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The role of the ERMES complex in the assembly of mitochondrial outer membrane proteins in the filamentous fungus *Neurospora crassa*

by

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ABSTRACT

The ERMES (endoplasmic reticulum-mitochondria encounter structure) is composed of the Mdm10, Mdm12, Mmm1 and Mmm2 proteins, and acts as a tether between mitochondria and the endoplasmic reticulum (ER). Mutations affecting any of the proteins in the structure are associated with a variety of phenotypes including altered mitochondrial morphology and defects in mitochondrial protein import. I have focused on ERMES components and their roles in the import and assembly of proteins into the mitochondrial outer membrane in the filamentous fungus *Neurospora crassa*.

In one study I investigated the relationship between the TOM (translocase of the mitochondrial outer membrane) complex subunit Tom7 and Mdm10 with respect to their roles in the assembly of the β-barrel proteins, Tom40 and porin. Previous work showed that mitochondria lacking Tom7 assemble Tom40 more efficiently, and porin less efficiently, than wild-type mitochondria. Here I demonstrate that mutants lacking Mdm10 assemble both Tom40 and porin less efficiently than wild-type mitochondria. My analysis of *mdm10* and *tom7* single and double mutants, demonstrated that the effects of the two mutations are additive. Loss of Tom7 partially compensated for the decrease in Tom40 assembly resulting from loss of Mdm10 while porin assembly is more severely reduced in the double mutant than in either single mutant. The additive effects observed in the double mutant suggest that different steps in β-barrel assembly are affected in the individual mutants. I also show that loss of Mdm12, Mmm1 or Mmm2 results

in reduced assembly of porin and Tom40 into the outer membrane and that loss of any ERMES component results in aberrant mitochondrial morphology.

I further examined the role of Mmm1 in the assembly of β-barrel proteins by mutational analysis. I showed that the *N. crassa* Mmm1 protein is an ER protein containing a Cys residue near its N-terminus that is conserved in the class Sordariomycetes. The residue is within the predicted ER lumen domain of the protein and is involved in disulphide bond formation. Mutation of the residue resulted in a defect in Tom40 assembly, but did not affect porin assembly or mitochondrial morphology. This demonstrates a specificity of function and suggests a direct role for Mmm1 in Tom40 assembly. Taken together, my research has provided evidence suggesting that the ERMES complex plays a direct role in the assembly of specific mitochondrial outer membrane proteins.

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LIST OF ABBREVIATIONS

3D	three dimensional
A	adenine
Å	Angstroms
AAC	ATP/ADP carrier
ADP	adenosine diphosphate
ATOM	archaic translocase of the outer mitochondrial membrane
ATP	adenosine triphosphate
Ben	benomyl
BLAST	basic local alignment search tool
BNGE	blue native gel electrophoresis
bp	base pair
BPA	benzoylphenylalanine
BSA	bovine serum albumin
C	cytosine
°C	degrees Celsius
cDNA	complimentary DNA
CL	cardiolipin
Cys	cysteine
Δ	deletion
Da	Dalton
DMSO	dimethylsulfoxide
EM	electron microscopy
ERM	ER membrane
ERMES	endoplasmic reticulum-mitochondria encounter structure
ETC	electron transport chain
$F_1\beta$	β subunit of the F_1 ATP synthase
$FADH_2$	flavin adenine dinucleotide (reduced form)
Fe-S	iron sulfur
fpa	fluorophenylalanine
G	guanine
g	gravity
gDNA	genomic DNA
GFP	green fluorescent protein
GIP	general import
GTP	guanine triphosphate
His	histidine
hr	hour
Hsp	heat shock protein
Hyg	hygromycin

IMS	intermembrane space
IP	immunoprecipitate
kbp	kilobasepair
kDa	kiloDalton
L	litre
µg	microgram
µL	microliter
µm	micrometer
M	molar
MAPL	mitochondrial-anchored protein ligase
Mas	mitochondrial assembly protein
mdm	mitochondrial distribution and morphology
MDV	mitochondria-derived vesicle
mg	milligrams
MIM	mitochondrial inner membrane
Mim1/2	mitochondrial import 1 and 2
mL	milliliter
mM	millimolar
mmm	maintaining mitochondrial morphology
min	minute
Mia	mitochondria IMS assembly
MOM	mitochondrial outer membrane
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide reduced form
Ni-NTA	nickel-nitrilotriacetic acid
OM	outer membrane
OMV	outer membrane vesicles
OXA	oxidase assembly
OXPHOS	oxidative phosphorylation
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PAM	presequence translocase associated protein import motor
PBR	peripheral-type benzodiazepine receptor
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PK	proteinase K
PMSF	phenylmethylsulfonyl fluoride

POTRA	polypeptide transit associated (domain)
PS	phosphatidylserine
PVDF	polyvinylidenefluoride
RIP	repeat induced point mutations
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolutions per minute
SAM	sorting and assembly machinery
SDS	sodium dodecyl sulfate
SUMO	small ubiquitin-like modifier
T	thymine
TCA	trichloroacetic acid
TEV	tobacco etch virus
TMD	transmembrane domain
TOB	topogenesis of outer membrane β-barrels
TOM	translocase of the outer mitochondrial membrane
TPR	tetratricopeptide repeat
Tris	tris (hydroxymethyl) aminomethane
TSPO	translocator protein
UTR	untranslated region

1. INTRODUCTION

1.1 Mitochondrial function and roles within the cell

Mitochondria are double membrane bound organelles consisting of four distinct compartments: the mitochondrial outer membrane (MOM), the intermembrane space (IMS), the matrix, and the mitochondrial inner membrane (MIM). The MIM can be further subdivided into the inner boundary membrane (IBM) and the cristae membrane (CM) (Vogel *et al.*, 2006). The IBM is functionally linked with the MOM forming contact sites for cooperation in mitochondrial protein import, ATP transport out of the matrix, as well as mitochondrial fusion and division (Reichert and Neupert, 2002). The cristae are MIM infoldings and are the location of the electron transport chain (ETC) and the site of oxidative phosphorylation (OXPHOS). While mitochondria are best known for their role in ATP production, they are essential for numerous other processes such as Fe-S cluster synthesis (Lill, 2009), calcium homeostasis (Drago *et al.*, 2011), lipid synthesis and signaling (Osman *et al.*, 2011; Toulmay and Prinz, 2011), steroid biosynthesis (Midzak *et al.*, 2011), and programmed cell death (Wang and Youle, 2009).

The ETC in the MIM is composed of four complexes and two mobile components [reviewed in (Lenaz and Genova, 2009)]. Complex I (NADH dehydrogenase) and Complex II (Succinate dehydrogenase) are responsible for oxidizing the electron carriers $NADH + H^+$ and $FADH_2$, respectively. The electrons from $NADH + H^+$ and $FADH_2$ are passed from Complexes I and II to the mobile element, ubiquinone (coenzyme Q). Electrons then pass from ubiquinol to Complex III (cytochrome c reductase) to cytochrome c to Complex IV (cytochrome oxidase) and to the final electron receptor, oxygen. As electrons pass through Complexes I, III, and IV protons are pumped into the IMS. The resulting proton gradient is harnessed by Complex V (F_1F_0 ATP synthase) to produce ATP. The vast majority of ATP in most eukaryotes is produced in this manner.

Due to their endosymbiotic origins and the presence of mitochondrial DNA (mtDNA), mitochondria are often thought of as semi-autonomous.

However, it is known that communication and crosstalk between mitochondria and the nucleus are required for proper mitochondrial function. The nucleus encodes the majority of mitochondrial proteins and therefore is intimately involved in virtually all mitochondrial processes and their biogenesis (Neupert and Herrmann, 2007; Kutik *et al.*, 2009; Schmidt *et al.*, 2010). However, mitochondria are capable of influencing these processes by retrograde signaling [reviewed in (Jazwinski, 2012)]. This retrograde communication occurs in the normal functioning of the cell, but can also respond to situations affecting mitochondrial function such as loss of mtDNA or a blockage of the ETC. Perhaps the most well studied example of retrograde signaling occurs in Saccharomyces cerevisiae. The system involves the transcription of specific nuclear genes that are activated upon the loss of mtDNA. In this pathway CIT2, a gene whose protein product is targeted to the peroxisome, is upregulated to compensate for the inability to generate α-ketoglutarate in the mitochondrion (Jazwinski, 2012).

Retrograde signaling is also known to occur in many organisms in response to the production of damaging reactive oxygen species (ROS) in mitochondria. For example, certain ROS species can act as messengers by reversibly reacting with Cys residues, thus altering the target protein's activity (Collins *et al.*, 2012). Such changes can result in activation or deactivation of transcription factors, kinases and other proteins. Ultimately such changes can have major effects on a variety of cellular activities. The primary ROS formed in mitochondria is superoxide ($O_2^{\cdot-}$), but it is converted to hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase. It is thought that hydrogen peroxide is the major ROS signalling molecule from mitochondria (Collins *et al.*, 2012).

Mitochondria also have relationships with other organelles of the cellular endomembrane system. It is well known that there is a coupling of mitochondria with the endoplasmic reticulum (ER) that is thought to facilitate exchange of lipids and calcium between the two organelles (Hayashi *et al.*, 2009; Grimm, 2012; Michel and Kornmann, 2012). The functional significance of this coupling is more fully discussed in section 1.3.4. It has also been reported that mammalian mitochondria in various tissue culture cell lines produce mitochondria-derived

vesicles (MDVs) with specific cargoes that are targeted to lysosomes or peroxisomes (Neuspiel *et al.*, 2008; Andrade-Navarro *et al.*, 2009; Soubannier *et al.*, 2012). Interestingly, depending on the cargo and destination, it appears that different MDVs are produced by different mechanisms (Braschi *et al.*, 2010). Peroxisome-targeted MDVs are enriched in MAPL (mitochondrial-anchored protein ligase), an E3 SUMO (small ubiquitin-like modifier) ligase proposed to be involved in the regulation of mitochondrial fission (Neuspiel *et al.*, 2008; Braschi *et al.*, 2009). On the other hand, the formation of lysosome targeted MDVs, which are enriched in the MOM protein Tom20, is induced by oxidative stress. These MDVs are thought to be involved in the turnover of mitochondrial proteins that have been oxidatively damaged (Soubannier *et al.*, 2012). It is currently unknown if the production of MDVs is a conserved process among eukaryotes or if it represents a derived feature restricted to metazoans. Bacteria are known to produce outer membrane vesicles (OMV) involved in toxin deployment and quorum sensing (Kulp and Kuehn, 2010), and it will be of great interest to determine if MDVs are homologous or analogous to bacterial OMV.

Since mitochondria are involved in so many different cellular processes, it is not surprising that many different diseases can arise from mutations in genes that code for mitochondrial proteins (Breuer *et al.*, 2012; Koopman *et al.*, 2012; Nunnari and Suomalainen, 2012). There is currently considerable interest in the relationship between mitochondria and age-related diseases. For example, there is a correlation with mitochondrial dysfunction in both Alzheimer's and Parkinson's diseases (Arduino *et al.*, 2011; Silva *et al.*, 2011; Nunnari and Suomalainen, 2012; Ylikallio and Suomalainen, 2012). The amyloid and amyloid precursor proteins both accumulate in Alzheimer's disease and are thought to inhibit mitochondrial dynamics and block the mitochondrial general import pore (GIP) (Anandatheerthavarada *et al.*, 2003; Devi *et al.*, 2006). The presenilins associated with familial forms of Alzheimer's have been found to localize at mitochondrial-ER contact points (Area-Gomez *et al.*, 2009; Schon and Area-Gomez, 2010). Furthermore, the PINK1 and Parkin proteins that are associated with familial forms of Parkinson's function in mitochondrial protein turnover and the autophagy of

dysfunctional mitochondria (Kitada *et al.*, 1998; Rogaeva *et al.*, 2004; Pilsl and Winklhofer, 2012). The functions of PINK1 and Parkin are further discussed in section 1.3.5. Natural aging has also been correlated with a decline in mitochondrial function in humans (Bratic and Trifunovic, 2010; Cui *et al.*, 2012), and mice expressing proofreading deficient mitochondrial DNA polymerase have been shown to have a rapid aging phenotype (Wallace and Fan, 2009).

1.2 Mitochondrial evolution

The idea that mitochondria (and other organelles) originally evolved from a bacterial precursor was first proposed in the early 1900s by Constantin Mereschkowsky (O'Malley, 2010), but this idea was not accepted by the scientific community until it was reintroduced by Lynn Margulis in 1967 (Sagan, 1967). There is now much evidence to support the concept. Comparison of mitochondrial 16S rRNA to 16S rRNA from eukaryotes, bacteria and archaea has led to acceptance of the hypothesis that mitochondria evolved from a single event involving an ancient α-proteobacterial endosymbiont (Spencer *et al.*, 1984; Yang *et al.*, 1985; Cavalier-Smith, 2006). This is supported by the observation that several extant α-proteobacteria are obligate intracellular parasites of eukaryotes (Bowman, 2011; McCutcheon and Moran, 2012). Since the original event establishing a relationship between the endosymbiont and host, endosymbiont-derived organelles have undergone massive genome reduction with most genes being either lost outright or transferred to the host nuclear genome. Therefore, the original symbiont genome must have been much different from extant mitochondria as mtDNA only codes for (on average) ~10-20 proteins while even the highly reduced genomes of obligate intracellular α-proteobacteria code for only approximately 1000 proteins (Andersson *et al*., 2003; Khachane *et al.*, 2007).

While the α-proteobacterial origin of mitochondria is well accepted, the origin of eukaryotes—all of which contain mitochondria (or related organelles)—is still a current debate (Martin, 2011; Vesteg and Krajcovic, 2011). It is generally agreed that eukaryotes are more closely related to archaea than eubacteria, however the nature of the relationship is still under scrutiny. Two general ideas

have been put forward. In the first, the three domains of life are taken as three monophyletic groups (Woese *et al.*, 1990), with eukaryotes and archaea sharing a common ancestor (Figure 1.1A). Alternatively, it has been suggested that eukaryotes arose from a specific branch of archaea, the eocytes (Lake *et al.*, 1984). This gives rise to the eocyte tree, and makes archaea paraphyletic (Figure 1.1B). Recent evidence in support of the eocyte tree has been advanced (Cox *et al.*, 2008; Foster *et al.*, 2009), but a consensus on the matter has not yet been reached. Understanding the roots of the eukaryotes gives us clues into the nature of the organism that hosted the mitochondrial precursor.

Several hypotheses have been proposed for the evolution of eukaryotes [Reviewed in (Embley and Martin, 2006)] all of which can be grouped into two categories (O'Malley, 2010): phagotrophic or syntrophic hypotheses (Figure 1.2). Phagotrophic hypotheses assume that phagocytosis evolved before the acquisition of mitochondria. Since no prokaryotes have been discovered with this ability, it is assumed that the host was a proto-eukaryote, with most eukaryotic features but lacking mitochondria. The original endosymbiont is regarded as phagocytosed prey that somehow escaped digestion within the host. Several years ago, phagotrophic hypotheses were supported by the existence of what were thought to be amitochondriate eukaryotes that were considered to be living fossils from a time before the evolution of mitochondria (van der Giezen, 2009). However, phagotrophic theories of mitochondrial evolution were weakened when it was discovered that these amitochondriate eukaryotes actually contain organelles—hydrogenosomes and mitosomes—that have mitochondrial ancestry and have evolved independently several times in highly divergent lineages (Tovar *et al.*, 1999; Embley *et al.*, 2003; van der Giezen, 2009). These discoveries made it apparent that there may never have been a eukaryote or proto-eukaryote before the acquisition of mitochondria and led to the development of a number of syntrophic hypotheses about the origin of eukaryotes (Figure 1.2B) [Reviewed in (Embley and Martin, 2006)].

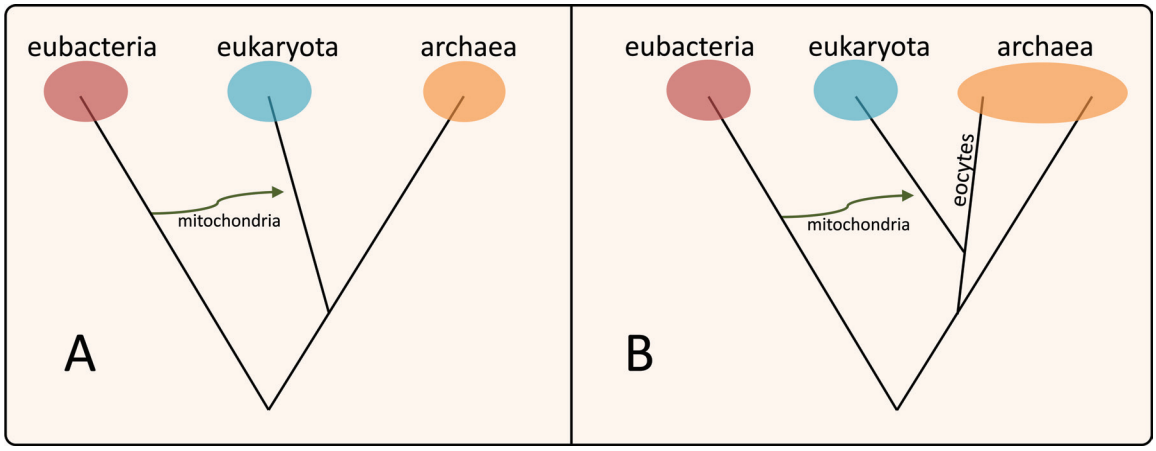


Figure 1.1. Two different hypotheses for the origin of eukaryotes. (A) The three domains tree. Archaea, eukaryotes and eubacteria are shown as three monophyletic branches. (B) The eocyte tree showing archaea as a paraphyletic group with eukaryotes emerging from the eocyte branch of the archaea.

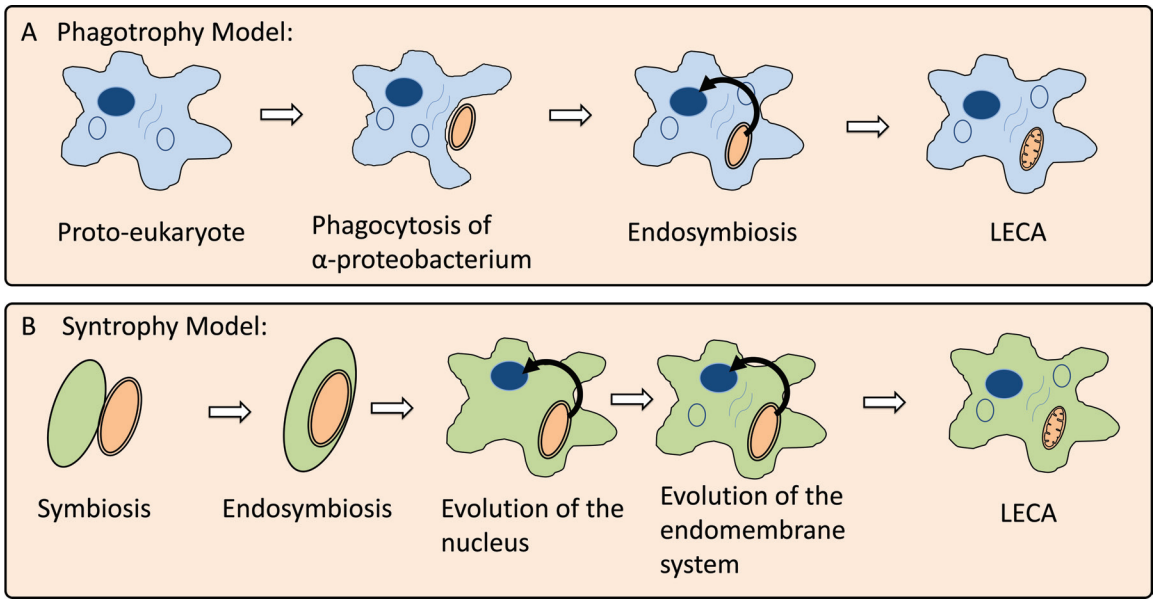


Figure 1.2. Phagotrophic and syntrophic models for the evolution of mitochondria. (A) Phagotrophy Model. A proto-eukaryote containing a nucleus (dark blue oval) and other eukaryotic features (small circles, wavy lines) evolved the process of phagocytocis and engulfed an early α-proteobacteria (orange oval). Normally the α-proteobacterium would have become prey, but in this instance it escaped digestion and an endosymbiotic relationship evolved. The symbiosis became obligate and transfer of genetic information (black arrow) from the symbiont to the host resulted in the reductive evolution of the symbiont into the mitochondrion. (B) Syntrophy Model. A nutritional symbiosis between an archaea (green) and an ancestral α-proteobacterium (orange) eventually leads to the engulfment of the eubacteria by the archaea. As time progresses genetic information is transferred (black arrow) from the symbiont to the host. Other eukaryotic features such as the nucleus (dark blue oval) and endomembrane components (small circles, wavy lines) evolve after the acquisition of the symbiont mitochondrion. Both phagotrophic and syntrophic models culminate in the LECA (last eukaryote common ancestor).

The syntrophic hypothesis that has gained the most support is the hydrogen hypothesis which is based largely on metabolic and biochemical considerations (Martin and Muller, 1998). The hydrogen hypothesis suggests that the original host was a methanogenic archaea that entered a symbiotic relationship with a hydrogen producing α-proteobacterium. The methanogenic archaeal host required an influx of H_2 for energy and CO_2 as a carbon source. While these were originally supplied by natural environmental sources, at some point in time the host entered a symbiotic relationship with an α-proteobacterial heterotroph that consumed organic compounds from the environment and produced H_2 and CO_2 as waste products. The α-proteobacterium may have benefitted from the presence of the archaea as its metabolic reactions were likely slowed in the presence of excess hydrogen. As time passed and evolution proceeded, the symbiotic relationship became obligate and the host began to surround the symbiont to maximize hydrogen uptake culminating in complete engulfment. For proponents of the hydrogen hypothesis, the acquisition of the endosymbiont was the key event that led to the evolution of other eukaryotic features such as the nucleus and endomembrane system. Currently, there is vigorous debate on this and other models for mitochondrial acquisition and the evolution of eukaryotes (Cavalier-Smith, 2006; Martin and Koonin, 2006; Poole, 2006; Dagan and Martin, 2007; de Duve, 2007; Poole and Penny, 2007a; Poole and Penny, 2007b; Cavalier-Smith, 2009, 2010; O'Malley, 2010; Forterre, 2011; Martin, 2011) There is still not enough evidence in firm support of any specific theory.

1.3 Mitochondrial morphology, dynamics, lipid transfer and the ERMES complex

This thesis centres on the function of the ERMES (ER-mitochondria encounter structure) and its components in relation to MOM protein import and assembly in *Neurospora crassa*. However, ERMES components are known to be involved in many other aspects of cell function. In section 1.3, I review the major cellular processes that have been shown to involve the ERMES complex. In section 1.4, I

present a review of protein import into mitochondria focusing on MOM protein import and assembly.

1.3.1 Mitochondrial morphology

Mitochondria exist as tubules in most cell types. In *Saccharomyces cerevisiae* mitochondria localize to the cell cortex in a tubular network or reticulum (Hoppins *et al.*, 2007), whereas *N. crassa* mitochondria exist as tubules that extend parallel to the growing hyphae (Westermann and Prokisch, 2002). In mammalian cells mitochondria generally exist as tubules that surround the nucleus and extend into the surrounding cytoplasm. They can reticulate and undergo drastic changes in morphology and distribution dependent upon cellular conditions and tissue type (Karbowski and Youle, 2003).

Numerous mutations in genes that affect mitochondrial distribution and morphology (*mdm* mutants) have been identified in screens of *S. cerevisiae* (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Berger *et al.*, 1997; Hermann *et al.*, 1997; Dimmer *et al.*, 2002; Youngman *et al.*, 2004; Altmann and Westermann, 2005). Detailed characterization revealed that many of these genes are involved in seemingly unrelated processes like mitochondrial dynamics (Fzo1, Dnm1, Mdm33) (Hoppins *et al.*, 2007), lipid trafficking and biosynthesis (Mdm31, Mdm32, Mdm35, Ups1, Ups2, Fmp30, Mdm10, Mdm12, Mmm1, Mmm2) (Dimmer *et al.*, 2002; Dimmer *et al.*, 2005; Kornmann *et al.*, 2009; Osman *et al.*, 2009a; Tamura *et al.*, 2009; Kuroda *et al.*, 2011), mtDNA maintenance and connection to the actin cytoskeleton (Mdm10, Mdm12, Mmm1, Mmm2) (Boldogh *et al.*, 2003; Youngman *et al.*, 2004), ER-mitochondria-plasma membrane tethering (Num1 and Mdm36) (Hammermeister *et al.*, 2010), mitochondrial inheritance (Gem1) (Frederick *et al.*, 2004), and mitochondrial protein import (Tom7, Tob37, Tob55, Tom70, Tom71, Mdm10, Mdm12, Mmm1, Mmm2) (Grad *et al.*, 1999; Meisinger *et al.*, 2004; Meisinger *et al.*, 2007; Kondo-Okamoto *et al.*, 2008; Wideman *et al.*, 2010). However, recent studies (discussed in the following sections) have demonstrated that most of these processes are highly inter-related and that the pathways that control mitochondrial morphology

are complex and surprising in nature. For example, Mdm10, Mdm12, Mmm1 and Mmm2 have been implicated in a number of cellular processes (see above) and have recently been shown to form the ERMES complex, which tethers the ER to mitochondria.

1.3.2 Mitochondrial dynamics

While it was long assumed that mitochondria were merely the powerhouses of the cell and largely static entities, it is now known that mitochondria are constantly moving, fusing and dividing (Okamoto and Shaw, 2005; McBride *et al.*, 2006; Hoppins *et al.*, 2007). Mitochondrial dynamics are tightly regulated and affect many processes including apoptosis, mitophagy, maintaining the quality of mitochondria and mtDNA quality, and metabolic efficiency (Nunnari and Suomalainen, 2012). The fusion and division of mitochondria require the coordination of proteins and lipids in the inner and outer mitochondrial membranes to ensure that the mitochondrial compartments are maintained throughout fission and fusion events. The major protein players of both mitochondrial fusion and fission are dynamin-related GTPases (Hoppins *et al.*, 2007). In fungi, fusion requires the dynamin-related GTPases Fzo1 (Mfn1/Mfn2 in mammals) in the MOM and Mgm1 (Opa1 in mammals) in the MIM (Hoppins and Nunnari, 2009). Outer and inner membrane fusion is coordinated by the MOM localized Ugo1 which is thought to form a double membrane spanning complex with Fzo1 and Mgm1 in fungi (Hoppins and Nunnari, 2009). In mitochondrial fission, another dynamin related GTPase, Dnm1 (Drp1 in mammals), is recruited to the mitochondrial outer membrane by Fis1 and Mdv1 (Lackner and Nunnari, 2009). Fission proceeds by Dnm1 complex assembly around constricted mitochondrial tubules followed by GTPase activity and membrane scission (Lackner and Nunnari, 2009; Friedman *et al.*, 2011). In *S. cerevisiae*, depletion of fusion components leads to fragmented mitochondria whereas depletion of fission components leads to hyper-connected or condensed mitochondria (Hoppins *et al.*, 2007). Interestingly, if components of both machineries are deleted nearly normal mitochondrial morphology results (Sesaki

and Jensen, 1999). It has been recently demonstrated in both *S. cerevisiae* and mammalian tissue culture that constricted ER-mitochondrial contact sites recruit Dnm1/Drp1 and mark the site of mitochondrial division (Friedman *et al.*, 2011). It will be interesting to determine if the ERMES complex plays a role in the recruitment of components of the mitochondrial division apparatus to these sites in fungi.

1.3.3 The ERMES complex and mitochondrial morphology and inheritance

The close apposition of the ER and mitochondria has been observed for several decades. The significance of this has only recently come to light [reviewed in (Raturi and Simmen, 2012)]. The identification of a distinct ER fraction that associates with mitochondria, termed the mitochondria-associated membranes (MAMs) provided biochemical evidence for the close association of the two organelles seen via microscopy. Currently, MAMs have been primarily implicated in lipid metabolism and transport, as well as calcium signaling. It has been suggested that MAM dysfunction may play a role in many diseases including diabetes, cancer, Alzheimer's and Parkinson's.

Although it was known for many years that the physical connection between ER and mitochondria was proteinaceous in nature, it was only recently that the proteins responsible for these interactions were identified. In mammals, two sets of interactions have been reported. The first involves an interaction between the ER calcium channel IP_3R (inositol 1,4,5-triphosphate receptor) and the mitochondrial VDAC (voltage dependent ion channel) protein that also involves a chaperone (Szabadkai *et al.*, 2006). In the second, ER-localized Mfn2 tethers mitochondria to the ER by homotypic and heterotypic interactions with mitochondrially-localized Mfn2 or Mfn1 (de Brito and Scorrano, 2008).

The identification of the tether in *S. cerevisiae* was found in a screen for mutants that could not grow on non-fermentable medium in the absence of an artificial mitochondria-ER tether (Kornmann *et al.*, 2009). The *mdm12* gene was found to be required to maintain ER-mitochondria contacts. Further work demonstrated that Mdm10, Mdm12, Mmm1 and Mmm2 (Mdm34) are

components of the ERMES complex which tethers the two organelles together (Kornmann *et al.*, 2009). Mmm1 is a transmembrane protein of the ER, Mdm10 and Mmm2 are MOM proteins, and Mdm12 acts as a bridge interacting with all three of the other proteins. ERMES components localize to between 1 and 10 punctate foci within the cell and each contains hundreds of molecules (Kornmann *et al.*, 2009). However, a recent report has suggested that the number of ERMES per cell may be much higher and each structure may contain fewer molecules of the components (Nguyen *et al.*, 2012).

Though the identification of the ERMES complex and its components is a relatively recent event, the same components were identified years earlier in screens for genes affecting mitochondrial morphology (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Berger *et al.*, 1997; Youngman *et al.*, 2004). Knocking out or mutating the genes encoding ERMES proteins was found to result in the formation of giant mitochondria accompanied by various growth defects in *S. cerevisiae*. Mutants lacking Mdm10 grew slowly at 23°C and not at all at 37°C. Even at permissive temperatures, almost all cells were found to contain only giant mitochondria (Sogo and Yaffe, 1994; Meisinger *et al.*, 2006). *S. cerevisiae* cells lacking Mmm1 grew slowly and contained only giant mitochondria at both 23°C and 37°C (Burgess *et al.*, 1994). Loss of Mdm12 gave cells that grew slowly at 23°C and were unable to grow at 37°C with large spherical mitochondria present at both temperatures (Berger *et al.*, 1997). Each of the ERMES component deletion strains grow very poorly on non-fermentable carbon sources (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Berger *et al.*, 1997; Youngman *et al.*, 2004). Mutations in the homologues of these genes in other organisms give rise to related but not always identical growth and morphological phenotypes. Deletion of the *mdm10* gene in *Aspergillus nidulans* resulted in a strain that grew at the same rate as wild type at 37°C, but grew slightly slower at 20°C. Mitochondria existed as identical tubular networks in both mutant and wild type cells grown at 37°C, but in mutant cells grown at 20°C more than 90% of hyphal compartments contained some large circular mitochondria in addition to normal tubular mitochondria (Koch *et al.*, 2003). An *mdm10* missense mutant of *Podospora anserina* grew

more slowly than wild type at 35°C and all mitochondria were enlarged. No differences in growth or mitochondrial morphology were seen at 18°C (Jamet-Vierny *et al.*, 1997). A *Neurospora crassa mmm1* mutant was inviable at 40°C and grew slowly at 21°C, 30°C or 37°C. Giant long mitochondria were observed in hyphae and giant circular mitochondria were seen in conidiaspores in this strain (Prokisch *et al.*, 2000). The variation in phenotypes among different organisms suggests that the functions of the proteins may be slightly different in different species or that some organisms may contain other proteins with a degree of functional overlap.

Mdm10, Mdm12 and Mmm1 were originally shown to exist in a complex that was thought to be involved in mitochondrial inheritance and mitochondrial motility via attachment to the actin cytoskeleton in *S. cerevisiae* (Boldogh *et al.*, 1998; Boldogh *et al.*, 2003). A role in mtDNA segregation was also suggested by the observation that ERMES components localized to punctate foci adjacent to mitochondrial nucleoids (Burgess *et al.*, 1994; Hobbs *et al.*, 2001; Boldogh *et al.*, 2003; Meeusen and Nunnari, 2003; Youngman *et al.*, 2004). These findings are supported by the observations that loss of any ERMES component results in a mitochondrial motility defect and a preferential loss of mtDNA (Hanekamp *et al.*, 2002). Thus, ERMES mutants in *S. cerevisiae* are generally ρ^0. Since ρ^0 mitochondria move twice as fast as ρ^+ mitochondria in *S. cerevisiae* (Boldogh *et al.*, 2003) there may be a selective advantage to losing mtDNA in ERMES mutants, as the increase in mitochondrial mobility may make it easier to transfer large mitochondria into the daughter bud. A recent study has shown that the mitochondrial motility and inheritance defects seen in *S. cerevisiae* lacking ERMES proteins are a secondary consequence of large mitochondrial size (Nguyen *et al.*, 2012), suggesting that other phenotypes such as the loss of mtDNA may be secondary consequences.

In addition to the four ERMES proteins discussed above, the highly conserved homologue of mammalian Miro, Gem1, has been shown to be a substoichiometric member of the ERMES complex (Kornmann *et al.*, 2011; Stroud *et al.*, 2011b). However, there is controversy regarding this result as a third

group has recently reported that Gem1 could not be coimmunoprecipitated with tagged ERMES components (Nguyen *et al.*, 2012). In mammals, Miro is involved in attaching mitochondria to the microtubule network via the adaptor protein Milton (Glater *et al.*, 2006). In fungi, the function of Gem1 is currently unresolved but it is thought to have a regulatory role because it consists of two calcium binding EF-hands flanked by two GTPase domains (Fransson *et al.*, 2003). Koshiba *et al*. (2011) demonstrated that in *S. cerevisiae* both GTPase domains are required for efficient mitochondrial inheritance, while the EF-hands are dispensable for this function; however, the first EF-hand is necessary for protein stability. Kornmann *et al*. (2011) demonstrated that both GTPase domains as well as both EF-hand domains are necessary for proper recruitment of Gem1 to ERMES foci. However, Nguyen *et al*. (2012) did not see a difference in ERMES complex size or number in strains lacking Gem1. In *S. cerevisiae,* other ERMES mutants exhibit enlarged spherical mitochondria under all tested growth conditions, and are virtually unable to grow on non-fermentable carbon sources. While mutants lacking Gem1 contain large globular mitochondria when grown on fermentable carbon sources, when grown on glycerol media they contain short tubular mitochondria (Frederick *et al.*, 2004). Further work will be required to assess the role of Gem1 in ERMES complex biology in *S. cerevisiae*.

1.3.4 The ERMES complex and lipid transport

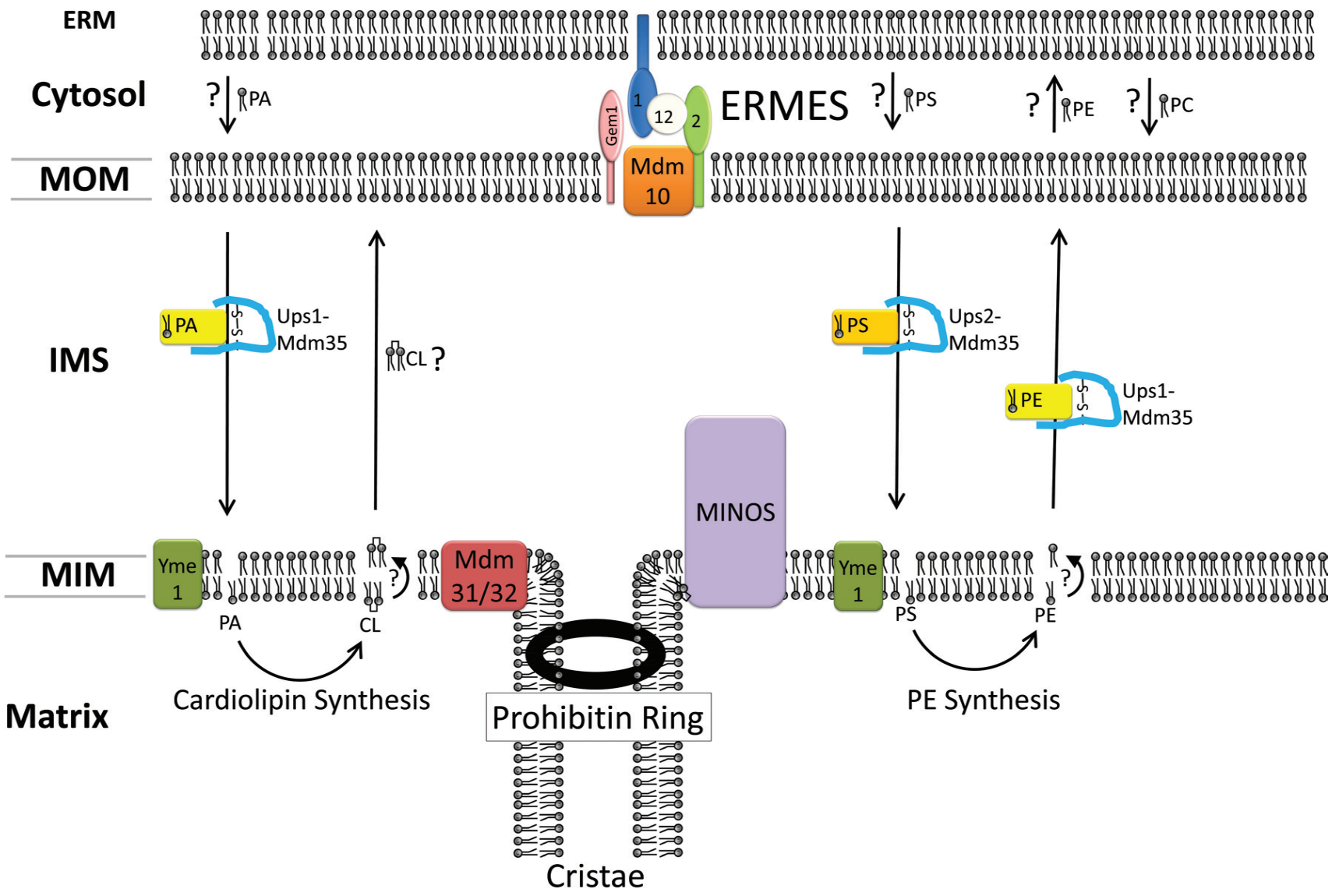
As mentioned in section 1.3.3, MAMs are the site of lipid exchange between the ER and mitochondria. Since the ERMES complex tethers the ER to the mitochondrial outer membrane (Kornmann *et al.*, 2009), it has been hypothesized that this interaction facilitates the transfer of lipids between the two organelles. Phospholipid trafficking is an important cellular process necessary to maintain proper membrane composition and function. Most phospholipids are synthesized in the ER and then transported to different membranes via the endomembrane system. An exception to this rule is the mitochondrial membranes. Mitochondrial membranes receive their phospholipids via the ER through ER-mitochondrial contact sites, mostly by unknown mechanisms. While the ER synthesizes most

phospholipids, mitochondria synthesize phosphatidylethanolamine (PE) from phosphatidylserine (PS) for transport to the rest of the cell (Osman *et al.*, 2011) (Figure 1.3). PS is transported from the ER to the MIM where Psd1 (PS decarboxylase) decarboxylates PS to form PE which is then transported back to the ER for export to other cellular membranes (Osman *et al.*, 2011). PE in the ER is converted to phosphatidylcholine (PC) and transported back into mitochondria (Figure 1.3).

Mitochondrial membranes are unique in their membrane composition. They lack sphingolipids and contain a large proportion of non-bilayer phospholipids like PE and the mitochondrial specific phospholipid, cardiolipin (CL) (Osman *et al.*, 2011). CL is unique to mitochondrial and bacterial membranes and, along with PE, is thought to be instrumental in forming and maintaining cristae architecture (Osman *et al.*, 2011). Although it is unclear exactly what the functions of PE and CL are, it is clear that they are important in proper mitochondrial function as depletion of both is lethal in *S. cerevisiae* (Gohil *et al.*, 2005). For the synthesis of CL, phosphatidic acid (PA) is transferred from the ER to the MIM where CL is synthesized by a complex chain of reactions (Figure 1.3) (Osman *et al.*, 2011).

ERMES complex members exhibit genetic interactions with several components of the mitochondrial lipid biosynthesis and transport pathways. These interactions are complex and the nature of the relationships is not always clear. Furthermore conflicting results have also been reported. Two groups demonstrated that strains lacking members of the ERMES complex have been shown to exhibit CL and PE deficiencies (Kornmann *et al.*, 2009; Osman *et al.*, 2009a). A more recent study showed that ERMES mutants were primarily deficient in CL (Kuroda *et al.*, 2011) and another study concluded that the ERMES had no dramatic effect on the rate of mitochondrial PE biosynthesis (Nguyen *et al.*, 2012). Yet another group showed that elevated PS levels can be seen in the mitochondria of ERMES mutants (Tamura *et al.*, 2012). ERMES components were found to genetically interact with conserved prohibitin proteins

Figure 1.3. Lipid transport between mitochondrial and ER membranes. The ERMES complex tethers the MOM to the ER membrane and potentially facilitates transport of PA (phosphatidic acid), PS (phosphatidylserine), PE (phosphatidylethanolamine) and PC (phosphatidylcholine) between the ER and mitochondrial membranes. PA is transferred from the MOM to the MIM by the Ups1-Mdm35 complex to synthesize CL in the MIM. After docking with the MIM it is thought that Ups1 is degraded by proteases like Yme1 to prevent bidirectional transfer of PA. It is unknown how CL is transported from the MIM to the MOM. PS is thought to be transported from the MOM to the MIM by the Ups2-Mdm35 complex for the production of PE. PE is then shuttled back to the MOM by Ups1-Mdm35 for transport into the ER. The MIM scaffolding complexes (Mdm31/32, MINOS and the Prohibitins) thought to be involved in MIM lipid architecture are also shown. ERM, ER membrane; MOM, mitochondrial outer membrane; IMS, intermembrane space; MIM, mitochondrial inner membrane.


ERM
Cytosol
MOM
IMS
MIM
Matrix
?
PA
Gem1
1
12
2
Mdm
10
ERMES
?
PS
?
PE
?
PC
PA
Ups1-
Mdm35
CL ?
PS
Ups2-
Mdm35
PE
Ups1-
Mdm35
MINOS
Yme
1
Mdm
31/32
Yme
1
PA
CL
PS
PE
Cardiolipin Synthesis
Prohibitin Ring
PE Synthesis
Cristae

in the MIM, Phb1 and Phb2 (Berger and Yaffe, 1998; Osman *et al.*, 2009a). Phb1 and Phb2 were also shown to be high copy suppressors of *mdm10* and *mdm12* mutations (Berger and Yaffe, 1998). Although the exact mechanism and function of Phb1 and Phb2 are unknown, they are thought to be involved in scaffolding and lipid organization in the MIM (Osman *et al.*, 2009b). As well as the ERMES genes, many other genes involved in both PE and CL biosynthesis were found to genetically interact with Phb1 and Phb2 (Osman *et al.*, 2009a). Some of these genes were later found to also interact with ERMES components (Tamura *et al.*, 2012).

Among the genes identified to interact with the prohibitins were IMS proteins that have been implicated in the transfer of lipids between the MIM and the MOM: Ups1, Ups2 and Mdm35. Ups proteins form a complex with Mdm35 and require Mdm35 for their efficient import and maintenance in the IMS (Tamura *et al.*, 2009; Potting *et al.*, 2010). Mdm35 is also required for Ups protein function as cells lacking Mdm35 resemble *Δups1Δups2Δups3* triple knockouts (Tamura *et al.*, 2012). Mutants lacking Ups1 are deficient in CL, while mitochondria in mutants lacking Ups2 are deficient in PE. Due to the reciprocal phenotypes seen in mutants lacking Ups1 or Ups2, an attractive model would be that Ups1-Mdm35 catalyzes transfer of a particular class of phospholipids between membranes while Ups2-Mdm35 catalyzes transfer of a different class of phospholipids, thus keeping mitochondrial membrane composition in equilibrium (Figure 1.3). Tamura et al. (2012) showed that this is likely the case as cells lacking Ups1 have a slower rate of conversion of PE to PC. And although cells lacking Ups2 have less PE in the mitochondria, they have a normal PE to PC conversion rate, indicating that PE export out of mitochondria is not impaired. These results suggest firstly, that mitochondria lacking Ups1 cannot efficiently export PE to the ER for PC production; and secondly, that mitochondria lacking Ups2 cannot efficiently import PS to the MIM for PE production. Yme1 and Atp23 have been shown to degrade Ups proteins at the MIM (Potting *et al.*, 2010). Therefore, it is conceivable that these proteases function to degrade Ups proteins after they have delivered their phospholipid cargo to the MIM, thus ensuring

unidirectionality of lipid transport. However, this hypothesis cannot explain how lipid transport from the MIM to the MOM is ensured.

Tamura *et al*. (2012) showed that ERMES mutants and mitochondria lacking Ups1 all had similar CL deficiencies. The deletion of an ERMES gene in *Δups1* cells resulted in slightly worsened phenotypes whereas the deletion of ERMES genes in *Δmdm35* or *Δups2* cells partially suppressed ERMES growth and lipid defects. Additionally, there is over 2-fold the amount of PS in ERMES mutant mitochondria than in wild-type mitochondria (Tamura *et al.*, 2012), suggesting that transport of PS into mitochondria is not affected but export of PE is impaired in ERMES mutants. Consistent with this idea, it has recently been reported that mitochondria lacking any ERMES component can efficiently synthesize PE and thus these proteins are not directly involved in PS transport from the ER (Nguyen *et al.*, 2012). In further support of this model Kornmann *et al*. (2009) observed a decrease in the rate of PC accumulation in ERMES mutants. Taken together, these results suggest that ERMES and Ups1 act in parallel pathways directing PA to the MIM and PE to the ER, whereas ERMES and Ups2 work in opposing pathways with respect to PS import into mitochondria.

Two more genetic interactors of prohibitins are *mdm31* and *mdm32*, which code for two homologous proteins found in the MIM of *S. cerevisiae*. Any ERMES deletion combined with the deletion of either Mdm31 or Mdm32 results in synthetic lethality (Dimmer *et al.*, 2005). While both Mdm31 and Mdm32 are necessary for proper mitochondrial morphology in *S. cerevisiae*, other fungal species contain only one orthologue. Recent results have demonstrated that mutants lacking Mdm31 exhibit CL deficiencies comparable to ERMES mutants (Tamura *et al.*, 2012). Similar to what was observed for Phb1 and Phb2 (Berger and Yaffe, 1998), the overexpression of Mdm31 partially rescued the growth, lipid, and mitochondrial morphology defects in ERMES mutants even though ERMES foci are not restored (Tamura *et al.*, 2012). Since the morphology defects were corrected by the overexpression of an inner membrane protein, this suggests that the lack of connection to the ER is not the cause of mitochondrial morphology defects in ERMES mutants.

With respect to mitochondrial morphology, however, ERMES and Mdm31/32 mutants exhibit rather different phenotypes. While all mutants exhibit enlarged mitochondria, ERMES mutants are nearly always spherical (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Berger *et al.*, 1997; Dimmer *et al.*, 2002; Youngman *et al.*, 2004), whereas Mdm31/32 mutants are often doughnut shaped (Dimmer *et al.*, 2005). Additionally, in ERMES mutants, cristae structures are visible (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Hobbs *et al.*, 2001) whereas in mutants lacking Mdm31 or Mdm32 no cristae can be seen (Dimmer *et al.*, 2005). Taken together, these findings suggest that while ERMES and Mdm31/32 both affect steady state levels of mitochondrial lipids, only Mdm31/32 are required for cristae formation. Alternatively, these differences could reflect differential/opposing influences on the trafficking of lipids between the MIM and MOM. Thus, while the overall membrane composition is similar in ERMES mutants compared to mutants lacking Mdm31, the mitochondrial membrane ultrastructures are very different.

1.3.5 The ERMES complex, mitophagy and mitochondrial protein turnover

While fission and fusion are thought to help monitor and maintain mitochondrial quality, components of this machinery are also involved in autophagy of mitochondria (mitophagy). In mammalian cells, one form of mitophagy is mediated by PINK1 and Parkin, two proteins associated with Parkinson's disease (Jin and Youle, 2012). PINK1 is normally imported into mitochondria and degraded; however, if mitochondrial function is compromised PINK1 accumulates in the MOM (Lazarou *et al.*, 2012) and recruits the E3 ubiquitin ligase Parkin to mitochondria. This results in the ubiquitylation of several MOM proteins including members of the fusion/fission machinery (Mfn1, Mfn2 and Fis1), subunits of the TOM (translocase of the mitochondrial outer membrane) complex (Tom40, Tom20 and Tom70), and the motility factor Miro (Yoshii *et al.*, 2011). PINK1/Parkin have been shown to cause both K48 ubiquitylation (involved in proteosome-dependent degradation) and K63 ubiquitylation (involved in mitophagy) (Jin and Youle, 2012). Proteosome-dependent protein

degradation has been shown to precede mitophagy in mammalian cells (Chan *et al.*, 2011; Yoshii *et al.*, 2011).

In fungi, PINK1 and Parkin sequence homologues have not been identified. However, it has been shown that the Mdm30 protein in *S. cerevisiae* is involved in maintaining mitochondrial morphology and is responsible for ubiquitylation of Fzo1 (homologue of Mfn1/2) (Escobar-Henriques *et al.*, 2006) and Mmm2 (Ota *et al.*, 2008). In *N. crassa* the E3 ubiquitin ligase Mus10 has been shown to be involved in maintaining proper mitochondrial morphology (Kato *et al.*, 2010) and could be the functional analogue of Mdm30. Furthermore, in a recent proteomics screen in *S. cerevisiae*, Tom70, Mmm2 and Mdm12 were identified as ubiquitylated proteins (Starita *et al.*, 2012), demonstrating that a similar set of MOM proteins are ubiquitylated in fungi and mammals. It is interesting to note that in both mammals and fungi, members of the ER-mitochondria tethering complex (Mfn1 and Mfn2 in mammals and ERMES components in *S. cerevisiae*) are ubiquitylated. This suggests that mitochondrial protein turnover at ER-mitochondrial contacts may be conserved. Further supporting this possibility, essential proteins of the ubiquitin/26S proteasome pathway (Pre1, Pre3, Pre5 Pre6, Rpn8, Rpt2, Rpt4, Cdc34, Cdc53) are involved in the maintenance of mitochondrial morphology in *S. cerevisiae* (Altmann and Westermann, 2005). While the protein players may not share sequence homology, there may be a conserved mechanism of MOM protein turnover via ubiquitylation and degradation by the proteasome in both mammals and fungi.

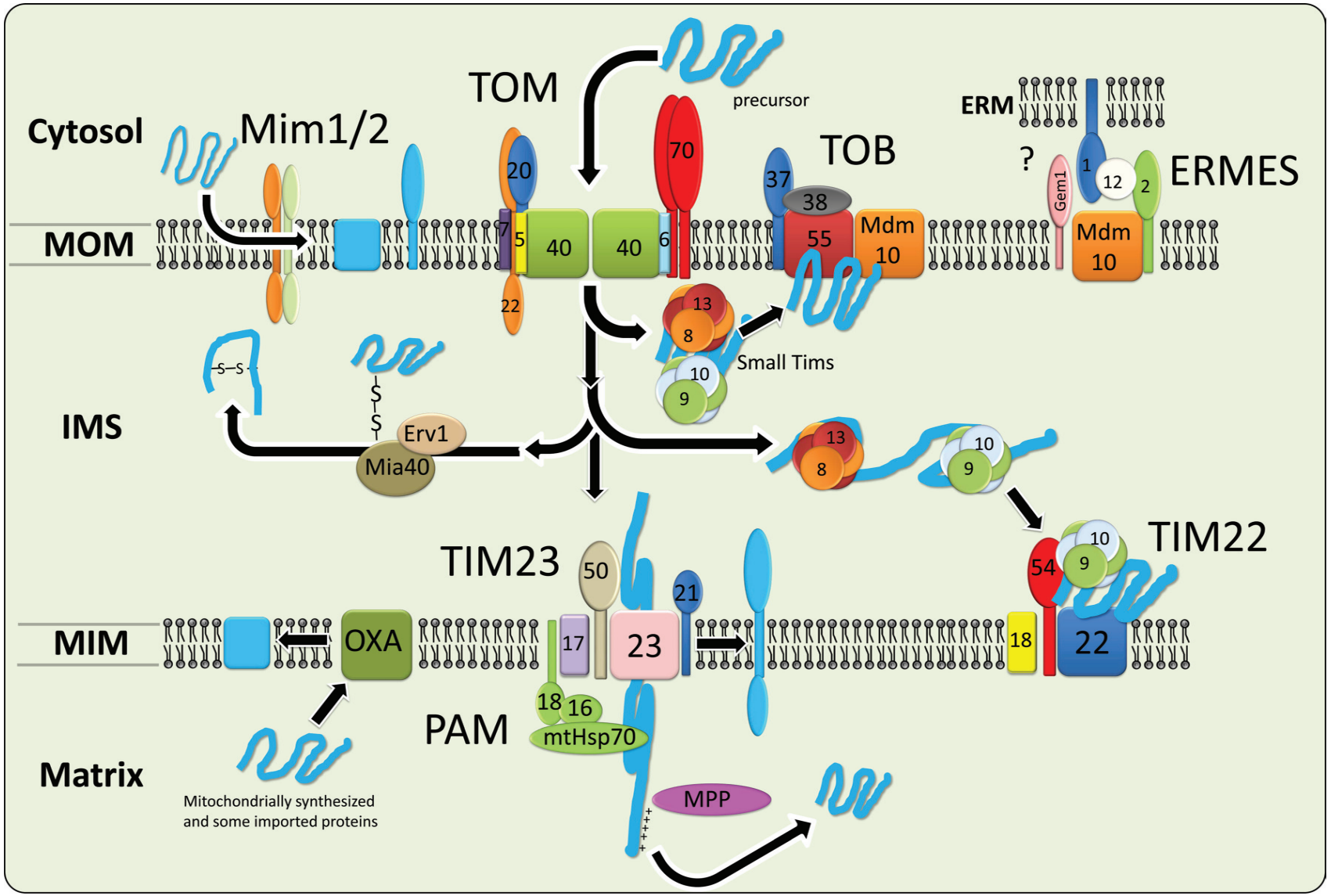
In addition to ubiquitylation and possible degradation, the ERMES complex has another connection with the 26S proteasome. The *rpn11-m1* mutation of the 26S proteasomal lid subunit Rpn11 causes a severe synthetic growth defect when combined with the loss of Mmm2 (Rinaldi *et al.*, 2008). Surprisingly, overexpression of Cdc14 [a dual specificity phosphatase and component of the mitotic exit network (MEN)] suppresses both the cell cycle defect and the mitochondrial morphology defect seen in *rpn11-m1* (Esposito *et al.*, 2011) and partially suppresses the mitochondrial morphology defects seen in mutants lacking Mmm2, but not other ERMES mutants (Esposito *et al.*, 2011).

This suggests a link exists between Mmm2, the 26S proteasome and cell cycle progression. Consistent with this idea, Cdc14 has been shown to be maintained in the nucleolus delaying the MEN in cells lacking Mmm2 (Esposito *et al.*, 2011) or Mdm10 (Garcia-Rodriguez *et al.*, 2009). However, like its *S. pombe* homologue (Mocciaro and Schiebel, 2010), Cdc14 is not essential in *N. crassa* (the knockout is reported as viable in the *N. crassa* knockout library) and may not interact with ERMES components in the same fashion as in *S. cerevisiae*. Studying the relationship between the MEN pathway, 26S proteasome and ERMES in a system other than *S. cerevisiae* may help understand these complex relationships.

1.4 Protein import into mitochondria (Figure 1.4).

Mitochondria in *S. cerevisiae* and *N. crassa* contain approximately 1000 proteins. Only a few are encoded by the mtDNA while the rest are encoded by the nuclear genome. This requires that roughly 99% of mitochondrial proteins be translated on cytosolic ribosomes, targeted to mitochondria and imported and assembled into the appropriate mitochondrial compartment (Figure 1.4). Import and assembly is accomplished by the action of several protein complexes existing in the MOM, MIM and IMS. The action of these complexes is discussed in several recent reviews (Schmidt *et al.*, 2010; Dukanovic and Rapaport, 2011; Endo *et al.*, 2011; Gebert *et al.*, 2011). The TOM complex functions as the general import pore (GIP) through which nearly all mitochondrial proteins must pass. The TOB (topogenesis of outer membrane β-barrels) complex, also known as the SAM (sorting and assembly machinery) complex, also exists in the MOM and functions to assemble several proteins into the MOM including all β-barrel proteins. The Mim1/2 complex functions to assemble single and multi-transmembrane domain (TMD) containing α-helical proteins into the MOM (Becker *et al.*, 2008a; Popov-Celeketic *et al.*, 2008; Becker *et al.*, 2011a; Papic *et al.*, 2011; Dimmer *et al.*, 2012). Two TIM (translocase of the inner mitochondrial membrane) complexes exist. The TIM22 complex functions to assemble carrier proteins into the MIM, while the TIM23 complex facilitates both the translocation of proteins into the matrix as well as the integration of some proteins into the

Figure 1.4. Protein import into mitochondria. Several complexes are required for the import and assembly of mitochondrial proteins into the different mitochondrial compartments. The TOM complex serves as the general import pore for nearly all preproteins destined for incorporation into mitochondria. The TOB complex functions to assemble MOM β-barrel proteins as well as tail-anchored TOM complex subunits. The Mim1/2 complex is responsible for assembling proteins with multiple and single α-helical TMDs into the MOM. The small Tim complexes (Tim8/13 and Tim9/10 complexes) guide both incoming β-barrel precursors to the TOB complex as well as carrier proteins to the TIM22 complex. The TIM22 complex assembles MIM carrier proteins into the inner membrane while the TIM23 complex is required for the import of matrix targeted proteins. The TIM23 complex also functions to insert proteins with single α-helical TMDs into the MIM by a stop-transfer mechanism. The OXA complex inserts components of the OXPHOS machinery into the MIM while the Mia40/Erv1 disulphide relay system assembles a class of Cys bonded IMS proteins. The ERMES tethers the ER to mitochondria and has been implicated in the import and assembly of several outer membrane proteins, but it is unknown if this is due to primary or secondary effects.


Cytosol
Mim1/2
TOM
precursor
ERM
?
ERMES
TOB
MOM
20
70
37
38
7
5
40
40
6
55
Mdm
10
Gem1
1
12
2
Mdm
10
22
13
8
10
9
Small Tims
IMS
Erv1
Mia40
13
8
10
9
TIM22
TIM23
50
21
54
10
9
MIM
OXA
17
23
18
22
18
16
mtHsp70
PAM
Matrix
MPP
Mitochondrially synthesized
and some imported proteins

MIM. The OXA (oxidase assembly) complex functions by incorporating members of the ETC and some other multi-TMD containing proteins into the MIM from the matrix side of the MIM. The ERMES complex and/or its components has also been implicated in the biogenesis of certain MOM proteins. The TOM, TOB, Mim1/2, TIM, and OXA complexes are discussed more fully in sections 1.4.2, 1.4.3, 1.4.4, 1.4.8, and 1.4.9, respectively.

1.4.1 Targeting signals

Proteins are targeted to different mitochondrial compartments by a variety of different sorting signals [reviewed in (Neupert and Herrmann, 2007; Bolender *et al.*, 2008; Chacinska *et al.*, 2009)]. While en route to their final destination in the correct mitochondrial subcompartment, the proteins are referred to as mitochondrial precursor proteins or preproteins. The best understood mitochondrial targeting signal is the matrix-targeting presequence peptide which is composed of an amphipathic α-helix in which one face is positively charged while the other face is predominantly hydrophobic. The matrix-targeted signal sequence is removed from the preprotein in the matrix by the matrix processing peptidase (MPP). Other targeting signals are less well understood. They are not removed from the precursor protein and persist within the mature protein sequence. β-barrels are a class of MOM protein that consist of an anti-parallel β-sheet that is wrapped into a barrel shape. The outside of the barrel is hydrophobic while the inside is hydrophilic. β-barrels have a C-terminal β-signal required for their recognition and integration into the MOM, but the signals that target them to the mitochondrial import receptors remain elusive. α-helical transmembrane MOM proteins are thought to be targeted to the MOM by their C- or N-terminal TMDs. For proper targeting, some of these proteins require positive charges flanking their TMDs. MIM proteins with multiple α-helical TMDs like the metabolite carrier proteins have multiple internal signals that direct them to the MIM. Finally, IMS proteins contain the MISS (mitochondrial intermembrane space signal) consisting of conserved Cys and hydrophobic residues important for their targeting to the IMS.

1.4.2 The TOM complex

As mentioned above, the TOM complex serves as the general import pore for nearly every mitochondrial protein. It is composed of the central pore forming β-barrel Tom40; the receptors Tom20, Tom22 and Tom70; and the small Toms Tom5, Tom6 and Tom7 (Becker *et al.*, 2008b; Chacinska *et al.*, 2009; Dukanovic and Rapaport, 2011).

Tom70 is a tetratrico peptide repeat (TPR) protein anchored to the MOM by an N-terminal TMD (Hase *et al.*, 1984), which also functions as a dimerization domain (Millar and Shore, 1993). Tom70 is responsible for the recognition of carrier proteins like the ADP/ATP carrier (AAC). In *N. crassa* and *S. cerevisiae*, mutants lacking Tom70 are deficient in the import of AAC. *Δtom70* mutants in *S. cerevisiae* contain enlarged mitochondria but do not exhibit growth defects (Schlossmann *et al.*, 1996) whereas Tom70 null mutants in *N. crassa* exhibit both a growth defect and altered mitochondrial morphology (Grad *et al.*, 1999).

In *S. cerevisiae* a homologue of Tom70, called Tom71 exists in the MOM (Bomer *et al.*, 1996; Schlossmann *et al.*, 1996). Although its expression level is low compared to other TOM proteins, Tom71 may be important for efficient protein import at high temperatures (Schlossmann *et al.*, 1996). Cells lacking the E3 ubiquitin ligase Mfb1 exhibit mitochondrial morphology defects similar to cells lacking Tom70 or Tom71 (Durr *et al.*, 2006; Kondo-Okamoto *et al.*, 2006) and it was shown that Tom70 and Tom71 are required to recruit Mfb1 to mitochondria to maintain mitochondrial morphology (Kondo-Okamoto *et al.*, 2008) and thus lack of Mfb1 recruitment in cells lacking Tom70 or Tom71 likely causes the morphology defects seen in these mutants. Since *N. crassa* has no obvious homologue of Mfb1 the mitochondrial morphology defects seen in *N. crassa Δtom70* mutants (Grad *et al.*, 1999) must arise in a different manner.

While both Tom22 and Tom20 are essential in *N. crassa* (Harkness *et al.*, 1994a; Nargang *et al.*, 1995), *S. cerevisiae* mutants lacking Tom20 are viable (Moczko *et al.*, 1994). Tom22 was originally thought to be essential in *S. cerevisiae*, but it has been shown that after a recovery period, Tom20 and Tom70 can partially compensate for the loss of Tom22 (van Wilpe *et al.*, 1999). The

difference in the essentiality of these proteins between organisms likely reflects the importance of OXPHOS in *N. crassa* while *S. cerevisiae* can survive via fermentation.

Understanding the substrate specificity of Tom20 and Tom22 has been the subject of ongoing research over the last couple of decades (Sollner *et al.*, 1989; Kiebler *et al.*, 1993; Moczko *et al.*, 1993; Harkness *et al.*, 1994b; Moczko *et al.*, 1994; Haucke *et al.*, 1995; Mayer *et al.*, 1995b; Terada *et al.*, 1997; Yano *et al.*, 2000). Recently, an elegant study in *S. cerevisiae* using selectively degradable Tom22 and Tom20 demonstrated that in the absence of the cytosolic domain of either receptor nearly identical import phenotypes were seen for all precursors tested (Yamano *et al.*, 2008). Although the role that the Tom20-Tom22 receptor plays may be different for different preproteins [see (Sollner *et al.*, 1989)], the contribution of both Tom20 and Tom22 to a single receptor site is now clear. Tom22 contributes an acidic domain to the receptor while Tom20 contributes a hydrophobic domain. The acidic domain of Tom22 binds to basic residues of the signal sequence helix, whereas Tom20 recognizes the amphipathic sequence WHxBHH; where H, W and B represent hydrophobic, hydrophilic and basic residues, respectively (Abe *et al.*, 2000; Muto *et al.*, 2001; Obita *et al.*, 2003). As receptors, Tom20, Tom22 and Tom70 are all required for efficient precursor binding to mitochondria (Hines *et al.*, 1990; Sollner *et al.*, 1990; Kiebler *et al.*, 1993; Mayer *et al.*, 1995a), but along with Tom40, they also exhibit some chaperone-like activity keeping precursor proteins in an unfolded state as they pass through the TOM complex channel (Esaki *et al.*, 2003; Yamamoto *et al.*, 2009; Yamamoto *et al.*, 2011).

While the Tom receptors have always had an obvious role in the function of the TOM complex, the function of the small Toms has been more elusive. *Δtom5* mutants are deficient in the import of all precursors tested in *S. cerevisiae* (Dietmeier *et al.*, 1997), but the negatively charged cytosolic domain of Tom5 is not necessary for complementation of *Δtom5 S. cerevisiae* (Horie *et al.*, 2003). This suggests that Tom5 is not required for precursor binding. Furthermore, *N. crassa* mitochondria lacking Tom5 do not have import or growth phenotypes even

though *N. crassa* Tom5 can complement the TOM complex stability, growth, and import defects seen in *S. cerevisiae Δtom5* strains (Schmitt *et al.*, 2005). Thus, it is likely that lack of Tom5 somehow disrupts the structure and/or function of the TOM receptors or the TOM pore in *S. cerevisiae*, but not in *N. crassa*.

Similarly, *S. cerevisiae* mitochondria lacking Tom6 are impaired in the import of both matrix and MIM targeted precursors whereas *N. crassa* mitochondria lacking the protein have no noticeable import phenotypes (Alconada *et al.*, 1995; Sherman *et al.*, 2005). Unlike Tom5, Tom6 precursors from either *S. cerevisiae* or *N. crassa* only assemble into the TOM complex of the corresponding organism (Dembowski *et al.*, 2001), suggesting a divergence in the assembly mechanisms of Tom6 compared to Tom5. The import phenotypes seen in both *N. crassa* and *S. cerevisiae* mutants lacking Tom7 are similar (Honlinger *et al.*, 1996; Sherman *et al.*, 2005). Mutants from both organisms exhibit no or only very minor defects in the import of matrix or MIM targeted precursors. On the other hand, in both organisms import and assembly of the MOM β-barrel porin is reduced while the assembly of another β-barrel, Tom40, is increased (Honlinger *et al.*, 1996; Sherman *et al.*, 2005; Meisinger *et al.*, 2006). In human tissue culture experiments, knockdown of any small Tom did not impair import of any precursor tested, however, when more than one small Tom was depleted effects were seen on both TOM complex stability and ability to import matrix targeted precursors (Kato and Mihara, 2008). From these and other data that will be discussed in section 1.6.1 and chapter 5, the primary functions of the small Toms appear to be in the stability and assembly of the TOM complex. Import defects seen in these mutants are probably a secondary consequence of the structural changes in the TOM complex.

In *S. cerevisiae,* protein import into mitochondria is modulated by the phosphorylation of different components of the TOM complex by cytosolic kinases (Schmidt *et al.*, 2011). When cells are grown by fermentation, Tom70 and Tom40 are phosphorylated at S174 and S54, respectively by protein kinase A (PKA) (Schmidt *et al.*, 2011; Rao *et al.*, 2012). Phosphorylation of Tom70 reduces the efficiency of carrier import into the MIM while phosphorylation of

the Tom40 preprotein impairs the assembly of Tom40 into the TOM complex. Not surprisingly, these sites are not conserved in *N. crassa* as the organism cannot grow by fermentation and thus does not need to modulate the assembly of carrier proteins or the TOM complex in the same manner as *S. cerevisiae*. Tom22 is constitutively phosphorylated at Ser44 and Ser46 by casein kinase 2 (CK2) in *S. cerevisiae*. This increases the rate of import and assembly of both Tom20 and Tom22 in vitro (Schmidt *et al.*, 2011). Similar sites are conserved in *N. crassa* at Ser31 and Ser37, however it is unknown if they are phosphorylated. It will be interesting to see if TOM complex activity is modulated by post-translational modification in organisms other than *S. cerevisiae*.

1.4.3 The TOB complex

The TOB core complex is composed of the β-barrel protein, Tob55; the transmembrane spanning protein, Tob37; and the peripheral membrane protein, Tob38 (Kozjak *et al.*, 2003; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004; Habib *et al.*, 2005; Lackey *et al.*, 2011). In *S. cerevisiae*, the SAM complex nomenclature for the subunits is used. Tob55 is Sam50, Tob37 is Sam37 and Tob38 is Sam35. The *S. cerevisiae* Tob37/Sam37 does not have a TMD. In this thesis I will use the TOB nomenclature. Even though Tob37 and Tob38 show only weak similarity to their mammalian counterparts (Metaxin1 and Metaxin2, respectively), there is evidence to suggest that the mammalian homologues interact with the mammalian homologue of Tob55 and have similar functions as their fungal counterparts (Armstrong *et al.*, 1997; Armstrong *et al.*, 1999; Xie *et al.*, 2007). The TOB core complex can also bind another β-barrel protein, Mdm10 to form the TOB holo complex (Meisinger *et al.*, 2004; Wideman *et al.*, 2010; Lackey *et al.*, 2011). In *N. crassa*, the stoichiometry of the TOB core and TOB holo complexes is 1:1:1 and 1:1:1:1, respectively (Klein et al. 2012 submitted).

Along with its essential role in the assembly of MOM β-barrel proteins, the TOB complex is also required for the assembly of several α-helical TOM complex proteins into the TOM complex, including Tom22, Tom5, and Tom6

(Stojanovski *et al.*, 2007; Becker *et al.*, 2010; Thornton *et al.*, 2010; Becker *et al.*, 2011b). The TOB holo complex is thought to function in the membrane integration of Tom22 into the MOM as well as its subsequent assembly into the TOM complex (Stojanovski *et al.*, 2007; Thornton *et al.*, 2010; Becker *et al.*, 2011b). Interestingly, in the pathogenic fungus *Candida albicans*, Tob37 has been implicated in phospholipid homeostasis, cell wall synthesis and virulence, making it a potential target for antifungal drugs (Dagley *et al.*, 2011; Qu *et al.*, 2012). Severe synthetic growth defects are seen in mutants lacking both Tob37 and ERMES components in *S. cerevisiae* suggesting a potential conserved role in phospholipid homeostasis (Stojanovski *et al.*, 2007; Hoppins *et al.*, 2011). The function of the TOB complex will be further discussed in sections 2, 3 and 5.

1.4.4 The Mim1/2 complex

The recently discovered Mim1/2 complex is composed of Mim1 and Mim2 in fungi (Dimmer *et al.*, 2012). Mim1 was originally discovered for its involvement in assembling Tom40 (Ishikawa *et al.*, 2004; Waizenegger *et al.*, 2005) and is known to be involved in the membrane integration of both the signal anchored TOM receptors, Tom20 and Tom70 as well as the tail anchored small Toms (Becker *et al.*, 2008a; Hulett *et al.*, 2008; Popov-Celeketic *et al.*, 2008; Lueder and Lithgow, 2009; Becker *et al.*, 2011a; Dimmer *et al.*, 2012). Mim2 is essential for the biogenesis of Mim1 and functions with Mim1 in the integration of multi-pass MOM proteins like Ugo1 (Becker *et al.*, 2011a; Papic *et al.*, 2011; Dimmer *et al.*, 2012). Interestingly, the Mim1/2 complex is required for proper stability of the TOM complex and the assembly of Tom40 (Ishikawa *et al.*, 2004; Waizenegger *et al.*, 2005; Dimmer *et al.*, 2012). However the TOM complex stability and Tom40 assembly defects seen in mutants lacking Mim1 are thought to be a secondary consequence due to inefficient assembly of Tom5 (Becker *et al.*, 2010). In *S. cerevisiae*, *Δmim1* mutants exhibit moderate growth defects and *Δmim2* mutants exhibit strong growth defects, whereas in *N. crassa* loss of Mim1 (and likely Mim2) is lethal (Nargang lab, unpublished observations). The

differences in the essentiality of these proteins may reflect the greater importance of Tom20 and Tom70 in *N. crassa* compared to *S. cerevisiae*.

Membrane proteins like Ugo1 that have multiple α-helical TMDs require Tom70 and the Mim1/2 complex for MOM integration (Becker *et al.*, 2011a; Papic *et al.*, 2011; Dimmer *et al.*, 2012). In human cell culture the multipass MOM protein PBR [(peripheral-type benzodiazepine receptor) also known as TSPO (translocator protein)] requires Tom70 but no other known mitochondrial import factor for its assembly (Otera *et al.*, 2007). Thus, it will be interesting to see if organisms outside of fungi have a complex homologous or analogous to Mim1/2 to assist in the integration of α-helical proteins into the MOM.

1.4.5 Alternative pathways to the MOM

It has been reported that some signal anchored proteins like OM45, Mcr1 and Fis1 can self integrate into the MOM without the help of a protein cofactor (Kemper *et al.*, 2008; Meineke *et al.*, 2008; Merklinger *et al.*, 2012). It was suggested that integration of these proteins into the MOM is directed by the unique composition of the MOM. The MOM of *S. cerevisiae* contains nearly no ergosterol and a high lipid to protein ratio compared to most other cellular membranes (Zinser *et al.*, 1991; Schneiter *et al.*, 1999) which could serve to allow targeting and integration of proteins like OM45 and Mcr1 (Merklinger *et al.*, 2012). Interestingly, the *N. crassa* MOM contains over tenfold the amount of ergosterol compared to the MOM of *S. cerevisiae* (Bay *et al.*, 2008; Bay and Court, 2009), but still contains homologues of Fis1 and Mcr1 suggesting that membrane composition may not be the only factor guiding these proteins to the MOM. A possible explanation is mRNA localization, as it is known that some mitochondrial transcripts are tethered to the MOM (Eliyahu *et al.*, 2010; Gadir *et al.*, 2011). Thus, MOM integration could be favoured if these signal-anchored proteins are translated near the MOM. However, Mim1/2 complex involvement was not considered in these studies. Thus, either the Mim1/2 complex or another as yet undiscovered complex could offer an alternative explanation for the integration of these proteins.

1.4.6 The small Tims: Tim8, Tim9, Tim10, (Tim12) and Tim13

All five small Tim proteins are derived from a common ancestral IMS protein and have very similar core structures (Gentle *et al.*, 2007; Alcock *et al.*, 2012). However, there are differences in their sequence which contribute to differential substrate specificities (Webb *et al.*, 2006; Beverly *et al.*, 2008). Tim8 and Tim13 form a heterodimer (Koehler *et al.*, 1999; Hoppins and Nargang, 2004) as do Tim9 and Tim10 (Koehler *et al.*, 1998). In both *S. cerevisiae* and *N. crassa* Tim9 and Tim10 are essential for viability whereas absence of Tim8 or Tim13 has no effect on growth in either organism (Hoppins and Nargang, 2004; Chacinska *et al.*, 2009). Tim12 is unique to *S. cerevisiae* and binds to the TIM22 complex and is essential for viability (Chacinska *et al.*, 2009; Petrakis *et al.*, 2009). The Tim8/13 and Tim9/10 complexes act as chaperones that guide incoming carrier proteins to the TIM22 complex for assembly into the MIM (Koehler *et al.*, 1998; Sirrenberg *et al.*, 1998) and incoming β-barrels to the TOB complex for assembly into the MOM (Hoppins and Nargang, 2004; Wiedemann *et al.*, 2004; Chacinska *et al.*, 2009; Petrakis *et al.*, 2009). Mutations in the human homologue of Tim8 (DPP) have been correlated with deafness dystonia syndrome (Koehler *et al.*, 1999).

1.4.7 The disulfide relay system of the IMS

Mia40 and Erv1 are essential components of the IMS disulfide relay system [reviewed in (Herrmann and Riemer, 2012)]. An 8 kDa region of Mia40 is highly conserved in both animals and fungi. Mia40 in some species of fungi including *S. cerevisiae*, but not *N. crassa*, has a non-essential N-terminal TMD that anchors it in the MIM. During translocation through the TOM complex, IMS proteins like the small Tims or Mdm35 that contain twin CX_3C motifs interact with Mia40 via the formation of mixed disulfide bonds. As a result of these interactions, Mia40 orients substrates to form intramolecular disulfide bonds thus breaking the mixed disulfide bond and releasing the oxidized substrates into the IMS. This results in the concomitant reduction of the disulfide bonds in Mia40. Erv1 functions to recycle Mia40 by oxidizing the reduced Cys residues formed in Mia40 so that it

can interact with newly imported IMS precursor proteins. Reduced Erv1 is reoxidized by passing electrons to molecular oxygen or cytochrome c in the ETC (Chacinska *et al.*, 2009; Herrmann and Riemer, 2012).

1.4.8 The TIM23 and TIM22 complexes

The TIM23 complex is responsible for translocating matrix targeted proteins across the MIM. It can also assemble a class of presequence-containing proteins that contain a TMD into the MIM by a stop-transfer mechanism (Mokranjac and Neupert, 2010; van der Laan *et al.*, 2010). A subclass of these proteins is further processed by IMS or MIM proteases releasing mature proteins into the IMS. The TIM23 complex further associates with PAM (presequence translocase-associated motor) which provides the force and energy to drive translocation across the MIM. After the N-terminal signal sequence is in the matrix it is removed by MPP to form the mature protein (Chacinska *et al.*, 2009; Mokranjac and Neupert, 2010; van der Laan *et al.*, 2010). While proteins are traversing the MOM and MIM via the TOM and TIM23 translocases, the two complexes form a supercomplex thought to tether the two membranes at mitochondrial contact sites (Schulke *et al.*, 1997; Schulke *et al.*, 1999; Chacinska *et al.*, 2009; Mokranjac and Neupert, 2010; van der Laan *et al.*, 2010). This interaction is dependent upon the IMS domains of Tom22 and Tim50 (Chacinska *et al.*, 2009).

The TIM22 complex is responsible for assembly of mitochondrial carrier proteins (like AAC) into the MIM. The TIM22 complex is composed of the pore forming protein Tim22, Tim54 which stably interacts with the Tim9/10 complex (and Tim12 in *S. cerevisiae*) and Tim18 which is involved in the assembly of the complex (Chacinska *et al.*, 2009). The mechanism by which carrier proteins are inserted into the membrane is unclear but it is known that it occurs in a membrane-potential dependent manner (Neupert and Herrmann, 2007). Carriers are then released laterally into the membrane and assembled into functional dimers.

1.4.9 The OXA complex

In addition to the import complexes there is a single “export” complex called the OXA (oxidase assembly) complex. The term “export” is applied because substrates of OXA are inserted into the MIM from the matrix side; that is, in the opposite direction to the action of TOM and TIM complexes. The OXA complex is responsible for the assembly of multiple TMD-containing mtDNA encoded proteins as well as some presequence-containing nuclear encoded proteins (Neupert and Herrmann, 2007). These nuclear encoded proteins are first fully translocated into the matrix before they are inserted into the MIM by the OXA complex. Some multipass transmembrane proteins are assembled cooperatively first by the TIM23 complex followed by the action of the OXA complex (Bohnert *et al.*, 2010).

1.4.10 The MINOS Complex

Recently, an inner membrane scaffolding complex termed MINOS (mitochondrial inner membrane organizing system) (von der Malsburg *et al.*, 2011), MICOS (mitochondrial contact site) (Harner *et al.*, 2011), or MitOS (mitochondrial organizing structure) (Hoppins *et al.*, 2011) was discovered simultaneously by three different groups. This complex consists of Fcj1, Mio27, Mio10, Aim37, Aim13, and Aim5. Fcj1 and Mio10 are highly conserved in eukaryotes, however other members have only weak homology to proteins found in higher eukaryotes (Harner *et al.*, 2011). Fcj1 (Mitofilin in mammals) was originally found to be required for the proper formation of MIM cristae junctions, the part of the MIM that links the CM to the IBM (John *et al.*, 2005; Rabl *et al.*, 2009). MINOS is likewise proposed to be primarily involved in the formation of cristae junctions and the architecture of the MIM in relation to the MOM (Korner *et al.*, 2012). MINOS is enriched at MOM-MIM contact sites (Harner *et al.*, 2011) and has been shown to interact with MOM proteins including Ugo1 (Harner *et al.*, 2011), porin (Hoppins *et al.*, 2011) Tob55 (Xie *et al.*, 2007; Darshi *et al.*, 2011; Harner *et al.*, 2011; Alkhaja *et al.*, 2012; Ott *et al.*, 2012; Zerbes *et al.*, 2012) as well as Tom40 (von der Malsburg *et al.*, 2011; Zerbes *et al.*, 2012). The interaction of

MINOS with both the TOM and TOB complexes raises the possibility that MINOS is involved in protein import. In support of this it has been demonstrated that MINOS mutants are deficient in the import of IMS proteins via the Mia40 import pathway (von der Malsburg *et al.*, 2011). Additionally, Tob55 interacts with Fcj1 via the C-terminal conserved region of Fcj1 and overexpression of Fcj1 has minor effects on the import and assembly of β-barrels (Korner *et al.*, 2012). Alternatively, since reduced levels of TOB complex components impairs the formation of cristae junctions (Korner *et al.*, 2012), the link between the TOB and MINOS complexes may be primarily structural in nature and the effects on β-barrel import may be a secondary phenotype. In an attempt to synthesize data on several systems, van der Laan et al (2012) propose that MINOS, along with ERMES and other MOM complexes, is part of a multi-membrane spanning, multiorgannellar network of complexes termed ERMIONE (ER-mitochondria organizing network). Coordination of membranes and complexes could help explain the pleiotropic effects seen in cells lacking individual components of this network.

1.5 Evolution of the protein import machines

During the evolution of the early endosymbiont to a modern mitochondrion, many genes were transferred from the symbiont to the host genome. Once transferred, genes had to be expressed and the resulting proteins had to be directed to the evolving pre-mitochondrial symbiont, imported through pre-existing machinery, and assembled into the appropriate compartment. Some components of the import machinery must have evolved from existing proteins in the symbiont whereas other components would be novel contributions from either the host or the symbiont. The first step to import proteins into the evolving mitochondrion would likely have been the evolution of the core component of the TOM complex, the β-barrel protein, Tom40. β-barrel proteins are found exclusively in the outer membranes of mitochondria, chloroplasts and Gram-negative bacteria and could have potentially served as primitive protein import pores in early mitochondrial evolution. Although the efficiency is very poor compared to purified TOM

complex, reconstituted Tom40 has the ability to translocate presequence peptides in vitro (Becker *et al.*, 2005). Thus it is conceivable that a single subunit primitive TOM complex may have existed in the early pre-mitochondrial endosymbiont.

A crystal structure has been determined for one mitochondrial β-barrel protein, porin [also called VDAC (voltage dependent anion channel)]. The structure revealed that porin contains 19 β-strands (Bayrhuber *et al.*, 2008; Ujwal *et al.*, 2008; Hiller *et al.*, 2010). Modeling and comparative studies suggest a related structure for Tom40 (Zeth, 2010) and that Tom40 and porin share an evolutionary history (Bay *et al.*, 2011), though a bacterial homologue that might represent an ancestor of the proteins has not been identified. Interestingly, the newly discovered MOM β-barrel ATOM (archaic translocase of the outer mitochondrial membrane) family in trypanosomes descended from a YtfM (TamA) homologue, a bacterial outer membrane protein in the OMP85 protein family (Pusnik *et al.*, 2011), which is required for the export of autotransporter proteins in pathogenic bacteria (Selkrig *et al.*, 2012). ATOM is required for mitochondrial protein import in trypanosomes and requires trTob55 (trypanosome Tob55) for its biogenesis (Pusnik *et al.*, 2011). It is not known if ATOM and Tom40 have the same ancestry, but it seems unlikely since ATOM/Omp85 proteins have 16 β-strands and it is difficult to imagine the evolution of 3 extra β-strands in ATOM to reach the 19 β-strands in Tom40. Alternatively, it has been suggested that the crystal structure of porin (from which the Tom40 structure was modeled) does not represent the native structure. The basis for the criticism of the crystal structure is that it does not align with much of the existing biochemical data on the protein (Colombini, 2009). All other TOM complex proteins are thought to be host innovations as no homologues have been identified in bacteria. Tom40, Tom70, Tom7 and Tom22 are thought to have been components of the TOM complex of the LECA because homologues have been found in extremely divergent eukaryote supergroups (Macasev *et al.*, 2004; Tsaousis *et al.*, 2011).

Similar to ATOM, Tob55 is the homologue of another OMP85 protein, BamA. BamA functions to incorporate β-barrels into the outer membrane (OM) of bacteria (Gentle *et al.*, 2004; Wu *et al.*, 2005; Hewitt *et al.*, 2011). The process of

β-barrel insertion into the OM of bacteria and mitochondria has been conserved through evolution since mitochondrial β-barrels can be assembled into bacterial OMs in vivo (Walther *et al.*, 2010) and bacterial β-barrels can be targeted and assembled into the MOM in vivo (Walther *et al.*, 2009). However the mechanism of β-barrel insertion into the membrane is slightly different because, in bacteria, BamA has 5 POTRA (polypeptide transport associated) domains that extend into the periplasmic space that are required for the incorporation of β-barrels into the MOM. On the other hand, Tob55 has only one POTRA domain in the IMS which, has been shown to bind β-barrels in vitro (Habib *et al.*, 2007) and is required for efficient substrate release (Stroud *et al.*, 2011a) but not for β-barrel incorporation into the MOM (Kutik *et al.*, 2008). A further difference between bacterial and mitochondrial β-barrel insertion is that Tob37 and Tob38 are required for the efficient incorporation of eukaryotic β barrels but they are dispensable for the assembly of bacterial β-barrels into the MOM (Jiang *et al.*, 2011).

Although components of the TOM and TOB complexes have obvious bacterial origins, homologues of the IMS import components in mitochondria have not been identified in bacteria. Instead, Mia40, Erv1 and the small Tims have functional analogues in the periplasmic space of bacteria suggesting that the IMS import components arose via convergent evolution (Hewitt *et al.*, 2011).

The inner membrane translocases are all thought to have arisen from α-proteobacterial homologues. The OXA complex is homologous to bacterial YidC which is responsible for the assembly of proteins into the inner membrane of bacteria (Hewitt *et al.*, 2011). Tim22 and Tim23, while being themselves homologous, have been shown to be homologous to the bacterial amino-acid transporter LivH (Rassow *et al.*, 1999), though this claim has recently been challenged (Gross and Bhattacharya, 2011). In bacteria, SecY cooperates with YidC, to incorporate inner membrane IM proteins. Interestingly, a protist in the order Jakobida, *Reclinomonas americana* has maintained SecY in its mtDNA (Tong *et al.*, 2011) which could potentially function with OXA or YidC-like proteins in the MIM. The maintenance of SecY in the mtDNA of *R. americana* suggests that perhaps the TIM23 complex has relatively recently evolved to take

over the role of SecY in mitochondria as, similar to the cooperation of SecY and YidC, the TIM23 complex cooperates with the OXA complex to insert some MIM proteins with multiple TMDs (Bohnert *et al.*, 2010).

Tim22 and Tim23 likely diverged from a common ancestor after a gene duplication event. Subsequent evolutionary 'tinkering' of existing symbiont proteins with host novelties could lead to the invention of new import apparatuses (Alcock *et al.*, 2010). It has been shown that two components of the TIM23 translocase, Tim44 and Pam18 are homologous to α-proteobacterial proteins TimA and TimB, respectively. In bacteria TimA functions in membrane quality control while the function of TimB is currently unknown. With a single point mutation, TimB can complement the loss of Pam18 (an essential protein) in *S. cerevisiae* (Clements *et al.*, 2009).

1.6 Deeper into the MOM: Function of the TOM complex and the assembly of β-barrel proteins into the MOM by the TOB complex

1.6.1 Structure of the TOM complex

All components of the TOM complex found in fungi are conserved in metazoans suggesting that the common ancestor between fungi and animals had a complete TOM complex. While the components are known, the stoichiometry and physical arrangement of the TOM complex is still being investigated. It is known that Tom70 is only loosely associated with other TOM complex components and is barely detected in coimmunoprecipitation experiments using antibodies directed to other TOM components (Dekker *et al.*, 1998). Tom20 is also rather loosely associated and dissociates from the TOM complex during blue native gel electrophoresis (BNGE) (Dekker *et al.*, 1998) (and my unpublished observations see appendix Figure A.1). However, Tom20 is a quantitative member under mild conditions of isolation when using 0.1% digitonin rather than the usual 1% used to solubilize mitochondrial membranes (Meisinger *et al.*, 2001). The other TOM complex components interact stably to form a ~400 kDa complex (Dekker *et al.*, 1996).

Tom40 and Tom22 form a very stable complex (Meisinger *et al.*, 2001) and benzoylphenylalanine (BPA) crosslinking analysis has shown that the TMD of a single Tom22 binds two molecules of Tom40 (Shiota *et al.*, 2011), this agrees with Ni-NTA-gold labeling and cryo-electron microscopy (EM) modeling placing Tom22 between two TOM pores (Model *et al.*, 2008). Tom40 is the essential hydrophilic pore-forming component of the TOM complex (Vestweber *et al.*, 1989; Baker *et al.*, 1990; Hill *et al.*, 1998; Kunkele *et al.*, 1998b; Ahting *et al.*, 2001; Taylor *et al.*, 2003). Since double helical DNA that is conjugated to protein presequences can be imported into mitochondria, the TOM complex pore must be at least 20 Å in diameter (Vestweber and Schatz, 1989). This is similar to the pore diameter (22 Å) calculated in experiments using reconstituted Tom40 and a polymer exclusion method (Hill *et al.*, 1998). These data are also consistent with the measurement of pores seen in purified TOM complex examined by EM (electron microscopy) tomography and cryo-EM experiments which gave estimates of about 15-25 Å (Kunkele *et al.*, 1998a; Ahting *et al.*, 1999; Model *et al.*, 2002).

In *S. cerevisiae* the TOM-core complex lacking Tom70 and Tom20 has been shown to exhibit a two-pore structure by EM and cryo-EM (Model *et al.*, 2002; Model *et al.*, 2008). When Tom20 is present three-pore TOM complexes are observed in both *S. cerevisiae* and *N. crassa* (Kunkele *et al.*, 1998a; Model *et al.*, 2002; Model *et al.*, 2008). In *S. cerevisiae*, even in the absence of both Tom20 and Tom70 a 400 kDa TOM complex can be detected by BNGE that behaves as a two-pore structure electrophysiologically (Meisinger *et al.*, 2001). The most recent model for TOM complex structure suggests a triple-pore model of the TOM complex (Figure 1.5A) that readily disassembles into a two-pore structure with the loss of Tom20 (Figure 1.5B). How or why (or if) a Tom40 subunit is also released in the absence of Tom20 is not clear.

The small Toms have been suggested to be important in the stability and assembly of the TOM complex. Tom6 and Tom40 are in contact (Rapaport *et al.*, 1998) and Tom6 is required for the assembly of Tom22 into a stable TOM complex (Dekker *et al.*, 1998). In *S. cerevisiae*, Tom5, Tom6 and Tom7 form a

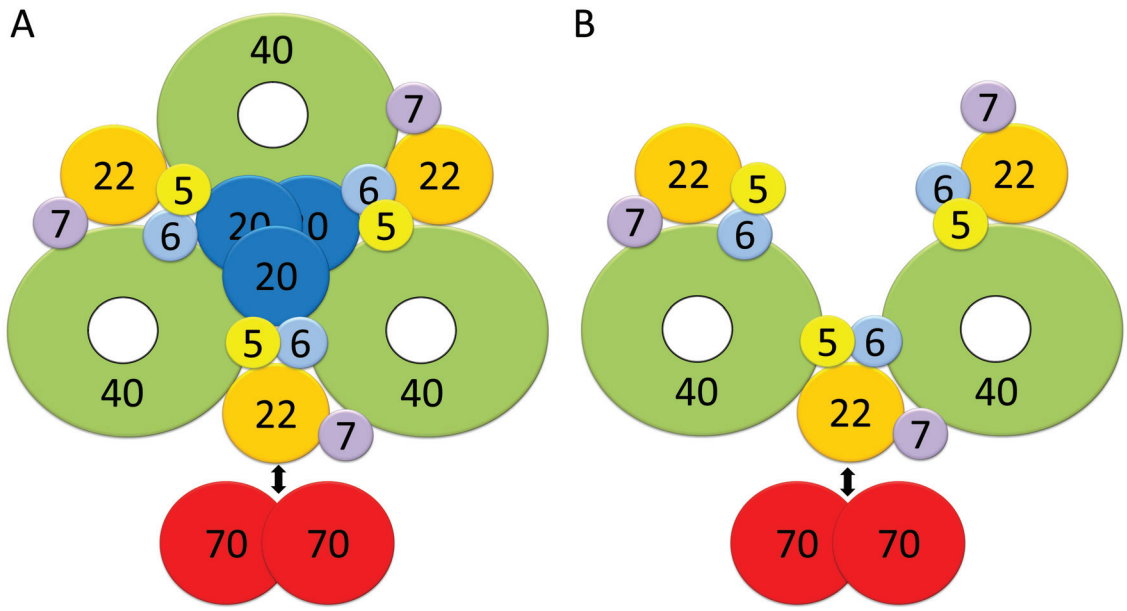


Figure 1.5. Possible models for the structure of the TOM complex. (A) Tripartite model based on Model *et al*. 2008. (B) Bipartite model lacking Tom20 based on Ahting *et al.*, 1999; Model *et al.*, 2002; Becker *et al.*, 2005 and Model *et al.*, 2008.

100 kDa complex with Tom40 in the absence of Tom22 (Dekker *et al.*, 1998) and in *N. crassa* Tom6 and Tom7 are in close proximity to Tom40 (Dembowski *et al.*, 2001). Thus, I have placed the small TOMs in contact with Tom40 and near Tom22 in TOM complex models (Figure 1.5). Lack of any of the individual small Toms in *N. crassa* causes only minor destabilization of the TOM complex (Schmitt *et al.*, 2005; Sherman *et al.*, 2005). Similarly, human cell culture cells deficient in any of the small Toms exhibit nearly normal TOM complex stability (Kato and Mihara, 2008). In *S. cerevisiae* the loss of Tom5 has little effect on TOM complex stability (Schmitt *et al.*, 2005), but loss of Tom6 greatly reduces stability (Alconada *et al.*, 1995; Model *et al.*, 2001). Deletion of *S. cerevisiae* Tom7 actually stabilizes the interaction between Tom20, Tom22 and Tom40 (Honlinger *et al.*, 1996). Lack of both Tom6 and Tom7 leads to a slight increase in stability of Tom40 with Tom22 compared with loss of only Tom6 even though the loss of both proteins causes a severe growth phenotype (Dekker *et al.*, 1998). In contrast, loss of both Tom6 and Tom7 in *N. crassa* causes major TOM complex destabilization, steady-state reductions of Tom5, Tom22 and Tom20, and a major growth defect. Loss of all three small Toms is lethal in *N. crassa* (Sherman *et al.*, 2005). In human cell culture, the TOM complex is slightly heat labile when Tom7 is knocked down and shows greater instability when two or more small Toms are depleted (Kato and Mihara, 2008). Although the small Toms have been conserved in both animals and fungi their functions appear to differ between organisms.

1.6.2 Sequence of events: TOM complex function during preprotein recognition and translocation: Break on through to the other side

Before arrival at the TOM complex, cytosolic chaperones like Hsp70 (and Hsp90 in mammals) target preproteins to mitochondria and then associate with the first TPR domain of Tom70 (Young *et al.*, 2003; Fan *et al.*, 2006). After transfer from chaperones, preprotein-Tom70 multimers form (Young *et al.*, 2003) and precursors are transferred to the so-called cis-receptor site of the TOM complex on the cytosolic side of the MOM. This site is composed primarily of

Tom20-Tom22, though Tom5 and Tom40 have also been suggested to be involved in the binding of precursors at this site (Dietmeier *et al.*, 1997; Rapaport *et al.*, 1997). After cis-site binding the preprotein is translocated through the Tom40 pore to the trans-site (Mayer *et al.*, 1995c) which is composed primarily of the IMS domains of Tom22 and Tom7 (Honlinger *et al.*, 1996; Esaki *et al.*, 2003) with some involvement of the IMS face of Tom40 (Rapaport *et al.*, 1997; Stan *et al.*, 2000; Gabriel *et al.*, 2003).

As N-terminal mitochondrial targeting sequences are rich in basic residues and many components of the TOM and TIM23 import complexes contain acidic patches, it was hypothesized that precursor proteins may follow an acid chain into the matrix. While there are varying reports for or against the involvement of various proteins or residues that might provide the "acids" for the chain (Bolliger *et al.*, 1995; Nakai *et al.*, 1995; Court *et al.*, 1996; Moczko *et al.*, 1997; Nargang *et al.*, 1998), there is excellent evidence suggesting that presequence-containing precursor proteins do follow a sequential chain into the matrix. In vitro assays have shown that transfer of precursors from cytosolic chaperones to Tom70, Tom70 to Tom20, Tom20 to Tom22 and the IMS domain of Tom22 to Tim23 each occur unidirectionally, with forward transfer of precursors favoured compared to the reverse reactions (Komiya *et al.*, 1998).

1.6.3 Assembly of β-barrel proteins by the TOB complex

Incoming β-barrel proteins are recognized by TOM complex receptors in a partially folded state suggesting that the 3-dimensional conformation, rather than a specific signal sequence is the important factor for the recognition and import of β-barrels into mitochondria (Rapaport and Neupert, 1999). While β-barrels were originally thought to require only Tom20 for recognition, this has been extended to the cytosolic domains of both Tom20 and Tom22 (Yamano *et al.*, 2008). Additionally, the import of β-barrels through the TOM complex requires a functional TOB complex suggesting a receptor role for the TOB complex (Walther *et al.*, 2009). This opens up the possibility that Tob37 and Tob38, which have large cytosolic domains, might function as β-barrel receptors on the outer

face of the MOM. After translocation through the TOM complex, β-barrel precursors associate with the small Tim complexes (Hoppins and Nargang, 2004; Wiedemann *et al.*, 2004) and are directed to the TOB complex. While the mechanism of insertion into the membrane is unclear, it is known that Tob38 contains the β-recognition sequence that recognizes the signal found in the last β-strand present in all mitochondrial β-barrels. The β-signal is a modestly conserved sequence; xPoxGxxHyxHy, where Po and Hy represent polar and hydrophobic residues, respectively (Kutik *et al.*, 2008). Assembly of β-barrels into the MOM proceeds by an unknown mechanism but could involve the use of Tob55 as a template or scaffold (Dimmer and Rapaport, 2012).

The role of Mdm10 in β-barrel assembly is currently unclear, though it is known that lack of Mdm10 decreases Tom40 assembly (Meisinger *et al.*, 2004). One model suggests that Mdm10 is required for release of Tom40 from the complex (Yamano *et al.*, 2010b, a). Another model suggests that Mdm10 is primarily involved in the assembly of Tom22 into the TOM complex (Thornton *et al.*, 2010; Becker *et al.*, 2011b). The need for Tom22 in Tom40 assembly would explain the decrease in Tom40 assembly in Mdm10 mutants. A third model suggests that Mdm10 is required for the efficient assembly of all β-barrels (Wideman *et al.*, 2010). The role of Mdm10 in MOM protein assembly is further discussed in Chapters 2, 3, and 5.

Two other ERMES proteins, Mmm1 and Mdm12, have also been shown to be required for proper β-barrel assembly into the outer membrane (Meisinger *et al.*, 2007; Wideman *et al.*, 2010), however it is unknown if this phenotype is due to primary or secondary effects. Interestingly, in *mmm1-1* temperature sensitive mutants, it takes at least 1.5 hr of growth at the non-permissive temperature for mutants to exhibit mitochondrial morphology defects (Hobbs *et al.*, 2001; Meisinger *et al.*, 2007) and even longer to observe aberrant mitochondrial ultrastructure (Hobbs *et al.*, 2001), while mitochondrial protein import defects can be seen after shifting to the non-permissive temperature for only 30 min (Meisinger *et al.*, 2007). These results suggest that the ERMES complex may actually be directly involved in the assembly of β-barrels into the MOM. On the

other hand, secondary effects due to possible alterations in MOM lipid composition (section 1.3.4) may also affect TOM and TOB complex function. It has been reported that CL is required for efficient assembly of certain MOM proteins and that both the TOM and TOB complexes from mutants lacking components of the CL biosynthesis pathway have altered mobility when analyzed by BNGE (Gebert *et al.*, 2009). It will be interesting to see if CL or other lipids are actively assembled into MOM complexes, perhaps by the ERMES complex.

1.7 Focus of this study: Involvement of ERMES in the assembly of the TOM complex

The first goal of my project (Chapter 2) was to determine if there was a relationship between Mdm10 and Tom7 in *N. crassa*. Experiments from *S. cerevisiae* suggested that mutants lacking these proteins exhibit mitochondrial morphology defects. However, they also exhibited reciprocal defects in the assembly of β-barrel proteins (Meisinger *et al.*, 2006). Mitochondria isolated from mutants lacking Tom7 were shown to assemble Tom40 faster but porin slower than mitochondria isolated from a control strain. Strains lacking Mdm10 exhibited the exact opposite phenotypes with enhanced porin assembly but impaired Tom40 assembly. Based on these data, a model was developed suggesting that the TOB holo complex preferentially assembles Tom40 and the TOB core complex preferentially assembles porin. The equilibrium between the two was thought to be controlled by Tom7, which was shown to interact with Mdm10. This model suggested that loss of Mdm10 would be epistatic to the loss of Tom7 with respect to TOB holo/TOB core equilibrium. To test this hypothesis, I examined *N. crassa* single and double deletion mutants for mitochondrial morphology and protein import phenotypes. I found that *Δmdm10* and *Δtom7* mutants had reciprocal Tom40 assembly phenotypes but when both genes were deleted, the phenotypes observed were additive. These data suggested that at least some details of *N. crassa* Mdm10 and Tom7 function are different than their *S. cerevisiae* counterparts.

The original reports of Mdm10 interaction with the TOB complex in *S. cerevisiae* were controversial because: 1) *mdm10* mutants were initially isolated as mitochondrial morphology mutants and 2) Only very small amounts of Mdm10 were involved in TOB complex interaction. Therefore, my next project (Chapter 3) was to determine if Mdm10 interacted with the TOB complex in *N. crassa*. Using strains expressing tagged versions of TOB complex components I was able to show that Mdm10 is a substoichiometric component of the TOB complex. Further analysis revealed that five different Tob55-containing complexes exist in the MOM of *N. crassa*. Two of these complexes contained Tob37 and Tob38, and it was one of these that contained Mdm10. I went on to analyze a strain expressing reduced levels of Tob55 that exhibited altered ratios of the five Tob55 complexes. Analyses demonstrated that Tom22 (a supposed substrate of TOB holo) assembly was drastically affected in this strain while the assembly of other precursors was only marginally affected.

The last part of my project (Chapter 4) involved characterizing the role of the other ERMES components in the assembly of the TOM complex. ERMES mutants are characterized by an array of phenotypes including defective assembly of β-barrel proteins into the MOM. I have shown that they are all also defective in Tom22 assembly. In an attempt to determine if specific domains of the proteins are responsible for specific phenotypes, I constructed strains expressing different alleles of *mmm1* and demonstrated that certain regions of the prot ein are required for the efficient assembly of Tom40 but do not affect mitochondrial morphology or assembly of Tom22. This suggests that Mmm1 could be a multifunctional protein that is directly involved in the assembly of MOM proteins.

Mmm2 (and possibly Gem1) has been shown to be an ERMES protein, but mitochondrial protein import has not been investigated in mutants lacking the protein. Here I show that mutants lacking Mmm2 exhibited β-barrel and Tom22 assembly defects similar to mutants lacking Mdm12 or Mmm1, but mutants lacking Gem1 had no detectable defects in mitochondrial protein import. I also foundthat ERMES mutants in *N. crassa* did not contain altered mitochondrial lipid profiles.

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CHAPTER 2. Roles of the Mdm10, Tom7, Mdm12, and Mmm1 proteins in the assembly of mitochondrial outer membrane proteins in *Neurospora crassa*

Based on:

Wideman, J.G., Go, N.E., Klein, A., Redmond, E., Lackey, S.W., Tao, T., Kalbacher, H., Rapaport, D., Neupert, W., and Nargang, F.E. (2010). Roles of the Mdm10, Tom7, Mdm12, and Mmm1 proteins in the assembly of mitochondrial outer membrane proteins in Neurospora crassa. Mol Biol Cell *21*, 1725-1736.

Note: Westerns and import/assembly assays in Figure 2.10B-E were done by N. E. Go. The *Δtom7* and *Δmdm10* strains were also made by N. E. Go.

2.1 INTRODUCTION

As discussed in sections 1.4.3 and 1.4.6, the TOB complex is responsible for the import and assembly of MOM β-barrels and certain TOM complex proteins with single α-helical TMDs and it has been shown to interact with the Mdm10 protein. As mentioned in section 1.6.3, the *mdm10* mutant was originally discovered as a mitochondrial morphology mutant. Therefore, the role of Mdm10 with respect to TOB complex function in *S. cerevisiae* was initially controversial. Similarly, the finding that the loss of *S. cerevisiae* Mmm1 and Mdm12 affected β-barrel assembly was unexpected. Thus, I wished to determine if Mdm10, Mdm12, and Mmm1 were involved in the assembly of β-barrel proteins into the mitochondrial outer membrane of another organism, *N. crassa*.

In addition to its involvement in the TOB and ERMES complexes, Mdm10 has been shown to interact with Tom7 in *S. cerevisiae* (Meisinger *et al.*, 2006). While it has been shown that *S. cerevisiae* mutants lacking Tom7 contain a large proportion of condensed aggregated mitochondria (79%) and strains lacking Mdm10 contain virtually 100% giant spherical mitochondria when grown at 28 ˚C (Sogo and Yaffe, 1994; Dimmer *et al.*, 2002; Meisinger *et al.*, 2006), it has also been demonstrated that these mutants have reciprocal phenotypes with respect to the import of β-barrel proteins (Meisinger *et al.*, 2004; Meisinger *et al.*, 2006). Mutants lacking Mdm10 exhibit impaired Tom40 assembly and increased porin assembly (Meisinger *et al.*, 2004). The opposite effects on the rate of assembly of Tom40 and porin have been described in Tom7 mutants of both *S. cerevisiae* and *N. crassa* (Sherman *et al.*, 2005; Meisinger *et al.*, 2006). It was also demonstrated in *S. cerevisiae* that Tom7 and Mdm10 form a complex and that lack of Tom7 increases the amount of Mdm10 available for interaction with TOB (Meisinger *et al.*, 2006). These results led to the development of a hypothesis suggesting that the TOB core complex preferentially incorporates porin whereas TOB holo preferentially incorporates Tom40. The import phenotypes seen in mutants lacking either of these proteins were suggested to be caused by changes in the relative ratio of TOB core to TOB holo. I reasoned that this model would predict that the deletion of *mdm10* would be epistatic to the deletion of *tom7* and

double mutant should exhibit import phenotypes equivalent to *mdm10* single mutants. To test this hypothesis and further explore the relationship between Mdm10 and Tom7 I have analyzed assembly of these β-barrel proteins in single and double *mdm10* and *tom7* mutants in *N. crassa*.

2.2 MATERIALS AND METHODS

2.2.1 Strains and growth of *N. crassa*

The strains used in this study are shown in Table A.1. Growth, crossing and general handling of *N. crassa* strains were as described previously (Davis and De Serres, 1970). For growth tests, colonial growth was induced by the presence of 1% sorbose in place of sucrose as a carbon source.

I wanted to construct a knockout of the *N. crassa mdm10* gene. Since it was possible that the *mdm10* knockout would be lethal in *N. crassa* I used a sheltered heterokaryon approach (Nargang and Rapaport, 2007) as described below (Figure 2.1). The 'wildtype' HP1 heterokaryon strain was constructed by superimposing conidia of strains 76-26 and 71-18 on minimal medium. 76-26 is auxotrophic for histidine (His) and carries resistance to p-fluorophenylalanine (fpaR) while 71-18 is auxotrophic for pantothenate (pan) and carries resistance to benomyl (BenR). Thus, conidial anastomosis of the two auxotrophic strains is required for growth in the absence of His and pan. The auxotrophies are complemented in the resulting heterokaryon so that the strain can grow on minimal media. To create a mutant lacking the *mdm10* gene, a construct consisting of a hygromycin (Hyg) resistance cassette flanked by 3000 bp of sequence upstream and downstream of the *mdm10* open reading frame (ORF) was transformed into HP1 multinucleate macroconidia using split marker fragments developed by PCR (Goswami, 2012). This split marker approach gives rise to about 40-50% homologous replacements and 50-60% ectopic integration (Colot *et al.*, 2006). Usually only one nucleus in multinucleate conidia is transformed (Grotelueschen and Metzenberg, 1995). Transformants were grown on hygromycin plates containing minimal Vogel's media with sorbose as the carbon

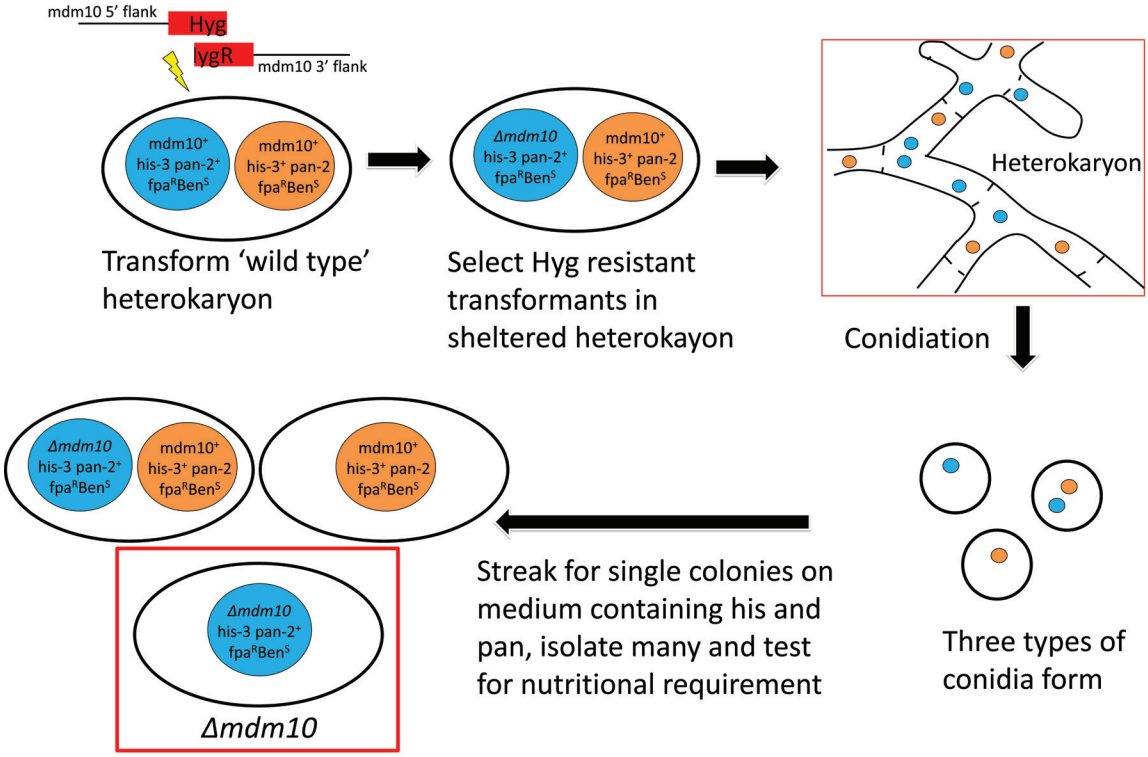


Figure 2.1. Construction of the *Δmdm10* strain in a sheltered heterokaryon using a split marker approach. The HP1 heterokaryon (oval) containing two different nuclei (His-requiring, blue circle and pan requiring, orange circle) was transformed with the *mdm10* knockout construct consisting of a hygromycin (Hyg) resistance cassette as a split marker flanked by 3000 bp of sequence upstream and downstream of the *mdm10* open reading frame (ORF). Transformants were incubated on plates containing Vogel's minimal medium plus Hyg. Single colonies were selected and transferred to slants and allowed to grow and conidiate. Southern analysis was performed and strains that contained both the replacement cassette and the endogenous *mdm10* gene were selected for further analysis. These strains were streaked on minimal medium plus His and pan to allow growth of all possible types of conidia. Resulting strains were tested for nutrient requirements and Hyg resistance. A strain in which the replacement of the *mdm10* gene occurred in the His-requiring nucleus was selected for further analysis.

source. Single colonies were selected, transferred to slants, and allowed to grow and conidiate. These transformants are considered to be "sheltered" heterokaryons since loss of the *mdm10* gene in one nucleus type is "sheltered" or complemented by the other nucleus type. Transformants were examined by Southern analysis (not shown). Those with both the knockout construct at the *mdm10* locus and the endogenous gene were chosen for future work since they should be the desired heterokaryons.

The sheltered heterokaryon should randomly give rise to three types of macroconidia. One type should contain only His-requiring nuclei, another should contain only pan-requiring nuclei, while the third should contain both. To determine if *mdm10* was essential in *N. crassa*, conidia from one heterokaryotic transformant were streaked on medium containing His and pan. Colonies that formed on the media were picked to slants with medium containing His and pan. The conidia that formed in the slants were subsequently tested for nutritional requirements to determine if the isolates were homokaryons that required His or pan, or if they were heterokaryons capable of growth on minimal medium. The presence of both types of homokaryons revealed that *mdm10* was not an essential gene. The His-requiring and pan-requiring homokaryons were then tested for hygR to determine which nucleus of HP1 had undergone the *mdm10* replacement. A homokaryotic strain (mdm10-1) in which the *mdm10* gene was replaced by the HygR cassette in the His-requiring nucleus was chosen for further work.

2.2.2 Fluorescence microscopy of mitochondria

Examination of mitochondria in hyphae was done using a previously described method (Hickey et al. 2004) with modifications. Conidia were inoculated in the centre of petri plates containing a thin layer of medium (~ 1 mm thick) that was solidified with agarose. The plates were incubated at 30°C for 6 to 8 hr. After incubation 20 µl of 500 nM MitoTracker Green (Molecular Probes, Eugene, Oregon) in liquid medium was applied to the hyphal tips of the growing mycelium. Incubation was allowed to continue for an additional 30 min. Agarose blocks containing growing hyphal tips were cut with a scalpel and placed on a

microscope slide, covered with a coverslip and visualized by epifluorescent microscopy. Samples were viewed using a Planapochromat 63X oil immersion objective (numerical aperture, 1.4; working distance, 0.19 mm) of an AxioImager M1 (Carl Zeiss, Oberkochen, Germany) equipped with an ORCA-ER digital camera (Hamamatsu Photonics K.K. Hamamatsu, Japan). MitoTracker Green was visualized using a 50% attenuated HBO103 mercury vapour short-arc lamp (Osram, Munich, Germany), with a BP470/40 excitation filter, an FT 495 beam splitter and a BP525/50 emission filter (Carl Zeiss). A higher dye concentration than generally recommended (500 nM rather than 20-200 nM) was used to account for diffusion into the surrounding media. Images were cropped and adjusted for brightness and contrast in Adobe Photoshop.

2.2.3 Measuring mitochondrial diameter and statistical analysis

The diameter of 50 mitochondrial tubules from each strain were measured using ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). The average diameter and standard deviation for mitochondria of each strain were calculated.

2.2.4 Isolation of mitochondria, alkaline extraction and whole cell extracts

Unless specified otherwise, mycelia were grown at 30° C, harvested by filtration, and ground in the presence of sand and SEMP isolation buffer (0.25 M sucrose; 10 mM MOPS, pH 7.2; 1 mM EDTA; 1 mM PMSF) using a mortar and pestle. Mitochondria were isolated by differential centrifugation as described (Nargang and Rapaport, 2007).

To determine if proteins were integral membrane proteins, alkaline extraction was performed. Mitochondria (50 μg protein) were suspended in 1 ml of 0.1 M sodium carbonate (pH 11.0) and left on ice for 30 min. The mixture was then centrifuged at 50,000 rpm in a TLA55 rotor using a tabletop ultracentrifuge (Beckman Instruments, Palo Alto, CA) at 2°C for 30 min. The pellets were processed for electrophoresis. Proteins in the supernatant were precipitated by adding 70% trichloroacetic acid to a final concentration of 7%, allowing the mixture to sit for 1 hr on ice, and then centrifuging as described above. The

supernatant was discarded and the pellets were washed with acetone, dried, and processed for electrophoresis.

Whole cell extracts were prepared by grinding mycelia in the presence of sand and protein isolation buffer (10 mM MOPS, pH 7.2; 1 mM EDTA; 1 % SDS; 1 mM PMSF) followed by centrifugation (3000 x g, 20 min, 4°C) to remove sand and cellular debris. The supernatant was further clarified by centrifugation (12,000 x g, 20 min, 4°C) to produce the whole cell extract and then assayed for protein concentration using the BCA-200 protein assay system (Pierce, Rockford, IL).

2.2.5 Preparation of damaged mitochondria

To create mitochondria from wild type cells that were similar to those isolated from strains containing enlarged mitochondria, we used a modified osmotic shock treatment. Isolated wild-type mitochondria in SEMP buffer (500 μg of mitochondrial protein) were pelleted by centrifugation (16000 x g, 15 min, 4°C), resuspended in 1 ml of swelling buffer (1 mM KPO_4, 1 mM EDTA, pH 7.2), and incubated on ice for 30 min. Every 5 min the mitochondria were vortexed at high speed for 10 sec. Mitochondria were reisolated by centrifugation (16000 x g, 15 min, 4°C) and resuspended in fresh SEMP. We refer to the mitochondria obtained by this treatment as "damaged" mitochondria.

2.2.6 Standard procedures

Blue native gel electrophoresis (BNGE) was used to analyze intermediates and complexes found in mitochondrial membranes and has been described previously (Schägger and von Jagow, 1991; Schägger *et al.*, 1994). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), Western blotting (Good and Crosby, 1989) and outer membrane vesicle (OMV) isolation (Mayer *et al.*, 1995) were performed as described previously. In some cases, irrelevant lanes were cropped from scanned blots.

2.2.7 Protein import into isolated mitochondria

Import of precursor proteins into isolated mitochondria was performed as described previously (Harkness *et al.*, 1994). Briefly, radiolabelled precursor proteins were synthesized in vitro using rabbit reticulocyte lysate in the presence of ^{35}S methionine (Promega TnT reticulocyte lysate system, Madison WI). Isolated mitochondria were incubated with radioactive precursor protein. After incubation unimported precursor was digested by proteinase K. Mitochondria were then washed, reisolated, subjected to SDS-PAGE and transferred to a nitrocellulose membrane for analysis by autoradiography. To test for bypass import, MOM receptors were digested by pretreatment with trypsin prior to incubation of mitochondria with lysate.

For analysis by BNGE, after import, mitochondria were washed and solublized in 1% digitonin. The samples were subjected to BNGE and transferred to polyvinylidenefluoride (PVDF) membrane for analysis by autoradiography.

2.3 RESULTS

2.3.1 Mutant construction and mitochondrial morphology in *mdm10* and *tom7* mutant strains

We identified the NCU07824 protein as the homologue of the *S. cerevisiae* Mdm10 protein from the *N. crassa* genome sequence (Galagan *et al.*, 2003). Comparisons of the protein predicted at the *N. crassa* database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html) with that of *S. cerevisiae* revealed the existence of large regions containing little sequence similarity. This prompted us to examine twenty *N. crassa mdm10* cDNAs because of the possibility that introns were misidentified in the genome sequence. Comparison of the cDNAs to the predicted genomic coding sequence showed that one region near the C-terminus was predicted to be an intron but was found to be present in all twenty cDNAs. Another region that encoded 24 amino acid residues near the N-terminus was included in the predicted coding sequence but was found in only two of the twenty cDNAs. Thus, this sequence is removed

```
Nc  MREFMNYITNAFYGATGWNEDNKYNELNATSRELIDFPLPRGLRLTLSSLATPHFATSYQ 60
Sc  MLPYMDQVLRAFYQSTHWSTQNSYEDITATSRTLLDFRIPSAIHLQISNKSTPNTFNSLD 60
    *  :*: : .*** :* *. :*.*:::.**** *:** :* .::* :*. :**:  .* :

Nc  LGSVGVVDGSISYLHS-SIPLTHIAAQSDKIPLPALLRCYRRLHDLRSPGQQHYILDADP 119
Sc  FSTRSRINGSLSYLYSDAQQLEKFMRNSTDIPLQDATETYRQLQ----PNLNFSVSSANT 116
    :.: . ::**:***:* :  * ::  :* .***    . **:*:    *. :. : .*:.

Nc  LSGLPPPPQSARALLGAASDAAVAGGALDGGNTDQDLGIYTHSLLYGRLYLPKSLLEGMI 179
Sc  LSSDNTTVDNDKKLL------------------HDSKFVKKSLYYGRMYYPSSDLEAMI 157
    **.  .. :. : **                  :*  : .:** ***:* *.* **.**

Nc  IKRFTQALQVQVRAVSEQSLRNGGTILGLVQYDKGKYGLEGLYSTDGGLLGFRGLYNFGG 239
Sc  IKRLSPQTQFMLKGVSSFKESLN-VLTCYFQRDSHRNLQEWIFSTSDLLCGYRVLHNF-- 214
    ***::   *. ::.**. .   . .:   .* *. :   * ::**.. * *:* *:**

Nc  DASSSTCDPWTPTPGENNNNNNNNNNNNNGNAQAGEKERIYGRFSVGGELYYGTLNKSGG 299
Sc  ----------LTTPSKFNTSLYNNSS----------------LSLGAEFWLGLVSLSPG 247
              .**.: *..  **..                 :*:*.*:: * :. * *

Nc  MSLGARFATLPAHRGTPLTATLTINPLMGNINATYALLAREYCSLATRVDFNVYSYESEW 359
Sc  CSTTLRYYTHSTNTGRPLTLTLSWNPLFGHISSTYSAKTGTNSTFCAKYDFNLYSIESNL 307
     *   *: * .:: * *** **: ***:*:*.:**:  :   .::.:: ***:** **:

Nc  AVGMELWSNRRPAGFLLGASPSNDFEPEPHPPRKKERSFQAKMEWRLDDPEPEPEPQP-- 417
Sc  SFGCEFWQKKH---HLLETNKNNNDKLEPISDELVDINPNSRATKLLHENVPDLNSAVND 364
    :.* *:*.:::   .** :. .*: : ** . .  : . :::    *.:  *: :.

Nc  ----------------------TPKTRKNDEYKGVLKARLDNN---------------- 438
Sc  IPSTLDIPVHKQKLLNDLTYAFSSSLRKIDEERSTIEKFDNKINSSIFTSVWKLSTSLRD 424
                          :.. ** ** :..::   ::

Nc  LRMGLLWEGRAKSLIFSIGTGIDLHKLGEPFRS-------------------------- 471
Sc  KTLKLLWEGKWRGFLISAGTELVFTRGFQESLSDDEKNDNAISISATDTENGNIPVFPAK 484
      : *****: :.:::* ** : : :  :   *

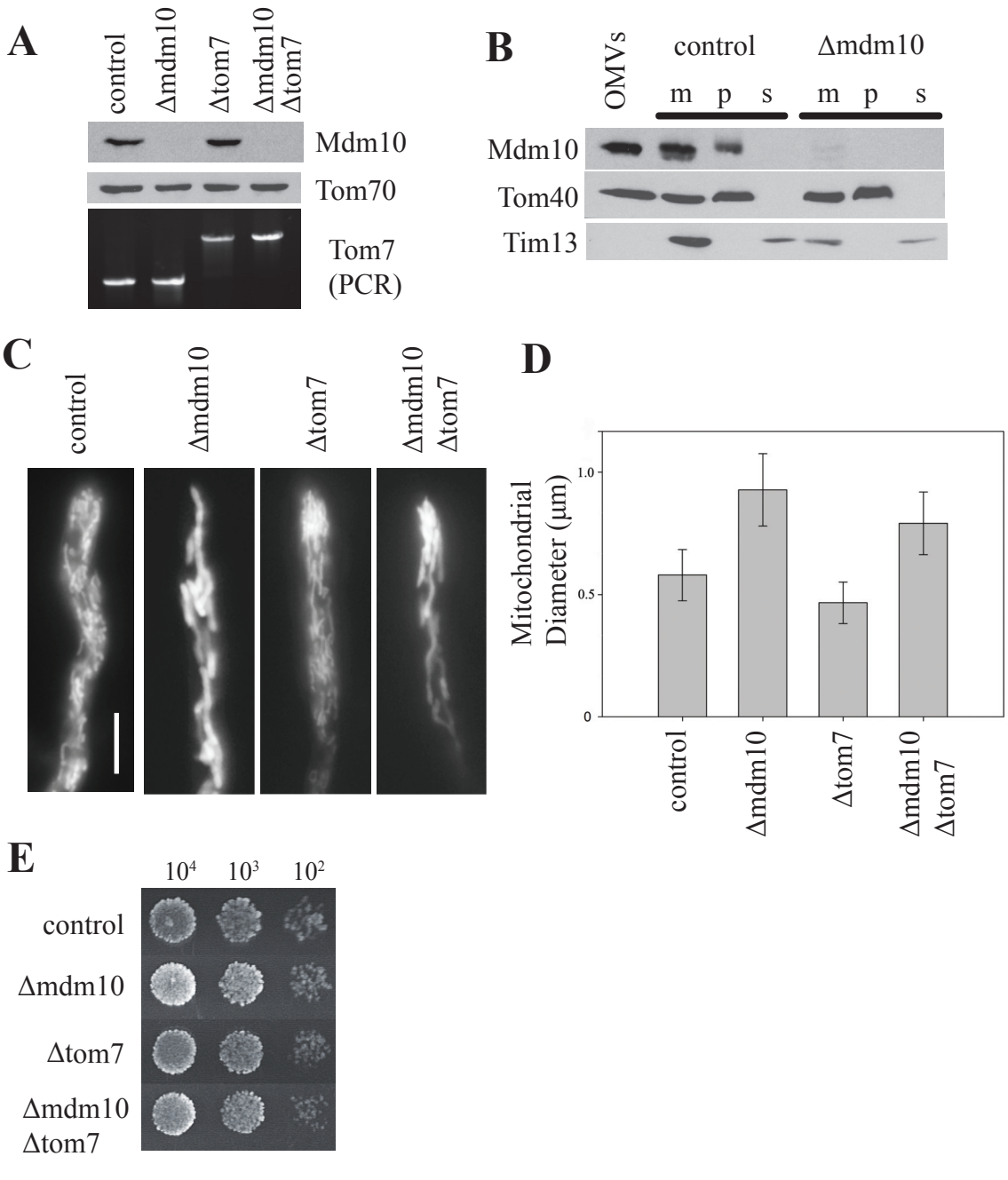
Nc  LGLEVQYSS 480
Sc  FGIQFQYST 493
    :*::.***:
```

Figure 2.2. Alignment of *S. cerevisiae* (S.c.) and *N. crassa* (N.c.) Mdm10 proteins. ***** indicates identical residues, : indicates similar residues.

as an intron in the majority of transcripts, but a low level of alternative splicing appears to occur in the *mdm10* transcript. The major cDNA would give rise to a 480 amino acid *N. crassa* protein. When this sequence was compared to the 493 amino acid yeast Mdm10 protein it was found to be 27% identical and 48% similar (Figure 2.2).

We constructed a knockout of the *N. crassa mdm10* gene in a sheltered heterokaryon using a hygromycin resistance cassette and a split marker approach to replace the *mdm10* coding sequence (Colot *et al.,* 2006; Nargang and Rapaport, 2007). Subsequent analysis of conidiaspores produced by the heterokaryon showed that the gene was not essential and a homokaryotic strain in which the *mdm10* gene had been replaced by hygromycin resistance was isolated from the heterokaryon for further experiments (details in section 2.2.1). We refer to this strain as *Δmdm10*. We also constructed a *Δmdm10Δtom7* double mutant by crossing *Δmdm10* with our previously described *Δtom7* strain (Sherman *et al.*, 2005) so that we could examine the combined effects of these two mutations on the assembly of β-barrel proteins. Western blots of mitochondria isolated from the single and double mutants confirmed the lack of Mdm10 (Figure 2.3A). We do not have an antibody against *N. crassa* Tom7, but the deletion of the *tom7* gene was demonstrated by PCR analysis of genomic DNA (Figure 2.3A). *N. crassa* Mdm10 was found to be located in mitochondrial OMV and exhibited behaviour similar to Tom40 following alkaline extraction of mitochondria (Figure 2.3B). We conclude that *N. crassa* Mdm10 is an integral protein of the mitochondrial outer membrane. Mutations affecting the Mdm10 protein are known to result in the formation of giant mitochondria in various organisms and mitochondria in *S. cerevisiae* strains lacking Tom7 have been shown to have a similar morphological phenotype (Dimmer *et al.*, 2002; Meisinger *et al.*, 2006). We examined our *N. crassa* mutants by fluorescence microscopy following growth at 30° C and staining with mitotracker green. Although circular giant mitochondria were not present in *Δmdm10* mutant cells, we did observe that cells lacking Mdm10 contained tubular mitochondria that were larger in diameter than the thin tubules present in wild-type cells (Figure 2.3C and D). Mitochondria in the *N. crassa*

Figure 2.3. Characterization of *Δmdm10*, *Δtom7*, and double mutants and topology of Mdm10. (A) The top and middle rows show Western blots of mitochondria isolated from the control strain (76-26), and the indicated mutant strains decorated with antibodies to the proteins indicated on the right. The lower panel shows ethidium bromide stained PCR products obtained from genomic DNA of the indicated strains using primers flanking the *tom7* gene. The knockout allele contains a larger product because the disrupting hygromycin cassette is larger than the *tom7* gene that it has replaced. (B) Alkaline extraction of proteins from mitochondria isolated from a control strain (76-26) and the *Δmdm10* mutant. Isolated mitochondria were resuspended in 0.1 M sodium carbonate (pH 11.0) and incubated on ice for 30 min. Membrane sheets were pelleted (p) by centrifugation and proteins in the supernatant (s) were precipitated with trichloroacetic acid. The fractions were subjected to SDS-PAGE, blotted to nitrocellulose, and immunodecorated with the antibodies indicated on the left. Untreated mitochondria (m) from each strain, and OMV from a control strain (76-26) were included as controls. Tom40 is a MOM protein, and Tim13 is found in the IMS (C) The indicated strains were grown on a thin layer of solid medium, stained with mitotracker green, and hyphal tips were examined by fluorescence microscopy. The bar in the control picture indicates 10 μm. (D) The diameter of mitochondrial tubules was measured in photographs of each of the different strains. Fifty tubules were measured for each strain and the average diameter was calculated. The error bars show one standard deviation. (E) Strains were grown on Vogel's solid medium containing sorbose at 30°C for 48 hr. The number of conidia spotted is indicated at the top.


A
control
Δmdm10
Δtom7
Δmdm10 Δtom7
Mdm10
Tom70
Tom7 (PCR)
B
OMVs
control
Δmdm10
m p s m p s
Mdm10
Tom40
Tim13
C
control
Δmdm10
Δtom7
Δmdm10 Δtom7
D
Mitochondrial Diameter (μm)
1.0
0.5
0
control
Δmdm10
Δtom7
Δmdm10 Δtom7
E
10⁴ 10³ 10²
control
Δmdm10
Δtom7
Δmdm10 Δtom7

strain lacking Tom7 did not resemble mitochondria lacking Mdm10 and even appeared to be slightly thinner than those in wild type cells (Figure 2.3C and D). Mitochondria in the double mutant lacking both Mdm10 and Tom7 appeared to be of intermediate diameter (Figure 2.3C and D)—smaller than in the *mdm10* mutant, but larger than in the *tom7* mutant. All four strains exhibited comparable growth rates (Figure 2.3E)

2.3.2 Import and assembly of precursor proteins into mitochondria lacking Mdm10

We next examined the ability of mitochondria lacking Mdm10 to import and assemble mitochondrial precursor proteins *in vitro*. Import of the β-subunit of the F_1 ATP synthase ($F_1\beta$, a matrix targeted precursor) was similar in all the strains examined (Figure 2.4A). On the other hand, import of the ADP/ATP carrier protein (AAC, an inner membrane targeted precursor) was reduced in both the *mdm10* mutant and the *mdm10 tom7* double mutant (Figure 2.4A), although this is likely not due to involvement of Mdm10 in the import of AAC (see below). To determine if *N. crassa* Mdm10 plays a role in the biogenesis of β-barrel proteins, the assembly of the precursor of Tom40 into the TOM complex was examined by BNGE following import into isolated mitochondria.

The assembly of Tom40 (summarized in Figure 2.5) progresses through known stages that are observable when analyzed by blue native gel electrophoresis (BNGE) (Rapaport and Neupert, 1999; Model *et al.*, 2001; Taylor *et al.*, 2003). After import through the TOM complex and association with the small Tim complexes, radiolabeled Tom40 precursor can first be seen when it interacts with the TOB complex as intermediate I at 250 kDa. After its association with the TOB complex, incoming Tom40 is inserted into the outer membrane where it interacts with an endogenous molecule of Tom40 as well as Tom5 in a 100 kDa complex termed intermediate II. Intermediate II is then assembled into the mature 400 kDa TOM complex (Rapaport and Neupert, 1999; Model *et al.*, 2001; Taylor *et al.*, 2003).

Figure 2.4. Import and assembly of precursor proteins into mitochondria of mutant strains. (A) Import of radiolabeled matrix-targeted β-subunit of mitochondrial F_1 ATP synthase ($F_1\beta$) and the inner membrane targeted ATP/ADP carrier (AAC) into mitochondria isolated from the indicated strains. Following import, the mitochondria were treated with proteinase K, re-isolated, electrophoresed, transferred to nitrocellulose membranes, and examined by autoradiography. The time (min) of each import reaction is indicated above the lanes. Lys, 33% of the input lysate containing radiolabeled protein used in each reaction; tryp, mitochondria pre-treated with trypsin prior to 8 min of import with precursor protein. Lack of import under these conditions serves as a control to demonstrate that import is dependent on mitochondrial surface receptors. For $F_1\beta$, arrows indicate the positions of the precursor (p) and mature (m) forms of the protein. (B) Assembly of radiolabeled Tom40 precursor into mitochondria isolated from the indicated strains. Following import, the mitochondria were re-isolated and dissolved in 1% digitonin. The samples were subjected to BNGE, transferred to PVDF membrane, and analyzed by autoradiography. The time (min) of each import reaction is indicated above the lanes. The molecular masses of complexes are indicated in kiloDaltons and correspond to intermediate I (250 kDa) and Intermediate II (100 kDa) as described in Figure 2.5. The "*" indicates a band previously characterized as a non-productive intermediate (Taylor *et al.*, 2003). (C) As in panel B, but radio-labeled porin precursor was imported. The molecular mass of the major bands are indicated. The faint bands of highest molecular mass in the *Δmdm10* and *Δtom7* lanes appear in some import experiments but not others and have not been characterized.

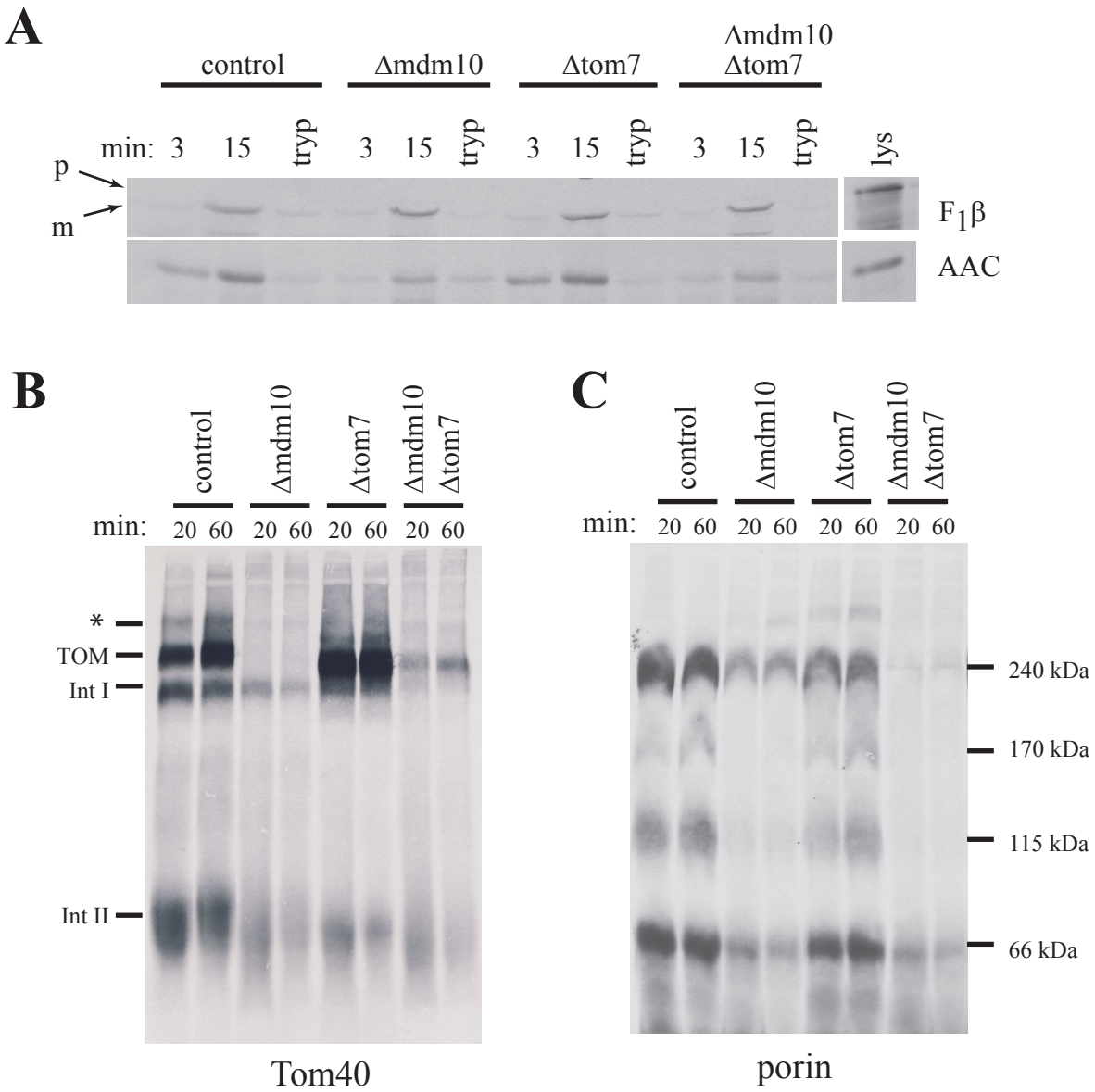
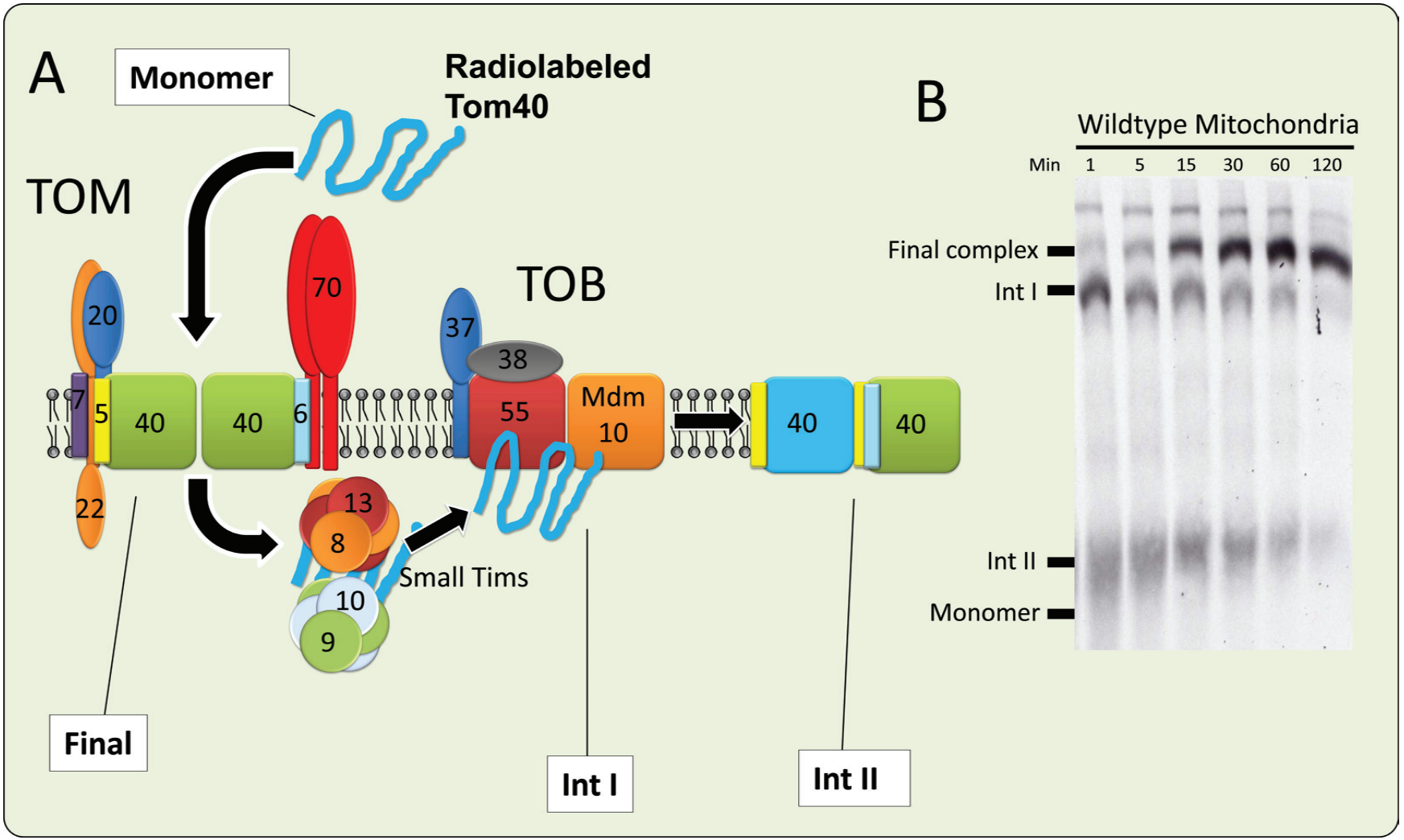

A
control
Δmdm10
Δtom7
Δmdm10
Δtom7
min: 3 15 tryp 3 15 tryp 3 15 tryp 3 15 tryp lys
p
m
F1β
AAC
B
control
Δmdm10
Δtom7
Δmdm10
Δtom7
min: 20 60 20 60 20 60 20 60
*
TOM
Int I
Int II
Tom40
C
control
Δmdm10
Δtom7
Δmdm10
Δtom7
min: 20 60 20 60 20 60 20 60
240 kDa
170 kDa
115 kDa
66 kDa
porin

Figure 2.5. Radiolabelled Tom40 passes through observable import intermediates when analyzed by BNGE. (A) Tom40 can first be seen as a monomer that is likely loosely associated with the MOM prior to import (Monomer). The amount of monomer actually seen following BNGE of Tom40 assembly assays is somewhat variable and usually quite small. After Tom40 passes through the TOM complex, and after association with the small Tim complexes, Tom40 can be observed interacting with the TOB complex as intermediate I (Int I) at 250 kDa. After association with the TOB complex, Tom40 is released into the MOM where it interacts with an endogenous molecule of Tom40 as well as Tom5 forming intermediate II (Int II) at 100 kDa. Intermediate II then assembles with other TOM complex components forming the mature 400 kDa complex (Final). (B) BNGE analysis of a pulse-chase time course of Tom40 import and assembly showing the structures described in A. The pulse-chase time course was taken from R. Taylor, Ph.D. thesis, University of Alberta 2004.


A
Monomer
Radiolabeled
Tom40
TOM
TOB
20
70
37
38
7
5
40
40
6
55
Mdm
10
40
40
22
13
8
10
9
Small Tims
Final
Int I
Int II
B
Wildtype Mitochondria
Min
1
5
15
30
60
120
Final complex
Int I
Int II
Monomer

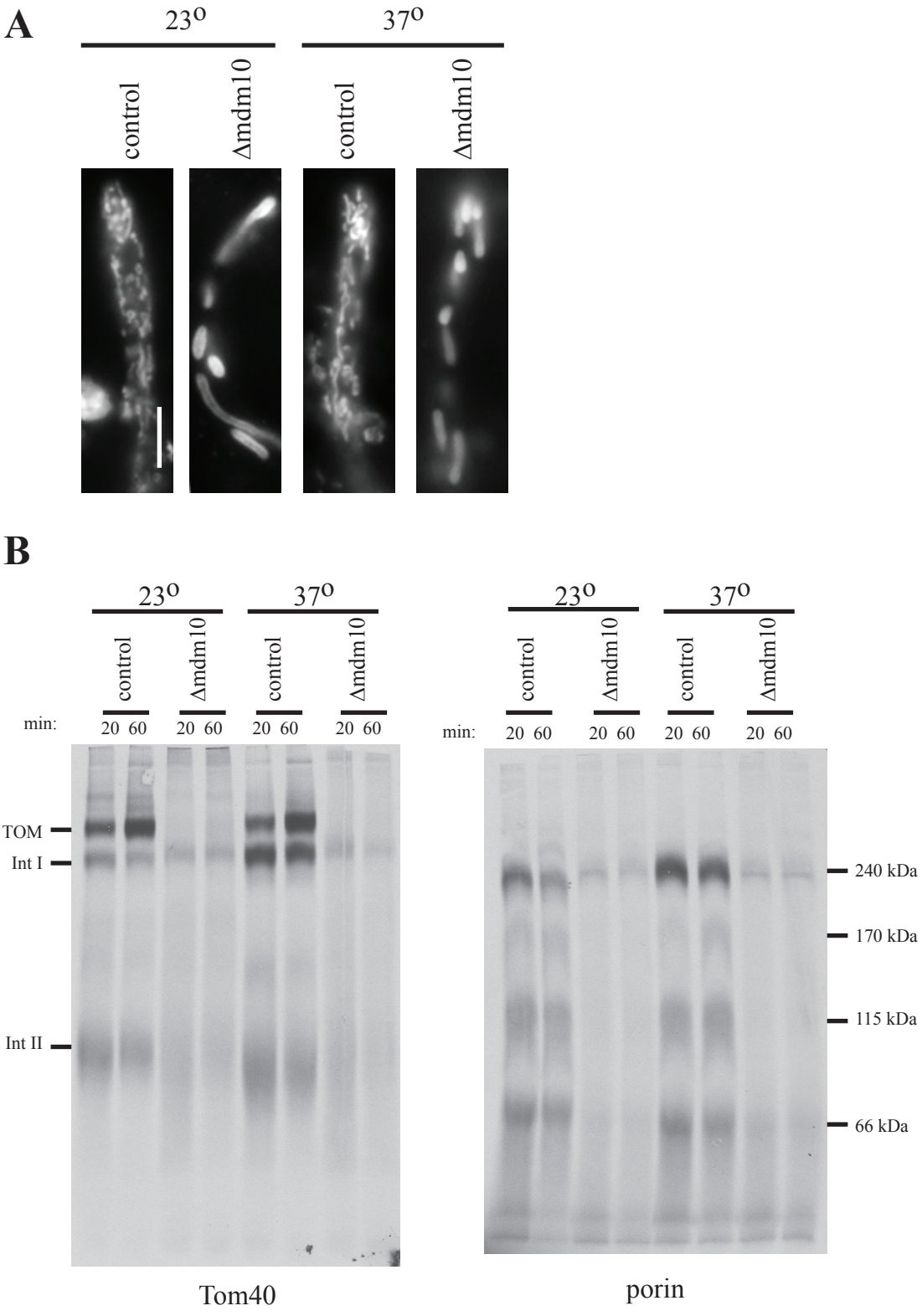
With somewhat reduced efficiency in mitochondria lacking Mdm10, the Tom40 precursor was found to reach the first (250 kDa) assembly intermediate, where it is associated with the TOB complex (Figure 2.4B). Similarly, the precursor was also found in reduced amounts at the second intermediate (100 kDa) where it has been inserted into the membrane by the TOB complex and has associated with pre-existing molecules of Tom40 and Tom5 (Model *et al.*, 2001; Wiedemann *et al.*, 2003; Meisinger *et al.*, 2007). Very low amounts of Tom40 precursor were found to assemble into the final TOM complex at 400 kDa in mitochondria lacking Mdm10 (Figure 2.4B). This suggests that the absence of Mdm10 most strongly affects a post-TOB complex stage of assembly. However, the reduced levels of the intermediates suggests that the interaction of the TOB complex with the Tom40 precursor may also be affected.

I also examined the assembly of porin, another β-barrel protein. The wild type control for porin assembly shows four distinct bands after 20 to 60 min of import (Figure 2.4C). Although we have not completely defined an assembly pathway for porin in *N. crassa*, it has previously been shown that the 240 kDa complex represents the porin precursor in association with the TOB complex (Hoppins *et al.*, 2007). Western blot analysis of mitochondrial proteins following BNGE, shows that most porin is detected in a low molecular mass band that corresponds to the 66 kDa band seen on import blots, with minor amounts in the two complexes of approximately 115 and 170 kDa (unpublished observations). In mitochondria lacking Mdm10 all four bands seen in the wild type assembly pattern were reduced (Figure 2.4C). The largest amount of imported precursor was present at 240 kDa, representing an early intermediate associated with the TOB complex (Hoppins *et al.*, 2007), and in the 66 kDa form. The presence of precursor at the 240 kDa intermediate suggests that the effect on the assembly of porin also occurs at least partially at a stage following association with the TOB complex. However, as with Tom40 the amount of precursor at this stage is reduced relative to the control, and interaction of the porin precursor with the TOB complex may not be optimal. (Additional lanes in Figure 2.4 involving *Δtom7* and *Δmdm10Δtom7* will be discussed in section 2.3.3).

The import results described above were performed on mitochondria isolated from strains grown at 30° C. Previous work in *S. cerevisiae* demonstrated that *mdm10* mutants exhibit stronger phenotypes at higher temperatures and weaker phenotypes at lower temperatures. This does not appear to be the case in *N. crassa* since in microscopy experiments large tubules were also observed in *Δmdm10* cells grown at either 23° C or 37° C (Figure 2.6A) and virtually identical porin and Tom40 assembly patterns were seen using mitochondria isolated from *Δmdm10* cells grown at either 23° C or 37° C (Figure 2.6B).

The reduction in import of AAC and the difference in effects on porin assembly compared to those reported for *S. cerevisiae* mitochondria lacking Mdm10 prompted us to investigate the possibility that factors other than the absence of Mdm10 were responsible for the observed phenotypes. Since the small Tim complexes (Tim9/10 and Tim8/13) are known to be required for the efficient import of β-barrel proteins (Hoppins and Nargang, 2004; Wiedemann *et al.*, 2004; Habib *et al.*, 2005) and AAC (Curran *et al.*, 2002a; Curran *et al.*, 2002b; Vasiljev *et al.*, 2004; Webb *et al.*, 2006), their loss could have an effect on import and assembly of these proteins. The large size of mitochondria in the mutant strain suggested that they might be more susceptible to damage during the process of isolation. Broken mitochondrial membranes could result in the loss of proteins such as the small Tim complexes from the intermembrane space. Western blot analysis revealed that the levels of the intermembrane space proteins, Tim8 and Tim13, as well as the matrix proteins, Hsp60 and Hsp70, were reduced in mitochondria isolated from the *Δmdm10* mutant (Figure 2.7A). To determine if this was due to loss of these proteins during the isolation procedure, versus an inherently lower level of these proteins in the mutant, we examined whole cell extracts for levels of Tim8 and Tim13. In this case, the levels of the proteins appeared to be similar to those in controls (Figure 2.7B), supporting the notion that the large mitochondria in *Δmdm10* mutant cells are physically damaged during isolation. The steady state levels of most other proteins in mitochondria lacking Mdm10 appeared to be similar to those in wild type. Exceptions were

Figure 2.6. Temperature does not affect the *Δmdm10* phenotype in *N. crassa*. (A) Hyphae from the indicated strains were incubated with mitotracker green and examined by fluorescence microscopy. The bar represents 10 μm. (B) Import of Tom40 or porin precursor was performed as described in the legend to Figures 2.4B and C, respectively.


A
23°
37°
control
Δmdm10
control
Δmdm10
B
23°
37°
control
Δmdm10
control
Δmdm10
min:
20 60 20 60 20 60 20 60
TOM
Int I
Int II
Tom40
23°
37°
control
Δmdm10
control
Δmdm10
min:
20 60 20 60 20 60 20 60
240 kDa
170 kDa
115 kDa
66 kDa
porin

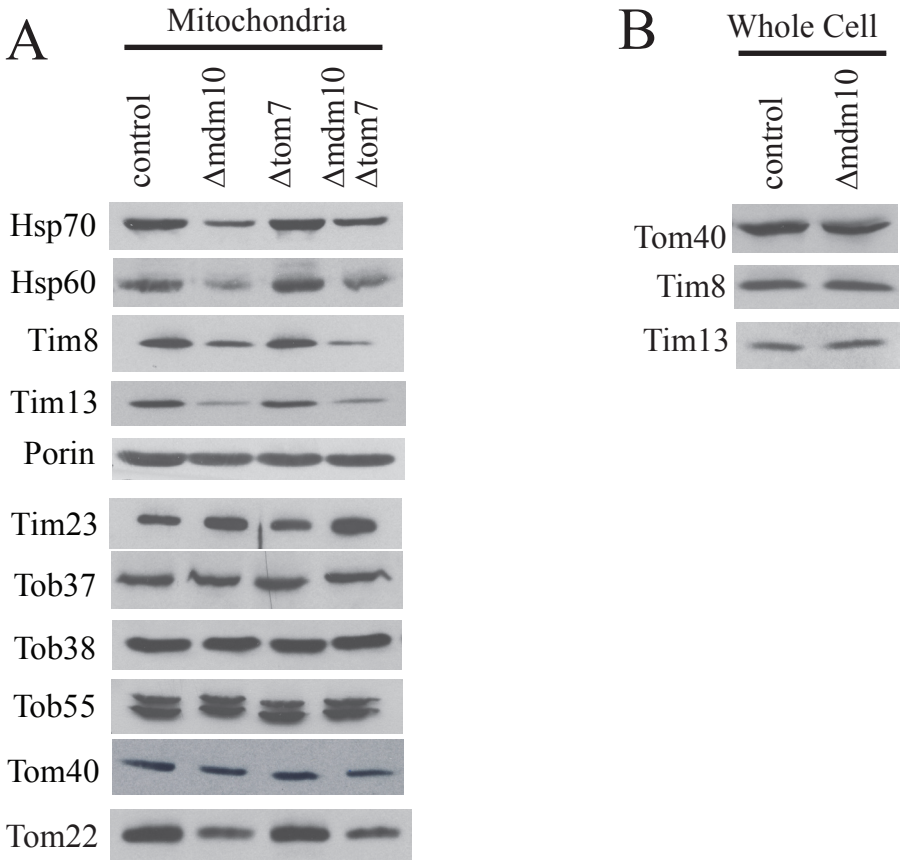


Figure 2.7. Mitochondrial proteins in mutant strains. (A) Mitochondria (30 μg protein) isolated from the strains indicated were analyzed on Western blots with antibodies to the indicated proteins. Two bands are seen for Tob55 because alternative splicing gives rise to different forms of the protein (Hoppins *et al.*, 2007) (B) Whole cell extracts were prepared from the control (76-26) and *Δmdm10* strains as described in section 2.2.4. The extracts were examined (100 μg protein per lane) using Western blots with antibodies against the indicated proteins.

Tim23, which is slightly increased in the mutant, as well as Tom40 and Tom22 which appear slightly reduced (Figure 2.7A).

We next determined if the defects in import and assembly of AAC, Tom40 and porin that were observed in *Δmdm10* mitochondria were due to lack of Mdm10 or the decreased levels of intermembrane space and matrix proteins observed to be lost from the mutant mitochondria during isolation. Therefore, we created damaged wild type mitochondria to act as a control, by subjecting isolated mitochondria to periodic vortexing while in the presence of a hypotonic buffer. Western blot analysis of these damaged wild-type mitochondria showed that the levels of the Tim8, Tim13, and Hsp70 proteins were similar to those in mitochondria isolated from the *Δmdm10* strain (Figure 2.8A), while the levels of other proteins examined were similar to wild type. Import of the $F_1\beta$ and AAC precursors into the damaged wild type mitochondria was slightly reduced in comparison to the Mdm10 deficient mitochondria (Figure 2.8B). This suggests that the inefficient import of AAC in the *mdm10* mutant is likely due to the deficiency of small Tim proteins. The pattern of assembly for the Tom40 precursor into damaged wild type mitochondria did not resemble the pattern seen in the Mdm10 deficient mitochondria. Although assembly into the final 400 kDa TOM complex in damaged wild type mitochondria was slightly reduced compared to the wild type control, it was obviously much more efficient than in the *Δmdm10* mutant mitochondria (Figure 2.8C). The presence of the Tom40 precursor at the first two intermediate stages in Mdm10 deficient mitochondria also argues against the import defects being due to reduced levels of the small Tim proteins as these intermembrane space chaperones are required to guide the incoming precursor to the first intermediate stage (Hoppins and Nargang, 2004; Wiedemann *et al.*, 2004). A similar result was seen for porin where all complexes containing porin were found to be formed more efficiently in the damaged wild type mitochondria than in those lacking Mdm10 (Figure 2.8D). We conclude that the lack of Mdm10 is responsible for the majority of the defects relating to Tom40 and porin assembly while the deficiency of the small Tim proteins explains the decrease in AAC import.

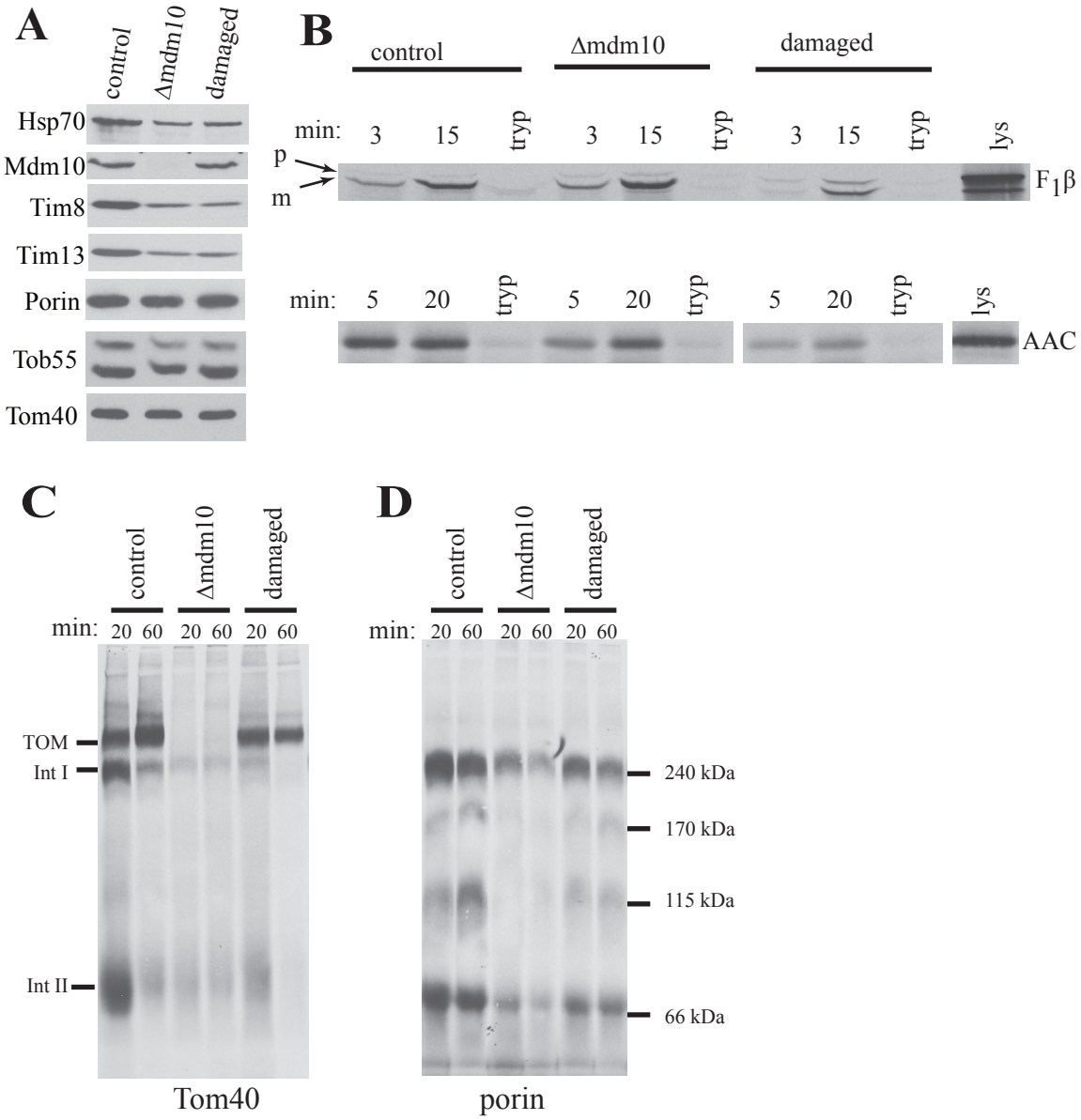


Figure 2.8 . Comparison of mitochondria isolated from the *Δmdm10* strain and damaged wild type mitochondria. (A) Mitochondria isolated from the control strain 76-26, the *Δmdm10* strain, and damaged 76-26 mitochondria (damaged) were compared on Western blots using antibodies directed against the proteins indicated. Each lane contained 30 μg of mitochondrial protein. (B, C, and D) Import of $F_1\beta$ and AAC, Tom40, and porin were as described in the legends to Figures 2.4A, B, and C, respectively.

2.3.3 Mdm10 and Tom7 affect different steps in the assembly of β-barrel proteins

Previous work in both *N. crassa* and *S. cerevisiae* had shown that mitochondria lacking Tom7 assembled porin less efficiently and Tom40 more efficiently than wild type mitochondria (Model *et al.*, 2001; Sherman *et al.*, 2005; Meisinger *et al.*, 2006). A model involving a relationship of Mdm10 with Tom7 was proposed to explain these observations in *S. cerevisiae* (Meisinger *et al.*, 2006). To explore a possible functional relationship of these two proteins in *N. crassa* we compared the assembly of Tom40 and porin in mitochondria isolated from Δ*tom7* and Δ*mdm10* single and double mutant strains. As reported previously (Sherman *et al.*, 2005), assembly of Tom40 into the final TOM complex occured more rapidly in mitochondria lacking Tom7 and a TOM complex of slightly smaller size was produced (Figure 2.4B). However, in mitochondria lacking both Mdm10 and Tom7, assembly of Tom40 to the final 400 kDa form appears to occur less efficiently than in wild-type or Tom7-deficient mitochondria, but more efficiently than in the single Mdm10 mutant (Figure 2.4B). The final TOM complex in the double mutant has the same size as the single *Δtom7* mutant. For porin, there was a slight decrease in the formation of all the complexes in mitochondria lacking Tom7 which was less severe than the defects seen in mitochondria lacking Mdm10. However, the defects in porin assembly in the double mutant were greater than in either single mutant (Figure 2.4C) demonstrating that the deficiencies observed in the single mutants are additive.

2.3.4 Decreased levels of assembled TOM complex in mitochondria lacking Mdm10

We were struck by the apparent discrepancy between the almost normal steady state, carbonate-resistant, levels of Tom40 in the mitochondrial outer membrane of the *Δmdm10* strain (Figures 2.1B and 2.5A), compared to the reduced ability of mitochondria from this strain to assemble the protein to the final TOM complex *in vitro* (Figure 2.4B). It seemed plausible that the steady-state levels of Tom40 observed by western blot analysis following standard SDS-PAGE might not

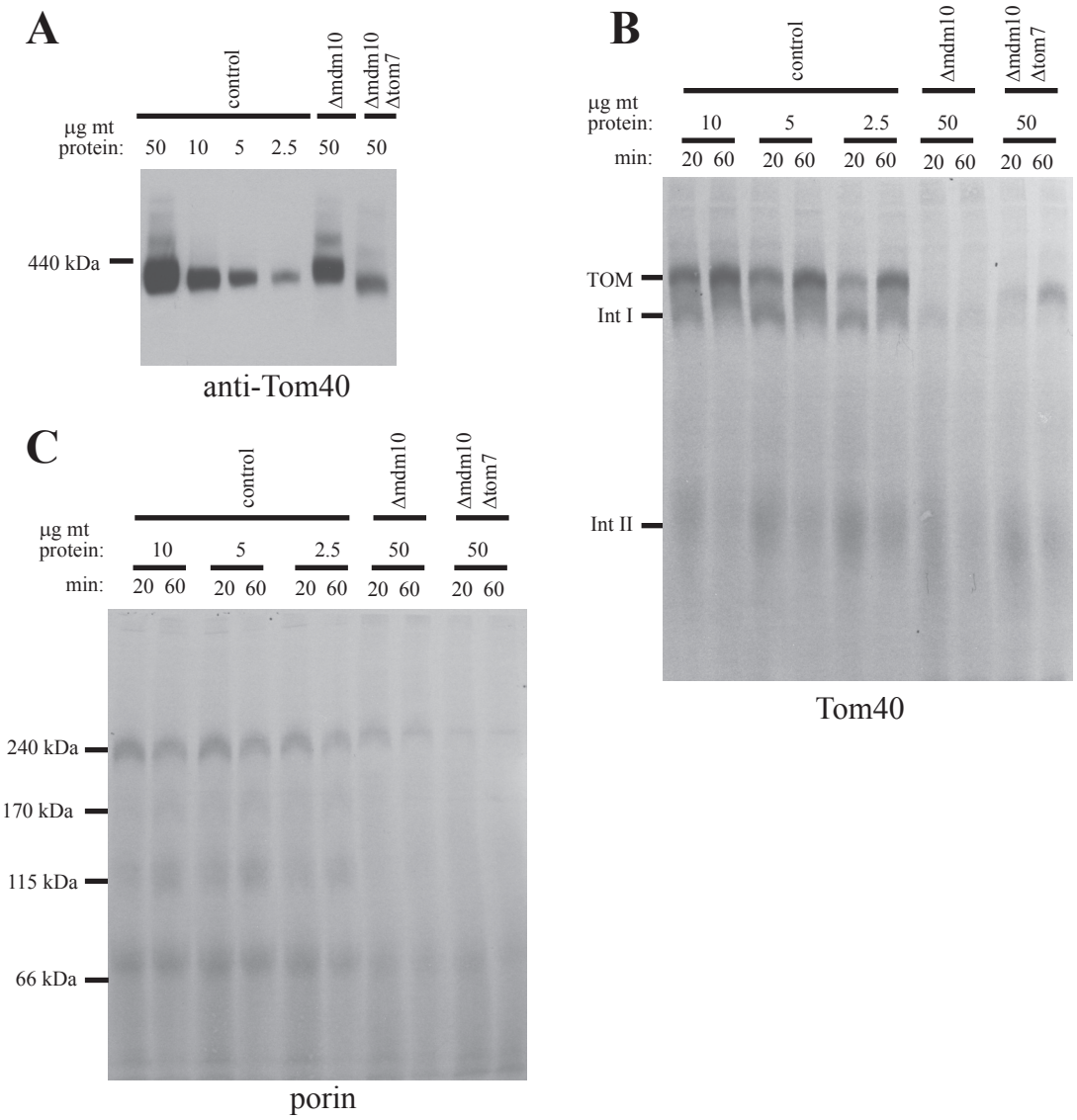


Figure 2.9. Reduced amounts of TOM complex in strains lacking Mdm10. (A) Mitochondria from the indicated strains were subjected to BNGE, blotted to PVDF membrane, and decorated with Tom40 antibodies. Different amounts of control mitochondria were loaded to allow estimation of the degree of deficiency in the mutants. (B) Import of Tom40 precursor into differing amounts of wild type mitochondria and standard amounts (50 µg) of mutant mitochondria. Further processing was as described in the legend to Figure 2.4B. (C) As in B, except that the radiolabeled precursor of porin was imported.

reflect the amount of the protein actually assembled into full sized, functional TOM complex. Lack of Tom40 assembly in vivo could be caused by the absence of Mdm10 and/or the somewhat reduced level of the core TOM complex component, Tom22 (Figure 2.7A). To test for levels of fully formed TOM complex we prepared blots following BNGE and decorated these with Tom40 antibodies. Under these conditions it was apparent that mitochondria lacking Mdm10 or both Mdm10 and Tom7 contained a reduced level of TOM complex (Figure 2.9A, compare lanes with 50 μg mitochondrial protein). To estimate the approximate level of TOM complex in the mutants relative to wild-type mitochondria, we included a dilution series of control mitochondria on the BNGE (Figure 2.9A). Mitochondria lacking Mdm10 contained about 50% of the control level of TOM complex while the double mutant contained only about 15-20%. Since the amount of Tom40 observed in standard western blots following SDS-PAGE did not suggest a large difference in the amounts of the protein between the different strains, we expected to see subcomplexes or monomers of Tom40 on the native gels. However, only small amounts of subcomplexes were seen on long exposures (unpublished observations). Thus, it is not apparent where the rest of Tom40 is on the native gels. Conceivably, some Tom40 in the mutants is present in many different forms that do not migrate as one or two discrete bands that are easily detected. It is also conceivable that aggregates form that do not enter the blue gel when the membranes are dissolved in digitonin.

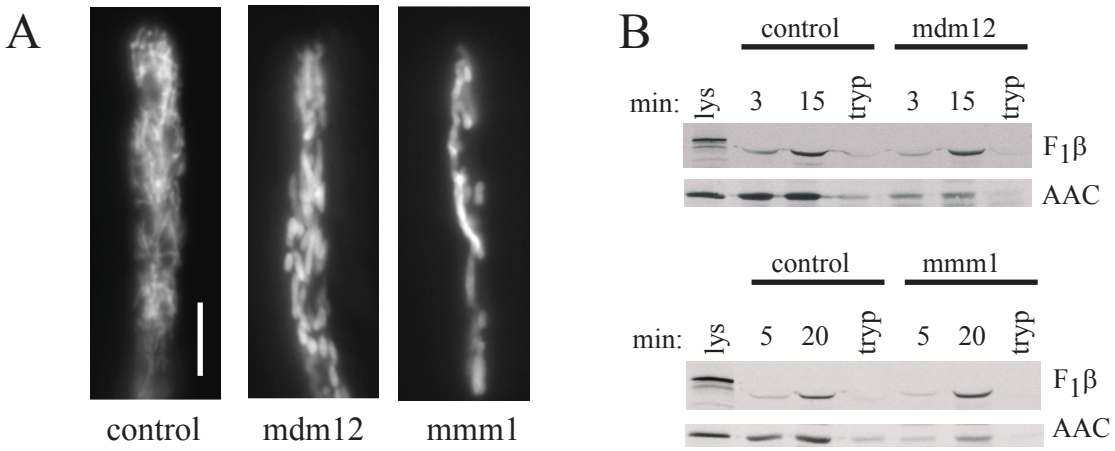
Since there were reduced levels of TOM complex in the mutants we compared the efficiency of import in the mutants to reduced amounts of control mitochondria. Even when only 5% of the amount of control mitochondria typically present in an import reaction were used, they still imported and assembled Tom40 and porin more efficiently than the mutant mitochondria (Figure 2.9B and C). This is likely due to the fact that TOM complexes/mitochondria are present in great excess to the number of radiolabeled precursor molecules in the standard reactions. Taken together, these data show that the amount of TOM complex is reduced in the mutants, but the reduction does not account for the deficiencies of β-barrel assembly seen in vitro.

2.3.5 *N. crassa* mitochondria lacking Mdm12 or Mmm1 are deficient in β-barrel assembly

The finding that *N. crassa* mitochondria lacking Mdm10 assembled β-barrel proteins less efficiently made it of interest to determine if removing two other ERMES proteins originally identified as required for maintenance of mitochondrial morphology would have a similar effect. A *N. crassa* mutant in the *mdm12* gene had previously been isolated as a hyphal growth morphology mutant (Seiler and Plamann, 2003) and a repeat induced point mutant of the *N. crassa mmm1* gene had been previously described as a mitochondrial morphology mutant containing large mitochondrial tubules (Prokisch *et al.*, 2000). We found that mitochondria in the *mdm12* strain existed as large diameter tubules similar to those of the *mdm10* and *mmm1* mutants (Figure 2.10A). We then examined import of the $F_1\beta$ and AAC precursors into mitochondria isolated from these mutant strains. The pattern of import was similar to that in the *mdm10* mutant with $F_1\beta$ similar to the control and AAC import somewhat reduced (Figure 2.10B). Again, the large size of these mitochondria suggested that breakage might occur during isolation and, as in the *Δmdm10* mitochondria, a deficiency of the small Tim proteins and Hsp70 was observed in both mutants (Figure 2.10C). Other mitochondrial proteins were found at levels similar to those in wild type. Tom40 assembly in the *mdm12* mutant was found to be deficient, but unlike in mitochondria lacking Mdm10, at least some assembly into the final 400 kDa form was observed (Fig 2.8D). The assembly of porin resembled the pattern seen in mitochondria lacking Mdm10 (Figure 2.10D). The assembly of Tom40 and porin was reduced in mitochondria from the *mmm1* mutant (Figure 2.10E) in a fashion similar to mitochondria lacking Mdm12. Thus, loss of either the Mdm12 or Mmm1 proteins leads to defects in the assembly of β-barrel proteins in *N. crassa*.

Comparison of growth rates of the three mutants revealed no differences from wild type for the *Δmdm*10 strain while the *mdm12* mutant has a slight growth defect at 30°C and 37°C, which was exacerbated when grown at 16°C (Figure 2.10F). As shown previously (Prokisch *et al.*, 2000), the *mmm1* mutant

Figure 2.10. Characterization of mitochondria from *mdm12* and *mmm1* mutants. (A) Hyphae were incubated with mitotracker green and examined by fluorescence microscopy. The bar represents 10 μm. (B) Precursors of $F_1\beta$ and AAC were imported into mitochondria isolated from the *mdm12* and *mmm1* mutant strains as described in Figure 2.4A (C) Mitochondrial proteins in the *mdm12* and *mmm1* mutants. Mitochondria were isolated from the indicated strains and subjected to Western blot analysis (30 μg mitochondrial protein per lane) using antibodies directed against the indicated proteins. (D) Import of radiolabeled Tom40 (left) and porin (right) into mitochondria isolated from the *mdm12* mutant as described in the legend to Figure 2.4B and C (E) Import of Tom40 (left) and porin (right) into mitochondria isolated from the *mmm1* mutant as described in the legend to Figure 2.4B and C (F) Growth rate of mutant strains. Conidiaspores from the control (76-26) and indicated mutant strains were counted and diluted to the desired concentrations. 10^4, 10^3, and 10^2 conidia from each strain were spotted on plates containing Vogel's medium with sorbose. The plates were incubated at 16°C for 72 hr, 30°C for 48 hr, or 37°C for 48 hr and then photographed.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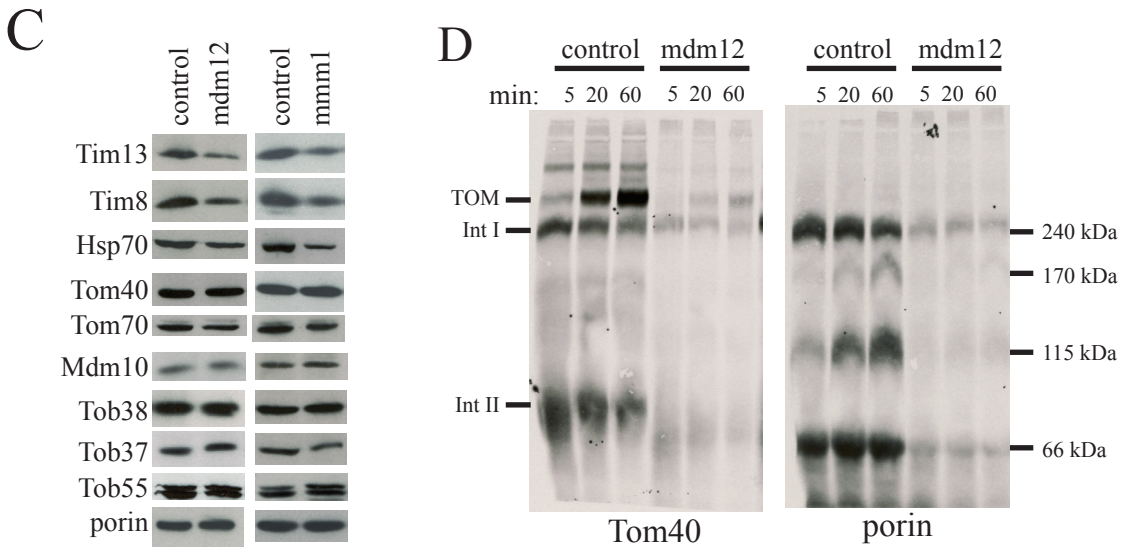
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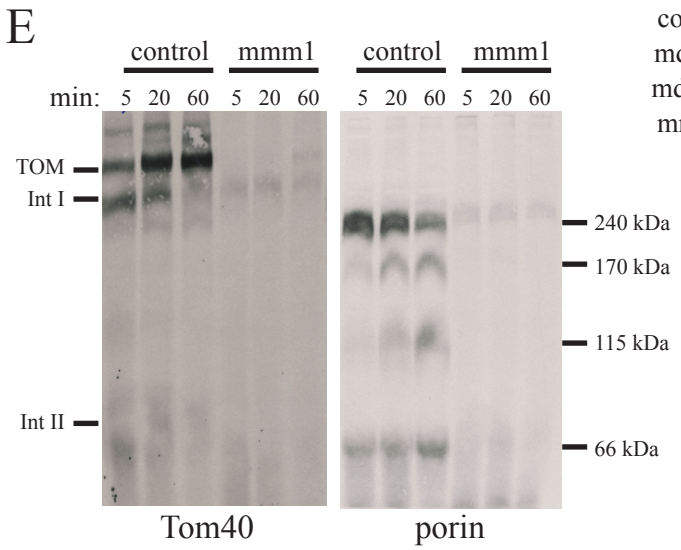
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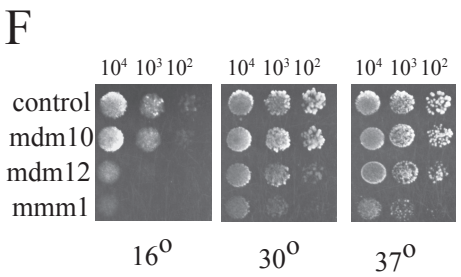
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has a more severe growth defect that is apparent at all temperatures examined (Figure 2.10F). Considering that all these mutants have similar morphological and import characteristics, the severe growth defect of the *mmm1* mutant may indicate additional functions for Mmm1 that are not shared with the other two proteins.

2.4 DISCUSSION

I have shown that *N. crassa* mitochondria lacking either Mdm10, Mdm12, or Mmm1 exist as large diameter tubules and do not efficiently assemble the β-barrel proteins Tom40 and porin into the mitochondrial outer membrane. Absence of any one of the proteins results in decreased formation of all four porin complexes that are observed in assembly assays with wild type mitochondria. For Tom40 assembly, mitochondria lacking Mdm12 or Mmm1 are deficient in formation of the 250 kDa and 100 kDa intermediates of the assembly pathway, as well as the final 400 kDa TOM complex. In mitochondria lacking Mdm10 the amount of Tom40 assembled into the final 400 kDa TOM complex is more dramatically reduced relative to the reduction in the amount of Tom40 in the intermediates.

I also characterized the relationship between Mdm10 and Tom7 in the assembly of Tom40 and porin. *N. crassa* and *S. cerevisiae* mitochondria lacking Tom7 show an increased rate of Tom40 assembly and a decreased ability to assemble porin (Sherman *et al.*, 2005; Meisinger *et al.*, 2006). Here I demonstrate that *N. crassa* mitochondria lacking both Mdm10 and Tom7 show an additive effect of the two single mutations with respect to the assembly of both porin and Tom40. Thus, in the double mutant the rate of incorporation of Tom40 into the TOM complex is faster than in mitochondria lacking Mdm10 and slower than in mitochondria lacking Tom7. For porin, the inefficient assembly observed in the *Δmdm10Δtom7* mutant mitochondria is greater than the decrease seen in either single mutant. These results suggest that Mdm10 and Tom7 affect different steps of the assembly pathways for these β-barrel proteins.

My findings with *N. crassa* differ in several respects from observations in *S. cerevisiae*. For example, mitochondria lacking Mdm10 in *S. cerevisiae* are also

inefficient at assembling Tom40 into the final TOM complex but, unlike *N. crassa*, they are more efficient in assembling porin into the outer membrane (Meisinger *et al.*, 2004; Meisinger *et al.*, 2006). In addition, the *N. crassa* results do not fit with a model developed to explain the assembly patterns of Tom40 and porin in yeast mitochondria lacking either Mdm10 or Tom7 (Meisinger *et al.*, 2006) . The basis of the model for *S. cerevisiae* is the existence of two forms of the TOB complex—a TOB holo complex containing Mdm10 that favors assembly of Tom40, and a TOB core complex without Mdm10 that favors porin assembly. The equilibrium between the two forms is thought to be influenced by Tom7 which was shown to be capable of forming a complex with Mdm10 (Meisinger *et al.*, 2006). Thus, in wild type mitochondria, Tom7 would sequester a certain amount of Mdm10 and the ratio of the two forms of the TOB complex would be maintained at a standard level. However, in mitochondria lacking Tom7 the normally sequestered population of Mdm10 would be available for other interactions resulting in an increase in the level of the TOB holo complex and a decrease in the level of the TOB core complex. Examination of this model suggests that the import phenotype of a *Δmdm10Δtom7* double mutant should not differ from the single *Δmdm10* mutant, since in both cases the level of TOB holo complex would be reduced to a similar extent due to lack of Mdm10. However, as described above, our results with *N. crassa* clearly show that mitochondria lacking both Mdm10 and Tom7 assemble Tom40 and porin in a fashion that suggests the effects of the two mutations are additive. Interestingly, while *Δmdm10Δtom7* exhibit synthetic growth defects in *S. cerevisiae* (Hoppins *et al.*, 2011), here I show that *N. crassa* lacking both proteins exhibit wild-type growth but synthetic effects in the assembly of β-barrels.

The Nargang lab has previously suggested a model to explain the assembly of Tom40 and porin in mutants of *N. crassa* lacking Tom7 (Sherman *et al.*, 2005). Newly imported Tom40 subunits might simply be incorporated into the TOM complex more easily in mitochondria lacking Tom7 since loss of the protein results in decreased complex stability (Sherman *et al.*, 2005) and assembly of new subunits into the TOM complex occurs by replacement of existing subunits

(Rapaport and Neupert, 1999; Model *et al.*, 2001; Rapaport *et al.*, 2001). Decreased porin import was suggested to be due to a specific role of Tom7 in the import or assembly of the porin precursor. To incorporate my current findings into this model, I suggest that lack of Mdm10 results in a general effect on the function of the TOB complex which is manifested in a reduced rate of incorporation of β-barrel precursors into the membrane. Since the amount of Tom40 precursor that reaches the final TOM complex is most seriously affected, it is conceivable that some of the molecules put into the membrane by the compromised TOB complex in the absence of Mdm10 are improperly inserted so that fewer precursors exist in the correct conformation to complete the assembly pathway. Loss of Mdm12 or Mmm1 may also result in general defects of TOB complex function.

Further differences between *N. crassa* and *S. cerevisiae* were also noted. Mitochondria in *N. crassa Δtom7* cells resemble those in wild type cells and may even be slightly thinner than the wild type tubules. In *S. cerevisiae*, mitochondria lacking Tom7 are enlarged and unevenly distributed in cells (Dimmer *et al.*, 2002; Meisinger *et al.*, 2006). Interestingly, the effects of Tom7 depletion in human cells more closely resemble those in *N. crassa* with respect to TOM complex stability and mitochondrial morphology (Kato and Mihara, 2008). The growth phenotypes of the *N. crassa mdm10*, *mdm12*, and *mmm1* mutants are generally less severe than those seen in the corresponding yeast mutants. This may be due to the effects the mutations have on mitochondrial distribution in yeast (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Berger *et al.*, 1997) where a daughter cell must receive a mitochondrion to insure its survival. The coenocytic nature and hyphal extension growth character of *N. crassa* may mitigate possible effects on mitochondrial distribution since growth of the mycelium does not involve formation of new free living daughter cells or cellular compartments.

My results clearly show that *N. crassa* cells lacking either Mdm10, Mdm12, or Mmm1 contain mitochondria with altered morphology that are unable to efficiently assemble porin and Tom40 into the outer membrane. However, virtually nothing is known about the actual mechanism of action of these proteins

and how the disruption of their function gives rise to multiple phenotypes. Mdm10 has been the most actively studied of the three proteins and in *S. cerevisiae* it has been shown to have a variety of interactions including association with the TOB complex, binding to Tom7, and formation of the ERMES complex with Mdm12, Mmm1, and Mmm2. Each of these interactions may explain aspects of the phenotypes observed in strains lacking Mdm10. Thus, some of the observed phenotypes may be secondary effects that result from loss of a single primary function. One possibility for the primary function of Mdm10 might be its role in the formation of ERMES complex (Kornmann *et al.*, 2009). The ERMES complex has been suggested to be involved in the transport of lipids between the ER and mitochondria and alterations in membrane lipid composition are known to have effects on the topogenesis of membrane proteins (Dowhan and Bogdanov, 2009). Additionally, several reports have described alterations in mitochondrial membrane protein function as the result of changes in lipid composition. For example, changes in cardiolipin levels have been shown to affect the assembly and activity of AAC and the TIM23 complex (Jiang *et al.*, 2000; Kutik *et al.*, 2008; Claypool, 2009; Klingenberg, 2009; Tamura *et al.*, 2009). Furthermore, it has been demonstrated that the activities of the TOM and TOB complexes are altered in mutants affecting cardiolipin synthesis (Gebert *et al.*, 2009). Altered lipid composition might also affect mitochondrial morphology. Much more work will be required to define the precise functions and mechanisms of action of Mdm10, Mdm12, and Mmm1 and to distinguish between primary and secondary phenotypic effects observed in mutants.

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CHAPTER 3. The *N. crassa* TOB complex.

Based on parts of:

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and

Lackey, S.W., **Wideman, J.G.**, Kennedy, E.K., Go, N.E., and Nargang, F.E. (2011). The Neurospora crassa TOB complex: analysis of the topology and function of Tob38 and Tob37. PLoS One *6*, e25650.

and unpublished findings.

Note: Westerns and import/assembly assay in Figure 3.2F-G were done by S.W.K. Lackey. Growth test in Figure 3.3F was done by F.E. Nargang. Strains expressing His-tagged versions of TOB components were constructed by N.E. Go

3.1 INTRODUCTION

As discussed in section 1.4, the TOB complex is required for efficient assembly of mitochondrial outer membrane β-barrels as well as C-terminally inserted outer membrane α-helical proteins of the TOM complex (Kozjak *et al.*, 2003; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004; Stojanovski *et al.*, 2007; Thornton *et al.*, 2010). In *S. cerevisiae* the major forms of the TOB complex are a core complex composed of Tob55, Tob37, and Tob38 (Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004), and the TOB holo complex that forms when the TOB complex interacts with Mdm10 (Meisinger *et al.*, 2004; Meisinger *et al.*, 2006). As discussed in section 1.8, the original reports in *S. cerevisiae* that Mdm10 interacted with the TOB complex were controversial for two reasons. Firstly, Mdm10 was originally identified as a protein involved in maintaining mitochondrial morphology, and secondly, only very small amounts of Mdm10 were shown to associate with the TOB complex. In this chapter I use strains expressing tagged versions of TOB complex components to investigate the nature of the relationship between Mdm10 and the TOB complex in *N. crassa*.

In addition to the TOB core and TOB holo complexes, Tob55 has been shown to be a member of a number of other larger complexes. In HeLa cells and human heart mitochondria Tob55 exists in a large complex and interacts with mitofilin (Xie *et al.*, 2007; Darshi *et al.*, 2011; Ott *et al.*, 2012), a MIM protein that is involved in the biogenesis of cristae junctions (John *et al.*, 2005; Rabl *et al.*, 2009) (see section 1.4.10). A similar interaction has recently been reported in *S. cerevisiae* (Harner *et al.*, 2011; Korner *et al.* 2012). The *S. cerevisiae* TOB complex forms additional high molecular weight TOB complexes that may occur as intermediates during the import and assembly of OM proteins: one by interacting with Tom40 and Tom5 (Thornton *et al.*, 2010), another by interacting with Mim1 (Stojanovski *et al.*, 2007; Becker *et al.*, 2008; Stroud *et al.*, 2011), and a third composed of TOB holo also interacting with Tom22 (Thornton *et al.*, 2010). Although likely transient in nature, these three complexes contribute to the complex equilibrium of protein complexes that exist in the MOM.

In this chapter I use 9 x histidinyl tagged versions of Tob55, Tob37, and Tob38 and BNGE to investigate the nature of the TOB complexes of *N. crassa*. Interestingly, *N. crassa* and other *Neurospora* species have evolved to express three different isoforms of Tob55 (termed short, intermediate and long) (Hoppins *et al.*, 2007). Strains expressing only the short or intermediate forms of Tob55 have no noticeable phenotype, while a strain expressing only the long isoform exhibited a growth phenotype at 37°C. The latter strain also exhibited Tom40 and porin assembly defects when mitochondria were isolated from cultures grown at 37°C. I have begun an investigation into the effects that the different isoforms have on the ability of Tob55 to assemble into different TOB complexes. Since the TOB complex is involved in the biogenesis of Tom22 (Stojanovski *et al.*, 2007; Thornton *et al.*, 2010; Becker *et al.*, 2011) I also investigate the roles that the different Tob55 isoforms have on the biogenesis of Tom22 in *N. crassa*. Some of the data in this chapter are preliminary but may lead to more detailed future investigations.

3.2 MATERIALS AND METHODS

3.2.1 Growth and handling of *N. crassa* strains

Strains used are listed in Table A.1. Growth and handling was performed as described in section 2.2.1.

3.2.2 Affinity purification of TOB complexes using Ni-nitrilotriacetic acid (Ni-NTA) columns

Mitochondria were isolated, as described in section 2.2.4, from strains expressing 9x-histidinyl-tagged versions of Tob37, Tob38, or Tob55 rather than the wild type proteins. Sample preparation and Ni-NTA chromatography was performed as described previously (Meisinger *et al.*, 2004) except that 20 mM imidazole was included during binding of complexes to the Ni-NTA resin. Mitochondria were dissolved in pulldown buffer (20mM Tris-HCl, pH 7.4; 0.1 mM EDTA; 50mM NaCl; 10% [w/v] glycerol) containing 1% digitonin. After a clarifying spin,

proteins were incubated with Ni-NTA agarose for 2 hr at 4 °C and then loaded onto a column. The flow through was collected and the bound proteins were washed with 10 column volumes of wash buffer (20mM Tris-HCl, pH 7.4; 0.1 mM EDTA; 200mM NaCl; 10% [w/v] glycerol; 30 mM imidazole) containing 0.2% digitonin and then with 20 column volumes of wash buffer containing 40 mM imidazole. For elution, a concentration of 200 mM imidazole was used. Proteins in the collected flow through, wash and elution fractions were analyzed in two different ways. In the first, proteins in the various fractions following Ni-NTA purification were precipitated in 15% trichloroacetic acid, washed with acetone and then subjected to SDS-PAGE and Western blotting. Second, for BNGE of purified His-tagged proteins, one tenth volume of 10x BNGE sample buffer (100 mM bis-Tris, pH 7.0; 500 mM 6-aminocaproic acid; 5% Coomassie brilliant blue G250) was added directly to the elution fractions. After electrophoresis, gels were transferred to PVDF for Western blot analysis.

3.2.3 Two-dimensional gel electrophoresis of proteins

For two-dimensional electrophoresis, lanes from the first dimension BNGE were excised and soaked in SDS-PAGE cracking buffer (0.06 M Tris-HCl, pH 6.8, 2.5% SDS, 5% sucrose and 5% β-mercaptoethanol) for 5 min. Treated lanes were then placed between the glass gel plates, onto the stacking gel, of a pre-made gel for second dimension SDS-PAGE. Following electrophoresis, gels were transferred to nitrocellulose and immunodecorated with specific antibodies.

3.2.4 Mitochondrial protein import and carbonate extraction

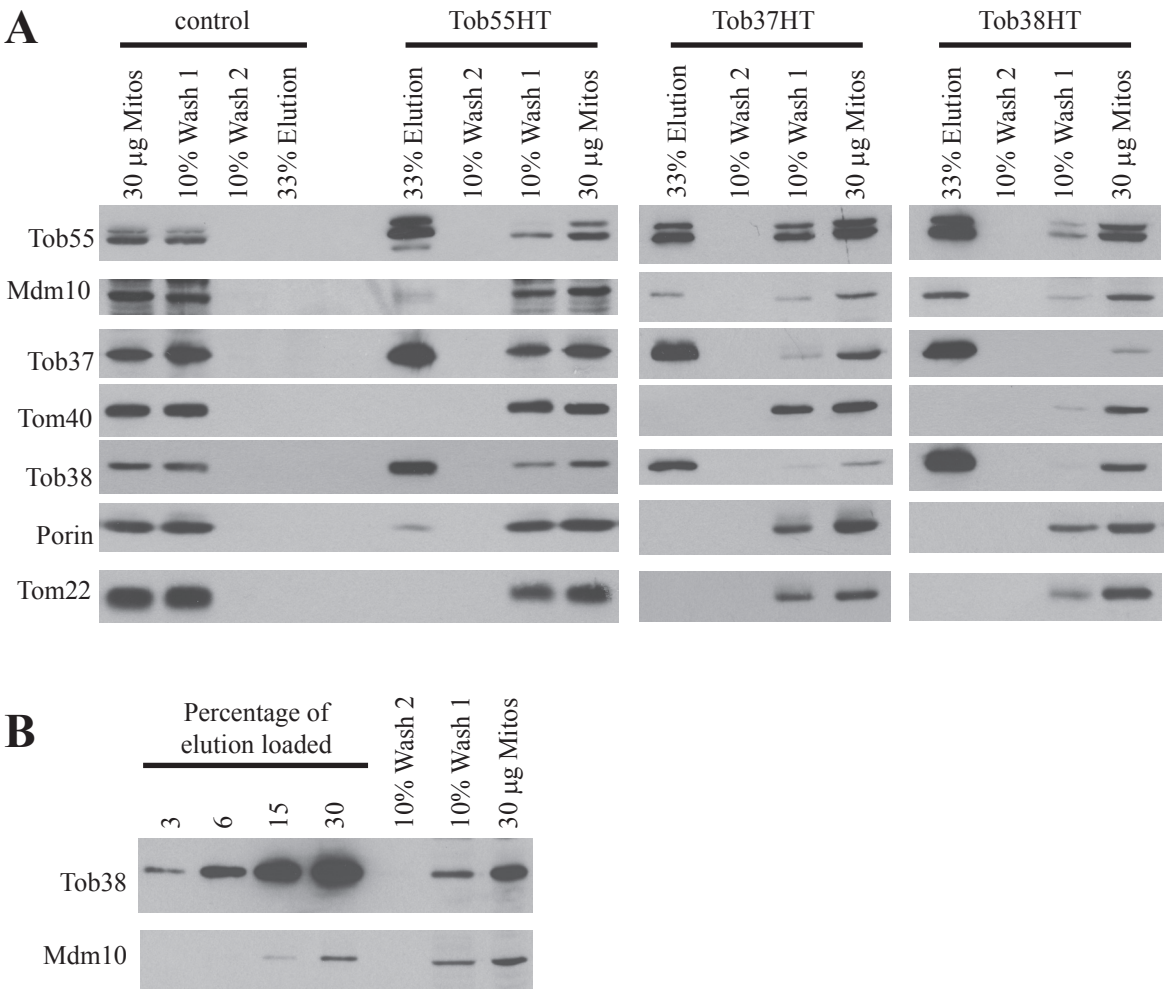
Import of radioactive precursors was performed as described in section 2.2.7. In some cases, after import of radiolabelled precursors, mitochondria were subjected to a carbonate extraction step (as described in section 2.2.4). Comparative quantification of radiolabeled protein was performed on scanned X-ray film using Adobe Photoshop.

3.3 RESULTS

3.3.1 Small amounts of Mdm10 are associated with the TOB complex

To determine if Mdm10 associates with the TOB complex in *N. crassa* I performed affinity binding co-purification experiments using strains that express only His-tagged versions of the individual components of the TOB core complex, Tob55, Tob38, or Tob37. Isolated mitochondria from each of these strains assemble Tom40 and porin at rates indistinguishable from wild type (N.E. Go, unpublished observations). Mitochondria from each strain were dissolved in digitonin and subjected to Ni-NTA chromatography. Fractions were eluted with imidazole and analyzed by SDS-PAGE and immunodecoration (Figure 3.1A). For the Tob55 His-tagged strain (Tob55HT), each of the three proteins of the TOB core complex was enriched in the elution fraction relative to the amounts in a standard mitochondrial load lane, suggesting a strong association of Tob37 and Tob38 to the His-tagged Tob55. A small amount of porin was also co-eluted which may reflect its abundance in the outer membrane. Alternatively, small amounts of porin could co-elute as an assembly intermediate trapped in the isolated TOB complex. It is also conceivable that a fraction of Tob55 exists in a stable complex with porin. None of the other outer membrane components examined were detected in the elutions with the exception of Mdm10. However, the ratio of Mdm10 in the elution, relative to the amount in the standard whole mitochondrial load lane was many fold lower than the ratio for the TOB proteins. With the tagged Tob37 strain (Tob37HT), both Tob37 and Tob38 were enriched several fold in the elution, while Tob55 was present at roughly the same level in the standard mitochondrial and elution lanes. Eluted Mdm10 was reduced about two or three fold, relative to the standard lane. All components of the TOB core complex were enriched several fold when His-tagged Tob38 (Tob38HT) mitochondria were examined. Although Mdm10 was present in the elution, its enrichment was again many fold lower than the TOB proteins. To quantify the relative fold purification of a TOB component relative to Mdm10, I prepared a dilution series of the elution fraction from a co-purification experiment using

Figure 3.1. Co-purification of outer membrane proteins with TOB core complex components. (A) Mitochondria were isolated from a control strain (76-26) containing no His-tagged proteins and strains expressing His-tagged versions of Tob37 (Tob37HT), Tob38 (Tob38HT), or all three isoforms of Tob55 (Tob55HT). The mitochondria (2.5 mg) were solubilized in 1% digitonin in the presence of 50 mM NaCl and 20 mM imidazole. Following clarification by centrifugation, the supernatants were bound to Ni-NTA resin and loaded onto columns for affinity purification of His-tagged TOB proteins and any associated proteins. The column was washed with 10 column volumes of buffer containing 30 mM imidazole and twenty volumes of buffer containing 40 mM imidazole. Elution was performed using two column volumes of buffer containing 200 mM imidazole. Samples from the indicated fractions were precipitated with trichloroacetic acid and subjected to SDS-PAGE and Western blot analysis using antibodies to the proteins indicated. The lane containing 30 μg of mitochondria serves as a standard to compare relative levels of proteins present in the elution. (B) Comparison of amounts of Tob38 and Mdm10 purified from Tob37HT mitochondria. Elution fractions were obtained as described in panel A. Dilutions of the elution fraction were also examined to estimate the relative fold purifications of Tob38 and Mdm10 relative to the amounts in a standard total mitochondrial proteins lane.

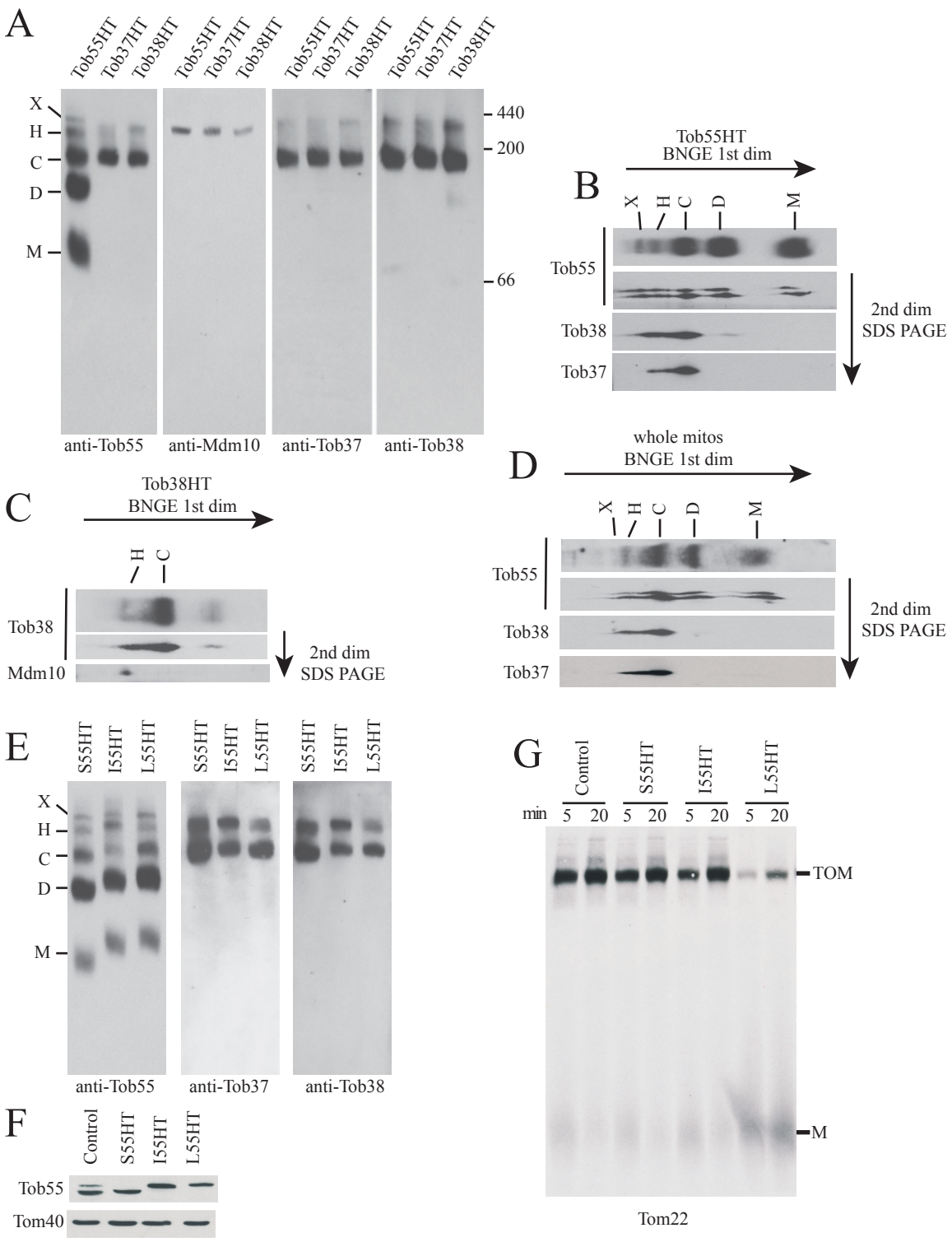

A
control
Tob55HT
Tob37HT
Tob38HT
30 µg Mitos
10% Wash 1
10% Wash 2
33% Elution
33% Elution
10% Wash 2
10% Wash 1
30 µg Mitos
33% Elution
10% Wash 2
10% Wash 1
30 µg Mitos
33% Elution
10% Wash 2
10% Wash 1
30 µg Mitos
Tob55
Mdm10
Tob37
Tom40
Tob38
Porin
Tom22
B
Percentage of elution loaded
3
6
15
30
10% Wash 2
10% Wash 1
30 µg Mitos
Tob38
Mdm10

Tob37HT mitochondria. The dilutions were subjected to SDS-PAGE and Western blot analysis using antibodies to Mdm10 and Tob38 (Figure 3.1B). Relative to the standard mitochondrial load lane, the amount of Mdm10 in the undiluted elution lane was about two fold decreased. On the other hand, Tob38 was enriched between five and ten fold. Thus, the enrichment of Tob38 over Mdm10 is between ten and twenty fold. Taken together these data suggest that Mdm10 associates with the TOB complex, but it is present at a much reduced level compared to the core components.

3.3.2 Multiple TOB complexes exist in *N. crassa* mitochondria

The number of TOB complexes, their components, and their size has not been investigated in *N. crassa*. BNGE examination of the complex purified from mitochondria containing His-tagged versions of the different TOB complex components revealed that Tob55, Tob38, and Tob37 are all found together in two complexes of about 280 kDa and 190 kDa (Figure 3.2A). In addition, Tob55 appears alone in two smaller complexes of about 75 and 140 kDa and one larger complex of 370 kDa. These latter three complexes are virtually devoid of Tob37 or Tob38, though a small amount of Tob38 is detectable in the 140 kDa form. Two dimensional gel electrophoresis (BNGE followed by SDS-PAGE) of TOB complex purified from mitochondria containing His-tagged Tob55 confirms these observations (Figure 3.2B), as does the finding that the 370 kDa, 140 kDa, and 75 kDa complexes are not observed when purification is performed using His-tagged Tob37 or Tob38 mitochondria (Figure 3.2A). Mdm10 appears only in the 280 kDa complex (Figure 3.2A). This was also confirmed by two-dimensional gel analysis (Figure 3.2C) of TOB complex purified from mitochondria containing His-tagged Tob38. Thus, the 190 and 280 kDa complexes appear to correlate with the TOB core and TOB holo complexes, respectively, that have been defined in *S. cerevisiae* (Ishikawa *et al.*, 2004; Meisinger *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004; Meisinger *et al.*, 2007; Yamano *et al.*, 2010a). It should be noted that in mock purifications, using control mitochondria without His-tagged proteins, no bands are observed when blots are immunodecorated with

Figure 3.2. The *N. crassa* TOB complex. (A) Mitochondria were isolated from strains expressing only His-tagged versions (instead of the endogenous versions) of either Tob55 (Tob55HT), Tob38 (Tob38HT), or Tob37 (Tob37HT) as indicated. TOB complexes were purified using Ni-NTA resin. Purified complexes were subjected to BNGE, transferred to PVDF membrane, and decorated with antibodies to Tob55, Mdm10, Tob37, or Tob38 as indicated at the bottom of each panel. The position of molecular weight markers (kDa) is shown on the right and the position of the 370 kDa (X) 280 kDa (H) 190 kDa (C) 140 kDa (D) and 75 kDa (M) TOB complexes are indicated on the left. The 370 kDa form is also indicated (B) TOB complex was purified from a strain carrying His-tagged Tob55 expressed from a genomic tob55 sequence that results in the expression of all three isoforms. The purified complex was subjected to first dimension BNGE (1st dim) in two separate lanes of the gel. One lane was transferred to PVDF, and decorated with antibody to Tob55 (top lane in panel). The second lane was removed for second dimension (2nd dim) electrophoresis by SDS-PAGE as described in section 3.2.3. Following SDS-PAGE, the gel was transferred to nitrocellulose and analyzed by Western blot for the presence of the indicated proteins. TOB complexes are marked as in A. (C) As in panel B, except mitochondria containing His-tagged Tob38 were used and the SDS-PAGE blot was examined with antibodies to Tob38 and Mdm10. (D) As in panel B except that whole mitochondria were examined for the presence of TOB complexes. (E) As in panel A, except TOB complex was purified from mitochondria isolated from cells expressing only His-tagged versions of different Tob55 isoforms: short Tob55 (S55HT), intermediate Tob55 (I55HT), or long Tob55 (L55HT) as indicated at the top of the panels. Blots were immunodecorated with the antibodies indicated at the bottom of the panels. (F) Western blot analysis of whole mitochondria from the indicated strains following SDS-PAGE. (G) Mitochondria from the indicated strains were incubated with radiolabeled Tom22, reisolated, dissolved in 1% digitonin and subjected to BNGE. Proteins were then transferred to PVDF and analyzed by autoradiography. Tom22 monomer (M) and the position of the assembled TOM complex (TOM) are indicated.


A
Tob55HT
Tob37HT
Tob38HT
X
H
C
D
M
440
200
66
anti-Tob55
anti-Mdm10
anti-Tob37
anti-Tob38
B
Tob55HT
BNGE 1st dim
Tob55
Tob38
Tob37
2nd dim
SDS PAGE
C
Tob38HT
BNGE 1st dim
Tob38
Mdm10
D
whole mitos
BNGE 1st dim
E
S55HT
I55HT
L55HT
F
Control
Tob55
Tom40
G
min
5
20
TOM
M
Tom22

antibodies to Tob55, Tob38, Tob37, or Mdm10 (Figure 3.1A). When whole mitochondria were examined by Western blot for Tob55 following BNGE or two-dimensional gel electrophoresis, a pattern similar to that observed for purified complexes was seen (Figure 3.2D). Thus, it appears unlikely that any of the complexes observed are artefacts of the purification procedure. The possibility that some complexes are breakdown products resulting from BNGE also seems unlikely, since they are not observed when complexes purified from Tob37HT or Tob38HT mitochondria are analyzed by BNGE. Recently, our collaborators have used isotope dilution mass spectrometry to determine that the TOB core complex contains Tob37, Tob38 and Tob55 in 1:1:1 stoichiometry and that the TOB holo complex has a similar 1:1:1:1 stoichiometry between these three subunits plus Mdm10. In addition they have shown that the 140 kDa complex is a Tob55 dimer and the band appearing at 75 kDa is a Tob55 monomer (Klein *et al*. 2012, submitted). The nature of the 370 kDa complex is not yet understood.

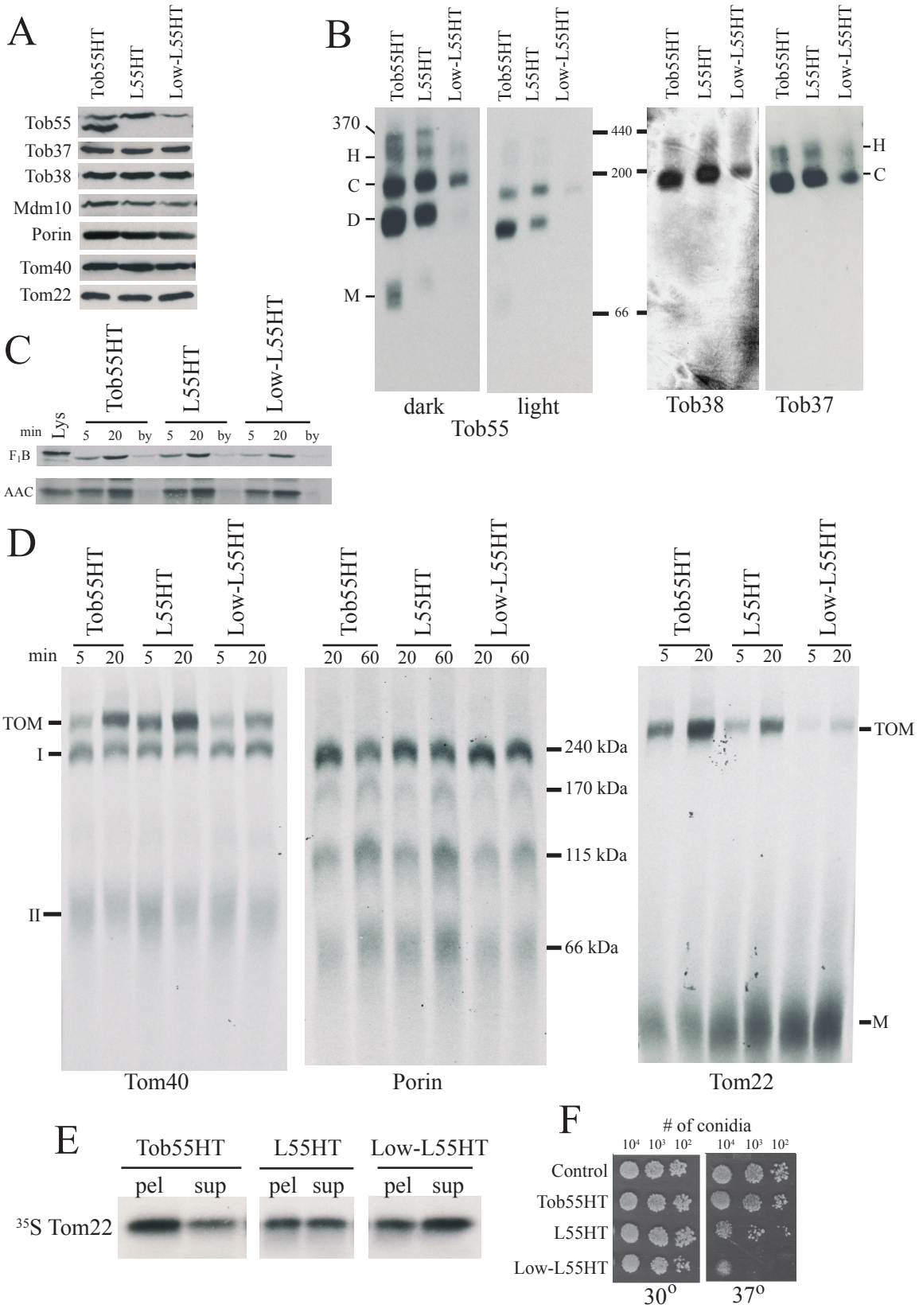
N. crassa contains three different isoforms of Tob55 (Hoppins *et al.*, 2007). To determine if any of these was specific for a given complex or set of complexes, mitochondria were isolated from strains expressing only the His-tagged versions of either the short, intermediate, or long form of Tob55 (S55HT, I55HT, and L55HT, respectively). TOB complexes were purified and analyzed by BNGE and Western blot. In each case, all five TOB complexes described above were present (Figure 3.2E). The only consistent difference observed between the isoforms was the greater ratio of the complex corresponding to TOB holo (H) in I55HT compared to the TOB core complex (C). Since I55HT and S55HT import β-barrel proteins similar to a control strain (N. E. Go, personal communication), it seems unlikely that the minor variations seen in the ratios of TOB complexes affect the import of β-barrels. Western blot analysis shows that the levels of Tob55 are roughly similar in all three strains (Figure 3.2F). However, the level of Tob55 in L55HT appears to be slightly reduced and, for unexplained reasons, is more obviously reduced in some isolations. When Tom22 is imported into mitochondria from the strains expressing the three different isoforms, assembly is basically unaffected in S55HT and I55HT, but there is an obvious defect in

L55HT (Figure 3.2G). This suggests that the long isoform of Tob55 is less efficient at assembling Tom22. However, a definite conclusion cannot be made because the slight deficiency of Tob55 in L55HT might also be the cause of the Tom22 assembly defect.

3.3.3 Assembly of TOB complex substrates in mitochondria containing different Tob55 isoforms

While constructing strains expressing the His-tagged version of the long isoform of Tob55, it was noted that one transformant expressed a very low level of Tob55 and it was reasoned that this strain could be of value in investigating the relationship between the different complexes and possibly their functions. Mitochondria were isolated from the low expressing strain (Low-L55HT), the strain expressing higher levels of the His-tagged long form (L55HT), which was used in the previous experiments (Figure 3.2), and a strain carrying a His-tagged genomic version that expresses normal levels of all three His-tagged isoforms of the protein (Tob55HT) (Figure 3.3A). Western blot analysis revealed that other proteins of the TOB complex were present in normal amounts in all strains except for a slight deficiency of Mdm10 in the two long isoform strains (Figure 3.3A). Other mitochondrial proteins tested (porin, Tom40, Tom22) were present at similar levels in all three strains (Figure 3.3A). BNGE of TOB complex purified from the three strains demonstrated that only the TOB core and TOB holo complexes were detected when Tob55 levels were very low (Figure 3.3B). The lower molecular weight forms that contain only Tob55 and no other TOB complex member could not be detected. Since the Tob55 dimer at 140 kDa is the predominant species in Tob55HT, these data show that all the forms present are not simply reduced in the same ratio. Rather, Tob55 appears to be preferentially assembled into the TOB and TOB holo complexes. In this regard it is also of note that in L55HT, there appears to be little to no difference in the levels of core and holo complex compared to controls but the dimer and monomer are slightly reduced (Figure 3.3B). This observation is slightly different than in Figure 3.2E

Figure 3.3. Effect of reduced levels of Tob55 on formation of TOB complexes and assembly of outer membrane proteins. (A) Mitochondria were isolated from the indicated strains, subjected to SDS-PAGE, and examined by Western blot analysis using the antibodies indicated. (B) Mitochondria were dissolved in 1% digitonin and Tob55 containing complexes were purified using Ni-NTA resin. Purified complexes were subjected to BNGE, transferred to PVDF membrane and immunodecorated with antibodies to Tob55, Tob37, and Tob38. Molecular mass markers are indicated as are the position of the TOB core and TOB holo complexes as well as the dimeric and monomeric forms of Tob55; H, C, D, and M, respectively. (C) Mitochondria isolated from the indicated strains were incubated with radiolabeled precursors of $F_1\beta$ and AAC. The import times are indicated. 'by' indicates bypass import (explained in section 2.2.7). (D) Mitochondria from the indicated strains were incubated with radiolabeled precursors of Tom40, porin and Tom22. Mitochondria were reisolated, dissolved in 1% digitonin and subjected to BNGE. Proteins were then transferred to PVDF and analyzed by autoradiography. The positions of the mature TOM complex as well as intermediates I and II are indicated. Positions of porin complexes are indicated by their molecular weight. Tom22 monomer and the position of the assembled TOM complex are also indicated. (E) Mitochondria isolated from the indicated strains were incubated with radiolabeled Tom22 for 20 min. Mitochondria were washed and subjected to alkaline extraction using 0.1M sodium carbonate at pH 11.0. pel, pellet; sup, supernatant. (F) Growth of strains on solid Vogel's sorbose medium at either 30 or 37°C. Numbers of conidia spotted are indicated at the top.


A
Tob55HT
L55HT
Low-L55HT
Tob55
Tob37
Tob38
Mdm10
Porin
Tom40
Tom22
B
370
H
C
D
M
440
200
66
dark
light
Tob55
Tob38
Tob37
C
Lys
min
5 20 by
F1B
AAC
D
TOM
I
II
240 kDa
170 kDa
115 kDa
66 kDa
M
Tom40
Porin
Tom22
E
pel sup
35S Tom22
F
# of conidia
10⁴ 10³ 10²
Control
Tob55HT
L55HT
Low-L55HT
30°
37°

where all complexes were present in normal ratios in L55HT. This points again to the small culture by culture differences observed for the strain.

Mitochondria from each strain were examined for their ability to import and assemble mitochondrial precursor proteins. Import assays using $F_1\beta$ and AAC revealed a minor defect in $F_1\beta$ import in the Low-L55HT mitochondria most easily seen at the 5 min time point (Figure 3.3C). Minor differences among the strains were also observed with respect to the assembly of the β-barrel proteins Tom40 and porin (Figure 3.3D). Surprisingly, L55HT appeared to assemble Tom40 into the TOM complex somewhat more efficiently than Tob55HT, while Low-L55HT assembled the protein with slightly decreased efficiency. Porin was assembled into complexes approximately equally in all three strains with perhaps a slight decrease in Low-L55HT. Mitochondria from Low-L55HT exhibited a striking deficiency in the assembly of Tom22 into the TOM complex, while a somewhat milder defect was seen for L55HT (Figure 3.3D). Quantitation of the bands representing Tom22 in the assembled TOM complex revealed that L55HT had approximately 40% of the level of Tom22 in the TOM complex when compared to Tob55HT while Low-L55HT had only 15%. A high level of radiolabeled Tom22 was seen near the bottom of the Tom22 assembly gel corresponding to the position of Tom22 monomer. To determine if the monomers had been assembled into the membrane or were simply non-specifically bound to the mitochondrial surface, alkaline extraction was performed following 20 minutes of import/assembly (Figure 3.3E). For Tob55HT, most of the radiolabeled Tom22 in the assembly assay was found in the pelleted membrane sheets. For L55HT, the pellet contained about half of the total while for Low-L55HT most of the total material was found in the supernatant, but a significant fraction was present in the pellet. These data suggest that mitochondria with low levels of the long isoform are able to integrate at least some Tom22 into the membrane but are deficient in the subsequent step of assembling it into the TOM complex. Surprisingly, growth rates of the three strains are virtually identical at 30°C (Figure 3.3F). As shown previously (Hoppins *et al.*, 2007), strains expressing only the long form of Tob55 exhibit temperature sensitive growth

(Figure 3.3F). This effect is exacerbated in the Low-L55HT strain. Taken together, these import/assembly results suggest that the long form of Tob55 preferentially assembles Tom40 into the TOM complex, however it cannot efficiently assemble Tom22. However, as noted in section 3.3.2 and for Figure 3.2, there are variations in the levels of Tob55 and its complexes in the L55HT strain. Therefore, Tom22 assembly may be dependent on overall Tob55 levels. A final possible interpretation comes from the observation that the Tob55 dimer and monomer are reduced slightly in L55HT and almost totally absent in Low-L55HT (Figure 3.3B) making it conceivable that a deficiency of one or both of these forms is responsible for the Tom22 assembly defects.

3.4 DISCUSSION

As in *S. cerevisiae* (Meisinger *et al.*, 2004; Meisinger *et al.*, 2006), our data show an association of Mdm10 with the TOB complex. However, Mdm10 only forms the TOB holo complex with a small fraction of the TOB components present in the cell. Since Mdm10 is a β-barrel it is possible that the Mdm10 that purifies with the TOB complex is interacting with the TOB complex as an assembly intermediate. However, this is unlikely as Tom40, which is much more abundant than Mdm10 (Yamano *et al.*, 2010a), does not purify with the TOB complex (Figure 3.1A).

Interestingly, other TOB complexes have been shown to exist in small amounts in *S. cerevisiae*. It has been demonstrated that TOB holo interacts with Tom22 when chemical amounts of Tom22 are imported into mitochondria (Thornton *et al.*, 2010), while another high molecular weight TOB complex requires Mim1 for its formation during the import of Tom6 (Becker *et al.*, 2008). A third TOB complex, the TOB-Tom40/Tom5 complex has also been proposed to exist in small but detectable amounts in the MOM (Becker *et al.*, 2010; Thornton *et al.*, 2010). Since these complexes all contain known substrates of the TOB complex, it is likely that these complexes represent assembly intermediates present in small amounts in the MOM. The TOM complex is composed of several proteins. Since TOM proteins are assembled into intermediates composed of some

TOM components but not others, imported TOM components cannot simply replace corresponding subunits in assembled TOM complexes. Instead, several converging assembly pathways must be coordinated to effectively assemble even a single TOM complex. Thus, it is likely that assembly pathways are not unidirectional pathways and instead can be best represented by an interconnected web of equilibrium reactions. This idea is supported by the fact that newly imported TOM complex subunits are assembled with pre-existing TOM subunits and that existing TOM complexes can exchange subunits (Rapaport *et al.*, 2001). Further evidence for this notion has been demonstrated in that the ratios between TOB core and TOB holo change depending upon how much Mdm10 is available for interaction with the TOB complex (Meisinger *et al.*, 2006; Yamano *et al.*, 2010b, a). These changes in complex ratios correlate with changes in the assembly rates of TOM precursors.

While various complexes in the MOM (TOM, TOB, Mim1/2) are assigned functions in various assembly pathways, no protein or protein complex has been assigned a role in facilitating the exchange of complex subunits. It may be the case that the TOB complex facilitates both the exchange and assembly of different TOM complex subunits. The efficiency of TOB-mediated exchange and assembly of subunits may be influenced by the amount of any given TOB complex member or substrate. A change in the amount of any given complex member, or substrate, could shift the equilibrium of complexes in the MOM and potentially increase or decrease the assembly rate of certain precursors. Understanding these equilibrium relationships may be important in understanding many of the import phenotypes seen in TOM and TOB complex mutants. Thus it would be interesting to determine how the relative ratio of the different TOB complexes is affected by the deletion or overexpression of different TOB components or TOB complex substrates in *N. crassa*. This would require the construction of deletion and overexpression strains harbouring His-tagged versions of TOB complex components.

Intriguingly, as discussed in section 1.4.10 it has been demonstrated that a portion of Fcj1 [a MIM protein involved in the formation of cristae junctions

(John *et al.*, 2005; Rabl *et al.*, 2009)], interacts with Tob55 (Xie *et al.*, 2007; Darshi *et al.*, 2011; Harner *et al.*, 2011; Korner *et al.*, 2012; Ott *et al.*, 2012). This opens up the possibility that the organization of the MIM is linked to the MOM and that these interactions could be regulated by one or more of the various TOB complexes. The Tob55-mitofilin complex may function as a scaffold to position and maintain cristae junctions at the appropriate positions and the relative amount of TOB complexes may be important for the proper formation of MIM architecture. It is conceivable that the 370 kDa complex seen following BNGE (Figure 3.2A) may represent this interaction in *N. crassa*.

It is unknown if the alternative splicing of Tob55 occurs in other genera or if it is limited to the *Neurospora* genus. Regardless, determining if the isoforms play specific roles is of considerable interest. Similarly, determining the significance of the abundant Tob55 dimeric form is also of interest. Here I demonstrate that a strain expressing very low levels of only the long isoform of Tob55 has slight reductions in Mdm10 and major alterations in the steady-state ratios of Tob55-containing complexes. I also demonstrate that a decrease in long Tob55 levels seems to primarily affect the assembly of Tom22 while β-barrel assembly is only slightly affected. Future work will be aimed at determining if it is the isoform or the deficiency of Tob55 that leads to altered complex ratios and the Tom22 assembly defects.

Given that the Tob55 dimer and monomer are more reduced as Tob55 levels decrease and that L55HT contains the TOB core and holo complexes at levels that are similar to wild type, a possible role for the dimeric and/or monomeric forms of Tob55 in Tom22 assembly is supported by observations from knockdown strains. When Tob55 is depleted to very low levels in *N. crassa*, the steady state levels of Tom22 also become very low (Hoppins *et al.*, 2007). However, when Tob37 or Tob38 levels are depleted steady state levels of Tom22 are much higher than in the Tob55 depletion experiments (Lackey *et al.*, 2011). Steady state levels of the β-barrel proteins, Tom40 and porin are similar in all three knockdown strains. Knockdown of Tob37 or Tob38 will primarily affect the TOB core and holo complexes, while knockdown of Tob55 will affect all

complexes including the dimeric and monomeric forms of Tob55. Thus, Tob55 and possibly the "Tob55 only" forms of the TOB complex, may play a crucial role in Tom22 assembly.

If we accept the hypothesis that the long isoform of Tob55 is inefficient in the assembly of Tom22 it follows that the import and assembly of Tom22 into the TOM complex is normally achieved by the action of the short and long isoforms of Tob55 in *N. crassa*. The long isoform may preferentially incorporate Tom40 into the MOM. This hypothesis is supported by the increased efficiency of Tom40 assembly in the L55HT compared to the control. Although the TOB holo complex has been shown to interact with Tom22 precursor, the slight reduction of Mdm10 seen in L55HT and Low-L55HT cannot account for the Tom22 import defects seen in these strains since *mdm10* mutants that express slightly decreased amounts of Mdm10 do not exhibit drastic Tom22 import defects (K. A. Norton and J.G. Wideman, unpublished observations).

Additionally, I demonstrated that the ratio of the different TOB complexes is dynamically regulated. At lower expression levels, Tob55 was seen primarily in the TOB core and TOB holo complexes. These results suggest that when Tob55 levels are low, formation of TOB core and TOB holo complexes is favoured over the formation of other TOB complexes. Tom22 assembly defects exist in strains with reduced Tob55 levels. At this point it is unclear if it is the change in complex ratios, the absence of the short and long isoforms of Tob55, or simply the reduced level of Tob55 that is responsible for the Tom22 assembly defect in these strains. To further understand the role that the TOB complex plays in the assembly of Tom22, Tom22 assembly experiments must be conducted using strains expressing lower amounts of Tob55. Low-S55HT and Low-I55HT strains must also be constructed to compare import phenotypes to the L55HT and Low-L55HT strains to determine if the isoform present has an effect on Tom22 assembly. Clearly, much more research is required to understand the dynamics and regulation of the different TOB complexes in *N. crassa*.

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CHAPTER 4. The *N. crassa* ERMES complex.

Based on:

Wideman, J.G., Lackey, S.W., Srayko, M.A., and Nargang, F.E. (2012). Lack of disulphide bond formation in *Neurospora crassa* Mmm1 leads to defects in Tom40 assembly but does not affect mitochondrial morphology. Submitted to the Journal of Biological Chemistry

Note: Microscopy in Figures 4.1B, 4.2, 4.5C, 4.6C, 4.7B, 4.8A was performed by MA Srayko. Western blot and protein import assays in Figure.4.8C-E were done by SWK Lackey.

4.1 INTRODUCTION

As discussed in section 1.3, regions of close apposition between the endoplasmic reticulum (ER) and mitochondria have been observed for several years and are thought to be required for lipid and calcium exchange between the two organelles [reviewed in (Hayashi *et al.*, 2009; Grimm, 2012; Michel and Kornmann, 2012)]. As outlined in section 1.3.3, in *S. cerevisiae* the ERMES complex has been shown to tether the two organelles (Kornmann *et al.*, 2009; Nguyen *et al.*, 2012) and is composed of Mdm10, Mdm12, Mmm1, and Mmm2 (Kornmann *et al.*, 2009; Stroud *et al.*, 2011). The Gem1 protein has also been found to co-purify with the ERMES complex and it may play a role in regulating the size, organization, and function of the complex (Kornmann *et al.*, 2011; Stroud *et al.*, 2011). However, a different study concluded that Gem1 is not involved in ERMES assembly or maintainence (Nguyen *et al.*, 2012).

The genes encoding the four structural ERMES proteins of *S. cerevisiae* were originally identified in genetic screens for mutants with defects in mitochondrial distribution and morphology (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Berger *et al.*, 1997; Dimmer *et al.*, 2002; Youngman *et al.*, 2004). As discussed in section 1.3.3, localization studies and analysis of mutants have suggested that each of the proteins is involved in several cellular functions. Unexpectedly, mutants lacking Mmm1, Mdm12, or Mdm10 were also shown to have defects in the assembly of β-barrel proteins into the MOM. As discussed in section 1.4.3 the process of β-barrel assembly is accomplished by the TOB complex, and as shown in Chapter 3, the Mdm10 protein has been shown to associate with the TOB complex (Meisinger *et al.*, 2004; Meisinger *et al.*, 2007; Wideman *et al.*, 2010; Lackey *et al.*, 2011) in addition to being a component of the ERMES complex. The outer membrane protein Tom22, which has one α-helical membrane spanning domain, has also been identified as a TOB complex substrate (Stojanovski *et al.*, 2007; Thornton *et al.*, 2010; Becker *et al.*, 2011) and defects in the assembly of this TOM complex protein have also been noted in *S. cerevisiae* strains lacking Mdm10 (Meisinger *et al.*, 2004; Meisinger *et al.*, 2006).

The array of possible functions for ERMES components has led to the problem of distinguishing primary effects from those which might be an indirect consequence of others. In this regard, it has been shown that in a strain containing a temperature-sensitive allele of *mmm1*, β-barrel assembly defects precede morphological alterations following a shift from the permissive to the restrictive temperature (Meisinger *et al.*, 2007). Another study concluded that the primary function of ERMES was to maintain the link between ER and mitochondria (Nguyen *et al.*, 2012). In the absence of this link, mitochondrial morphology is altered which leads to secondary effects on mitochondrial inheritance. In the present study I have reasoned that if ERMES proteins are involved in different functions, then it should be possible to define domains in each protein responsible for individual functions. I have examined the effects of mutations in two regions of the *N. crassa* Mmm1 protein with respect to mitochondrial morphology and TOB complex function. In addition, since there is currently controversy as to the role of the *S. cerevisiae* Gem1 protein in ERMES composition and function (Kornmann *et al.*, 2011; Stroud *et al.*, 2011; Nguyen *et al.*, 2012) a *Δgem1 N. crassa* strain is also examined with respect to phenotypes characteristic of ERMES mutants. I extended my analysis to a strain lacking Mmm2 since mitochondrial protein import/assembly defects have never been reported for mutants lacking this ERMES complex member.

4.2 MATERIALS AND METHODS

4.2.1 Strains and growth of *N. crassa*

Strains used in this study are listed in Table A.1. Strains were grown and handled as described in section 2.1.1. The deletion strains used in this study (*Δmmm1*, *Δmmm2*, and *Δgem1)* were developed by the *N. crassa* gene knockout project. The target genes were replaced with a hygromycin resistance cassette (Colot *et al.*, 2006). The strains were obtained from the fungal genetics stock center (FGSC). PCR analysis was used to confirm the replacements in each strain.

4.2.2 Microscopy and measurement of mitochondrial diameter

N. crassa samples were prepared for visualization as described in section 2.2.2 (Wideman *et al.*, 2010). Imaging was done on an Olympus IX81 (60X, NA 1.42 oil objective) inverted microscope with a Yokogawa CSU-10 spinning disc confocal head modified with a condenser lens in the optical path (Quorum Technologies). Z-stacks (16 images, 0.2 μm spacing, ASI Nanodrive) were acquired for each hyphae. Digital images were obtained with a Hamamatsu Orca R2 camera, controlled by MetaMorph software. Post image processing and mitochondrial measurements were performed manually using Metamorph offline software. The length of individual mitochondria varied, therefore, to obtain width measurements of long mitochondria (>2 microns), multiple lines approximately 1 micron apart were drawn perpendicular to the long axis of the mitochondria. Optical planes used for width measurements were chosen based on maximum fluorescence intensity of MitoTracker. Measurements were obtained for all mitochondria visible from at least 4 hyphae for each experiment.

4.2.3 Creation of strains harboring mutant versions of Mmm1

The *mmm1* gene plus 500 bp of upstream and downstream sequence was cloned into AscI sites in a modified Bluescript plasmid containing Basta resistance (pBasc) (Pall and Brunelli, 1993). Using site directed PCR mutagenesis, a NotI site was inserted into the 3' region of *mmm1* just before the stop codon. I obtained another plasmid (FN-NotI-HA3) containing three repeats of the hemaglutinin (HA) epitope (YPYDVPDYA) flanked by NotI sites in a kanamycin resistance vector synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa). One extra base pair was added on each side of the triple HA sequence to correct the +1 frameshift caused by the insertion of two eight bp NotI sites. The additional nine bp (NotI site plus 1 bp) extensions on either side of the 3xHA sequence code for Ala-Ala-Ala and Gly-Gly-Arg, respectively. The triple HA tag was cut out using NotI and ligated into the NotI site that had been engineered in the *mmm1* gene. The resulting plasmid was called pMmm1-HA. Subsequently, pMmm1-HA was subjected to site directed PCR mutagenesis to encode the desired mutant forms of Mmm1 described in Table A.1. For C5S, C179S, and C319S Cys residues 5, 179,

and 319 were changed to Ser, respectively. For CS-123, all three Cys residues were changed to Ser. For A116-124 residues 116-124 were each mutated to Ala. Plasmids containing mutations were confirmed by sequence analysis, linearized and used to transform *Δmmm1*. The transformation mixtures were plated onto media containing Basta. Strains were purified by one round of single colony isolation. Mutations were confirmed by sequence analysis of genomic DNA isolated from the transformants.

4.2.4 Cellular fractionation experiments

Crude mitochondria were isolated as described previously (Nargang and Rapaport, 2007). Crude mitochondria were further purified in flotation sucrose gradients as described (Lambowitz, 1979) except that the buffer used was SEMP (0.25 M sucrose, 1 mM EDTA, 10 mM MOPS, 1 mM phenylmethylsulfonyl fluoride (PMSF)) containing protease inhibitors (2μg/mL aprotinin, 1 μg/mL leupeptin and 1 μg/mL pepstatin A). Crude mitochondria were resuspended in SEMP buffer containing 60% sucrose and overlayed with SEMP containing 55% and 44% sucrose. Samples were spun at 75, 000 x g for 2 hr. The band at the interface of the 44% and 55% SEMP was collected and diluted in SEMP. Mitochondria were repelleted and resuspended in SEMP for analysis. The supernatant fraction from the initial crude mitochondrial preparation was further fractionated by subjecting 1 ml samples to ultracentrifugation at 130,000 x g at 4°C for 1.5 hr in a TLA55 rotor in a Beckman Optima^{TM} MAX tabletop ultracentrifuge. The supernatant was collected as the cytosolic fraction. The pellet, which contains the ER as microsomes, was termed the post mitochondrial pellet (PMP). Control marker proteins for the fractions were a TOM complex component for mitochondria, KAR2 for PMP (Addison, 1998), and arginase for the cytosol (Borkovich and Weiss, 1987; Marathe *et al.*, 1998).

4.2.5 Phospholipid analysis by TLC (thin layer chromatography)

Mitochondrial phospholipids were extracted from isolated crude mitochondria (300 μg protein) resuspended in 100 μL water with 200 μL 1:1 chloroform:

methanol (v/v) using a procedure modified from Osman *et al*. (2009). Briefly, samples were vortexed for 30 sec and then shaken for 30 min. Samples were then centrifuged at max speed on a table top centrifuge to separate aqueous and organic phases. The aqueous phase was discarded and the organic phase was allowed to dry in a fume hood. The lipid pellet was then dissolved in 30 μL 2:1 chloroform methanol (v/v) and subjected to TLC as previously described (Vaden *et al.*, 2005). Briefly, samples were spotted onto TLC plates (5729-6 Merck KGaA, Darmstadt, Germany) that were prewashed in 1:1 chloroform methanol (v/v) and developed in chloroform: water: ethanol: triethylamine (30:7:35:35) until the liquid front neared the top of the plate (approximately 1.5 hr at room temperature). The plate was then allowed to dry in a fume hood and the developing step was repeated to increase resolution. Plates were dried a second time and sprayed with molybdenum blue spray reagent (M1942-100ML, Sigma).

4.2.6 General Procedures

Blue native gel electrophoresis (Schägger and von Jagow, 1991; Schägger *et al.*, 1994), Western blotting (Good and Crosby, 1989), import of proteins into isolated mitochondria (Harkness *et al.*, 1994), alkaline extraction (Wideman *et al.*, 2010; Lackey *et al.*, 2011), isolation of outer membrane vesicles (Mayer *et al.*, 1995), and transformation of *N. crassa* (Hoppins *et al.*, 2007) were performed as described previously. Mitochondrial proteins were analyzed by SDS-PAGE as previously described (Laemmli, 1970). However, where indicated, proteins were prepared for SDS-PAGE by dissolving in cracking buffer (0.06 M Tris-HCl, pH 6.8; 2.5% SDS; 5% sucrose) with (reducing) or without (non-reducing) 5% β-mercaptoethanol. In some cases irrelevant lanes were electronically removed from gel blots or autoradiograms.

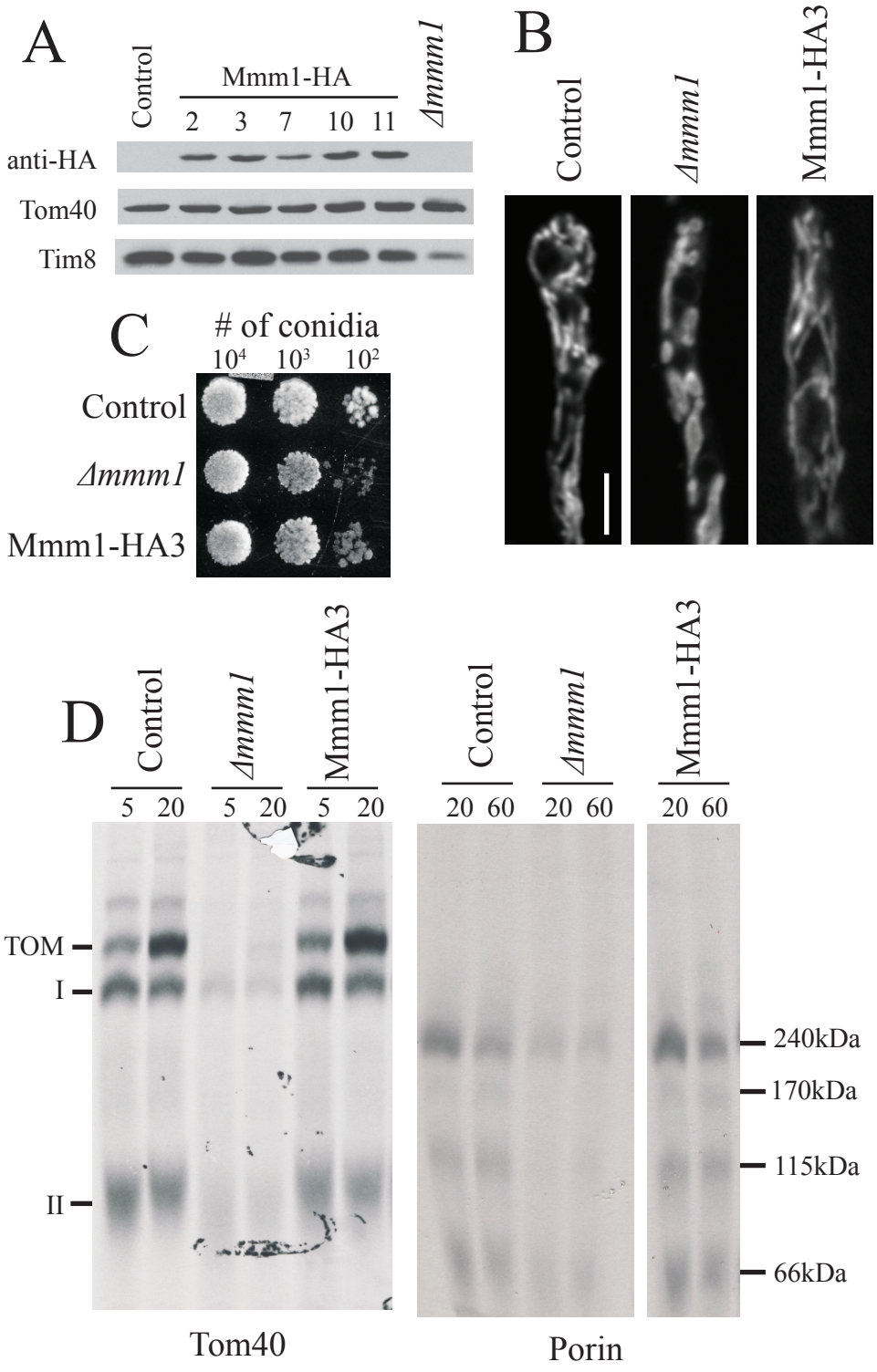
4.3 RESULTS

4.3.1 Mmm1 fractionates with both purified mitochondria and the ER but not with purified mitochondrial outer membrane vesicles.

The Mmm1 protein was originally characterized as a MOM protein in both *S. cerevisiae* and *N. crassa* (Prokisch *et al.*, 2000; Hobbs *et al.*, 2001; Kondo-Okamoto *et al.*, 2003; Youngman *et al.*, 2004; Meisinger *et al.*, 2007). However, more recent studies in *S. cerevisiae* have concluded that the protein resides in the ER membrane and associates with MOM proteins to form the ERMES complex (Kornmann *et al.*, 2009; Kornmann *et al.*, 2011; Stroud *et al.*, 2011). I wished to investigate the location of Mmm1 in *N. crassa* by cell fractionation. Since no antibody to *N. crassa* Mmm1 is available, I constructed a plasmid containing the *N. crassa mmm1* gene with a C-terminal triple hemaglutinin (HA) epitope-tag expressed from the endogenous *mmm1* promoter. This construct was transformed into a *Δmmm1* strain and several transformants were examined on Western blots for the presence of Mmm1-HA. Although there was variation in the level of expression of the protein among the different transformants most expressed similar levels of the protein (Figure 4.1A). One such strain, Mmm1-HA3, was chosen for further work. The defects in mitochondrial morphology, growth rate, and β-barrel assembly that are seen in the *Δmmm1* strain are all rescued in Mmm1-HA3 (Figures 4.1B-D and 4.2).

Isolation of subcellular fractions from the tagged strain revealed that Mmm1-HA fractionated with both flotation gradient purified mitochondria and the post-mitochondrial pellet (PMP), which contains the ER as shown by the presence of the Kar2 marker protein (Addison, 1998) in this fraction (Figure 4.3A). These data are consistent with the notion that Mmm1 is an ER-membrane protein that interacts with MOM proteins but do not exclude the possibility that it is a MOM protein that interacts with the ER. To distinguish between these two alternatives I examined mitochondrial OMV for the presence of Mmm1-HA. If Mmm1 was a bona fide MOM protein it would fractionate with OMV. On the other hand, it seemed likely that if Mmm1 was an ER protein, then its association

Figure 4.1. Mmm1-HA rescues *Δmmm1* phenotypes. (A) Mitochondria isolated from the indicated transformant strains were subjected to SDS-PAGE and analyzed by Western blot for detection of the indicated proteins. (B) The control, *Δmmm1*, and Mmm1-HA3 strains were grown on solid Vogel's media, stained with MitoTracker Green FM and examined by confocal fluorescence microscopy. Bar indicates 10 µm (C) Conidiaspores from the indicated strains were spotted as tenfold dilutions on Vogel's sorbose medium plates. Plates were incubated at 30°C for 48h. (D) Assembly of Tom40 and Porin. Radiolabled precursors were incubated with isolated mitochondria for the indicated times. Mitochondria were then reisolated and dissolved in 1% digitonin. The samples were subjected to BNGE, transferred to PVDF membrane and analyzed by autoradiography. The mature TOM complex and intermediates I and II are indicated. The four major complexes containing radiolabeled porin are indicated by their estimated molecular masses.


A
Control
Mmm1-HA
2 3 7 10 11
Δmmm1
anti-HA
Tom40
Tim8
B
Control
Δmmm1
Mmm1-HA3
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10⁴ 10³ 10²
Control
Δmmm1
Mmm1-HA3
D
Control
Δmmm1
Mmm1-HA3
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20 60 20 60 20 60
TOM
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II
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170kDa
115kDa
66kDa
Tom40
Porin

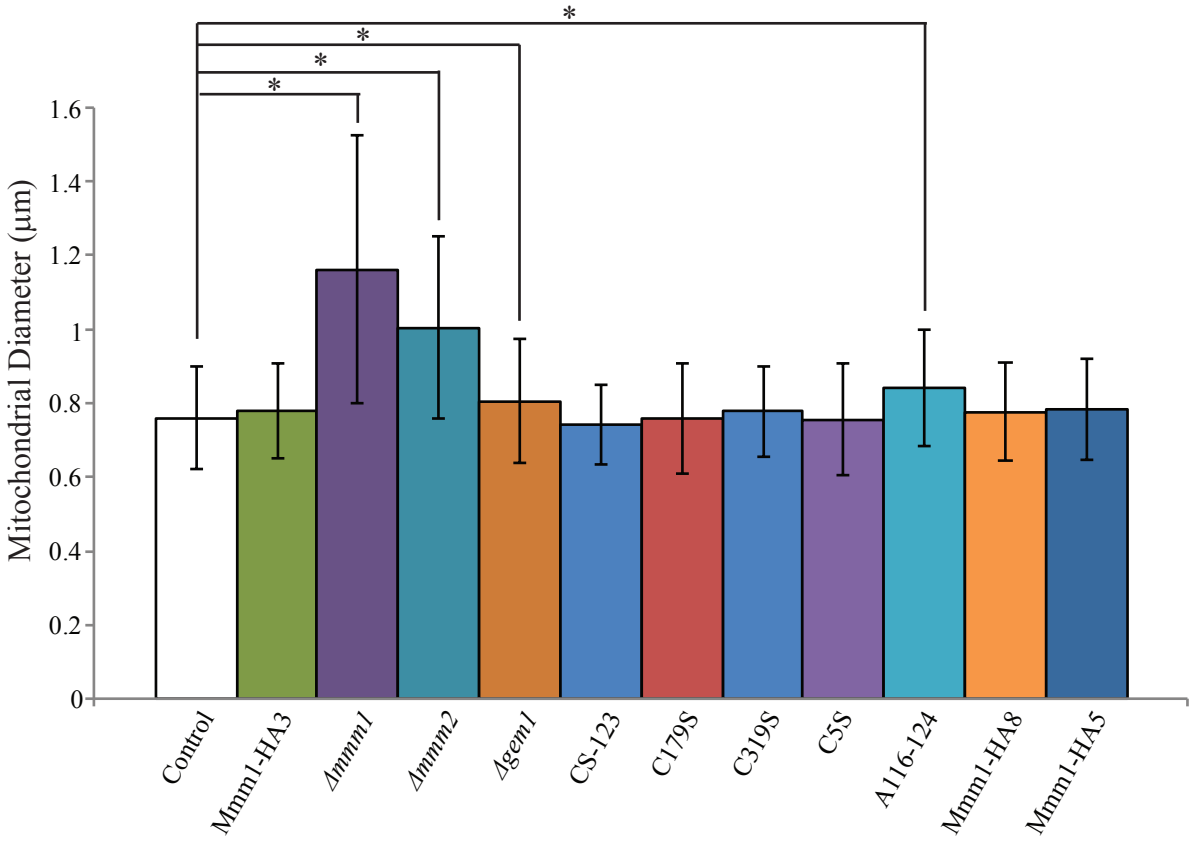
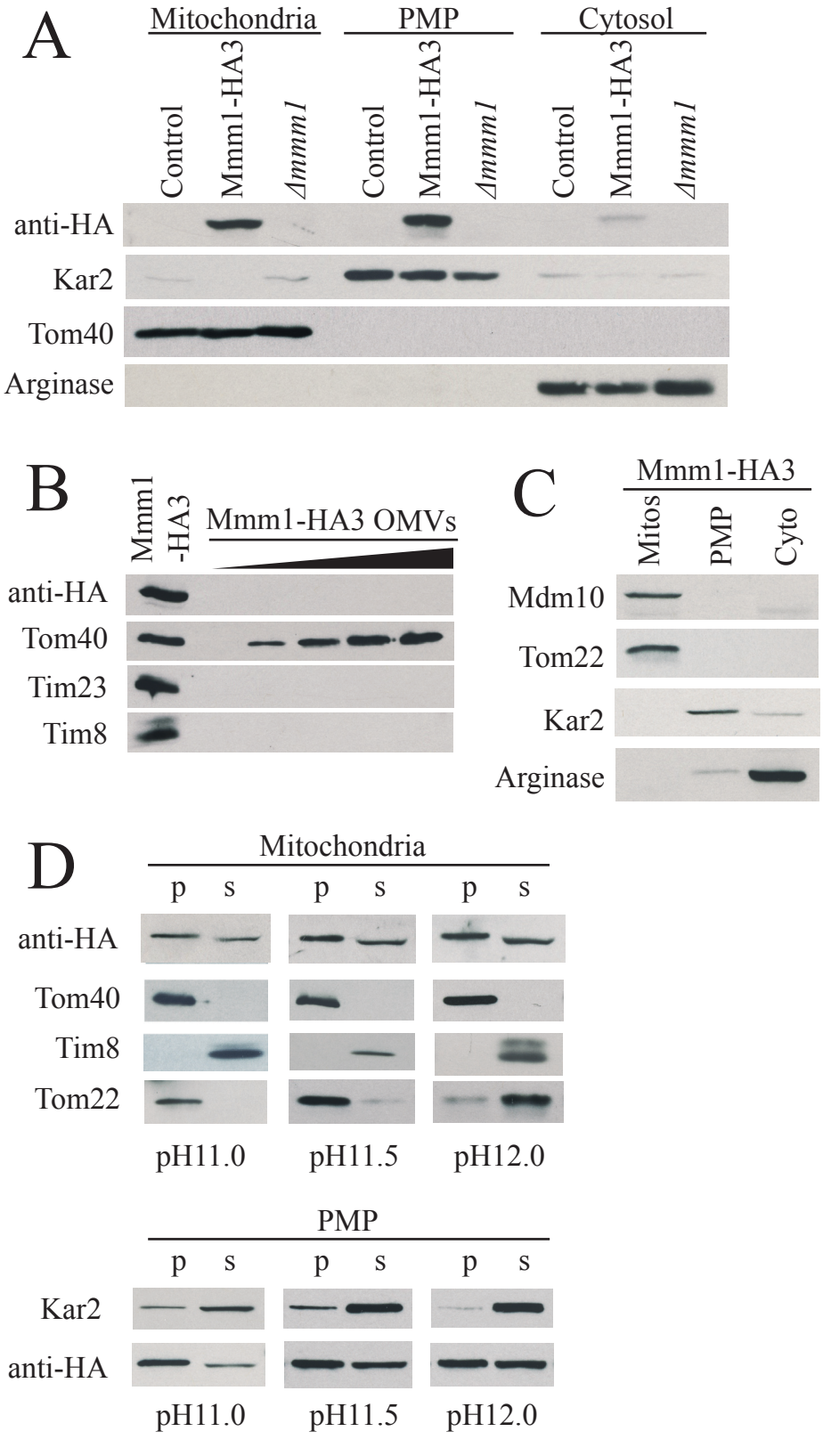


Figure 4.2. Mutants lacking Mmm1 or Mmm2 contain mitochondria with larger diameters than other strains. Mitochondrial diameter was measured using Metamorph offline software. Measurements were obtained for all visible mitochondria from at least four hyphae for each experiment. When measuring longer mitochondria, measurements were taken approximately every micrometer. Error bars indicate standard deviation. * indicates significant difference between two indicated strains as analyzed by a student's T-test ($p < 0.01$).

Figure 4.3. Subcellular localization of HA-tagged Mmm1. (A) Cell fractionation by differential and sucrose flotation gradient centrifugation was performed on the indicated strains. Purified mitochondrial, post mitochondrial pellet and cytosolic fractions (30µg) were subjected to SDS-PAGE followed by Western blot analysis using antibodies to the indicated proteins. Kar2, ER marker; Tom40, mitochondrial marker; Arginase, cytosolic marker. (B) Outer membrane vesicles (OMV) were isolated from cells expressing Mmm1-HA protein, subjected to SDS-PAGE and analysed by western blot for the presence or absence of the indicated proteins. (C) Cell fractionation and detection of indicated proteins. As in panel A but Tom22 is the mitochondrial marker. (D) Gradient purified mitochondria and PMP isolated from the Mmm1-HA strain were treated with 0.1 M sodium carbonate at pH 11.0, 11.5 or 12.0. Membrane sheets were pelleted by ultracentrifugation. Proteins in the supernatant were precipitated with trichloroacetic acid. Pellet (pel) and supernatant (sup) fractions were then subjected to SDS-PAGE, transferred to nitrocellulose and analyzed by Western blot using antibodies to the indicated proteins.


A
Mitochondria
PMP
Cytosol
Control
Mmm1-HA3
Δmmm1
anti-HA
Kar2
Tom40
Arginase
B
Mmm1-HA3
Mmm1-HA3 OMVs
anti-HA
Tom40
Tim23
Tim8
C
Mmm1-HA3
Mitos
PMP
Cyto
Mdm10
Tom22
Kar2
Arginase
D
Mitochondria
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s
anti-HA
Tom40
Tim8
Tom22
pH11.0
pH11.5
pH12.0
PMP
Kar2
anti-HA

with gradient purified mitochondria in our fractionation experiments would be due to small patches of ER membrane that stay associated with mitochondria at ERMES attachment points during isolation and gradient purification. However, since OMV are isolated by shearing the MOM from mitochondria followed by purification based on density in sucrose gradients (Mayer *et al.*, 1995), membrane fragments containing both MOM and ER would be expected to separate from fragments made up solely of MOM. Western blot analysis of OMV prepared from Mmm1-HA3 revealed the presence of the MOM protein Tom40, whereas Mmm1-HA, the inner mitochondrial membrane protein Tim23, and the intermembrane space protein Tim8 could not be detected (Figure 4.3B). This result is consistent with the observation that Mmm1 was undetectable in *N. crassa* OMV by mass spectrometry (Schmitt *et al.*, 2006). Conversely, I have previously shown that Mdm10, another ERMES component, does fractionate with mitochondrial OMV (Wideman *et al.*, 2010). Here I have also shown that Mdm10 only fractionates with gradient purified mitochondria and not the PMP (Figure 4.3C). Taken together, these data suggest that during subcellular fractionation, fragments of ER membrane stay attached to mitochondria at ERMES junctions, but mitochondrial membrane fragments do not remain attached to the ER. They also suggest that there are two populations of Mmm1. One population is tightly associated with mitochondria while the other is not, and fractionates with the post mitochondrial pellet.

We also performed alkali extractions to determine if Mmm1 behaves differently with respect to membrane integration in the gradient purified mitochondria versus the PMP fractions. Mmm1 was approximately 50% extractable at pH 12.0 in both fractions (Figure 4.3D). Since the known membrane spanning protein Tom22 is almost completely extracted from mitochondria at this pH it appears that Mmm1 is strongly anchored to the ER membrane. From these data and previous data from *S. cerevisiae* (Kornmann *et al.*, 2011; Stroud *et al.*, 2011) we conclude that while a population of Mmm1 molecules fractionates with mitochondria, Mmm1 is actually an ER protein.

4.3.2 Mmm1 structure and function.

Mmm1 proteins are quite well conserved among different fungi. Most species we examined contain a small N-terminal domain (Figure 4.4A) followed by a predicted membrane spanning domain (Figure 4.4B), and then a large C-terminal domain (Figure A.2). The latter has been shown to exist in the cytosol in *S. cerevisiae* and *N. crassa* (Burgess *et al.*, 1994; Prokisch *et al.*, 2000) and is known to be essential for function (Burgess *et al.*, 1994; Kondo-Okamoto *et al.*, 2003; Stroud *et al.*, 2011). The cytosolic domain contains a synaptotagmin-like-mitochondrial-lipid binding protein (SMP) domain, which may be involved in lipid transfer between ER and mitochondria (Kopec *et al.*, 2010; Toulmay and Prinz, 2012), and has been shown to be essential for protein targeting to organelle contact sites (Toulmay and Prinz,2012). The N-terminal region of *S. cerevisiae* Mmm1 that precedes the membrane spanning domain is located in the ER lumen and is known to be N-glycosylated (Kornmann *et al.*, 2009; Stroud *et al.*, 2011) (Figure 4.4A). However, in many species the ER-lumen domain is much smaller than in *S. cerevisiae* and lacks any predicted N-glycosylation sites (Figure 4A). Surprisingly, species from the subphylum Mucormycotina and the phylum Chytridiomycota appear to lack a membrane spanning domain and therefore must also lack the ER-lumen domain.

Further examination of the Mmm1 alignment revealed that two Cys residues were conserved in many Ascomycetes (position 179 and 319 of the *N. crassa* protein) while a third, at position 5 of the *N. crassa* protein, was conserved in the class Sordariomycetes (Figure 4.4A and Figure 4.2). Since the domain containing the C5 residue is predicted to be located in the ER lumen, it was possible that it might be involved in disulphide bond formation. When isolated mitochondria (Figure 4.5A) or PMP (data not shown) were analyzed on Western blots following non-reducing SDS-PAGE, a band was detected at approximately 100 kDa, twice the molecular weight of Mmm1-HA. A small amount of material was still seen at 50 kDa, but the majority of the Mmm1 protein appears to be in a disulphide bonded form, most likely a dimer.

Figure 4.4. Alignments of fungal Mmm1 proteins. (A) Alignment of Mmm1 N-terminal regions from several Ascomycetes. Known *S. cerevisiae* (N50, N55 and N59) (Stroud et al. 2011) and potential N-glycosylation sites in other species are highlighted in black. Cys residue conserved in Sordariomycetes is shaded in grey. (B) Predicted transmembrane domain of Mmm1is present in most fungi, but absent in Mucormycotina and Chytridiomycota. The predicted *N. crassa* transmembrane domain is highlighted in grey. Identify/similarity symbols are for the alignment of the Ascomycota and Basidiomycota only. * indicates conserved residues, : indicates conservation of groups with strongly similar properties (score of >0.5 in the Gonnet PAM 250 matrix), . indicates conservation of groups with weakly similar properties (score of <0.5in the Gonnet PAM 250 matrix). (C) Alignment of the highly conserved region of Mmm1 chosen for mutation analysis. The nine amino acid region that was chosen for mutation is highlighted in the *N. crassa* protein (residues 116-124). Symbols (as in panel B) are for the alignment of all proteins. Abbreviations: N.c., *Neurospora crassa*; G.z., *Gibberella zeae*; C.g., *Chaetomium globosum*; S.m., *Sordaria macrospora*; M.o., *Magnaporthe oryzae*; P.a., *Podospora anserina*; F.o. *Fusarium oxysporum*; V.d., *Verticillium dahliae*; A.n., *Aspergillus nidulans*; T.t.,*Trichophyton tonsurans*; C.i., *Coccidioides immitis*; P.b., *Paracoccidioides brasiliensis*; S.s., *Sclerotinia sclerotiorum*; B.f., *Botryotinia fuckeliana*; P.t., *Pyrenophora teres*; S.c., *Saccharomyces cerevisiae*; K.l., *Kluyveromyces lactis*; C.a., *Candida albicans*; S.p., *Schizosaccharomyces pombe*; U.m., *Ustilago maydis*; C.n., *Cryptococcus neoformans*; R.o., *Rhizopus oryzae*; B.d., *Batrachochytrium dendrobatidis*.

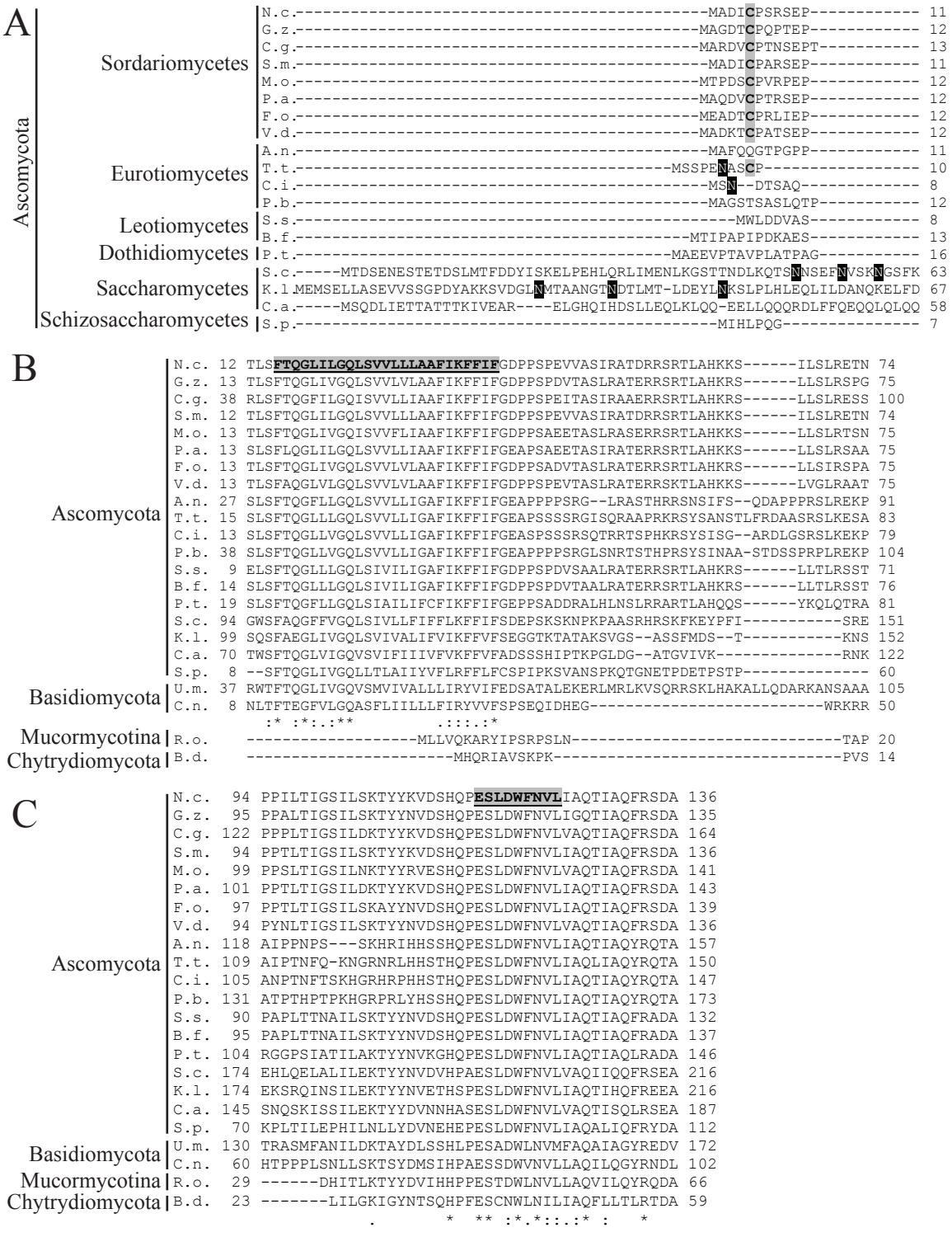
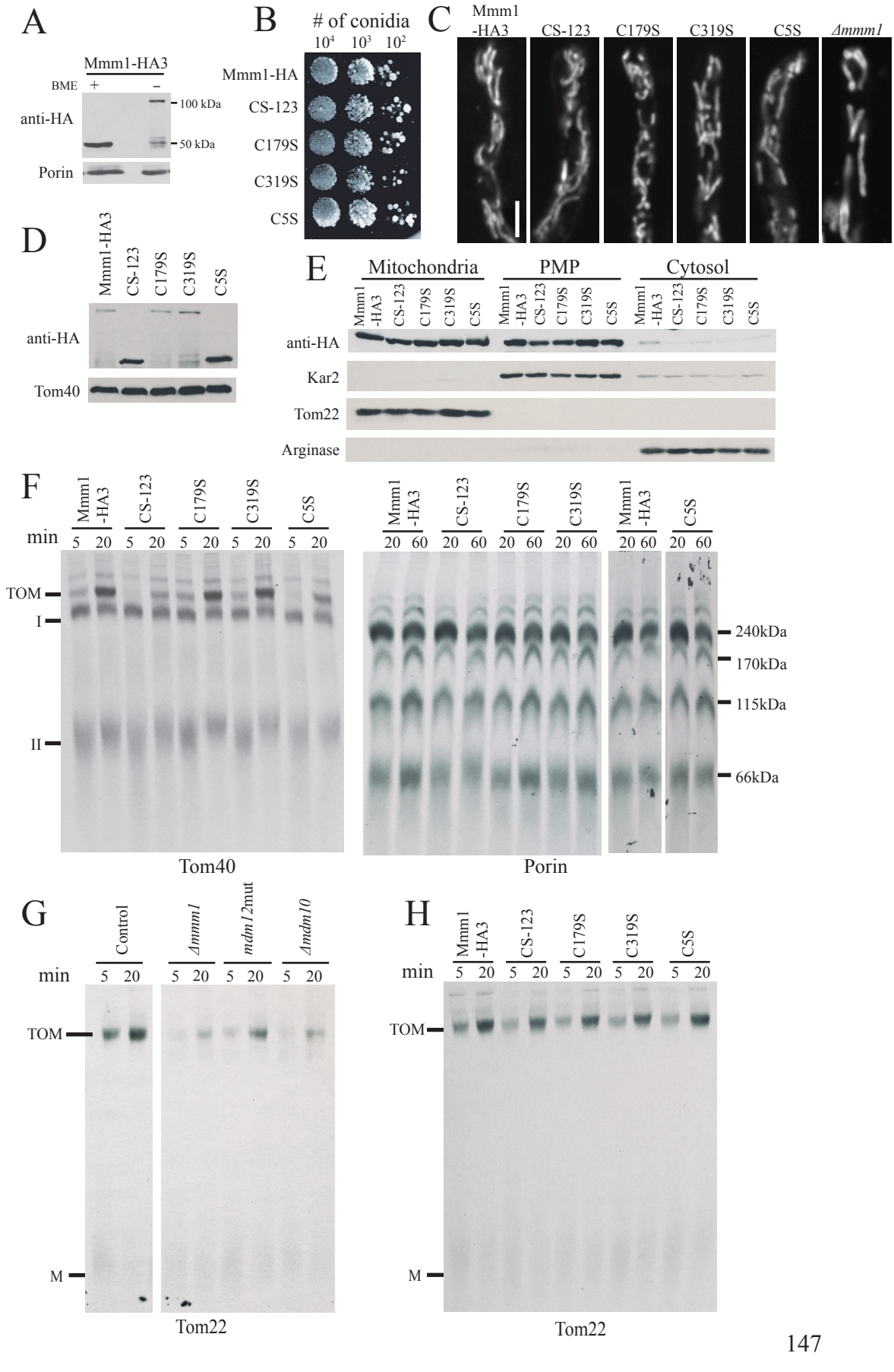

A
Ascomycota
Sordariomycetes
Eurotiomycetes
Leotiomycetes
Dothidiomycetes
Saccharomycetes
Schizosaccharomycetes
N.c.----------MADICPSRSEP---------- 11
G.z.----------MAGDTCPQPTEP---------- 12
C.g.----------MARDVCPTNSEPT---------- 13
S.m.----------MADICPARSEP---------- 11
M.o.----------MTPDSCPVRPEP---------- 12
P.a.----------MAQDVCPTRSEP---------- 12
F.o.----------MEADTCPRLIEP---------- 12
V.d.----------MADKTCPATSEP---------- 12
A.n.----------MAFQQGTPGPP---------- 11
T.t.----------MSSPENASCP---------- 10
C.i.----------MSN--DTSAQ---------- 8
P.b.----------MAGSTSASLQTP---------- 12
S.s.----------MWLDDVAS---------- 8
B.f.----------MTIPAPIPDKAES---------- 13
P.t.----------MAEEVPTAVPLATPAG---------- 16
S.c.-----MTDSENESTETDSLMTFDDYISKELPEHLQRLIMENLKGSTTNDLKQTSNNSEFNVSKNGSFK 63
K.l.MEMSELLASEVVSSGPDYAKKSVDGLNMTAANGTNDTLMT-LDEYLNKSLPLHLEQLILDANQKELFD 67
C.a.-----MSQDLIETTATTTKIVEAR----ELGHQIHDSLLEQLKLQQ-EELLQQQRDLFFQEQQLQLQQ 58
S.p.----------MIHLPQG---------- 7
B
Ascomycota
Basidiomycota
Mucormycotina
Chytrydiomycota
N.c. 12 TLSFTQGLILGQLSVVLLLAAFIKFFIFGDPPSPEVVASIRATDRRSRTLAHKKS------ILSLRETN 74
G.z. 13 TLSFTQGLIVGQLSVVLVLAAFIKFFIFGDPPSPDVTASLRATERRSRTLAHKRS------LLSLRSPG 75
C.g. 38 RLSFTQGFILGQISVVLLIAAFIKFFIFGDPPSPEITASIRAAERRSRTLAHKRS------LLSLRESS 100
S.m. 12 TLSFTQGLILGQLSVVLLLAAFIKFFIFGDPPSPEVVASIRATDRRSRTLAHKKS------ILSLRETN 74
M.o. 13 TLSFTQGLIVGQISVVFLIAAFIKFFIFGDPPSAEETASLRASERRSRTLAHKKS------LLSLRTSN 75
P.a. 13 SLSFLQGLILGQLSVVLLIAAFIKFFIFGEAPSAEETASIRATERRSRTLAHKKS------LLSLRSAA 75
F.o. 13 TLSFTQGLIVGQLSVVLVLAAFIKFFIFGDPPSADVTASLRATERRSRTLAHKRS------LLSIRSPA 75
V.d. 13 TLSFAQGLVLGQLSVVLVLAAFIKFFIFGDPPSPEVTASLRATERRSKTLAHKKS------LVGLRAAT 75
A.n. 27 SLSFTQGFLLGQLSVVLLIGAFIKFFIFGEAPPPPSRG--LRASTHRRSNSIFS--QDAPPPRSLREKP 91
T.t. 15 SLSFTQGLLLGQLSVVLLIGAFIKFFIFGEAPSSSSRGISQRAAPRKRSYSANSTLFRDAASRSLKESA 83
C.i. 13 SLSFTQGLLVGQLSVVLLIGAFIKFFIFGEASPSSSRSQTRRTSPHKRSYSISG--ARDLGSRSLKEKP 79
P.b. 38 SLSFTQGLLVGQLSVVLLIGAFIKFFIFGEAPPPPSRGLSNRTSTHPRSYSINAA-STDSSPRPLREKP 104
S.s. 9 ELSFTQGLLLGQLSIVILIGAFIKFFIFGDPPSPDVSAALRATERRSRTLAHKRS------LLTLRSST 71
B.f. 14 SLSFTQGLLLGQLSIVILIGAFIKFFIFGDPPSPDVTAALRATERRSRTLAHKRS------LLTLRSST 76
P.t. 19 SLSFTQGFLLGQLSIAILIFCFIKFFIFGEPPSADDRALHLNSLRRARTLAHQQS------YKQLQTRA 81
S.c. 94 GWSFAQGFFVGQLSIVLLFIFFLKFFIFSDEPSKSKNPKPAASRHRSKFKEYPFI-----------SRE 151
K.l. 99 SQSFAEGLIVGQLSVIVALIFVIKFFVFSEGGTKTATAKSVGS--ASSFMDS--T-----------KNS 152
C.a. 70 TWSFTQGLVIGQVSVIFIIIVFVKFFVFADSSSHIPTKPGLDG--ATGVIVK-------------RNK 122
S.p. 8 --SFTQGLIVGQLLTLAIIYVFLRFFLFCSPIPKSVANSPKQTGNETPDETPSTP-------------- 60
U.m. 37 RWTFTQGLIVGQVSMVIVALLLIRYVIFEDSATALEKERLMRLKVSQRRSKLHAKALLQDARKANSAAA 105
C.n. 8 NLTFTEGFVLGQASFLIILLLFIRYVVFSPSEQIDHEG-------------------------WRKRR 50
:* :*:.:** .:::.:*
R.o. ------------------MLLVQKARYIPSRPSLN------------------------------TAP 20
B.d. ----------------------MHQRIAVSKPK--------------------------------PVS 14
C
Ascomycota
Basidiomycota
Mucormycotina
Chytrydiomycota
N.c. 94 PPILTIGSILSKTYYKVDSHQPESLDWFNVLIAQTIAQFRSDA 136
G.z. 95 PPALTIGSILSKTYYNVDSHQPESLDWFNVLIGQTIAQFRSDA 135
C.g. 122 PPPLTIGSILDKTYYKVDSHQPESLDWFNVLVAQTIAQFRSDA 164
S.m. 94 PPTLTIGSILSKTYYKVDSHQPESLDWFNVLIAQTIAQFRSDA 136
M.o. 99 PPSLTIGSILNKTYYRVESHQPESLDWFNVLVAQTIAQFRSDA 141
P.a. 101 PPTLTIGSILDKTYYKVDSHQPESLDWFNVLIAQTIAQFRSDA 143
F.o. 97 PPTLTIGSILSKAYYNVDSHQPESLDWFNVLIAQTIAQFRSDA 139
V.d. 94 PYNLTIGSILSKTYYNVDSHQPESLDWFNVLVAQTIAQFRSDA 136
A.n. 118 AIPPNPS---SKHRIHHSSHQPESLDWFNVLIAQTIAQYRQTA 157
T.t. 109 AIPTNFQ-KNGRNRLHHSTHQPESLDWFNVLIAQLIAQYRQTA 150
C.i. 105 ANPTNFTSKHGRHRPHHSTHQPESLDWFNVLIAQTIAQYRQTA 147
P.b. 131 ATPTHPTPKHGRPRLYHSSHQPESLDWFNVLIAQTIAQYRQTA 173
S.s. 90 PAPLTTNAILSKTYYNVDSHQPESLDWFNVLIAQTIAQFRADA 132
B.f. 95 PAPLTTNAILSKTYYNVDSHQPESLDWFNVLIAQTIAQFRADA 137
P.t. 104 RGGPSIATILAKTYYNVKGHQPESLDWFNVLIAQTIAQLRADA 146
S.c. 174 EHLQELALILEKTYYNVDVHPAESLDWFNVLVAQIIQQFRSEA 216
K.l. 174 EKSRQINSILEKTYYNVETHSPESLDWFNVLIAQTIHQFREEA 216
C.a. 145 SNQSKISSILEKTYYDVNNHASESLDWFNVLVAQTISQLRSEA 187
S.p. 70 KPLTILEPHILNLLYDVNEHEPESLDWFNVLIAQALIQFRYDA 112
U.m. 130 TRASMFANILDKTAYDLSSHLPESADWLNVMFAQAIAGYREDV 172
C.n. 60 HTPPPLSNLLSKTSYDMSIHPAESSDWVNVLLAQILQGYRNDL 102
R.o. 29 ------DHITLKTYYDVIHHPPESTDWLNVLLAQVILQYRQDA 66
B.d. 23 -------LILGKIGYNTSQHPFESCNWLNILIAQFLLTLRTDA 59
. * ** :*.*::.:* : *

Figure 4.5. Characterization of *N. crassa* Cys to Ser Mmm1 mutants. (A) Western blot of mitochondria isolated from Mmm1-HA. Mitochondria (30μg) were treated with cracking buffer that either did (+BME) or did not (-BME) contain β-mercaptoethanol. Samples were subjected to SDS-PAGE, transferred to nitrocellulose and analyzed by Western blotting for the indicated proteins. (B) Growth of strains was examined as in Figure 4.1C (C) Mitochondrial morphology was examined in Cys to Ser Mmm1 mutants as in Figure 4.1B. Bar represents 10 μm. (D) Western blot analysis of Mmm1 Cys mutant mitochondria. As in Figure 4.5A, but mitochondria were only analyzed by non-reducing SDS-PAGE. (E) Cell fractionation as in Figure 4.3A. (F) Import and assembly of Tom40 and porin as in Figure 4.1D (G) As in F, but radiolabeled Tom22 precursor was imported into isolated mitochondria from the indicated strains. Mature TOM complex (TOM) and monomeric Tom22 (M) are indicated. (H) Assembly of Tom22 into mitochondria from the indicated strains as in panel G.


A
Mmm1-HA3
BME + –
anti-HA
100 kDa
50 kDa
Porin
B
# of conidia
10^4 10^3 10^2
Mmm1-HA
CS-123
C179S
C319S
C5S
C
Mmm1 -HA3
CS-123
C179S
C319S
C5S
Δmmm1
D
Mmm1-HA3
CS-123
C179S
C319S
C5S
anti-HA
Tom40
E
Mitochondria
PMP
Cytosol
Mmm1 -HA3
CS-123
C179S
C319S
C5S
anti-HA
Kar2
Tom22
Arginase
F
min
5 20
20 60
TOM
I
II
240kDa
170kDa
115kDa
66kDa
Tom40
Porin
G
Control
Δmmm1
mdm12mut
Δmdm10
min
5 20
TOM
M
Tom22
H
Mmm1 -HA3
CS-123
C179S
C319S
C5S
min
5 20
TOM
M
Tom22

4.3.3 Loss of disulphide bond formation in the ER-lumen domain of Mmm1 affects Tom40 assembly.

To determine if Mmm1 disulphide bonding is functionally relevant in *N. crassa* I used the HA-tagged wild-type gene to construct mutant versions of the protein in which each of the three Cys residues was changed individually to Ser (C5S, C179S and C319S) as well as a version in which all three Cys were changed to Ser (CS-123). The *Δmmm1* strain was transformed with plasmids encoding these mutant versions of the protein. Mitochondria were isolated from transformants and analysed by non-reducing SDS-PAGE. As expected, the Cys responsible for disulphide bond formation of Mmm1 was the one predicted to occur in the oxidizing environment of the ER, at position 5 of the *N. crassa* protein (Figure 4.5B). None of the four Cys mutant strains exhibited alterations with respect to mitochondrial morphology (Figure 4.5C and Figure 4.2), or growth rate (Figure 4.5D). Fractionation experiments showed that none of the Cys mutations result in mislocalization of Mmm1 (Figure 4.5E).

I next examined mitochondria isolated from the Cys mutants for their ability to assemble Tom40 and porin into the MOM, since these β-barrels are known to have reduced assembly in Mmm1 mutants [(Wideman *et al.*, 2010), Figure 4.1]. I found that the inability of Mmm1 to form disulphide bonds in the C5S and CS-123 mutants caused a moderate defect in the incorporation of Tom40 into the final TOM complex (Figure 4.5F). Several complexes are also detected when porin import/assembly is assessed by BNGE. Four prominent bands and other minor bands are seen, though the nature of the different forms is not well understood. Assembly of porin (Figure 4.5F) did not appear to be altered in the C5S or CS-123 mutants. Since Tom22 is also a substrate of the TOB complex (Meisinger *et al.*, 2004; Stojanovski *et al.*, 2007; Thornton *et al.*, 2010; Becker *et al.*, 2011) I also wished to assess its assembly in the C5S mutant. However, *N. crassa* ERMES mutants had not been previously tested for Tom22 assembly, so I first examined the *Δmmm1* mutant as well as the previously described *mdm12* mutant and *Δmdm10* strain. Mitochondria from all three mutant strains exhibited reduced ability to assemble Tom22 into the TOM complex (Figure 4.5G). The

Cys mutants of Mmm1 all assembled Tom22 as effectively as the wild-type control (Figure 4.5H). Thus, the ability of Mmm1 to form disulphide bonds differentially affects aspects of the *Δmmm1* phenotype.

4.3.4 Mutation of a conserved region of Mmm1 affects interaction with mitochondria and assembly of mitochondrial outer membrane proteins.

The Mmm1 alignment revealed a region highly conserve in all fungi at position 116 to 124 of the *N. crassa* protein (Figure 4.4C and Figure 4.2). We constructed a mutant version of the HA-tagged *mmm1* gene in which all nine codons of the region were mutated to Ala residues. The mutant allele was transformed into *Δmmm1*. None of the resulting transformants contained the mutant form of the protein at the same level as the control strain Mmm1-HA3 in crude mitochondrial preparations (data not shown). We chose one strain (A116-124) for further analysis, but to allow functional assessment of the conserved region we required a strain with reduced levels of the HA-tagged wild type protein to serve as a control. Therefore, we re-examined strains from our original transformation of *Δmmm1* with plasmid pMmm1-HA to look for isolates with low expression of Mmm1-HA. Two strains, Mmm1-HA8 and Mmm1-HA5, were selected because A116-124 crude mitochondria contained levels of Mmm1HA intermediate between these two strains (Figure 4.6A).

Strain A116-124 exhibited a slight growth defect (Figure 4.6B) and contained mitochondria with a slightly larger diameter than those of the control strains (Figure 4.6C and Figure 4.2). When mitochondria isolated from these strains were subjected to non-reducing SDS-PAGE, Mmm1 in both low-expression control strains was found in the high molecular weight disulfide bonded form of the protein. However, in mitochondria from A116-124 no high molecular weight form could be detected (Figure 4.6D). Despite the fact that the mutant protein was readily detected in crude mitochondrial preparations (Figure 4.6A), only much reduced amounts were seen in flotation gradient purified mitochondria (compare ratios of Mmm1 in A116-124, Mmm1-HA8, and Mmm1-HA5 in the crude mitochondrial preparation of Figure 4.6A to those in the gradient

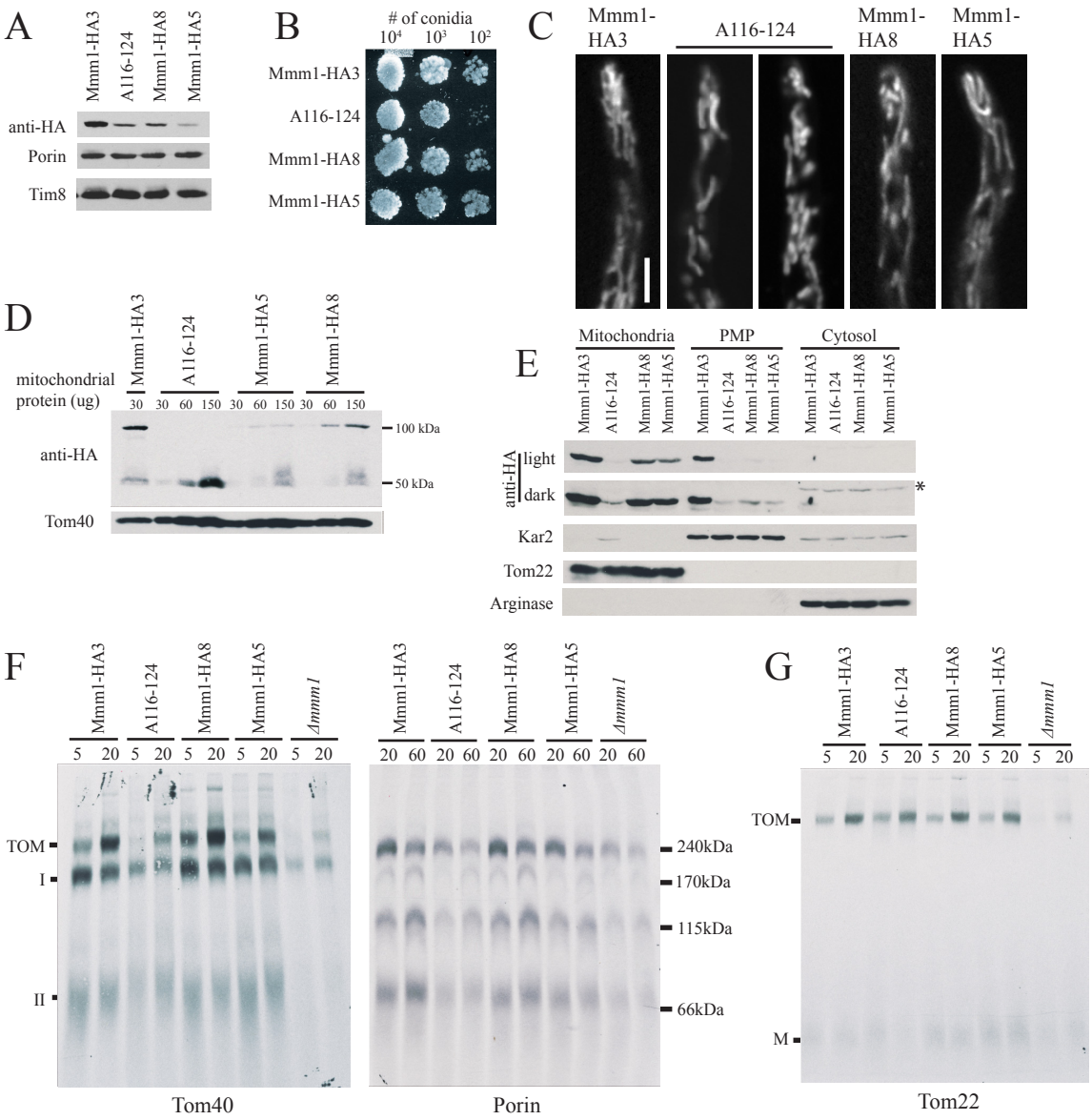


Figure 4.6. Characterization of *N. crassa* Mmm1 conserved region mutant A116-124. (A) Western blot analysis of crude mitochondria (30 ug) from the indicated strains. Samples were subjected to SDS-PAGE, transferred to nitrocellulose and analyzed by Western blotting for the indicated proteins. (B) Measurement of growth as in Figure 4.1C. (C) Confocal microscopy of mitochondria as in Figure 4.1B. (D) Non-reducing SDS-PAGE followed by Western blot analysis as in Figure 4.5B, but 30, 60, and 150µg of mitochondria isolated from the indicated strains were loaded. (E) Cell fractionation as in Figure 4.3A. Asterisk indicates a contaminating band seen in cytosolic fractions that reacts with the HA antibody. (F) Assembly of radiolabeled β-barrels as in Figure 4.5F. (G) Assembly of radiolabeled Tom22 as in Figure 4.5G.

purified mitochondrial fraction of Figure 4.6E). Thus, it appears that the A116-124 mutant form of Mmm1 is lost from crude mitochondria during gradient purification and it is conceivable that residues 116-124 of Mmm1 may be involved in physically connecting Mmm1 to the mitochondria via interactions with other ERMES proteins. Interestingly, in the strains containing lower levels of the wild type protein (Mmm1HA-5, Mmm1HA-8), Mmm1-HA is almost exclusively found associated with mitochondria rather than in the ER containing PMP (Figure 4.6E). It is possible that when Mmm1 levels are low the protein may preferentially localize to mitochondrial attachment sites, whereas at higher levels additional Mmm1 might also localize to sites in the ER that are not involved in ERMES formation.

Mitochondria from A116-124 were impaired in their ability to assembly the β-barrel proteins Tom40 and porin to an extent between *Δmmm1* and the controls (Figure 4.6F). On the other hand, the assembly of Tom22 was not affected (Figure 4.6G). This suggests that regions of the Mmm1 protein differentially affect the assembly of TOB complex substrates.

4.3.5 Mutants lacking Mmm2 exhibit phenotypes similar to other ERMES mutants.

S. cerevisiae mutants lacking Mmm2 are similar to mutants in other ERMES components in that they exhibit giant condensed mitochondria, decreased levels of mitochondrial phosphatidylethanolamine and cardiolipin, and a growth defect on non-fermentable carbon sources (Youngman *et al.*, 2004; Osman *et al.*, 2009). Examination of a *Δmmm2 N. crassa* strain revealed a slight growth defect (Figure 4.7A) and the presence of enlarged mitochondria (Figure 4.7B and Figure 4.2). However, the mitochondrial morphology defect seen in *Δmmm2* is not as severe as that in *Δmmm1* (Figure 4.7B and Figure 4.2). Mitochondria isolated from *Δmmm2* contain wild-type levels of all mitochondrial proteins examined with the exception of the intermembrane space proteins Tim8 and Tim13 which are slightly reduced (Figure 4.7C). We have previously shown that these proteins are

partially lost because of rupture of the MOM during the mitochondrial isolation procedure in cells containing enlarged mitochondria (Wideman *et al.*, 2010).

Since it has not yet been shown if lack of Mmm2 results in deficiencies of β-barrel assembly in *S. cerevisiae* or *N. crassa*, we examined the assembly of Tom40 and porin in *Δmmm2*. The formation of both Tom40 intermediate complexes and the final TOM complex was reduced in *Δmmm2* mitochondria (Figure 4.7D). Similarly, formation of all porin import complexes in mitochondria from the mutant strain were reduced (Figure 4.7D). Import of the matrix targeted protein $F_1\beta$ into mitochondria isolated from *Δmmm2* was similar to the control, while import of the inner membrane targeted AAC was slightly reduced (Figure 4.7E). This result is similar to our previous findings with *mmm1*, *mdm12* and *mdm10* mutants where we demonstrated that reduced levels of intermembrane space proteins are responsible for the AAC deficiency but not the β-barrel import phenotype (Wideman *et al.*, 2010; Lackey *et al.*, 2011). Assembly of Tom22 was also moderately reduced in mitochondria lacking Mmm2 (Figure 4.7F). We also examined whole mitochondria for levels of mitochondrial phospholipids cardiolipin (CL), phosphatidylethanolamine (PE), and phosphatidylcholine (PC). No obvious deficiencies were seen in *Δmmm2*, or other ERMES mutants (Figure 4.7G).

4.3.6 Loss of Gem1 results in partial ERMES mutant phenotypes.

Mutants lacking the Gem1 protein in *S. cerevisiae* exhibit some phenotypic characteristics of ERMES component mutants. They have altered mitochondrial morphology and delays in mitochondrial inheritance (Frederick *et al.*, 2004). We analyzed a *N. crassa* strain lacking Gem1 and found minor alterations in mitochondrial morphology (Figure 4.8A and Figure 4.2), but no alteration in growth rate (Figure 4.8B), steady state mitochondrial protein levels (Figure 4.8C), assembly of mitochondrial outer membrane β-barrel proteins (Figure 4.8D), assembly of Tom22 (Figure 4.8E), or mitochondrial phospholipid content (Figure 4.8F).

Figure 4.7. Characterization of the *Δmmm2* strain. (A) Measurement of growth rate as in Figure 4.1C. (B) Visualization of mitochondria as in Figure 4.1B. (C) Mitochondria (30 ug) isolated from the control and mutant strains were subjected to SDS-PAGE, transferred to nitrocellulose and analyzed on Western blots with antibodies to the indicated proteins (30μg mitochondrial protein per lane). (D) Assembly of radiolabeled β-barrel proteins as in Figure 4.1D. (E) As in D, but radiolabeled $F_1\beta$ and AAC were imported into mitochondria isolated from the indicated strains. After import, the mitochondria were treated with proteinase K, reisolated, electrophoresed, transferred to nitrocellulose membranes, and examined by autoradiography. Lys, 33% of the input lysate containing radiolabeled protein used in each reaction; by, mitochondria pretreated with trypsin before 3 min of import with precursor proteins. This lane serves as a control to show no import occurs when mitochondrial surface receptors have been removed; p, preprotein; m, mature protein. (F) Assembly of radiolabeled Tom22 as in Figure 4.5G (G) Total mitochondrial lipids were extracted from isolated mitochondria (300 μg protein) from the indicated strains in 1:1 chloroform : methanol. Lipids were then analyzed by TLC and stained with molybdenum blue and photographed. PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin.

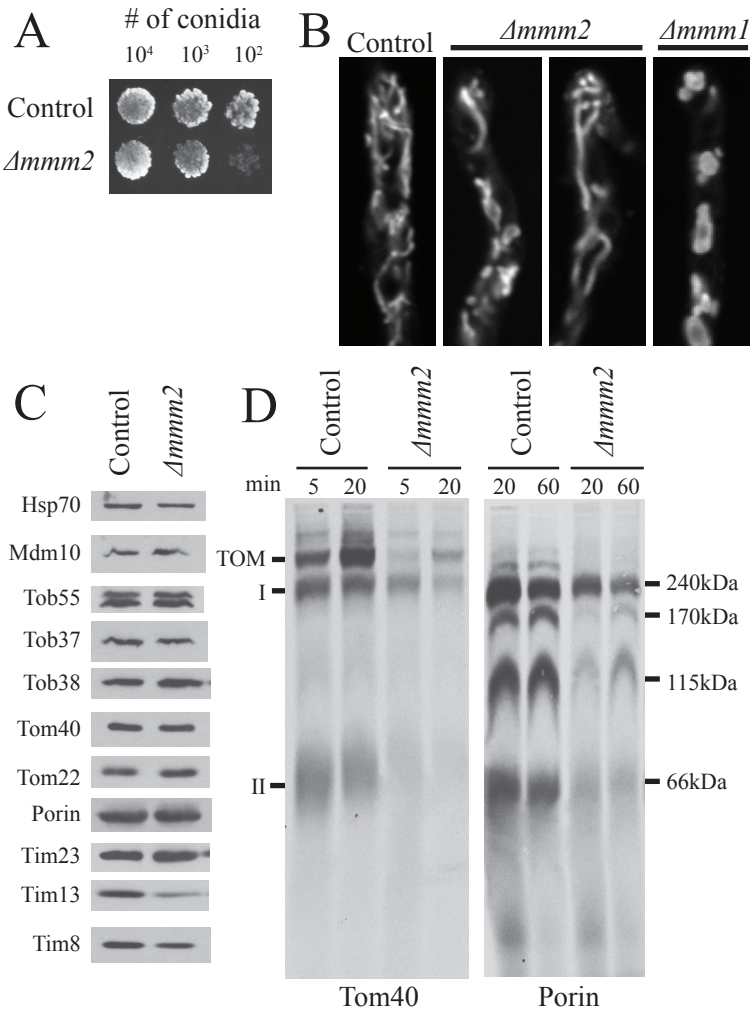
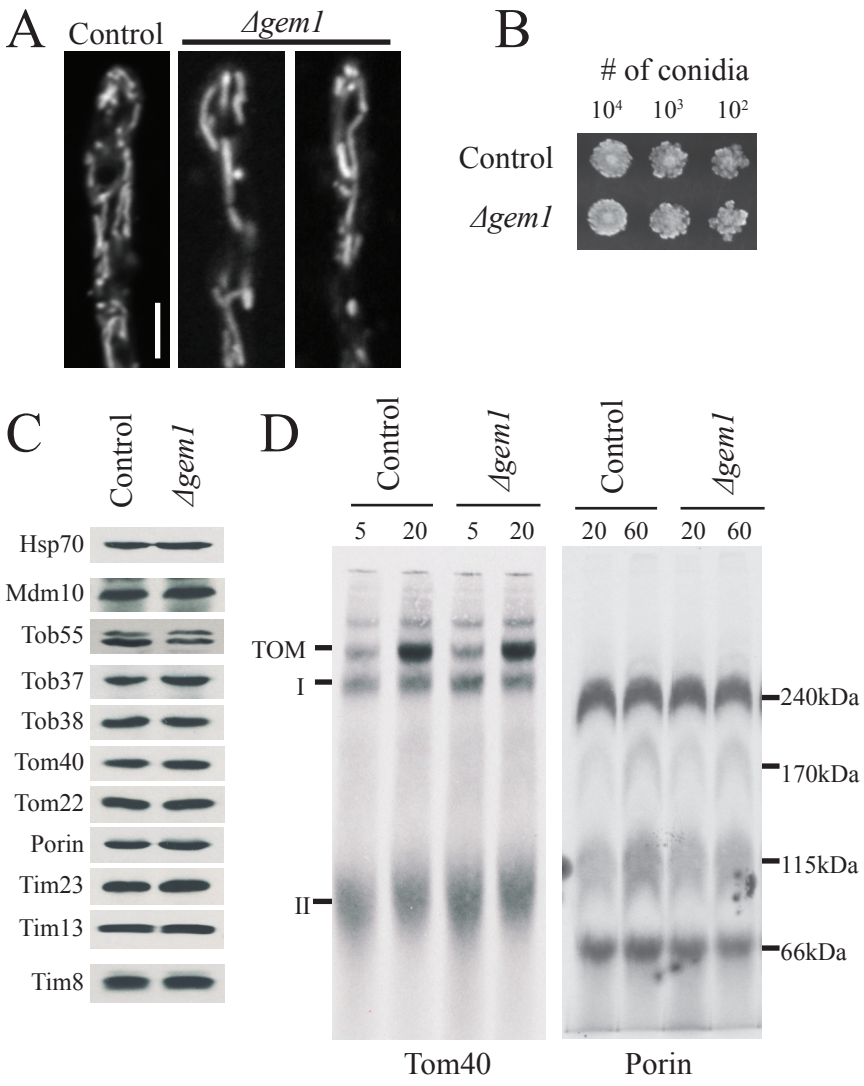

A
# of conidia
10⁴ 10³ 10²
Control
Δmmm2
B
Control
Δmmm2
Δmmm1
C
Control
Δmmm2
Hsp70
Mdm10
Tob55
Tob37
Tob38
Tom40
Tom22
Porin
Tim23
Tim13
Tim8
D
Control
Δmmm2
Control
Δmmm2
min 5 20 5 20 20 60 20 60
TOM
I
II
240kDa
170kDa
115kDa
66kDa
Tom40
Porin
E
Control
Δmmm2
min Lys 1 3 5 by 1 3 5 by
F1B
AAC
F
Control
Δmmm2
min 5 20 5 20
TOM
M
Tom22
G
Control
Δmmm2
Δmmm1
mdm12mut
Δmdm10
CL
PE
PC

Figure 4.8. Characterization of the *Δgem1* strain. (A) Examination of mitochondrial morphology as in Figure 4.1B. (B) Growth rates as in Figure 4.1C. (C) Steady state levels of mitochondrial proteins as in Figure 4A. (D) β-barrel protein assembly as in Figure 4.1D. (E) Tom22 assembly as in Figure 4.5G. (F) Mitochondrial phopholipid content was examined as in Figure 4.7G.


A
Control
Δgem1
B
# of conidia
10⁴ 10³ 10²
Control
Δgem1
C
Control
Δgem1
Hsp70
Mdm10
Tob55
Tob37
Tob38
Tom40
Tom22
Porin
Tim23
Tim13
Tim8
D
Control
Δgem1
Control
Δgem1
5 20 5 20
20 60 20 60
TOM
I
II
240kDa
170kDa
115kDa
66kDa
Tom40
Porin
E
Control
Δgem1
5 20 5 20
TOM
M
Tom22
F
Control
Δgem1
CL
PE
PC

4.4 DISCUSSION

Though *N. crassa* Mmm1 was originally thought to be a MOM protein, my fractionation studies have shown that it is most likely a membrane anchored protein of the ER, in agreement with recent findings for *S. cerevisiae* Mmm1 (Kornmann *et al.*, 2009; Kornmann *et al.*, 2011; Stroud *et al.*, 2011). This conclusion is supported by the observation that the Cys residue at position five of the protein is predicted to occur in the oxidizing environment of the ER lumen and is involved in disulphide bond formation. The C5S mutation results in loss of disulphide bond formation and a Tom40 assembly defect. However, no other defects characteristic of strains lacking Mmm1 were observed in the mutant strain. This demonstrates that the set of typical phenotypes observed in cells lacking ERMES components can be separated, at least for Mmm1, and that defects in Tom40 assembly are not secondary to other aspects of the phenotype. Furthermore, since the *Δmmm1* strain has defects in Tom40, porin, and Tom22 assembly while the C5A mutation affects only Tom40, an effect on specific aspects of TOB complex function is implied in the latter case. The mechanism whereby a missing disulfide bond in the ER lumen would affect Tom40 assembly in the MOM is not immediately obvious but may be related to the structure and topology of Mmm1. Since the disulphide bonded form of Mmm1 is twice the molecular weight of the monomer, it seems likely that the bond results in the formation of an Mmm1 dimer. Dimerization in the ER lumen would not be sufficient to dimerize the cytosolic domain, but it would keep two subunits in close proximity to each other. Subunits acting in tandem may be important for interaction with other ERMES components or other possible Mmm1 functions. Another difficulty in assessing how Mmm1 could directly influence TOB complex function is based on simple physical constraints. ERMES in *S. cerevisiae* are seen in only a small number of punctae in fluorescence microscopy studies (Kornmann *et al.*, 2009; Kornmann *et al.*, 2011), whereas the TOB complex would be expected to have a more random distribution throughout the MOM. However, the distribution of the ERMES and its components has not been investigated in *N. crassa* and it is conceivable that the arrangement of ERMES is

different in the organism. In this regard, it has been suggested that there may be many smaller ERMES in *S. cerevisiae* than originally thought (Nguyen *et al.*, 2012).

Our data demonstrate that the ER-lumen domain of *N. crassa* Mmm1 has functional importance. However, it is not clear that this would extend to other fungal species. There is considerable variation within the N-terminal ER-lumen domain of Mmm1 among different fungal groups (Figure 4.4 and Figure A.2). In all the Sordariomycetes examined, the domain is short (14 residues in *N. crassa*), lacks potential N-glycosylation sites, and contains a Cys residue. However, members of the class Saccharomycetes have a comparatively long ER-lumen domain that contains no Cys residues. In *S. cerevisiae* the domain is 96 residues long and is N-glycosylated at three sites (Kornmann *et al.*, 2011; Stroud *et al.*, 2011). However, the functional significance of glycosylation is not known since Mmm1 lacking the glycosylation sites can still rescue the inability of the *Δmmm1* strain to grow on a non-fermentable carbon source (Stroud *et al.*, 2011). Earlier work demonstrated that the first 73 residues of the yeast protein are not required to complement the mitochondrial morphology and growth defects seen in a temperature-sensitive mmm1 mutant (Burgess *et al.*, 1994). A separate study showed that the Mmm1 membrane spanning domain was required for proper mitochondrial morphology and mtDNA maintenance, but the N-terminal 90 residues were not required (Kondo Okamoto *et al*. 2003). It was also demonstrated that the *N. crassa* Mmm1 protein could complement the mitochondrial morphology and growth defects of the *Δmmm1 S. cerevisiae* mutant phenotype (Kondo-Okamoto *et al.*, 2003). However, these studies did not examine Tom40 assembly and it remains to be determined if changes in the ER-lumen domain affect the process of mitochondrial protein import/assembly in *S. cerevisiae*. Surprisingly, the Mmm1 protein in fungi from the subphylum Mucormycotina and the phylum Chytridiomycota lacks the membrane spanning domain, which would also eliminate the ER-lumen domain (Figure 4.4 and Figure A.2). If these proteins serve the same function as their *N. crassa* and *S. cerevisiae*

homologs, they may act as as strongly associated peripheral membrane proteins of the ER.

The phenotype of the A116-124 mutant is complex. No transformants expressing the mutant protein at the level of the control HA-tagged Mmm1 protein could be identified. Furthermore, the amount of Mmm1 in crude mitochondrial preparations from the mutant strain is reduced even further upon gradient purification of mitochondria. One possibility is that the stability of the mutant protein may be reduced. Surprisingly, even though the A116-124 mutation occurs in the cytosolic domain of Mmm1, it eliminates disulphide bond formation in the ER lumen domain. Alkali extraction (not shown) suggests that the protein is inserted into the ER membrane making it difficult to suggest a mechanism whereby the altered cytosolic domain affects disulphide bond formation in the ER. Despite the much reduced level of the protein observed in purified subcellular fractions, it still partially rescues the mitochondrial morphology, Tom40 assembly, and porin assembly defects seen in the *Δmmm1* strain, while fully rescuing the Tom22 assembly defect.

Although Mmm2 has been recognized as an ERMES component, it has not been examined with respect to mitochondrial import/assembly. Here we show that deletion of the gene results in a defective assembly phenotype for Tom40 and porin that resembles the defects seen in *mmm1* or *mdm12* mutants of *N. crassa*. We have also shown that mitochondria isolated from cells lacking any of the ERMES structural components are defective in the assembly of Tom22. This differs from *S. cerevisiae* where mitochondria lacking Mdm12 were shown to assemble Tom22 normally (Meisinger *et al.*, 2004).

Mutants lacking Gem1 in *S. cerevisiae* contain large globular mitochondria, exhibit a growth defect when grown on non-fermentable carbon sources (Frederick *et al.*, 2004) and are delayed in the transfer of mitochondria to daughter buds (Frederick *et al.*, 2008). The Gem1 protein is well conserved among eukaryotes and contains two GTPase and two calcium binding EF-hand domains (Fransson *et al.*, 2003). There is debate as to whether *S. cerevisiae* Gem1 is involved in ERMES function or regulation. Two groups have identified Gem1

as a member of the ERMES and it was suggested that the protein plays a role in the regulation of the size and number of ERMES complexes (Kornmann *et al.*, 2011; Stroud *et al.*, 2011). However, another group could not find evidence of Gem1 associating with the ERMES and found no alterations in ERMES size or numbers in strains lacking the protein (Nguyen *et al.*, 2012). Here we have shown that while the *Δgem1* mutant has a minor mitochondrial morphology defect in *N. crassa*, it does not display other ERMES phenotypes, such as inefficient β-barrel and Tom22 assembly. If Gem1 is an ERMES member in *N. crassa*, its role is different than the other proteins in the complex.

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5. GENERAL DISCUSSION

5.1 The Tom7-Mdm10 relationship: new developments

Prior to the publication of my work in Chapter 2 a relationship between Mdm10 and Tom7 had been reported to exist in *S. cerevisiae* (Discussed in section 2.4). Mutants lacking Tom7 or Mdm10 were shown to have reciprocal phenotypes with respect to the assembly of the β-barrels Tom40 and porin (Meisinger *et al.*, 2006). As discussed in section 2.1, a model was presented that suggested that Tom7 and Mdm10 formed a complex that functioned to maintain a steady-state ratio of TOB core to TOB holo, where TOB core preferentially assembles porin, and TOB holo preferentially assembles Tom40. My results demonstrate that, similar to *S. cerevisiae*, *N. crassa* mutants lacking Tom7 or Mdm10 exhibit reciprocal phenotypes with respect to their ability to assemble Tom40. However, I found that the mutants exhibited similar phentoypes with respect to the assembly of porin. Additionally, the model presented for *S. cerevisiae* (Meisinger *et al.*, 2006), would have predicted that loss of Mdm10 would be epistatic to the loss of Tom7 so that the *Δmdm10* phenotype should be seen in the double mutant. In contrast, my results show that *Δmdm10Δtom7* mutants exhibit additive phenotypes with respect to the assembly of both Tom40 and porin demonstrating that they likely participate in different pathways affecting the assembly of β-barrels.

After the publication of my work in Chapters 2 and 3 further investigations into the relationship between Mdm10 and Tom7 in *S. cerevisiae* have been published by two different groups (Yamano *et al.*, 2010b, a; Becker *et al.*, 2011). The first group focused on overexpression studies involving both Mdm10 and Tom7. They found that overexpression of Mdm10 caused an increase in the TOB holo to TOB core ratio and impaired the assembly of all β-barrels (Tom40, porin, Tob55 and Mdm10) (Yamano *et al.*, 2010a). This result led to the development of the following model. Endogenous Mdm10 competes with imported preproteins for a shared binding site in the TOB complex thus releasing the preprotein for further assembly. The various β-barrel preproteins are thought to have different binding affinities for the TOB complex. Porin was suggested to

have a relatively low affinity for the TOB complex and readily dissociates since a TOB complex-porin assembly intermediate cannot be detected in *S. cerevisiae*. Therefore in the absence of Mdm10, porin assembly is not impaired. However, since Tom40 binds more tightly to the TOB complex, Mdm10 is required to displace Tom40 precursor proteins. This explains why in the absence of Mdm10, a Tom40 assembly defect is observed. If too much Mdm10 is present in the MOM, then the TOB complexes become saturated with Mdm10 and no β-barrel precursor binding site is available. Thus, the assembly of all β-barrels is inhibited (Tom40, porin, Tob55 and Mdm10). Overexpression of Mdm10 had no effect on the assembly of any other TOM complex protein (including Tom22) (Yamano *et al.*, 2010a). Taken together, these results suggest that Mdm10 plays a direct role in the assembly of β-barrels. At first glance, my results appear inconsistent with this model as I have shown that mutants lacking Mdm10 exhibit defects in the assembly of Tom40 as well as porin. However, in *N. crassa*, both the TOB complex-Tom40 and the TOB complex-porin assembly intermediate can be detected (Hoppins *et al.*, 2007) suggesting that porin binds the TOB complex more strongly in this organism. Thus, this model could be true in *N. crassa*.

The second group performed experiments in which chemical amounts of mitochondrial proteins were imported into isolated mitochondria in vitro. I will refer to these experiments as "overimport". I am making the assumption that in these experiments the amount of protein imported results in much more than the endogenous amount of the protein existing in the MOM. This would result in effects similar to overexpression except that no in vivo secondary effects due to changes in cellular responses are observed (i.e. the only difference between control and test mitochondria is the excess of a particular protein). This group demonstrated that the overimport of Tom22, but not Mdm10, rescues the Tom40 assembly defect seen in *Δmdm10* mutants (Becker *et al.*, 2011). Thus, direct effects of Mdm10 on Tom40 assembly were not considered. Rather, a model was developed suggesting that the Tom40 assembly defect seen in *Δmdm10* mutants is indirect and primarily due to deficiencies in Tom22 assembly because it had previously been shown that Tom22 enhances Tom40 assembly (Humphries *et al.*,

2005) and that steady state levels of Tom22 were reduced in mutants lacking Mdm10 (Meisinger *et al.*, 2004). However, the conclusions drawn from this result appear to be logically inconsistent for two reasons. First, as mentioned above, overexpression of Mdm10 causes Tom40 assembly defects (Yamano *et al.*, 2010a). Thus, the Tom40 assembly defects seen when Mdm10 is overimported into isolated *Δmdm10* mitochondria could be explained by the "shared binding site" hypothesis described above. Second, although steady state levels of Tom22 are reduced in *S. cerevisiae* mutants lacking Mdm10 (Meisinger *et al.*, 2004), it was shown that when wild-type levels of Tom22 were restored in vivo by overexpression, the Tom40 import defect was not rescued. Furthermore, experiments in human tissue culture have shown that overexpression of Tom22 increases the assembly of both Tom40 and Tom7 into the TOM complex (Johnston *et al.*, 2002; Humphries *et al.*, 2005). The increase in the availability of Tom22 in the MOM could "prime" the MOM for Tom40 assembly, thus accelerating the Tom40 assembly rate. The priming of the MOM with Tom22 would likely accelerate Tom40 assembly in most mutant backgrounds. Overimport of Tom22 may also "prime" the MOM for Tom40 assembly, thus complementing the Tom40 assembly defect seen in mutants lacking Mdm10. In my opinion, contrary to the model put forth by the authors, their results do not discount the possibility that Mdm10 has a direct role in the assembly of Tom40. Import of Mdm10 into mitochondria lacking Mdm10 such that endogenous levels are restored (but not exceeded) has not been performed—this experiment is essential for determining if Mdm10 is directly or indirectly involved in the assembly of Tom40.

Both groups have also performed Tom7 overexpression and overimport experiments that contribute to their models of Mdm10 function. Tom7 overexpression or overimport both resulted in the dissociation of TOB holo into TOB core, and impaired the assembly of Tom40 (Yamano *et al.*, 2010b; Becker *et al.*, 2011). Overimport of Tom7 impaired Tom22 assembly (Becker *et al.*, 2011), but Tom22 assembly was not examined in the Tom7 overexpression study. Lack of Tom7 increased the rate of Tom22 assembly in both *S. cerevisiae* (Becker *et*

al., 2011) and *N. crassa* (my unpublished observations). The group that used overexpression developed a model suggesting that Tom7 functions to recruit Mdm10 and promote the dissociation of the TOB holo complex, thus regulating TOB function and β-barrel assembly. The group that used overimport have a similar model except that the role of Tom7 is in the regulation of Tom22 assembly. Both models suggest that Tom7 and Mdm10 act in opposition in the same assembly pathway. Interestingly, a synthetic growth defect is observed in strains lacking both proteins in *S. cerevisiae* (Hoppins *et al.*, 2011), suggesting that the proteins function in different parallel pathways. Consistent with this idea, the Nargang lab has previously put forth a much simpler model suggesting that lack of Tom7 destabilizes the TOM complex and promotes the exchange of TOM complex subunits (Sherman *et al.*, 2005; Wideman *et al.*, 2010). The Nargang lab model accounts for the synthetic/additive effects observed when both Mdm10 and Tom7 are absent in *N. crassa* (Chapter 2) and *S. cerevisiae*.

Surprisingly, Tom7 was found to coimmunoprecipitate with tagged Mdm12 suggesting that Tom7 is a member of the ERMES complex (Yamano *et al.*, 2010b). However, Tom7 was not detected in mass spectrometry analysis performed by other groups using tagged Mmm1 or Mmm2 (Kornmann *et al.*, 2011; Stroud *et al.*, 2011). Furthermore, deletion or overexpression of Tom7 did not cause alterations in the mitochondrial phospholipid content, suggesting that if it is a member, the function of Tom7 in the ERMES complex is not similar to the undisputed ERMES members.

In strains expressing lower amounts of Mdm10, Mdm10 preferentially associated with the ERMES complex rather than the TOB complex (Yamano *et al.*, 2010a). Isolated mitochondria from this strain assemble Tom40 nearly normally, have normal lipid levels and "nearly normal mitochondrial morphology" (Yamano *et al.*, 2010a). These results suggest that the TOB holo complex is either not required for the assembly of β-barrels or only miniscule amounts of the TOB holo complex are required for the proper assembly of β-barrels. I have made similar observations in *N. crassa* strains expressing lower amounts of Mdm10 (K.A. Norton and J.G. Wideman unpublished observations).

These results seem to suggest that Mdm10 plays an indirect role in TOM complex assembly.

Since so many seemingly conflicting interpretations of data exist, it is very difficult to derive a mechanism that can explain all the phenotypes seen in *tom7* and *mdm10* mutants. Thus far the models proposed cannot account for all of the data produced suggesting that some aspects of each model are likely false.

5.2 Role of the ERMES complex in the assembly of MOM proteins

The exact mechanism by which ERMES components affect the assembly of TOB complex substrates is not known. Investigations into the mechanism have focused on Mdm10 since it is known to associate with the TOB complex (Meisinger *et al.*, 2004; Meisinger *et al.*, 2006; Meisinger *et al.*, 2007; Wideman *et al.*, 2010; Yamano *et al.*, 2010b, a; Lackey *et al.*, 2011). As discussed above in section 5.1, two models for the role of Mdm10 in Tom40 assembly have been proposed. The first was based on the effects of overexpression of Mdm10 which led to the suggestion that because of different affinities of preproteins for the TOB complex, Mdm10 is needed to displace Tom40, but not porin, from the TOB complex (Yamano *et al.*, 2010a). The second model suggests that the role of Mdm10 in the assembly of the TOM complex primarily involves its role in the assembly of Tom22 (Becker *et al.*, 2011). The Tom40 assembly defects seen in mutants lacking Mdm10 are suggested to be caused by deficiencies in the assembly of Tom22 during Tom40 import. This model cannot be extended to other ERMES components as mutants lacking Mdm12 in *S. cerevisiae* do not have Tom22 assembly defects (Meisinger *et al.*, 2004), but do exhibit β-barrel protein assembly defects (Meisinger *et al.*, 2007). However, despite the fact that no interaction of TOB with any component of the ERMES complex other than Mdm10 has been reported, the first model might explain β-barrel protein import deficiencies in other ERMES mutants if excess Mdm10 at the TOB complex was the result of a lack of the ERMES complex. Here I demonstrate that mutants lacking ERMES components in *N. crassa* have β-barrel and Tom22 assembly defects.

If the TOB core and TOB holo complexes have different functions, then TOM complex assembly defects seen in mutants lacking Mdm12, Mmm1 or Mmm2 could be explained by the change in the ratio of TOB holo to TOB core. Lack of Mdm12, Mmm1 or Mmm2 may release Mdm10 into the MOM to interact with TOB core to form more TOB holo complexes. An increase in the TOB holo to TOB core ratio could lead to Tom40 assembly defects similar to Mdm10 overexpression mutants in which the TOB holo to TOB core ratio is increased substantially (Yamano *et al.*, 2010a). Remarkably, the import phenotypes seen in experiments using mitochondria isolated from strains overexpressing Mdm10 look very similar to *S. cerevisiae* mutants lacking either Mdm12 or Mmm1 (Meisinger *et al.*, 2007; Yamano *et al.*, 2010a). However, no study has reported on TOB holo to TOB core ratios in mutants lacking Mdm12, Mmm1 or Mmm2.

The Tom40 assembly defect seen in mutants lacking Mdm10 differs from that seen in other ERMES mutants (Meisinger *et al.*, 2004; Meisinger *et al.*, 2006; Meisinger *et al.*, 2007; Wideman *et al.*, 2010). In radiolabeled import experiments using mitochondria isolated from *mdm10* mutants, the Tom40 precursor fails to progress to the 400 kDa TOM complex while in mutants lacking either Mdm12 or Mmm1, the final 400 kDa TOM complex is observed, though at reduced levels. The difference in Tom40 assembly phenotypes seen in different ERMES mutants could be explained by the "indirect model" mentioned above. The role of Mdm10 in the assembly of Tom22 may be the cause of the different phenotype. As mentioned in section 5.1 mutants lacking Mdm10 in *S. cerevisiae* exhibit decreased steady state levels of Tom22. Steady state levels of Tom22 are also decreased in the *N. crassa Δmdm10* mutant. The levels of Tom22 in other ERMES mutants have not been investigated in *S. cerevisiae*. However, I show that mitochondria isolated from *Δmmm2* contain Tom22 levels comparable to wild-type levels and still exhibit both Tom22 and β-barrel assembly defects. Since all ERMES mutants exhibit a Tom22 assembly defect, the Tom22 defect cannot account for the unique Tom40 assembly defect seen in mutants lacking Mdm10.

In *S. cerevisiae*, the lack of Mdm12 results in a slight decrease in the steady state levels of Mdm10, Tom40 and porin (Meisinger *et al.*, 2007). Loss of

Mmm1 causes major reduction in the steady state levels of both Mdm12 and Mdm10 as well as milder reductions in steady state levels of Tom40, Tob55 and Tob37 (Meisinger *et al.*, 2007). The reduced levels of Tom40 and TOB complex proteins could explain the β-barrel assembly defects observed in both *Δmdm12* and *Δmmm1 S. cerevisiae* mutants. However, steady state levels of TOB components are not altered in *N. crassa* mutants lacking any ERMES component (Figures 2.7A, 2.10C and 4.7C) indicating that assembly defects in these mutants are independent of a decrease in the levels of TOB components. In *S. cerevisiae*, the best evidence suggesting that ERMES complex components play a direct role in β-barrel assembly was an experiment utilizing the *mmm1-1* temperature sensitive mutant. Temperature shifting this mutant revealed Tom40 assembly defects that occurred before mitochondrial morphology defects could be detected (Meisinger *et al.*, 2007). Here, I similarly demonstrate that a mutation (*N. crassa*, C5S) in *mmm1* specifically causes impaired assembly of Tom40 but no other ERMES associated defect. This result suggests that ERMES mutant phenotypes are separable and that ERMES may play a direct role in MOM protein assembly.

Membrane composition has been shown to affect the mobility of reconstituted TOM core complex when analyzed by Western blot after BNGE (Stan *et al.*, 2000). Similarly, the TOM complex exhibits altered mobility and defects in assembly in mutants affecting CL biosynthesis (Gebert *et al.*, 2009). In addition, the TOM complex has been shown to exhibit altered mobility/stability in mutants lacking ERMES components in both *S. cerevisiae* (Meisinger *et al.*, 2004; Meisinger *et al.*, 2007) and *N. crassa* (Figure 2.9A, and my unpublished observations). Since *S. cerevisiae* mutants lacking ERMES complex components have been shown to have altered mitochondrial lipid profiles (Kornmann *et al.*, 2009; Osman *et al.*, 2009; Tamura *et al.*, 2012), it is possible that altered MOM lipid composition is responsible for alterations in TOM complex mobility as well as defects in TOM complex assembly. While I have shown that the total mitochondrial lipid content of ERMES mutants is unaffected in *N. crassa*, our collaborators have demonstrated that the phospholipid to protein ratio in isolated OMV from mutants lacking Mdm10 is lower than in OMV isolated from a wild-

type strain (Wideman *et al.*, 2010). These results seem contradictory; however, it is likely that a change in the MOM lipid to protein ratio cannot be detected within the background of whole mitochondria. Thus, it is conceivable that all ERMES mutants exhibit altered MOM phospholipid to protein ratios. In order to make solid conclusions on whether or not the absence of ERMES complex components affects MOM lipid content in *N. crassa*, OMV must be isolated from all ERMES mutants and analyzed for phospholipid content and phospholipid to protein ratios.

Although arguments for secondary effects can still be made, my research has provided evidence suggesting that the ERMES complex plays a direct role in the assembly of the TOM complex. Nonetheless, much more work must be done before we can fully understand the interconnected relationships that exist between the TOM, TOB and ERMES complexes.

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APPENDIX

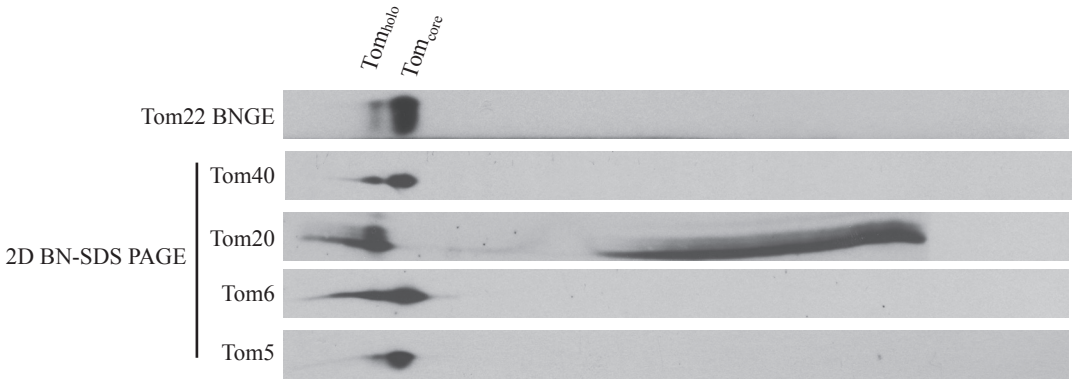
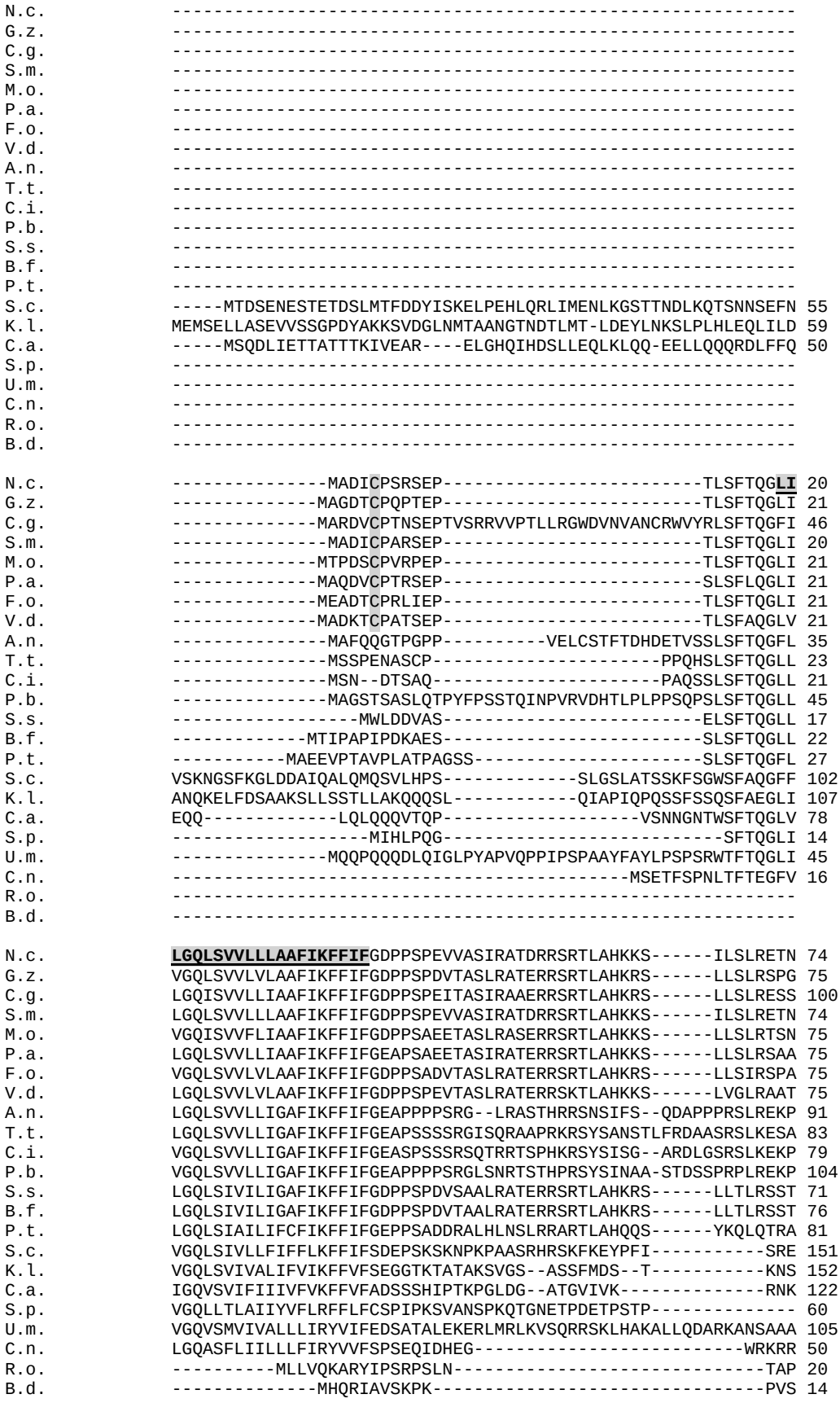


Figure A.1. *N. crassa* Tom20 is loosely associated with the TOM complex. TOM complex was purified from a strain expressing 6x His tagged Tom22 by Ni-NTA affinity chromatography and subjected to BNGE in two separate lanes. The first lane was transferred to PVDF and analyzed by Western blot for Tom22 (top lane in panel). The second lane was removed and subjected to a second dimension of SDS-PAGE. After electrophoresis the proteins were transferred to nitrocellulose and analyzed by western blot for the TOM complex proteins indicated.

Figure A.2. Alignments of fungal Mmm1 proteins. Conserved cysteines are shaded in grey. A116-124 is shaded in grey in the *N. crassa* protein. The predicted TMD in *N. crassa* is shaded in grey and underlined. Abbreviations: N.c., *Neurospora crassa*; G.z., *Gibberella zeae*; C.g., *Chaetomium globosum*; S.m., *Sordaria macrospora*; M.o., *Magnaporthe oryzae*; P.a., *Podospora anserina*; F.o. *Fusarium oxysporum*; V.d., *Verticillium dahliae*; A.n., *Aspergillus nidulans*; *Trichophyton tonsurans*; C.i., *Coccidioides immitis*; P.b., *Paracoccidioides brasiliensis*; S.s., *Sclerotinia sclerotiorum*; B.f., *Botryotinia fuckeliana*; P.t., *Pyrenophora teres*; S.c., *Saccharomyces cerevisiae*; *Kluyveromyces lactis*; C.a., *Candida albicans*; S.p., *Schizosaccharomyces pombe*; U.m., *Ustilago maydis*; C.n., *Cryptocuccus neoformans*; R.o., *Rhizopus oryzae*; B.d., *Batrachochytrium dendrobatidis*.


N.c. ----------
G.z. ----------
C.g. ----------
S.m. ----------
M.o. ----------
P.a. ----------
F.o. ----------
V.d. ----------
A.n. ----------
T.t. ----------
C.i. ----------
P.b. ----------
S.s. ----------
B.f. ----------
P.t. ----------
S.c. -----MTDSENESTETDSLMTFDDYISKELPEHLQRLIMENLKGSTTNDLKQTSNNSEFN 55
K.l. MEMSELLASEVVSSGPDYAKKSVDGLNMTAANGTNDTLMT-LDEYLNKSLPLHLEQLILD 59
C.a. -----MSQDLIETTATTTKIVEAR----ELGHQIHDSLLEQLKLQQ-EELLQQQRDLFFQ 50
S.p. ----------
U.m. ----------
C.n. ----------
R.o. ----------
B.d. ----------
N.c. ----------MADICPSRSEP----------TLSFTQGLI 20
G.z. ----------MAGDTCPQPTEP----------TLSFTQGLI 21
C.g. ----------MARDVCPTNSEPTVSRRVVPTLLRGWDVNVANCRWVYRLSFTQGFI 46
S.m. ----------MADICPARSEP----------TLSFTQGLI 20
M.o. ----------MTPDSCPVRPEP----------TLSFTQGLI 21
P.a. ----------MAQDVCPTRSEP----------SLSFLQGLI 21
F.o. ----------MEADTCPRLIEP----------TLSFTQGLI 21
V.d. ----------MADKTCPATSEP----------TLSFAQGLV 21
A.n. ----------MAFQQGTPGPP----------VELCSTFTDHDETVSSLSFTQGFL 35
T.t. ----------MSSPENASCP----------PPQHSLSFTQGLL 23
C.i. ----------MSN--DTSAQ----------PAQSSLSFTQGLL 21
P.b. ----------MAGSTSASLQTPYFPSSTQINPVRVDHTLPLPPSQPSLSFTQGLL 45
S.s. ----------MWLDDVAS----------ELSFTQGLL 17
B.f. ----------MTIPAPIPDKAES----------SLSFTQGLL 22
P.t. ----------MAEEVPTAVPLATPAGSS----------SLSFTQGFL 27
S.c. VSKNGSFKGLDDAIQALQMQSVLHPS----------SLGSLATSSKFSGWSFAQGFF 102
K.l. ANQKELFDSAAKSLLSSTLLAKQQQSL----------QIAPIQPQSSFSSQSFAEGLI 107
C.a. EQQ----------LQLQQQVTQP----------VSNNGNTWSFTQGLV 78
S.p. ----------MIHLPQG----------SFTQGLI 14
U.m. ----------MQQPQQQDLQIGLPYAPVQPPIPSPAAYFAYLPSPSRWTFTQGLI 45
C.n. ----------MSETFSPNLTFTEGFV 16
R.o. ----------
B.d. ----------
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G.z. VGQLSVVLVLAAFIKFFIFGDPPSPDVTASLRATERRSRTLAHKRS------LLSLRSPG 75
C.g. LGQISVVLLIAAFIKFFIFGDPPSPEITASIRAAERRSRTLAHKRS------LLSLRESS 100
S.m. LGQLSVVLLLAAFIKFFIFGDPPSPEVVASIRATDRRSRTLAHKKS------ILSLRETN 74
M.o. VGQISVVFLIAAFIKFFIFGDPPSAEETASLRASERRSRTLAHKKS------LLSLRTSN 75
P.a. LGQLSVVLLIAAFIKFFIFGEAPSAEETASIRATERRSRTLAHKKS------LLSLRSAA 75
F.o. VGQLSVVLVLAAFIKFFIFGDPPSADVTASLRATERRSRTLAHKRS------LLSIRSPA 75
V.d. LGQLSVVLVLAAFIKFFIFGDPPSPEVTASLRATERRSKTLAHKKS------LVGLRAAT 75
A.n. LGQLSVVLLIGAFIKFFIFGEAPPPPSRG--LRASTHRRSNSIFS--QDAPPPRSLREKP 91
T.t. LGQLSVVLLIGAFIKFFIFGEAPSSSSRGISQRAAPRKRSYSANSTLFRDAASRSLKESA 83
C.i. VGQLSVVLLIGAFIKFFIFGEASPSSSRSQTRRTSPHKRSYSISG--ARDLGSRSLKEKP 79
P.b. VGQLSVVLLIGAFIKFFIFGEAPPPPSRGLSNRTSTHPRSYSINAA-STDSSPRPLREKP 104
S.s. LGQLSIVILIGAFIKFFIFGDPPSPDVSAALRATERRSRTLAHKRS------LLTLRSST 71
B.f. LGQLSIVILIGAFIKFFIFGDPPSPDVTAALRATERRSRTLAHKRS------LLTLRSST 76
P.t. LGQLSIAILIFCFIKFFIFGEPPSADDRALHLNSLRRARTLAHQQS------YKQLQTRA 81
S.c. VGQLSIVLLFIFFLKFFIFSDEPSKSKNPKPAASRHRSKFKEYPFI----------SRE 151
K.l. VGQLSVIVALIFVIKFFVFSEGGTKTATAKSVGS--ASSFMDS--T----------KNS 152
C.a. IGQVSVIFIIIVFVKFFVFADSSSHIPTKPGLDG--ATGVIVK----------RNK 122
S.p. VGQLLTLAIIYVFLRFFLFCSPIPKSVANSPKQTGNETPDETPSTP---------- 60
U.m. VGQVSMVIVALLLIRYVIFEDSATALEKERLMRLKVSQRRSKLHAKALLQDARKANSAAA 105
C.n. LGQASFLIILLLFIRYVVFSPSEQIDHEG----------WRKRR 50
R.o. ----------MLLVQKARYIPSRPSLN----------TAP 20
B.d. ----------MHQRIAVSKPK----------PVS 14

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G.z.          NR----QDR---ELNRKKSTVLRN----PPALTIGSILSKTYYNVDSHQPESLDWFNVLI 124
C.g.          TRRAGQQP----TLNRKKSSILRPS---PPPLTIGSILDKTYYKVDSHQPESLDWFNVLV 153
S.m.          ALQLVQNP----ALNKKH--VLRPG---PPTLTIGSILSKTYYKVDSHQPESLDWFNVLI 125
M.o.          QRPGSQQQQS--VLNRKKSSILRSG---PPSLTIGSILNKTYYRVESHQPESLDWFNVLV 130
P.a.          TQRQGSQPPALPALNKKKSSILRSN---PPTLTIGSILDKTYYKVDSHQPESLDWFNVLI 132
F.o.          NRGDRSQDR---SLSRKKSTVLRN----PPTLTIGSILSKAYYNVDSHQPESLDWFNVLI 128
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T.t.          S-SNVLRPVP--SSSTNTKSILRKTYYNAIPTNFQ-KNGRNRLHHSTHQPESLDWFNVLI 139
C.i.          S-SNVLRPVP--SSSTNTRSILRKTYYSANPTNFTSKHGRHRPHHSTHQPESLDWFNVLI 136
P.b.          STSNILRPVP--SSSTNTRSILRKTYYSATPTHPTPKHGRPRLYHSSHQPESLDWFNVLI 162
S.s.          PRHASQS------LNRKRSSVLRN----PAPLTTNAILSKTYYNVDSHQPESLDWFNVLI 121
B.f.          PRRASQP------LNRKRSSVLRN----PAPLTTNAILSKTYYNVDSHQPESLDWFNVLI 126
P.t.          NSTSLSLR------HKPSTSIIRKGEETRGGPSIATILAKTYYNVKGHQPESLDWFNVLI 135
S.c.          FLTSLVRKGA------KQHYELNEEAENEHLQELALILEKTYYNVDVHPAESLDWFNVLV 205
K.l.          ILSTIIKRGG------KDGLEVDDK-DNEKSRQINSILEKTYYNVETHSPESLDWFNVLI 205
C.a.          NKKHSNGQFA------NDGENEDDTSLDSNQSKISSILEKTYYDVNNHASESLDWFNVLV 176
S.p.          -------------------LSNNKKRYKKPLTILEPHILNLLYDVNEHEPESLDWFNVLI 101
U.m.          SAAAAAAPSP----ASHPLRKSHRLASDTRASMFANILDKTAYDLSSHLPESADWLNVMF 161
C.n.          AERADLLSN-------------------HTPPPLSNLLSKTSYDMSIHPAESSDWVNVLL 91
R.o.          SVAAAALPN-------------------------DHITLKTYYDVIHHPPESTDWLNVLL 55
B.d.          AQQRRTIDS--------------------------LILGKIGYNTSQHPFESCNWLNILI 48
                                                  .       *  ** :*.*::.

N.c.          AQTIAQFRSDA-----QHDDAILSSLSKALNG--------------------TARPDFLD 160
G.z.          GQTIAQFRSDA-----QHDDAILDSLSKALNG--------------------GSRPDFID 159
C.g.          AQTIAQFRSDA-----QHDDAILTSLTKALNG--------------------TSRPDFVD 188
S.m.          AQTIAQFRSDA-----QHDDAILSSLSKALNG--------------------TARPDFLD 160
M.o.          AQTIAQFRSDA-----QHDDAILTSLTKALNG--------------------TSRPSFLD 165
P.a.          AQTIAQFRSDA-----QHDDAILTSLSKTLNG--------------------TSRPDFVD 167
F.o.          AQTIAQFRSDA-----QHDDAILDSLTKALNG--------------------DSRPDFID 163
V.d.          AQTIAQFRSDA-----QHDDAILSSLTKALNG--------------------TSRPDFVD 160
A.n.          AQTIAQYRQTAYLLKDSPTSSILHSLTAALNNP--------------------EKKPSFID 187
T.t.          AQLIAQYRQTAYLLKDSPTSSILDSLTDTLNNV--------------------EKKPSWID 180
C.i.          AQTIAQYRQTAYILKDSPTSSILESLATTLNNP--------------------EKKPSFID 177
P.b.          AQTIAQYRQTAYILKDSPTSSILASLSETLNNP--------------------EKKPSFID 203
S.s.          AQTIAQFRADA-----QHDDAILTSLTKALNG--------------------GNRPDFLD 156
B.f.          AQTIAQFRADA-----QHDDAILTSLTKVLNG--------------------GNRPDFLD 161
P.t.          AQTIAQLRADA-----RQDDAILTSLTEVLNT--------------------GSKPDWIG 170
S.c.          AQIIQQFRSEA-----WHRDNILHSLNDFIGRKS-------------------PDLPEYLD 242
K.l.          AQTIHQFREEA-----LQKNNILNSLNDFIERRS-------------------NELPQYLD 242
C.a.          AQTISQLRSEA-----LLKDNIYHSLNNFLTN--------------------AKLPDFID 211
S.p.          AQALIQFRYDA-----CSNDVALRKLETVLNKG-------------------AQDKSMVD 137
U.m.          AQAIAGYREDVLTGG--VSSHHHTASDAIPSPNPLEPQKERTARDLMEEILNRATSSFLD 219
C.n.          AQILQGYRNDLLSEG--GEEGARQRIEGWLNPK--------------------GENLSWLD 130
R.o.          AQVILQYRQDAS-----INNRMSCALDSVFNSG--------------------VRPSFVG 90
B.d.          AQFLLTLRTDAEFG-----LKSVSMLDSILNSA--------------------WKPSFLG 83
              .* :   *                                                . :.

N.c.          EIKVTELSLGEDFPIFSNCRIIPVDEDGLSFGTGKAFDANMATREGARLQARMDVDLS-D 219
G.z.          EIRVTELSLGEDFPILSNCRIIPVDEDGVSLGHGKKFDPDKAARDGTRLQARMDVDLS-D 218
C.g.          EIRVTELSLGEDFPIFSNCRIIPVDEDGLNLQSGKGLDANMATREGARLQARMDVDLS-D 247
S.m.          EIKVTELSLGEDFPIFSNCRIIPVDEDGLSFGTGKAFDANMATREGARLQARMDVDLS-D 219
M.o.          EIRVTELSLGEDFPIFSNCRIIPVDEDGLDFGPGKAFDANMATRDGGTLQARMDVDLS-D 224
P.a.          EIRVSELSLGEDFPIFSNCRIIPVDEDGLQFGAGKAFDPKQAAREGARLQARMDVDLS-D 226
F.o.          EIRVTELSLGEDFPILSNCRIIPVDEDGVSLAPGKKFDPATAARDGLRLQARMDVDLS-D 222
V.d.          EIRVTKLSLGEDFPIFSNCRIIPVDEDGLSLGNSKTIDASAATREGAKLQARMDVDLS-D 219
A.n.          KITVTDISLGEEFPIFSNCRIIAVDDP---------------MSDGGRLQALLDVDMSDD 232
T.t.          RINVTDISIGEEFPIFSNCRVIAVDDP---------------NSDGGRLQALMDVDLSDD 225
C.i.          DITVTDISLGEEFPIFSNCRVIAIDDP---------------SSDGGRLQALMDVDLSDD 222
P.b.          IIKVTDISLGEEFPIFSNCRVIAVEDP---------------NSDGGRLQALMDVDLSDD 248
S.s.          EIKVTELSLGEDFPIFSNCRVIPVDEDGITLGR----EGGAAGREHGRLQARMDVDLS-D 211
B.f.          EIKVTELSLGEDFPIFSNCRVIPVDEDGMTLGR----EGGAAGREHGRLQARMDVDLS-D 216
P.t.          EIKVTEIALGDEFPIFSNCRVMPA-EDGFWYGPG------NTGNDKERLQARMDVDLS-D 222
S.c.          TIKITELDTGDDFPIFSNCRIQYSP-----------------NSGNKKLEAKIDIDLN-D 284
K.l.          QIKITEVDIGDDFPIFSNCIIQYSP-----------------NSNKKRLEAKIDIDLS-D 284
C.a.          TINLTEIDIGDDFPIFSNCRIKYG-------------------EDLKRLEAKIDVDLS-D 251
S.p.          HIYVRDLSLGDGFPVFSHCRVLPHQHN-----------------SSQLRAEMLVSLT-D 178
U.m.          PIRVTEADFGDAYPIFTNARVRPADDT-----------------GRTRIEIDVDYS-D 259
C.n.          PIDVTSLSLGTSYPLLSNARIRPADGQ-----------------GRLRAEIDVDYL-D 170
R.o.          PIHVTELNLGQEFPIFSRARIRPSDEA-----------------GSTRAEIDFEYS-D 130
B.d.          DISITNFSLGEEYPTLKNARVRFAEPE-----------------SGMKIHVDFSFD-D 123
               * : .   *  :* :... :                          .  : ..   *
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N.c.          MITLAVETKLLLNYPKRLSAVLPVALAVSVVRFSGTLSISFIPSN--------------- 264
G.z.          MLTLAVETKLLLNYPKKLSAVLPVALAVSVVRFSGTLSISFIPSN--------------- 263
C.g.          MITLAVETKLLLNFPKRLSAVLPVALAVSVVRFSGTLSISFVPSN--------------- 292
S.m.          MITLAVETKLLLNYPKRLSAVLPVALAVSVVRFSGTLSISFIPSN--------------- 264
M.o.          MITLALETKLLLNYPKKLTAVLPVALAVSVVRFSGTLAISFIPSN--------------- 269
P.a.          MITLAVETKLLLNFPKRLSAVLPVALAVSVVRFSGTLSISFNPSN--------------- 271
F.o.          MLTLAVRTKLILNYPKKLSAVLPVELAVSVIRFSGTLSISFIPSN--------------- 267
V.d.          LLTLALETKLLLNYPKKLSAVLPVALTVSVRRFSGTLSVSFIPSN--------------- 264
A.n.          NLSIAVETSLVLNYPKPCSAILPVALSISVVRFSGTLCISLVPAS--------------T 278
T.t.          NLSLAIETNLLLNYPKPASAVLPVALSVSVVRFSGTLCISFVPSP--------------G 271
C.i.          NLSLAIETNLVLNYPKPYSAILPVALSVSVVRFSGTLCISFVPGT--------------T 268
P.b.          NLSLAIETSLLLNYPKPFSAVLPVALAVSVVRFSGTLCISFVPGP--------------R 294
S.s.          FITLAVETKLLLNYPKPLVAVLPVALAVSVMRFSGTLSISFVPGS--------------- 256
B.f.          FITLAVETKLLLNYPKPLVAVLPVALAVSVVRFSGTLSISFVPGS--------------- 261
P.t.          VITIGVETTLNLNWPKPMSAVLPVALAVSIVRFSGTLAMSFIPSSSPPSTTAPMPSPTCN 282
S.c.          HLTLGVETKLLLNYPKPGIAALPINLVVSIVRFQACLTVSLTN----------------- 327
K.l.          RLALGIETKLLLNYPKPFSAALPIKLTVSIVRFQACLTVSLTT----------------- 327
C.a.          TLTLGIATKLLLNQPRPLTAVLPVSMTVSIVRFSGCLTVSLINTKDIDLKNVDKTSNMNG 311
S.p.          NINCTVDTKLLLNFPKPAFATLPLSITVRICKFVGKVSLFTTLIVYS------------- 225
U.m.          QITLAIDTKLLINFPKPRFAVLPVSLGLTIVRFSGTLAIELFSSD--------------- 304
C.n.          SLSMTLSTAVLVNFPKPRFAVLPVTLGVELVSIGGTMSVQLHEPI--------------- 215
R.o.          QVTLGIETQLILNWPKQAFAVLPVSLVLSVVRFSGTLTIELINP---------------- 174
B.d.          QLTLGIDTQMLINYPKPGMAALPISIVLSIVKFSGTFVIEFVSKP--------------- 168
                :   : * : :* *:   * **: : : :  : . . :

N.c.          ---------PSNNE-------------------------------------------PAK 272
G.z.          ---------PSQST-------------------------------------------PTR 271
C.g.          ---------PSENT-------------------------------------------PTK 300
S.m.          ---------PSNNE-------------------------------------------PAK 272
M.o.          ---------PSQST-------------------------------------------PTK 277
P.a.          ---------PSENT-------------------------------------------PTK 279
F.o.          ---------PSQST-------------------------------------------PTR 275
V.d.          ---------PSQST-------------------------------------------PTM 272
A.n.          PPLHTPSPMPSPPTAGAQPAAGAQPTDGGDIP----PKSSS---------------KSN 318
T.t.          TTS----ESSSNPPPHSEGQNEP---RPGQED----PRRPSSKDGIRS------GIPKTS 314
C.i.          QTSTHLATSPSNIDPTLQTNDYSGANRRGNRR----QERTDTEQATQANNAGTTGIPKTS 324
P.b.          TSDQTMSPIPTPHDTTSEAIDDQSSDQPSPAQNPDGPKDAHANTSNTTDASSKHGIPKTS 354
S.s.          ---------PLNGS-------------------------------------------PTT 264
B.f.          ---------PLNGS-------------------------------------------PTT 269
P.t.          THRSSSPSRPTSSSGAPP---------------------------------------HRPTT 305
S.c.          --AEEFASTSNGSSSENGMEGNS----------------------------------GYF 351
K.l.          --DEQFVPTSEETN-DDEMGNDK----------------------------------GYY 350
C.a.          YSKENANGDGASSSNNDEDEDDG----------------------------------GTA 337
S.p.          ---LYDSLHPSPGGLKHQVLNRITVIMIYFSP------------------SNGAGQPAY 263
U.m.          ------------------------------------------------------PNATV 309
C.n.          ------------------------------------------------------EDRQH 220
R.o.          ------------------------------------------------------PETTT 179
B.d.          ------------------------------------------------------ILPVC 173

N.c.          MIFTFLDDYRLDFSIRS----------------------LLGSRSRLQDVPKIAQLVE 308
G.z.          MIFNFLDDYRLDFSIRS----------------------LLGSRSRLQDVPKIAQLIE 307
C.g.          MTFTFLDDYRLDFSIRS----------------------LMGSRSRLQDVPKIAQLVE 336
S.m.          MIFTFLDDYRLDFSIRS----------------------LLGSRSRLQDVPKIAQLVE 308
M.o.          MVFNFLDDYRLDFSIRS----------------------LVGSRSRLQDVPKIAQLVE 313
P.a.          MTFTFLDDYRLDFSIRS----------------------LLGSRSRLQDVPKIAQLVE 315
F.o.          MIFNFLDDYRLDFEIRS----------------------LLGSRSKLHNVPKIAQLVE 311
V.d.          MTFNFLDDYRLDFSIGS----------------------LLGSRSKLEDVPKIAQLIE 308
A.n.          IAFSFLPDYRLDLSVRS----------------------LIGSRSRLQDVPKVAQLVE 354
T.t.          LAFSFLPDYRLDISVRS----------------------LIGSRSRLQDVPKVAQLVE 350
C.i.          LAFSFLPDYRLDLSVRS----------------------LIGSRSRLQDVPKVAQLVE 360
P.b.          LAFSFLPDYRLDLSVRS----------------------LIGSRSRLQDVPKVAQLVE 390
S.s.          LAFCFLDDYRLDLSIRS----------------------LVGSRSRLQDVPKIAQLIE 300
B.f.          LAFCFLDDYRLDLSIRS----------------------LVGSRSRLQDVPKIAQLIE 305
P.t.          LAFTFLDDYRLDLSVRS----------------------LVGSRSRLQDVPKIAQLIE 341
S.c.          LMFSFSPEYRMEFEIKS----------------------LIGSRSKLENIPKIGSVIE 387
K.l.          LMFSFNPEYRMELEVKS----------------------LIGARSKLENIPKIASLIE 386
C.a.          LMFSFSPDYRLEFIVKS----------------------LIGSRAKLQDVPKISSLIE 373
S.p.          MNLSFDPNFVISLQVSS----------------------LVGARSKLQDIPKITQLIE 299
U.m.          LPTANPNPSSSSSSSATPPRSRHQLHFSLHPDFALEASATSLLGSRAKLQDIPKIEQLLI 369
C.n.          IHVNLLPDFHLNLKVTS----------------------LLGSRAKLQDIPKLEQLIV 256
R.o.          KP-------------------------------------------KIPLERYIAI 191
B.d.          QQPLYKSQASETSTS---------------------------------DLGHHTYVSV 198
```

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                                                          .:     :

N.c.            SRLHRWFDERCVEPRFQEIALPNMWPRKKNTRG---GDETISDVER------------S 352
G.z.            SRLHRWFDERAVEPRFQEIALPSLWPRKKNTRG---PDDGLAEGSV------------P 351
C.g.            SRLHRWFDERCVEPRFQEIALPSMWPRKKNTRG---GDEAIADAER------------S 380
S.m.            SRLHRWFDERCVEPRFQEIALPNMWPRKKNTRG---GDETISDVER------------S 352
M.o.            SRLHRWFDERCVEPRFQEIPLPSMWPRKKNARG---GDDAIADVER------------S 357
P.a.            SRLHRWFDERCVEPRFQEIELPSMWPRKKNTRG---GDEIIANIEQ------------S 359
F.o.            ARLHRWFDERAVEPRFQEIALPSLWPRKKNTRG---PEDASTERSE------------S 355
V.d.            ARLHRWFDERCVEPRFQEIALPSLWPRKKNTRG---PDDGMAEGNIG------------S 353
A.n.            ARVHAWFEERVVEPRVQVVGLPDLWPRMGRTGVRT-GDESETGSNTASRPAMSVDMS--S 411
T.t.            ARVQSWFEDRVVEPRVQLVALPGIWPRMGRTGVRA-QEDHDAVSDSDDP-EAKAAKSGFT 408
C.i.            ARVQAWFEERVVEPRVQVVALPGIWPRMGRTGVRG-QEEQQEVGSSGNAGVSTANVSMLG 419
P.b.            ARVQSWFEERVVEPRVQVVGLPNIWPRMGRTGLRSSQEEPEAGSGSVEIPVMTSPGADGV 450
S.s.            ARLHTWFDERCVEPRFQQIELPSLWPRKKNTRG---GEDLDTGSEAG------------G 345
B.f.            ARLHTWFDERCVEPRFQQIELPSLWPRKKNTRG---GEDLDTGSDAG------------G 350
P.t.            SRVHAWFDERAVEPRFQQIVLPSLWPRKHNTRGGA-PEDTEAAVEGEEGLDEDDFAVVDG 400
S.c.            YQIKKWFVERCVEPRFQFVRLPSMWPRSKNTRE---EKPTEL------------------ 426
K.l.            YQISKWFVERCVEPRFQFVKLPSMWPRSKNTRK---EKTDTDDSVS-------------- 429
C.a.            NQLRTWFIERCVEPRFQVVRLPSLWPRTKNTREPVTKKTTTTPSTT-------------- 419
S.p.            SRIRQWFTNRCVSPQFQQIAIPNLWPTSAKEGHARSHAPQEESSNED------------- 346
U.m.            SRLRGWIMDRFVWPRYWSLTLPNLVPSPAASRSFSAAAAANRHTDAASVGSSGGHGQINV 429
C.n.            SRLRNLVQDRFVHPNHISLALPRILSPSVSSTPILEGLGEGAVDAMKDAVSDGMKRMVED 316
R.o.            SSYSDFILD-------LQIKVPNMWK---------------------------------- 210
B.d.            SVLDDFLLD---------FDVRSLLG---------------------------------- 215
                      . :         . :  :
N.c.            MSKAKG----------VDIAKDVREEARKEIEAEAHGGADRV---------------PDS 387
G.z.            IGRSKG----------RDASRDPQ-EEARG---DAEIRDR-----------------GT 379
C.g.            MGKSKG----------MEMAKEMRTEARREVEAETDVRADRG---------------HES 415
S.m.            MNKTKG----------VDIAKDVREEARKEIEAEAHGGADRV---------------PDS 387
M.o.            MSKAKG----------ADVARDLR----REVLVEAEARQAAQ---------------RDS 388
P.a.            INKAHG----------GAIAKEAR----QELDTETDG----------------------- 382
F.o.            IGRSRG----------NDAGRDHR-EEIRGRGGDAEIRDRGA---------------GGT 389
V.d.            VGRSKG----------RDVGQDIR-AEARK-EVEAEASSRTD---------------RAQ 386
A.n.            PGHLQGD--------GGNHEEELRFRGLGPRPPLPFDAVSRTSSYQVE---------TGA 454
T.t.            PVNANRD--------GPQTPRDLNMDGLRYRRGNGGGQSTPDNYENLP---------QGD 451
C.i.            ARDAGAE--------GSHATRDADMEGLRYRRNASPGDETSGVRYSPQ---------NQD 462
P.b.            SGGGGSG--------GGSGGGGGGMRGIDRGLSGREAGYEALRYRHAA---------CGG 493
S.s.            IGRARS----------RDVERDLREEARKEVEAETGVRVGRSKLGVSLDVPDVGLD-GGS 394
B.f.            IGRARS----------RDVERDLREEARKEVEAETGIRVGRSKLGVSLDVPDE-----GS 395
P.t.            NGTAPGSTSYIPTPIAENATLEERIEAEGAKMREAEIRAGVRKPSASQERSRGRDDRADG 460
S.c.            ------------------------------------------------------------
K.l.            ----------------VKSND---------------------------------------- 434
C.a.            ----------------VNGTSAATVTTPGEYVNSNI----------------------- 439
S.p.            ------------------------------------------------------------
U.m.            GTGVERTDASVIQPGHHESARQEMLHAASERPS------LASSRPPHVRSSSSGLRANGM 483
C.n.            FMGENPVEGALNGQGEEQWLDDDFPPTPLVQPPGTFPTLSVSSRQSHRQSLPPSRPQSTT 376
R.o.            -----------------EQEEQEE----------------------------------- 217
B.d.            ----------------HRTKVKDLPKLTSLISN------SLRSVFISEMVFPACKMIKIP 253

N.c.            LRYRHRPRADEEFPGAGSMPGSMPG-SMP----------------------------- 415
G.z.            IRQRRGTRGSDSDD--FSMPGSLPSFQIPTP--------------------------- 408
C.g.            LRYRRRPQTDDSFSTSGSMPGSMPDLDIPT---------------------------- 445
S.m.            LRYRHRPRAEEEFPGAGSMPGSMPG-SMP----------------------------- 415
M.o.            LRYRR-PRADDAFP----MPGSLAVDD------------------------------- 410
P.a.            LRYRRRPVGDDTYSVSGSMPGSLPGIDMPT---------------------------- 412
F.o.            VRHRRGTRSNDSDD--FSMPGSLPDFQVAAS--------------------------- 418
V.d.            ESLRR-RRLRSEDE--LIMPGSLPDFVQ------------------------------ 411
A.n.            PRSPSLTRERSLG-DDFHMPGSMPEAPGAQ---------------------------- 483
T.t.            GHPQPTSG------EQFRIPGSLPGTSAIA---------------------------- 475
C.i.            SREQACRD------DPFRIPGSLPDVVPVT---------------------------- 486
P.b.            HQNQSGRDGGRGGNEQFAMPGSMPDTVTET---------------------------- 523
S.s.            EEGLRFRRRSRGRGDEYAMPGSMPGLSMA----------------------------- 423
B.f.            EDGLRFRRKSKGR-DEYAMPGSMPGLSMT----------------------------- 423
P.t.            MRWRGEQRERTTGPGRPKLQSRTTTGIVKAIPGALPR---------------------- 497
S.c.            ------------------------------------------------------------
K.l.            ------------------------------------------------------------
C.a.            ------------------------------------------------------------
S.p.            ------------------------------------------------------------
U.m.            GSVEAWRAHAAGVYNQPSHQHATSLNQAALLRAHVGGGGATDCAPGSDVNIEVPGSYRGS 543
C.n.            QGQPQLFYRRPLIHPTQSYPHYNTYTLDPQIPHSVSYRHPPRGSHVHNPPETPVPQRPSH 436
R.o.            ------------------------------------------------------------
```

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B.d.            NGEELFGDIANAVDENSHDVFLNQENGSETLKSA-------------------------- 287


N.c.            ---------------------------------------
G.z.            ---------------------------------------
C.g.            ---------------------------------------
S.m.            ---------------------------------------
M.o.            ---------------------------------------
P.a.            ---------------------------------------
F.o.            ---------------------------------------
V.d.            ---------------------------------------
A.n.            ---------------------------------------
T.t.            ---------------------------------------
C.i.            ---------------------------------------
P.b.            ---------------------------------------
S.s.            ---------------------------------------
B.f.            ---------------------------------------
P.t.            ---------------------------------------
S.c.            ---------------------------------------
K.l.            ---------------------------------------
C.a.            ---------------------------------------
S.p.            ---------------------------------------
U.m.            VAGAGIELASSSNASSVLPTGAHHSSGLRNRPGFVQ--- 579
C.n.            GQGRMSTTSSLTPSQSQSQFRFRGQFASGVTPGQVGTSR 475
R.o.            ---------------------------------------
B.d.            ---------------------------------------
```

Table A.1. Strains used in this study.

Strain	Genotype	Origin or reference
76-26	*his-3 mtrR a* (*mtrR* imparts fluorophenylalanine (fpa) resistance)	R.L. Metzenberg
71-18	*pan-2 Ben^R* (*Ben^R* imparts benomyl resistance)	R.L. Metzenberg
HP1	Heterokaryon of 76-26 and 71-18	R.L. Metzenberg
NCN251	*A*	FGSC[1] #2489
Δmdm10	*his-3 mtrR Δmdm10 ::hygR a*	Replacement of *mdm10* gene in 76-26 with hygromycin resistance (HygR) cassette
Δtom7	*Δtom7::hygR A*	Nargang lab (Sherman *et al.*, 2005)
Δmdm10Δtom 7	*Δtom7::hygR Δmdm10::hygR*	Cross of *Δmdm10* with *Δtom7.*
*mdm12*mut	*mdm12*	FGSC #9852 (Seiler and Plamann, 2003)
mmm1-RIP23	*mmm1^{RIP}*	Neupert lab (Prokisch *et al.*, 2000)
Tob55HT (H6C4-5)	*his-3 mtrR Δtob55::hygR a* Contains an ectopic copy of genomic *tob55* with an N-terminal nine x His tag. Also bleomycin resistant.	Nargang lab (Wideman *et al.*, 2010)
Tob37HT	*his-3 mtrR Δtob37::hygR a* Contains an ectopic copy of genomic *tob37* with an C-terminal nine x His tag. Also bleomycin resistant.	Nargang lab (Wideman *et al.*, 2010)
Tob38HT	*his-3 mtrR Δtob38::hygR a* Contains an ectopic copy of genomic *tob38* with an C-terminal nine x His tag. Also bleomycin resistant.	Nargang lab (Wideman *et al.*, 2010)
S55HT	*his-3 mtrR Δtob55::hygR a* contains an ectopic copy of N-terminal 9x His tagged tob55 cDNA specific for the	Nargang Lab (Lackey *et al.*, 2011)

short form.

I55HT	*his-3 mtrR Δtob55::hygR a* contains an ectopic copy of N-terminal 9x His tagged tob55 cDNA specific for the intermediate form.	Nargang Lab (Lackey *et al.*, 2011)
L55HT	*his-3 mtrR Δtob55::hygR a* contains an ectopic copy of N-terminal 9x His tagged tob55 cDNA specific for the long form.	Nargang Lab (Lackey *et al.*, 2011)
Low-L55HT	As L55HT.	This study
Δmmm2	*Δmmm2::hygR a*	FGSC #19795
Δmmm1	*Δmmm1::hygR a*	FGSC #21180
Δgem1	*Δgem1::hygR a*	FGSC #19466
Mmm1-HA3	*Δmmm1::hygR a* Contains an ectopic copy of *mmm1* with a C-terminal 3xHA tag. Also Basta resistant	This study.
Mmm1-HA5	As Mmm1-HA3 but is reduced in the expression of Mmm1-HA	This study.
Mmm1-HA8	As Mmm1-HA3 but is reduced in the expression of Mmm1-HA	This study.
CS-123	As Mmm1-HA3 but cysteine residues 5, 179 and 319 were mutated to serines	This study.
C5S	As Mmm1-HA3 but cysteine residue 5 was mutated to serine	This study.
C179S	As Mmm1-HA3 but cysteine residue 179 was mutated to serine	This study.
C319S	As Mmm1-HA3 but cysteine residue 319 was mutated to serine	This study.
A116-124	As Mmm1-HA3 but residues 116-124 were mutated to alanines	This study

[1]Fungal Genetic Stock Center

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Table A.2. Complementation and synthetic phenotypes of TOM, TOB or ERMES mutants in *S. cerevisiae*

Mutations	Effect	Reference
rpn11-m1Δmmm2	Synthetic GD	(Rinaldi *et al.*, 2008)
tom40ts mutations overexpression of Tom6,7,22	Suppression of ts phenotype	(Kassenbrock *et al.*, 1993; Gabriel *et al.*, 2003)
Overexpression of Tom22 in *Δtom20Δtom70*	Suppression	(Lithgow *et al.*, 1994; Honlinger *et al.*, 1995; Dekker *et al.*, 1998)
Overexpression of Tom70 in *Δtom20*	Suppression	(Ramage *et al.*, 1993)
Δtom20Δtom70	Synthetic GD	(Ramage *et al.*, 1993)
*Δ*IMS*tom22Δtom7*	Synthetic GD	(Esaki *et al.*, 2004)
Δtom6Δtom70	Synthetic GD	(Alconada *et al.*, 1995)
Δtom6Δtom20	Synthetic GD	(Alconada *et al.*, 1995)
Δtom7Δtom20	Synthetic Lethal	(Honlinger *et al.*, 1996)
Δtom7Δtom70	No synthetic effect	(Honlinger *et al.*, 1996)
Δtom6Δtom7	Synthetic GD	(Honlinger *et al.*, 1996)*
Δtom5Δtom6Δtom7	Lethal	(Honlinger *et al.*, 1996)*
tom70mdm10ts	Synthetic GD	(Jamet-Vierny *et al.*, 1997)**
overexpression of Mmm1, Mdm12, Mdm10, Tob55, Tob38 in *Δtob37*	Suppression (greater suppression for Tob55 and Tob38) at 30°C	(Meisinger *et al.*, 2007)
Overexpression of Tom5 or Tom6 in *Δtob37*	Suppression even at 37°C	(Dukanovic *et al.*, 2009; Becker *et al.*, 2010)
Δtom5Δtob37	Synthetic GD	(Becker *et al.*, 2010)
Δtom6Δtob37	Synthetic GD	(Becker *et al.*, 2010)

Tom6 overespression in *Δtom5*	Mostly suppresses *Δtom5* growth defect and import defect	(Becker *et al.*, 2010)
Overexpression of Tom7 simultaneously with Tom40	Growth Defect	(Yamano *et al.*, 2010b)
Overexpression of Tom40 in *mdm10KFY* or *mdm10KY*	Growth Defect	(Yamano *et al.*, 2010b)
Overexpression of Mdm10	Major growth defect	(Yamano *et al.*, 2010a)
Δfmp30 with deletion of any ERMES gene or *Δfzo1*	CL defects and growth defects	(Kuroda *et al.*, 2011)
Δfmp30Δmdm35 or *Δfmp30Δups1*	Synthetic Lethal	(Kuroda *et al.*, 2011)
ERMES deletion and *Δphb1* or *Δphb2*	Synthetic Lethal	(Berger and Yaffe, 1998; Osman *et al.*, 2009)
Overexpression of Phb1 or Phb2 in *Δmdm10*, *Δmdm12* or *Δmmm1*	Partial suppression of growth and mitochondrial motility	(Berger and Yaffe, 1998)
Overexpression of Mdm31 in any ERMES deletion	Partial suppression of growth and mitochondrial motility	(Tamura *et al.*, 2012)
Δmdm10Δtom7	Strong synthetic GD No GD in *N. crassa*	(Wideman *et al.*, 2010; Hoppins *et al.*, 2011)
Δmdm10Δtob37	Strong synthetic defect	(Stojanovski *et al.*, 2007)
Δtom70Δcrd1 Δtom7Δcrd1 Δtob37Δcrd1 Δtob37Δtaz1 Δmdm10Δcrd1 mdm10Δtaz1	temperature sensitive	(Gebert *et al.*, 2009)
Δtom5Δcrd1 or	lethal	(Gebert *et al.*, 2009)

Δtom70Δtaz1

*Similar phenotype seen in *N. crassa* (Sherman *et al.*, 2005) **In *P. anserina*

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Table A.3. Oligonucleotide primers used in this study.

Primer name	Sequence	Used For
JWmdm10-5'new	CCACACGGGGAGCACTCCAT	+-500bp *mdm10*
JWmdm10-3'new	GGATGTTTGTCGTTCGATGA	+-500bp *mdm10*
pBR322 NotI-AscI	/5Phos/CGTCTTCAAGAATTCAGCG GCGCGCCTGAGAATTCTCATGTTT G	Mutate NotI site in pBR322 to AscI
JWm10-3'Eco	AATCCAGAGAATTCTCAAGAGGA GTACTGAACCTCAAGGC	Cloning *mdm10*
Mdm10 cDNA 5'UTR 50bp	GCAATTAGAAGCTTGACTCGCGA CTACGTTAACC	*mdm10* cDNA cloning
JWm10-5'UTRHind	ACTCTTCAAGCTTCCACACGGGG AGCACTCCATTATGCGCGAGTTC ATGAACTAC	*mdm10* cDNA cloning
Bleo seq1F	CATCGCCTTCTATCG	BleoR sequencing
Bleo seq2F	GGTTACCGCCTGTTG	BleoR sequencing
Ben seq1F	ATCACCACTTCCATC	BenR sequencing
Ben seq2F	GCAACAAGTATGTCC	BenR sequencing
Ben seq3F	AGCAGATGTTCGACC	BenR sequencing
JW Mmm1 cDNA HindIII Fwd	CCAATCGTTAAGCTTCGGACAGA TGCTTTTGCTCC	*mmm1* cDNA cloning
JW Mmm1 cDNA HindIII Rev	TTAGCATAGAATTCCGTGTTCCTT AGCCCGTTCG	*mmm1* cDNA cloning
JWmim1 cDNA F	TGATTTGAGGATCCCCTTATACCG TTCCTTTGTCG	*mim1* cDNA cloning
JWmim1 cDNA R	TGATTTGAGAATTCGTGGCCTGC GGAAGACGACG	*mim1* cDNA cloning
JW Mmm1 AscI 5'	ATACTTCCGGCGCGCCGCTTTAG CGGATTGGCACC	*mmm1* +-500bp cloning

JW Mmm1 AscI 5'-2	GTACATCTGGCGCGCCGGGGCTTTAGCGGATTGGC	*mmm1* +-500bp cloning fixed
JW Mmm1 AscI 3'	ATACTTCCGGCGCGCCCTGAACCCAGACTCCCTGG	*mmm1* +-500bp cloning
JW Mmm1 Not I Mut 5'	/5Phos/CCCTCGATAACCCCAAGAAGATGGCGGCCGCGCCGACATTTGCCCATCAAGG	Insert NotI site into 5' *mmm1*
JW Mmm1 Not I Mut 3'	/5Phos/GGTTCTATGCCCGGTTCTATGCCCGCGGCCGCTGAATGAGCATACCCTTTCCG	Insert NotI site into 3' *mmm1*
JWngo29	GTGACGACGAAAGGTGCAGTCGCG	+-500bp *tom7* from Nancy
JWngo30	CGATCCGAAGCTTGCGACTGTCCC	+-500bp *tom7* from Nancy
JW7-FNA362	GACGTTTTGTTCAAGACCATAACTC	Sequencing *tom7*
JW Tom7 AscI 5'	ATACTTGAGGCGCGCCGCCAGAGTATGTTAATGTGC	*tom7* +-500bp cloning
JW Mmm1 Tom7 5'-2	ATACTTGAGGCGCGCCTAACGTCGCAGGATATCGGC	*tom7* +-500bp cloning fixed
JW Tom7 AscI 3'	ATACTTGAGGCGCGCCAACAGAGAGCTCATGCCTGG	*tom7* +-500bp cloning
JW Tom7 NotI Mut	/5Phos/CAGACCAAATCAGGCAACATGGCGGCCGCTTCGCCCTTTCGGAAGAGTCC	Insert NotI site into 5' *tom7*
JW Mdm10 AscI 5'	ATACTTCCGGCGCGCCTCTTTGCTGACGGGCGTGAGG	*mdm10* +-500bp cloning
JW Mdm10 AscI 3'	ATACTTCCGGCGCGCCCGCTATCAAAGACGCATCCG	*mdm10* +-500bp cloning
JW Mdm10 CTerm 9xHis	/5Phos/CTTGAGGTTCAGTACTCCTCTCATCACCATCACCATCACCATCACCATTGATCATCGAACGACAAAC	Insert 9x his codons into 3' *mdm10*

JW Mdm10 C term NotI	/5Phos/CTTGAGGTTCAGTACTCCTCTGCGGCCGCTGATCATCGAACGACAAAC	Insert NotI site into 3' *mdm10*
JW M10 NotI	/5Phos/CGGGGAGCACTCCATTATGGCGGCCGCCGCGAGTTCATGAACTACATC	Insert NotI site into 5' *mdm10*
JW Mmm1 seq1	GGTCGATAAGATAAGG	Sequence *mmm1*
JW Mmm1 seq2	CATATTACAAGGTCG	Sequence *mmm1*
JW Mmm1 seq3	TCCGCAGTCTTTTGG	Sequence *mmm1*
JW Mmm2 AscI 5'	ATACATCAGGCGCGCCAAGCCACACAGCGTCCCA	*mmm2* +-500bp cloning
JW Mmm2 AscI 3'	GTACATCCGGCGCGCCTGCTTGTCTGTCCGTTCG	*mmm2* +-500bp cloning
JW Mmm2 cDNA(+50) F ClaI	ACGATGTAATCGATGCAACCATTATTCAACACC	*mmm2* cDNA cloning
JW Mmm2 cDNA(+50) R EcoRI	TCGTTGATGAATTCGATCGGCACATGGAAGAGC	*mmm2* cDNA cloning
JW Mdm10 cDNA(+50) F HindIII	TCGTTGATAAGCTTCCAGGTGCGATAGACTCG	*mdm10* cDNA cloning
JW Mdm10 cDNA(+50) R EcoRI	TGCATGATGAATTCGTCTGAAGAACAGCTGTACG	*mdm10* cDNA cloning
JW Mmm1 C->S 1	/5Phos/CCATCTTCAGCAACTCCCGCATCATCCCGG	Codon 179 was changed to code for Ser
JW Mmm1 C->S 2	/5Phos/GGTTCGATGAGCGCTCTGTGGAGCCCAGGTTCC	Codon 319 was changed to code for Ser
JW Mmm1 ESLDWFN -> A	/5Phos/CAAGGTCGACAGCCATCAACCCGCAGCTGCCGCCGCGGCCGCCGTGCTGATCGCCCAAACCATCGC	Codons 116-124 were changed to code for Ala

JW Mdm12 BglII 5'	/5Phos/CCCGATTTTTACGAAGAGCAGATCTGAATAGACTCCTCGGAAGAATC	Insert BglII site into 5' *mdm12*
JW Mdm12 BglII 3'	/5Phos/GAAGAAAGGCAACGGTTTCGAGATCTTAGGGTGGAAGACATCCAAGC	Insert BglII site into 3' *mdm12*
JW Mmm2 seq1	CCATTATTCAACACC	Sequence *mmm2*
JW Mmm2 seq2	GTGCTCCGAACATGG	Sequence *mmm2*
JW Mmm2 seq3	TCGAGATGAAGTTGC	Sequence *mmm2*
JW Mmm2 seq4	CTGCGTAGCAAGACG	Sequence *mmm2*
JW Mmm2 seq5	GGGACGTGCTGGTCC	Sequence *mmm2*
Mmm2- N-Term 9xhis	/5Phos/CTAAAGACAACGCCAACTCAATATGCATCACCATCACCATCACCATCACCATGCCTTCAACTTCAACTGGTCGCC	Insert 9x his codons 5' *mmm2*
Mmm2- C-Term 9xhis	/5Phos/CCACCGGCTTATGAACCAAGACATCACCATCACCATCACCATCACCATTAGATGAGCCTGGGGACTGTCATATTGCG	Insert 9x his codons 3' *mmm2*
Mmm2- N-Term NotI	/5Phos/CTAAAGACAACGCCAACTCAATATGGCGGCCGCGCCTTCAACTTCAACTGGTCGCC	Insert NotI site 5' *mmm2*
Mmm2- C-Term NotI	/5Phos/CCACCGGCTTATGAACCAAGAGCGGCCGCTAGATGAGCCTGGGGACTGTCATATTGCG	Insert NotI site 3' *mmm2*

Tob55 HT 5’ F (AscI)	TATTTACTGGCGCGCCGGAGGGG TGGGCAGTGATGTACG	Constructing Tob55 N-term Histag Knock-In plasmid
Tob55 HT 5’ R (NotI)	GGCTGACGTGGCGGCCGCCATTG AGATGAAAGCCGACGG	Constructing Tob55 N-term Histag Knock-In plasmid
Tob55 HTNF (NotI)	CATCTCAATGGCGGCCGCCACGT CAGCCACACGGGCAGCC	Constructing Tob55 N-term Histag Knock-In plasmid
Tob55 HTNR (AscI)	TATTTACTGGCGCGCCCGTCGAG CTCGGTGCGGTCGGTGG	Constructing Tob55 N-term Histag Knock-In plasmid
JW Mmm2 ab N BglII F	AACATGTAAGATCTGCCTTCAAC TTCAACTGGTCG	Mmm2 fusion protein for Ab production
JW Mmm2 ab N PstI R	AACATGTACTGCAGCTCGGCAGG ATCCAGACGATGG	Mmm2 fusion protein for Ab production
JW Mmm2 ab C BglII F	TTCCTGTAAGATCTATTTCGTCAC TATCTCTTGAC	Mmm2 fusion protein for Ab production
JW Mmm2 ab C PstI R	TTCCTGTACTGCAGGGCCTTTGGC ATCTGCGTAGATCG	Mmm2 fusion protein for Ab production
JW BarKO R ClaI	ATTCAATAATCGATTCAGATCTC GGTGACGG	*mdm10*KO barR plasmid
JW BarKO F ClaI	ATTCAATGATCGATAGAAGATGA TATTGAAGGAGC	*mdm10*KO barR plasmid
JW BarKO F SpeI	TAACAATGACTAGTAGAAGATGA TATTGAAGGAGC	*mdm10*KO barR plasmid

AO mdm10 YGR->AAA mut	/5Phos/GCATATACACACACTCTCTTCTAGCCGCCGCTCTGTATCTGCCCAAATCACTGC	Codons 165-167 were changed to code for Ala
AO mdm10 GSIS->AAAA mut	/5Phos/GGCAGTGTTGGCGTCGTCGACGCTGCTGCAGCATACCTACATAGCTCCATTCC	Codons 69-72 were changed to code for Ala
AO mdm10 BglII 3'wards mut	/5Phos/CTAGGCATATACACACACTCTAGATCTTACGGCCGTCTGTATCTG	Insert BglII sites into 5' region of *mdm10*
AO mdm10 BglII 5'wards mut	/5Phos/CATGAACTATATCACCAATGCCTTCTACAGATCTACGGGGTGGAACGAAGACAACAAG	Insert BglII sites into 5' region of *mdm10*
JW M10TGWNEDNKY-A	/5Phos/CTACATCACCAATGCCTTCTACGGAGCCGCGGGGGCGAACGAAGCCGCCAAGGCCAACGAGCTGAATGCGACATCTAGAGG	Codons 18,21, 22, 24 were changed to code for Ala
JW M10 FNVYSYES-A	/5Phos/TACTGTTCGCTCGCAACGCGGGTCGATGCCGCGGCGGCCGCTGCTGCAGCCGAGTGGGCGGTAGGTATGGAGCTCTGG	Codons 350-357 were changed to code for Ala
JW M10Karl-Earl	/5Phos/GAAAGAACGACGAGTACAAGGGGGTCCTGGAAGCTCGCCTTGACAACAACCTTCGCATGGG	Codon 432 changed to code for Glu
JW M10 Karl-aarl	/5Phos/GAAAGAACGACGAGTACAAGGGGGTCCTGGCAGCTCGCCTTGACAACAACCTTCGCATGGG	Codon 432 changed to code for Ala
JW M10 WEGR-AAAA	/5Phos/CTTGACAACAACCTTCGCATGGGGTTGCTGGCCGCGGCAGCAGCCAAGTCCCTTATTTTCAGTATAGGAACG	Codons 445-448 were changed to code for Ala
JW M10 WEGR-R	/5Phos/CTTGACAACAACCTTCGCATGGGGTTGCTGAGAGCCAAGTCCCTTATTTTCAGTATAGGAACG	Codons 445-447 were deleted
JW M10 G-H	/5Phos/GTGAGCCCTTTAGGAGCCTTCATCTTGAGGTTCAGTACTCCTCTTG	β-signal Gly changed to His

JW M10 GFRGLYNF-A	/5Phos/TACTCGACCGACGGTGGTC TGCTGGGTTTTGCTGGTGCCGCG GCCGCTGGCGGCGACGCCTCTTC CTCGACCTGTG	Codons 232, 234-237 were changed to code for Ala
Fwd M10KO-Bar	CGATCACTGTGAGTGGATGTTTTC TTACACAGAAGATGATATTGAAG GAGC	*mdm10*KO barR plasmid fix
Rev M10KO-Bar	ATGATACAGAGTCTCAACAAGTC TTGAGTCAGATCTCGGTGACGG	*mdm10*KO barR plasmid fix
JW mmm1 CS 3	/5Phos/GATGGCCGACATTTCCCCA TCAAGGTCGG	Codon 5 changed to code for Ser
JW10seq5' aka M10seq1	CAACGACCGCTCTTC	Sequencing *mdm10*
JWMdm10mid-seq5' aka M10seq2	CGTCGACGGCTCTAT	Sequencing *mdm10*
JW10seqmidF3' aka 10seq3	CCGACGGTGGTCTGCTGGGTTTTC	Sequencing *mdm10*
JW M10seq 1.5	GTTCATGAACTACTCACC	Sequencing *mdm10*
JW M10seq 2REV	ATAGAGCCGTCGACGACG	Sequencing *mdm10*
JW M10seq 2.5	GGCCGTCTGTATCTGC	Sequencing *mdm10*
JW M10seq 3.5	GGTCCTGAAAGCTCGCC	Sequencing *mdm10*
JW Gem1 check 5'	GGCATCCTCTGAGCTTCATGG	*mmm2*KO confirmation
JW Gem1 check 3'	TCACAGGTTGGTGGTTACGC	*mmm2*KO confirmation

Table A.4. Plasmids used in this study.

Plasmid name	Origin or Source	Description
pHis3	FGSC	*his3* plasmid from FGSC
pHis3 AscI	This Study	pHis3 with AscI site
pBR322-AscI	This Study	pBR322 with NotI site mutated to AscI
pBS520-AscI	This Study	pBS520 with NotI site mutated to AscI
pBarks	FGSC	Contains Bar Resistance cassette
pBASC	Frank	pBarks with NotI site mutated to AscI
BL-21-M10N	This Study	5' region of *mdm10* cloned into pQE40 for antigen production
BL-21-M10C	This Study	3' region of *mdm10* cloned into pQE40 for antigen production
Nancy's Mdm10HT-Bleo	Nancy	Mdm10 with 6xHT at N-term in pBR322
Nancy's Mdm10 Rip Bleo	Nancy	Plasmid for ripping with BleoR in pBR322
pQE40 M10N	This Study	N-terminal region of Mdm10 in pQE40 for antibody production
M10 9xHT Bleo	This Study	Nancy's Mdm10-HT Bleo with 9x HT at N-term
Mdm10- pGEM	This Study	mdm10 cDNA cloned into HindIII and EcoR1 in pGEM for SP6 lysate production
pBR322 AscI Bleo	This Study	pBR322 with Bleo resistance Not1 site changed to AscI
Mdm10 AscI	This Study	*mdm10* +500bp into pBR322 AscI Bleo

Mdm10 NotIN	This Study	Mdm10 AscI with a NotI site after ATG codon
Mdm10 NotIC	This Study	Mdm10 AscI with a NotI site before stop codon
Mdm10 HA N term	This Study	HA into Mdm10 NotIN
Mdm10 HA C term	This Study	HA into Mdm10 NotIC
Mdm10 9xHis C term	This Study	9xHis into Mdm10 AscI
Mdm10 YGR->AAA	This Study	YGR mutated to AAA in Mdm10 AscI
Mdm10 GSIS->AAAA	This Study	GSIS mutated to AAA in Mdm10 AscI
Mdm10 N-term deletion	This Study	BglII sites were introduced into Mdm10 to generate an N-terminal deletion. caused a frame shift
Mdm10 YGR->AAA HIS3	This Study	Mdm10 YGR->AAA subcloned into HIS3 vector
Mdm10 GSIS->AAAA HIS3	This Study	Mdm10 GSIS->AAAA subcloned into HIS3 vector
Mdm10 N-term deletion HIS3	This Study	Mdm10 N-term deletion subcloned into HIS3 vector
Mdm10KO plasmid	This Study	plasmid used to KO mdm10 mdm10 ORF is replaced with HygR
Mdm10 AscI Basta	This Study	Mdm10 +500bp into pBASC (Bar Resistance)
M10 GF	This Study	Codons 230-237 were changed to code for Ala in Mdm10 AscI Basta
M10 GH	This Study	β-signal glycine codon changed to His in Mdm10 AscI Basta
M10 WA	This Study	Codons 445-448 were changed to code for Ala in Mdm10 AscI Basta
M10 WR	This Study	Codons 445-447 were deleted in Mdm10 AscI Basta

M10 GW	This Study	Codons 17-24 were changed to code for Ala in Mdm10 AscI Basta
M10 FN	This Study	Codons 350-357 were changed to code for Ala in Mdm10 AscI Basta
M10 KA	This Study	Codon 432 changed to code for Ala in Mdm10 AscI Basta
M10 KE	This Study	Codon 432 changed to code for Glu in Mdm10 AscI Basta
Tom7 AscI	This Study	*tom7* +500bp into AscI of pBS520 AscI in Mdm10 AscI Basta
Tom7 N-NotI	This Study	Tom7 AscI with NotI site added after ATG codon in Mdm10 AscI Basta
Tom7 Myc Nterm	This Study	Myc into Tom7 N-NotI
Tom7 HA Nterm	This Study	HA into Tom7 N-NotI
Tom7KO plasmid	Nancy	Plasmid used to KO tom7. tom7 ORF replaced with HygR
Mmm1 AscI	This Study	Mmm1 in AscI site of pBR322 AscI
Mmm1 NotI C term	This Study	NotI site inserted before stop codon in Mmm1 AscI
Mmm1 HA C	This Study	HA tag inserted into Mmm1 NotI C term
Mmm1 ESDWFN->A	This Study	Mmm1 AscI with conserved region mutated to 9xAla
Mmm1 C->S C->S	This Study	Codon 179 and 319 were changed to code for Ser
Mmm1 C->S 1	This Study	Codon 179 was changed to code for Ser
Mmm1 C->S 2	This Study	Codon 319 was changed to code for Ser
Mmm1 HA C Basta	This Study	Mmm1 HA C subcloned into pBASC Bar resistance

Mmm1 ESDWFN->A Basta	This Study	Mmm1 AscI with conserved region mutated to 9xAla in Mmm1 HA C Basta
Mmm1 C->S C->S Basta	This Study	Codons 179 and 319 were changed to code for Ser in Mmm1 HA C Basta
Mmm1 C->S 1 Basta	This Study	Codon 179 was changed to code for Ser in Mmm1 HA C Basta
Mmm1 C->S 2 Basta	This Study	Codon 319 was changed to code for Ser in Mmm1 HA C Basta
Mmm1 C->S 3 Basta	This Study	Codon 5 was changed to code for Ser in Mmm1 HA C Basta
Mmm1 CS123	This Study	Codons 5, 179 and 319 were changed to code for Ser in Mmm1 HA C Basta
Mdm12 N-HA Basta	This Study	subclone of Mdm12 N-HA BarR
Mdm12 BglII	This Study	BglII sites introduced to delete non-conserved region frameshifted! never used!!
pMmm2 gDNA (AscI)	This Study	Mmm2 in pAscI-322
pMmm2 C-His9x	This Study	C term 9xHis tagged Mmm2 in pAscI322
pMmm2 N-His9x	This Study	N term 9xHis tagged Mmm2 in pAscI322
m3-2 CHT basta	This Study	pMmm2 C-His9x subcloned into pBASC
m3-2 basta	This Study	gMmm2 subcloned into pBASC

m3-2 N-NotI	This Study	NotI site inserted after ATG codon in m3-2 basta
m3-2 N-myc basta	This Study	N-terminal Myc tag inserted into m3-2N-NotI
pT55fusBLEO	This Study	N-term histag of Tob55 with Bleo resistance upstream (to knock in the tag)
M12Ab	This Study	Full length mdm12 in pQE40 for Ab production in BL-21
m3-1 Ab	This Study	Full length mmm1 in pQE40 for Ab production in BL-21
m3-2 NAb	This Study	N-terminal fragment of mmm2 in pQE40 for Ab production in BL-21
m3-2 CAb	This Study	C-terminal fragment of mmm2 in pQE40 for Ab production in BL-21
m3-2 WAb	This Study	Full length mmm2 in pQE40 for Ab production in BL-21
mim1 pGEM	This Study	mim1 cDNA in pGEM for in vitro TnT
mdm10 pGEM	This Study	mdm10 cDNA in pGEM for in vitro TnT
mmm2 pGEM	This Study	mmm2 cDNA in pGEM for in vitro TnT