded, far-UV CD spectra of Tim9C1,4S and Tim9C2,3S were measured and compared with that of the Tim9wt (Figure 4. 6). The far-UV (190-250nm) CD is sensitive to the secondary structure of proteins, with α-helix, β-sheet, and random coil structures giving different and characteristic CD spectrum. The CD spectrum of the Tim9wt was the same as previously published, showing the protein was folded in a predominantly α-helical structure (Vial et al., 2002). Both Tim9C1,4S and Tim9C2,3S showed similar spectra as that of the with Tim9wt, with two peaks at 209nm and 222nm respectively. This suggested that two mutants were folded with α-helical structure, but the intensity of two mutants were less than that of Tim9wt. Thus, both mutants were partially folded compared with the Tim9wt.

![Figure 4. 6 Far-UV CD spectra of Tim9wt and double cysteine mutants.](image)

10mM of each protein in 15mM Tris buffer (pH7.4), was scanned by the far-UV CD from 200nm to 260nm. The measurement was performed at 25°C.
4.4 The effect of mutation on interaction between Tim9 and Tim10

Because the secondary structures of double cysteine mutated Tim9 are not as compactly folded as the WT, it is interesting to know whether the mutants can interact with Tim10 and form a functional Tim9-Tim10 complex. To address this, the binding affinity of the WT proteins was analysed using the isothermal titration calorimetry (ITC) before. However, ITC needs much protein at high concentration, which was difficult for two mutants. Here, the microscale thermophoresis (MST) was used, because of low sample consumption and easy handling (Figure 4. 7). As described in the chapter of Materials and Methods, 2µM Tim10 was labeled by fluorescence dye for detecting, while unlabeled 20µM Tim9wt and mutants were serial diluted for affinity with Tim10. The result showed that that the interaction between WT Tim9 and Tim10 at the ratio of 1:1, the dissociation constant (K_D) was 0.17µM (Figure 4. 7 A), which was similar or the same to that obtained by ITC (K_D=0.2µM, Table 4. 1) (Vial et al., 2002). It demonstrates that MST is reliable for protein-protein interaction measurement. Next, MST was used to study interaction between Tim10 and mutants of Tim9. It showed that the K_D of Tim10 with Tim9C1,4S was 43µM, and K_D of Tim10 with Tim9C2,3S was 9.1µM (Figure 4. 7 B and C). The higher K_D of a complex has, the weaker interactions between the two proteins are. Thus, it can be concluded that the disulphide bonds of Tim9 are not only important for folding, but also affect interactions of Tim9 with Tim10.

Table 4. 1 Protein interactions by ITC and MST

<table>
<thead>
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<th>Interaction</th>
<th>ITC (K_D)</th>
<th>MST (K_D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tim9wt and Tim10</td>
<td>0.2µM</td>
<td>0.17µM</td>
</tr>
<tr>
<td>Tim9C1,4S and Tim10</td>
<td>N/A</td>
<td>43µM</td>
</tr>
<tr>
<td>Tim9C2,3S and Tim10</td>
<td>N/A</td>
<td>9.1µM</td>
</tr>
</tbody>
</table>
Figure 4. 7 MST analysis of the interactions of Tim9wt and mutants with Tim10. (A) Tim9wt interacted with Tim10; (B) Tim9C1,4S interacted with Tim10; (C) Tim9C2,3S interacted with Tim10. 2µM Tim10 was labeled by fluorescence dye, while 20µM Tim9wt and mutants were serially 2-fold diluted for 15 dilutions without labeling. The interaction was analysed using 1:1 mixture by MST, which was performed by by Stephen Blanker and Petra Schliack from Nano Temp Technologies. $K_D$ values were generated by fitting of the yielded data.
4.5 The effect of mutation on Tim9-Tim10 complex formation

Although the MST thermophoresis analysis showed that both Tim9C1,4S and Tim9C2,3S mutants can interact with Tim10wt, it is unknown if the same size of hexameric complex is formed with the mutants. Thus, gel filtration chromatography was utilised to answer this question. Tim9wt, Tim9C1,4S and Tim39,55S were respectively mixed with Tim10wt at the ratio of 1:1, at room temperature for 30 minutes, and then loaded to a Superdex 75 column for analysis.

In the case of mixing Tim9wt and Tim10wt, a peak with higher molecular weight appeared at 1.1~1.2ml (P_{WT1}), compared with the individual protein eluted at 1.3~1.4ml (P_{WT2}) (Figure 4. 8 A). The result showed that a complex formed between the proteins. To confirm and understand the complex further, P_{WT1} and P_{WT2} were collected and analysed by western-blot with using anti-Tim9 and anti-Tim10 antibodies (Figure 4. 8 B). For the P_{WT1} in the Tim9wt-Tim10wt profile, it contains Tim9wt and Tim10wt detected by antibodies. Regardless of reducing or nonreducing conditions, Tim9wt and Tim10wt have only 10KDa bands, indicating they are monomer. Thus, this suggests that the P_{WT1} is a complex formed by monomeric Tim9wt and Tim10wt. And the size of this complex is 75kDa according to previous study (Lu et al., 2004a). For the P_{WT2} in this profile, it also is composed by the extra monomeric Tim9wt and Tim10wt, which are unassembled components.
Figure 4.8 The complex formation of Tim9wt and Tim10. (A) Gel filtration profiles of Tim9wt, Tim10wt and the complex formed by 1:1 mixing the two proteins. The experiment was carried out on the Superdex 75 column. (B) Western-blot analysis for panel A. The peaks shown in the gel filtration profile were analysed by SDS-PAGE under reduced (+DTT) and non-reduced (-DTT) conditions, followed by immunoblotting using anti-Tim9 and anti-Tim10 antibodies respectively.
The same experiments were done with Tim9C1,4S (Figure 4. 9). In the profile of gel filtration, two peaks were observed, one at 1.2~1.3ml and the other at 1.3~1.4ml (Figure 4. 9 A). Interestingly, the size of this complex ($P_{OUT1}$) is smaller than the complex formed by Tim9wt and Tim10wt. The second peak ($P_{OUT2}$) appears at 1.3~1.4ml, corresponding to unassembled Tim9wt or Tim10wt. From analysis of western-blot, the $P_{OUT1}$ formed by Tim9C1,4S and Tim10wt contains the monomeric Tim9C1,4S and Tim10wt, as there is no difference with reducing and without nonreducing treatments (Figure 4. 9 B). Additionally, the $P_{OUT2}$ of Tim9C1,4S is mainly composed by unassembled Tim10wt, suggesting that almost all Tim9C1,4S has participated in the formation of complex. So the monomeric Tim9C1,4S and Tim10wt can form complex like WT but with smaller-size.
**Figure 4.9** The complex formation of Tim9C1,4S and Tim10. (A) Gel filtration profiles of Tim9C1,4S, Tim10wt and the complex formed by 1:1 mixing the two proteins. The experiment was carried out on the Superdex 75 column. (B) Western-blot analysis for panel A. The peaks shown in the gel filtration profile were analysed by SDS-PAGE under reduced (+DTT) and non-reduced (-DTT) conditions, followed by immunoblotting using anti-Tim9 and anti-Tim10 antibodies respectively.
In the case of Tim9C2,3S, two peaks at 1.1~1.2ml (\(P_{IN1}\)) and 1.3~1.4ml (\(P_{IN2}\)) were observed (Figure 4. 10 A). This profile is similar to that of Tim9wt-Tim10wt complex, in which the \(P_{IN1}\) eluted at the same position as the Tim9wt-Tim10wt complex. Furthermore, western-blot shows that both \(P_{IN1}\) and \(P_{IN2}\) of Tim9C2,3S are composed by monomeric components (Figure 4. 10 B). This is in line with the complex formation of Tim9wt and Tim10wt. However, comparing to the \(P_{IN1}\), the \(P_{IN2}\) containing unassembled components has much more Tim9C2,3S and Tim10wt than the source proteins in the complex formation.
Figure 4. 10 The complex formation of Tim9C2,3S and Tim10. (A) Gel filtration profiles of Tim9C2,3S, Tim10wt and the complex formed by 1:1 mixing the two proteins. The experiment was carried out on the Superdex 75 column. (B) Western-blot analysis for panel A. The peaks shown in the gel filtration profile were analysed by SDS-PAGE under reduced (+DTT) and non-reduced (-DTT) conditions, followed by immunoblotting using anti-Tim9 and anti-Tim10 antibodies respectively.
To directly compare the complexes formed by Tim9wt, Tim9C1,4S and Tim9C2,3S, three profiles were shown together in Figure 4. It demonstrated clearly the both double cysteine mutated Tim9 can form complexes (P\textsubscript{OUT1} and P\textsubscript{IN1}) with Tim10 \textit{in vitro}. However, the Tim9 with inner disulphide bond (Tim9C1,4S) seems to form a smaller-size complex (P\textsubscript{OUT1}), when compared to the WT Tim9-Tim10 complex (P\textsubscript{WT1}). Although the Tim9 mutant containing the outer disulphide bond (Tim9C2,3S) can form the same size complex (P\textsubscript{IN1}) as the WT proteins, the yield of the complex is low.

![Figure 4](image)

**Figure 4.** The comparison for formed complexes by Tim9wt and double cysteine mutants. The gel filtration profiles of formed complexes by Tim9wt, Tim9C1,4S and Tim9C2,3S were combined together for comparison.
Next, BN-PAGE was applied to analyse the size of the complexes formed between these proteins (Figure 4. 12). The Tim10wt was incubated with Tim9wt, Tim9C1,4S and Tim9C2,3S at different ratios, on ice for 45 minutes, followed by mixing with BN sample buffer. It was analysed using 6%~16% gradient BN-PAGE and visualised by Coomassie Blue staining. At the ratio of 10:10, the Tim9wt and Tim10wt formed ~75kDa complex as expected (Figure 4. 12 lane 1). At the same ratio, no complex formed by Tim9C1,4S was observed (Figure 4. 12 lane 2), but it was observed that Tim9C2,3S formed ~75kDa complex with WT Tim10 (Figure 4. 12 lane 3). Due to the concentration of each protein was not very accurate, in order to verify the complexes formed by mutants on gel filtration chromatography, the mutants were mixed with WT Tim10 at various molar ratios. At the ratio of 7:14, the situation was same with the 10:10 ratios. no Tim9C1,4S formed complex was detected (Figure 4. 12 lane 4), while the Tim9C2,3S formed ~75kDa complex can be observed (Figure 4. 12 lane 5). However, at the ratio of 14:7, it was observed that the Tim9C1,4S may form an ~70kDa complex (Figure 4. 12 lane 6), and the ~75kDa complex formed by Tim9C2,3S was clearly observed as before (Figure 4. 12 lane 7). Therefore, this result was consistent with that of gel filtration.
Figure 4. **12 BN-PAGE analysis of complexes formed by Tim9wt and double cysteine mutants.** Tim9wt, Tim9C1,4S and Tim9C2,3S were mixed with Tim10 at various molar ratios. The samples were analysed by 6-16% BN-PAGE, and visualised by Coomassie Blue staining.
Because the BN-PAGE is not sensitive enough for detecting all the complexes, the cross-link method was utilised to understand the oligomer state or size of the complexes formed between Tim10 and the double cysteine mutants of Tim9. The proteins were prepared in the same way as that for BN-PAGE. The 0.1% (v/v) gluteraldehyde (GA) was used as chemical cross-linker, which reacts mainly with amino groups of lysine residues resulting in forming stable inter- and intra-subunit covalent bonds. The cross-link reaction was performed at room temperature for 10 minutes, followed by adding 100mM Tris-HCl to quench the reaction. Then, the samples were analysed by 16% Tricine SDS-PAGE, followed by Coomassie Blue staining (Figure 4. 13). Using the same ratios of proteins analysed in BN-PAGE, the cross-link of Tim9wt and Tim10 showed there were 6 oligomers, such as monomer, dimer, trimer, tetramer, pentamer and hexamer. The predominant oligomers were dimers, monomers and trimers, with relative low levels of higher oligomers present. This evidence verified that the complex formed by Tim9wt and Tim10 was in the form of hexamer. The cross-link of Tim9C2,3S and Tim10wt showed the similar high oligomers like WT, suggesting Tim9C2,3S may form a WT-like complex with Tim10. However, no higher oligomers than trimers were observed in the cross-link of Tim9C1,4S and Tim10, indicating the Tim9C1,4S can not form the same complex as the WT, or the formed complex was smaller than WT as shown in the gel filtration and BN-PAGE. Due to the western-blot was lacking after the analysis of cross-link, this result was limitary to confirm the evidences of gel filtration and BN-PAGE.
Figure 4. 13 SDS-PAGE analyses of hetero cross-link of Tim9wt and double cysteine mutants with Tim10wt. Purified Tim9wt and single disulphide mutants were mixed with Tim10wt at ratios of 1:1, 1:2 and 2:1 respectively. Then 0.1% (v/v) gluteraldehyde (GA) was added for cross-linking, and reacted at room temperature for 10 min. 16% Tricine SDS-PAGE was performed for the analysis of samples, and visualised by Coomassie Blue staining.
4.6 Discussion

The purification of double cysteine mutants of Tim9 (Tim9C1,4S and Tim9C2,3S) had the problem that the expressed recombinant mutants were less soluble and form inclusion bodies (Figure 4. 2 and Figure 4. 3). The same situation happened with the purification of Tim9wt in a previous study. The GST-Tim9wt was intrinsically insoluble and up to 75% of the protein accumulated in inclusion bodies (Vial et al., 2002). It was shown that the correct folding of Tim9 depends on the formation of intramolecular disulphide bonds (Lu et al., 2004a). There are four conserved cysteines in Tim9, the properly folded Tim9 has formed two intramolecular disulphide bonds between all four cysteines. In the absence of intramolecular disulphide bonds, Tim9 is unfolded as shown by CD (Morgan et al., 2009). In this study, far-UV CD studies showed that both single disulphide bond mutants were not as folded as WT Tim9 (Figure 4. 6). They are largely unfolded, which may be a reason why the mutants are less soluble and easy to form inclusion bodies.

Previous in vitro study has suggested a model about biogenesis and assembly of Tm9-Tim10 complex. The reduced Tim9 and Tim10 import into the IMS followed by oxidative folding that is catalysed by Mia40-Erv1 disulphide relay system. Only the oxidatively folded Tim9wt can form a hexameric complex with Tim10 (Lu et al., 2004a, Lu et al., 2004b). In this study, it is shown for the first time that both outer and inner disulphide bond mutants of Tim9 can interact with WT Tim10 (Figure 4. 7). In terms of binding affinity, both mutants have a higher K_d and thus lower affinity than the WT proteins. In particular, Tim9C1,4S is weaker than Tim9C2,3S to interact with Tim10.

Furthermore, gel filtration gave the direct evidence on the complex formation. It showed that both mutants can form complexes with Tim10, and Tim9C1,4S
formed smaller sized complex, while Tim9C2,3S formed a complex with a size similar to that of Tim9wt with Tim10 (Figure 4. 11). Moreover, the BN-PAGE result was consistent with that of gel filtration, which detected the full sized complex formed by the Tim9C2,3S, and also detected a smaller complex formed by Tim9C1,4S (Figure 4. 12). Although the hetero cross-link can not fully confirm the result of gel filtration, it showed the Tim9C2,3S tended to form hexameric complex with Tim10, and the similar high oligomers were hard to be formed by Tim9C1,4S and Tim10 (Figure 4. 13). All in all, Tim9 double cysteine mutants can not efficiently form a WT-like Tim9-Tim10 complex in vitro, and the formation of Tim9-Tim10 complex is less dependent on the present of the inner disulphide bond of Tim9, but is more dependent on the formation of outer disulphide bond of Tim9. In order to answer whether the complexes formed by Tim9 double cysteine mutants can function as the WT, in vivo studies on the roles of cysteines of Tim9 were performed and are reported in the next chapter.
5 RESULTS AND DISCUSSION III:

In vivo study of the role of cysteines in the biogenesis of small Tim proteins

5.1 Introduction

In previous work, small Tim protein biogenesis has been studied using in vitro or in organelle methods (Lu et al., 2004b, Sideris and Tokatlidis, 2007, Ivanova et al., 2008). These works have indicated that particular cysteines of Tim9 or Tim10 are very important for protein import and complex formation. Specially, it was found that the most N-terminal cysteine mediated the interaction between imported small Tim proteins and Mia40. During this process, Mia40 traps the incoming small Tim proteins in the IMS, and completes their oxidative folding which is then followed by assembly of the Tim9-Tim10 complex (Milenkovic et al., 2007, Sideris and Tokatlidis, 2007). However, in vivo data for the mechanisms of the oxidation of substrates in the IMS is lacking. Especially, the understanding about the role of disulphide bonds in the formation of small Tim complexes is limited.

On the basis of the in vitro results of Chapter 4, which investigated effects of Tim9C1,4S or Tim9C2,3S mutants on protein folding and complex formation, I wanted to assess whether mutation of individual cysteines and disulphide bonds influence the viability of yeast cells by using yeast genetic methods. The CD study has shown that in vitro Tim9C1,4S and Tim9C2,3S are partially folded compared to Tim9wt. Furthermore, Tim9C1,4S forms an intermediate complex and Tim9C2,3S can not efficiently assemble the hexamer Tim9-Tim10 complex. Thus, the next step is to study if Tim9 with a single disulphide bond affects in vivo assembly of Tim9-Tim10 complex, resulting in defects in cell viability, or whether
the intermediate complex formed by Tim9C1,4S contributes the same function as
the hexamer complex for cell viability.

In yeast cells, the endogenous TIM9, TIM10 genes were individually regulated by
the integration of a KanMX-tetO2 or KanMX-pMET3 cassette. The cysteines of
small Tim proteins were mutated, and then transformed into the constructed
yeast strains. Spotting tests were performed to investigate the effects of cysteine
mutants on cell viability. Meanwhile, the temperature sensitivity and redox stress
were tested for mutant strains and protein levels in these mutants were tested by
western-blots and pulse labeling. Finally, in order to avoid trace of endogenous
proteins affecting results, a TIM9 knock-out yeast strain was applied to the in vivo
study of single disulphide Tim9 mutants, which allowed isolation and analysis
of mitochondria from TIM9 knock-out WT and Cys mutant strains.

5.2 Phenotypes of small Tim cysteine mutants

In the in vitro studies, purified double cysteine mutated proteins of Tim9 or
Tim10 do not form normal complexes, making it probable that these mutants
might be lethal in vivo. In order to investigate whether and how cysteine mutants
can affect mitochondrial biogenesis in vivo, yeast cells must be kept alive and
have compact structures and functions. Thus, in this study, two yeast systems
have been constructed to regulate expression of endogenous small TIM genes,
which apply different mechanisms for regulation. But the common aspect of their
constructions is the integration of KanMX4-tetO2 or KanMX4-pMET3 cassette into
the relatively short upstream regions of target genes. The promoter of tetO
allows doxycycline-repressible expression, while the other one, pMET3, can switch
off gene expression in the presence of methionine (Yen et al., 2003).
5.2.1 Phenotypes of *KanMX4-tetO*₂ regulating strains

For construction of TetO regulated Tim9, the oligonucleotides tetO.P1 and tetO.P2 are used to amplify the promoter-replacement cassettes (Figure 5. 1). The oligonucleotides used in this study are listed in the Appendix 8. 4. To identify successfully integrated cassettes, a pair of oligonucleotides was designed for yeast colony PCR screening. For the 5’ region, tetO.P3 annealed about 200bp further upstream of tetO.P1, and tetO.P4 for the 3’ region is downstream of tetO.P2. All oligonucleotides were designed using OLIGO 6.0 software (http://www.oligo.net/oligo updates.htm). The promoter-replacement cassette, *KanMX4-tetO*₂ was cloned by PCR using P1/P2 primer pairs for TIM9, TIM10 and TIM12 respectively. The pCM324 vector containing the *KanMX4-tetO*₂ cassette was used as template. Transformation of PCR product into parent yeast stain, CML476 (*MAT a ura3-52 leu2Δ1 his3Δ200 GAL2 CMVp(tetR’-SSN6)::LEU2 trp::CMVp-tTA*), was performed by LiAc method as described in the methods Chapter (Gietz et al., 1992). The PCR product underwent homologous recombination with genomic DNA during yeast transformation, so that the cassette of *KanMX4-tetO*₂ was integrated into the genome of yeast. The selection of positive transformed strains employed resistance to 200 or 400um/ml of geneticin (G418), used to select for the *KanMX4* marker in yeast. In order to further confirm whether transformation of cassette is successful, yeast colony PCR screening was applied with designed diagnostic oligonucleotides (Figure 5. 1 A).
Figure 5. 1 The construction scheme and regulation mechanism of *KanMX4-tetO* yeast strain.

(A) The construction scheme of *KanMX4-tetO* yeast strain. The regulation cassette of *KanMX4-tetO* is amplified using designed primers. With the transformation into CML476 cells, the cassette is inserted between the small TIM ORF and TIM promoter. In order to diagnose if the cassette is successfully recombined in the yeast genomic DNA, yeast colony PCR with diagnostic primers is performed.

(B) The regulation mechanism of *KanMX4-tetO* in cell. The tTA is expressed by the gene integrated in the CML476 genomic DNA. In the absence of Doxycycline, the tTA will bind to tetO in the transformed cassette triggering transcription of TIM gene. In the presence of Doxycycline, the tTA will bind to Doxycycline, resulting in the failure of transcription of target gene.
The mechanism of expression regulation by tetO₂ promoter employs the action of a tetR-VP16 (tTA) hybrid transactivator. In this study, tTA gene has been integrated into the parent yeast strain, CML476. The expressed tTA composes two moieties. One is tetR moiety that is responsible for tetO recognition and binding, the other is VP16, the activator moiety. In the absence of doxycycline (Dox), tTA can recognise and bind to the tetO₂ promoter via the tetR moiety, and then transcription is activated by the VP16 moiety so that tetO-driven expression occurs. In the presence of doxycycline or other molecules of the tetracycline antibiotic family can particularly bind to tTA with high affinity, the transcription is blocked, leading to silencing the expression of the following gene (Figure 5. 1 B) (Belli et al., 1998).

In this study, TIM9, TIM10 and TIM12 were individually regulated by the integrated KanMX4-tetO₂ cassette. According to the scheme described in Figure 5. 1, transformation of the amplified cassette was performed followed by the first round selection with G418 (data not shown). In order to make certain of KanMX4-tetO₂ cassette integrating into the yeast genome, yeast colony PCR was utilised with tetO.P3 and tetO.P4 primers (Figure 5. 2). The KanMX4-tetO₂ cassette is 2kb; the ORF of TIM9 is 0.26Kb; tetO.P3 is located at 0.2Kb upstream of the cassette; and tetO.P4 is located at 200Kb downstream of the ORF. Thus, adding three fragments together, the target band is about 2.6Kb for the TIM9 strains. In 20 candidate TIM9 colonies which have been selected by G418, at least 6 colonies can produce the target size bands, but these bands look smeared and possible non-specific (Figure 5. 2 A). This problem may be caused by non-specific priming, or be caused the long fragment is not efficient is amplified under the uniform conditions of yeast colony PCR. There are also two bands below 1Kb, indicating these two strains did not contain an integrated cassette. In the case of the TIM10 strains, given the ORF of TIM10 is about 0.3Kb, the target size is about 2.7Kb. Among 20 candidates, only 3 strains have bright and correctly sized bands.
(Figure 5.2 B). Although this yield is less than that of TIM9 screening, positive bands are very clear without any contamination. The TIM12 transformation has more positive colonies than TIM10, with at least 6 positive strains in 20 candidates. Meanwhile, the PCR product is as specific as TIM10 (Figure 5.2 C). All in all, the yeast colony PCR provides a more accurate and sensitive method to diagnose the false-positive strains after G418 selection. All of the true-positive strains identified by yeast colony PCR required further phenotype tests for final evaluation.
Figure 5. 2 Yeast colony PCR screening of KanMX4-tetO₂ TIM9, TIM10 and TIM12 strains. Colonies were collected from G418 YPD plates, and dissolved in 10µl ddH₂O. Yeast colony PCR was performed as described in methods followed by 1% Agarose gel analysis. The PCR primers for screening are tetO.P3 and tetO.P4. (A) Screening of TIM9 strains. The target size is ~2.6Kb. (B) Screening of TIM10 strains. The target size is ~2.7Kb. (C) Screening of TIM12 strains. The target size is ~2.7Kb.
To demonstrate whether endogenous small TIM genes can be regulated by the tetO promoter-substitution cassette, the phenotype of the constructed strains was analyzed by using spotting tests on YPD media in the presence or absence of 50μg/ml doxycycline at 30°C (Figure 5. 3). In the absence of doxycycline, the tetO regulated TIM12 grew the same as the non-regulated strain (CML476), but the TIM9 and TIM10 strains grew slightly slower (Figure 5. 3, left panel). In contrast, the TIM9 and TIM10 strains cannot grow in the presence of doxycycline, but there was some growth of the TIM12 strain at a high concentration of cells (Figure 5. 3, right panel). Overall, the results showed that the expression of these genes under the regulation of tetO could be suppressed by the adding of doxycycline. The slightly slow growth of the TIM9 and TIM10 strains in the absence of doxycycline may be caused by a manipulative error during sample dilution, or because the introduced exogenous promoter decreases the growth rate of cell. Importantly, these results confirmed that Tim9, Tim10 and Tim12 are essential for yeast viability, which is consistent with previous studies (Koehler et al., 1998, Tokatlidis and Schatz, 1999), and that expression of endogenous small Tim genes can be regulated by the tetO promoter.

**Figure 5. 3 Phenotypes of endogenous Tim9, Tim10 and Tim12 regulated by KanMX4-tetO2 in yeast.** Cells were grown at 30°C in YPD media until stationary phase before dilution to OD600= 1, followed by 10-fold gradient dilutions using fresh YPD media. 5μl diluted cells were spotted onto YPD plates with or without 50μg/ml of doxycycline (Dox) and incubated at 30°C for 48 hours. The original strain CML476 treated in the same manner was used as control.
Having created stains in which expression of Tim9/10/12 was regulated, we could use these strains to test the requirement for the small Tim cysteine residues in vivo. The in vitro studies have demonstrated that the cysteines of the small Tim proteins are important for protein folding and complex assembly. As Tim9, Tim10 and Tim12 are essential for cell viability, plasmid-based approach was employed to investigate the roles of individual cysteines and their intramolecular disulphide bonds. To study single cysteines, each cysteine residue was mutated into serine, for example, generating Tim9C1S, Tim9C2S, Tim9C3S and Tim9C4S. Based on the single mutants, double cysteine mutants were created for inner or outer disulphide bond, like Tim9C1,4S and Tim9C2,3S. All shuttling plasmids used in this study have been listed in the Appendix 8.3. Plasmids encoding the cysteine mutated proteins were transformed into the appropriate KanMX4-tetO2 cassette regulated strains. The parental strain has the endogenous tryptophan gene mutated, so the cell can not survive in medium without Trp provided. However, the shuttling vector pRS414 can express Trp for the requirement of host cell growth. Therefore, using the synthetic defined minus tryptophan media (SD-Trp), the host strain without any plasmid can be eliminated to confirm the transformation of plasmids.
In the presence of doxycycline, the phenotype of TIM9 single cysteine mutants showed that C1S was identical to WT; C3S, C4S grew slower than WT, but C2S could not grow at all (Figure 5.4, left panel). As expected, all single cysteine mutants grew as normal in the absence of doxycycline (Figure 5.4, right panel). Therefore, this result suggested that C2 of Tim9 was essential for cell viability. For the double cysteine mutants, C1,4S is healthy, whereas C2,3S is lethal. C3 being viable suggests C2 is important, but not the C2-C3 disulphide bond.

Figure 5.4 Spotting test of Tim9 cysteine mutation in the KanMX4-tetO2 yeast strains. Tim9 cysteine mutated plasmids were constructed with the pRS414 shuttling vector, and individually transformed into tetO2 regulated TIM9 yeast strains. Each strain was grown in SD-Trp media at 30°C to mid-log phase (OD600=0.5~0.6), then gradiently diluted by 10-folds with fresh SD-Trp media. 5µl of diluted cells were spotted on SD-Trp with or without 50µg/ml DOX, followed by incubating at 30°C for 2 days. Note: WT: wild type; VT: vector only; cysteines were indicated by the order.
For Tim10, the spotting test showed that only the C4S mutant can not grow in the presence of doxycycline (Figure 5.5, left panel). In the absence of doxycycline, all strains were healthy (Figure 5.5, right panel). Interestingly, the normal C1,4S mutant did not have a similar growth defect to C4S, suggesting that only C4 of Tim10 is required for cell viability, and its role may be more important than that of disulphide bonds in vivo. More than that, it also suggests that C1S rescues the growth defect of the C4S mutation.

Figure 5.5 Spotting test of Tim10 cysteine mutation in the KanMX4-tetO2 yeast strains. Tim10 cysteine mutated plasmids were constructed with the pRS414 shuttling vector, and individually transformed into tetO2 regulated TIM10 yeast strains. Each strain was grown in SD-Trp media at 30°C to mid-log phase (OD600=0.5~0.6), then gradiently diluted by 10-folds with fresh SD-Trp media. 5µl of diluted cells were spotted on SD-Trp with or without 50µg/ml DOX, followed by incubating at 30°C for 2 days. Note: WT: wild type; VT: vector only; cysteines were indicated by the order.
In the case of Tim12, because it contains two extra non-conserved cysteine residues as well as four cysteine residues in the twin CX$_3$C motif, six single cysteine mutants were tested for whether they have important roles in vivo. With repression of the genomic copy by doxycycline, the phenotypes of single cysteine mutants showed that the C2S and C6S of Tim12 can not survive like WT (Figure 5. 6). The double cysteine mutant containing C2 and C6 could also not grow in the presence of doxycycline. Therefore, this suggests that the outer disulphide bond of Tim12 might have an essential function in yeast. In contrast, both the inner disulphide cysteines C3 and C5, and the non-conserved cysteines C1 and C4 were not essential.

**Figure 5. 6 Spotting test of Tim12 cysteine mutation in the KanMX4-tetO$_2$ yeast strains.** Tim12 cysteine mutated plasmids were constructed with the pRS414 shuttling vector, and individually transformed into tetO$_2$ regulated TIM12 yeast strains. Each strain was grown in SD-Trp media at 30°C to mid-log phase (OD$_{600}$=0.5~0.6), then gradiently diluted by 10-folds with fresh SD-Trp media. 5μl of diluted cells were spotted on SD-Trp with or without 50μg/ml DOX, followed by incubating at 30°C for 2 days. Note: WT: wild type; VT: vector only; cysteines were indicated by the order.
As the used yeast genome DNA was modified by integration of the *KanMX4-tetO*$_2$ cassette, it was possible to induce yeast cells into petite (Senapin et al., 2003). In order to known if the constructed strains were petite, the growth of strains was tested by the non-fermentable medium (e.g. YPEG and lactate medium), since the respiratory deficient or petite mutants can not grow without dextrose as carbon source. Unfortunately, the constructed mutations, VT and WT of Tim9, Tim10 and Tim12 can not grow on plate of YPEG, indicating these strains were petite (Figure 5. 7).

![Figure 5. 7](image)

*Figure 5. 7 Phenotype of the KanMX4-tetO2 regulated yeast strains on non-fermentable medium.* The strains were inoculated on YPD and YPEG plates from stock culture, followed the incubation at 30°C for 5 days.
5.2.2 Phenotypes of KanMX4-pMet3 regulating strains

Subsequent to the work in section 5.2.1, we discovered that the constructed KanMX4-tetO yeast strains are petites and cannot grow on non-fermentable medium, e.g. YPEG and lactate medium. It appears that the parental strain CML476 may be petite prone, and petite seems to be induced by the integration of tetO. Thus, it was better to use a different background parental strain. The new parental strain in this study is BY4742, whose genotype is MAT alpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0. Instead of KanMX4-tetO, the cassette of KanMX4-pMET3 was applied to regulate expression of the target genes, and was also integrated upstream of the ORF. However, these two cassettes use completely different mechanisms for regulation. ATP sulfurylase, an enzyme implicated in methionine biosynthesis in yeast, is encoded by MET3, which catalyzes the initial step of the sulfur assimilation pathway where inorganic sulphate and ATP generate adenosine 5'-phospho-sulphate (APS). Exogenous methionine (Met) and S-adenosyl methionine (SAM) can block expression of MET3, and SAM can also be formed from Met. Therefore, Met is a strong repressor for the transcription of MET3. MET3 has been used as a regulatory promoter for a long time, because it works stringently leading to critical control of the expression of target gene. Moreover, comparing to the commonly used GAL1 promoter, the addition or removal of Met causes less metabolic disturbance than switching carbon source (Care et al., 1999). KanMX4 works as selectable marker, which responds to G418. The cassette of KanMX4-pMET3 is amplified from pRS33, which also contains the Ampicillin antibiotic resistance gene. The shuttling vector for carrying mutated genes is pRS416, which can complement URA for growth and selection. The scheme for constructing KanMX4-pMET3 regulation yeast strain is presented in Figure 5.8.
Figure 5. The scheme of constructing KanMX4-pMET3 yeast strains. The pRS416-Tim9 plasmid was transformed into BY4742. After the selection of SD-URA, KanMX4-pMET3 cassette was transformed into the positive strain, followed by the selection of G418. The pRS416-Tim9 was removed by the 5-FOA medium without methionine. The cassette integrated strains can be selected by defective growth on SD+Met medium. Furthermore, the selected strains would be confirmed again by yeast colony PCR.
Here, the construction of \textit{KanMX4-pMET3} TIM9 yeast strain is used as an example to describe the detail of process (Figure 5.8). Due to the essential nature of TIM9 in yeast, exogenous TIM9 should be transformed into the strain to complement the silenced endogenous TIM9 by methionine in the YPD+G418 media which is used to select the positive colonies containing the \textit{KanMX4-pMET3} cassette. This is required because G418 is not effective in SD-Met media. The ORF of TIM9 plus 800bp of upstream and 500bp of downstream sequence was cloned into the pRS416 vector, and then transformed into BY4742. pRS416 vector contains URA3 that encodes orotidine 5-phosphate decarboxylase (ODCase), an enzyme involved in the synthesis of pyrimidine ribonucleotides. Loss of ODCase activity leads to a lack of cell growth unless uracil or uridine is added to the media. So, SD-URA media was employed to select the successful transformant of pRS416-Tim9 plasmid.

The cassette of \textit{KanMX4-pMET3} was amplified from the pPR33 plasmid using PCR. The oligonucleotides for PCR are pMET.P1 and pMET.P2, sequences of which are listed in the Appendix 8.4. The amplified cassette was transformed into the BY4742 pRS416-Tim9wt strain, and positive transformants were selected by YPD+G418 media. The surviving colonies indicate the cassette has been successfully integrated in the yeast genome, as \textit{KanMX4} allows G418 resistance. Although YPD contains Met that could regulate expression of the genomic Tim9 gene, previous transformation of pRS416-Tim9 plasmid would complement the lack of endogenous Tim9. Therefore, the successful transformant can still survive on YPD+G418 plates.

5-FOA is usually used to remove plasmids containing URA gene. This chemical is converted into the toxic compound 5-fluorouracil by ODCase which is encoded by the URA3 of pRS416-Tim9. After eliminating pRS416-Tim9 (plasmid for the complement of URA), yeast with the \textit{KanMX4-pMET3} cassette already integrated
would be dead in the presence of Met, as Tim9 is an essential protein for yeast. Thus, the cassette integrated strains can be selected by survival growth on SD-Met+5FOA and defective growth on SD+Met. For further conformation of integration of KanMX4-pMET3, yeast colony PCR was applied with pMET.P3 and pMET.P4 primers, producing a 2.4kb expected band.

**Figure 5.9 The process of constructing TIM9 KanMX4-pMET3 yeast strain.** (A) After transformation of KanMX4-pMET3 cassette into strains containing pRS416-Tim9 plasmid, strains were selected by using YPD+G418. (B) The pRS416-Tim9 was removed from the selected strains by SD-Met+5FOA plate. (C) The same strains in B were checked for the removal of plasmid and regulation of Tim9 by SD+Met plate. (D) The lethal strain on SD+Met plate (No.1 in A) was further identified for the integrated cassette by yeast colony PCR diagnosis with Tim9.pMET.P3 and Tim9.pMET.P4 primers. M: Marker; S: selected strain with the expected size of 2.4kb.
According to the above description, the strain with TIM9 regulated by KanMX4-pMET3 was constructed step by step (Figure 5. 9). The pRS416-Tim9 plasmid was successfully transformed into BY4742 strain (data not shown), followed by the transformation of the KanMX4-pMET3 cassette. The 8 candidate colonies containing pRS416-Tim9 and cassette were streaked on the YPD G418 plate (Figure 5. 9 A). The No.1 and No.2 strains can resist 400µg/ml G418, suggesting the KanMX4-pMET3 cassette has integrated into their genome. However, other strains can survive at the locations of initial streaks. The probable reason is that the cell density is the highest at the first streak, and the limited G418 can not efficiently inhibit growth of a large amount of cells. In order to remove pRS416-Tim9 from the above selected strains, the first four strains were streaked on SD-Met+5FOA plate (Figure 5. 9 B). Without Met in the media, the endogenous Tim9 was not regulated by the pMET3 promoter, allowing expression of Tim9 despite loss of pRS416-Tim9. To confirm removal of the exogenous Tim9 plasmid, the same strains were then streaked on SD+Met plate, which should silence the endogenous Tim9 expression (Figure 5. 9 C). Only the No.1 strain could not survive on the SD+Met plate, indicating that the pRS416-Tim9 was removed from the strain by 5-FOA and that Tim9 was expressed from the pMET3 promoter. For further confirm this strain, yeast colony PCR diagnosis displayed that a band around 2.4kb was generated as expected (Figure 5. 9 D). Therefore, the TIM9 KanMX4-pMET3 yeast strain had been successfully constructed.

As with the tetO-Tim9 strain, construction of pMET-Tim9 allow us to test the ability of plasmid-borne Tim9 mutants to support growth. Mutant plasmids were transformed into the TIM9 KanMX4-pMET3 yeast strain followed by spotting test with and without Met regulation (Figure 5. 10). On the SD+Met-Ura plate, with the suppression of endogenous Tim9 expression, Tim9C2S and Tim9C2,3S can not grow like Tim9wt, and the other mutants grow normally. These growth phenotypes are in the agreement with the tetO-regulated mutants. In order to
know whether cysteine mutated to serine in particular was causing the lethal phenotype, the second cysteine of Tim9 was mutated into alanine (A). However, C2A was lethal just like C2S, suggesting the serine residue does not affect the phenotype of cysteine mutants. In summary, the phenotypes of TIM9 KanMX4-pMET3 yeast strains are the same as that of the TIM9 KanMX4-tetO2 yeast strains, suggesting that C2 is essential for cell viability (Figure 5.4).

**Figure 5. 10 Spotting test of KanMX4-pMET3 regulated Tim9 cysteine mutant strains.** Cysteine mutant plasmids were constructed with pRS416 shuffling vector, and individually transformed into KanMX4-pMET3 regulated TIM9 yeast strains. Each strain was grown in SD-Met-Ura media at 30°C to mid-log phase (OD600=0.5~0.6), then gradually diluted by 10-folds with fresh SD-Met-Ura media. 5µl of diluted cells were spotted on both SD-Met-Ura and SD+Met-Ura plates, followed by incubating at 30°C for 2 days. WT: wild type; VT: vector only.
Next, the TIM10 *KanMX4*-*pMET3* yeast strain was constructed by the same method. For the phenotype of Tim10 cysteine mutants in which the genomic copy is regulated by *KanMX4*-*pMET3* promoter, only Tim10C4S cannot grow on the SD+Met-Ura plate (Figure 5. 11). This result matches that obtained from the spotting test of TIM10 *KanMX4*-tetO2 strains (Figure 5. 5). These two methods suggest that the C4 of Tim9 is essential for cell growth. According to the phenotypes of Tim9 and Tim10 mutants across the tetO2 or pMET3 regulated strains, it is clear that the important cysteine residues of Tim9 or Tim10 do not change, whether the yeast cells are petites or fully respiratory-competent.

Although the TIM12 *KanMX4*-*pMET3* yeast strain was not constructed, it can be hypothesised that the essential cysteines for Tim12 would be C2 and C6, which form the outer disulphide bond of Tim12.

**Figure 5. 11** Spotting test of *KanMX4*-*pMET3* regulated Tim10 cysteine mutant strains. Cysteine mutant plasmids were constructed with pRS416 shuttling vector, and individually transformed into *KanMX4*-*pMET3* regulated TIM10 yeast strains. Each strain was grown in SD-Met-Ura media at 30°C to mid-log phase (OD600=0.5~0.6), then gradiently diluted by 10-folds with fresh SD-Met-Ura media. 5µl of diluted cells were spotted on both SD-Met-Ura and SD+Met-Ura plates, followed by incubating at 30°C for 2 days. WT: wild type; VT: vector only.
5.3 Temperature sensitivities of Tim9 and Tim10 cysteine mutants

Yeast can grow at 16°C to 37°C, with faster growth at higher temperatures. Depending on the species, the optimal temperatures vary, but most around 30°C (Arthur and Watson, 1976). Genetic mutations can cause yeast to lose the capability to grow at elevated temperatures, often due to an inability to respond to stress or to a lack of protein stability. To study if there are some cysteine mutants sensitive to various temperatures, spotting tests of TIM9 KanMX4-tetO2, TIM9 KanMX4-pMET3 strains (Figure 5. 12) and TIM10 KanMX4-pMET3 strains were performed at different temperatures (Figure 5. 13).

For TIM9 KanMX4-tetO2 strains, the spotting tests were individually incubated at 24°C, 30°C and 37°C (Figure 5. 12). At 24°C, strains grew a little slower than at other temperatures. At 37°C, all mutants appeared stressed on SD-Trp+Dox as all grew slower than WT, especially Tim9C3S. It is possible that Tim9 mutants at 37°C may lead mitochondrial biogenesis to be slow. Overall, the phenotypes of lethal Tim9C2S and Tim9C2,3S are not rescued by various temperatures.

Figure 5. 12 Temperature sensitivities of TIM9 KanMX4-tetO2 strains. The strains were pre-cultured in liquid medium at 30°C for 16 hours, followed by adjusting culture to OD600=1.0. The cultures were serially diluted until OD600=10^-5 and spotted on plates. The incubations were at 24°C, 30°C and 37°C for 2~3 days.
The temperature sensitivities of TIM9 mutants were also tested at 30°C and 37°C with the *pMET3*-regulated strains (Figure 5.13). At 37°C, the Tim9C2S and Tim9C2,3S can not grow on SD+Met-Ura plate, as at 30°C, but the Tim9C3S showed sensitivity at 37°C, growing slower compared to the phenotype at 30°C. Because all TIM9 *KanMX4-tetO2* strains did not grow vigorously at 37°C, the specific defect of Tim9C3S wasn’t shown obviously in this strain (Figure 5.12), but can be seen in the *pMET3*-regulated strain (Figure 5.13).

**Figure 5.13 Temperature sensitivities of TIM9 KanMX4-pMET3 strains.** The strains were pre-cultured in liquid medium at 30°C for 16 hours, followed by adjusting culture to OD600=1.0. The cultures were serially diluted until OD600=10⁻³ and spotted on plates. The incubations were at 24°C, 30°C and 37°C for 2~3 days.
The temperature sensitivities of Tim10 cysteine mutants were tested at 30°C and 37°C in the KanMX4-pMET3 strains (Figure 5. 14). Tim10C4S was lethal at both 30°C and 37°C. Tim10C2S which grew well at 30°C was sensitive at 37°C. On the other hand, neither Tim10C1,4S nor Tim10C2,3S showed any defect at 37°C, suggesting that the loss of either inner or outer disulphide bond of Tim10 does not affect the cell viability. These Tim10 mutants may be enough stable to resistant to the elevated temperature. In contrast to double cysteine mutants, the single C2 mutant became slow growing at 37, meaning Tim10 without C2 result in a defect of some mechanisms in vivo, causing the temperature sensitivity.

Figure 5. 14 Temperature sensitivities of TIM10 KanMX4-pMET3 strains. The strains were pre-cultured in liquid SD-Met medium at 30°C for 16 hours, followed by adjusting culture to OD600=0.1. The cultures were serially diluted until OD600=10^-5 and spotted on SD+Met-Ura plates. The incubations were individually taken at 30°C and 37°C for 2~3 days.
5.4 Redox stress sensitivities of Tim9 cysteine mutants

As described in the biogenesis of small Tim proteins (The section 1.3.2), the redox state of the small Tim proteins is important for their import into mitochondria and the oxidative folding in the IMS. The reduced small Tim9 is imported into the mitochondrial IMS, where the proteins switch into the oxidised form, helped by the MIA pathway. The cysteine residues of small Tim proteins are sensitive to the redox states of cellular or mitochondrial environments, which are maintained by enzymes or factors in the cytosol or the IMS. A recent in vitro study showed that the cytosolic Trx system can reduce double cysteine mutants (C1,4S and C2,3S) of Tim9 and Tim10 (Durigon et al., 2012). To understand whether the phenotypes of Tim9 cysteine mutants can be affected by redox stress, H$_2$O$_2$ and DTT were added into plates, which were used for the spotting tests of TIM9 KanMX4-tetO$_2$ strains (Figure 5. 15). Dithiothreitol (DTT) is a common strong reducing agent, and usually used to reduce the disulphide bonds of proteins, and preventing cysteine residues from forming intramolecular and intermolecular disulphide bonds. Here, 1mM and 10mM DTT were applied to test the reductive stress sensitivity. The phenotypes of TIM9 KanMX4-tetO$_2$ WT and mutant strains on DTT plates didn’t have any differences with those on -DTT plates, though 10mM DTT repressed the growth of all strains (Figure 5. 15 A), indicating the phenotypes of Tim9 cysteine mutants were not sensitive to the reducing reagent DTT.

Hydrogen peroxide (H$_2$O$_2$) is often used as an oxidising agent in biology, and its strong oxidising power can make most proteins oxidised in vitro. To test oxidative stress sensitivity, H$_2$O$_2$ was used at concentrations of 0.5mM and 1mM (Figure 5. 15 B). In the case of low concentration of H$_2$O$_2$ (0.5mM), the phenotypes of TIM9 KanMX4-tetO$_2$ strains did not show any obvious differences with or without the regulation of Dox. However, 1mM H$_2$O$_2$, we observed some slow growth for
Tim9C3S and C4S on the +Dox plate. Unexpectedly, we also observed slow growth on –Dox plate for Tim9C2S, C3S and C4S, suggesting a general slight effect of high concentration of H$_2$O$_2$. However, as with sensitivity to reductive stress, the phenotypes of Tim9 cysteine mutants under oxidative stress are barely different to WT.

**Figure 5. 15 Redox stress sensitivities of TIM9 KanMX4-tetO$_2$ strains.**
Both SD-Trp and SD-Trp+Dox plates were prepared by adding various concentrations of DTT or H$_2$O$_2$. Cells for spotting tests were incubated in liquid SD-Trp media until mid-log phase (OD600=0.5~0.6). The pre-culture was gradiently diluted with liquid SD-Trp media and 5µl was spotted on SD-Trp and SD-Trp+Dox plates. Then the plates were incubated at 30°C for 2 days. (A) Sensitivity test against 1mM and 10mM DTT. (B) Sensitivity test against 0.5mM and 1mM H$_2$O$_2$. 

151
Because previous studies showed that the oxidised form of Tim9 can reduce the efficiency of its mitochondrial import, contrasted to the sensitivity of reductive stress, oxidative stress might affect small Tim proteins more (Morgan and Lu, 2008). After observing the small H₂O₂ effect on tetO-regulated strains, we would like to see if it is confirmed with pMET3-regulated strains. The sensitivities to oxidative stress were therefore tested with the TIM9 KanMX4-pMET3 strains (Figure 5. 16). The results showed that all Tim9 cysteine mutants were not sensitive to 1mM and 2mM H₂O₂. Therefore, this result confirms again that oxidising agent has little effect on the phenotypes of the Tim9 cysteine mutants. It also suggests that the small defects of tetO strains are due to them being petites.

**Figure 5. 16 Oxidative stress sensitivities of TIM9 KanMX4-pMET3 strains.** The 1mM and 2mM H₂O₂ were added into SD+Met-Ure and SD-Met-Ura plates in advance. Cells for spotting tests were incubated in liquid SD-Met-Ura media until mid-log phase (OD600=0.5~0.6). The pre-culture was gradiently diluted with liquid SD-Met-Ura media and spotted 5µl on SD+Met-Ure and SD-Met-Ura plates, followed by incubation at 30°C for 2 days.
5.5 Steady-state levels of Tim9 and Tim10 in Tim9 cysteine mutants

The growth defects of the small Tim mutants may be caused by many factors in vivo, but the protein levels of mutants should be the simplest to test. To assess the steady-state levels of Tim9 and Tim10 in cells, yeast proteins were extracted from KanMX4-pMET3 strains carrying Tim9 or Tim10 on plasmids, followed by analysis by western-blot with Tim9 or Tim10 antibodies (Figure 5. 17). Mitochondria numbers and size differ between log and stationary phases, so both these two phases were tested for cells grown in SD+Met media. As a control, in the SD-Met media, both Tim9 and Tim10 can be detected in the extracts from all KanMX4-pMET3 strains at stationary phase (Figure 5. 17 A). By contrast, in the SD+Met-Ura, Tim9 can not be detected at either log or stationary phase in the lethal Tim9C2S and Tim9C2,3S mutants. For the complex partner Tim10, although it can be detected in all strains, its levels in Tim9C2S and Tim9C2,3S mutants were less than in WT and other non-lethal cysteine mutants. Additionally, the Tim9C3S mutant is temperature sensitive at 37°C, so it is interesting to know if its Tim9 level is also decreased like the lethal cysteine mutants. Thus, total yeast proteins were extracted from the culture of Tim9C3S incubated at 37°C (Figure 5. 17 B). Indeed, at 37°C, the protein level of Tim9C3S was nearly as hard to detect as Tim9C2S in the SD+Met media. All in all, the above results indicate that the steady-state levels of Tim9 in the Tim9 cysteine mutants is relevant to the growth phenotypes, and the levels of Tim9 directly affect the steady-state levels of Tim10 in vivo.
Figure 5. 17 Protein steady-state levels of TIM9 KanMX4-pMET3 strains. (A) Cells were harvested at log and stationary phase of cultures that were incubated at 30°C. (B) Cells were harvested at stationary phase of cultures that were incubated at 37°C. Total proteins were extracted from the harvested cells, and analysed by western-blot and immunodecoration with the indicated antibodies.
According to the growth phenotypes of the TIM10 *KanMX4*-p*MET3* strains, Tim10C4S is lethal for cell viability, and the Tim10C2S mutant is temperature sensitive at 37°C (Figure 5. 14). To study if the lethality of the Tim10 cysteine mutants is caused by the lack of protein, the steady-state levels of Tim9 and Tim10 in these strains were analysed from the cultures grown at 30°C and 37°C (Figure 5. 18). In the SD-Met media, Tim10 can be detected in all the 30°C cultures of TIM10 *KanMX4*-p*MET3* strains (Figure 5. 18 A). With the regulation of genomic Tim10 by SD+Met, Tim10 can still be detected in the extracts from the lethal Tim10C4S and temperature sensitive Tim10C2S mutant, which were incubated at both 30°C and 37°C (Figure 5. 18 B and C). Meanwhile, the Tim9 levels in the Tim10C4S mutant are down, compared to Tim9 mutants (Figure 5. 18). This means that the defects of the lethal Tim10 cysteine mutants are probably not caused by the lack of Tim10 and Tim9, which is different to the Tim9 cysteine mutants.

Figure 5. 18 Protein steady-state levels of TIM10 *KanMX4*-p*MET3* strains. (A) Cell were harvested at stationary phase of SD-Met-Ura culture at 30°C, and analysed for Tim10 levels by western-blot. Cells were harvested at stationary phase of SD+Met-Ura culture at 30°C (B) and 37°C (C), followed by the analysis of Tim9 and Tim10 levels using western-blot.
The lethality of the Tim9 mutants appears to be due to low levels of protein. This could be due to low levels of expression, or because the protein is unstable. In order to investigate the reason why Tim9 can not be detected in the lethal Tim9 cysteine mutants, TIM9 KanMX4-pMET3 WT, C2S and C2,3S strains were pulse labeled with \(^{35}\)S, chased for several hours followed by immunoprecipitation (IP) with Tim9 antibody (Figure 5. 19 left panel). Because \(^{35}\)S is incorporated during synthesis of protein, the newly synthesised protein carrying the radioactive label would subsequently be imported into mitochondria where it would complete its biogenesis. Thus, this method is useful for tracking the location and expression of a specific protein. As expected, the Tim9 level in the WT strain gradually decreased during the chase. For Tim9C2S and C2,3S, the Tim9 levels seemed to not decrease with time, but instead there was less accumulation of labeled Tim9 after pulse labelling. However, it is difficult to judge whether the lethal Tim9 cysteine mutants were poorly expressed by exogenous plasmids, or whether the expressed proteins were degraded less quickly by protease due to improper conformations.

To compare with the IP for pulse labeling, the same samples were also analysed for their total Tim9 content, which was detected by western-blot using Tim9 antibody (Figure 5. 19 right panel). The result showed that the Tim9 level for WT was steady over the time course, which confirmed that Tim9 was expressed from the Tim9 plasmid. As in the result shown in Figure 5. 17 A, levels of Tim9C2S and Tim9C2,3S were scarcely detectable in SD+Met. The weak Tim9 levels in SD+Met may be caused by the loss of proteins during the extraction from cells.
Figure 5. 19 IP of $^{35}$S pulse labeled Tim9 in TIM9 KanMX4-pMET3 WT and lethal cysteine mutants. TIM9 KanMX4-pMET3 WT, C2S and C2,3S were cultured in SD-Met-Ura until log phase. After pulse labeling by $^{35}$S for 10 minutes, cells were switched into SD+Met-Ura and individually harvested after incubations of 2 hours, 4 hours and 8 hours. The Tim9 levels were detected by IP using Tim9 antibody and visualising with autoradiography (left panel). The samples were analysed for Tim9 levels by using western-blot with Tim9 antibody (right panel).
5.6 Studies of disulphide bond mutants in TIM9 KO strains

5.6.1 Phenotypes of disulphide bond mutants in TIM9 KO strains

The results of the previous section suggested that the Tim9C2S and Tim9C2,3S mutants were expressed at a low level, but stable. Since autoradiography of $^{35}$S is much more sensitive than western-blots visualised by fluorescent antibodies, it is possible that the minimal Tim9 detected after $^{35}$S pulse labeling, was expressed from the genomic copy of the gene, even though the expression of endogenous Tim9 in KanMX4-pMET3 yeast strains was repressed using SD+Met media. The reason for this problem is that shut down of genomic TIM9 expression is not 100% efficient, so it is inevitable that some proteins are expressed. In order to eliminate this problem, the same parental strain used to make KanMX4-pMET3 yeast (BY4742) was used to knock-out the genomic TIM9 ORF. The Tim9 ORF was replaced with a PCR fragment containing the bleR antibiotic resistance gene, and positive colonies were selected by YPD+bleomycin media. Prior to that, as Tim9 is an essential gene, Tim9 in the pRS416 vector was transformed into BY4742. The TIM9 KO was done by the help of Dr. M. Spiller. Subsequently, disulphide bond mutants (Tim9C1,4S and Tim9C2,3S) and WT within pRS415 vectors were transformed into previous TIM9 KO strains, FOA media was then employed for the removal of pRS416 Tim9wt plasmids (Figure 5.20 A). In contrast to the control of empty vector (VT), WT and both of mutants are alive on the FOA plate. It is a surprise Tim9C2,3S grew well. This contrasts strongly with the phenotype of KanMX4- tetO2 (Figure 5.4) Tim9C2,3S and KanMX4- pMET3 Tim9C2,3S (Figure 5.10).

Next, the growth phenotypes of WT and mutants were analysed by incubations at 25°C, 30°C and 37°C on YPD plates (Figure 5.20 B). As expected C1,4S grew at
all temperatures, although slightly slower than WT. However, Tim9C2,3S was temperature sensitive as it did not grow at 37°C.

**Figure 5. 20 Phenotypes of TIM9 KO disulphide bond mutants.** (A) The pRS416-Tim9wt plasmid was transformed into BY4742 in advance. The ORF of TIM9 was replaced in the genome of BY4742 by PCR product containing the bleR antibiotic resistance gene. The Tim9wt, C1,4S and C2,3S plasmids within pRS415 were individually transformed into the TIM9 KO strains, and selected by FOA media. (B) The growth phenotypes at various temperatures. The selected WT and mutants were incubated at 25°C, 30°C and 37°C on YPD plates.
5.6.2 The protein stabilities of single disulphide bond mutants

The constructions of Tim9 single disulphide bonds mutant in Tim9 KO background allowed the isolation of mitochondria to analyse the Tim9C1,4S and C2,3S mutants in organelle. Based on the phenotypes of TIM9 knock-out strains, the mitochondria of WT, Tim9C1,4S were isolated from the stationary phase of cultures grown at 30°C. However, Tim9C2,3S mitochondria were isolated from the stationary phase of cultures grown with and without shift from 30°C to 37°C.

To investigate the steady-state levels of Tim9 and Tim10 in the TIM9 KO disulphide bond mutants, isolated mitochondria were solubilised and analysed by western-blot with Tim9 and Tim10 antibodies (Figure 5. 21). The Tim9 and Tim10 levels in the Tim9C1,4S mitochondria were similar with that to WT. In contrast, the Tim9C2,3S mitochondria which were isolated at from cells grown at 30°C had reduced levels of both Tim9 and Tim10, and they were almost undetectable after temperature shift to 37°C. This correlates with the lethality of this mutant at 37°C, and with the steady-state levels of Tim9 and Tim10 in the KanMX4-pMET3 TIM9C2,3S strain which is also lethal for cell viability (Figure 5. 17 A). Therefore, this is further evidence that the growth phenotype of Tim9C2,3S is caused by low-levels of Tim9 and Tim10 in vivo.
Figure 5. Steady-state levels of Tim9 and Tim10 in the isolated mitochondria of TIM9 KO strains. The mitochondria of Tim9 WT and Tim9C1,4S were isolated from the stationary phase of culture grown at 30°C. The Tim9C2,3S cells were pre-cultured at 30°C, followed by the isolation of mitochondria from the stationary phase of culture grown at 30°C and 37°C. The harvested cells were solubilised using 1% digitonin. Western-blot with Tim9 and Tim10 antibodies was performed for analysis of protein steady-stat levels.
It appears that the temperature sensitive growth phenotype of Tim9C2,3S is due
to very low protein levels. To further investigate how the protein levels in Tim9
disulphide mutants were affected by high temperature, we used the isolated
mitochondria to test the stability of Tim9 mutants at 37°C (Figure 5. 22). Using
the same amount of mitochondria isolated at 30°C, the Tim9 levels after
mitochondria incubation at 37°C for various time were analysed by western-blot
(Figure 5. 22 A). First, at time 0, without the influence of 37°C, the protein level
in the Tim9C2,3S mutant was relatively low, suggesting that Tim9C2,3S at 30°C
was still less stable than Tim9C1,4S. Next, to compare the Tim9 stabilities at
37°C, the relative intensities of bands were qualified by AIDA analysis and plotted
against time (Figure 5. 22 B). The graph showed that both Tim9C1,4S and
Tim9C2,3S were less stable than WT. The stabilities of Tim9 in Tim9C1,4S and
Tim9C2,3S mutants at 37°C were almost same, and Tim9 levels were significantly
decreased with time.
Figure 5. Temperature stability of Tim9 in mitochondria of WT and mutants. (A) The mitochondria of WT, Tim9C1,4S and Tim9C2,3S were isolated from cell grown at 30°C, and incubated at 37°C for 160 minutes. The Tim9 levels in these mitochondria were checked at several time points by αTim9 western-blot analysis of solubilised mitochondria. (B) The curves of Tim9 stability with extension of time were made by AIDA analysis, depending on the relative intensity of bands in A. The bands at 0 minute time point were used as 100%.
5.7 Discussion

5.7.1 Phenotypes of cysteine mutants of small Tim proteins

With the regulation of small Tim expression by *KanMX4-tetO2* or *KanMX-pMET3*, the growth phenotypes of Tim9, Tim10 and Tim12 cysteine mutants have been obtained by spotting tests. Initially, tetO-regulated strains were used. They showed that C2S and C2,3S are lethal for Tim9, C4S for Tim10, C1S and C1,4S for Tim12 (Table 5. 1). Although all *KanMX4-tetO2* strains were not used for further experiments with isolated mitochondria because of petite formation, Tim9 and Tim10 have previously been found to be required for petite-survival (Senapin et al., 2003). Thus, this suggests that the growth phenotypes observed for the Tim9/10/12 mutants have some relevance. To avoid the petite problem associated with the tetO strains, the growth phenotypes of Tim9 and Tim10 mutants have been reproduced using *KanMX-pMET3* yeast strains, which confirms the tetO2-regulated ones. It suggests that not all the conserved cysteine residues are equally important for cell viability, and that the essential cysteine residues are located in different positions in Tim9, Tim10 and Tim12. Previously, it has been shown that the most N-terminal cysteine of Tim9 or Tim10 is very important for their interaction with Mia40, which catalyses the import and oxidative folding of small Tim proteins in the IMS (Milenkovic et al., 2007, Sideris and Tokatlidis, 2007). In this study, only the growth phenotype of Tim12 mutants suggests that the first conserved cysteine is essential for cell viability. Similarly, in a recent study of the cysteine of Tim9 and Tim10, the phenotypes of single cysteine mutants of Tim9 and Tim10 showed that mutation of the first cysteines did not cause growth defects (Baker et al., 2012). Therefore, the interaction between small Tim proteins and Mia40 might not be relevant to cell viability. The defects of cysteine mutants of Tim9, Tim10 and Tim12 may be caused by many factors,
not only the import of mitochondrial small Tim proteins, but also related to other processes, such as Tim9-Tim10 complex formation.

Table 5. 1 Phenotypes of cysteine mutations of Tim9, Tim10 and Tim12

<table>
<thead>
<tr>
<th>Conserved Cys</th>
<th>Tim9</th>
<th>Tim10</th>
<th>Tim12</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1S (outer) (Tim12C2S)</td>
<td>Healthy</td>
<td>Healthy</td>
<td>Lethal</td>
</tr>
<tr>
<td>C2S (inner) (Tim12C3S)</td>
<td>Lethal</td>
<td>Temperature sensitive</td>
<td>Healthy</td>
</tr>
<tr>
<td>C3S (inner) (Tim12C5S)</td>
<td>Temperature sensitive</td>
<td>Healthy</td>
<td>Healthy</td>
</tr>
<tr>
<td>C4S (outer) (Tim12C6S)</td>
<td>Healthy</td>
<td>Lethal</td>
<td>Lethal</td>
</tr>
<tr>
<td>C1,4S (outer) (Tim12C2,6S)</td>
<td>Healthy</td>
<td>Healthy</td>
<td>Lethal</td>
</tr>
<tr>
<td>C2,3S (inner) (Tim12C3,5S)</td>
<td>Lethal</td>
<td>Healthy</td>
<td>Healthy</td>
</tr>
</tbody>
</table>
5.7.2 Effects of temperature and redox on the phenotypes of mutants

The mature forms of small Tim proteins contain two intramolecular disulphide bonds, which are formed by the four cysteine residues of CX3C motifs. In this study, the temperature sensitivities of the cysteine mutants of Tim9 and Tim10 have been investigated at 37°C. The mutants with one or two cysteines deleted would be expected to have only one disulphide bond. This single instead of double disulphide bond might make the protein more sensitive to being unstable at higher temperatures. In terms of growth phenotype, most cysteine mutants of Tim9 and Tim10 were not sensitive with 37°C, except for the Tim9C3S and Tim10C2S (Table 5.1). This situation is too complicated to be explained for the defects of growth phenotypes.

In general, disulphide bonds are not readily formed, as most cellular compartments including mitochondria possess reducing environments, unless sulfhydryl oxidases catalyse the formation of disulphide bonds (Hatahet et al., 2010). In vitro experiments have shown that the reducing agent DTT can disrupt the disulphide bonds of small Tim proteins resulting in the failure of Tim9-Tim10 complex formation. On the other hand, the oxidised forms of small Tim proteins can not be imported into mitochondria. So, small Tim proteins require keeping in the reduced form before import into mitochondria, then to be oxidatively folded in mitochondrial IMS. The oxidative folding process is catalysed by the MIA system. Then the oxidised proteins form small Tim complexes (Lu et al., 2004a, Ivanova et al., 2008, Grumbt et al., 2007). Clearly, the switch of cysteine redox states is very important for the biogenesis of small Tim proteins. Mutation of the cysteine residues of the small Tim proteins might be expected to disrupt the biogenesis process or the balance of cysteine redox state, which would make these proteins sensitive to either reducing agent or oxidative stress. However, in this study, the growth phenotypes of the cysteine mutants of Tim9 and Tim10 were not affected.
by either DTT or \( \text{H}_2\text{O}_2 \). This indicates that there may be complicated mechanisms to maintain the redox states of small Tim in vivo. This is also in agreement with the results discussed above, suggesting that the mutated disulphide bonds themselves are not responsible for any growth defects observed.

5.7.3 Protein steady-state levels in cysteine mutants of Tim9 and Tim10

According to the test of protein steady-state levels, the Tim9 levels in the Tim9C2S and Tim9C2,3S strains were almost too low to be detected by western-blot. A similar recent study has suggested the growth defect of Tim9C2,3S at 37°C was caused by the lack of Tim9 (Baker et al., 2012). Therefore, the lack of protein may be a reason for the growth defects of Tim9 and Tim10 cysteine mutants. Furthermore, it is suggested that improperly folded Tim9 may be degraded by the i-AAA protease in mitochondria (Baker et al., 2012). Moreover, as the partner of the Tim9 in Tim9-Tim10 complex, the steady-state levels of Tim10 in Tim9C2S and Tim9C2,3S cells are also decreased. This observation raises a question why the Tim10wt in the defect Tim9 mutants can not exist stably. According to the in vitro results (Chapter 4), both Tim9C1,4S and Tim9C2,3S can complex with Tim10, but not efficiently as the WT. Moreover, Tim9C1,4S seems form a smaller sized complex than the WT proteins. Therefore, we may hypothesise that the Tim9-Tim10 complex play an important role in stabilise both proteins. Additionally, based on the growth defects of Tim9C2,3S, it suggests that loss of the inner disulphide bond means Tim9 may form very little functional complex with Tim10 in vivo, resulting in the degradation of both proteins and death of cells. Otherwise, the complex formed by Tim9 only with the inner disulphide bond, may contribute functions in a hexamer independent mechanism.
6 CONCLUSIONS AND MODEL

This study aimed to investigate the structures and functions of yeast mitochondrial Tim9, Tim10 and Tim12 focusing on the role of conserved cysteine residues. As there is little known about Tim12 from in vitro studies, purification of Tim12 was attempted with several methods in this study, but Tim12 was not purified in sufficient quantities to be useful. For the role of cysteines in Tim9, the purified double cysteine mutants of Tim9 were used to study how mutated cysteine residues in Tim9 impacted on protein folding, interaction with Tim10 and formation of Tim9-Tim10 complex. From the aspect of in vivo study, the cysteine mutants of Tim9, Tim10 and Tim12 were studied for their effects on cell viability using yeast genetic and in organelle methods.

In the first stage of the research, the purification of Tim12 has been tried using GST-tag, N-terminal His-tag and C-terminal His-tag, and expression was tested in two separate E. coli strains. However, all of the expressed recombinant proteins were insoluble, which might be caused by the intrinsic hydrophobicity and incorrect folding after renaturation. The 6M urea solubilised Tim12 can not efficiently become into the native form, and even the purified Tim12 in the form of redox-dependent dimer is easy to become aggregations. Although a little Tim12 in the monomer form was separated after gel filtration, all the six cysteines of Tim12 monomer were oxidised. These results made sense with a previous study, in which N-terminal His-tagged Tim12 was solubilised under denatured condition of 8M urea and the purified protein was existed in a mixed state of disulfide-bonded dimer and monomer (Baud et al., 2007). For the function of Tim12, it has been found that its C-terminus is important for the lipid integration via binding with subunits of TOM22 complex and association with Tim9-Tim10 complex (Gebert et al., 2008, Lionaki et al., 2008). Nevertheless, the intrinsic characterisation of Tim12 is still mystery.
Despite the lack of Tim12, the role of disulphide bonds in the assembly of Tim9-Tim10 complex was studied using double cysteine mutants of Tim9 in the second stage. Although the large majority of GST-tagged recombinant protein was insoluble under the optimal expression conditions, the double cysteine mutants of Tim9 were still purified from the soluble fractions with low yields. Far-UV circular dichroism showed both mutants were less folded than the WT Tim9, which may be resulted by the low solubility. Based on the results of MST, both double cysteine mutants of Tim9 were found to poorly interact with Tim10, particularly Tim9C1,4S (Tim9C35,59S). Furthermore, gel filtration, BN-PAGE and crosslinking showed both mutants can form complexes with Tim10. However, Tim9C1,4S formed a small amount of intermediate-sized complex, while Tim9C2,3S (Tim9C39,55S) formed a hexameric complex. Thus, it appears that the absence of either disulphide bond in Tim9 can affect the protein conformation, which results in weak interactions with Tim10 and less complex formed.

In the third stage, in vivo and in organelle methods were used to expand upon the in vitro work described in chapter 4. With the help of yeast genetic methods, it was found that the cysteines of Tim9, Tim10 and Tim12 were not equally essential for cell viability. The growth defects are caused by Tim9C2S, Tim9C2,3S (inner disulphide bond), Tim10C4S (outer disulphide bond), Tim12C2S, Tim12C6S and Tim12C2,6S (inner disulphide bond). Additionally, there were no effects of reducing and oxidising agents observed on the growth phenotypes of the Tim9 and Tim10 mutants, suggesting the in vivo cysteine redox states of small Tim proteins are maintained by complicated mechanisms. Importantly, with using western-blot, the Tim9 and Tim10 steady-state levels in the lethal mutants were found to be decreased or undetectable, suggesting that the growth defects of lethal cysteine mutants might be due to the lack of Tim9 and Tim10. Based on the in vitro and in vivo studies, it was summarised that the Tim9C1,4S forming the intermediate complex had no effect on the cell viability, but the Tim9C2,3S
forming hexameric complex was not stable to function in cell at high temperatures. It suggests that the role of Tim9-Tim10 complex may be independent of the hexamer form, but requires the presence of inner disulphide bond of Tim9 for the stability of Tim9 and Tim10 proteins. Overall, this study indicates the formation of disulphide bonds is not only important for structures of small Tim proteins, and also essential to form a functional small Tim complex. It was suggested by this study that the Tim9-Tim10 complex does not only play like chaperon to facilitate translocation of mitochondrial protein, it is also a stable form for the existing of individual Tim9 and Tim10 in the mitochondrial IMS.

Based on the results of this study and current views, a model for the biogenesis and complex formation of small Tim is developed (Figure 6.1). Following the red line, there are 5 steps for the normal process. The reduced protein is imported into mitochondria via the TOM pathway, and directly docks onto Mia40. The inner disulphide bond is formed by the transfer of a disulphide bond form one Mia40 (Milenkovic et al., 2007). The formation of outer disulphide is transferred from the unreduced Mia40. The fully folded small protein (e.g. Tim9) is released and the oxidised protein forms the functional complex with its oxidised partner protein (e.g. Tim10). In addition, there are two alternative ways for the complex formation. Following the blue line of the first alternative way, the partially folded protein is released at step 3 with the outer disulphide bond, instead of the inner disulphide bond. The protein with only outer disulphide bond becomes not stable as WT, so it would be degraded by the i-AAA protease Yme1, leading to the failure of complex formation (Baker et al., 2012). However, following the green line of the second alternative way, the partially folded protein with the inner disulphide bond is released at step 3. Because the stability of protein with inner disulphide bond is better than the protein composing with outer disulphide bond, the former protein can be involved in complex formation. In this model, since Mia40 dimer can contribute disulphide bonds one by one, the formation of two
disulphide bonds of small Tim protein become more efficient. Furthermore, the partially folded protein with inner disulphide bond can form the functional complex, creating an alternative way for the functional complex formation. Unstable folded protein such as the protein with outer disulphide bond would be cleaned by the i-AAA protease, Yme1. After the selection of Yme1, the stable protein can be used for the formation of complex. Thus, this mechanism can cater the requirement of small Tim complexes for the mitochondrial biogenesis.

**Figure 6. 1 Model for the biogenesis and complex formation of small Tim.** Step 1, the reduced small Tim imports into mitochondria via the TOM complex. Step 2, the imported protein docks onto the Mia40 dimer with the interactions of two terminal cysteines. Step 3, the inner disulphide is transferred from one Mia40 to protein. Step 4, the outer disulphide is transferred from the rest Mia40, and is released into the IMS. Step 5, the functional complex is formed by fully folded proteins. Alternative way 1 (A1), the partially folded protein with the outer disulphide bond is released at step 3, but it is degraded by i-AAA protease Yme1 due to the less stability so that it can not be involved in the complex formation (Baker et al., 2012). Alternative way 2 (A2), the partially folded protein with the inner disulphide bond is released at step 3, and continue to form the functional complex.
7 REFERENCES


Molecular recognition and substrate mimicry drive the electron-transfer process between MIA40 and ALR. *Proc Natl Acad Sci U S A*, 108, 4811-6.


### 8.1 List of used yeast strains

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<td>Tim9.C59S R</td>
<td>GCT TCA AGA ACT TTT CTG AGG ACT TCA TGA TGC ATG TTT G</td>
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<tr>
<td>Tim9.C59A F</td>
<td>GAA AGA TGT TTC ACA GAC GCT GTC AAT GAC TTC ACA AC</td>
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<tr>
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<tr>
<td>Tim10.C40S F</td>
<td>TAA ATT GTT TAA TAA CTA CCT GTA TAA AAA ATG TAT C</td>
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<tr>
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<td>GAT ACA TGG TTT TAT ATA CGA G1T ATT ACAA TTT A</td>
</tr>
<tr>
<td>Tim10.C44S F</td>
<td>CTG TTA TAA AAA ATC CAT CAA TAC TCC TTA TCC</td>
</tr>
<tr>
<td>Tim10.C44S R</td>
<td>GAA TAA GGA GTA TTT GTG ATG TTT TTA TAA CAG</td>
</tr>
<tr>
<td>Tim10.C61S F</td>
<td>GAA TAA GGA TGA ATC TCC GAG CCT AGA CAG ATG TGG GG</td>
</tr>
<tr>
<td>Tim10.C61S R</td>
<td>CCA CAC ATC TGT TAG GCT CGA AGA TAA TTT ATG AT TAT C</td>
</tr>
<tr>
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<td>CTT CGT GCC TAG ACA GAA GTG TGG CCA AAT ATT TTG</td>
</tr>
<tr>
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<td>CAA ATG TGG CCA CAC TTC TCT GTA GGC AGC AAG</td>
</tr>
<tr>
<td>Tim12.C30S F</td>
<td>CAA TTC GAT GCG ATG AGC TCG ACT TTC AAC</td>
</tr>
<tr>
<td>Tim12.C30S R</td>
<td>GGT GAA ATG CGA GCT CAT CTC ATG GAA CTG</td>
</tr>
<tr>
<td>Tim12.C40S F</td>
<td>CAA TAT TCT CAG TAC GTT CCT TGA GAA ATG TAT TCC G</td>
</tr>
<tr>
<td>Tim12.C40S R</td>
<td>CGG AAT ACA TTT CTC AAG GGA CGT ACT GAG AAT ATT G</td>
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<tr>
<td>Tim12.C44S F</td>
<td>CGT GTC TTT AGA AAT CCA TTC CGC ATG AGG G</td>
</tr>
</tbody>
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8.5 List of used buffer solutions

<table>
<thead>
<tr>
<th>Buffer Name</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer A</td>
<td>50mM Tri- HCl, 150mM NaCl, pH7.4</td>
</tr>
<tr>
<td>Buffer B</td>
<td>50mM Tri- HCl, 150mM NaCl, pH 7.4, 6M Urea, 10mM DTT</td>
</tr>
<tr>
<td>Buffer AE</td>
<td>50mM Tri-HCl, 150mM NaCl, 1mM EDTA, pH7.4</td>
</tr>
<tr>
<td>Binding Buffer</td>
<td>50mM Tri-HCl, 150mM NaCl, 5mM imidazole, pH7.4</td>
</tr>
<tr>
<td>Washing Buffer 1</td>
<td>50mM Tri-HCl, 150mM NaCl, 20mM imidazole, pH7.4</td>
</tr>
<tr>
<td>Washing Buffer 2</td>
<td>50mM Tri-HCl, 150mM NaCl, 40mM imidazole, pH7.4</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>50mM Tri-HCl, 150mM NaCl, 250mM imidazole, pH7.4</td>
</tr>
<tr>
<td>TAE Buffer</td>
<td>37mM Tris base, 1.74M Acetic acid, 1.26mM EDTA</td>
</tr>
<tr>
<td>Tricine SDS-PAGE Cathode buffer</td>
<td>0.1M Tris pH8.25, 0.1M Tris-Tricine, and 0.05% (w/v) SDS</td>
</tr>
<tr>
<td>Tricine SDS-PAGE Anode buffer</td>
<td>0.2M Tris pH8.9</td>
</tr>
<tr>
<td>2×SDS-PAGE sample buffer</td>
<td>4% (w/v) SDS, 8% (v/v) Glycerol, 0.02% (w/v) Bromophenol blue, 80mM Tris-HCl, pH 6.8, 100mM DTT</td>
</tr>
<tr>
<td>BN-PAGE Cathode buffer</td>
<td>50mM Tricine, 15mM Bis-Tris, 0.02% (w/v) Coomassie blue, pH7.0</td>
</tr>
<tr>
<td>BN-PAGE Anode buffer</td>
<td>50mM Bis-Tris, pH7.0</td>
</tr>
<tr>
<td>10×BN-PAGE sample buffer</td>
<td>0.5M 6-Aminocaproic acid pH 7.0, 100mM Bis-Tris pH7.0, 20mM DTT and 5% Coomassie blue</td>
</tr>
<tr>
<td>Western-blot transfer buffer</td>
<td>25mM Tris, 192mM Glycine, 20% (v/v) Methanol</td>
</tr>
<tr>
<td>PBS buffer</td>
<td>137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 2mM NaH₂PO₄, pH 7.4, 0.1% Tween-20</td>
</tr>
<tr>
<td>Spheroplast buffer</td>
<td>1.4M sorbitol, 50mM Tris-HCl pH7.4, 2mM MgCl₂</td>
</tr>
<tr>
<td>IP lysis buffer</td>
<td>1% SDS, 50mM Tris-HCl pH7.4, 5mM EDTA</td>
</tr>
<tr>
<td>IP buffer</td>
<td>187.5mM NaCl, 62.5mM Tris-HCl pH7.4, 6.25mM EDTA, 1.25% Triton X100</td>
</tr>
<tr>
<td>Tris-DTT buffer</td>
<td>100mM Tris-DTT, pH 9.4, 10mM DTT</td>
</tr>
<tr>
<td>Sorbitol buffer</td>
<td>1.2M Sorbitol, 7mM K₂HPO₄</td>
</tr>
<tr>
<td>Digestion buffer</td>
<td>1.2M Sorbitol, 20mM K₂HPO₄, 1.5 mg/ml Zymolase 20T</td>
</tr>
<tr>
<td>Homogenisation buffer</td>
<td>0.6M Sorbitol, 20mM HEPES, 1mM PMSF</td>
</tr>
<tr>
<td>S-buffer</td>
<td>20mM Bis-Tris pH 7.0, 50mM NaCl, 10% Glycerol, 1mM PMSF, 1% Digitonin</td>
</tr>
</tbody>
</table>