Mcp1 and Mcp2 Two Novel Proteins Involved in Mitochondrial Lipid Homeostasis

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Dekan: Prof. Dr. Wolfgang Rosenstiel

1. Berichterstatter: Prof. Dr. Doron Rapaport

2. Berichterstatter: Prof. Dr. Ralf-Peter Jansen

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst habe und keine anderen als die angegeben Hilfsmittel und Quellen verwendet habe.

Tübingen, den 28. August. 2013

Tao Tan

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1. Introduction

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1.1.1 The origin of mitochondria

Mitochondria are essential organelles of eukaryotic cells. They participate in many important metabolic processes. A bulk of discoveries favors the endosymbiotic origin of mitochondria (Embley and Martin 2006). This hypothesis states that the endosymbiosis event happened between a nucleus-containing host cell and eubacteria ancestors from the subgroup of α-Proteobacteria over 1.5 billion years ago (Gray et al. 1999, Andersson et al. 2003, Gray 2012). According to a recent advanced endosymbiosis theory called the hydrogen hypothesis, a strict anaerobic autotrophic archaeon (host cell) adopted fermentative α-Proteobacteria generating hydrogen and carbon dioxide as waste products. The initial host cell might have been a methanogen and consumed hydrogen produced by the symbiont (Martin and Muller 1998, Moreira and Lopez-Garcia 1998). With more exchange of metabolic products host cells gradually developed membrane transport systems for exogenous substances and started to depend on the proteobacteria. Finally organisms became irreversible heterotroph, containing ancestral mitochondria, independent from hydrogen and an aerobic metabolism in the cell evolved (Lang et al. 1999, Lopez-Garcia and Moreira 1999). In contrast to aerobic eukaryotes, two membranes containing organelles-hydrogenosomes, deriving from mitochondrial ancestors-occur in diverse anaerobic microbial eukaryotes, which produce large amount of hydrogen (van der Giezen 2005, Embley et al. 2003).

1.1.2 Mitochondrial genome diversity

Mitochondria contain their own genome called mitochondrial DNA (mtDNA), which is packaged to avoid deleterious and oxidative damage in nucleotides-protein complex named nucleoids. Mitochondrial DNA nucleotides have close contacts to the mitochondrial inner membrane. The mitochondrial genomes of all eukaryotes are generally divided into two groups according to the extreme genome size difference, animal and plant mitochondrial genomes, respectively. The size of animal mtDNAs ranges from 14 kb to 42 kb, and they contain circular genomes with a typical uniform

architecture and are short in intron DNA. In contrast, land plants possess larger mitochondrial DNA and most complex genome architecture. The sizes of heterogeneous genomes of plants range from 184 kb to 2400 kb with low mutation rate. The size of mitochondrial genomes of fungi is in between that of animals to that of plants. Their circular molecules range from 17 to 180 kb (Bullerwell and Gray 2004). Mitochondrial genomes vary greatly in different lineages in respect to protein encoding genes from maximal 67 to 3 (Gray et al. 2001). One-third of the mitochondrial genome encoding proteins was shown to encode vital mitochondrial functions such as subunits of ATP synthase complex, cytochrome b and the cytochrome c oxidase complex (Andersson et al. 2003). Mitochondrial ribosomal genes are poorly conserved in mitochondrial genomes and are normally located in the nucleus of animals and most fungi (Leblanc et al. 1997). About 800 genes were predicted to encode mitochondrial proteins in yeast cells (Reinders et al. 2006, Meisinger et al. 2008). Among them 50% have no bacterial homologues and are from eukaryotic origin, yet 10% can be definitely traced to an α-Proteobacterial origin (Kurland and Andersson 2000). Mitochondrial proteins with prokaryotic origin predominantly take part in biosynthesis, protein synthesis and bioenergetics, whereas mitochondrial proteins of eukaryotic origin are mainly responsible for regulation, transport and serve as membrane components (Adams and Palmer 2003). The mitochondrial genome of *Saccharomyces cerevisiae* encodes 2 rRNAs, 24 tRNAs and 8 hydrophobic proteins (Foury et al. 1998, Sickmann et al. 2003, Andersson et al. 2003). The copy number of mtDNA depends on the cell types. Haploid yeast cells contain around 30 copies (Chen and Butow 2005, Spelbrink 2010). Nucleotides can be stained by DNA binding fluorescent dyes and are visualized as many small foci of around 0.3 µm diameter along the mitochondrial tubules (MacAlpine et al. 2000). Mutation or loss of mtDNA leads to defect of mitochondrial morphology and impaired growth on non-fermentable medium (Okamoto and Shaw 2005).

1.1.3 Mitochondrial genome and proteome evolution

Only a small number of mitochondrial proteins are still encoded by the mitochondrial genome. Most proteins are encoded in the nuclear genome and are recruited and imported into the organelle to complement the functions of lost genes from the proteobacterial ancestor (Gray et al. 2001, Karlberg et al. 2000, Karlberg and

Andersson 2003). This phenomenon implies that modern mitochondria underwent a genome reduction process after symbiosis (Saccone et al. 2000). The driving force for extended migration of genes from the mitochondrial to the nuclear genome, derives mainly from higher mutation rates in mitochondria than in nucleus (Andersson and Kurland 1998, Graack and Wittmann-Liebold 1998, Berg and Kurland 2000). Vacuole-dependent transfer (autophagy and degradation of mitochondria in cellular vacuoles) is suggested to be the primary mechanism to result in the uptake of mitochondrial DNA sequences by the nucleus (Berg and Kurland 2000). Some proteins lost from the mitochondrial proteome were retargeted to other organelles, such as the enzymes of beta-oxidation in peroxisomes in yeast cells (Gabaldon and Huynen 2004). Interestingly loss of complex I of the respiratory chain in *Saccharomyces cerevisiae* was not functionally complemented by a nuclear gene counterpart (Gray et al. 2001). Compared to nuclear encoded proteins, mitochondrial encoded proteins are unique due to their higher hydrophobicity. Because highly hydrophobic proteins are difficult to be recruited to mitochondria and transferred across both membranes, it is thought that these proteins remained mitochondrially encoded (Popot and de Vitry 1990).

1.2 Structure and functions of mitochondria

1.2.1 Structure of mitochondria

Mitochondria have a double-membrane structure resulting in four distinct compartments: mitochondrial outer membrane (MOM), intermembrane space (IMS), mitochondrial inner membrane (MIM) and the matrix. According to topology analysis the mitochondrial inner membrane can be divided into two contiguous components. The inner boundary membrane is one component which lies in close proximity to the outer membrane. The invagination of the mitochondrial inner membrane forms so called cristae, which protrude from the inner boundary membrane into the matrix and increase the surface of inner membrane. The short tubular or slot-like structure, which is found in connection between cristae and inner boundary membrane, is called cristae junction (CJ) (Harner et al. 2011). The structure of cristae, their distribution in the matrix, the number of cristae and their surface area depend highly on cell types, tissues and organisms (Korner et al. 2012). A novel complex, mitochondrial contact site (MICO) complex was identified to contain a couple of membrane proteins (including inner

membrane Fcj1 (Formation of CJ protein 1)) and to be localized at site of CJs. This complex is essential for formation of contact sites in combination with outer membrane fusion proteins Ugo1 and Fzo1 (Harner et al. 2011).

Relatively high concentrations of pore forming proteins called porin (yeast) or VDAC (mammals) are located in the outer membrane. These pore forming proteins make the outer membrane semipermeable to small ions and molecules, whereas the inner membrane is permeable for hydrophilic species only by specific transport proteins (Perkins et al. 1997).

1.2.2 Cellular functions of mitochondria

Mitochondria take part in a lot of fundamental metabolic processes in cells, such as oxidative phosphorylation and ATP generation, heme biosynthesis, Krebs cycle, pyruvate oxidation, electron transport and biosynthesis of iron–sulphur (FeS) clusters. Acetyl-CoA is derived from oxidation of glucose, fatty acid and amino acids. Oxidation of the acetyl moiety into CO_2 in the citric acid cycle passes electrons to NADH and protein-bound $FADH_2$. The resulting electrons are used in the mitochondrial respiratory chain to pump the protons through the mitochondrial inner membrane and generate a proton gradient. This gradient is then used to synthesize energy in the form of ATP by oxidative phosphorylation in the F_1F_o-ATPase complex (Green et al. 2004). Mitochondria also contribute to several process central to cellular function and dysfunction including cell growth and differentiation, calcium signaling, cell cycle control and cell death (Zick et al. 2009).

Defects of mitochondrial functions are often known to cause a wide variety of diseases including endocrine related disorders, such as diabetes mellitus. Mitochondrial dysfunction gives distinct influence on different cells and tissues because specific cell types respond to particular functions of mitochondria. Mitochondria rich regions of the synapse of an axon probably reflect the intense ATP demand of synaptic transmission between active neurons. Neurons seem to be vulnerable to mitochondrial dysfunction and thus defects in mitochondrial dynamics result often in neurodegeneration (Chan 2006). Since the normal mitochondrial biogenesis needs coordination of nuclear and mitochondrial encoded gene expression, mutation of mtDNA leads to many diseases (Osellame et al. 2012). Several developmental and cell differentiation processes are

regulated by mitochondrial divisions including embryonic development in *C. elegans* and formation of synapses and dendritic spines in neurons (Westermann 2008). Accumulated evidences show that altered mitochondrial functions are potential early events of apoptosis, while the release of cytochrome c from the mitochondria to the cytosol triggers the late steps of apoptosis (Murphy et al. 1999, Turcotte 2003). An age-related increase of oxygen radicals (reactive oxygen species: ROS) was observed in the aging process and can subsequently lead to accumulation of mtDNA damage and oxidative stress for mitochondria leading to a vivious circle (Wei et al. 2009, Seo et al. 2010).

1.3 Overview of protein translocation into mitochondria

The major part of mitochondrial proteins are encoded in nucleus and synthesized in the cytosol. Protein precursors therefore have to be imported into mitochondria, processed and assembled in one of the different subcompartments of the organelle (Borgese et al. 2007, Neupert and Herrmann 2007). Many precursors of mitochondrial proteins have hydrophobic segments and are prone to aggregation in aqueous environment during the import process. To prevent these consequences different chaperons and other cytosolic factors are involved in stabilizing the precursor in an import competent state (Endo and Yamano 2009). The recruitment of complexes Tim9-Tim10 and Tim8-Tim13 in the intermembrane space (IMS), and different chaperons in the mitochondrial matrix (Hsp70, Mdj1, Hsp60/10, Hsp78 and Zim17) were likewise identified to be responsible for the protein folding and stabilization in the IMS or matrix, respectively (Young et al. 2003).

Besides the help of chaperons, specific mitochondrial targeting signals are also required to guide proteins to their right final destination in one of the subcompartments of the organelle. An N-terminal presequence, which serves as a specific targeting signal, was identified in the majority of nuclear-encoded mitochondrial precursors. Presequences possess 10-80 amino acids that form amphipathic helices with one hydrophobic pace and another positive charged side (Neupert and Herrmann 2007). Specific presequence peptidases are adapted to cleave the signal sequence upon import and sorting. They are localized to the matrix, the inner membrane and the intermembrane space. The mitochondrial processing peptidase (MPP) is located in the

matrix, whereas inner membrane peptidase (IMP) was found in the mitochondrial inner membrane (MIM) with its catalytic domain exposed to the IMS (Mossmann et al. 2012). In contrast, some IMS proteins, inner membrane proteins and all outer membrane proteins, which lack an N-terminal presequence, were found to have internal signals. Such internal signals, which are partially still uncharacterized, can guide the precursors to different mitochondrial subcompartments (Ferramosca and Zara 2013).

Although post-translational import of mitochondrial proteins seems to be the main mechanism for most mitochondrial precursors, recent studies have shown that co-translational or translation-coupled import of proteins into mitochondria is of importance in certain cases (Yogev et al. 2007). Translationally active ribosomes and mRNA, which encode mitochondrial precursor proteins, were observed to accumulate on the surface of mitochondria (Funfschilling and Rospert 1999). Specific mRNAs in proximity to the mitochondrial were shown to contain a certain 3´-UTR stretch conferring the mitochondria-targeting properties (Marc et al. 2002).

In general, mitochondrial proteins are distributed to their place of destination by six different import machineries (Pfanner et al. 2004, Neupert and Herrmann 2007). Import of most proteins from cytosol depends on passage through the general gate, the translocase of the outer membrane (TOM) complex. Exceptions are signal and tail-anchored proteins, as well as helical multi-span proteins, all reside in the outer membrane (Setoguchi et al. 2006, Kemper et al. 2008).

1.3.1 The translocase of the outer membrane (TOM) complex

Most mitochondrial proteins encoded by nuclear genes are first recognized by the TOM complex and subsequently transported through this major entrance gate to their target destinations. Studies on TOM complexes from different species indicate that they are evolutionary conserved from fungi to mammalian (Dagley et al. 2009). The holo-TOM complex possesses molecular size of 490-600 kDa (Kunkele et al. 1998, Meisinger et al. 2001, Model et al. 2002). The core subunit of this complex is Tom40 with a β-barrel structure, which is responsible for the protein-conducting channel of this complex (Ahting et al. 2001, Stan et al. 2003). Investigations on the stoichiometry of TOM complex show that several Tom40 copies are present in the complex (Ahting et al. 2001, Stan et al. 2000, Rapaport et al. 1998). Tom70, Tom20 and Tom22 are three preprotein receptors with distinct functions. They are exposed to the cytosol and

recognize mitochondrial specific signals. Tom70 is not only receptor for polytopic inner membrane proteins such as metabolite carriers (Brix et al.1999), but also serves as a docking site for cytosolic chaperones and aggregation-prone substrates (Yamamoto et al. 2009). Recently, Tom70 was found to interact with multi-span helical mitochondrial outer membrane (MOM) proteins to guide them during the transfer to the mitochondrial import (MIM) complex (Papic et al. 2011), another protein insertion complex of the MOM. Tom20 and Tom22 play a role in recognition of proteins containing classical presequences and β-barrel proteins of the MOM (Krimmer et al. 2001, Saitoh et al. 2007). Compared to the other two receptors Tom22 has additional functions in downstream biogenesis processes. Tom22 can relay the precursor proteins to the conducting pore (Chacinska et al. 2009). Since Tom22 contains not only a cytosolic domain but also a long segment exposed to the IMS, the latter segment can interact with substrate proteins on the trans side of TOM complex and facilitate their release to the IMS (Dukanovic and Rapaport 2011).

The small subunits of the TOM complex named Tom5, Tom6 and Tom7 are α-helical proteins. In combination with Tom22 and Tom40 they form the TOM core complex with a molecular mass of 450-500 kDa (Ahting et al. 1999). Although Tom5, Tom6 and Tom7 are subunits of TOM complex, they are not responsible for protein tanslocation but rather contribute to the biogenesis, assembly and stability of TOM complex. Tom5 participates in assembly and stability of TOM complex and helps preproteins move from Tom22 through the channel (Dietmeier et al. 1997, Wiedemann et al. 2003, Schmitt et al. 2005). Tom6 is proposed to stabilize the TOM complex in cooperation with Tom22 and Tom40, whereas Tom7 favors disassembly of the complex (Alconada et al. 1995, Honlinger et al. 1995, Dembowski et al. 2001).

1.3.2 The TOB/SAM complex

The topogenesis of mitochondrial outer membrane β-barrel proteins (TOB) complex is another translocation machinery of the MOM. It serves as a translocase and insertase for the biogenesis of β-barrel proteins and is alternatively called sorting and assembly machinery (SAM) complex (Paschen et al. 2003, Gentle et al. 2004). There are three major subunits present in the TOB complex of yeast mitochondria: Tob55/Sam50, Tob38/Sam35 and Mas37/Sam37 (Ishikawa et al. 2004, Milenkovic et al. 2004, Wideman et al. 2010). The molecular mass of this complex is around 200-250 kDa as

determined by Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE), in which Tob55 is the largest subunit with β-barrel structure. The TOB complex is evolutionary conserved (see 1.5). The topology of Tob55 indicates that its N-terminal segment localizes into the IMS containing one POTRA (ploypeptide transport-associated) domain (Kutik et al. 2008), while the C-terminal part of Tob55 forms β-barrel structure embedded in the MOM (Stroud et al. 2011). Although the precise structure of the TOB pore still needs to be unveiled, the investigation of purified Tob55 shows that the pore structure has an inner diameter of 7-8 nm and an outer diameter of 15 nm. Reduced expression of Tob55 leads to reduced steady-state levels of β-barrel proteins, while other mitochondrial protein levels stay unchanged (Paschen et al. 2003).

The second component of TOB complex is Tob38 that together with Tob55 forms the TOB core complex with a molecular mass of 160 kDa (Waizenegger et al. 2004). It is not an integral mitochondrial outer membrane protein. Although both termini of Tob38 are found in the cytosol, the most part of Tob38 is embedded in a proteinaceous environment (Kutik et al. 2008). Deletion or reduced levels of Tob38 lead to lower steady-state levels of β-barrel proteins and reduce expression levels of other subunits of the TOB complex (Milenkovic et al. 2004, Waizenegger et al. 2004). Tob38, with the help of Tob55, is proposed to take a receptor-like role by binding precursors to the TOB complex (Habib et al. 2007, Chacinska et al. 2009). The functional paralog of Tob38 in mammalia is Metaxin-2, but sequence analysis shows low level of homology between the two proteins (Kozjak-Pavlovic et al. 2007).

The third member of the TOB complex, Mas37 is a peripheral membrane protein (Gratzer et al. 1995, Wiedemann et al. 2003). The function of Mas37 is attributed to the release of precursor proteins from the TOB complex (Dukanovic et al. 2009). The deletion or reduced expression of Mas37 inhibits the insertion and assembly of Tom40 in the MOM (Wiedemann et al. 2003). The impaired assembly of Tom40 leads to reduced mature TOM complex and further hinders the import of β-barrel proteins (Chan and Lithgow 2008, Dukanovic et al. 2009). In mammals, Metaxin-1 as homolog of Mas37, forms a complex with Metaxin-2 and participates in the biogenesis of β-barrel proteins (Kozjak-Pavlovic et al. 2007).

Mdm10 is a β-barrel protein involved in mitochondrial distribution and morphology. Some reports indicate that Mdm10 is a subunit of a subpopulation of the TOB complex and promotes the assembly of Tom40 with the receptor protein Tom22 (Meisinger et

al. 2004). Tom7 was identified to cooperate with Mdm10 in the process of releasing Tom40 precursor from the TOB complex (Yamano et al. 2010).

1.3.3 The MIM complex

Recently, a new complex termed mitochondrial import (MIM) complex was identified in yeast MOM (Waizenegger et al. 2005, Becker et al. 2008, Popov-Celeketic et al. 2008). Until now two proteins, Mim1 and Mim2 were shown to belong to the MIM complex and they form a higher molecular complex with a size of 200-300 kDa (Dimmer et al. 2012). Mim1 and Mim2 are small integral proteins of MOM with a molecular mass of 13 and 11 kDa, respectively (Waizenegger et al. 2005, Lueder and Lithgow 2009, Dimmer et al. 2012). Mim1 is genetically and physically an interaction partner of Mim2. The primary function of the MIM complex seems to be the insertion of multi-span outer membrane proteins such as Ugo1 as well as single span proteins like Tom20 (Papic et al. 2011, Dimmer et al. 2012).

1.3.4 The translocation machinery in the intermembrane space of mitochondria

The IMS of mitochondria harbors certain proteins enriched in cysteine residues that contain conserved twin Cx_3C or twin Cx_9C motifs. They show hairpin-like structures through disulfide bonds within the monomers (Arnesano et al. 2005, Webb et al. 2006). Mia40/Tim40 and the flavin-dependent oxidase Erv1 are the central components found in the novel translocation pathway for this type of IMS proteins (Mesecke et al. 2005, Deponte and Hell 2009). Mia40 possesses a highly conserved segment of about 60 amino acids residues that exhibits two redox states, a partially reduced and an oxidized state (Mesecke et al. 2005, Terziyska et al. 2009). The cysteine-containing IMS proteins are oxidized and become correctly folded proteins by the reduction of Mia40 in the IMS (Muller et al. 2008). The function of Erv1 is to re-oxidize Mia40 to regenerate its active state. In this process Erv1 transfers the electrons to molecular oxygen via cytochrome c and complex IV (Grumbt et al. 2007, Terziyska et al. 2009).

The import of polytopic proteins of the mitochondrial inner membrane (IM) is facilitated by Tim9p/Tim10p and Tim8p/Tim13p protein complexes in IMS. These small proteins are chaperons that inhibit protein aggregation (Davis et al. 2007).

1.3.5 The translocases of the mitochondrial inner membrane

1.3.5.1 The TIM23 complex

The TIM23 complex is the major translocase in the MIM to import all matrix proteins, most MIM proteins and some IMS proteins (Mokranjac and Neupert 2005, Chacinska et al. 2009). The TIM23 complex comprises two functional parts: the membrane-integrated translocation channel and the import motor facing the matrix. Four components Tim17, Tim21, Tim23 and Tim50 are found in membrane-integrated translocation channel. Tim50 is the major receptor of this complex and recognizes the proteins containing presequences emerging from the TOM complex (Geissler et al. 2002, Yamamoto et al. 2002, Mokranjac et al. 2003). Tim17 and Tim23 are integral multi-transmembrane proteins and build a protein translocation channel (Milisav et al. 2001, Meier et al. 2005). Tim23 contains an N-terminal domain with receptor function that interacts with presequences. Tim21 specifically binds Tom22 with its IMS domain, indicating that TIM23 and TOM complexes cooperate during translocation (Chacinska et al. 2005).

The TIM23 translocase recognizes the proteins containing N-terminal positively charged mitochondrial presequences. The driving force of translocation depends on the membrane potential ($\Delta\psi$) and ATP consumed by the import motor of TIM23 complex. Five different proteins were identified in the import motor: Tim14, Tim16, Tim44, mtHsp70 and Mge1. As a central organizer of the import motor, Tim44 interacts with Mge1, Tim14/Tim16 and mtHsp70 to bring the proteins to the import channel (Moro et al. 1999, Bukau et al. 2006). The chaperone mtHsp70 switches between ATP-bound and ADP-bound state. The substrate can easily be bound or released in an ATP-bound state, while the ADP-bound state forms a closed binding pocket and the substrate is tightly bound to the chaperone. The function of Mge1 is as nucleotide exchange factor for mtHsp70, and it helps in the exchange of the ADP-bound state of mtHsp70 to the ATP-bound state. Using such a mechanism the substrate can be targeted step by step into the matrix. In addition to Mge1 there are two other proteins

containing J-domains that can regulate mtHsp70. Tim14 stimulates the ATPase activity of mtHsp70, while the activity of Tim14 is regulated by Tim16 (Kozany et al. 2004, Li et al. 2004).

1.3.5.2 The TIM22 complex

The TIM22 complex is located in the mitochondrial inner membrane. It imports membrane-embedded TIM proteins (Tim17, Tim22 and Tim23) and solute carrier proteins, such as ADP/ATP and phosphate carries. These precursors, targeted to the mitochondrial inner membrane, contain an internal non-cleavable targeting sequence and their import depends on the inner membrane potential. The central component of TIM22 complex is Tim22, which forms the translocation channel and mediates the import (Rehling et al. 2003). Small Tim proteins Tim9, Tim10 and Tim12 are regarded as a chaperone complex and bind Tim54, another subunit of the TIM22 complex. They conduct the precursor proteins in the IMS and target them to Tim22 in a way that they can be inserted and assembled in the inner membrane (Peixoto et al. 2007, Mokranjac and Neupert 2008, Wagner et al. 2008). The exact functions of Tim54 and Tim18 (yet another component of TIM22 complex) still need to be investigated.

1.3.5.3 The OXA1 complex

Proteins, which are synthesized in the matrix and inserted in the mitochondrial inner membrane, are recognized by the OXA1 complex (Baumann et al. 2002, Nargang et al. 2002). Analysis of all proteins inserted by OXA1 complex shows that they contain negatively charged N-terminal residues and the import force of these proteins depends on the inner membrane potential (Rojo et al.1995, Herrmann et al.1997). The exact components of the OXA complex and the way by which it mediates membrane insertion remain unclear. But Oxa1, Mba1 and Cox18 were identified with several other proteins to be involved in this import pathway. In the presence of Cox18, Oxa1 mediates insertion of some proteins in the inner membrane, whereas Mba1 together with Oxa1 facilitates the association of translating mitochondrial ribosomes at the inner membrane (Stuart 2002, Yi and Dalbey 2005, Ott et al. 2006).

1.4 Morphology and dynamics of mitochondria

1.4.1 Overview of mitochondrial morphogenesis

Under normal physiological conditions mitochondria from most fungi, animals and plants share the basic similar morphology, which is organized into networks composed of filamentous tubules that undergo frequent shape changes, e.g., elongation, shortening, branching, buckling and swelling (Bereiter-Hahn and Voth 1994). In active metabolic cells the large mitochondrial networks facilitate the transmission of mitochondrial membrane potential from oxygen-rich to oxygen-poor regions and thus transfer energy inside the cells (Westermann 2008). The shape of the mitochondrial membranes changes enormously in different organisms and tissues. Enlarged mitochondria can be observed under a wide range of abnormal conditions and as a result of different deficiencies (Bereiter-Hahn and Voth 1994). ER and plasma membrane are associated with the mitochondrial outer membrane. The change of mitochondrial shape often affects ER and peroxisomes to influence many additional cell processes (Rambold et al. 2011). Extensive connections between mitochondrial membranes and the fibers of the cytoskeleton exist and mitochondria continuously move along cytoskeleton tracks (Bereiter-Hahn et al. 2008, Hammermeister et al. 2010). Moreover, the fibers may participate in determining the form of mitochondria. The interaction between mitochondria and microtubules was identified to be responsible for mitochondrial motility in higher eukaryotes (Bereiter-Hahn and Voth 1994).

1.4.2 Dynamics of mitochondria

The number and morphology of mitochondria are precisely regulated in cells by rates of fusion and fission (Karbowski and Youle 2003). Fusion and fission of mitochondrial tubules are dynamic events that not only mix the outer and inner membranes but also the content of the mitochondrial matrix and mitochondrial DNA (mtDNA). A balanced frequency between fusion and fission can in principle maintain relatively constant tubular morphology of mitochondria. If the tubulation process of mitochondria is blocked, mitochondria are converted to large spheres (Okamoto and Shaw 2005). Mitochondrial fragmentation appears in case of unbalanced fission, and unbalanced fusion results in mitochondrial elongation (Chan 2006, Bereiter-Hahn et al. 2008).

1.4.2.1 Mitochondrial fusion

Fzo1 in budding yeast and the homologous mitofusin family in mammals (Mfn1 and Mfn2), are conserved GTPases, that act as the principal regulators of MOM fusion (Rapaport et al. 1998, Santel and Fuller 2001). During fusion they anchor two MOMs together and form homo- or hetero-protein complexes (Okamoto and Shaw 2005). Additionally Mfn2 is found in the mitochondria-associated membranes (MAM) of the ER and thought to be essential for interorganellar bridges (Otera and Mihara 2011). Mgm1 in yeast and the mammalian homolog OPA1 are dynamin family GTPases and locate to the IMS. They regulate inner membrane fusion and cristae maintenance (Okamoto and Shaw 2005, Chan 2006). In addition, another protein Ugo1 identified only in fungi interacts with Fzo1 and Mgm1 to form a fusion complex between the inner and outer membranes (Karbowski and Youle 2003, Westermann 2003). Charcot-Marie-Tooth type 2A and autosomal dominant optic atrophy classified as inherited neuropathies are caused by mutations of mitofusin 2 and OPA1, respectively (Chen and Chan 2005).

1.4.2.2 Mitochondrial fission

Dnm1 in yeast and its mammalian homologue Drp1 are dynamin-related proteins that assemble on the mitochondrial surface to mediate organelle division (Westermann 2008). The other key component Fis1 is a conserved tail-anchored outer membrane protein in yeast and human, with its N-terminal domain exposed to the cytosol and is essential for mitochondrial fission (Okamoto and Shaw 2005). Further studies revealed additional proteins named Mdv1 and Caf4 that interact with Fis1/Dnm1 and necessary for mitochondrial fission in yeast (Griffin et al. 2005, Nagotu et al. 2008).

1.4.2.3 Tubulation of mitochondria

Components of tubulation process are detected in fungi but not in higher eukaryotes (Okamoto and Shaw 2005). One tubulation mediator is the outer membrane β-barrel protein Mdm10 (see below). Mitochondrial morphology maintenance (Mmm) 1 is an ER membrane protein. Mutation or loss of Mmm1 results in defect in mitochondrial morphology, a severe mtDNA instability and impairment of the inheritance of mtDNA during bud growth. Mmm1 is therefore proposed to be indirectly involved in mtDNA maintenance and interaction of mitochondria with the actin cytoskeleton (Hanekamp et al. 2002, Garcia-Rodriguez et al. 2009). Furthermore Mmm1 is a subunit of the ER mitochondria encountering structure (ERMES) complex present at the contact sites of

ER and mitochondria (Kornmann et al. 2009). The exact mechanism leading to giant mitochondria in case of loss of Mmm1 is so far unknown.

Mitochondrial distribution and morphology (Mdm) 12 is a cytosolic protein that is found in adjacent to mtDNA nucleotide (Boldogh et al. 2003). Moreover in a systematic genome-wide screen a series of novel genes named *MDM* were unveiled to take an important role in mitochondrial distribution and morphology (Dimmer et al. 2002). Among these *MMM2* encodes a mitochondrial outer membrane protein. Its homologues are only found in fungi (Youngman et al. 2004). Deletions of *Mdm10*, *Mdm12* or *Mmm2* result in similar mitochondrial morphology changes and growth defect as upon loss of Mmm1 (Hermann and Shaw 1998). Accordingly, the encoded proteins are subunits of the ERMES complex too. Two additional mitochondrial inner membrane proteins Mdm31 and Mdm32 are also essential for tubulation of mitochondria as their loss results in large spherical mitochondria, impaired mitochondrial motility and instability of mtDNA (Dimmer et al. 2005).

1.4.3 Intracellular transport of mitochondria

It is important for cellular function to move mitochondria throughout the cell and to target them to locations with high energy demand. In mammalian cells, mitochondria are principally transported to different regions along the microtubule network, whereas in yeast cells actin microfilaments are used to distribute mitochondria properly. In mammals, syntabulin interacts with kinesin 1 and carries out anterograde transport of mitochondria in neurons (Tanaka et al. 1998). Another three mammalian proteins, KBP (KIF1 binding protein) and Milt1/Milt2 locate in MOM and interact with different kinesin proteins to affect mitochondrial cargoes. Miro 1 and Miro 2 from mammals are Rho-like GTPases necessary to keep normal mitochondrial morphology. Their orthologue in *S. cerevisiae*, Gem1p, influences the mitochondrial shape in a manner independent of fission and fusion (Frazier et al. 2006) and was recently shown to be an additional subunit of ERMES complex (Kornmann et al. 2011, Stroud et al. 2011) .

1.5 Beta-barrel proteins

1.5.1 Overview on beta-barrel proteins

Membrane proteins constitute around one-third of all proteins encoded by the genome of living organisms. They are classified in two major structural motifs, α-helices and β-barrel. α-helical proteins are detected in all intra-cellular membranes, while β-barrel proteins are restricted to the outer membrane of Gram-negative bacteria, mitochondria and chloroplasts (Schulz 2000, Galdiero et al. 2007). All β-barrel proteins are composed of antiparallel sheets and the number of β-strands range from eight-stranded small barrels to large 22-stranded existing. It is always an even number in bacterial β-barrels but recent structures of mitochondrial VDAC demonstrated a barrel with 19 strands (Schulz 2000, Paschen et al. 2005, Qiu et al. 2013). *E. coli* has around 60 different β-barrel membrane proteins. In contrast, only a few mitochondrial β-barrel proteins have been identified in the yeast MOM. Among them there are two isoforms of porin, and subunits of translocases of the outer membrane such as Tom40 and Tob55. In addition, Mdm10 is another β-barrel protein of the mitochondrial outer membrane. Of note, no homologues of the latter one protein have so far been found in mammals (Walther et al. 2009). β-barrel membrane proteins have many different functions, such as mediating non-specific, nutrient intake (such as maltose and sucrose), passive transport of ions and small molecules, membrane anchors, voltage dependent anion transport, membrane-bound enzymes and defense against invasive proteins (Gromiha and Suwa 2007).

1.5.2 Beta-barrel proteins in bacterial cells

The topology of β-barrel proteins is such that the hydrophilic residues of the β-strands face towards the interior of the proteins, while the hydrophobic residues point towards the lipid environment of the membrane (Tommassen 2010). Omp85/BamA is a predicted integral β-barrel protein that plays a central role in insertion of β-barrels into the outer membrane in bacteria. It consists of two domains, an N-terminal periplasmic domain and a C-terminal domain embedded as a β-barrel into the outer membrane. The N-terminal polypeptide transport-associated (POTRA) domain is located to the periplasmic space with a few repeated domains, which seem to interact with substrate outer membrane proteins. BamA is evolutionarily conserved across all Gram-negative

bacteria and its homologues have a different number of POTRA domains, which range from one to five in different organisms (Hagan et al. 2011). Tob55 was identified as homologue of BamA in yeast (Paschen et al. 2003) and it is involved in the assembly of the β-barrel proteins in the mitochondrial outer membrane (Endo et al. 2011, see above). In bacteria the β-barrel outer membrane proteins are synthesized in the cytoplasm and delivered by the Sec channel to the periplasm, then immature β-barrel proteins are translocated and held in an unfolded state with help of chaperons like SurA, which prevents such proteins from aggregating and misfolding in the periplasm. β-barrel proteins are then inserted and folded into the outer membrane by the BAM complex.

1.5.3 Beta-barrel proteins in yeast mitochondria

Mitochondrial β-barrel outer membrane proteins are synthesized in yeast cells in the cytosol and imported through outer membrane into the IMS via the pore formed by Tom40. The small Tim chaperons help the β-barrel proteins by escorting them through the IMS and they are then assembled into the outer membrane by the TOB/SAM complex (Davis et al. 2007, Endo et al. 2011, see above). β-barrel membrane proteins of Gram-negative bacteria and of chloroplasts can be imported and correctly assembled in yeast mitochondria (Müller et al. 2011, Ulrich et al. 2012). These results suggest that the β-barrel machinery is functionally conserved in evolution. Although the POTRA domains of BamA are necessary for recognition and assembly of outer membrane proteins, the precise function of POTRA domain of Tob55 is still unclear (Habib et al. 2007). Mitochondrial β-barrel proteins were suggested to be recognized by the TOB complex through a signal, termed β-signal, which was recently discovered. It is the main component of the most C-terminal β-strand of the precursor proteins and is conserved in mitochondrial β-barrel proteins of all eukaryotes (Kutik et al. 2008). Tob55 has one POTRA domain that faces the mitochondrial IMS, the equivalent part of bacterial periplasm. In TOB complex Tob38 but not Tob55 is adapted as a receptor to specially recognize the β-signal (Kutik et al. 2008). Deletion of the POTRA domain of Tob55 results in severe growth phenotype and reduction of release of precursors. The POTRA lacking mutant of Tob55 has also effect on the kinetics of β-barrel assembly (Habib et al. 2007). The POTRA domain of Tob55 seems to facilitate the release of the precursor of β-barrel proteins from TOB complex into the outer

membrane. Collectively, the POTRA domain may play a chaperon-like function in the process of β-barrel maturation (Habib et al. 2007, Stroud et al. 2011). Tob38 and Mas37 interact with Tob55 as subunits of TOB complex (Jiang et al. 2012). Mdm10 is predicted to be a subunit of a subpopulation of the TOB/SAM complex (Meisinger et al. 2004, see above).

1.5.4 Beta-barrel proteins in the chloroplast

Toc75 is the central component of the protein-conducting channel of the translocase (TOC complex) of outer membrane of chloroplasts (Paschen et al. 2005). In *Arabidopsis*, at least four Toc75 coding genes have been reported, Toc75-III, Toc75-IV, and Toc75-V and one pseudo-gene Toc75-I. Their names are according to their chromosome locations. Toc75 from chloroplasts and cyanobacteria shares homology with Omp85 and Tob55. β-barrel proteins were identified in chloroplast to be important as solute channels (Andres et al. 2010).

1.5.5 Functions of Mdm10

The mitochondrial β-barrel protein Mdm10 is proposed to play crucial roles in different mitochondrial processes. It was first identified in 1994 as a protein that can regulate mitochondrial morphology and inheritance (Sogo and Yaffe 1994). So far no functional homologue of Mdm10 was identified in higher eukaryotes. Yeast cells lacking Mdm10 show aberrant mitochondrial distribution and have an abnormally large mitochondrial structure in the mother cell, whereas mitochondria are rarely observed in daughter buds. This suggests that cells lacking Mdm10 are deficient in proper transfer of the mitochondria to daughter buds for inheritance. Mutant cells show a defective growth phenotype on either fermentable or non-fermentable carbon source (Sogo and Yaffe 1994). In addition *mdm10*Δ cells are linked to loss of mtDNA (Berger et al. 1997, Boldogh et al. 2003). Later, Mmm1, Mdm10 and Mdm12 were shown to be in a higher molecular mass complex which is functionally linked with mtDNA inheritance. Furthermore it affects actin-dependent mitochondrial motility (Boldogh et al. 1998, Boldogh et al. 2003, Boldogh et al. 2005). Additionally, Mdm10 was reported to be a subunit of a special subpopulation of the TOB complex and be involved in the final assembly step of TOM complex. It affects the assembly of Tom40, Tom22 and small

Tom proteins but not other β-barrel proteins like porin (Meisinger et al. 2004, Wideman et al. 2010). Yet over-expression of Mdm10 inhibits the association of TOB complex with all β-barrel proteins, not only Tom40. Increasing amount of Mdm10 definitely stabilize Mas37 in the TOB complex. Mas37 on the other hand promotes the release of Tom40 from TOB complex (Yamano et al. 2010b). Lack of Mdm10 impairs the interaction between Tom5/Tom40 and TOB complex (Thornton et al. 2010). It was shown that Tom7 as a conserved subunit of the TOM complex, directly interacts with Mdm10 to become a binding partner and has a function in an antagonistic manner to Mdm10. Increase of Tom7 leads to trapping of Mdm10 and impairment of the TOB-Mdm10 complex with delaying the late assembly steps of Tom22 and Tom40 (Becker et al. 2011). Deletion of Tom7 results in elevated Mdm10 levels available for the TOB complex and promotes the TOM complex assembly (Meisinger et al. 2006, Wideman et al. 2010, Yamano et al. 2010).

1.6 Respiratory chain complexes of mitochondria

1.6.1 Mitochondrial respiratory chain and its function

Mitochondria are primarily involved in the energy production through synthesis of ATP molecules. They do so by employing the proton gradient across the MIM generated by the respiratory chain supercomplexes, which are larger structural and functional units (Smeitink et al. 2001). The mitochondrial oxidative phosphorylation system (OXPHOS) contains five major multi-subunit complexes: NADH-Coenzyme Q (CoQ) reductase (Complex I), succinate-CoQ reductase (Complex II), ubiquinol-cytochrome c reductase (Complex III), cytochrome c oxidase (Complex IV) and F_1F_o–ATP synthase (complex V). Additionally, two connecting redox-active molecules take part in reactions in the respiratory chain and are used as electron shuttles between the complexes (Schagger 2002, Berardo et al. 2011). One of them named Coenzyme Q (CoQ) is a lipophilic quinone embedded in the inner membrane lipid bilayer. CoQ possesses three redox states, fully oxidized (ubiquinone), one electron reduced semiquinone radical (ubisemiquinone) and fully reduced state (ubiquinol). The other one is a hydrophilic heme-containing protein, cytochrome c, which is encoded by a nuclear gene and is localized on the external surface of the inner membrane (Elston et al. 1998, Noji and Yoshida 2001).

The electrons emerging from the oxidation of NADH and succinate are passed to complex III and finally to complex IV where they are used to reduce oxygen to water. This electron transport is accompanied by a vectorial proton transport across the mitochondrial inner membrane to generate an electroosmotic potential. This gradient is used by complex V to synthesize ATP from ADP (Thomas et al. 2008). After treatment with the chemical inhibitors for respiratory chain complexes I-IV or electron transport chain (ETC), ATP synthesis is suppressed and the mitochondrial morphology is disrupted (Galloway et al. 2012). Some small proteins in the MIM are additionally required for electron transfer activity. There are electron transfer flavoprotein-glycerol-3-phosphate dehydrogenase, ubiquinone oxidoreductase, dihydroorotate dehydrogenase, choline dehydrogenase and alternative NADH dehydrogenase (Lenaz and Genova 2009).

1.6.2 Supramolecular organisation of the mitochondrial respiratory chain

In contrast to most eukaryotes, complex I was not identified in the yeast *S. cerevisiae*. However, the absent complex I is replaced by three NADH dehydrogenases associated with the inner mitochondrial membrane (De Vries et al. 1992, Small and McAlister-Henn 1998). In bovine heart mitochondria complex I forms various supercomplexes under physiological conditions. About 17% of total complex I was characterized in form of I_1III_2, and further two major supercomplexes containing complex IV were found with $I_1III_2IV_1$ and $I_1III_2IV_2$, respectively (Lenaz and Genova 2009). It is reported that a functional association between complex III and IV in yeast exists (Cruciat et al. 2000, Schagger and Pfeiffer 2000). Complex V is present in a dimeric state in both yeast and mammalian mitochondria (Arnold et al. 1998, Wittig and Schagger 2009). A few of the central subunits of complex III and IV are candidates for a direct interaction between the two complexes. For example, cytochrome b and cytochrome c1 from complex III, and Cox1, Cox2 and Cox3 from complex IV (Cruciat et al. 2000). The constitution of respiratory chain supercomplexes depends on growth conditions. Under common growth conditions the amount of respiratory complex IV is relatively reduced. If *S. cerevisiae* cells are grown on glucose medium and their mitochondria are analyzed by BN-PAGE, two bands have been observed as

supercomplexes III_2IV_1 with 750 kDa and III_2V_2 with 1000 kDa. In contrast, the content of complex IV is increased by growing yeast on non-fermentative carbon source and supercomplex III_2V_2 is increased in amount to substitute for supercomplex III_2IV_1 as major form (Cruciat et al. 2000). Mitochondrial F_1F_o-ATPase (Complex V) shows dimeric and higher oligomeric structures in the mitochondrial inner membrane. Furthermore, a direct physical interaction between complex IV and V has been identified (Wittig and Schagger 2009).

Cardiolipin is an essential phospholipid for respiratory supercomplex formation. Loss of cardiolipin in yeast results in functional deficiency of complex IV. Cytochrome *c*1 is involved at the interface of complexes III and IV. Through neutralization of lysine residues in the transmembrane helix of cytochrome *c*1, cardiolipin influences the interaction of complex III and IV and stabilizes such supercomplexes under the conditions of BN-PAGE (Zhang et al. 2002, Pfeiffer et al. 2003).

1.7 ER and mitochondrial association sites

The ER was found to have membrane contact sites with other organelles and these regions are called membrane contact sites (MCSs) (Elbaz and Schuldiner 2011, Toulmay and Prinz 2011). Mitochondria and ER have a close contact region but the membranes still maintain their identities without fusion (Rowland and Voeltz 2012). The physical association between ER and mitochondria are called mitochondria-associated ER membrane (MAM) (Poston et al. 2013). MAM has a restricted location, and only a small area of the MOM (12%) is proposed to contact with ER (Achleitner et al. 1999). The distance between the two organelles was suggested to be within ca. 10-25 nm (Achleitner et al. 1999, Csordas et al. 2006). The close proximity between the ER and mitochondria promotes rapid and efficient exchange pathways for signals and metabolisms, so that MAM can play several pivotal roles in cellular functions, including regulation of Ca^{2+} transport from the ER to mitochondria, lipid transport, energy metabolism and cellular survival (Hayashi et al. 2009). Elevation of calcium concentration in mitochondria has effects on mitochondrial division and movement (Szabadkai et al. 2006a and b). The process of calcium transport has three roles, activation of a series of metabolic steps to produce ATP; control of induction of

mitochondrial permeability transition to control apoptosis; and modulation of cytosolic calcium transients or waves (Gunter and Gunter 2001).

Previous investigations showed that diverse enzymes involved in lipid and glucose metabolism are enriched in MAM. These enzymes comprise phosphatidylserine (PS) synthase, phosphatidylethanolamine (PE) methyltransferase-2, diacylglycerol acyltransferase (DGAT), glucose-6-phosphatase and acyl-CoA: cholesterol acyltransferase (ACAT). Other observations also indicate that the MAM might contain enzymes required for cholesterol and ceramide biosynthesis (Rusinol et al. 1994, Bionda et al. 2004). Recently, chaperones and ubiquitin ligases were discovered at this ER-mitochondria interface. These results bring us new insights into the communication of two classes of stress proteins between the two organelles (Hayashi et al. 2009).

The mechanistic details of ER-mitochondria contact sites remain unclear. In mammalian cells several proteins are identified as tethering candidates to maintain this connection. One suggested structure was the voltage-dependent anion channel (VDAC1) of MOM in association with the ER Ca^{2+} release channel IP_3R (inositol (1,4,5)-trisphosphate receptor) through the chaperone glucose-regulated protein 75 (GRP75) (Szabadkai et al. 2006b). A component of another tethering structure was identified as protein mitofusin 2 (Mfn2), which is a mitochondrial outer membrane dynamin-like GTPase mediating mitochondrial fusion (de Brito and Scorrano 2008). Mfn2 was also shown to be localized to the ER membrane.

In yeast, a complex termed ERMES (ER-mitochondria encounter structure) complex was revealed between ER and mitochondria by a synthetic biology screen, in which mutants could be complemented a synthetic protein named ChiMERA designed to artificially tether the ER and mitochondria. Four components were identified in this complex, including the mitochondrial outer membrane proteins Mdm10 and Mmm2, the membrane protein Mmm1 of the ER and the cytosolic protein Mdm12 (Kornmann et al. 2009). The calcium-binding MOM Miro GTPase Gem1 was later proposed to be the fifth component of ERMES complex. It is supposed to have regulatory function (Kornmann et al. 2011, Stroud et al. 2011). Its mammalian homolog, MIRO, can also be found on sites of ER–mitochondria proximity in mammalian cell culture. Mutants of the components of ERMES complex cause mitochondrial morphology defects and growth defect on non-fermentable medium. The main function of ERMES complex

seems to be a mechanical link between the ER and mitochondria (Kornmann and Walter 2010). Three subunits of the ERMES complex contain the yet uncharacterized SMP (synaptotagmin-like, mitochondrial and lipid-binding protein) domain identified by bioinformatic analysis. This domain is also found in some eukaryotic proteins that bind lipids or hydrophobic ligands. Therefore it was predicted that ERMES complex plays a putative role in the lipid transport between ER and mitochondria (Kopec et al. 2010). Indeed, the PS transfer from ER to mitochondria is impaired and the conversation ratio of PS/PE is greatly reduced, if one subunit of ERMES complex is missing (Kornmann et al. 2009, Kornmann and Walter 2010, Voss et al. 2012).

1.8 Biogenesis of mitochondrial lipids in yeast

1.8.1 Synthesis of membrane lipids

Biological membranes contain primary structural elements named phospholipids, which consist of a glycerol backbone esterified by two fatty acids and a phosphate group (Schneiter and Kohlwein 1997). Except for phospholipids, sterols and sphingolipids are two other classes of lipids found in biological membranes. In yeast and other eukaryotes the majority of phospholipid synthesis enzymes are located in the ER, where accordingly most phospholipids are synthesized (Pichler et al. 2001, van Meer et al. 2008). In addition, some enzymes are found to produce certain phospholipids in mitochondria, such as phosphatidylserine decarboxylase (Psd1) which convert the PS to PE, and enzymes for phosphatidylglycerol and cardiolipin biosynthesis (Leber et al. 1995). Generally, phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylethanolamine (PE) are synthesized in mitochondria, whereas phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and sterols are imported from other organelles (Flis and Daum 2013). Mitochondria from different organisms share similar characteristics in respect to their lipid composition. In contrast to most other organelles overall lower phospholipid to protein ratio is observed in mitochondria. This is the outcome of the minor contribution of the outer membrane with a higher ratio of phospholipid/protein of 0.67 together with that of the inner membrane with a value of about 0.18 (Zinser and Daum 1995, Dolis et al. 1996, Schneiter et al. 1999). Although most biosynthesis pathways of lipids are identical to higher eukaryotes, some specific pathways can be identified only in yeast. For example,

yeast cells have two PS decarboxylases named Psd1 in mitochondria and Psd2 in Golgi/vacuolar compartment, whereas mammalian cells use only one enzyme Psd to synthesize the PE in mitochondria. In addition, the Golgi apparatus and lipid particles were also shown to participate in phospholipid and sterol synthesis (Trotter and Voelker 1995, Leber et al. 1998). Phospholipids are crucial to maintain mitochondrial ultrastructure, so alteration of composition of phospholipid may cause mitochondrial disorders, such as Barth syndrome which is caused by mutation in tafazzin, a protein involved in cardiolipin synthesis (Zick, Rabl et al. 2009). Phospholipids participate in critical mitochondrial functions such as oxidative phosphorylation (the role of cardiolipin in supercomplex assembly, see 1.6.2) and apoptosis (Schlame et al. 2000, Joshi et al. 2009), biogenesis of mitochondria (Jiang et al. 2000, Kutik et al. 2008) and fusion of mitochondria (Choi et al. 2006).

In yeast cells, PS is synthesized from the initial substrates CDP-diacylglycerol and serine through phosphatidylserine synthase Pss1/Cho1 (Letts et al. 1983). In contrast, in higher eukaryotes PS is synthesized by base exchange mechanism using two phosphatidylserine synthases Pss-1 and Pss-2 and a calcium dependent reaction (Vance 2008). PS has low concentration in mitochondrial membrane, but it is substrate for the enzyme phosphatidylserine decarboxylase in the inner membrane (Zinser and Daum 1995). PS is an important precursor for the synthesis of two major phospholipids, PE and PC (Achleitner et al. 1995, Achleitner et al. 1999).

PC is the most abundant phospholipid constituent of mitochondrial membranes and comprises almost 40% of their total phospholipids (van Meer et al. 2008). It is synthesized in ER and then transferred to mitochondria. In the absence of exogenous choline the synthesis of PC in yeast depends on the triple methylation of PE (Carman and Zeimetz 1996). An enzyme named phosphatidylethanolamine methyltransferase catalyzes these three methylation steps. Through the Land´s cycle PC is obtained from lyso-PC and fatty acids by LPCAT (lyso-phosphocholine acyltransferase) (Shindou et al. 2009). PC can also be found to be formed through CDP-choline branch of the Kennedy pathway (Gibellini and Smith 2010). The PC transport between the inner and outer membranes of mitochondria is coupled to its synthesis (de Kroon et al. 2003).

PE is a zwitterionic phospholipid that promotes non-bilayer structure and the second most abundant phospholipid in eukaryotic cells. It comprises 30% of total phospholipid in mitochondria (de Kruijff 1997). There are three spatial separate pathways to

synthesize PE in yeast. First way includes the Kennedy pathway where the precursor cytidyldiphosphate (CDP)-ethanolamine reacts with diacylglycerol (DAG) and is transformed to PE by Ept1 outside mitochondria (Kanfer and Kennedy 1964). Biosynthesis of PE can be additionally accomplished by two de novo pathways. One option is by phosphatidylserin (PS) synthesis and finally decarboxylation to PE. To this end, PS is transferred from the ER to mitochondria or Golgi/vacuole and is decarboxylated by phosphatidylserine decarboxylase 1 (Psd1) in the mitochondrial inner membrane and by phosphatidylserine decarboxylase 2 (Psd2) in the Golgi/vacuole compartment, respectively (Osman et al. 2011). Finally, yeast cells can also use enzymes of the Land´s cycle to catalyze acylation of lyso-PE to obtain PE (Riekhof and Voelker 2006, Deng et al. 2010). In the mitochondrial associated membranes (MAM) of the ER some PE migrates from mitochondria to ER and is converted into phosphatidylcholine (PC) by methylation reactions. PE is essential for yeast growth since mutant strains defective in PE synthesis show reduced growth on non-fermentable carbon sources, but depletion of PE results in no obvious changes of the mitochondrial membrane (Birner et al. 2001). Moreover, respiration-deficient cells (*petite* mutants) are a possible consequence of reducing the level of PE in the cell.

The biosynthesis of glycerophospholipids and triacylglycerols require phosphatidic acid (PA) as an essential substrate. PA is the precursor of CDP-diacylglycerol which is the basis substrate for synthesis of PI, PG, PS and CL. Mitochondria are able to produce their own PA (Chakraborty et al. 1999). PA can be synthesized in two different pathways: the Gro3P (glycerol 3-phosphate) or the GrnP (dihydroxyacetone phosphate) pathway. But the GrnP pathway only occurs in yeast (Athenstaedt and Daum 1999).

Phosphatidylglycerol (PG) is a minor phospholipid component in cells. Phosphatidyl-glycerolphosphate is synthesized from CDP-diacylglycerol and glycerol-3-phosphate, then it is dephosphorylated to PG. Similarly to cardiolipin it is regarded as mitochondria-specific phospholipid (Daum and Vance 1997).

Phosphatidylinositol (PI) is another abundant phospholipid in mitochondria with 7-15% of total phospholipids. This lipid is enriched in the mitochondrial outer membrane in comparison to the inner membrane (Daum and Vance 1997). PI is synthesized from CDP-diacylglycerol and inositol (Gardocki et al. 2005). Disruption of phosphatidylinositol synthase gene *PIS1* in yeast cells results in lethality indicating the essential role of PI (Nikawa et al. 1987). PI and its derivatives are known to be

precursors for certain cell signaling molecules and involved in vesicle traffic (Daum 2004). The biosynthesis of GPI anchors is also affected by PI (Serricchio and Butikofer 2011).

Cardiolipin (CL) is characterized by two 1, 2-diacyl-sn-glycero-3-phosphoryl parts linked by a glycerol bridge. It can be found in different membranes and organisms, e.g., mitochondrial inner and outer membranes, hydrogenosomes and bacterial membranes (Mileykovskaya et al. 2005, Schlame 2008, Acehan et al. 2011). In eukaryotes, cardiolipin is regarded as a special mitochondrial phospholipid. The CL synthase in eukaryotic cells is only found in mitochondria to synthesize CL in the multistep reaction sequence (Schlame et al. 1993). Recently, a fraction of cardiolipin and the protein tafazzin were identified in mitochondrial outer membrane by a quantitative analysis of highly pure yeast outer membrane (Gebert et al. 2009). CL together with PE are interaction partners of many mitochondrial proteins and contribute to the stability of their conformation (Osman et al. 2011, Joshi et al. 2012). In yeast cells cardiolipin plays an essential role in stabilizing the association of respiratory complex III and IV to form supercomplexes in the mitochondrial inner membrane (Zhang et al. 2005). Since the mitochondrial inner membrane is in charge of energy transformation and regulation of mitochondrial volume, defects of cardiolipin metabolism, which include alterations in CL fatty acyl composition, the accumulation of monolysocardiolpin and reduced amounts of total CL, inhibit the energetic coupling at the maximal rate of oxidative phosphorylation through decreased activity of mitochondrial proteins, such as the ATP/ADP antiporter (Koshkin and Greenberg 2002). A functional ERMES complex is required for cardiolipin biosynthesis (Kornmann et al. 2011). Defective synthesis of CL also causes swollen vacuole morphology, loss of vacuolar acidification, and impairment of V-ATPase activity and proton pumping in isolated vacuoles (Chen et al. 2008).

Sterols are another integral component of eukaryotic membranes and minor lipids of mitochondria. Certain enzymatic steps of the ergosterol synthesis in yeast don't exist in mammals and biosynthesis of sterols in plants takes place in the ER (Trotter and Voelker 1995, Leber et al. 1998). In a complex pathway of reactions cholesterol is the final product in mammalian cells, while ergosterol is the major sterol in yeast (Miller and Bose 2011, Jacquier and Schneiter 2012). The enzymes for ergosterol biosynthesis have dual localization in yeast cells, namely the ER and lipid particles

(Leber et al. 1998). Three enzymes, namely Erg1, Erg6 and Erg7, are localized in lipid particles and thus lack of these compartments affects sterol homeostasis in yeast cells (Sorger et al. 2004). Mitochondria exhibit a very low sterol to protein ratio compared with other subcellular fractions. The ergosterol is unequally present in the mitochondrial outer and inner membrane. In yeast mitochondria the concentration of ergosterol is six times higher in the inner membrane in comparison of the outer membrane, whereas in higher eukaryotes most sterols are found in the mitochondrial outer membrane (Zinser et al. 1993, Daum et al. 1998, Tuller and Daum 1995, Schneiter et al. 1999).

1.8.2 Intracellular traffic of lipids

Mitochondria and ER are recognized as the two subcellular compartments that are involved in most phospholipids biosynthesis pathways in yeast. ER plays a dominant role in phospholipids synthesis, but significant amounts of PC and PI synthesis also occurs in the Golgi (Voelker 2003). In eukaryotes most other cell organelles have no capacity to synthesize lipids and obtain them by intracellular lipid transport. The lipid transport between organelles requires specific carrier proteins, membrane contact sites, tethering complexes or vesicle flux (Flis and Daum 2013).

A specific subfraction of the ER, MAM has been identified to be involved in the lipid translocation to mitochondria (Shiao et al. 1998). MAM are not only fractions with high capacity to synthesize certain phospholipids, like PS, with phosphatidylserine synthase (Pss1), but also are proposed to directly supply phospholipids to mitochondria through contact sites (Gaigg et al. 1995, Pichler et al. 2001). The investigation of phospholipid translocation demonstrates that contact sites between the ER and mitochondria are crucial for exchange of phospholipids in yeast and mammalian cells. Import of PS in mitochondria is energy-independent in yeast cells and depends on the PS concentration in the ER or Golgi membrane. In contrast, ATP is necessary to transport PE from MAM to the MOM in mammalian cells (Voelker 1990, Shiao et al. 1998). Treatment of isolated MAM and mitochondria with protease *in vitro* has no effect on the import rate of PS into mitochondria, which indicates that surface proteins of mitochondria and MAM are only necessary for the association of the two compartments (Achleitner et al. 1999). The aforementioned ERMES complex not only physically

tethers the ER/MAM and mitochondria, but also influences phospholipid transport. The absence of ERMES complex results in reduced levels of CL and PS/PC conversion rate, suggesting that ERMES complex play an essential role in exchange of phospholipid between ER and mitochondria (Kornmann et al. 2009). Of note, defects of ER-shaping proteins, such as Rth1, Yop1 and Sey1, not only change the ER tubules morphology, but also impair the phospholipid transport between ER and mitochondria (Voss et al. 2012).

The precise import mechanism of PC and PI is unclear, yet previous reports using radiolabeled PC or PI, which are imported from unilamellar donor vesicles to isolated yeast mitochondria, suggest that contact sites between mitochondrial outer and inner membranes are involved in intramitochondrial phospholipid transport and the lipid translocation is rapid and bidirectional (Lampl et al. 1994, Janssen et al. 1999). Moreover, there is transport of different phospholipids between the outer and inner mitochondrial membranes. Mitochondrial contact sites (CS) are made up by close connection of outer membrane and inner boundary membrane (Perkins et al. 1997, Frey and Mannella 2000). In this case, the contact sites of both membranes become the hot spots for lipid transport (Schatz and Dobberstein 1996, Osman et al. 2011, Flis and Daum 2013). Some proteins such as Ups1 and Ups2 localized in IMS were also found to affect the lipids synthesis and trafficking in the mitochondria. The CL level is antagonistically regulated by Ups1 and Ups2 (Tamura et al. 2009), while the PE level is controlled by Ups2 (Potting et al. 2010). Moreover, experiments indicated that Ups1 facilitated the conversation of PE to PC and export of PE from the mitochondrial inner membrane, whereas Ups2 inhibited this process (Tamura et al. 2012). Recently, another IMS protein Mdm35 was identified as an interaction partner of Ups1 and both proteins were shown to form a complex and to mediate the PA transport between the mitochondrial outer and inner membranes. This process promotes the conversation of PA to CL in the inner membrane, while high concentration of CL keeps Ups1 bound to the membrane, resulting in proteolysis of Ups1 and decreased transport of PA and CL synthesis (Connerth et al. 2012).

In mammalia, the sterol transport is associated with the so-called steroidogenic acute regulatory protein (StAR), which is regulated by cAMP. StAR binds cholesterol and transports it to the MOM (Petrescu et al. 2001, Benmessahel et al. 2004). In yeast the transport of sterols from ER to mitochondria is different from the import machinery used

by phospholipids. The import of ergosterol into mitochondria is enhanced by a cytosolic factor and the transfer to the MIM takes place in an energy-independent manner (Tuller and Daum 1995). Most intramitochondrial transport of sterol appears to take place at the contact sites of outer and inner membranes. Yet the exact transport mechanism is still obscure (Tuller and Daum 1995).

1.9 Aim of study

The mitochondrial outer membrane protein Mdm10 is involved in many different processes in yeast cells. It influences the mitochondrial protein biogenesis, inheritance and morphogenesis of mitochondria, and lipid homeostasis. All processes are connected with each other. Although Mdm10 furthermore was recently identified as a subunit of the ERMES complex that tethers the ER and mitochondria, the primary function of Mdm10 is so far uncovered.

The aim of the present study was to decipher the precise function of Mdm10 and hence to obtain detailed insight into the biogenesis of the mitochondrial outer membrane. To that aim a multi-copy suppressor screen of the *mdm10*Δ growth phenotype was performed and the identified suppressors were characterized.

2. Materials and Methods

2.1 Materials

2.1.1 Media

The following tables summarize frequently used media in this study.

Table 1: Media for *S. cerevisiae*

Name	Composition
Lactate medium	0.3% (w/v) yeast extract, 0.05% (w/v) glucose, 0.05% (w/v) NaCl, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgCl_2 \times 6H_2O$, 0.05% (w/v) $CaCl_2 \times 2H_2O$, 2.5% (v/v) 80% lactic acid, 0.8% (w/v) NaOH, pH adjusted to 5.5 with NaOH, add water to 10 l
SLac medium	1.7 g yeast nitrogen base without ammonium sulfate, 5 g ammonium sulfate, 55 mg adenine sulfate, 55 mg uracil, 24.75 ml 80% (v/v) lactic acid, pH adjusted to 5.5 with KOH, add 100×stock amino acid and H_2O to 990 ml
YPD medium	2% (w/v) bacto peptone, 1% (w/v) yeast extract, 2% (v/v) glucose, pH adjusted to 5.5
YPG medium	2% (w/v) bacto peptone, 1% (w/v) yeast extract, 3% (v/v) glycerol, pH adjusted to 5.5
YP agar	2% (w/v) bacto peptone, 1% (w/v) yeast extract, 2% (w/v) agar, 2% (v/v) glucose or 3% (v/v) glycerol, pH adjusted to 5.5
Synthetic medium	0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 0.0055% (w/v) adenine sulfate, 0.0055% (w/v) uracil, pH adjusted to 5.5 with NaOH (uracil is omitted for S-Ura). Autoclaved carbon sources were added to 2% (v/v) glucose (SD), 2% galactose (SGal) or 3% (v/v) glycerol (SG) final concentration. Corresponding amino acids were added from 100×stock amino acid solution
D-Glucose stock solution	40% (w/v) D-glucose
Glycerol stock solution	100% glycerol
D-Galactose stock solution	40% (w/v) D-galactose
100×stock amino acid	0.2% (w/v) arginine, 0.4% (w/v) tryptophan, 1% (w/v) leucine, 0.4% (w/v) lysine, 0.2% (w/v) histidine, 0.6% (w/v) phenylalanine, 0.2% (w/v) methionine
2.4 M sorbitol	437.2 g/l sorbitol
Sporulation medium	1% (w/v) potassium acetate, 0.1% (w/v) bacto-yeast extract, 0.05% (w/v) glucose, 2% (w/v) bacto agar
G418	Stock: 100 mg/ml, 200 µl on one YP agar plate

Table 2: Media for *E. coli*

Name	Composition
LB medium	1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl in ddH_2O, pH 7.0, add 2% (w/v) agar for solid media before autoclaving

LB-Amp medium	LB-medium was supplemented with 100 µg/ml ampicillin. Stock solution of ampicillin (100 mg/ml) sterilized by filtration. Add to autoclaved medium or agar solutions below 50°C.

2.1.2 Buffers

The following tables summarize frequently used buffers in this study

Buffers for molecular biology

Table 3: Buffers for agarose-gel electrophoresis

Name	Components
1×TAE buffer	40 mM Tris-Base, 1.14 ml/l acetic acid, 1 mM EDTA, pH 8
10×Loading buffer	6% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol

Table 4: Buffers for small-scale plasmid isolation from *E. coli* (Miniprep)

Name	Components
E1	50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A
E2	200 mM NaOH, 1% (w/v) SDS in water
E3	3 M potassium acetate, pH adjusted to 5.5 with acetic acid

Table 5: Buffers for PCR

Name	Components
10×Pfu buffer with $MgSO_4$	100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% (v/v) Triton X-100, 1 mg/ml BSA, 20 mM $MgSO_4$, 200 mM Tris, pH adjusted to 8.8 with HCl
10×Taq buffer with $(NH_4)_2SO_4$	200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 750 mM Tris, pH adjusted to 8.8 with HCl

Table 6: Buffers for chemical competent *E. coli* cells

Name	Components
Tfb 1 buffer	30 mM potassium acetate, 100 mM RbCl, 100 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, pH adjusted to 5.8 with acetic acid
Tfb 2 buffer	100 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) glycerol, pH adjusted to 6.5 with NaOH.

Table 7: Buffers for preparation of electro-competent yeast cells

Name	Components
DTT-buffer	30 mM DTT, 0.6 M sorbitol, 20 mM Hepes , pH adjusted to 7.5 with KOH
Freezing buffer	0.6 M Sorbitol, 10 mM $CaCl_2$, 10 mM Hepes, pH adjusted to 7.5 with KOH

Table 8: Buffer for genomic DNA-extraction from yeast cells

Name	Components
DNA extraction buffer	50 mM Tris/HCl, 20 mM EDTA containing 1% (w/v) SDS, pH adjusted to 7.4 with HCl

Table 9: T4 DNA ligase buffer

Name	Components
T4 DNA ligase buffer	100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, 400 mM Tris, pH adjusted to 7.8 with HCl

Table 10: Buffer for isolation of plasmid DNA from yeast cells

Name	Components
Prep buffer	2% (v/v) Triton X-100, 1% SDS, 0.1 M NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA

Buffers for mitochondrial isolation

Table 11: Buffers for isolation of mitochondria from yeast cells

Name	Components
Resuspension buffer	100 mM Tris, 10 mM DTT, without pH adjustion
Spheroplasting buffer	1.2 M Sorbitol, 20 mM potassium phosphate buffer pH 7.2
Homogenization buffer	0.6 M sorbitol, 1 mM EDTA, 1 mM PMSF, 0.2% (w/v) fatty acid free BSA, 10 mM Tris adjusted to pH 7.4 with HCl
SEM buffer	250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH adjusted to 7.4 with KOH
Percoll gradient buffer	25% (v/v) Percoll (Sigma, Germany), 250 mM sucrose, 10 mM MOPS/KOH pH7.2, 1 mM EDTA, 2 mM PMSF

Table 12: Buffer for purification of isolated mitochondria by sucrose gradient centrifugation

Name	Components
Sucrose	20%, 30%, 40%, 50% and 60% (w/v) sucrose in 10 mM MOPS/KOH pH7.4, 100 mM KCl, 1 mM EDTA, 1 mM PMSF

Buffers for protein analysis

Table 13: Buffers for silver staining

Name	Components
Fixation solution	40% (v/v) ethanol, 10% (v/v) acetic acid
Sensitizer solution	30% (v/v) ethanol, 8 mM sodium thiosulfate, 500 mM sodium acetate
Silver solution	30 mM silver nitrate

Developing solution	235 mM sodium carbonate, 0.02% (v/v) formaldehyde, 0.003% sodium thiosulfate
Stopping solution	5% (v/v) acetic acid

Table 14: Buffers for nitrocellulose and PVDF membrane stripping

Name	Components
Stripping buffer	62.5 mM Tris-HCl (pH6.8), 2% (w/v) SDS, 0.7% (v/v) β-metacaptoethanol
TBST buffer	0.05% (v/v) Tween 20 in TBS buffer

Table 15: Buffers for SDS-PAGE

Name	Components
2×Lämmli loading buffer	4% (w/v) SDS, 20% (v/v) glycerol, 0.02% (w/v) bromphenol-blue, 5% (v/v) 2-mercaptoethanol, 160 mM Tris, pH adjusted to 6.8 with HCl
SDS running buffer	190 mM glycine, 24.8 mM Tris, 0.1% (w/v) SDS
1.0 M Tris-HCl, pH 6.8	
1.0 M Tris-HCl, pH 8.8	

Table 16: Buffer for Western blotting

Name	Components
Blotting buffer	20 mM Tris, 150 mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol

Table 17: Buffers for immunodetection

Name	Components
Ponceau staining buffer	0.4 g Ponceau S and 8.5 ml 72% (w/v) TCA for 200ml final volume
TBS buffer	5 mM Tris, 150 mM NaCl, pH adjusted to 7.5 with HCl
TBS buffer + 0.05% (v/v) Triton X-100	TBS buffer + 0.05% (v/v) Triton X-100
Blocking buffer	5% (w/v) dry skim milk in TBS buffer
ECL	0.2 mM p-coumaric acid, 1.25 mM Luminol, 100 mM Tris, pH adjusted to 8.5 with HCl; 30% (w/v) H_2O_2 was mixed with ECL in ratio 1:1000 before usage.

Table 18: Buffers for immunostaining

Name	Components
Spheroplasting premix	1.2 M sorbitol, 0.1 M potassium phosphate buffer pH 7.4, 0.5 mM $MgCl_2$
Spheroplasting solution	1 ml spheroplasting premix, 2 µl mercaptoethanol per ml, zymolyase 20T (100 µg/ml)
BSA-PBS-NaAzid buffer	1% (w/v) crude BSA, 0.04 M K_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl, 0.05% (w/v) NaN_3

Table 19: Buffers for *in vitro* import of proteins into isolated mitochondria

Name	Components
Transcription buffer	40 mM Tris-HCl pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine
F5-import buffer	250 mM sucrose, 10 mM MOPS, 80 mM KCl, 5 mM $MgCl_2 \times 6H_2O$, 3% (w/v) fatty acid free BSA, pH adjusted to 7.2 with KOH
SEM-K80 buffer	SEM buffer + 80 mM KCl

Table 20: Buffers for BN-PAGE

Name	Components
3×Gel buffer	200 mM ε-Amino-n-caproic acid, 150 mM Bis-Tris, pH adjusted to 7.0 with HCl
10×loading buffer	5% (w/v) Coomassie blue G, 500 mM ε-Amino-n-caproic acid, 100 mM Bis-Tris, pH 7.0
Solubilization buffer	0.1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF, 20 mM Tris, pH adjusted to 7.4 with HCl
10×Cathode buffer A	500 mM Tricine, 150 mM Bis-Tris, 0.2% (w/v) Coomassie G, pH 7.0
10×Cathode buffer B	500 mM Tricine, 150 mM Bis-Tris, pH adjusted to 7.0
10×Anode buffer	500 mM Bis-Tris, pH adjusted to 7.0

2.1.3 Enzymes

Restriction endonucleases and buffer systems were supplied by New England Biolabs (Frankfurt am Main, Germany). *ExactRun*-DNA polymerase with buffer system was from GENAXXON bioscience (Ulm, Germany). T4-DNA-Ligase, Shrimp Alkaline Phosphatase, *Taq*-DNA polymerase and *Pfu*-DNA polymerase were products from Fermentas (Schwerte, Germany). Proteinase K (Roche, Germany), Zymolyase 20T (MP Biomedicals, U.S.A) and SP6-RNA-polymerase (Progema, Germany) were purchased from the indicated companies. All experiments were performed in accordance with recommended protocols of the manufacturers.

2.1.4 Antibodies

All antibodies used in this study are summarized in the following two tables. All primary antibodies were obtained from laboratory stock. Except for anti-Flag antibody from mouse and anti-HA antibody from rat, all the other antibodies are raised in rabbits.

Table 21: Primary antibodies

Primary antibodies	Marker for	Dilution
α-Tom40	MOM	1:10.000
α-Tom20	MOM	1:1000
α-Tom22	MOM	1:1000
α-Tom70	MOM	1:1000
α-Tob55	MOM	1:1000
α-Mas37	MOM	1:1000
α-Dld1	MIM	1:1000
α-Fis1	MOM	1:200
α-Cor1	MIM	1:2000
α-Cox2	MIM	1:1000
α-Erv2	ER	1:1000
α-Mcr1	MOM/IMS	1:1000
α-HA	HA-Tag	1:1500
α-Flag	Flag-Tag	1:2000
α-Mia40	IMS	1:1000
α-Sec61	ER	1:1000
α-Hep1	Matrix	1:1500
α-AAC	MIM	1:500
α-Bmh1	Cytosol	1:1000
α-Hexokinase	Cytosol	1:5000
α-Mge1	Matrix	1:500
α-Mdm10	MOM	1:500
α-Oxa1	IMS	1:1000
α-Porin	MOM	1:4000

Table 22: Secondary antibodies

Name	Producer
Goat anti-Rabbit IgG (H+L)-HRP conjugate	Bio Rad
Goat anti-Mouse IgG (H+L)-HRP conjugate	Bio Rad
Goat polyclonal secondary antibody to Rat IgG- H&L (HRP)	ab6845, Abcam

2.1.5 Yeast and *E. coli* strains

The *E. coli* strain XL1-blue was used for cloning purposes and plasmid preparations.

Yeast strains used in this study are listed in the following table.

Table 23: *S. cerevisiae* strains

Strain	Genotype	Reference
CW130	W303a; *ups2::NAT*	Gift from Thomas Langer
CW143	W303a; *ups1::NAT*	Gift from Thomas Langer
YKD034	W303a; *MATa; ura3-1; trp1Δ2; leu2-3,112; his3-11,15; ade2-1; can1-100*	Euroscarf

YKD035	W303α; *MATα; ura3-1; trp1Δ2; leu2-3,112; his3-11,15; ade2-1; can1-100*	Euroscarf
YKD037	YPH499; *MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his1-Δ200 leu2-Δ1*	Euroscarf
YKD228	W303a; *mmm1::KanMX4*	by Dr. Kai Stefan Dimmer
YKD293	W303a; *mmm2::HIS3MX6*	by Dr. Kai Stefan Dimmer
YKD294	W303α; *mmm2::HIS3MX6*	by Dr. Kai Stefan Dimmer
YKD301	W303a; *mdm12*::*HIS3MX6*	by Dr. Kai Stefan Dimmer
YKD450	W303a; *ylr253w::KanMX4*	by Dr.Kai Stefan Dimmer
YKD472	W303a; *psd1::KanMX4*	by Dr. Kai Stefan Dimmer
YKD478	W303a; *psd1::HIS3MX6*	by Dr. Kai Stefan Dimmer
YKD553	W303a; *mdm10::HIS3MX6*	by Dr. Kai Stefan Dimmer
YTT004	W303α; *yor228c::HIS3MX6*	This study
YTT006	W303α; *ylr253w::HIS3MX6*	This study
YTT010	W303α; *mdm10*::*HIS3MX6*	This study
YTT011	W303a; *ylr253w::HIS3MX6*	This study
YTT124	W303α; *yor228c::HIS3MX6; ylr253w::KanMX4*	This study
YTT184	BY4741*; MATa; his3Δ 1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf

2.1.6 Oligonucleotides

Oligonucleotides used are listed in Table 24. They were designed to delete the genes of interest and to clone gene sequences (ORFs) into different vectors. In addition, Flag-tag was genomically introduced at the C-terminus of Mcps. To this end special primers were designed and used to amplify the tag sequence.

Table 24: List of oligonucleotides

Primers for deletion by gene-targeting	
mdm10fwd	5’ AAA TAT ACG TTA GGA AAA AGA CAC GAA CAG AGA AGA CCG ATC TTG *CGT ACG CTG CAG GTC GAC* 3’
mdm10rev	5’ ATT TTT TAA CCT GTA TAT TAA AAC CTT TAT TTT ATT TCA CAT TAC *ATC GAT GAA TTC GAG CTC G* 3’
mcp1fwd	5’ ATT ACT AGG GCA TAT ACC AGT TAG CCC AGA GTT TTG TTT ATT ACG *CGT ACG CTG CAG GTC GAC* 3’
mcp1rev	5’ GAA TTG TCG CAA ATT AAT AGC TTC ACA CTA CTC CCA CAA GGA TTC *ATC GAT GAA TTC GAG CTC G* 3’
mcp2fwd	5’ GAG CAA GAT TAT AGT TGA ATG TTT CTT ATT CGG TGT TGA TAG TAG *CGT ACG CTG CAG GTC GAC* 3’

mcp2rev	5’ TAT AAT TTT ACG TAT ATA TTT ACA AGT AGA AAG AAC GCT AAC GAT *ATC GAT GAA TTC GAG CTC G* 3’
Primers for introducing open reading frames into pYX plasmids	
pYXmdm10s	5’ GGG *CCA TGG* CCA TGC TAC CCT ATA TGG ACC AA 3’
pYXmdm10a1	5’ GGG *CCC GGG* TCA TGT GGA GTA CTG GAA TTG 3’
pYXmdm10a2	5’ GGG *CCC GGG* TGT GGA GTA CTG GAA TTG TAT 3’ (omitting the stop codon for a plasmid-borne HA-Tag)
pYXmmm1s	5’ GGG *CCA TGG* CCA TGA CTG ATA GTG AGA ATG AAT C 3’
pYXmmm1a1	5’ GGG *CCC GGG* TTA TAA CTC TGT AGG CTT TTC TT 3’
pYXmmm2s	5’ GGG *CCA TGG* CCA TGT CAT TTA GAT TCA ACG AAG C 3’
pYXmmm2a1	5’ GGG *AAG CTT* TTA ATG ATA TGG TGG GGG GC 3’
pYXmdm12s	5’ GGG *GAA TTC* ATG TCT TTT GAT ATT AAT TGG AGT 3’
pYXmdm12a1	5’ GGG *AAG CTT* TTA CTC ATC ACC ATC GTT GAA A 3’
pYXmcp1s	5’ GGG *GGA TCC* CAT AAA GTT GCA TGA AGT GCC T 3’
pYXmcp1a1	5’ GGG *AAG CTT* CTA ATT CAC GTG CAA CAG CAA 3’
pYXmcp1a2	5’ GGG *AAG CTT* ATT CAC GTG CAA CAG CAA CC 3’ (omitting the stop codon for a plasmid-borne HA-Tag)
pYXmcp2s	5’ GGG *CCA TGG* CCA TGA TGA CCA AAG CTT TTT TTA A 3’
pYXmcp2a1	5’ GGG *CCC GGG* TTA AGA TGA CAA CCA AGT CTT C 3’
pYXmcp2a2	5’ GGG *CCC GGG* AGA TGA CAA CCA AGT CTT CGG 3’ (omitting the stop codon for a plasmid-borne HA-Tag)
pYXsym1s	5’ GGG GAA TTC ATG AAG TTA TTG CAT TTA TAT GAA G 3’
pYXsym1a	5’ GGG AAG CTT TTA TTC GAC CAC GGG TGG ATA 3’
pYXmdm31s	5’ GGG GAA TTC ATG TCC CTT TTT ACC AGG CC 3’
pYXmdm31a	5’ GGG AAG CTT CGC GAT AGC ACC GAG ACT C 3’
Primers for FLAG-tagging by gene-targeting	
Flagmcp1fwd	5’ TTC GAA AGC ATT TTC AAA AAG ATC CGG TTG CTG TTG CAC GTG AAT *ACT AGT GGA TCC CCC GGG* 3’
Flagmcp1rev	5’ GAA TTG TCG CAA ATT AAT AGC TTC ACA CTA CTC CCA CAA GGA TTC *ATC GAT GAA TTC GAG CTC G* 3’
Flagmcp2fwd	5’ GTG TTG TGG TGG AAA AAA TTT ATT CCG AAG ACT TGG TTG TCA TCT *ACT AGT GGA TCC CCC GGG* 3’
Flagmcp2rev	5’ TAT AAT TTT ACG TAT ATA TTT ACA AGT AGA AAG AAC GCT AAC GAT *ATC GAT GAA TTC GAG CTC G* 3’

2.1.7 Plasmids

Plasmids used in this study are listed in Table 25.

Table 25: List of plasmids

Plasmid name	Promoter	Marker	Reference
pFL44L	Lac	URA	(Bonneaud et al.1991)
pGEM4	SP6 and T7	Amp	Promega
pGEM4-TOM22	SP6	Amp	(Dukanovic et al. 2009)
pGEM4-TOM40	SP6	Amp	(Paschen et al. 2003)
pRS316-CherryFis1	TEF2	URA	From Dr. Katrin Krumpe
pRS416-mtRFP	TPI	URA	Plasmid collection, Rapaport group
pYX132	TPI	TRP	AG Rapaport

pYX132-MCP1	TPI	LEU	This study
pYX132-MCP2	TPI	LEU	This study
pYX142	TPI	LEU	AG Rapaport
pYX142-MCP1	TPI	LEU	This study
pYX142-MCP1-HA	TPI	LEU	This study
pYX142-MCP2	TPI	LEU	This study
pYX142-MCP2(D223A)	TPI	LEU	This study
pYX142-MCP2(D223A)-HA	TPI	LEU	This study
pYX142-MCP2(E256A)-HA	TPI	LEU	This study
pYX142-MCP2-HA	TPI	LEU	This study
pYX142-MDM10	TPI	LEU	This study
pYX142-MDM10-HA	TPI	LEU	This study
pYX142-MDM12	TPI	LEU	This study
pYX142-MDM31	TPI	LEU	This study
pYX142-MMM1	TPI	LEU	This study
pYX142-MMM2	TPI	LEU	This study
pYX142-mtGFP	TPI	LEU	Plasmid collection, Rapaport group
pYX142-SYM1	TPI	LEU	This study

2.2 Methods

2.2.1 Molecular biology methods

2.2.1.1 Polymerase chain reaction (PCR)

Polymerase chain reaction was applied to amplify specific DNA sequences (Saiki et al. 1988). DNA templates were genomic or plasmid DNA. 1-2 units *Taq*-DNA-polymerase or *Pfu*-DNA-polymerase were used with two specific primers (100 pmol) and 0.02 mM dNTPs, the appropriate 10×Taq or Pfu-buffer containing 1-2 mM Mg^{2+} (Table 5).

PCR was carried out in a thermal cycler (Biometra, Germany) to repeat programmed heating and cooling steps for DNA melting and enzymatic replication. A typical PCR reaction is divided in three parts. First DNA template was denatured at 95°C for 5 to 10 min. Second in 30-35 thermal cycles, consisting of 1 min DNA denaturation at 95°C, 1 min annealing of oligonucleotide primers at 50°C-60°C and a few minutes of synthesis of the new DNA sequence at 72°C (time depends on length of target sequence and processivity of polymerase). A final step of incubation at 72°C for 10 min enabled that all replication processes were finished. At the end the PCR reaction was cooled to 4°C and agarose gel electrophoresis (2.2.1.2) was employed to further analyze amplified DNA fragments.

2.2.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis was employed to analyze and purify DNA fragments. Different agarose gel concentrations (0.8%-2% in TAE buffer) (Table 3) were applied depending on the size of DNA fragments of interest. DNA samples were mixed with 10×loading buffer (Table 3) and applied to an agarose gel containing 0.5 µg/ml ethidium bromide. TAE buffer was used for electrophoresis. A 1 kb DNA ladder from Fermentas (Schwerte, Germany) was used to determine the length of DNA molecules by comparison. DNA fragments in the gel were visualized under UV light.

2.2.1.3 Isolation of DNA from agarose gels

DNA fragments of interest were separated by electrophoresis and cut from agarose gels. They were isolated by a silica column using the peqGOLD Gel Extraction Kit (peqLAB, Germany) according to the manufacturer´s protocol.

2.2.1.4 Determination of DNA concentration

DNA concentration was determined by spectrophotometric analysis. The concentration of DNA is measured by UV light at 260 nm (Eppendorf BioPhotometer, Germany). An OD value of 1.0 equals 50 µg/ml of pure double-stranded DNA, 33 µg/ml single-stranded DNA, 40 µg/ml RNA and 20 µg/ml oligonucleotide. The ratio of absorbance at 260 nm and to that at 280 nm is used to assess purity of DNA and RNA. A ratio of around 1.8 is generally sufficient for DNA, while the ratio of around 2 corresponds to pure RNA.

2.2.1.5 Restriction digestion of DNA

For cloning or identification of plasmids, DNA was digested with specific restriction endonucleases. Restriction digestion conditions were applied according to the protocol of the manufacturer. Analysis and purification of restricted DNA fragments was performed by agarose gel electrophoresis (2.2.1.2 and 2.2.1.3).

2.2.1.6 Dephosphorylation of digested DNA fragments

To prevent re-ligation of linearized plasmid DNA, dephosphorylation of plamid DNA was performed. Shrimp Alkaline Phosphatase (Fermentas, Germany) was employed

to catalyze the release of 5'-phosphates on linear plasmid DNA according to the manufacturer's specification. Dephosphorylated plasmids were purified as in 2.2.1.3.

2.2.1.7 Ligation of DNA

Dephosphorylated plasmid DNA and insert DNA were ligated by T4 DNA ligase (Fermentas, Germany) at a molar ratio of 1:3 to 1:7. Ligation was performed with 1 U T4 DNA ligase and 1×T4 ligase buffer (Table 9) at 14°C for 16h. Ligase was inactivated by incubation at 65°C for 15 min.

2.2.1.8 Preparation of chemical competent *E. coli* cells

E. coli cells of the XL-1 blue strain were inoculated in LB medium overnight at 37°C. A 2 ml pre-culture was diluted to an OD_{600} of 0.1 in 200 ml LB medium and cells were grown to an OD_{600} of 0.5. The culture was transferred to pre-cooled 50 ml sterile Falcon tubes and cells were harvested by centrifugation (3000 g, 5 min, 4°C). The cell pellet was resuspended in 80 ml pre-cooled TfbI (Table 6) and incubated on ice for 15 minutes. After centrifugation (4000 g, 10 min, 4°C) pellet was resuspended in 80ml pre-cooled TfbII (Table 6) and kept on ice for another 15 minutes. The cells were divided in 100 µl aliquots and shock frozen using liquid nitrogen to be kept at -80°C for storage.

2.2.1.9 Transformation of *E. coli* cells

Plasmids and ligation reactions were added to a reaction tube containing 100 µl competent cells and incubated on ice for 30 min. After heat shock at 42°C for 45 s cells were incubated on ice for another 10 min. 750 µl sterile LB medium was added to the bacteria and the tube was kept shaking for 60 min at 37°C. Finally, cells were collected by centrifugation (10000 g, 30 sec, RT). The cell pellet was resuspended in 50 µl sterile water and plated on solid selective LB-Amp medium at 37°C overnight. Single colonies were obtained for plasmid isolation (2.2.1.10).

2.2.1.10 Isolation of plasmid DNA from *E. coli* cells

2.2.1.10.1 Small scale isolation of plasmid DNA from *E. coli* cells

On the basis of the method of alkaline lysis small scale preparations of plasmid DNA were carried out from *E. coli* cells (Birnboim and Doly 1979). Single bacterial colonies containing plasmids were picked from LB-Amp plates and incubated overnight in 5 ml LB-Amp at 37°C. The cells were collected by centrifugation (3000 g, 5 min, RT). The pellet was resuspended in 300 µl buffer E1 (Table 4). The tube was inverted 5 times to lyse cells after the addition of 300 µl buffer E2 (Table 4). The mixture was incubated for 5 minutes at room temperature. After addition of 300 µl neutralization buffer E3 (Table 4) the samples were inverted carefully 5 times. After centrifugation (15000 g, 20 min, RT) supernatants were transferred in new tubes and mixed with 600 µl 96% (v/v) isopropanol. DNA pellets were precipitated by centrifugation (15000 g, 30 min, 2°C) and washed with 200 µl 70% ice-cold ethanol. After centrifugation (15000 g, 10 min, 2°C) the DNA pellet was collected and dried at room temperature for 10 minutes. Finally the isolated DNA was resuspended in 40 µl sterile water and stored at -20°C.

2.2.1.10.2 Large-scale isolation of plasmid DNA from *E. coli* cells

PureYield Plasmid Midiprep System with vacuum manifold (Promega, Germany) was used to isolate large-scale plasmid DNA from *E. coli* cells. Bacteria with target plasmid were inoculated and incubated in 50 ml LB-Amp overnight at 37°C. The cells were harvested by centrifugation (3000 g, 10 min, RT). Purification of the plasmids was performed according to the protocol of the manufacturer. The concentration of DNA plasmids was measured by spectrophotometry (2.2.1.4). Plasmids were stored at -20°C.

2.2.1.11 Preparation of genomic DNA from *S. cerevisiae*

A yeast culture was inoculated in 50 ml YPD medium and grown overnight at 30°C. Cells were harvested by centrifugation (5000 g, 5 min, RT). The cell pellet was resuspended in 3 ml 0.9 M sorbitol solution containing 0.1 M EDTA (pH 7.5). To turn the cells into spheroplasts 900 µg zymolyase (300 µg/ml final concentration) was added to the mixture and incubated for 1h at 30°C followed by centrifugation of the spheroplasts (5000 g, 5 min, RT). The pellet was resuspended in DNA extraction buffer (Table 8). The suspension was incubated at 65°C for 30 min. Then 1.5 ml of 5 M potassium acetate were added for 1 h on ice the solution was centrifuged (12000 g, 15

min, RT). The supernatant was mixed with 2 volumes 100% (v/v) ethanol and centrifuged again (15000 g, 15 min, RT). The pellet was dissolved in 3 ml TE buffer (10mM Tris/HCl, pH 7.4, 1 mM EDTA) and centrifuged (12000 g, 15 min, RT). The supernatant was transferred to a new tube and the pellet was discarded. 150 µl 50 mM potassium acetate solution (pH 5.5) containing 1 mg/ml RNase A was added to a new tube and incubated for 30 min at 37°C. One volume of 100% isopropanol was added and mixed. DNA precipitate (a loose ”cocoon” of fibers) was carefully removed by sterile pipette tip. The DNA was air-dried and dissolved in TE buffer (pH 7.4). Finally the concentration of genomic DNA was determined by spectrophotometry (2.2.1.4).

2.2.1.12 Quick extraction of genomic DNA from yeasts for PCR-based applications

Cells (OD_{600}=0.4) collected from 100 µl liquid YPD culture (or single yeast colonies) were resuspended in 100 µl 200 mM lithium acetate (LiOAc) containing 1% (w/v) SDS. Cells were treated according to a published protocol (Looke et al. 2011). Shortly, the tube was vortexed and incubated for 5 min at 70°C. Then, 300 µl of 96%-100% (v/v) ethanol was added to the sample to precipitate DNA by brief vortexing and DNA was collected by centrifugation (15000 g, 3 min, RT). The pellet was washed with 500 µl 70% (v/v) ethanol after the supernatant was removed. The pellet was next dissolved in 100 µl of H_2O, the cell debris was removed by centrifugation (15000 g, 15 sec, RT). 1-2 µl of the DNA solution was used for PCR.

2.2.2 Methods in yeast genetics

2.2.2.1 Cultivation of *S. cerevisiae*

S. cerevisiae was cultured in YPD, YPG, lactose- or galactose-containing medium according to published procedures (Sambrook et al.1989) (Table 1). YPG medium was applied to determine mitochondrial function or to maintain their mtDNA. To select yeast cells for auxotrophic marker (uracil, leucine and tryptophan) cells were grown in synthetic medium lacking the regarding marker. Yeast suspension cultures were incubated at 120 rpm at 30°C. For isolation of mitochondria from yeast cells YPGal and SGal-leu media were employed. The OD_{600} for isolation of mitochondria did not exceed 2.3 in galactose containing medium, while it was lower than 1.5 in lactose containing

medium. Yeast strains were kept on YPD or YPG plate at 4°C for not longer than two months. For long-term preservation yeast cells were stored in 15% (v/v) glycerol at -80°C. All media used are listed in Table 1.

2.2.2.2 Preparation of electro-competent yeast cells

Yeast cells were inoculated and incubated overnight in 50 ml of YPD medium. In the morning OD_{600} value was measured, and the culture was diluted to an OD_{600} of 0.2. Cells were grown to an OD_{600} of 0.8 and then harvested by centrifugation (1600 g, 5 min, RT). The supernatant was discarded and the pellet was resuspended in DTT buffer (Table 7), in 1/10 of the initial volume of the culture (5 ml). Cells were harvested again as above. From this moment on all steps were performed on ice or at 4°C. Supernatant was discarded and ice-cold 1 M sorbitol (25 ml, 50% original volume of culture) was added and cells in the pellet were resuspended. Cells were centrifuged (1600 g, 5 min, 2°C) and the same procedure was repeated for two additional times. Finally, the pellet was resuspended in 2 ml ice-cold freezing buffer (Table 7). A total culture of 400 ml is enough for the whole screening process. Aliquots of 1 ml were stored at -80°C (at least one night, not longer than 1 month).

2.2.2.3 Electro-transformation and high copy suppressor screen of yeast cells

A 1 ml aliquot of electro-competent yeast cells lacking Mdm10 was thawed in a water bath at 30°C for 1-2 min. Immediately after thawing cells were transferred on ice, and centrifuged (1600 g, 5 min, 4°C). The cell pellet was resuspended in 1 ml ice-cold 1 M sorbitol. This step was repeated once. The pellet was resuspended in 300 µl of ice-cold 1 M sorbitol. Then, 100 µl of cells were combined in pre-cooled sterile cuvettes with 400 ng yeast genomic DNA library constructed in the plasmid pFL44L (Stettler et al. 1993). Electroporation was carried out by Gene Pulser Xcell (BioRad, U.S.A) under the following conditions: 2.2 KV, 25 µF, 200 Ω. The cell suspension was mixed well with 1 ml of sterile 1 M sorbitol and 100 µl of the mixture was spread on each SD-Ura plate. For initial selection of plasmids plates were incubated 3-4 days at 30°C. Then clones were replica plated on glycerol-containing full medium and further incubated for selection at 37°C. Plasmid DNA was extracted from the clones grown at 37°C and transformed into *E. coli* cells. DNA inserts of plasmids isolated from the transformed *E. coli* cells were analyzed by sequencing.

2.2.2.4 Small scale isolation of plasmid DNA from yeast cells

Glass beads 0.75-1 mm (Roth, Germany) were washed in HNO_3 for 1-2 hours followed by washing with water and TE buffer. Glass beads were then dried at 80°C for a few hours. Yeast colonies picked from selective plates were incubated overnight at 30°C in 10 ml SD-Ura. The cells were harvested by centrifugation (3000 g, 5 min, RT). After discarding the supernatant the pellet was resuspended in residual liquid in a 2 ml reaction tube. Next, 600 µl prep buffer (Table 10) and the same volume of phenol: chloroform: isoamylaalcohol 25:24:1 mixture were added together with 300 mg of glass beads. The tube was vortexed for 2 min. Centrifugation at maximal speed in a table top centrifuge was carried out for 5 min. The aqueous layer was transferred to new 2 ml plastic tubes and 2.5 volumes of 100% ethanol were added. Samples were kept at -20°C for 20 min before the mixture was centrifuged (15000 rpm, 15 min, 4°C). The supernatant was carefully discarded and 900 µl 70% ethanol was added to wash the pellet. After another centrifugation (15000 rpm, 10 min, RT) the pellet was air-dried and dissolved in 40 µl water. Isolated yeast plasmids were transformed in competent *E. coli* cells.

2.2.2.5 Transformation of yeast cells with lithium acetate

A modified protocol for transformation of *S. cerevisiae* with lithium acetate (Gietz et al. 1995) was applied in this work. Yeast cells were incubated on YPD solid medium at 30°C. Enough cells were scraped from the plate with a sterile loop and washed with 1 ml sterile water. After centrifugation (10000 g, 30 sec, RT) cells were harvested and resuspended in 1 ml of 100 mM sterile lithium acetate. Cells were incubated 5 min at 30°C shaking at 400 rpm. Cells were collected by centrifugation (8000 g, 1 min, RT) and resuspended in 400 µl 100 mM sterile lithium acetate. Cells were pelleted (3000 g, 5 min, RT) and different solutions in the following order were added on top of the pellet: 240 µl 50% (w/v) PEG 3350, 36 µl 1 M lithium acetate, 25 µl salmon sperm DNA (5 mg/ml heat denatured at 95°C, 10 min), 50 µl sterile water containing 0.1-10 µg plasmid DNA. The tube was mixed well and incubated at 42°C for 20 min while gently shaking at 800 rpm. Cells were collected by centrifugation (10000 g, 30 sec, RT) and the pellet was resuspended in 100 µl water. The suspension was plated on solid synthetic medium lacking appropriate selective markers. The plate was incubated at 30°C for 3-6 days until appearance of colonies.

2.2.2.6 Construction of deletion strains of *S. cerevisiae* (non-essential genes)

In this study yeast genes were deleted by a PCR approach in the isogenic strains W303a and W303α by introducing the Kanamycin and Histidin3 markers. The HIS3MX6 cassette was amplified from the plasmid pFA6a-HIS3MX6 (Wach et al. 1997) and the KanMX4 cassette from pFA6a-KanMX4 (Wach et al. 1994) with gene-specific primers (Table 24). The complete open reading frames were deleted by homologous recombination. Cells with deleted genes were selected on SD-His or YPD supplied with G418. Double-deletion strains were obtained by tetrad dissection. All deletion strains were confirmed by genome based PCR with specific primers (Table 24).

2.2.2.7 Mating of yeast strains and tetrad analysis

To achieve double-deletion strains in *S. cerevisiae*, cell strains with different single deletion were mated by using opposite mating types (*MATa* and *MATα*). The two opposite mating type strains were separately incubated overnight at 30°C as parallel lines on two YPD plates. With a velvet-covered disk the first plate was replicated on a new YPD plate, while cell lines of the second plate were replicated on the same new plate perpendicular to the previous cells. After one day incubation at 30°C cells were then replica plated on selective medium to allow growth of only mated diploid cells. After growing the heterologous diploid knock out cells for a couple of days on YPD, the cells were transferred for a few days at room temperature onto sporulation medium to stimulate sporulation. 100 µg/ml zymolyase was applied to digest the cell wall of asci by incubation for 10 min at 30°C in sterile 1.2 M sorbitol. One drop of these treated asci was spotted on a new YPD plate. With help of a micromanipulator single ascis were selected and divided in four spores, each of which was shifted by a tiny needle and located at a certain position on the plate. After incubation at 30°C for a few days the spores grew out to yeast colonies, whose growth phenotype and auxotrophic markers were analyzed on selective media. The mating type of single colonies was determined by Mating-type-PCR method using yeast cell directly as template (Huxley et al. 1990).

2.2.3 Cell biology methods

2.2.3.1 Drop dilution assay

Drop dilution assays were employed to compare the growth phenotype of different yeast strains under the same test conditions. Yeast cells were picked from a plate and incubated overnight at 30°C in 20 ml YPD or synthetic medium with sucrose. The culture was diluted to an OD_{600} value of 0.2 with the same medium. Cells continued to grow at 30°C to exponential growth phase with an OD_{600} of around 1. Next cells were harvested by centrifugation (3000 g, 5 min, RT) and washed with 1 ml sterile water. Cells were obtained by the same centrifugation step and resuspended with sterile water to an OD_{600} of 2.0. The OD_{600} of all strains was measured again and adjusted to the same value with sterile water. Suspensions of different strains were diluted in five-fold series with water and 5µl of each dilution was spotted on different solid media of interest. Plates were incubated at 15°C, 30°C and 37°C for several days.

2.2.3.2 Isolation of mitochondria by lysis with glass beads

Yeast cells were inoculated from plate and incubated overnight at 30°C in liquid medium. Cells were diluted to an OD_{600} of 0.2 in 200 ml of the same medium and grown to an OD_{600} value of 0.8-1.0. Cells were harvested (3000 g, 5min, RT) and the cell pellet was washed once with sterile water and centrifuged (3000 g, 5 min, RT) again. The pellet was resuspended in 1.5 ml ice-cold SEM buffer with 2 mM PMSF and divided in two 2 ml tubes containing 300 mg glass beads (0.75-1 mm) (Roth, Germany). To break the cells each tube was vortexed for 30 s with 30 s break intervals on ice. This procedure was repeated 4 times. Cell lysates were clarified by centrifugation (1000 g, 3 min, 4°C) and protein concentration of the supernatant was determined by the Bradford method (2.2.4.1). The amount of crude mitochondria was roughly estimated to be around 8% of total protein for YPD medium or 15% for lactate or galactose medium. Mitochondria were sedimented by centrifugation (13,200 g, 10 min, 2°C) and resuspended in 2×Lämmli buffer for further analysis.

2.2.3.3 Isolation of mitochondria by enzymatic spheroplastation

Large scale isolation of mitochondria from *S. cerevisiae* was performed by enzymatic spheroplastation according to a published protocol (Daum et al. 1982). Cells in 2 to

20 l liquid culture were harvested by centrifugation (3000 g, 5 min, RT) when the OD_{600} value was between 1.2 and 1.5 (OD_{600} was under 3.0 for SGal-Leu medium). After washing with water the cell pellet was resuspended in resuspension buffer (2 ml per gram of cells, Table 11) and incubated for 10 min at 30°C with shaking. Cells were sedimented again and washed in spheroplasting buffer (Table 11), which was discarded after centrifugation. For every gram of cells the pellet was resuspended in 6.6 ml spheroplasting buffer and supplied with 4 mg zymolyase to subsequently digest the cell wall at 30°C for 1 h while shaking (120 rpm). Spheroplasts were collected by centrifugation (2000 g, 5 min, 4°C) and resuspended in ice-cold homogenization buffer (Table 11). Disruption of the spheroplasts was achieved by a Dounce tissue grinder by 10 strokes on ice with a tight fitting glass pestle in a glass mortar. The homogeneous cell lysate was clarified by two sequential centrifugation steps (2000 g, 5 min, 4°C) and mitochondria were harvested by another high-speed centrifugation (18000 g, 12 min, 2°C). Afterwards the mitochondrial pellet was resuspended in SEM buffer with 2mM PMSF for further centrifugation steps. First, two times low-speed centrifugation steps (2000 g, 5 min, 2°C) for removal residual cell debris and the mitochondria were obtained in a final high-speed centrifugation (18000 g, 12 min, 2°C). Finally the crude mitochondrial pellet was resuspended in 300 µl - 500 µl of SEM buffer with 2 mM PMSF, aliquoted, and frozen in liquid nitrogen before stored at -80°C.

2.2.3.4 Preparation of subcellular fractionations of yeast cells

Yeast cultures were incubated overnight in SLac-Leu media until an OD_{600} of 1.2 - 1.6 was reached. Isolation of mitochondria was performed by the standard isolation method (2.2.3.3). Whole cell extract was obtained after homogenization during the mitochondrial preparation. After the sedimentation of mitochondria the cell lysate was centrifuged again (18000 g, 15 min, 4°C) to get rid of all residual mitochondria. Through centrifugation of the resulting supernatant (200000 g, 90 min, 2°C) the cytosolic fraction was collected as the supernatant and the ER fraction was obtained in the pellet. The ER pellet was resuspended in 3 ml SEM buffer with 2 mM PMSF and treated with dounce homogenizer. The ER mixture was clarified by centrifugation (18000 g, 20 min, 4°C). Protein amounts were determined by Bradford (2.2.4.1). Finally, cytosolic and ER proteins were harvested by TCA precipitation (2.2.4.2) and dissolved in 2×Lämmli buffer (5 min, 95°C).

2.2.4 Biochemical methods

2.2.4.1 Determination of protein concentration

The Bradford method (Bradford 1976) was used to determinate protein concentration in this study. Aliquots of 2-10 µl of 1 mg/ml bovine serum albumin (BSA) were prepared to acquire a standard curve. The protein solution was diluted 20 fold and 10 µl of diluted protein solution and different amount of BSA were mixed with 1 ml of five times diluted commercially available Roti-Quant reagent (Roth, Germany) in 1×Phosphate buffered saline (PBS) and incubated for 5 min at RT. The absorbance of the samples was measured at 595 nm and protein concentration was calculated according to standard curve.

2.2.4.2 TCA protein precipitation

Stock solution of 72% (w/v) trichloroacetic acid (TCA) was added to a final TCA concentration of 12% (v/v) to the aqueous solution of proteins. The mixture was incubated on ice for 30 min and centrifuged (36000 g, 20 min, 2°C). After discarding the supernatant, 1 ml pre-cooled acetone (-20°C) was slowly added to the pellet without disturbing it. Centrifugation was repeated as above and the protein pellet was air-dried for 15 min. The pellet was dissolved in 2×Lämmli buffer and denatured for 5 min at 95°C.

2.2.4.3 Chloroform-methanol precipitation

Chloroform-methanol precipitation is a fast method applied to precipitate soluble proteins (Wessel et al. 1984). This method is especially suitable for solutions with high sucrose concentrations. Four volumes of methanol were added to the sample and vortexed. Then one volume of chloroform and three volumes of water were added. The samples were vortexed for another 20 sec, the mixture was centrifuged at maximal speed in a table centrifuge at room temperature. The upper layer was discarded as completely as possible without disturbing the interface. Then three volumes of methanol were added. After vortexing the mixture was centrifuged at maximal speed for 2 min. Protein pellets were obtained by discarding supernatants and air-drying for 15 min. The pellets were dissolved in 2×Lämmli buffer and boiled for 5 min at 95°C.

2.2.4.4 Carbonate extraction

Carbonate extraction was performed according to a previously described protocol to distinguish membrane proteins from soluble and loosely membrane-attached proteins (Fujiki et al. 1982). Mitochondria were pelleted by centrifugation (8000 g, 15 min, 2°C) and resuspended in 50 µl of ice-cold 20 mM Hepes/KOH pH 7.5. Afterwards cold 50 µl 200 mM sodium carbonate solution (freshly prepared) was added. Samples were vortexed and incubated on ice for 30 min. Membranes were sedimented by ultracentrifugation (120000 g, 60 min, 4°C) and resuspended in 2×Lämmli buffer. The soluble proteins were obtained by TCA precipitation (2.2.4.2) of the supernatant.

2.2.4.5 DAPI-staining of mitochondrial DNA

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that is used for visualization of DNA in fluorescence microscopy. Mitochondrial DNA can also be visualized by DAPI staining (McConnell et al.1990; Williamson and Fennell 1979). Yeast cells were incubated in medium containing glucose to an OD_{600} of 1.0 and one ml of the culture was harvested by centrifugation (13000 rpm, 20 sec, RT). The cell pellet was incubated with 700 µl methanol for 5 min, cells were centrifuged (13000 rpm, 20 sec, RT) and washed with one ml PBS buffer. Cells were resuspended in one ml PBS with one µl DAPI (1 mg/ml in water) and incubated shaking at RT for one hour. After centrifugation (8000 g, 1 min, RT) and washing with PBS, the cell pellet was collected and resuspended in 100 µl - 300 µl PBS for analysis by fluorescence microscopy.

2.2.4.6 Fluorescence microscopy of yeast cells

For visualization of mitochondria, yeast cells were transformed with an expression vector harboring the mitochondrial presequence of subunit 9 of F_o-ATPase of *N. crassa* fused to GFP or RFP (Mozdy et al. 2000; Westernmann and Neupert 2000). Yeast cells containing such fluorescent marker proteins were incubated overnight in 20-40 ml synthetic selective medium. The culture was diluted to an OD_{600} of 0.2 and cells were grown further to an OD_{600} of 1.0. One ml of the culture was harvested by centrifugation (10000 g, 1 min, RT) and resuspended in the same medium to concentrate cells if necessary. Five µl of 1% (w/v) low melting point agarose was mixed with five µl cell suspension, applied to a microscope slide and covered with a coverslip.

Microscopic images were acquired with an Axioskop20 fluorescense microscope with an Axiocam MRm camera using the 43 cy3 filter set and AxioVision software (Zeiss, Germany). Fluorescence of GFP was excited at 488 nm (blue light) and its emission peak is at 509 nm (green light). RFP was excited at 580 nm (green light) and emission was measured at 600 nm (red light).

2.2.4.7 *In situ* immunofluorescence of epitope tagged proteins in yeast

Immunofluorescence is applied to allow the visualization of a specific protein or antigen in cells or tissue sections by staining a specific antibody chemically conjugated with a fluorescent dye. In this study a modified version of an original protocol of the group of Prof. Dr. R. Jansen was used (Nasmyth et al. 1990). Yeast cells with overexpression of either Mcp1-HA or Mcp2-HA were grown to exponential phase. Five ml of a dense culture were treated with formaldehyde (final concentration 3.7% (v/v)) and incubated for 60 min while shaking at 30°C. The cell pellet was obtained by centrifugation and resuspended in spheroplasting premix (Table 18). To achieve ghostlike spheroplasts cells were treated with spheroplasting solution (Table 18) for 60 min at 30°C. The spheroplasts were collected by centrifugation (3000 g, 3 min, RT) and washed carefully once in spheroplasting premix. Next, spheroplasts were centrifuged, resuspended in 50-500 µl spheroplasting premix and stored at -80°C or kept one night at 4°C.

Cells were washed with BSA-PBS-NaAzid (1×PBS-1% (w/v) BSA-0.05% (w/v) NaAzid) and covered at RT for two hours with primary antibody in BSA-PBS-NaAzid solution on a prepared slide, which was coated with 0.02% ploylysine and rinsed with water. Primary antibody was washed away with 5-10 µl BSA-PBS-NaAzid solution containing 0.05% (v/v) Triton X-100 for three times. Florescent dye conjugated- secondary antibody was added to the cells, which were kept for one hour at RT in darkness and moist to prevent bleaching of fluorescence. Cells were rinsed as above to remove secondary antibody and incubated with PBS containing 10 µg/ml DAPI for 5-10 min at RT. The solution was aspirated off and rinsed with PBS. Finally the slide was covered with a drop of mounting solution (80% glycerol in 1×PBS) and the assembled coverslip was sealed with nail polish. The fluorescence signals were analyzed by microscopy.

2.2.4.8 Treatment of mitochondria with Proteinase K

The topology of mitochondrial proteins was analyzed by using Proteinase K protection assays. Isolated mitochondria (60 µg-100 µg) were harvested by centrifugation (8000 g, 10 min, 2°C) and resuspended in one of the following buffers: SEM buffer, hypoosmotic buffer (10 mM HEPES/KOH, pH 7.4) or SEM buffer with 0.5% (v/v) Triton X-100 buffer. The mixture was incubated on ice for 30 min and Proteinase K was added to a final concentration of 100 µg/ml. Samples were incubated on ice for additional 30 min. The reaction was terminated by addition of two µl of 200 mM PMSF resulting in inactivation of Proteinase K. The tube was kept on ice for 10 min and TCA precipitation was employed to acquire the proteins for further analysis (2.2.4.2).

2.2.4.9 Purification of isolated mitochondria by Percoll gradient

Mitochondria obtained by the standard isolation protocol (2.2.3.3) were mixed by three strokes in a loose-fitting Douncer with five ml SEM containing 2 mM PMSF. The suspension was loaded on the top of 15 ml of 25% (v/v) Percoll solution (10 ml Percoll, 5 ml 2 M Sucrose, 4 ml 100 mM MOPS/KOH pH 7.2, 400 µl 100 mM EDTA, 400 µl 200 mM PMSF in 40 ml). After centrifugation in a Beckman 60Ti rotor with slow acceleration and brake off setting (80000g, 30 min, 2°C) the mitochondrial fraction was collected and resuspended in 30 ml SEM buffer containing 2 mM PMSF. The solution was centrifuged (6500 g, 10 min, 4°C). Next the mitochondrial pellet was resuspended in SEM buffer, aliquoted and frozen in liquid nitrogen.

2.2.4.10 Purification of isolated mitochondria by trypsin digestion and sucrose step gradient

The protein concentration of mitochondria from standard mitochondria isolation (2.2.3.3) was first determined by Bradford (2.2.4.1). Mitochondria were incubated for 20 min on ice with five µg trypsin (Sigma, Germany) per mg of mitochondrial protein. The digestion reaction was stopped by addition of soybean trypsin inhibitor (Sigma, Germany) (10 µg trypsin inhibitor per µg trypsin) and the mixture was incubated further on ice for another 30 min. Next trypsin digested mitochondria were mixed with two to three ml SEM buffer containing 1 mM PMSF and loaded on a sucrose step gradient (20%, 30%, 40%, 50% and 60% (w/v) sucrose in 10 mM MOPS/KOH pH7.4, 100 mM

KCl, 1 mM EDTA, 1 mM PMSF). Pure mitochondria were obtained after centrifugation with a Beckman SW40Ti rotor (210000g, 19 min, 2°C) by harvesting the fraction between the 40% and 50% sucrose phase. Harvested mitochondria were diluted with 35 ml SEM buffer containing 2 mM PMSF. Finally, mitochondria were collected by centrifugation (17500g, 15 min, 2°C). The mitochondrial pellet was resuspended in SEM buffer containing 2 mM PMSF and used directly or frozen in liquid nitrogen and storage at -80°C.

2.2.4.11 SDS-PAGE and Western blotting

SDS-PAGE was used to separate the denatured proteins according to their molecular size. Proteins (15 µg-100 µg) were dissolved in 20-40 µl of 2×Lämmli buffer and denatured (5 min, 95°C) to be subjected to SDS-PAGE. The bottom, running and stacking gels were casted according to Table 26. Electrophoresis was performed with an initial current of 16 mA until the samples completely entered the gel, then the current was increased to 20-30 mA until the end of the run. Buffers and detergents are listed in Table 15. After SDS-PAGE the semi-dry western blotting system was used. The buffer is listed in Table 16. Proteins were transferred from an SDS gel to nitrocellulose membrane at 220 mA for one hour.

2.2.4.12 Coomassie staining of gels

To visualize proteins after electrophoresis gels were stained with Coomassie staining solution. The gel was washed shortly with water and incubated for 30 min at RT with shaking in staining solution containing 30% (v/v) methanol, 10% (v/v) acetate and 0.1% (w/v) Coomassie Brilliant Blue R-250. The visualization of gel bands was performed by washing with destaining solution (30% (v/v) methanol, 10% (v/v) acetate) until defined bands were observable. If necessary, bands of protein of interest were cut and analyzed by MS-MS.

Table 26: Components of SDS gels

Gel Component	Bottom gel	Stacking gel	Running gel (%AA/%bis-AA)			
			10%/0.6%	12%/0.6%	14%/0.6%	16%/0.6%
30% acrylamide	5 ml	830 µl	5 ml	6 ml	8.4 ml	10.6 ml
2% bis-acrylamide	2 ml	550 µl	2.14 ml	2.57 ml	3.6 ml	4.54 ml
H_2O	675 µl	2.92 ml	3.8 ml	2.33 ml	1.1 ml	-
1.5 M Tris/ HCl pH 6.8	-	630 µl	-	-	-	-
1.5 M Tris/ HCl pH 8.8	2.23 ml	-	3.8 ml	3.8 ml	4.5 ml	4.5 ml
10% SDS	100 µl	50 µl	150 µl	150 µl	180 µl	200 µl
10% APS	50 µl	25 µl	150 µl	150 µl	180 µl	200 µl
TEMED	25 µl	10 µl	6 µl	6 µl	12 µl	15 µl
Volume	10 ml	5 ml	15 ml	15 ml	18 ml	20 ml

2.2.4.13 Immunodetection of proteins on membranes

After western blotting membranes were incubated for one hour at RT in blocking buffer (Table 17) to block unspecific protein-binding. Next membranes were incubated with primary antibody for one hour at RT or overnight at 4°C. Afterwards membranes were washed three times with TBS buffer for five min. Next the corresponding secondary antibody (Table 22) diluted in blocking buffer was added and the membrane was incubated for one or two hour at RT. The same washing steps as above were repeated. The signals on the membranes were detected by the ECL system (Table 17) and exposition of Super RX X-ray films (Fuji, Japan).

2.2.4.14 Membrane stripping

Nitrocellulose membrane that were already used for Western blotting can be reused for an additional immunodecoration. To that end, the membrane was washed for five min four times in TBST (Table 14). Next the membrane was incubated in a water bath at 50°C for 30 min in stripping buffer (Table 14). After the stripping buffer was discarded, the membrane was washed with TBST buffer for another six times (5 min each). In the

end the membrane was again blocked with 5% (w/v) dry skim milk in TBS for one hour and antibody incubation was performed as in 2.2.4.12.

2.2.4.15 Blue Native PAGE

BN-PAGE was developed to separate with high resolution membrane protein complexes under native conditions using mild neutral detergents (Schägger et al. 1991). Isolated mitochondria were collected by centrifugation (8000 g, 15 min, 2°C) and were lysed in 40 µl buffer containing 0.5% (v/v) Triton X-100 or 1% (w/v) digitonin. After incubation on ice for 15 min to solubilize the mitochondrial membranes the solution was clarified (30000 g, 15 min, 2°C). Solubilized sample was mixed with five µl sample buffer containing 5% (w/v) Coomassie blue G and loaded on 6-13% or 4-8% gradient blue native gels (Schägger 2002) for electrophoresis at 4°C.

In the beginning gels were run with cathode buffer B (Table 20) at 150 V and 15 mA for around two hours until the blue front reached half of the gel. Then buffer B was exchanged with cathode buffer A (Table 20) and the gel continued to run at 50 V overnight or at 600 V and 15 mA. Gels were blotted on polyvinylidene fluoride membranes and proteins were further analyzed by autoradiography or immunodecoration.

Table 27: Blue Native system

Gel components	Bottom gel	Stacking gel	3.5%	4%	6%	8%	10%	13%
48%AA /1.5% Bis-AA	5 ml	0.417 ml	0.44 ml	0.5 ml	0.75 ml	1 ml	1.25 ml	1.625 ml
3×Gel buffer	3.3 ml	1.67 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
H_2O	1.7 ml	2.88 ml	3.4 ml	3.47 ml	3.22 ml	1 ml	0.75 ml	-
60% (v/v) glycerol	-	-	-	-	-	2 ml	2 ml	2.355 ml
10% APS	50 µl	30 µl	50 µl	30µl	25 µl	20 µl	20 µl	20 µl
TEMED	25 µl	3 µl	25 µl	3 µl	2.5 µl	2 µl	2 µl	2 µl

2.2.4.16 *In vitro* synthesis of radiolabeled proteins

Genes of interest were cloned into the vector pGEM4 containing the Sp6 promoter for *in vitro* transcription. The plasmid was first linearized by restriction digestion on the C-

terminal cloning site. The mRNA of target gene sequence was synthesized at 37°C for one hour by SP6-RNA-polymerase (Promega, Germany) in a transcription reaction according to the protocol of the manufacturer. Precursor proteins were obtained by translation of mRNA in rabbit reticulocyte lysate (Promega, Germany) for one hour at 30°C in presence of ^{35}S-methionine according to the manufacturer's protocol. To avoid associated ribosomes in the import reactions the radiolabeled proteins were centrifuged (41000 g, 45 min, 2°C). The supernatant was used directly in import experiments or aliquoted and stored at -80°C.

2.2.4.17 Import of proteins into isolated mitochondria

Isolated mitochondria (350 µg) were resuspended in 650 µl F5 import buffer (Table 19). Then 185 µl of such a mixture were incubated at 25°C for different time intervals (5 min, 40 min or 90 min) with 20 µl rabbit reticulocyte containing radiolabeled proteins and the import reaction was incubated. The incubation was supplemented every 20 min with 10 µl E-mix reaction buffer (4 µl of 0.2 M ATP (pH 7.0), 2 µl of 0.2 M NADH, 1 µl of 5 mM creatine phosphate, 2 µl of 100µg/ml creatine kinase). Import reaction was terminated by addition of 600 µl SEM-K80 buffer and mitochondria were harvested by centrifugation (13200 g, 10 min, 2°C). Import was analyzed by either SDS-PAGE and autoradiography or proteins were extracted by 1% (w/v) digitonin or 0.05% (v/v) Triton X-100 and further analyzed by Blue Native PAGE and autoradiography.

2.2.4.18 Autoradiography and quantification

Radiolabeled proteins can be analyzed and quantified by autoradiography. To this end, the dried nitrocellulose or PVDF membranes with radiolabeled proteins were exposed to X-ray film (Kodak Bio Max MM, U.S.A) and kept in sealed photo boxes for different exposure times, which depended on signal intensity and varied from two days to a month. The exposed films were developed and quantified by scanning (MICROTEK ScanMaker i800). The intensity of bands were quantified by AIDA Image Analyzer v 4.19 (Straubenhardt, Germany).

2.2.4.19 Silver staining of proteins in SDS-PAGE

Silver staining is a sensitive method for detection of trace amounts of proteins in gels (Blum et al. 1987). All solutions used were freshly prepared. After electrophoresis the gel was incubated with fixing buffer containing 40% (v/v) ethanol and 10% (v/v) acetic acid overnight at 4°C or two hours at RT. The gel was rinsed with 20% (v/v) ethanol twice and soaked for 30 min in sensitizing solution (Table 13). After three washes of five minutes in water, the gel was treated for 30 min with silver solution (30 mM silver nitrate). The adequate degree of staining was achieved by washing the gel with water to remove residual silver solution and by incubation of the gel with developing solution (Table 13). The reaction was terminated by direct addition of 200 ml 5% (v/v) acetic acid to the gel. The stained gel was kept at 4°C in 1% (v/v) acetic acid.

2.2.4.20 Thin layer chromatography (TLC) with mitochondrial lipid

The phospholipid composition of mitochondria was analyzed by lipid extraction and thin layer chromatography (TLC). Purified mitochondria (300 µg) were suspended in 300 µl SEM buffer and mixed with 300 µl chloroform and 300 µl methanol and extraction of lipids was performed by vortexing for two to three minutes. The mixture was centrifuged (15000 rpm, 15 min, 4°C) and three layers were present in the tube. First the upper supernatant fraction and then the protein layer were discarded. Then the bottom phase was transferred to new tube and dried by a MiVac concentrator (Genevac, UK). The lipid pellet was dissolved in 30 µl loading buffer (chloroform:methanol (v/v)=2:1). Standard lipids and samples were loaded on a TLC plate (Silica gel 60 F_{254} HPTLC) (Merck, Germany) by the automatic loading device Linomat 5 (CAMAG, Germany). Chromatography was carried out with running buffer (chloroform: water: ethanol: triethylamine =30:7:35:35) in a glass chamber until the liquid front reached a height of 8.1 cm. Then the plate was air-dried for 10 min and the above step was repeated. For the visualization of phosphate groups in phospholipids the plate was stained with molybdenum blue (Sigma, Germany).

3. Results

3.1 *Mcp1* and *Mcp2* are high-copy suppressors of *mdm10*Δ

3.1.1 Genetic screen for suppressors of *mdm10*Δ growth phenotype

Mdm10 is a β-barrel protein of the mitochondrial outer membrane (MOM). Deletion or mutation of *MDM10* results in condensed mitochondrial morphology and reduced growth of yeast cells under all conditions. Most obvious growth defects appear on non-fermentable carbon sources. Mdm10 is furthermore involved in processes as biogenesis of β-barrel proteins of the MOM, lipid homeostasis of mitochondria and mitochondria-ER organelle contacts as outlined in the Introduction section. Of note, *mdm10*Δ cells were shown to lose the mitochondrial genome (mtDNA) (Sogo and Yaffe 1994). Therefore, in this study, all haploid *mdm10*Δ strains were tested in advance to confirm that they still contain mtDNA. To better understand the precise primary molecular functions of Mdm10 and to identify its genetic interaction partners, we screened for high-copy suppressors of *mdm10*Δ cells. The *mdm10*Δ cells were transformed with a yeast genomic library cloned in the high copy number yeast vector pFL44L (2µ based) (Stettler et al. 1993). Transformants were initially grown on selective medium for the plasmid pFL44L and then transferred to plates containing glycerol medium. The suppressor colonies were subsequently incubated on glycerol medium at 37°C. The isolated clones were monitored for growth at elevated and reduced temperature. In sum, over 30000 colonies were screened and finally five clones showed significant rescue capacity in *mdm10*Δ cells at 37°C in two independent rounds of selection.

The plasmids from the five yeast clones were isolated and retransformed in *mdm10*Δ cells. Using a plasmid encoding *MDM10* (pYX142-Mdm10) as control, the growth phenotypes were compared by drop dilution assay at 30°C and 37°C on plates containing glycerol (Fig. 3.1A). In the meantime, the inserts DNA of the five isolated plasmids were sequenced. Two of the five plasmids contain the *MDM10* open reading frame. Another two clones were identified to contain a DNA fragment encoding three open reading frames *YOR228C* and *WTM2* as well as *ARS1523.* According to the *Saccharomyces. cerevisiae* genome database (SGD, www.yeastgenome.org) *WTM2* is a transcription regulator involved in regulation of meiosis and its subcellular location isn't linked to mitochondria. Furthermore, *ARS1523* is an autonomously replicating

sequence. *YOR228C* encodes a protein of unknown function, which was identified in the mitochondrial outer membrane by proteomic analysis (Zahedi et al. 2006). The plasmid isolated from the fifth clone contains four gene sequences *SYM1*, *YLR252W*, *YLR253W* and *NDL1*. Overexpression of this plasmid showed good rescue capacity at 30°C but only a very weak growth rescue at 37°C (Fig. 3.1A, #13). *NDL1* is the homologue of nuclear distribution factor and has no direct functional connection to mitochondria according to SGD. *YLR252W* is a dubious ORF that unlikely encodes a protein. *SYM1* forms an import channel in the mitochondrial inner membrane and is required for ethanol metabolism (Trott and Morano 2004, Dallabona et al. 2010, Reinhold et al. 2012). *YLR253W* was detected in the mitochondrial proteome and its function is uncharacterized so far (Huh et al. 2003, Tu et al. 2005, Reinders et al. 2006).

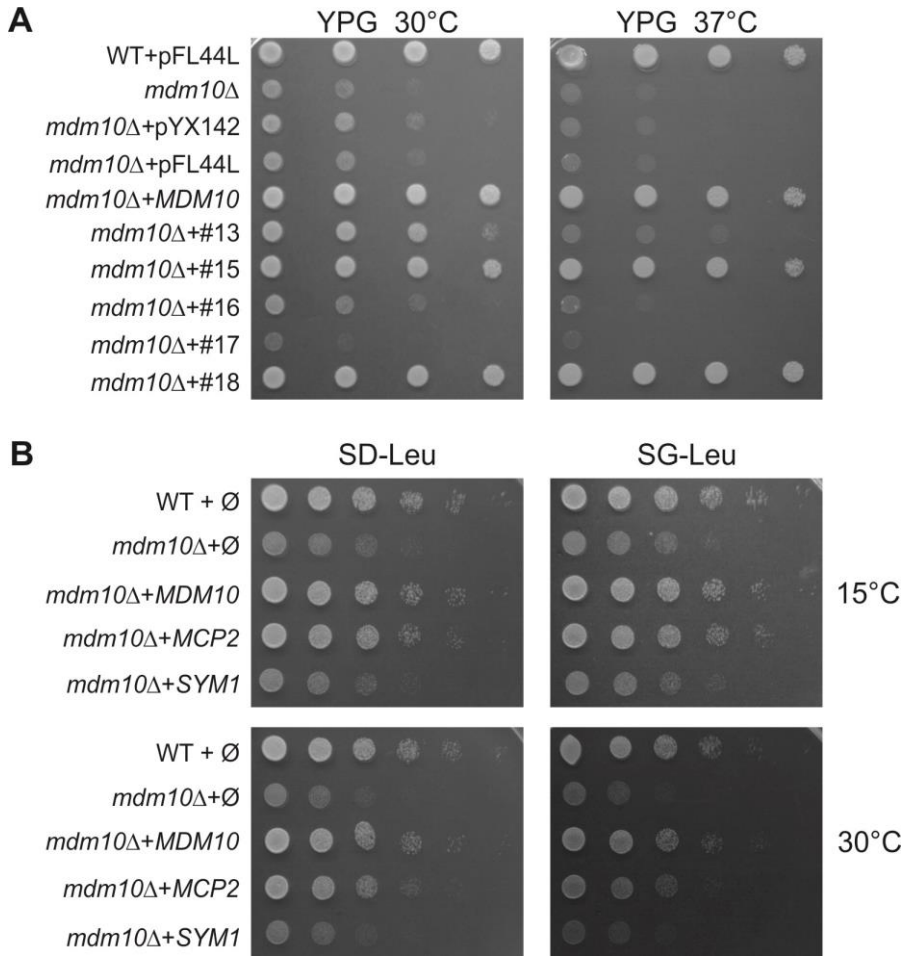


Figure 3.1. Mcp1 and Mcp2 are multi-copy suppressors of *mdm10*Δ growth defect. **(A)** Wild-type and *mdm10*Δ cells were transformed with empty plasmids (pFL44L or pYX142) or a plasmid containing *MDM10* as control. Cells lacking Mdm10 were

furthermore transformed with plasmids of the candidates of the genetic screen (#13, #15-18). Cells were grown to logarithmic phase and spotted on an YPG plate in a 1:5 dilution series. Plasmid #13 contains *YLR253W*, #15 contains *YOR228C* and #18 contains *MDM10*. **(B)** Over-expression of Mcp2 but not Sym1 rescues the growth defect of *mdm10*Δ cells. Wild-type and *mdm10*Δ cells were transformed with empty plasmid pYX142 or a plasmid containing Mdm10, Sym1 or Ylr253w (Mcp2, see below). The indicated cells were incubated overnight and diluted in synthetic medium containing glucose and lacking leucine (SD-Leu). Serial five-fold dilutions of the same cell amount were spotted on SD or SG medium lacking leucine and incubated at 15°C and 30°C.

Thus, the insert of plasmid #13 contains with the ORFs *YLR253W* and *SYM1*, two mitochondrial proteins. To know which one is the suppressor of *mdm10*Δ*,* they were separately cloned in a centromeric yeast expression vector pYX142. Over-expression of *YLR253W* rescued the growth defect of *mdm10*Δ cells at 15°C and 30°C in synthetic medium (SD-Leu and SG-Leu), while over-expression of *SYM1* had no rescue capacity of *mdm10*Δ cells (Fig 3.1B). Taken together, *YLR253W* is the suppressor of *mdm10*Δ encoded on plasmid #13. Finally, the two verified suppressor proteins were named Mcp1 (Mdm10 complementing protein 1, Yor228c) and Mcp2 (Mdm10 complementing protein 2, Ylr253w).

3.1.2 Mcp1 is a stronger suppressor than Mcp2

In Fig. 3.1A expression of plasmid #15 (encoding Yor228c, Mcp1) showed a stronger rescue of the growth phenotype of *mdm10*Δ cells on YPG at 30°C and 37°C than expression of plasmid #13 (encoding Ylr253w, Mcp2). To identify whether Mcp1 is indeed the stronger suppressor for *mdm10*Δ*,* plasmids encoding *MCP1* or *MCP2* were transformed into *mdm10*Δ cells. Mcp1 showed stronger rescue capacity than Mcp2 at 15° and 30°C on YP medium containing glucose or glycerol. Over-expression of Mcp1 or Mcp2 hardly complement the growth defect of *mdm10*Δ cells on YPG at 37°C, while they partially rescued *mdm10*Δ cells on medium containing glucose at 37°C (Fig. 3.2A). Similar results were obtained by incubation of cells expressing Mcp1 or Mcp2 on synthetic medium at 15°C and 30°C (Fig. 3.2B). Taken together, Mcp1 is a stronger suppressor for *mdm10*Δ than Mcp2 under all tested conditions.

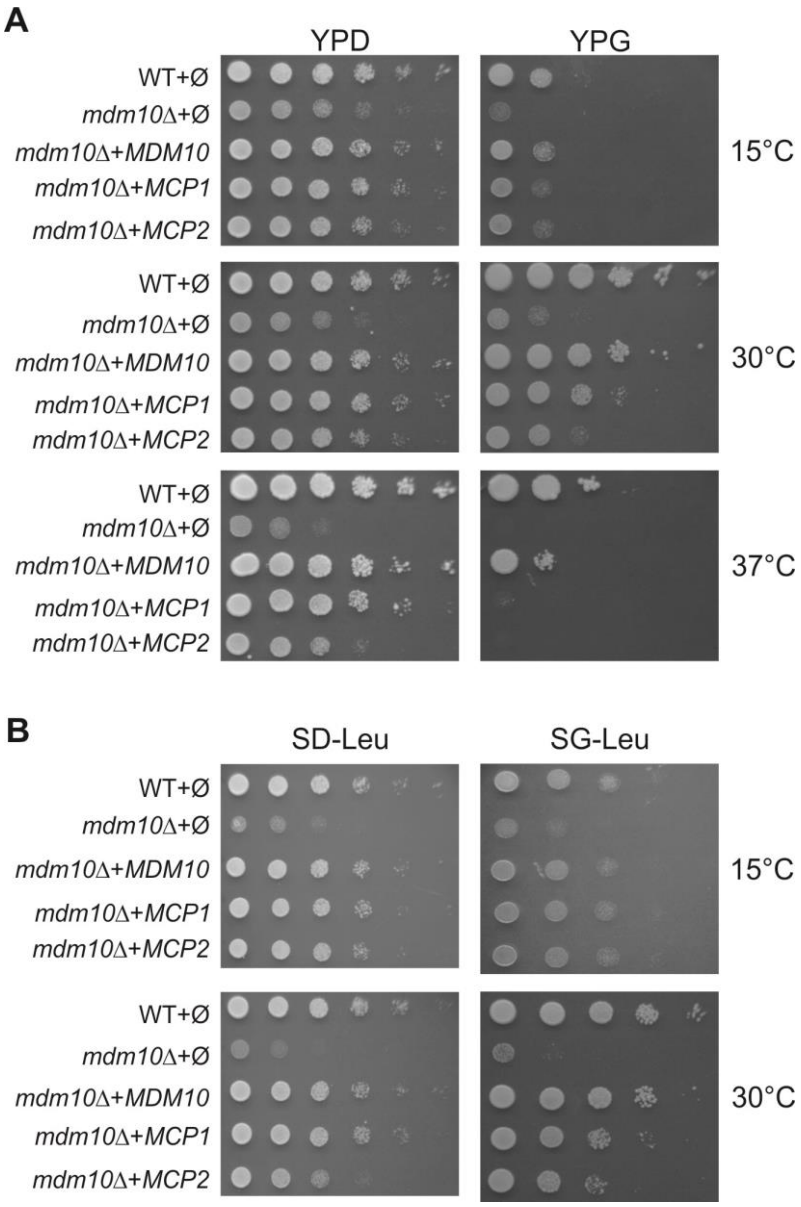


Figure 3.2. Mcp1 is a stronger suppressor than Mcp2. (A) Over-expression of Mcp1 has stronger rescue capacity than that of Mcp2. The indicated cells were incubated overnight in medium containing glucose (YPD). The diluted cells were grown to logarithmic phase and spotted on plates containing glucose (YPD) or glycerol (YPG) in serial 5-fold dilution and then cells were further incubated at 15°C, 30°C or 37°C. **(B)** Over-expression of Mcp1 and to a lesser extent Mcp2 rescue the growth defect of *mdm10*Δ cells on synthetic medium. The strains described in (A) were analyzed by drop dilution at 15°C or 30°C on synthetic medium containing either glucose (SD-Leu) or glycerol (SG-Leu).

3.2 Over-expression of both Mcp1 and Mcp2 has no additive rescue effect

Over-expression of either Mcp1 or Mcp2 never shows complete rescue capacity of *mdm10*Δ growth defect. To investigate whether over-expression of Mcp1 together with Mcp2 could lead to additive rescue effect, *MCP1* and *MCP2* were constructed in two plasmids with the same promoter but different selective markers (pYX142 and pYX132 containing the LEU2 and the TRP1 marker, respectively). The two plasmids were concurrently transformed in *mdm10*Δ cells to over-express both proteins together. Under all tested conditions (for example at 30°C on synthetic medium), there was no difference between co-expression of both proteins and expression of only Mcp1 (Fig. 3.3 and data not shown). Hence, over-expression of both Mcp1 and Mcp2 together cannot further increase the growth rate of *mdm10*Δ cells than over-expression of Mcp1 alone.

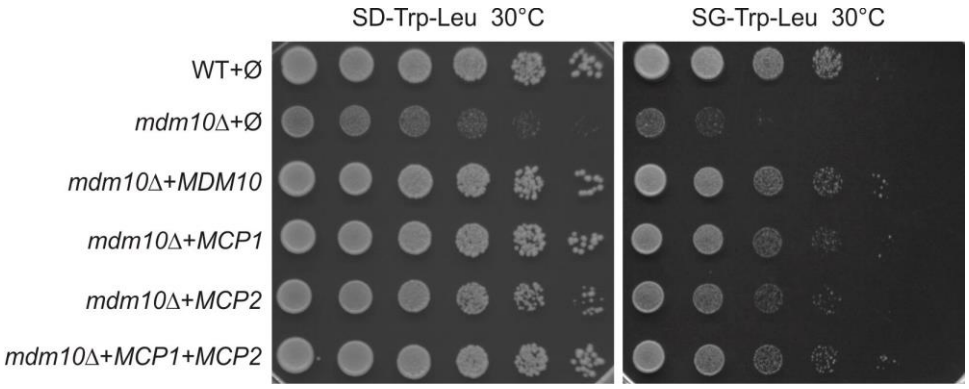


Figure 3.3. Concurrent over-expression of Mcp1 and Mcp2 shows no additive rescue effect on *mdm10*Δ cells growth phenotype. The indicated strains were incubated in selective medium lacking leucine and tryptophan and containing glucose or glycerol (SD-Trp-Leu or SG-Trp-Leu respectively) and grown to logarithmic phase. The cells were spotted on the indicated media in 5-fold serial dilutions and further incubated at 30°C.

3.3 C-terminally HA-tagged Mcp1 or Mcp2 show equal rescue capacity as native Mcps

Since Mcp1 and Mcp2 are uncharacterized proteins, there are no available antibodies against the two proteins. Therefore Mcp1 and Mcp2 were expressed with a C-terminal

HA-tag to be used in biochemical experiments. To exclude the loss or change of functionalities by the addition of a C-terminal HA-tag, the rescue capacities of Mcp1-HA or Mcp2-HA was compared to that of the native proteins. The two HA-tagged proteins showed on all tested conditions equal capacity to rescue growth phenotype of *mdm10*Δ cells as their native proteins (Fig. 3.4 and data not shown). Taken together the HA tagged proteins show the same functionality as unmodified Mcp1 and Mcp2.

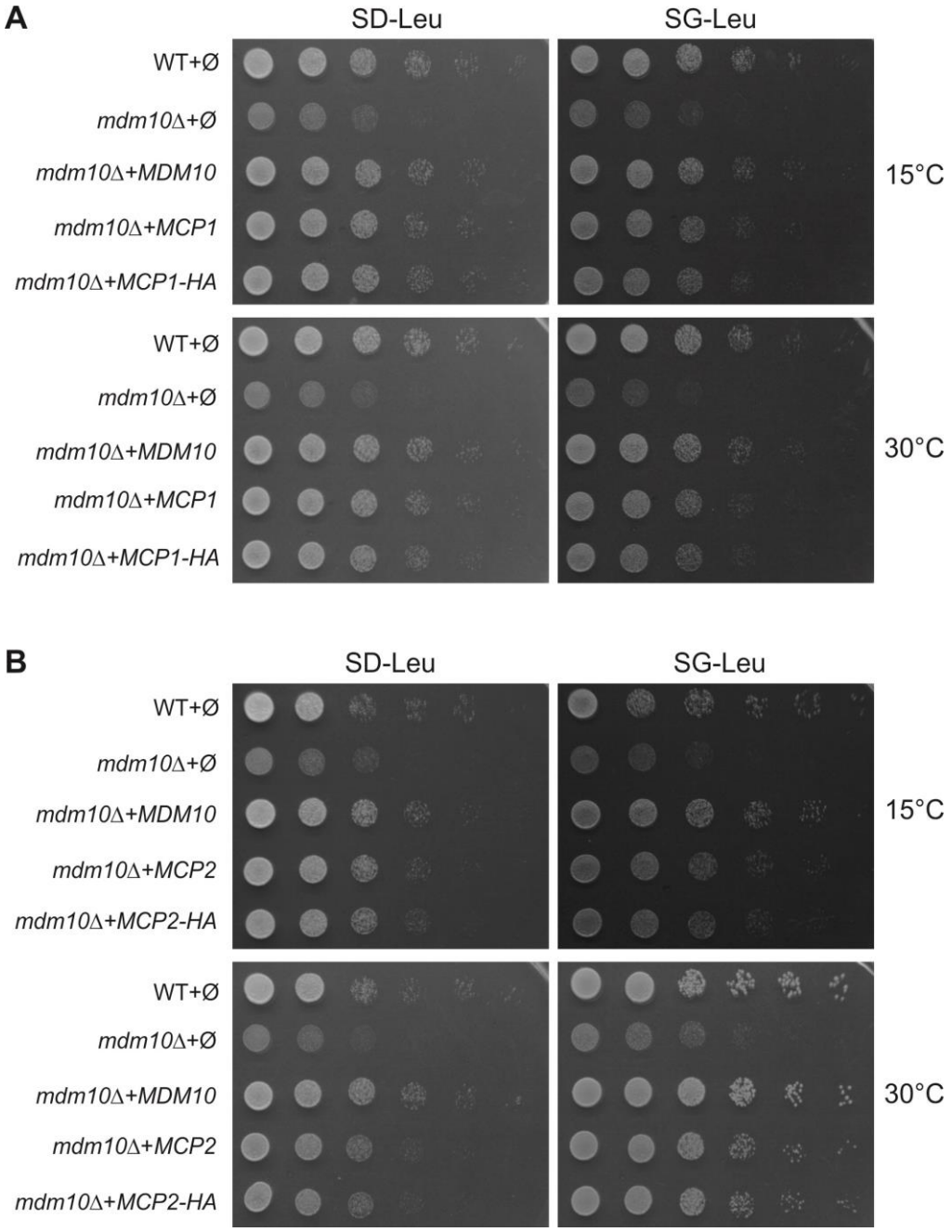


Figure 3.4. Over-expression of Mcp1 and Mcp2 with C-terminally HA-tag rescues the growth phenotype of *mdm10*Δ cells. The indicated cells were incubated in

synthetic medium containing glucose (lacking leucine) for overnight and diluted to grow to logarithmic phase. Serial 5-fold dilutions were spotted on selective medium containing glucose (SD-Leu) or glycerol (SG-Leu) and cells were further incubated at 15°C or 30°C.

3.4 Mcp1 and Mcp2 are mitochondrial membrane proteins

Mcp1 is a protein of 303 amino acid residues and is predicted to contain five α-helical transmembrane domains (TMDs) (http://www.cbs.dtu.dk/services/TMHMM/). Homologue proteins of Mcp1 are found only in fungi. Mcp2 is a protein of 569 amino acid residues and at least one TMD was predicted within its primary sequence by several programs (Biegert et al. 2006). Compared to Mcp1 homologues of Mcp2 can be found in all higher eukaryotes. In mammalian cells the highest homology shows so called aarF domain containing kinase 1 (ADCK-1) that shares 33% identity with 92% coverage of the sequence of Mcp2 (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4.1 Mcp1 and Mcp2 are mitochondrial proteins

Previous high-throughput studies suggested that Mcp1 and Mcp2 are localized to mitochondria. To study the subcellular localization of Mcp1 or Mcp2 on a single-gene level, cells expressing HA-tagged Mcp1 or Mcp2 (described in 3.3) were transformed with plasmids encoding the MOM marker protein Cherry-Fis1. Cell were converted to

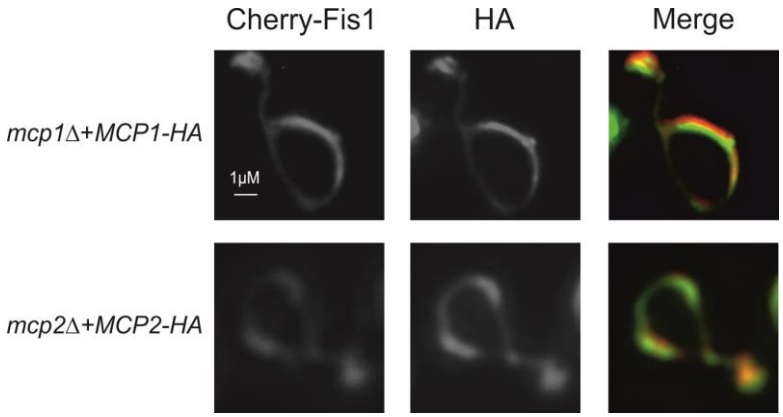


Figure 3.5. Mcp1 and Mcp2 are located in mitochondria. C-terminally HA-tagged Mcp1 or Mcp2 were over-expressed in *mcp1*Δ or *mcp2*Δ cells. Both strains were additionally transformed with a plasmid encoding the MOM marker Cherry-Fis1. The HA-tags were visualized by *in situ* immunofluorescence microscopy.

spheroplasts by zymolyase treatment and fixed with formaldehyde. Finally, treated cells were analyzed by fluorescence microscopy. As expected, the visualization of Cherry-Fis1 by fluorescence microscopy showed that it is localized to mitochondria (Fig. 3.5, left). The fluorescence signal of the HA-tagged over-expressed Mcp1 or Mcp2 (Fig. 3.5, middle) overlapped with the signal Cherry-Fis1 (Fig. 3.5, right). These observations confirm that Mcp1 and Mcp2 are yeast mitochondrial proteins.

3.4.2 Subcellular localization of Mcp1 and Mcp2

To further test the subcellular localization of Mcp1 and Mcp2 in yeast cells, cells expressing Mcp1-HA or Mcp2-HA were fractionated into cytosolic, mitochondrial and microsomal fractions by differential centrifugation. The different fractions were analyzed by SDS-PAGE and immunodecoration with specific antibodies against the indicated proteins (Fig. 3.6.). Both Mcp1-HA and Mcp2-HA were detected in the mitochondrial fraction as the integral MOM protein Tom70 (Fig. 3.6A and B), whereas no Mcp1 or Mcp2 signal was found in the cytosolic or microsomal fraction. These findings confirm those of the fluorescence microscopy that Mcp1 and Mcp2 are mitochondrial proteins.

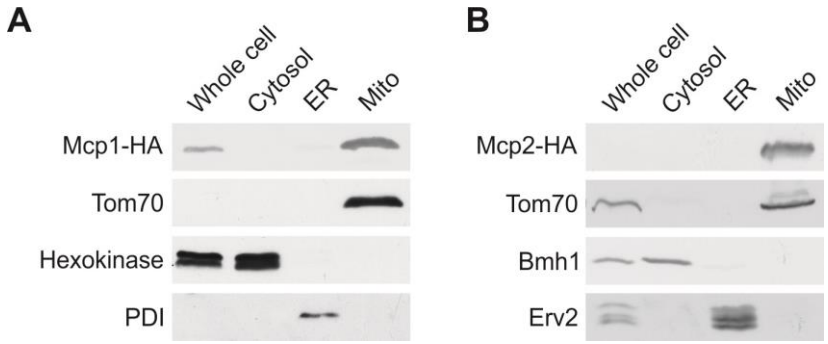


Figure 3.6. Mcp1 and Mcp2 are identified as mitochondrial proteins. (A) and **(B)** Whole cell lysate and cytosol, light microsomal fraction (ER) and mitochondrial (Mito) fraction were isolated by differential centrifugation from cells expressing either Mcp1-HA (A) or Mcp2-HA (B). They were analyzed by SDS-PAGE and immunodecoration with antibodies against the HA-tag, the mitochondrial protein Tom70, a marker protein for the cytosol (Hexokinase or Bmh1) and an ER marker protein (disulfide isomerase, PDI or Erv2).

3.4.3 Submitochondrial localization of Mcp1 and Mcp2

Since for both proteins transmembrane domains were predicted (Biegert et al. 2006), next I investigated whether Mcp1 or Mcp2 are indeed integral membrane proteins. To that goal, mitochondria isolated from cells expressing Mcp1-HA or Mcp2-HA were subjected to alkaline extraction. By this treatment soluble and peripheral membrane proteins can be found in the supernatant after centrifugation, whereas the integral membrane proteins are detected in the pellet. Mcp1-HA and Mcp2-HA were detected after SDS-PAGE and immunodecoration in the pellet fraction similarly to the integral MOM proteins Tom70 and Porin (Fig. 3.7A and B). As expected, the matrix soluble proteins Mge1 and Hep1 were found in the supernatant. These results show that both Mcps are integral membrane proteins.

Since mitochondria possess two distinct membranes, we wondered in which membrane both proteins are embedded. Mitochondria containing Mcp1-HA or Mcp2-HA were treated with PK under different conditions as indicated in Fig. 3.7C and D. PK degrades exposed proteins on the surface of intact mitochondria. Upon rupture of the mitochondrial outer membrane by treatment with hypo-osmolar buffer (swelling, SW), proteins facing the IMS are degraded as well. In presence of Triton X-100 all proteins can be degraded by PK since all membranes are solubilized. As shown in Fig. 3.7C, Mcp1-HA behaved similar to the integral MOM protein Tom70. After the proteolytic treatment of intact mitochondria, full length protein of Mcp1-HA (around 40 kDa) was not detected and two fragments of around 15 kDa appeared (Fig. 3.7C, second lane asterisk). Mcr1 served as a control for an intact mitochondrial outer membrane. In the presence of PK the outer membrane isoform of Mcr1 is degraded in intact mitochondria, whereas IMS isoform stays intact. After treatment with swelling buffer the rupture of MOM leads to degradation of the IMS isoform of Mcr1 by PK. Mcp1-HA as well as the matrix protein Mge1, a control for the intactness of the MIM, were completely degraded after the solubilization of membranes by the detergent Triton X-100 (Fig. 3.7C, last lane). These results suggest that Mcp1 is an integral MOM protein with its C-terminus facing the IMS, while its N-terminus is located in the cytosol. This conclusion is in agreement with the detection of Mcp1 in a proteomic analysis of the MOM (Zahedi et al. 2006).

In contrast, Mcp2-HA was not degraded as long as the MOM was intact. However, upon rupture of the MOM by hypo-osmolar treatment, the amount of Mcp2-HA was reduced due to degradation with PK (Fig. 3.7D). The results of Fig. 3.7D suggest that

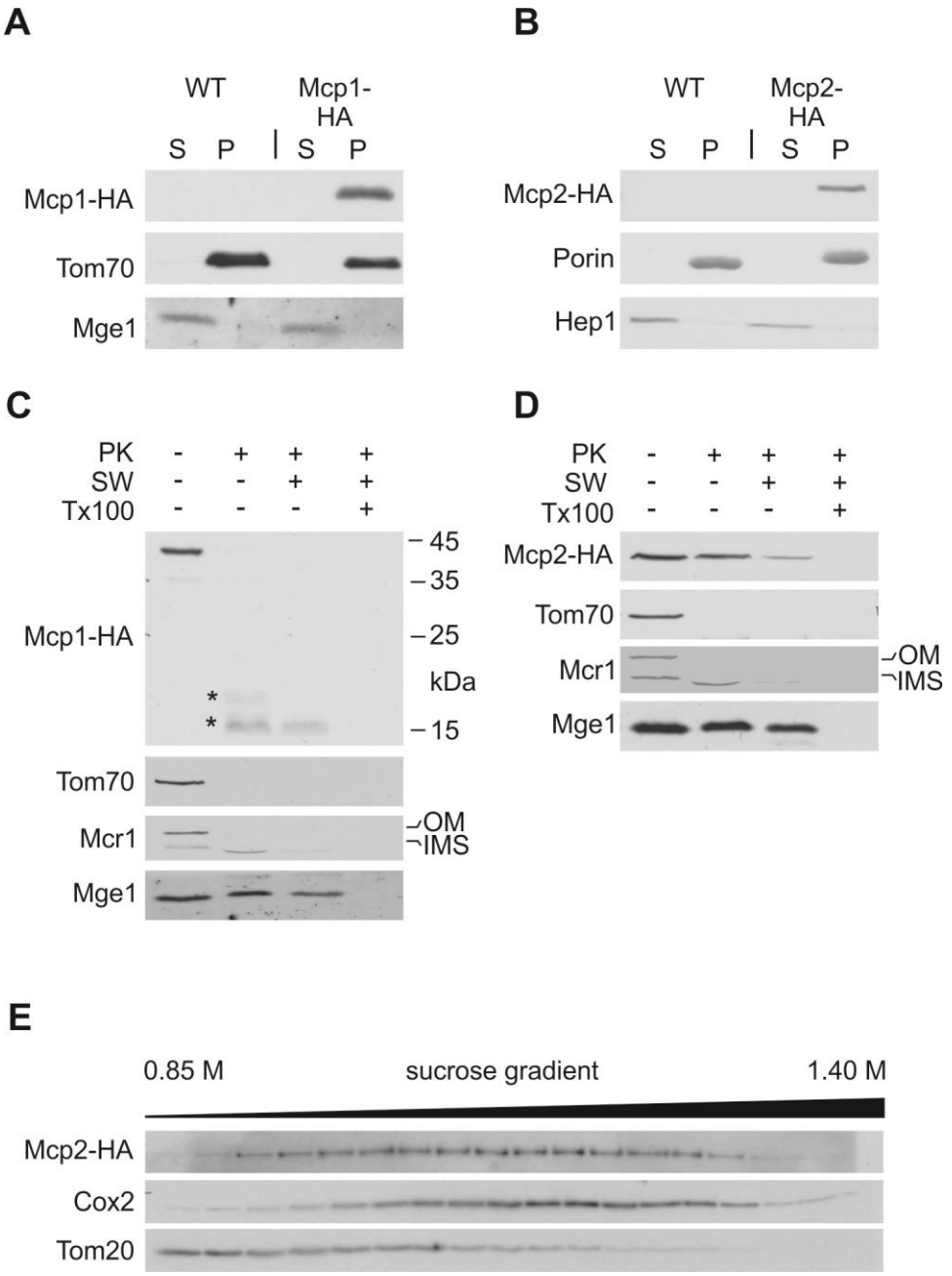


Figure 3.7. Submitochondrial localization of Mcp1 and Mcp2. (A) and **(B)** Mcp1 and Mcp2 are mitochondrial membrane proteins. Mitochondria were isolated from wild-type and cells expressing Mcp1-HA (A) or Mcp2-HA (B). After treatment by alkaline extraction, the supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE and immunodecoration. Tom70 and Porin, integral MOM proteins; Mge1 and Hep1, soluble matrix proteins. **(C)** Mcp1 is a MOM protein. Mitochondria isolated from cells

expressing Mcp1-HA were treated as indicated. Hypo-osmolar swelling (SW) led to rupture of the MOM. Addition of the detergent Triton X-100 (Tx100) was used to lyse the mitochondria completely. Samples were collected by TCA precipitation and analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins. Proteolytic fragments are marked by an asterisk. Tom70, a MOM protein; Mcr1, a dually located mitochondrial protein with an isoform in the MOM and another in the IMS (Hahne et al. 1994); Mge1, a soluble matrix protein. **(D)** Mcp2 in intact mitochondria is protected from protease digestion, while it is degraded after the rupture of the MOM. Mitochondria isolated from cells expressing Mcp2-HA were treated and analyzed as described in (C). **(E)** Mitochondria isolated from cells expressing Mcp2-HA were ruptured into vesicles by swelling and sonication. Mitochondrial vesicles were subjected to sucrose density gradient centrifugation (0.85–1.40 M sucrose) and the fractions of the gradient were collected and analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins. Cox2, mitochondrial inner membrane protein; Tom20, mitochondrial outer membrane protein.

Mcp2 is a membrane protein with its main part facing the IMS localized either in the inner or the outer membrane. To identify in which membrane Mcp2 is localized, mitochondrial vesicles containing Mcp2-HA were separated in collaboration with Dr. K.S. Dimmer by sucrose density gradient centrifugation. The migration behavior of Mcp2-HA was investigated by immunodecoration of the resulting fractions. Tom20 served as a marker for integral MOM proteins, and Cox2 (subunit of cytochrome c oxidase) for MIM proteins (Fig. 3.7E). Mcp2-HA could be found in nearly the same fractions as Cox2, whereas Tom20 was found in fractions with lower density (Fig. 3.7E). These results suggest that Mcp2 is an integral protein of the mitochondrial inner membrane with its main part facing the IMS.

3.4.4 Genomically C-terminal Flag-tagged Mcp2 is localized to the mitochondrial membrane

The experiments described so far used over-expressed Mcps, a situation that can lead to inaccurate results. To investigate the subcellular and submitochondrial localization of Mcp1 and Mcp2 under their endogenous expression level, I aimed to genomically modify the proteins with a Flag-tag. The Flag-tag could successfully be inserted at the C-terminus of Mcp2 in the yeast genome. Although the same strategy was used, no endogenously labelled Mcp1 could be obtained. Nevertheless, Mcp2-Flag could be

detected with antibody against Flag-tag under the endogenous expression level (Fig. 3.8 and data not shown). The growth phenotype of cells containing Mcp2-Flag was similar to wild-type cells as assessed by drop dilution assay. No defect of growth was observed on media with fermentable (YPD) or non-fermentable (YPG) carbon sources at 30°C and 37°C (Fig. 3.8A). Next subcellular fractions of cells expressing Mcp2-Flag were prepared by differential centrifugation and analyzed by SDS-PAGE and immunodecoration. Mcp2-Flag was detected in whole cell lysate and the mitochondrial fraction as the integral MOM protein Tom40 (Fig. 3.8B). Furthermore, mitochondria isolated from cells expressing Mcp2-Flag and wild-type cells were subjected to alkaline extraction treatment to confirm that Mcp2 is an integral membrane protein. Mcp2-Flag behaved as the integral MOM protein Tom20 and was found in the pellet. The soluble matrix protein Hep1 was detected in the supernatant (Fig. 3.8C). These results confirm that Mcp2 is a mitochondrial membrane protein.

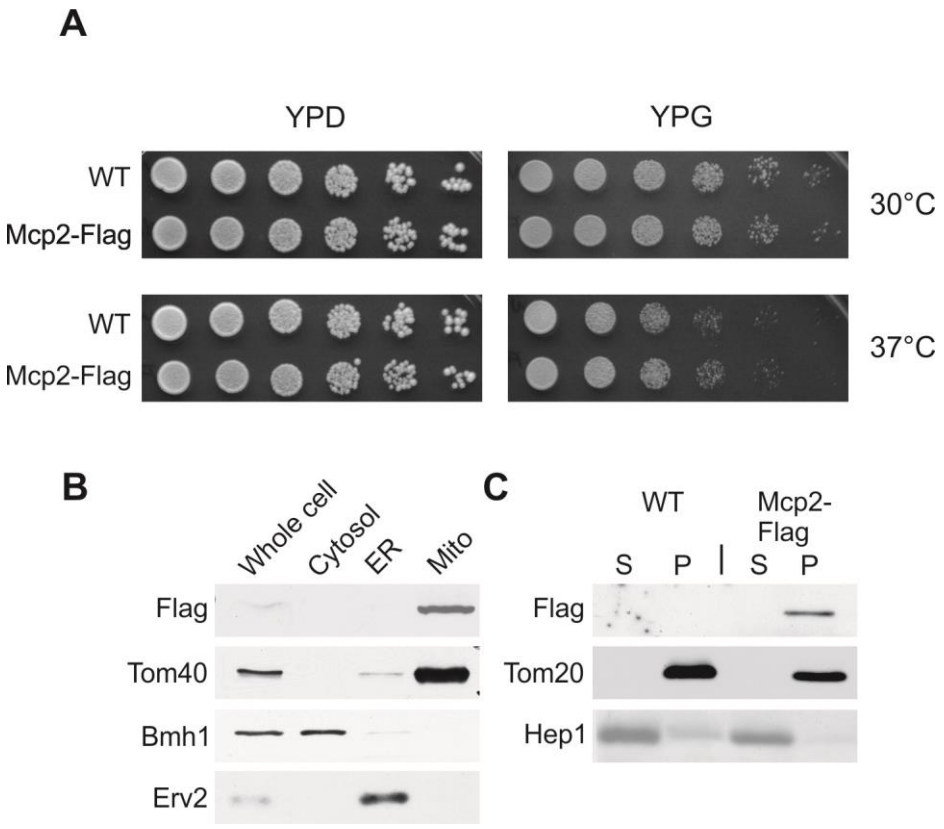


Figure 3.8. Mcp2 is a mitochondrial membrane protein. (A) Cells with endogenous C-terminally Flag-tagged Mcp2 and wild-type cells were incubated overnight in medium containing glucose (YPD). Cells were diluted, grown to the logarithmic phase and spotted on plates containing glucose (YPD) or glycerol (YPG) in serial 5-fold dilutions.

Cells were incubated further at 30°C or 37°C. **(B)** Cells described in (A) were subjected to subcellular fractionation. The indicated fractions were analyzed by SDS-PAGE and immunodecoration with antibodies against Flag-tag, the mitochondrial protein Tom40, the cytosolic protein Bmh1 and an ER marker Erv2. **(C)** Mitochondria isolated from wild-type and cells containing Flag-tagged Mcp2 were subjected to alkaline extraction. The supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE and immunodecoration with antibodies against Flag-tag, the mitochondrial MOM protein Tom20 and the soluble matrix protein Hep1.

3.5 Alteration of the expression levels of *Mcp1* and *Mcp2* has no influence on the growth phenotype of yeast cells

To study further the function of Mcp1 and Mcp2, the growth phenotypes of cells with the single deletion of *MCP1* or *MCP2* and double deletion of both genes were investigated. Since over-expression of Mcp1 or Mcp2 can rescue the growth defect of *mdm10*Δ, it was furthermore of interest whether over-expression of Mcps in wild-type cells might cause a growth phenotype.

As shown in Fig. 3.9A, the deletion strains *mcp1*Δ and *mcp2*Δ showed under all tested conditions the same growth behavior as wild-type cells. The double deletion *mcp1*Δ*/mcp2*Δ exhibited at 30°C on YPG a slightly slower growth than wild-type and the two single deletion strains (Fig. 3.8A). The over-expression of either Mcp1 or Mcp2 in wild-type cells had no significant effects on the growth rate (Fig. 3.8B). In brief, both proteins Mcp1 and Mcp2 seem to have no essential function for optimal cellular growth under all tested conditions.

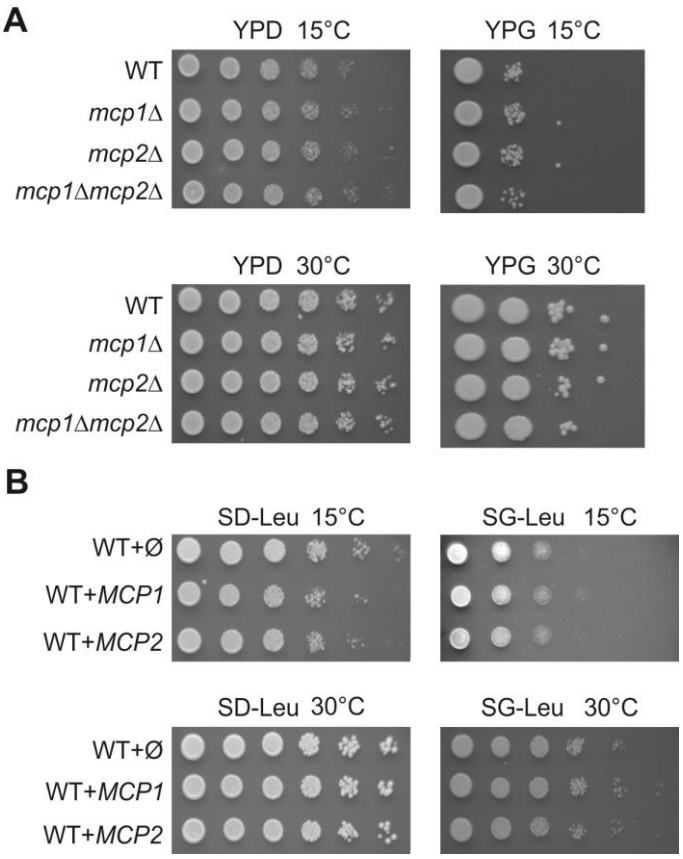


Figure 3.9. Growth of yeast cells is not affected by alterations in Mcp1 and Mcp2 expression levels. (A) Wild-type cells and the indicated deletion strains were incubated overnight in YPD medium, diluted and grown to logarithmic phase. Growth was analyzed by drop dilution assay. Serial five-fold dilutions were spotted on YPD or YPG medium and incubated further at 15°C or 30°C. **(B)** Wild type cells with empty plasmid pYX142 (Ø) or over-expressing either Mcp1 or Mcp2 were analyzed by drop dilution assay at the indicated temperatures on synthetic medium containing glucose (SD-Leu) or glycerol (SG-Leu) and lacking leucine.

3.6 Effects on mitochondrial morphogenesis by altered expression levels of Mcp1 or Mcp2

3.6.1 Loss of Mcp1 or Mcp2 has no effect on mitochondrial morphology

Deletion of *MCP1* or *MCP2* showed no effects on yeast growth under all tested conditions. To better understand the molecular functions of Mcp1 and Mcp2 we next

investigated the influence of their deletion on mitochondrial morphology. The deletion strains *mcp1*Δ and *mcp2*Δ were transformed with a plasmid encoding matrix targeted GFP (mtGFP, Westermann and Neupert 2000) to visualize mitochondria (Fig. 3.10A) and their morphology was analyzed by microscopy. No alterations of mitochondrial morphology were observed in the cells lacking Mcp1 or Mcp2 (Fig. 3.10A). Statistical analysis of mitochondrial morphology showed that the number of cells harboring mitochondria with normal morphology was the same for wild-type and deletion strains (Fig. 3.10B). These results suggest that deletion of *MCP1* or *MCP2* has no effects on mitochondrial morphology. This conclusion is in agreement the fact that both proteins were not identified in a high throughput screen to find proteins involved in mitochondrial morphology (Dimmer et al. 2002).

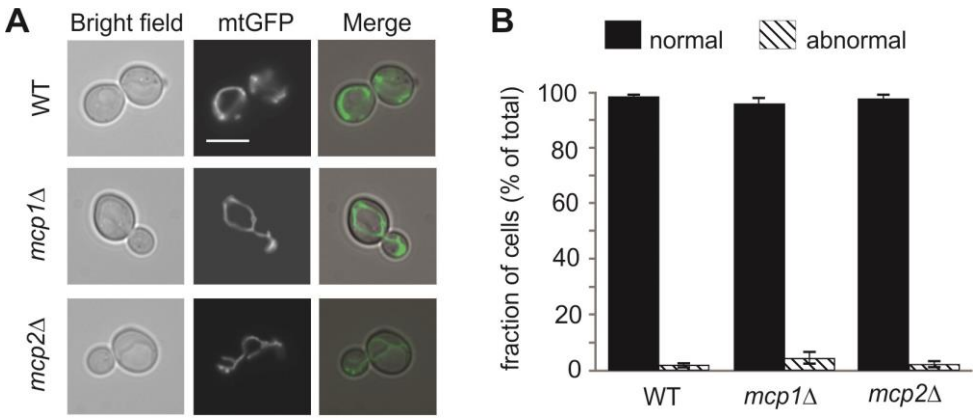


Figure 3.10. Deletion of *MCP1* or *MCP2* has no effects on mitochondrial morphology. (A) Deletion of *MCP1* or *MCP2* has no influence on mitochondrial morphology. Mitochondrially targeted GFP (mtGFP) was expressed in wild-type, *mcp1*Δ and *mcp2*Δ cells. Mitochondria were analyzed by fluorescence microscopy. Typical images of mitochondrial morphology of the depicted yeast strains are shown (scale bar = 5 µM). **(B)** Statistical analysis of mitochondrial morphology of cells described in (A). Three independent experiments were carried out with at least n=100 cells. The average values are shown with standard deviation bars.

3.6.2 Over-expression of Mcp2 causes changes in mitochondrial morphology

To better understand the effects of Mcp1 and Mcp2 on mitochondrial morphology, either Mcp1 or Mcp2 was over-expressed in wild-type cells together with mitochondrially targeted RFP (mtRFP). Cells were analyzed by fluorescence microscopy. The over-expression of Mcp1 had no effects on the mitochondrial morphology, whereas shortened, condensed or aggregated mitochondria were observed in around 20% cells with over-expression of Mcp2 (Fig. 3.11A and B). These results show that elevated levels of Mcp2 affect mitochondrial morphology.

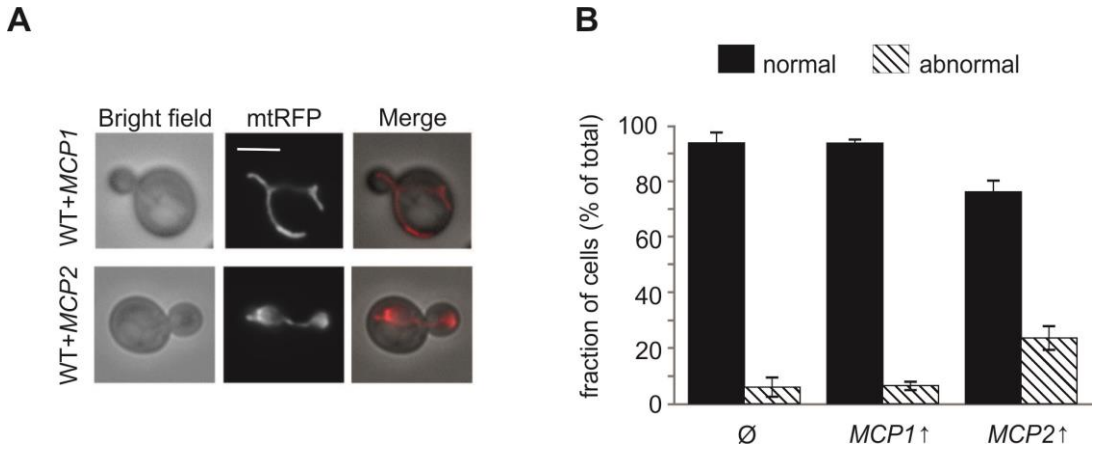


Figure 3.11. Over-expression of Mcp2 influences the mitochondrial morphology of wild-type cells. (A) Wild-type cells with over-expression of Mcp1 or Mcp2 were transformed with mitochondrially targeted RFP (mtRFP) to visualize the morphology of mitochondria by fluorescence microscopy. Typical images of the two different strains are shown (scale bar=5 µm). **(B)** Statistical analysis of mitochondrial morphology of cells described in (A). Three independent experiments were carried out with at least n=100 cells each. The mean with standard deviation bars is shown.

3.6.3 Over-expression of Mcp1 and Mcp2 rescues the mitochondrial morphology phenotype of *mdm10*Δ cells

To test whether Mcp1 or Mcp2 rescues the mitochondrial morphology phenotype of *mdm10*Δ cells, cells lacking Mdm10 were co-transformed with two different plasmids,

a mtRFP plasmid and a plasmid encoding either Mcp1 or Mcp2. Then, mitochondrial morphology was visualized by fluorescence microscopy. As shown in Fig. 3.12 the mitochondrial morphology of around 90% of *mdm10*Δ cells were rescued by over-expression of Mcp1. The rescue was similar to re-introduction of Mdm10 itself. In contrast, Mcp2 showed a much weaker rescue capacity for cells lacking Mdm10. The statistical analysis showed that only around 20% of *mdm10*Δ cells contained tubular mitochondrial networks in case of over-expression of Mcp2. Still 40% of the cells kept the condensed mitochondrial morphology, yet another 40% of the cells exhibited variable morphology phenotypes ranging from elongated condensed structures to short tubules (Fig. 3.12A and B). Taken together, both Mcps rescue the mitochondrial morphology phenotype of *mdm10*Δ cells. In agreement with the results from growth defect complementation of *mdm10*Δ cells by Mcps, Mcp1 is also a stronger suppressor than Mcp2 regarding rescue of mitochondrial morphology.

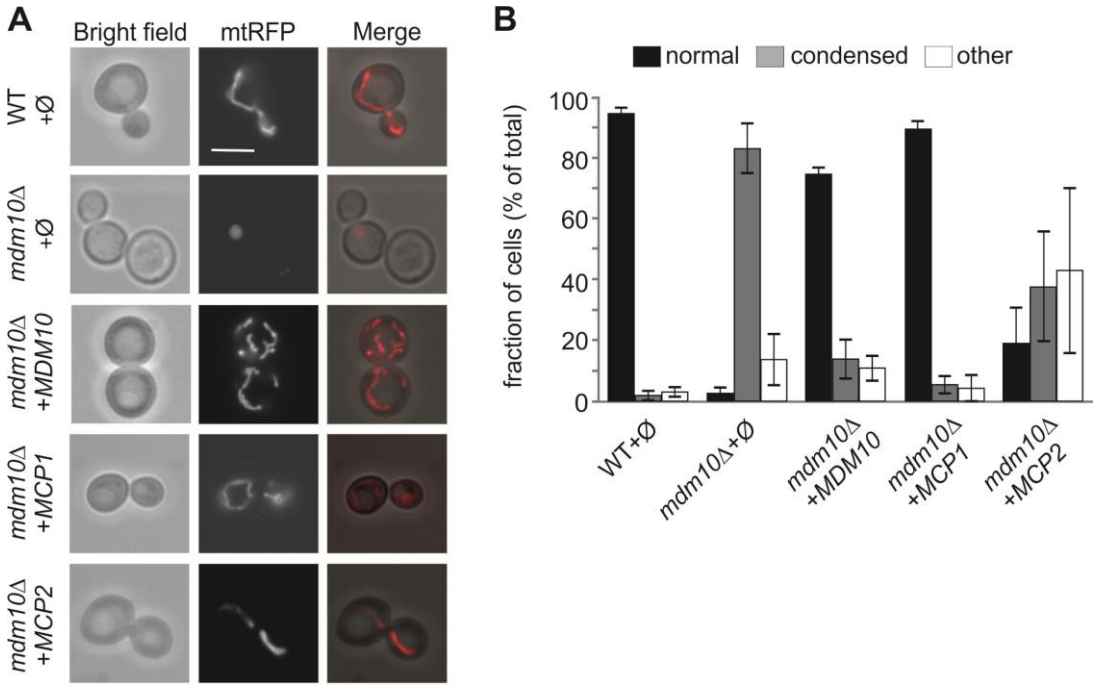


Figure 3.12. Over-expression of Mcp1 and Mcp2 complements the mitochondrial morphology defects of *mdm10*Δ cells. (A) The mitochondrially targeted RFP (mtRFP) was co-transformed into wild-type and *mdm10*Δ cells together with an empty plasmid (Ø) or a plasmid expressing Mdm10, Mcp1 or Mcp2. Cells were grown to logarithmic phase on synthetic medium and analyzed by fluorescence microscopy. Typical images of the five different strains are shown (scale bar=5 µm). **(B)** The statistical analysis of the cells described in (B). Three independent experiments were carried out with at least

n=100 cells each. The mean of these experiments with standard deviation bars is shown.

3.7 Over-expression of Mcp1 and Mcp2 does not affect the steady-state levels of mitochondrial proteins

Reduced amounts of Tom22 and Tom40 of the TOM complex were previously reported in the mitochondria lacking Mdm10 (Meisinger et al. 2004). One possible way by which Mcp1 and Mcp2 could rescue the growth phenotype of *mdm10*Δ cells, could be the restoration of the steady-state levels of these protein import components. To test whether over-expression of Mcp1 or Mcp2 influences the steady-state levels of mitochondrial proteins, mitochondria were isolated from wild-type or *mdm10*Δ cells transformed with an empty plasmid and from *mdm10*Δ cells over-expressing Mcp1, Mcp2 or Mdm10 itself (as control). These organelles were subjected to SDS-PAGE and immunodecoration. Of note, our results showed that no significantly reduced levels of Tom22 and Tom40 were observed in mitochondria lacking Mdm10 (Fig. 3.13). A potential explanation is ascribed to the different genetic background of the employed deletion strains. The wild-type background W303 was applied by us, whereas Meisinger et al. utilized the BY4741/2/3 background. Indeed it was previously reported, that the steady-state levels of TOM and TOB components vary between *mdm10*Δ mutants of different genetic backgrounds (Yamano et al. 2010b). Furthermore, the steady-state levels of other TOM components like Tom20 or Tom70 showed no changes as well as the amounts of Mas37 and Tob55, both subunits of the TOB complex (Fig. 3.13). Over-expression of Mcp1 or Mcp2 did not result in a change of any of the analyzed steady-state levels of mitochondrial proteins (Fig. 3.13 and data not shown). These results suggest that Mcp1 or Mcp2 has no direct influence on the import and biogenesis of mitochondrial proteins.

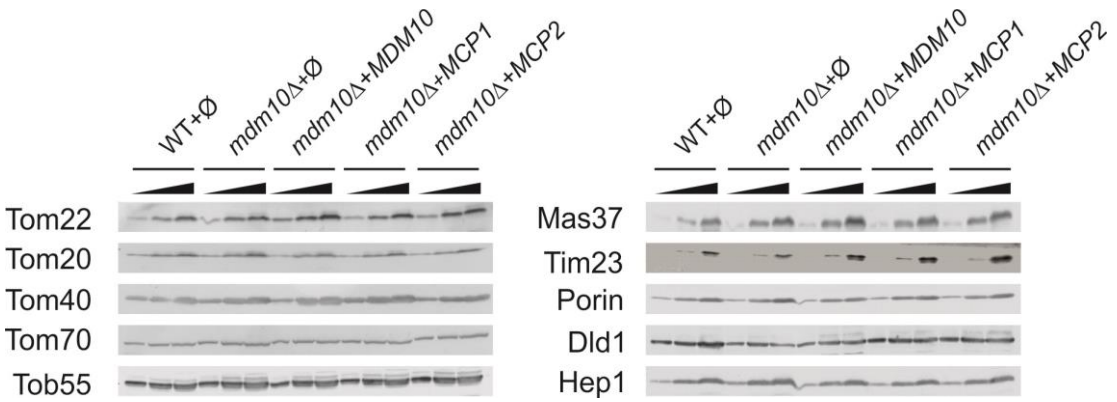


Figure 3.13. Over-expression Mcp1 or Mcp2 in *mdm10*Δ cells has no influence on steady-state levels of mitochondrial proteins. Mitochondria (10, 30 and 60 µg) isolated from the indicated strains were subjected to SDS-PAGE and the levels of different mitochondrial proteins were analyzed by immunodecoration with the indicated antibodies. A representative experiment of three independent repeats is shown.

3.8 Mcp1 and Mcp2 do not influence the assembly of TOM and TOB complexes

Mdm10 was reported to be a component of a subpopulation of TOB complexes and primarily involved in the biogenesis of the TOM subunit Tom40 (Meisinger et al. 2004). The lack of Mdm10 leads to an impaired assembly of TOM complex as assessed by BN-PAGE analysis (Meisinger et al. 2004, Yamano et al. 2010b). To test whether over-expression of Mcp1 and Mcp2 complements the assembly defect of TOM complex in *mdm10*Δ cells, we analyzed by BN-PAGE the steady-state levels of the complex in different cells (Fig. 3.14A). To this end, mitochondria isolated from the indicated strains were treated with 1% digitonin (Fig. 3.14A). Furthermore, radiolabeled Tom40 and Tom22 precursors were imported into mitochondria isolated from the indicated strains to investigate the assembly of TOM precursors into TOM complex. After termination of import reactions mitochondria were treated with 1% digitonin and then subjected to BN-PAGE. The radiolabeled proteins were analyzed by autoradiography.

Deletion of *MDM10* leads to a reduction of the levels of TOM complex and results in an additional unassembled species of Tom40 with a molecular mass of around 100 kDa in BN-PAGE (Fig. 3.14A, second lane). Over-expression of Mdm10 in *mdm10*Δ cells rescued the assembly of TOM complex and the unassembled species of Tom40

disappeared. In contrast, over-expression of Mcp1 or Mcp2 had no influence on the presence of the unassembled species and fully assembled TOM complex is still mildly reduced (Fig. 3.14A, last two lanes). Taken together, Mcp1 and Mcp2 have no effects on the stability of TOM complex.

Since Mcp1 and Mcp2 have no influence on the steady-state level of TOM complex, the next step was to investigate whether over-expression of Mcp1 or Mcp2 facilitate the TOM complex assembly. In previous reports the absence of Mdm10 leads to a reduction of TOM complex assembly. Imported radiolabeled Tom40 accumulated as a 250 kDa intermediate I and a 100 kDa intermediate II, and it was less efficiently incorporated into mature TOM complex of around 450 kDa (Model et al. 2001, Meisinger et al. 2004, Yamano et al. 2010b). We observed the same import pattern of radiolabeled Tom40 in mitochondria isolated from *mdm10*Δ cells of W303 genetic background. In contrast to the situation in control organelles, only a small amount of radiolabeled Tom40 was assembled into the mature complex after 90 minutes of *in vitro* import into mitochondria lacking Mdm10 (Fig. 3.14B and C). Re-introduction of Mdm10 rescued the assembly defect (Fig. 3.14B). Mitochondria isolated from *mdm10*Δ cells with plasmids encoding Mcp1 or Mcp2 were used to test whether Mcp1 or Mcp2 has the capacity to rescue the assembly of TOM complex. In spite of increased amounts of assembly intermediates I and II upon over-expression of Mcp1 and especially Mcp2 (Fig. 3.14C), the amount of fully assembled mature TOM complex was still decreased as in mitochondria from cells lacking Mdm10. Therefore, Mcp1 and Mcp2 do not rescue the assembly of TOM complex in the absence of Mdm10. The increased assembly intermediates upon over-expression of Mcp1 and especially Mcp2 might be an indirect effect of partially restored mitochondrial membrane lipid composition (see below).

Import of Tom22 *in vitro* is another way to assess TOM complex assembly. The import of Tom22 *in vitro* results in incorporation of the precursor into the 450 kDa mature TOM complex. A 400 kDa assembly intermediate named assembly intermediate III could be observed in mitochondria lacking Mdm10 (Meisinger et al. 2004). The radiolabeled precursor Tom22 was imported into mitochondria isolated from cells of interest and after import the mitochondria were treated with 1% digitonin and subjected to BN-PAGE (Fig. 3.14D and E). In agreement with previous report reduced amounts of 450 kDa mature TOM complex and accumulation of the 400 kDa assembly intermediate III

were observed in mitochondria lacking Mdm10 (Fig. 3.14D and E, lane 4-6). Again re-introduction of Mdm10 rescued the assembly defect (Fig. 3.14D). Over-expression of Mcp1 or Mcp2 resulted in a slight restoration of TOM complex amounts but intermediate III at around 400 kDa was still visible even with increased amount. Of note, in contrast to over-expression of Mcp1, the amounts of labeled Tom22 molecules in the different complexes were in total increased in mitochondria from *mdm10*Δ cells with over-expression of Mcp2. However, we could not find any support for a direct role of Mcp2 in the import of Tom22. This experiment again suggests that Mcp1 and Mcp2 have no direct function in assembly of the TOM complex.

As mentioned before, Mdm10 was identified as a component of a subpopulation of TOB complex and the expression level of Mdm10 affects the assembly of the TOB complex (Thornton et al. 2010, Yamano et al. 2010a, Yamano et al. 2010b, Becker et al. 2011). Thus we wanted to analyze the migration behavior in BN-PAGE of the TOB complex in mitochondria harboring different Mdm10 levels. To this end we isolated mitochondria from the strains of interest and solubilized them with 0.5% (v/v) Triton X-100. Only TOB core complex (including Tob55, Tob38 and Mas37) could be found in the wild-type cells containing empty plasmid (Fig. 3.14F, Ø). In mitochondria isolated from cells lacking Mdm10 a larger complex marked with an asterisk was visible (Fig. 3.14F, second lane). According to an earlier report this complex resembles a TOB-Tom5/Tom40 species (Thornton et al. 2010). Over-expression of Mdm10 in *mdm10*Δ cells led to an additional TOB complex species, which is indicated by an arrowhead and behaves like the previously reported TOB-Mdm10 holo-complex (Meisinger et al. 2004, Yamano et al. 2010b). To test whether Mcp1 or Mcp2 influence the TOB-complex composition, mitochondria from cells over-expressing Mcp1 or Mcp2 in the absence of Mdm10 were analyzed. In both cases the same TOB-complex pattern as in *mdm10*Δ cells was found (Fig. 3.14F, last two lanes). This observation suggests that the rescue effect of Mcp1 and Mcp2 is not due to restored biogenesis or assembly of the TOB complex.

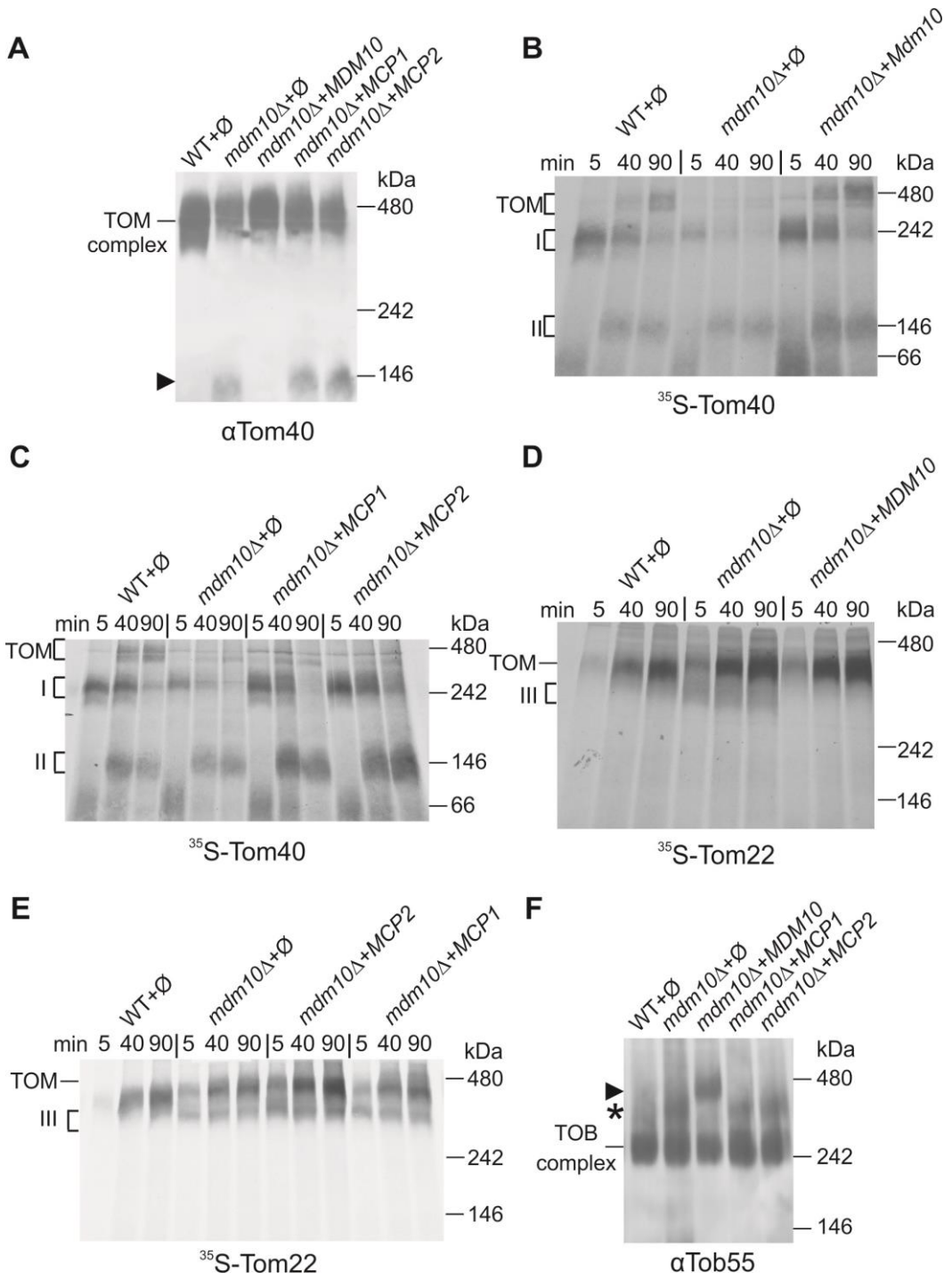


Figure 3.14. The assembly of TOM or TOB complex is not affected by over-expression of Mcp1 or Mcp2 in *mdm10*Δ cells. (A) Mitochondria isolated from the indicated strains were treated with 1% digitonin and the protein complexes were separated by BN-PAGE. The assembled TOM complex and unassembled Tom40 species were detected with an antibody raised against Tom40. The arrowhead at around 100 kDa indicates the unassembled Tom40 species. **(B)** and **(C)** Radiolabeled Tom40 precursor was imported for different time periods into mitochondria isolated from the indicated strains. The mitochondria were lysed with digitonin and analyzed by BN-

PAGE and autoradiography. Two assembly intermediates (I and II) of Tom40 are present at around 240 kDa and 150 kDa, respectively. The assembled TOM core complex (TOM) is indicated at around 450 kDa. **(D)** and **(E)** Radiolabelled precursor of Tom22 was imported into the indicated mitochondria as described in (B). The assembly intermediate of Tom22 (III) and the assembled TOM core complex (TOM) are indicated at around 300 kDa and 450 kDa, respectively. **(F)** Mitochondria isolated from the indicated strains were treated with 0.5% Triton X-100 and analyzed by BN-PAGE and immunoblotting with antibody raised against Tob55. The TOB core complex is indicated at around 240 kDa. Two additional species of TOB complex with higher molecular weight are indicated by an arrowhead and an asterisk and described in the text.

3.9 Over-expression of other ERMES subunits does not rescue the growth phenotype of *mdm10*∆ cells

Mdm10 is a subunit of the ERMES complex, which is localized at contact sites between ER and mitochondria in yeast. The mitochondrial protein Mmm2, the cytosolic protein Mdm12 and the ER protein Mmm1 are also components of this complex. To analyze whether over-expression of the other ERMES components rescues the growth defect

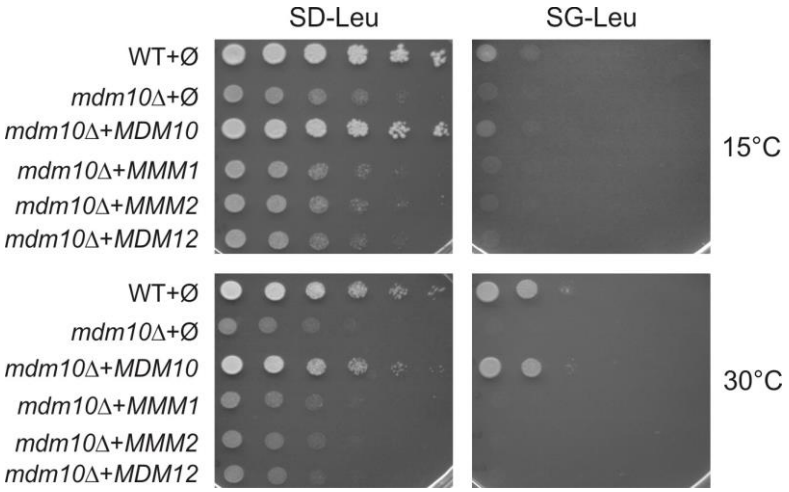


Figure 3.15. Over-expression of other ERMES subunits can not rescue *mdm10*∆ growth phenotype. The indicated cells were incubated overnight in selective medium, diluted in synthetic medium containing glucose and grown to logarithmic phase. Serial 5-fold dilutions were spotted on plates with synthetic medium containing glucose (SD-Leu) or glycerol (SG-Leu) and plates were incubated further at 15°C or 30°C.

of *mdm10*Δ cells, plasmids encoding Mmm1, Mmm2 or Mdm12 were transformed into *mdm10*Δ cells and transformed cells were analyzed by drop dilution assays. Over-expression of any single subunit Mmm1, Mmm2 or Mdm12 showed no rescue of the growth of *mdm10*Δ cells under all test conditions (Fig. 3.15 and data not shown). These results suggest that the function of Mdm10 cannot be replaced by another subunit of the ERMES complex and further indicate that the cellular functions of Mcp1 and Mcp2 is different from the functions of the components of ERMES complex.

3.10 Mcp1 and Mcp2 suppress the growth defect of cells lacking Mmm1, Mmm2 or Mdm12

Since the previous investigations showed that Mcp1 and Mcp2 are suppressors for loss of Mdm10, the next question was whether Mcp1 and Mcp2 have similar functional relationship with the other subunits of the ERMES complex. To that end, rescue capacity of over-expression of Mcp1 or Mcp2 in *mmm1*Δ, *mmm2*Δ and *mdm12*Δ cells was analyzed by drop dilution assays (Fig. 3.16). These results demonstrate that Mcp1 or Mcp2 could partially or completely rescue the growth defects in the deletions of other ERMES components (Fig. 3.16A-C). Therefore both proteins are also the suppressors of loss of Mmm1, Mmm2 and Mdm12. Of note a stronger rescue ability of Mcp1 than Mcp2 was observed in the cells lacking Mmm1 or Mmm2 (Fig. 3.16A and B), whereas Mcp2 behaved as a stronger suppressor in cells lacking Mdm12 (Fig. 3.16C). The reason for this different rescue potential is so far unknown. Taken together Mcp1 and Mcp2 are suppressor of deletions in each of the known ERMES subunits.

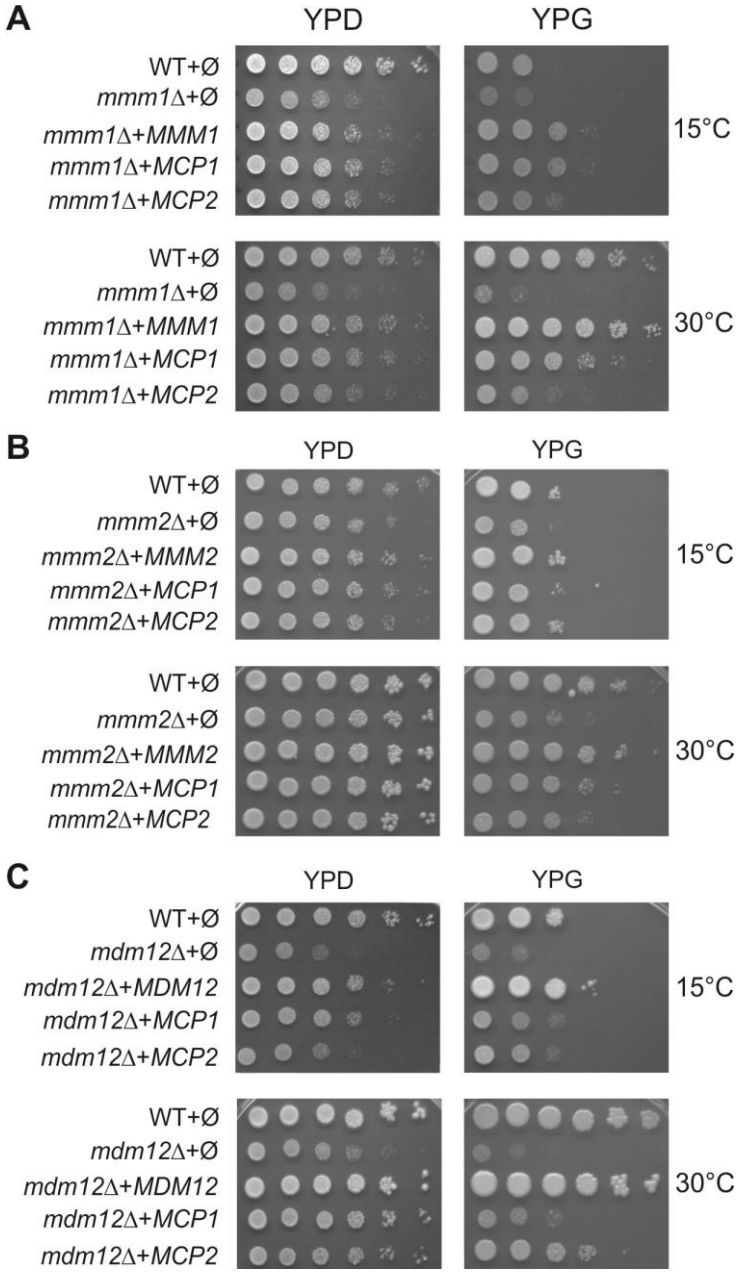


Figure 3.16. Mcp1 and Mcp2 are suppressors of the other subunits of ERMES complex. (A) Plasmids encoding Mcp1 or Mcp2 were transformed into *mmm1*Δ cells, whereas wild-type and *mmm1*Δ cells transformed with empty plasmid pYX142 (Ø) served as control. All cells were grown to logarithmic phase and spotted on plates containing either glucose (YPD) or glycerol (YPG) and further incubated at 15°C or 30°C. **(B)** Wild-type and *mmm2*Δ cells were transformed with indicated plasmids and analyzed as described in (A). **(C)** Wild-type and *mdm12*Δ cells were transformed with indicated plasmids and analyzed as described in (A).

3.11 Mutations of the putative kinase domains of Mcp2 leads to loss of rescue capacity

Bioinformatic analysis of Mcp2 proposed that it contains the (http://browse.yeastgenome.org/fgb2/gbrowse/scproteome/?src=scproteome&name=YLR253W) ABC1 family protein kinase domain. In addition, amino acids sequence of Mcp2 was subjected to BLAST algorithm provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Putative conserved domains have been detected in Mcp2 by this analysis. These domain hits are associated with ABC1 superfamily, Aminoglycoside 3'-phosphotransferase (APH), Choline Kinase (ChoK) family and AarF protein. Hence, it is tempting to speculate that the putative conserved kinase domain is essential for the function of Mcp2.

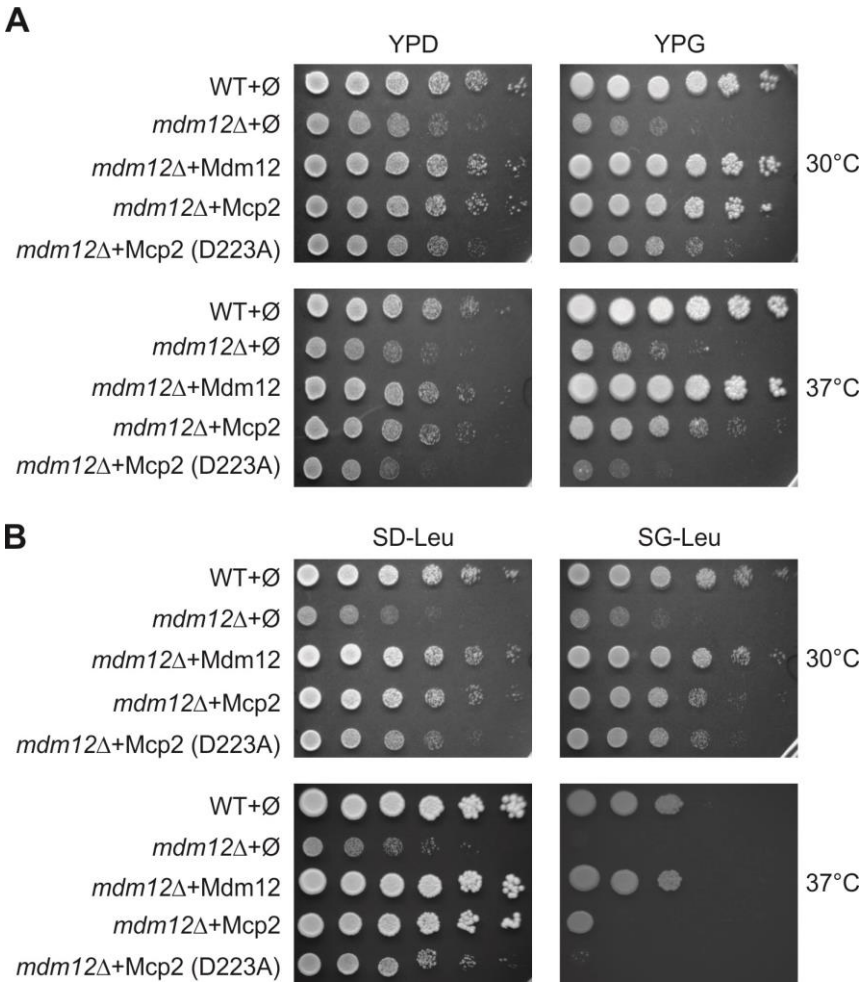
As a functional assay for Mcp2 we used the observation that over-expression of Mcp2 showed good complementation of growth phenotype of *mdm12*Δ cells (Fig. 3.16C). To test whether the kinase domain is essential for the function of Mcp2 we mutated the amino acids of two conserved residues in the nucleotide binding pocket. Three conserved stretches of the kinase domain were detected and amino acid 223 and 256 of Mcp2 were chosen for point mutation. Next, the constructs Mcp2(D223A), Mcp2(D223A)-HA and Mcp2(E256D)-HA were obtained by site directed mutagenesis. The plasmids, which encode mutated Mcp2, as well as plasmids encoding Mcp2, Mcp2-HA, Mdm12 or Mdm12-HA, were transformed into *mdm12*Δ cells and the rescue of the growth defect of *mdm12*Δ cells was analyzed by drop dilution assay (Fig. 3.17)

Over-expression of Mcp2 in *mdm12*Δ cells showed almost complete rescue of the growth phenotype on both media (YPD and YPG) at 30°C and on YPD plate at 37°C (Fig 3.17A), whereas rescue on YPG plate at 37°C was less pronounced. Similar results were observed on synthetic medium (Fig.3.17B). Over-expression of kinase mutant Mcp2(D223A) in *mdm12*Δ cells could not rescue the growth phenotype in cells lacking Mdm12 (Fig. 3.17A and B). Therefore mutation of the conserved kinase domain of Mcp2 seems to lead to a loss of function.

Next, we analyzed the capacity of HA-tagged Mcp2 variants to complement the growth phenotype of *mdm12*Δ cells. Surprisingly, the rescue capacity of Mcp2-HA and its Mcp2(D223A)-HA variant were similar under all tested conditions. The reason for this difference between Mcp2(D223A) and Mcp2(D223A)-HA is so far unknown. Over-

expression of Mcp2(E256A)-HA in *mdm12*Δ cells showed that this variant has reduced complementation ability as compared to native Mcp2 on YPD and YPG medium at 30°C and 37°C, while no significant difference was observed on synthetic medium (Fig.3.17C and D).

To analyze whether mutation of Mcp2 leads to lower expression of Mcp2 or degradation of Mcp2 in cells, mitochondria (50 µg) isolated from the indicated cells were analyzed by SDS-PAGE and immunodecoration with antibodies against the HA-tag or Tom40 (as loading control). Similar amounts of the Mcp2 variant were observed (Fig.3.17E). These results suggest that Mcp2 with mutations in the conserved kinase domain is still localized to mitochondria and no increased degradation takes place. Yet the functional analysis of untagged Mcp2 versus Mcp2-HA is contradictory and further investigations are necessary.



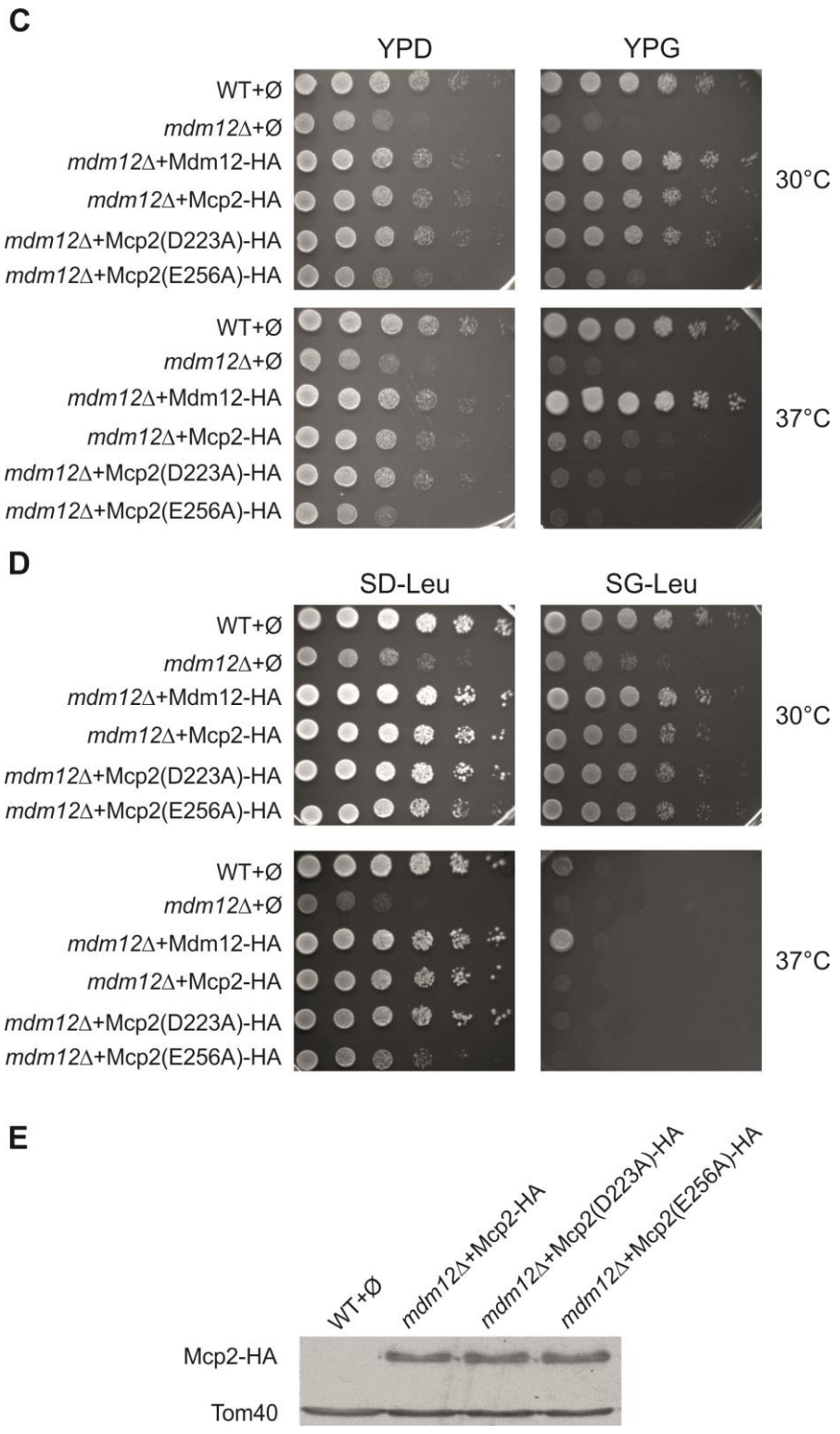


Figure 3.17. Rescue capacity of Mcp2 with mutated kinase domain. (A) Wild-type cells transformed with an empty plasmid pYX142 (Ø) served as control. Cells lacking Mdm12 were transformed with empty plasmid pYX142 (Ø), or plasmids encoding Mdm12, Mcp2 or Mcp2(D223A). Indicated strains were incubated overnight, diluted and grown to logarithmic phase in medium containing glucose (YPD). Serial 5-fold dilutions were spotted on plates containing glucose (YPD) or glycerol (YPG) and cells were

further incubated at 30°C or 37°C. **(B)** The same cells as described in (A) were incubated in synthetic medium containing glucose (SD-Leu) and were treated as in (A). Growth phenotypes were analyzed at 30°C or 37°C on synthetic medium containing glucose (SD-Leu) or glycerol (SG-Leu). **(C)** Cells lacking Mdm12 were transformed with empty plasmid, plasmids encoding Mdm12-HA, Mcp2-HA, Mcp2(D223A)-HA and Mcp2(E256A)-HA and were treated as described in (A). The growth phenotypes were analyzed at 30°C or 37°C. **(D)** Indicated cells were treated as described in (B). **(E)** Mitochondria (50 µg) isolated from the indicated cells were subjected to SDS-PAGE and analyzed by immunodecoration with antibodies against the HA-tag and the mitochondrial outer membrane protein Tom40.

3.12 Over-expression of Mcp1 or Mcp2 rescues the assembly defects of respiratory chain supercomplexes in *mdm10*∆ cells

The supercomplexes of the respiratory chain have been successfully investigated by BN-PAGE in combination with silver or Coomassie staining or immunodecoration (Schagger, 2001). Previous works reported that the assembly of TOM and TOB complexes is impaired upon loss of Mdm10. Thus, it can be assumed that other protein complexes in yeast mitochondria lacking Mdm10 are also destabilized. Mcp1 or Mcp2 could functionally compensate this destabilization effect. To assess this question, mitochondria isolated from wild-type and cells lacking Mdm10 together with *mdm10*∆ cells over-expressing Mcp1, Mcp2 or Mdm10 itself were analyzed by BN-PAGE (Fig. 3.18A).

In contrast to control organelles, the total amount of protein complexes was significantly reduced in mitochondria lacking Mdm10. Furthermore, altered complex pattern was observed in higher molecular mass regions (above 480 kDa). Re-introduction of Mdm10 led to complete rescue of the pattern of protein complexes whereas over-expression of Mcp1 or Mcp2 led to partial rescue (Fig. 3.18A). Most of the protein complexes visible in this molecular mass range by Coomassie staining correlate to the supercomplexes of respiratory chain.

Antibodies against subunits of respiratory complex III (Cor1) and complex IV (Cox2) in the oxidative phosphorylation pathway, were applied to identify the specific

supercomplexes influenced by loss of Mdm10 (Fig. 3.18B and C). Immunodecoration with antibody against Cor1 indicated increased amount of the dimer of complex III in the range between 480 and 720 kDa, while a hetero-trimeric complex III_2IV and the hetero-tetramer complex III_2IV_2 were dramatically decreased in the cells lacking Mdm10. In addition, a supercomplex of around 700 kDa (arrowhead) of unknown composition was observed. There were no significant differences in rescue of complex patterns between over-expression of Mcp1 and re-introduction of Mdm10 itself as both lead to a nearly complete restoration of migration pattern (Fig. 3.18B and C). In contrast, over-expression of Mcp2 led to restoration of supercomplex III_2IV_2 and supercomplex III_2IV, but the novel 700 kDa specie was still visible (Fig. 3.18B, right lane). By decoration with an antibody against complex IV subunit Cox2, similar restoration effects could be observed by over-expression of Mdm10, Mcp1 and Mcp2 in *mdm10*Δ cells. Over-expression of Mdm10 or Mcp1 could recover the amount of supercomplexes III_2IV_2 and III_2IV and the lost complex IV in cells lacking Mdm10. An additional band also appeared at 700 kDa in all cells lacking Mdm10 (Fig. 3.18C arrowhead).

To test whether reduced steady-state levels of the subunits Cor1 and Cox2 are responsible for the observed assembly defects effects, their quantity was assessed in mitochondria isolated from the corresponding cells by SDS-PAGE and immunodecoration (Fig. 3.18D). Only the amount of Cox2 was found to be slightly decreased in *mdm10*Δ cells. This very minor reduction cannot be responsible for the absence of complexes as observed in the BN-PAGE analysis. Over-expression of Mdm10 or Mcps could complement the reduced amount of protein steady-state levels (Fig. 3.18D). Since Cox2 is encoded on the mitochondrial genome, the fact that cells lacking Mdm10 exhibit lower expression level can be explained by the reported altered nucleoid structure and stability of *mdm10*Δ cells (Sogo and Yaffe, 1994). Taken together, over-expression of Mcp1 or Mcp2 can complement the defect in assembly of protein complexes of the respiratory chain in cells lacking Mdm10.

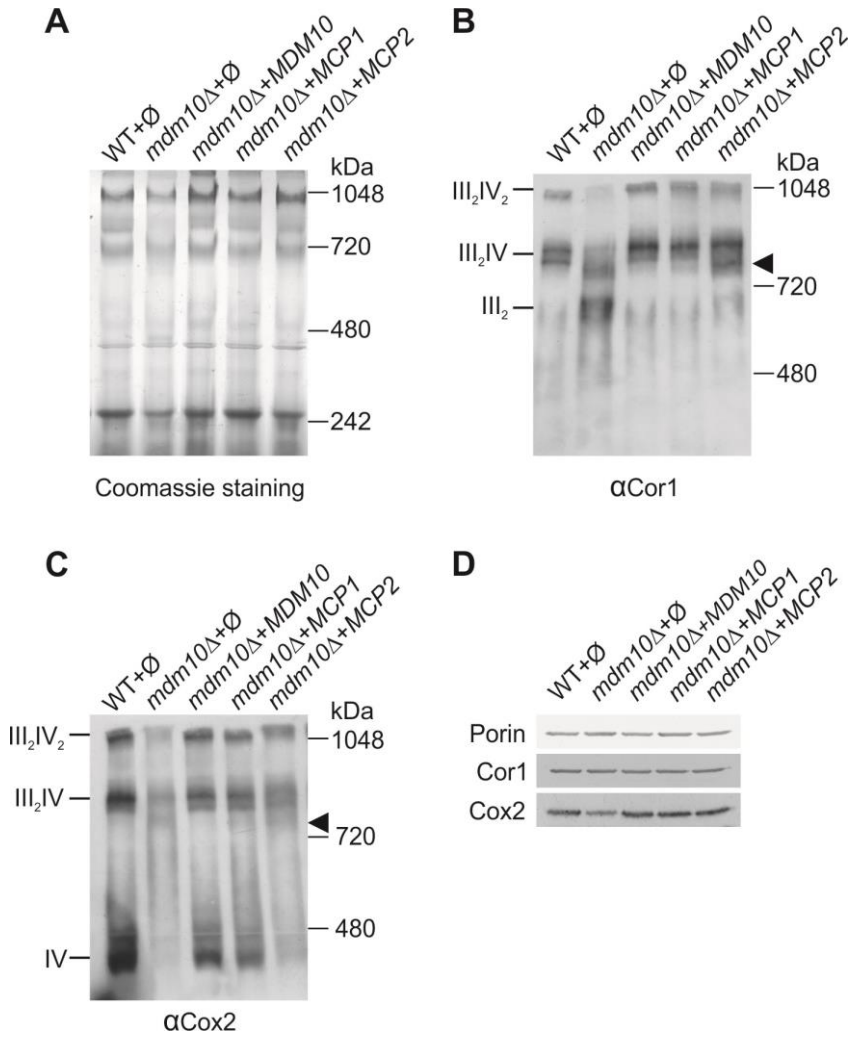


Figure 3.18. Over-expression of Mcp1 or Mcp2 in *mdm10*Δ cells rescues the assembly defect of mitochondrial respiratory chain supercomplexes. (A) Mitochondria isolated from indicated cells were treated with 1% digitonin and subjected to 4-8% BN-PAGE. Coomassie Brilliant Blue G-250 was used to stain the gel. **(B)** and **(C)** Mitochondria were treated as described in (A). Afterwards, blue native gels were blotted on a PVDF membrane and protein complexes were analyzed by immunodecoration with antibodies against the subunit Cor1 of complex III (B) or the subunit Cox2 of complex IV (C). An uncharacterized complex at 700 kDa is indicated with an arrow head. **(D)** Isolated mitochondria (20 µg) from indicated strains were lysed with 1% digitonin and the solubilized proteins were analyzed by SDS-PAGE and immunodecoration with antibodies against porin, Cor1 and Cox2.

3.13 Over-expression of Mcp1 and Mcp2 partially complements the phospholipid composition of mdm10Δ mitochondria

3.13.1 Evaluation of phospholipid composition of mitochondria over-expressing Mcp1 or Mcp2 by thin layer chromatography (TLC)

According to previous reports qualitative and quantitative changes of lipid components of mitochondria can influence the biogenesis, assembly and function of protein complexes in mitochondrial membranes. Further investigations on the role of cardiolipin showed its close functional association with the respiratory chain and import complexes in mitochondria (Jiang et al. 2000, Schagger 2002, Zhang et al. 2002, Pfeiffer et al. 2003, Zhong et al. 2004, Nury et al. 2005, Zhang et al. 2005, Kutik et al. 2008). In our study, over-expression of Mcp1 or Mcp2 can rescue the growth defect of *mdm10*Δ cells by complementation of the mitochondrial morphology and supercomplex assembly of the respiratory chain. To investigate whether these effects of Mcp1 or Mcp2 over-expression are due to an influence on lipid homeostasis of mitochondria, the different mitochondrial phospholipid species were analyzed by thin layer chromatography (Fig. 3.19). With the method used (see Material and Methods section) the phospholipids phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC) and cardiolipin (CL) could be discriminated and identified (Fig. 3.19). Only very minor changes could be observed among the tested strains. Cells lacking Mdm10 displayed marginal reduced amounts of PE and PS, whereas CL, PC and other phospholipids (lyso-phospholipid) were not changed in comparison with wild-type. Re-introduction of Mdm10 and over-expression of Mcp1 or Mcp2 seemed to rescue the minor loss of PS and PE in *mdm10*Δ cells. However, reliable quantitative results cannot be obtained by this method. Therefore as described in 3.13.2, quantitative mass spectrometry was applied for further phospholipids analysis.

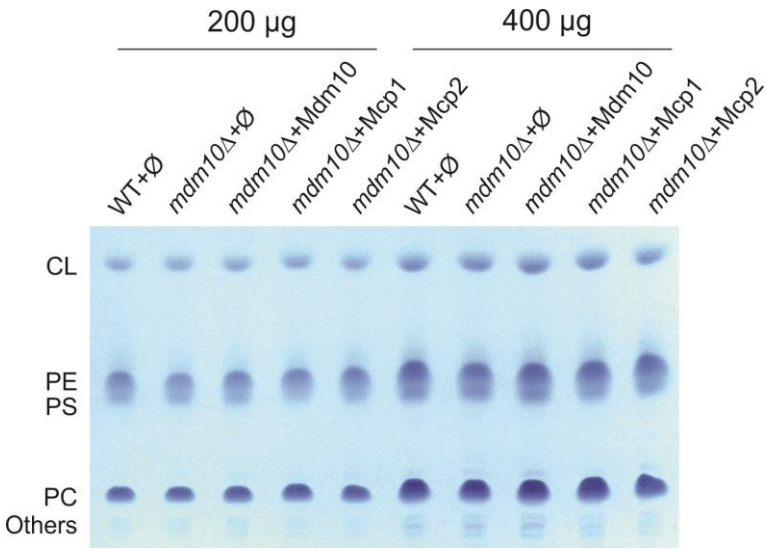


Figure 3.19. Phospholipid composition of mitochondria from wild-type and *mdm10*Δ cells over-expressing Mdm10, Mcp1 and Mcp2. Mitochondria isolated from the indicated strains (200 µg and 400 µg) were purified via Percoll gradient and treated with chloroform and methanol to extract lipids. The isolated lipids were analyzed by thin layer chromatography. The phospholipids were stained by molybdenum blue. Cadiolipin (CL); Phosphatidylcholine (PC), Phosphatidylethanolamine (PE); Phosphatidylserine (PS).

3.13.2 Quantification of phospholipids by mass spectrometry

To analyze quantitatively changes of phospholipid composition of mitochondria, it is crucial to obtain highly pure organelles. To that end, the isolated mitochondria were further treated with trypsin and subsequentially sucrose step gradient centrifugation to remove other membranous contaminations (especially ER). To exclude major contaminations of the mitochondrial fraction by ER, purified mitochondria were analyzed by SDS-PAGE and immunodecoration. The applied purification protocol lead to highly pure mitochondria, as only trace amounts of the ER protein Erv2 could be detected in the mitochondrial preparations (Fig. 3.20A). Mass spectrometric analysis and quantification of the lipids of highly purified mitochondria was performed in a collaboration with the laboratory of Britta Brügger in the Biochemistry Center of the University of Heidelberg. Fig. 3.20 shows the results of the changes of the phospholipid composition of mitochondria lacking Mdm10, Mcp1 or Mcp2. Similarly mitochondria isolated from *mdm10*Δ cells over-expressing either the two suppressors or Mdm10 itself and from wild-type cells were compared. Among five tested phospholipid species,

the levels of phosphatidylethanolamine (PE) and cardiolipin (CL) were reduced, whereas the amount of phosphatidylserine (PS) was greatly increased in *mdm10*Δ cells (Figure 3.20B, D and E). The analysis of different species of the single phospholipids showed that deletion of *MDM10* led to an increase of 34:2 species of PC, PE, PS and PI and a decrease of the 32:1 species (suppl. Table S2).

The amount of phosphatidylcholine (PC) in *mdm10*Δ cells was at the same level as for wild-type cells (Fig. 3.20B). Over-expression of Mcp1 or Mcp2 in *mdm10*Δ cells and deletion of *MCP1* lead to a reduction of the PC amount of around 20-25% in comparison to wild-type. Loss of Mcp2 had no effect on PC levels (Fig. 3.20B). PE levels in cells with deletion of *MCP1* or *MCP2* were similar as in wild-type cells. Yet as reported before, loss of Mdm10 led to a 35% reduction of PE in comparison to organelles from wild-type cells. This reduction could be compensated by over-expression of Mdm10 itself, Mcp1 and to a lesser extent Mcp2 (Fig. 3.20C). The loss of Mdm10 as well as Mcp2 has no influence on PI levels (Fig. 3.20D). Also deletion of *MCP1* leaves PI levels unaltered. Interestingly, over-expression of Mdm10 and Mcp1 leads to decreased PI levels which could be a compensation mechanism to the changes in the other phospholipid amounts.

Mitochondria lacking Mdm10 show twice the relative amount of PS as compared to control organelles (Fig. 3.20E). Over-expression of Mcp1 or Mdm10 could completely reverse PS alteration, while over-expression of Mcp2 leads to a statistically insignificant slight PS reduction. Deletion of *MCP1* or *MCP2* again had no effect on the level of PS (Fig. 3.20E). The same was observed for the amount of CL (Fig. 3.20F). Yet again as for the other non-bilayer forming lipid PE, greatly reduced relative CL levels were observed in *mdm10*Δ mitochondria. This effect could be rescued by re-introduction of Mdm10, whereas over-expression of Mcp1 or Mcp2 in contrast to PE increased the CL amount only impalpably (Fig. 3.20F).

In general, the amounts of different phospholipids in mitochondria of cells with deletion of *MCP1* or *MCP2* showed only minor changes in comparison to wild-type cells. PC and PS were insignificantly slightly reduced by deletion of *MCP1*, whereas very minor reductions of PE and PI were present in case of *mcp2*Δ mitochondria. This is in agreement with previous results, that the loss of Mcp1 and Mcp2 had no effect on the growth phenotype of yeast cells and on their mitochondrial morphology. Taken together, the changes in the phospholipid composition of mitochondria lacking Mdm10

that we observed are in agreement with previous studies (Jiang et al. 2000, Schagger 2002, Zhang et al. 2002, Pfeiffer et al. 2003, Zhong et al. 2004, Nury et al. 2005, Zhang et al. 2005, Kutik et al. 2008). Over-expression of Mcp1 and to a lesser extent, Mcp2 can partially complement the altered mitochondrial phospholipids composition phenotype of *mdm10*Δ cells.

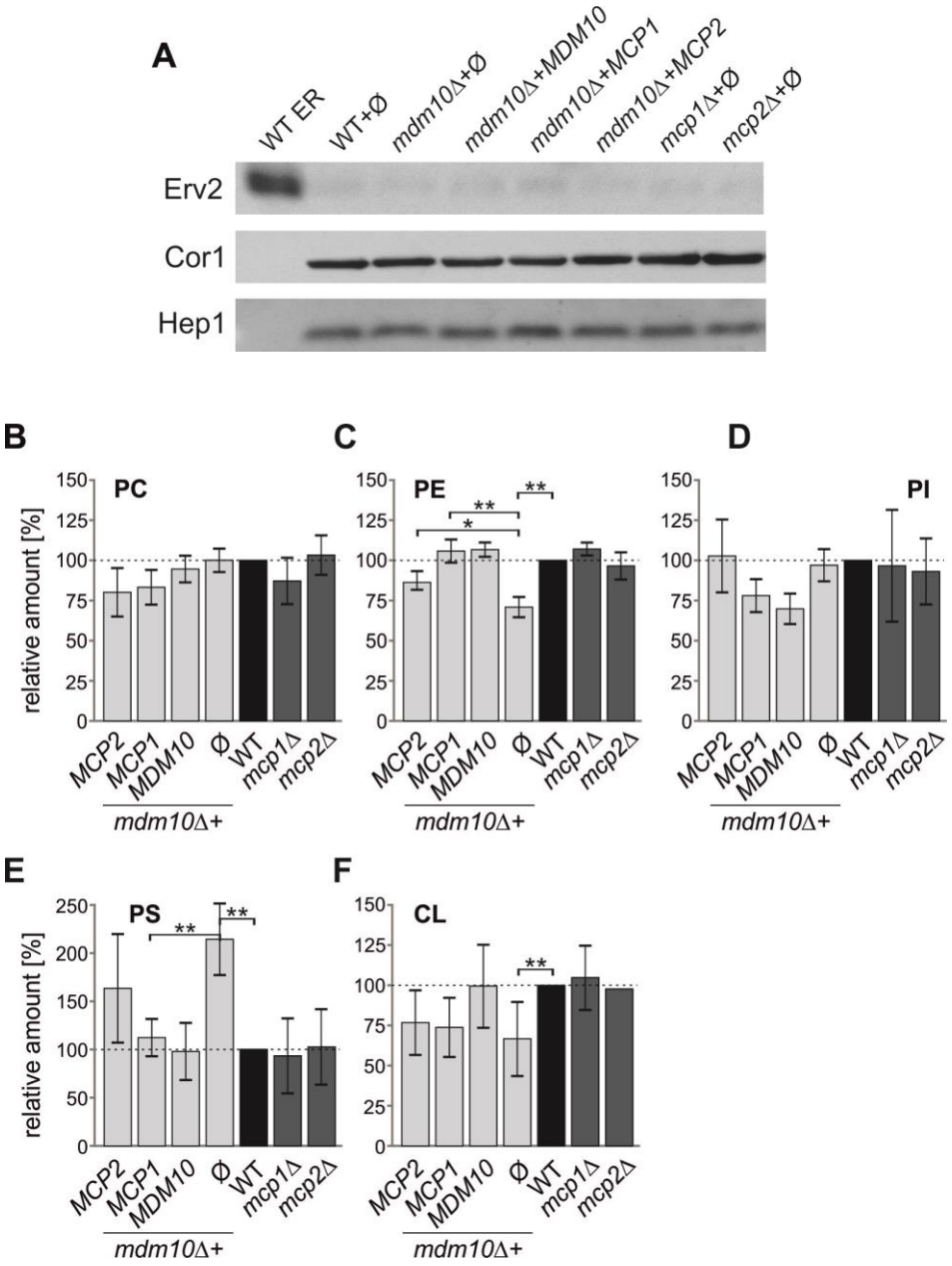


Figure 3.20. Alterations of phospholipids in *mdm10*Δ cells can be partially restored by over-expression of Mcp1 or Mcp2. Mitochondria isolated from indicated strains were purified by Percoll or sucrose step gradient centrifugation after treatment with trypsin. **(A)** ER contamination levels of purified mitochondria. Mitochondria isolated from indicated cell strains were purified by sucrose step gradient centrifugation and

subjected to SDS-PAGE (30 µg per lane). The purified mitochondria and an ER fraction from wild-type cells (WT ER) were analyzed by immunodecoration with antibody against ER protein Erv2 and mitochondrial proteins Cor1 and Hep1. **(B)-(F)** Lipids were extracted from mitochondria to be quantified by mass spectrometry analysis. The amount of each phospholipid species from the wild-type mitochondria was set to 100% and relative values of different phospholipids in the mitochondria from other cells are presented. The mean with standard deviations of three biological repeats with two technical repeats for each (n=6) is given. *P<0.05, **P<0.005 (unpaired t-test, two tailed). Quantification of phospholipids by mass spectrometry was performed in collaboration with the group of Britta Brügger in Heidelberg.

3.13.3 Deletion of subunits of ERMES complex leads to elevated mitochondrial ergosterol levels

As shown above for Mdm10 and as was reported recently, overall defects of the ERMES complex lead to changed mitochondrial biogenesis and the alterations of phospholipid composition of the mitochondria (Kornmann et al. 2009, Osman et al. 2009, Yamano et al. 2010, Nguyen et al. 2012, Tamura et al. 2012). Upon loss of any subunit of ERMES complex, mitochondrial phospholipid composition was altered. The main steroid lipid of yeast mitochondria is ergosterol. To answer the question whether deletion of *MDM10*, its suppressors or other ERMES subunits lead also to changes in mitochondrial ergosterol levels, mass spectrometry analysis and quantification of ergosterol from highly purified mitochondria were performed (in collaboration with the laboratory of Britta Brügger). Mitochondria were isolated from the corresponding strains and purified by trypsin treatment and sucrose step gradient centrifugation. Fig. 3.21 shows the results of the mass spectrometric analysis. Mitochondria lacking Mdm10, Mcp1 or Mcp2 as well as mitochondria isolated from *mdm10*Δ cells over-expressing either the two suppressors or Mdm10 were analyzed in comparison to wild-type mitochondria. A minor increase of ergosterol was identified in mitochondria from cells with deletion of *MCP2*, whereas deletion of *MCP1* had no effect (Fig. 3.21A and Suppl. Table S1). Strikingly double relative amount of ergosterol levels in comparison to wild-type were found in mitochondria lacking Mdm10. The ergosterol levels were reduced to normal amounts by re-introduction of Mdm10 itself and by over-expression of Mcp1, but over-expression of Mcp2 only slightly reduced the ergosterol levels. A possible contamination of ergosterol by ER impurities could be the reason for the

observed higher ergosterol levels. However, this possibility can be excluded since there no contamination of ER in the tested mitochondrial samples was observed (Fig. 3.20A).

Furthermore, the influence of other ERMES components on the traffic of ergosterol to and in mitochondria was tested. Deletion strains for *MDM10*, *MMM1* and *MDM12* and wild-type cells were grown on rich medium. The amount of ergosterol from isolated purified mitochondria was determined by mass spectrometry. The relative ratio of ergosterol to protein was compared between the mitochondria isolated from deletion strains and the wild-type cells. An increase of 50% of ergosterol was found in mitochondria from cells lacking Mdm10, Mdm12 or Mmm1 (Fig. 3.21B). Again the purification of mitochondria was tested by immunodecoration with the antibodies against the ER marker Erv2 and mitochondrial protein Hep1 (Fig. 3.21C). These results suggest that the subunits of the ERMES complex play a yet unrecognized role in mitochondrial homeostasis of ergosterol in yeast and Mcp1 may participate in this process.

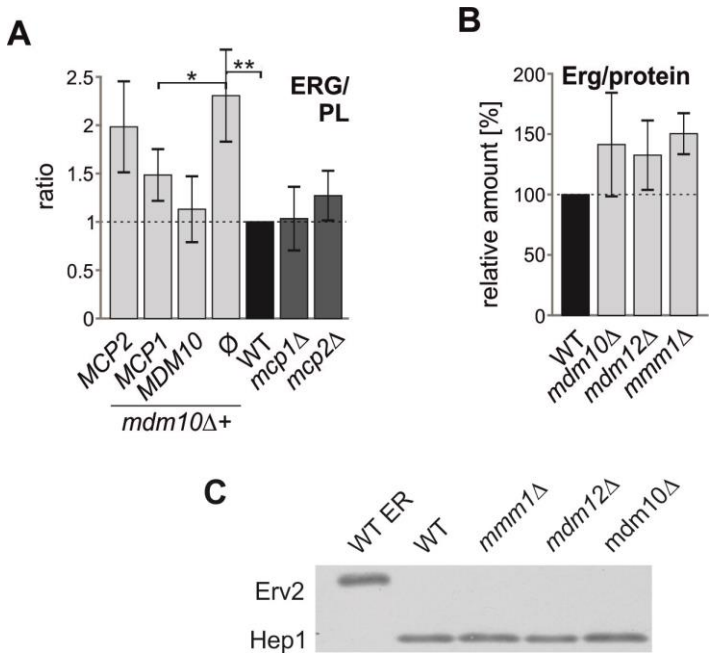


Figure 3.21. Mcps and subunits of the ERMES complex influence mitochondrial ergosterol levels. (A) Mitochondria as described in 3.13.2 were analyzed for the ratio of ergosterol to phospholipid and relative values are presented. The value of wild-type was set to 1.0. The mean with standard deviation of three biological repeats with two technical repeats for each (n=6) are shown *P<0.05, **P<0,005 (unpaired t-test, two

tailed). (Ergosterol analysis was performed by the group of Britta Brügger in Heidelberg) **(B)** Mitochondria isolated from cells with deletion of *MDM10*, *MMM1*, *MDM12* and wild type cells were prepared and analyzed as described in 3.13.2. The ergosterol levels are present as ERG/protein ratio. Relative amount of ergosterol in wild-type was set to 100%. The mean with standard deviation of three independent mitochondrial samples are shown. (Ergosterol analysis was performed by the group of Britta Brügger in Heidelberg) **(C)** The same amount of mitochondria as described in (B) (each 50 µg) were analyzed by SDS-PAGE and immunodecoration with antibodies raised against the ER marker Erv2 and the mitochondrial matrix protein Hep1.

4. Discussion

Mdm10 is a β-barrel protein and localized in the mitochondrial outer membrane (MOM) of *S. cerevisiae*. It is a subunit of a subpopulation of TOB complex and influences the biogenesis of β-barrel proteins in the MOM. Mdm10 is also required to link mitochondria and actin filaments of yeast. Loss of Mdm10 function has direct consequences for cytoskeleton involved processes, such as mitochondrial movement, inheritance and morphogenesis. Loss of Mdm10 was previously reported to affect the lipid composition of mitochondria (Kornmann et al. 2009, Osman et al. 2009, Yamano et al.2010b, Osman et al. 2011, Nguyen et al. 2012).

Recently, ERMES complex was identified as a tethering structure between the ER and mitochondria in yeast. This structure comprises the mitochondrial proteins Mdm10, Mmm2, the ER protein Mmm1, and the soluble cytosolic protein Mdm12. Mutation or loss of each subunit of ERMES complex results in altered mitochondrial morphology, growth defect, mtDNA loss and changes in lipid composition of mitochondria. All processes in which Mdm10 was shown to be involved are connected with each other. For instance, reduced import of β-barrel proteins leads to decreased assembly of TOM complex, which can consequently impair the import of many proteins in charge of mitochondrial morphology, inheritance and lipid transport. On the other hand, alterations in the lipid composition of a membrane has implication on its biophysical characteristics and affect some processes, such as protein insertion capacity and curvature of the membrane. These changes can result in alterations of the morphology of the regarding organelle. These difficulties suggest why the primary function of Mdm10 still needs to be unveiled.

In this study a genetic screen for suppressors of *mdm10*Δ growth phenotype has been performed. Two novel mitochondrial proteins, which were named Mcp1 and Mcp2, were identified as high-copy suppressors of the growth phenotype of *mdm10*Δ cells. Both of them can completely or partly complement the growth defect and mitochondrial morphology in the absence of Mdm10. In contrast to Mcp1 and Mcp2, over-expression of components of TOM or TOB complex in *mdm10*Δ cells had no rescue effects of their growth phenotype (data not shown). These findings suggest that Mcp1 and Mcp2 play different roles than the subunits of TOM or TOB complex in mitochondria. In addition, over-expression of any other subunits of ERMES complex also failed to complement

the growth phenotype of *mdm10*Δ cells. Therefore the function of Mdm10 cannot be replaced by other subunits of the ERMES complex and Mdm10 seems to play a unique role in this complex. These observations also hint that Mcp1 and Mcp2 have distinct functions than the ERMES subunits. Recently, Mdm31 was reported as a suppressor of *mdm10*Δ cells of FY833 strains (Tamura et al. 2012). Mdm31 was previously found to be involved in mitochondrial morphogenesis, inheritance (Dimmer et al. 2005) and ion homeostasis (Kucejova et al. 2005). During the process of the current genetic screen around 30000 colonies were tested, which corresponds to at least five times the number of genes of *S. cerevisiae*. Mdm31 was not found among our suppressor candidates. Furthermore over-expression of Mdm31 as a single copy gene in *mdm10*Δ cells could not suppress the loss of Mdm10 in the W303 background used in this work (data not shown). The potential reason for this discrepancy is the usage of different wild-type backgrounds in the two studies (Tamura et al. 2012, Tan et al. 2013). Indeed, it was reported that ERMES component deletion has different impact in different wild-type backgrounds (Yamano et al. 2010b).

The subcellular localization of Mcp1 and Mcp2 was investigated by analysis of subcellular fractions of yeast cells and immunofluorescence microscopy with epitope tagged proteins. Mcp1 was identified as a multi-span protein with five proposed transmembrane domains localized in MOM, while Mcp2 is a mitochondrial inner membrane protein. Loss of Mdm10 was reported to impair the structural tethering between the ER and mitochondria. In regard to the localization of Mcp1 in the outer membrane, over-expression of Mcp1 is in the perfect spatial position to reconstitute or by-pass this connection between the two organelles as a potential mechanism of the rescue in *mdm10*Δ cells. Intra-mitochondrial contact sites are made up by close connections of outer membrane and inner boundary membrane (Perkins et al. 1997, Frey and Mannella 2000). The contact sites of both membranes are hot spots for intra-mitochondrial lipid transport (Schatz and Dobberstein 1996, Osman et al. 2011, Flis and Daum 2013). Mcp2 behaved very similar to the mitochondrial inner membrane protein Cox2 in the sucrose step gradient centrifugation, yet it was found to be slightly shifted to submitochondrial vesicles with lower density. It is possible that over-expression of Mcp2 could modify the contact sites of both mitochondrial membranes and this capacity contributes to its rescue function.

Over-expression of Mcp1 or Mcp2 can partially or completely restore the growth phenotype of cells lacking Mdm10. Mitochondrial morphology was investigated in the *mdm10*Δ cells over-expressing Mdm10, Mcp1 and Mcp2. Re-introduction of Mdm10 and over-expression of Mcp1 almost completely complemented the mitochondrial morphology, yet over-expression of Mcp2 did so only partially. Hence, Mcp1 is a stronger suppressors than Mcp2, which is in agreement with the better rescue capacity of Mcp1 regarding cell growth. No additive rescue effect was observed for over-expression of both Mcp1 and Mcp2. This might be explained by the assumption that Mcp1 and Mcp2 are involved in the same functional system. Their contributions to the complement of *mdm10*Δ cells are not superimposed but only depend on the stronger one. An influence on mitochondrial morphology was identified in wild-type cells over-expressing Mcp2 but not Mcp1. It might well be that over-expression of Mcp2, which is located to the inner membrane of mitochondria, is harmful for mitochondrial function since Mcp2 actively changes the properties of the MIM therefore influencing the mitochondrial morphology.

According to previous reports, deletion of *Mdm10* leads to the reduction of the steady-state levels of Tom22 and Tom40. To decipher their function, the effect of over-expression of Mcp1 or Mcp2 on the steady-state levels of mitochondrial proteins in *mdm10*Δ cells was investigated. Of note, in our wild-type background W303 no significantly reduced levels of Tom22 and Tom40 were observed in *mdm10*Δ cells. This probably might result from the different wild-type backgrounds used in the two studies. Meisinger et al. conducted their experiments in the BY background (Meisinger et al. 2004). Importantly, in cells lacking Mdm10 the steady-state levels of IMS proteins, mitochondrial inner membrane proteins and components of TOM or TOB complex were not significantly changed by over-expression of Mcp1 or Mcp2. Taken together, these results suggest that the reason for rescue of the growth phenotype by over-expression of Mcp1 or Mcp2 and the defective growth of *mdm10*Δ cells themselves is not due to altered steady-state levels of mitochondrial proteins.

Mdm10 was previously shown to be essential for β-barrel protein insertion, especially for the assembly of precursor Tom40 into functional TOM complex (Meisinger et al. 2004). Yet over-expression of Mcp1 or Mcp2 had no direct effects on the assembly of TOM and TOB complex in cells lacking Mdm10. In comparison to *mdm10*Δ cells the amounts of assembly intermediate species of Tom40 were increased in case of over-

expression of Mcp1 or Mcp2, yet the mature TOM complex was still absent. In summary Mcp1 and Mcp2 did not rescue altered steady-state levels of TOM complex or its assembly kinetics as assessed by import of radiolabelled Tom40 precursor *in vitro*. Furthermore, steady-state levels of the TOB complex subpopulations was not influenced by Mcp1 or Mcp2, therefore their main functions do not relate to TOM and TOB complex assembly or stability.

By analysis by BN-PAGE of total mitochondrial complexes of cells lacking Mdm10 greatly reduced steady-state levels of complexes with high molecular mass were observed. The regular complex pattern as in wild-type cells was restored by re-introduction of Mdm10, over-expression of Mcp1 or Mcp2. The supercomplexes of respiratory chain are the most abundant higher molecular mass complexes of the organelle and are localized in the inner membrane of mitochondria. Using antibodies raised against subunits of complex III and complex IV it became clear that Mcp1 and Mcp2 as well as Mdm10 were able to rescue the assembly defect in respiratory chain supercomplex of *mdm10*Δ cells.

It was previously reported that the phospholipid composition of MIM can affect the assembly of respiratory chain supercomplexes. Especially cardiolipin (CL), which is mainly found in MIM, was shown to be an essential phospholipid for respiratory chain supercomplex formation. Cardiolipin influences the interaction of complex III and IV and stabilizes such supercomplexes. Loss of CL in yeast results in functional deficiency of the complex IV (Zhang et al. 2002, Pfeiffer et al. 2003). It is tempting to assume that Mcp1 and Mcp2 may complement the assembly of supercomplexes of respiratory chain through an effect on lipid composition of yeast mitochondria.

Indeed analyzing alterations of the lipid composition in *mdm10*Δ mitochondria by quantitative mass spectrometry were observed. Of note, similar alterations were reported before (Jiang et al. 2000, Schagger 2002, Zhang et al. 2002, Pfeiffer et al. 2003, Zhong et al. 2004, Nury et al. 2005, Zhang et al. 2005, Kutik et al. 2008). The amounts of PE, PS and CL showed a significant difference between mitochondria from *mdm10*Δ and those from wild-type cells. It is assumed that reduced synthesis level of PE partly results in the accumulation of PS in the mitochondria, which would be toxic to the cells. Over-expression of Mcp1 or re-introduction of Mdm10 greatly changes the PE and PS levels in *mdm10*Δ cells and brings them back to normal levels. Mcp2 shows a much lower rescue effect in agreement with its lower cell growth and mitochondrial

morphology rescue capabilities. Deletion of *MCP1* or *MCP2* did not lead to significant changes in the content of phospholipids. This is in agreement with the fact that no defect in growth phenotype was observed for *mcp1*Δ or *mcp2*Δ cells.

Mcp1 and Mcp2 are also found to be suppressors of deletions of other subunits of the ERMES complex. Mcp1 acts as a stronger suppressor of *mdm10*Δ, *mmm1*Δ and *mmm2*Δ cells, whereas mitochondrial inner membrane protein Mcp2 is the stronger suppressor in *mdm12*Δ cells. Although the exact mechanism for this difference is still unclear, this evidence indicates that Mcp1 plays a different role as Mcp2 does in mitochondria.

In collaboration with Dr. Kai Stefan. Dimmer the genetic interactions of Mcp1 or Mcp2 with any of the four classical subunits of ERMES complex were studied. It was shown by tetrad analysis that *mcp1*Δ in combination with *mdm10*Δ, *mmm1*Δ or *mmm2*Δ and *mcp2*Δ in combination with *mmm1*Δ results in synthetic lethality. Severe synthetic growth defects were found by *mcp1*Δ in combination with *mdm12*Δ and *mcp2*Δ in combination with *mdm10*Δ, *mdm12*Δ and *mmm2*Δ by drop dilution (Tan et al. 2013).

All aforementioned findings support a hypothesis that the pathway of mitochondrial lipid exchange by Mcp1 or Mcp2 is a “bypass route” of the default pathway by the ERMES complex. In the presence of ERMES complex loss of Mcp1 and Mcp2 has no impact on mitochondrial lipid composition. However, the loss of ERMES as functional complex by deletion of any single subunit can be compensated by over-expressing the “back-up system” of Mcp1 or Mcp2. But deletion of both lipid exchange pathways results in complete blockage of lipid exchange, greatly impairs the mitochondrial functions and leads to extreme negative synthetic genetic effects or lethality. In line with this “by-pass” hypothesis, no physical interactions of Mcp1 or Mcp2 with any subunits of the ERMES complex was found in different pull-down approaches (data not shown). Of note, two other groups have studied the composition of ERMES complex by affinity purification and mass spectrometry analysis (Kornmann et al. 2011, Stroud et al. 2011a). In both independent studies only the four canonical components Mdm10, Mdm12, Mmm1, Mmm2 and the novel regulatory subunit Gem1 were shown to form this complex. Of note, in higher eukaryotes the ERMES complex is absent. Two other systems, the voltage-dependent anion channel (VDAC1) together with IP3-receptor in the ER and MFN2, which is dually localized in the MOM and the ER, were identified as tethering candidates to maintain ER-mitochondrial connection (Szabadkai

et al. 2006a and b, de Brito and Scorran 2010, Toulmay and Prinz 2011). It is well possible that an additional ER-mitochondria connection exists also in yeast. As a by-pass system, Mcp1 and Mcp2 may have no physical connections with ERMES complex and functions independently of this complex. We propose that Mcp1 and Mcp2 maybe form another pathway to regulate the lipid homeostasis. Although defective ERMES complex results in the impaired phospholipids transport between ER and mitochondria, lipid transport from ER to mitochondria still takes place in such mutated yeast cells (Kornmann et al. 2009). A recent report even suggested that the ERMES complex does not directly influence the PS transport from the ER to mitochondria and that maintaining mitochondrial morphology is its main function (Nguyen et al. 2012).

Although the main lipid exchanges take place between ER and mitochondria, proposed alternative lipid transport pathways could also be between mitochondria and other cell organelles. A recent report showed a functional connection between mitochondria and vacuoles within the cardiolipin biosynthesis pathway (Chen et al. 2008). Moreover, lipid particles are also in contact with mitochondria and transport lipid exchange takes place (Beller et al. 2010).

Which are the main classes of phospholipids that are influenced by ERMES and the Mcp1/2 system? The levels of CL and PE were reduced dramatically in mitochondria lacking Mdm10. Over-expression of Mcp1 or Mcp2 only restored PE levels to a certain extend. CL and PE both are so called non-bilayer forming lipids due to their cone-like shape. It was reported that PE and CL can compensate for each other (Gohil et al. 2005). Furthermore yeast cells with simultaneous reduction of CL and PE levels leads to defective mitochondria (Osman et al. 2011). In line with these observations and our results we propose that functional mitochondria regarding morphogenesis and assembly of respiratory chain supercomplexes need a certain threshold of PE and CL levels. Over-expression of Mcp1 and a lower extend of Mcp2 lead to an increase of PE and therefore the threshold of non-bilayer forming lipids exceed the critical amount in *mdm10*Δ cells. Supporting this assumption, Mdm31 that was reported as a suppressor of *mdm10*Δ cells (Tamura et al. 2012) increased the level of cardiolipin in mitochondria and thus rescued the growth phenotype of *mdm10*Δ cells.

Recently, two proteins Ups1 and Ups2 localized in IMS were also found to affect the lipid homeostasis mitochondria. They control the transport of PA from MOM to MIM and regulate the conversation of PA to CL (Tamura et al. 2009, Potting et al. 2010,

Tamura et al. 2012, Connerth et al. 2012). Therefore they are involved in the intra-mitochondrial lipid traffic. We showed that Mcp1 and Mcp2 are contributing to the lipid homeostasis of whole mitochondria. Yet over-expression of Mcp1 or Mcp2 in cells lacking Ups1 or Ups2 could not rescue the defective growth phenotype of *ups1*Δ and *ups2*Δ mutants (data not shown). This suggests that Mcp1 and Mcp2 are not functionally related to Ups1 or Ups2. Mcps seem to control the global mitochondrial lipid composition through lipid transport to mitochondria, rather than to play a role in the lipid exchange in the mitochondria.

Ergosterol is the major sterol in yeast cells and the enzymes for ergosterol biosynthesis have dual localization, namely the ER and lipid particles (Leber et al. 1998). To test whether deletion of subunits of ERMES complex affect also the ergosterol levels of mitochondria, quantification of ergosterol levels in *mdm10*Δ, *mmm1*Δ and *mdm12*Δ was performed. In comparison to wild-type cells higher ergosterol levels were observed in these mutated cells. Deletion of *MCP1* or *MCP2* has almost no effect on the ratios of ergosterol /phospholipid in comparison to wild-type cells. Re-introduction of Mdm10 or over-expression of Mcp1 in *mdm10*Δ cells lead to a reduction of ergosterol levels to those found in wild-type cells. In contrast, Mcp2 showed only a minor rescue of the ergosterol amounts. Taken together, these findings indicate that Mdm10 and Mcp1 are involved in ergosterol homeostasis of mitochondria. Membrane with higher ergosterol levels has reduced fluidity, thus insertion of membrane proteins will be inhibited. It was reported that down-regulation of genes for ergosterol biosynthesis results in changed composition of membranes and subsequently causes a severe mitochondrial morphology phenotype (Altmann and Westermann, 2005). Thus, it appears that ergosterol plays an important role in regulation of organelle morphogenesis. Moreover, recent studies suggest that some single-span mitochondrial outer membrane proteins can be rightly targeted to mitochondria and inserted into the MOM depending on its low ergosterol content (Kemper et al.2008, Krumpe et al. 2012, Merklinger et al. 2012). Except for ergosterol, the change of other lipid can influence the functions of the protein translocases and insertases embedded in those membrane. For instance, the function of protein import complexes like TOM, TIM22 and TIM23 depend on the amount of cardiolipin (Kutik et al. 2008, Gebert et al. 2009). Finally, this work provides for the first time new evidence that deletion of *MDM10* leads to an alteration of ergosterol levels in the mitochondrial membranes. The high ergosterol levels can explain some defects

especially in mitochondrial morphology and import impairment in mitochondria of *mdm10*Δ cells.

It was shown in this work that Mcp1 and Mcp2 rescue mitochondrial morphology, assembly of respiratory chain supercomplexes and the lipid homeostasis of mitochondria lacking Mdm10, yet the precise molecular mechanism of both proteins is so far unknown. By bioinformatics analysis the primary sequences of Mcp1 and Mcp2 were also analyzed (Biegert et al. 2006). The closest homologue of Mcp1 is quinol-fumarate reductase diheme cytochrome B subunit C of *Wolinella succinogenes*, a subclass of proteobacteria. Quinol-fumarate reductase is a member of the complex II (succinate dehydrogenase, succinate:quinone reductase) family. It is identified as a terminal electron acceptor during anaerobic respiration. The reduction of fumarate to succinate is coupled with the oxidation of quinol to quinone by this reductase (Madej et al. 2006, Singh et al. 2013). Mcp1 is predicted to contain the heme binding domain and therefore might play a role in electron transfer due to the heme prosthetic groups. Since Mcp1 is a MOM protein, it is unlikely that Mcp1 could be involved in electron transport during oxidative phosphorylation. Yet a hypothetical function might be the involvement in desaturation reactions which modify the fatty acid composition of mitochondrial lipids. Mcp1 is in optimal position as MOM protein to mediate the contact between mitochondria and ER or other cellular structures. Increased amount of Mcp1 by over-expression could increase such contact areas to facilitate lipid trafficking. In contrast to over-expression of ERMES subunits, that accumulate in punctuate structures on mitochondria (Kornmann et al. 2009), Mcp1 distributed evenly in the mitochondrial membrane as shown by immunofluorescence microscopy. This observation favors a global enzymatic function of Mcp1 in the MOM rather than a localized contact-mediating role.

Mcp2 is predicted to contain a putative kinase domain. The closest homologue in higher eukaryotes is the uncharacterized AarF Domain Containing Kinase 1 (ADCK1). Mcp2 most probably possesses kinase activity. Indeed our previous results indicate that mutations of conserved residues of kinase domain of Mcp2 influence its functionality. The potential substrates of kinase Mcp2 are yet unknown. But Mcp2 could either phosphorylate proteins to therefore harbor a regulative function or small molecules by phosphate transferring, such as lipid or lipid precursors. In this case Mcp2 would be involved directly in lipid metabolism. In addition, Mcp2 is localized in

the MIM. Sucrose density gradient separation of mitochondrial vesicles and immunodetection showed a small shift of Mcp2 to vesicles with lower density in comparison to classical MIM proteins. This suggests that Mcp2 slightly enriches at the so called inner boundary membrane. Therefore Mcp2 could get the contact between the MOM and MIM closer.

Taken together, two novel mitochondrial proteins Mcp1 and Mcp2 were identified in our study. They complement the alteration of mitochondrial morphology and stabilize the mitochondrial respiratory chain supercomplexes in *mdm10*Δ cells. Mdm10 was discovered to play a crucial role in the ergosterol homeostasis of the organelle. Importantly, Mcp1 and Mcp2 are also found to be involved in the mitochondrial lipid homeostasis and are maybe involved in a lipid transport pathway between ER and mitochondria that is independent of the ERMES complex.

5. Summary

Mdm10 is a β-barrel protein that is localized to the mitochondrial outer membrane (MOM). It is found in fungi but is not conserved in higher eukaryotes. Mdm10 is involved in several processes of mitochondrial biogenesis. It is a subunit of a subpopulation of the TOB complex and affects the insertion of β-barrel proteins. In addition, loss of Mdm10 directly impairs mitochondrial genome stability, movement, inheritance, morphogenesis and lipid composition. Recently, Mdm10 was identified as a subunit of the ERMES complex involved in formation of contact sites between the ER and mitochondria in yeast. Despite its importance for mitochondria, the primary function of Mdm10 is so far unclear.

To decipher the precise function of Mdm10, a screen for high-copy suppressors of the growth phenotype of *mdm10*Δ cells was performed. Two novel mitochondrial proteins named Mcp1 and Mcp2 were identified as such multi-copy suppressors. Mcp1 is a multi-span protein in the MOM, whereas Mcp2 is located to the mitochondrial inner membrane (MIM). Over-expression of Mcp1 or Mcp2 can completely or partly complement the growth defect and alteration of mitochondrial morphology in cells lacking Mdm10. Accordingly, such over-expression leads to rescue of the assembly phenotype of respiratory chain supercomplexes observed in *mdm10*Δ cells. However, over-expression of Mcp1 or Mcp2 cannot restore the reduced assembly of TOM and TOB complexes in *mdm10*Δ cells. Mcp1 and Mcp2 are also found to be suppressors of the deletion growth phenotype of other subunits of the ERMES complex. Lipid analysis by quantitative mass spectrometry showed that over-expression of Mcp1 or Mcp2 partly complements the alterations in phospholipid composition in mitochondria isolated from cells lacking Mdm10. Furthermore, high ergosterol levels were observed in cells lacking any subunit of the ERMES complex and these elevated amounts can be reduced by over-expression of Mcp1.

Collectively, a working hypothesis can be proposed in which Mcp1 and Mcp2 build a “bypass route” to the default lipid transport pathway mediated by the ERMES complex in yeast. Both proteins seem to play an important role in mitochondrial lipid homeostasis. In addition, this work adds new insight into the function of Mdm10 and the ERMES complex by demonstrating that this complex takes part in the homeostasis of ergosterol in mitochondria.

6. Supplementary Table

Table S1: Amount of phospholipids [mol% ± SD] and ergosterol/phospholipid ratio [ERG/PL ± SD] of mitochondria isolated from the indicated strains:

	WT +pYX142	*mdm10*Δ +pYX142	*mdm10*Δ +MDM10	*mdm10*Δ +MCP1	*mdm10*Δ +MCP2	*mcp1*Δ +pYX142	*mcp2*Δ +pYX142
PC	17.06 ± 1.71	17.38 ± 2.67	16.20 ± 2.61	14.18 ± 2.09	13.56 ± 2.33	14.96 ± 3.14	17.70 ± 3.05
PE	43.06 ± 4.37	30.26 ± 0.93	46.06 ± 5.98	45.59 ± 5.99	37.13 ± 4.38	46.04 ± 4.46	41. 25 ± 2.38
PI	11.02 ± 2.85	10.58 ± 2.51	7.73 ± 2.28	8.44 ± 1.69	11.16 ± 3.09	9.95 ± 2.07	9.73 ± 1.30
PS	3.68 ± 1.21	7.57 ± 1.84	3.65 ± 1.54	4.17 ± 1.57	5.98 ± 2.33	3.16 ± 0.87	3.47 ± 0.96
PG	0.67 ± 0.23	0.85 ± 0.36	0.96 ± 0.35	0.91 ± 0.42	0.61 ± 0.27	0.83 ± 0.20	0.93 ± 0.31
CL	10.94 ± 1.66	7.24 ± 2.49	10.82 ± 2.98	8.00 ± 1.81	8.43 ± 2.47	11.28 ± 1.58	10.59 ± 1.74
ERG	13.55 ± 1.48	26.13 ± 3.98	14.58 ± 2.74	18.72 ± 3.16	23.13 ± 3.28	13.78 ± 4.11	16.33 ± 1.62
Ergosterol/phospholipid ratio							
ERG/PL	0.16 ± 0.02	0.36 ± 0.07	0.17 ± 0.04	0.23 ± 0.05	0.30 ± 0.05	0.16 ± 0.06	0.20 ± 0.02

Table S2. Relative amount of different species of the single phospholipid

Phosphatidylcholine

>0.2%	mean						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PC 28:1	1.03	0.40	0.80	0.90	0.93	1.29	0.99
PC 30:2	0.43	0.26	0.57	0.48	0.35	0.52	0.32
PC 30:1	1.94	1.44	1.97	1.99	1.71	2.57	2.16
PC 30:0	0.24	0.05	0.19	0.20	0.23	0.31	0.28
PC 32:2	27.79	27.73	30.30	27.86	24.05	27.13	28.06
PC 32:1	12.49	9.42	12.47	12.36	11.74	14.49	14.28
PC 34:2	35.53	41.46	34.25	35.68	38.12	32.47	33.23
PC 34:1	11.61	9.48	10.96	10.89	12.17	12.56	12.43
PC 36:2	5.15	6.42	5.01	5.69	6.49	4.85	4.52
PC 36:1	3.32	2.99	2.92	3.25	3.61	3.29	3.19

PC 36:0	0.16	0.12	0.20	0.20	0.15	0.16	0.19
PC 38:0	0.24	0.17	0.29	0.40	0.38	0.28	0.26

	Standard deviation (SD)						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PC 28:1	0.21	0.04	0.21	0.43	0.08	0.16	0.12
PC 30:2	0.05	0.08	0.27	0.09	0.10	0.14	0.05
PC 30:1	0.01	0.02	0.36	0.35	0.11	0.44	0.17
PC 30:0	0.02	0.07	0.06	0.04	0.07	0.07	0.08
PC 32:2	0.11	2.61	6.19	2.06	2.41	4.32	4.09
PC 32:1	1.31	2.49	6.01	4.08	2.43	1.14	2.34
PC 34:2	1.20	2.01	5.42	4.62	2.11	0.57	0.55
PC 34:1	0.20	1.97	4.84	2.89	2.32	2.06	2.61
PC 36:2	0.15	0.07	0.38	1.10	0.33	1.23	0.13
PC 36:1	0.08	0.52	0.82	0.24	0.24	0.08	0.18
PC 36:0	0.03	0.17	0.00	0.04	0.03	0.11	0.03
PC 38:0	0.09	0.00	0.07	0.07	0.06	0.20	0.14

Phosphatidylethanolamine

>0.5%	mean						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PE 32:2	21.25	21.52	21.80	23.64	23.43	23.46	24.35
PE 32:1	7.26	3.29	9.06	6.01	4.33	8.68	7.60
PE 34:2	56.13	61.69	51.66	56.75	60.88	51.22	52.43
PE 34:1	4.89	2.98	6.84	4.28	3.53	5.90	4.73
PE 36:2	9.04	9.55	9.07	8.17	6.78	9.18	9.45

	Standard deviation (SD)						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PE 32:2	0.26	3.55	4.11	2.29	0.15	0.28	1.38
PE 32:1	0.52	0.01	2.07	0.17	0.22	1.06	0.07

PE 34:2	1.15	0.33	2.51	0.71	0.30	1.80	0.21
PE 34:1	0.55	0.79	3.05	0.71	0.01	1.14	0.20
PE 36:2	1.17	3.04	0.30	0.09	0.14	2.21	0.24

Phosphatidylserine

>0,5%	mean						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PS 32:2	9.08	9.94	7.59	10.21	11.09	10.78	11.21
PS 32:1	9.57	5.47	9.81	8.90	8.01	11.04	10.48
PS 34:2	56.81	64.39	50.72	57.22	57.35	52.06	54.78
PS 34:1	21.46	17.53	28.33	20.11	20.03	21.99	20.57
PS 36:2	0.60	1.13	1.08	0.81	1.19	1.18	0.65
PS 36:1	0.39	0.26	0.35	0.74	0.62	0.28	0.62

	Standard deviation (SD)						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PS 32:2	1.20	3.29	2.38	0.05	1.44	0.66	3.87
PS 32:1	2.16	1.48	6.23	1.36	0.18	2.92	3.96
PS 34:2	8.03	4.49	17.22	7.21	2.02	10.29	11.87
PS 34:1	0.90	2.08	9.66	2.18	0.47	4.30	8.22
PS 36:2	0.51	0.36	0.21	0.41	0.05	0.45	0.47
PS 36:1	0.31	0.37	0.49	0.47	0.34	0.39	0.68

Cardiolipin

>1%	mean						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
CL 64:4	2.08	2.15	1.83	1.93	1.98	2.59	2.33
CL 64:3	1.83	2.05	1.73	1.75	1.75	2.05	1.79
CL 64:2	1.06	1.16	1.16	1.19	1.13	1.25	1.02

CL 66:7	1.48	1.61	1.45	1.51	1.59	1.73	1.54
CL 66:6	1.21	1.27	1.03	1.12	1.21	1.25	1.08
CL 66:5	1.01	1.09	0.95	1.00	1.10	1.17	0.97
CL 66:4	7.10	6.97	6.24	6.13	6.47	8.75	8.31
CL 66:3	4.89	5.71	5.28	5.26	5.19	5.66	5.07
CL 66:2	3.47	3.70	4.12	3.72	3.32	3.80	3.37
CL 66:1	1.07	1.03	1.36	1.23	1.32	1.35	1.12
CL 68:7	2.67	2.48	2.14	2.36	2.50	2.62	2.58
CL 68:6	2.10	2.21	1.80	2.14	2.06	1.92	1.82
CL 68:5	1.69	1.67	1.82	1.80	1.72	1.61	1.55
CL 68:4	8.35	7.36	7.16	6.79	7.25	10.24	9.91
CL 68:3	10.57	11.24	11.35	11.78	11.54	10.42	10.72
CL 68:2	5.74	5.29	7.19	5.89	5.35	5.55	5.27
CL 70:7	3.15	2.71	2.33	2.53	2.73	2.83	3.03
CL 70:6	4.81	4.93	4.27	5.30	5.01	3.41	4.03
CL 70:5	2.71	2.47	3.15	2.84	2.64	2.16	2.29
CL 70:4	5.46	4.58	4.64	4.25	4.80	6.56	6.62
CL 70:3	11.16	12.05	12.02	13.12	12.83	9.27	10.57
CL 70:2	4.14	3.89	5.13	4.21	4.24	3.66	3.78
CL 72:7	1.36	1.26	1.03	1.08	1.23	1.27	1.34
CL 72:6	3.66	3.88	3.17	3.89	3.88	2.13	2.67
CL 72:5	1.37	1.27	1.63	1.43	1.44	1.08	1.15
CL 72:4	1.26	1.23	1.17	1.02	1.21	1.55	1.55
CL 72:3	3.15	3.40	3.59	3.38	3.06	2.61	3.14

	Standard deviation (SD)						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
CL 64:4	0.00	0.01	0.13	0.16	0.63	0.10	0.49
CL 64:3	0.40	0.29	0.07	0.16	0.16	0.04	0.02
CL 64:2	0.35	0.19	0.24	0.42	0.00	0.01	0.15
CL 66:7	0.45	0.45	0.40	0.24	0.45	1.16	0.42
CL 66:6	0.39	0.46	0.37	0.39	0.48	0.91	0.45
CL 66:5	0.19	0.26	0.26	0.13	0.31	0.78	0.28

CL 66:4	0.62	1.07	0.85	0.61	1.74	0.95	1.07
CL 66:3	1.34	1.30	1.24	1.25	0.30	0.92	0.64
CL 66:2	1.32	1.43	0.95	1.57	0.21	0.36	0.73
CL 66:1	0.02	0.17	0.16	0.18	0.04	0.42	0.01
CL 68:7	0.61	0.23	0.18	0.47	0.66	0.90	0.67
CL 68:6	0.41	0.64	0.19	0.27	0.44	0.98	0.41
CL 68:5	0.31	0.30	0.34	0.11	0.26	0.90	0.14
CL 68:4	1.51	1.63	1.44	0.62	1.31	3.17	0.44
CL 68:3	1.73	0.88	2.11	2.06	2.42	2.40	1.85
CL 68:2	1.33	1.87	0.42	1.53	1.17	0.48	1.08
CL 70:7	0.59	0.21	0.21	0.73	0.86	0.80	0.78
CL 70:6	1.64	2.12	0.46	0.69	0.15	1.48	0.24
CL 70:5	0.91	0.59	1.26	0.64	0.25	1.26	0.36
CL 70:4	1.02	1.01	0.94	0.37	0.51	2.50	0.19
CL 70:3	0.66	1.50	0.16	0.56	3.23	1.40	1.40
CL 70:2	0.10	0.16	1.62	0.01	0.76	0.34	0.40
CL 72:7	0.33	0.14	0.18	0.34	0.40	0.31	0.31
CL 72:6	1.76	1.84	0.76	0.91	0.40	0.79	0.09
CL 72:5	0.67	0.51	0.98	0.61	0.08	0.74	0.24
CL 72:4	0.07	0.21	0.07	0.10	0.15	0.40	0.06
CL 72:3	0.24	0.22	0.64	0.08	0.53	0.30	0.40

Phosphatidylinositol

	mean						
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PI 32:2	5.11	4.88	7.05	5.76	5.29	5.82	5.61
PI 32:1	24.42	17.71	23.77	21.37	19.27	24.24	24.30
PI 34:2	11.67	15.00	11.44	13.76	12.57	10.44	10.12
PI 34:1	44.09	40.17	39.74	42.32	44.64	43.91	44.19
PI 36:1	14.18	20.75	17.38	16.15	16.99	14.97	15.13

Standard deviation (SD)							
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PI 32:2	1.16	2.90	0.03	0.54	0.91	0.69	0.53
PI 32:1	0.80	0.85	5.50	0.63	0.31	2.69	0.90
PI 34:2	0.35	0.85	3.94	0.58	1.93	1.20	2.99
PI 34:1	0.08	3.20	2.81	1.42	0.92	1.23	0.73
PI 36:1	1.37	6.16	2.15	1.67	1.99	0.56	2.29

Phosphatidylglycerol

mean							
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PG 32:2	5.77	7.87	6.75	7.55	8.09	8.05	6.76
PG 32:1	23.37	21.57	24.36	26.59	21.70	23.03	25.66
PG 34:2	15.04	20.64	14.19	13.85	17.50	16.01	14.51
PG 34:1	46.58	41.13	46.17	43.31	37.09	41.74	45.99
PG 36:2	2.13	2.42	2.10	1.75	3.77	2.69	1.34
PG 36:1	3.67	2.33	3.75	3.11	5.87	2.58	1.65

Standard deviation (SD)							
	WT + pYX142	*mdm10*Δ +pYX142	*mdm10*Δ + *MDM10*	*mdm10*Δ + *MCP1*	*mdm10*Δ + *MCP2*	*mcp1*Δ + pYX142	*mcp2*Δ + pYX142
PG 32:2	3.10	3.14	5.36	0.63	0.99	1.72	1.55
PG 32:1	0.59	0.92	5.17	4.93	2.39	2.85	1.52
PG 34:2	1.41	5.93	8.46	4.24	2.25	2.89	0.86
PG 34:1	12.61	3.56	13.69	6.15	2.54	5.83	6.51
PG 36:2	1.97	1.01	0.99	1.39	0.46	0.90	1.23
PG 36:1	0.67	1.75	0.25	0.78	0.48	1.32	1.31

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8. Abbreviations

Δ	deletion
AA	acrylamide
ABC1	ATP-binding cassette 1
ADCK	aarF domain containing kinase
ADP	adenosine diphosphate
Amp	ampicillin
APH	aminoglycoside 3'-phosphotransferase
ATP	adenosine triphosphate
BAM	β-barrel assembly machinery
BN-PAGE	blue native polyacrylamide gel electrophoresis
bp	base pairs
BSA	bovine serum albumin
C-	carboxyl-
CDP	cytidine diphosphate
ChiMERA	construct helping in mitochondria-ER association
ChoK	choline Kinase
CJ	cristae junction
CL	cardiolipin
CoQ	Coenzyme Q
Cor	CORe protein of QH2 cytochrome c reductase
Cox	cytochrome c oxidase
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
Dnm1	dynamin 1
Drp1	human dynamin-related protein 1
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
Erg/ERG	ergosterol
ERMES	the ER mitochondria encountering structure
Erv	essential for respiration and viability
ETC	electron transport chain
FADH	flavin adenine dinucleotide
Fcj1	formation of CJ protein 1
Fig.	figure
Fis1	mitochondrial fission protein 1
Fzo1	FuZzy Onions homolog 1
g	standard gravity
Gem	GTPase EF-hand protein of Mitochondria
GFP	green fluorescent protein
GrnP	dihydroxyacetone phosphate
GTP	guanosine triphosphate
HA	Hemagglutinin

HEPES	4-(2-Hydroxyethyl)-piperazine-N'-ethane-2-sulfonic acid
HIS	histidine
HRP	horse raddish peroxidase
Hsp	heat shock protein
IMP	inner membrane peptidase
IMS	Intermembrane space
Kan	kanamycin
kDa	kilodalton
l	liter
LB	Luria Bertani
Leu	leucine
MAM	mitochondria-associated membranes
MAT	mating type
Mcp	Mdm10 complementing protein
MCS	membrane contact site
Mdj1	mitochondrial DnaJ 1
Mdm	mitochondrial distribution and morphology
Mfn	mitofusin family
Mgm	mitochondrial genome maintenance
Mia40	mitochondrial intermembrane space import and assembly
MICO	mitochondrial contact site
Mim	mitochondrial import
MIM	mitochondrial inner membrane
Miro	mitochondrial Rho-GTPase
Mmm1	mitochondrial morphology maintenance 1
MOM	mitochondrial outer membrane
MOPS	3-(N-morpholino)propane sulphonic acid
MPP	mitochondrial processing peptidase
mRNA	messenger RNA
mt	mitochondrial
mtDNA	mitochondrial DNA
N-	aminio-
NADH	nicotinamide adenine dinucleotide
NDL	NuDeL homolog
OD	optical density
Omp25	outer membrane protein 25
ORF	open reading frame
OXA1	cytochrome oxidase activity protein 1
OXPHOS	mitochondrial oxidative phosphorylation system
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
Pfu	*Pyrococcus furiosus*
PG	phosphatidylglycerol
PI	phosphatidylinositol

PK	proteinase K
PL	phospholipid
PMSF	phenylmethylsulfonyfluoride
PORTA	polypeptide transport associated
PS	phosphatidylserine
Psd	phosphatidylserine decarboxylase
Pss	phosphatidylserine synthase
PVDF	polyvinylidene difluoride
RFP	red fluorescent protein
Rho	ras homolog
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SAM	sorting and assembly machinery
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SMP	synaptotagmin-like, mitochondrial and lipid-binding protein
SW	swelling
SYM	stress-inducible Yeast Mpv17
Taq	*Thermophilus aquaticus*
TBS	Tris buffered saline
TBST	Tris buffered saline with tween 20
TCA	trichloroacetic acid
TEMED	N,N,N‘,N‘-tetramethylene diamine
TIM	translocase of the inner mitochondrial membrane
TLC	thin layer chromatography
TMD	transmembrane domain
TOB	topogenesis of mitochondrial outer membrane β-barrel proteins
TOC	translocon of outer membranes of chloroplasts
TOM	the translocase of the outer membrane
Tom40	subunit of TOM complex 40
TPI	triose phosphate isomerase
tRNA	transfer RNA
Trp	tryptophan
TX-100	Triton X-100
Ugo	UGO (Japanese for fusion)
UTR	untranslated region
UV	ultra violet
v/v	volume per volume
VDAC	the voltage-dependent anion channel
w/v	weight per volume
wt/WT	wild type
WTM	WD repeat containing Transcriptional Modulator
α	antibody

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10. Curriculum Vitae

Tao Tan (谈涛)

Personal data:

Name, surname:	Tao, Tan
Date of birth:	28.11.1979
Place of birth:	Wuxi, Province Jiangsu, China
Nationality:	Chinese

School education:

1986-1992	Nanjing Youfu West Road Primary School
1992-1998	Nanjing No.1 High School
June 1998	High school diploma

Academic education:

1998-2002	Studies of biotechnology at Jiangnan University, China (Bachelor engineer)
2004-2008	Studies of biology at Technische Universität Kaiserslautern, Germany
2009-2013	PhD research in the lab of Prof. Dr. Doron Rapaport at Interfaculty Institute of Biochemistry, Eberhard Karls Universität Tübingen, Germany The topic of the PhD thesis: Mcp1 and Mcp2, two novel proteins involved in mitochondrial lipid homeostasis