The mitochondrial outer membrane protein assembly machinery of the human fungal pathogen *Candida albicans*

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Notice 1

Declaration

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Abstract

Mitochondria are essential organelles with protein complexes embedded in their membranes. These act as molecular machines that produce energy and carefully regulate what goes in to and out of the organelle. The outer membrane machinery provides the interface between the mitochondrion and the rest of the cell and coordinates the activity of each mitochondrion in keeping with the metabolic requirements of the cell. Research over the last twenty years has identified many of the components and complexes that assemble this membrane machinery, but many questions remain about how these machines function and how they are assembled.

While we have learnt a great deal about these molecular machines in the tractable model system *Saccharomyces cerevisiae*, its unusual regulation of mitochondrial function makes it difficult to glean general principles from discoveries in this organism alone. By comparing findings in other organisms we can determine which features are conserved and learn more about the evolutionary origins of the molecular components. The work in this thesis focuses on the protein import machinery of the mitochondrial outer membrane, in particular the sorting and assembly machinery (SAM) complex.

My thesis is the first in-depth study of the mitochondrial import machinery of the human fungal pathogen *Candida albicans*. I outline the mitochondrial protein import pathways with a focus on the roles and partnerships of the SAM complex. Examining the import pathways in *C. albicans* in comparison to *S. cerevisiae* revealed interesting examples of rewiring of the protein import pathways into the intermembrane space. These preliminary studies also show that mitochondria from *C. albicans* more efficiently assembles proteins into the mitochondrial outer membrane, which leads to a more detailed investigation of the composition and behaviour of the SAM complex. This reveals new roles for the SAM complex components; Sam37, Sam35 and Mdm10 and a new component of the *C. albicans* SAM complex, Sam51. The work presented here describes the functions and interactions of *C. albicans* SAM complex components, a detailed characterisation of their roles in the import and assembly of outer membrane proteins, and the partnerships between the *C. albicans* SAM complex and other outer membrane protein complexes.

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Preface

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Hewitt, V., Alcock, F. and Lithgow, T. (2011). "Minor modifications and major adaptations: the evolution of molecular machines driving mitochondrial protein import." **Biochimica et Biophysica Acta** 1808(3): 947-54. Included as Appendix 9.

Qu, Y., Jelicic, B., Pettolino, F., Perry, A., Lo, T. L., **Hewitt, V. L**., Bantun, F., Beilharz, T. H., Peleg, A. Y., Lithgow, T., Djordjevic, J. T. and Traven, A. (2012). "Mitochondrial Sorting and Assembly Machinery Subunit Sam37 in *Candida albicans*: Insight into the Roles of Mitochondria in Fitness, Cell Wall Integrity, and Virulence." **Eukaryotic Cell** 11(4): 532-44.

Alcock, F., Webb, C. T., Dolezal, P., **Hewitt, V.**, Shingu-Vasquez, M., Likic, V. A., Traven, A. and Lithgow, T. (2012). "A small Tim homohexamer in the relict mitochondrion of *Cryptosporidium*." **Molecular Biology and Evolution** 29(1): 113-22.

Hewitt, V. L., Heinz, E., Shingu-Vazquez, M., Qu, Y., Jelicic, B., Lo, T. L., Beilharz, T. H., Dumsday, G., Gabriel, K., Traven, A. and Lithgow, T. (2012). "A model system for mitochondrial biogenesis reveals evolutionary rewiring of protein import and membrane assembly pathways." **Proceedings of the National Academy of Sciences of the United States of America** 109(49): E3358-66. Included as Appendix 10.

Hewitt, V. L., Gabriel, K. and Traven, A. (2014). "The ins and outs of the intermembrane space: Diverse mechanisms and evolutionary rewiring of mitochondrial protein import routes." **Biochimica et Biophysica Acta** 1840(4): 1246-1253. Included as Appendix 11.

1 Introduction

The evolution of mitochondria from endosymbiotic bacteria helped arm eukaryotic cells with the ability to revolutionise life on Earth. A pivotal step in mitochondrial evolution was the transfer of endosymbiont proteins to the host cell nucleus (Timmis, Ayliffe et al. 2004). Thus a key step in the transition of mitochondria from endosymbiont to organelle was the evolution of protein transport machinery to translocate proteins, synthesised in the cytosol, across the mitochondrial membranes and into their appropriate mitochondrial location. This transport machinery escorts proteins into and through the lipid inner and outer membranes that separate the mitochondrial compartments (Chacinska, Koehler et al. 2009). Some components of this machinery can be traced back to their bacterial origins (Hewitt, Alcock et al. 2011), so to understand the evolution of mitochondrial import machinery we can look for clues, not only at the diversity of machinery in modern day mitochondria across different species, but also at extant bacterial protein transport machinery.

1.1 Mitochondrial protein import machinery

Mitochondria encode only a few of the proteins required for their biogenesis and function. This means that most mitochondrial proteins are targeted to the mitochondria after translation at cytosolic ribosomes. Mitochondrial protein import is mediated by a series of molecular machines in the outer and inner mitochondrial membranes, and soluble factors in the mitochondrial intermembrane space and matrix. As well as import pathways that recognise and target proteins to the correct mitochondrial compartments, additional mechanisms are required to ensure the proteins remain in suitable conformations for transport and assume the correct conformation once they are at their required location. In addition, integration of proteins into the membranes or into complexes may require further scaffolding or chaperone activity to ensure the structure and topology is maintained and that these processes occur on the time scales required by an actively growing and respiring cell. Most of what we know about these import pathways and this machinery comes from studies in *Saccharomyces cerevisiae* and is summarised in Figure 1.1 and the following paragraph.

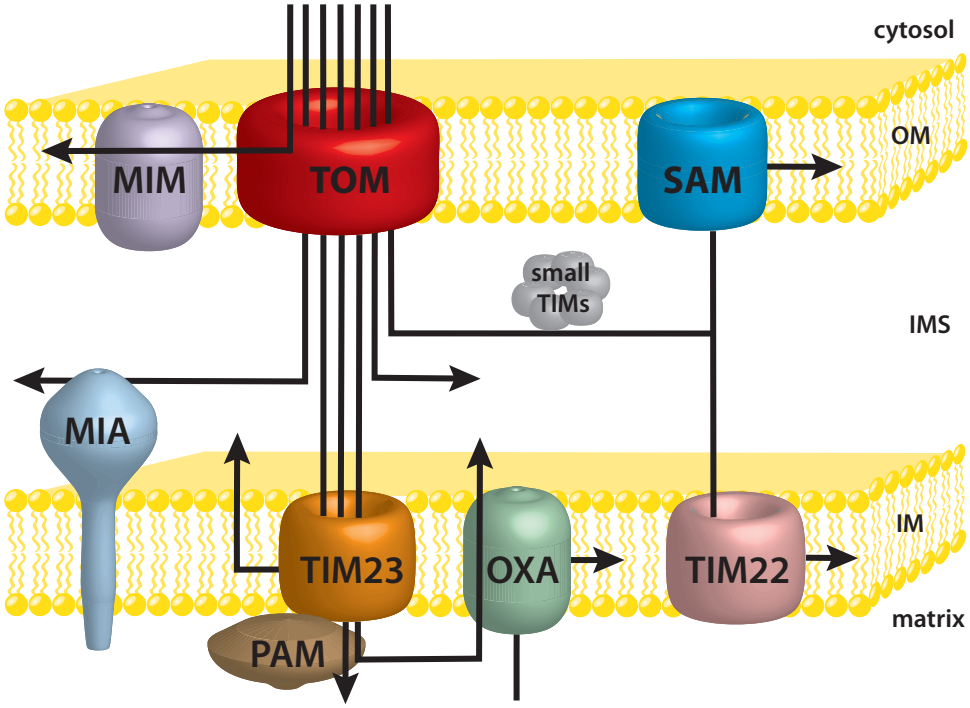


Figure 1.1 Overview of the mitochondrial import and assembly machinery
Protein import pathways diverge at the outer membrane (OM) after recognition by the translocase of the outer membrane complex (TOM, red). Some proteins are inserted directly into the outer membrane by the mitochondrial import machinery (MIM, purple), but most pass through the outer membrane through the TOM complex. Some intermembrane space (IMS) proteins use the mitochondrial intermembrane space transport and assembly pathway (MIA, pale blue). The small translocase of the inner membrane complex (TIM, grey) escorts proteins to the sorting and assembly machinery complex (SAM, blue), or to the TIM22 machinery (pink) to be assembled into the outer membrane or inner membrane (IM) respectively. The TIM23 complex (orange) coordinates import into the matrix with the help of presequence translocase-associated protein import motor (PAM, brown). The oxidase assembly machinery (OXA, green) mainly assembles proteins translated in the matrix into the inner membrane.

The first transport machine to engage most precursor proteins is the TOM (translocase of the outer membrane) complex (red, Figure 1.1) (Neupert 1997, Hill, Model et al. 1998, Chacinska, Koehler et al. 2009). This multi-subunit complex includes receptors that recognise a wide range of substrates (Vestweber, Brunner et al. 1989, Kiebler, Pfaller et al. 1990). Some alpha helical outer membrane (OM) proteins are integrated directly into the membrane by the mitochondrial import complex (MIM) (purple, Figure 1.1) (Becker, Pfannschmidt et al. 2008), but most proteins are imported through the TOM complex before the import pathways to the different

mitochondrial compartments diverge. The SAM (sorting and assembly machinery) complex helps insert beta barrel proteins into the outer membrane (blue, Figure 1.1) (Wiedemann, Kozjak et al. 2003). This assembly process is aided by small TIM (translocase of the inner membrane) chaperones which bind SAM complex substrates in the intermembrane space (Wiedemann, Truscott et al. 2004). Small TIM chaperones also escort inner membrane proteins to the TIM22 complex for assembly into the inner membrane (pink, Figure 1.1) (Sirrenberg, Bauer et al. 1996, Koehler, Jarosch et al. 1998). The main role of the TIM23 complex (orange, Figure 1.1) is to translocate proteins across the inner membrane into the matrix (Dekker, Martin et al. 1997, Chacinska, Rehling et al. 2003). This movement is a membrane potential dependent process driven by the PAM (presequence translocase-associated protein import motor) module of the TIM23 complex (D'Silva, Schilke et al. 2003, Chacinska, Lind et al. 2005). In some cases, this transport process is interrupted and the precursor proteins are laterally released from TIM23 into the inner membrane. A small number of inner membrane proteins are inserted from the matrix side of the inner membrane and have been found to require the oxidase assembly (OXA) translocation machinery (green, Figure 1.1) (Bonnefoy, Chalvet et al. 1994), which is primarily involved in the insertion of proteins synthesised within the mitochondria into the inner membrane (Hell, Herrmann et al. 1998, Ott and Herrmann 2010). The most recently discovered transport machinery is the MIA (mitochondrial intermembrane space transport and assembly) machinery (pale blue, Figure 1.1), which ensures proper import and folding of a number of intermembrane space proteins (Chacinska, Pfannschmidt et al. 2004).

1.2 Outer membrane assembly machinery

The main role of the SAM complex is the assembly of beta barrel proteins into the mitochondrial outer membrane (Wiedemann, Kozjak et al. 2003). Beta barrel proteins are made up of beta strands that fold to form a tubular beta sheet embedded in the membrane (Murzin, Lesk et al. 1994). The beta barrel assembly machinery (BAM) complex performs this role in the bacterial outer membrane (Voulhoux, Bos et al. 2003, Hagan, Silhavy et al. 2011). The core components of both these complexes are the beta barrel proteins BamA and Sam50 (Genevrois, Steeghs et al. 2003, Paschen, Waizenegger et al. 2003). The conserved roles, structures and sequence

similarities of these proteins indicate that Sam50 evolved directly from a bacterial BamA-like protein (Gentle, Gabriel et al. 2004). This evolutionary relationship is also apparent in the way the SAM complex functions, assembling beta barrel proteins into the outer membrane from the intermembrane space. In bacteria the topology of the assembly process is the same; beta barrel proteins are assembled into the outer membrane from the periplasm after being translocated across the inner membrane (Figure 1.2). The details of the SAM complex composition are discussed in more detail in the next section.

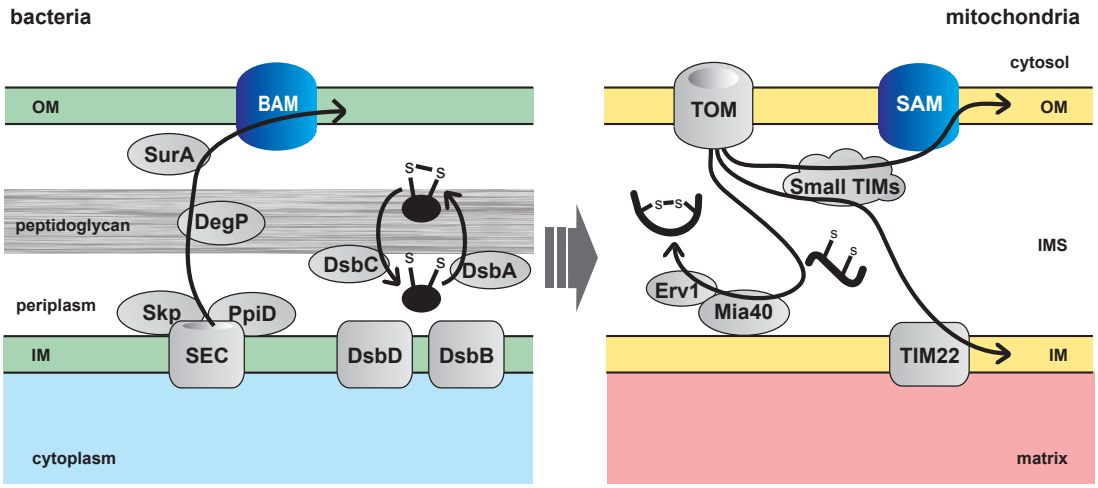


Figure 1.2 Evolution of the mitochondrial protein import machinery
Evolution of the intermembrane space (IMS) and beta barrel protein assembly machinery in mitochondria from an endosymbiotic ancestor. Molecular chaperones (Skp, DegP, PpiD and SurA) in bacteria translocate proteins across the periplasm to the beta barrel assembly machinery (BAM) complex after they emerge from the SEC complex. The Dsb redox system acts as a folding catalyst for disulfide-containing periplasmic proteins. In mitochondria the small translocase of the inner membrane (TIM) chaperones fulfill a molecular chaperone function and transfer membrane protein precursors from the translocase of the outer membrane (TOM) complex to the TIM22 and sorting and assembly machinery (SAM) complexes. The Mia40/Erv1 machinery couples a Dsb-like redox activity with import of precursor proteins into the intermembrane space.

Much of the bacterial periplasmic machinery that is also important for beta barrel protein assembly has been replaced with eukaryote-specific proteins. The small TIM chaperones play a SurA-like role in mitochondria (Alcock, Grossmann et al. 2008), and the MIA/Erv disulfide relay has replaced the bacterial Dsb system

(Herrmann, Kauff et al. 2009). Primitive mitochondria must have found a way to target and import the beta barrel proteins. They were then able to make use of and improve the existing bacterial machinery to correctly assembly the proteins into the mitochondrial outer membrane.

Beta barrel proteins must first pass through the outer membrane using the TOM complex before they can be assembled by the SAM complex (Pfaller, Steger et al. 1988). The TOM complex is found in all mitochondria as the receptor and import gateway for entry of proteins into mitochondria (Kiebler, Pfaller et al. 1990). The core of the complex is Tom40 (Isp40), a beta barrel protein that forms a channel through the outer membrane (Vestweber, Brunner et al. 1989, Dekker, Ryan et al. 1998, Hill, Model et al. 1998, Kunkele, Juin et al. 1998). Tom40 forms a complex with Tom20 (Mas20), Tom22 (Mas22) and Tom70 (Mas70) that are also embedded in the membrane but have domains exposed at the mitochondrial surface that act as receptors for TOM complex substrates (Kiebler, Pfaller et al. 1990). The small proteins Tom5, Tom6 (Kassenbrock, Cao et al. 1993) and Tom7 (Honlinger, Bomer et al. 1996) have important and varied roles in stabilising the complex and engaging and releasing substrates (Dietmeier, Honlinger et al. 1997).

A further contribution to efficient beta barrel protein assembly is made by the ER-mitochondrial encounter structure (ERMES) components (Kornmann, Currie et al. 2009). The ERMES is a molecular tether between the endoplasmic reticulum and mitochondria, composed of Mdm10, Mdm12, Mmm1 and Mdm34 (Mmm2) (Kornmann, Currie et al. 2009). It appears to play a role in beta barrel protein assembly downstream of the SAM complex function (Meisinger, Rissler et al. 2004, Meisinger, Pfannschmidt et al. 2007, Thornton, Stroud et al. 2010). Disrupting the ERMES complex alters various aspects of cellular physiology including mitochondrial morphology, phospholipid biosynthesis, calcium homeostasis and mitochondrial DNA replication making it difficult to dissect the primary function of the complex (Kornmann and Walter 2010).

1.3 Core SAM complex components

While Sam50 is clearly derived from BamA, the other components of the beta barrel protein assembly pathway have diverged considerably. Both the BAM and

SAM complexes have multiple components; BamA forms a complex with lipoprotein partners but mitochondria have lost the ability to synthesize lipoproteins and Sam50's SAM complex partners, Sam35 and Sam37, do not share sequence similarity with any of the BAM complex lipoproteins (Ono, Wang et al. 2010, Webb, Heinz et al. 2012, Cartron, Petit et al. 2014).

Together with Sam50, Sam35 and Sam37 make up the core of the SAM complex in *S. cerevisiae.* Sam37 was the first component of the SAM complex to be identified (Mas37, (Gratzer, Lithgow et al. 1995)), followed by Sam50 (Tob55, (Kozjak, Wiedemann et al. 2003, Paschen, Waizenegger et al. 2003, Gentle, Gabriel et al. 2004)). Sam35 was identified last in co-immunopurification experiments with Sam50 and Sam37 (Tob38, (Ishikawa, Yamamoto et al. 2004, Milenkovic, Kozjak et al. 2004, Waizenegger, Habib et al. 2004)). Sam50 and Sam35 are essential in *S. cerevisiae* (Kozjak, Wiedemann et al. 2003, Ishikawa, Yamamoto et al. 2004), but Sam37 can be deleted (Gratzer, Lithgow et al. 1995). However, in *Neurospora crassa* all three are essential (Lackey, Wideman et al. 2011). A preliminary study in *Candida albicans* indicates the role of *C. albicans* Sam37 may fall somewhere in between, with Sam37 deletion resulting in a serious growth defect (Dagley, Gentle et al. 2011). The putative mammalian versions of Sam35 and Sam37, the metaxins, have diverged considerably in sequence and it is not yet clear the extent to which functions of Sam35, Sam37 and the metaxins are conserved across different eukaryotic species (Armstrong, Komiya et al. 1997).

Sam35 and Sam37 are degraded if protease is added to mitochondria and are not tightly associated with the *S. cerevisiae* outer membrane so they are often described as peripheral membrane proteins that are associated with the outside of the mitochondrial outer membrane (Pfanner and Neupert 1990, Gratzer, Lithgow et al. 1995, Ishikawa, Yamamoto et al. 2004, Chan and Lithgow 2008, Kutik, Stojanovski et al. 2008, Dagley, Gentle et al. 2011). Such a location is perplexing given our limited knowledge of the evolution and function of these components (Chacinska, Koehler et al. 2009). While they are not direct descendants of the BAM complex lipoproteins, it is not unreasonable to expect that Sam35 and Sam37 have similar roles in the assembly process. However, it is hard to imagine how these proteins might all perform similar functions to the BAM complex lipoproteins from the opposite side of

the outer membrane. Adding to the confusion, Sam35 acts as a receptor (Chan and Lithgow 2008) that is involved in the initial binding of beta barrel protein substrates to the SAM complex, but this process occurs in the intermembrane space. Recent work from our lab and others' suggests the solution to this enigma is that at least one of the BAM complex lipoproteins crosses the outer membrane and is exposed on the bacterial cell surface (Webb, Selkrig et al. 2012). Studies in *C. albicans* and *N. crassa* show Sam35 and Sam37 may both have transmembrane domains that extend through the outer membrane and into the intermembrane space (Lackey, Wideman et al. 2011, Hewitt, Heinz et al. 2012) (Figure 1.3).

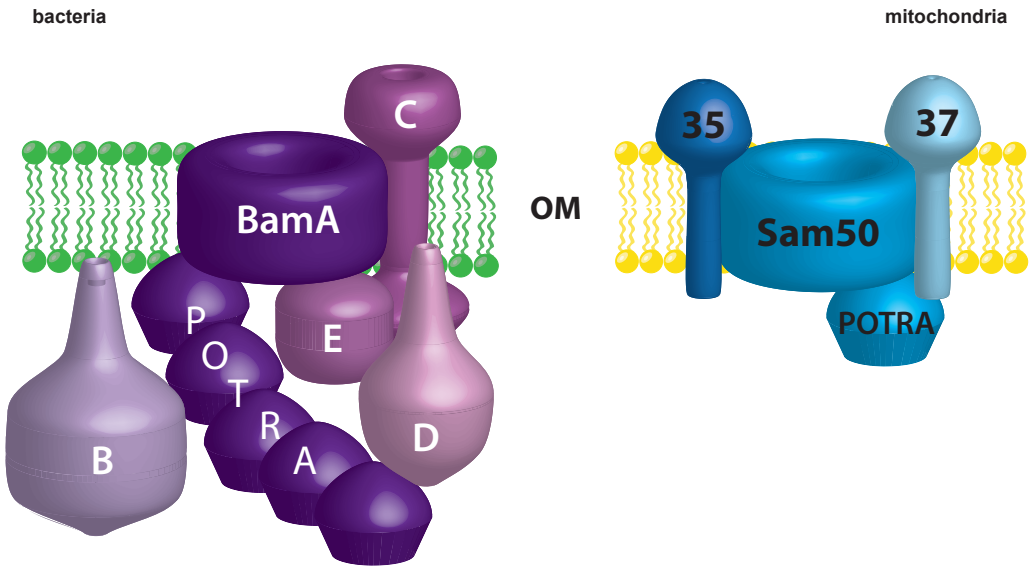


Figure 1.3 Comparison of BAM and SAM complex components
The main interactions and components in the core BAM (purple) and SAM (blue) complexes are shown including the polypeptide translocation associated (POTRA) domains attached to each beta barrel protein. Green and yellow show bacterial and mitochondrial outer membrane (OM) lipids respectively.

1.4 Function of the SAM complex

While the primary role of the SAM complex is in assembly of beta barrel proteins, the mechanism of this assembly process is unknown (Kozjak, Wiedemann et al. 2003, Paschen, Waizenegger et al. 2003, Wiedemann, Kozjak et al. 2003). How beta barrel proteins are inserted into the membrane remains an unsolved question that is fundamental to our understanding of both mitochondrial and bacterial biogenesis (Webb, Heinz et al. 2012, Noinaj, Kuszak et al. 2013). Hypotheses for beta barrel

protein folding mechanisms have to explain the order and coordination of the folding and insertion of the barrel into the membrane (Noinaj, Kuszak et al. 2014). While the hydrophobic exterior residues mean that some beta barrel proteins can fold and insert spontaneously into a lipid bilayer, the importance of the BAM and SAM complexes indicate that this does not occur in bacterial or mitochondrial membranes *in vivo* (Gessmann, Chung et al. 2014). The lipid composition of the membrane is also an important factor in the assembly and stability of beta barrel proteins in bacterial and mitochondrial outer membranes (Gebert, Joshi et al. 2009, Becker, Horvath et al. 2013, Gessmann, Chung et al. 2014). The recent crystal structure of BamA gives us important clues as to how the BAM and SAM complexes might function to aid beta barrel protein assembly. However, the assembly mechanism of either the bacterial or eukaryotic complex is still unknown, especially with the respect to the contribution of the partner proteins (Noinaj, Kuszak et al. 2013).

More recent work has suggested that the SAM complex also functions in the import, membrane insertion and assembly of alpha helical outer membrane proteins, particularly components of the TOM complex (Stojanovski, Guiard et al. 2007, Becker, Pfannschmidt et al. 2008, Thornton, Stroud et al. 2010). The following section discusses the different substrates of the SAM complex and what we know about their assembly pathways.

1.5 Substrates of the SAM complex

Beta barrel proteins are a key feature of the mitochondrial outer membrane proteome and provide the basis for communication between mitochondria and their host. In yeast, beta barrel proteins play important roles in metabolite exchange (porin/VDAC), protein transport (Tom40), membrane protein assembly (Sam50) and inter-organelle communication (Mdm10). In multicellular organisms these proteins also have important functions in mitochondrial regulation of cell death pathways (Martinou and Youle 2011, Toulmay and Prinz 2011). The SAM complex recognises a four-residue motif in the last beta strand of outer membrane beta barrel proteins called the beta signal (Kutik, Stojanovski et al. 2008). This is the sorting signal that the SAM complex uses to recognise beta barrel proteins. This short, conserved motif is found in the last strand in all mitochondrial beta barrel proteins studied to date

(Kutik, Stojanovski et al. 2008); another feature inherited from the BAM complex (Walther, Bos et al. 2010). Since the interactions between SAM and its substrates are key to many of the experiments in this thesis, here I will briefly discuss the SAM complex substrates and any unique features of their import. Although these studies were performed mainly in *S. cerevisiae,* the limited information we have about this machinery in other organisms is also included.

1.5.1 Porin

Porin or VDAC (voltage-dependent anion-selective channel (Kozjak-Pavlovic, Ross et al. 2010)) is the most abundant beta barrel protein in the mitochondrial outer membrane (Freitag, Janes et al. 1982). The mitochondrial porins have 19 strands and have no clear relationship in amino acid sequence to the bacterial porins with even numbers of beta strands (Forte, Guy et al. 1987, Bayrhuber, Meins et al. 2008, Hiller, Garces et al. 2008, Ujwal, Cascio et al. 2008). The beta barrel structure of porin, also predicted for Tom40 and Mdm10 (Pusnik, Charriere et al. 2009, Gessmann, Flinner et al. 2011, Flinner, Ellenrieder et al. 2013), suggests they may have evolved from a single ancestor inherited from the endosymbiotic predecessor of mitochondria (Saccone, Caggese et al. 2003, Young, Bay et al. 2007).

S. cerevisiae and *N. crassa* both contain porins of ~30 kDa. These form oligomers that can be separated using blue native protein gel electrophoresis (BN-PAGE) (Freitag, Janes et al. 1982, Gasser and Schatz 1983, Krimmer, Rapaport et al. 2001, Hoppins, Go et al. 2007). Even larger oligomers have been detected but their biological relevance is unclear (Hoogenboom, Suda et al. 2007). ATP may be required *in vivo* for porin import (Gasser and Schatz 1983, Kleene, Pfanner et al. 1987, Hwang and Schatz 1989), possibly to help release the protein from chaperones that help maintain the protein in an import competent form in the cytoplasm (Pfanner, Pfaller et al. 1988).

1.5.2 Tom40

There are a number of steps required for the assembly of the ~42 kDa Tom40 into the ~450 kDa TOM complex (Baker, Schaniel et al. 1990, Model, Meisinger et al. 2001): import of the Tom40 precursor by existing TOM complex (Kiebler, Pfaller et al. 1990), the escort of the precursor to the SAM complex (Wiedemann, Truscott et

al. 2004), the folding and insertion of the Tom40 beta barrel protein (Rapaport and Neupert 1999), and incorporation into the mature complex (Kiebler, Pfaller et al. 1990). With so many components with varied topologies, the assembly of Tom40 into the TOM complex is the most intricate beta barrel protein assembly problem (Becker, Vogtle et al. 2008).

Assembly of Tom40 into the TOM complex has been shown to occur through two main stages. The import of radiolabelled Tom40 can be monitored by BN-PAGE and autoradiography and shows the Tom40 precursor first assembles into ~250 kDa complex of (Intermediate I) before forming a smaller ~100 kDa complex (Intermediate II) (Model, Meisinger et al. 2001) (Figure 1.4). The amount of Tom40 in these complexes decreases as the protein is assembled into the mature ~450 kDa TOM complex (Model, Meisinger et al. 2001). Radiolabelled Tom40 in Intermediate I can be chased to Intermediate II and go on to form mature TOM complex if the precursor is removed from the import assay and the mitochondria are incubated in favourable import conditions (Wiedemann, Truscott et al. 2004).

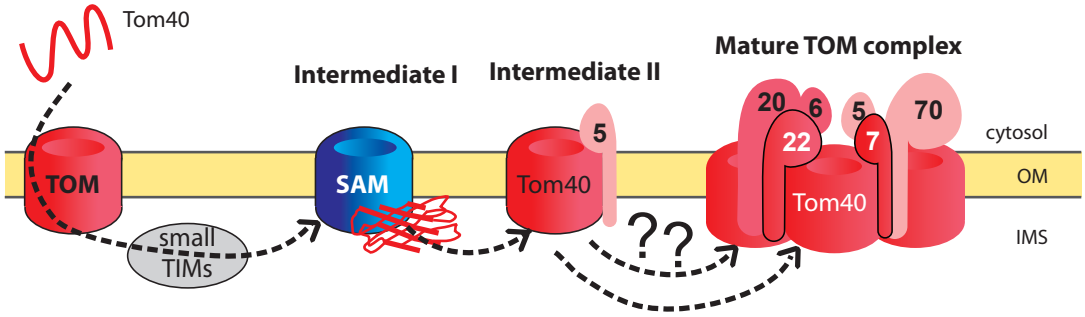


Figure 1.4 Tom40 assembly into the TOM complex
Tom40 is imported through the TOM complex and escorted to the SAM complex by the small TIM proteins. The beta barrel structure forms in association with the SAM complex (Intermediate I). Tom40 is released from the SAM complex to form Intermediate II with Tom5 and the final steps of Tom40 assembly into mature TOM complex occur rapidly.

While the literature often discusses a single Intermediate I of ~250 kDa, there are multiple complexes around this size that are detected with various levels of resolution when Tom40 import reactions are analysed by BN-PAGE (Meisinger, Rissler et al. 2004, Stojanovski, Guiard et al. 2007, Becker, Pfannschmidt et al. 2008,

Thornton, Stroud et al. 2010). An attempt to delineate the composition of these intermediates labelled them SAM1a and SAM1b, with SAM1b the larger complex that forms after SAM1a (Becker, Guiard et al. 2010). In mitochondria lacking Tom5, Tom40 is unable to form SAM1b or Intermediate II, but assembly is rescued when the mitochondria are incubated with chemical quantities of Tom5 before the Tom40 assembly assay is performed. Together with antibody shift experiments, this provides good evidence that SAM1b contains Sam50, Tom40 and Tom5 and/or Tom6.

In Intermediate II, Tom40 is no longer associated with the SAM complex and is inserted into the membrane (Dekker, Ryan et al. 1998, Model, Meisinger et al. 2001). Intermediate II is thought to consist of two Tom40 barrel proteins associated with Tom5 and possibly Tom6 (Model, Meisinger et al. 2001, Wiedemann, Kozjak et al. 2003). An N-terminal truncated version of ScTom40 accumulates in SAM1a (Kutik, Stojanovski et al. 2008), implying Tom5 is not attaching and associating with Tom40 as it should, and that the binding of other TOM complex components may help release Tom40 from the SAM complex.

The mature TOM complex is thought to contain either two (Ahting, Thun et al. 1999) or three Tom40 pores (Kunkele, Heins et al. 1998). Newly imported Tom40 can assemble with pre-existing TOM complex (Model, Meisinger et al. 2001) and the TOM complex itself can dissociate into more stable sub-complexes in mutant strains (*S. cerevisiae* (van Wilpe, Ryan et al. 1999); *N. crassa* (Taylor, McHale et al. 2003)). A very similar series of assembly intermediates and therefore a similar assembly process occurs in *N. crassa* (Rapaport and Neupert 1999).

1.5.3 Mdm10

The ~55 kDa Mdm10 protein was discovered through the mitochondrial distribution of morphology defects when it is deleted in *S. cerevisiae* (Sogo and Yaffe 1994). Mdm10 was predicted to be a beta barrel protein and finding that Mdm10 import depended on the SAM complex provided further evidence for this prediction (Paschen, Waizenegger et al. 2003). Mdm10 was later found to contribute to beta barrel protein assembly and associate with the SAM complex (Meisinger, Rissler et al. 2004) and to form part of the ERMES (Kornmann, Currie et al. 2009).

When Mdm10 is incubated with isolated *S. cerevisiae* mitochondria, it rapidly assembles into a ~300 kDa complex (No sizes given in (Paschen, Waizenegger et al. 2003), described as ~350 kDa in (Wiedemann, Kozjak et al. 2003), described as ~250 kDa in (Waizenegger, Habib et al. 2004)), and a small amount of Mdm10 is detected at ~100 kDa and decreases over time (Wiedemann, Kozjak et al. 2003). The ~300 kDa complex is labelled as an assembly intermediate and a larger complex ~500 kDa is also visible in BN gels and labelled as non-specific (Fig 3E, (Meisinger, Rissler et al. 2004), Fig 4d, (Paschen, Waizenegger et al. 2003)) or uncharacterised (Fig 4E, (Yamano, Tanaka-Yamano et al. 2010)) and has not been investigated further. In some publications the amount of this larger complex appears constant (Paschen, Waizenegger et al. 2003), while in others the intensity decreases with time (Fig 3E, (Meisinger, Rissler et al. 2004)) or increases (Fig 4E, (Yamano, Tanaka-Yamano et al. 2010)). The role of Mdm10 as a module of SAM complex, as well as a substrate, complicates the study of Mdm10. This is discussed in more detail in Section 1.6.1.

1.5.4 Sam50

While Sam50 requires existing SAM complex to assemble into the outer membrane (Waizenegger, Habib et al. 2004) as both a substrate and a component of the complex, it is difficult to interpret import results monitoring radiolabelled Sam50 assembly by BN-PAGE. Despite these limitations, it is sometimes used as a substrate to look specifically at the contributions of other SAM complex components such as Sam35 or Sam37 (Chan and Lithgow 2008), and more recently to investigate the role of Mdm10 in beta barrel protein assembly (Yamano, Tanaka-Yamano et al. 2010).

1.5.5 Alpha helical SAM complex substrates

Detailed studies of TOM complex assembly have revealed that import of other TOM complex components besides Tom40 are impaired in SAM complex mutants. Tom22 is anchored in the outer membrane by an N-terminal alpha helix. The integration of Tom22 into the membrane (measured by carbonate extraction) and its assembly into the TOM complex are defective in *S. cerevisiae sam35* and *sam50* mutants as well as the *sam37Δ* strain (Stojanovski, Guiard et al. 2007). No other N-terminal alpha helical outer membrane proteins examined in this study were similarly affected, indicating that the SAM complex does not have a general role in assembly of

alpha helical proteins. However, the authors suggest that SAM helps to insert Tom22 into the membrane and helps to assemble Tom22 into the TOM complex (Stojanovski, Guiard et al. 2007). They were able to show that the Tom22 assembly defect is not simply due to reduced levels of Tom40 but impaired Tom40 assembly and loss of Mdm10 could explain these results. The SAM-Mdm10 complex binds chemical quantities of imported Tom22, and steady state levels and membrane insertion of Tom22 are reduced in the *mdm10Δ* strain (Meisinger, Rissler et al. 2004, Thornton, Stroud et al. 2010, Klein, Israel et al. 2012). Together these experiments suggest that the binding of Tom22 to SAM-Mdm10 mediates its assembly into the TOM complex.

In *N. crassa* both Sam35 and Sam37 are essential, but even reduced levels of these proteins result in less of the other SAM_{core} components, less Mdm10 as well as less of the TOM complex components Tom40, Tom22 Tom5 and Tom6 (Lackey, Wideman et al. 2011). Assembly of Tom40 and Tom22 are both drastically reduced in the mitochondria with depleted Sam37 or Sam35 but the reduced amounts of so many other TOM complex components makes it impossible determine if the slowed assembly is due to the SAM complex defect or the lack of assembly partners.

Assembly of the small TOM complex proteins Tom5, Tom6 and Tom7 is also slower in the *S. cerevisiae sam37Δ* strain but not the *sam35* or *sam50* mutants (Stojanovski, Guiard et al. 2007). While this could be due to a specific role for Sam37 in small TOM protein assembly, Mdm10 levels are also severely depleted in the *sam37Δ* strain (Meisinger, Rissler et al. 2004). Since the assembly of small TOM complex proteins is also slowed in the Mdm10 deletion strains the lack of Mdm10 could be contributing to the assembly defect (Meisinger, Rissler et al. 2004). To rule this out, assembly of Tom5 in mitochondria from the *sam37Δmdm10Δ* strain was tested and found to be even more defective (Stojanovski, Guiard et al. 2007). However, the steady state levels of the TOM and SAM complex components are not reported for this strain and may well be severely altered so a more specific contribution from Mdm10 and/or a general TOM complex assembly defect cannot be ruled out.

Tom6 assembles into the TOM complex in *S. cerevisiae* mitochondria via a SAM intermediate that is larger than the normal Tom40 Intermediate I (Thornton,

Stroud et al. 2010). This is likely to contain Tom40 that is bound to both Tom5 and Tom6, but still bound to the SAM complex. This Tom6-intermediate cannot form in the absence of the outer membrane protein Mim1 (Tom13, (Ishikawa, Yamamoto et al. 2004, Mnaimneh, Davierwala et al. 2004, Becker, Pfannschmidt et al. 2008)), suggesting the role of the MIM components is to help arrange the small TOM components correctly into the mature complex.

1.6 SAM complex partnerships

This section discusses the interactions between the SAM complex and the outer membrane proteins Mdm10 and Mim1 as well as the recently discovered interactions between the SAM complex, the TOM complex and the MICOS (mitochondrial contact site and cristae organizing system) complex in the mitochondrial inner membrane (Ishikawa, Yamamoto et al. 2004, Meisinger, Rissler et al. 2004, Bohnert, Wenz et al. 2012, Qiu, Wenz et al. 2013). The accumulated information about the interactions between the SAM complex and other ER and inner membrane complexes has lead to the hypothesis that these interactions constitute an ER–mitochondria organizing network (ERMIONE) that connects and regulates functions in the mitochondrial compartments and the ER (van der Laan, Bohnert et al. 2012).

This section introduces these additional protein complexes and their potential functions. These have been almost exclusively studied in *S. cerevisiae* and the extent to which these interactions are conserved across fungi, or eukaryotes more broadly, is largely unexplored. Section 6.1 discusses the sizes of these complexes and their hypothesized mechanisms in more detail, with the rest of the chapter exploring the *C. albicans* SAM complex and its partners. Finally, the implications of my findings for our understanding of these partnerships are discussed in Chapter 6.

1.6.1 SAM and Mdm10; a beta barrel protein assembly partnership

In addition to the core SAM complex (SAM_{core}) containing Sam50, Sam35 and Sam37, Mdm10 has been found to associate with these components as part of a larger form of the SAM complex (SAM_{holo}) (Meisinger, Rissler et al. 2004). The deletion of Mdm10 results in defects in TOM complex assembly but the precise function of this larger form of the SAM complex is still being debated (Yamano,

Tanaka-Yamano et al. 2010, Becker, Wenz et al. 2011). Mdm10 seems to be important for the assembly of the TOM complex components Tom40 and Tom22, but how and at what stage it does so remains unclear (Meisinger, Rissler et al. 2004, Yamano, Tanaka-Yamano et al. 2010). It may play a role in the release of beta barrel protein substrates from the SAM complex, perhaps by displacing the precursor from the complex (Meisinger, Wiedemann et al. 2006, Yamano, Tanaka-Yamano et al. 2010). Others have suggested a regulatory function that may influence substrate specificity or block beta barrel protein assembly by the SAM complex (Meisinger, Rissler et al. 2004, Meisinger, Wiedemann et al. 2006).

The role of Mdm10 could be specifically important in the intricate assembly of Tom40, via its multiple assembly intermediates, into the TOM complex. In the *S. cerevisiae mdm10Δ* strain, imported Tom40 accumulates slowly in the ~100 kDa assembly Intermediate II (Fig 2A, (Meisinger, Rissler et al. 2004)) and there is little or no assembly into the mature TOM complex. While the *S. cerevisiae mdm10Δ* strain has defective assembly of Tom40, the assembly of porin and Mdm10 improves (Meisinger, Rissler et al. 2004), and mutations in the beta signal of Mdm10 slow the assembly of Tom40 into the TOM complex but not the assembly of porin, Sam50 or Mdm10 itself into their respective oligomers (Yamano, Tanaka-Yamano et al. 2010).

In *S. cerevisiae,* this impaired assembly of Tom40 in *mdm10Δ* mitochondria may be due to a direct role of Mdm10 or may be an indirect result of reduced levels of Tom22. Both assembly and steady state levels of Tom22 are reduced in the *mdm10Δ* strain (Meisinger, Rissler et al. 2004), as well as in strains with mutations in the core SAM complex components (Stojanovski, Guiard et al. 2007). Import of chemical quantities of Tom22 but not Mdm10 prior to radiolabelled Tom40 import rescues the assembly of Tom40 into the TOM complex in the *mdm10Δ* strain (Fig 5a & 6, (Becker, Wenz et al. 2011)). Together with the lack of evidence for any Mdm10-Tom40 interaction, this argues against a direct role of Mdm10 in Tom40 assembly and suggests Mdm10 helps in the later assembly of Tom22 into the complex. When Tom22 is incubated with mitochondria from *mdm10Δ* and *tom22Δ* strains it accumulates in a ~400 kDa complex which can be chased into a mature TOM complex, but similar import assays were not performed in the Mdm10 beta signal mutant strain (Meisinger, Rissler et al. 2004, Yamano, Tanaka-Yamano et al. 2010).

Pre-incubation chemical quantities of Mdm10 with mitochondria from these mutant strains restores assembly of Tom22 to wild type levels and imported Tom22 co-purifies with Mdm10 (Fig5b & 5d, (Becker, Wenz et al. 2011)) suggesting that Mdm10 is directly involved in assembly of Tom22 into the TOM complex.

1.6.2 SAM and MIM; coordinating outer membrane protein insertion

Mim1 (mitochondrial import protein 1), also known as Tom13 (Waizenegger, Schmitt et al. 2005), like Mdm10, is described as a modular subunit of the SAM complex (Ishikawa, Yamamoto et al. 2004, Becker, Pfannschmidt et al. 2008, Hulett, Lueder et al. 2008, Lueder and Lithgow 2009, Thornton, Stroud et al. 2010). Mim1 is anchored in the outer membrane with its N-terminus exposed to the cytosol and the C-terminus to the intermembrane space (Lueder and Lithgow 2009) and forms a 100-150 kDa MIM complex with another recently discovered C-terminal outer membrane-anchored protein Mim2 (Dimmer, Papic et al. 2012). This complex is lost if Mim1 is deleted and steady state Mim1 levels decrease if Mim2 is deleted (Dimmer, Papic et al. 2012).

Mim1 interacts with the SAM complex (Becker, Pfannschmidt et al. 2008) but is not a permanent or stable subunit of the SAM complex or SAM import intermediates (Ishikawa, Yamamoto et al. 2004, Becker, Pfannschmidt et al. 2008, Thornton, Stroud et al. 2010). Both Mim1 and Mim2 contribute to TOM complex assembly. Loss of Mim1 impairs the integration of newly imported Tom40, Tom20, Tom6 and Tom70 (but not Tom22) into the TOM complex and the Mim2 deletion strain has impaired assembly of Tom40 into the TOM complex (Ishikawa, Yamamoto et al. 2004, Waizenegger, Schmitt et al. 2005, Thornton, Stroud et al. 2010, Dimmer, Papic et al. 2012). Imported Tom6 forms intermediates that contain SAM complex components and are an appropriate size for SAM-Mim1 complexes, but there is no further evidence that these intermediates contain Mim1 (Becker, Pfannschmidt et al. 2008, Thornton, Stroud et al. 2010). The import of Tom20 was slowed and there is less mature TOM complex (detected by radiolabelled Tom20) in the *mim1Δ* deletion strain (Hulett, Lueder et al. 2008). The amount of the TOM complex detected by Tom22 and Tom40 antibodies is also drastically reduced in the *mim1Δ* and *mim2Δ* deletion strains (Dimmer, Papic et al. 2012).

A broader search for Mim1 substrates revealed a number of proteins with multiple segments spanning the outer membrane (Ugo1, Scm4 and Fzo1, (Becker, Wenz et al. 2011, Dimmer, Papic et al. 2012)). Mim1 phosphorylation also contributes to its activity as mutations of the phosphorylated residues slow the assembly of Tom20 and Tom70 (Schmidt, Harbauer et al. 2011). Tom70 also contributes to the import of these multiple membrane-spanning substrates of the MIM complex (Becker, Wenz et al. 2011) suggesting regulatory posttranslational modifications are an intriguing new aspect of the coordination of mitochondrial protein import. The Mim proteins have no close homologues outside fungi (Hulett, Lueder et al. 2008, Lueder and Lithgow 2009) and it is not known whether their roles are conserved across different fungal species or if proteins with a similar function but unrelated sequences exist in other eukaryotes. Together these results suggest the role of the MIM complex is in the insertion of outer membrane-spanning proteins and that it may function in close proximity to the SAM complex or other fungal-specific import components to coordinate the association of the other TOM complex components with newly assembled Tom40.

1.6.3 The TOM-SAM super-complex; outer membrane partnership

Recent work using fusion proteins has stabilised a long-hypothesised TOM-SAM super-complex in action by using substrates that get trapped in the TOM complex (Qiu, Wenz et al. 2013). This complex joins TOM to the SAM complex through Tom22 and the proximity of the complexes is thought to help coordinate import and assembly of beta barrel proteins. Mdm10 is notably missing from the TOM-SAM super-complex, suggesting that the interaction between the SAM complex and Tom22 blocks the binding of Mdm10 to either or both of these proteins. Whether this complex is conserved in other species has not yet been reported. However, the SAM complex intermediate in Tom40 assembly in human mitochondria is larger than the mature complex and may represent a similar super-complex (Humphries, Streimann et al. 2005).

1.6.4 SAM and MICOS; coordinating intermembrane cooperation

Another recently identified super-complex partnership is the interaction between SAM and the mitochondrial contact site and cristae organizing system (MICOS) complex (Harner, Korner et al. 2011, Hoppins, Collins et al. 2011, von der

Malsburg, Muller et al. 2011). The simultaneous publication of the discovery of this complex by various research groups resulted in a profusion of alternative names for the components and complex. This has recently been resolved through a collective agreement (Pfanner, van der Laan et al. 2014). As the agreed name suggests, the MICOS complex is located at contact sites where the inner and outer membranes are in close proximity. The main role of the complex is thought to be in the arrangement of the membrane contact sites, thereby helping to establish and maintain the folds of the inner membrane that form the mitochondrial cristae.

Components of both the SAM and TOM complexes have also been purified in conjunction with tagged MICOS complex components (Bohnert, Wenz et al. 2012). Deletion of Mic60, the MICOS complex component that interacts with Sam50, results in slower beta barrel protein assembly, but disrupting the Mic60-SAM complex interaction does not give the same defect. This suggests it is the interaction of Mic60 with the TOM complex and not with the SAM complex that is helping with beta barrel protein assembly. Tom40 takes longer to reach a protected location in the mitochondria lacking Mic60 consistent with a defect early in the Tom40 import pathway.

Deletion of Mic60 impairs the import of proteins into the intermembrane space via the MIA pathway as well as the import of beta barrel proteins (von der Malsburg, Muller et al. 2011, Bohnert, Wenz et al. 2012). For the MIA pathway substrates, the interaction between the TOM complex and the MICOS complex was suggested to improve the efficiency of the import process by keeping the MIA complex in close proximity to the TOM complex to receive its substrates (von der Malsburg, Muller et al. 2011). I hypothesise Mic60 could be playing a similar role in the beta barrel protein import pathway; holding the TOM-SAM super-complex in close proximity to the MIA complex. This would supply the small TIM complex chaperones that are needed for beta barrel protein assembly (Chacinska, Pfannschmidt et al. 2004).

1.7 *Candida albicans* as a model organism

Much of what we know about these mitochondrial protein import pathways has been discovered using the model yeast *S. cerevisiae*. Its tractable genetic system

coupled with biochemical analyses has led to a detailed understanding of many of the components that drive protein import into mitochondria. While *S. cerevisiae* is an excellent model organism to determine how cellular metabolism is integrated with signalling pathways and networks, its regulation of mitochondrial biogenesis is somewhat peculiar. *S. cerevisiae* ferments glucose anaerobically, whether or not oxygen is available (Piskur and Langkjaer 2004), and many of the genes involved in growth in the absence of oxygen arose through a whole-genome-duplication event, after which duplicated genes were modified to provide new functions or new signalling switches (Wolfe and Shields 1997, Gojkovic, Knecht et al. 2004, Piskur and Langkjaer 2004, Wolfe 2004). During optimal, rapid growth in glucose, mitochondrial function is repressed, and transcriptional networks have been re-organized in *S. cerevisiae* to enable regulation of mitochondrial biogenesis by different carbon sources (Schüller 2003, Ihmels, Bergmann et al. 2005). These peculiarities of *S. cerevisiae* also make it difficult to determine which aspects of the import processes are particular to *S. cerevisiae* and which are general features of the import machinery that evolved in the earliest mitochondria.

In this thesis I discuss the development of the human fungal pathogen, *Candida albicans,* as a new model system to study these mitochondrial protein import processes. *C. albicans* is an attractive model system because it differs substantially from *S. cerevisiae* in regards to the requirement for mitochondrial function during growth. Unlike *S. cerevisiae*, *C. albicans* grows aerobically and there is no repression of mitochondrial function when grown in glucose. The genome of *C. albicans* has been completely sequenced (Braun, van Het Hoog et al. 2005) and various tools have been developed for gene deletion and epitope-tagging, facilitating genetic manipulation and sequence-based analysis (Berman and Sudbery 2002, Bruno and Mitchell 2004, Lavoie, Sellam et al. 2008, Nobile and Mitchell 2009). *C. albicans* has recently been found to have a haploid form, but it grows and causes disease primarily in diploid form (Hickman, Zeng et al. 2013). Strains and manipulations used in this work are therefore all diploid.

Since *C. albicans* is a budding yeast like *S. cerevisiae*, I expected many protein import components to be conserved. However, since these yeasts diverged around 300 million years ago (Taylor and Berbee 2006), by comparing their protein

import pathways I hoped to gain an insight into which features are conserved and which were inherited from an ancestral mitochondrion. Comparing these pathways would also identify which pathways evolved specifically to accommodate the unique evolutionary pressures and ecological niches of these two organisms.

Finally, recent work suggests that mitochondria play key roles in resistance of *C. albicans* to antifungal drugs, as well as in its ability to cause disease (Dagley, Gentle et al. 2011, Qu, Jelicic et al. 2012). Two mitochondrial proteins that are important for pathogenicity include the *Candida*-specific mitochondrial protein Goa1, which does not have orthologs in *S. cerevisiae* (Bambach, Fernandes et al. 2009, She, Zhang et al. 2013) and Ptc4 (Zhao, Sun et al. 2010). A better understanding of the mitochondrial components and metabolic pathways that are specific to fungi could also provide a route to developing new antifungal agents (Akimenko, Golovchenko et al. 1974, Brown, Brown et al. 2014). Therefore, beyond its usefulness as a new model system for understanding fundamental mechanisms and regulation of mitochondrial biogenesis, studying these processes in *C. albicans* also has the potential to improve our understanding of virulence mechanisms and help us to combat this major human pathogen (Shingu-Vazquez and Traven 2011).

1.8 Thesis Outline

In this thesis I identify the components of the mitochondrial protein import machinery of *C. albicans* (Chapter 3). I discuss key differences in this import machinery comparing *C. albicans* and *S. cerevisiae* and the implications of these differences for the evolution of protein import pathways. I examine the components of the *C. albicans* sorting and assembly machinery (SAM) complex in detail (Chapters 4-6). This includes characterizing the sequences, functions and biochemistry of the homologues of core and modular components of the SAM complex. I finish by discussing the implications of these discoveries for the potential mechanisms of the SAM complex function, SAM complex partners and the evolution of the outer membrane protein assembly machinery (Chapter 6).

2 Materials and Methods

This section describes the general protocols used throughout the thesis. The specific conditions used for each experiment are provided in the results section in the text, figures or figure legends as appropriate.

2.1 Bioinformatics

2.1.1 Identification of homologous proteins

I identified *Candida albicans* homologues of mitochondrial proteins using BLAST (Basic Local Alignment Search Tool) searches in the Candida Genome Database (CGD) (http://www.candidagenome.org) (Arnaud, Inglis et al. 2014) using the *Saccharomyces cerevisiae* protein sequence obtained from the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/) (Cherry, Hong et al. 2012).

BLAST searches are limited to single sequence alignments, and combined with the sequence divergence of membrane proteins, this makes homologues in more distantly related species difficult to identify (Martelli, Fariselli et al. 2002, Likic, Dolezal et al. 2010). Hidden Markov models (HMMs) can be used to search for homologues based on multiple sequence alignments. I constructed HMMs for the SAM complex components Sam35, Sam37 and Sam50 and Mdm10 using the fungal homologues of these proteins in the species listed in Appendix 1: Table 8.1 (Likic, Dolezal et al. 2010). BLAST searches using the *S. cerevisiae* sequences were used to detect the fungal homologues of the various SAM and ERMES components used to construct the HMMs. These sequence sets were used to create HMMs according to previously devised methods (Likic, Dolezal et al. 2010). These HMMs were used to scan UniProt (Release 12.4, containing Swiss-Prot Release 54.4 and TrEMBL Release 37.4) to identify open reading frames in the *C. albicans* genome (strain SC5314 and Assembly 21) and identify homologues in species where BLAST searches did not identify a single clear hit.

Expectation values (E values) for the hits are used to indicate the probability the hit would be detected by chance (a value of 1 would be equally likely to be scored by chance). While this value does depend on the size of the database searched, with the large databases used in these studies, E values much lower than one are a good

indication of the significance of the hit and the likelihood that the protein sequence detected shares an evolutionary relationship to the sequences used to construct the model (Finn, Bateman et al. 2014) (http://pfam.xfam.org/help).

2.1.2 Domain identification

Domain information for proteins of interest was gathered from the CGD (http://www.candidagenome.org) (Arnaud, Inglis et al. 2014) and SGD (http://www.yeastgenome.org/) (Cherry, Hong et al. 2012) databases as well as the Pfam (http://pfam.xfam.org/) (Finn, Bateman et al. 2014) and NCBI conserved domains database (http://www.ncbi.nlm.nih.gov/cdd/) (Marchler-Bauer, Zheng et al. 2013). Version numbers and access dates are given in the Results sections where appropriate. Accession numbers for the sequences used for the domain characterisation of the Sam35, Sam37 and metaxin proteins (Figure 4.1) are listed in Appendix 1: Table 8.2 if not already listed in Appendix 1: Table 8.1.

2.1.3 Multiple sequence alignments

Coloured multiple sequence alignments were made in SeaView v4.4.2 (Gouy, Guindon et al. 2010) (http://doua.prabi.fr/software/seaview) using Clustal Omega. Text only alignments were made using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (McWilliam, Li et al. 2013). Accession numbers for the sequences used for multiple sequence alignments of the Sam35 and Sam37 proteins are listed in Appendix 1: Table 8.2 if not already listed in Appendix 1: Table 8.1.

2.1.4 Phylogenetic tree construction

Trees were constructed from MUSCLE multiple sequence alignments (Edgar 2004) using PhyML 3.0 (Guindon, Dufayard et al. 2010) run in SeaView v4.4.2. Sequences are listed in Appendix 1: Table 8.3. After alignment, Gblocks was used to remove non-conserved sites with stringency settings allowing smaller final blocks, gap positions within the final blocks and less stringent flanking positions (Talavera and Castresana 2007). Tree calculations were performed using default settings with the LG exchange matrix and the Best of NNIs and SPRs option and 500 bootstrap calculations.

2.2 Strains, plasmids and growth conditions

The *C. albicans* strains used in this research are derivatives of BWP17 (Wilson, Davis et al. 1999) and part of the Traven Lab Collection, except those constructed as described below. The full genotypes of all the *C. albicans* strains used are listed in Appendix 2: Table 8.4. The wild type strain used for *S. cerevisiae* experiments was haploid W303a unless otherwise stated. The full genotypes of all the *S. cerevisiae* strains used are listed in Appendix 2: Table 8.5.

2.2.1 Media and growth conditions

Unless otherwise stated, *C. albicans* strains were grown in YPD (1 % yeast extract, 2 % peptone, 2 % glucose) supplemented with 0.1 % uridine (Cheng, Nguyen et al. 2003). *S. cerevisiae* strains were grown in YPAD (YPD with 0.1 % adenine) unless otherwise stated, to compensate for a mutation in *ADE2* in the adenine biosynthesis pathway in the W303 strain of *S. cerevisiae.* Depletion strains were grown in minimal synthetic media (SD) (0.67 % w/v yeast nitrogen base without ammonium sulphate (US Biologicals, Y2025)) with 2 % glucose (D) or glycerol (G) and supplemented with amino acids (Burke, Dawson et al. 2000).

Mutant growth assays used the following chemicals at the concentrations indicated in the results section (Section 5.1.6); Calcofluor white (Sigma, F3543), iron sulphate (Chem-Supply, FA001), iron citrate (Sigma, F3388), the iron chelator bathophenanthroline disulfonic acid disodium salt (BPS, MP Biomedicals, 150112), mitochondrial membrane potential inhibitors: antimycin (Sigma, A8674), valinomycin (Sigma, V0627), and oligomycin (Sigma, O4876) or CCCP (carbonyl cyanide m-chlororophenyl hydrazone, Sigma).

2.2.2 Epitope tagging of SAM complex components

Since antibodies that recognised Sam37, Sam35, Sam50 and Mdm10 in *C. albicans* were not available at the start of the project, I tried to introduce a hemaglutinin peptide (HA) tag to the C-termini of these proteins using the methods described by Lavoie and colleagues (Lavoie, Sellam et al. 2008). The tagging plasmids pFA-HA-URA3 (BAT13-Traven Lab Plasmid Collection) and pFA-HA-ARG4 (BAT15-Traven Lab Plasmid Collection) were used as templates. Forward

primers included the last ~100 bases of the open reading frame and a 20 base sequence matching the tagging plasmids to amplify the tag and selectable marker (Primers listed in Appendix 3: Table 8.6). Reverse primers included the first ~100 bases of the 3'-untranslated region downstream of the gene of interest and a 20 base sequence matching the tagging plasmids.

Insertion of this construct via homologous recombination was used to put in a chromosomal C-terminal HA tag after the gene of interest. A second round of homologous recombination with a different selectable marker amplified by the same long primers was used to tag the second copy of the gene by selection for growth on plates lacking both arginine and uracil. Forward checking primers were designed to recognise the sequence just upstream of the region of the open reading frame used in the forward construction primers and the reverse checking primers recognise a sequence just downstream of the reverse construction primers. Internal primers matching the *URA3* or *ARG4* cassettes were used in combination with the appropriate forward or reverse checking primers as further confirmation when the sizes of the fragments amplified by the PCR check of the whole insert were not clear.

Screening by PCR to amplify the C-terminus of the positive clones indicated a genome duplication event occurred in those constructs where a tag was introduced to the second copy of Sam50. This suggests the addition of a tag to the C-terminus of these proteins is detrimental to Sam50 function, thus no further attempts were made to create C-terminal tagged Sam50 via this method and instead I produced antibodies to this protein (Section 2.5).

I also confirmed that the epitope-tagging was successful by western blots of mitochondria from HA-tagged strains compared to the parent strain using anti-HA antibodies. In all strains I detected bands corresponding to a 3.6 kDa (the size of a triple HA tag) increase in the protein size in (Figure 2.1A). The HA tag on Mdm10 did not alter complex stability on BN-PAGE (Figure 2.1B).

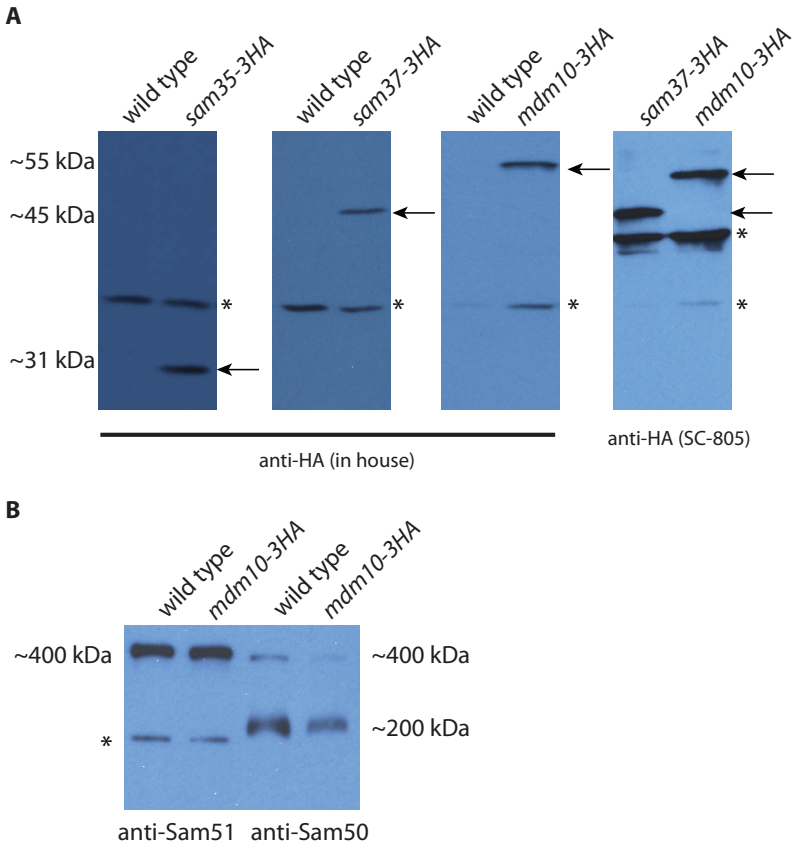


Figure 2.1 Confirming HA-tagged strains
(A) Mitochondrial samples (50 µg per lane) from the indicated strains were analysed by SDS-PAGE and western blotting with the indicated antibodies. Anti-HA recognises a ~45 kDa band in the *sam37-3HA* strain (untagged protein 41.6 kDa), a ~31 kDa band in the *sam35-3HA* strain (untagged protein 28.1 kDa), and a ~55 kDa band in the *mdm10-3HA* strain (untagged protein 53.1 kDa). **(B)** Mitochondrial samples (100 µg per lane) from the indicated strains were analysed by BN-PAGE and western blotting with the indicated antibodies. Arrows indicate specific signals detected at the appropriate size. Asterisks indicate non-specific bands.

2.2.3 Characterisation of Sam37 deletion strains

The *sam37ΔΔ* and *sam37ΔΔSAM35↑* strains were constructed by my colleagues in the Traven Lab (Qu, Jelicic et al. 2012). My characterisation of the *sam37ΔΔ* strain is described in Section 5.2. With no antibodies to confirm the up regulation of *SAM35* in the *sam37ΔΔSAM35↑* strain, I used quantitative polymerase chain reaction (qPCR) to detect *SAM35* transcripts (Figure 2.2 & Methods Section 2.10). This confirmed that the *sam37ΔΔSAM35↑* strain expresses high levels of

SAM35 and also showed that *SAM35* transcript levels were not reduced in the *sam37ΔΔ* strain (less Sam35 is associated with the SAM complex in the *S. cerevisiae* *sam37Δ* strain (Chan and Lithgow 2008)).

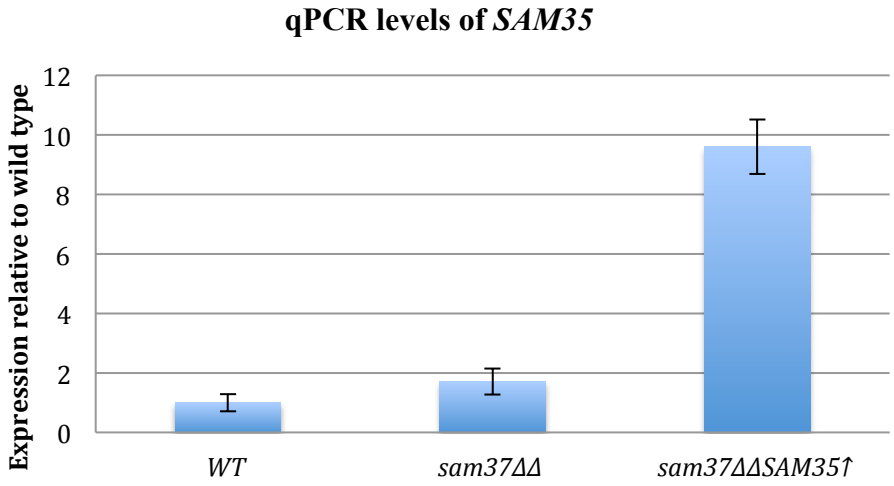


Figure 2.2 Expression of *SAM35* in the *sam37ΔΔ* strains
The expression levels of the *SAM35* gene were measured by quantitative PCR in the indicated strains. Levels were normalised to *ACT1* and averages of two independent biological repeats assayed in duplicate and the standard errors are shown.

2.2.4 Plasmid transformation into *S. cerevisiae*

Two investigations in this thesis required the expression of high levels of proteins in various *S. cerevisiae* strains (See Sections 2.2.5 and 2.2.6). Transformation of *S. cerevisiae* strains with high copy number vectors was performed using the following technique (modified from (Keszenman-Pereyra and Hieda 1988)). Four fresh colonies were resuspended in 100 µL of transformation buffer (0.2 M lithium thiocyanate, 1 mM magnesium acetate, 0.2 mM calcium acetate, 1 M Tris buffer pH 7.5) and mixed by vortexing. The vector was prepared by miniprep (Wizard ® Plus SV Miniprep DNA purification system, Promega, A1460) and eluted in 100 µL. The vector (1 µL of miniprep), 2 µL of 2 mg/mL Salmon sperm DNA (preheated 10 min then cooled on ice) and 15 µL triacetin were added to the cells and mixed by vortexing. Finally 350 µL of PEG solution (PEG 3350 (Sigma) 700 g/L in 10 mM Tris-HCl pH 7.5) was mixed using a 10 s vortex. The cells were left to recover at 30 °C (wild type) or 23 °C (mutants with growth defects) for 1 h before being collected

using a 30 s, 12000 rpm spin at room temperature. The cell pellet was resuspended in 100 µL of sterile MilliQ water and plated onto the appropriate selective media.

2.2.5 Yeast over-expression plasmids

C. albicans Tom40 did not assemble into the *S. cerevisiae* TOM complex. To identify the cause of this assembly block I tried to rescue the assembly of *C. albicans* Tom40 into the TOM complex in *S. cerevisiae* mitochondria by transforming wild type *S. cerevisiae* (W303a) with plasmids expressing high levels of *S. cerevisiae* Tom22, Tom20 and Mim1. I obtained these yeast expression plasmids for over-expression of *S. cerevisiae* Tom22, Tom20 and Mim1 from the Lithgow lab collection. Their construction is outlined briefly below, as it is not described elsewhere in published work. However, previous researchers confirmed that these plasmids produce high levels of the encoded protein. The Tom22 plasmid (pTT28) contains a 2.9 kb fragment that includes *S. cerevisiae TOM22* cloned into the EcoRI restriction site of YEplac195 (Gietz and Sugino 1988), a 2 micron yeast expression vector with a *URA3* selectable marker (Lithgow, Junne et al. 1994). The Tom20 plasmid (pTT32) contains a 2.6 kb fragment that includes *S. cerevisiae TOM20* cloned into the BamHI restriction site of YEplac181 (Gietz and Sugino 1988), a 2 micron yeast expression vector with a *LEU2* selectable marker (Gratzer, Lithgow et al. 1995). The Mim1 plasmid (YEpMim1) contains *S. cerevisiae MIM1*, including DNA from 1.5 kb upstream and 1 kb downstream, inserted between the BamHI and SphI restriction sites of YEplac181.

2.2.6 *CYB2* plasmid construction

To investigate the functions of the *CYB2* genes from *S. cerevisiae* and *C. albicans* I designed experiments to determine whether growth assays with L-lactate as the carbon source could be used as a measure of Cyb2 function. I found growth of the *S. cerevisiae cyb2Δ* strain (BY4741 background, (Winzeler, Shoemaker et al. 1999) (www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html)) on minimal media with L-lactate as the carbon source was restored when transformed using the multicopy plasmid pGR401 with *S. cerevisiae CYB2* inserted between the EcoRI and HindIII sites (Beasley, Muller et al. 1993). I designed primers to incorporate these restriction sites at the 5' and 3' ends of the open reading frame of

the *C. albicans CYB2* gene and used them to amplify the gene from genomic DNA and clone it into the same vector to test for complementation. Although this construct failed to complement the deletion strain for growth on L-lactate, the sequence of this construct was not ideal to test the function of the *C. albicans* Cyb2. The *C. albicans CYB2* gene has three ctg codons that would be translated as lysine in *S. cerevisiae* but would be translated as serine in *C. albicans* (Santos and Tuite 1995).

To confirm *C. albicans* Cyb2 was unable to complement the growth defect of the *S. cerevisiae cyb2Δ* mutant I cloned a codon optimised *C. albicans CYB2* (codon optimised for expression in *S. cerevisiae* and to correct codon usage, purchased from GenScript) from the storage vector pUC57 into the expression vector. The *S. cerevisiae* CYB2 gene was also cloned into this vector from pGR401 above. An N-terminal truncation of the *S. cerevisiae CYB2* gene mimicking the shorter targeting sequence of Cyb2 in *C. albicans* was also constructed using primers designed to amplify a truncated version of *S. cerevisiae CYB2* from pGR401 (ScCyb2ΔN) and inserted between the EcoRI and HindIII restriction sites of pYX212 (primers listed in Appendix 3: Table 8.7). The constructs were confirmed by restriction digest mapping and DNA sequencing. I was able to confirm expression of Cyb2 by western blotting as antibodies raised against *S. cerevisiae* Cyb2 cross-reacted with *C. albicans* Cyb2 (Figure 3.5). These constructs were assayed for growth on minimal media with L-lactate as the carbon source. Plasmids for Cyb2 import experiments were made by inserting the codon optimised *C. albicans CYB2* gene into pSP64 between the EcoRI and BamHI restriction sites.

2.2.7 *In vitro* transcription plasmid construction

For cloning of *C. albicans* genes the open reading frame was first checked for CTG codons. Where these codons were present, the gene was purchased in a codon-optimised form from GenScript. Where possible the gene of interest was amplified from genomic DNA. The *C. albicans TOM40* open reading frame was cloned into pSP64 between the HindIII and XbaI restriction sites using primers listed under pSP64-CaTom40 in Appendix 3: Table 8.7. The *C. albicans POR1* gene was cloned into pSP64 (between the BamHI and EcoRI sites), and into pSP73 (between the EcoRI and XbaI sites) and amplified using primers that include the Kozak sequence (making translation more efficient) and SP6 promoter, but none of these constructs

produced protein. The codon optimised *C. albicans MDM10* gene was cloned into pSP64 between XbaI and EcoRI from the pET28 construct described in Section 2.5.

To elucidate the roles of two mitochondrial-targeted proteins associated with the pathogenicity of *C. albicans* I made plasmids for *in vitro* transcription of Goa1 and Ptc4 (Bambach, Fernandes et al. 2009, Zhao, Sun et al. 2010). *GOA1* contains CTG codons, so a codon optimised copy of the open reading frame was purchased from GenScript in pUC57 and cloned into pSP64 between the XbaI and BamHI restriction sites. *PTC4* does not contain CTG codons so was amplified from genomic DNA and inserted into pSP64 between the HindIII and XbaI restriction sites using the primers listed under pSP64-CaPtc4 in Appendix 3: Table 8.7. While these constructs produced proteins of the appropriate sizes, I was unable to find any import reaction conditions where the proteins do not aggregate when added to mitochondria.

2.3 Protein and complex separation

2.3.1 SDS-PAGE

To analyse protein content of whole cells, cells from liquid cultures (volume (mL) = 1/OD600) were collected (1 min, 14000 rpm, room temperature) and resuspended in 200 µL 0.1M NaOH. Samples were left at room temperature for 5 min and then reisolated by centrifugation (30 s, 14000 rpm, room temperature). The pellet was resuspended in 80 µL SDS loading dye (50 mM Tris pH 6.8, 1 % SDS, 10 % glycerol, 100 mM dithiothreitol (DTT), 0.0005 % bromophenol blue) and heated to 95 °C for 5 min. The insoluble material was removed by centrifugation and 20 µL of sample was loaded per lane.

For separation by SDS-PAGE mitochondrial samples (typically 50 µg per lane) were solubilised in SDS loading dye as above. Where indicated mitochondria were incubated with proteases; 100 µg/mL trypsin or 50 µg/mL proteinase K (Promega, V3021) in the buffer containing 0.6 M sorbitol and 20 mM HEPES (pH 7.4) for 20 min on ice before addition of protease inhibitors (1 mg/mL soya bean trypsin inhibitor or 1 mM PMSF). Where indicated, mitochondria were subject to osmotic shock to create mitoplasts by incubation for 10 min at room temperature with the same buffer lacking sorbitol. Relative sizes were determined using Amersham LMW Calibration Kit (17-0446-01, GE Healthcare). Gels were made using 12 %

acrylamide (40 % acrylamide:bis 29:1, Biorad) with a 4 % stacker (separating buffer: 1.5 M Tris pH 8.8, 0.4 % SDS, 2 mM EDTA, stacking buffer: 1 M Tris pH 8.0, 0.08 % SDS, 2 mM EDTA) and electrophoresis was performed at 50-120 V at room temperature (running buffer: 30.3 g/L tris, 71.3 g/L glycine, 5 g/L SDS).

2.3.2 BN-PAGE

BN-PAGE was used to separate and analyse complexes using slight modifications of published techniques (Schagger and von Jagow 1991, Wittig, Braun et al. 2006). Typically for each lane, 100 μg of mitochondria were solubilised in 50 μL blue native lysis buffer (20 mM Tris pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10 % glycerol, 0.5 mM PMSF, 1 % digitonin (Calbiochem, 300410) or 1 % n-Dodecyl-β-D-maltoside (DDM)) at 4 °C for 15-20 min with occasional agitation. PMSF was added just prior to use by diluting a 100 mM stock made in ethanol. Insoluble material was removed by centrifugation (4 °C, 13000-15000 rpm, 10 min) and the supernatant was transferred to a new tube containing 10x BN loading dye (0.5 M aminocaproic acid, 0.1 M Bis Tris pH 7.0, 10 % Coomassie Brilliant Blue G250).

BN-PAGE gels were 4-14 % acrylamide gradients (made from 40 % acrylamide:bis 37.5:1, Biorad (Cat # 161-0148) with 2 % w/v bisacrylamide added, 67 mM 6-aminocaproic acid, 50 mM Bis Tris pH 7.0) poured in 179x82 mm plates. BN-PAGE was performed at 120 V at 4 °C for 850 min (cathode buffer: 50 mM tricine, 15 mM Bis Tris pH 7.0, 0.02 % Coomassie blue G250, anode buffer: 50 mM Bis Tris pH 7). Solutions were all stored at 4 °C except Coomassie blue, which was stored at room temperature and added to the cathode buffer immediately before electrophoresis.

Stability of the *S. cerevisiae* SAM complex has been shown to depend on the salt concentration of the lysis buffer and the concentration of detergent. I tested for optimal conditions for detection of the *C. albicans* SAM complex using western blotting (Figure 2.3). No variations on the standard lysis conditions improved the signal or relative amounts of any of the complexes detected, so the BN-PAGE experiments in this thesis all use a buffer containing 50 mM NaCl and 1 % digitonin.

Initial testing using the in-house monoclonal anti-HA antibody detected the two expected forms of the SAM complex (Figure 2.3A) but later batches of this

antibody did not detect any complexes. The rabbit antibodies produced against *C. albicans* Sam50, Sam51 and Mdm10 as part of this project all detect complexes on BN-PAGE. The Mdm10 antibody must be affinity purified against purified inclusion bodies before it is suitable for use in conjunction with BN-PAGE. Affinity purification also helped to remove non-specific bands detected by Sam51 crude serum (compare Figure 2.3C and Figure 6.3A).

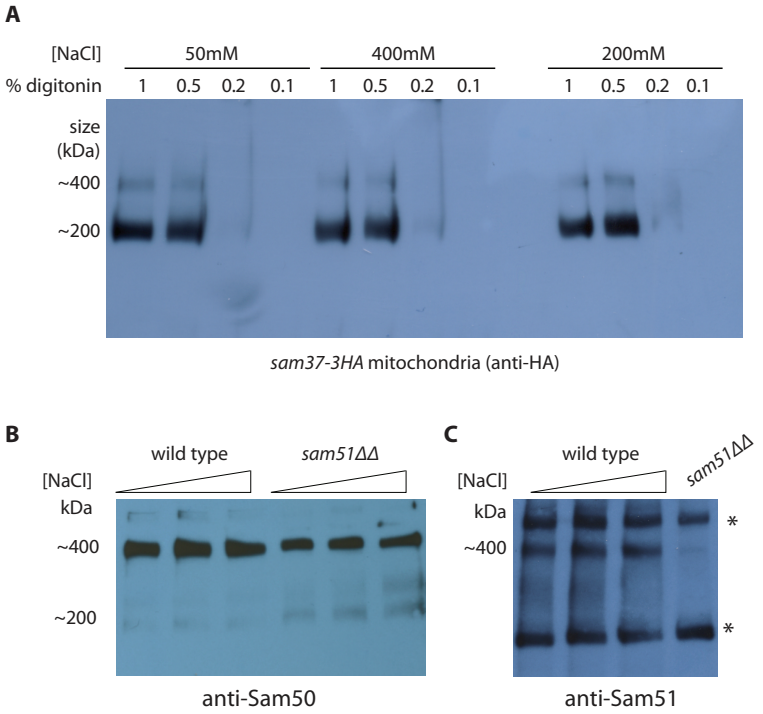


Figure 2.3 Testing SAM complex stability in different BN-PAGE conditions
(A) Complexes detected mitochondria isolated from a *sam37-3HA* strain of *C. albicans* separated by BN-PAGE (in the indicated salt and digitonin conditions) and detected using in house antibodies against the HA epitope (anti-HA). **(B)** Mitochondrial complexes in wild type and *sam51ΔΔ* strains of *C. albicans* were separated by BN-PAGE (with NaCl concentrations of 50 mM, 200 mM and 400 mM) and detected using anti-Sam50 antibodies. **(C)** Mitochondria were separated as described in (B) and detected using crude anti-Sam51 serum. Mitochondria from the *sam51ΔΔ* strain were solubilised in lysis buffer. Asterisks indicate non-specific bands.

2.4 Antibodies and western blotting

Proteins separated using SDS-PAGE were transferred onto nitrocellulose (0.45 µm, 1 Amp, 1 h) and detected by antibodies using chemiluminescence using the Fuji FPM 100A film processor and SuperRX Fuji Medical X-ray film (100 NIF). Western blots of samples separated by BN-PAGE were transferred to PVDF after soaking for up to 5 min in SDS running buffer (0.45 µm, 1 Amp, 1.5 h) to enable removal of Coomassie using 100 % methanol.

Antibody shift assays with epitope tagged components or polyclonal antibodies can be used to determine the constituents of complexes identified by BN-PAGE. The solubilised protein complexes are incubated with antibodies as described previously (Thornton, Stroud et al. 2010).

I tested the cross reactivity of antibodies raised against *S. cerevisiae* mitochondrial proteins in the Lithgow Lab Collection against their *C. albicans* homologues to identify those that could be used to study these components in *C. albicans*. Using western blotting of mitochondrial proteins separated by SDS-PAGE, I compared the positions of the signals from mitochondria isolated from *S. cerevisiae* or *C. albicans*. Antibodies giving a clear signal at a position corresponding to a size near to the predicted size of the *C. albicans* protein were deemed specific. The results are summarised in Appendix 4: Table 8.9.

2.5 Antibody production

Antibodies to Sam50, Sam51 and Mdm10 were produced in house at the Monash Large Animal Facility (Ethics approval SOBS/B/2009/18 – Chief Investigator Kip Gabriel). The genes were corrected for codon use and codon-optimised for expression in *Escherichia coli* (GenScript). Constructs were provided by the Buchanan Lab (NIH, NIDDK, USA) and cloned into a pET28 expression vector with a C-terminal 6xHis tag between the NdeI and BamHI sites. A summary of the steps in this process is shown in Figure 2.4.

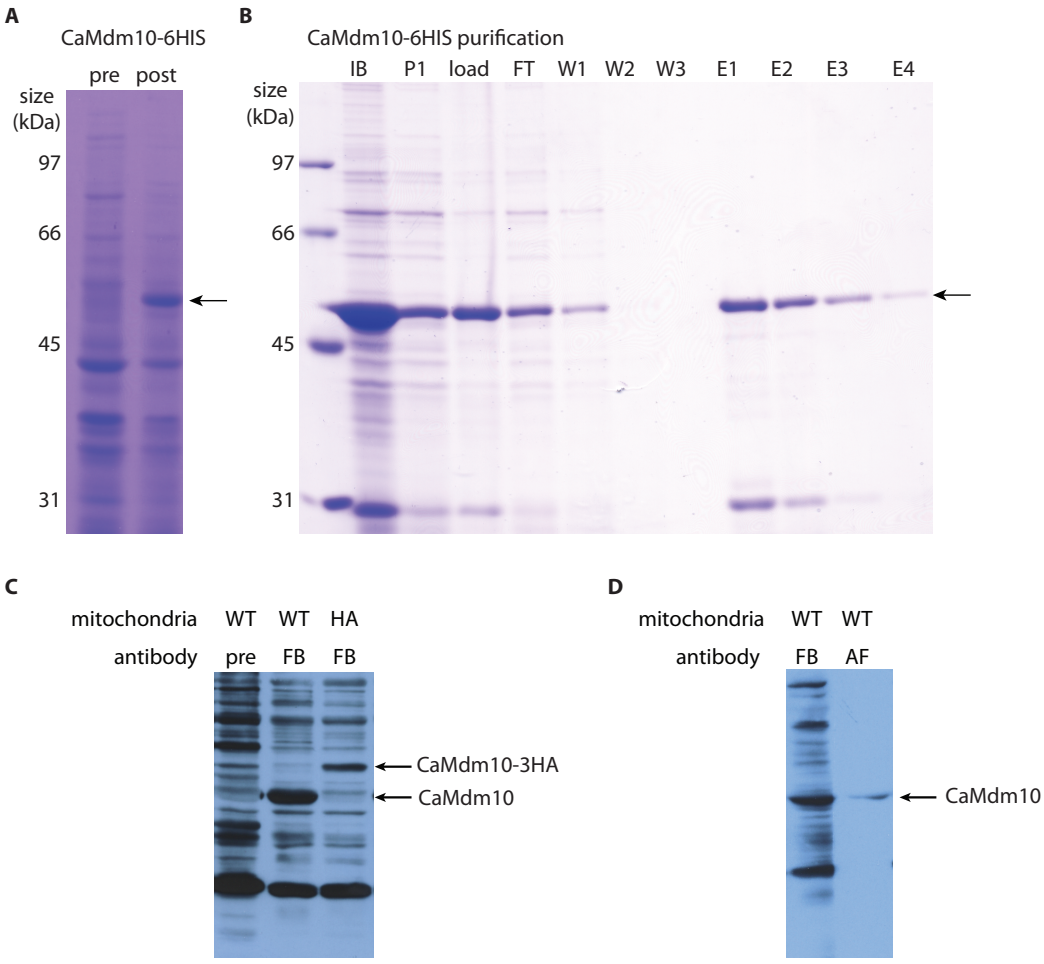


Figure 2.4 Mdm10 antibody production workflow
(A) Commassie stained gel showing expression of HIS tagged Mdm10 in BL21 DE3* (CaMdm10-6HIS) pre-induction (pre) and post-induction (post) with IPTG. **(B)** Commassie stained gel showing purification of inclusion bodies (IB) where P1 is the inclusion body pellet after washing with 10 % (w/v) TritonX-100 in PBS and load shows the inclusion body pellet after the second triton-X100 wash, FT (flow through), W1-3 (Ni-NTA agarose column washes), E1-4 elutions. **(C)** Antibody testing showing signals for pre-immune serum (pre) and final bleed (FB), in mitochondrial samples from wild type (WT) and *mdm10-3HA* (HA) strains. **(D)** Comparison of final bleed serum (FB) and affinity purified (AF) Mdm10 antibody (1:1000 dilutions).

Recombinant expression in *E. coli* produced inclusion bodies that contained the proteins of interest. This material was purified by washing with 10 % (w/v) TritonX-100 in PBS. The inclusion bodies were then dissolved in 8 M urea buffer and the solubilised protein purified using Ni-NTA agarose (Qiagen) as per manufacturers' instructions. The samples were diluted into PBS and mixed with Freund's adjuvant and then used to immunise rabbits. Antibodies were tested in comparison with the

pre-immune bleed from the rabbit against purified protein and isolated mitochondria, and where possible against mitochondria from the deletion strain lacking the protein as a negative control.

2.6 Isolation of mitochondria

Mitochondria were isolated by differential centrifugation from cells grown to an OD600 2-7 and collected by centrifugation at 3000 xg for 10 min at room temperature. The cell pellet was resuspended in water then centrifuged at 3000 xg for 5 min at room temperature. Cell pellets were weighed and then resuspended in 0.1 M Tris-SO_4, pH 9.4 at a weight:volume ratio of around 1 g : 40 mL with DTT added give a 10 mM solution just prior to use. Cell suspensions were incubated with shaking at 120 rpm for 15 min at 30 °C then centrifuged at 6500 xg for 5 min at room temperature. Cells were washed in sorbitol buffer (1.2 M sorbitol, 20 mM KH_2PO_4/K_2HPO_4, pH 7.4) to remove DTT then centrifuged at 6500 xg for 5 min at room temperature. Cells were incubated in sorbitol buffer with 0.1 mg/g cells lyticase (Sigma, L2524) using 2 mL/g of wet cells for 1 h shaking at 100 rpm at 30 °C. Cells were collected by centrifugation at 6500 xg for 5 min at room temperature then resuspended in 4 °C breaking buffer ~3 mL/g wet cells (0.6 M sorbitol, 20 mM potassium 2-(*N*-morpholino)ethanesulfonate pH 6.0, 1 mM phenylmethylsulfonyl fluoride (PMSF) added just prior to use diluting a 100 mM stock made in ethanol). Cells were homogenised 12 times using a tight dounce and the homogenate was centrifuged at 10000 xg for 5 min at 4 °C. The supernatant containing mitochondria was kept on ice while the pellet was resuspended and the douncing and spin steps repeated. The combined supernatants were centrifuged at 10000 xg for 5 min at 4 °C to clear more of the contaminating membranes. This supernatant was then centrifuged at 36500 xg for 10 min at 4 °C to collect the mitochondria. The brown mitochondrial pellet was resuspended in a small amount of buffer (0.6 M sorbitol, 20 mM potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate pH 7.4). The mitochondrial concentration was determined by adding 10 µL of the resuspended mitochondria to 990 µL 0.6 % SDS and calculated from the absorbance measurement at 280 nm measured in a Quartz cuvette (A280/0.021=mitochondrial concentration in mg/mL). Mitochondria were then stored in aliquots at -80 °C.

2.7 Import of radiolabelled proteins into isolated mitochondria

One efficient and well-established technique used to investigate protein import pathways into mitochondria is the incubation of *in vitro* synthesised radiolabelled proteins with isolated mitochondria (Maccecchini, Rudin et al. 1979). The steps used in this process are described below.

2.7.1 *In vitro* transcription

The templates for *in vitro* transcription were linearised plasmids encoding *S. cerevisiae* or *C. albicans* proteins (Table 8.10) which had been purified by phenol-chloroform extraction (using phenol:chloroform:isoamylalcohol (25:24:1) pH 8 Tris), followed by ethanol precipitation. Reactions were performed in 50 µL using transcription buffer and DTT as per manufacturers instructions, 1.2 µL SUPERase-In (Ambion, AM2696) 0.2 mM rNTPs (without GTP), 0.5 units G-cap (m^7G(5')ppp(5')G) (Roche, 10904988001), 5 µg linear DNA, 2.5 µL SP6 polymerase (Promega, P108B) and incubated at 40 °C for 15 min before addition of 2.5 µL GTP (10 mM) and incubation for a further 90 min at 40 °C.

2.7.2 *In vitro* translation

Radiolabeled precursor proteins were translated *in vitro* in rabbit reticulocyte lysates (Promega, L4960) in the presence of radiolabelled ^{35}S-methionine/^{35}S-cysteine (Express 35S labeling mix, Perkin Elmer, #NEG072007MC) at 30 °C for 50 min. Reaction mix for 10 µL reticulocyte lysate includes 1 µL amino acid mix (1 mM each) 1 µL RNA prepared by *in vitro* translation, 1 µL ^{35}S-labelled amino acids (1 mM each) and 1µL RNAsin SUPERase-In (up to a maximum of 2.5 µL).

Some precursor proteins aggregate when the lysate is added to the import buffer. This can be particularly problematic when samples are analysed on SDS-PAGE as the aggregated protein can be protease resistant. To reduce this the synthesised precursor proteins can be pre-incubated in the import buffer and centrifuged to remove insoluble protein before addition of mitochondria to the assay (Thornton, Stroud et al. 2010).

2.7.3 Protein import method

Mitochondrial import assays were performed using 50 µg of mitochondria per time point in 100 µL of import buffer (0.6 M sorbitol, 50 mM HEPES, pH 7.4, 2 mM KPi (1 M KH_2PO_4 mixed with 1 M K_2HPO_4 to give pH 7.4), 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 10 mg/mL bovine serum albumin (BSA) and 1 mM DTT supplemented with 5 mM b-nicotinamide adenine dinucleotide (NADH, Sigma, N8129) and 4 mM ATP (Sigma, A7699) from a stock in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM potassium 3-(N-morpholino)propanesulfonate (K^+MOPS) pH 7.2).

When necessary, the membrane potential was dissipated by the addition of a 100x solution of antimycin (8 µM, Sigma, A8674), valinomycin (1 µM, Sigma, V0627), and oligomycin (20 µM, Sigma, O4876) to the import buffer. To remove ATP, mitochondria and precusor proteins were incubated in import buffer containing 10 Units/mL apyrase (10 min, 25 °C).

For samples to be analysed by SDS-PAGE 5 % v/v of reticulocyte lysate was used in the import reaction. For samples to be analysed by BN-PAGE 2.4 M sorbitol and water were added to reticulocyte lysate mix after translation to make a 0.6 M sorbitol solution and 15 % v/v of this mix was used for the import reaction. Samples were removed at indicated time points and left on ice until further processing.

2.7.4 Sample analysis

After the stated reaction time an aliquot of the import reaction was diluted into cold import buffer on ice. Where indicated, protease treatment included 100 µg/mL trypsin or 50 µg/mL proteinase K in the import buffer and samples were left on ice for 15-20 min before addition of protease inhibitors (1 mg/mL soya bean trypsin inhibitor or 1 mM PMSF). Samples were separated by SDS-PAGE or BN-PAGE as described in Section 2.3. Radioactivity was detected using a phosphorscreen (GE Healthcare) and phosphorimage analysis was carried out using a Typhoon TRIO variable mode imager (GE Healthcare).

2.8 Co-immunopurification

To address the protein-protein interactions within the *C. albicans* SAM complex I used the HA-tagged Sam37 strain co-immunopurification of the SAM complex. Using 300 µL of Protein A/G-Plus-Agarose beads (Santa Cruz) cross-linked to our in house HA antibody using dimethyl pimelimidate according to the manufacturers' instructions I attempted to co-purify the tagged Sam37 with its partner proteins from 1 mg of isolated mitochondria. Despite various optimisation attempts the efficiency of this method remained very low and I was only ever able to detect proteins by western blot. Sam37-3HA was co-purified with a very small amount of Sam50 that was not detected in the wild type control (Figure 2.5).

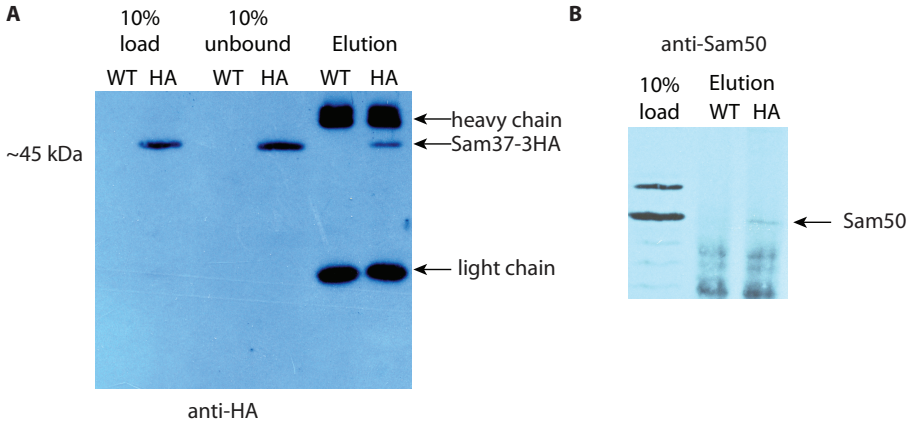


Figure 2.5 Co-immunopurification using *sam37-3HA* mitochondria
(**A**) Immunopurification of Sam37-3HA protein detected using HA antibodies showing 100 % of eluted material from mitochondria isolated from wild type (WT) and *sam37-3HA* (HA) *C. albicans* strains. Method as described above. (**B**) Co-immunopurification using the same conditions as (A) detected using antibodies against Sam50.

2.9 Microscopy

Mitochondria were visualised by fluorescence imaging using 1 µM MitoTracker Red CMXRos (Life Technologies, M7512) stock diluted into the growth media to give a final concentration of 0.1 µM and used as per the manufacturer's instructions. Images were taken using an Olympus BX60 fluorescence microscope

with the 100x objective and analysed using Spot Advanced Software (http://www.spotimaging.com/software/).

2.10 qPCR

RNA was extracted from liquid cultures of *C. albicans* using the hot acidic phenol method as described by Collart and Oliviero (2001). DNase treatment of RNA using TURBO DNase (Ambion) was performed according to the manufacturer's instructions and the samples were then chloroform-extracted using phase lock gel heavy tubes (5 PRIME). Treated RNA was precipitated with 10 µL of 3 M sodium acetate and 2 µL GlycoBlue (Invitrogen) before precipitation with 2.5x volume of 100 % ethanol, incubation at -20 °C for 15 min and centrifugation at 14,000 rpm for 15 min at 4 °C. The RNA pellet was then washed with 80 % ethanol before being dried.

The cDNA was made as follows: 5 µL 200 ng/µL RNA, 1 µL 50:50 mix random decamer dT20vn and 5.5 µL water were incubated at 65 °C for 10 min before being placed on ice. The following were then added in order: 4 µL 5x First Strand Buffer (Invitrogen), 0.5 µL RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), 2 µL 10 mM dNTPs, 1 µL 0.1 M DTT and 1 µL SuperScript III Reverse Transcriptase (Invitrogen). The cDNA synthesis was done using the following conditions: 42 °C (15 min), 47 °C (15 min), 52 °C (15 min), 80 °C (15 min), 4 °C indefinitely.

For qPCR, the FastStart Universal SYBR Green Master Rox (Roche) reagent mix was used. Reactions were prepared as follows: 10 µL SYBR Green, 2 µL 10 µM forward and reverse primers, and 10 ng cDNA to a total volume of 20 µL. The reaction was performed using the Eppendorf Realplex 4 Mastercycler with the following conditions: 95 °C (10 min); 95 °C (20 s), 60 °C (20 s), 72 °C (20 s); (40x cycles); 95°C (15 s); 60 °C (15 s); 95 °C (15 s); 12 °C indefinitely.

The qPCR primers were designed using Primer3 (v0.4.0, http://frodo.wi.mit.edu/) and are listed in Appendix 3: Table 8.8. Results were analysed using LinRegPCR v12.18 (Ruijter, Ramakers et al. 2009). Primers designed by this method failed to amplify SAM51 in qPCR conditions, however products were successfully amplified from cDNA using these primers in a PCR machine (T3000

Thermocycler, Biometra) using a longer extension time than is available in the qPCR machine.

2.11 Membrane potential measurements

The membrane potential of mitochondria were measured using the drop in fluorescence of the cationic dye TMRM (tetramethylrhodamine methyl ester, Invitrogen Molecular Probes) on addition of AVO cocktail to mitochondria. The AVO solution was made at 100x in ethanol and used at the following final concentrations: antimycin (8 μM), valinomycin (1 μM) and oligomycin (20 μM). The measurements were performed at 25 °C using a BMG Fluostar Optima spectrophotometer in a 96 well Lab Systems fluoro plate using 540 nm excitation and 590 nm emission. Mitochondria were isolated by differential centrifugation from the wild type (YCAT19) and *sam37ΔΔ* (YCAT248) strains grown in YPD. Mitochondria at 5 mg/mL (0.6 M sorbitol, 20 mM HEPES pH 7.4, 10 mg/ml BSA) were diluted to 1.67 mg/mL in buffer with succinate and malate (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, 10 mM succinate pH 7, 10 mM malate pH 7) then 25 μg of these mitochondria were added to 300 μL of potential buffer (0.6 M sorbitol, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.1 % (w/v) BSA, 20 mM KPi pH 7.2) containing TMRM (25 nM final concentration).

3 *C. albicans* mitochondrial import machinery

This chapter summarises the experiments used to establish and characterise *C. albicans* as a useful model organism for the study of mitochondrial protein import and biogenesis. Here I show that the main import processes and components are conserved between *S. cerevisiae* and *C. albicans* and note a few key differences, particularly in the import proteins into the intermembrane space (Section 3.2) (Hewitt, Heinz et al. 2012, Hewitt, Gabriel et al. 2014) and in the properties and components of the outer membrane assembly machinery (Section 3.3) (Hewitt, Heinz et al. 2012). These experiments facilitated development of hypotheses and more specific questions relating to the assembly of mitochondrial outer membrane proteins and complexes, which are explored in Chapters 4-6 of this thesis.

3.1 *C. albicans* mitochondrial functions and components

3.1.1 Identification of mitochondrial import components

The first step to establishing *C. albicans* as a model organism to study mitochondrial import machinery was to determine whether homologues of the well-studied *S. cerevisiae* proteins existed using BLAST searches in the *Candida* Genome Database (CGD, http://www.candidagenome.org) (Arnaud, Inglis et al. 2014). Table 8.11 identifies the *C. albicans* open reading frames of the closest *S. cerevisiae* homologues of all the proteins involved in mitochondrial protein import discussed in this thesis. The proteins are grouped according to their function and/or membership in a complex as identified in *S. cerevisiae*.

3.1.2 Metabolic cycling comparisons

The oscillation between glycolytic and respiratory growth phases complicates the study of mitochondrial biogenesis in *S. cerevisiae* as expression of many of the nuclear-encoded mitochondrial genes change during these cycles (Tu, Kudlicki et al. 2005). *Neurospora crassa,* another organism used in many early mitochondrial protein import studies (Hartl, Schmidt et al. 1986, Sollner, Griffiths et al. 1989, Neupert 1997), also regulates expression of a number of metabolic genes in a circadian rhythm (Correa, Lewis et al. 2003). These limitations hinder our ability to investigate the genetic and biochemical integration of metabolic control globally

across multiple cellular pathways (Rao, Schmidt et al. 2012). I wanted to determine if *C. albicans* could be used as a model to investigate such global metabolic regulation. In *C. albicans,* mitochondrial function is not repressed during optimal growth, but it was not known whether it undergoes metabolic cycling like *S. cerevisiae*. Such a model system would enable rapid progress in the study of the switching events involved in metabolic control, in a similar fashion to *N. crassa* and *S. cerevisiae* that were used to determine the key components of mitochondrial protein import (Herrmann and Neupert 2000) and *Schizosaccharomyces pombe* to understand the cell cycle (Nurse and Bissett 1981).

In collaboration with Dr. Traude Beilharz (Monash University, Department of Biochemistry and Molecular Biology) and Dr. Geoff Dumsday (Commonwealth Scientific and Industrial Research Organisation, Biotransformation section) I made use of a continuous culture system with oxygen consumption monitored to replicate the metabolic cycling results in *S. cerevisiae* seen by Tu and colleagues ((Tu, Kudlicki et al. 2005) Figure 3.1A). A repeating cycle of ~3.5 h with oscillations in dissolved oxygen saturation indicated the consumption of oxygen was changing as the cells switched between glycolytic to respiratory growth. Despite cells growing to a comparable density the oxygen consumption remained constant in the *C. albicans* cultures (Figure 3.1B) suggesting this wild type strain (DAY185) does not undergo metabolic cycling. This wild type strain is derived from BWP17, which is widely used in laboratories including to monitor virulence in mouse models of *C. albicans* infections (Davis, Edwards et al. 2000).

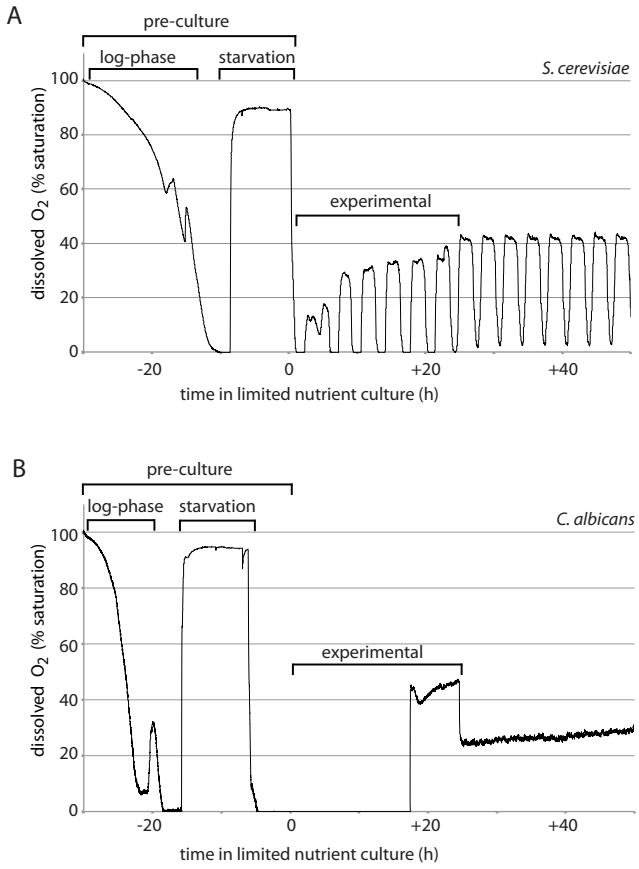


Figure 3.1 Continuous yeast cultures in nutrient limited conditions
Chemostat cultures of **(A)** *S. cerevisiae* CEN.PK and **(B)** *C. albicans* DAY185 showing oxygen concentration to indicate the oxygen usage of the cultures. Figure created by Victoria Hewitt for (Hewitt, Heinz et al. 2012) from data generated by Dr. Traude Beilharz (Monash University, Department of Biochemistry and Molecular Biology) and Dr. Geoff Dumsday (Commonwealth Scientific and Industrial Research Organisation, Biotransformation section).

3.1.3 Protein import into isolated mitochondria

To establish whether mitochondria isolated from *C. albicans* were functional I began by testing their import capacity using *S. cerevisiae* proteins. Such heterologous import reactions have been used previously to evaluate the conservation of import processes between species (Kiebler, Pfaller et al. 1990). Isolated mitochondria were incubated with ^{35}S radiolabelled proteins made by *in vitro* transcription and translation (Methods Section 2.7).

I tested a range of available precursor proteins with well-characterised import pathways in *S. cerevisiae* and analysed the results by SDS-PAGE. The 10 % lysate control represents 10 % of the total precursor protein added to an assay and therefore

allows the efficiency of the process to be evaluated (Figure 3.2A). The addition of protease degrades any protein aggregated on the surface of the mitochondria, giving a clearer indication of whether the proteins are properly imported (Figure 3.2). Aggregated proteins are not always accessible to protease and import assays may appear to show import of the protein but with no change over time, which is indistinguishable from rapid import. To distinguish between aggregation and rapid import, assays can be performed at lower temperatures. This slows the import process and makes it possible to detect increasing amounts of mature protein over time (Figure 3.2B).

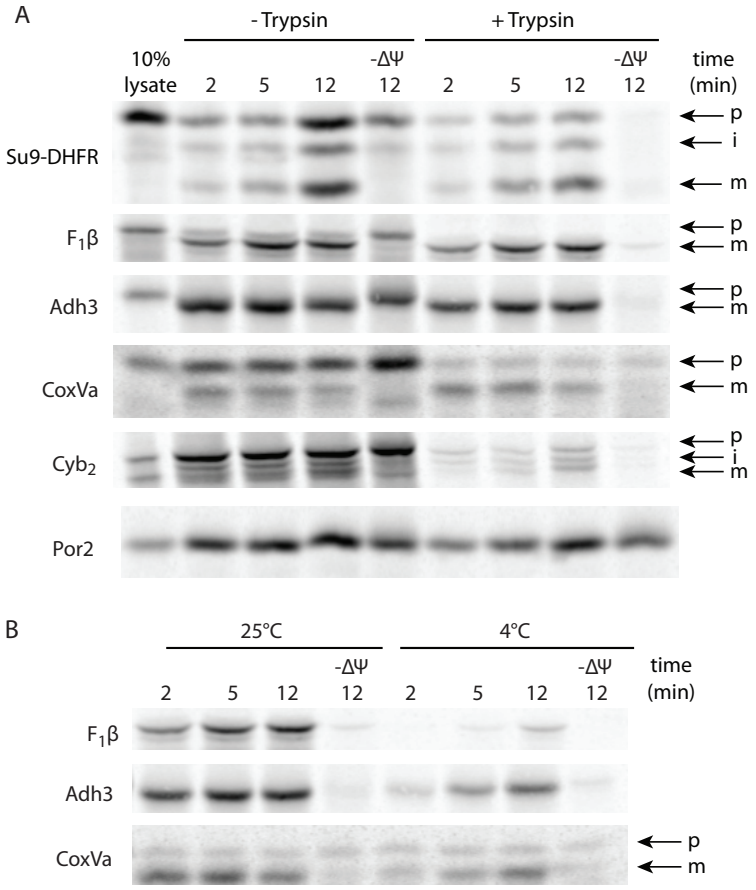


Figure 3.2 Testing mitochondrial protein import in *C. albicans*
(A) Mitochondria isolated from *C. albicans* were incubated with the radiolabelled su9-DHFR, $F_1\beta$, Adh3, CoxVa, Cyb_2 and Por2 precursor proteins at 25 °C and samples taken at the indicated time points and treated with trypsin where indicated. The mitochondrial membrane potential was disrupted before addition of precursor in the -ΔΨ lanes. 10 % lysate shows 10 % of the protein available in the import reaction. Where protein processing steps are visible the precursor (p), intermediate (i) and mature (m) forms are labelled. **(B)** Import reactions performed as described in (A) but at 25 °C and 4 °C and both treated with trypsin. Samples were separated by SDS-PAGE and detected by autoradiography.

The first protein I used to test the import capabilities of isolated *C. albicans* was the precursor protein Su9-DHFR, a fusion-protein originally synthesised to determine the role of the mitochondrial presequence in protein import (Pfanner, Muller et al. 1987). In *S. cerevisiae* Su9-DHFR is translocated across the inner and outer membranes where it undergoes two cleavage steps catalysed by the mitochondrial processing peptidase (Schmidt and Neupert 1984, Pfanner, Tropschug et al. 1987, Pollock, Hartl et al. 1988). When incubated with mitochondria from *C. albicans* the same steps are observed, the precursor (Figure 3.2A p), intermediate (Figure 3.2A i) and mature (Figure 3.2A m) forms of su9-DHFR were clearly distinguishable, with the intermediate and mature forms being protected from trypsin by the intact membranes (Figure 3.2A + Trypsin lanes). The mature form was not produced when the membrane potential (-ΔΨ) had been dissipated (Figure 3.2).

In agreement with the literature (Gartner, Voos et al. 1995), I saw a similar membrane potential–dependent import but with a single protease processing step for the other matrix proteins $F_1\beta$ (Atp2, (Maccecchini, Rudin et al. 1979)), Adh3 (Pilgrim and Young 1987) and CoxVa (Cox5, (Miller and Cumsky 1991)). These proteins were very rapidly imported at 25 °C, but by performing the import at 4 °C I was able to show a time-dependent import reaction (Figure 3.2B). These experiments show that mitochondria isolated from *C. albicans* are robust and import proteins across the outer and inner membranes in a time- and membrane potential-dependent manner, similar to the import processes observed in *S. cerevisiae* (Lithgow and Schatz 1995).

Next I compared the relative import efficiency of these proteins into an equal amount of mitochondria isolated from either *C. albicans* or *S. cerevisiae.* The extent of import into mitochondria from *C. albicans* was similar or better for $F_1\beta$, Adh3 and CoxVa, but Cyb2 was imported and processed much less efficiently in mitochondria from *C. albicans* (Figure 3.3). A large proportion of this intermembrane space protein associated with the mitochondria but very little was processed into its mature form (Figure 3.2A). In *S. cerevisiae*, this protein is imported via the stop-transfer pathway (Glick, Brandt et al. 1992), so I tested the ability of the *C. albicans* mitochondria to import Cyt1 (Cytc), another protein that follows the stop-transfer pathway. Figure 3.3 shows Cyt1 also had reduced import efficiency, with significantly less intermediate

and mature forms detected. The implications of these differences are discussed and investigated further in Section 3.2.

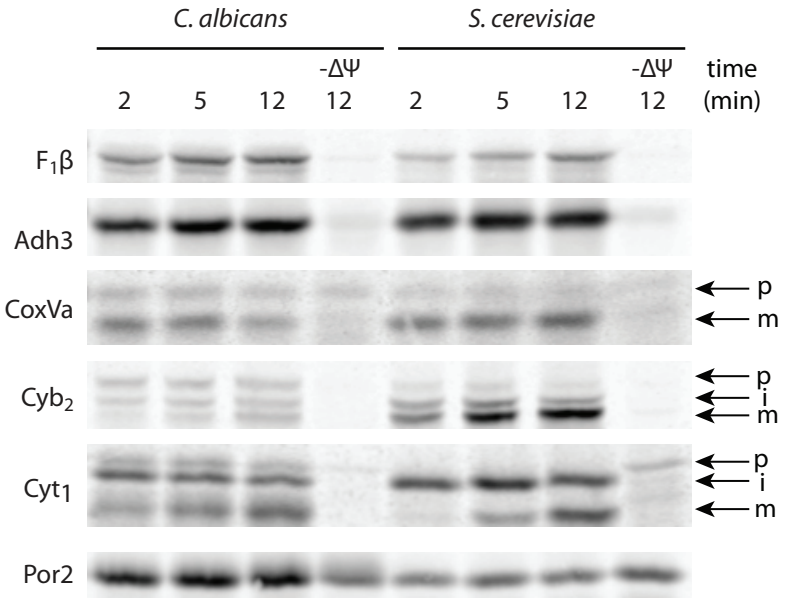


Figure 3.3 Import into *S. cerevisiae* mitochondria compared to *C. albicans*
Mitochondria isolated from *C. albicans* or *S. cerevisiae* were incubated with radiolabelled F1β, Adh3, CoxVa, Cyb2, Cyt1, and Por2 precursor proteins at 25 °C and samples were taken at the indicated time points and treated with trypsin. The mitochondrial membrane potential was disrupted before addition of precursor in the -ΔΨ lanes. Samples were separated by SDS-PAGE and detected by autoradiography. Where protein processing steps are visible the precursor (p), intermediate (i) and mature (m) forms are labelled.

3.2 Stop-transfer intermembrane space import pathway

The significant differences in the efficiency of import and processing of the intermembrane space proteins, Cyb2 and Cyt1, prompted a more detailed investigation of these proteins and their import pathway in *C. albicans*. In *S. cerevisiae* these proteins are both imported through a complicated series of transfer and processing steps called the stop-transfer pathway (Glick, Brandt et al. 1992).

3.2.1 Comparison of *S. cerevisiae* and *C. albicans* Cyb2 proteins

The *S. cerevisiae* Cyb2 (ScCyb2) precursor contains a two-part signal sequence used to target it to the intermembrane space (Glick, Brandt et al. 1992). I aligned the *C. albicans* Cyb2 (CaCyb2) sequence with Cyb2 from other yeast species (Figure 3.4A). This shows it lacks a significant portion of the two-part targeting sequence found in ScCyb2, so must be targeted to the mitochondria via another pathway. Similarly, CaCyt1 lacks a large segment from within the N-terminal stop-transfer sorting sequence found on ScCyt1 (Figure 3.4B).

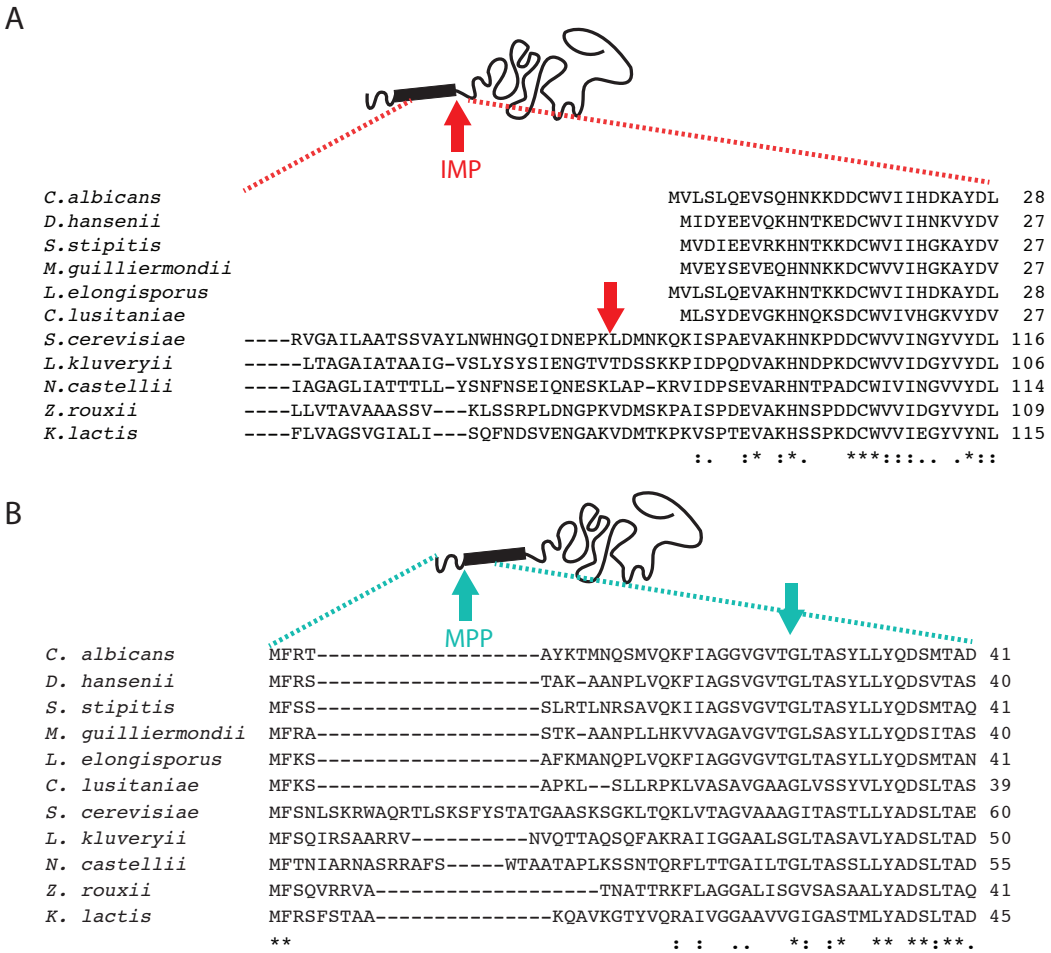


Figure 3.4 Sequence alignments of the N-terminal regions of Cyb2 and Cyt1
(A) Cyb2 sequences of the organisms listed below with the processing site that generates the mature form of ScCyb2 shown between residues L84 and D85, four residues upstream from Q89. **(B)** Cyt1 sequences from the organisms listed below. Sequences from *C. albicans*, *Debaryomyces hansenii* CBS767, *Scheffersomyces stipitis* CBS 6054, *Meyerozyma guilliermondii* ATCC 6260, *Lodderomyces elongisporus* NRRL YB-4239, *Clavispora lusitaniae* ATCC 42720, *S. cerevisiae*, *Lachancae kluveryii, Naumovozyma castellii* CBS 4309, *Zygosaccharomyces rouxii* and *Kluyveromyces lactis* NRRL Y-1140. Sequence identity is indicated by asterisks and sequence similarity by semicolons or dots respectively.

3.2.2 Growth complementation by CaCyb2 and ScCyb2

To determine what import pathway was being used to get CaCyb2 into the intermembrane space I wanted to use previously characterized *S. cerevisiae* mutant strains. I began by checking if CaCyb2 could be imported into mitochondria isolated from *S. cerevisiae* and if CaCyb2 could complement for the loss of ScCyb2. Growth of *S. cerevisiae* on minimal synthetic media with L-lactate as a carbon source requires Cyb2 activity (Guiard 1985). While the expression of ScCyb2 from a high copy number vector was able to complement growth of *cyb2Δ* mutants of *S. cerevisiae* for growth on L-lactate, the open reading frame encoding CaCyb2 cloned from genomic DNA in the same vector did not (Hewitt, Heinz et al. 2012). A codon-optimized version of CaCyb2 was also unable to restore the growth on L-lactate in the *S. cerevisiae* YPH499 strain but did complement growth in the *S. cerevisiae* BY4741 background (Figure 3.5A & C) despite comparable levels of protein expression in each strain (Figure 3.5B & D). Processed ScCyb2 (~58 kDa predicted size) and CaCyb2 (~63 kDa predicted size) can be detected at the expected positions by western blotting. I hypothesized CaCyb2 could not support growth of the *cyb2Δ* mutant on L-lactate in the YPD499 strain due to inefficient import in this strain.

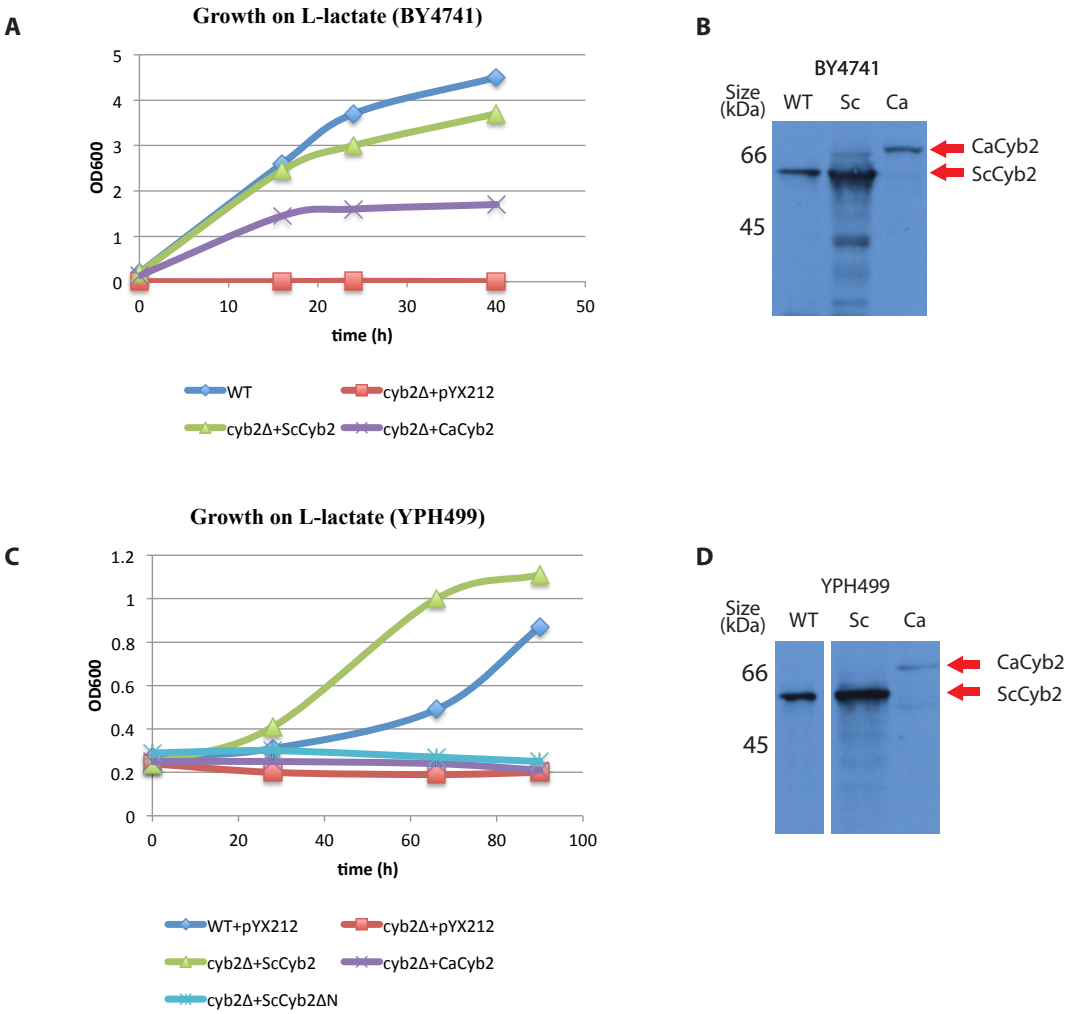


Figure 3.5 Growth of Cyb2 deletion strains on L-lactate
(A) Growth of *S. cerevisiae* BY4741 wild type compared to Δ*cyb2* strain made in the BY4741 background with and without vectors encoding ScCyb2 or CaCyb2. Empty vector control pYX212. **(B)** Western blot showing Cyb2 levels in strains shown in (A). **(C)** Growth of *S. cerevisiae* YPH499 wild type compared to Δ*cyb2* strain made in the YPH499 background with and without vectors encoding ScCyb2, CaCyb2 or ScCyb2 with an N-terminal truncation (ScCyb2ΔN). **(D)** Western blots showing Cyb2 levels in strains shown in (C) (lane excised for clarity but blot was performed all on the same membrane).

3.2.3 Mia40 and intermembrane space protein import

The other major facilitator of protein import into the intermembrane space is the MIA pathway (Chacinska, Pfannschmidt et al. 2004). To investigate whether Cyb2 and Cyt1 might use this pathway I obtained *S. cerevisiae* mutants in the MIA pathway from the Pfanner lab (Institute for Biochemistry and Molecular Biology, University of Freiburg (Chacinska, Pfannschmidt et al. 2004)). The *mia40-3* strain, which has a defect in binding precursor proteins to Mia40 (Chacinska, Pfannschmidt et al. 2004), behaved as expected; with no change in su9-DHFR import efficiency but a defect in Tim9 import. I also found the import and processing of both ScCyb2 and ScCyt1 were impaired when radiolabelled precursors were incubated with the *mia40-3* mutant strain, a result that has not been reported in the literature. I performed import assays using CaCyb2 and but could find no import conditions where the translated protein did not aggregate.

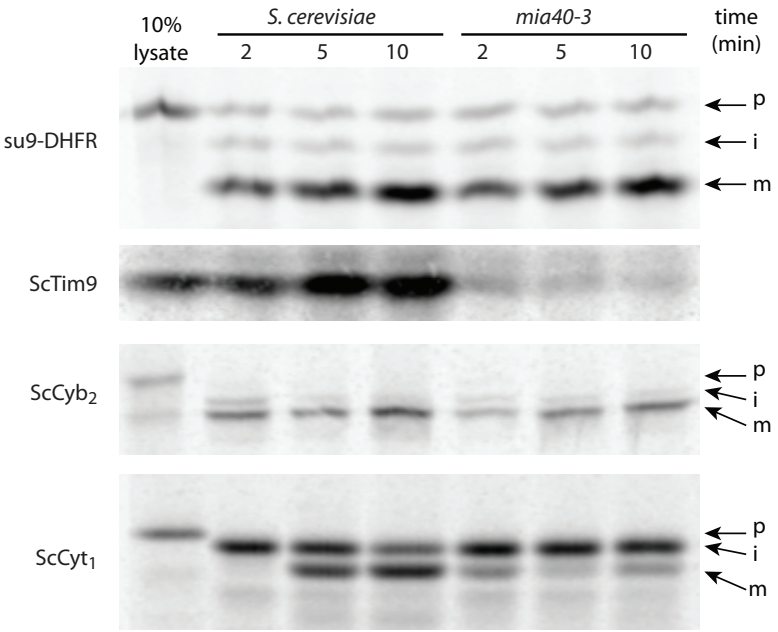


Figure 3.6 Protein import in the *S. cerevisiae mia40-3* strain
Mitochondria isolated from *S. cerevisiae* wild type and *mia40-3* mutant strains were incubated with radiolabelled su9-DHFR, Tim9, Cyb2 and Cyt1 precursor proteins at 25 °C and samples were taken at the indicated time points and treated with trypsin. The 10 % lysate lane shows 10 % of the total protein available in the import reaction. Samples were separated by SDS-PAGE and detected by autoradiography. Where protein processing steps are visible the precursor (p), intermediate (i) and mature (m) forms are labelled.

3.3 Beta barrel protein assembly in *C. albicans* mitochondria

Mitochondrial proteins assembled into the outer membrane do not have cleavable presequences (Chacinska, Koehler et al. 2009). Since there is no change in size to confirm the import and processing of the precursor, it can be difficult to distinguish between import and the aggregation of proteins on the surface of the mitochondria. This is because the processes look the same when separated by SDS-PAGE and analyzed using autoradiography to detect proteins. Analysis of samples using BN-PAGE and autoradiography can be used instead to monitor the assembly of precursor proteins into complexes providing more information than simple import assays. The detection sensitivity of the radioactive signal means that this technique can sometimes even identify intermediate complexes formed in very small amounts during the assembly process.

There are two classes of outer membrane proteins: (1) proteins with alpha helical transmembrane segments that anchor them in the membrane, and (2) beta barrel proteins made of multiple beta strands embedded in the membrane to form a barrel-like structure (Chacinska, Koehler et al. 2009). In collaboration with Dr Miguel Shingu-Vazquez, I examined insertion of some tail-anchored proteins in *C. albicans* mitochondria but our studies were thwarted by aggregation of the radiolabelled precursor proteins as found by previous researchers (Thornton, Stroud et al. 2010). Thus while the import of some alpha-helical protein components of the TOM complex are discussed in Chapter 5, here I focus primarily on the assembly of the more experimentally tractable beta barrel proteins. The main protein components of the beta barrel assembly machinery in mitochondria have been identified (Gratzer, Lithgow et al. 1995, Kozjak, Wiedemann et al. 2003, Meisinger, Rissler et al. 2004, Milenkovic, Kozjak et al. 2004), but the mechanisms of this assembly pathway are still under investigation (Stroud, Becker et al. 2011).

Porin is a major component of the mitochondrial outer membrane and the first predicted mitochondrial beta barrel protein (Forte, Guy et al. 1987). As the core of the TOM complex, Tom40 is the other key beta barrel protein of the mitochondrial outer membrane. I therefore began my studies of beta barrel protein assembly with a comparison of the import and assembly of these two proteins in mitochondria isolated

from *S. cerevisiae* and *C. albicans* using radiolabelled time course experiments analysed by SDS-PAGE and BN-PAGE.

3.3.1 A stable porin trimer in *C. albicans*

The *C. albicans* genome encodes only one porin (CaPor1, orf19.1042) in contrast to *S. cerevisiae*, which contains two paralogues (ScPor1 and ScPor2 (Colombini 1979, Forte, Blachly-Dyson et al. 1996)). The import of ScPor2 into mitochondria isolated from *C. albicans* and monitored by SDS-PAGE appeared slightly faster than mitochondria isolated from *S. cerevisiae* (Figure 3.3). Although in *S. cerevisiae,* porin is largely protease inaccessible (Figure 3.2A), this assay cannot distinguish between protease inaccessible aggregates and assembled proteins that are protected by the membrane.

When monitored using BN-PAGE, porin is inserted into the outer membrane of mitochondria that were isolated from *S. cerevisiae* in a time-dependent manner and various, still not fully-characterised, oligomers form a ladder of different sized complexes (~440 kDa, ~400 kDa, ~200 kDa, ~100 kDa and ~70 kDa) (Figure 3.7A) (Krimmer, Rapaport et al. 2001, Hewitt, Heinz et al. 2012). In contrast, when ScPor2 was incubated with mitochondria from *C. albicans,* only minor amounts of the smaller forms were detected, with the majority of the protein in a single stable oligomer that migrated at ~440 kDa (Figure 3.7A) (Hewitt, Heinz et al. 2012).

While the SAM complex is required for the efficient import of porin (Kozjak, Wiedemann et al. 2003), in *S. cerevisiae* porin has only been detected bound to the SAM complex when the beta signal of porin is mutated (Kutik, Stojanovski et al. 2008). This intermediate was identified by antibody shift experiments where antibodies to SAM components are added to solubilised mitochondria after import. The antibodies bind to the radiolabelled complexes that contain the epitopes they recognise and form larger complexes that migrate more slowly in the gel. In *N. crassa* the ~240 kDa complex formed during porin import is the SAM intermediate (Hoppins, Go et al. 2007). In agreement with previous work in *S. cerevisiae* (Kutik, Stojanovski et al. 2008), antibody shifts using Sam50 antibodies show none of these complexes are porin assembly intermediates in which the imported protein is associated with the SAM complex (Figure 3.7B).

I wanted to perform *in vitro* import experiments using CaPor1, however, several attempts using various methods (outlined in the Methods Section 2.2.7) were ultimately unsuccessful.

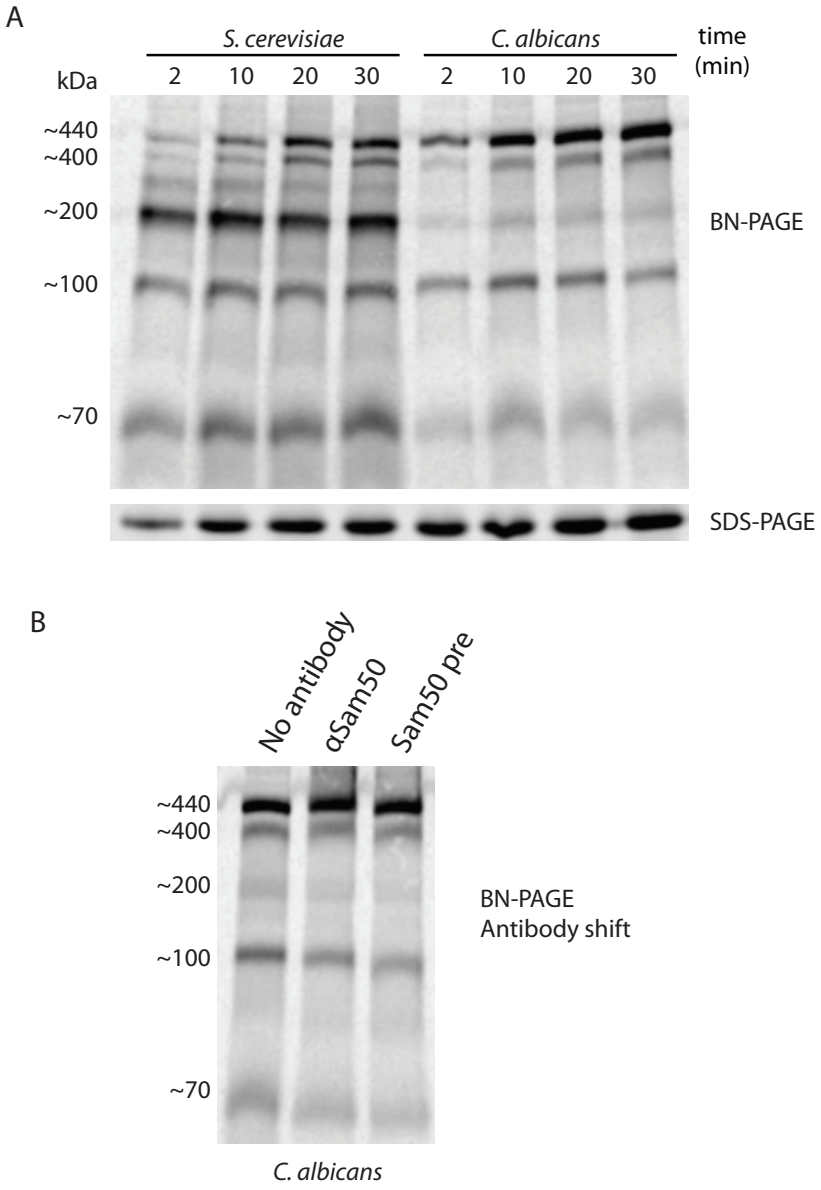


Figure 3.7 ScPor2 assembly assays in *C. albicans* vs. *S. cerevisiae* mitochondria
(A) Mitochondria isolated from *C. albicans* or *S. cerevisiae* were incubated with ScPor2 at 25 °C and samples taken at the indicated time points. Samples were separated by SDS-PAGE or BN-PAGE as indicated and detected by autoradiography. Samples separated by SDS-PAGE were treated with trypsin directly after import. **(B)** Mitochondria isolated from *C. albicans* were incubated with ScPor2 for 20 min before being solubilised in lysis buffer containing digitonin. The sample was then divided in three and one sample was left untreated, another incubated with Sam50 antibody and the third incubated with the Sam50 pre-immune serum. Complexes were then separated by BN-PAGE and detected by autoradiography.

3.3.2 Role of ATP in porin import

The robust assembly of porin oligomers in mitochondria isolated from *C. albicans* enabled me to address the role of ATP in beta barrel protein assembly. While apyrase treatment to remove ATP from import reactions was shown to greatly diminish import (Kleene, Pfanner et al. 1987), it has been unclear which step in the import and assembly pathway requires this ATP. To address this point, apyrase was used to remove ATP from the mitochondria and the translation reactions. Import of porin was measured by assessing its resistance to trypsin proteolysis over time (lower panels, Figure 3.8). Assembly of porin oligomers assessed by BN-PAGE and autoradiography showed porin assembled slower in mitochondria that are depleted of ATP from both *S. cerevisiae* and *C. albicans* (Figure 3.8).

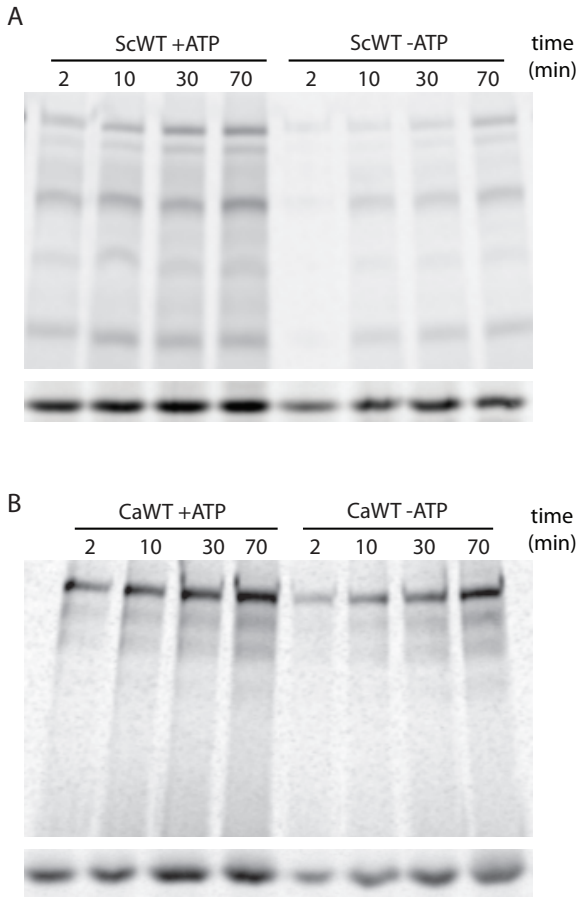


Figure 3.8 ATP dependence of porin import
Mitochondria isolated from **(A)** *S. cerevisiae* or **(B)** *C. albicans* were incubated with ScPor2 at 25 °C and samples taken at the indicated time points. Complexes were separated by BN-PAGE (upper panels) or SDS-PAGE (lower panels) and detected by autoradiography. Apyrase was used to remove ATP from the isolated mitochondria and the translation reactions in the indicated time courses.

3.3.3 TOM complex assembly is accelerated *C. albicans* mitochondria

Using mitochondria isolated from *S. cerevisiae*, the TOM complex assembles via two intermediates into a mature oligomer (Model, Meisinger et al. 2001, Chan and Lithgow 2008). I found mitochondria from *C. albicans* could assemble ScTom40 into the mature TOM complex much more rapidly (Figure 3.9).

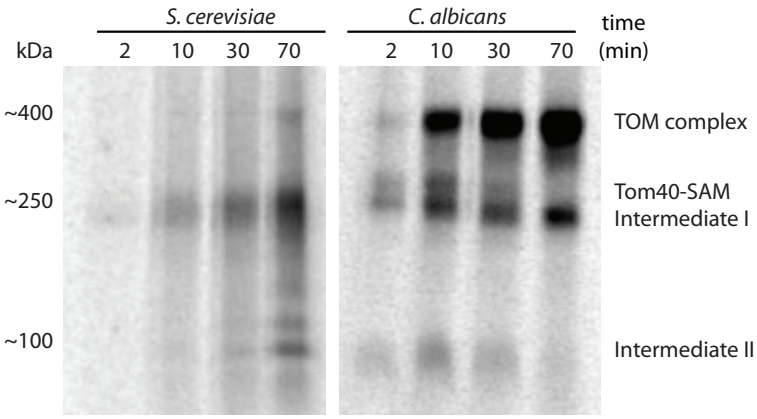


Figure 3.9 ScTom40 assembly assays
Mitochondria isolated from *C. albicans* or *S. cerevisiae* were incubated with ^{35}S-ScTom40 at 25 °C and samples taken at the indicated time points. Complexes were separated by BN-PAGE and detected by autoradiography.

When mitochondria from *C. albicans* were incubated with ^{35}S-ScTom40 a ~250 kDa complex formed within two minutes. This complex represents Tom40 bound in the SAM complex and is known as Intermediate I. The imported Tom40 was rapidly transferred into a mature ~400 kDa TOM complex within 10 minutes. Very little of the smaller intermediate form (Intermediate II), which represents Tom40 in contact with one or more of the small TOMs (Wiedemann, Kozjak et al. 2003), is detected in this reaction suggesting the other TOM complex components rapidly dock with the newly imported Tom40.

To confirm that this difference in import efficiency was not due to using ScTom40 rather than the *C. albicans* protein, I cloned a construct for *in vitro* production of CaTom40 (See Methods Section 2.2.7). Importing CaTom40 into mitochondria from *C. albicans* produced a similar import profile but with a more defined SAM-sized import intermediate (Figure 3.10A). To confirm this was the same SAM intermediate seen in import of ScTom40 I performed an antibody shift using antibodies against Sam50, which selectively shifted only the ~250 kDa SAM

intermediate (Intermediate I) (Figure 3.10B). To confirm the presence of Sam37 in this intermediate, I constructed a *C. albicans* strain with a triple HA tag at the C-terminus on both copies of the gene (See Methods Section 2.2.2). The import profile of Tom40 into mitochondria from this strain was the same as for *C. albicans* wild type mitochondria, and addition of HA antibody shifted both the ScTom40 and CaTom40 complexes at ~250 kDa, thus confirming the presence of Sam37 in this intermediate (Figure 3.10C).

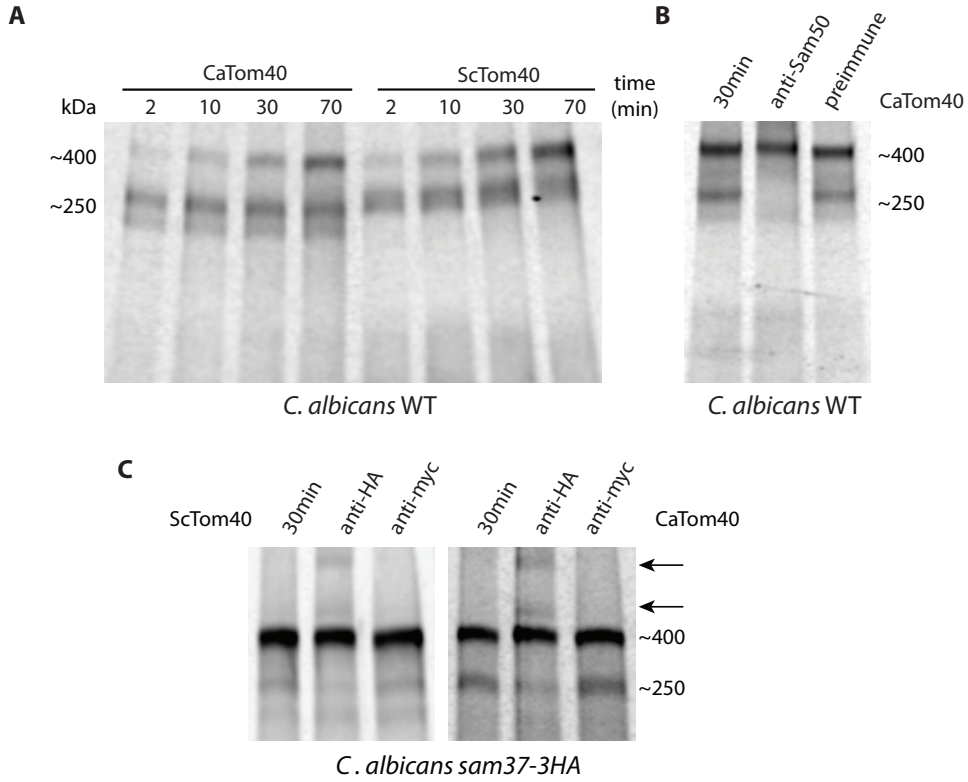


Figure 3.10 ScTom40 and CaTom40 assembly in *C. albicans* mitochondria
(A) Mitochondria isolated from wild type *C. albicans* were incubated with ^{35}S-ScTom40 or ^{35}S-CaTom40 at 25 °C with samples taken at the indicated times, separated by BN-PAGE and detected by autoradiography. (**B)** Mitochondria isolated from wild type *C. albicans* were incubated with ^{35}S-CaTom40 at 25 °C for 30 min, solubilised in lysis buffer containing digitonin and then incubated with antibodies against CaSam50 or the corresponding pre-immune serum, before separation by BN-PAGE and detection by autoradiography. (**C)** Mitochondria isolated from a *C. albicans sam37-3HA* strain were incubated with ^{35}S-ScTom40 or ^{35}S-CaTom40 at 25 °C for 30 min, solubilised in lysis buffer containing digitonin and incubated with antibodies against the HA tag or myc tag. Complexes where then separated by BN-PAGE and detected by autoradiography. Arrows indicate bands shifted due to antibody binding.

3.3.4 Heterologous Tom40 assembly

Out of curiosity and for the sake of completeness, I performed the converse heterologous import assays, incubating mitochondria from *S. cerevisiae* with CaTom40. To my surprise the result was strikingly different, with no CaTom40 assembled into the mature TOM complex under any conditions (Figure 3.11). Considerably more CaTom40 accumulated in the ~100 kDa intermediate than is usually seen in Tom40 assembly and a ladder of intermediate sized complexes can be seen between the ~100 kDa and ~250 kDa bands.

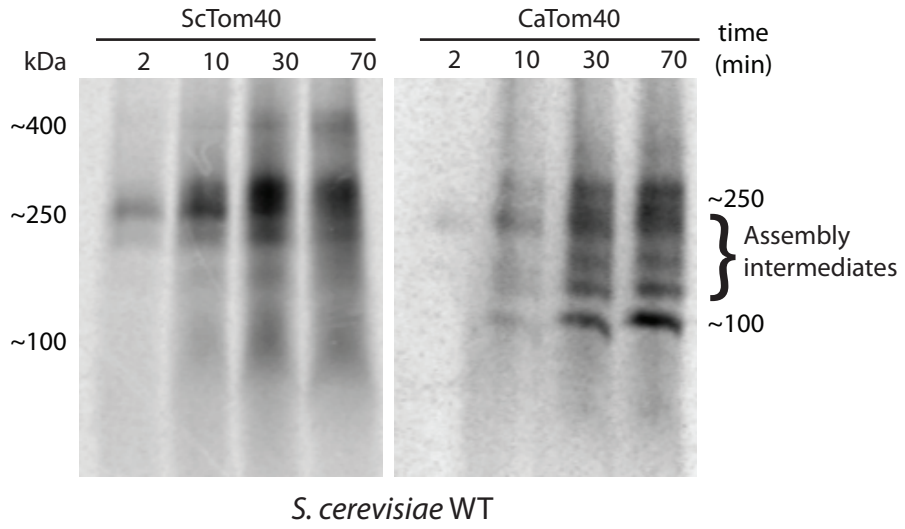


Figure 3.11 ScTom40 and CaTom40 import into *S. cerevisiae* mitochondria
^{35}S-ScTom40 or ^{35}S-CaTom40 were incubated with mitochondria isolated from *S. cerevisiae* at 25 °C with samples taken at the indicated times and separated by BN-PAGE before detection using autoradiography.

To identify the other components of these intermediate sized complexes I made use of previously constructed yeast strains from the Lithgow Lab collection to produce mitochondria with tags on potential partner proteins (See Methods Section 2.2.5). Mitochondria from the *S. cerevisiae tom40-3HA* strain produced the same import profile as mitochondria from wild type *S. cerevisiae* when incubated with CaTom40 (Figure 3.12A). The addition of HA antibody did not shift any of the intermediate sized bands suggesting CaTom40 is not associated with endogenous Tom40 in any of these complexes (Figure 3.12B). Mitochondria from the *S. cerevisiae sam35-3HA* strain were treated in the same way and clearly show the rapid association of CaTom40 with ScSam35 in the ~250 kDa SAM complex intermediate

(Figure 3.12). These mitochondria all failed to produce the ladder of intermediates observed in the import of CaTom40 into mitochondria from wild type *S. cerevisiae* (Figure 3.12C). This may be due to the HA tag interfering with the release of substrate from the SAM complex.

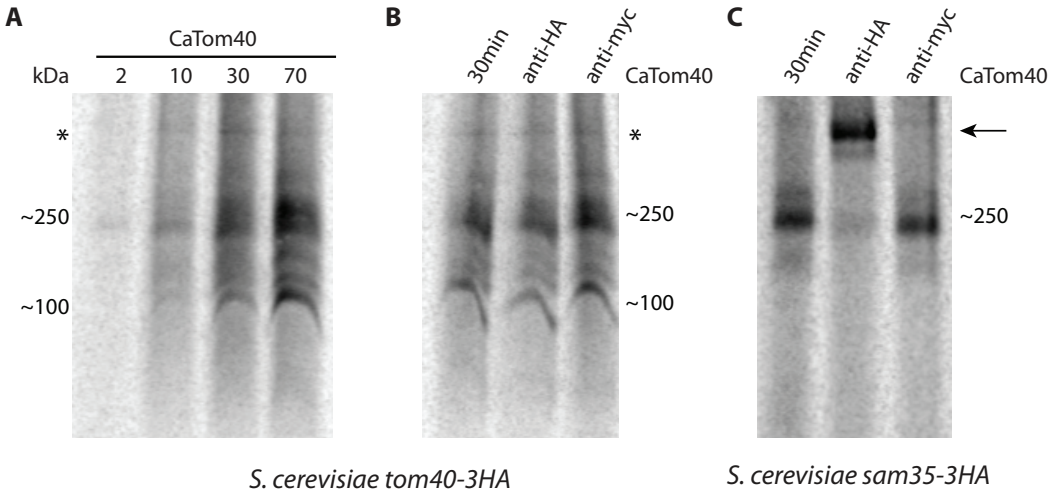


Figure 3.12 Antibody shifts designed to identify tagged components in trapped CaTom40 assembly intermediates
Mitochondria isolated from a *S. cerevisiae tom40-3HA* strain were incubated with ^{35}S-CaTom40 at 25 °C. **(A)** Samples were taken at the indicated times and separated by BN-PAGE before detection using autoradiography. **(B)** Samples incubated for 30 min were solubilised in lysis buffer containing digitonin and incubated with antibodies against HA or myc tags before the complexes were separated by BN-PAGE and detected using autoradiography. **(C)** As for (B) except using mitochondria isolated from a *S. cerevisiae sam35-3HA* strain. Arrow indicates band shifted due to antibody binding. Asterisks denote non-specific bands.

To identify the block in the Tom40 assembly pathway, I tested whether expressing high levels of components of the TOM complex or its assembly factors could rescue the assembly process. High copy number plasmids with strong promoters expressing Tom20, Mim1 or Tom22 were transformed into *S. cerevisiae* and these transformants were used to create mitochondria with increased levels of these proteins (See Methods Section 2.2.5). Again, these mitochondria failed to produce the ladder of intermediates observed in the import of CaTom40 into mitochondria from wild type *S. cerevisiae* (Figure 3.13).

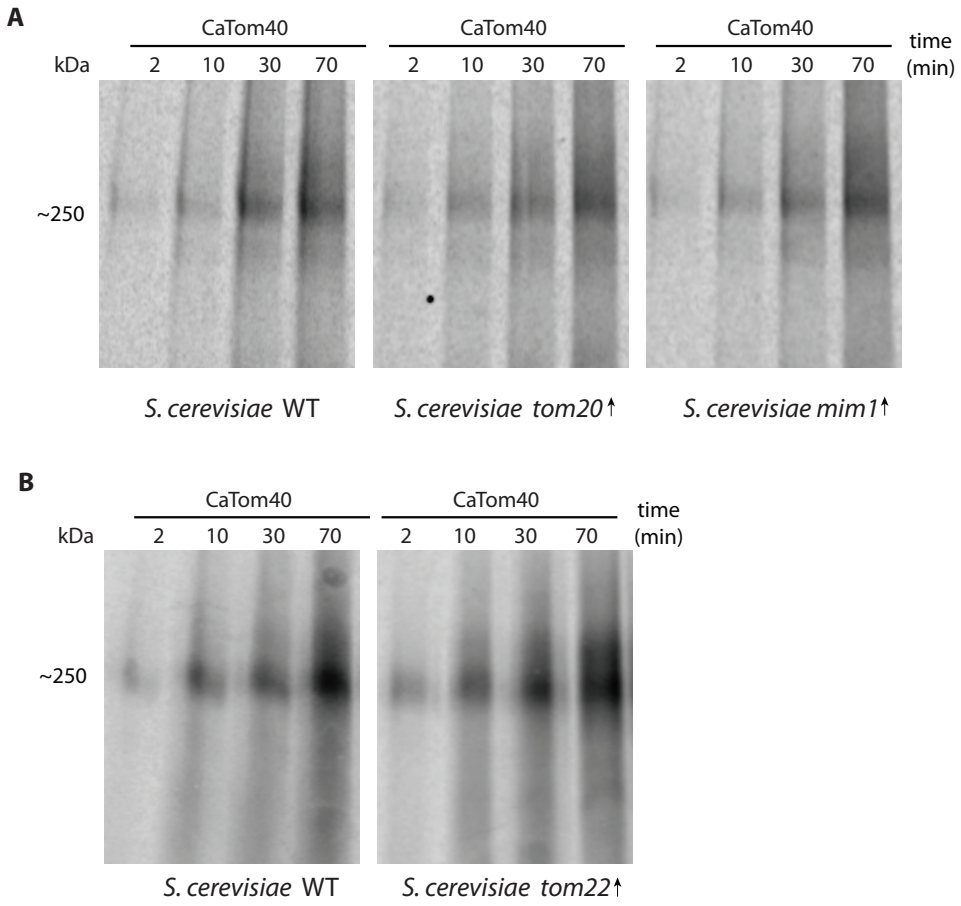


Figure 3.13 Import of CaTom40 into *S. cerevisiae* mitochondria with high levels of Tom20, Mim1 or Tom22
(A) Mitochondria isolated from *S. cerevisiae* strains producing high levels of Tom20 (*tom20*↑), and Mim1 (*mim1*↑) and their corresponding wild type (WT) strain were incubated with ^{35}S-CaTom40 for the indicated times. **(B)** Mitochondria isolated from an *S. cerevisiae* strain producing high levels of Tom22 (*tom22*↑) and its corresponding wild type strain were incubated with ^{35}S-CaTom40 for the indicated times. The complexes were separated by BN-PAGE and detected using autoradiography.

Characterising the subunits of these intermediate complexes by isolation of mitochondria from various strains and optimising import into mitochondria from each strain is extremely labour intensive. As an alternative I devised more direct methods to address the composition of these complexes in mitochondria from wild type *S. cerevisiae*. Previous work has shown small Tom proteins assembling into intermediate sized complexes (Model, Meisinger et al. 2001, Thornton, Stroud et al. 2010) similar to those seen in the trapped CaTom40 import assay. By repeating these small Tom import assays I was able to detect a similar ladder of intermediates (Figure 3.14), but unfortunately there were no differences between the import of Tom5, Tom6

or Tom7 at the resolution of the assay that could help identify the composition of the intermediate complexes.

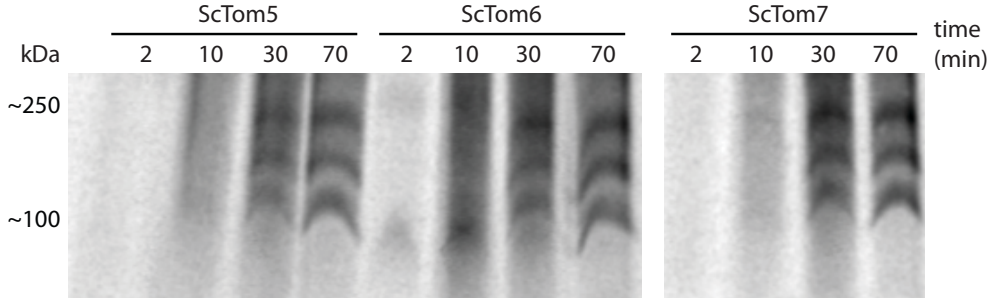


Figure 3.14 Small Tom protein assembly in *S. cerevisiae* mitochondria
Mitochondria isolated from *S. cerevisiae* were incubated with ^{35}S-ScTom5, ^{35}S-ScTom6 or ^{35}S-ScTom7 for the indicated times. The complexes were separated by BN-PAGE and detected using autoradiography.

Rather than attempting further optimisation of import conditions to tease out small import differences I decided to use antibodies against specific *S. cerevisiae* mitochondrial components which, if the epitope was present, would shift specific bands in the assembly assay as described in previous sections. Despite trying a range of antibodies against a number of TOM complex components, there was no shift in any of the complexes formed during CaTom40 import into *S. cerevisiae* wild type mitochondria (Figure 3.15).

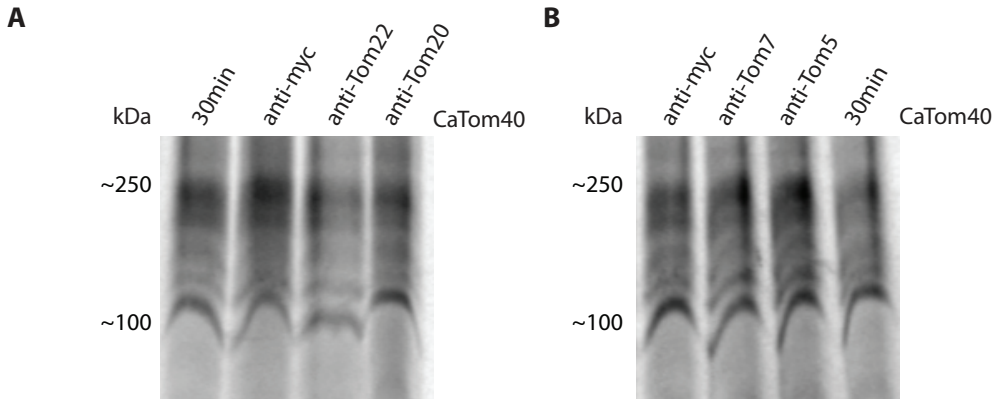


Figure 3.15 Antibody shifts designed to identify other TOM components in trapped CaTom40 assembly intermediates
Mitochondria were isolated from *S. cerevisiae* and were incubated with ^{35}S-CaTom40 at 25 °C for 30 min, solubilised in lysis buffer containing digitonin. The complexes were separated by BN-PAGE and detected using autoradiography. **(A)** Samples were incubated with antibodies against Tom20 or Tom22 or with no antibody or with antibody recognising the myc tag. **(B)** Samples were incubated with antibodies against Tom5 or Tom7 or with no antibody or with antibody recognising the myc tag.

3.4 Discussion

This chapter demonstrates that mitochondria from *C. albicans* have similar protein import machinery to *S. cerevisiae* mitochondria (Section 3.1.1), and that they can be efficiently harvested and used for import assays in a similar fashion to those described for the well-established model system *S. cerevisiae* (Section 3.1.3). This section will discuss the key differences I identified between *C. albicans* and *S. cerevisiae* genes encoding the protein transport machinery (Section 3.4.1, (Hewitt, Heinz et al. 2012)), the implications of my results investigating the import of Cyb2 and the *C. albicans* stop-transfer pathway (Section 3.4.2, (Hewitt, Gabriel et al. 2014)), and my observations of beta barrel protein import and TOM complex assembly in *C. albicans* (Section 3.4.3, (Hewitt, Heinz et al. 2012)). It will also include the hypotheses that lead to the experiments performed in the remaining chapters of this thesis.

3.4.1 Protein import into mitochondria from *C. albicans*

The components of the protein import pathway as discovered in *S. cerevisiae* are highly conserved in *C. albicans* and BLAST searches are sufficient to identify candidate homologues in most cases (Table 8.11). Proteins of overall similar size and domain structures were detected. Even such a simple comparison can be informative, for example Mdj2 was thought to be a result of the ancient whole genome duplication (WGD) in the *Saccharomyceateae* (Wolfe and Shields 1997), and of uncertain importance (Westermann and Neupert 1997, Hayashi, Schilke et al. 2011). The presence of both Mdj2 and Pam18 in *C. albicans* (Table 8.11), which diverged from the *Saccharomyceateae* before the WDG event (Fitzpatrick, Logue et al. 2006), suggests a more fundamental role of Mdj2 and an alternate evolutionary history. In contrast, finding just two Tim18/Sdh4 isoforms in *C. albicans* confirms the origin of Sdh4 in the WGD event (Gebert, Gebert et al. 2011).

In some cases hidden Markov models (HMM) proved essential for identification of homologues: for example, an open-reading frame encoding a Tom5-related sequence was previously unidentified and unannotated due to its small size coupled to sequence divergence. I also discovered an additional Sam50-related

protein in *C. albicans* with no homolog in *S. cerevisiae* using a HMM search (See Section 4.5 for further characterisation).

By comparing the sequences of the imported proteins and pathways used to assemble them into mitochondria in different organisms we can gain new insight into how these proteins and pathways evolved. *C. albicans* is more closely related to *S. cerevisiae* than *N. crassa* (Fitzpatrick, Logue et al. 2006), the only other model system developed to study mitochondrial protein import (Neupert 1997), and mitochondria isolated from *C. albicans* can efficiently import a range of heterologous *S. cerevisiae* proteins (Figure 3.2). Compared to mitochondria isolated from other sources, the *in vitro* import assays shown here are as efficient or better (*S. cerevisiae* (Glick, Brandt et al. 1992); *N. crassa* (Kiebler, Keil et al. 1993, Sherman, Go et al. 2005); *S. pombe,* (Khalimonchuk, Ott et al. 2006)).

The ability of *C. albicans* to import and process *S. cerevisiae* proteins also means the import pathways are sufficiently similar that experiments can make use of the advantages of both systems to study the details of the import processes. I took advantage of these benefits in my newly developed system to investigate the differences I observed in the import of Cyb2 and Cyt1 in *C. albicans* compared to *S. cerevisiae* (Figure 3.3). This discussion begins with an introduction to the details of the more complex stop-transfer import pathway required to explain the significance of these results.

3.4.2 Rewiring of protein import pathways

Like many intermembrane space proteins, the cytochromes Cyb2 and Cyt1 are synthesized with a cleavable N-terminal presequence that contains matrix-targeting information (van Loon, Brandli et al. 1986). In proteins destined for the mitochondrial matrix this targeting information leads them to, and through the TOM complex and TIM23 complex (Schneider, Berthold et al. 1994) (Figure 3.16A). In intermembrane space destined proteins like Cyb2 and Cyt1 the charged presequence targets the precursor for membrane potential-dependent insertion into the Tim23 (Truscott, Kovermann et al. 2001), but this charged sequence is also followed by a hydrophobic sorting signal (van Loon, Brandli et al. 1986) (Figure 3.16B). This sorting signal is recognised by the TIM23 complex and results in a lateral transfer of the sequence into

the inner membrane, thereby preventing translocation into the matrix (Glick, Brandt et al. 1992, Botelho, Osterberg et al. 2011) (Figure 3.16A).

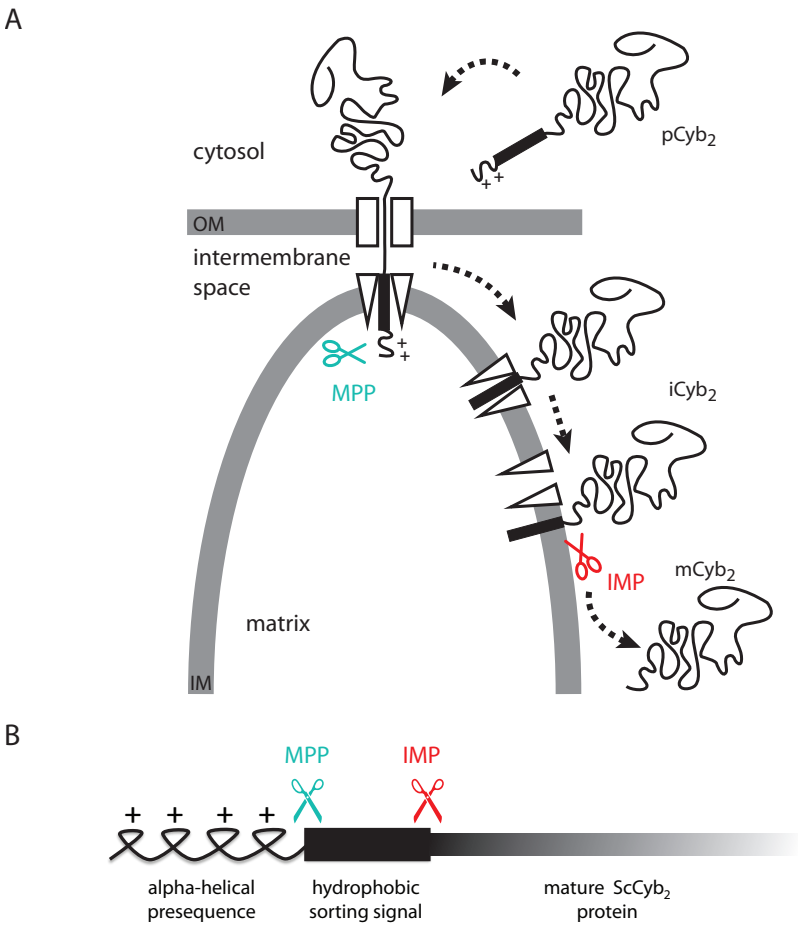


Figure 3.16 Stop-transfer protein import pathway
(A) Import and processing of ScCyb2 via the stop-transfer pathway occurs by the sequential interaction with the TOM complex (white rectangles) and the TIM23 complex (white triangles), and the sequential processing by the mitochondrial processing peptidase (MPP) in the mitochondrial matrix and the inner membrane peptidase (IMP) in the inner membrane. **(B)** Cartoon showing the cleavage steps in the processing of ScCyb2 in the stop-transfer pathway. Figure adapted from (Hewitt, Heinz et al. 2012).

In the stop-transfer pathway, the positively charged presequence protruding into the matrix through the pore of the TIM23 complex is cleaved by a matrix protease, in most cases, the mitochondrial processing peptidase (MPP) (reviewed in (Mossmann, Meisinger et al. 2012)). The now terminal hydrophobic sorting signal crossing the inner membrane acts as a brake in the pathway, preventing the translocation of the precursor into the matrix (van Loon and Schatz 1987).

Hydrophobic and proline residues in the transmembrane domain (Meier, Neupert et al. 2005), and charged residues following this hydrophobic region (Rojo, Guiard et al. 1998), have all been shown to play a role in directing precursor proteins into the stop-transfer pathway, but the mechanistic basis behind these observations is still unclear (Botelho, Osterberg et al. 2011).

Further cleavage of the stop-transfer sequence by specific proteases can release soluble proteins into the intermembrane space (Figure 3.16A). In the case of Cyb2 the hydrophobic sequence is cleaved by the inner membrane protease (IMP) (Schneider, Behrens et al. 1991). A variation on this stop-transfer pathway anchors Cyt1 in the inner membrane via its C-terminus. Cleavage of the N-terminal signal by the IMP occurs only after the insertion of the C-terminus into the inner membrane (Arnold, Folsch et al. 1998). Once released into the intermembrane space, a Cyt1-specific heme lyase incorporates heme into Cyt1, ensuring correct folding of the protein into its final form and rendering it unable to escape from the intermembrane space (Corvest, Murrey et al. 2010).

While *C. albicans* encodes homologues of all the components of TOM, TIM23 and peptidase machinery that is required for import via the stop-transfer pathway (Table 8.11), it does not efficiently import and process Cyb2 and Cyt1 (Figure 3.3). Comparative sequence analysis shows that CaCyb2 does not have a stop-transfer sorting sequence and Cyt1 is missing a section of its presequence (Figure 3.4A). The Cyt1 sequence of the parasite *Trypanosoma brucei* is also lacking the stop-transfer sequence (Diekert, de Kroon et al. 2001). These differences in signal sequences mean that Cyb2 and Cyt1 must make their way to the intermembrane space via an alternative route in these organisms, making it likely that evolutionary rewiring of import pathways has occurred in these organisms (Hewitt, Gabriel et al. 2014).

This evolutionary rewiring may be due to the metabolic constraints that are imposed by the environmental niches inhabited by these different organisms. As a commensal of humans, commonly inhabiting the gastrointestinal and vaginal tracts, the natural niches of *C. albicans* contain very little oxygen. Such a hypoxic environment has been shown to alter the redox state of proteins in the intermembrane space (Waypa, Marks et al. 2010) and impair the generation of the mitochondrial membrane potential (Chandel, Budinger et al. 1997, Lefebvre-Legendre, Balguerie et

al. 2003, Frezza, Zheng et al. 2011). The stop-transfer pathway, which *S. cerevisiae* uses to import Cyb2 into the intermembrane space, critically depends on the magnitude of the mitochondrial membrane potential (Gasser, Ohashi et al. 1982). However, this pathway may not function as efficiently in the hypoxic niche of *C. albicans*. This may have selected for proteins that are able to engage with other pathways.

A better understanding of the changes in oxygen levels, ATP availability, redox states of translocase components, and membrane potential for cells growing in environments, such as host niches or multicellular biofilms, would help determine which factors or pathways might be most compatible. With the limited information available, I hypothesised that the MIA pathway was involved in the import of *C. albicans* Cyb2 into the intermembrane space. Assays in Mia40 mutants using *C. albicans* homologues of Cyt1 and Cyb2 would have identified whether the MIA pathway plays a role in their import, but I was unable to find conditions where the *C. albicans* proteins did not aggregate. The protocols that are sometimes used successfully to circumvent this common problem in mitochondrial import studies were unsuccessful and are outlined in the Methods (Section 2.7.2, (Thornton, Stroud et al. 2010)). The delayed import and processing of the *S. cerevisiae* Cyt1 and Cyb2 precursors (Figure 3.6) is already an indication that even in *S. cerevisiae* the MIA pathway, in addition to the stop-transfer processing, might contribute to the import of these proteins. This adds further substrates to the already diverse intermembrane space proteins that make use of the MIA pathway (Chacinska, Pfannschmidt et al. 2004, Gabriel, Milenkovic et al. 2007, Varabyova, Topf et al. 2013, Wrobel, Trojanowska et al. 2013).

The complexities of the import machinery suggest the pressure to evolve a more efficient import process in response to environmental stimuli must be high (Rainbolt, Atanassova et al. 2013). *CYB2* encodes L-lactate cytochrome-c oxidoreductase, an enzyme essential for the utilization of L-lactate (Guiard 1985). In the human host, while lactate is available as a carbon source, the niches of *C. albicans* are very poor in glucose. Subsequently, there would be a significant growth advantage to *C. albicans* able to import Cyb2 into the intermembrane space and make efficient use of lactate. The function of Cyb2 in *C. albicans* biology has not yet been studied.

However, in another pathogenic yeast species *Candida glabrata*, which is also a human commensal and occupies similar niches to *C. albicans*, Cyb2 is essential for utilisation of lactate. The use of this carbon source is thought to be involved in the ability of *C. glabrata* to grow in the gastrointestinal tract of its host, as determined in the murine infection model using wild type and *cyb2* mutants (Ueno, Matsumoto et al. 2011). Importantly, *C. glabrata* and *C. albicans* are better at utilising lactate than *S. cerevisiae* when grown in low oxygen conditions *in vitro* (Ueno, Matsumoto et al. 2011). This mimics the hypoxia present in the host niches and fits with our model that *Candida* can import Cyb2 in the intermembrane space in hypoxic conditions, likely utilising import pathways that are different to the stop-transfer pathway. As we identify more examples and characterise this rewiring process we will gain insights into the evolutionary pressures on these pathways. It is only comparative studies of mitochondrial protein import in more diverse organisms that can reveal the significance of these variations for the regulation of protein import and of mitochondrial metabolism and the ability of organisms to thrive in their environmental niches.

3.4.3 Porin import and assembly

Porin exists in *S. cerevisiae* in numerous packing densities and oligomeric forms from monomers and trimers (likely corresponding to the complexes detected in Figure 3.7A), up to patches of around 20 clustered pores (Goncalves, Buzhynskyy et al. 2007). Porins from *N. crassa* and humans also form ladders of different sized complexes when import is monitored by BN-PAGE (human (Kozjak-Pavlovic, Ross et al. 2007); *N. crassa* (Habib, Waizenegger et al. 2007)). Results from cross-linking porin in rat mitochondria suggest a mixture of dimer, trimers and tetramers (Zalk, Israelson et al. 2005). The situation in *C. albicans* is quite distinct with what appears to be a more regular arrangement of porin in a predominant oligomeric form of ~440 kDa (Figure 3.7A). This large oligomeric form is thought to correspond to the porin trimer in *S. cerevisiae*, similar to what has been observed for bacterial porins (Mannella 1998). Regardless of the identity of the porin oligomers the assembly of porin into a large complex in mitochondria from *C. albicans* is very efficient, with significant amounts of the ~440 kDa complex detected after just 2 min of import (Figure 3.7A).

When ATP is removed from the import reaction, the import and assembly of porin into mitochondria from both *S. cerevisiae* and *C. albicans* slowed, but there was no accumulation of any particular complex that could represent an intermediate form (Figure 3.8). These assays suggest that ATP is needed at an early stage in the import process, perhaps in the release of precursor from the receptors or chaperones (Lithgow, Hoj et al. 1993), rather than in the insertion into the membrane or oligomerisation. Reduced ATP levels are not sufficient to explain the differences in porin import and assembly efficiency in mitochondria from *S. cerevisiae* and *C. albicans* (Figure 3.8). Instead, the different oligomeric forms may be a way *C. albicans* uses to regulate porin function as gating of the channel is thought to depend on its oligomeric state (Mannella 1998).

3.4.4 Intermediates in Tom40 assembly

I was surprised to find that while mitochondria isolated from *C. albicans* were able to rapidly assemble ScTom40 into the TOM complex, mitochondria from *S. cerevisiae* were unable to do so with CaTom40. I hypothesised that the ladder of intermediate size complexes represented intermediates in TOM complex assembly that are not normally visible in these efficient later steps of the TOM assembly pathway in wild type mitochondria from *S. cerevisiae* ((Model, Meisinger et al. 2001, Paschen, Waizenegger et al. 2003, Wiedemann, Kozjak et al. 2003, Meisinger, Wiedemann et al. 2006, Lueder and Lithgow 2009) or *N. crassa* (Wideman, Go et al. 2010)). Such intermediates are sometimes shown in the literature for the import of Tom40 or other TOM components (Tom40 (Dembowski, Kunkele et al. 2001, Stojanovski, Guiard et al. 2007); Tom5 (Stojanovski, Guiard et al. 2007); Tom6 (Stojanovski, Guiard et al. 2007, Thornton, Stroud et al. 2010)). These intermediate sized complexes have not been well characterised partly due to their low abundance (hence the difficulties in detection) and variability in the BN-PAGE assembly assays (Stojanovski, Guiard et al. 2007). The clear signal and reproducible results from my heterologous assay showing import of CaTom40 into mitochondria isolated from *S. cerevisiae* suggested it could be a useful system to characterise these intermediates. I tried four approaches to identify other components of these intermediates: (1) monoclonal antibody shifts using tagged *S. cerevisiae* strains, (2) increased protein expression to rescue assembly, (3) comparison with import of radiolabelled potential

partner proteins, and (4) antibody shifts with polyclonal antibodies against potential partner proteins.

Antibody shift experiments have previously been used to identify components of import intermediates (Paschen, Waizenegger et al. 2003, Kutik, Stojanovski et al. 2008). Monoclonal antibodies have a defined binding site and as a consequence often produce clearer results than antibody shifts using polyclonal antibodies (Becker, Pfannschmidt et al. 2008, Yamano, Tanaka-Yamano et al. 2010). The addition of HA antibody did not shift any of the intermediate sized complexes formed when CaTom40 was incubated with mitochondria from the *tom40-3HA* strain, suggesting that CaTom40 may not associate with endogenous Tom40 in any of these complexes (Figure 3.15). This was surprising as there is a strong interaction between Tom40 monomers to form dimers of ~80 kDa and in the absence of Tom22 a ~100 kDa complex forms containing Tom40, Tom5 and probably Tom6 and Tom7, suggesting the ~100 kDa Tom40 assembly intermediate also contains two Tom40 molecules (van Wilpe, Ryan et al. 1999). It is possible the HA epitope is not exposed in the endogenous complex so the antibody cannot bind. Since this experiment did not produce a result consistent with the literature and I was unable to reproduce the ladder of intermediates in the SAM tagged strain, I tried instead to rescue Tom40 assembly by creating yeast strains expressing TOM complex components in high copy number.

I hypothesised that CaTom40 may have been unable to assemble into mature TOM complex due to impaired interactions with one of the other *S. cerevisiae* TOM complex components. When the complex assembly is impaired, expression of high levels of other components of the complex can improve the assembly process possibly just by increasing the likelihood of temporarily forming productive intermediates (Chan and Lithgow 2008, Dukanovic, Dimmer et al. 2009). Neither the strains expressing high levels of Tom20, Mim1 or Tom22 nor the empty vector controls produced a ladder of bands on import of CaTom40 (Figure 3.13). This suggests the plasmids or the transformation process may have been affecting the mitochondrial import process in some way. I decided not to go through lengthy troubleshooting to optimise the import behaviour of these plasmids as I had alternative methods to attempt.

Previous results show that variable amounts of imported small TOM proteins form intermediates that are suggested to be Tom40-Tom7 or Tom40-Tom6 intermediates (Thornton, Stroud et al. 2010). I wanted to examine these small TOM intermediates in more detail to see if comparisons with my trapped CaTom40 intermediates might suggest which components were present in each complex (Stojanovski, Guiard et al. 2007). While I did see a ladder of intermediates suggesting small TOM proteins are present in these complexes, there were no differences in ladders of intermediates that could be exploited to suggest which other TOM complex components were present in the distinct trapped intermediates (Figure 3.14). This may indicate that the assembly order of the additional TOM complex components after Intermediate II is not critical and assembly can proceed with whichever subunits are available.

Polyclonal antibodies can also be used to identify assembly intermediates but can obscure other complexes in the gel (Wiedemann, Kozjak et al. 2003, Meisinger, Rissler et al. 2004, Humphries, Streimann et al. 2005). I was not able to detect shifts in any of the intermediates formed in CaTom40 import using any of the available antibodies against TOM complex components. As none of these methods seemed likely to produce more promising results, I returned my focus to characterising the *C. albicans* import and outer membrane assembly system.

3.4.5 Efficient beta barrel protein assembly in *C. albicans* mitochondria

The Tom40 assembly process is highly efficient with the majority of the imported protein found in the mature TOM complex after 70 minutes (Figure 3.9). This is rarely observed for TOM complex assembly reactions using mitochondria from *S. cerevisiae* (Model, Meisinger et al. 2001, Paschen, Waizenegger et al. 2003, Waizenegger, Schmitt et al. 2005) and efficient import in *S. cerevisiae* requires addition of creatine kinase and creatine phosphate to regenerate ATP (Model, Meisinger et al. 2001). In *S. cerevisiae* the formation of the ~250 kDa intermediate is also rapid but the formation of the ~100 kDa intermediate and mature complex is much slower (Model, Meisinger et al. 2001). The rapid assembly of Tom40 into the TOM complex with only a small amount of Tom40 seen in the 100 kDa intermediate in *C. albicans* is similar to the assembly of NcTom40 into *N. crassa* mitochondria (Rapaport, Taylor et al. 2001).

One possible reason for the difference in the assembly rates in *S. cerevisiae* compared to mitochondria from *C. albicans* is a difference in lipid composition as beta barrel protein assembly has recently been shown to be highly dependant on the specific lipid composition of the membranes they reside in (Gessmann, Chung et al. 2014). The transition from the ~250 kDa intermediate to the ~100 kDa intermediate is very temperature sensitive (Model, Meisinger et al. 2001), with the latter unable to occur at low temperature, suggesting that the mobility of the lipid bilayer may contribute to the assembly efficiency (Rapaport 2002). Since sensitive evaluation of the lipid composition requires highly purified mitochondrial outer membranes (Daum and Vance 1997), a technique not optimised for mitochondria from *C. albicans*, I decided not to pursue this hypothesis experimentally and focus on the biochemical characterisation of the *C. albicans* SAM complex.

3.4.6 Conclusions and Future Directions

Together with the results from assembly of porin and Tom40, I concluded that assembly of beta barrel proteins into the mitochondrial outer membrane is highly efficient in *C. albicans.* This efficient assembly of Tom40 in mitochondria from *C. albicans* also provides an exciting opportunity to re-evaluate the import process using a more robust assay system. SAM intermediates in the Tom40 assembly pathway are still not clearly understood (Section 1.5.2), partly due to the low abundance and resolution of these complexes in BN-PAGE import assays.

The sequences of the *C. albicans* SAM complex components are examined in more detail in Chapter 4. In Section 5.1, I investigate the role of *C. albicans* Sam51, a novel protein I identified in Section 3.1.1. The contributions of Sam37 and Mdm10 to beta barrel protein and TOM complex assembly are explored in Sections 5.2 and Section 5.3. Finally, Chapter 6 explores the biochemical properties of the SAM complex including contribution of Sam51 and investigates the relationship between the SAM and ERMES complexes in *C. albicans*.

4 Bioinformatic analysis of SAM complex components

This chapter provides a more detailed introduction to the SAM complex components Sam35, Sam37, Sam50 and Mdm10. I compare the sequences of the *C. albicans* SAM complex components with those of *S. cerevisiae* and other organisms and discuss what these comparisons can tell us about the function and evolution of these components. Section 4.5 includes the details of my discovery of the novel SAM complex component, Sam51, which falls into a previously unidentified family of Omp85 proteins.

I constructed hidden Markov models (HMMs) using fungal sequences of Sam35, Sam37, Sam50 and Mdm10 to confirm the identity of each of the *C. albicans* SAM complex components identified by BLAST search and to identify any more distant homologues. The top BLAST and HMM hits identified the same open reading frames for Sam35, Sam37, Sam50 and Mdm10 (Appendix 6: Table 8.11). Further details and interesting features of these sequences are discussed in the following sections.

4.1 Sam37

The Candida Genome Database (CGD, http://www.candidagenome.org, (Arnaud, Inglis et al. 2014)) identifies two domains in the *C. albicans* Sam37 sequence; Tom37 (PF10568, dark blue Figure 4.1) and Tom37_C (PF11801, red Figure 4.1), which are also identified in the *S. cerevisiae* Sam37 protein in the Saccharomyces Genome Database (SGD, http://www.yeastgenome.org (Cherry, Hong et al. 2012)). The names of these domains do not reflect current usage as they are based on an out-dated reference (Bowers and Ardehali 2006). The Tom37 domain falls into the Thioredoxin-like super family of domains and the Tom37_C falls into the Glutathione S-transferase C-terminal domain family (GST_C_family) (NCBI Conserved domains database, (http://www.ncbi.nlm.nih.gov/cdd/, (Marchler-Bauer, Zheng et al. 2013)). A Thioredoxin-like domain near the N-terminus and a GST-C domain near the C-terminus is the domain structure shared by all Sam37 sequences that could be identified by BLAST searches using the *S. cerevisiae* or *C. albicans* Sam37 sequences (Figure 4.1). Multiple sequence alignments of Sam37 homologues

confirm that the most conserved sections of the proteins fall into these conserved domains and the most significant sequence variation occurs near their C-termini beyond the GST-C domain (Figure 8.1).

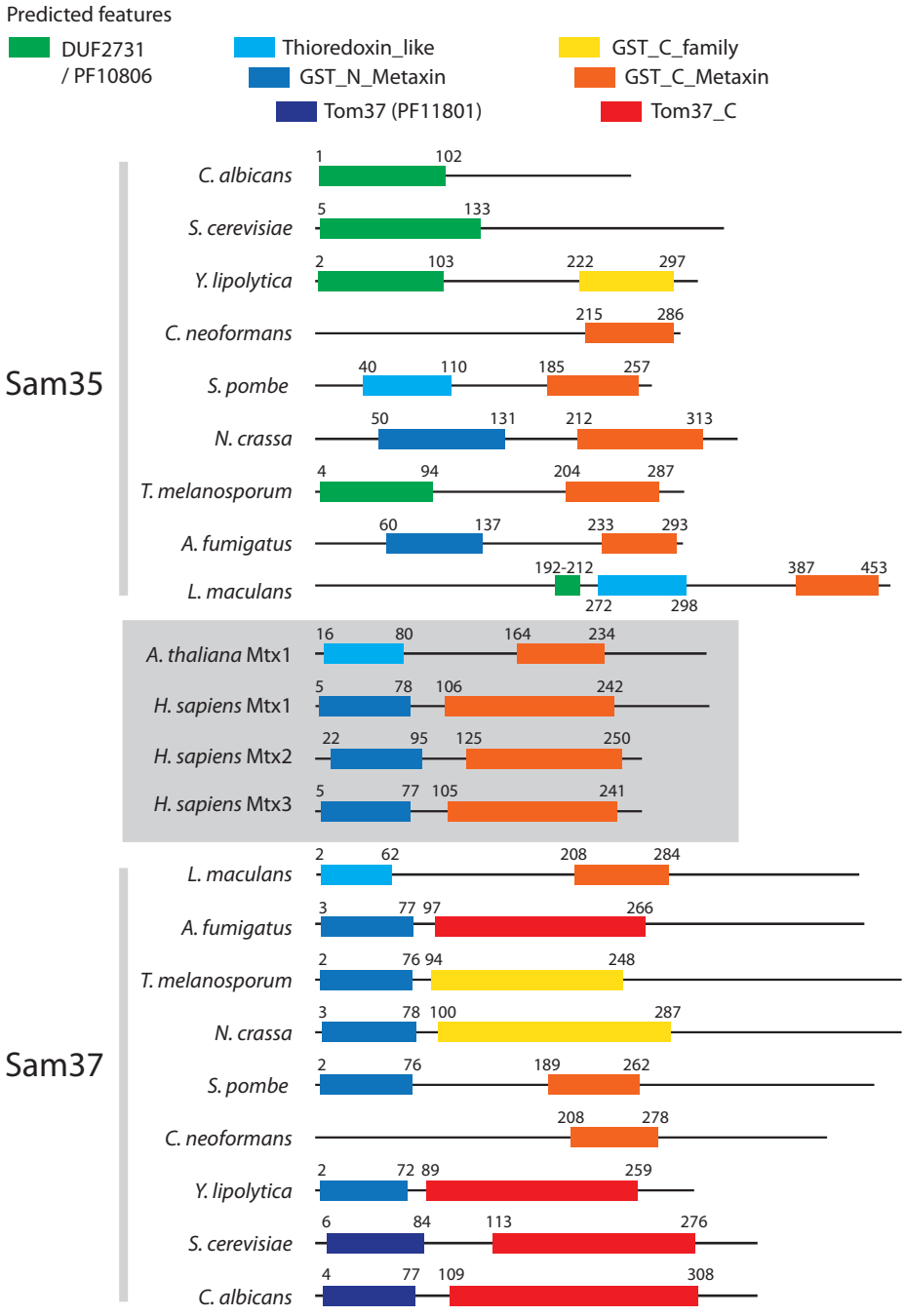


Figure 4.1 Domain organisation of Sam35 and Sam37 and the metaxins
Numbers indicate start and end residues of the *C. albicans* homologue or domain. Green - DUF2731 (Pfam10806); Thioredoxin like: pale blue – no specified subfamily, blue - GST_N_Metaxin, dark blue – Tom37 (Pfam10568); GST_C_family: yellow – no specified subfamily, orange GST_C_Metaxin (Pfam00043), red – Tom37_C (Pfam11801).

I used the fungal BLAST function of the SGD to find closely related Sam37 homologues to compare with *C. albicans* Sam37. BLAST searches using *S. cerevisiae* or *C. albicans* Sam37 sequences finds hits for *Ascomycota* Sam37 sequences but recognize only a single *Basidiomycota* sequence (EJU05326.1 from a *Dacryopinax* species). Other more divergent *Basidiomycota* sequences (such as those from *Cryptococcal* species) were identified using the fungal Sam37 HMM search with the presence of a GST_C_family domain near the C-termini of these proteins used as a further indication that these are true Sam37 homologues.

The second highest hit for the fungal Sam37 HMM in the *C. albicans* genome was an uncharacterised open reading frame (orf19.1265) with a HMM score of 5.70×10^{-4}. This large open reading frame has a high expectation value, does not contain either of the typical Tom37 domains and its closest homologue in *S. cerevisiae* is Trs103, a protein involved in ER-Golgi vesicle trafficking (Sacher, Barrowman et al. 2000). Together these factors strongly suggest that this second hit is not a *C. albicans* Sam37 homologue.

4.2 Sam35

The CGD identifies a single domain of unknown function in the *C. albicans* Sam35 sequence (DUF2731, PF10806), which is also identified in the *S. cerevisiae* homologue in the SGD (green, Figure 4.1). All the *Saccharomycetales* Sam35 sequences contain this domain but it is not found in all *Ascomycota* Sam35 sequences (Figure 4.1). Most Sam35 sequences lacking this domain have a Thioredoxin-like domain near their N-terminus instead, and in many sequences, both in and beyond the *Ascomycota*, there is also a GST C-terminal domain as seen in the domain organisation of most Sam37 sequences (Figure 4.1).

I used the fungal BLAST function of the SGD to find Sam35 sequences to compare with *C. albicans* Sam35. A BLAST search using *S. cerevisiae* or *C. albicans* Sam35 sequences only identified close Sam35 homologues in the *Saccharomycotina* (see Figure 4.3 for a simple phylogenetic tree). This reflects the greater sequence variation of the Sam35 proteins compared to Sam37 proteins, a feature that is also clear in multiple sequence alignments of Sam35 homologues (Figure 8.2). I identified more divergent Sam35 homologues using the fungal Sam35 HMM and again used

domain organization to confirm the true Sam35 homologues. The HMM based on Sam35 fungal sequences detected no other hits in the *C. albicans* genome below the 1×10^{-3} threshold.

The Sam35 HMM identified both Sam35 and metaxin sequences and the Sam37 HMM identified Sam37, Sam35 and metaxin sequences from various species (e.g. *Aspergillus flavus* Sam35 and human metaxin-1). These results show the weak sequence similarity extends across the Sam35, Sam37 and metaxin proteins. The metaxin proteins perform similar functions in mammals to Sam37 and Sam35, but their limited sequence similarity with the fungal proteins makes it unclear whether they are homologous (Bornstein, McKinney et al. 1995, Armstrong, Komiya et al. 1997, Kozjak-Pavlovic, Ross et al. 2007).

The shared domain organization with a Thioredoxin-like domain near the N-terminus and a GST-C domain near the C-terminus of most of these protein sequences shown in Figure 4.1 supports the idea that Sam35, Sam37 and the metaxins may have shared ancestry. The sequence similarity between these proteins has also been shown by Andrew Perry, using CLANS (CLuster ANalysis of Sequences) analysis (Frickey and Lupas 2004) that presents a visual representation of BLAST scores ((Qu, Jelicic et al. 2012), Figure 6). These domain super families are quite inclusive; there is considerable sequence variation between family members, no indication that Sam37, Sam35 or the metaxins have redox or transferase activity, and the residues responsible for binding glutathione are not conserved (Armstrong, Komiya et al. 1997). This variation may reflect the evolution of these components to include more specialised roles, for example the mammalian metaxins may modulate cell death pathways (Ono, Wang et al. 2010, Cartron, Petit et al. 2014) and in *C. albicans* Sam37 is important for the link between the SAM and ERMES complexes via Mdm10 (Section 6).

The common domain organisation points towards shared evolutionary origins and perhaps similar folds (Koonin, Mushegian et al. 1994, Armstrong, Komiya et al. 1997). While the evolutionary relationships between these proteins are still unclear, this analysis supports the trend in the recent literature to discuss these proteins as homologues (Kozjak-Pavlovic, Ross et al. 2007, Becker, Vogtle et al. 2008, Endo and Yamano 2010). This is also consistent with more recent publications, which suggest the metaxins can be found in a complex with Sam50 (Xie, Marusich et al. 2007).

Together these results make a strong case for renaming the metaxins to reflect their role in conjunction with the SAM complex in place of a name derived from the location of metaxin-1 between two other genes in the mouse chromosome 3 (Bornstein, McKinney et al. 1995).

4.3 Mdm10

In *S. cerevisiae*, Mdm10 is thought to be a component, or at least a transient module of the SAM complex I therefore included this protein in my investigation of the *C. albicans* SAM complex (Meisinger, Rissler et al. 2004). The *C. albicans* Mdm10 homologue (CaMdm10, orf19.184) is easily identified by BLAST search using the *S. cerevisiae* Mdm10 sequence. The second highest hit in the *C. albicans* genome using the Mdm10 HMM was an uncharacterised open reading frame (orf19.2476) with a HMM score of 4.20×10^{-4}. This gene is annotated as an ortholog of *ECM5* in *S. cerevisiae* and a BLAST search using the predicted sequence of the *C. albicans* open reading frame identifies Ecm5 in *S. cerevisiae* not Mdm10. My Mdm10 HMM also identifies Tom40 or VDAC sequences with scores indicating limited sequence similarity, in keeping with the suggested evolutionary relationship of Mdm10 to these other "eukaryotic porins" (Bay, Hafez et al. 2012, Flinner, Ellenrieder et al. 2013).

In *C. albicans* Mdm10, like all other Mdm10 sequences, includes a DUF3722 (Pfam12519) domain near its N-terminus (Figure 4.2). Mdm10 proteins are found in diverse fungi, and these sequences vary considerably in the predicted loops (Flinner, Ellenrieder et al. 2013), particularly the internal loops 3, 9 and 15 and the last external loop 18 (Appendix 7: Figure 8.3). There is also more variation in the first residue of the Mdm10 beta signal, a conserved sequence at the C-terminus of beta barrel proteins (Kutik, Stojanovski et al. 2008), than in other beta signals (Imai, Fujita et al. 2011) (Figure 8.3). The recently identified particularly divergent Mdm10 sequences in the Amoebozoan, *Acanthamoeba castellanii* and Choanozoan, *Capsaspora owczarzaki* (an Opisthokont that diverged from the Metazoa after Fungi) (Wideman, Gawryluk et al. 2013) also contain the DUF3722 domain but have very limited sequence similarity beyond the beta signal (Figure 8.3). If they are true homologues,

this suggests that a version of Mdm10 may have been present in the last eukaryotic common ancestor but has been lost from Metazoans.

There is considerable interest in the regulation of protein import by post-translational modification (Schmidt, Harbauer et al. 2011). ScMdm10 has a number of hydroxylated amino acid residues (Sogo and Yaffe 1994) so could potentially be phosphorylated, but there are currently no reports of such a modification. ScMdm10 is not glycosylated (Stroud, Oeljeklaus et al. 2011) but using the same program to predict glycosylation sites in CaMdm10 (http://www.cbs.dtu.dk/services/NetNGlyc/) shows very few of these predicted sites are conserved between these two species (2 of 11) making it impossible to extrapolate from ScMdm10 to CaMdm10.

4.4 Sam50

Sam50 is the essential core of the SAM complex (Kozjak, Wiedemann et al. 2003) and a direct descendant of BamA, the core of the bacterial BAM (beta barrel assembly machinery) complex (Gentle, Gabriel et al. 2004). The Sam50 C-terminus is predicted to form a 16-stranded beta barrel (Gentle, Gabriel et al. 2004, Walther, Rapaport et al. 2009, Noinaj, Kuszak et al. 2013). The *C. albicans* and *S. cerevisiae* Sam50 proteins both contain the same single C-terminal Pfam domain Bac_surface_Ag (bacterial surface antigen, Pfam01103, purple Figure 4.2) found in the bacterial Omp85 proteins. This domain is characterised by the immune response to the *Haemophilus influenzae* Omp85 bacterial surface protein (Flack, Loosmore et al. 1995, Moslavac, Mirus et al. 2005).

The N-terminal region of Sam50 is predicted to include a single POTRA (polypeptide translocation associated) domain that is also found in bacterial Omp85 proteins (Sánchez-Pulido, Devos et al. 2003, Gentle, Burri et al. 2005). These domains share limited sequence similarity but have conserved folds and a secondary structure with two beta strands followed by two alpha helices and a final beta strand (Sánchez-Pulido, Devos et al. 2003, Kim, Malinverni et al. 2007, Arnold, Zeth et al. 2010). Based on PSIPRED secondary structure predictions the *C. albicans* Sam50 POTRA domain extends from residue 55 - 128 (pink, Figure 4.2) (incorrectly annotated in (Arnold, Zeth et al. 2010)). A more accurate Sam50 model based on the recent BamA structures confirms these features (Noinaj, Kuszak et al. 2013).

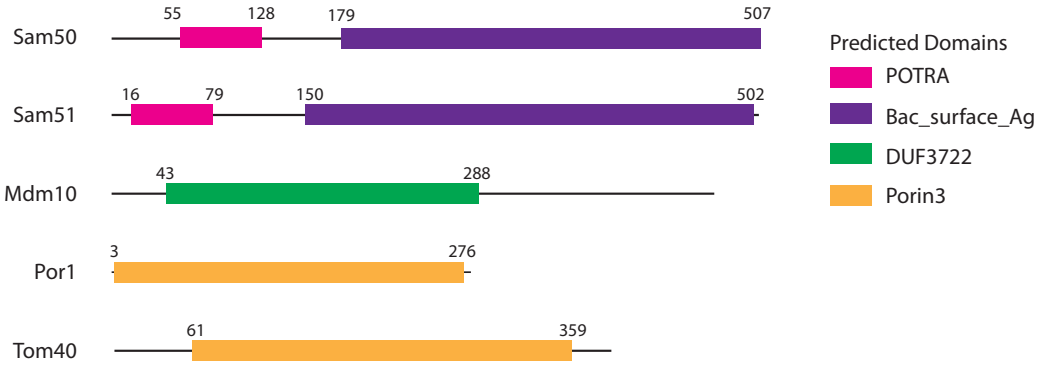


Figure 4.2 Domain organisation of *C. albicans* beta barrel proteins
Numbers indicate start and end residues of the *C. albicans* domain. Purple – Bac_surface_Ag (Pfam01103); Pink – POTRA domain (based on PSIPRED secondary structure prediction); Green – DUF3722, Yellow – Porin3 (Pfam01459)

4.5 Sam51

The second highest hit in the *C. albicans* genome using the Sam50 HMM was an uncharacterised open reading frame (orf19.8540) with a HMM score of 7.80×10^{-9}. This score indicates this protein sequence is considerably more divergent than the 2.10×10^{-218} Sam50 hit, but is still a sufficiently low score to investigate further. Entering this sequence into the NCBI Conserved Domains database shows it contains the characteristic Omp85 bacterial surface antigen domain and secondary structure prediction indicates the presence of a single POTRA domain as is found in Sam50 (Figure 4.2). We named this Sam50-like protein Sam51 in keeping with the naming convention in yeast mitochondrial proteins (Schlossmann, Lill et al. 1996), and the CGD entry has since been updated based on our findings (Hewitt, Heinz et al. 2012).

I found homologues of Sam51 throughout the *Saccharomycotina* using the fungal Sam50 HMM (Figure 4.3). There are no Sam51 candidates in *S. cerevisiae* or its close relatives, suggesting that the gene has been lost from *S. cerevisiae* (Figure 4.3). My analysis indicated the reconstructed ancestor that underwent a whole genome duplication (WGD) event includes both Sam50 and Sam51 (Byrne and Wolfe 2005)(Figure 4.3). Some of the yeasts that underwent the WGD event and all of the CTG clade (the yeasts, like *C. albicans*, which use CTG to encode serine not leucine) have kept their copy of Sam51 (Figure 4.3). *N. crassa* does not have a *SAM51* gene, but it does express multiple isoforms of Sam50 with alternative splicing at the N-

terminus (Hoppins, Go et al. 2007). Like Sam51 the alternatively spliced versions of *N. crassa* Sam50 are shorter than most Sam50 proteins but there is considerable variation in the N-terminal region of both Sam50 and Sam51 proteins (Figure 8.4).

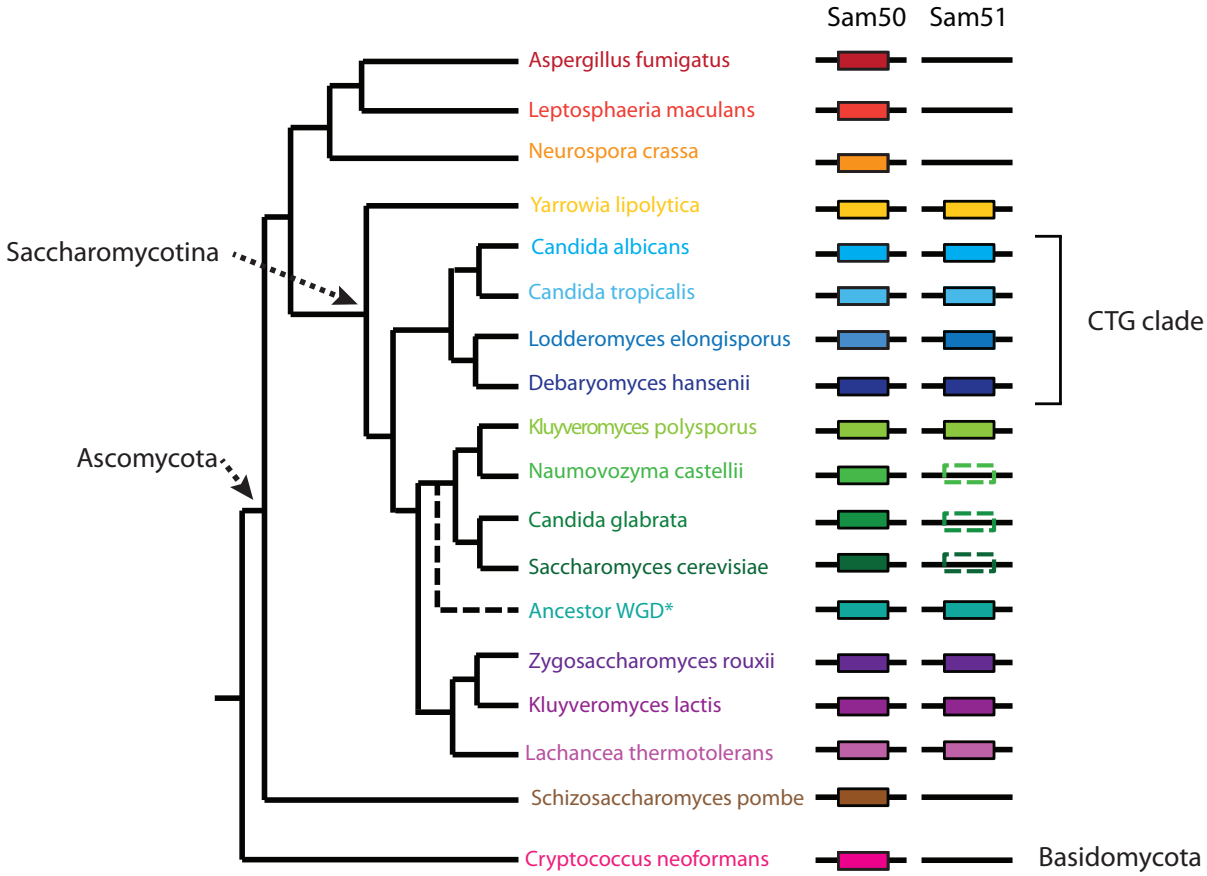


Figure 4.3 Distribution of Sam50 and Sam51
The distribution of *SAM50* and *SAM51* genes in fungi was analysed by Eva Heinz (Figure S2C, (Hewitt, Heinz et al. 2012)). An extended version of this analysis is shown here incorporating information from Figure 1 (Wang, Xu et al. 2009) to show the range of fungi that possess both *SAM50* and *SAM51*. *This branch indicates the inferred progenitor species that underwent the whole genome duplication (WGD) event (Wolfe and Shields 1997).

4.6 Phylogenetic analysis of Omp85 proteins

To form a beta barrel structure the primary sequence of the beta strands of a protein must have alternating hydrophobic and hydrophilic residues. There is low selection pressure on the specific residues in the beta strands that span the membrane; therefore there is a lot of primary sequence variation. This makes it hard to create

multiple sequence alignments and therefore phylogenetic trees to compare shared features of homologues in different organisms, let alone more distantly related proteins (Gessmann, Flinner et al. 2011, Flinner, Schleiff et al. 2012). So while the bacterial origin of the Omp85 proteins is clear it is hard to determine the characteristics of the ancestral Sam50 or the details of the evolutionary origin of Sam51.

The branching pattern of the phylogenetic tree made from Omp85 sequences by Eva Heinz shows that Sam51 proteins are as similar to bacterial Omp85 BamA proteins as they are to other Sam50 sequences (Hewitt, Heinz et al. 2012). This result suggests that Sam51 proteins form a separate Omp85 family. Repeating this experiment using more stringent criteria for regions (G-blocks selection with less stringent options selected) shows Sam51 branching from within the Sam50 sequences (Figure 4.4). Both trees show Sam51 forms a monophyletic branch that does not include any Sam50 sequences but my analysis suggests the Sam51 proteins could have evolved from an ancient Sam50-like protein. This type of maximum likelihood tree construction is prone to long branch attraction, so more divergent sequences tend to flip out of the main groupings. The more stringent sequence selection means the tree is based on less total conserved sites, placing more emphasis on these conserved residues. A different tree calculation method (e.g. Bayesian) might help to distinguish between a separate, more ancient origin for Sam51 from BamA or a more recent divergence from the Sam50 proteins, but the divergent sequences of these proteins makes the origins of Sam51 unclear.

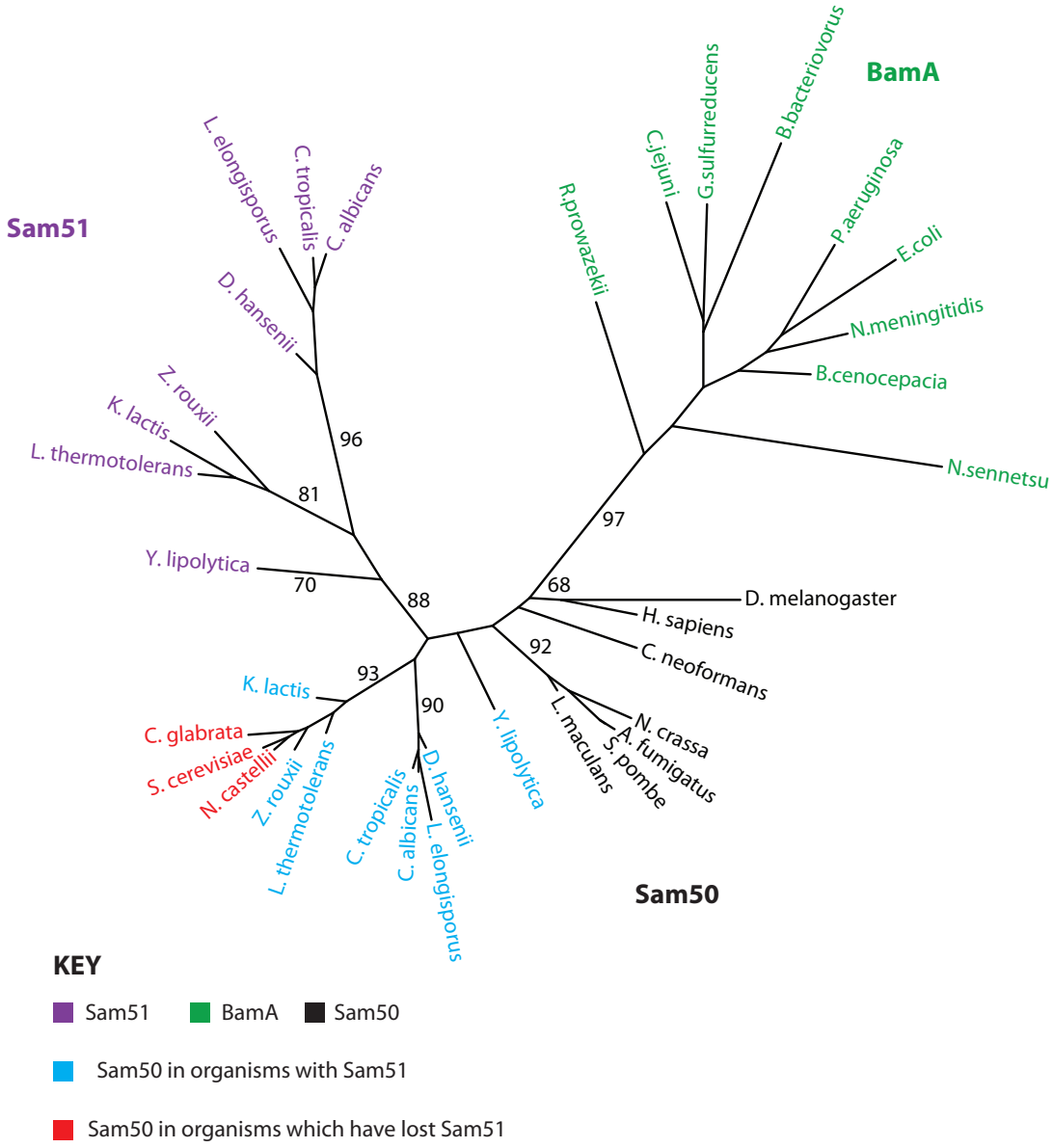


Figure 4.4 Phylogenetic analysis of Omp85 protein families
The tree was calculated as described in the Methods Section 2.1.4. Branch supports are shown as percentage bootstrap values for the main divisions. Green shows BamA sequences and purple shows Sam51 sequences. Organisms which have both Sam50 and Sam51 have Sam50 shown in blue. Organisms with no Sam51 are shown in black and organisms which have lost Sam51 are shown in red.

5 Functional analysis of SAM complex components

This chapter examines the roles of Sam51 and Mdm10 to see if they contribute to the robust beta barrel protein assembly in *C. albicans*. Deletion of Sam37 in *C. albicans* gives a more severe growth defect than in *S. cerevisiae* (Dagley, Gentle et al. 2011) so I also investigate the roles of Sam37 in protein import in *C. albicans* in this chapter. I use *C. albicans* mutants to investigate the functions of Sam51 (Section 5.1), Sam37 (Section 5.2) and Mdm10 (Section 5.3), and radiolabelled protein import assays into isolated mitochondria to characterise their roles in the import and assembly of outer membrane proteins.

5.1 Functional characterisation of Sam51

This section investigates the function of Sam51 in *C. albicans*. I show evidence that *SAM51* RNA is produced at comparable levels to *SAM50* (Figure 5.1), that Sam51 is not essential and that Sam51 cannot compensate for the loss of Sam50 (Figure 5.2). I also show there are no mitochondrial morphology defects (Figure 5.3), changes in transcript levels of other proteins (Figure 5.4) or growth defects (Section 5.1.6) associated with the *sam51ΔΔ* strain. Finally I show Sam51 is needed for efficient assembly of Tom40 in *C. albicans* (Figure 5.8).

5.1.1 *SAM51* is expressed

To determine the function of Sam51, I first wanted to determine whether the gene was expressed in normal growth conditions in *C. albicans*. None of the three attempts to design qPCR primers using the Primer3 program (as described in the Methods Section 2.10) produced a primer product in the qPCR conditions. I was able to find PCR conditions where primers amplifying the Sam50 and Sam51 sequences produced detectable PCR product. I used these primers with cDNA from *C. albicans* as a template to show that in rich media with a glucose carbon source *C. albicans* expressed both Sam50 and Sam51 at comparable levels (Figure 5.1).

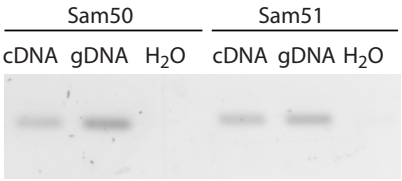


Figure 5.1 *SAM50* and *SAM51* expression levels
Primers were used to amplify *SAM50* and *SAM51* from cDNA. These products migrated at a position corresponding to a ~1.5 kilobase product on a 1 % agarose gel with SYBR Safe DNA gel stain (Life Technologies) and imaged using Gel Doc EZ imager (BioRad).

5.1.2 Sam51 is not essential

Having established that Sam51 was expressed we attempted to delete both copies of *SAM51* to determine whether the gene was essential. As these strains were created using homologous recombination in collaboration with Miguel Shingu-Vazquez and Tricia Lo (Traven Lab), the details of this method are therefore reported in our joint publication (Hewitt, Heinz et al. 2012). The homozygous diploid mutant *sam51ΔΔ* could be created indicating that the gene is not essential.

5.1.3 Sam51 cannot compensate for loss of Sam50

Since *C. albicans* Sam50 and Sam51 are closely related Omp85 proteins, I hypothesised that Sam51 might be able to replace Sam50 in the SAM complex. To test this we attempted to recover a homozygous diploid *sam50* deletion strain. This was unsuccessful, consistent with an essential function for Sam50 (Brachat, Liebundguth et al. 2000). Instead we deleted one copy of *SAM50* and placed the other copy under the control of the *MET3* repressible promoter. In the absence of cysteine and methionine the gene transcribed and protein is produced. This strain showed a near total loss of viability when grown in repressive conditions (Hewitt, Heinz et al. 2012). I confirmed *SAM50* was repressed in this strain after 5 h of growth in repressive conditions using quantitative PCR (Figure 5.2), and this repression was maintained for at least 30 h.

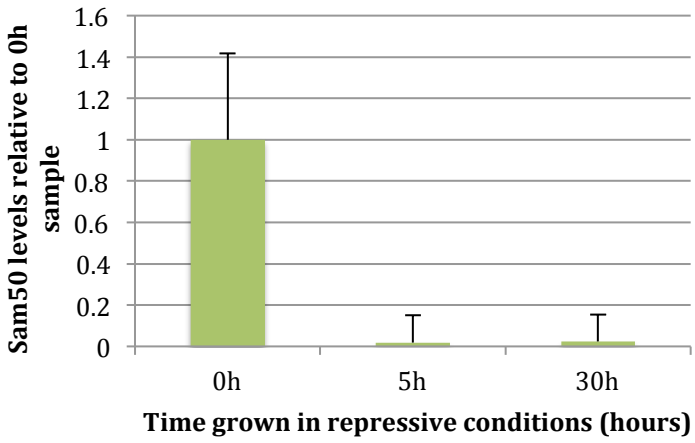


Figure 5.2 Expression of *SAM50* in *SAM50* repressive strain
Repression of *SAM50* expression was determined from RNA extracted from cells grown in permissive conditions (0 h) or for either 5 h or 30 h in the presence of 2.5 mM methionine and 0.5 mM cysteine to repress the *MET3* promoter. Results were normalised relative to *ACT1* and expression levels are shown relative to the sample grown in permissive conditions. Error bars show the standard deviation.

5.1.4 Mitochondrial morphology is unaffected in the *sam51ΔΔ* strain

To determine whether loss of Sam51 had any effect on mitochondrial morphology I used Mitotracker dye to stain the mitochondrial network and compared them in wild type and *sam51ΔΔ* strains (Figure 5.3). There were no differences in the reticulated network of mitochondria detected in both wild type and deletion strains.

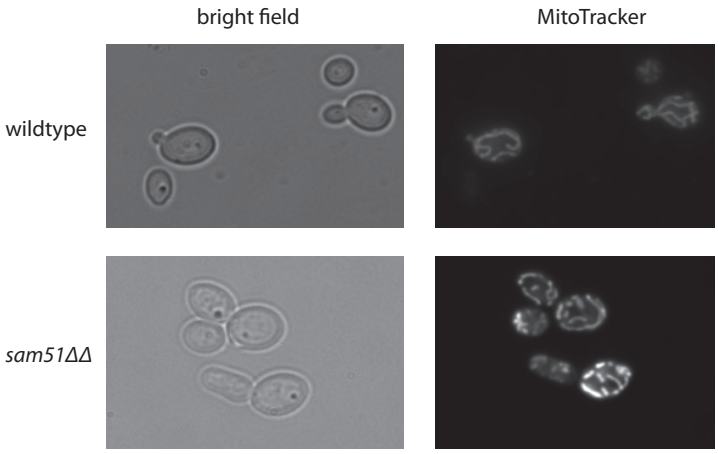


Figure 5.3 Mitochondrial morphology is unaffected in the *sam51ΔΔ* strain
Representative fluorescent images of cells of wild type (DAY185) and *sam51ΔΔ* strains stained with Mitotracker dye as described in the Materials and Methods Section 2.9.

5.1.5 No changes in protein or transcript levels in the *sam51ΔΔ* strain

To narrow down which aspects of mitochondrial function might be affected by loss of Sam51, I again used qPCR to determine transcript levels of a number of mitochondrial import components (Figure 5.4). Again there were no significant changes in any of the measured transcripts.

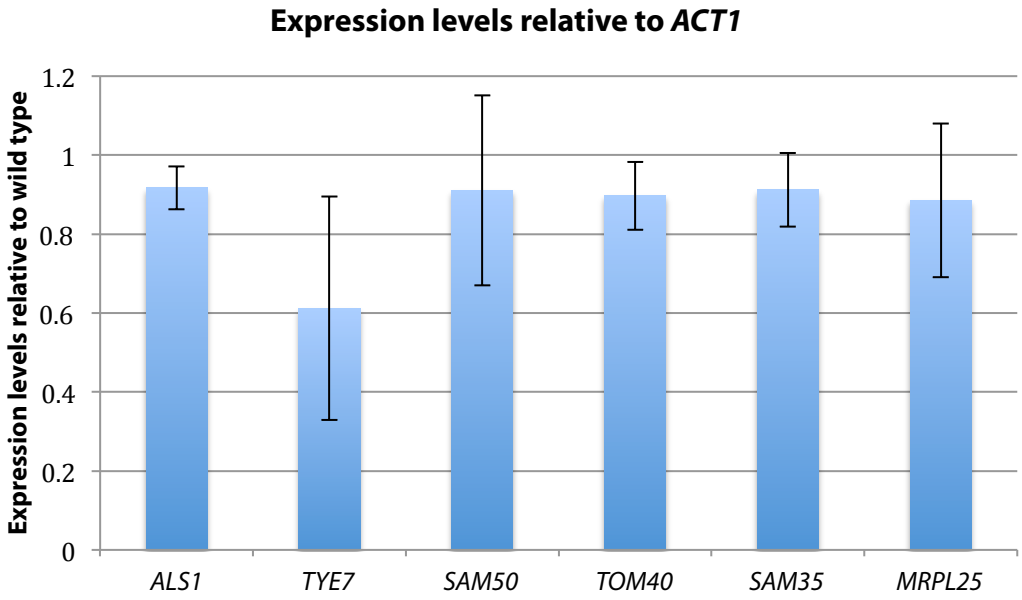


Figure 5.4 Transcripts in *sam51ΔΔ* strain compared to wild type
ALS1, TYE7, SAM50, TOM40, SAM35 and *MRPL25* expression levels were determined from RNA extracted from cells grown in rich media with glucose as a carbon source. Averages are shown from three independent cultures and the error bars show the standard deviation. Primers are listed in Appendix 3: Table 8.8.

These results were confirmed using western blots with available antibodies (Figure 5.5). No changes in steady state levels of any of the mitochondrial proteins were detected.

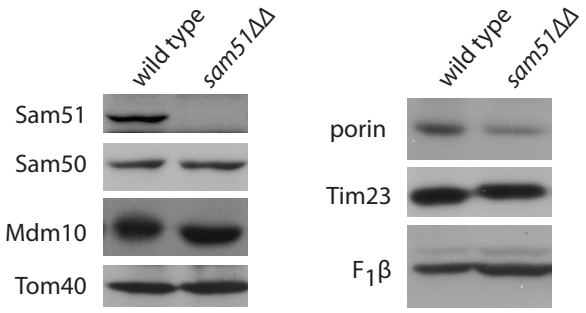


Figure 5.5 Mitochondrial protein levels in the *sam51ΔΔ* strain
Mitochondrial samples (50 µg per lane) from the wild type and *sam51ΔΔ* strains were analysed by SDS-PAGE and western blotting with the indicated antibodies. Tom70 (outer membrane), Tim10 (intermembrane space), Tim23 (inner membrane) and $F_1\beta$ (matrix) proteins are shown as loading controls.

5.1.6 No growth defect in the *sam51ΔΔ* strain

My preliminary growth tests showed no differences in the growth of the *sam51ΔΔ* strains compared to wild type on rich or minimal media at 30 °C or 37 °C (Figure 5.6). As Sam51 is a mitochondrial protein I screened growth conditions where alterations in mitochondrial fitness might be detected in the *sam51ΔΔ* strain. I included the *ccr4* deletion strain as it is both temperature sensitive and sensitive to cell wall targeting drugs due to the role of Ccr4 in post-translational regulation (Dagley, Gentle et al. 2011). These screens included mitochondrial membrane potential inhibitors (valinomycin, antimycin, oligomycin, CCCP, Appendix 8: Figure 8.5), altered iron concentrations (iron sulfate, iron citrate, the iron chelator BPS, Appendix 8: Figure 8.6), and antifungal drugs (calcofluor white, Appendix 8: Figure 8.7). There were no differences in the growth of the *sam51ΔΔ* strain compared to wild type in any of the conditions tested (nor additional assays testing other antifungal drugs performed by Tricia Lo).

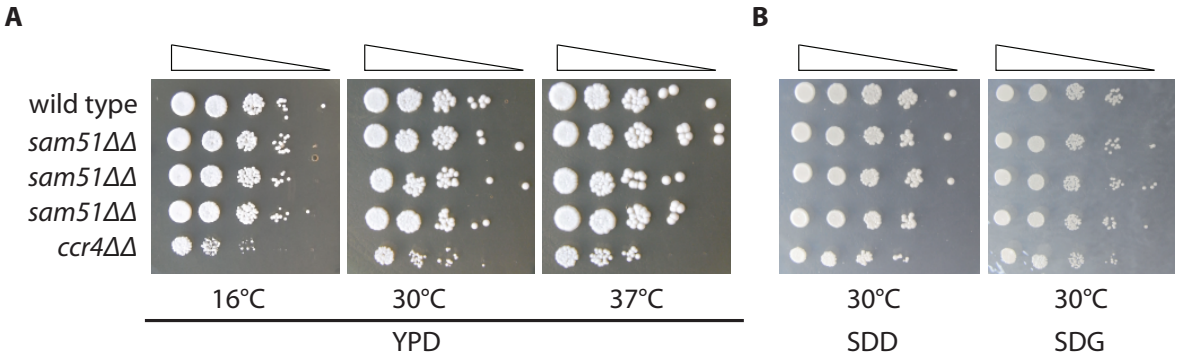


Figure 5.6 Drop tests comparing growth of wild type and *sam51ΔΔ* strains
Wild type *C. albicans* (DAY186) and three clones of the *sam51ΔΔ* strain and *ccr4ΔΔ* were grown on (**A**) rich (YPD) or (**B**) minimal media (SDD or SDG) at the indicated temperatures. Cells were grown overnight in the same media on which they were to be plated and diluted to an OD600 of 0.2 and grown for a further 3 h before ten-fold serial dilutions were plated.

5.1.7 Protein import assays in the *sam51ΔΔ* strain

Due to the sequence similarity between Sam51 and Sam50, I predicted that Sam51 would play a role in beta barrel protein import and assembly in *C. albicans*. While I saw no assembly defect in the porin import assay at either 25 °C or 16 °C (Figure 5.7), there was a slight defect in the Tom40 assembly assay even at 25 °C (Figure 5.8A).

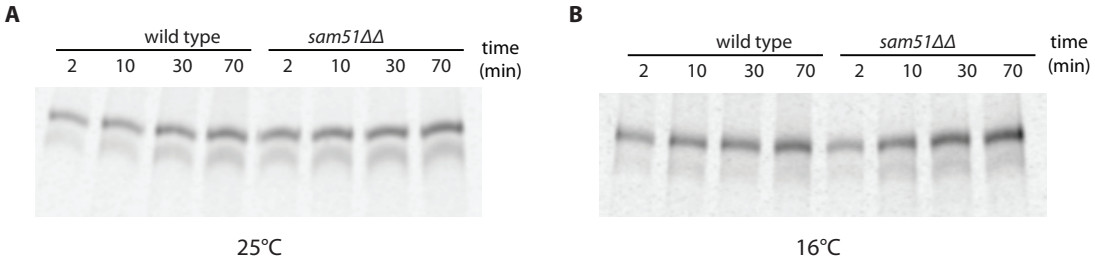


Figure 5.7 Porin assembly assays in *sam51ΔΔ* mitochondria
Mitochondria isolated from wild type *C. albicans* were incubated with ^{35}S-ScPor2 at **(A)** 25 °C or **(B)** 16 °C and samples taken at the indicated time points. Samples were separated by BN-PAGE and detected using autoradiography.

Lowering the temperature of import assay reduced the TOM complex assembly rate and made the Tom40 assembly defect in the *sam51ΔΔ* mitochondria clearer (Figure 5.8B). The defect was also clearer in the pulse chase experiment (Figure 5.8C), and shows that Tom40 associates more slowly with the SAM complex

and is released more slowly from the SAM complex in the *sam51ΔΔ* mitochondria. Antibody shift experiments show that the size and behaviour of the SAM intermediate containing Sam50 is the same in the *sam51ΔΔ* mitochondria as wild type (Figure 5.8D compared to Figure 3.10) and that Sam51 is not present in this intermediate.

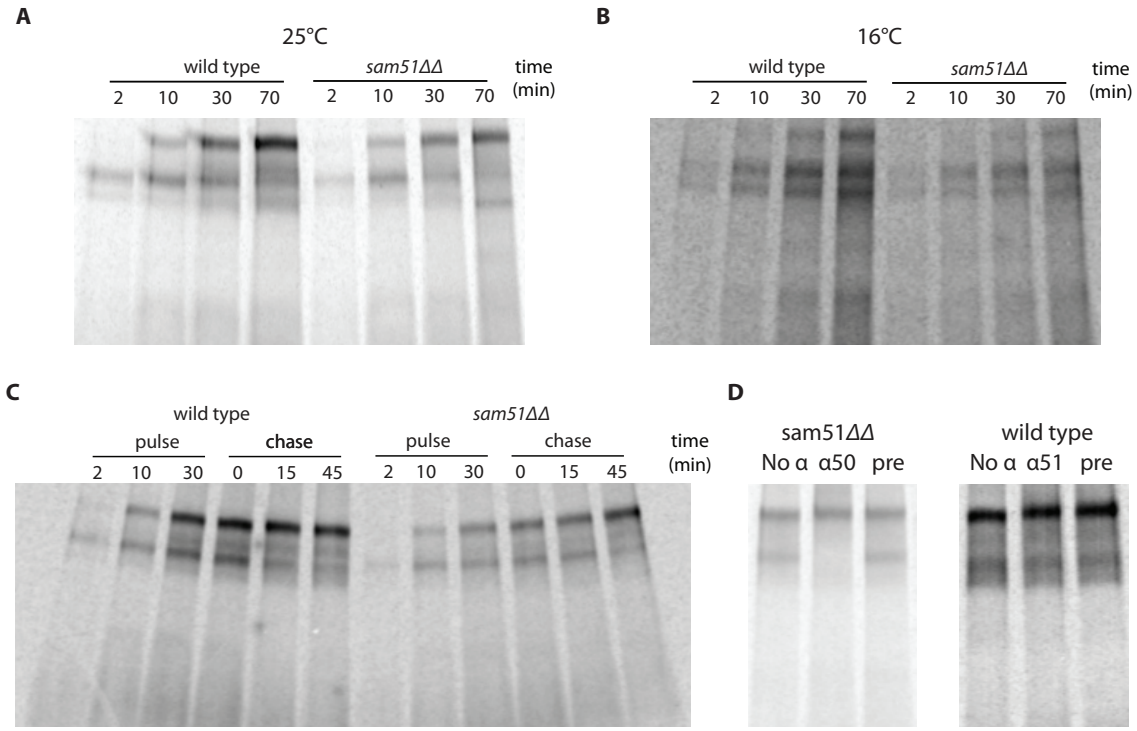


Figure 5.8 Tom40 assembly assays in *C. albicans sam51ΔΔ* mitochondria
Mitochondria isolated from wild type or *sam51ΔΔ C. albicans* strains were incubated with ^{35}S-CaTom40 at **(A)** 25 °C or **(B)** 16 °C and samples taken at the indicated time points. For the pulse chase experiment **(C)** samples were taken from mitochondria incubated with ^{35}S-CaTom40 at 25 °C at the indicated time points in the "pulse" samples. After 30 min the mitochondria were washed and resuspended in import buffer at 25 °C and samples were taken at the indicated "chase" times. Samples were separated by BN-PAGE and detected using autoradiography. For the antibody shift experiments **(D)** mitochondria were incubated with ^{35}S-CaTom40 at 25 °C for 30 min, solubilised in digitonin and incubated with antibodies against Sam50 (α50) or Sam51 (α51), no antibody (no α) or the corresponding pre-immune serum (pre) as indicated, before separation by BN-PAGE and detection using autoradiography.

5.2 Characterisation of the Sam37 deletion strains

In *S. cerevisiae* the roles of Sam35 and Sam37 are interdependent and loss of Sam37 also results in less Sam35 in a complex with Sam50 (Ishikawa, Yamamoto et al. 2004, Chan and Lithgow 2008). In *S. cerevisiae* the mild defects associated with loss of Sam37 can be mitigated by the expression of high levels of Sam35, but

expression of high levels of Sam37 cannot compensate for the loss of Sam35 (Chan and Lithgow 2008). In *C. albicans* the deletion of Sam37 results in a more severe growth defect than in *S. cerevisiae* and makes *S. cerevisiae* and *C. albicans* more sensitive to antifungal drugs (Dagley, Gentle et al. 2011).

5.2.1 Growth is partially rescued by increased *SAM35* expression

To investigate the specific role of Sam37 in the *C. albicans* SAM complex in more detail I used a *C. albicans* strain where *SAM35* was expressed at high levels (*sam37ΔΔSAM35*↑). The high levels of Sam35 partially rescue this growth defect (Figure 5.9) (Qu, Jelicic et al. 2012).

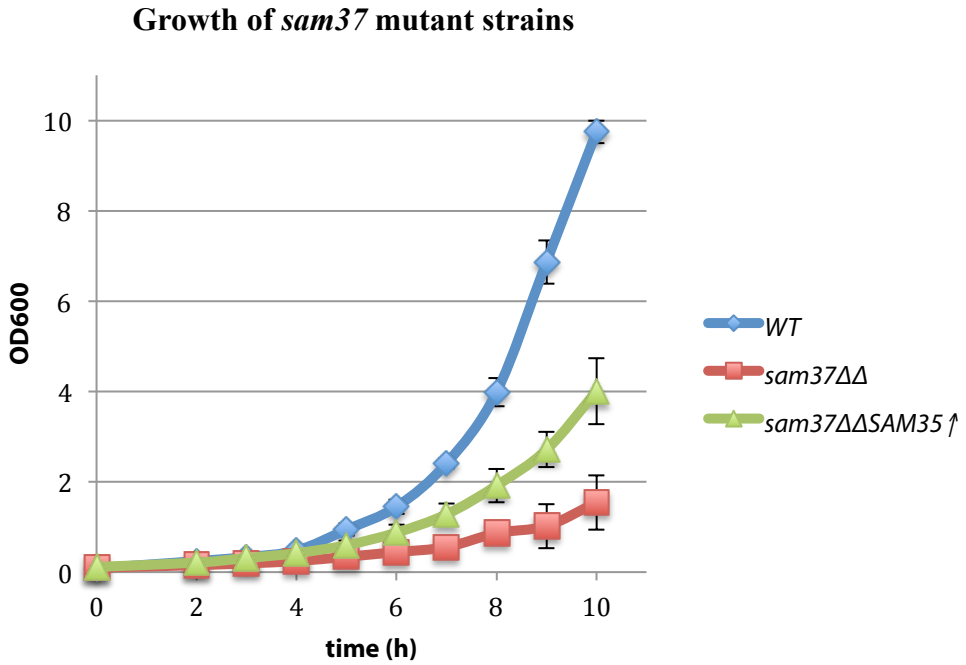


Figure 5.9 Growth rates of *sam37ΔΔ* strains
The indicated strains were grown at 30 °C in overnight cultures, diluted to an OD600 of 0.2 and then growth was monitored for a further 10 h. Error bars show standard deviation for three biological repeats.

5.2.2 Reduced steady state levels of Mdm10

I used western blotting used to determine whether reduced levels of other mitochondrial proteins might be contributing to the fitness defects of the *sam37ΔΔ* strains (Figure 5.10). I found the steady state levels of Mdm10 were drastically reduced in the *sam37ΔΔ* strain but returned to wild type levels in the

sam37ΔΔSAM35↑ strain and in the strain with a single copy of Sam37 restored (*sam37ΔΔSAM37*). The steady state levels of the other ERMES component Mdm34 were also altered, but Mdm34 levels were increased relative to wild type in both the *sam37ΔΔ* and *sam37ΔΔSAM35↑* strains. This suggests that changes to the SAM complex can affect the ERMES independently of Mdm10 levels. Steady state levels of Sam51 were also reduced in both the *sam37ΔΔ* and *sam37ΔΔSAM35↑* strains but returned to wild type levels in the *sam37ΔΔSAM37* strain. The steady state levels of the other beta barrel proteins Sam50 and porin were unaffected but there was a slight reduction in the Tom40 levels.

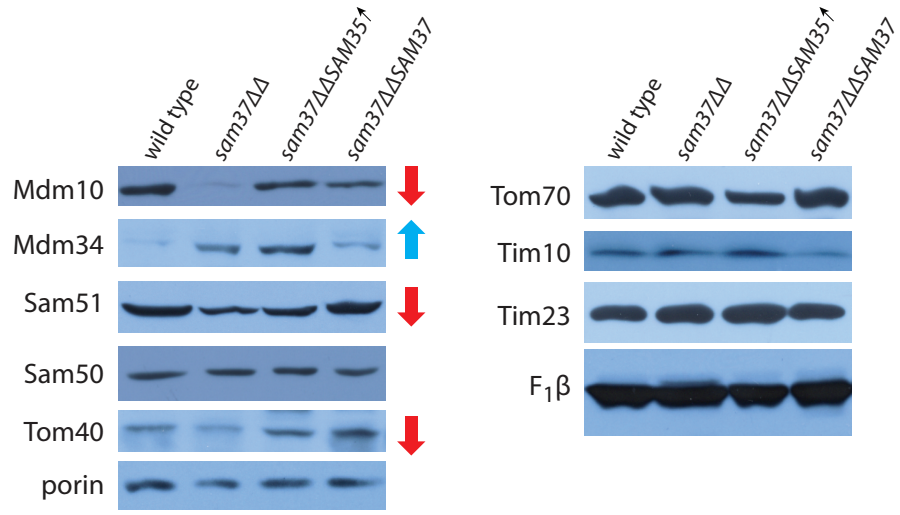


Figure 5.10 Steady state protein levels in *sam37ΔΔ* strains
Mitochondrial samples (50 µg per lane) from the indicated strains were analysed by SDS-PAGE and western blotting with the indicated antibodies. Tom70 (outer membrane), Tim10 (intermembrane space), Tim23 (inner membrane) and $F_1\beta$ (matrix) proteins are shown as loading controls. Arrows indicate substantial changes in the protein levels.

5.2.3 Protein import into the matrix is unaffected

I assayed the import of various matrix-targeted proteins to investigate whether an import defect may be the cause of the growth defects in the *sam37ΔΔ* strain. There was no defect in the import of su9-DHFR into the *sam37ΔΔ* strain (Figure 5.11A). To confirm if this was true of other matrix proteins in the absence of Sam37 I performed the remaining assays using the *sam37ΔΔSAM35↑* strain since the extremely poor

growth of the *sam37ΔΔ* strain made it difficult to isolate sufficient mitochondria. The other matrix proteins $F_1\beta$, Adh3 and CoxVa were all imported into the *sam37ΔΔSAM35↑* strain with no defect (Figure 5.11B).

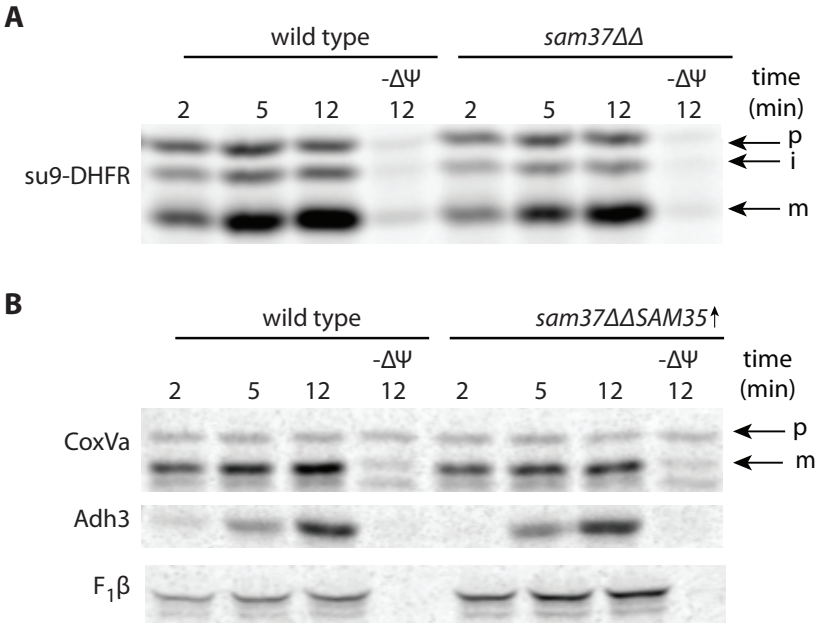


Figure 5.11 Matrix protein import in *sam37ΔΔ* strains
(A) Isolated mitochondria from wild type and *sam37ΔΔ* strains were incubated with the labelled precursor proteins at 25 °C and samples taken at the indicated time points and treated with trypsin. Samples were separated by SDS-PAGE and detected by autoradiography. The mitochondrial membrane potential was disrupted before addition of precursor in the -ΔΨ lanes. **(B)** As for (A) but comparing wild type and the *sam37ΔΔSAM35↑* strains. Where protein processing steps are visible the precursor (p), intermediate (i) and mature (m) forms are labelled.

I also assessed the membrane potential by measuring the drop in fluorescence produced by TMRM (tetramethylrhodamine methyl ester) on addition of a membrane potential inhibitor cocktail (Methods Section 2.11). The drop in membrane potential was 9.0 % ± 2.7 for the wild type mitochondria and 7.5 % ± 1.3 for the *sam37ΔΔ* strain. These results are averages (with standard deviations) for three separate mitochondrial preparations and indicate there is no difference in the membrane potential of these strains.

5.2.4 Import of porin is mildly impaired

The import and assembly of porin is radically impaired in *S. cerevisiae sam37Δ* mitochondria (Wiedemann, Kozjak et al. 2003). Despite the severe growth defects of the *C. albicans sam37ΔΔ* strain, the import of porin as measured by SDS-PAGE is unaffected and assembly of porin is only mildly impaired (Figure 5.12).

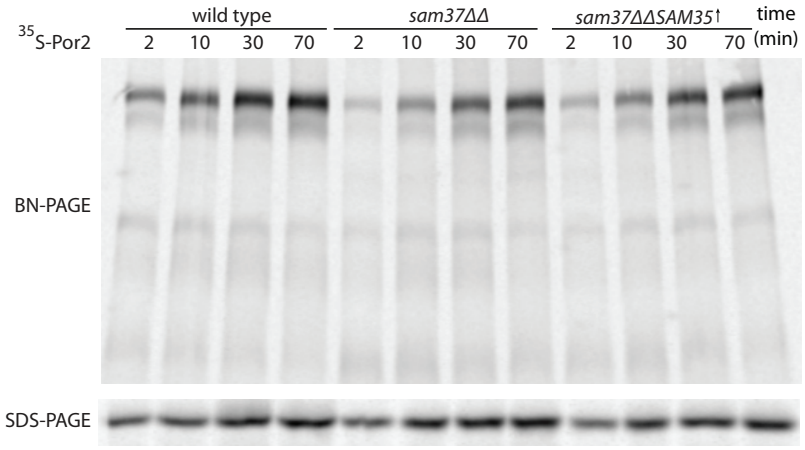


Figure 5.12 Porin assembly in *sam37ΔΔ* mitochondria
Mitochondria isolated from the indicated strains were incubated with ^{35}S-ScPor2 at 25 °C and samples taken at the indicated time points. Samples were separated by BN-PAGE (top panel) or SDS-PAGE (bottom panel) and detected using autoradiography.

5.2.5 Import of Tom40 is drastically impaired

Previous work has shown that Sam35 is required for entry of substrates into the SAM complex, and Sam37 is required for exit of substrates from the SAM complex (Chan and Lithgow 2008, Kutik, Stojanovski et al. 2008). Mitochondria isolated from the *C. albicans sam37ΔΔ* strain have a compound defect, with less Tom40 being imported into the mitochondria as determined by SDS-PAGE and slower association and release of Tom40 from the SAM complex (Figure 5.13). When Sam35 is expressed at high levels in the *sam37ΔΔ* background (*sam37ΔΔSAM35*↑) there is a slight improvement in Tom40 assembly, but the assembly rate is still much slower than in wild type mitochondria.

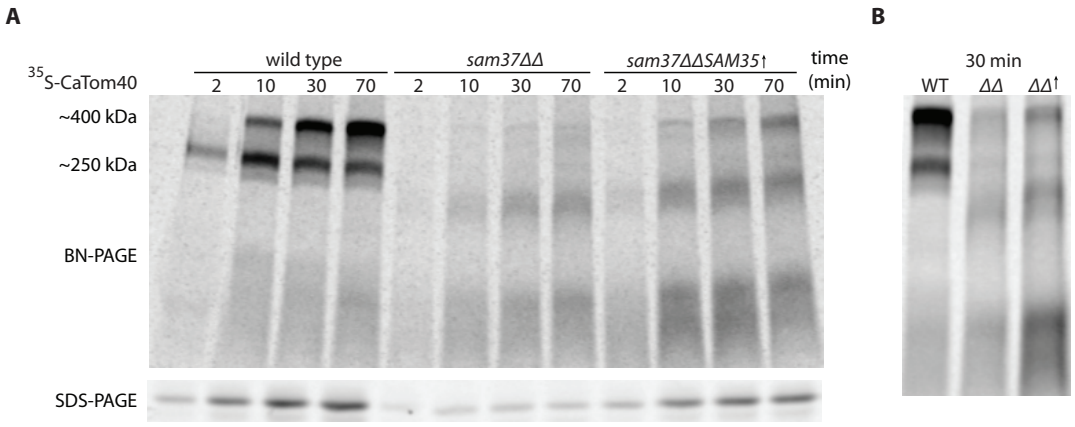


Figure 5.13 Tom40 assembly in *sam37ΔΔ* mitochondria
(A) Mitochondria isolated from the indicated strains were incubated with ^{35}S-CaTom40 at 25 °C and samples taken at the indicated time points. Samples were separated by BN-PAGE (top panel) or SDS-PAGE (bottom panel) and detected using autoradiography. **(B)** Additional samples taken at 30 min from each strain are shown in the right panel for comparison.

5.3 Mdm10 import and assembly roles in *C. albicans* mitochondria

While its association with the SAM complex during import has been known for some time (Meisinger, Rissler et al. 2004), the contributions of the individual SAM complex components to the Mdm10 import pathway are not known. Ten years after Mdm10 was found to associate with the SAM complex (Meisinger, Rissler et al. 2004), its precise role in protein import and assembly also remains unclear (Thornton, Stroud et al. 2010, Wideman, Go et al. 2010, Yamano, Tanaka-Yamano et al. 2010, Yamano, Tanaka-Yamano et al. 2010). After finding a dramatic reduction in Mdm10 levels in the *sam37ΔΔ* strain (Figure 5.10), I wanted to determine whether the loss of Mdm10 was contributing to the phenotype of this strain and use *C. albicans* to investigate the role of Mdm10 in protein import and assembly more generally.

5.3.1 Import of Mdm10 is drastically impaired but rescued by Sam35

Comparing import assays of CaMdm10 into mitochondria from wild type *C. albicans* with *S. cerevisiae* assays shows a similar pattern of complexes at ~300 and ~500 kDa but no significant radioactive material at ~100 kDa (Figure 5.14). Like in *S. cerevisiae,* the ~300 kDa complex is protease resistant in whole mitochondria (Figure 5.14C) (Paschen, Waizenegger et al. 2003), indicating it is protected by the

mitochondrial outer membrane. I introduced a washing step to remove un-imported material on the surface of the mitochondria and used bovine serum albumin in the import reaction to reduce the surface accumulation of precursor proteins. This is likely the reason I do not detect large amounts of the ~100 kDa, protease accessible radiolabelled material (Paschen, Waizenegger et al. 2003).

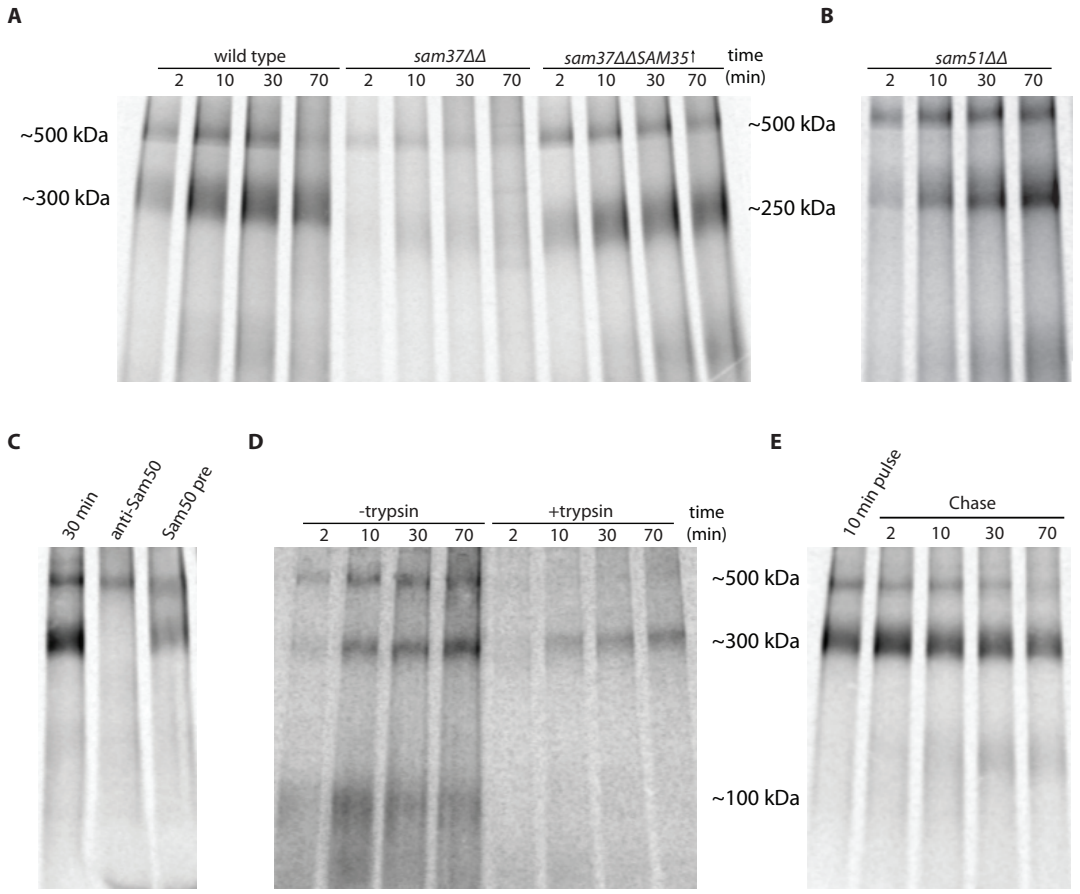


Figure 5.14 Mdm10 assembly in *sam37ΔΔ* mitochondria
(A) Mitochondria isolated from the indicated strains were incubated with ^{35}S-CaMdm10 at 25 °C and samples taken at the indicated time points. Samples were separated by BN-PAGE and detected using autoradiography. **(B)** Antibody shift experiment where wild type mitochondria were incubated with ^{35}S-CaMdm10 at 25 °C for 30 min, solubilised in digitonin and incubated with antibodies against Sam50 or pre-immune serum, before separation by BN-PAGE and detection using autoradiography. **(C)** Incubation of ^{35}S-CaMdm10 with wild type mitochondria as in (A) but with trypsin treatment after the reaction. **(D)** Pulse chase experiment where ^{35}S-CaMdm10 was incubated with wild type mitochondria for 10 min before mitochondria were re-isolated and washed and the reaction allowed to continue with samples taken at the indicated times, separated by BN-PAGE and detected using autoradiography.

I confirmed that the ~300 kDa complex in *C. albicans* also contains Sam50 (Figure 5.14B) and found the formation of this complex was severely impaired in the *sam37ΔΔ* strain (Figure 5.14A). The Mdm10-SAM complex assembly levels are restored to almost wild type levels by the over-expression of Sam35 (Figure 5.14). The complex is smaller reflecting the loss of Sam37, but the restored binding in the *sam37ΔΔSAM35↑* strain indicates that Sam37 is not required for Mdm10 to associate with the SAM complex. This fits well with previous findings showing Sam35 acts as a receptor; Sam35 mutations reduced Tom40 binding to the SAM complex (Chan and Lithgow 2008) and Sam35 depletion reduced the rate the ~300 kDa complex formed in *S. cerevisiae* (Fig 4B, (Waizenegger, Habib et al. 2004)).

In *S. cerevisiae* this ~500 kDa complex is depleted when incubated with beads bound to antibodies against Fis1 (Figure 4d, (Paschen, Waizenegger et al. 2003)), suggesting Mdm10 may interact with this outer membrane protein. This ~500 kDa complex is labelled as "non-specific" (Paschen, Waizenegger et al. 2003, Wiedemann, Kozjak et al. 2003), so this result is not discussed in the paper. No explanation is provided in the literature for this labelling and more recent papers describe this complex as "uncharacterised" (Yamano, Tanaka-Yamano et al. 2010), so I took the opportunity to characterise this complex in more detail. The complex is protease accessible (Figure 5.14C), suggesting at least the Mdm10 component is not completely protected by the outer membrane. The reduced amount of the ~500 kDa complex in the Sam37 deletion strains suggests the complex is not simply non-specific binding of Mdm10 as it requires SAM complex components for its formation. The pulse chase experiment shows a decreasing amount of Mdm10 in both the ~300 and ~500 kDa forms (Figure 5.14E), suggesting neither form represents a stable, mature Mdm10 complex.

Results in *S. cerevisiae* were similar, however, the authors interpret this import assay as showing Mdm10 assembling into the mature SAM_{holo} complex (Yamano, Tanaka-Yamano et al. 2010). In my results the mature SAM_{holo} complex containing Mdm10 (see Section 6.3) is ~400 kDa and a different size from the complexes formed in the ^{35}S-Mdm10 assembly assay, so cannot be the same complex. While this size difference may be clearer in my assay, it is also noticeable in the results of Yamano and colleagues in *S. cerevisiae* (compare Figures 3A and 1SE,

(Yamano, Tanaka-Yamano et al. 2010)). I favour the interpretation of other researchers that the ~300 kDa form is an assembly intermediate where Mdm10 associates with the SAM complex (Paschen, Waizenegger et al. 2003), but is not released from the SAM complex in mitochondrial extracts. The fact that Mdm10 is not released from the SAM complex may explain why Sam37, important in release of SAM substrates, is less important for Mdm10 to be imported or associate with the SAM complex. This also indicates Mdm10 cannot assemble into the ~400 kDa complex in isolated mitochondria, perhaps requiring components of the ERMES to assemble properly.

5.3.2 Mdm10 depletion did not alter other protein levels

To investigate the role of Mdm10 in protein import and assembly I collaborated with Miguel Shingu-Vazquez and the Traven lab in an attempt to construct an *mdm10* deletion strain. This was unsuccessful so instead we constructed a conditional Mdm10 depletion strain (*mdm10*↓) by deleting one copy of the gene and placing the second copy under the *MET3* promoter. Addition of cysteine and methionine to the growth media represses gene expression resulting in protein levels diminishing over time (top panel, Figure 5.15). My work characterising the defects in mitochondrial function when Mdm10 is depleted are discussed below, and further characterisation of the strain was performed in collaboration with the Traven Lab.

To separate the direct and downstream effects of Mdm10 depletion I used western blotting of isolated mitochondria to detect changes in steady state levels of proteins associated with these mitochondria (Figure 5.15). The amount of Mdm10 present in the isolated mitochondria decreased when the cells were grown in repressive conditions and after 15 hours no Mdm10 was detected by western blotting (top panel, Figure 5.15). The only other protein that changed in conjunction with Mdm10 depletion was another component of the ERMES complex, Mdm34, which increased as Mdm10 was depleted (Figure 5.15). The levels of Tom40 and Tom22 are reduced in the *S. cerevisiae mdm10Δ* strain (Meisinger, Rissler et al. 2004), whereas the levels of Tom40 and Tom20 in the *C. albicans mdm10*↓ strain were unchanged after 15 hours in repressive conditions. The *S. cerevisiae mdm10Δ* strain also has altered phospholipid content (Kornmann, Currie et al. 2009, Yamano, Tanaka-Yamano et al. 2010), which could also alter assembly of proteins into the outer

membrane. Together these features make the *C. albicans mdm10↓* strain an excellent system in which to determine the specific role of Mdm10 in protein import and assembly.

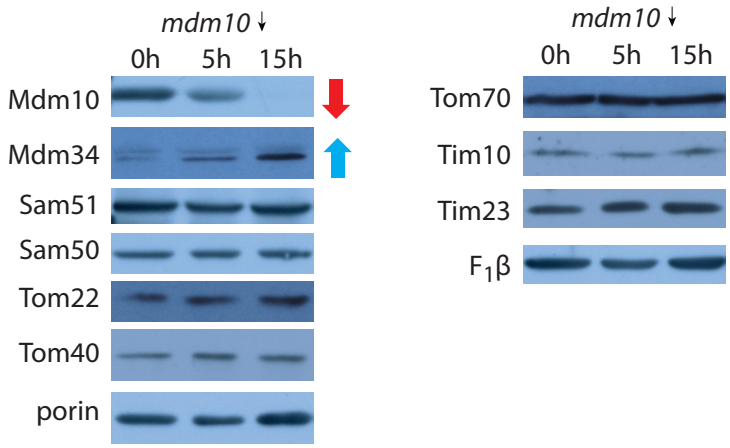


Figure 5.15 Steady state protein levels in *mdm10* depletion strain
Mitochondria (50 µg per lane) were prepared from the *mdm10↓* strain grown in permissive (0 h) or repressive conditions for 5 h or 15 h. Mitochondrial samples were analysed by SDS-PAGE and western blotting with the indicated antibodies. Tom70 (outer membrane), Tim10 (intermembrane space), Tim23 (inner membrane) and $F_1\beta$ (matrix) proteins are shown as loading controls. Arrows indicate substantial changes in the protein levels.

5.3.3 Reduced levels of Mdm10 impair beta barrel protein assembly

Since Mdm10 levels were dramatically reduced in the *sam37ΔΔ* strain I used the *mdm10↓* strain to determine whether depletion of Mdm10 alone caused similar beta barrel protein assembly defects. Import of the model protein Su9-DHFR shows the membrane potential and matrix import processes are unaffected by the depletion of Mdm10 (Figure 5.16A). Assembly of porin into a trimer and association of newly imported Mdm10 with the SAM complex are also both slower when Mdm10 levels are depleted (Figure 5.17B and C).

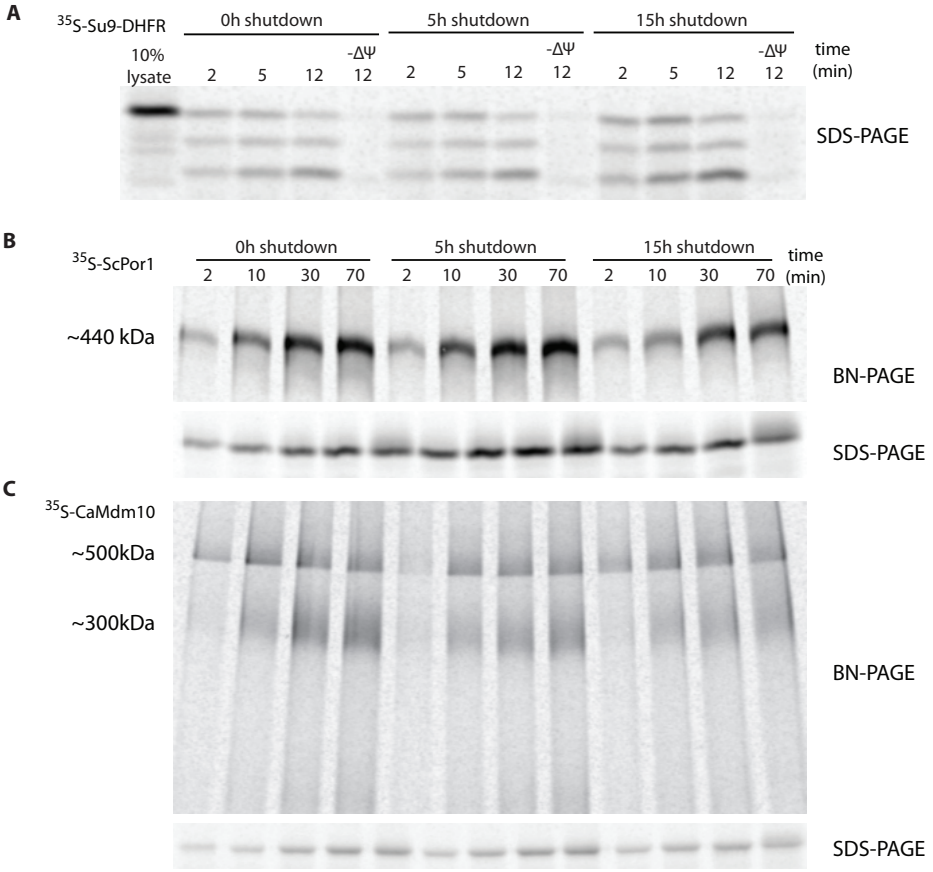


Figure 5.16 Depletion of Mdm10 slows beta barrel protein assembly
Mitochondria prepared from the *mdm10*↓ strain after growth in permissive (0 h) or repressive conditions for 5 h or 15 h. **(A)** These mitochondria were incubated with ^{35}S-Su9-DHFR for the indicated times. In the –ΔΨ lanes, the membrane potential was dissipated before the precursor protein was added. Import was then analysed by SDS-PAGE and phosphorimaging. **(B)** These mitochondria were incubated with ^{35}S-ScPor1 and samples taken at the indicated time points. Samples were separated by BN-PAGE (top panel) or SDS-PAGE (bottom panel) and detected using autoradiography. **(C)** As for (B) but using ^{35}S-CaMdm10.

5.3.4 Slowed TOM complex assembly after Mdm10 depletion

In *S. cerevisiae* the deletion of Mdm10 causes a defect in the assembly of Tom40 (Meisinger, Rissler et al. 2004). I used the *C. albicans mdm10*↓ strain to analyse the assembly of the TOM complex components Tom40, Tom22 and Tom5 into the TOM complex and I found a general defect in TOM complex assembly (Figure 5.17).

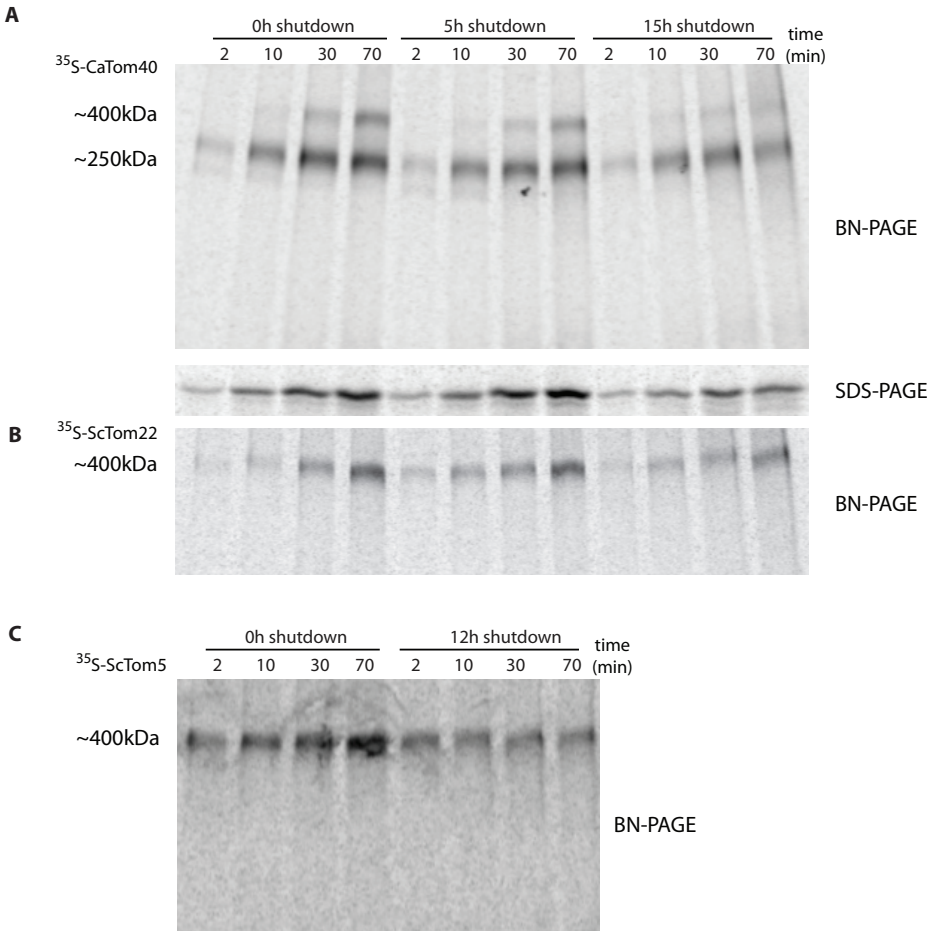


Figure 5.17 Depletion of Mdm10 slows TOM complex assembly
Mitochondria prepared from the *mdm10*↓ strain after growth in permissive (0 h) or repressive conditions for 5 h or 15 h. **(A)** These mitochondria were incubated with ^{35}S-CaTom40 and samples taken at the indicated times. Samples were separated by BN-PAGE (top panel) or SDS-PAGE (bottom panel) and detected using autoradiography. **(B)** As for (A) but using ^{35}S-ScTom22. **(C)** As for (A) ^{35}S-ScTom5.

5.4 Discussion

Here I discuss the implications of the growth and import defects reported in this chapter and their relationship to published work.

5.4.1 Sam51 has a minor role in TOM complex assembly

The deletion of Sam51 resulted in no detectable growth defect in any of the conditions tested. The only difference I identified in the *sam51ΔΔ* strain was a slower association of Tom40 with the SAM complex intermediate, which also delays assembly into the mature TOM complex (Figure 5.8). This assembly defect was specific to Tom40 assembly and leaves porin and Mdm10 assembly unaffected. This suggests Sam51 may play a specific role in the early stages of assembly of Tom40

into the TOM complex and/or association of Tom40 with the SAM intermediate. The antibody shift experiment (Figure 5.8) shows that Sam51 does not function in the place of Sam50 in the Tom40 assembly intermediate.

Given that *C. albicans* and a number of other yeast species have maintained *SAM51* genes it seems likely that this protein has a specific or important regulatory role in growth conditions that we have not yet identified. For example, the role of mitochondria in the development of hyphae and filaments in *C. albicans* has not yet been determined and there may be specific requirements for regulation of protein import into mitochondria in these growth forms of *C. albicans*.

5.4.2 Import defects reduce steady state protein levels

The severe growth defect in the *C. albicans sam37ΔΔ* strain at the optimal growth temperature of 30 °C suggests the role of Sam37 in *C. albicans* is more important and/or it has additional functions not required in *S. cerevisiae.* The loss of Sam37 resulted in reduced levels of Mdm10 and Sam51 so the loss of these proteins might be contributing to the phenotypes of the *sam37ΔΔ* strain. The role of Sam37 in phospholipid trafficking both in *S. cerevisiae* (Gratzer, Lithgow et al. 1995) and *C. albicans* (Dagley, Gentle et al. 2011), may also be explained by the loss of Mdm10.

Since the Sam51 deletion strain has no growth defect and only a mild import defect it is unlikely that the loss of Sam51 in the *sam37ΔΔ* is solely responsible for the growth defect. However, since the levels of Sam51 are not restored in the *sam37ΔΔSAM35↑* strain, Sam37 may be particularly important for import and/or assembly of Sam51. There was also no change in the assembly of Mdm10 in the Sam51 deletion strain, suggesting the reduced levels of Sam51 are a direct result of loss of Sam37 and not dependant on Mdm10.

The ^{35}S-Mdm10 import assays in the *sam37ΔΔ* strains are consistent with the reduced steady state levels of Mdm10; import is dramatically reduced in the *sam37ΔΔ* strain and restored in the *sam37ΔΔSAM35↑* strain showing the loss of Mdm10 is due to the import defect. This also shows a distinct role for Sam35 in the binding of Mdm10 to the SAM complex similar to what was seen in the Tom40 import assays in *sam37ΔΔSAM35↑* mitochondria (Chan and Lithgow 2008). In the *S. cerevisiae sam37Δ* strain there are reduced levels of Mdm10 (Meisinger, Rissler et al. 2004) and

over-expression of Mdm10 partially suppresses the growth defect in this strain at 37 °C (Meisinger, Pfannschmidt et al. 2007). These results, in *C. albicans* and *S. cerevisiae,* are also consistent with the idea that some of the defects in the *sam37* deletion strains are due to loss of Mdm10.

5.4.3 Tom40 assembly defect in the Sam37 deletion strains

The loss of Sam37 has a major impact on the assembly of the TOM complex (Figure 5.13), with only a very small amount of Tom40 associating with the SAM complex in Intermediate I. There is a slight improvement in the assembly process when *SAM35* is overexpressed but the improvement in binding of Tom40 to the debilitated SAM complex is only minor. In *S. cerevisiae* the over-expression of *SAM35* restores Tom40 binding to the SAM complex and Tom40 is assembled into the TOM complex at nearly wild type levels. Therefore this result in *C. albicans* is quite different from the equivalent experiment in *S. cerevisiae* where the over-expression of *SAM35* completely restored Tom40 assembly (Chan and Lithgow 2008), and suggests the functions of Sam37 and Sam35 in Tom40 assembly are not as distinct as seen in *S. cerevisiae.* While over-expression experiments have not been reported in *N. crassa,* the fact that both Sam37 and Sam35 are essential (Lackey, Wideman et al. 2011) shows that the importance of these proteins in the assembly process varies considerably between species.

SAM35 over-expression can suppress the Mdm10 assembly defect in the *sam37Δ* strain but not the Tom40 assembly defect. This may be due to the intimate coordination required between the TOM and SAM complexes. Over-expression of both *MDM10* and *TOM6* can also partially suppress the growth defect at 37 °C in the *S. cerevisiae sam37Δ* strain (Dukanovic, Dimmer et al. 2009). There is also some evidence that this may be because additional Tom6 helps stabilise the TOM complex in the *sam37Δ* strain but only minor differences were observed (Dukanovic, Dimmer et al. 2009). I hypothesise these growth and assembly defects seen in the *sam37* deletion strains may be due to the reduced stability of the recently discovered TOM-SAM super-complex (Qiu, Wenz et al. 2013). This super-complex is completely destabilised in the absence of Sam37 (or Tom22) and appears to be particularly important for assembly of Tom40 into the TOM complex.

5.4.4 Assembly defects in the Mdm10 depletion strain

In *C. albicans* I found similar assembly defects for Tom40, Tom22 and Tom5 import into the *mdm10*↓ strain (Figure 5.17). Similar assembly defects are seen for the TOM complex components in the *S. cerevisiae* Mdm10 deletion strain (Meisinger, Rissler et al. 2004). In *S. cerevisiae mdm10Δ*, Tom40 accumulates in assembly Intermediate II after being released from the SAM complex (Fig 2G, (Meisinger, Rissler et al. 2004)). Tom22 assembly into the TOM complex is also impaired in the *mdm10Δ* strain and accumulates in an intermediate form ~400 kDa (labelled Intermediate III) but can be chased into mature TOM complex (Fig 4B, (Meisinger, Rissler et al. 2004)). There are also severe defects in Tom6 and Tom7 assembly and a milder defect in Tom5 assembly (Fig 4F, (Meisinger, Rissler et al. 2004)). While the impact on the overall assembly rate of these components is similar in the two systems, I observed no accumulation of Tom40 in Intermediate II or accumulation of Tom22 into the ~400 kDa Intermediate III, perhaps indicating that the assembly of Tom22 in *C. albicans* is more efficient.

The differences been *S. cerevisiae* and *C. albicans* were even greater when I examined the role of Mdm10 in the import of the other beta barrel proteins porin and Mdm10 itself (Figure 5.16). Depletion of Mdm10 in *C. albicans* resulted in slower assembly of the porin trimer and slower association of Mdm10 with the SAM complex, while in *S. cerevisiae* the import of these proteins appeared to improve slightly in the absence of Mdm10 (Meisinger, Rissler et al. 2004). There is also considerable variation in the phenotypes of the *mdm10* deletion strains in different species. Large spherical mitochondria are seen in *S. cerevisiae mdm10Δ* (Meisinger, Wiedemann et al. 2006) but are not observed in the *Aspergillus nidulans mdm10Δ* strain unless the cells are grown at low temperature (Koch, Suelmann et al. 2003) and *N. crassa mdm10Δ* strain forms thicker, but still tubular mitochondria (Wideman, Go et al. 2010). One possible explanation for these differences is that the importance or specific role of Mdm10 within the SAM and/or ERMES complexes is different in different species. To investigate this further I examined the steady state complex compositions of the *C. albicans* complexes containing Mdm10 (See Chapter 6).

6 Biochemical characterisation of the SAM complex

Having explored the functions of the *C. albicans* SAM complex proteins Sam51, Sam37 and Mdm10 in Chapter 5, this chapter examines the composition and organization of the SAM complex itself. I begin by reviewing the current understanding of the structure and organization of the SAM complex (Section 6.1). This is followed by a biochemical examination of the *C. albicans* SAM complex and makes use of the mutants, antibodies and BN-PAGE methods developed in the rest of this thesis to gain a better understanding of the composition of the SAM complex. I investigate the size, stability and steady state composition of the wild type *C. albicans* SAM complex, the contribution of Sam51 to the *C. albicans* SAM complex, the effects of deleting Sam37 and the relationship between the SAM complex and Mdm10.

6.1 Forms of the SAM complex

There are two main forms of the SAM complex: a SAM_{core} complex of ~200 kDa and a larger SAM_{holo} complex of ~400 kDa. The *S. cerevisiae* SAM_{core} complex migrates at a size corresponding to a ~200 kDa complex on BN-PAGE when solubilised in 1 % digitonin (210 kDa (Wiedemann, Kozjak et al. 2003); 220-250 kDa (Paschen, Waizenegger et al. 2003), 200 kDa (Kozjak, Wiedemann et al. 2003, Yamano, Tanaka-Yamano et al. 2010)). The SAM_{core} complex in *N. crassa* and humans is a similar size (200-210 kDa (Waizenegger, Habib et al. 2004), ~200 kDa (Humphries, Streimann et al. 2005)).

The SAM_{holo} complex can be stabilised and detected in some conditions but variable ratios of the SAM_{holo} and SAM_{core} complex are reported (Meisinger, Rissler et al. 2004, Becker, Guiard et al. 2010, Yamano, Tanaka-Yamano et al. 2010). In *S. cerevisiae* the SAM_{holo} complex is more stable in high salt concentrations and in the *tom22Δ* and *tom7Δ* strains (~350 kDa (Wiedemann, Kozjak et al. 2003, Meisinger, Rissler et al. 2004, Yamano, Tanaka-Yamano et al. 2010)). In *N. crassa* strains with truncations in the Sam50 POTRA domain stabilise larger forms of the SAM complex (~350 kDa (Habib, Waizenegger et al. 2007), 370 & 280 kDa (Lackey, Wideman et al. 2011)). In human mitochondria lowering the detergent concentration also stabilises

a larger form of the SAM complex (Kozjak-Pavlovic, Ross et al. 2007). The stability of the large SAM complex also appears to be strain dependant (Habib, Waizenegger et al. 2005). Both the ~200 and ~400 kDa forms are detected by BN western using anti-Sam50 in the *S. cerevisiae* strain 273-10B but only the ~200 kDa form in the YPH499 strain. A ~170 kDa complex without Sam37 as well as some larger forms are detectable in the W303-1A strain (Ishikawa, Yamamoto et al. 2004).

The SAM_{holo} complex also contains Mdm10 (Meisinger, Rissler et al. 2004) but the large size indicates it may include other components yet to be identified. Mdm10 antibodies detect a ~400 kDa complex which is not present in the *sam37ΔΔ* strain (Meisinger, Rissler et al. 2004) and tagged components of the SAM_{core} complex or Mdm10 can all be co-purified with one another (Meisinger, Pfannschmidt et al. 2007, Becker, Wenz et al. 2011). The first report of Mdm10 in the SAM_{holo} complex and recent work using *N. crassa* showed that no smaller complexes were detected by the Mdm10 antibody and complete loss of Mdm10 in a *S. cerevisiae sam37Δ* strain (Meisinger, Rissler et al. 2004, Lackey, Wideman et al. 2011). Other papers show variable amounts of Mdm10 detected in a smaller complex ~150 kDa (*S. cerevisiae* (Meisinger, Wiedemann et al. 2006, Becker, Pfannschmidt et al. 2008, Becker, Guiard et al. 2010, Yamano, Tanaka-Yamano et al. 2010), *N. crassa* (Klein, Israel et al. 2012)). Studying the forms of the SAM complex in other organisms such as *C. albicans* will help to distinguish which of these forms are conserved and could help to unravel the processes regulating the relative stability of these two forms.

6.2 Surface exposure of the SAM complex components

To establish the topology of the SAM complex components in *C. albicans* I performed protease shaving of intact mitochondria and mitoplasts (Figure 6.1). Mitoplasts are mitochondria that have been subjected to osmotic shock, which ruptures the outer membrane enabling intermembrane space components to escape and protease to access the intermembrane space.

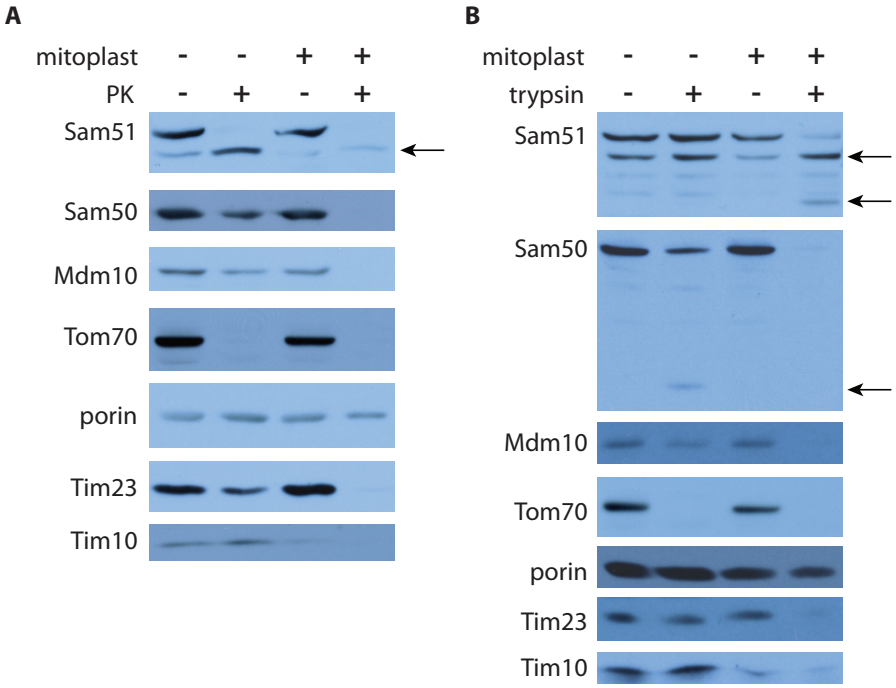


Figure 6.1 Protease shaving of mitochondria and mitoplasts
(A) Mitochondrial samples from wild type *C. albicans* were treated with 5 mg/mL proteinase K (PK) and/or the outer membrane ruptured to produce mitoplasts, as indicated. After addition of PMSF, the samples were analysed by SDS-PAGE and western blotting with the indicated antibodies. **(B)** As for (A) except using 100 µg/mL trypsin. Images are cropped to show all specific bands. Arrows indicate protease resistant fragments.

I used mitochondrial proteins Tom70, Por1, Tim23 and Tim10 as controls for the mitoplasting and protease treatment. Tom70 is on the mitochondrial surface and is accessible to protease in whole mitochondria (Hines, Brandt et al. 1990). The Tom70 control shows both proteases efficiently degrade domains on the surface of mitochondria (Figure 6.1). Since porin is embedded in the outer membrane it is protected from protease (Figure 6.1). Some Tim23 spans the both the inner and outer membranes, making a small proportion of the protein protease accessible in intact mitochondria (Donzeau, Kaldi et al. 2000). In mitochondria that are isolated from *C. albicans,* this surface exposed domain was cleaved by proteinase K but not by trypsin and the protein was completely degraded in mitoplasts (Figure 6.1). Since Tim10 is an intermembrane space protein protected by an intact outer membrane it is not affected by the addition of protease to intact mitochondria (Jarosch, Tuller et al. 1996). In *C.*

albicans, when the outer membrane was ruptured, protein levels decreased as the protein began to leak out of the intermembrane space and the protein was completely degraded when protease was added to mitoplasts (Figure 6.1).

Proteinase K but not trypsin completely cleaved ~3 kDa of Sam51 from the surface of intact mitochondria (Figure 6.1). This confirms Sam51 is a mitochondrial outer membrane protein, as expected from its sequence similarity with Sam50. This fragment appeared to be particularly sensitive to protease as even without adding protease a small amount of the faster migrating fragment was often detected, presumably due to action of endogenous mitochondrial proteases released when the mitochondria were lysed. When the outer membrane was ruptured Sam51 was completely degraded by proteinase K but the trypsin treated samples still contained significant amounts of the ~47 kDa clipped form of the protein as well as smaller fragments (Figure 6.1). These results indicate that the Sam51 POTRA domains, in contrast to those of Sam50, are in an orientation where they are not readily cleaved by trypsin, perhaps protected by components interacting with the POTRA domains. *C. albicans* Sam50 showed the same protease accessibility reported for *S. cerevisiae* Sam50, with a small amount of the full-length protein cleaved by both proteases (Figure 6.1)(Paschen, Waizenegger et al. 2003). Unlike its *S. cerevisiae* homologue, the *C. albicans* Mdm10 protein was not significantly accessible to protease in intact mitochondria (Figure 6.1, (Flinner, Ellenrieder et al. 2013)).

If Sam51, Sam50 or Mdm10 are in a complex with Sam37 then I hypothesised that the protease accessibility of these proteins may be altered if Sam37 was deleted. Proteinase K accessibility was no different for any of these outer membrane proteins or the controls in the *sam37* deletion strains (Figure 6.2).

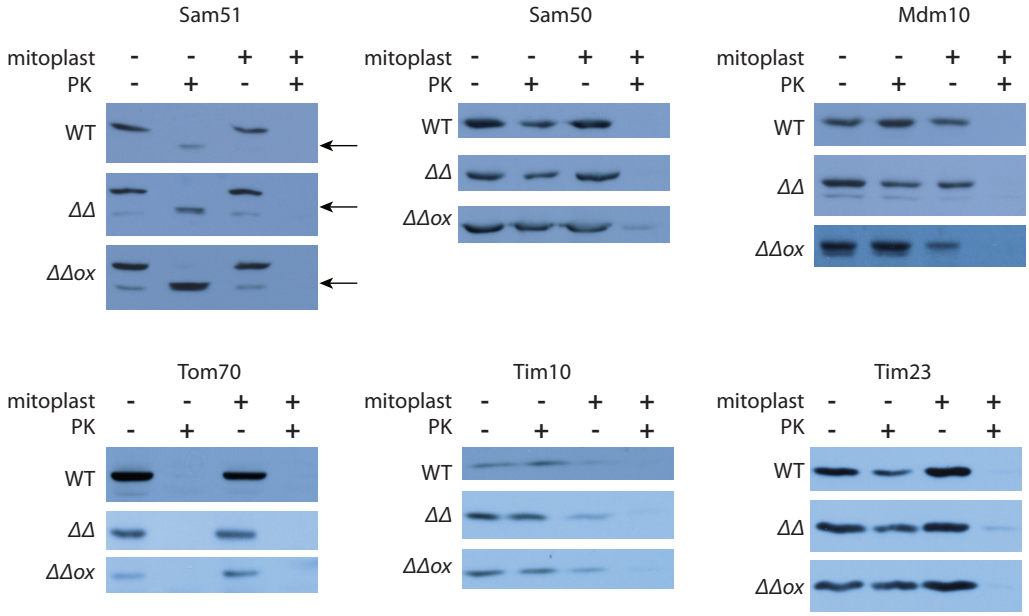


Figure 6.2 Protease shaving of mitochondria from *sam37ΔΔ* strains
Mitochondrial samples from wild type (WT), *sam37ΔΔ* (*ΔΔ*) and *sam37ΔΔSAM35↑* (*ΔΔ*ox) *C. albicans* were treated with 5 mg/mL proteinase K (PK) and/or the outer membrane ruptured to produce mitoplasts, as indicated. After addition of PMSF, the samples were analysed by SDS-PAGE and western blotting with the indicated antibodies. Arrows indicate protease resistant fragments.

6.3 Steady state forms of the SAM complex

I examined the SAM complex composition of *C. albicans* in steady state conditions using western blotting to detect the complexes in wild type mitochondria separated by BN-PAGE and solubilised in digitonin (Section 2.3.2). When mitochondria were solubilised in digitonin, the three beta barrel proteins Sam50, Sam51 and Mdm10 were all found in ~400 kDa complexes (SAM_{holo}, Figure 6.3A). The amount of the smaller of these two ~400 kDa complexes varied between experiments, thus the discussion that follows will focus on the more prominent form which I will refer to as SAM_{holo}. A proportion of Sam50 was also found in a ~200 kDa complex (SAM_{core}, Figure 6.3A) corresponding to the core SAM complex containing Sam50, Sam35 and Sam37 (Milenkovic, Kozjak et al. 2004) (Figure 2.3 and Figure 2.5). Like in *N. crassa* and *S. cerevisiae*, I sometimes detected another

large but less abundant form of the SAM complex ~350 kDa (Figure 6.3-Figure 6.6, (Becker, Pfannschmidt et al. 2008, Klein, Israel et al. 2012)).

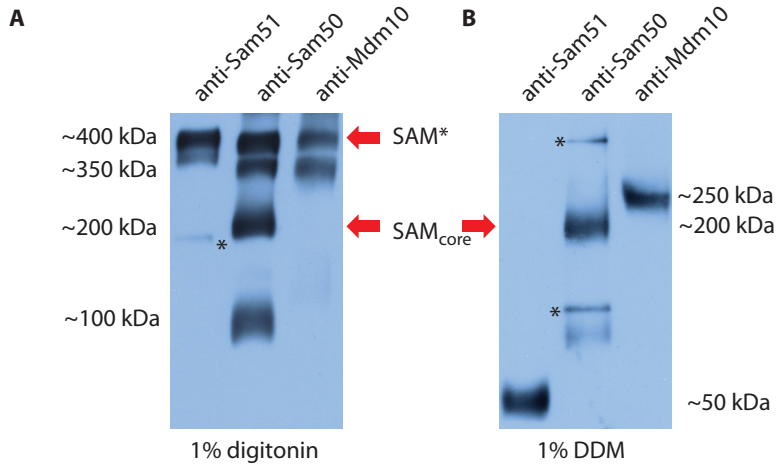


Figure 6.3 Native complexes in *C. albicans* mitochondria
(A) Mitochondrial samples were solubilised in lysis buffer containing 1 % digitonin and analysed by BN-PAGE and western blotting with the indicated antibodies. Asterisks denote non-specific bands detected by the antibody. **(B)** As for (A) but using 1 % DDM.

To investigate the stability of these complexes I compared the sizes of the complexes solubilised in buffer containing digitonin to those solubilised in buffer containing another detergent, n-Dodecyl-β-D-maltoside (DDM) (Figure 6.3B). Previous studies of membrane protein complexes in the mitochondrial outer membrane have shown digitonin disrupts less of the protein-protein interactions within complexes than DDM allowing separation of larger complexes (Paschen, Waizenegger et al. 2003, Wiedemann, Kozjak et al. 2003, Ishikawa, Yamamoto et al. 2004, Meisinger, Rissler et al. 2004). Antibodies against Sam50, Sam51 and Mdm10, all detected different sized complexes in mitochondria solubilised in DDM. All of the Sam50 is detected in the ~200 kDa complex in keeping with this being the SAM_{core} complex (Figure 6.3B, (Wiedemann, Kozjak et al. 2003)). The complex detected by the Sam51 antibodies migrates rapidly through the gel, indicating Sam51 has dissociated from the larger complexes and is migrating as a monomer, suggesting it is not as strongly associated with its partner proteins as Mdm10 or Sam50. Mdm10 antibodies detected a single ~250 kDa complex distinct from the SAM_{core} complex.

To determine whether Sam51 was in a complex with the other SAM components, I used antibody shift experiments in strains with an HA tag introduced at the C-terminus of Sam37 or Mdm10 (Section 2.2.2). Having confirmed the tag did not affect the stability of the complexes (Figure 2.3), I added anti-HA antibodies to the solubilised mitochondria before detecting with antibodies against Sam50 or Sam51 (Figure 6.4). The increased incubation time in these experiments increased the proportion of Sam50 found in the more stable ~200 kDa SAM_{core} complex. The loss of a large form of the SAM_{holo} complex in lane 2 (*sam37-3HA*) confirms Sam37 is found in this SAM_{holo} complex with Sam50. The decreased intensity in lanes 4 (*sam37-3HA*) and 6 (*mdm10-3HA*) indicates Sam51 is present in a SAM_{holo} complex with both Sam37 and Mdm10 (Figure 6.4). Sam51 is never seen in the smaller SAM_{core} complex, consistent with its inability to compensate for the loss of Sam50. Together this data suggests Sam51 is part of an alternative version of the SAM_{holo} complex in *C. albicans*.

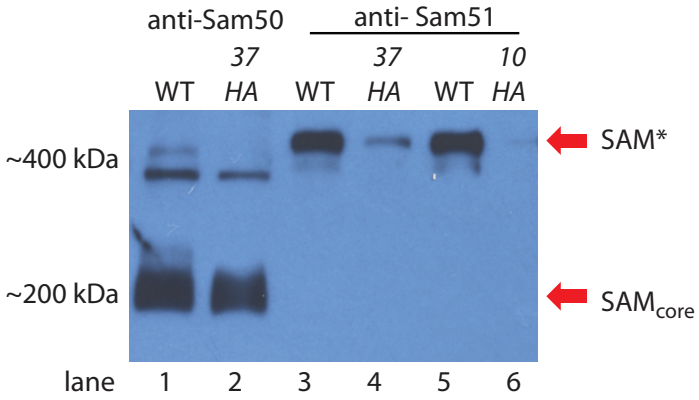


Figure 6.4 Antibody shifts reveal Sam51 partners
Mitochondrial samples isolated from a wild type (WT), *sam37-3HA* (*37HA*) strain or *mdm10-3HA* (*10HA*) strain of *C. albicans* were solubilised in lysis buffer containing 1 % digitonin. These solublised mitochondria were then incubated with 4 µL of anti-HA antibody and analysed by BN-PAGE and western blotting with the indicated antibodies.

6.4 The SAM complex in the Sam51 deletion strain

To determine whether Sam51 was responsible for increasing the stability of the ~400 kDa SAM_{holo} complex in *C. albicans,* I analysed the complexes containing Sam50 and Mdm10 in the *sam51ΔΔ* strain (Figure 6.5). Surprisingly, deletion of Sam51 does not change the size or protease accessibility of the complex containing

Sam50. Even though the Sam51 protein is clipped by protease treatment (Figure 6.1), protease treatment does not alter the size or stability of the complex containing Sam51 detected by western blotting on BN-PAGE (Figure 6.5B).

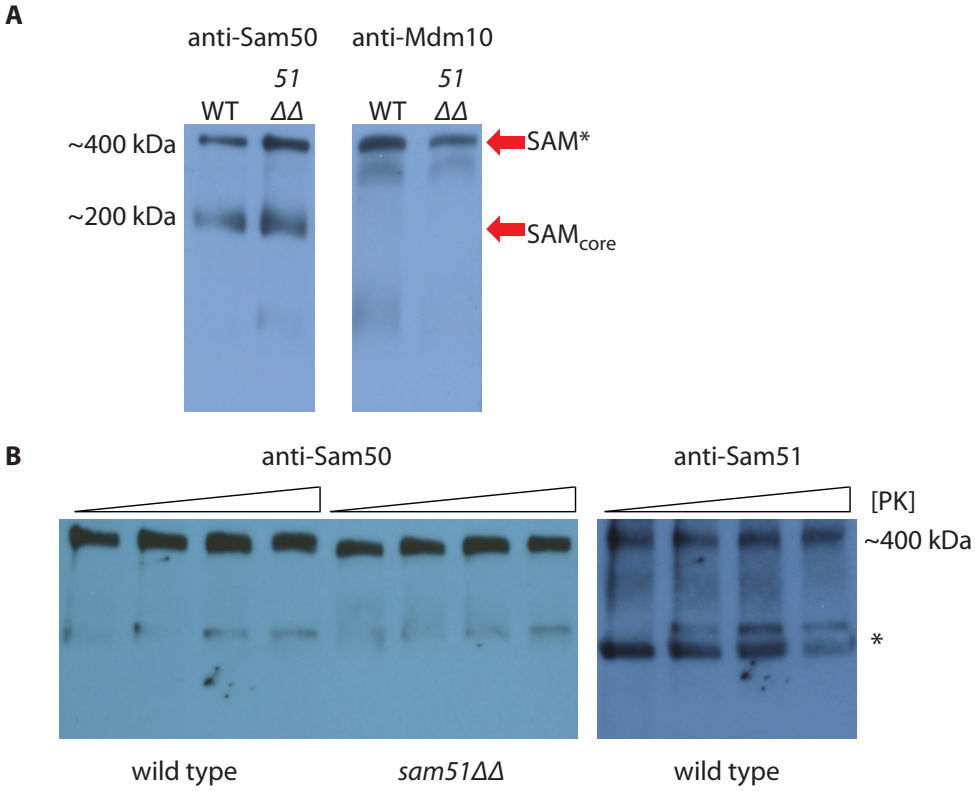


Figure 6.5 The sizes of the SAM complexes in the *sam51ΔΔ* strain
(**A**) Mitochondrial samples from wild type (WT) or *sam51ΔΔ* (*51ΔΔ*) strains were solubilised in lysis buffer containing 1 % digitonin and analysed by BN-PAGE and western blotting with the indicated antibodies. (**B**) Mitochondria from wild type or *sam51ΔΔ* strains were treated with 0, 0.1, 1 or 10 mg/mL proteinase K (increasing concentration of PK indicated by triangle) and solubilised and analysed as in (A). Asterisks denote non-specific bands detected by the antibody.

6.5 The SAM complex in the Sam37 deletion strains

To understand the role of Sam37 in the different forms of the SAM complex I examined the complexes in the *sam37* deletion strains (*sam37ΔΔ* and *sam37ΔΔSAM35↑*) to see if the loss of Sam37 was altering the size or interactions of the SAM complexes (Figure 6.6). I isolated mitochondria from the *sam37ΔΔ* strain, solubilised the complexes in digitonin buffer and separated them using BN-PAGE. Without Sam37, Sam35 and Sam50 still associated in a crippled version of the

SAM_{core} complex of ~150 kDa. There was much less of this Sam50-Sam35 complex than of the SAM_{core} complex in wild type mitochondria and it was unable to efficiently assemble Tom40 or Mdm10 (Sections 5.2.5 and 5.3.1). The amount of this complex was dramatically increased when Sam35 was over-expressed but the crippled complex was still unable to efficiently assemble Tom40 (Figure 5.13).

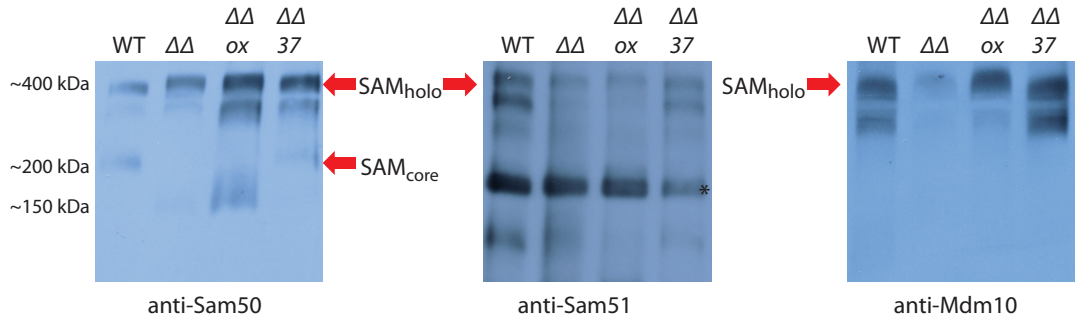


Figure 6.6 The sizes of the SAM complexes in the *sam37ΔΔ* strains
Mitochondrial samples from wild type (WT), *sam37ΔΔ* (*ΔΔ*) and *sam37ΔΔSAM35↑* (*ΔΔ*ox) and the complemented *sam37ΔΔSAM37 (ΔΔ37) C. albicans* strains were solubilised in lysis buffer containing 1 % digitonin and analysed by BN-PAGE and western blotting with the indicated antibodies.

The reduced levels of the complexes containing Mdm10 and Sam51 in the *sam37ΔΔ* strains reflected the differences in the protein levels measured by western blotting of samples separated by SDS-PAGE (Figure 5.10). There was very little Mdm10 detected in the *sam37ΔΔ* strain but when Sam35 over-expression restored Mdm10 levels, the Mdm10 was also returned to the larger form of the SAM_{holo} complex. The amount of Sam51 present was also reduced in the *sam37ΔΔ* strain but the levels remained unchanged when Sam35 was over-expressed, indicating that Sam51 and Sam37 interact. The dissociation of Sam51 from the large SAM complex in DDM (Figure 6.3B) along with its protease accessibility (Figure 6.1) suggests that Sam51 is not as tightly integrated into the complex as Sam50. Since the deletion of Sam51 does not change the sizes of the complexes detected by Sam50 or Mdm10 antibodies, Sam50 may be able to take the place of the missing Sam51 (Figure 6.5A).

Surprisingly, the large SAM complexes detected by Sam50 antibodies were unaffected by the loss of Sam37 (Figure 6.6). The majority of Sam50 is detected in a ~400 kDa form of the complex in the absence of Sam37, suggesting at least one large form of the *C. albicans* SAM complex does not include Sam37.

6.6 Forms of the SAM complex

Based on the evidence in this section, I hypothesise that in *C. albicans* Sam51 can be found in place of Sam50 in an alternative form of the SAM_{holo} complex with Mdm10. As well as the SAM_{holo} complex described above (Section 6.1 & (Meisinger, Rissler et al. 2004)), small amounts of other large complexes containing SAM components with different partner proteins have also been detected in other organisms. These complexes contain the components of the SAM_{core} complex and additional partner proteins including Mim1, Tom40 and Tom5. All the known forms of the SAM complex with its partner proteins are summarised in Figure 6.7, along with the hypothesised Sam51 complex.

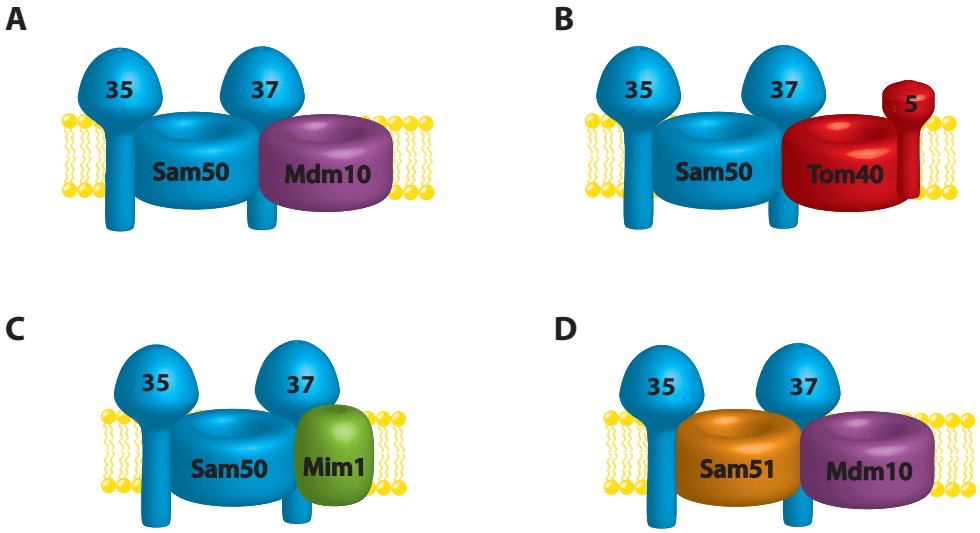


Figure 6.7 Large forms of the SAM complex
(A) The SAM-Mdm10 (SAM_{holo}) complex (Meisinger, Rissler et al. 2004) **(B)** The SAM-Tom40-Tom5 complex (Thornton, Stroud et al. 2010) **(C)** The Mim-SAM complex (Becker, Pfannschmidt et al. 2008) **(D)** The Sam51 complex.

In *S. cerevisiae*, the SAM_{core} components were co-purified with protein A tagged Mim1 and deletion of Mim1 reduces the size of a ~350 kDa complex containing the SAM_{core} components (Becker, Pfannschmidt et al. 2008). The size of this complex indicated it was possible that Mim1 might be part of the SAM_{holo} complex but the size of the complex detected by Mdm10 antibodies did not change in the *mim1Δ* strain (Becker, Pfannschmidt et al. 2008). A further ~350 kDa complex containing Sam50 and Tom5 has also been reported (Thornton, Stroud et al. 2010).

The authors co-purified Tom40 and Tom5 with epitope tagged Sam35 and showed that less Tom40 and Tom5 associated with the SAM complex in the absence of Mdm10.

The ~250 kDa complex detected by the Mdm10 antibody in mitochondria solubilised in DDM (Figure 6.3) suggests another possible interpretation for the arrangement of the protein complexes in the mitochondrial outer membrane. This complex does not include the SAM complex components and is around the same size as the complex containing Mmm1 detected in mitochondria isolated from *S. cerevisiae* (Meisinger, Rissler et al. 2004) (our Mmm1 and Mdm34 antibodies do not work on BN-PAGE). This *S. cerevisiae* complex is unaffected by the deletion of Mdm10, suggesting Mdm10 is either not a part of this complex or that under these conditions Mdm10 may preferentially associate with the SAM complex. In contrast, associations between the ERMES components Mdm10 and Mmm1 have been shown by co-immunopurification and co-localisation in fluorescence microscopy (Boldogh, Nowakowski et al. 2003, Kornmann, Currie et al. 2009, Stroud, Becker et al. 2011). In *S. cerevisiae* Mdm34 and Mmm1 have both been detected in large but different mobility complexes >667 kDa by gel filtration (Youngman, Hobbs et al. 2004), but no stable form of ERMES has been conclusively identified by western blotting of complexes separated by BN-PAGE.

Taken together with studies in *S. cerevisiae*, my data suggests that the ~250 kDa complex in *C. albicans* detected by Mdm10 antibodies may represent a stable form of the ERMES complex. This also suggests that at least one of the larger SAM complexes detected in *C. albicans* that also contains Mdm10 could represent a SAM-ERMES super-complex. Future research could make use of glycerol gradient centrifugation, 2D gel analysis, co-immunopurification and antibody shifts to validate these hypotheses and provide a biochemical characterisation of the ERMES.

7 The SAM complex as a platform for protein assembly

This chapter summarises, combines and compares the results examining the *C. albicans* SAM complex composition, size and function from all four results chapters and explains their significance in the context of the current literature.

The assembly of beta barrel proteins into the *C. albicans* outer membrane is a rapid process efficiently facilitated by the SAM complex. Tom40 and Mdm10 were rapidly imported and associated with the SAM complex with a similarly robust assembly of porin. The deletion of *SAM37* resulted in dramatic delays in beta barrel protein assembly. The over-expression of *SAM35* in the *sam37ΔΔ* strain promoted the formation of a Sam50-Sam35 sub-complex and restored import of porin and Mdm10 as well as Mdm10 binding to the SAM complex. However, unlike in *S. cerevisiae,* this crippled complex was unable to efficiently bind Tom40 precursors so does not restore Tom40 assembly. The divergent roles of Sam35 and Sam37 in different yeasts suggest that the sequence variation in these proteins throughout eukaryotes is a result of evolution of specialised roles in the import and/or regulation of SAM complex functions.

Western blotting detected Mdm10 and Sam51 in large forms of the *C. albicans* SAM complex. Mdm10 works with the SAM complex to help assemble beta barrel proteins and other TOM complex components. The depletion of Mdm10 caused delays in assembly of Tom40, Tom22 and Tom5 into the TOM complex and slowed assembly of porin and Mdm10 itself. Deletion of *SAM51* specifically impacted Tom40 assembly but not the assembly of other beta barrel proteins. None of these alterations to the SAM complex or its partners contributed to the import of proteins into the other compartments of the mitochondria.

The canonical role of the SAM_{core} complex is in the assembly of beta barrel proteins. As newly imported beta barrel proteins emerge from the TOM complex they must engage with the SAM machinery to be efficiently assembled. Both the TOM-SAM super-complex and the small TIM complexes help with this transition (Wiedemann, Truscott et al. 2004, Qiu, Wenz et al. 2013). The *in vitro* binding of Tom40 to purified SAM complex is inefficient (Klein, Israel et al. 2012) and suggests

that other SAM complex partners, such as Mdm10, play important roles in the efficient binding of Tom40 even if they are not part of the intermediate complexes.

When Mdm10 was depleted in *C. albicans* mitochondria, beta barrel proteins assembled more slowly and Tom40 and Mdm10 were slower to associate with the SAM complex. This suggests that the Mdm10-SAM complex has an early role in beta barrel protein assembly in *C. albicans,* perhaps priming the SAM complex to receive substrates by holding it in a certain conformation. In *S. cerevisiae* Mdm10 deletion slows assembly of Tom40 by trapping it in the SAM complex but accelerates the assembly of porin and Mdm10. This suggests different mechanisms may be regulating these assembly processes in these organisms.

In *S. cerevisiae* the binding of Mdm10 to the SAM complex is modulated by Tom7; Mdm10 cannot bind both the SAM complex and Tom7. Thus Tom7 is hypothesised to bind to Mdm10 and prevent it from prematurely displacing Tom40 from the SAM complex before the partner proteins have assembled with it (Yamano, Tanaka-Yamano et al. 2010). This involves the formation of an Mdm10-Tom7 complex that is not detected in *C. albicans,* suggesting *C. albicans* uses a different mechanism to regulate the association between Mdm10 and the SAM complex. Since Sam51 is found exclusively in a SAM_{holo} complex with Mdm10 it may be functioning as an alternative regulator of SAM complex function in *C. albicans.*

In *S. cerevisiae,* Tom5 and/or Tom6 associate with Tom40 before or as it leaves the SAM complex and these components accumulate in Intermediate II (Thornton, Stroud et al. 2010). If the binding of Tom40 to the SAM complex is impaired the small TOM proteins are unable to efficiently associate with Tom40, conversely mutant *tom40* is unable to bind Tom5 or escape from the SAM complex (Kutik, Stojanovski et al. 2008), suggesting the association with Tom5 may help disengage Tom40 from the SAM complex. In *C. albicans* the steps following release from the SAM complex occurred rapidly and very little of Intermediate II was detected. This indicates the arrangement of the SAM complex and its partners facilitates the rapid assembly of the newly imported Tom40 with its partner proteins.

The rapid import and assembly of Tom40 and the stable SAM_{holo} complex in *C. albicans* are reminiscent of the properties of the *S. cerevisiae tom7Δ* strain where

the SAM_{holo} complex is stabilised (Meisinger, Rissler et al. 2004) and Tom40 assembles faster and forms less Intermediate II (Meisinger, Wiedemann et al. 2006). This suggests that in *C. albicans* Mdm10 plays an important role after Tom40 associates with the SAM complex, perhaps, as suggested by Yamano and colleagues, displacing Tom40 to facilitate its release from the SAM complex (Yamano, Tanaka-Yamano et al. 2010).

The SAM complex also helps coordinate the association of Tom40 with the other components of the TOM complex (Stojanovski, Guiard et al. 2007, Thornton, Stroud et al. 2010). As well as Tom40, the integration of Tom5 and Tom22 into the *C. albicans* TOM complex was delayed in the *mdm10*↓ strain. Since the steady state levels of other TOM complex components are unaffected in this strain, this result adds further evidence for a specific role of Mdm10 in the assembly of Tom22 and Tom5 later on in the TOM assembly pathway. This may explain why Meisinger and colleagues found Tom40 accumulated in Intermediate II when Mdm10 was deleted (Meisinger, Wiedemann et al. 2006); Tom22 was not able to efficiently associate with the newly imported Tom40 without the help of Mdm10.

Collectively these results suggest that the SAM complex behaves as an assembly platform for beta barrel proteins with the help of partner proteins and complexes. I suggest the following model that incorporates these functions of the SAM complex and its interacting partners, as shown in cartoon form in Figure 7.1. By interacting with the MICOS complex (Bohnert, Wenz et al. 2012), the TOM complex is held close to the import pathway for small TIM proteins (Chacinska, Pfannschmidt et al. 2004), which would ensure the small TIM chaperones were available to associate with the beta barrel protein as it exits the TOM complex (Wiedemann, Truscott et al. 2004). The interaction of the TOM and SAM complexes ensures there is a SAM complex nearby to aid insertion of the protein into the membrane (Qiu, Wenz et al. 2013). For the assembly of the TOM complex, small TOM proteins can then assemble with membrane-embedded Tom40 with the help of the MIM proteins (Thornton, Stroud et al. 2010), and Tom22 is integrated into the complex later with the help of a SAM-Mdm10 complex. The SAM-ERMES interaction brings the ERMES complex into this assembly hub where it may contribute directly to beta barrel protein assembly or perhaps help regulate the local lipid environment, thereby

indirectly facilitating protein insertion into the outer membrane (Meisinger, Pfannschmidt et al. 2007, Kornmann, Currie et al. 2009). The newly discovered lipid transfer sites the vacuole and mitochondria patch (vCLAMP, (Elbaz-Alon, Rosenfeld-Gur et al. 2014, Honscher, Mari et al. 2014)) and endoplasmic reticulum membrane protein complex (EMC, (Lahiri, Chao et al. 2014)) may also contribute to the composition of the mitochondrial membranes and thereby contribute to the efficiency of beta barrel protein assembly (Gessmann, Chung et al. 2014).

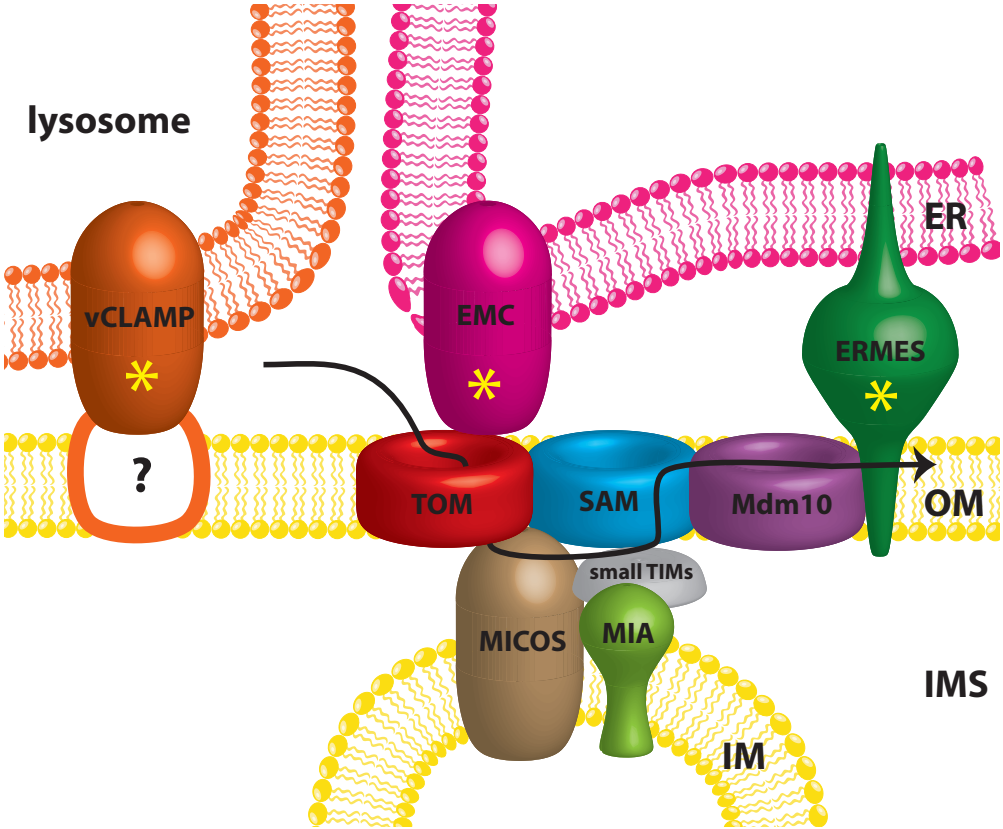


Figure 7.1 Beta barrel protein assembly pathway
Hypothesised import pathway showing locations and coordination between the complexes important for beta barrel protein import and assembly. The protein first passes through the translocase of the outer membrane (TOM, red) complex that interacts with the mitochondrial contact site and cristae organizing system (MICOS, brown). This interaction keeps the TOM complex close to mitochondrial intermembrane space transport and assembly pathway (MIA, pale green) for the small translocase of the inner membrane (TIM, grey) chaperones. The sorting and assembly machinery (SAM, blue) complex then inserts the beta barrel protein with the help of Mdm10 (purple) and the rest of the ER-mitochondrial encounter structure (ERMES, dark green). The transfer of lipids between the mitochondria and other organelles is thought to occur at the locations indicated by the yellow asterisks. These sites include the vacuole and mitochondria patch (vCLAMP, orange) and the ER membrane protein complex (EMC, pink).

The efficient beta barrel protein assembly and stable large forms of the SAM and ERMES complexes in *C. albicans* mitochondria make it a useful model to continue studies of the interactions between these complexes. My research presents examples of re-wiring of protein import pathways, the discovery of Sam51 and differences in the subunits contributing to the activity of the SAM complex, illustrating the utility studies of the mitochondrial import machinery using *C. albicans*. I anticipate that future investigations into the mitochondrial protein import machinery could implement similar comparative studies in both *C. albicans* and *S. cerevisiae* to provide a more comprehensive mechanistic picture of the import pathways and the links between metabolism, the cell cycle and mitochondrial biogenesis.

8 Appendices

Appendix 1: Protein sequence accession numbers

Table 8.1 Accession numbers for Hidden Markov Model sequences

Species	Sam37	Sam35	Sam50	Mdm10
Saccharomyces cerevisiae	NP_013776.1	NP_011951.1	NP_014372.1	YAL010C
Yarrowia lipolytica	XP_502993.1	XP_504008.1	XP_501539.2	XP_502348.1
Lachancea thermotolerans	XP_002551980.1	XP_002553638.1	XP_002555504.1	XP_002552850.1
Kluyveromyces polysporus	XP_001643746.1	XP_001646511.1	XP_001644603.1	XP_001643985.1
Ashbya gossypii	NP_984232.1	NP_984399.2	NP_987058.1	NP_984525.2
Kluyveromyces lactis	XP_456252.1	XP_454721.1	XP_454343.1	XP_455444.1
Clavispora lusitaniae	XP_002615682.1	XP_002614194.1	XP_002615880.1	XP_002618882.1
Debaryomyces hansenii	XP_461684.2	XP_461435.2	XP_002770022.1	XP_458340.2
Scheffersomyces stipitis	XP_001384419.2	XP_001385699.2	XP_001382599.2	XP_001384015.2
Pichia pastoris	XP_002489783.1	XP_002493671.1	XP_002491993.1	XP_002489898.1
Meyerozyma guilliermondii	XP_001482253.1	XP_001487684.1	XP_001486328.1	XP_001487642.1

Table 8.2 Sequences used in domain analysis and multiple sequence alignments

Species	Sam37	Sam35	Mdm10
Cryptococcus neoformans	AFR97266.1	AFR94126.1	P0CO67.1
Schizosaccharomyces pombe	CAB52621.1	CAC19761.1	NP_593586.1
Neurospora crassa	XP_964749.1	ESA42570.1	NCU07824.7
Tuber melanosporum	CAZ83161.1	CAZ81749.1	XP_002840558.1
Aspergillus fumigatus	XP_749516.1	XP_752688.1	Q4WVV6.1
Leptosphaeria maculans	XP_003838025.1	XP_003839914.1	XP_003840150.1
Naumovozyma castellii	XP_003673756.1	XP_003677837.1	XP_003677019.1
Candida glabrata	CAG59172.1	CAG59822.1	XP_445294.1
Lodderomyces elongisporus	XP_001528587.1	XP_001525739.1	XP_001528482.1
Candida tropicalis	XP_002547109.1	XP_002545942.1	XP_002547319.1
Cryptococcus gattii	XP_003195527.1	XP_003196772.1	ADV25733.1
Homo sapiens (MTX1)	NP_002446.3		
Homo sapiens (MTX2)	NP_006545.1		
Homo sapiens (MTX3)	NP_001161213.1		
Arabidopsis thaliana (MTX)	NP_565446.1		

Table 8.3 Sequences used in phylogenetic analysis of Sam50 and Sam51

Species	Sam50	Sam51
Saccharomyces cerevisiae	NP_014372.1	
Lachancea thermotolerans	XP_002555504.1	XP_002554882.1
Kluyveromyces polysporus	XP_001644603.1	From YGOB*
Ashbya gossypii	NP_987058.1	NP_985258.2
Kluyveromyces lactis	XP_454343.1	XP_454048.1
Clavispora lusitaniae	XP_002615880.1	XP_002614919.1
Scheffersomyces stipitis	XP_001382599.2	XP_001386142.2
Pichia pastoris	XP_002491993.1	CCA37572.1
Meyerozyma guilliermondii	XP_001486328.1	XP_001484036.1
Lodderomyces elongisporus	XP_001526227.1	XP_001524708.1
Candida tropicalis	XP_002548154.1	XP_002550983.1
Yarrowia lipolytica	XP_501539.2	XP_502409.1
Debaryomyces hansenii	XP_002770022.1	XP_460145.2
Zygosaccharomyces rouxii	XP_002494473.1	XP_002497635.1
Cryptococcus neoformans	CNBF0850	
Schizosaccharomyces pombe	NP_594600.1	
Neurospora crassa	XP_960555.1	
Tuber melanosporum	XP_002836483.1	
Aspergillus fumigatus	XP_746648.2	
Leptosphaeria maculans	XP_003843565.1	
Naumovozyma castellii	XP_003677614.1	
Candida glabrata	XP_449645.1	

*http://ygob.ucd.ie/ (Byrne and Wolfe 2005)

Appendix 2: Strain genotypes

Table 8.4 *C. albicans* strains

Strain	Genotype	Collection reference	Literature Reference
BWP17	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/his1Δ::hisG*	YCAT15	(Wilson, Davis et al. 1999)
DAY185	*ura3Δ::λimm434/ura3Δ::λimm434::URA3 arg4Δ::hisG/arg4Δ::hisG::ARG4 his1Δ::hisG/his1Δ::hisG::pHIS1*	YCAT19	(Davis, Edwards et al. 2000)
DAY286	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1::hisG /his::hisG*	YCAT14	(Davis, Edwards et al. 2000)
ccr4ΔΔ	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/hisΔ::hisG::pHIS1 ccr4Δ::URA3/ccr4Δ::ARG4*	YCAT133	(Dagley, Gentle et al. 2011)
mdm10↓	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/his::hisG::pHIS1 mdm10Δ::ARG4/URA3-*P_{MET3}*-MDM10*	YCAT597	Miguel Shingu-Vazquez
sam37ΔΔ	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/hisΔ::hisG::pHIS1 sam37Δ::URA3/sam37Δ::ARG4*	YCAT248	(Dagley, Gentle et al. 2011)
sam37ΔΔSAM35↑	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/hisΔ::hisG::pHIS1-TEF1-SAM35 sam37Δ::URA3/sam37Δ::ARG4*	YCAT84	(Qu, Jelicic et al. 2012)
sam37ΔΔSAM37	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/hisΔ::hisG::pHIS1-SAM37 sam37Δ::URA3/sam37Δ::ARG4*	YCAT249	(Qu, Jelicic et al. 2012)
sam37-3HA	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/hisΔ::hisG::pHIS1 sam37-3HA-ARG4/sam37-3HA-URA3*	YCAT608	This study Section 2.2.2
mdm10-3HA	*ura3Δ::λimm434/ura3Δ::λimm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/hisΔ::hisG::pHIS1 mdm10-3HA-ARG4/mdm10-3HA-URA3*	YCAT506	This study Section 2.2.2

Table 8.5 *S. cerevisiae* strains

Strain	Genotype	Reference
W303a	MATa *leu2-3 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11*	
cyb2Δ	MATa *cyb2Δ::KanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler, Shoemaker et al. 1999)
YPH499-BG-FOMP2 (wild type)	MATa *ade2-101 his3-200 leu2-1 ura3-52 trp1-63 lys2-801 mia40::ADE2 (pFL39-FOMP2/MIA40-WT)*	(Chacinska, Pfannschmidt et al. 2004)
mia40-3	MATa *ade2-101 his3-200 leu2-1 ura3-52 trp1-63 lys2-80 mia40::ADE2 (pFL39-FOMP2-8ts/mia40-3)*	(Chacinska, Pfannschmidt et al. 2004)
tom40-3HA	MATa *leu2-3 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11 tom40Δ::tom40-3HA-KANMX6*	(Chan 2008)
sam35-3HA	MATa *leu2-3 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11 sam35Δ::HIS-MX6 (p416-MET25-Sam35-3HA)*	(Chan 2008)

Appendix 3: Primers

Table 8.6 Primers used for *C. albicans* strain construction

Primer	Sequence
Sam37 forward primer	TAAGAATCAAATAAGTAAAAATAAATTTAGAAATCCAATTGG AAACGAAATACCGAGTTTAACAAATGAAGTTAAATATTGGAT TGGATCAATTGAATATGGTCGACGGATCCCCGGGTA
Sam37 reverse primer	CTTCTTCTATGTTTAACTATATACTTATTTCTTTTTTTTATACAA GATTACTATTAATATTTATGCTATCCCCCCCCCTTGGTGTATTC CTCTTCTTTTATCGATGAATTCGAGCTCGTT
Sam37 forward check	TCTATGCTTACATTTTCTGT
Sam37 reverse check	TGAGAGATAGAGAGATGAGA
Sam35 forward primer	CAACCAGCTCTTAAGCTCTACAAGACTTGGAAAAATAGTGTGT ACCAAACATGCTTTGCTTAGCCGCTCCTACTCCCTACTTAACCT CCACACAACTATAGGTCGACGGATCCCCGGGTA
Sam35 reverse primer	ATGACGATTATGTAGCCAGCGAGTTTGAGGTTGATTATGGGGT GGTCGTACGATTCTAGCAAGTCCAAGTACTCCTCAAATTCGAC AAGTTGTTGGGAGTTCGATGAATTCGAGCTCGTT
Sam35 forward check	ACTCCCAACAACTTGTCGAA
Sam35 reverse check	GTTGTGCCACCGAAGTATTT
Mdm10 forward primer	AAAAGAATTTTTAGTTAGTACAGGAGTTAAATTATCTTTAAAT CCAATTACAAATACTCCTGAATTCAACAAATTAGGTGTATCTT TTTCATATGCTCTTGGTCGACGGATCCCCGGGTA
Mdm10 reverse primer	GTAATATTTAATGCTACACTTACTCTATCTTCATCTCTATCTAT CTATCAAAGGTATGGTTCTATAGTATTAAACTAACCTATCTAG CAACTTACTTCTTTCGATGAATTCGAGCTCGTT
Mdm10 forward check	GCGAGACAGTGACAGCCGC
Mdm10 reverse check	GTTGGTGGTTCTGATTTG
Reverse URA3 checking primer (CAT18)	ATGGCACTACAGCAACTTTC
Reverse *ARG4* checking primer (CAT37)	GGAATTGATCAATTATCTTTTGAAC

Table 8.7 Primers used for plasmid construction

ScCyb2ΔN forward	GGGGGAATTCATGGATATGAATAAACAA
ScCyb2ΔN reverse	GGGAAGCTTTCATGCATCCTCAAATTCTG
pSP64-CaTom40 forward	GGGGAAGCTTATGTCTCAACAGATTAATCC
pSP64-CaTom40 reverse	GGTCTAGATTACAAAGCACCTGGAGCTG
pSP64-CaPtc4 forward	GGGGAAGCTTATGGGTCAACTATTATCACA
pSP64-CaPtc4 reverse	GGTCTAGATCACCTGGTAATCCCCTTTT
pSP64-CaPor1 forward	GGGAATTCATGGCTCCAGCTGCTTATTC
pSP64-CaPor1 reverse	GGGTCTAGATTAAGCAGCAAAAGACAAAG

Table 8.8 Primers used for qPCR

Gene	Primer sequence	Traven Collection Reference
ACT1 forward	CCCAGGTATTGCTGAACGTA	qC01
ACT1 reverse	GAACCACCAATCCAGACAGA	qC02
SAM50 forward	CCCGATTTCATGGTGATATT	qC151
SAM50 reverse	GCATTGATTTACTCAGGTGGTT	qC152
SAM51 forward	AGTTGTTGGTGGTGAATCATTT	qC153
SAM51 reverse	TTCAGTTGCAGGGATTTGAT	qC154
SAM35 forward	TCCTTGCCCACAAGAATAAA	qC67
SAM35 reverse	ATAAGGAGCGGGAGTGTGTT	qC68
ALS1 forward	ACCAATCCAGTTCCAACTGTGGCA	qC45
ALS1 reverse	TGGATGCTGATTCATGAGAACCGCT	qC46
TYE7 forward	AGAACCAGGTACGAAGGCAGCT	qC29
TYE7 reverse	TGCCGGCAATCTTGGCATTAATGT	qC30
TOM40 forward	ACGGATGGGACAAATCAAAC	qC83
TOM40 reverse	GACTGCCAACTTGGATGACA	qC84
MRPL25 forward	CCACGACCATTTGCACAGTA	qC99
MRPL25 reverse	TGGAACTTTCTTGGCGTAGG	qC100

Appendix 4: Antibodies

Table 8.9 Antibody cross reactivity

Name	Size (kDa)		Cross-reacts with *C. albicans* protein?	Reference code: Lithgow Antibody Collection	Dilution for use with *C. albicans*
	Sc	Ca			
Tim23	23.2	23.1	Yes	NK665 / KT140 /KT141	1:2000
Tim10	10.3		Yes	CK81	1:2000
Atp2 ($F_1\beta$)	54.8	53.9	Yes	SS85	1:10000
Porin	30.4	29.8	Yes	KS424	1:2000
Tom70	70.1	69.7	Yes	UM9	1:2500
mtHsp70	70.6	69.7	Yes	SH3	1:6000
Tom22	16.8	16.7	Yes	From Shiota Takuya	1:1000
Tom22	16.8	16.7	No	TL3	1:1000
Tom40	42.3	42.0	Yes	From Shiota Takuya	1:1000
Tom20	20.3	19.0	No	TL652	
Cyb2	65.5	63.0	No	SS91	
HA-probe			See Section 2.2.2	Santa Cruz Biotechnology HA-probe (Y-11 X SC-805) rabbit polyclonal	1:1000
Tom5	6.0	5.4	No	115-6	
Tom5	6.0	5.4	No	From Pfanner Lab	
Tom6	6.4	6.0	No	From Pfanner Lab	
Tom7	6.7	7.2	No	H68.2	
Sam50	54.4	58.1	Yes	Raised against *C. albicans* protein (Section 2.5)	1:1000
Sam51		57.4	Yes		1:2000
Mdm10	56.3	53.1	Yes		1:500
Mdm34	52.0	70.1	Yes	Raised against *C. albicans* protein (by Miguel Shingu-Vazquez)	1:1000

Appendix 5: Plasmids used for *in vitro* transcription/translation

Table 8.10 Plasmids for *in vitro* transcription/translation

Protein	Plasmid/ promoter	Cut with	Notes	Lithgow Plasmid #	References
ScTom40	pSP64	PvuII	Has a EcoRI site in the gene	Box 5 P4	TJL R26 B3 #71 (B1 p84)
Bovine PiC	SP6	HindIII			(Runswick, Powell et al. 1987) (B1 p80)
Su9DHFR 1-69	pGEM3	EcoRI		Box 5 P27	TJL R25 B2 #25 (IC8 Schatz Plasmids) (Pfanner, Muller et al. 1987)
ScPor2	pSP64	HindIII		Box 5 P2	(B1 p82)
ScAac1	pSP64	EcoRI		Box 5 P28	(B1 p79)
$ScF_1\beta$ (ATP2)	pSP65	HindIII	subcloned from pGR207 as a ~1850bp EcoRI-HindIII fragment by Graeme Reid	Box 5 P15	VIIG3 Schatz Plasmids
ScCoxVa	pSP64	EcoRI	600bp inserted between SalI and EcoRI	Box 5 P16	TJL R25 B2 #26 (Ca297 B7 p42)
ScAdh3	pSP65	HindIII		Box 5 P18	TJL R25 B2 #18 (Ca297 B7 p42)
ScCyb2	pSP65	HindIII	Between EcoRI and HindIII sites	Box 5 P17	pSP65-CL1, Clemens Wachter, (VIA2 Schatz Plasmids) (Ca297 B7 p42)
ScTom22	pGEM4z-Tom22	NA	PCR reaction using primers Sp6-f and Sp6-r (gene between EcoRI and HindIII)		Shiota Takuya, Lithgow Lab
CaTom40	pSP64	SacI	Amplified from genomic DNA and cloned between HindIII and XbaI	Box 5 P25	This study Section 2.2.7
CaMdm10	pSP64	EcoRI	Cloned out of pET28 (See section 2.5) between XbaI & EcoRI	Box 5 P24	This study Section 2.2.7 (Ca686 B15 p3)
ScTom5	pSP65	EcoRI		Box 5 P26	Nickie Chan, Lithgow Lab
ScCyt1	pSP64	EcoRI	SpeI-EcoRI fragment of pDVL43 (van Loon, Brandli et al. 1986) digested with XbaI and EcoRI and inserted in these sites of pSP64	Box 3 P17	BG20 (Ca686 B15 p3) (EV38 Schatz Plasmids) (Wachter, Schatz et al. 1992)
CaGoa1	pSP64	PvuII		Box 2 P17	This study Section 2.2.7
CaPtc4	pSP64	SacI		Box 2 P28	This study Section 2.2.7

Appendix 6: Components of the *C. albicans* import machinery

Table 8.11 Mitochondrial protein import components in *C. albicans*
Proteins are grouped in accordance with their function and/or membership in a complex. Open reading frame (orf) and the name of the closest *S. cerevisiae* homologue were obtained from the Candida Genome Database (Arnaud, Inglis et al. 2014). Shading indicates open reading frames where there was uncertainty with respect to the homology. Resolutions are noted in the following footnotes.

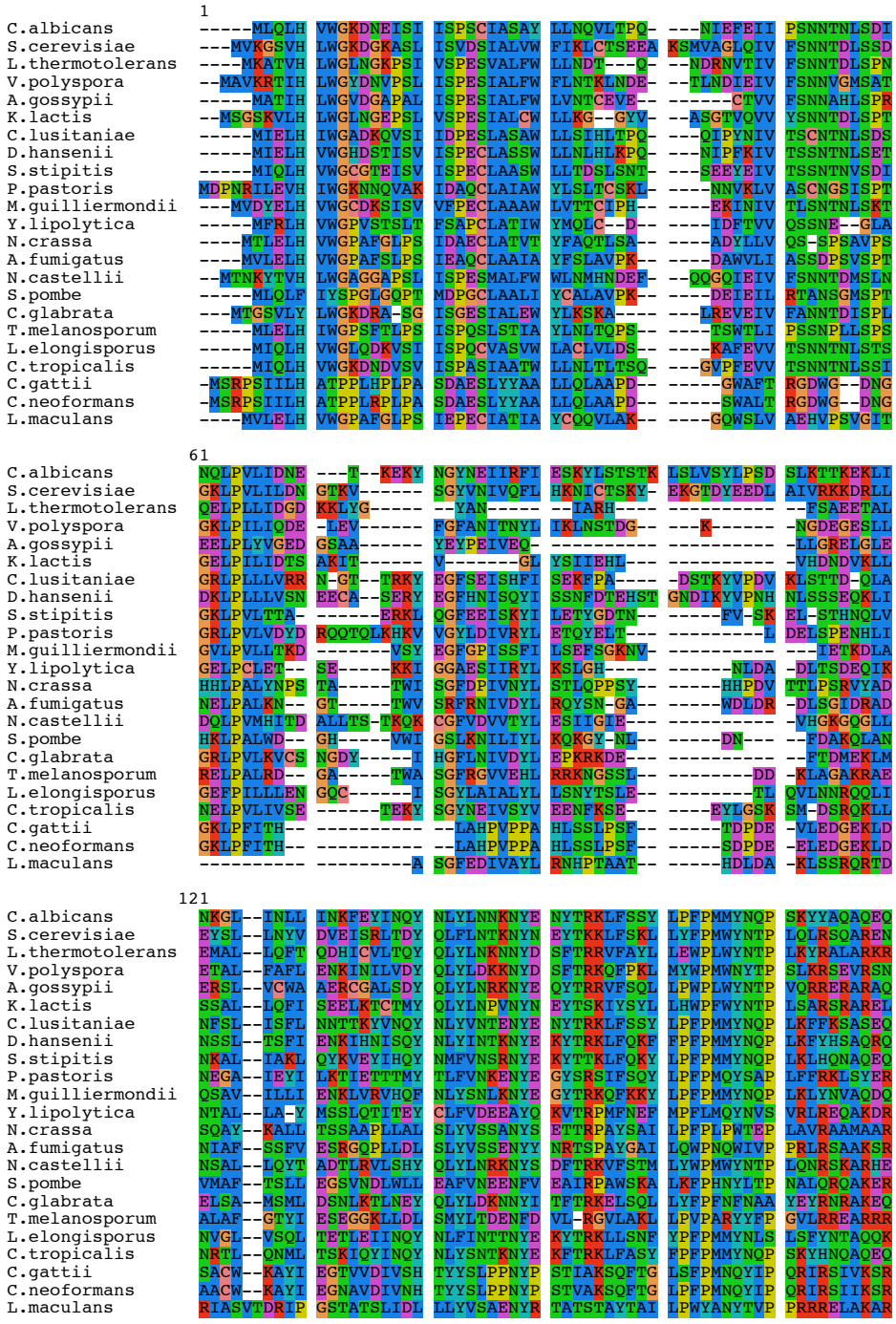
Function	Protein	Gene in *C. albicans*	Notes
TOM complex in outer membrane for protein translocation into mitochondria	Tom5	orf19.6247.1	Annotated as Tom5[1]
	Tom6	orf19.1650	Annotated as Tom6
	Tom7	orf19.6531.1	Annotated as Tom7[2]
	Tom20	orf19.2953	Annotated as Tom20
	Tom22	orf19.3696	Annotated as Tom22
	Tom40	orf19.6524	Annotated as Tom40
	Tom70	orf19.3700	A single isoform is present[3]
	Tom71	None	
Chaperones in inter-membrane space for beta barrel (and other) membrane proteins	Tim9	orf19.6696	Annotated as Tim9
	Tim10	orf19.4577.3	Annotated as Tim10
	Tim8	orf19.6183	Annotated as Tim8
	Tim13	orf19.2754	Annotated as Tim13
SAM complex for beta barrel protein assembly	Sam35	orf19.7267	Annotated as Sam35
	Sam37	orf19.1532	Annotated as Sam37
	Sam50	orf19.7358	Annotated as Sam50
	Sam51	orf19.925	Annotated as Sam51[4]
ERMES complex for beta barrel protein assembly	Mdm10	orf19.184	Annotated as Mdm10
	Mdm34	orf19.6900	Annotated as Mdm34
	Mdm12	orf19.6900	Annotated as Mdm12
	Mmm1	orf19.4187	Annotated as Mmm1
	Gem1	orf19.6016	Annotated as Gem1
Alpha helical protein insertion into outer membrane	Mim1	orf19.542.2	Annotated as Mim1
	Mim2	orf19.3265.1	Annotated as uncharacterised ORF[5]
Intermembrane space import components	Mia40	orf19.2977	Annotated as Mia40
	Erv1	orf19.2863.1	Annotated as Erv1
Inner membrane peptidase	Imp1	orf19.3061	Annotated as Imp1
	Imp2	orf19.1981	Annotated as Imp2
Carrier translocase of the inner mitochondrial membrane (TIM22 complex)	Tim22	orf19.1352	Annotated as Tim22
	Tim18/ Sdh4/Shh4	orf19.4022/ orf19.4468	Sequences both annotated as Sdh4[6]
	Tim54	orf19.5143	Annotated as Tim54
	Tim12	orf19.4620	Annotated as Tim12
Translocase of the Inner Mitochondrial Membrane (TIM23 complex)	Tim23	orf19.1361	Annotated as Tim23
	Tim17	orf19.150	Annotated as Tim17
	Tim50	orf19.680	Annotated as Tim50
PAM engagement	Tim21	orf19.3691	Annotated as Tim21
Presequence Translocase-Associated Motor (PAM)	Pam16	orf19.7222	Annotated as Pam16
	Pam17	orf19.240	Annotated as Pam17
	Pam18	orf19.4190	Annotated as Pam18
	Mdj2	orf19.3574	Annotated as Mdj2
	Ssc1	orf19.1869	Annotated as Ssc1

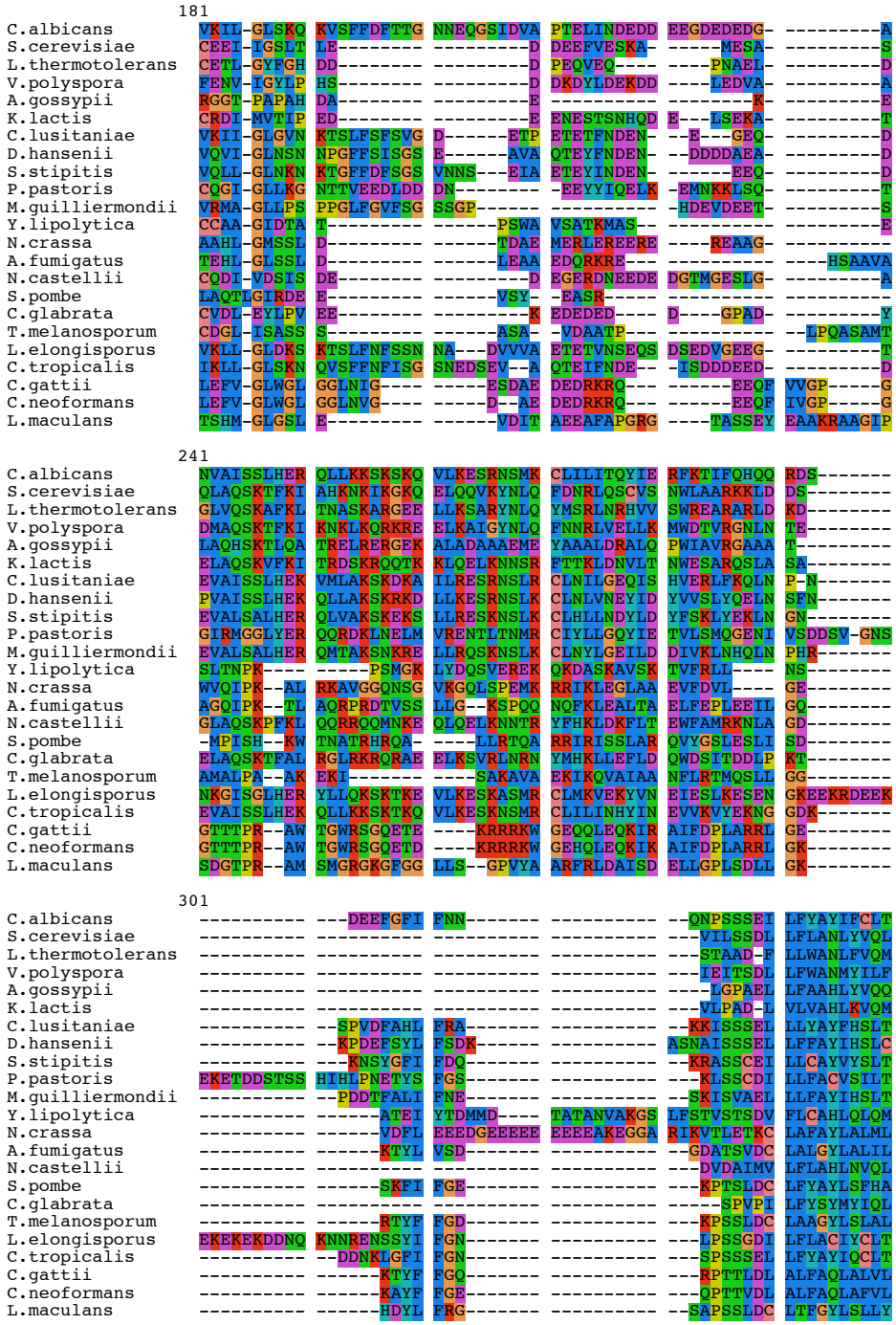
Matrix processing peptidase	Mas1	orf19.3026	Annotated as Mas1
	Mas2	orf19.6295	Annotated as Mas2

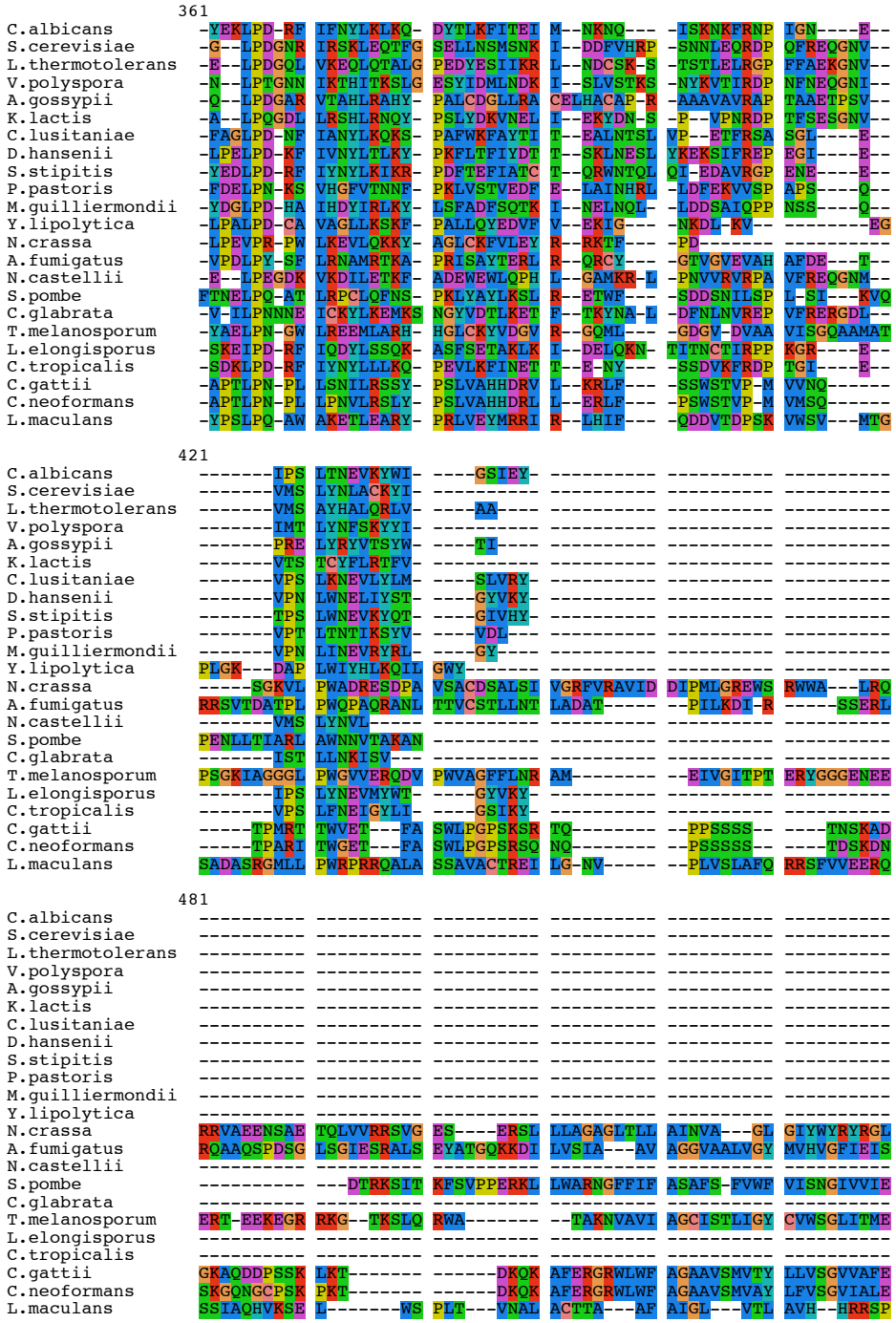
1. Tom5: In 2009 a BLAST search using ScTom5 in the CGD did not identify a Tom5 homologue (Top hit was orf19.5553, E value 0.18). A Hidden Markov Model search performed by Nermin Celik identified a transcribed (Sellam, Hogues et al. 2010) open reading frame in the region Ca21Chr1.1421439-14215821. The 47 residue open reading frame was probably too small to be captured by automated gene assignments. In 2013 an update to the database meant that a BLAST search using *S. cerevisiae* Tom5 in the CGD now identifies orf19.6247.1 as the top hit with an E value $3x10^{-4}$ and located at Ca21Chr1.1421581-1421438 in agreement with the HMM search (Maguire, OhEigeartaigh et al. 2013).
2. Tom7: In 2009 a BLAST search using the *S. cerevisiae* Tom7 sequence identified orf19.6531.1 annotated as Tom71. Our HMM search confirmed the BLAST search and identified an open-reading frame in this region (101 aa residues). CGD was alerted to this incorrect annotation and the annotation has been updated citing our 2012 publication (Hewitt, Heinz et al. 2012).
3. Tom70 and Tom71 are paralogs in *S. cerevisiae*, the relic of an ancient genome duplication event (Chan, Likic et al. 2006). *C. albicans* has a single form of the protein annotated as Tom70.
4. Sam51: Sam51 is member of a new group of Omp85 proteins first identified in my research (See Section 4.5) (Hewitt, Heinz et al. 2012).
5. Mim2: A BLAST search using *S. cerevisiae* Mim2 identifies this open reading frame with an E value of $8x10^{-8}$.
6. Tim18, Sdh4 and Shh4 are isoforms with related but overlapping functions all found in the *S. cerevisiae* mitochondrial inner membrane (Gebert, Gebert et al. 2011). Two forms present in *C. albicans* but multiple sequence alignments cannot distinguish between these forms, therefore functional analysis is required to distinguish between Tim18 and Sdh4 isoforms.

Appendix 7: Multiple sequence alignments

Figure 8.1 Sam37 multiple sequence alignments
Accession numbers of sequences and full species names are listed in Appendix 1: Table 8.1 and Table 8.2.




181
C.albicans VKIL-GLSKQ KVSFFDFTTG NNEQGSIDVA PTELINDEDD EEGDEDEDG- ----------A
S.cerevisiae CEEI-IGSLT LE--------- ---------D DEEFVESKA- -----MESA- ---------S
L.thermotolerans CETL-GYFGH DD--------- ---------D PEQVEQ----- ----PNAEL- ---------D
V.polyspora FENV-IGYLP HS--------- ---------D DKDYLDEKDD ----LEDVA- ---------A
A.gossypii RGGT-PAPAH DA--------- ---------E ---------- --------K- ---------E
K.lactis CRDI-MVTIP ED--------- ---------E ENESTSNHQD E---LSEKA- ---------T
C.lusitaniae VKII-GLGVN KTSLFSFSVG D------ETP ETETFNDEN- --E---GEQ- ---------D
D.hansenii VQVI-GLNSN NPGFFSISGS E------AVA QTEYFNDEN- --DDDDAEA- ---------D
S.stipitis VQLL-GLNKN KTGFFDFSGS VNNS---EIA ETEYINDEN- ------EEQ- ---------D
P.pastoris CQGI-GLLKG NTTVEEDLDD DN--------- -EEYYIQELK -EMNKKLSQ- ---------T
M.guilliermondii VRMA-GLLPS PPGLFGVFSG SSGP------ ---------- ---------- -HDEVDEET- ---------S
Y.lipolytica CCAA-GIDTA T--------- ------PSWA VSATKMAS-- ---------- ---------- ---------E
N.crassa AAHL-GMSSL D--------- ------TDAE MERLEREERE ----REAAG- ---------
A.fumigatus TEHL-GLSSL D--------- ------LEAA EDQRKRE--- ---------- ----HSAAVA
N.castellii CQDI-VDSIS DE--------- ---------D EGERDNEEDE DGTMGESLG- ---------A
S.pombe LAQTLGIRDE E--------- ------VSY- -EASR----- ---------- ----------
C.glabrata CVDL-EYLPV EE--------- ---------K EDEDED---- D----GPAD- ---------Y
T.melanosporum CDGL-ISASS S--------- ------ASA- -VDAATP--- ---------- --LPQASAMT
L.elongisporus VKLL-GLDKS KTSLFNFSSN NA---DVVVA ETETVNSEQS DSEDVGEEG- ---------T
C.tropicalis IKLL-GLSKN QVSFFNFISG SNEDSEV--A QTEIFNDE-- --ISDDDEED- ---------D
C.gattii LEFV-GLWGL GGLNIG---- -----ESDAE DEDRKRQ--- ------EEQF VVGP-----G
C.neoformans LEFV-GLWGL GGLNVG---- -----D--AE DEDRKRQ--- ------EEQF IVGP-----G
L.maculans TSHM-GLGSL E--------- ------VDIT AEEAFAPGRG ----TASSEY EAAKRAAGIP
241
C.albicans NVAISSLHER QLLKKSKSKQ VLKESRNSMK CLILITQYIE RFKTIFQHQQ RDS-------
S.cerevisiae QLAQSKTFKI AHKNKIKGKQ ELQQVKYNLQ FDNRLQSCVS NWLAARKKLD DS--------
L.thermotolerans GLVQSKAFKL TNASKARGEE LLKSARYNLQ YMSRLNRHVV SWREARARLD KD--------
V.polyspora DMAQSKTFKI KNKLKQRKRE ELKAIGYNLQ FNNRLVELLK MWDTVRGNLN TE--------
A.gossypii LAQHSKTLQA TRELRERGEK ALADAAAEME YAAALDRALQ PWIAVRGAAA T---------
K.lactis ELAQSKVFKI TRDSKRQQTK KLQELKNNSR FTTKLDNVLT NWESARQSLA SA--------
C.lusitaniae EVAISSLHEK VMLAKSKDKA ILRESRNSLR CLNILGEQIS HVERLFKQLN P-N-------
D.hansenii PVAISSLHEK QLLAKSKRKD LLKESRNSLK CLNLVNEYID YVVSLYQELN SFN-------
S.stipitis EVALSALHER QLVAKSKEKS LLRESKNSLK CLHLLNDYLD YFSKLYEKLN GN--------
P.pastoris GIRMGGLYER QQRDKLNELM VRENTLTNMR CIYLLGQYIE TVLSMQGENI VSDDSV-GNS
M.guilliermondii EVALSALHER QMTAKSNKRE LLRQSKNSLK CLNYLGEILD DIVKLNHQLN PHR-------
Y.lipolytica SLTNPK---- -----PSMGK LYDQSVEREK QKDASKAVSK TVFRLL---- NS--------
N.crassa WVQIPK--AL RKAVGGQNSG VKGQLSPEMK RRIKLEGLAA EVFDVL---- GE--------
A.fumigatus AGQIPK--TL AQRPRDTVSS LLG--KSPQQ NQFKLEALTA ELFEPLEEIL GQ--------
N.castellii GLAQSKPFKL QQRRQQMNKE QLQELKNNTR YFHKLDKFLT EWFAMRKNLA GD--------
S.pombe -MPISH--KW TNATRHRQA- -----LLRTQA RRIRISSLAR QVYGSLESLI SD--------
C.glabrata ELAQSKTFAL RGLRKRQRAE ELKSVRLNRN YMHKLLEFLD QWDSITDDLP KT--------
T.melanosporum AMALPA--AK EKI------- ----SAKAVA EKIKQVAIAA NFLRTMQSLL GG--------
L.elongisporus NKGISGLHER YLLQKSKTKE VLKESKASMR CLMKVEKYVN EIESLKESEN GKEEKRDEEK
C.tropicalis EVAISSLHEK QLLKKSKTKQ VLKESKNSMR CLILINHYIN EVVKVYEKNG GDK-------
C.gattii GTTTPR--AW TGWRSGQETE -----KRRRKW GEQQLEQKIR AIFDPLARRL GE--------
C.neoformans GTTTPR--AW TGWRSGQETD -----KRRRKW GEHQLEQKIK AIFDPLARRL GK--------
L.maculans SDGTPR--AM SMGRGKGFGG LLS--GPVYA ARFRLDAISD ELLGPLSDLL GK--------
301
C.albicans ---------- ---DEEFGFI FNN------- ---------- --QNPSSSEI LFYAYIFCLT
S.cerevisiae ---------- ---------- ---------- ---------- ---VILSSDL LFLANLYVQL
L.thermotolerans ---------- ---------- ---------- ---------- ---STAAD-F LLWANLFVQM
V.polyspora ---------- ---------- ---------- ---------- ---IEITSDL LFWANMYILF
A.gossypii ---------- ---------- ---------- ---------- ----LGPAEL LFAAHLYVQQ
K.lactis ---------- ---------- ---------- ---------- ---VLPAD-L VLVAHLKVQM
C.lusitaniae ---------- --SPVDFAHL FRA------- ---------- --KKISSSEL LLYAYFHSLT
D.hansenii ---------- --KPDEFSYL FSDK------ ---------- ASNAISSSEL LFFAYIHSLC
S.stipitis ---------- ---KNSYGFI FDQ------- ---------- --KRASSCEI LLCAYVYSLT
P.pastoris EKETDDSTSS HIHLPNETYS FGS------- ---------- ---KLSSCDI LLFACVSILT
M.guilliermondii ---------- --PDDTFALI FNE------- ---------- --SKISVAEL LLFAYIHSLT
Y.lipolytica ---------- ------ATEI YTDMMD---- TATANVAKGS LFSTVSTSDV FLCAHLQLQM
N.crassa ---------- ------VDFL EEEDGEEEEE EEEEAKEGGA RIKVTLETKC LAFAYLALML
A.fumigatus ---------- ------KTYL VSD------- ---------- --GDATSVDC LALGYLALIL
N.castellii ---------- ---------- ---------- ---------- ---DVDAIMV LFLAHLNVQL
S.pombe ---------- ------SKFI FGE------- ---------- ---KPTSLDC LFYAYLSFHA
C.glabrata ---------- ---------- ---------- ---------- -----SPVPI LFYSYMYIQL
T.melanosporum ---------- ------RTYF FGD------- ---------- ---KPSSLDC LAAGYLSLAL
L.elongisporus EKEKEKDDNQ KNNRENSSYI FGN------- ---------- ---LPSSGDI LFLACIYCLT
C.tropicalis ---------- --DDNKLGFI FGN------- ---------- ---SPSSSEL LFYAYIQCLT
C.gattii ---------- ------KTYF FGQ------- ---------- ---RPTTLDL ALFAQLALVL
C.neoformans ---------- ------KAYF FGE------- ---------- ---QPTTVDL ALFAQLAFVL
L.maculans ---------- ------HDYL FRG------- ---------- --SAPSSLDC LTFGYLSLLY


361
C.albicans -YEKLPD-RF IFNYLKLKQ- DYTLKFITEI M--NKNQ--- -ISKNKFRNP IGN----E--
S.cerevisiae -G--LPDGNR IRSKLEQTFG SELLNSMSNK I--DDFVHRP -SNNLEQRDP QFREQGNV--
L.thermotolerans -E--LPDGQL VKEQLQTALG PEDYESIIKR L--NDCSK-S -TSTLELRGP FFAEKGNV--
V.polyspora -N--LPTGNN IKTHITKSLG ESYIDMLNDK I--SLVSTKS -NYKVTIRDP NFNEQGNI--
A.gossypii -Q--LPDGAR VTAHLRAHY- PALCDGLLRA CELHACAP-R -AAAVAVRAP TAAETPSV--
K.lactis -A--LPQGDL LRSHLRNQY- PSLYDKVNEL I--EKYDN-S -P--VPNRDP TFSESGNV--
C.lusitaniae -FAGLPD-NF IANYLKQKS- PAFWKFAYTI T--EALNTSL VP--ETFRSA SGL----E--
D.hansenii -LPELPD-KF IVNYLTLKY- PKFLTFIYDT T--SKLNESL YKEKSIFREP EGI----E--
S.stipitis -YEDLPD-RF IYNYLKIKR- PDFTEFIATC T--QRWNTQL QI-EDAVRGP ENE----E--
P.pastoris -FDELPN-KS VHGFVTNNF- PKLVSTVEDF E--LAINHRL -LDFEKVVSP APS----Q--
M.guilliermondii -YDGLPD-HA IHDYIRLKY- LSFADFSQTK I--NELNQL- -LDDSAIQPP NSS----Q--
Y.lipolytica -LPALPD-CA VAGLLKSKF- PALLQYEDVF V--EKIG---- -NKDL-KV-- --------EG
N.crassa -LPEVPR-PW LKEVLQKKY- AGLCKFVLEY R--RKTF--- -PD-------- ----------
A.fumigatus -VPDLPY-SF LRNAMRTKA- PRISAYTERL R--QRCY--- -GTVGVEVAH AFDE---T--
N.castellii -E--LPEGDK VKDILETKF- ADEWEWLQPH L--GAMKR-L -PNVVRVRPA VFREQGNM--
S.pombe FTNELPQ-AT LRPCLQFNS- PKLYAYLKSL R--ETWF--- -SDDSNILSP L-SI---KVQ
C.glabrata -V-ILPNNNE ICKYLKEMKS NGYVDTLKET F--TKYNA-L -DFNLNVREP VFRERGDL--
T.melanosporum -YAELPN-GW LREEMLARH- HGLCKYVDGV R--GQML--- -GDGV-DVAA VISGQAAMAT
L.elongisporus -SKEIPD-RF IQDYLSSQK- ASFSETAKLK I--DELQKN- TITNCTIRPP KGR----E--
C.tropicalis -SDKLPD-RF IYNYLLLKQ- PEVLKFINET T--E-NY--- -SSDVKFRDP TGI----E--
C.gattii -APTLPN-PL LSNILRSSY- PSLVAHHDRV L--KRLF--- -SSWSTVP-M VVNQ------
C.neoformans -APTLPN-PL LPNVLRSLY- PSLVAHHDRL L--ERLF--- -PSWSTVP-M VMSQ------
L.maculans -VPSLPQ-AW AKETLEARY- PRLVEYMRRI R--LHIF--- -QDDVTDPSK VWSV---MTG
421
C.albicans -------IPS LTNEVKYWI- ----GSIEY- ---------- ---------- ----------
S.cerevisiae -------VMS LYNLACKYI- ---------- ---------- ---------- ----------
L.thermotolerans -------VMS AYHALQRLV- -----AA---- ---------- ---------- ----------
V.polyspora -------IMT LYNFSKYYI- ---------- ---------- ---------- ----------
A.gossypii -------PRE LYRYVTSYW- ----TI---- ---------- ---------- ----------
K.lactis -------VTS TCYFLRTFV- ---------- ---------- ---------- ----------
C.lusitaniae -------VPS LKNEVLYLM- ----SLVRY- ---------- ---------- ----------
D.hansenii -------VPN LWNELIYST- ----GYVKY- ---------- ---------- ----------
S.stipitis -------TPS LWNEVKYQT- ----GIVHY- ---------- ---------- ----------
P.pastoris -------VPT LTNTIKSYV- -----VDL-- ---------- ---------- ----------
M.guilliermondii -------VPN LINEVRYRL- ----GY---- ---------- ---------- ----------
Y.lipolytica PLGK---DAP LWIYHLKQIL GWY------- ---------- ---------- ----------
N.crassa -----SGKVL PWADRESDPA VSACDSALSI VGRFVRAVID DIPMLGREWS RWWA---LRQ
A.fumigatus RRSVTDATPL PWQPAQRANL TTVCSTLLNT LADAT----- --PILKDI-R -----SSERL
N.castellii -------VMS LYNVL----- ---------- ---------- ---------- ----------
S.pombe PENLLTIARL AWNNVTAKAN ---------- ---------- ---------- ----------
C.glabrata -------IST LLNKISV--- ---------- ---------- ---------- ----------
T.melanosporum PSGKIAGGGL PWGVVERQDV PWVAGFFLNR AM-------- --EIVGITPT ERYGGGENEE
L.elongisporus -------IPS LYNEVMYWT- -----GYVKY- ---------- ---------- ----------
C.tropicalis -------VPS LFNEIGYLI- ----GSIKY- ---------- ---------- ----------
C.gattii -----TPMRT TWVET---FA SWLPGPSKSR TQ-------- --PPSSSS-- -----TNSKAD
C.neoformans -----TPARI TWGET---FA SWLPGPSRSQ NQ-------- --PSSSSS-- -----TDSKDN
L.maculans SADASRGMLL PWRPRRQALA SSAVACTREI LG-NV----- --PLVSLAFQ RRSFVVEERQ
481
C.albicans ---------- ---------- ---------- ---------- ---------- ----------
S.cerevisiae ---------- ---------- ---------- ---------- ---------- ----------
L.thermotolerans ---------- ---------- ---------- ---------- ---------- ----------
V.polyspora ---------- ---------- ---------- ---------- ---------- ----------
A.gossypii ---------- ---------- ---------- ---------- ---------- ----------
K.lactis ---------- ---------- ---------- ---------- ---------- ----------
C.lusitaniae ---------- ---------- ---------- ---------- ---------- ----------
D.hansenii ---------- ---------- ---------- ---------- ---------- ----------
S.stipitis ---------- ---------- ---------- ---------- ---------- ----------
P.pastoris ---------- ---------- ---------- ---------- ---------- ----------
M.guilliermondii ---------- ---------- ---------- ---------- ---------- ----------
Y.lipolytica ---------- ---------- ---------- ---------- ---------- ----------
N.crassa RRVAEENSAE TQLVVRRSVG ES----ERSL LLAGAGLTLL AINVA---GL GIYWYRYRGL
A.fumigatus RQAAQSPDSG LSGIESRALS EYATGQKKDI LVSIA---AV AGGVAALVGY MVHVGFIEIS
N.castellii ---------- ---------- ---------- ---------- ---------- ----------
S.pombe ---------- ---DTRKSIT KFSVPPERKL LWARNGFFIF ASAFS-FVWF VISNGIVVIE
C.glabrata ---------- ---------- ---------- ---------- ---------- ----------
T.melanosporum ERT-EEKEGR RKG--TKSLQ RWA------- --TAKNVAVI AGCISTLIGY CVWSGLITME
L.elongisporus ---------- ---------- ---------- ---------- ---------- ----------
C.tropicalis ---------- ---------- ---------- ---------- ---------- ----------
C.gattii GKAQDDPSSK LKT------- ------DKQK AFERGRWLWF AGAAVSMVTY LLVSGVVAFE
C.neoformans SKGQNGCPSK PKT------- ------DKQK AFERGRWLWF AGAAVSMVAY LFVSGVIALE
L.maculans SSIAQHVKSE L-------WS PLT---VNAL ACTTA---AF AIGL---VTL AVH--HRRSP

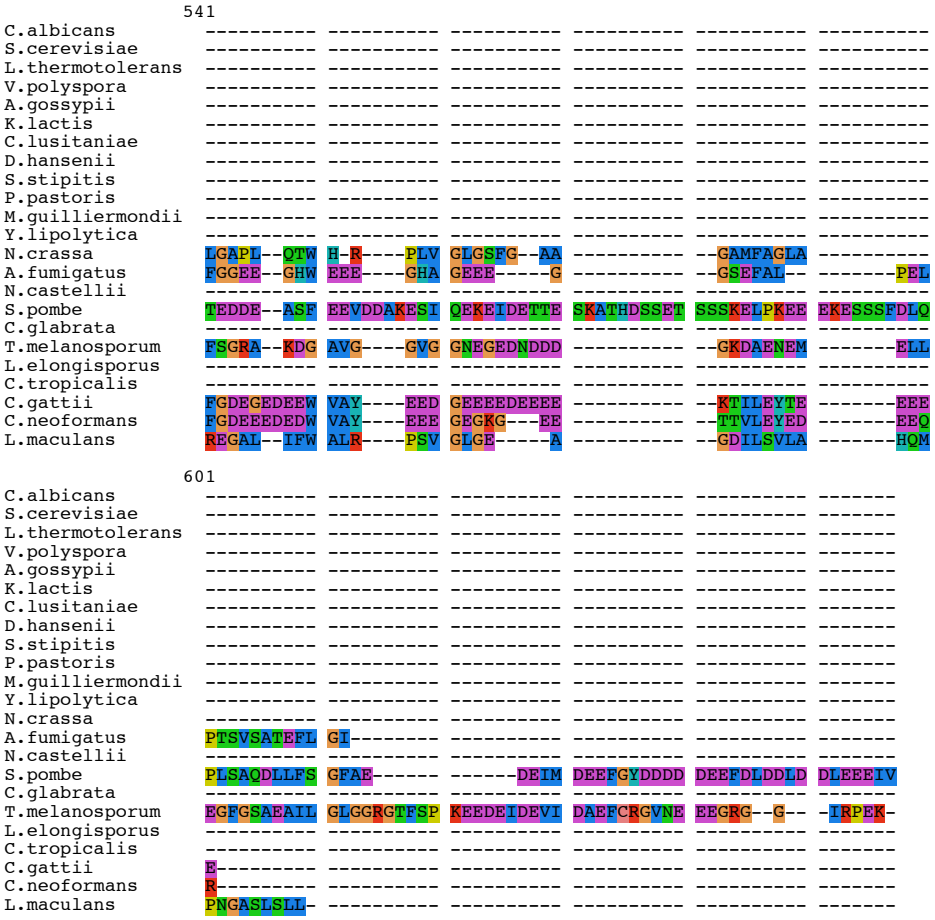
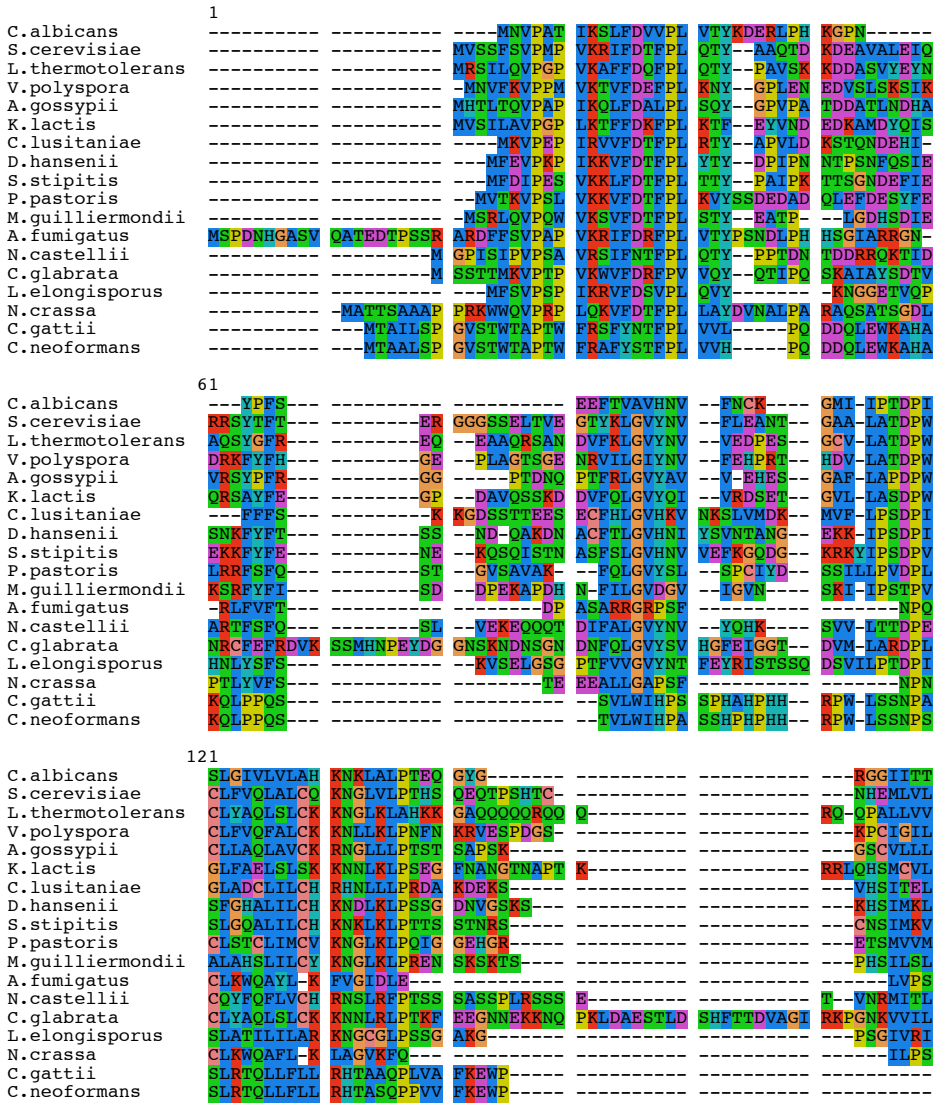
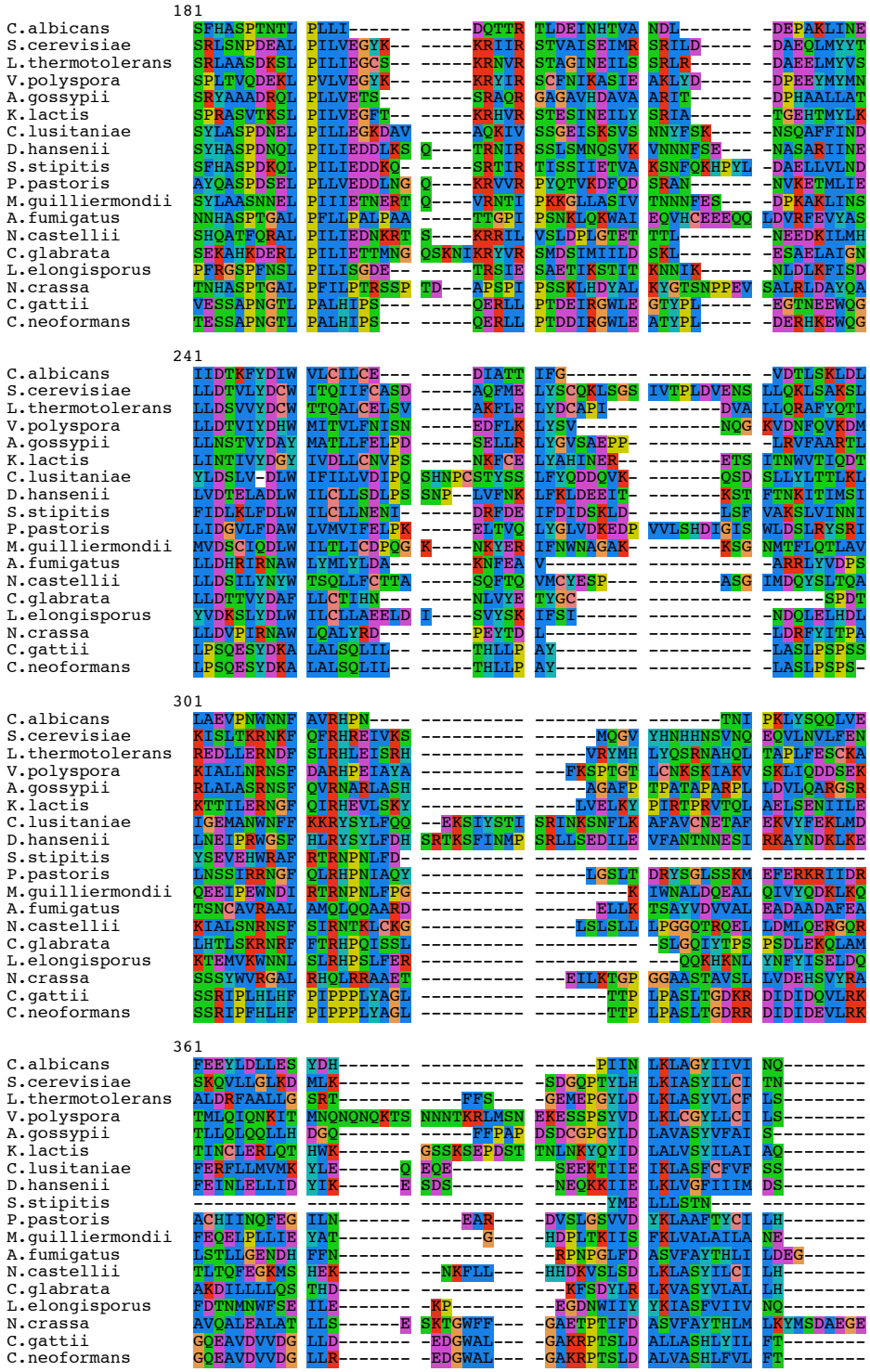

541
C.albicans
S.cerevisiae
L.thermotolerans
V.polyspora
A.gossypii
K.lactis
C.lusitaniae
D.hansenii
S.stipitis
P.pastoris
M.guilliermondii
Y.lipolytica
N.crassa
A.fumigatus
N.castellii
S.pombe
C.glabrata
T.melanosporum
L.elongisporus
C.tropicalis
C.gattii
C.neoformans
L.maculans
601
C.albicans
S.cerevisiae
L.thermotolerans
V.polyspora
A.gossypii
K.lactis
C.lusitaniae
D.hansenii
S.stipitis
P.pastoris
M.guilliermondii
Y.lipolytica
N.crassa
A.fumigatus
N.castellii
S.pombe
C.glabrata
T.melanosporum
L.elongisporus
C.tropicalis
C.gattii
C.neoformans
L.maculans

Figure 8.2 Sam35 multiple sequence alignments
Accession numbers of sequences and full species names are listed in Appendix 1: Table 8.1 and Table 8.2.




181
C.albicans SFHASPTNTL PLLI------ -----DQTTR TLDEINHTVA NDL------- -DEPAKLINE
S.cerevisiae SRLSNPDEAL PILVEGYK-- -----KRIIR STVAISEIMR SRILD----- -DAEQLMYYT
L.thermotolerans SRLAASDKSL PILIEGCS-- -----KRNVR STAGINEILS SRLR------ -DAEELMYVS
V.polyspora SPLTVQDEKL PVLVEGYK-- -----KRYIR SCFNIKASIE AKLYD----- -DPEEYMYMN
A.gossypii SRYAAADRQL PLLVETS--- -----SRAQR GAGAVHDAVA ARIT------ -DPHAALLAT
K.lactis SPRASVTKSL PILVEGFT-- -----KRHVR STESINEILY SRIA------ -TGEHTMYLK
C.lusitaniae SYLASPDNEL PILLEGKDAV -----AQKIV SSGEISKSVS NNYFSK---- -NSQAFFIND
D.hansenii SYHASPDNQL PILIEDDLKS Q----TRNIR SSLSMNQSVK VNNNFSE--- -NASARIINE
S.stipitis SFHASPDKQL PILIEDDKQ- -----SRTIR TISSIIETVA KSNFQKHPYL -DAELLVLND
P.pastoris AYQASPDSEL PLLVEDDLNG Q----KRVVR PYQTVKDFQD SRAN------ -NVKETMLIE
M.guilliermondii SYLAASNNEL PIIIETNERT Q----VRNTI PKKGLLASIV TNNNFES--- -DPKAKLINS
A.fumigatus NNHASPTGAL PFLLPALPAA -----TTGPI PSNKLQKWAI EQVHCEEEQQ LDVRFEVYAS
N.castellii SHQATFQRAL PILIEDNKRT S----KRRIL VSLDPLGTET TTI------- -NEEDKILMH
C.glabrata SEKAHKDERL PILIETTMNG QSKNIKRYVR SMDSIMIILD SKL------- -ESAELAIGN
L.elongisporus PFRGSPFNSL PILISGDE-- -----TRSIE SAETIKSTIT KNNIK----- -NLDLKFISD
N.crassa TNHASPTGAL PFILPTRSSP TD---APSPI PSSKLHDYAL KYGTSNPPEV SALRLDAYQA
C.gattii VESSAPNGTL PALHIPS--- -----QERLL PTDEIRGWLE GTYPL----- -EGTNEEWQG
C.neoformans TESSAPNGTL PALHIPS--- -----QERLL PTDDIRGWLE ATYPL----- -DERHKEWQG
241
C.albicans IIDTKFYDIW VLCILCE--- -----DIATT IFG------- ---------- -VDTLSKLDL
S.cerevisiae LLDTVLYDCW ITQIIFCASD -----AQFME LYSCQKLSGS IVTPLDVENS LLQKLSAKSL
L.thermotolerans LLDSVVYDCW TTQALCELSV -----AKFLE LYDCAPI--- -------DVA LLQRAFYQTL
V.polyspora LLDTVIYDHW MITVLFNISN -----EDFLK LYSV------ -------NQG KVDNFQVKDM
A.gossypii LLNSTVYDAY MATLLFELPD -----SELLR LYGVSAEPP- ---------- -LRVFAARTL
K.lactis LINTIVYDGY IVDLLCNVPS -----NKFCE LYAHINER-- -------ETS ITNWVTIQDT
C.lusitaniae YLDSLV-DLW IFILLVDIPQ SHNPCSTYSS LFYQDDQVK- -------QSD SLLYLTTLKL
D.hansenii LVDTELADLW ILCLLSDLPS SNP--LVFNK LFKLDEEIT- -------KST FTNKITIMSI
S.stipitis FIDLKLFDLW ILCLLNENI- -----DRFDE IFDIDSKLD- -------LSF VAKSLVINNI
P.pastoris LIDGVLFDAW LVMVIFELPK -----ELTVQ LYGLVDKEDP VVLSHDIGIS WLDSLRYSRI
M.guilliermondii MVDSCIQDLW ILTLICDPQG K----NKYER IFNWNAGAK- -------KSG NMTFLQTLAV
A.fumigatus LLDHRIRNAW LYMLYLDA-- -----KNFEA V---------- ---------- -ARRLYVDPS
N.castellii LLDSILYNYW TSQLLFCTTA -----SQFTQ VMCYESP--- -------ASG IMDQYSLTQA
C.glabrata LLDTTVYDAF LLCTIHN--- -----NLVYE TYGC------ ---------- ------SPDT
L.elongisporus YVDKSLYDLW ILCLLAEELD I----SVYSK IFSI------ ---------- -NDQLELHDL
N.crassa LLDVPIRNAW LQALYRD--- -----PEYTD L---------- ---------- -LDRFYITPA
C.gattii LPSQESYDKA LALSQLIL-- -----THLLP AY-------- ---------- -LASLPSPSS
C.neoformans LPSQESYDKA LALSQLIL-- -----THLLP AY-------- ---------- -LASLPSPS-
301
C.albicans LAEVPNWNNF AVRHPN---- ---------- ---------- -------TNI PKLYSQQLVE
S.cerevisiae KISLTKRNKF QFRHRREIVKS ---------- ------MQGV YHNHHNSVNQ EQVLNVLFEN
L.thermotolerans REDLLERNDF SLRHLEISRH ---------- -----VRYMH LYQSRNAHQL TAPLFESCKA
V.polyspora KIALLNRNSF DARHPEIAYA ---------- ---FKSPTGT LCNKSKIAKV SKLIQDDSEK
A.gossypii RLALASRNSF QVRNARLASH ---------- -----AGAEP TPATAPARPL LDVLQARGSR
K.lactis KTTILERNGF QIRHEVLSKY ---------- -----LVELKY PIRTPRVTQL AELSENIILE
C.lusitaniae IGEMANWNFF KKRYSYLFQQ --EKSIYSTI SRINKSNFLK AFAVCNETAF EKVYFEKLMD
D.hansenii LNEIPRWGSF HLRYSYLFDH SRTKSFINMP SRLLSEDILE VFANTNNESI RKAYNDKLKE
S.stipitis YSEVEHWRAF RTRNPNLFD- ---------- ---------- ---------- ----------
P.pastoris LNSSIRRNGF QLRHPNIAQY ---------- -----LGSLT DRYSGLSSKM EFERKRIIDR
M.guilliermondii QEEIPEWNDI RTRNPNLFPG ---------- ----------K IWNALDQEAL QIVYQDKLKQ
A.fumigatus TSNCAVRAAL AMQLQQAARD ---------- ------ELLK TSAYVDVVAL EADAADAFEA
N.castellii KIALSNRNSF SIRNTKLCKG ---------- -----LSLSLL LPGGQTRQEL LDMLQERGQR
C.glabrata LHTLSKRNRF FTRHPQISSL ---------- ---------- -SLGQIYTPS PSDLEKQLAM
L.elongisporus KTEMVKWNNL SLRHPSLFER ---------- ---------- ---QQKHKNL YNFYISELDQ
N.crassa SSSYWVRGAL RHQLRRAAET ---------- ---EILKTGP GGAASTAVSL LVDEHSVYRA
C.gattii SSRIPLHLHF PIPPPLYAGL ---------- -------TTP LPASLTGDKR DIDIDQVLRK
C.neoformans SSRIPFHLHF PIPPPLYAGL ---------- -------TTP LPASLTGDRR DIDIDEVLRK
361
C.albicans FEEYLDLLES YDH------- ---------- ------PIIN LKLAGYIIVI NQ--------
S.cerevisiae SKQVLLGLKD MLK------- ---------- -SDGQPTYLH LKIASYILCI TN--------
L.thermotolerans ALDRFAALLG SRT------- ----FFS--- -GEMEPGYLD LKLASYVLCF LS--------
V.polyspora TMLQIQNKIT MNQNQNQKTS NNNTKRLMSN EKESSPSYVD LKLCGYLLCI LS--------
A.gossypii TLLQLQQLLH DGQ------- -----FFPAP DSDCGPGYLD LAVASYVFAI S---------
K.lactis TINCLERLQT HWK------- GSSKSEPDST TNLNKYQYID LALVSYILAI AQ--------
C.lusitaniae FERFLLMVMK YLE------Q EQE------- --SEEKTIIE IKLASFCFVF SS--------
D.hansenii FEINLELLID YIK------E SDS------- --NEQKKIIE LKLVGFIIIM DS--------
S.stipitis ---------- ---------- ---------- -------YME LLLSTN---- ----------
P.pastoris ACHIINQFEG ILN------- ----EAR--- -DVSLGSVVD YKLAAFTYCI LH--------
M.guilliermondii FEQELPLLIE YAT------- -------G--- -HDPLTKIIS FKLVALAILA NE--------
A.fumigatus LSTLLGENDH FFN------- ---------- --RPNPGLFD ASVFAYTHLI LDEG------
N.castellii TLTQFEGKMS HEK------- --NKFLL--- -HHDKVSLSD LKLASYILCI LH--------
C.glabrata AKDILLLLQS THD------- ---------- ---KFSDYLR LKVASYVLAL LH--------
L.elongisporus FDTNMNWFSE ILE------- -KP------- --EGDNWIIY YKIASFVIIV NQ--------
N.crassa AVQALEALAT LLS------E SKTGWFF--- -GAETPTIFD ASVFAYTHLM LKYMSDAEGE
C.gattii GQEAVDVVDG LLD------- -EDGWAL--- -GAKRPTSLD ALLASHLYLL FT--------
C.neoformans GQEAVDVVDG LLR------- -EDGWAL--- -GAKRPTSLD ALVASHLFVL FT--------

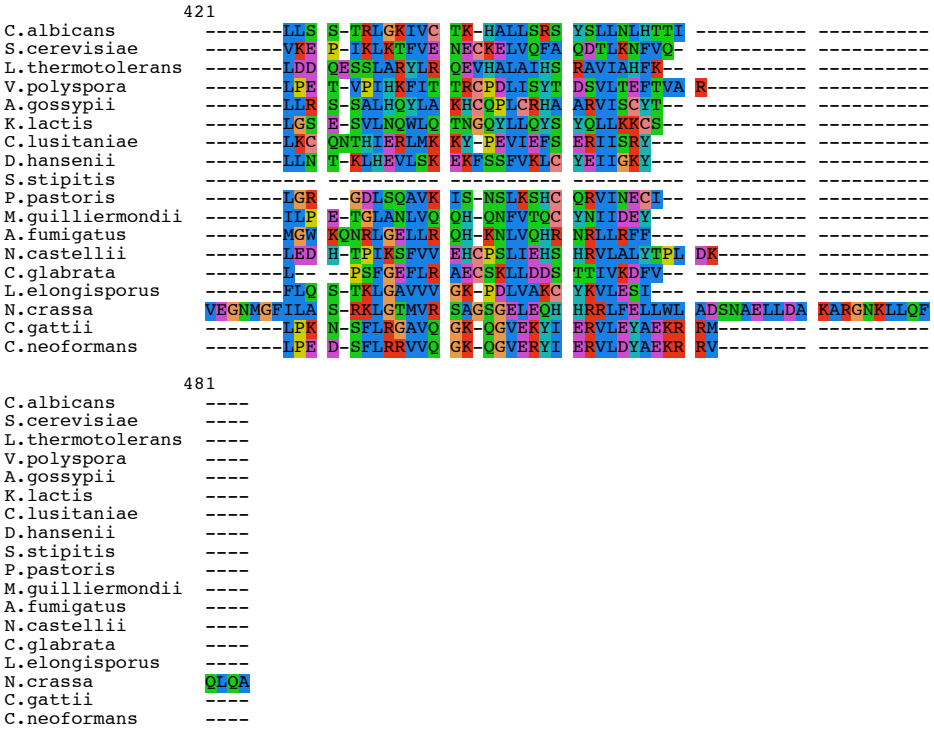
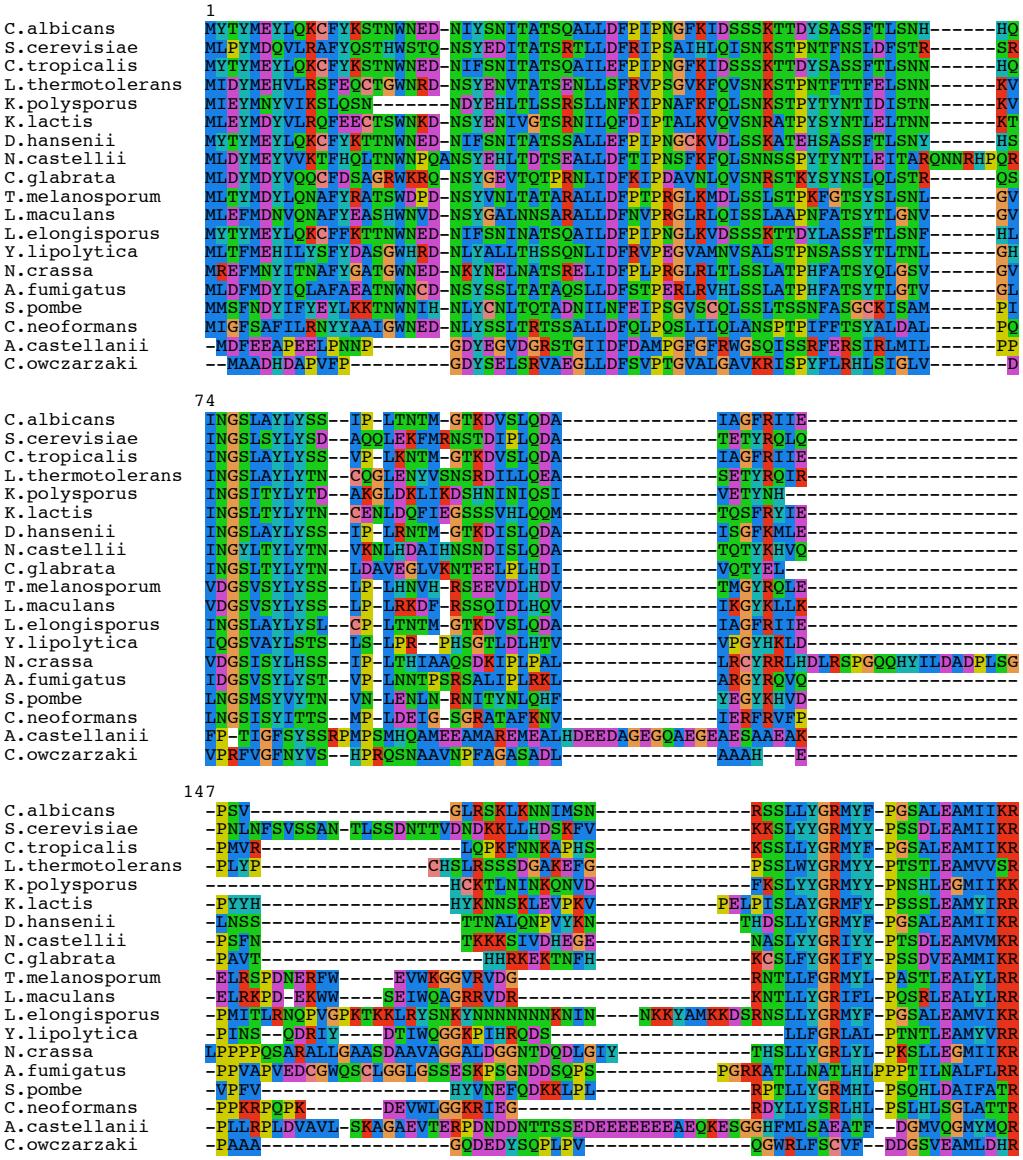
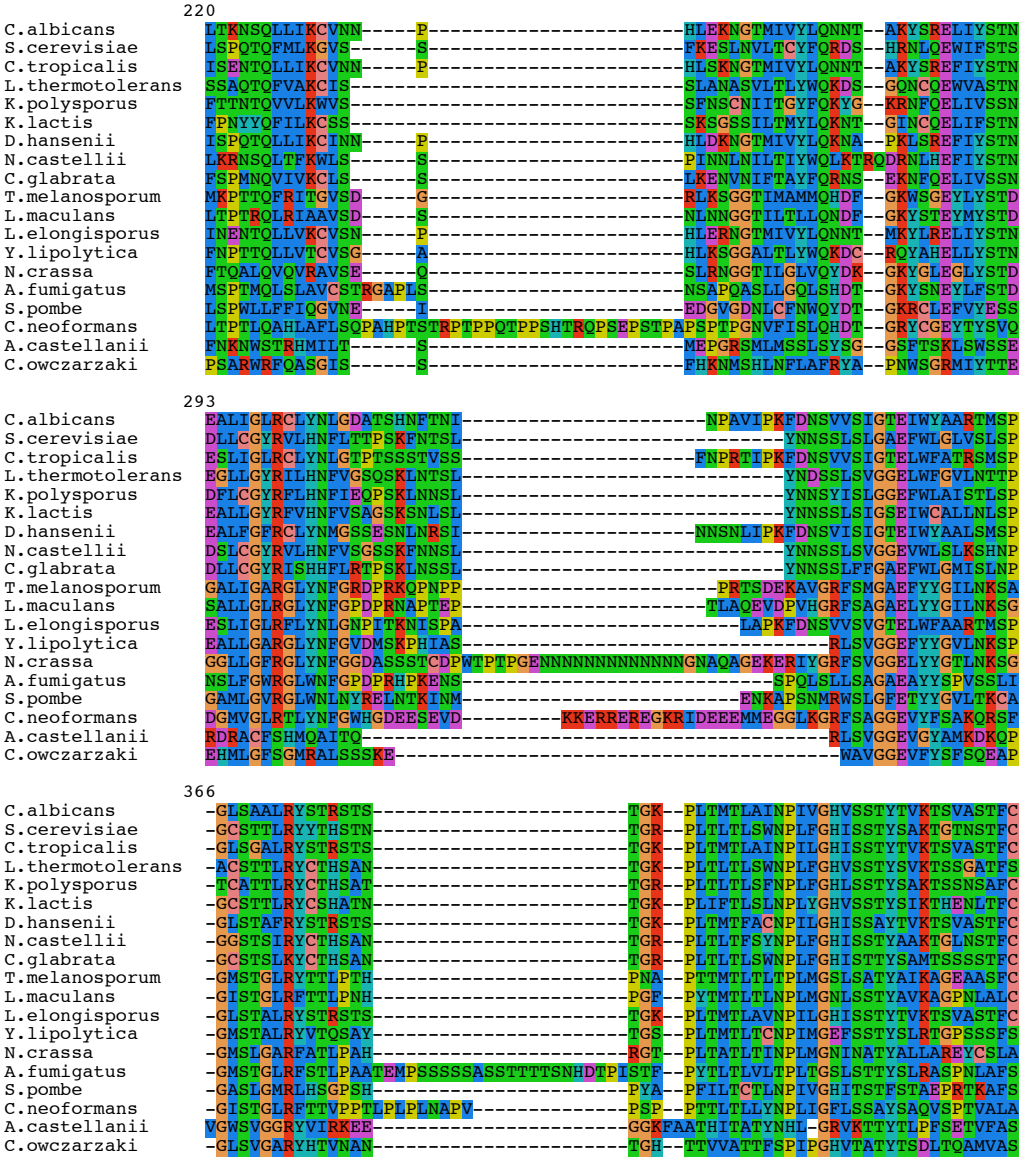
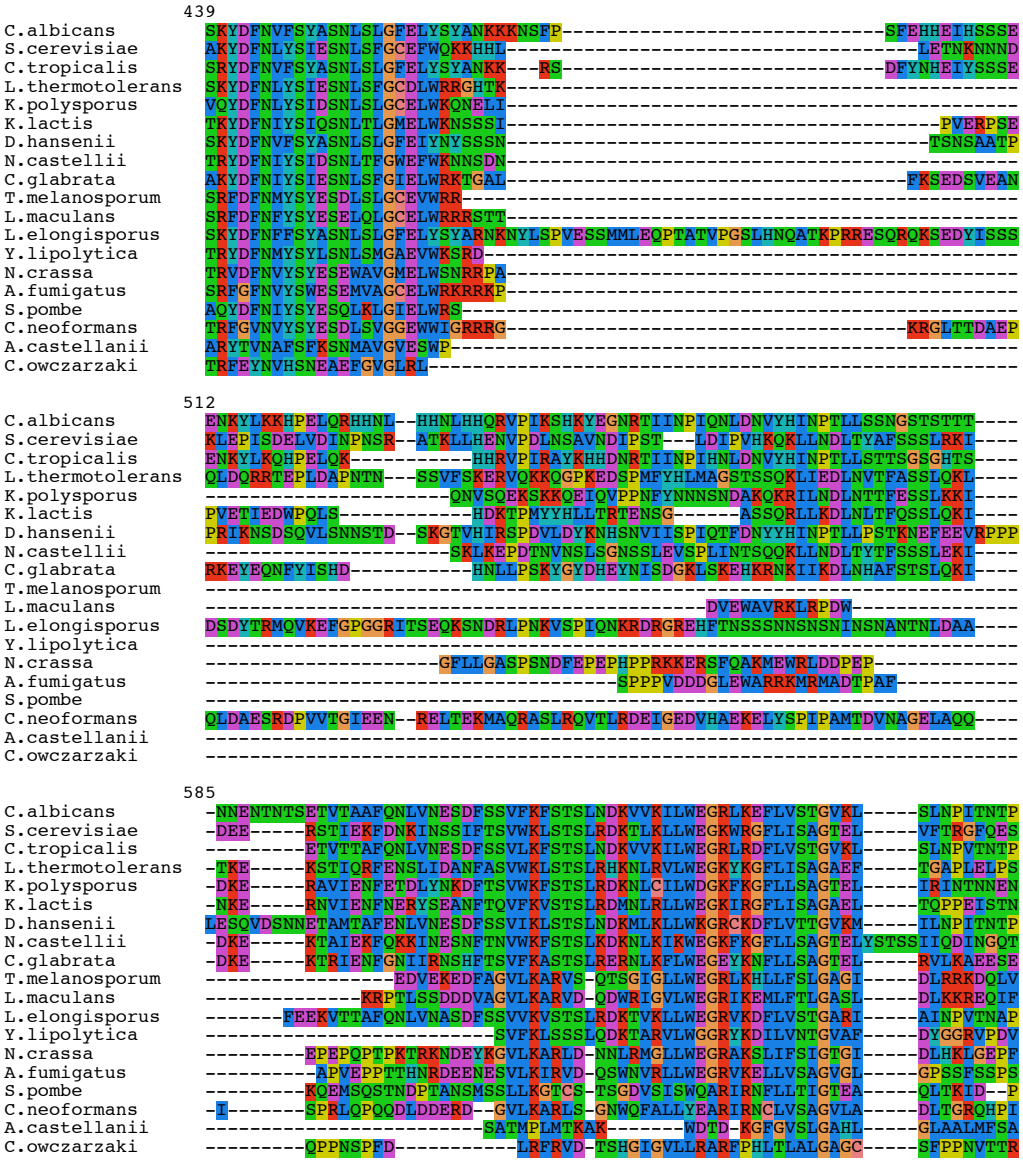
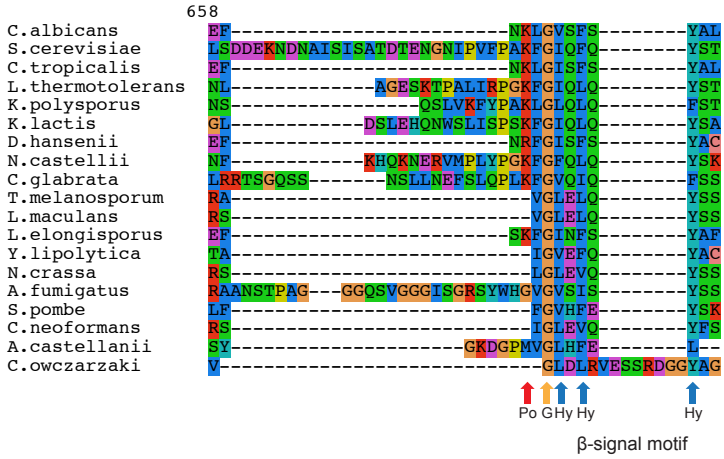

421
C.albicans -------LLS S-TRLGKIVC TK-HALLSRS YSLLNLHTTI ---------- ----------
S.cerevisiae -------VKE P-IKLKTFVE NECKELVQFA QDTLKNFVQ- ---------- ----------
L.thermotolerans -------LDD QESSLARYLR QEVHALAIHS RAVIAHFK-- ---------- ----------
V.polyspora -------LPE T-VPIHKFIT TRCPDLISYT DSVLTEFTVA R--------- ----------
A.gossypii -------LLR S-SALHQYLA KHCQPLCRHA ARVISCYT-- ---------- ----------
K.lactis -------LGS E-SVLNQWLQ TNGQYLLQYS YQLLKKCS-- ---------- ----------
C.lusitaniae -------LKC QNTHIERLMK KY-PEVIEFS ERIISRY--- ---------- ----------
D.hansenii -------LLN T-KLHEVLSK EKFSSFVKLC YEIIGKY--- ---------- ----------
S.stipitis ---------- ---------- ---------- ---------- ---------- ----------
P.pastoris -------LGR --GDLSQAVK IS-NSLKSHC QRVINECI-- ---------- ----------
M.guilliermondii -------ILP E-TGLANLVQ QH-QNFVTQC YNIIDEY--- ---------- ----------
A.fumigatus -------MGW KQNRLGELLR QH-KNLVQHR NRLLRFF--- ---------- ----------
N.castellii -------LED H-TPIKSFVV EHCPSLIEHS HRVLALYTPL DK--------- ----------
C.glabrata -------L-- --PSFGEFLR AECSKLLDDS TTIVKDFV-- ---------- ----------
L.elongisporus -------FLQ S-TKLGAVVV GK-PDLVAKC YKVLESI--- ---------- ----------
N.crassa VEGNMGFILA S-RKLGTMVR SAGSGELEQH HRRLFELLWL ADSNAELLDA KARGNKLLQF
C.gattii -------LPK N-SFLRGAVQ GK-QGVEKYI ERVLEYAEKR RM--------- ----------
C.neoformans -------LPE D-SFLRRVVQ GK-QGVERYI ERVLDYAEKR RV--------- ----------
481
C.albicans ----
S.cerevisiae ----
L.thermotolerans ----
V.polyspora ----
A.gossypii ----
K.lactis ----
C.lusitaniae ----
D.hansenii ----
S.stipitis ----
P.pastoris ----
M.guilliermondii ----
A.fumigatus ----
N.castellii ----
C.glabrata ----
L.elongisporus ----
N.crassa QLQA
C.gattii ----
C.neoformans ----

Figure 8.3 Mdm10 multiple sequence alignment
Beta signal motif shows conserved residues normally found in the indicated positions of the beta signal (Imai, Gromiha et al. 2008, Kutik, Stojanovski et al. 2008). Accession numbers of sequences and full species names are listed in Appendix 1: Table 8.1 and Table 8.2.




220
C.albicans LTKNSQLLIKCVNN-----P----------HLEKNGTMIVYLQNNT--AKYSRELIYSTN
S.cerevisiae LSPQTQFMLKGVS-----S----------FKESLNVLTCYFQRDS--HRNLQEWIFSTS
C.tropicalis ISENTQLLIKCVNN-----P----------HLSKNGTMIVYLQNNT--AKYSREFIYSTN
L.thermotolerans SSAQTQFVAKCIS----------SLANASVLTLYWQKDS--GQNCQEWVASTN
K.polysporus FTTNTQVVLKWVS----------SFNSCNIITGYFQKYG--KRNFQELIVSSN
K.lactis FPNYYQFILKCSS----------SKSGSSILTMYLQKNT--GINCQELIFSTN
D.hansenii ISPQTQLLIKCINN-----P----------HLDKNGTMIVYLQKNA--PKLSREFIYSTN
N.castellii LKRNSQLTFKWLS-----S----------PINNLNILTIYWQLKTRQDRNLHEFIYSTN
C.glabrata FSPMNQVIVKCLS-----S----------LKENVNIFTAYFQRNS--EKNFQELIVSSN
T.melanosporum MKPTTQFRITGVSD-----G----------RLKSGGTIMAMMQHDF--GKWSGEYLYSTD
L.maculans LTPTRQLRIAAVSD-----S----------NLNNGGTILTLLQNDF--GKYSTEYMYSTD
L.elongisporus INENTQLLVKCVSN-----P----------HLERNGTMIVYLQNNT--MKYLRELIYSTN
Y.lipolytica FNPTTQLLVTCVSG-----A----------HLKSGGALTLYWQKDC--RQYAHELLYSTN
N.crassa FTQALQVQVRAVSE-----Q----------SLRNGGTILGLVQYDK--GKYGLEGLYSTD
A.fumigatus MSPTMQLSLAVCSTRGAPLS----------NSAPQASLLGQLSHDT--GKYSNEYLFSTD
S.pombe LSPWLLFFIQGVNE-----I----------EDGVGDNLCFNWQYDT--GKRCLEFVYESS
C.neoformans LTPTLQAHLAFLSQPAHPTSTRPTPPQTPPSHTRQPSEPSTPAPSPTPGNVFISLQHDT--GRYCGEYTYSVQ
A.castellanii FNKNWSTRHMILT------S----------MEPGRSMLMSSLSYSG--GSFTSKLSWSSE
C.owczarzaki PSARWRFQASGIS------S----------FHKNMSHLNFLAFRYA--PNWSGRMIYTTE
293
C.albicans EALIGLRCLYNLGDATSHNFTNI----------NPAVIPKFDNSVVSIGTEIWYAARTMSP
S.cerevisiae DLLCGYRVLHNFLTTPSKFNTSL----------YNNSSLSLGAEFWLGLVSLSP
C.tropicalis ESLIGLRCLYNLGTPTSSSTVSS----------FNPRTIPKFDNSVVSIGTELWFATRSMSP
L.thermotolerans EGLLGYRILHNFVGSQSKLNTSL----------YNDSSLSVGGELWFGVLNTTP
K.polysporus DFLCGYRFLHNFIEQPSKLNNSL----------YNNSYISLGGEFWLAISTLSP
K.lactis EALLGYRFVHNFVSAGSKSNLSL----------YNNSSLSIGSEIWCALLNLSP
D.hansenii EALFGFRCLYNMGSSESNLNRSI----------NNSNLIPKFDNSVISIGTEIWYAALSMSP
N.castellii DSLCGYRVLHNFVSGSSKFNNSL----------YNNSSLSVGGEVWLSLKSHNP
C.glabrata DLLCGYRISHHFLRTPSKLNSSL----------YNNSSLFFGAEFWLGMISLNP
T.melanosporum GALIGARGLYNFGRDPRKQPNPP----------PRTSDEKAVGRFSMGAEFYYGILNKSA
L.maculans SALLGLRGLYNFGPDPRNAPTEP----------TLAQEVDPVHGRFSAGAELYYGILNKSG
L.elongisporus ESLIGLRFLYNLGNPITKNISPA----------LAPKFDNSVVSVGTELWFAARTMSP
Y.lipolytica EALLGARGLYNFGVDMSKPHIAS----------RLSVGGEFYYGVLNKSP
N.crassa GGLLGFRGLYNFGGDASSSTCDPWTPTPGENNNNNNNNNNNNNGNAQAGEKERIYGRFSVGGELYYGTLNKSG
A.fumigatus NSLFGWRGLWNFGPDPRHPKENS----------SPQLSLLSAGAEAYYSPVSSLI
S.pombe GAMLGVRGLWNLNYRELNTKINM----------ENKAPSNMRWSLGFETYYGVLTKCA
C.neoformans DGMVGLRTLYNFGWHGDEESEVD---------KKERRREREGKRIDEEEMMEGGLKGRFSAGGEVYFSAKQRSF
A.castellanii RDRACFSHMQAITQ----------RLSVGGEVGYAMKDKQP
C.owczarzaki EHMLGFSGMRALSSSKE----------WAVGGEVFYSFSQEAP
366
C.albicans -GLSAALRYSTRSTS----------TGK--PLTMTLAINPIVGHVSSTYTVKTSVASTFC
S.cerevisiae -GCSTTLRYYTHSTN----------TGR--PLTLTLSWNPLFGHISSTYSAKTGTNSTFC
C.tropicalis -GLSGALRYSTRSTS----------TGK--PLTMTLAINPILGHISSTYTVKTSVASTFC
L.thermotolerans -ACSTTLRYCTHSAN----------TGK--PLTLTLSWNPLFGHVSSTYSVKTSSGATFS
K.polysporus -TCATTLRYCTHSAT----------TGR--PLTLTLSFNPLFGHLSSTYSAKTSSNSAFC
K.lactis -GCSTTLRYCSHATN----------TGK--PLIFTLSLNPLYGHVSSTYSIKTHENLTFC
D.hansenii -GLSTAFRYSTRSTS----------TGK--PLTMTFACNPILGHISSAYTVKTSVASTFC
N.castellii -GGSTSIRYCTHSAN----------TGR--PLTLTFSYNPLFGHISSTYAAKTGLNSTFC
C.glabrata -GCSTSLKYCTHSAN----------TGR--PLTLTLSWNPLFGHISTTYSAMTSSSSTFC
T.melanosporum -GMSTGLRYTTLPTH----------PNA--PTTMTLTLTPLMGSLSATYAIKAGEAASFC
L.maculans -GISTGLRFTTLPNH----------PGF--PYTMTLTLNPLMGNLSSTYAVKAGPNLALC
L.elongisporus -GLSTALRYSTRSTS----------TGK--PLTMTLAVNPILGHISSTYTVKTSVASTFC
Y.lipolytica -GMSTALRYVTQSAY----------TGS--PLTMTLTCNPIMGEFSSTYSLRTGPSSSFS
N.crassa -GMSLGARFATLPAH----------RGT--PLTATLTINPLMGNINATYALLAREYCSLA
A.fumigatus -GMSTGLRFSTLPAATEMPSSSSSASSTTTTSNHDTPISTF--PYTLTLVLTPLTGSLSTTYSLRASPNLAFS
S.pombe -GASLGMRLHSGPSH----------PYA--PFILTCTLNPIVGHITSTFSTAEPRTKAFS
C.neoformans -GISTGLRFTTVPPTLPLPLNAPV----------PSP--PTTLTLLYNPLIGFLSSAYSAQVSPTVALA
A.castellanii VGWSVGGRYVIRKEE----------GGKFAATHITATYNHL-GRVKTTYTLPFSETVFAS
C.owczarzaki -GLSVGARYHTVNAN----------TGH--TTVVATTFSPIPGHVTATYTSDLTQAMVAS


439
C.albicans SKYDFNVFSYASNLSLGFELYSYANKKKNSFP----------SFEHHEIHSSSE
S.cerevisiae AKYDFNLYSIESNLSFGCEFWQKKHHL----------LETNKNNND
C.tropicalis SRYDFNVFSYASNLSLGFELYSYANKK---RS----------DFYNHEIYSSSE
L.thermotolerans SKYDFNLYSIESNLSFGCDLWRRGHTK----------
K.polysporus VQYDFNLYSIDSNLSLGCELWKQNELI----------
K.lactis TKYDFNIYSIQSNLTLGMELWKNSSSI----------PVERPSE
D.hansenii SKYDFNVFSYASNLSLGFEIYNYSSSN----------TSNSAATP
N.castellii TRYDFNIYSIDSNLTFGWEFWKNNSDN----------
C.glabrata AKYDFNIYSIESNLSFGIELWRKTGAL----------FKSEDSVEAN
T.melanosporum SRFDFNMYSYESDLSLGCEVWRR----------
L.maculans SRFDFNFYSYESELQLGCELWRRRSTT----------
L.elongisporus SKYDFNFFSYASNLSLGFELYSYARNKNYLSPVESSMMLEQPTATVPGSLHNQATKPRRESQRQKSEDYISSS
Y.lipolytica TRYDFNMYSYLSNLSMGAEVWKSRD----------
N.crassa TRVDFNVYSYESEWAVGMELWSNRRPA----------
A.fumigatus SRFGFNVYSWESEMVAGCELWRKRRKP----------
S.pombe AQYDFNIYSYESQLKLGIELWRS----------
C.neoformans TRFGVNVYSYESDLSVGGEWWIGRRRG----------KRGLTTDAEP
A.castellanii ARYTVNAFSFKSNMAVGVESWP----------
C.owczarzaki TRFEYNVHSNEAEFGVGLRL----------
512
C.albicans ENKYLKKHPELQRHHNL--HHNLHHQRVPIKSHKYEGNRTIINPIQNLDNVYHINPTLLSSNGSTSTTT----
S.cerevisiae KLEPISDELVDINPNSR--ATKLLHENVPDLNSAVNDIPST---LDIPVHKQKLLNDLTYAFSSSLRKI----
C.tropicalis ENKYLKQHPELQK----------HHRVPIRAYKHHDNRTIINPIHNLDNVYHINPTLLSTTSGSGHTS----
L.thermotolerans QLDQRRTEPLDAPNTN---SSVFSKERVQKKQGPKEDSPMFYHLMAGSTSSQKLIEDLNVTFASSLQKL----
K.polysporus ----------QNVSQEKSKKQEIQVPPNFYNNNSNDAKQKRILNDLNTTFESSLKKI----
K.lactis PVETIEDWPQLS----------HDKTPMYYHLLTRTENSG-----ASSQRLLKDLNLTFQSSLQKI----
D.hansenii PRIKNSDSQVLSNNSTD--SKGTVHIRSPDVLDYKNHSNVIISPIQTFDNYYHINPTLLPSTKNEFEEVRPPP
N.castellii ----------SKLKEPDTNVNSLSGNSSLEVSPLINTSQQKLLNDLTYTFSSSLEKI----
C.glabrata RKEYEQNFYISHD----------HNLLPSKYGYDHEYNISDGKLSKEHKRNKIIKDLNHAFSTSLQKI----
T.melanosporum ----------
L.maculans ----------DVEWAVRKLRPDW----------
L.elongisporus DSDYTRMQVKEFGPGGRITSEQKSNDRLPNKVSPIQNKRDRGREHFTNSSSNNSNSNINSNANTNLDAA----
Y.lipolytica ----------
N.crassa ----------GFLLGASPSNDFEPEPHPPRKKERSFQAKMEWRLDDPEP----------
A.fumigatus ----------SPPPVDDDGLEWARRKMRMADTPAF----------
S.pombe ----------
C.neoformans QLDAESRDPVVTGIEEN--RELTEKMAQRASLRQVTLRDEIGEDVHAEKELYSPIPAMTDVNAGELAQQ----
A.castellanii ----------
C.owczarzaki ----------
585
C.albicans -NNENTNTSETVTAAFQNLVNESDFSSVFKFSTSLNDKVVKILWEGRLKEFLVSTGVKL-----SLNPITNTP
S.cerevisiae -DEE-----RSTIEKFDNKINSSIFTSVWKLSTSLRDKTLKLLWEGKWRGFLISAGTEL-----VFTRGFQES
C.tropicalis ---------ETVTTAFQNLVNESDFSSVLKFSTSLNDKVVKILWEGRLRDFLVSTGVKL-----SLNPVTNTP
L.thermotolerans -TKE-----KSTIQRFENSLIDANFASVWKLSTSLRHKNLRVLWEGKYKGFLISAGAEF-----TGAPLELPS
K.polysporus -DKE-----RAVIENFETDLYNKDFTSVWKFSTSLRDKNLCILWDGKFKGFLLSAGTEL-----IRINTNNEN
K.lactis -NKE-----RNVIENFNERYSEANFTQVFKVSTSLRDMNLRLLWEGKIRGFLISAGAEL-----TQPPEISTN
D.hansenii LESQVDSNNETAMTAFENLVNESDFSSVIKLSTSLNDKMLKLLWKGRCKDFLVTTGVKM-----ILNPITNTP
N.castellii -DKE-----KTAIEKFQKKINESNFTNVWKFSTSLKDKNLKIKWEGKFKGFLLSAGTELYSTSSIIQDINGQT
C.glabrata -DKE-----KTRIENFGNIIRNSHFTSVFKASTSLRERNLKFLWEGEYKNFLLSAGTEL-----RVLKAEESE
T.melanosporum ----------EDVEKEDFAGVLKARVS-QTSGIGLLWEGRLKHLLFSLGAGI-----DLRRKDQLV
L.maculans ----------KRPTLSSDDDVAGVLKARVD-QDWRIGVLWEGRIKEMLFTLGASL-----DLKKREQIF
L.elongisporus -------FEEKVTTAFQNLVNASDFSSVVKVSTSLRDKTVKLLWEGRVKDFLVSTGARI-----AINPVTNAP
Y.lipolytica ----------SVFKLSSSLQDKTARVLWGGRYKDILVNTGVAF-----DYGGRVPDV
N.crassa ---------EPEPQPTPKTRKNDEYKGVLKARLD-NNLRMGLLWEGRAKSLIFSIGTGI-----DLHKLGEPF
A.fumigatus ----------APVEPPTTHNRDEENESVLKIRVD-QSWNVRLLWEGRVKELLVSAGVGL-----GPSSFSSPS
S.pombe ---------KQEMSQSTNDPTANSMSSLLKGTCS-TSGDVSISWQARIRNFLLTIGTEA-----QLTKID--P
C.neoformans -I-------SPRLQPQQDLDDERD--GVLKARLS-GNWQFALLYEARIRNCLVSAGVLA-----DLTGRQHPI
A.castellanii ----------SATMPLMTKAK-------WDTD-KGFGVSLGAHL-----GLAALMFSA
C.owczarzaki ---------QPPNSPFD----------LRFRVD-TSHGIGVLLRARFPHLTLALGAGC-----SFPPNVTTR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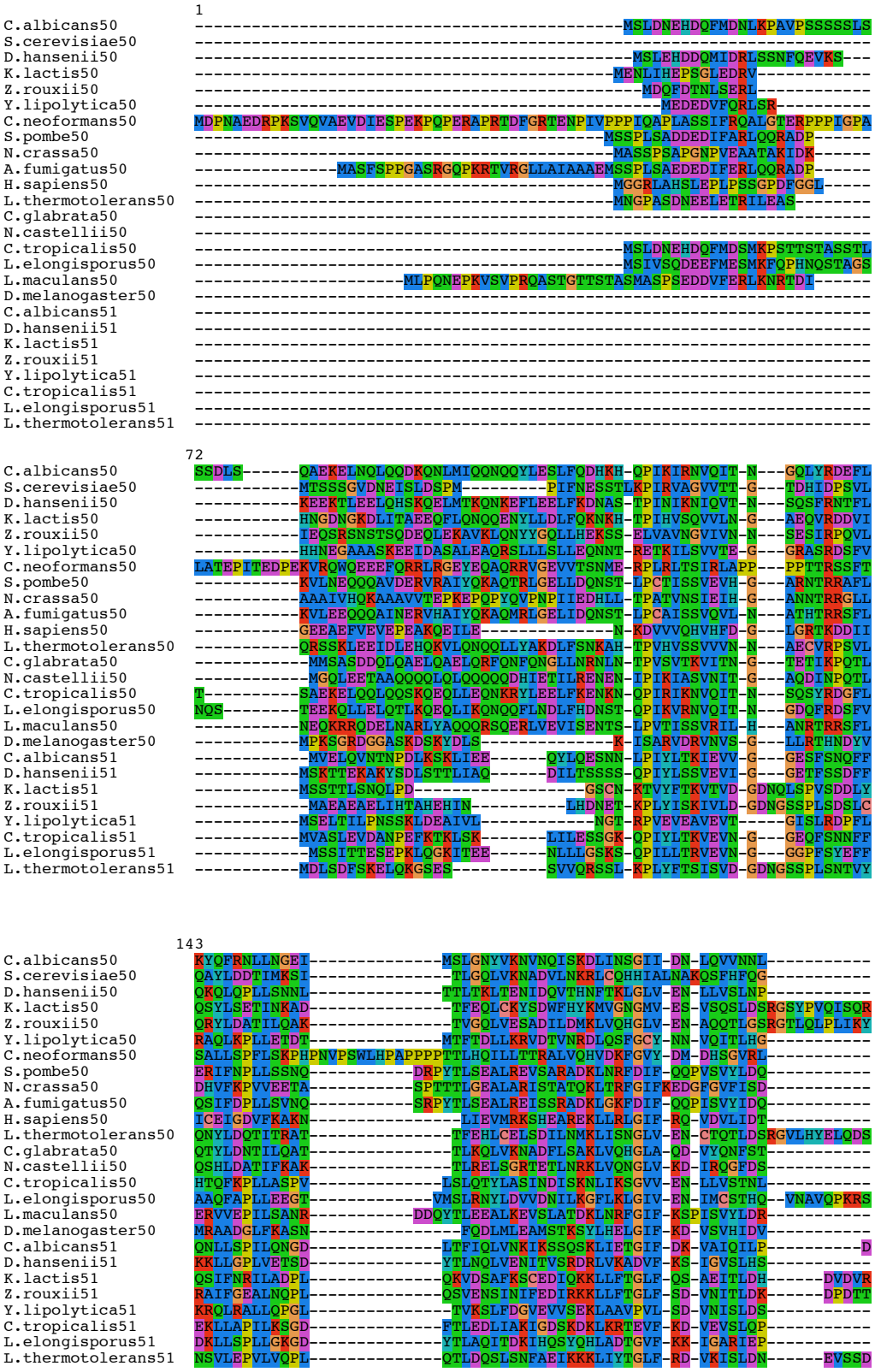
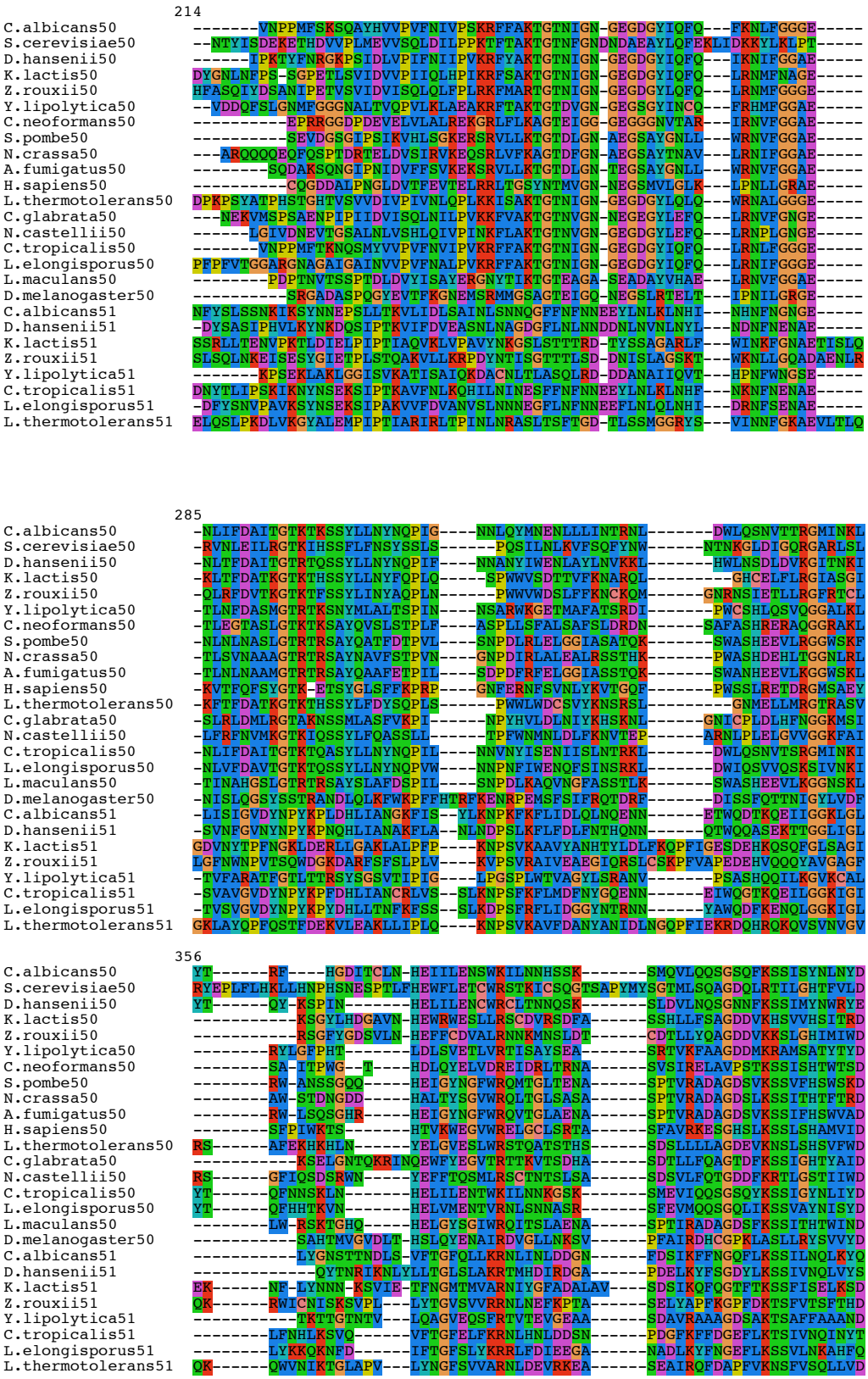
K.polysporus
K.lactis
D.hansenii
N.castellii
C.glabrata
T.melanosporum
L.maculans
L.elongisporus
Y.lipolytica
N.crassa
A.fumigatus
S.pombe
C.neoformans
A.castellanii
C.owczarzaki
Po G Hy Hy
Hy
β-signal motif

Figure 8.4 Sam50 and Sam51 multiple sequence alignment
Accession numbers of sequences and full species names are listed in Appendix 1: Table 8.3.

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S.cerevisiae50 --NTYISDEKETHDVVPLMEVVSQLDILPPKTFTAKTGTNFGNDNDAEAYLQFEKLIDKKYLKLPT-----
D.hansenii50 ------IPKTYFNRGKPSIDLVPIFNIIPVKRFYAKTGTNIGN-GEGDGYIQFQ---IKNIFGGAE-----
K.lactis50 DYGNLNFPS-SGPETLSVIDVVPIIQLHPIKRFSAKTGTNIGN-GEGDGYIQFQ---LRNMFNAGE-----
Z.rouxii50 HFASQIYDSANIPETVSVIDVISQLQLFPLRKFMARTGTNIGN-GEGDGYLQFQ---LRNMFGGGE-----
Y.lipolytica50 --VDDQFSLGNMFGGGNALTVQPVLKLAEAKRFTAKTGTDVGN-GEGSGYINCQ---FRHMFGGAE-----
C.neoformans50 ----------EPRRGGDPDEVELVLALREKGRLFLKAGTEIGG-GEGGGNVTAR---IRNVFGGAE-----
S.pombe50 ----------SEVDGSGIPSIKVHLSGKERSRVLLKTGTDLGN-AEGSAYGNLL---WRNVFGGAE-----
N.crassa50 ---ARQQQQEQFQSPTDRTELDVSIRVKEQSRLVFKAGTDFGN-AEGSAYTNAV---LRNIFGGAE-----
A.fumigatus50 ---------SQDAKSQNGIPNIDVFFSVKEKSRVLLKTGTDLGN-TEGSAYGNLL---WRNVFGGAE-----
H.sapiens50 ----------CQGDDALPNGLDVTFEVTELRRLTGSYNTMVGN-NEGSMVLGLK---LPNLLGRAE-----
L.thermotolerans50 DPKPSYATPHSTGHTVSVVDIVPIVNLQPLKKISAKTGTNIGN-GEGDGYLQLQ---WRNALGGGE-----
C.glabrata50 ---NEKVMSPSAENPIPIIDVISQLNILPVKKFVAKTGTNVGN-NEGEGYLEFQ---LRNVFGNGE-----
N.castellii50 ------LGIVDNEVTGSALNLVSHLQIVPINKFLAKTGTNVGN-GEGDGYLEFQ---LRNPLGNGE-----
C.tropicalis50 --------VNPPMFTKNQSMYVVPVFNVIPVKRFFAKTGTNIGN-GEGDGYIQFQ---LRNLFGGGE-----
L.elongisporus50 PFPFVTGGARGNAGAIGAINVVPVFNALPVKRFFAKTGTNIGN-GEGDGYIQFQ---LRNIFGGGE-----
L.maculans50 --------PDPTNVTSSPTDLDVYISAYERGNYTIKTGTEAGA-SEADAYVHAE---LRNVFGGAE-----
D.melanogaster50 ----------SRGADASPQGYEVTFKGNEMSRMMGSAGTEIGQ-NEGSLRTELT---IPNILGRGE-----
C.albicans51 NFYSLSSNKIKSYNNEPSLLTKVLIDLSAINLSNNQGFFNFNNEEYLNLKLNHI---NHNFNGNGE-----
D.hansenii51 -DYSASIPHVLKYNKDQSIPTKVIFDVEASNLNAGDGFLNLNNDDNLNVNLNYL---NDNFNENAE-----
K.lactis51 SSRLLTENVPKTLDIELPIPTIAQVKLVPAVYNKGSLSTTTRD-TYSSAGARLF---WINKFGNAETISLQ
Z.rouxii51 SLSQLNKEISESYGIETPLSTQAKVLLKRPDYNTISGTTTLSD-DNISLAGSKT---WKNLLGQADAENLR
Y.lipolytica51 -------KPSEKLAKLGGISVKATISAIQKDACNLTLASQLRD-DDANAIIQVT---HPNFWNGSE-----
C.tropicalis51 DNYTLIPSKIKNYNSEKSIPTKAVFNLKQHILNINESFFNFNNEEYLNLKLNHF---NKNFNENAE-----
L.elongisporus51 -DFYSNVPAVKSYNSEKSIPAKVVFDVANVSLNNNEGFLNFNNEEFLNLQLNHI---DRNFSENAE-----
L.thermotolerans51 ELQSLPKDLVKGYALEMPIPTIARIRLTPINLNRASLTSFTGD-TLSSMGGRYS---VINNFGKAEVLTLQ
285
C.albicans50 -NLIFDAITGTKTKSSYLLNYNQPIG----NNLQYMNENLLLINTRNL-------DWLQSNVTTRGMINKL
S.cerevisiae50 -RVNLEILRGTKIHSSFLFNSYSSLS------PQSILNLKVFSQFYNW------NTNKGLDIGQRGARLSL
D.hansenii50 -NLTFDAITGTRTQSSYLLNYNQPIF----NNANYIWENLAYLNVKKL-------HWLNSDLDVKGITNKI
K.lactis50 -KLTFDATKGTKTHSSYLLNYFQPLQ-----SPWWVSDTTVFKNARQL---------GHCELFLRGIASGI
Z.rouxii50 -QLRFDVTKGTKTFSSYLINYAQPLN------PWWVWDSLFFKNCKQM------GNRNSIETLLRGFRTCL
Y.lipolytica50 -TLNFDASMGTRTKSNYMLALTSPIN----NSARWKGETMAFATSRDI-------PWCSHLQSVQGGALKL
C.neoformans50 -TLEGTASLGTKTKSAYQVSLSTPLF----ASPLLSFALSAFSLDRDN------SAFASHRERAQGGRAKL
S.pombe50 -NLNLNASLGTRTRSAYQATDTPVL----SNPDLRLELGGIASATQK-------SWASHEEVLRGGWSKF
N.crassa50 -TLSVNAAAGTRTRSAYNAVFSTPVN----GNPDIRLALEALRSSTHK-------PWASHDEHLTGGNLRL
A.fumigatus50 -TLNLNAAMGTRTRSAYQAAFETPIL----SDPDFRFELGGIASSTQK-------SWANHEEVLKGGWSKL
H.sapiens50 -KVTFQFSYGTK-ETSYGLSFFKPRP----GNFERNFSVNLYKVTGQF-------PWSSLRETDRGMSAEY
L.thermotolerans50 -KFTFDATKGTKTHSSYLFDYSQPLS------PWWLWDCSVYKNSRSL---------GNMELLMRGTRASV
C.glabrata50 -SLRLDMLRGTAKNSSMLASFVKPI------NPYHVLDLNIYKHSKNL------GNICPLDLHFNGGKMSI
N.castellii50 -LFRFNVMKGTKIQSSYLFQASSLL------TPFWNMNLDLFKNVTEP------ARNLPLELGVVGGKFAI
C.tropicalis50 -NLIFDAITGTKTQASYLLNYNQPIL----NNVNYISENIISLNTRKL-------DWLQSNVTSRGMINKI
L.elongisporus50 -NLVFDAVTGTKTQSSYLLNYNQPVW----NNPNFIWENQFSINSRKL-------DWIQSVVQSKSIVNKI
L.maculans50 -TINAHGSLGTRTRSAYSLAFDSPIL----SNPDLKAQVNGFASSTLK-------SWASHEEVLKGGNSKL
D.melanogaster50 -NISLQGSYSSTRANDLQLKFWKPFFHTRFKENRPEMSFSIFRQTDRF-------DISSFQTTNIGYLVDF
C.albicans51 -LISIGVDYNPYKPLDHLIANGKFIS--YLKNPKFKFLIDLQLNQENN------ETWQDTKQEIIGGKLGL
D.hansenii51 -SVNFGVNYNPYKPNQHLIANAKFLA--NLNDPSLKFLFDLFNTHQNN------QTWQQASEKTTGGLIGL
K.lactis51 GDVNYTPFNGKLDERLLGAKLALPFP----KNPSVKAAVYANHTYLDLFKQPFIGESDEHKQSQFGLSAGI
Z.rouxii51 LGFNWNPVTSQWDGKDARFSFSLPLV----KVPSVRAIVEAEGIQRSLCSKPFVAPEDEHVQQQYAVGAGF
Y.lipolytica51 -TVFARATFGTLTTRSYSGSVTIPIG----LPGSPLWTVAGYLSRANV-------PSASHQQILKGVKCAL
C.tropicalis51 -SVAVGVDYNPYKPFDHLIANCRLVS--SLKNPSFKFLMDFNYGQENN------EIWQGTKQEILGGKIGI
L.elongisporus51 -TVSVGVDYNPYKPYDHLLTNFKFSS--SLKDPSFRFLIDGGYNTRNN------YAWQDFKENQLGGKIGL
L.thermotolerans51 GKLAYQPFQSTFDEKVLEAKLLIPLQ----KNPSVKAVFDANYANIDLNGQPFIEKRDQHRQKQVSVNVGV
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C.albicans50 YT------RF----HGDITCLN-HEIILENSWKILNNHSSK-------SMQVLQQSGSQFKSSISYNLNYD
S.cerevisiae50 RYEPLFLHKLLHNPHSNESPTLFHEWFLETCWRSTKICSQGTSAPYMYSGTMLSQAGDQLRTILGHTFVLD
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Y.lipolytica50 --------RYLGFPHT--------LDLSVETLVRTISAYSEA-------SRTVKFAAGDDMKRAMSATYTYD
C.neoformans50 --------SA-ITPWG--T-----HDLQYELVDREIDRLTRNA------SVSIRELAVPSTKSSISHTWTSD
S.pombe50 --------RW-ANSSGQQ-----HEIGYNGFWRQMTGLTENA------SPTVRADAGDSVKSSVFHSWSKD
N.crassa50 --------AW-STDNGDD-----HALTYSGVWRQLTGLSASA------SPTVRADAGDSLKSSITHTFTRD
A.fumigatus50 --------RW-LSQSGHR-----HEIGYNGFWRQVTGLAENA------SPTVRADAGDSVKSSIFHSWVAD
H.sapiens50 --------SFPIWKTS--------HTVKWEGVWRELGCLSRTA------SFAVRKESGHSLKSSLSHAMVID
L.thermotolerans50 RS------AFEKHKHLN-----YELGVESLWRSTQATSTHS------SDSLLLLAGDEVKNSLSHSVFWD
C.glabrata50 ----------KSELGNTQKRINQEWFYEGVTRTTKVTSDHA------SDTLLFQAGTDFKSSIGHTYAID
N.castellii50 RS------GFIQSDSRWN-----YEFFTQSMLRSCTNTSLSA------SDSVLFQTGDDFKRTLGSTIIWD
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L.elongisporus50 YT------QFHHTKVN--------HELVMENTVRNLSNNASR-------SFEVMQQSGQLIKSSVAYNISYD
L.maculans50 --------LW-RSKTGHQ-----HELGYSGIWRQITSLAENA------SPTIRADAGDSFKSSITHTWIND
D.melanogaster50 ----------SAHTMVGVDLT-HSLQYENAIRDVGLLNKSV------PFAIRDHCGPKLASLLRYSVVYD
C.albicans51 ----------LYGNSTTNDLS-VFTGFQLLKRNLINLDDGN------FDSIKFFNGQFLKSSILNQLKYQ
D.hansenii51 ----------QYTNRIKNLYLLTGLSLAKRTMHDIRDGA------PDELKYFSGDYLKSSIVNQLVYS
K.lactis51 EK------NF-LYNNN-KSVIE-TFNGMTMVARNIYGFADALAV----SDSIKQFQGTFTKSSFISELKSD
Z.rouxii51 QK------RWICNISKSVPL---LYTGVSVVRRNLNEFKPTA------SELYAPFKGPFDKTSFVTSFTHD
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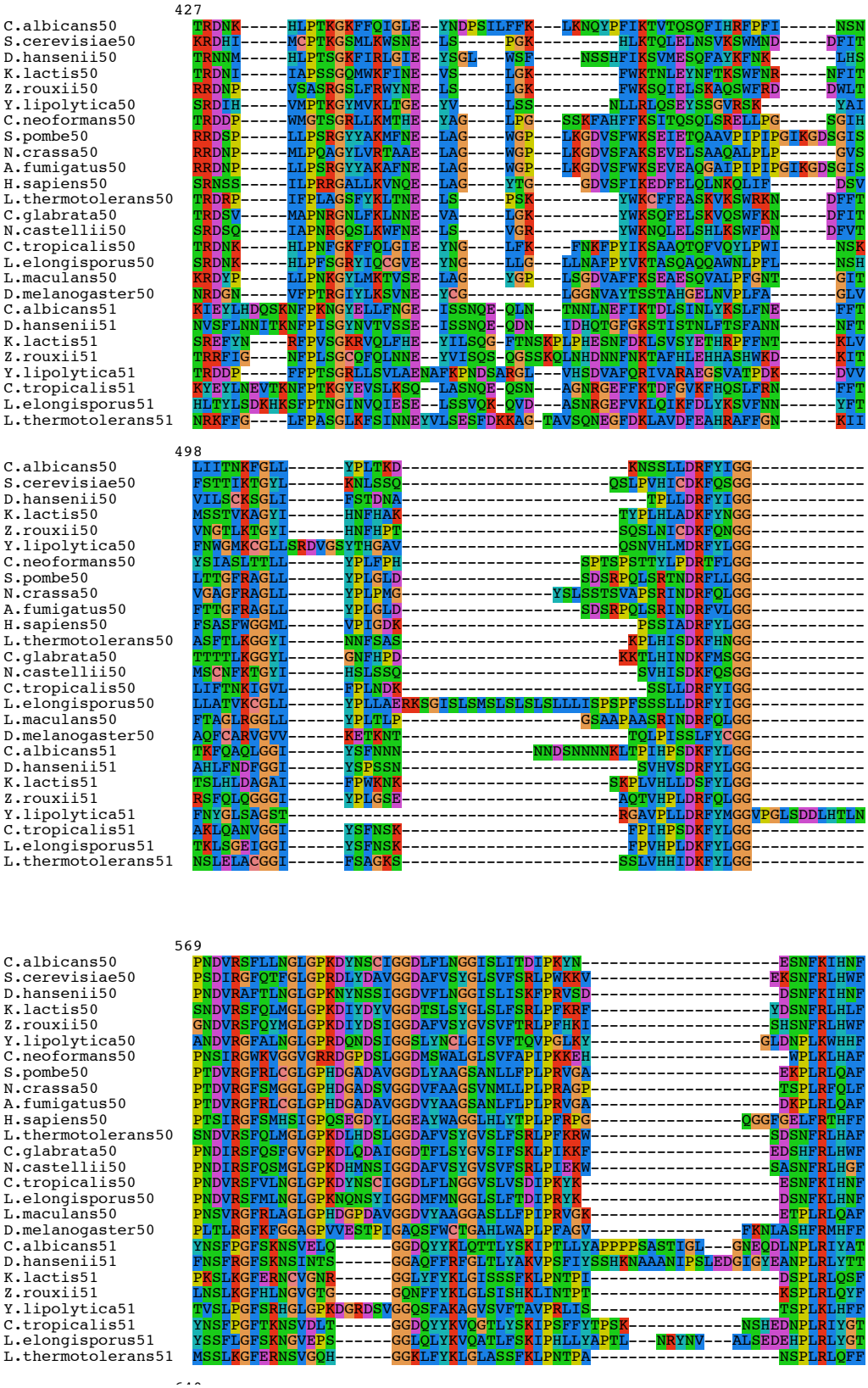
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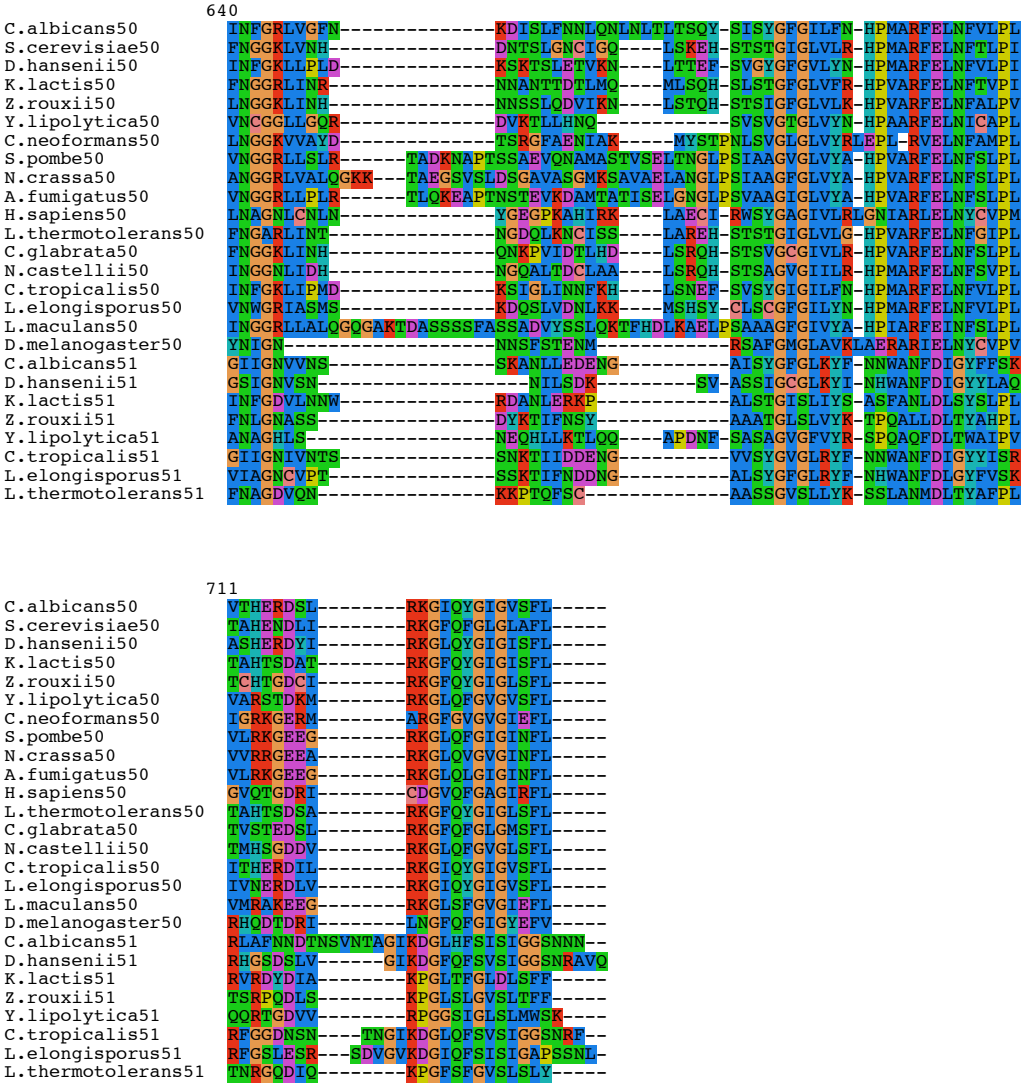
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Appendix 8: Drop tests of *sam51* deletion strains

Figure 8.5 Drop tests of *sam51ΔΔ* sensitivity to mitochondrial targeting drugs

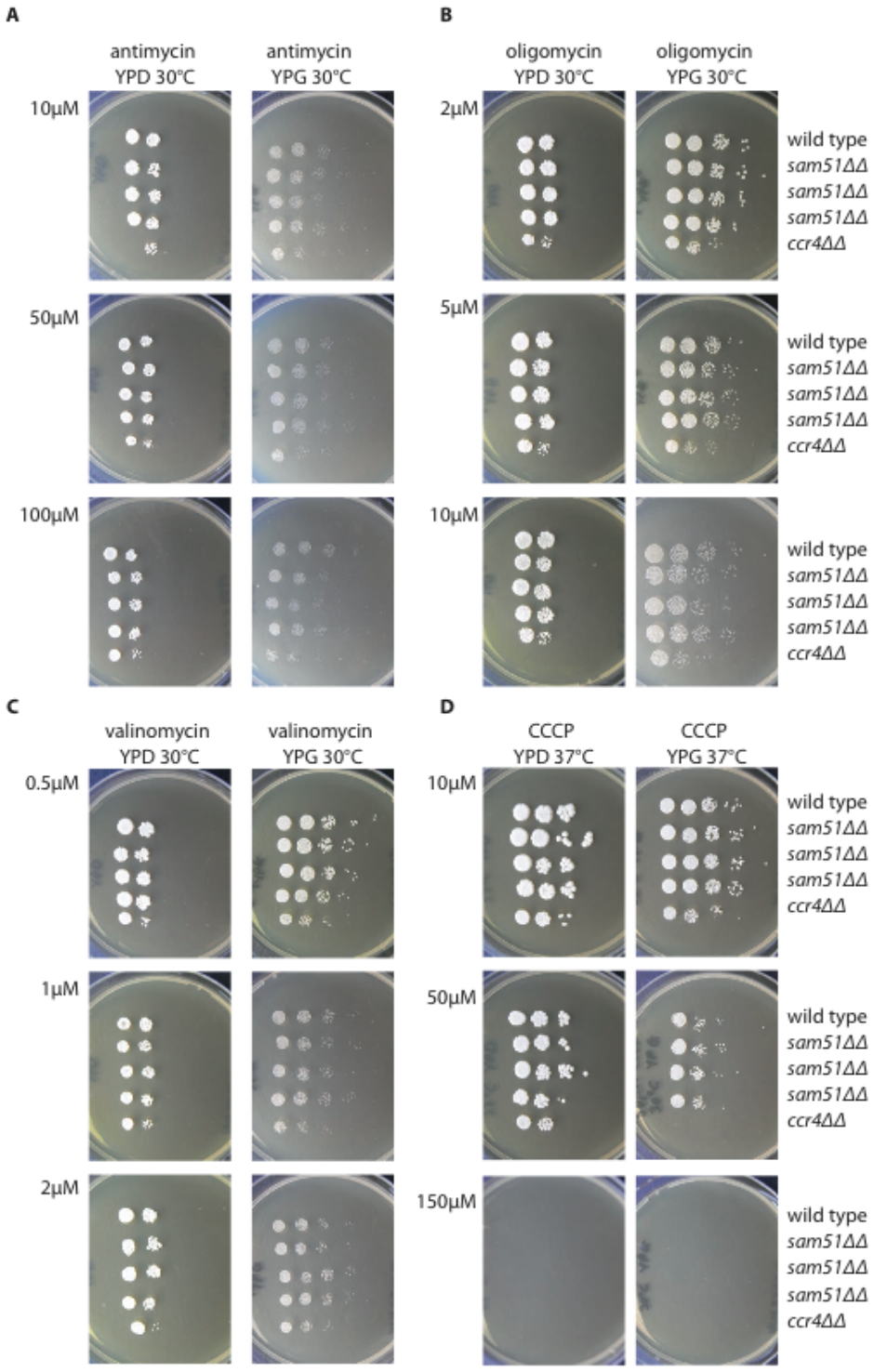


Figure 8.6 Drop test assay testing *sam51ΔΔ* iron metabolism

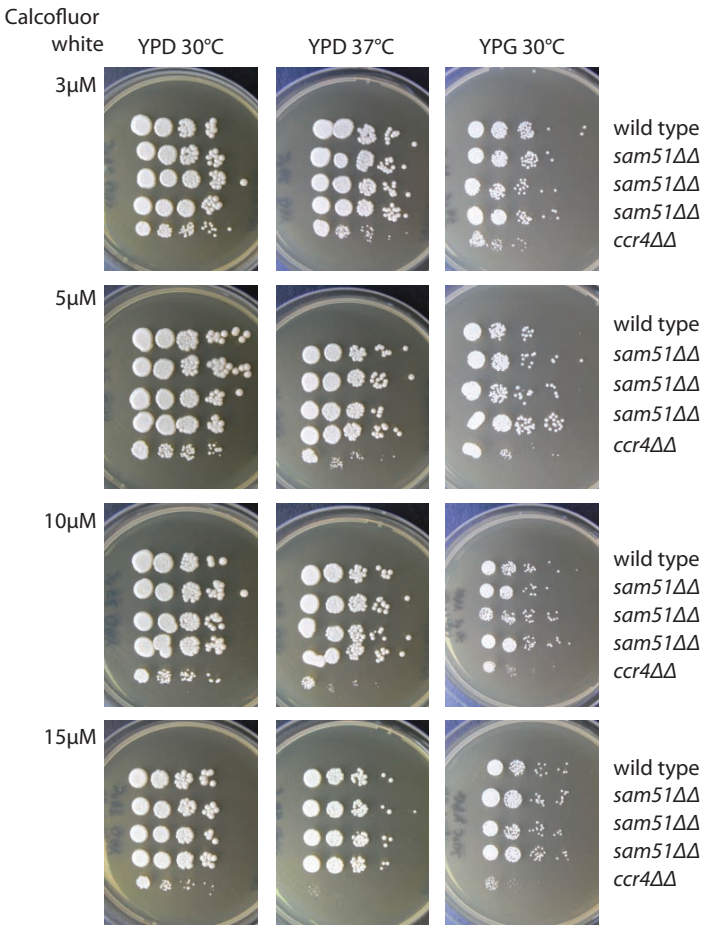
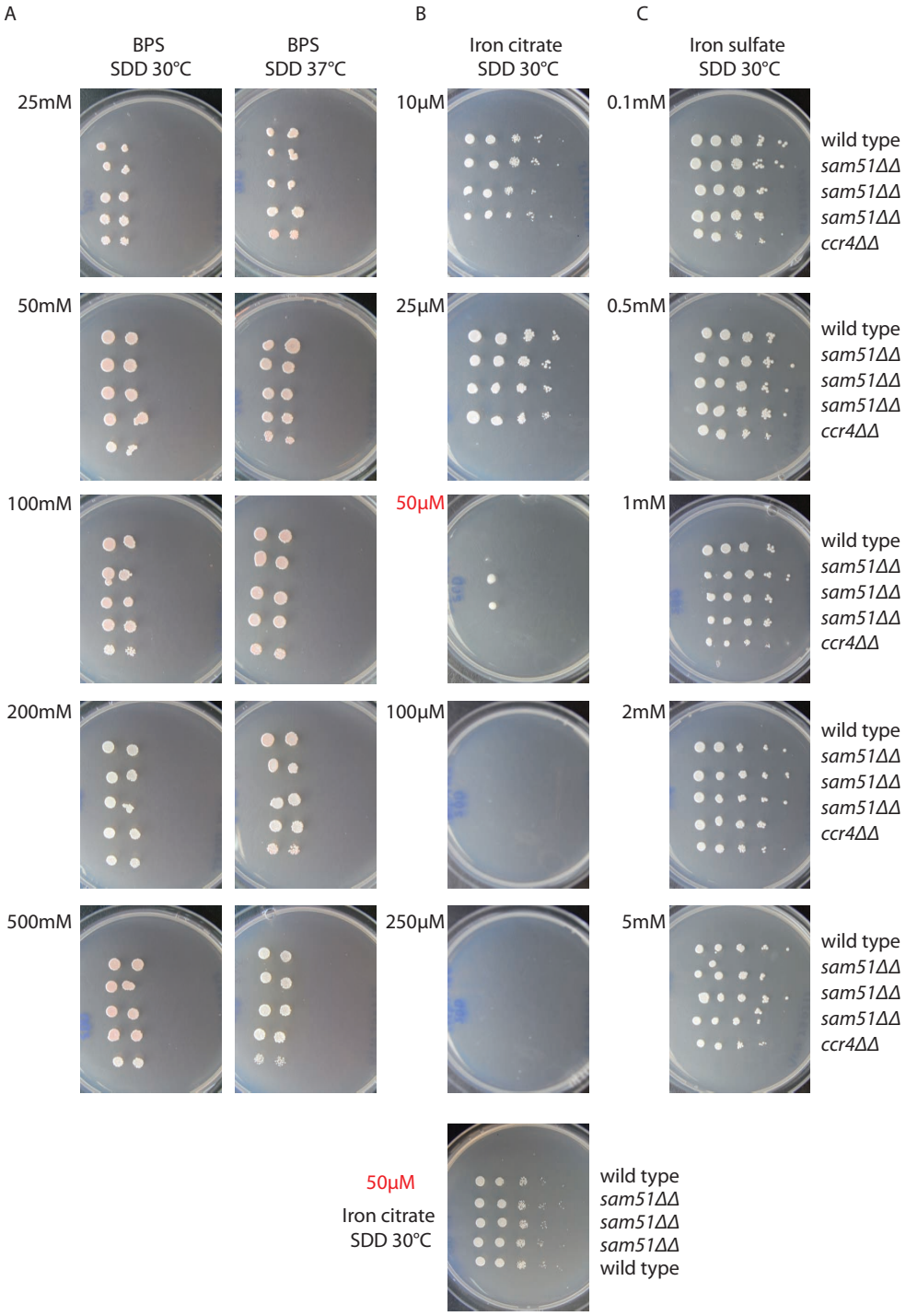


Figure 8.7 Drop tests of *sam51ΔΔ* sensitivity to calcofluor white



Appendix 9: Hewitt et. al. (2011)

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Review

Minor modifications and major adaptations: The evolution of molecular machines driving mitochondrial protein import☆

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ABSTRACT

Bacterial endosymbionts gave rise to mitochondria in a process that depended on the acquisition of protein import pathways. Modification and in some cases major re-tooling of the endosymbiont's cellular machinery produced these pathways, establishing mitochondria as organelles common to all eukaryotic cells. The legacy of this evolutionary tinkering can be seen in the homologies and structural similarities between mitochondrial protein import machinery and modern day bacterial proteins. Comparative analysis of these systems is revealing both possible routes for the evolution of the mitochondrial membrane translocases and a greater understanding of the mechanisms behind mitochondrial protein import. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

The conversion of ancestral α-proteobacteria to mitochondria involved the transfer of genes from the bacterial endosymbiont to the host cell genome [1–9]. Once relocated to the nucleus the gene products, translated in the cytosol, had to be recognized, targeted, translocated and assembled in mitochondria. An account of the evolution of such sophisticated molecular machinery should explain how the components could plausibly be established in a stepwise fashion with modifications to and the support of existing mechanisms.

Three main themes emerge when investigating the evolution of the mitochondrial protein transport machinery: (i) modifications of an existing system, wherein the ancestral function is conserved in bacteria and mitochondria; (ii) reorganisation and modification of bacterial proteins giving rise to machinery with new functions; (iii) use of structural or functional homologues to provide insight into components where sequence similarity does not illuminate the evolutionary path. In this review we examine the evolutionary implications of each of these cases, and the impact this has on our understanding of how the protein import machinery functions in mitochondria.

2. Mitochondrial protein translocation pathways

The majority of proteins targeted to mitochondria have a presequence, a short extension of the polypeptide which forms a positively charged amphipathic α-helix, and directs translocation across the outer and inner mitochondrial membranes [10,11]. The Translocase of the Outer mitochondrial Membrane (TOM complex) is

☆ This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

* [redacted]

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a large multimeric machine, whose major subunit–the β-barrel Tom40–forms a channel across the membrane. With the exception of a few peripheral, outward-facing outer membrane proteins, all mitochondrial proteins are imported via the TOM channel. Translocation of matrix proteins is dependent on the presequence, which is drawn through the TOM complex by sequential interaction with negatively charged sites within the complex (acid chain hypothesis: [12–15]). The inner membrane presequence translocase, TIM23, receives the presequence as it exits the TOM channel and cooperates with the ATP-driven Presequence-Associated Motor (PAM) to complete translocation into the matrix (Fig. 1).

Alternative pathways exist to target proteins to the inner membrane, outer membrane and intermembrane space, often depending on as yet poorly defined targeting signals. In many organisms, a second inner membrane translocase, TIM22, assembles polytopic proteins into the inner membrane [16,17]. In all eukaryotes, the outer membrane Sorting and Assembly Machinery (SAM complex) assembles outer membrane β-barrels [18–20]. Both types of precursor proteins require assistance from the small Tim chaperones in the intermembrane space for delivery to TIM22 or SAM [21–23]. These chaperones are imported by another pathway, using the Mitochondrial Intermembrane space import and Assembly (MIA) machinery, which couples precursor import with oxidation [24–26]. A further translocase in the inner membrane, termed OXA (OXidase Assembly), inserts proteins from the mitochondrial matrix into the inner membrane [27–29]. As far as we know, the majority of OXA substrates are encoded in the mitochondrial genome, and inserted co-translationally.

The TOM, TIM23, TIM22, SAM and MIA molecular assemblies have been intensively studied in yeast, and we have a remarkable understanding of many of the mechanistic intricacies of this highly evolved system. By combining these insights with sequence and functional analyses of mitochondrial import systems in diverse eukaryotes, a picture is emerging of the minimal requirements of each import machine, and how the original, simplest versions of each might first have come to be.

3. Re-vamping bacterial protein translocases for continued function

The Oxa1 protein is the core component of the OXA translocase and is a direct descendant of the bacterial YidC translocase, which also inserts inner membrane proteins (Fig. 2) [27,30,31]. Like OXA, the mitochondrial SAM complex also has a direct counterpart in bacteria; the BAM complex, found in the outer membrane of all Gram-negative bacteria. The core component of the SAM complex, Sam50, is a member of the Omp85 family of proteins that also includes BamA, the core component of the BAM complex [4,18–20,32,33].

While Sam50 is clearly derived from BamA, and the SAM and BAM complexes are functionally homologous, significant evolutionary divergence is evident in the mitochondrial SAM complex (Fig. 2). Mitochondria have lost whole aspects of envelope biogenesis including the ability to synthesise lipoproteins, events that likely determined the loss of the lipoprotein partners, BamD and BamE, of the endosymbiont's BAM complex [34]. These have been replaced, either during or subsequent to this period of lipoprotein loss, by proteins of uncertain ancestry. From functional studies in several organisms we know of at least three types of these proteins: the metaxins, Mim1 and Mdm10. These "modules" of the SAM complex are not conserved across eukaryotes [35,36] and we anticipate that a better understanding of the precise function of these components will give insight into how this modular system evolved.

The metaxins are proteins with a predicted glutathionine-S-transferase type fold and are associated with the SAM complex in fungi (Sam35 and Sam37; [37–41]), animals (metaxin-1 and metaxin-2; [42,43]) and plants (metaxin; [44,45]). Given the divergence between these groups the metaxins may be found in other eukaryotic lineages too, but bioinformatics alone has not been able to resolve this issue. Work in the yeast system shows that the metaxin Sam35 is responsible for substrate docking/entry into the SAM complex [46,47]), while Sam37 is required for efficient release of substrates from the SAM complex [47]. Presumably, the metaxins associated with the SAM complex in animals and plants play similar roles.

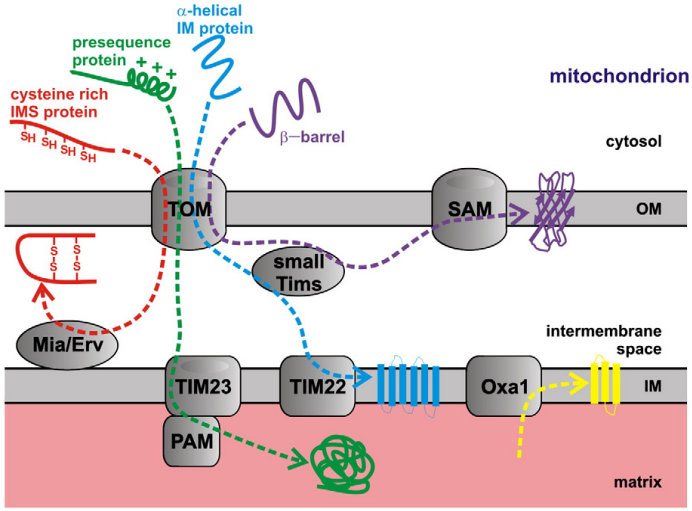

Fig. 1. An overview of mitochondrial protein import routes. Four classes of protein precursor are translocated across the TOM channel in the outer membrane (OM). Proteins with a presequence (green) are transferred from TOM to TIM23, and imported into the matrix with the help of the import motor, PAM. α-helical proteins (blue) are inserted into the inner membrane by TIM22 and β-barrel proteins (purple) are inserted into the outer membrane by SAM. Precursors of both β-barrel and α-helical membrane proteins are chaperoned in the intermembrane space by the small Tims. Small, cysteine-rich proteins (red) of the intermembrane space are imported by the MIA/Erv machinery, which also mediates their oxidation and folding. Oxa1 in the inner membrane inserts mitochondrially-encoded proteins into the inner membrane (yellow).

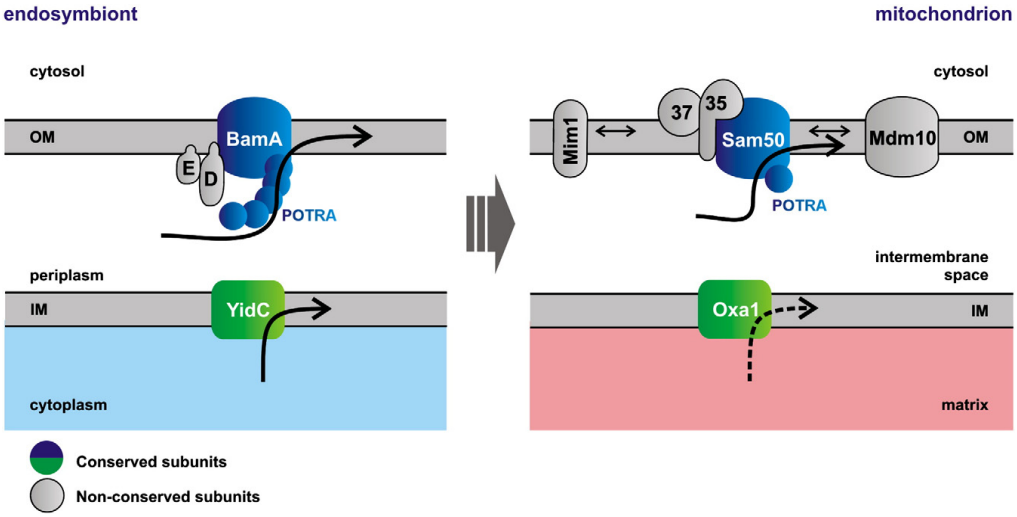

Fig. 2. Re-vamping the BAM and YidC translocases to function in mitochondria. The bacterial BAM complex consists of the core subunit BamA, and several lipoprotein partners, two of which (BamD and BamE) are conserved in all α-proteobacteria. BamA consists of a transmembrane β-barrel domain and five periplasmic POTRA domains. Sam50 is the core component of the SAM complex in mitochondria, and was derived from the endosymbiont BamA but has a truncated N-terminal domain with what is perhaps a single POTRA. However, the other components of these outer membrane complexes have been extensively remodeled. The SAM complex incorporates Sam35 and Sam37, and the modular subunits Mim1 and Mdm10. At least some of these SAM subunits may have analogous roles to the lipoprotein subunits of the BAM complex. Oxa1 inserts proteins into the inner membrane from the matrix, and evolved from the bacterial YidC. In mitochondria, relatively few proteins are assembled by Oxa1 (indicated by dashed arrow).

In addition, the SAM complex can engage with two outer membrane proteins found only in fungi: Mdm10 and Mim1. Mdm10 is a modular component of two complexes which seem to function in distinct pathways for assembly of outer membrane proteins. A SAM-Mdm10 complex assists in assembly of the TOM complex, while a second complex, containing Mdm10, Mdm12 and Mmm1, appears to function sequentially after the SAM complex in the β-barrel assembly pathway [48–50]. This second complex has also recently been described as ERMES (ER-Mitochondria Encounter Structure), a molecular tether between the endoplasmic reticulum and mitochondria, composed of Mdm10, Mdm12, Mmm1 and Mdm34 [51]. ERMES impacts on various aspects of cellular physiology including mitochondrial morphology, phospholipid and calcium homeostasis, and mitochondrial DNA replication [52]. This intriguing link between protein import and mitochondria-ER tethering machinery suggests a network of connections that might regulate mitochondrial biogenesis in response to higher-level cellular cues.

Mim1 is another modular subunit of the yeast SAM complex [53] which, like Mdm10, functions in assembly of the multimeric TOM complex [39,50,53–55]. Mim1 and Mdm10 are each required for integration of different subunits into the TOM complex. Despite their roles in assembling the TOM complex, neither Mdm10 nor Mim1 is directly involved in the import of β-barrel proteins [39,49]. Several subunits of the TOM complex evolved after the divergence of the eukaryotic lineages [56,57], consistent with the fungal-specific distribution of Mdm10 and Mim1. It seems highly likely that analogous, but non-homologous, proteins function in place of Mdm10 and Mim1 in other organisms.

4. The ultimate in evolutionary tinkering: a new machine for protein transport

The core of the TIM23 complex is the Tim23 subunit, a multi-topic membrane protein that forms the protein import channel [58,59]. It is widely accepted that Tim23 and Tim22 (the core of the TIM22 complex) are related to each other by sequence. While there is agreement that one was derived from the other by gene duplication and modification, which complex arose first has not been determined. By the principle of Occam's razor we favor the idea that the TIM23 complex was established first, and that it was cobbled together from existing bacterial proteins (Fig. 3).

Rassow et al. have suggested that the Tim23 channel was derived from an amino-acid transporter called LivH [60]. These transporters import large, bulky hydrophobic amino acids via an aqueous channel and might require relatively little modification in order to transport polymers of amino acids. Indeed, the OEP16 protein, found in the chloroplast outer envelope, is a member of the Tim23 protein family [60,61] and has been shown to transport amino acids when reconstituted in liposomes [62]. Not surprisingly, given the evolutionary distance and the sequence-based changes driven by interactions with multiple subunits in the TIM23 complex, pair-wise sequence conservation between LivH and Tim23 family proteins is low. However, a signature PReprotein and Amino acid Transporters (PRAT) motif found in the Tim23-type mitochondrial translocases and in OEP16 is also present in the LivH protein of bacteria.

The TIM23 import motor, mtHsp70, drives protein translocation across the inner membrane through successive rounds of ATP hydrolysis, and is derived from an Hsp70 (DnaK) protein found in extant species of α-proteobacteria [63]. The import motor is docked to the TIM23 translocase by the Tim44 subunit [64,65] and Pam18 (also known as Tim14) regulates motor ATPase activity [66–68]. Recent work has shown that α-proteobacteria carry inner membrane proteins with strong sequence similarity to the Tim44 (TimA) and Pam18 (TimB) [69]. Studies on the α-proteobacterium *Caulobacter crescentus* showed that these two proteins function distinctly, yet both are found in the same compartment and have the same topology as their mitochondrial counterparts. Furthermore, a single point mutation in the J-domain of an α-proteobacterial Pam18 homologue is sufficient to convert it to a functional TIM23 translocase subunit [69].

It is reasonable to infer from these findings that relatively little evolutionary tinkering would be required to derive a core TIM23

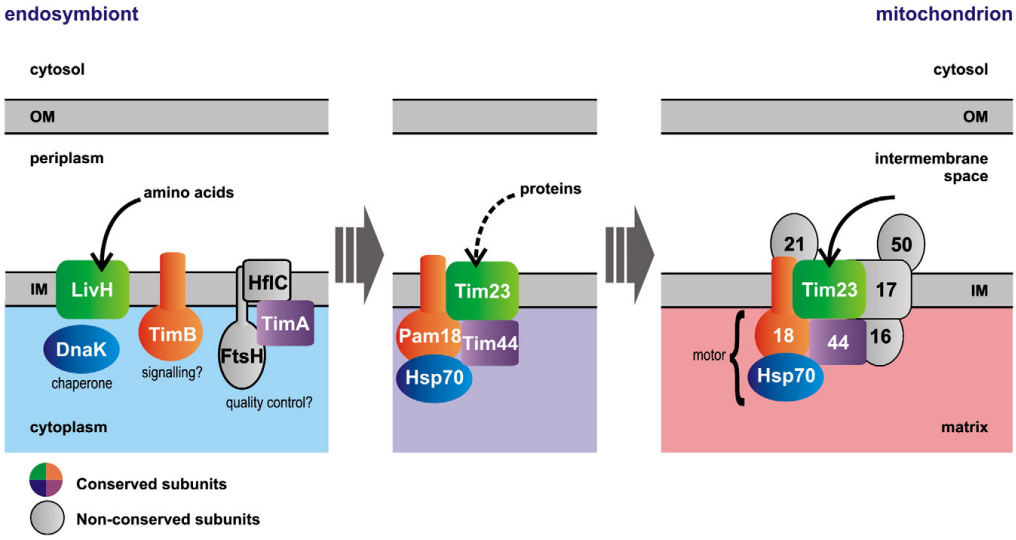

Fig. 3. A model for the evolution of the TIM23 complex. In many organisms, the TIM23 complex is composed from eight or more subunits and translocates all presequence-containing proteins (right panel). The core subunits of the TIM23 complex (Tim23, Pam18, Tim44 and mHsp70) are common to all lineages of eukaryotes [35]. Tim23 forms a transmembrane channel, and Pam18, Tim44 and mHsp70 are part of the presequence-assisted motor (PAM). Each of these components can be traced back to an ancestral protein in bacteria. The Tim23 subunit is related to LivH-type amino acid transporters [60]. The mitochondrial Hsp70 is clearly derived from α-proteobacterial DnaK [63] and the Tim44 and Pam18 subunits from the α-proteobacterial proteins TimA and TimB, respectively [35,69,97]. Harnessing the mitochondrial Hsp70 to the inner membrane provided a motive force to the transporter, providing a means to translocate proteins through the inner membrane. Initially, this would have been relatively inefficient (dashed arrow). Subsequent, lineage-specific evolution of some components (shown in grey) has provided further efficiency and sophistication to TIM23 function.

complex from components already present in the ancestral endosymbiont. With a rudimentary TIM23 translocase in place and the continued presence of both Sec and YidC translocases [35,69], the "proto-mitochondrion" would have had a functional system for import of both matrix and inner membrane proteins. A primitive system such as this would provide the basis for the evolution of the highly specialized, and diverse, TIM translocases in extant organisms.

5. The origin of the TOM complex: cultivating the endosymbiont–host interaction

It has been suggested that the first protein translocase system in the proto-mitochondrion would have involved a primitive set-up: a β-barrel protein in the outer membrane and substrates in the host cytosol predisposed for targeting to mitochondria [69,70] (Fig. 4).

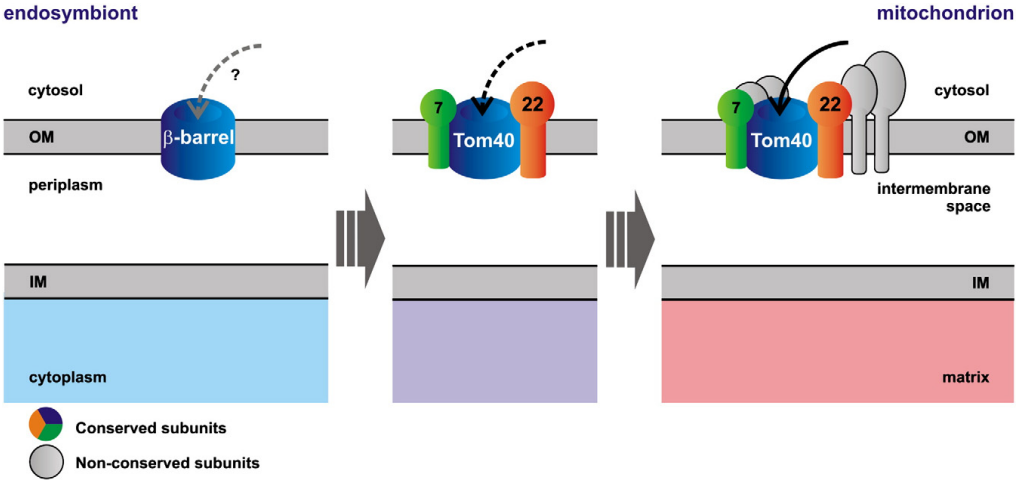

Fig. 4. Evolution of the TOM complex from an ancestral β-barrel. This model proposes a β-barrel protein in the outer membrane of the endosymbiont served as a binding site for proteins with basic, amphipathic N-termini [98]. Such a simple protein import system can be envisaged by analogy with the simplified, core complexes seen in some parasites. Tom7 and Tom22 are common to all lineages of eukaryotes suggesting they constitute the first partner proteins to have arisen in early eukaryotes [99]. Optimization of protein transfer, both in terms of efficiency and versatility, required the later addition of TOM complex subunits, after the divergance of some lineages.

Simple TOM complexes have recently been identified in both *Giardia* and microsporidians, consisting of Tom40 and perhaps a single partner subunit [71–73]. In the case of *Giardia*, as with many other organisms, it remains possible that additional, lineage-specific subunits of the TOM complex remain to be discovered. However, microsporidians provide a true proof-of-principle example of a simple TOM complex. Microsporidia are allied with fungi phylogenetically and in the case of the protein import machinery, sequence similarity of all TOMs and TIMs are extremely high [71,73]. Only two TOM proteins are encoded in the complete genome of *Encephilizoon cuniculi*: Tom70 and Tom40. Given the function of Tom70 as a receptor, acting prior to the translocation reaction, this says that Tom40 alone can form a functional protein translocase. While clearly a result of secondary gene loss in microsporidians, it demonstrates the feasibility of a primitive, "Tom40-only" TOM complex in the ancestral endosymbiont.

Phylogenetic analysis does not establish the ancestry of Tom40. Based on its β-barrel topology it is broadly accepted that Tom40 was derived from the genome of the endosymbiont. Like all bacteria with two membranes, the endosymbiont would have had a range of β-barrel outer membrane proteins [4,33,74]. Initially synthesised within the endosymbiont, a primitive TOM translocase could have been transported to the periplasm using the bacterial export pathway. Assembly of this β-barrel subunit into the outer membrane would then have been mediated by the endosymbiotic BAM complex. This primitive β-barrel would have been the founding member of the "mitochondrial porin" family of proteins, which includes both the protein translocation channel Tom40 and the mitochondrial outer membrane metabolite transporter VDAC [75]. Whether the first mitochondrial porins functioned in metabolite transport or protein translocation is as yet unknown.

What family of bacterial proteins gave rise to the first mitochondrial porins? There are numerous, divergent metabolite transporters in bacterial outer membranes that transfer charged substrates by virtue of "chains" of charged residues lining the inner surface of the pore. The acidic sugar-specific porin KdgM provides a beautiful illustration of how a "basic chain" of residues in the pore channel can lead a negatively-charged sugar through the outer membrane [76]. The transfer of a positively charged mitochondrial targeting sequence might likewise have followed an acid chain through a β-barrel protein in the outer membrane of the endosymbiont. The principles of the acid chain hypothesis were established based on acidic domains on Tom40's partner subunits [12,14,15,77,78], however a recent model of the structure of Tom40 shows such an "acid chain" of residues in the pore lining, with the net charge being greatest at the intermembrane space exit site [74].

An alternative proposition for the ancestry of Tom40 comes from a tantalizing observation of sequence signatures shared by the YdeK autotransporter (SwissProt accession P32051) and the Tom40 family of proteins [79]. Autotransporters are simple β-barrel protein translocation channels commonly found in the outer membrane of bacteria. The crystal structure of the β-domain of autotransporter EspP shows that the barrel pore can accommodate a positively charged α-helical segment, which is stabilised by complementary charged surfaces on the inside wall of the barrel [80]. If Tom40 was derived from such an autotransporter channel one caveat might be the directionality of substrate translocation, as the TOM complex imports proteins, rather than exports. However, this difference need not matter as biochemical analysis of purified mitochondrial outer membrane vesicles has shown that purified proteins can move in either direction through the TOM channel [81].

While the specific ancestry of Tom40 remains to be determined, it is reasonable to predict that with that first translocase subunit in place, additional subunits were sequestered from other activities to enhance the function of the primitive translocase. Both Tom7 and Tom22 are present in each of the major eukaryotic lineages, suggesting them to be the first partner proteins added into the primitive TOM complex (Fig. 4). There remains some uncertainty as to whether they are present or not in the Excavata (one of the six supergroups of the eukaryotes); the small size of the proteins makes their identification challenging (each protein is only ~50–70 residues in most lineages). In addition two other smaller proteins, Tom5 and Tom6, are present in many but not all lineages, and may have been added to the TOM complex later. Selection for further receptor subunits, Tom20 and Tom70, appears to have been a lineage-specific adaptation, with the "Tom20" receptor in opisthokonts (i.e. fungi and animals) being unrelated in sequence and ancestry to the function-ally-analogous "Tom20" in plants [56]. These receptors would have enabled the evolution of an increasing diversity of substrate proteins and targeting sequences, enhancing efficiency of the import process and overall fitness of the host organism.

6. Replacing the old order in the intermembrane space

The protein transport reactions, signalling networks, structural peptidoglycan and redox conditions of the bacterial periplasm make it a radically different environment from the mitochondrial intermembrane space. The bacterial periplasm is a highly oxidising environment, reinforced with a thick peptidoglycan meshwork. Bacterial networks for monitoring and responding to a fluctuating extracellular environment have vanished from mitochondria, replaced with new systems for signalling and quality control in an intracellular context. Thus much of the bacterial periplasmic machinery has been replaced with eukaryote-specific proteins as new pathways evolved. There are now two examples where the periplasmic machinery of the endosymbiont seems to have been superseded by protein import apparatus: the small Tim chaperones that play a SurA-like role in mitochondria, and the MIA/Erv disulfide relay that has replaced the bacterial Dsb system (Fig. 5).

The assembly of β-barrel proteins into the bacterial outer membrane requires assistance from chaperones found in the periplasm, which fulfill three functions: precursor release from the inner membrane, molecular chaperone activity during transit, and targeting/hand-off to the BAM complex for outer membrane insertion. Periplasmic chaperones such as SurA, Skp, DegP and PpiD all play a role in this pathway in *Escherichia coli* [82–87] (Fig. 5, left panel). Bioinformatic analysis of SurA and Skp distribution revealed that both chaperones are present in diverse bacterial species, including all proteobacterial lineages, but are not detected in eukaryotes [88] [our unpublished data].

The small Tim chaperones are found only in eukaryotes [89], where they transfer precursors of both inner and outer mitochondrial membrane proteins from the TOM complex to the appropriate downstream machinery [21–23,90,91]. Comparative analysis of SurA with the small Tims shows that while both chaperones can bind similar substrates, SurA cannot transfer mitochondrial inner membrane proteins to the TIM translocase for insertion [88]. The small Tim family may therefore have arisen to enhance transport of inner membrane proteins, and also proved competent in transfer of outer membrane precursors, leaving bacterial chaperones like SurA redundant.

The bacterial Dsb proteins, which catalyse formation and isomerisation of disulfide bonds in the periplasm, are absent from mitochondria. The only redox proteins identified to date in the mitochondrial intermembrane space constitute the MIA disulfide relay machinery, which mediates the import of small, cysteine-rich proteins into the mitochondrial intermembrane space [24–26]. In yeast, substrates translocated through the TOM complex are bound by the oxidoreductase Mia40. Substrate and Mia40 together form a complex with the thiol oxidase Erv1, and electron flow from substrate via Mia40 to Erv1 is followed by release of the oxidised substrate [25,92–94].

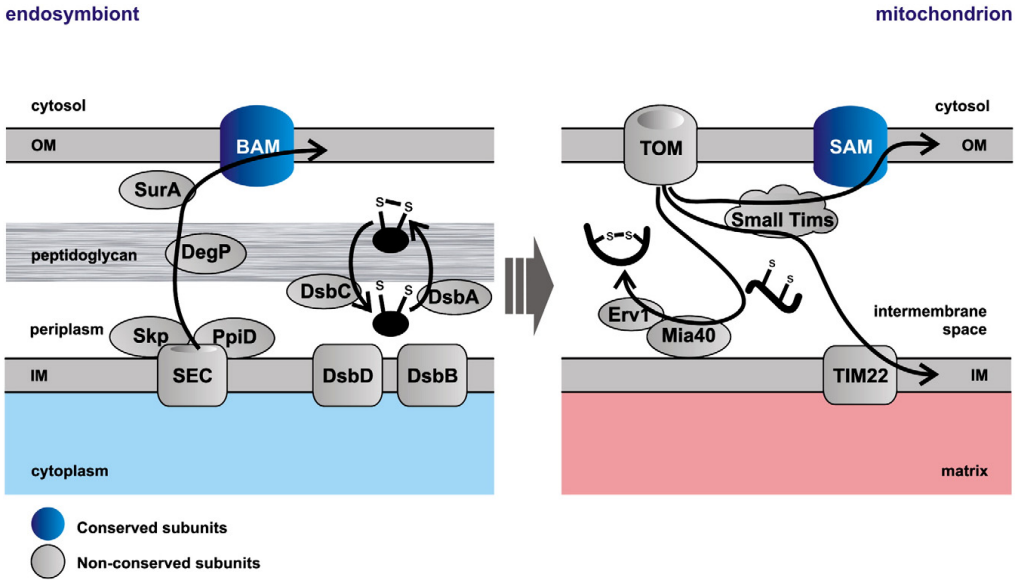

Fig. 5. Evolution of an intermembrane space in mitochondria. Molecular chaperones which translocate proteins across the periplasm (Skp, DegP, PpiD and SurA) also function in cellular stress responses. The Dsb redox system acts as a folding catalyst for several hundred predicted disulphide-containing periplasmic proteins [99]. These systems may have provided chaperone activity essential for the foundation of new protein import pathways, however it is likely that their inefficiency in these pathways meant they were replaced early in eukaryotic evolution. In mitochondria the small TIM chaperones fulfill a molecular chaperone function and transfer membrane protein precursors from the TOM complex to the TIM22 and SAM complexes. The MIA/Erv machinery couples a Dsb-like redox activity with import of precursors into the intermembrane space.

No homologue of either Mia40 or Erv1 has been identified in any prokaryotic genome, so it is difficult to determine the origin of this pathway. While some unicellular eukaryotes appear to lack both MIA pathway substrates and machinery, there are organisms, including the protozoan trypanosomatids, which do contain both classic MIA substrates and an Erv1 homologue, but seem to lack Mia40 itself [95]. Allen and colleagues suggest this minimalistic set-up reflects the ancestral pathway, where the redox cascade comprised only the substrate, Erv1 and molecular oxygen. This system would have used Erv1 to create disulfide bonds but might also have relied on the bacterial protein disulfide isomerase dsbC, homologues of which have been reported in α-proteobacteria. Mia40 could then have been added to the evolving eukaryote at a later date, making the bacterial isomerase dispensable and improving the efficiency and accuracy of the system.

7. Concluding remarks

Species of α-proteobacteria have conquered diverse environments, and show great breadth in the complexity of their genomes and proteomes [96]. We can assume that the endosymbiont that gave rise to mitochondria had a robust protein transport system for the assembly of proteins into both its outer and inner membranes. The existing bacterial protein folding and translocation pathways played a dual role in supporting the stepwise evolution of the mitochondrial machinery, providing both a source of building blocks for the evolution of new import systems and functional support to the fledgling translocases.

The mitochondrial SAM complex provides a prime example of the adaptation of a bacterial system to perform an equivalent role in mitochondria. The TIM23 complex appears to have been cobbled together from existing components to produce a sophisticated and versatile translocase, but is still assisted by the ancient inner membrane translocase Oxa1. Bacterial SurA-type chaperones, although unable to dock with newly established TOM and TIM machinery, might have provided chaperone activity essential for the passage of imported membrane proteins, until the later invention of the small Tim chaperones. Similarly, a rudimentary intermembrane space import pathway might have initially relied on the bacterial disulfide isomerase, DsbC [95]. With the current available evidence pointing towards proteins from the endosymbiont as progenitors for many of the translocase components, we suggest that these proteins played a central role in driving the evolution of the new protein transport pathways. The evolution of these import pathways eventually produced the mutually beneficial arrangement that became the first eukaryote.

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Appendix 10: Hewitt et al. (2012)

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A model system for mitochondrial biogenesis reveals evolutionary rewiring of protein import and membrane assembly pathways

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The controlled biogenesis of mitochondria is a key cellular system coordinated with the cell division cycle, and major efforts in systems biology currently are directed toward understanding of the control points at which this coordination is achieved. Here we present insights into the function, evolution, and regulation of mitochondrial biogenesis through the study of the protein import machinery in the human fungal pathogen, *Candida albicans*. Features that distinguish *C. albicans* from baker's yeast (*Saccharomyces cerevisiae*) include the stringency of metabolic control at the level of oxygen consumption, the potential for ATP exchange through the porin in the outer membrane, and components and domains in the sorting and assembling machinery complex, a molecular machine that drives the assembly of proteins in the outer mitochondrial membrane. Analysis of targeting sequences and assays of mitochondrial protein import show that components of the electron transport chain are imported by distinct pathways in *C. albicans* and *S. cerevisiae*, representing an evolutionary rewiring of mitochondrial import pathways. We suggest that studies using this pathogen as a model system for mitochondrial biogenesis will greatly enhance our knowledge of how mitochondria are made and controlled through the course of the cell-division cycle.

SAM complex | Omp85 | stop-transfer pathway | Sam51

Mitochondrial biogenesis is an essential aspect of the cell-division cycle and requires replication of the mitochondrial genome, synthesis of lipids to build new membranes, and the import of around 1,000 different proteins encoded in the nuclear genome (1, 2). The pathways for protein import have been defined and are mediated by a series of molecular machines in the outer and inner mitochondrial membranes, along with soluble factors in the intermembrane space and matrix (3, 4). One of these molecular machines, the translocase of the outer membrane (TOM) complex, forms a channel in the outer mitochondrial membrane that serves as the gateway for protein import into mitochondria. Recent work in the model yeast *Saccharomyces cerevisiae* has shown that phosphorylation of specific subunits of the TOM complex regulates the activity of the protein import channel. An elegant coordination of mitochondrial biogenesis with cellular metabolic needs was seen when glucose levels were high, with phosphorylation of TOM complex subunits coinciding with repression of mitochondrial activity (5, 6). *S. cerevisiae* responds to the presence of glucose through metabolic cycling, oscillating cycles of aerobic respiration and glycolytic activity. This process requires tight regulatory control of mitochondrial biogenesis cordoned into specific states of metabolic activity that are coordinated with the cell-division cycle (7–9).

Although *S. cerevisiae* has provided an excellent, genetically tractable model organism to determine how cellular metabolism is integrated with signaling pathways and networks, it is somewhat peculiar with regards to regulation of mitochondrial biogenesis. This yeast is subject to the Crabtree effect: It ferments glucose anaerobically, whether oxygen is available or not (10). Many of the genes required for anaerobiosis arose through a whole-genome-duplication (WGD) event, with duplicated genes modified to provide new functions or new signaling switches (10–13). During optimal, rapid growth in glucose, mitochondrial function is repressed, and transcriptional networks have been reorganized in *S. cerevisiae* to enable regulation of mitochondrial biogenesis by carbon source (14, 15).

In anticipation of work that soon will enable genetic and biochemical investigations of the integration of metabolic control globally with every cellular pathway, we sought to develop a yeast model in which mitochondrial function is not repressed during optimal growth and which does not show metabolic cycling. This model would provide a means to distinguish those control points that are linked specifically to the switching events in metabolic control. *Candida albicans* is a budding yeast that diverged from the lineage that gave rise to *S. cerevisiae* around 300 million years ago (16). The genome of *C. albicans* has been sequenced (17), and various tools have been developed for gene deletion, epitope tagging, and genetic studies in this organism (18–21). Unlike *S. cerevisiae*, *C. albicans* grows aerobically, and mitochondrial respiration is active during optimal growth in glucose (15, 22, 23). Thus, although mitochondrial activity is regulated through the course of the cell cycle in *C. albicans*, it is not subject to changes depending on the available carbon source (15, 23, 24).

Here we demonstrate that *C. albicans* does not undergo metabolic cycling and that mitochondria can be isolated from *C. albicans* and assayed for the import of mitochondrial proteins in vitro. Using this model system, we find several differences in the import reactions relative to *S. cerevisiae*, uncovering functional aspects relating to the mitochondrial sorting and assembly machinery (SAM) complex, an essential molecular machine that assembles β-barrel proteins into the mitochondrial outer membrane. We show that in *C. albicans* the core SAM complex comprises at least three integral membrane proteins: Sam35, Sam50, and a protein which we refer to as "Sam51." We demonstrate that Sam50 and Sam51 play similar roles in the assembly of the TOM complex, a process that is much more rapid in *C. albicans* than in *S. cerevisiae*. Moreover, consideration of the protein import pathways for components of the electron transport chain in *C. albicans* provides examples of evolutionary rewiring of mitochondrial import pathways in related species, showing that the particular pathway taken into mitochondria was not hard-wired early in evolution; instead, rewiring is open

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to organisms to take advantage of new metabolic or gene-regulatory opportunities.

Results

Protein Import Machinery in Mitochondria from *C. albicans*. The components of the protein import pathways as discovered in *S. cerevisiae* are highly conserved in *C. albicans*, and BLAST searches are sufficient to identify candidate homologs in most cases (Table S1). Proteins of overall similar size and domain structures were detected, as detailed in Table S1. Several distinguishing features of *C. albicans* also were observed, e.g., in respect to the TOM complex: *C. albicans* has no Tom70 and has extensions to the cytosolic domains of Tom6 and Tom7 (Table S1). With recent studies in *S. cerevisiae* showing that phosphorylation in the cytosolic domains of TOM subunits is crucial for regulation of TOM complex activity in response to changes in metabolic activity (5, 6), future analysis of sequence variations in *S. cerevisiae* and *C. albicans* could help show how these phosphorylation sites affect cell cycle and metabolism. In some cases hidden Markov models (HMM) proved essential for identifying homologs; for example, an ORF encoding a Tom5-related sequence previously had been unidentified and unannotated because of its small size and sequence divergence. The Mia40 homolog in *C. albicans* previously was known only as a Hap43-repressed gene, because it lacks the characteristic N-terminal transmembrane signature of the Mia40 from *S. cerevisiae*. Using an HMM search, we also found a Sam50-related protein in *C. albicans* with no homolog in *S. cerevisiae* (Table S1). In *S. cerevisiae*, Sam50, Sam35, Sam37, and Mdm10 form the SAM complex, a molecular machine required for the assembly of β-barrel proteins into the mitochondrial outer membrane (3, 4).

Oxidative Phosphorylation and Protein Import into *C. albicans* Mitochondria. During growth in glucose, *S. cerevisiae* represses mitochondrial function through changes in gene expression and metabolite levels via the yeast metabolic cycle (8). Chemostat cultures of *S. cerevisiae* revealed a cyclic change in oxygen consumption (Fig. 1*A*), consistent with that seen previously (8, 9). Metabolic cycling was not observed with cultures of *C. albicans*: These grew at comparably high cell density but had a constant requirement for oxygen consumption (Fig. 1*A*). We isolated mitochondria from cultures of *C. albicans* to test their capacity for protein import (Fig. 1*B*). The model precursor protein Su9-DHFR is destined to the mitochondrial matrix and is translocated across the outer and inner membranes to be processed in two steps, thereby generating an intermediate (i) form and then a mature (m) form of the imported protein (25). The intermediate and mature forms were protected from trypsin by the intact mitochondrial membranes (Fig. 1*B*). Membrane potential ($\Delta\Psi_m$)-dependent import was seen for Su9-DHFR and the other matrix proteins *Sc*F$_1$β, *Sc*Adh3, and *Sc*CoxVa ("*Sc*" denotes proteins from *S. cerevisiae*) (Fig. 1*B*). The precursor (p) form of these proteins binds to the surface of mitochondria from *C. albicans* but is not imported. Treatment of the isolated mitochondria with trypsin degrades the surface-located precursor forms of all four proteins (Fig. 1*B*). The import rates of some precursor proteins are so high that the reaction was complete within 2 min when assayed at 25 °C but could be slowed at lower temperature (4 °C) to demonstrate a time-dependent rate of import (Fig. 1*C*).

Stop-Transfer Pathway: Rewiring the Import of Cytochromes. A distinguishing feature of mitochondria isolated from *C. albicans* is the inefficiency with which they import Cyb2, a heme-containing dehydrogenase (L-lactate cytochrome-*c* oxidoreductase) essential for the utilization of L-lactate as a carbon source. Fig. 2*A* details the pathway for Cyb2 import via the stop-transfer sorting pathway as it occurs in *S. cerevisiae*. The import of *Sc*Cyb2 depends on active unfolding of the heme-binding domain by the action of matrix-located Hsp70 (26–30). All the necessary components for the

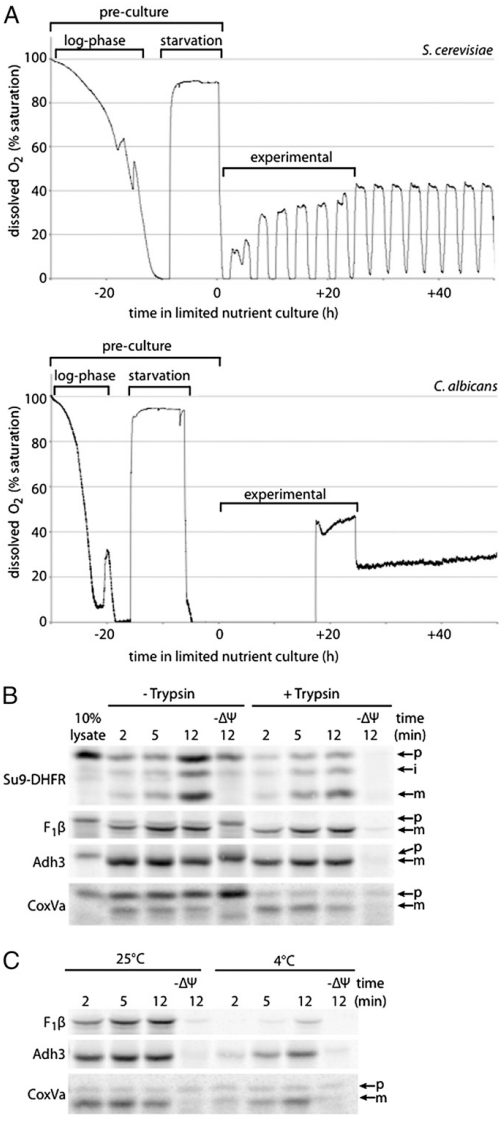

Fig. 1. Protein import into mitochondria from *C. albicans*. (*A*) Chemostat culture of *S. cerevisiae* CEN.PK and *C. albicans* DAY185 strains. (*B*) Mitochondria isolated from wild-type *C. albicans* (50 μg protein per lane) were incubated with the indicated proteins at 25 °C for the indicated times (min), treated with trypsin, and then analyzed by SDS/PAGE and phosphorimage analysis. The precursor (p), intermediate (i), and mature (m) forms are indicated where relevant. (*C*) Mitochondria isolated from *C. albicans* (50 μg protein per lane) were incubated with the indicated proteins at either 25 °C or 4 °C for the indicated times (min), treated with trypsin, and then analyzed by SDSPAGE and phosphorimage analysis.

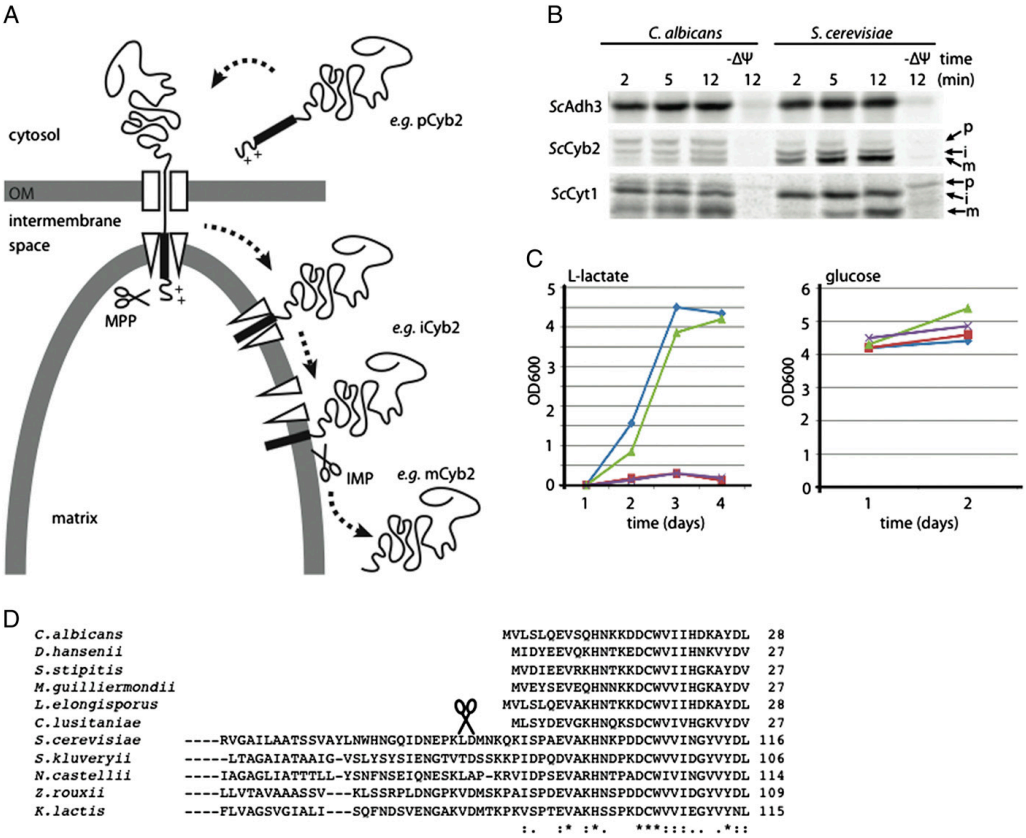

Fig. 2. The stop-transfer pathway for protein import into mitochondria. (*A*) The stop-transfer pathway occurs by the sequential interaction with the TOM complex (white rectangles) and the TIM23 complex (white triangles), and the sequential processing by the mitochondrial processing peptidase (MPP) in the mitochondrial matrix and the inner membrane peptidase (IMP) in the inner membrane. (*B*) Mitochondria isolated from *C. albicans* (50 μg protein per lane) were incubated with the precursor (p) forms of *Sc*Adh3 or *Sc*Cyb2 or *Sc*Cyt1 at 25 °C for the indicated times (min), treated with trypsin, and then analyzed by SDS/PAGE and phosphorimage analysis. The intermediate (i) and mature (m) forms are indicated where relevant. (*C*) The *CYB2* genes from *S. cerevisiae* and *C. albicans* were amplified and cloned into a plasmid for expression in a Δ*cyb2* strain of *S. cerevisiae*, and the transformed strains were monitored for growth in semisynthetic medium with either L-lactate or glucose as a carbon source. Wild type, blue; Δ*cyb2*, red; Δ*cyb2 /ScCYB2*, green; Δ*cyb2 /CaCYB2*; purple. All strains grew to comparable density in glucose medium. (*D*) Sequence alignment of the N-terminal 28 residues of Cyb2 from *C. albicans* (*Ca*Cyb2), Cyb2 from *S. cerevisiae* (*Sc*Cyb2), and related yeasts. Sequence identity and similarity are indicated by asterisks and dots, respectively. The processing site that generates the mature form of *Sc*Cyb2 is the peptidyl bond between residues L84 and D85, four residues upstream from Q89.

stop-transfer pathway are present in *C. albicans* (Table S1), but, as compared with *S. cerevisiae*, import of *Sc*Cyb2 is highly inefficient in mitochondria from *C. albicans*, with a greatly diminished processing to produce the intermediate ("iCyb2") and mature ("mCyb2") forms (Fig. 2*B*). Similarly, the import of cytochrome c_1 from *S. cerevisiae* (*Sc*Cyt1) also follows the stop-transfer pathway (31), and the conversion of p*Sc*Cyt1 to i*Sc*Cyt1 is less efficient in mitochondria from *C. albicans* (Fig. 2*B*).

The ORFs encoding *Ca*Cyb2 and *Sc*Cyb2 were cloned for expression from a plasmid, and Δ*cyb2* mutants of *S. cerevisiae* were transformed. On minimal growth medium with L-lactate as a carbon source, the Δ*cyb2* mutants depend on *Sc*Cyb2 activity to feed electrons from L-lactate into the electron transport chain (Fig. 2*C*). *Ca*Cyb2 could not support growth of the Δ*cyb2* mutant on L-lactate, although the transcript was expressed at high levels (Fig. S1*A*), and the functional domains of Cyb2 are highly homologous (Fig. S1*B*), suggesting that the *Candida* protein was not imported sufficiently well into the mitochondrial intermembrane space in *S. cerevisiae*. The import signals for delivery of the cytochromes Cyb2 and Cyt1 to the intermembrane space in *S. cerevisiae* are well characterized N-terminal extensions, and comparative sequence analysis shows that *Ca*Cyb2 and the Cyb2 homologs from related yeasts entirely lack this N-terminal stop-transfer sorting sequence (Fig. 2*D*). Similarly, *Ca*Cyt1 lacks a large segment from within the N-terminal stop-transfer sorting sequence found on *Sc*Cyt1 (Fig. S1*B*).

Sam35, Sam37, and the Assembly of Voltage-Dependent Anion-Selective Channel Oligomers in *C. albicans*. The assembly of β-barrel proteins into mitochondria can be measured readily in vitro using blue-native PAGE (BN-PAGE), and two model proteins

have been used as substrates in these assays: Tom40 and the voltage-dependent anion-selective channel (VDAC), also known as "porin." In the case of Tom40, the assembly reaction measures both the assembly of the Tom40 β-barrel and the recruitment of other TOM subunits to form the mature TOM complex. When mitochondria isolated from *C. albicans* are used, the assembly of Tom40 from *S. cerevisiae* occurs at a rate similar to that of the assembly of Tom40 from *C. albicans*, demonstrating that there is sufficient sequence similarity in the interactive surfaces so that the heterologous Tom40 can be assembled into the oligomeric TOM complex (Fig. 3*A*).

The major β-barrel protein in the outer mitochondrial membrane is the homo-oligomeric VDAC. Newly imported VDAC monomers assemble into oligomers with preexisting VDAC (32). *Sc*VDAC is inserted into the outer membrane in a time-dependent manner forming numerous oligomers, including a major 200-kDa form, which are resolved by BN-PAGE (Fig. 3*B*). In contrast, the oligomers of *Sc*VDAC in *C. albicans* mitochondria are of uniform stoichiometry and/or are less dynamic in nature: The imported *Sc*VDAC showed the majority of the protein in stable oligomers of ~400 and ~440 kDa (Fig. 3*B*). In *S. cerevisiae*, the import and assembly of VDAC depends largely on the SAM complex subunit Sam37 (33). In both *S. cerevisiae* and *C. albicans*, deletion of Sam37 leads to a concomitant reduction in levels of Sam35, and this reduction can be suppressed by overexpression of the *SAM35* gene (34, 35), With a *sam37ΔΔ* strain of *C. albicans* engineered to have one copy of the *SAM35* gene under the control of the strong *TEF1* promoter, a 9.5-fold up-regulation of *SAM35* transcript levels was measured in the mutant (*sam37ΔΔSAM35↑↑*) (Fig. 3*C*). That Sam35 then is assembled into the SAM complex in the *sam37ΔΔSAM35↑↑* strain is evident by the change in the size of Tom40 assembly intermediate I compared with the *sam37ΔΔ* strain (Fig. 3*D*). Only a minor defect in VDAC assembly is seen in mitochondria from the *C. albicans sam37ΔΔ* strain, and the increased levels of *SAM35* did not rescue this defect (Fig. 3*E*). The very mild phenotype seen in *C. albicans* is in contrast to the strong requirement for Sam37 in VDAC assembly measured in *S. cerevisiae* (33). For quality control, we show that mitochondria from the *sam37ΔΔSAM35↑↑* strain import *Sc*$F_1\beta$, *Sc*Adh3, and *Sc*Aac1 with efficiency comparable to that of mitochondria from the wild type strain under conditions where protein import was linear with respect to time and dependent on the membrane potential ($\Delta\Psi_m$) (Fig. 3*F*).

TOM Complex Assembly in *C. albicans*. In *S. cerevisiae*, the β-barrel core of the TOM complex, Tom40, is assembled via two intermediate forms into the mature TOM complex (Fig. 4*A*) in a time-dependent manner that, in vitro, takes more than 60 min to complete (34, 36). Mitochondria from *C. albicans* assemble Tom40 into the mature TOM complex much more rapidly: When [^{35}S]-labeled *Ca*Tom40 was imported into mitochondria isolated from *C. albicans*, Intermediate I (representing [^{35}S]-*Ca*Tom40 bound in the SAM complex) forms within 2 min and is transferred into the mature, ~400-kDa form of the TOM complex within 10 min (Fig. 4*B*). The loss of Sam37 has a major impact on the assembly of the TOM complex, including a decrease in the amount of [^{35}S]-*Ca*Tom40 entering the intermembrane space as judged by SDS/PAGE and BN-PAGE. These effects are suppressed partially by overexpression of *SAM35* (Fig. 4*B*). These results in *C. albicans* are consistent with the functions of Sam35 and Sam37 observed in the *S. cerevisiae* model system (34, 37).

The Sam35 subunit of the SAM complex is enigmatic, because it interacts selectively with substrate β-barrel proteins in the intermembrane space but behaves as if it were a peripheral membrane protein on the outer surface of mitochondria: It is largely accessible to proteolysis and is readily extracted from mitochondrial membranes using sodium carbonate treatment (37). Hydrophobicity analysis of *Ca*Sam35 suggested that it has potential membrane-spanning segments that are more hydrophobic than the amphipathic segments detected in *Sc*Sam35 (Fig. 4*C*). We sought to take advantage of this difference and use the more hydrophobic *C. albicans* protein to determine whether Sam35 has a transmembrane segment. Mitochondria isolated from a strain expressing N-terminally epitope-tagged HA-*Ca*Sam35 and from a strain expressing C-terminally epitope-tagged *Ca*Sam35-HA were treated with trypsin, and in both cases *Ca*Sam35 behaves as an outer membrane protein, exposed on the mitochondrial surface and sensitive there to protease (Fig. 4*C*). Incubation of mitochondria with sodium carbonate to strip peripheral membrane proteins revealed that both HA-*Ca*Sam35 (Fig. 4*D*) and *Ca*Sam35-HA (Fig. 4*E*) behave as an integral membrane protein.

***C. albicans* has a Second Member of the Omp85 Protein Family.** The feature distinguishing SAM complex components in *C. albicans* from those in *S. cerevisiae* is a second protein sharing 22.8% sequence identity and predicted domain composition with *Sc*Sam50 (Fig. S2*A*). We refer to this protein as "Sam51." This protein is not simply a result of gene duplication from the well-characterized WGD event, because we found homologs of Sam51 in most clades of yeast, including species prior and subsequent to the WGD event (Fig. S2*B*). Sam51 is found throughout the Ascomycotina, indicating that its absence in *S. cerevisiae* is the result of a secondary loss in this particular species (Fig. S2*C* and Fig. 5). Reconstruction of the ancestral lineage at the point of genome duplication predicts that the ancestor had both *SAM51* and *SAM50* genes, with data for the ancestor derived from the Yeast Gene Order Browser (38). In the post-WGD species, there are various permutations in the observed losses of the *SAM51* and *SAM50* genes (Fig. S2*C*). The Sam51 proteins are as similar to bacterial BamA proteins as to the Sam50 sequences, as shown by the clear branching pattern forming three independent subgroups of the Omp85 protein family (Fig. 5). This phylogenetic analysis demonstrates that the Sam51 group of sequences forms a monophyletic branch with the exclusion of all Sam50 sequences and therefore represents a separate subgroup of Omp85 proteins (Fig. 5).

RT-PCR shows that under standard growth conditions the *SAM51* gene is expressed at higher levels than *SAM50* in wild-type *C. albicans* (Fig. 5*B*). We raised antibodies to recombinant Sam50 and Sam51 and used a semiquantitative immunoblot, calibrated with titrations of purified Sam50 and Sam51, to determine that the steady-state protein levels of Sam50 and Sam51 are similar in *C. albicans* mitochondria (Fig. 5*C*).

SAM51 is not essential, and a homozygous diploid mutant *sam51ΔΔ* was created from which mitochondria were isolated. Tom40 is imported and assembled rapidly in mitochondria from *C. albicans* (Figs. 3*A* and 4*B*). To analyze the kinetics of assembly of the TOM complex in the absence of Sam51, [^{35}S]-*Ca*Tom40 was bound to mitochondria from wild-type *C. albicans* or the *sam51ΔΔ* mutant and was chased into the TOM complex (Fig. 6*A*) or was monitored in standard assembly assays run at 16 °C (Fig. 6*B*). Both assay systems showed that Sam51 plays a role in assembly of the TOM complex. Although the [^{35}S]-Tom40 substrate occupied the "assembly intermediate I" stage in mitochondria from wild-type cells, the assembly intermediate is somewhat more transient in mitochondria lacking Sam51 (Fig. 6*A*). The assembly of VDAC proceeded so rapidly, even at 16 °C, that no defect was observed in the kinetics of assembly (Fig. 6*C*), but a small decrease in the steady-state level of VDAC was seen in mitochondria from the *sam51ΔΔ* mutant (Fig. 6*D*). Thus, although Sam51 plays a role in β-barrel assembly, deletion of Sam51 results in only a mild defect of the assembly process.

Attempts to recover a homozygous diploid mutant *sam50ΔΔ* were unsuccessful, consistent with an essential role for the *SAM50* gene in viability of *C. albicans*. A strain was engineered with *SAM50* gene expression under the control of a repressible promoter, and growth on agar under repressive conditions showed a nearly total loss of viability (Fig. 6*E*). After only 3 h in liquid culture, gene

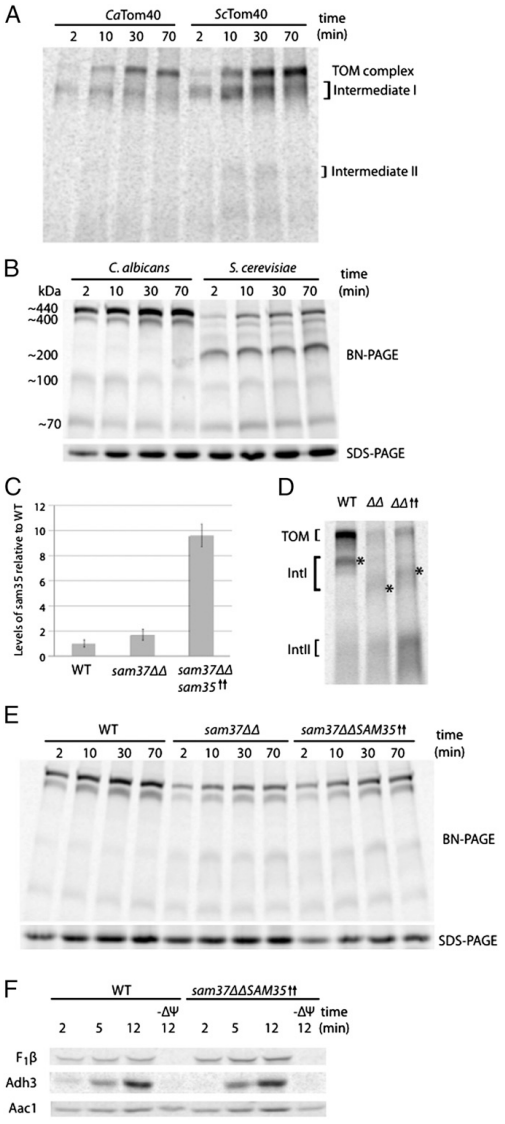

Fig. 3. Sam37 is a major determinant of SAM function in *C. albicans*. (*A*) Mitochondria (50 μg protein per lane) from *C. albicans* were isolated and assayed for import of [^{35}S]-*Ca*Tom40 and [^{35}S]-*Sc*Tom40, and were monitored by BN-PAGE and phosphorimage analysis. (*B*) Mitochondria were isolated from *S. cerevisiae* and *C. albicans*, assayed for import of *Sc*VDAC (Por2), and analyzed by BN-PAGE (*Upper*) or SDS/PAGE (*Lower*), followed by phosphorimage analysis. (*C*) The expression level of *SAM35* in the indicated strains was monitored by quantitative PCR. Shown are averages plus SE of three independent biological repeats assayed in duplicate (39). (*D*) Mitochondria from the same strains of *C. albicans* were isolated and assayed for import of [^{35}S]-*Ca*Tom40 for 30 min, visualized by BN-PAGE and phosphorimaging. The relative size of the SAM complex can be inferred from the migration of "intermediate I" (IntI), indicated by the asterisks. (*E*) Mitochondria from *C. albicans* wild-type, *sam37ΔΔ*, or *sam37ΔΔSAM35*↑↑ strains (50 μg protein per lane) were incubated with VDAC at 25 °C for the indicated times (min). Then samples were analyzed by BN-PAGE (*Upper*) or SDS/PAGE (*Lower*), followed by phosphorimage analysis. (*F*) Mitochondria from the indicated strains of *C. albicans* (50 μg protein per lane) were incubated with *Sc*F$_1$β or *Sc*Adh3 at 4 °C or with*Sc*Aac1 at 25 °C for the indicated time (min), treated with trypsin, and then analyzed by SDS/PAGE and phosphorimage analysis.

expression was shut down in this *sam50ΔSAM50*↓↓ strain, with transcriptional repression confirmed by quantitative PCR (39), so that expression was below the level of detection (Fig. 6*D*). Mitochondria were isolated after growth under repressive conditions for 3 h, and Western blots using antisera specific for either Sam50 or Sam51 show there is no compensatory up-regulation of Sam51 levels in the *sam50ΔSAM50*↓↓ strain (Fig. 6*D*). Mitochondria isolated from the *sam50ΔSAM50*↓↓ strain and the *sam51ΔΔ* strain grown on the equivalent minimal medium were assayed for TOM complex assembly and showed that the *sam51ΔΔ* mutant and the *sam50ΔSAM50*↓↓ mutants have equivalent rates and extent of assembly of the [^{35}S]-Tom40 subunit into the mature TOM complex (Fig. 6*F*). We conclude that both Sam50 and Sam51 participate in the assembly of Tom40 and have overlapping roles in the entry and exit of substrate proteins through the SAM complex.

Discussion

Characterization of mitochondrial protein import in *C. albicans* opened the way to using this yeast as a model for mitochondrial biogenesis. Distinctions in the protein import pathway in *C. albicans* include an evolutionary rewiring of mitochondrial protein import pathways between related species. Furthermore, the *C. albicans* system revealed aspects of the function of the mitochondrial SAM complex that can be exploited in experiments directed at understanding the role that each component plays in the assembly of the TOM complex and other proteins in the mitochondrial outer membrane.

Evolutionary Rewiring of Targeting Routes. It is well documented that *S. cerevisiae* has coevolved a forceful import motor and bipartite targeting sequences to deliver cytochromes into the intermembrane space via the stop-transfer pathway (reviewed in refs. 3 and 4). *C. albicans* has the capacity for import by the stop-transfer pathway, as evidenced by cytochrome c_1 from *S. cerevisiae* reaching the intermembrane space in *C. albicans* mitochondria and being processed there (Fig. 2*B*). Surprisingly, the cytochromes c_1 and b_2 in *C. albicans* and related yeasts lack the necessary targeting sequences to take the stop-transfer route into the mitochondrial intermembrane space, suggesting an evolutionary rewiring of targeting routes. Consistent with this model, the *C. albicans CYB2* gene could not rescue the *S. cerevisiae* Δ*cyb2* mutation, although it was expressed at high levels and the functional domains of Cyb2 are highly conserved between these two yeasts. How then do the cytochromes enter the mitochondrial intermembrane space in *C. albicans*? Three potential routes are available: (*i*) the disulfide-relay pathway, mediated by Mia40; (*ii*) the cytochrome *c* pathway, mediated by heme lyases; or (*iii*) another route, not previously described.

Both *Ca*Cyb2 and *Ca*Cyt1 have several cysteine residues that in principle could allow for interaction with Mia40. Although these residues are not the CX_3C or CX_9C motifs characteristic of most MIA substrates (3, 4), emerging data indicate that substrates that have CX_2C and other arrangements of cysteine residues (e.g., Erv1, Ccs1) use the MIA pathway for import into the intermembrane space (40–42). An alternative pathway would be driven by cytochrome heme lyases (e.g., Cyt2 and Cyc3), by analogy with the pathway for cytochrome *c* that is driven by the heme lyase Cyc3. In this import pathway the attachment of heme can serve to drive completion of polypeptide transfer across the outer membrane

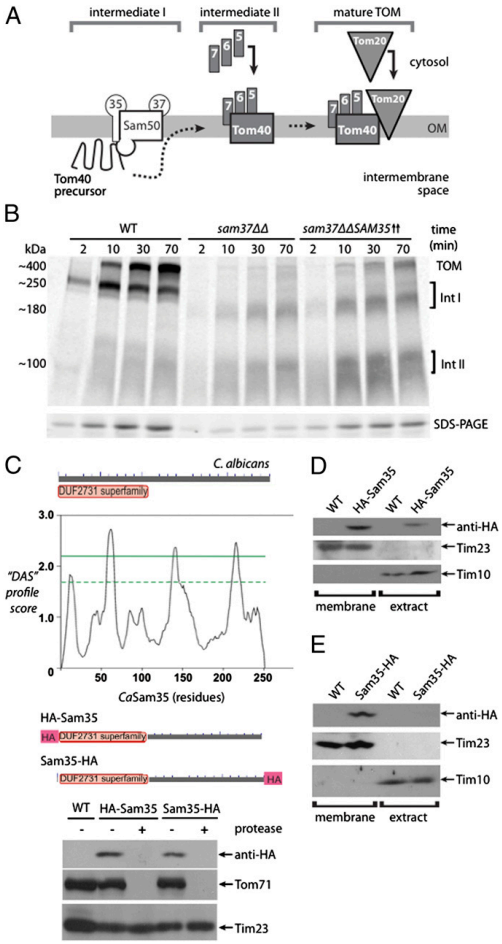

Fig. 4. Sam35 is an integral membrane protein functioning in substrate entry into the SAM complex. (*A*) Schematic of the assembly pathway for the Tom40 precursor, showing its incorporation into assembly intermediate I, assembly intermediate II, and the mature TOM complex. (*B*) Mitochondria (50 μg protein per lane) from the indicated strains of *C. albicans* were isolated and assayed for the import of *Ca*Tom40. Equal samples were withdrawn for analysis by BN-PAGE (*Upper*) or SDS/PAGE (*Lower*) and phosphorimaging. (*C*) Hydrophobicity analysis of *Ca*Sam35 with dense alignment surface method (DAS) showed it to have a more hydrophobic character than the prototypical *Sc*Sam35 and a conserved DUF2731 domain (35) indicated by the pink shape, with the gray bar representing the protein drawn to scale with the *x* axis. Schematics of HA-tagged versions of Sam35 are shown also. Mitochondria from *C. albicans HA-SAM35* strain and *SAM35-HA* were isolated, incubated with proteinase K for 20 min at 4 °C, and then analyzed by SDS/PAGE and immunoblotting. Tom71 is an outer membrane protein. Tim23 is an inner membrane protein. (*D*) Mitochondria from *C. albicans HA-SAM35* were isolated and incubated with 0.1 M Na_2CO_3 (pH 11.5), and the extracted membranes were reisolated by centrifugation. The extract supernatant ("extract") and the extracted membranes ("membrane") were analyzed by SDS/PAGE and immunoblotting. Tim10 is a soluble protein in the intermembrane space. (*E*) Mitochondria from *C. albicans SAM35-HA* were isolated and incubated with 0.1 M Na_2CO_3 (pH 11.5), and the extracted membranes were reisolated by centrifugation and analyzed by SDS/PAGE and immunoblotting.

(43, 44). The proposition that evolution has rewired targeting routes might provide a broad explanation to some curious past examples in mitochondrial protein import in diverse organisms. For example, the cytochrome c_1 found in the protozoan *Trypanosoma brucei* has no recognizable stop-transfer sequence, and in protist mitochondria *Tb*Cyt1 is imported into mitochondria in the absence of a membrane potential, leading to the suggestion that a route other than stop-transfer is used for its entry into the intermembrane space (45). Our results also shed light on differences observed between *S. cerevisiae* and humans. In *S. cerevisiae* the Mia40 protein is imported via the stop-transfer pathway, but in humans the homolog of Mia40 is imported via the Mia40 pathway (3, 4). This difference had been puzzling, initially calling into question the functional homology between the yeast and human Mia40 proteins. Based on our data for the *C. albicans* cytochromes, for which the functional and structural conservation with *S. cerevisiae* cannot be doubted, we suggest that the distinct import pathways are not an inevitable consequence of an event in the earliest eukaryotes that has been hard-wired into mitochondrial biogenesis but instead reflect rewiring options open to organisms to take advantage of new evolutionary opportunities.

Assembly of VDAC and Assembly of the TOM Complex. VDAC is required for metabolite exchange between the cytosolic and mitochondrial pools. In *S. cerevisiae* VDAC exists in numerous packing densities and oligomeric forms highly sensitive to metabolic conditions in the cell, ranging from monomers and trimers to regions of membrane where the packing density is 80% VDAC, functioning as a voltage-dependent molecular sieve (46). In *S. cerevisiae* the assembly of these dynamic VDAC oligomers is highly sensitive to the presence of Sam37 (33). The situation in *C. albicans* is quite distinct, with an apparently more regular arrangement of VDAC in a predominant oligomeric form and with little effect observed for Sam37 in the assembly of this VDAC oligomer.

Sam35 is necessary for newly imported [^{35}S]-Tom40 to engage productively with the SAM complex (34, 37) and functions as the receptor for the targeting signal present at the C terminus of Tom40, VDAC, and other β-barrel proteins (37). Until now, it has been difficult to rationalize how Sam35, thought to be a peripheral protein on the outer surface of the outer membrane, could perform this function on the inner surface of the outer membrane. The topology of *Ca*Sam35 explains this apparent contradiction: Although most of Sam35 is exposed to the cytosol, at least one transmembrane span is integrated into the outer membrane, leaving some part of Sam35 exposed in the intermembrane space and thus explaining how it can function as a receptor for β-barrel proteins. Sodium carbonate extraction is an empirical method that depends on the hydrophobicity of a transmembrane span to resist chaotropic forces at pH ~11 (47), and the relatively amphipathic nature of *Sc*Sam35 makes this integral membrane protein in this species prone to alkali extraction from the mitochondrial outer membrane.

Although at present Sam 51 has been described only in *C. albicans*, molecular phylogenetics (Fig. S2*B*) and comparative genome analysis (Fig. S2*C*) demonstrate that the presence of genes encoding both Sam50 and Sam51 is the ancestral yeast condition. In the course of evolutionary time, through the WGD event, *sam51* genes were lost from some yeasts, such as *S. cerevisiae*. Given that Sam51 seems to be dispensable, it is of great interest that none of the yeasts in the same category as *C. albicans* have, in fact, dispensed with its function. In *C. albicans*, assembly of newly imported [^{35}S]-Tom40 into the TOM complex is much faster than in *S. cerevisiae* and is driven by both Sam50 and Sam51. Mitochondria with a nearly complete depletion of Sam50 or complete depletion of Sam51 have similarly mild defects in TOM complex assembly. This result is consistent with Sam50 and Sam51 playing equivalent roles in β-barrel assembly but is in sharp contrast to the growth phenotypes of the two

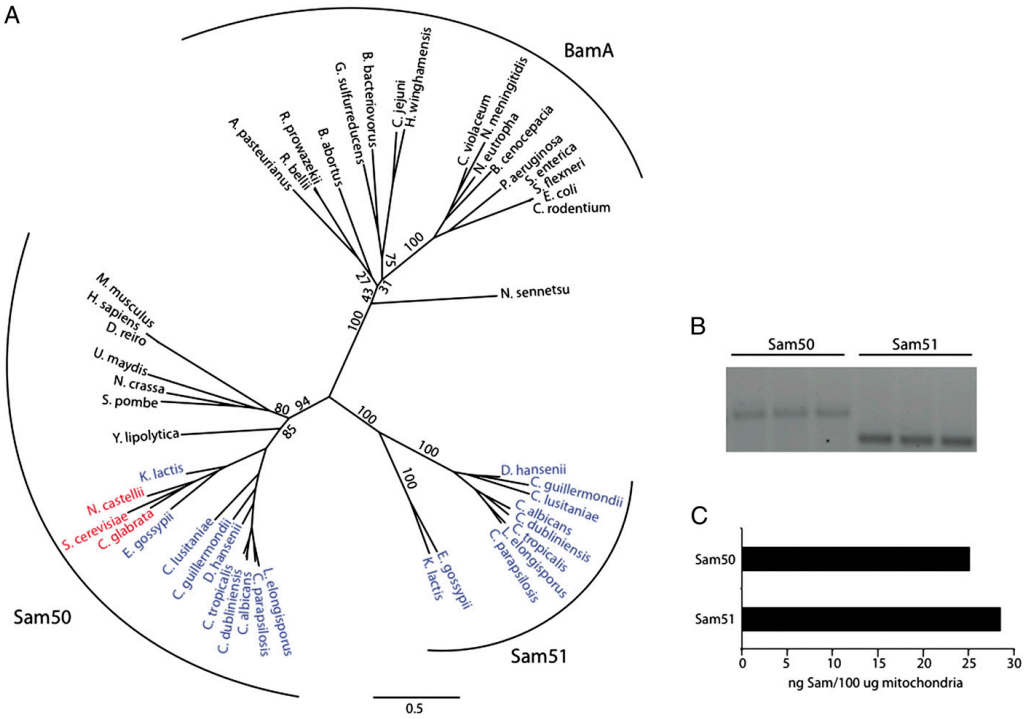

Fig. 5. The Omp85 protein family in *C. albicans*. (*A*) Phylogenetic analysis of Sam50, Sam51, and bacterial BamA sequences (*Materials and Methods*). The tree demonstrates that Sam51 sequences share a common ancestor to the exclusion of all Sam50 sequences as well as to the bacterial BamA sequences, recovered as the subgroup most similar to Sam51 based on BLASTP scores. Shown in blue are species that have both Sam50 and Sam51 genes; species with Sam50 but a secondary loss of Sam51 are indicated in red. Accession numbers for all sequences are given in Fig. S2*D*. (*B*) RT-PCR showing expression levels of *SAM50* and *SAM51* in *C. albicans*. PCR products from three independent cultures grown in rich medium (YPD) to log phase, with the linear range for each primer pair determined by assaying the appearance of the PCR products in time at different cycles. Shown are products that fell within the linear range of the reaction. (*C*) Purified Sam50 and Sam51 (0–100 ng protein) were used as standards alongside samples of mitochondria (25, 50, and 100 μg mitochondrial protein) analyzed by SDS/PAGE, immunoblot analysis, and phosphorimage quantitation to estimate the amount of Sam50 and Sam51 in 100 μg mitochondria.

mutant strains, with a loss of viability seen only for the deletion of the *SAM50* gene. Given the emergence of data from *S. cerevisiae* suggesting the involvement of subunits of the SAM complex in contacts between the mitochondrial outer membrane and endoplasmic reticulum (48, 49) and between the mitochondrial outer membrane and the mitochondrial inner membrane (50), *C. albicans* is a unique system in which to address the essential function of Sam50 that might distinguish it from Sam51.

Concluding Remarks. Taken together, the rewiring of protein import pathways, the less dynamic use of VDAC for metabolite exchange, and the subtle differences in the subunits contributing to the activity of the SAM complex show the utility of studying the mitochondrial import machinery in *C. albicans*. We anticipate that parallel investigations into the regulation points of TOM complex assembly and mitochondrial protein import using both *C. albicans* and *S. cerevisiae* will provide a more comprehensive picture of import pathway mechanisms and of the links among metabolism, the cell cycle, and mitochondrial biogenesis.

Materials and Methods

Yeast Strains. The *C. albicans* strains are derivatives of BWP17 (51). The wild-type strains were DAY185 (*URA3*$^+$ *ARG4*$^+$ *HIS1*$^+$) and DAY286 (*URA3*$^+$ *ARG4*$^+$) (52). The *C. albicans sam37ΔΔ* mutant and the *sam37ΔΔ* mutant overexpressing SAM35 (*sam37ΔΔ SAM35*↑↑) under the strong constitutive promoter *TEF1* have been described previously (35). The *sam51ΔΔ* mutant was constructed by standard methods using PCR and homologous recombination, with the *URA3* and *ARG4*-based selection cassettes (48). The conditional *C. albicans sam50* mutant (*sam50ΔSAM50*↓↓) was made by deleting the first allele with the *ARG4* marker cassette and placing the other allele under the control of the repressible *MET3* promoter (1,362 bp of the 5′ UTR of *MET3*) by constructing a *URA3-MET3* promoter fusion cassette. The *MET3* promoter was repressed by the addition of 2.5 mM methionine and 0.5 mM cysteine to synthetic growth medium. The epitope-tagged Sam35 strain was constructed by fusing a single HA tag to the N terminus of one of the alleles of *SAM35* under the *TEF1* promoter using the plasmid pCJN498 (53). The C-terminal fusion of the HA tag was performed as described in ref. 21. For complementation of the *S. cerevisiae Δcyb2* mutation, the *CYB2* gene was deleted in the YPH499 strain background (MATa *ade2-101 his3-200 leu2-1 ura3-52 trp1-63 lys2-801*), and the mutant was transformed with either the empty parental plasmid (2μ/*URA3*) or a plasmid containing either *ScCYB2* or *CaCYB2* driven by the *ADH1* promoter. Growth was monitored in synthetic medium lacking uracil at 30 °C, with either 2% (wt/vol) glucose or 2% (wt/vol) L-lactate (pH 4.6) as the carbon source.

For metabolic cycling experiments prototrophic strains of yeast were grown as previously described (8). The *S. cerevisiae* strain CEN.PK and the *C. albicans* strain DAY185 (52) were used in these experiments.

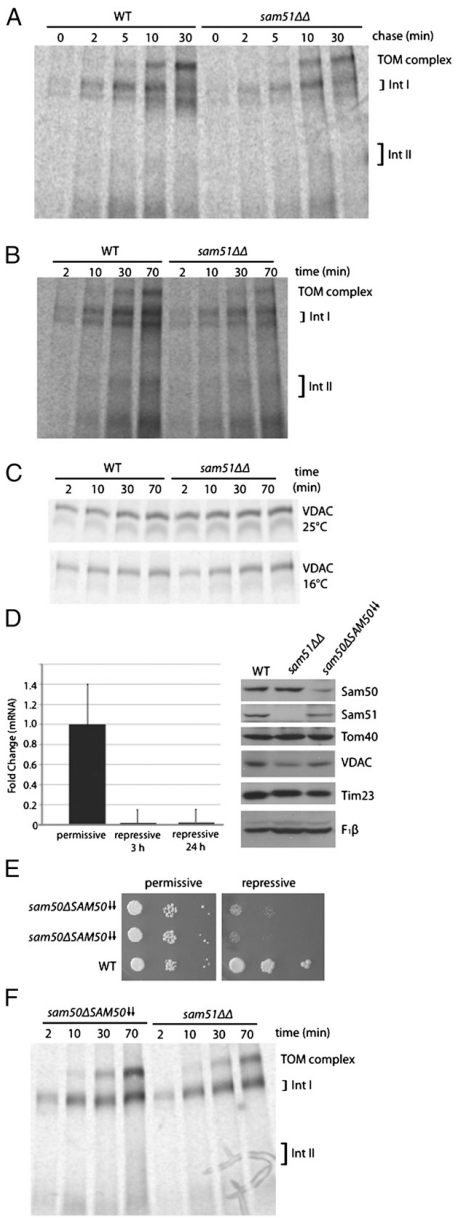

Fig. 6. Sam50 and Sam51 function in outer membrane protein assembly. (*A*) Mitochondria (50 μg protein per lane) from *C. albicans* wild-type or the *sam51ΔΔ* strains were incubated with *Ca*Tom40 for 10 min at 25 °C, isolated, and resuspended in import buffer at 25 °C. Samples were taken after the indicated time of chase for analysis by BN-PAGE and phosphorimaging. (*B*) Mitochondria (50 μg protein per lane) from *C. albicans* wild-type or *sam51ΔΔ* strains were isolated and assayed for import of *Ca*Tom40 at 16 °C and analyzed as above. (*C*) Mitochondria (50 μg protein per lane) from *C. albicans* wild-type or the *sam51ΔΔ* strains were isolated and assayed for import of VDAC at 25 °C or 16 °C. At the indicated times (min), a sample was removed for analysis by BN-PAGE and phosphorimaging. (*D*) Complete shutdown of *SAM50* expression in the *sam50ΔSAM50↓↓* strain was verified by quantitative PCR. RNA was extracted from cells grown for either 3 or 24 h in the presence of 2.5 mM methionine and 0.5 mM cysteine to repress the *MET3* promoter. Mitochondria were isolated from the *C. albicans sam50ΔSAM50↓↓* strain after 3 h incubation in repressive conditions for comparison with wild-type and the *sam51ΔΔ* strain using SDS/PAGE and immunoblotting with the indicated antisera. (*E*) Growth of the *C. albicans sam50ΔSAM50↓↓* strain on permissive (without methionine and without cysteine) and repressive (2.5 mM methionine/0.5 mM cysteine) medium. Growth at 30 °C was assessed after 3 d incubation. (*F*) The *C. albicans sam50ΔSAM50↓↓* strain and the *sam51ΔΔ* strain were grown in parallel liquid cultures and shifted to repressive conditions for the final 3 h of incubation. Mitochondria then were isolated and assayed for import of *Ca*Tom40 at 25 °C. At the indicated times (min), a sample was removed for analysis by BN-PAGE and phosphorimaging. The position of the assembly intermediates I and II and the mature TOM complex are indicated.

Sequence Analysis. The methodology for HMM analysis has been described previously (54), and HMMs were used to scan UniProt (Release 12.4, containing Swiss-Prot Release 54.4 and TrEMBL Release 37.4). Searches of the *C. albicans* genome refer to the type strain SC5314 and Assembly 21 of the genome information. The *C. albicans* Sam51 sequence was identified originally by HMM searches, and further homologs were identified by BLASTP and are available in Fig. S2*D*. Alignments were calculated using MUSCLE (55) under the default settings, and poorly conserved sites were removed manually. The best-fitting rate exchange matrix was determined with ProtTest under the Akaike information criterion (AIC) and resulted in LG + G for both datasets; tree calculations were performed with PhyML 3.0 (56) with the LG rate exchange matrix (57), tree topology search was performed with the Best of NNIs and SPRs option, 500 bootstrap calculations, and all other settings as default. Bootstrap support is shown as percentage values.

Preparation of Mitochondria. For the preparation of mitochondria, cultures of *S. cerevisiae* strain W303 were grown in rich medium [YPAGal; 2% (wt/vol) yeast extract, 1% peptone, 0.01% adenine, 2% (wt/vol) galactose]. *C. albicans* strains were grown in rich yeast extract peptone dextrose (YPD) medium [2% (wt/vol) glucose] at 30 °C. Mitochondria were isolated by differential centrifugation based on the method of Daum et al. (58). Homogenization was performed in 0.6 M sorbitol, 20 mM K^+ Mes (pH 6.0), 1 mM PMSF. The mitochondrial pellet was resuspended in 0.6 M sorbitol, 20 mM K^+ Hepes (pH 7.4), 10 mg/mL BSA.

Protein Import Assays and Electrophoresis. In vitro translation and import assays were performed as described (34). When necessary, the membrane potential was dissipated by the addition of a 100× solution of antimycin (8 μM), valinomycin (1 μM), and oligomycin (20 μM) to mitochondrial samples. Proteins were analyzed by SDS/PAGE or by BN-PAGE as previously described (34).

Antibody Production. Open-reading frames encoding Sam50 and Sam51 were synthesized in codon-optimised form for expression in *Escherichia coli* (GenScript) and cloned into a pET9 expression vector with an N-terminal 10×His tag. Recombinant expression in *E. coli* produced inclusion bodies containing the proteins. This material was purified by washing with 10% (wt/vol) TritonX-100 in PBS and using Ni-NTA agarose (Qiagen) as per manufacturers instructions, and the material was emulsified for immunization of rabbits. Other antisera used were raised against mitochondrial proteins from *S. cerevisiae* and cross-react with the homologous protein in *C. albicans*.

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Supporting Information

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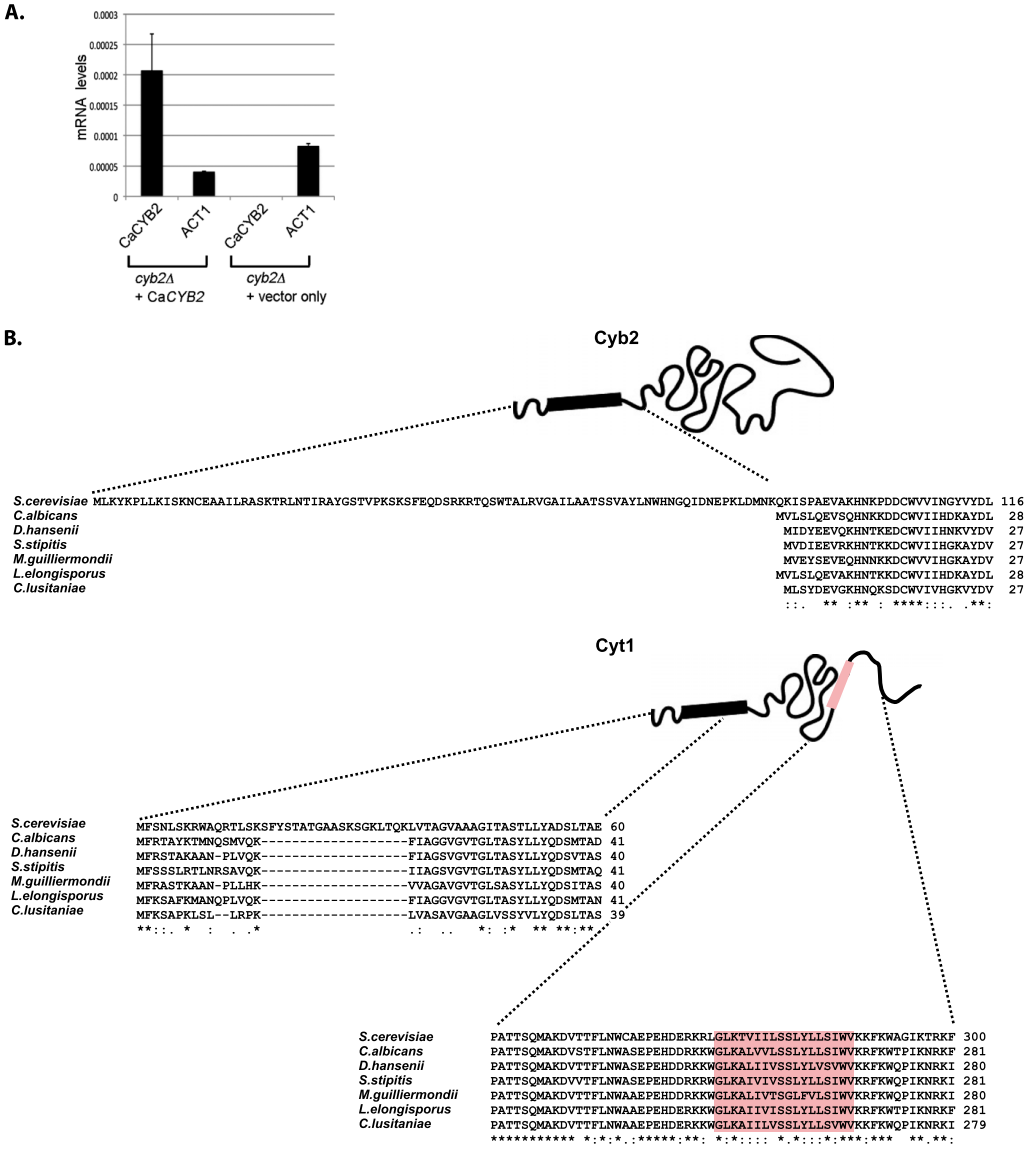

Fig. S1. Multiple sequence alignment for Cyb2 and Cyt1 from yeast. (*A*) The expression levels of the *CaCYB2* gene were measured by quantitative PCR in the *Saccharomyces cerevisiae* Δ*cyb2* mutant strain transformed either with an empty vector or with a vector containing the *CaCYB2* gene. The LinReg program (1, 2) was used to calculate the starting concentration (NO) of the *CaCYB2* transcript. Levels of the *ACT1* transcript in the same samples are shown to demonstrate

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that the *CaCYB2* transcript was expressed at higher levels than *ACT1*. Data shown are average plus SE of two independent biological repeats assayed in duplicate. (*B*) Schematic representation of Cyb2 from Fig. 2 is shown, and sequence alignments of yeasts in the group that did not undergo whole-genome duplication (WGD) are compared with the sequence from *S. cerevisiae*. The species are *Candida albicans, Debaryomyces hansenii*, *Scheffersomyces stipitis*, *Meyerozyma guilliermondii*, *Lodderomyces elongisporus*, and *Clavispora lusitaniae*. The alignment was prepared with ClustalW, and sequence identity and similarity are indicated by asterisks and dots, respectively. The N terminus of Cyb2 from *C. albicans* aligns well with Cyb2 from *S. cerevisiae*, but starting with residue Q89. The processing site that generates the mature form of *Sc*Cyb_2 is the peptidyl bond between residues L84 and D85, four residues upstream from Q89. An equivalent analysis for the cytochrome c_1 (Cyt1) is also shown. The N terminus of cytochrome c_1 from *C. albicans* aligns well with cytochrome c_1 from residue *S. cerevisiae*, but a region is deleted from within the stop-transfer–targeting region. Note, the 16 N-terminal residues of *Sc*Cyt1 are sufficient to target a reporter protein into the mitochondrial matrix (3). A C-terminal region of cytochrome c_1 (pink) serves as a transmembrane segment for insertion into the inner membrane (4) and is conserved in *C. albicans* and related yeasts.

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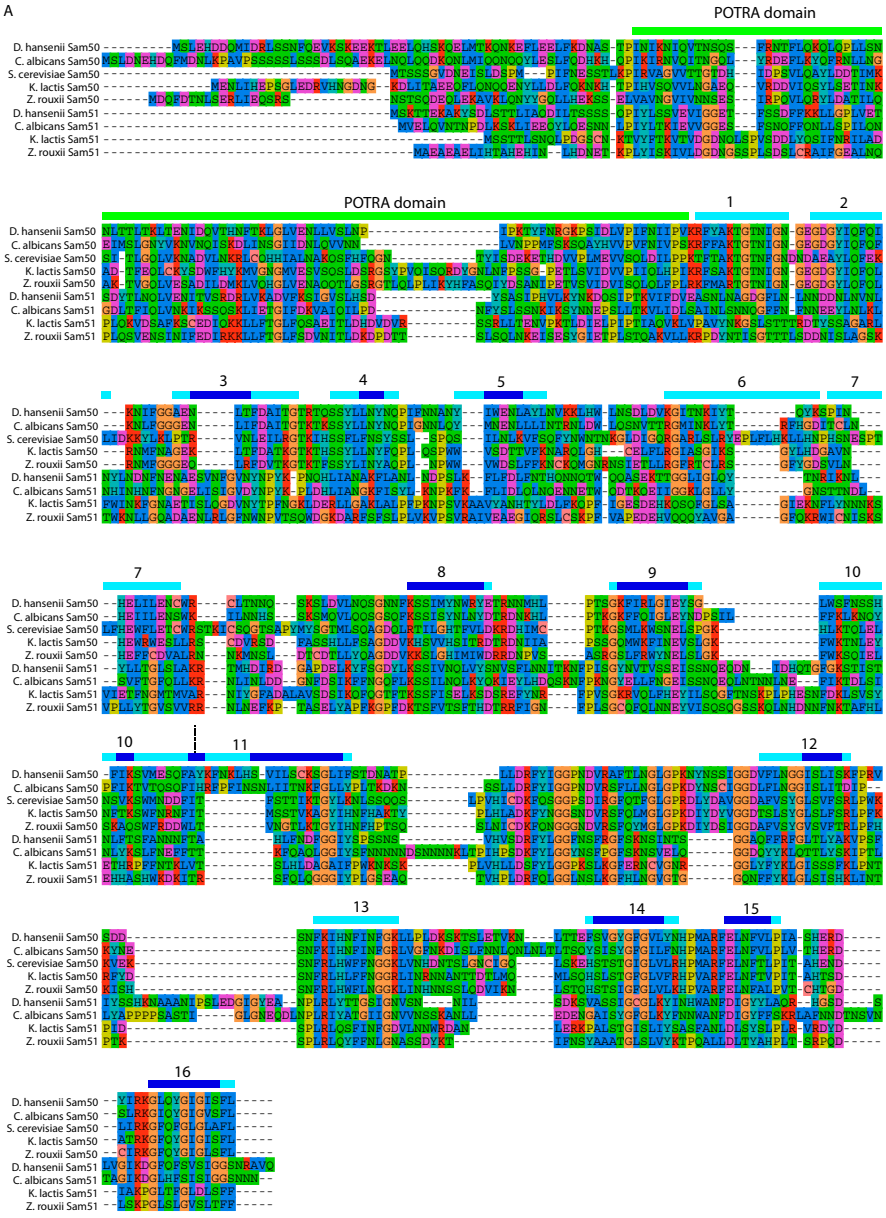

Fig. S2. (Continued)

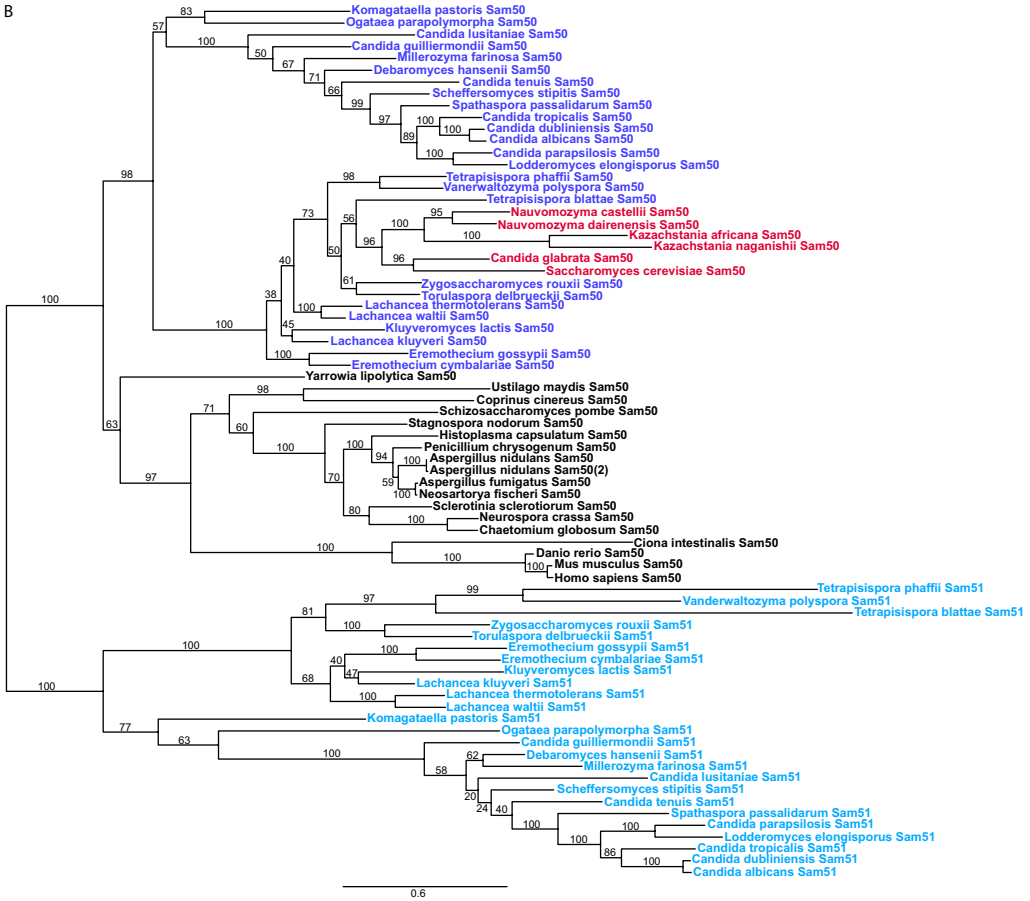

Fig. S2. (Continued)

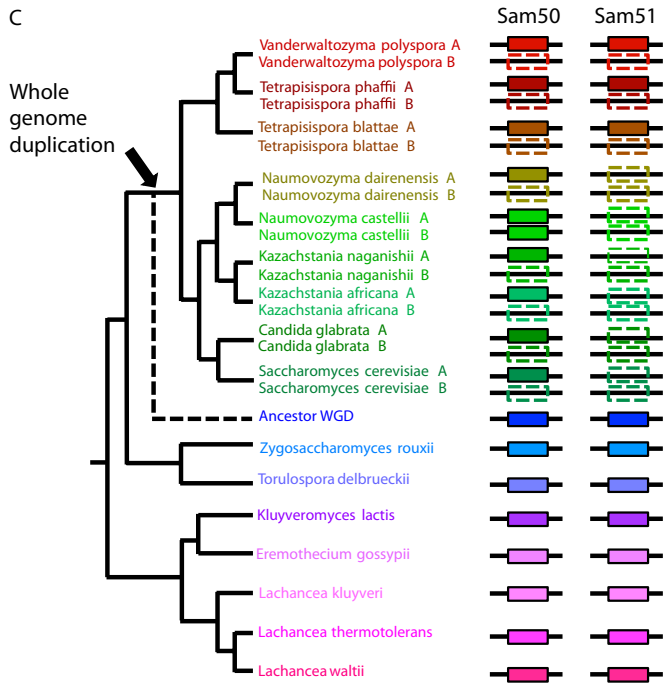

Fig. S2. (Continued)

D

Species	Sam50	Sam51	BamA
Clavispora (Candida) lusitaniae ATCC 42720	XP_002615880.1	XP_002614919.1	
Meyerozyma (Candida) guilliermondii ATCC 6260	EDK37901.2	EDK39319.2	
Debaryomyces hansenii CBS767	XP_002770022.1	XP_460145.2	
Candida parapsilosis	CCE42161.1	CCE41383.1	
Candida tropicalis MYA-3404	XP_002548154.1	XP_002550983.1	
Candida dubliniensis CD36	XP_002419475.1	XP_002420368.1	
Candida albicans SC5314	XP_716599.1	XP_716824.1	
Kluyveromyces lactis NRRL Y-1140	XP_454343.1	XP_454048.1	
Ashbya (Eremothecium) gossypii ATCC 10895	NP_987058.1	NP_985258.2	
Lodderomyces elongisporus NRRL YB-4239	XP_001526227.1	XP_001524708.1	
Scheffersomyces stipitis CBS 6054	XP_001382599.2	XP_001386142.2	
Spathaspora passalidarum NRRL Y-27907	EGW31681.1	EGW32011.1	
Candida tenuis ATCC 10573	EGV63016.1	EGV63290.1	
Millerozyma farinosa CBS 7064	CCE79338.1	CCE85839.1	
Ogataea parapolymorpha DL-1	EFW96696.1	EFW94959.1	
Komagataella pastoris GS115	XP_002491993.1	XP_002490790.1	
Lachancea thermotolerans	XP_002555504.1	XP_002554882.1	
Eremothecium cymbalabiae DBVPG#7215	XP_003645648.1	XP_003646589.1	
Zygosaccharomyces rouxii	XP_002494473.1	XP_002497635.1	
Torulaspora delbrueckii	XP_003682812.1	XP_003681643.1	
Tetrapisispora blattae	XBLA0B06060*	XBLA0A02810*	
Tetrapisispora phaffii	TPHA0G00830*	TPHA0E01750*	
Kluyveromyces polysporus	Kpol_1003.51*	Kpol_1062.28*	
Saccharomyces kluyveri	SAKL0E07194g*	SAKL0G02574g*	
Kluyveromyces (Lachancea) waltii	Kwal_27.11456*	Kwal_55.21418*	
Naumovozyma (Saccharomyces) castellii CBS 4309	XP_003677614.1		
Candida glabrata CBS 138	XP_449645.1		
Saccharomyces cerevisiae 288c	NP_014372.1		
Yarrowia lipolytica	XP_501539.2		
Ustilago maydis 521	XP_759051.1		
Coprinus cinereus okayama7 # 130	XP_001840415.1		
Schizosaccharomyces pombe 972-	NP_594600.1		
Ajellomyces (Histoplasma) capsulatum H143	EER44783.1		
Aspergillus nidulans FGSC A4	CBF79308.1, EAA61635.1+		
Phaeosphaeria (Stagonospora) nodorum SN15	XP_001798373.1		
Neurospora crassa OR74A	XP_960555.1		
Sclerotinia sclerotiorum 1980	XP_001594561.1		
Mus musculus	Q8BGH2.1		
Danio rerio	Q803G5.1		
Homo sapiens	Q9Y512.3		
Kazachstania africana	XAFR0H03250*		
Kazachstania naganishii	XNAG0H01940*		
Naumovozyma dairenensis	NDAI0L00700*		
Acetobacter pasteurianus IFO 3283-01			YP_003187185.1
Neorickettsia sennetsu str. Miyayama			YP_506594.1
Brucella abortus bv. 3 str. Tulya			ZP_05928429.1
Rickettsia prowazekii str. Madrid E			NP_220550.1
Rickettsia bellii RML369-C			YP_538287.1
Nitrosomonas eutropha C91			YP_748215.1
Chromobacterium violaceum ATCC 12472			NP_901874.1
Burkholderia cenocepacia AU 1054			YP_625904.1
Neisseria meningitidis alpha14			YP_003083995.1
Escherichia coli str. K-12 substr. MG1655			NP_414719.1
Shigella flexneri 2a str. 301			NP_706122.1
Salmonella enterica subsp. arizonae serovar 62:z4,z23:-- str. RSK2980			YP_001571769.1
Citrobacter rodentium ICC168			YP_003363826.1
Pseudomonas aeruginosa PAO1			NP_252338.1
Geobacter sulfurreducens PCA			NP_953317.1
Bdellovibrio bacteriovorus HD100			NP_968381.1
Helicobacter winghamensis ATCC BAA-430			ZP_04583808.1
Campylobacter jejuni subsp. jejuni HB93-13			ZP_01072108.1

Fig. S2. Sam51 was acquired at the separation of the *Candida* and *Saccharomyces* clades from the other *Saccharomycetales* families and was secondarily lost in the lineage of *S. cerevisiae* after the WGD event. (*A*) Sam50 and Sam51 share a similar domain structure with the classical Omp85 components: an N-terminal POTRA domain (green bar) and the C-terminal transmembrane β-barrel domains (dark blue bar: a barrel-domain predicted for all sequences shown; light blue bar: a barrel-domain predicted for at least two sequences). SeaView was used for the graphical alignment representation (1); assignment of the POTRA domains is based on the POTRA domain in *S. cerevisiae* (2); β-barrel regions were predicted using BOCTOPUS (3). (*B*) Phylogenetic analysis of Sam50 and Sam51 demonstrates the clear distinction between Sam50 and Sam51. More distantly related animal Sam50 sequences group together with the fungal Sam50 sequences to the exclusion of all Sam51 sequences. Fungal species with copies of both Sam50 (dark blue) and Sam51 (light blue) are indicated, and the Saccharomycetales subgroup in which Sam51 was secondarily lost is indicated in red. Fungal and animal species that diverged before the duplication/acquisition of Sam51 are indicated in black. Accession numbers for all sequences are given in *D*. The two Sam50 sequences from *A. nidulans* differ in that the second copy codes for an additional 313-amino acid residues at the C terminus compared with the canonical Sam50. (*C*) The distribution of Sam50 and Sam51 in the *Saccharomyces* clade, with the data obtained and the display adapted from the Yeast Gene Order Browser, YGOB (http://wolfe.gen.tcd.ie/ygob/; ref. 4). Species names followed by "A" and "B" represent the two copies of the genomes after WGD; dashed squares indicate an absence of the respective gene in the according region. Sam51 was still present during the WGD event and also is indicated as being present in the WGD ancestral sequence (data obtained from the YGOB website) but later was secondarily lost from several species. The two copies of Sam50 observed for *Naumovozyma castellii* derived from the WGD event are 100% identical and therefore are not represented separately in *B*. (*D*) Accession numbers for Sam50, Sam51, and BamA sequences. Sequences derived from the YGOB database are indicated by an asterisk. The plus sign (+) denotes that *A. nidulans* has two highly similar copies of Sam50.

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Table S1. Mitochondrial protein import machinery in *C. albicans*

Function	Protein	SC5314 ORF ID	% identity (similarity)	Notes
Translocase of the outer membrane (TOM) complex in outer membrane for protein translocation into mitochondria	Tom5	None	42 (65)	Hidden Markov model (HMM) search identified an open-reading frame (47aa residues) too small for annotation, in the region Ca21Chr1.1421439–1421582*
	Tom6	ORF19.1650	56 (67)	Annotated as Tom6
	Tom7	ORF19.6531.1	70 (81)	Inappropriately annotated as Tom71†
	Tom20	ORF19.2953	49 (72)	Annotated as Tom20
	Tom22	ORF19.3696	44 (62)	Annotated as Tom22
	Tom40	ORF19.6524	62 (78)	Annotated as Tom40
	Tom70	None	—	Single isoform (Tom71) is present in *C. albicans*‡
	Tom71	ORF19.3700	43 (64)	
Chaperones in intermembrane space for β-barrel (and other) membrane proteins	Tim9	ORF19.6696	81 (88)	Annotated as Tim9
	Tim10	ORF19.4577.3	69 (81)	Annotated as Tim10
	Tim8	ORF19.6183	57 (72)	Annotated as Tim8
	Tim13	ORF19.2754	64 (75)	Annotated as Tim13
Sorting and Machinery (SAM) complex for β-barrel protein assembly	Sam35	ORF19.7267	26 (41)	Annotated as Sam35
	Sam37	ORF19.1532	37 (56)	Annotated as Sam37
	Sam50	ORF19.7358	31 (49)	Annotated as Sam50
	Sam51	ORF19.925	25 (40)	Annotated as "uncharacterized ORF" §
Component of both SAM and ER-mitochondria encounter structure (ERMES) complexes	Mdm10	ORF19.184	35 (53)	Annotated as Mdm10
α-Helical protein insertion into outer membrane	Mim1	ORF19.542.2	41 (62)	Annotated as Mim1
Intermembrane space import components	Mia40	ORF19.2977	50 (72)	Annotated as "Hap43-repressed gene"¶
	Erv1	ORF19.2863.1	52 (75)	Annotated as Erv1
Inner membrane peptidase	Imp1	ORF19.3061	54 (70)	Annotated as Imp1
	Imp2	ORF19.1981	54 (71)	Annotated as Imp2
Carrier translocase of the inner mitochondrial membrane (TIM22 complex)	Tim22	ORF19.1352	64 (75)	Annotated as Tim22
	Tim18/ Sdh4	ORF19.4022/ ORF19.4468	44 (58)/ 38 (59)	Functional analysis required to distinguish between Tim18 and Sdh4 isoforms‖
	Tim54	ORF19.5143	39 (58)	Annotated as Tim54
	Tim12	ORF19.4620	42 (59)	Annotated as Tim12
Translocase of the Inner mitochondrial membrane (TIM23 complex)	Tim23	ORF19.1361	61 (77)	Annotated as Tim23
	Tim17	ORF19.150	82 (89)	Annotated as Tim17
	Tim50	ORF19.680	48 (63)	Annotated as Tim50
Presequence translocase-associated motor (PAM) engagement	Tim21	ORF19.3691	45 (64)	Annotated as Tim21
PAM	Pam16	ORF19.7222	50 (65)	Annotated as Pam16
	Pam17	ORF19.240	54 (73)	Annotated as Pam17
	Pam18	ORF19.4190	55 (67)	Annotated as Pam18. Has "intermembrane space" domain characteristic of Pam18**
	Mdj2	ORF19.3574	41 (61)	Annotated as Mdj2. Does not have "intermembrane space" domain of Pam18 proteins**
	Ssc1	ORF19.1869	78 (88)	Annotated as Ssc1

Shading denotes ORFs for which there was initial uncertainty with respect to functional homology.
*The 47-residue ORF is too small to be captured by automated gene assignments but shares high sequence similarity with Tom5 and has been shown to be transcribed (1).
†HMM search identified an ORF (101 aa residues) candidate Tom7. *Candida* Genome Database (www.candidagenome.org) has been alerted to this misannotation.
‡Tom70 and Tom71 are paralogs in *S. cerevisiae*, a relic of ancient genome duplication (2).
§Sam51 is characterized in this study.
¶The gene encoding Mia40 is one of a collection controlled by the iron-responsive transcription factor Cap2/Hap43 (3).
‖Tim18 and Sdh4 are isoforms of a related protein and are both found in the mitochondrial inner membrane (4).
**Although Mdj2 was previously thought to be found only as a result of the ancient genome duplication in the Saccharomyceateae (5), and of uncertain importance, its presence in *C. albicans* suggests a fundamental importance of the Mdj2 and an alternate evolutionary history.

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Review

The ins and outs of the intermembrane space: Diverse mechanisms and evolutionary rewiring of mitochondrial protein import routes☆

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ABSTRACT

Background: Mitochondrial biogenesis is an essential process in all eukaryotes. Import of proteins from the cytosol into mitochondria is a key step in organelle biogenesis. Recent evidence suggests that a given mitochondrial protein does not take the same import route in all organisms, suggesting that pathways of mitochondrial protein import can be rewired through evolution. Examples of this process so far involve proteins destined to the mitochondrial intermembrane space (IMS).
Scope of review: Here we review the components, substrates and energy sources of the known mechanisms of protein import into the IMS. We discuss evolutionary rewiring of the IMS import routes, focusing on the example of the lactate utilisation enzyme cytochrome b_2 (Cyb2) in the model yeast *Saccharomyces cerevisiae* and the human fungal pathogen *Candida albicans*.
Major conclusions: There are multiple import pathways used for protein entry into the IMS and they form a network capable of importing a diverse range of substrates. These pathways have been rewired, possibly in response to environmental pressures, such as those found in the niches in the human body inhabited by *C. albicans*.
General significance: We propose that evolutionary rewiring of mitochondrial import pathways can adjust the metabolic fitness of a given species to their environmental niche. This article is part of a Special Issue entitled Frontiers of Mitochondrial.

1. Introduction

Mitochondria are organelles that play a central role in metabolism and energy production. Mitochondrial activity and biogenesis is regulated in response to a variety of intracellular and environmental cues [1], including nutrient availability [2], oxidative stress [3] and presence of toxic compounds [4]. In order for mitochondria to replicate and adapt rapidly to their environment, they must be populated with functional proteins in their correct compartments. With the vast majority of mitochondrial proteins encoded in the nuclear genome, a key mechanistic component of mitochondrial biogenesis is the import of these proteins after their synthesis in the cytosol. These proteins are recognised and imported by the diverse transport machineries of mitochondria (Fig. 1) [5,6].

The first transport machine to engage most precursor proteins is the TOM (translocase of the outer membrane)[1] complex [7] (Fig. 1, brown). This multi-subunit complex includes the Tom20 [8] and Tom22 [9] receptors that recognise a range of TOM complex substrates. Some α-helical outer membrane (OM) proteins are integrated directly into the membrane by the mitochondrial import complex (MIM) [10], but most proteins are imported through the TOM complex before the import pathways to the different mitochondrial compartments diverge (Fig. 1). Proteins forming β-barrels in the OM are inserted by the SAM (sorting and assembly machinery) complex [11], recently shown to be directly coupled to the TOM complex [12]. SAM complex substrates in the intermembrane space (IMS) are also bound by the small TIM (translocase of the inner membrane) chaperones [13]. Small TIM chaperones also escort inner membrane (IM) proteins to the TIM22 complex

☆ This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

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[1] Abbreviations: translocase of the outer membrane (TOM), outer membrane (OM), mitochondrial import complex (MIM), sorting and assembly machinery (SAM), intermembrane space (IMS), translocase of the inner membrane (TIM), inner membrane (IM), presequence translocase-associated import motor (PAM), oxidase assembly (OXA), mitochondrial intermembrane space transport and assembly (MIA), mitochondrial processing peptidase (MPP), inner membrane protease (IMP), mitochondrial inner membrane organizing system (MINOS).

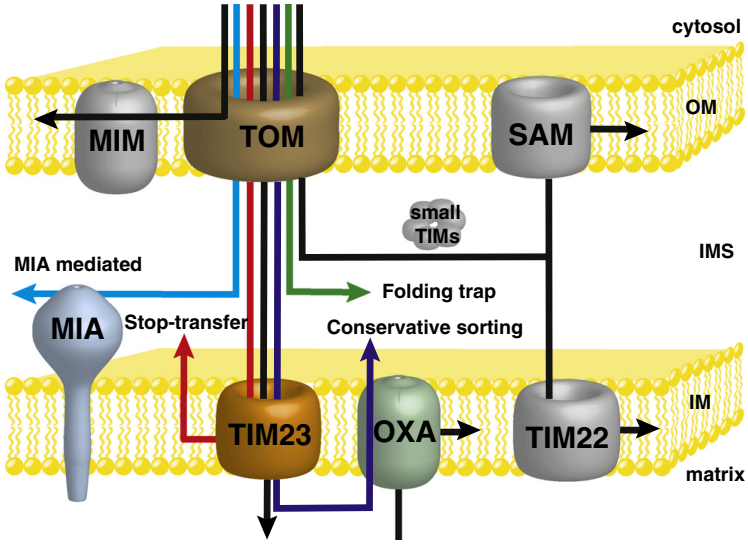

Fig. 1. Overview of mitochondrial protein import pathways. Pathways into the intermembrane space (IMS) are shown in colour and discussed in more detail in the text. Import machinery not discussed in depth in this review is shown in grey. Pathways diverge at the outer membrane (OM) after recognition by the TOM (translocase of the outer membrane) complex (brown). Some proteins are inserted directly into the OM by the mitochondrial import machinery (MIM), but most pass through the OM through the TOM complex. Proteins imported by the MIA (mitochondrial intermembrane space transport and assembly) pathway (bright blue), or folding trap pathway (green), remain in the IMS. Small TIM (translocase of the inner membrane) proteins escort proteins to the SAM (sorting and assembly machinery) complex, or TIM22 machinery to be assembled into the OM or inner membrane (IM) respectively. Proteins imported by a stop-transfer pathway (red) or conservative sorting pathway (dark blue) also use the TIM23 complex (orange) to traverse the IM. Proteins with domains that then pass back through the IM sometimes use the OXA (oxidase assembly) machinery, which also transports proteins translated in the matrix.

for assembly into the inner membrane [14,15]. In contrast, the TOM complex interacts directly with the TIM23 complex (Fig. 1, orange) to transport matrix-destined proteins through both membranes [16,17]. This movement is a membrane potential dependent process driven by the PAM (presequence translocase associated import) motor module of the TIM23 complex. In some cases this transport process is interrupted and the precursors are laterally released from TIM23 into the inner membrane. In a few cases insertion of inner membrane proteins from the matrix side has been found to require the oxidase assembly (OXA) translocation machinery (Fig. 1, green) [18], which is primarily involved in the insertion of proteins synthesised within the mitochondria into the IM [19,20]. The most recently discovered transport machinery is the MIA (mitochondrial intermembrane space transport and assembly) machinery (Fig. 1, blue), which ensures proper import and folding of a number of IMS proteins [21].

Recent work has identified regulatory controls on mitochondrial protein import, which modulate mitochondrial function and biogenesis in line with the metabolic needs of the cell [22,23]. However in addition to these acute regulatory responses to environmental stimuli, a much more drastic, long-term modulation of the import pathways seems to be at work. The import pathways for mitochondrial proteins appear to have been rewired over evolutionary time to accommodate the distinct metabolic circumstances of a given biological species in its environmental niche. This idea has received support from a recent study done in our laboratories comparing protein import into the IMS in distantly related fungal species [24], as well as from previous work on the IMS protein Mia40 in yeast and mammals [25]. Prompted by these discoveries, we use this review as an opportunity to provide an up-dated account of the different pathways used for protein import into the mitochondrial IMS and discuss the concept of evolutionary rewiring of these import pathways.

2. Unexpected journeys into the IMS

Proteins are targeted to the IMS by diverse mechanisms, perhaps reflecting the different roles of the proteins found in this compartment. Specific machinery for import through the IM or assembly in the IMS recognise further signal elements within precursor proteins after their import through the TOM complex. The IMS proteins discussed in this review include both soluble IMS proteins and IMS-exposed proteins with anchors in the inner membrane. We discuss known features of the stop-transfer, conservative sorting, folding trap and MIA pathways. Many of the mechanisms of import into the IMS are still being elucidated and new examples of interactions between these mechanisms and other import processes are still being identified.

2.1. Arrest at the inner membrane: the stop-transfer pathway

Some IMS proteins are synthesised with a cleavable N-terminal presequence, containing matrix-targeting information [26]. In proteins destined for the mitochondrial matrix this targeting information leads them to and through the TOM complex and TIM23 complex [27]. A positively charged presequence at the N-terminus is recognised by the receptors Tom20 and Tom22 and facilitates import through the outer membrane via the TOM complex [28] (Fig. 2A). The presequence also enables the protein to engage with Tim50 and Tim23 resulting in membrane potential-dependent insertion of the precursor into the Tim23 channel in the TIM23 complex [29] (Fig. 2A). However, in IMS-destined proteins, this charged sequence is followed by a hydrophobic sorting signal that is recognised by the TIM23 complex, resulting in a lateral transfer of the sequence into the IM thereby preventing translocation into the matrix [26,30,31] (Fig. 2B). This process may require rearrangement of the TIM23 complex [32], but unlike translocation into

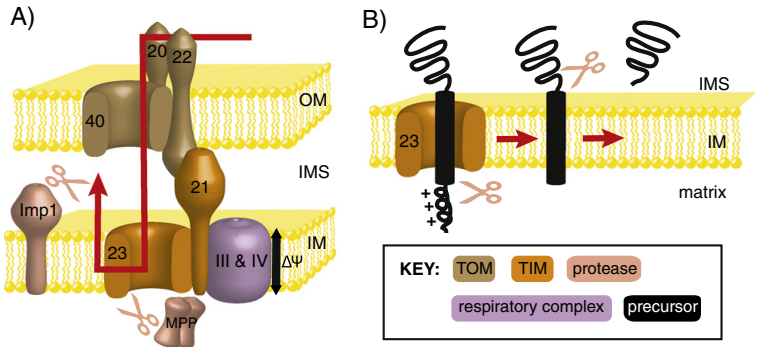

Fig. 2. General features of the stop-transfer pathway. (A) The components and interactions of the stop-transfer pathway are shown. The red arrow shows the path of a precursor through the TOM complex (brown) and on into the TIM23 complex (orange). This is followed by the lateral release of the precursor into the inner membrane (IM) and, in some cases, proteolysis to release the precursor into the intermembrane space (IMS). The locations of proteases (pink) and the coupling of the respiratory complexes (purple) to the TIM23 complex are also shown. The black arrow indicates that this pathway requires a membrane potential ($\Delta\Psi$) across the IM. (B) Processing of the bipartite signal sequence of the precursor (black) to produce membrane anchored or soluble IMS proteins.

the matrix, it is not dependent on the activity of the PAM module of the complex [33,34].

In the stop-transfer pathway, the positively charged presequence protruding into the matrix through the pore of the TIM23 complex is cleaved by a matrix protease, in most cases, the mitochondrial processing peptidase (MPP) (Fig. 2, pink) (reviewed in Refs. [35,36]). The now terminal hydrophobic sorting signal crossing the inner membrane acts as a brake in the pathway, preventing the translocation of the precursor into the matrix [37]. Hydrophobic and proline residues in the transmembrane domain [38], and charged residues following this hydrophobic region [39], have all been shown to play a role in directing precursor proteins into the stop-transfer pathway, but the mechanistic basis behind these observations is still unclear [31].

After the stop signal is recognised, proteins are laterally released from the TIM23 machinery (Fig. 2B). The mechanism of release from TIM23 is unclear, but the pathway is impaired in mutants of Tim17 (a TIM23 complex subunit), or if the interaction between the TOM complex and the TIM23 complexes via the Tom22 and Tim21 proteins is disrupted [40]. This membrane potential-dependent step is also stimulated by coupling of the TIM23 complex to the respiratory chain complexes III and IV via Tim21 [41] (Fig. 2A, purple). Proteins that do not undergo any further processing remain anchored by their N-terminal transmembrane domain in the inner membrane [5].

Further cleavage of the stop-transfer sequence by specific proteases can release soluble proteins into the IMS (Fig. 2B). Most commonly the hydrophobic sequence is cleaved by the inner membrane protease (IMP) (Fig. 2, pink) (reviewed in Refs. [5,42]). The key features of this pathway were identified through work on the *Saccharomyces cerevisiae* protein cytochrome b_2 (Cyb2) [30], but this protein has an additional requirement for Ssc1 (mitochondrial Hsp70) for proper folding of its heme-binding domain [43]. Mcr1 is another substrate of this pathway with an interesting variation [44]; the hydrophobic sequence can also be inserted in the OM, resulting in both OM-anchored and IMS forms of this protein [45].

A similar two-step, stop-transfer process produces the soluble IMS form of Mgm1, a dynamin-related GTPase with both soluble and membrane associated isoforms [46]. In this instance however, the MPP cleavage step is followed by the action of a different protease, Pcp1 [47]. The position of the transmembrane domain in this process is critical for the cleavage step to occur and is dependent on ATP levels within the mitochondria [48]. Thus the metabolic status of mitochondria regulates the import process and controls the relative amounts of the cleaved soluble forms and the laterally released membrane-anchored form of Mgm1. The second processing step of cytochrome c peroxidase (Ccp1) is also mediated by Pcp1 cleavage, in this case after an AAA protease (an ATPase associated with various cellular activities [49]) removes the first segment of the targeting signal [50]. The specific proteolytic processing of precursors has recently been recognised as a mechanism for the regulation of protein stability [51].

A final variation on the stop-transfer pathway anchors proteins in the inner membrane via their C-termini. In the case of cytochrome c_1 (Cyt1), cleavage of the N-terminal signal by the IMP occurs only after the insertion of the C-terminus into the inner membrane [52]. The sequences and predicted secondary structure of Shy1 [53] suggests that it may be imported via a similar pathway [52]. While Shy1 remains anchored to the IM via its C-terminus, its major functional domain is exposed to the IMS [54]. Shy1 is the yeast homologue of human Surf1, which is mutated in the mitochondrial disease, Leigh Syndrome. In contrast to findings in yeast, both hydrophobic targeting sequences of the human protein are important for its proper function [55], raising the possibility that defective import could play a role in the disease mechanism. A similar C-terminal transmembrane domain followed by an internal presequence-like signal is required for the IM insertion of Bcs1 [56]. This mechanism may further extend to other IMS-exposed substrates such as the J-proteins Tim14 [57], Mdj2 [58] and Jid1 [59].

2.2. There and back again: the conservative sorting pathway

In the conservative sorting pathway, proteins are firstly imported into the matrix via the TIM23 complex requiring ATP-dependent action of the PAM module [27] (Fig. 3) and stabilisation by the matrix-located isoform of Hsp60 [60]. This is followed by a further export-like step to relocate the protein back into the inner membrane [5,61]. This pathway was initially characterised through studies of the IMS-exposed Rieske iron sulfur protein (Rip1). Rip1 has a single N-terminal transmembrane domain anchoring the protein in the membrane, and a large C-terminal domain that is exposed to the IMS [61]. The protein is imported into the matrix, and the C-terminal domain is translocated back across the IM by the AAA-protease Bcs1 [62]. Other proteins that use the conservative sorting mechanism to translocate their N-termini back across the IM into an IMS-exposed location are subunit 9 of the *Neurospora crassa* F_1F_0-ATPase [63] and the polytopic inner membrane protein Oxa1 (discussed in more detail below) [64].

For many proteins this final step towards the IMS is mediated by Oxa1 [19], which is also part of the OXA machinery that assembles inner membrane proteins encoded on the mitochondrial genome into

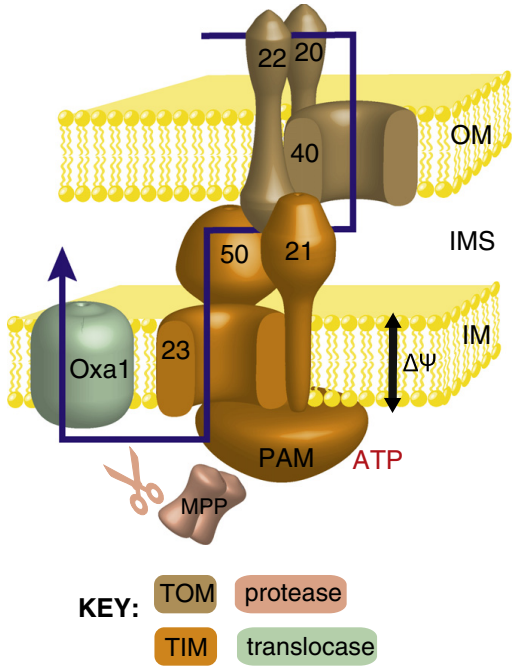

Fig. 3. General features of the conservative sorting pathway. The components and interactions of the conservative sorting pathway are shown. The blue arrow shows the path of a precursor through the TOM complex (brown) in the outer membrane (OM) and on into the TIM23 complex (orange). The precursor is imported into the matrix in a PAM (presequence translocase-associated import motor) dependent process that requires ATP and a membrane potential ($\Delta\Psi$) across the inner membrane (IM). Processing by a protease (pink), in most cases MPP (mitochondrial processing peptidase) precedes translocation back across in the IM by translocation machines (green), such as the Oxa1 machinery.

the IM [5,65]. This pathway may be particularly important for multi-topic membrane proteins originating from the bacterial endosymbiont [66], but it is not restricted to these substrates. Dependence on this endosymbiont-derived export machinery shows that there are limits to the more recently evolved systems in their ability to insert inner membrane proteins directly from the IMS [67]. Also implicated in the insertion of proteins into the IM from the matrix side are Mba1 [68], Cox18 (Oxa2) [69] and Bcs1 [56], but the general principles governing this step are still to be determined [70].

Recent work has revealed that pathways can cooperate to import proteins with multiple transmembrane domains. Containing six transmembrane domains and loops exposed to both the IMS and matrix, Mdl1 was the first protein shown to require both the stop-transfer and conservative sorting pathways [71]. Cleavage of the presequence and insertion of the first two transmembrane domains has the hallmarks of the stop-transfer process, whereas the insertion of the less hydrophobic transmembrane domains within the polypeptide requires both Ssc1 and Oxa1. Similarly, the two moderately hydrophobic transmembrane segments at the N-terminus of Sdh4 are imported into the matrix from where the transmembrane segments are inserted into the membrane, and the N-terminus passes back across the membrane making it IMS-exposed in the mature protein. The third transmembrane domain of Sdh4 is sufficiently hydrophobic to follow the stop-transfer pathway [70]. These are the first examples showing that the stop-transfer mechanism is able to process sufficiently hydrophobic transmembrane domains, but that mitochondria still require additional translocation machinery for the insertion of less hydrophobic transmembrane segments unable to be recognised for lateral sorting [72]. It is likely there are more examples and variations of cooperation between import pathways that remain to be discovered.

Some organisms retain other ancient components of their ancestral bacterial protein export machinery, namely components of the SecYEG translocon and the Tat machinery [73,74]. Proteins in these organisms could be assembled into the IM by the translocon or exported into the IMS by the Tat machinery, but this has not yet been demonstrated.

2.3. Thou shalt not pass: no escape from the folding trap

As well as travelling to the correct compartment, proteins must be properly folded and assembled with co-factors, enzymes or partner proteins in order to function. For example a Cyt1-specific heme lyase is found in the IMS and is needed to incorporate heme into Cyt1 [75]. It is not clear how important the protein folding reaction is in driving the translocation of such precursors through the TOM complex, but this probably varies depending on the substrate. Once folded, the protein is unable to escape from the IMS, at least as long as the outer membrane remains intact.

Another example of this folding trap mechanism is the retention of cytochrome c (Cyc1) in the IMS, which requires binding to cytochrome c heme lyase (Cyc3/CCHL). Cyc3 is found in the IMS associated with the inner membrane [76], and catalyses the attachment of a heme group to Cyc1. The superoxide dismutase, Sod1, is another metal-binding protein that is imported via a folding trap [77]. This protein reduces oxidative stress by reacting with superoxides, and overexpression of Sod1 has recently been shown to be protective in diabetes-prone mice [78]. Sod1 precursors interact with the copper-binding chaperone Ccs1, which helps assemble the apoprotein with zinc and copper co-factors [79]. Ccs1 and Sod1 may also have roles in assembly, import or folding of other metal-binding IMS proteins [80].

Unexpected flexibility in import pathways has been revealed by the recent publication of Ccs1-independent import of Sod1 [81]. Varabyova and colleagues show reduced and mutant forms of Sod1 can be imported into the IMS by the MIA pathway in conjunction with the mitochondrial inner membrane organising system (MINOS) [82]. The mutant forms of Sod1 are associated with amyotrophic lateral sclerosis in humans, presenting the intriguing possibility of connections between regulation of mitochondrial import and this disease [81].

2.4. More than just disulfide bonds: the MIA pathway

In 2004, Mia40 was discovered and named for its role in mitochondrial intermembrane space transport and assembly [21]. Initially, substrates were thought to be restricted to cysteine rich proteins containing conserved CX_3C or CX_9C disulfide bonded motifs [83,84]. The interaction of Mia40 with these canonical substrates ensures the proper formation of disulfide bonds before the oxidised substrate is released into the IMS ([21,85] and reviewed in [86–88]). Substrates with more diverse cysteine organisations have since been identified [42,84,89–91].

The importance of hydrophobic residues in the interaction of Mia40 with small IMS substrates [92,93] suggested an even broader range of substrates might use this pathway. This was confirmed with the recent discovery that import of Atp23 does not require Mia40-mediated disulfide formation [89]. Instead the hydrophobic interactions between Mia40 and Atp23 were shown to be more important for import. Hydrophobic interactions are also important for the role of Mia40 in the import of the IM protein Tim22 [91].

Mia40 works in conjunction with a number of other proteins in the IMS and IM (Fig. 4). The roles of these partners are described briefly below, as they have recently been reviewed in depth elsewhere [88]. The sulfhydryl oxidase Erv1 (ALR in mammals [94]) helps control the oxidative folding of disulfide-containing proteins in the IMS [85].

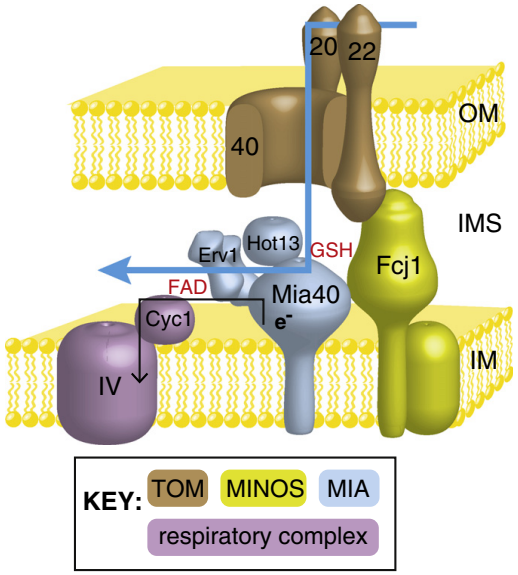

Fig. 4. General components of Mia40 mediated import. The import path of a precursor protein and its interactions with proteins associated with MIA import is shown by the blue arrow. The FAD-dependent transfer of proteins from Mia40 to the respiratory complexes (purple) via Erv1 (blue) is shown by the black arrow. Glutathione (GSH) is important in maintaining the redox states of Mia40 and Hot13 (blue) in the intermembrane space (IMS). The interaction of the Fcj1 (yellow) component of MINOS (mitochondrial inner membrane organising system) with the TOM complex (brown) in the outer membrane (OM), and Mia40 (blue) in the inner membrane (IM), links these three complexes and mitochondrial compartments.

Electron transfer to the respiratory chain via cytochrome c (Cyc1) re-oxidises Erv1 in an FAD-dependent process [80,95]. Hot13 has also been shown to contribute to the import of disulfide-containing IMS proteins [96], possibly by helping to maintain proteins in appropriate redox states during redox active MIA import [97,98]. Mia40 has also been found to associate with small amounts of the inner membrane protein Fcj1 (a component of MINOS), and the deletion of this component produces defects in IMS import [82]. The interaction of Fcj1 with both Mia40 and the TOM complex may help localise Mia40 near precursors as they exit the TOM complex [82], and may be even more important to localise the mammalian Mia40 as it lacks the transmembrane domain that anchors the yeast form of Mia40 in the IM [99].

The coupling of import and oxidative folding via the interaction between Mia40 and the TOM complex may help increase import rates via the MIA system [100]. The import rate is also increased by the presence of glutathione [85], which releases Mia40 from unproductive substrate intermediates both *in vitro* and *in vivo* [98,100]. The latter study by Fischer et al. also shows that in mammalian mitochondria, Cox19 import is greatly reduced when the membrane potential is disrupted. This contrasts with findings in yeast, where import into the IMS via the MIA pathway is independent of the membrane potential [101]. This may provide a mechanism to ensure only healthy mitochondria import some substrates, hinting at an import regulated quality control process that may only be functioning in multicellular organisms.

3. Many paths to tread: rewiring import pathways to the IMS

Studies of mitochondrial protein import in more diverse organisms have produced the surprising revelation that while many organisms share homologues of IMS proteins, these homologues may be imported by different mechanisms in different organisms. In a few cases the alternative mechanism has been identified. For example, *S. cerevisiae* Mia40 is imported by the stop-transfer pathway, but human Mia40 lacks the transmembrane sorting signal [102], so is imported by the MIA pathway [99]. Since other eukaryotes also lack the N-terminal signal, it is likely that interactions with other proteins, such as the Mia40-Fcj1 interaction, could compensate for or perhaps be even more effective in localising Mia40 in an appropriate position. Once able to engage with an alternative import pathway the Mia40 precursor would no longer have a need for a transmembrane sorting signal. Recent work also suggests that the import rate may be influenced by the folding state of the cytosolic form of Mia40, and consequently the available cofactors and chaperones which differ considerably between yeast and humans [103].

In most cases we have evidence alternative mechanisms must be functioning, but we have yet to identify which ones. In *S. cerevisiae* the F_1F_0-ATPase is encoded in the mitochondrial genome, but in *N. crassa* the nuclear encoded protein is imported via the conservative sorting pathway [63], where the final translocation step back through the inner membrane is yet to be elucidated. In *S. cerevisiae*, Cyt1 and Cyb2 use variations of the stop-transfer pathway to reach the IMS [30]. However, the lack of stop-transfer sequence in Cyt1 from *Trypanosoma brucei* [104], and the lack of bipartite targeting signal in the *Candida albicans* Cyb2 homologue both suggest that alternative import routes must be used [24]. In other words, it is likely that evolutionary rewiring of import pathways has occurred between these organisms.

Why rewire the mitochondrial protein import routes of a given protein between biological species? The answer might lie in the metabolic constraints that are imposed by the environmental niches inhabited by organisms. *S. cerevisiae* and *C. albicans* are two yeast species that diverged 100–300 million years ago [105]. As a commensal of humans, the natural niche of *C. albicans* is the hypoxic environment of the gastrointestinal tract [106]. These conditions have been shown to alter the redox state of proteins in the IMS [107] and impair the generation of a healthy mitochondrial membrane potential [108–110]. The stop-transfer pathway, which *S. cerevisiae* uses to import Cyb2 into the IMS, critically depends on the mitochondrial membrane potential [111]; however this pathway may not function as efficiently in the hypoxic niche of *C. albicans*. This may have given organisms with mutant proteins, able to engage with other pathways, a selective advantage and eventually resulted in the loss of the signal sequence. Whether the MIA pathway is indeed involved in the import of *C. albicans* Cyb2 into the IMS needs to be determined. A better understanding of the changes in oxygen levels, ATP availability, redox states of translocase components, and membrane potential for cells growing in environments, such as host niches or multicellular biofilms, is needed to suggest which factors or pathways might be most mutually compatible.

The complexities of the import machineries suggest that the pressure to evolve a more efficient import process in response to environmental stimuli must be high. Cyb2 encodes L-lactate cytochrome-c oxidoreductase, an enzyme essential for the utilisation of L-lactate [112]. In the human host, the niches of *C. albicans* are very poor in glucose, while lactate is available as a carbon source. Therefore, there would be a significant growth advantage to *C. albicans* able to efficiently import Cyb2 into the IMS and make efficient use of lactate. The function of Cyb2 in *C. albicans* biology and pathogenesis has not been studied yet. However, in another pathogenic yeast species *Candida glabrata*, which is also a human commensal and occupies similar niches to *C. albicans*, Cyb2 is essential for utilisation of lactate. The use of this carbon source is thought to be involved in the ability of *C. glabrata* to grow in the gastrointestinal tract of its host, as determined in the murine infection model using wild type and *cyb2* mutants [113]. Importantly, *C. glabrata* and *C. albicans* are better at utilising lactate than *S. cerevisiae* when grown in low oxygen conditions *in vitro* [113]. This mimics the hypoxia present in the host niches and fits with our model that *Candida* can import Cyb2 in the IMS in hypoxic conditions, likely utilising import

pathways different to the stop-transfer pathway. As we identify more examples and characterise this rewiring process we will gain insights into the evolutionary pressures on these pathways. This will also reveal the significance of these variations for the regulation of protein import and of mitochondrial metabolism and the ability of organisms to thrive in their environmental niches.

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