Characterization of the genetic interactome of prohibitins in *S. cerevisiae*

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Abstract

Prohibitins comprise an evolutionary conserved and ubiquitously expressed family of membrane proteins that is essential for development in higher eukaryotes. Large ring complexes formed in the inner mitochondrial membrane by prohibitins regulate mitochondrial dynamics and function. Roles of prohibitins in cell signaling events across the plasma membrane and transcriptional regulation in the nucleus have been proposed as well. The molecular mechanism of prohibitin function, however, remains elusive. In contrast to higher eukaryotes, prohibitin-deficient yeast cells are viable and exhibit a reduced replicative life-span.

To investigate the functional role of prohibitins in yeast and to identify redundant processes that fulfill the functions of prohibitins in their absence, an unbiased genetic approach was chosen. Synthetic genetic arrays were applied to identify genes showing synthetic lethal interactions with prohibitins. This approach revealed 35 genes required for cell survival in the absence of prohibitins. The assembly of the F_O-particle of the F_1F_O-ATP synthase was identified as one process essential in prohibitin-deficient cells. Atp23 was characterized as a novel processing peptidase with a dual function in maturation of the mitochondrially encoded subunit Atp6 and its assembly into the functional F_1F_O-ATP synthase. ~50% of the genes required in prohibitin-deficient cells, including the strongest genetic interactions, are demonstrated for the first time to be required for mitochondrial phospholipid homeostasis. Evidence is provided that members of a conserved protein family, Ups1 and Gep1, coordinately regulate the levels of the non-bilayer forming phospholipids cardiolipin and phosphatidyl-ethanolamine in mitochondria. Additionally, an uncharacterized putative phosphatase was identified that is required for cardiolipin biosynthesis and might represent the last missing enzyme in the biosynthesis pathway of cardiolipin in yeast.

The genetic interactome of prohibitins defined in this thesis suggests that prohibitins serve scaffolding functions in the inner mitochondrial membrane and define functional

microdomains composed of proteins and non-bilayer forming lipids. This function becomes essential, when levels of cardiolipin and phosphatidylethanolamine are limiting in mitochondria. In the absence of prohibitins and decreased levels of non-bilayer forming lipids, essential processes dependent on these microdomains are compromised.

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1 Introduction

1.1 Cellular compartmentalization

One of the key steps for life to become possible was the first sequestration of organic molecules that allowed the formation of a certain organization protected against the exterior environment. This very day the formation of aqueous compartments is still one of the most fundamental principles for the development of living cells. Besides a plasma membrane that demarcates a cell from the surrounding environment, eukaryotic cells have evolved an intricate system of intracellular compartments that separate and enable processes with complete distinct biochemical requirements. One of these specialized compartments is represented by the mitochondrium that distinguishes itself from other organelles (excluding chloroplasts) as it is surrounded by two different cellular membranes that originate from an endosymbiotic event between a eukaryotic cell and an α-proteobacterium (Wallace, 2005). Four different compartments can thus be distinguished in mitochondria: the outer mitochondrial membrane, the intermembrane space, the cristae forming inner mitochondrial membrane and the matrix space. The best known role of mitochondria is their function in the generation of cellular ATP by oxidative phosphorylation that is accomplished by high molecular weight multisubunit complexes in the inner membrane. Besides this, mitochondria also harbour the Krebs cycle, enzymes required for heme and Fe/S cluster biosynthesis as well as the metabolism of certain amino acids. Furthermore, mitochondria also exert crucial functions during programmed cell death and calcium signaling (Chan, 2006; McBride et al., 2006). Consistent with roles of mitochondria in all these processes, a variety of human disorders have been linked to mitochondrial dysfunction (Chan, 2006; Wallace, 2005). It has to be noted that mitochondria are essential organelles in all species (Lill and Kispal, 2000). Therefore, any severe perturbation of mitochondrial biogenesis impacts dramatically on the viability of the whole cell.

The traditional textbook view of mitochondria as bean-shaped and static organelles was renewed by studies in the last years, which show that mitochondria can adopt a variety

of different shapes in different cell types. Additionally, mitochondria constantly move and undergo fusion and fission events that are required for maintenance of mitochondrial morphology and inheritance. Mitochondria need to ensure the coordinated fusion or fission of the outer and the inner membrane. Consistently, elaborated molecular machines have evolved and several conserved components have been identified in the past years that mediate these steps (Hoppins et al., 2007). The importance of the regulation of mitochondrial dynamics is highlighted by the observation that defects in this process have been linked to a variety of diseases (Detmer and Chan, 2007).

1.2 Import of mitochondrial proteins

While compartmentalization is crucial for living cells, it also imposes the problem on cells to ensure import of proteins that are synthesized in the cytosol to their sites of action. The import of mitochondrial proteins has been extensively studied in yeast in the recent years and signal sequences and protein complexes required for the import of proteins into the four different mitochondrial compartments have been identified (Becker et al., 2008; Bolender et al., 2008). Proteins destined for mitochondria are synthesized on cytosolic ribosomes and are recognized by receptors at the outer membrane (Abe et al., 2000; Young et al., 2003). Subsequently, all mitochondrial proteins are transported through the outer membrane import pore, termed the TOM complex (Becker et al., 2005). At that stage, import routes diverge depending on the final localization of the protein to be imported. Many proteins resident in the outer mitochondrial membrane are released from the TOM complex into the lipid bilayer (Gabriel et al., 2003). For a different set of proteins that form β-barrel structures in the outer membrane the action of another complex is required, the SAM complex (Wiedemann et al., 2003). Inner membrane and matrix proteins are in contrast further transferred to the presequence translocase complex (TIM23 complex) in the inner membrane (Chacinska et al., 2005). Proteins taking this route typically have a positively charged N-terminal leader sequence that is responsible for recognition of the protein at the outer membrane and also for further import by the TIM23 complex. The energy that is required for import is derived from two sources. The membrane potential, formed by the respiratory chain, exerts an

electrophoretic effect on the positively charged presequence. Additionally, ATP is used by another complex, the presequence translocase-associated motor (PAM) complex, to either trap the imported protein in the matrix or to actively exert a pulling force (Krayl et al., 2007). Multispanning membrane proteins are integrated into the inner membrane by the help of yet another complex, termed TIM22 complex (Rehling et al., 2003). This import pathway is also dependent on the membrane potential. A fourth import route was identified recently that ensures efficient import of proteins into the intermembrane space. This pathway involves the reversible formation of disulfide bonds between components of the import machinery and the imported protein (Chacinska et al., 2004; Mesecke et al., 2005).

1.3 F_1F_O-ATP synthase assembly

In the course of evolution, the majority of the mitochondrial genome was lost or transferred to the nuclear DNA and only a small percentage of the genome, encoding for eight or thirteen proteins in *S. cerevisiae* or humans, respectively, is still present in mitochondria. Consequently, mitochondria still have transcription and translation machineries for the expression of mitochondrial genes (Chen and Butow, 2005). The respiratory chain complexes and the F_1F_O-ATP synthase in the inner membrane are composed of proteins that are encoded in the nuclear and the mitochondrial genome. Therefore, mitochondria not only have to ensure correct import of nuclearly encoded proteins but they also have to coordinate this import with the expression of mitochondrially encoded genes. This is exemplified by the F_1F_O-ATP synthase, a multisubunit complex in the inner membrane that utilizes the electrochemical gradient, formed by the respiratory chain complexes, for the generation of ATP. The functional F_1F_O-ATP synthase in yeast is composed of at least 21 different structural subunits of which three are mitochondrially encoded (Velours and Arselin, 2000). The complex can be structurally separated into the membrane embedded F_O-sector and the hydrophilic F_1-particle (**Figure 1**). The F_O-part contains the proton transducing channel and a peripheral stator stalk. The F_1-part contains a central stalk and the catalytic headpiece responsible for the catalysis of the reversible formation of ATP from ADP+P_i. For ATP synthesis, protons are translocated through the F_O-sector and this results in the rotation

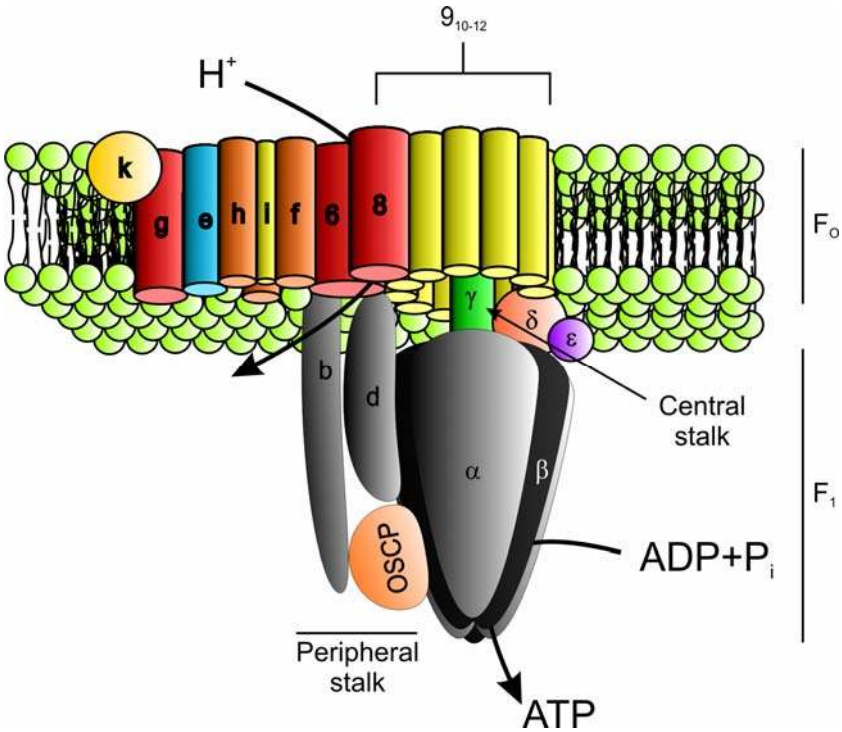

Figure 1 Schematic model of the F_1F_O-ATP synthase of *S. cerevisiae* (Adapted from Ackerman and Tzagoloff, 2005)

of the central stalk, which sequentially interacts with the six subunits constituting the headpiece, which causes conformational changes that result in the synthesis of ATP (Nakamoto et al., 1999). During this process the headpiece is fixed to the peripheral stator stalk to avoid futile rotation (Nakamoto et al., 1999).

The F_O- and the F_1-part appear to have the ability to assemble in the absence of the respective other one and both have their own set of assembly factors that act at different steps, ranging from gene transcription to the final assembly steps (Ackerman and Tzagoloff, 2005). While henceforth nine genes were identified to facilitate steps at mtDNA or RNA levels, only four genes function on the protein level. Atp11 and Atp12 are two proteins that are required for proper assembly of the F_1-part. Current models suggest that Atp11 and Atp12 maintain the unassembled subunits of the F_1-headpiece $F_1\beta$ and $F_1\alpha$, respectively, in an assembly-competent state (Ackerman, 2002).

The knowledge about the biogenesis of the membrane embedded F_O-moiety is likewise limited. Atp6, Atp8 and Atp9 are mitochondrially encoded subunits that form the core structure of the proton transducing channel and are believed to be inserted into the inner membrane co-translationally, but evidence for the responsible proteins mediating this insertion is lacking. Interestingly, yeast Atp6 is synthesized with a 10 amino acid long extension at the N-terminus, which is cleaved off following its integration into the inner membrane (Michon et al., 1988). The peptidase responsible for this maturation, however, has not been identified to date. Subsequent, to its integration into the membrane Atp9 has the ability to self-assemble into a ring structure (Arechaga et al., 2002). The next step of assembly is chaperoned by Atp10 that binds Atp6 and assists its assembly with the Atp9 ring (Tzagoloff et al., 2004). This step needs to be tightly controlled, as imprecise assembly could lead to proton leakage through the proton channel formed by the Atp9 oligomer and Atp6. How subsequent assembly processes are coordinated, specifically at what step the proton transducing channel assembles with the peripheral stalk and the F_1-moiety and whether this is under the control of specific proteins is currently unknown.

1.4 Mitochondrial quality control system

The above described considerations point out that biogenesis of mitochondrial protein complexes is a highly intricate process that requires assistance by a multitude of proteins. Removal of misfolded or damaged proteins by mitochondrial proteases is of high importance to maintain integrity of mitochondrial membranes. Many proteases have been identified in the proteome of mitochondria and it became evident that the function of these proteins is not restricted to control of protein quality but that they also play essential roles in regulatory processes. Most of the proteases identified in the mitochondrial proteome have been studied and can be grouped into three different classes: Processing peptidases, ATP-dependent proteases and oligopeptidases (Koppen and Langer, 2007). Several other proteases, though, still await their assignment into one of these functional classes.

Many mitochondrial proteins that are synthesized in the nucleus possess N-terminal signal sequences that ensure correct targeting, but are not required for the function of the proteins. These sequences are removed by the hetero-dimeric mitochondrial processing peptidase (MPP), a metallopeptidase in the mitochondrial matrix (Gakh et al., 2002). Subsequently, many proteins are further processed by the mitochondrial intermediate peptidase (MIP) that removes an octapeptide from the pre-proteins. The inner membrane protease IMP exposes its catalytic domains to the intermembrane space and is required for maturation of a variety of pre-proteins that are localized in this mitochondrial compartment (Gakh et al., 2002). A processing peptidase with a regulatory role is represented by the rhomboid protease Pcp1 in the inner membrane that cleaves polypeptide bonds within the membrane bilayer (McQuibban et al., 2003). Two substrates of Pcp1 have been identified in yeast: Mgm1, a GTPase required for mitochondrial fusion, is processed by Pcp1 which results in the generation of a short isoform. Processing of the reactive oxygen scavenger protein Ccp1, the other known substrate of Pcp1, is mediated by the help of the *m*-AAA protease that dislocates Ccp1 through the inner membrane to expose the target sequence to the catalytic center of Pcp1 (Esser et al., 2002; Tatsuta et al., 2007).

Members of the second class of proteases, the ATP-dependent proteases, have been identified in all mitochondrial compartments with the exception of the outer membrane. The homo-oligomeric PIM1 protease is present in the mitochondrial matrix (Van Dyck et al., 1994). The *m*-AAA protease and the *i*-AAA protease are anchored in the inner membrane and expose their catalytic centers to the matrix or the intermembrane space, respectively (Langer, 2000). Hydrolysis of ATP is used by these proteases for the unfolding of substrates or the extraction of proteins from membranes that has to precede degradation. Yeast cells devoid of either of these proteases display severe phenotypes (Van Dyck and Langer, 1999). The nature of all these phenotypes is, however, not fully understood to date, but they appear to reflect two roles of these proteases. On one hand, ATP-dependent proteases remove damaged or otherwise harmful proteins and on the other hand, they exert important regulatory activities that are indispensable for mitochondrial biogenesis (Koppen and Langer, 2007; Nolden et

al., 2005). Oligopeptidases represent a third class of mitochondrial proteases that encompasses members localized to the matrix and the intermembrane space of mitochondria. These enzymes function in the degradation of oligopeptides that are generated by processing peptidases and ATP-dependent proteases (Kambacheld et al., 2005).

1.5 Phospholipids, the major components of mitochondrial membranes

Research on the import of proteins into the various mitochondrial compartments, their assembly into functional complexes and their degradation has identified a plethora of components that facilitate all possible steps in these processes. Consequently, a fairly well defined picture has emerged. This, however, only represents half of the story of mitochondrial biogenesis. Phospholipids that are the major components of mitochondrial membranes have to be synthesized and assembled into functional membranes as well. Elucidation of these processes, however, has attracted less interest in the past years and lags behind (Voelker, 2004). Mitochondria, like other cellular membranes harbour many more lipid species than would be required for simply establishing water impermeable barriers. All these species have distinct properties that serve distinct functions, of which only the minority has been elucidated (van Meer et al., 2008).

The spectrum of mitochondrial lipids in *S. cerevisiae* comprises glycerophospholipids and to a minor extent the sterol ergosterol (Zinser and Daum, 1995). In contrast, sphingolipids that are present in other cellular membranes do not occur in mitochondria. Phosphatidic acid is the basic building block of glycerophospholipids and consists of a glycerol backbone whose three hydroxyl groups are esterified with two fatty acids and a phosphate. Different species of these phospholipids are defined by the attachment of different head groups to the phosphate, which comprise choline, ethanolamine, serine, inositol and glycerol (van Meer et al., 2008) (**Figure 2**). Additionally, mitochondria possess cardiolipin, a phospholipid that is not found elsewhere in the cell and is somewhat special in its architecture as it contains two phosphatidic acid moieties that are held together by a glycerol bridge (Schlame, 2008).

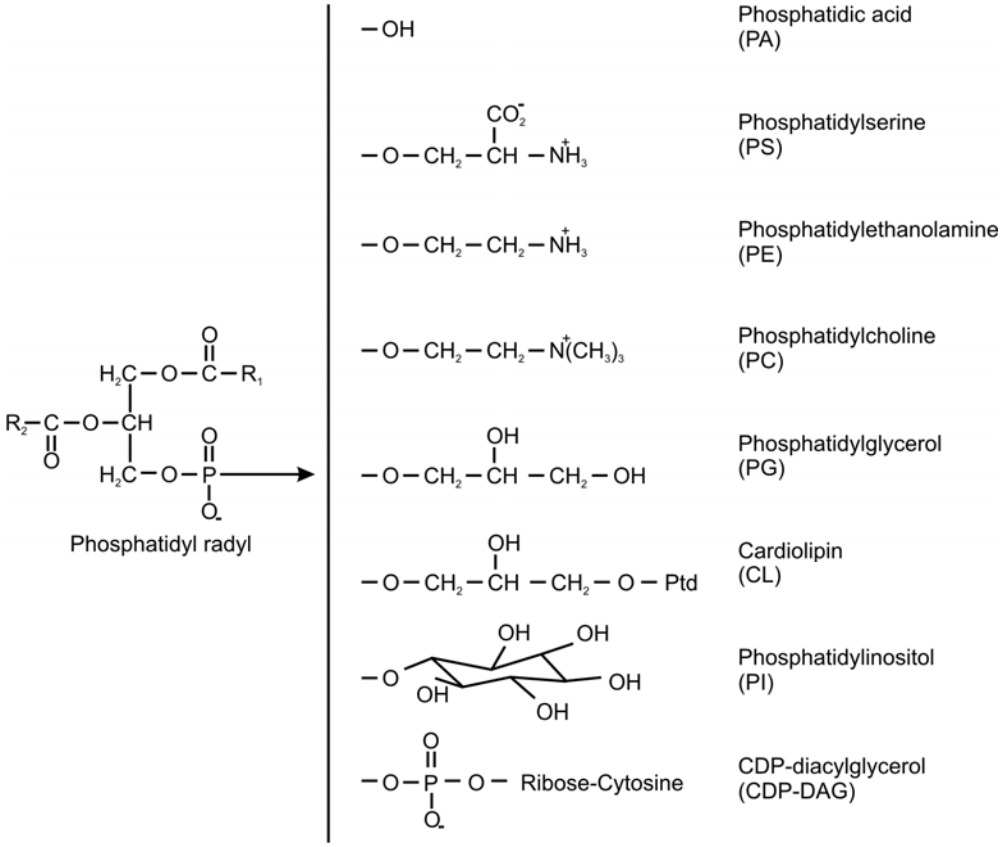

Figure 2 Representation of mitochondrial phospholipids. A glycerol backbone is esterified to two fatty acids (R_1 and R_2) that can vary in length and saturation. Different head groups are attached to the glycerol backbone via a phosphodiester, which gives rise to different phospholipids. (Adapted from Voelker, 2004)

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) account for ~70% of total mitochondrial phospholipids with roughly equal distributions between outer and inner mitochondrial membranes. The main synthesis pathway starts for both phospholipids in a specialized fraction of the endoplasmic reticulum, known as the MAM (mitochondria associated membrane) compartment, where the phosphatidylserine synthase Cho1 synthesizes phosphatidylserine (PS) from serine and CDP-diacylglycerol (CDP-DAG) (Achleitner et al., 1999; Gaigg et al., 1995) (**Figure 3**). Two PS-decarboxylases have been described in *S. cerevisiae* that are capable of converting PS into PE. None of these enzymes is localized in the endoplasmic reticulum and therefore PS needs to be transported to either the inner mitochondrial membrane or the Golgi apparatus to be decarboxylated by Psd1 or Psd2, respectively (Clancey et al., 1993; Trotter et al., 1993;

Trotter and Voelker, 1995). After this step, PE is either transported to its final target membrane or subjected to further modifications that result in PC formation. In this case, PE again requires retranslocation to the endoplasmic reticulum, where the final methylation reactions for the production of PC can take place (Kuchler et al., 1986; Zinser et al., 1991). Once synthesized, PC has to be shuttled to membranes of all cellular compartments. Besides this described biosynthesis pathway, PE and PC can also be formed via the so-called Kennedy pathway, where exogenously added ethanolamine or choline is linked to DAG by a different set of enzymes (Kent, 1995). This pathway, however, contributes very little to the mitochondrial phospholipid supply (Birner et al., 2001).

The third most abundant lipid within mitochondria is cardiolipin, which is preferentially localized to the inner membrane, but small amounts were also identified in the outer membrane (Schlame, 2008). Cardiolipin has been recognized to be required for a variety of different functions in yeast that include aging and apoptosis, mitochondrial protein import, mitochondrial bioenergetics, translational regulation and cell wall biogenesis in yeast (Joshi et al., 2008). Consequently, cardiolipin and its impact on several cellular processes are being studied extensively. For its synthesis, phosphatidic acid synthesized at the outer mitochondrial membrane, needs to cross the intermembrane space to be activated by Cds1 in the inner mitochondrial membrane to CDP-DAG (Kuchler et al., 1986), which together with glycerol-3-phosphate (G3P) in turn is converted to phosphatidylglycerolphosphate (PGP) by the enzyme Pgs1 (Chang et al., 1998a) (**Figure 3**). Dephosphorylation by a yet unidentified enzyme yields phosphatidylglycerol (PG). Finally, the cardiolipin synthase Crd1 catalyzes the synthesis of CL from PG and CDP-DAG (Chang et al., 1998b; Tuller et al., 1998). CL is afterwards subjected to postsynthetic modifications that lead to substantial changes in the acylchain composition. The best characterized enzyme involved in this remodeling process is a transacylase localized in the outer mitochondrial membrane and the outer leaflet of the inner membrane, which is termed Taz1 (Claypool et al., 2006). Considerable interest in Taz1 has recently arisen, when it was demonstrated that the human homolog is frequently mutated in patients suffering from Barth Syndrome, a

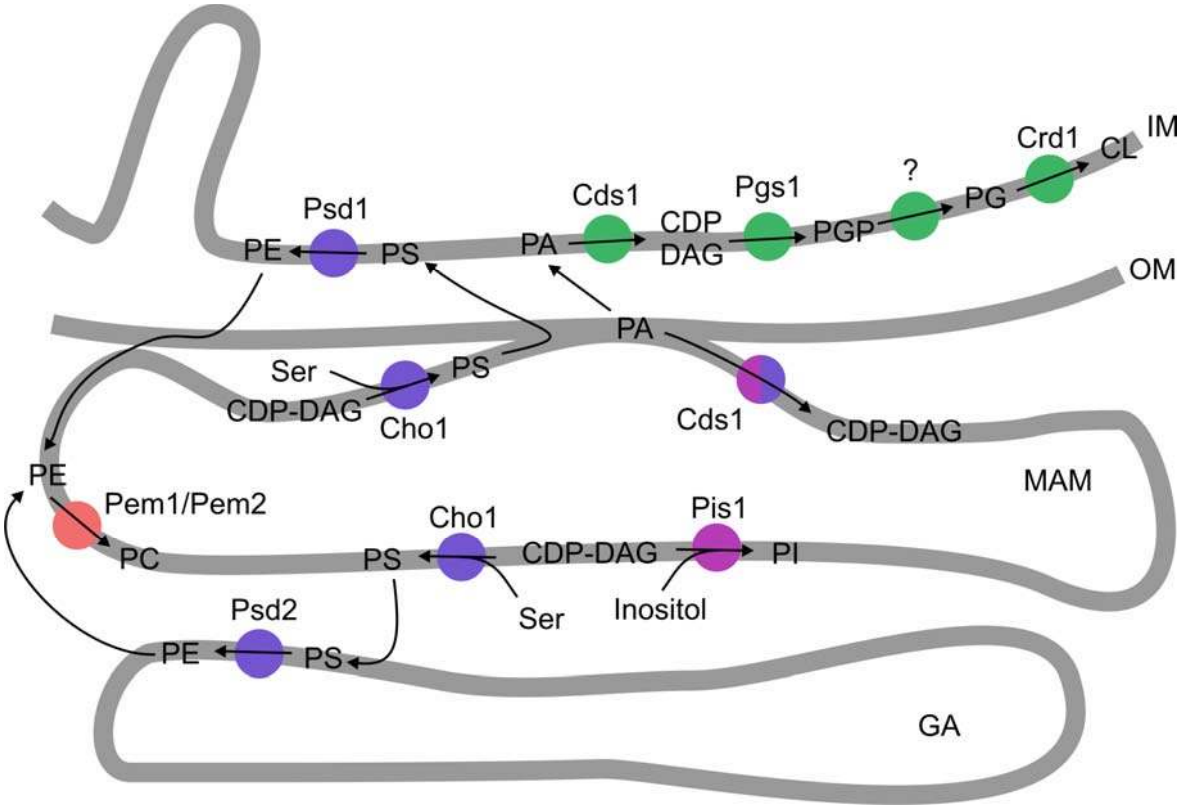

Figure 3 Phospholipid synthesis in *S. cerevisiae.* Enzymes required for CL, PE, PC and PI synthesis are shown in green, blue, red and violet, respectively. The synthesis of PA by a two-step acylation of glycerol-3-phosphate is not shown. Transport routes of PI and PC or PE, synthesized in the Golgi apparatus, to mitochondria are not indicated. IM – inner mitochondrial membrane, OM – outer mitochondrial membrane, MAM – mitochondrial associated membranes (subfraction of the endoplasmic reticulum), GA – Golgi apparatus (for phospholipid abbreviations see figure 2) (Adapted from Voelker, 2004)

disease characterized by cardiomyopathy, neutropenia and muscle weakness (Bione et al., 1996). The biosynthesis of phosphatidylinositol (PI) requires fewer steps than the CL synthesis. In a reaction that is similar to the production of PS by Cho1, inositol is attached to CDP-DAG in the MAM compartment (Gaigg et al., 1995). Like other lipids, PI requires transport to various target membranes within the cell.

As it is evident from the cellular localization of the enzymes involved in the biosynthesis of different phospholipid species, several translocation steps are required for efficient supply of different cellular organelles with lipids. While most organelles are connected to the vesicular transport system, which represents a way to also distribute lipids besides

proteins into various cellular compartments, mitochondria have to ensure their lipid supply by other means. In yeast this process is poorly understood and only one protein, namely Met30, has been demonstrated to be important for import of phospholipids into mitochondria. Met30 is an F-box protein and a subunit of an ubiquitin ligase complex (Schumacher et al., 2002). At what stage ubiquitination plays a role during import of lipids into mitochondria or if Met30 has a role independent of its function in the ubiquitin ligase complex, however, remains unresolved (Choi et al., 2006a). Similarly, it is not known how lipids cross the intermembrane space and if this is protein assisted or regulated to any extent. Another empty spot in our understanding of mitochondrial phospholipid homeostasis regards the regulation of the specific lipid composition of mitochondrial membranes that varies significantly under different growth conditions. It is unclear, whether this is determined exclusively on the level of transcription of genes encoding for enzymes required for lipid biosynthesis or also at later steps. Furthermore, the contribution of lipid turnover to mitochondrial lipid composition has not been studied extensively to date and awaits identification of components that mediate or regulate this step.

1.6 Functional membrane domains

Cellular membranes have long been looked at as homogenous fluids, where different lipids are equally distributed. This view, however, has been challenged with the proposal of functional domains within a membrane that are capable of separating processes occurring in the same membranes. Initially, the idea of a lipid raft was put forward to explain the differential sorting of proteins and lipids to the plasma membrane in morphologically polarized cells, where apical and basal membrane differ in their protein and lipid composition. This model proposes crucial roles for cholesterol and sphingolipids in forming membrane clusters destined for the apical membrane in the Golgi apparatus, which are then recognized as a unit and sorted to the target membrane by the cellular trafficking system (Simons and van Meer, 1988; van Meer and Simons, 1988). This idea was supported, when it was demonstrated that such domains survive extraction with the detergent Triton-X100 at 4°C, which raised the term “detergent resistant membranes” (DRM) (Brown and Rose, 1992). Additional evidence

was derived from experiments where cells were deprived of cholesterol, which prevented lipid raft isolation (Cerneus et al., 1993). Although, insolubility with Triton-X100 and cholesterol depletion have been used in a number of experiments to investigate lipid rafts, it is still a matter of debate, whether insights obtained with these approaches truly reflect the physiological state of a cell or whether these methods are prone to experimental artifacts (Heerklotz, 2002; Munro, 2003). Nevertheless, successful reconstitution of lipid rafts in model membranes and sophisticated optical microscopy based approaches that allow the direct detection of lipid rafts in living cells, greatly support the existence of lipid rafts (Brown and London, 1998; Dietrich et al., 2001; Jacobson et al., 2007; Samsonov et al., 2001). Questions still remain regarding the physiological relevance of lipid rafts as the latest models envision lipid rafts as almost invisible entities that are extremely small and short-lived (Edidin, 2003; Shaw, 2006; Simons and Vaz, 2004).

The current available methods have been extensively used for the characterization of lipid rafts and this has led to the identification of several proteins that are selectively enriched in these microdomains. The most studied protein is represented by caveolin that is responsible for the formation of characteristic structures known as caveolae (Thomas and Smart, 2008). Other examples are GPI-anchored proteins, acylated proteins, cholesterol or phospholipid binding proteins and heterotrimeric G proteins (Rajendran and Simons, 2005). Lipid rafts harbouring these proteins were demonstrated to play important roles in caveolae mediated endocytosis, non-caveolar internalization, sorting in polarized epithelial cells, virus budding or immune receptor signaling (Rajendran and Simons, 2005). Common to the function of lipid rafts in all these processes is the idea that they compartmentalize cellular processes in a membrane that leads to a concentration or segregation of specific proteins.

1.7 The SPFH-family of proteins

A group of proteins that share the same domain, termed SPFH-domain (named after the founding members **S**tomatin, **P**rohibitin, **F**lotillin and **H**flK/HflC) were repeatedly found in DRM's (Langhorst et al., 2005; Morrow and Parton, 2005). ~1800 proteins

harbouring this domain were identified in species ranging from prokaryotes to higher eukaryotes by computational analysis (Browman et al., 2007; Huber et al., 2006). Interestingly, it was recently reported that the SPFH-domain emerged independently in different proteins, which suggests that it plays a role in a membrane process of fundamental importance (Rivera-Milla et al., 2006). Proteins belonging to the SPFH-family share a few characteristics (Browman et al., 2007): First, all proteins studied to date are membrane attached, although some variations in the exact membrane association are evident. Second, SPFH-proteins form large homooligomeric or heterooligomeric complexes. Third, members of the SPFH-family appear to be lipid raft associated. Despite these similarities, cellular localization and proposed functions clearly vary among these proteins. Flotillins, stomatins and erlins, all members of the SPFH-family, have been studied in some detail and the current knowledge is summarized below.

The term flotillins is used for two highly homologous proteins, named flotillin-1 and flotillin-2 that are localized to the plasma membrane and endosomes (Lang et al., 1998; Morrow et al., 2002). Both proteins have been identified in DRM's and since then are commonly used as marker proteins for lipid microdomains (Lang et al., 1998). Many different roles have been attributed to both proteins that include signal transduction, vesicle trafficking and cytoskeletal rearrangement (Morrow and Parton, 2005). Recently, strong evidence has been provided that supports a function of flotillin-1 in a novel endocytosis pathway that is independent of the two known endocytosis mediating proteins clathrin and caveolin (Glebov et al., 2006). A different idea is emerging for the function of members of the stomatin-family within the SPFH-superfamily that encompasses podocin, stomatin and stomatin-like proteins (e. g. SLP-3, MEC2). All proteins have been localized to the plasma membrane (Huber et al., 2006; Morrow and Parton, 2005; Wetzel et al., 2007). The common model at this state of investigations ascribes a function to stomatins in the regulation of ion-channels that appears to be required for mechanosensation in several cases (Gillespie and Walker, 2001; Huber et al., 2006; Wetzel et al., 2007). Strikingly, it was demonstrated for MEC-2 and podocin that this function depends on the ability of these proteins to bind cholesterol that in turn

depends on a conserved proline residue in the N-terminal part of these proteins (Huber et al., 2006). The latest contribution to the SPFH-family of proteins was the identification of the two human proteins erlin-1 and erlin-2 that were recovered from DRM's (Browman et al., 2006). In contrast to the flotillins and stomatins, erlin-1 and erlin-2 are localized in the endoplasmic reticulum, which is at odds with the lipid raft definition that assumes a requirement for cholesterol and sphingolipids in lipid rafts. These lipids are, however, low abundant in the endoplasmic reticulum. These observations prompted the authors to conclude that lipid rafts are also present in the endoplasmic reticulum in the absence of large quantities of cholesterol and sphingolipids.

1.8 Prohibitins, proteins with a variety of functions

Further members of the SPFH-family are the prohibitins that encompass two proteins known as Phb1 and Phb2. These proteins are introduced in more detail below, because of their central role in the present thesis. Phb1 and Phb2 are homologous proteins that are evolutionary conserved and were identified in most eukaryotic species including plants (Merkwirth and Langer, 2008). Prohibitins are membrane attached proteins that form higher order oligomers. The N-termini of both proteins display hydrophobic regions that are required for membrane anchorage while C-terminal coiled-coil regions, present in both proteins, mediate hetero-oligomerization. A ring-shaped high molecular weight complex that is formed by Phb1 and Phb2 has recently been successfully purified and analyzed by single particle electron microscopy (Tatsuta et al., 2005). In this complex 16-20 subunits of each Phb1 and Phb2 are arranged in an alternative manner, which is supported by an interaction of both proteins that was demonstrated in yeast and mammalian cells (Coates et al., 1997; Steglich et al., 1999) and by crosslinks exclusively detected between different prohibitin subunits (Back et al., 2002) (**Figure 4**). Moreover, both proteins are interdependent and depletion of one protein results in the degradation of the respective other one in various organisms, which is in accordance with a quantitative assembly of both proteins observed in immune depletion experiments (Artal-Sanz et al., 2003; Berger and Yaffe, 1998; Coates et al., 2001; Kasashima et al., 2006). Hence, the prohibitin complex represents the physiologically active structure.

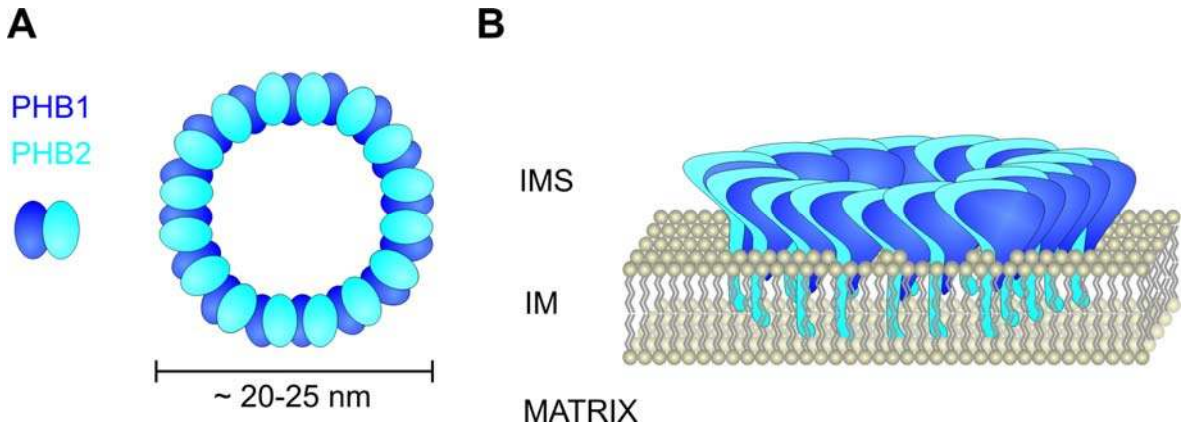

Figure 4 Schematic representation of the prohibitin complex **A** Dimers of Phb1 and Phb2 are the building blocks of the prohibitin complex. Heterodimers assemble into ring-like complexes. **B** The prohibitin complex is anchored to the inner mitochondrial membrane via N-terminal hydrophobic stretches. The C-terminal domains are exposed to the intermembrane space. (Courtesy of Carsten Merkwirth)

Two characteristics that are shared by SPFH-family members, namely membrane attachment and the formation of heterooligomeric complexes, also hold true for prohibitins. Lipid raft association that was demonstrated for various members of the SPFH-family, however, is currently less obvious for prohibitins. The bulk of information present supports a mitochondrial localization of prohibitins that is incompatible with the classical view of lipid rafts. According to this, the formation of lipid rafts depends on the presence of cholesterol and sphingolipids, two lipids that are either very low abundant or completely absent in mitochondria, respectively (Zinser and Daum, 1995).

Studies in yeast exclusively support a mitochondrial localization of prohibitins. The targeting signals of Phb1 and Phb2 have been identified and reside in the N-terminal part of both proteins (Tatsuta et al., 2005). Neither of the proteins is processed upon import (Nijtmans et al., 2000). Phb2 harbours a bipartite targeting signal that consists of an amphipathic helix and a hydrophobic domain, reminiscent of other intermembrane space proteins. Phb1, in comparison, does not display any characteristics of known mitochondrial targeting signals but, nevertheless, the N-terminal region is sufficient to target a hybrid protein to mitochondria (Tatsuta et al., 2005). Studies on the submitochondrial localization show that the prohibitin complex is anchored to the inner

membrane and exposes large domains into the intermembrane space (Berger and Yaffe, 1998; Steglich et al., 1999).

Despite extensive effort, the function of the prohibitin complex in mitochondria is not well understood, which is in part explained by the very mild phenotype of yeast cells lacking prohibitins that affects replicative life-span. Insights were provided by experiments that identified prohibitins as binding partners of the *m*-AAA protease in the inner membrane (Steglich et al., 1999). Interestingly, absence of prohibitins results in an accelerated degradation of substrates by the *m*-AAA protease suggesting an inhibitory function of prohibitins on *m*-AAA protease function (Steglich et al., 1999). This finding is reminiscent of the prokaryotic AAA-protease homolog FtsH that is regulated by the SPFH-proteins HflK and HflC (Kihara et al., 1996; Kihara et al., 1997). In addition to the biochemical interaction, synthetic lethal interactions between *PHB1* or *PHB2* and the genes encoding for the subunits of the *m*-AAA protease exist (Steglich et al., 1999). This suggests that prohibitins have functions beyond regulation of the *m*-AAA protease, because it appears counterintuitive that absence of a regulatory factor has an effect in the absence of the protein it regulates.

Genetic approaches have linked prohibitins also to other mitochondrial processes. It was demonstrated that prohibitins are essential in the absence of proteins encoded by *MMM1*, *MDM10* and *MDM12* (Berger and Yaffe, 1998). These proteins were initially identified to be essential for mitochondrial morphology maintenance (Berger et al., 1997; Burgess et al., 1994; Sogo and Yaffe, 1994). The function in this process, however, remained elusive. More recent work, suggests that disturbance of mitochondrial morphology in mutants lacking these proteins is a secondary consequence from defects in the primary function, which is the assembly of β-barrel proteins in the outer membrane together with the SAM complex (Meisinger et al., 2007). Yet another function has been linked to prohibitins, which is mitochondrial phospholipid metabolism. Psd1, required for PE biosynthesis in the inner membrane, is another protein essential in the absence of prohibitins (Birner et al., 2003). Although these genetic analyses provide first starting points for the investigation of the functional

process where prohibitins are involved, it is currently not possible to pinpoint the function of these proteins.

The current status of investigations in mammalian cells adds another degree of complexity to the field. Starting with the localization, prohibitins have been shown to be residents in the plasma membrane, the nucleus or mitochondria in mammalian cells (Fusaro et al., 2003; Kolonin et al., 2004; Merkwirth et al., 2008). A model uniting all these localizations is currently not available. Very recent work has closely examined the function of prohibitins within mouse embryonic fibroblasts (Merkwirth et al., 2008). Depletion of PHB2 in these cells results in a proliferation defect that can be rescued by the reexpression of PHB2. This complementation, however, strongly depends on the integrity of the mitochondrial targeting sequence. Mutation of a nuclear localization signal, present in the sequence, did not interfere with restoration of cell proliferation, suggesting a major role of prohibitins in mitochondria. Another phenotype in prohibitin depleted cells, identified in the same study, is a defect in mitochondrial cristae morphogenesis (Merkwirth et al., 2008). One of the key players in this process is Opa1, the functional homolog of the intermembrane space GTPase Mgm1 in yeast (Herzig and Martinou, 2008). Prohibitin depletion led to the selective loss of the longest isoform of Opa1. Expression of a non-cleavable long isoform of Opa1 was capable of rescuing the morphology defect, leading the authors to conclude that Opa1 is a major target of prohibitin function in mouse embryonic fibroblasts (Merkwirth et al., 2008).

Despite growing evidence for a mitochondrial function of prohibitins in a variety of cells, prohibitin functions in the nucleus or the plasma membrane are supported by a series of experiments from independent groups. For PHB1 as well as PHB2 a nuclear function in transcriptional regulation has been proposed. The available data suggest a scenario in which PHB1 associates with p53 or retinoblastoma and inhibits expression of genes that are under the control of the E2F transcription factor (Wang et al., 1999a; Wang et al., 1999b). Similar findings have been obtained for PHB2 that demonstrate an inhibitory effect of PHB2 on the transcriptional activity of estrogen receptors that involves physical association of both proteins (Montano et al., 1999). The repressive effect of PHB1 and

PHB2 on transcription was shown to be mediated by the recruitment of diverse co-repressors in both cases (Kurtev et al., 2004; Wang et al., 2002a; Wang et al., 2002b; Wang et al., 2004). It has to be stated, that evidence for a nuclear function of prohibitins was derived in many studies from experiments that involved overexpression of either PHB1 or PHB2 without concurrent overexpression of the respective other subunit. Accumulation of a single subunit might lead to an artificial situation because it has been shown that the prohibitin complex is the functional active structure (see above).

The proposed functions of prohibitins at the plasma membrane are all connected to signal transduction processes, where prohibitins either directly act as receptors or are associated with a receptor complex. Back in the early years of prohibitin research, PHB1 and PHB2 were co-purified together with the IgM antigen receptor (Terashima et al., 1994). Further reports demonstrated that a synthetic pro-apototic peptide is recognized directly by PHB1 in white fat vasculature of mice (Kolonin et al., 2004) and that prohibitin associates with a recognition complex responsible for binding to a virulence antigen of *Salmonella typhi* in a human intestinal epithelial cell line (Sharma and Qadri, 2004). The latest observation implicated prohibitin in Ras/Raf signaling and the authors suggested that prohibitin might localize to the inner leaflet of the plasma membrane for this function (Rajalingam and Rudel, 2005; Rajalingam et al., 2005). Interestingly, two of these studies isolated prohibitins from DRM's, which is reminiscent of other SPFH-proteins and raises the possibility that prohibitins might engage in the formation of lipid microdomains (Sharma and Qadri, 2004; Terashima et al., 1994). Clearly, further experimental evidence is required to establish such an activity of prohibitins.

2 Aim of thesis

Prohibitins comprise an evolutionary conserved and ubiquitously expressed family of membrane proteins that is essential for development in higher eukaryotes. This suggests an involvement of these proteins in a cellular process of fundamental importance. Despite extensive research during the last years, the molecular function of prohibitins remains poorly understood. The research on prohibitins in yeast is complicated by the very mild phenotype associated with the loss of prohibitins that is restricted to a shortened replicative life-span. A possibility to investigate the function of genes, whose deletion does not result in an apparent phenotype, is the discovery of synthetic lethal interactions that unmask redundant processes required in the absence of the protein under investigation.

The aim of this thesis was the identification and characterization of the genetic interactome of prohibitins. To achieve this, a comprehensive genetic approach was chosen that allowed a systematic analysis of the viability of double mutants with a deletion of prohibitin combined with the deletion of every non-essential gene. In the ideal case, such an approach should identify functionally related genetic interactors, which would link prohibitin function to a certain cellular process. Furthermore, the integration of so far uncharacterized genes into this genetic network would offer ideas to unravel their function as well. Every elucidation of an uncharacterized gene in this genetic interactome would further contribute to the functional characterization of prohibitins.

3 Material and methods

3.1 Molecular Biology

Standard methods of molecular biology were performed according to established protocols (Sambrook and Russell, 2001). Chemicals were purchased either from Sigma or Merck unless stated otherwise. Enzymes used in this study were purchased from NEB or Invitrogen.

3.2 Cloning procedures

For expression of myc-tagged Gep4 variants in yeast, the coding region of *GEP4* with 541 bp of the promoter was amplified from genomic DNA. The C-terminal myc-tag and restriction sites were introduced by polymerase chain reaction (PCR) using the oligonucleotides TL4072 and TL4073 (for oligonucleotide sequences see table 1). Subsequently, a *Bam*HI/*Pst*I DNA fragment containing the promoter and the myc-tagged ORF were cloned into the corresponding restriction sites of the digested 2µ plasmid Yep352 (for vectors used in this study see table 2). Point mutations were introduced with degenerated oligonucleotides listed in table 1 and the site-directed mutagenesis kit (Stratagene). *GEP2*, *UPS1* and *CHO1* were cloned into the vector Yeplac181 to confirm their suppressive effect on the lethality of *Δgep1Δphb1* cells. *GEP2*, including ~500 bp of the promoter, was amplified from genomic DNA. Restriction sites and a C-terminal myc-tag were introduced by PCR using the oligonucleotides TL3987 and TL3989. The fragment resulting from *Bam*HI/*Pst*I digestion was cloned into the respective restriction sites of Yeplac181. CHO1, including ~500 bp up- and ~150 bp downstream of the ORF, was amplified from genomic DNA. Restriction sites were introduced by PCR with the primer pair TL3990/TL3991 and the digested fragment was cloned into the *Bam*HI/*Pst*I restriction sites of Yeplac181. *UPS1*, including ~500 bp up- and ~250 bp downstream of the ORF was excised from the overexpression clone of the Yep13 library by *Bam*HI and *Nhe*I and cloned into the compatible *Bam*HI and *Xba*I sites of Yeplac181.

For overexpression, *GEP1*, *GEP2* and *UPS1* were cloned into the 2µ plasmid pESC-URA under the control of the strong galactose inducible promoters Gal1 and Gal10. The ORF's of *GEP1, GEP2* and *UPS1* excluding the stop codons were amplified from genomic DNA with the primer pairs TL3374/TL3375, TL3376/TL3377 and TL4604/TL4605, respectively. *GEP1*, *GEP2* and *UPS1* containing DNA fragments were cloned into the *Sal*I, the *Not*I or the *Bam*HI/*Sal*I restriction sites of pESC-URA, respectively. These cloning procedures resulted in an in-frame fusion of *GEP1* and *UPS1* to the sequence encoding a myc-tag in pESC-URA. *GEP2* cloning resulted in an in frame fusion to an FLAG-tag encoding sequence in pESC-URA.

Table 1 Oligonucleotides used in this study

Primer	Description	Sequence
TL224	Disruption *PHB1*, S1	GGTACGAAACTTACATTCAAAATCAATAATTTACTTTAGAAAAG ACGTACGCTGCAGGTCGAC
TL225	Disruption *PHB1*, S2	AAAAATTTTCTCCCCTAGTTTATTGTGTTCATAGCTTTTCCAGA ATCGATGAATTCGAGCTCG
TL226	Disruption *PHB2*, S1	AAATAAAAGCAAGCGGCTGCTAGAAAAGAATATAATTTAGTGC TGCGTACGCTGCAGGTCGAC
TL227	Disruption *PHB2*, S2	AGAGCTAGCCAAGATCTTTGCAATGTCCCTGGCTGTATCTAAT CTATCGATGAATTCGAGCTCG
TL2085	Disruption *ATP23*, S1	TTTGAGTGGTGGAGACGGACCATGCAGTACAAGACTGGTATG ACGTACGCTGCAGGTCGAC
TL2086	Disruption *ATP23*, S2	TTAGTTCTCGAGCTTTAGAGTCGTCTTCGTATCGAGTTTTATCG ATGAATTCGAGCTCG
TL2387	C-terminal tagging *ATP23*, S3	TTGCTTCGCCGATACGAGACCGTTTGATGAGATTTACAGACGG ATCCCCGGGTTAATTAA
TL2388	C-terminal tagging *ATP23*, S2	ATATTTTCTATTATAGAATATTGTCATTTATTACATTGGTGAATT CGAGCTCGTTTAAAC
TL2024	Disruption *GEP1*, S1	AGACTAAGATAAAATAATCGAGAATAATTAAAAGACGATACGTA CGCTGCAGGTCGAC
TL2023	Disruption *GEP1*, S2	GTAGTATGCAGTGCCATGCGGGATCAAGGAATTTGTATCTATC GATGAATTCGAGCTCG

Primer	Description	Sequence
TL2391	C-terminal tagging *GEP1*, S2	AAATATTGACTTGTTTAGAGACGCATACAACCACGAAAATCGGATCCCCGGGTTAATTAA
TL2392	C-terminal tagging *GEP1*, S3	GTAGTATGCAGTGCCATGCGGGATCAAGGAATTTGTATCTGAATTCGAGCTCGTTTAAAC
TL3367	Disruption or N-terminal tagging *GEP2*, S1	AAATATTTCTATTAGTCATATATCTCTCGAGCTTATATATTAATAATG
TL3368	Disruption or C-terminal tagging *GEP2*, S2	AAATCGTTGAGAAAAAATCGTTGAGAAAAGATGTATTTTTTTTTTAATCGATGAATTCGAGCTCG
TL3369	C-terminal tagging *GEP2*, S3	AGTATTCTAGCAATGTTCAACGATATCTGGAAAAATGCTAACGAACGTACGCTGCAGGTCGAC
TL4610	N-terminal tagging *GEP1,* S1	TCAGACTAAGATAAAATAATCGAGAATAATTAAAAGACGATAATGCGTACGCTGCAGGTCGAC
TL4611	N-terminal tagging *GEP1,* S4	CTGGTCCCATGGATAGTTGAAATCGTAACTGTTTTGAAACAATTTCATCGATGAATTCTCTGTCG
TL4612	N-terminal tagging *GEP2,* S4	TTTCTCCCATGGGTAATCGAATTCATAAGATTTTTGAAATGATTTCATCGATGAATTCTCTGTCG
TL4613	N-terminal tagging *UPS1,* S1	TCTGGCTTCTGAGACGGCGGTAAGATATCCTTAAGAGTTGCAATGCGTACGCTGCAGGTCGAC
TL4614	N-terminal tagging *UPS1,* S4	GGCAAAATCGGTAGGAAATATATGTGTGCTTTTGTGTAAAAGGACCATCGATGAATTCTCTGTCG
TL4072	*GEP4*-myc cloning	CGCGGATCCATATCTTTGACATAAAATCC
TL4073	*GEP4*-myc cloning	AAAACTGCAGTCACAAATCTTCTTCAGAAATCAACTTTTGTTCAAATCCCAAAAAGTTGTATAAT
TL4102	$GEP4^{D45N}$ forward mutagenesis primer	CGTGGTCTTGGATAAGAACAACTGCATCGCCTTCC
TL4103	$GEP4^{D45N}$ reverse mutagenesis primer	GGAAGGCGATGCAGTTGTTCTTATCCAAGACCACG
TL4104	$GEP4^{D47N}$ forward mutagenesis primer	CCGTGGTCTTGAATAAGGACAACTGC
TL4105	$GEP4^{D47N}$ reverse mutagenesis primer	GCAGTTGTCCTTATTCAAGACCACGG

Primer	Description	Sequence
TL3987	Cloning of *GEP2,* Yeplac181	CGCGGATCCTATGATGACAATGATAGTGC
TL3988	Cloning of *GEP2,* Yeplac181	AAGCTTTTACTCGAGGTCTTCTTCGGAAATCAACTTCTGTTCTTCGTTAGCATTTTTCCAGATATCG
TL3990	Cloning of *CHO1*	CGCGGATCCATGATTATAGAGCTTATAGC
TL3991	Cloning of *CHO1*	AAACTGCAGGTAACCATAGTCAGATGTGG
TL3374	Cloning of *GEP1*	GTCGACCGATAATGAAATTGTTTCAAAACAGTTACG
TL3375	Cloning of *GEP1*	GTCGACATTTTCGTGGTTGTATGC
TL3376	Cloning of *GEP2,* pESC-URA	GCGGCCGCTAATGAAATCATTTCAAA
TL3377	Cloning of *GEP2,* pESC-URA	GCGGCCGCTTCGTTAGCATTTTTCCAGATATCGTTG
TL4604	Cloning of *UPS1*	GGATCCATGGTCCTTTTACACAAA
TL4605	Cloning of *UPS1*	GTCGACAAACTGAGGATTTCTCGC

Table 2 Plasmids used in this study

Description	Reference
pFL38-*ATP23*	(Wilmes, 2006)
pFL38-*ATP23*^{H167A}	(Wilmes, 2006)
pFL38-*ATP23*^{E168Q}	(Wilmes, 2006)
pFL38-*ATP23*^{H171A}	(Wilmes, 2006)
Yep352	(Hill et al., 1986)
Yep352-*GEP4*	This study
Yep352-*GEP4*^{D45N}	This study
Yep352-*GEP4*^{D47N}	This study
pCM189-*PHB1*	(Osman, 2005)
pYX142-*PHB1*	(Tatsuta, T., unpublished)
Yeplac181	(Gietz and Sugino, 1988)
Yeplac181-*GEP2*-myc	This study
Yeplac181-*CHO1*	This study
Yeplac181-*UPS1*	This study

Description	Reference
Ycplac181-ADH	(Dip, 2008)
Ycplac181-ADH-SLMO1-myc	(Dip, 2008)
Ycplac181-ADH-SLMO2-myc	(Dip, 2008)
Ycplac181-ADH-PRELI-myc	(Dip, 2008)
Ycplac181-ADH-*GEP1*-myc	(Dip, 2008)
pESC-URA	(Stratagene)
pESC-URA-*GEP1*-myc	This study
pESC-URA-*GEP2*-myc	This study
pESC-URA-*UPS1*-myc	This study
pFA6a-kanMX6	(Longtine et al., 1998)
pFA6a-3HA-kanMX6	(Longtine et al., 1998)
pFA6a-hphNT1	(Janke et al., 2004)
pAG25	(Goldstein and McCusker, 1999)
pYM46	(Janke et al., 2004)
pYM-N23	(Janke et al., 2004)

3.3 Yeast genetic procedures

For transformation of yeast cells, a lithium acetate/single-stranded carrier DNA/polyethylene glycol method was used (Gietz and Woods, 2002). The SGA was performed as described previously (Osman, 2005; Tong et al., 2001). Synthetic genetic interactions were confirmed by sporulation and tetrad dissection. In order to identify high-copy suppressors of synthetic lethal interactions, *Δphb1Δgep1*[*PHB1*] cells were transformed with a genomic Yep13 high-copy library. After growth for one day at 30°C on SC-Leu, plates were replicated on plates containing 5'FOA (1 mg/ml) to counterselect against cells containing the pCM189-*PHB1* expression plasmid. Clones were analyzed after two days and the suppressor gene identified by subcloning.

3.4 Yeast strains and growth conditions

Yeast strains were grown according to standard procedures on complete (YP) or synthetic media (SC) supplemented with 2% (w/v) glucose, 2% (w/v) galactose and 0.5% (v/v) lactate or 3% (v/v) glycerol. When indicated, medium was supplemented with doxycycline (2 µg/ml). Yeast strains used in this study are derivatives of W303 (Rothstein, 1983) or S288c (Brachmann et al., 1998) strains (**Table 3**). Single mutants not listed in table 3 were obtained from the Euroscarf collection. Deletion strains were generated by PCR-based homologous recombination strategies (Goldstein and McCusker, 1999; Janke et al., 2004; Longtine et al., 1998).

Table 3 Yeast strains used in this study. CG strains were generated in this study, CW and PD strains were generated previously (Dip, 2008; Wilmes, 2006). [1)] Deletion cassette was amplified from genomic DNA isolated from the respective deletion strains of the Euroscarf library and introduced into a diploid strain. Single mutants were derived by tetrad dissection.

#	Strain name	Background	Mating type	Genotype
CG214	WT	S288c	a	*his3Δ1leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0*
CG409	*Δphb1*[*PHB1*]	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δphb1::nat1 pCM189-PHB1*
CG410	*Δgep1Δphb1*[*PHB1*]	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1*
CG210	*Δphb1*	S288c	a	*his3Δ1leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 Δphb1::nat1*
CG212	*Δphb2*	S288c	a	*his3Δ1leu2Δ0 (met15Δ0/MET15) lys2Δ0 ura3Δ0 Δphb2::kanMX6*
CW1	*Δatp23*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δatp23::His3MX6*
CG233	*Δgep1*	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0ura3Δ0 Δgep1::nat1*
CG240	*ATP23-3HA*	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 ATP23-3HA (kanMX4)*
CW18	*Δatp23 +ATP23*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δatp23::His3MX6 pfL38-ATP23*

#	Strain name	Background	Mating type	Genotype
CW33	*Δatp23 +ATP23^{H167A}*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δatp23::His3MX6 pfL38-ATP23^{H167A}*
CW26	*Δatp23 +ATP23^{E168Q}*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δatp23::His3MX6 pfL38-ATP23^{E168Q}*
CW20	*Δatp23 +ATP23^{H171A}*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δatp23::His3MX6 pfL38-ATP23^{H171A}*
CG421	*Δatp23Δphb1 +ATP23*	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 Δatp23::kanMX6 Δphb1::nat1 pFL38-ATP23*
CG422	*Δatp23Δphb1 +ATP23^{H167A}*	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 Δatp23::kanMX6 Δphb1::nat1 pFL38-ATP23^{H167}*
CG423	*Δatp23Δphb1 +ATP23^{E168Q}*	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 Δatp23::kanMX6 Δphb1::nat1 pFL38-ATP23^{E168Q}*
CG424	*Δatp23Δphb1 +ATP23^{H171A}*	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 Δatp23::kanMX6 Δphb1::nat1 pFL38-ATP23^{H171A}*
CG406	*Δgep4*[1)]	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 gep4Δ::kanMX4*
CG573	*Δgep4* +Yep352	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 gep4Δ::kanMX4 Yep352*
CG574	*Δgep4 +GEP4*	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 gep4Δ::kanMX4 Yep352-GEP4-myc*
CG575	*Δgep4 +GEP4^{D45N}*	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 gep4Δ::kanMX4 Yep352-GEP4^{D45N}-myc*
CG576	*Δgep4 +GEP4^{D47N}*	S288c	a	*his3Δ1 leu2Δ0 (met15Δ0/MET15?) (lys2Δ0/LYS2?) ura3Δ0 gep4Δ::kanMX4 Yep352-GEP4^{D47N}-myc*

#	Strain name	Background	Mating type	Genotype
CG206	WT	W303	a	*can1Δ100 his3Δ11,15 leu2Δ3,112 ura3Δ1 ade1Δ1 trp1Δ1*
CG202	*Δphb1*	W303	a	*can1Δ100 his3Δ11,15 leu2Δ3,112 ura3Δ1 ade1Δ1 trp1Δ1 Δphb1::nat1*
CG278	*Δphb1* [*PHB1*]	W303	a	*can1Δ100 his3Δ11,15 leu2Δ3,112 ura3Δ1ade1Δ1 trp1Δ1 Δphb1::nat1 pCM189-PHB1*
CG295	*Δgep1Δphb1* [*PHB1*]	W303	a	*can1Δ100 his3Δ11,15 leu2Δ3,112 ura3Δ1 ade1Δ1 trp1Δ1 Δphb1::nat1 Δgep1::kanMX4 pCM189-PHB1*
CG309	*Δgep1*[1)]	W303	?	*can1Δ100 his3Δ11,15 leu2Δ3,112 ura3Δ1 ade1Δ1 trp1Δ1 Δgep1::kanMX4*
CG547	*Δgep1Δphb1*[*PHB1*] +empty	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Yeplac181*
CG548	*Δgep1Δphb1*[*PHB1*] +*PHB1*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +pYX142-PHB1*
CG550	*Δgep1Δphb1*[*PHB1*] +*GEP2*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Yeplac181-GEP2-Myc*
CG551	*Δgep1Δphb1*[*PHB1*] +*UPS1*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Yeplac181-UPS1*
CG549	*Δgep1Δphb1*[*PHB1*] +*CHO1*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Yeplac181-CHO1*
CG608	*Δgep1 +GEP2*	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ ura3Δ0 Δgep1::nat1 +Yeplac181-GEP2-myc*
CG609	*Δgep1 +CHO1*	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ ura3Δ0 Δgep1::nat1 +Yeplac181-CHO1*
CG544	*Δgep1Δpsd1*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::nat1 Δpsd1::kanMX4*
CG545	*Δgep1Δpsd2*	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 Δgep1::nat1 Δpsd2::kanMX4*
PD43	*Δgep1Δphb1*[*PHB1*] +empty	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Ycplac111-ADH*

#	Strain name	Background	Mating type	Genotype
PD40	*Δgep1Δphb1*[*PHB1*] +SLMO1	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Ycplac111-ADH-SLMO1-myc*
PD42	*Δgep1Δphb1*[*PHB1*] +SLMO2	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Ycplac111-ADH-SLMO2-myc*
PD38	*Δgep1Δphb1*[*PHB1*] +PRELI	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Ycplac111-ADH-PRELI-myc*
PD72	*Δgep1Δphb1*[*PHB1*] +*GEP1*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δgep1::kanMX4 Δphb1::nat1 pCM189-PHB1 +Ycplac111-ADH-GEP1-myc*
CG624	*Δgep1* +empty	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 Δgep1::nat1 +Ycplac111-ADH*
CG601	*Δgep1* +SLMO1	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 Δgep1::nat1 +Ycplac111-ADH-SLMO1-myc*
CG602	*Δgep1* +SLMO2	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 Δgep1::nat1 +Ycplac111-ADH-SLMO2-myc*
CG600	*Δgep1* +PRELI	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 Δgep1::nat1 +Ycplac111-ADH-PRELI-myc*
CG603	*Δgep1 +GEP1*	S288c	α	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 Δgep1::nat1 +Ycplac111-ADH-GEP1-myc*
CG249	*GEP1-3HA*	S288c	a	*his3Δ1 leu2Δ ura3Δ0 GEP1-3HA (kanMX6)*
CG463	*Δgep2*	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 Δgep2::kanMX6*
CG464	*Δgep1Δgep2*	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δgep1:: nat1 Δgep2::kanMX6*
PD49	*Δups1*	S288c	α	*his3Δ1 leu2Δ0 ura3Δ0 Δups1::hphNT1*
PD65	*Δgep1Δups1*	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δgep1::nat1 Δups1::hphNT1*
PD63	*Δgep2Δups1*	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δgep2::kanMX6 Δups1::hphNT1*
PD61	*Δgep1Δgep2Δups1*	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δgep1::nat1 Δgep2::kanMX6 Δups1::hphNT1*

#	Strain name	Background	Mating type	Genotype
CG492	WT +empty↑	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 +pESC-URA*
CG490	*WT +GEP1*↑	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 +pESC-URA-GEP1-Myc*
CG491	*WT +GEP2*↑	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 +pESC-URA-GEP2-Myc*
CG636	*WT +UPS1*↑	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 +pESC-URA-UPS1-Myc*
CG626	P_{Gal}*-GEP1-Myc-7His*↑	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 P_{Gal}(natNT2)-GEP1-myc-7His(kanMX4)*
CG628	P_{Gal}*-GEP2-Myc-7His* ↑	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 P_{Gal}(natNT2)-GEP2-myc-7His(kanMX4)*
CG630	P_{Gal}*-UPS1-Myc-7His* ↑	S288c	a	*his3Δ1 leu2Δ0 met15Δ0 lys2Δ0 ura3Δ0 P_{Gal}(natNT2)-UPS1-myc-7His(kanMX4)*
CG345	*Δmmm1*[1)]	S288c	a	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δmmm1::kanMX4*
CG317	*Δmdm10*[1)]	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δmdm10::kanMX4*
CG407	*Δmdm12*[1)]	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δmdm12::kanMX4*
CG319	*Δmdm31*[1)]	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δmdm31::kanMX4*
CG385	*Δmdm32*[1)]	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δmdm32::kanMX4*
CG387	*Δmdm34*[1)]	S288c	a	*his3Δ1 leu2Δ0 ura3Δ0 Δmdm34::kanMX4*

3.5 Preparation of crude mitochondrial fractions

For the preparation of crude mitochondrial fractions, yeast cells were lysed using glass beads. 10 OD_{600} units of a yeast culture were centrifuged for 5 min at 3,000 rpm and RT. The yeast cells were then resuspended in 300 µl ice-cold SHKCl buffer (0.6 M sorbitol, 50 mM HEPES/KOH pH 7.4, 80 mM KCl, 2 mM PMSF) and glass beads (Ø 0.5 mm)

equivalent to a volume of 200 µl were added. The sample was mixed ten times for 30 s with a Vortex mixer and each time cooled on ice for another 30 s. Upon the addition of 700 µl SHKCl buffer, glass beads and non-lysed cells were removed by centrifugation for 3 min at 2,000 rpm and 4°C. The cellular membrane fraction was separated from the soluble supernatant by centrifugation for 10 min at 13,000 rpm and 4°C.

3.6 *In organello* labeling of mitochondrial translation products

Mitochondria isolated from cells grown in the presence of chloramphenicol (2 mg/ml) were resuspended in 1.5 ml translation buffer (0.6 M sorbitol, 150 mM KCl, 15 mM KP_i-buffer pH 7.4, 20 mM Tris/Cl pH 7.4, 13 mM $MgSO_4$, 0.3% (w/v) BSA (fatty acid free), 4 mM ATP, 0.5 mM GTP, 6 mM α-ketoglutarate, 5 mM phosphoenolpyruvate and 0.1 mM each amino acid except methionine) per mg mitochondria and pyruvate kinase was added to a final concentration of 0.04 mg/ml. After incubation for 3 min at 30°C, 0.25 µCi ^{35}S-methionine was added per µg mitochondria and samples were further incubated at 30°C for 20 min. Then synthesis of labeled protein was stopped by addition of non-labeled methionine to a final concentration of 50 mM and cooling the samples for 5 min on ice. Mitochondria were pelleted by centrifugation for 5 min at 13,000 rpm at 4°C, washed with SHKCl buffer and subjected to SDS-PAGE and Western blot analysis.

3.7 Blue native PAGE analysis

Blue native PAGE (BN-PAGE) was essentially performed as described previously (Schagger, 2001). Briefly, 100 µg mitochondria were solubilized in 20 µl solubilization buffer (50 mM NaCl; 5 mM 6-aminohexanoic acid, 50 mM imidazole/HCl pH 7.0; 50 mM KP_i-buffer pH 7.4 and 10% (v/v) glycerol) containing 1.875 % (w/v) digitonin for 20 min at 4°C. The sample was then centrifuged for 20 min at 18,000 rpm and 4°C and the supernatant was mixed with 2 µl 2% Coomassie blue G-250 (in solubilization buffer). 18 µl were loaded onto the BN-PAGE with an acrylamide concentration ranging from 3% to 11%. Gels were run at 4°C at 50 V with deep blue cathode buffer and anode buffer until the samples had completely entered the gel (deep blue cathode buffer: 50 mM Tricine, 7.5 mM imidazole, 0.02% (w/v) Coomassie blue G-250; anode buffer:

25 mM imidazole-HCl pH 7.0). Then, deep blue cathode buffer was exchanged against slightly blue cathode buffer (containing 1/10 Coomassie of the deep blue cathode buffer) and gels were run for further 2-3 hours at 300 V. Coomassie staining was performed with PageBlue protein staining solution (Fermentas) according to the manufacturer's instructions. Semidryblotting was performed with PVDF membranes that were preincubated with methanol before use.

3.8 Lipid analysis

3.8.1 TLC analysis

Phospholipids were extracted from mitochondria which were isolated from cells grown on YP medium containing 2% (w/v) galactose and 0.5% (v/v) lactate. The mitochondrial fraction was washed, resuspended in buffer A (0.6 M sorbitol, 5 mM MES pH 6.0) and loaded on a continuous sucrose gradient (20%-50% in buffer A). Mitochondria were harvested from the lower third of the gradient and diluted 1:5 in buffer A, pelleted, washed once in SEM buffer (250 mM sucrose, 10 mM MOPS pH 7.2, 1 mM EDTA) and finally resuspended in SEM buffer. The purity of the mitochondrial fraction and absence of contaminations by vacuolar and endoplasmic reticulum membranes was assessed by immunoblotting using Sec61- and Vac8-specific antisera. Purified mitochondria (1 mg) were mixed with 1.5 ml chloroform/methanol (1/1, v/v) and vigorously shaken for 60 min. H_2O (300 µl) was added and samples were vortexed for 60 s. After centrifugation (1,000 rpm, 5 min) the aqueous phase was removed and the solvent phase was washed with 250 µl methanol/H_2O (1/1, v/v). Then, samples were dried under a constant stream of air. Lipids were dissolved in chloroform and subjected to TLC analysis. TLC plates (HPTLC Silica gel 60 F_{254}, MERCK) were developed with chloroform/methanol/25% ammonia (50/50/3, v/v/v) when not stated differently and stained with 470 mM $CuSO_4$ in 8.5% *o*-phosphoric acid and subsequent incubation of the plates for 10 min at 180°C.

3.8.2 Quantification of PE and CL by mass spectrometry

Lipid extractions were performed according to the method of Bligh and Dyer (Bligh and Dyer, 1959). Phosphate determination was done according to Rouser (Rouser et al., 1970). For mass spectrometric analysis, 1.5 nmol phospholipids of mitochondrial fractions were extracted in the presence of cardiolipin (CL56:0, Avanti Polar Lipids, USA) and PE standard (50 pmol each) as described (Brugger et al., 2006). Dried lipids were redissolved in 10 mM ammonium acetate in methanol.

Quantification of PE was performed by neutral loss scanning, selecting for a neutral loss of 141 Da as described (Brugger et al., 2006). Quantification of CL was performed in negative mode on a quadrupole time-of-flight mass spectrometer (QStar Elite, Applied Biosystems). 10 µl of lipid extracts was diluted 1:2 with 0.1% piperidine in methanol and automatically infused (Triversa Nanomate, Advion Biosciences). Ionization voltage was set to -0.95 kV, gas pressure to 0.5 psi. CL's were detected as single charged molecules. CL species (all combinations of fatty acids 16:0, 16:1, 16:2, 18:0, 18:1 and 18:2) were analyzed by pseudo-multiple reaction monitoring (pMRM). The peak areas of CL-derived fatty acid fragments were extracted from the respective product ion spectra via the "Extract Fragments" script (Analyst QS 2.0). Isotope correction for M+2 ions was done manually; values were corrected for response factors of standards.

3.8.3 Determination of Psd1 activity

Mitochondria were resuspended in assay buffer B (0.1 M Tris/HCl pH 7.4, 10 mM EDTA, 2 µM PS-C6-NBD (Avanti polar lipids)) to a final concentration of 5 mg/ml and incubated at 25°C. At time points indicated, mitochondria (500 µg) were removed from the reaction mixture, phospholipids were extracted and analyzed by TLC (Developing solvent: $CHCl_3/C_2H_5OH/H_2O/N(CH_2CH_3)_2$ (30/35/7/35 v/v/v/v)). NBD signals were detected and quantified by fluorimaging (TyphoonTrio, GE Healthcare).

3.8.4 *In vivo* labeling of phospholipids

Logarithmic growing yeast cells were harvested and resuspended in medium supplemented with ^{3}H-serine (12.5 µCi/ml) to a final OD_{600}=5. After incubation at 30°C for 0, 20, 40 or 60 min cells corresponding to 10 OD_{600} were harvested and stored in liquid nitrogen. Phospholipids were extracted and analyzed by TLC. PE spots were recovered from the TLC plates and mixed with H_2O (400 µl) and scintillation cocktail (8 ml). Labeled PE was quantified by liquid scintillation counting. The stability of PE was monitored in pulse chase experiments. Yeast cells were labeled for 10 min as described before. Then, cells were harvested, resuspended in medium containing unlabeled serine (20 min) and incubated at 30°C for 0, 30, 60, 90, 120 or 150 min, before PE was quantified as above.

3.9 Microscopy

Fluorescence and electron microscopy was performed as described (Durr et al., 2006). For the determination of the cristae/mitochondrial contour ratio, ultrathin sections were viewed in a JEOL JEM-2100 transmission electron microscope (JEOL Ltd., Tokyo, Japan) and images were taken with a Gatan Model 782, Erlangshen ES500W digital camera (Gatan Inc., Pleasanton, USA). Approximately 100 micrographs of mitochondria were taken for each strain at the same magnification. For each image, the numbers of cristae were counted and the contour of each mitochondrion was measured in µm by using the measure function of ImageJ (Abramoff et al., 2004). For each strain the numbers of cristae were put into relation to the sum of the mitochondrial contours.

3.10 Miscellaneous

Mitochondria were prepared according to established protocols (Tatsuta and Langer, 2007). Submitochondrial localization, assessment of mitochondrial membrane potential, SDS-PAGE and Western blotting was performed as described previously (Geissler et al., 2000; Laemmli, 1970; Leonhard et al., 2000; Towbin et al., 1979).

4 Results

4.1 A synthetic lethal screen to integrate prohibitins into a genetic network

Despite the fact that prohibitins have been studied extensively over the past years in a variety of different model organisms, their molecular function remains elusive. In the yeast *Saccharomyces cerevisiae,* the study on prohibitins is hampered by the very mild phenotype associated with loss of prohibitins. It is believed that redundant processes are able to buffer the absence of prohibitins. In an attempt to unravel these processes, we performed a synthetic genetic array (SGA) analysis, in which either a *PHB1* or a *PHB2* deletion was combined with deletions of ~4750 non-essential genes to check the viability of the resulting double mutants. Inviablility or growth defects associated with neither single deletion but the respective double deletion strains were scored as genetic interactions, which were further confirmed by tetrad dissection. In addition, several genes were tested for synthetic lethality with prohibitins, which fell into functional groups that have already previously genetically been linked to prohibitins ([1)] in **Table 4**). In total, we uncovered 29 novel synthetic interactions of prohibitins and importantly reproduced six and therefore all previously published genetic interactions ([2)] in **Table 4**). Strikingly, 88% of these genes encoded protein products for which a mitochondrial or a dual localization has been reported. To test the importance of the genetic interactors of prohibitins for respiration, the majority of single deletion strains were spotted on media containing either glucose as a carbon source, which allows fermentative growth, or glycerol, a non-fermentable carbon source. ~45% of the strains showed no growth on glycerol containing plates and another ~20% exhibited a growth defect on this medium compared to growth on glucose medium (**Figure 5**). These observations are in line and further support a major role of prohibitins in mitochondria in *S. cerevisiae.*

19 of the genetic interactors fall into two functional classes: genes with functions during the assembly of the respiratory chain and genes required for the import of β-barrel proteins into the outer membrane and the maintenance of mitochondrial morphology.

Table 4 Identified synthetic genetic interactions with prohibitins, Loc. – Localization, IM - inner mitochondrial membrane, OM - outer mitochondrial membrane, M - mitochondrial matrix, IMS - mitochondrial intermembrane space, M – mitochondria, C - cytosol, N - nucleus, SL – synthetically lethal, GD – growth defect associated with double deletion, genes marked with [1)] were identified in candidate approach all others by SGA, genes marked with [2)] were already previously reported to genetically interact with prohibitins (Berger and Yaffe, 1998; Birner et al., 2003; Steglich et al., 1999)

	Gene	ORF	Loc.	
Respiratory chain assembly	*YTA10*[2)]	*YER017c*	IM	SL
	YTA12[2)]	*YMR089c*	IM	SL
	YME1	*YPR024w*	IM	SL
	OXA1	*YER154w*	IM	GD
	COX6	*YHR051w*	IM	GD
	ATP10	*YLR292w*	IM	GD
	ATP7	*YKL016c*	IM	GD
	ATP17	*YDR377w*	IM	GD
	COQ1	*YBR003w*	M	GD
	QRI5	*YLR204w*	IM	GD
Mitochondrial morphology/ β-barrel assembly	*MMM1*[1)2)]	*YLL006w*	IM/OM	SL
	MDM10[1)2)]	*YAL010c*	OM	SL
	MDM12[1)2)]	*YOL009c*	OM	GD
	MDM31[1)]	*YHR194w*	IM	GD
	MDM32[1)]	*YOR147w*	IM	SL
	MDM34[1)]	*YGL219c*	OM	SL
	MDM35	*YKL053c-A*	IMS	SL
	UPS1[1)]	*YLR193c*	IMS	SL
Unknown	*GEP1*	*YLR168c*	IMS	SL
	GEP3	*YOR205c*	M	GD
	GEP4	*YHR100c*	M	SL
	GEP5	*YLR091w*	M	GD
	GEP6	*YMR293c*	M	SL
	GEP7	*YGL057c*	M	GD
	GEP8	*YER093c-A*	-	SL
	GEP9	*YNL170w*	-	SL
	ATP23	*YNR020c*	IMS	GD
Diverse	*PSD1*[2)]	*YNL169c*	IM	SL
	CRD1[1)]	*YDL142c*	IM	SL
	HMI1	*YOL095c*	IM/M	GD
	MRE11	*YMR224c*	M, N, C	GD
	CTK3	*YML112w*	N, C	GD
	PIF1	*YML061c*	M, N	GD
	PIH1	*YHR034c*	N	GD
	EMI1	*YDR512c*	C	GD

Genes from the latter group, namely *MMM1*, *MDM10*, *MDM12*, *MDM31*, *MDM32* and *MDM34*, have been reported to genetically interact with each other and therefore

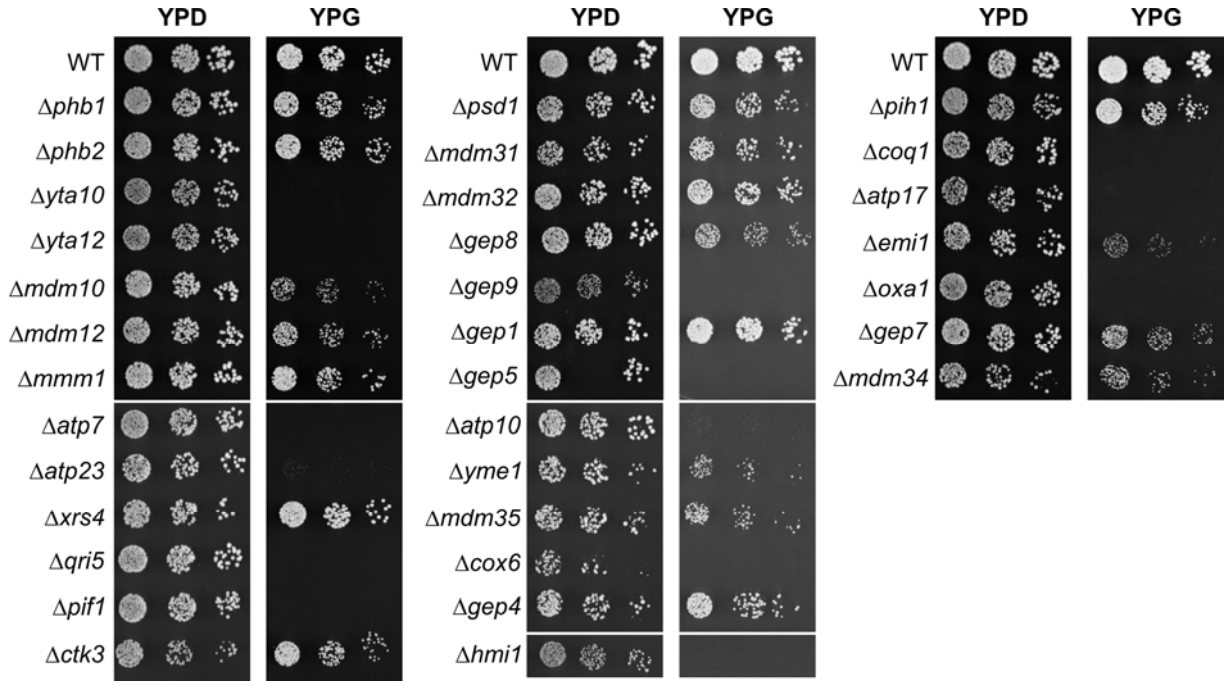

Figure 5 Phenotypic analysis of strains with deletion of genes required in the absence of prohibitins. Cells were grown on YPD or YPG medium at 30°C for two or four days, respectively.

represent a genetic entity (Dimmer et al., 2005). For eight other genes, diverse functions have been described. The strongest genetic interactions with prohibitins in this class were observed for the phosphatidylserine decarboxylase (*PSD1*) and the cardiolipin synthase (*CRD1*) encoding genes, required for the synthesis of phosphatidylethanolamine (PE) and cardiolipin (CL), respectively. No *Δpsd1Δphb1* spores were obtained by tetrad analysis. *Δcrd1Δphb1* spore clones sporadically formed little colonies that stopped proliferation upon further cultivation (**Figure 6**). A fourth discernable group comprised eight so far uncharacterized genes, which were termed *GEP1-9* (Genetic interaction with prohibitins).

4.2 Atp23, a metallopeptidase in the mitochondrial intermembrane space

The inspection of the uncharacterized genes essential in the absence of prohibitins, revealed an open reading frame that attracted our attention (**Figure 7 A**). *YNR020c*, which was later termed *ATP23* based on the experiments described below, was found

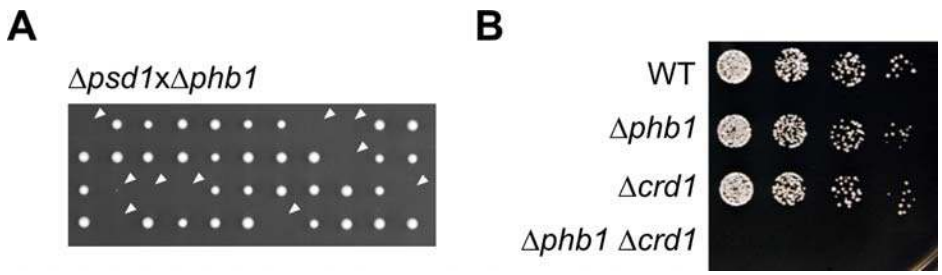

Figure 6 *PSD1* and *CRD1* are essential in the absence of prohibitins. **A** Tetrads derived from a diploid strain heterozygous for *PSD1* and *PHB1* deletion were dissected. Genotypes were inferred from the distribution of markers linked to the deletions. Inviable *Δpsd1Δphb1* double mutant haploid spore clones are indicated by arrowheads. **B** Serial dilutions of indicated yeast strains spotted on YPD plates were incubated at 30°C for two days.

to be essential for respiratory growth (**Figure 5**). Furthermore, the analysis of the protein sequence identified a consensus metal binding motif HEXXH formed by amino acid residues 167-171, which is characteristic of metalloproteases and which is highly conserved in homologous proteins present in various eukaryotic organisms including mammals and plants (**Figure 7 B**).

To examine the function of Atp23 in more detail, we first determined its subcellular localization. A strain that allowed the expression of a C-terminal hemagglutinin-epitope (HA) tagged Atp23 variant was generated. This strain grew on non-fermentable carbon sources, indicating the functionality of the modified protein (data not shown). In cellular fractionation studies, we exclusively detected Atp23-HA in the mitochondrial fraction together with the outer membrane protein Tom40, whereas Bmh1, a cytosolic protein, was only found in the postmitochondrial fraction (**Figure 8 A**). To discriminate between the possible submitochondrial localizations, we isolated mitochondria from Atp23-HA expressing cells and performed swelling experiments combined with trypsin treatment. In contrast to Tom70, an outer membrane protein exposed to the cytosol, Atp23-HA was protected against externally added trypsin in intact mitochondria. Under hypotonic conditions that lead to the disruption of the outer membrane, Atp23-HA was accessible and completely degraded upon trypsin treatment, similar to Yme1, an inner membrane protein exposed to the intermembrane space (**Figure 8 B**). The matrix protein Mge1

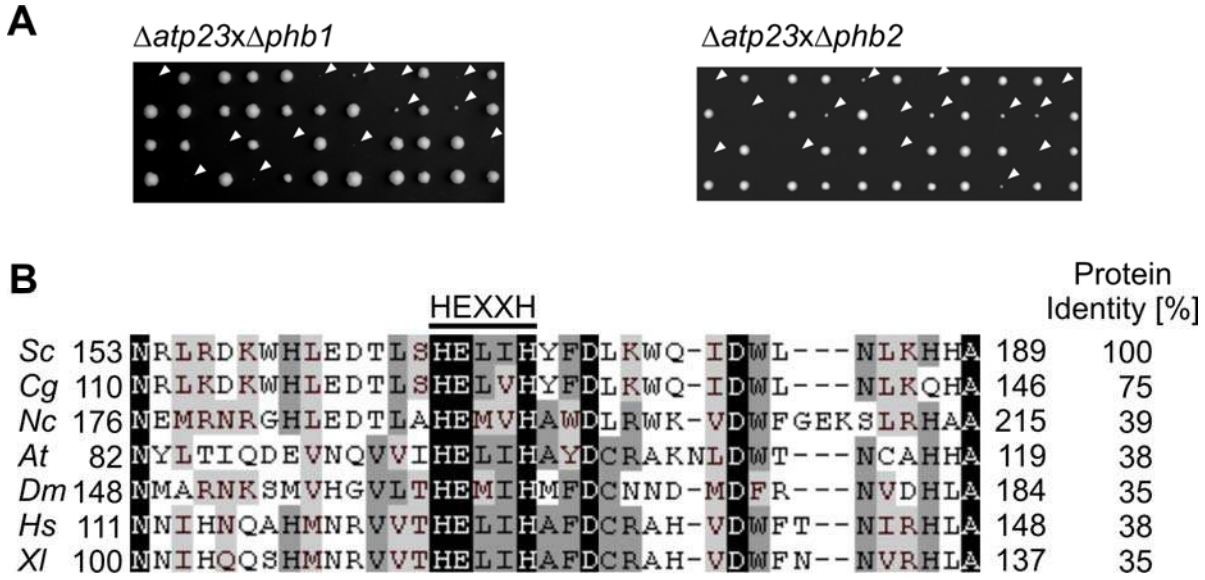

Figure 7 Prohibitins are synthetically lethal with the gene encoding the metallopeptidase Atp23 **A** Tetrads derived from a diploid strain heterozygous for *ATP23* and *PHB1* or *ATP23* and *PHB2* deletions were dissected. Genotypes were inferred from the distribution of markers linked to the deletions. Double mutant haploid spore clones are indicated by arrowheads. **B** Atp23 is a conserved metallopeptidase. Multiple sequence alignment of Atp23 with homologs found in other species. The alignment was performed with AlignX. Protein identities are according to Blastp search. *Sc Saccharomyces cerevisiae* Atp23 (Ynr020c), *Cg Candida glabrata* CAG26785, *Nc Neurospora crassa* XP322193, *At Arabidopsis thaliana* Ku70-binding family protein NP_566205, *Dm Drosophila melanogaster* CG5131-PA, *Hs Homo sapiens* KUB3, *Xl Xenopus laevis* KUB3-homologue. Identical amino acids are shown in black, conserved residues in dark gray, and similar residues in light gray.

was not degraded under any conditions. Thus, the C-terminus of Atp23 carrying the HA-epitope is exposed to the intermembrane space of mitochondria.

Sequence analysis of Atp23 with several protein prediction programs did not provide evidence for a membrane-spanning segment. Consistently, the majority of Atp23-HA was released into the soluble fraction after alkaline extraction of mitochondrial membranes at pH 11.5 (**Figure 8 C**). A similar behavior was observed for the soluble intermembrane space protein L-lactate dehydrogenase cytochrome b_2. As expected, the inner membrane protein Yme1 was recovered from the pellet fraction in these experiments. In summary, it can be concluded that Atp23 is a soluble protein resident in the intermembrane space of mitochondria.

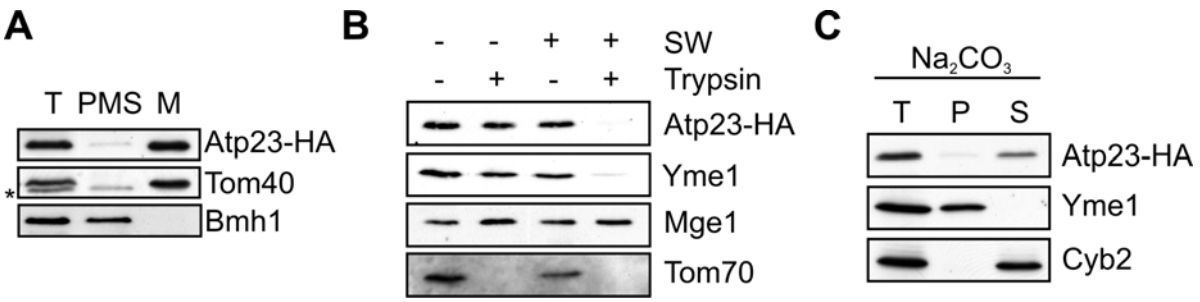

Figure 8 Atp23 is an intermembrane space protein of mitochondria. **A** Atp23 is a mitochondrial protein. Mitochondria were isolated from a strain expressing Atp23 with a C-terminal HA tag. Whole cell extracts (T), post-mitochondrial supernatant (PMS) and isolated mitochondria (M) were analyzed by SDS-PAGE and immunoblotting with indicated antisera. **B** Atp23 is localized in the intermembrane space of mitochondria. Mitochondria isolated from the yeast strain expressing Atp23-HA were treated with trypsin with or without hypotonic disruption (SW) of the outer membrane. Samples were subjected to SDS-PAGE followed by immunoblotting. **C** Atp23 is a soluble protein. Isolated mitochondria were treated with sodium carbonate (pH 11.5) (T) and subjected to ultracentrifugation to obtain soluble (S) and insoluble (P) fractions, which were analyzed by SDS-PAGE and immunoblotting.

To characterize the function of Atp23 within mitochondria, a new *ATP23* disruption strain was generated by homologous recombination. To avoid indirect effects on neighboring genes only base pairs 196-215 were replaced by a marker cassette. As observed with a strain from the Euroscarf collection, the new *Δatp23* strain was unable to grow on non-fermentable carbon sources like glycerol and formed small colonies on glucose containing medium (**Figure 9 A**). This *petite* phenotype indicated an essential function for *ATP23* in maintaining respiratory growth. We therefore examined steady-state levels of various subunits of the respiratory chain complexes in *Δatp23* mitochondria by immunoblotting. In line with the observed respiratory incompetence, a number of subunits accumulated in drastically reduced amounts in mitochondria lacking Atp23 (**Figure 9 B**). These included Atp6 and Atp18, subunits of the F_1F_O-ATP synthase (complex V), cytochrome b (complex III) and the subunits Cox1 and Cox3 of the cytochrome *c* oxidase (complex IV). The reduced amounts of various respiratory chain subunits indicate a rather profound defect in the assembly of respiratory chain complexes.

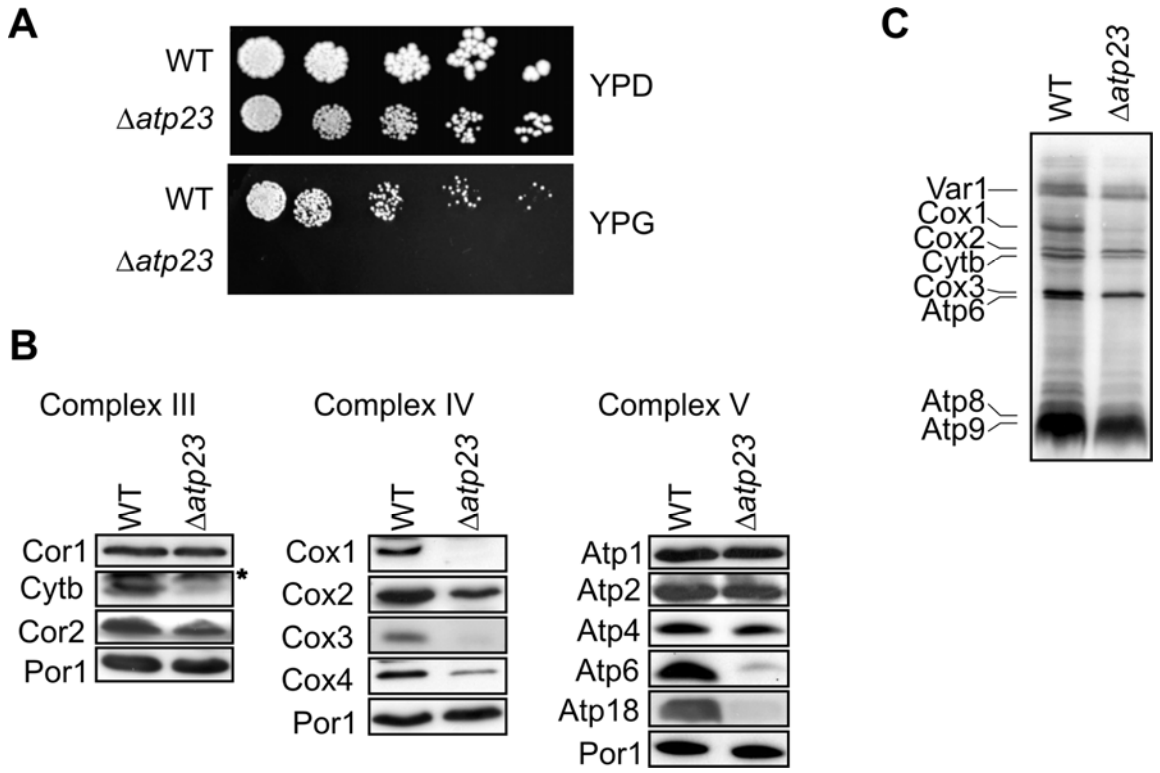

Figure 9 Atp23 is essential for respiratory growth. **A** Serial dilutions of wild-type (WT) and *⊿atp23* cells were spotted on YPD and YPG plates. Strains were grown at 30°C for three days. **B** Respiratory chain subunits are reduced in *⊿atp23* mitochondria. Steady-state levels of several respiratory chain subunits were examined by immunoblotting of mitochondrial proteins derived from *⊿atp23* and wild-type cells. **C** Mitochondrial translation is unaffected in mitochondria lacking Atp23. Mitochondrial translation competence of *⊿atp23* was compared to wild-type by *in vivo* labeling of mitochondrially encoded proteins. After inhibition of cytosolic translation with cycloheximide, cells were incubated in the presence of ^{35}S-methionine. Labeled mitochondrial proteins were separated by SDS-PAGE and analyzed by autoradiography. The efficiency of Cox1 labeling varied in different experiments (data not shown). (Performed in collaboration with Claudia Wilmes)

Respiratory growth is dependent on the correct import of nuclearly encoded subunits into mitochondria and their assembly with mitochondrially encoded subunits into respiratory chain complexes. Therefore, all proteins facilitating these assembly steps are required for efficient respiration. Additionally, the mitochondrial genome encodes eight proteins of which seven represent structural and essential subunits of the respiratory chain complexes. Consequently, functional maintenance of the mitochondrial genome is crucial for aerobic growth. To exclude a role of Atp23 in the maintenance of the mitochondrial genome or the expression of mitochondrially encoded genes, an *in*

vivo labeling experiment with ^{35}S-methionine was performed that revealed only slightly reduced synthesis of mitochondrially encoded proteins (**Figure 9 C**). Thus, *ATP23* is dispensable for mitochondrial gene expression and the pleiotropic defects observed upon its deletion appear to be the result of either a role of Atp23 for the assembly of all complexes or an indirect effect of the impaired assembly of one of these complexes.

4.3 Atp23 mediates maturation of newly synthesized Atp6

In the course of the experiments it was noted that Atp6 accumulated at a slightly larger molecular weight and in drastically reduced amounts in *⊿atp23* mitochondria (**Figure 9 B**). The same size shift was observed for newly synthesized Atp6 in *in organello* labeling experiments using wild-type and *⊿atp23* mitochondria. This difference became more apparent when a high resolution SDS-PAGE was performed after radioactively labeling mitochondrially encoded gene products in wild-type and *⊿atp23* mitochondria. Both, newly synthesized Atp6 and Atp6 detected with a specific antiserum migrated at higher molecular weights compared to the wild-type samples (**Figure 10 A**). This

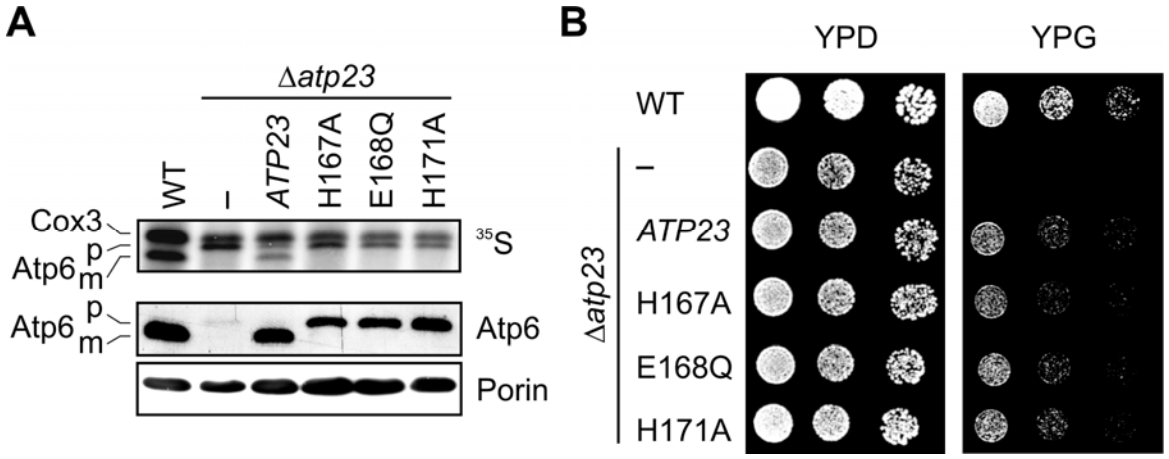

Figure 10 Atp6 processing depends on the proteolytic motif of Atp23. **A** Mitochondrial protein synthesis was carried out *in organello* in the presence of ^{35}S-methionine in isolated mitochondria from wild-type and mutant strains. Mitochondrial proteins were separated by SDS-PAGE followed by Western blotting, autoradiography (upper panel) and immunoblot analysis with indicated antisera (middle and lower panel). **B** Processing of Atp6 is dispensable for respiratory growth. The *⊿atp23* strain was transformed with plasmids carrying the wild-type gene or mutant variants, where the metalloprotease motif was disrupted. Serial dilutions of wild-type (WT) and mutant cells were spotted on YPD and YPG plates and incubated for two days at 30°C. (Performed in collaboration with Claudia Wilmes)

observation was in so far intriguing, as it was previously reported that Atp6 is synthesized as a precursor form which is matured by a yet unidentified protease prior to its assembly into the F_1F_O-ATP synthase (Michon et al., 1988). The presence of the metalloprotease motif in Atp23 and the altered running behavior of Atp6 from *⊿atp23* mitochondria suggested that Atp23 might be the missing peptidase. To test this hypothesis, we introduced point mutations into the conserved HEXXH motif that either led to the replacement of one of the histidines by alanine or the replacement of the glutamic acid by glutamine (*ATP23*^{H167A}, *ATP23*^{E167Q} or *ATP23*^{H171A}). These mutations were previously shown to completely inactivate other metalloproteases with a HEXXH motif. Atp23 and the mutant variants were expressed in *⊿atp23* cells to test for their ability to restore respiratory growth. To our surprise, the mutant variants as well as wild-type Atp23 complemented the respiratory deficiency of a strain lacking Atp23 (**Figure 10 B**). These findings suggested that either Atp23 does not exert proteolytic activity on Atp6 or that Atp23 mediates Atp6 maturation, which is not required for assembly of the functional ATP synthase.

To distinguish between these possibilities, mitochondria from strains expressing wild-type Atp23, the mutant variants or the empty vector control were isolated and subjected to *in organello* labeling with ^{35}S-methionine and subsequent SDS-PAGE to monitor Atp6 maturation. Newly synthesized Atp6 was processed in strains expressing a proteolytically active Atp23 protein (**Figure 10 A**). In strains expressing mutant Atp23, however, newly synthesized Atp6 was detected as the larger uncleaved form. An interesting observation was made, when we examined the steady-state levels of Atp6 in these samples by immunoblotting with Atp6-specific antiserum. While the processed form of Atp6 was absent, both in cells lacking Atp23 or expressing proteolytically inactive variants, steady-state levels differed significantly between these samples. In contrast to *⊿atp23* mitochondria, where Atp6 was barely detectable, the precursor form of Atp6 accumulated at wild-type levels in mitochondria harbouring the mutant Atp23 variants (**Figure 10 A**). These results therefore suggest a dual role for Atp23 in the biogenesis of Atp6. One is the processing of Atp6 and the other one is the stabilization

of Atp6 that is sufficient to allow respiratory growth and apparently independent of its proteolytic activity.

4.4 The assembly of the F_O-particle of the ATP Synthase depends on Atp23 but not its proteolytic activity

To further characterize the non-proteolytic role of Atp23, we examined the assembly of respiratory chain complexes in cells lacking Atp23 or expressing mutant variants by blue native PAGE (BN-PAGE) analysis. Gentle solubilization of mitochondrial complexes with digitonin preserves respiratory supercomplexes, namely the dimeric and monomeric form of the F_1F_O-ATP synthase as well as supercomplexes consisting of complexes III and IV (Arnold et al., 1998). These complexes can be identified by immunoblotting with specific antisera directed against subunits of the complexes. As it is already apparent in the Coomassie stained BN-PAGE, respiratory complexes are drastically reduced in mitochondria lacking Atp23 compared to the wild-type control (**Figure 11**). In contrast, all respiratory chain complexes accumulated at wild-type levels in mitochondria harbouring plasmid-borne Atp23 or the mutant variants. These results were further substantiated by immunoblotting with antisera directed against diverse subunits of the respiratory chain complexes. The assembled monomeric and dimeric F_1F_O-ATP synthase complexes were virtually undetectable in mitochondria lacking Atp23, using either an Atp6-specific antiserum or an antiserum directed against $F_1\alpha$, a subunit of the peripheral membrane-associated F_1-part of the ATP synthase. Regardless of the integrity of the metalloprotease motif, monomeric and dimeric F_1F_O-ATP synthase complexes were easily detectable in cells expressing Atp23. Notably, a major band corresponding to a native molecular mass of ~440 kDa accumulated in the absence of Atp23, which was detected with $F_1\alpha$-specific, but not Atp6-specific antibodies and therefore most likely represents the assembled F_1-particle.

A less dramatic effect, revealed by immunoblotting with a specific antiserum against the complex IV subunit Cox2, was also observed for the abundance of the supercomplexes consisting either of two complex III and two complex IV subcomplexes (III_2IV_2) or two complex III and one complex IV subcomplexes (III_2IV) (**Figure 11**). In summary, steady-

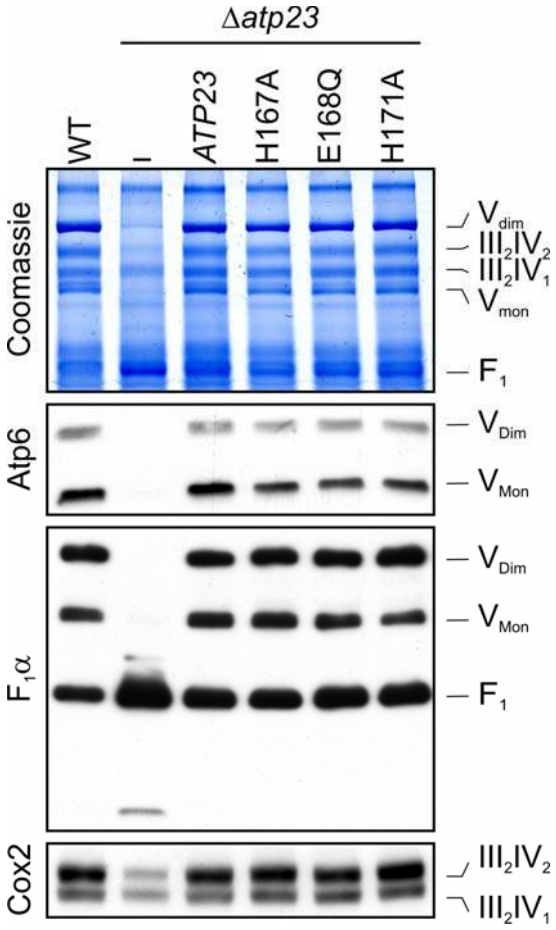

Figure 11 The assembly of the F_1F_O-ATP synthase and other respiratory chain complexes is reduced in the absence of ATP23. Mitochondria of *Δatp23* and wild-type strains were solubilized in digitonin and analyzed by BN-PAGE (3-11%) followed by Coomassie staining or immunoblotting with indicated antisera.

state levels of respiratory chain subunits, as wells as the abundance of the complexes themselves, are significantly reduced in *Δatp23* cells. Specifically, the assembly of the monomeric and dimeric F_1F_O-ATP synthase is most severely compromised in these cells. The defects can be rescued by the expression of proteolytically inactive Atp23 proteins, which suggests a role for Atp23 in ensuring respiratory chain complex assembly independent of its proteolytic function.

4.5 Atp23 and Atp10 affect F_O-assembly at a similar step

These results suggest two independent roles of Atp23: The maturation of newly synthesized Atp6 and the assembly of Atp6 into the F_1F_O-ATP synthase. To test which of these functions is essential in the absence of prohibitins, a diploid strain heterozygous for a *PHB1* and an *ATP23* deletion was transformed with plasmids allowing the expression of Atp23 or proteolytically defective variants. After sporulation and tetrad dissection of these strains, spore clones carrying both mutations and harbouring one of the plasmids were subjected to growth analysis. Irrespective of its proteolytic activity, Atp23 was able to complement the synthetic lethality of *Δphb1Δatp23* strains on media containing glucose or glycerol as the carbon source (**Figure 12 A**). Poor growing double mutant spores containing an empty vector stopped proliferation upon further cultivation. Thus, F_1F_O-ATP synthase assembly rather than maturation of Atp6 is required for growth of prohibitin-deficient cells. Additionally, the cleavage of a putative unidentified substrate of Atp23 cannot be essential in prohibitin-deficient cells.

The subunit-specific chaperone Atp10 can be cross-linked to newly synthesized Atp6 and assists its assembly into the F_O-particle (Tzagoloff et al., 2004). Strikingly, we identified *ATP10* as another genetic interactor in our search for genes essential in cells devoid of prohibitin (**Figure 12 B**). Therefore, Atp10 and Atp23, two chaperones required for efficient assembly of Atp6 into the F_1F_O-ATP synthase complex, are both required for growth of prohibitin-deficient cells.
Previous studies have demonstrated that Atp10 assists the binding of Atp6 to an oligomer of Atp9 at late stages of F_O-assembly (Tzagoloff et al., 2004). To examine whether Atp23 affects the biogenesis of this complex in a similar manner, we tested the assembly of the F_1F_O-ATP synthase in wild-type, *Δatp10* and *Δatp23* cells in parallel by BN-PAGE and immunoblotting of digitonin-solubilized mitochondria. Coomassie staining of the BN-PAGE revealed a comparable reduction of the monomeric and dimeric form of the F_1F_O-ATP synthase in both *Δatp10* and *Δatp23* mitochondria (**Figure 12 C**). This was substantiated by immunoblotting with antisera directed against $F_1\alpha$, Atp6 and Atp9.

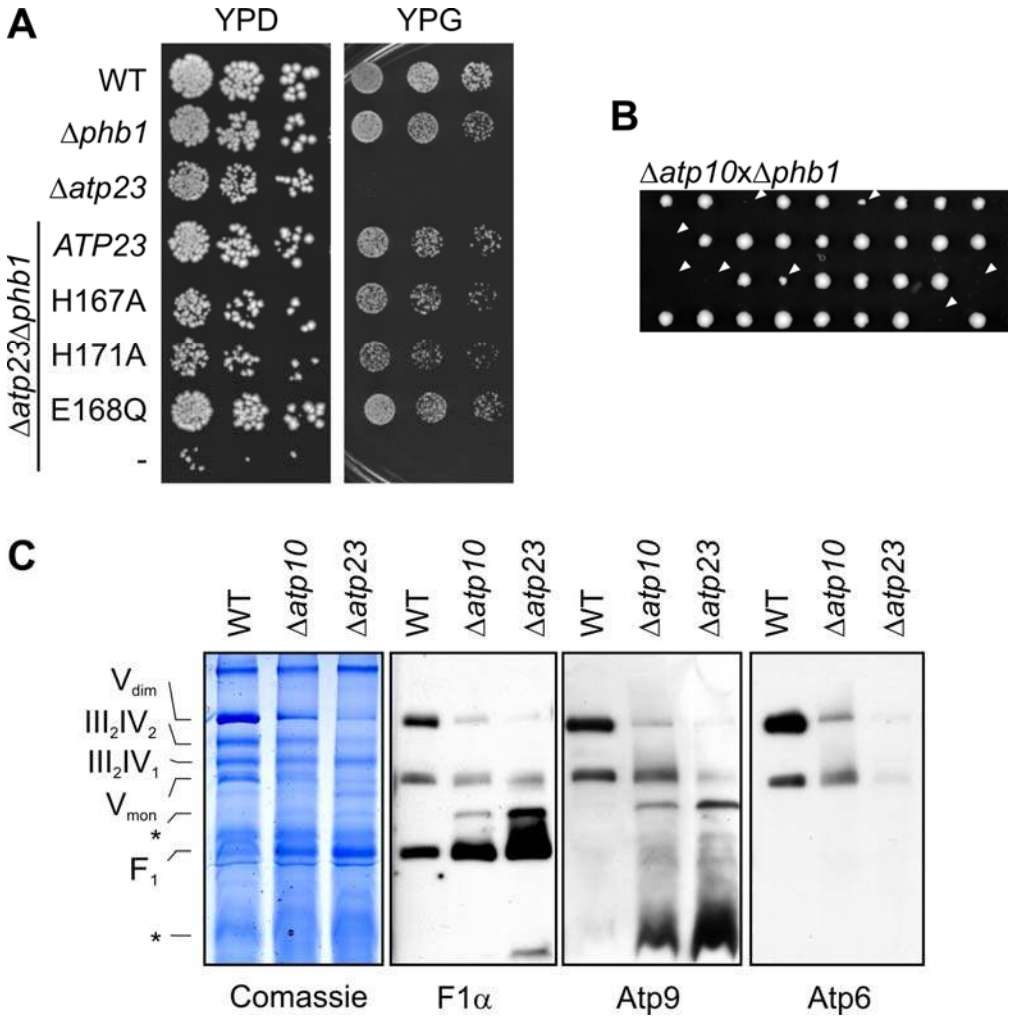

Figure 12 Genetic interaction of *PHB1* with *ATP23* and *ATP10*, both controlling Atp6 assembly into the F_O-particle. **A** Proteolycally inactive Atp23 allows growth of *Δatp23Δphb1* cells. Tetrads derived from diploid *Δatp23*/*ATP23* *Δphb1*/*PHB1* cells expressing Atp23 or its proteolytically inactive variants $Atp23^{H167A}$, $Atp23^{E167Q}$ or $Atp23^{H171A}$ were dissected. Ascospores carrying deletion of both *ATP23* and *PHB1* and expressing Atp23 or variants thereof were isolated and examined for growth on YPD or YPG at 30°C. **B** Synthetic lethal interaction of *ATP10* and *PHB1*. Tetrads derived from a diploid strain heterozygous for *ATP10* and *PHB1* deletions were dissected. Genotypes were inferred from the distribution of markers linked to the deletions. Inviable *Δatp10Δphb1* double mutant haploid spore clones are indicated by arrowheads. **C** Impaired assembly of F_1F_O-ATP synthase complexes in *Δatp10* and *Δatp23* mitochondria. Isolated mitochondria were solubilized in digitonin and complexes were separated by BN-PAGE followed by Coomassie staining or immunoblotting with indicated antisera. Assembly-intermediates detected in *Δatp23* and, to a lower extent, in *Δatp10* mitochondria are marked with asterisks.

Interestingly, the $F_1\alpha$- and Atp9- but not Atp6-specific antibodies detected two additional assemblies present in mitochondria lacking Atp23 or Atp10. One was slightly larger than

the F_1-particle and the other one was significantly smaller. Thus, our BN-PAGE analysis reveals the presence of similar-sized assembly-intermediates in the absence of Atp23 or Atp10 that contain $F_1\alpha$ and Atp9 but not Atp6. It therefore seems reasonable to assume that both proteins act at a very similar step in the assembly of the mature F_1F_O-ATP synthase, which most probably is the association of Atp6 with Atp9-oligomers already associated with the F_1-particle.

A recent publication provided evidence for the role of another protein during this process, namely the protein export component Oxa1 in the inner membrane. Not surprisingly at this stage of the analysis, Oxa1 was also found to interact genetically with prohibitins (**Table 4**).

4.6 Is efficient respiratory chain assembly crucial in prohibitin-deficient cells?

The requirement of Atp10, Atp23 and Oxa1 in prohibitin-deficient cells suggests that the fidelity of a certain step in the assembly of the ATP synthase is essential in the absence of prohibitins. Therefore, it was examined whether more strains with deletions of genes synthetically lethal with prohibitins show defects in the assembly of the respiratory chain complexes. Mitochondria from 27 of these strains were isolated and solubilized with the mild detergent digitonin and assembly of respiratory chain complexes was monitored by BN-PAGE analysis and immunoblotting with antibodies recognizing the F_O-ATP synthase subunit Atp6 or the complex IV subunit Cox2. Defects observed in this analysis varied among the tested strains (**Figure 13**). A specific defect for the F_1F_O-ATP synthase was only detected in *Δatp10* and *Δatp23* mitochondria. Assembly-intermediates of the F_1F_O-ATP synthase were not observed in any other strain using antibodies recognizing $F_1\alpha$ (data not shown). Five strains revealed an altered assembly of the supercomplexes consisting of two complex III copies and either one (III_2IV_1) or two (III_2IV_2) complex IV copies. Whereas the levels of these supercomplexes appeared to be reduced in mitochondria lacking Gep7 or Gep8, no supercomplexes were present in *Δcox6* mitochondria. The defects in *Δgep4* and *Δcoq1* strains were more peculiar in their occurrence. In mitochondria lacking Gep4, supercomplexes III_2IV_1 and III_2IV_2 were

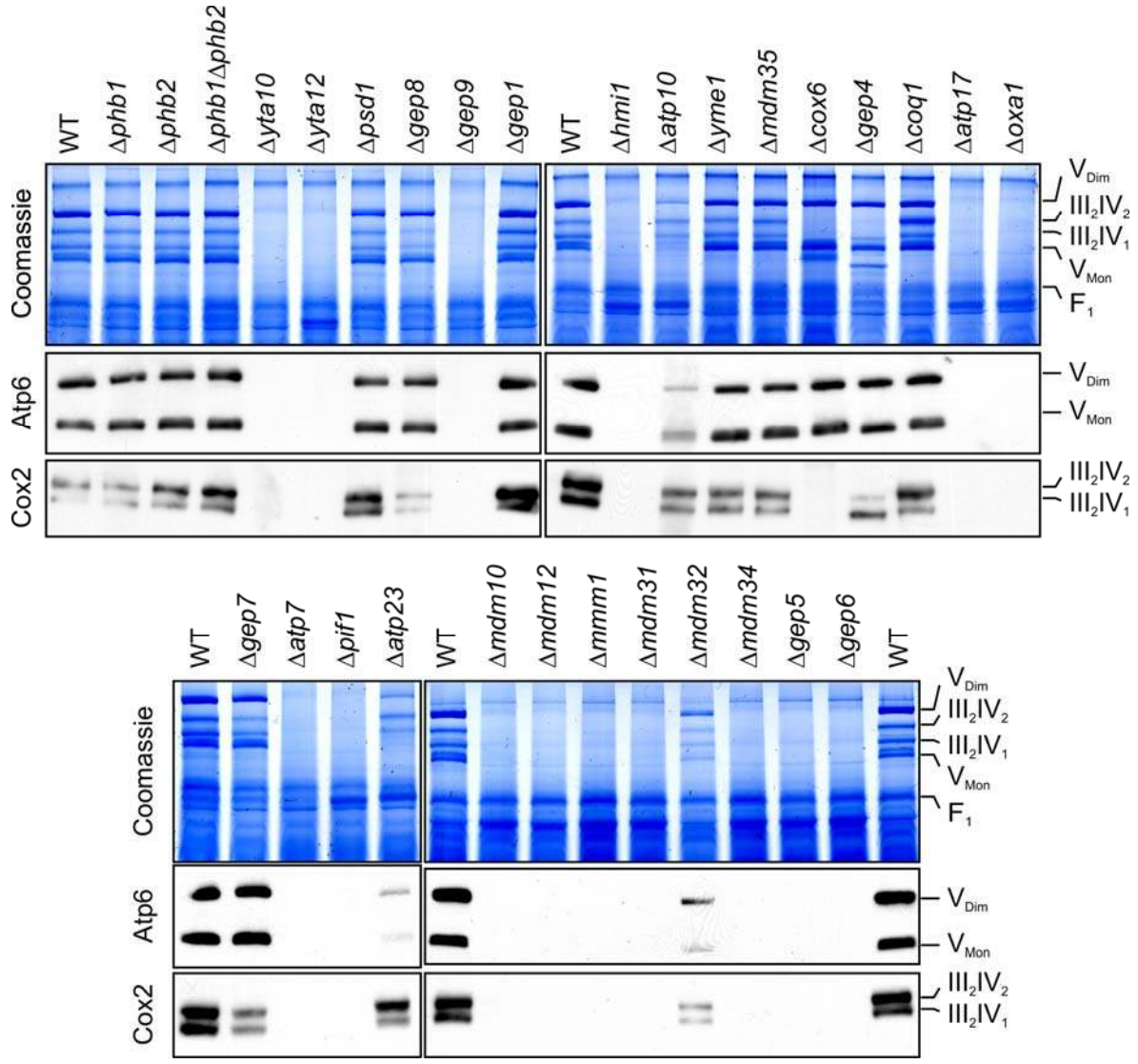

Figure 13 Analysis of respiratory chain assembly in mitochondria lacking proteins essential in the absence of prohibitins. Isolated mitochondria from indicated strains were solubilized with digitonin, and subjected to BN-PAGE analysis and immunoblotting with indicated antisera.

reduced in their abundance and both seemed to be shifted to a lower native molecular weight. Additionally, another band running slightly above the F_1-particle accumulated in these samples. LC/MS and PMF analysis of this band returned the highest scores for the proteins Cor1, Cor2, Rip1, Cyt1 and Qcr7, which are all subunits of the cytochrome *c* reductase complex (III). In contrast, no complex IV subunits were identified suggesting that the additional band observed in this sample most likely represents a monomeric or dimeric form of complex III. The *Δcoq1* mutant exhibited a more subtle defect, which

manifested itself in an increased ratio of supercomplex III_2IV_2 to supercomplex III_2IV_1 compared to the wild-type sample where this ratio is approximately 1:1 (**Figure 13**).

Several strains showed a complete absence (*Δyta10*, *Δyta12*, *Δgep9*, *Δhmi1*, *Δatp17*, *Δoxa1*, *Δatp7*, *Δpif1*, *Δmdm10*, *Δmdm12*, *Δmmm1*, *Δmdm31*, *Δmdm34*, *Δgep5*, *Δgep6*) or a severe reduction (*Δmdm32*) of all respiratory chain complexes, indicating a profound defect in the assembly of the respiratory chain (**Figure 13**). In summary, ~85% of the analyzed deletion strains exhibit a defect in respiratory chain assembly. The defects are, however, diverse in their nature, which makes it difficult to pinpoint a certain process that is essential in prohibitin-deficient cells.

4.7 Gep4, a putative phosphatase is essential for normal mitochondrial cardiolipin levels

The BN-PAGE analysis of strains with deletions of GEP-genes identified several genes required for normal assembly of the respiratory chain complexes. The deletion of *GEP4* led to a particularly interesting defect of the respiratory chain complexes as it specifically affected the assembly of complex III/complex IV supercomplexes (**Figure 13**, **Figure 14 A**). To analyze the function of Gep4 that has been localized to mitochondria previously (Kumar et al., 2002), we first blasted the protein sequence in a search for homologous proteins against a database containing non-redundant protein sequences. Gep4 turned out to have homologues among fungi and plants, whose functions have so far not been characterized (**Figure 14 B**). Further sequence examination identified Gep4 as a member of the hydrolase superfamily with an inverted DXDX(T/V) motif characteristic for phosphotransferases. It was reported that replacement of either aspartic acid in this motif leads to an inactivation of the phosphotransferase activity in other proteins displaying this motif (Collet et al., 1998). Notably this motif is conserved in homologous proteins.

To examine the importance of this motif for the function of Gep4, we cloned the C-terminally myc-tagged wild-type gene or mutant variants with replacements of either one of the aspartic acids by asparagines (*GEP4*^{D45N} and *GEP4*^{D47N}), expressed them in a

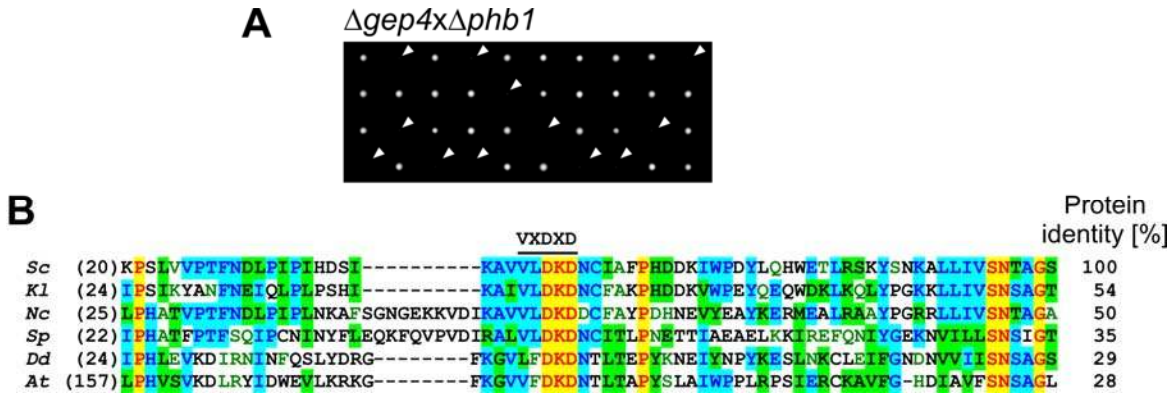

Figure 14 Gep4, a putative phosphotransferase required in prohibitin-deficient cells **A** Synthetic lethal interaction of *GEP4* with *PHB1*. Tetrads derived from a diploid strain heterozygous for *GEP4* and *PHB1* deletions were dissected. Genotypes were inferred from the distribution of markers linked to the deletions. Inviable *Δgep4Δphb1* double mutant haploid spore clones are indicated by arrowheads. AAT92669), *Kl Kluyveromyces lactis* (CAG99786), *Nc Neurospora crassa* (CAD01105), *Sp Schizosaccharomyces pombe* (CAB39898), *Dc Dictyostelium discoideum* (XP635149), *At Arabidopsis thaliana* (CAB88300).

Δgep4 strain and subjected isolated mitochondria to BN-PAGE analysis. Expression of the wild-type protein led to a restoration of the compromised assembly of the complex III/complex IV supercomplexes in *Δgep4* cells, which was indistinguishable from the wild-type cells (**Figure 15 A**). In this analysis, we also made use of an antibody specifically recognizing Cor1, a subunit of complex III. This antibody revealed the presence of the above described complex III assembly-intermediate in *Δgep4* mitochondria, which most likely represents a monomeric or dimeric form of the cytochrome bc_1 complex (III). Immunoblotting with an antibody detecting the complex IV subunit Cox2 uncovered a complex IV assembly-intermediate in cells lacking Gep4 that is not detected in wild-type mitochondria. This most likely represents a monomeric or dimeric form of complex IV as it is not detected with an antibody recognizing Cor1. Expression of the mutant variants, however, did not complement the absence of Gep4 (**Figure 15 A**). Thus, Gep4 is required for stability of the complex III/complex IV supercomplexes and relies on the integrity of its phosphotransferase motif for this function.

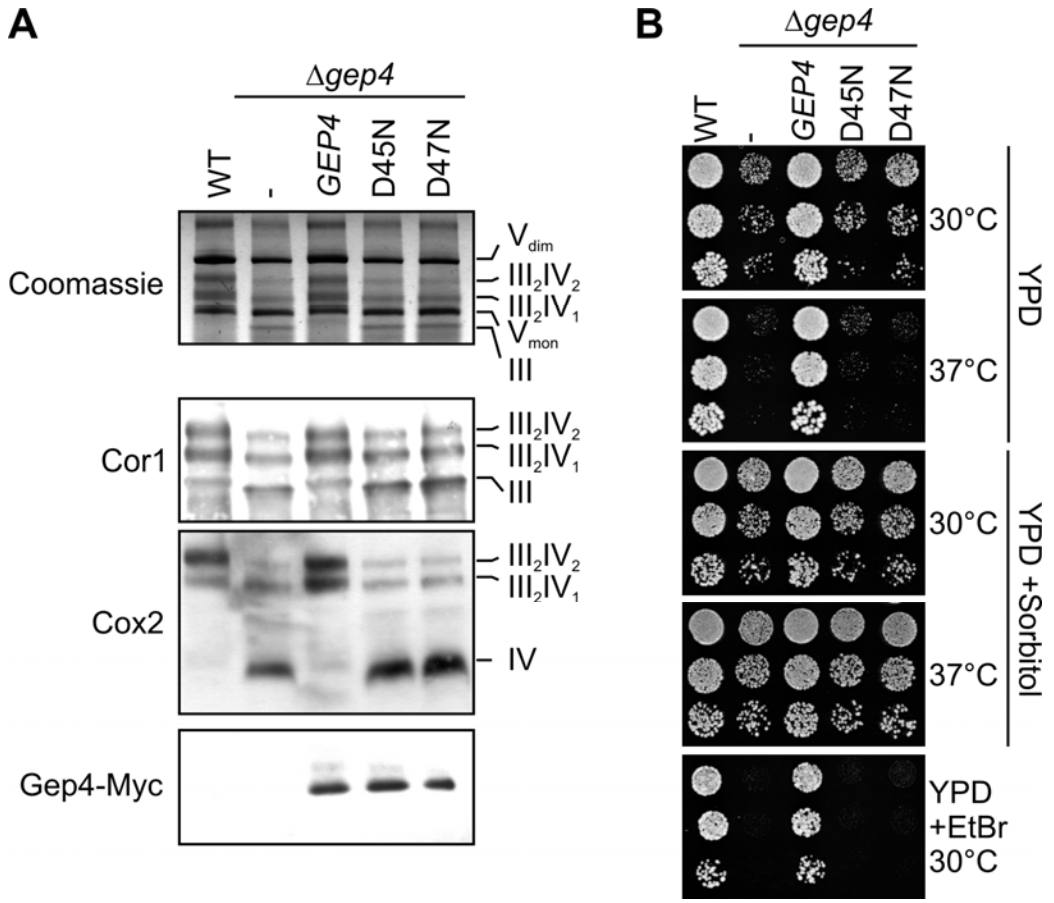

Figure 15 Gep4 is essential for supercomplex formation and growth at elevated temperatures **A** Gep4 is essential for supercomplex integrity. Mitochondria isolated from indicated strains were solubilized in digitonin and subjected to BN-PAGE analysis and immunoblotting with indicated antisera. Immunoblotting with myc-specific antibodies detected the native Gep4 complexes that did not differ in mitochondria harbouring wild-type or mutant variants. **B** *Δgep4* cells are sensitive to high temperatures and ethidium bromide. Serial dilutions of indicated strains were grown under various conditions; EtBr – ethidium bromide (25 µg/ml), sorbitol (0.6 M)

The further basic characterization of Gep4 included growth tests of the deletion strain expressing C-terminally myc-tagged wild-type and mutant variants under various conditions. Interestingly, *Δgep4* cells failed to grow at elevated temperatures and this growth defect was suppressed, when sorbitol was added to the growth medium to achieve isotonic conditions, a phenotype that is observed for cells with defects in normal cell wall biogenesis (**Figure 15 B**). Additionally, *Δgep4* cells displayed a *petite negative* phenotype as they did not grow on media containing ethidium bromide, which leads to loss of mitochondrial DNA. Under any conditions expression of Gep4 restored growth.

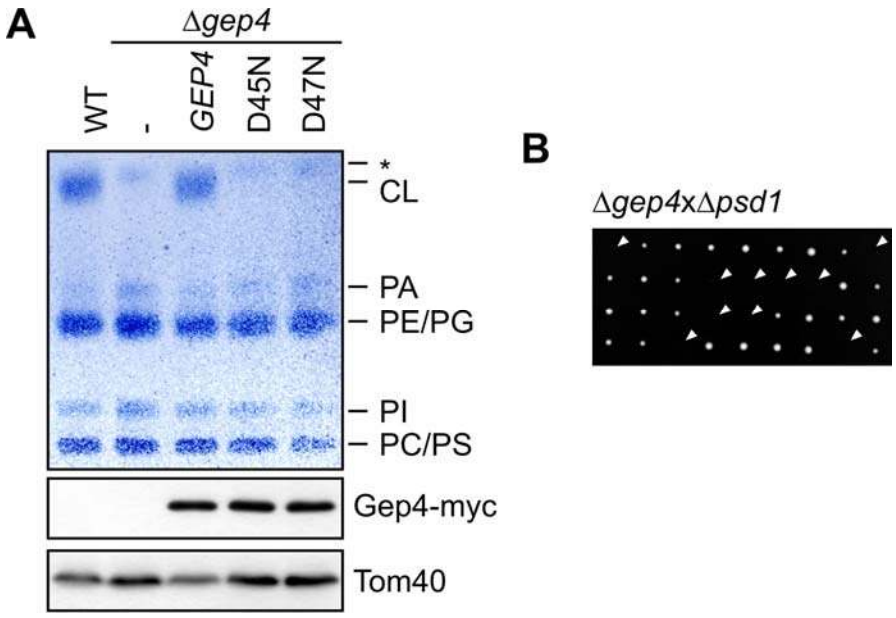

Figure 16 Gep4 is required for normal cardiolipin levels **A** Lipids were extracted from isolated mitochondria and analyzed by TLC (developing solvent: $CHCl_3/C_2H_5OH/H_2O/N(CH_2CH_3)_3$ (30/35/7/35 v/v/v/v)). Phospholipid species were identified by similar migration of synthetic marker lipids (* unidentified lipid species). Proteins from the same mitochondria were analyzed by SDS-PAGE and immunoblotting. Expression levels of Gep4 variants are identical. Tom40 served as a loading control. **B** *GEP4* and *PSD1* are synthetically lethal. Tetrads derived from a diploid strain heterozygous for *GEP4* and *PSD1* deletions were dissected. Genotypes were inferred from the distribution of markers linked to the deletions. Inviable *Δgep4Δpsd1* double mutant haploid spore clones are indicated by arrowheads.

Cells expressing mutant variants, however, exhibited identical phenotypes as cells lacking Gep4, confirming the importance of the phosphotransferase motif for Gep4 function.

The phenotypes of *Δgep4* cells, namely the disturbed assembly of the complex III/complex IV supercomplexes, the sensitivity to ethidium bromide and high temperatures that can be suppressed by osmotically stabilizing the cells, are highly reminiscent of those reported for *Δpgs1* or *Δcrd1* strains (Chang et al., 1998a; Dzugasova et al., 1998; Janitor et al., 1996; Jiang et al., 2000; Kronekova and Rodel, 2005; Zhong et al., 2004). Pgs1 catalyzes the synthesis of phosphatidylglycerol-phosphate from CDP-diacylglycerol and glycerol-3-phosphate, which is the first step in the synthesis of CL. Crd1 is the last enzyme in the biosynthesis cascade and produces

CL from CDP-DAG and PG. Consequently, *Δpgs1* or *Δcrd1* cells are devoid of any CL, the hallmark phospholipid of mitochondria. Therefore, we examined the phospholipid composition of *Δgep4* cells expressing C-terminally myc-tagged wild-type or mutant Gep4 proteins. Mitochondria were isolated and subjected to lipid extraction and thin layer chromatography (TLC) analysis. Strikingly, CL was undetectable in mitochondria lacking Gep4 or harbouring the mutant variants, whereas other phospholipids remained unaffected (**Figure 16 A**). In contrast, expression of wild-type Gep4 restored CL levels.

Genetic evidence for a role of Gep4 in CL biosynthesis was obtained, when we tested for a genetic interaction of *GEP4* and *PSD1*. It is well accepted that cells devoid of CL cannot tolerate a decrease of mitochondrial PE levels that can be achieved by deletion of *PSD1* (Gohil et al., 2005). Tetrad dissection of *Δgep4*/*GEP4 Δpsd1*/*PSD1* cells did not lead to viable double mutant offspring, indicating synthetic lethality (**Figure 16 B**). We conclude that Gep4 plays an essential role in the biosynthesis of CL that depends on a functional phosphotransferase motif. The phenotypes associated with deletion of *GEP4* most probably are the consequence of the inability of these cells to maintain normal CL levels. This assumption is based on the reported studies on *Δpgs1* or *Δcrd1* strains that exhibit very similar phenotypes. *GEP4* therefore represents the third gene, besides *PSD1* and *CRD1,* which is synthetically lethal with prohibitins and exerts a crucial function in ensuring normal supply of mitochondria with phospholipids.

4.8 Gep1, an uncharacterized open reading frame synthetically lethal with prohibitins

A reexamination of our list of genetic interactors of prohibitins prompted us to focus on two more genes, namely *GEP1* and *UPS1*. Interestingly, computational analysis revealed that Gep1 and Ups1 are both members of the PRELI/MSF1' protein family, which is characterized by the presence of the 170 amino acid encompassing PRELI/MSF1' domain (**Figure 17 A**). A fact increasing our interest in the elucidation of the function of Gep1 and Ups1 is the conservation of the PRELI/MSF1' proteins among higher eukaryotes (**Figure 17 A**). The PRELI/MSF1' domain encompasses almost the whole sequence of these proteins, but it is also found as an N-terminal domain in

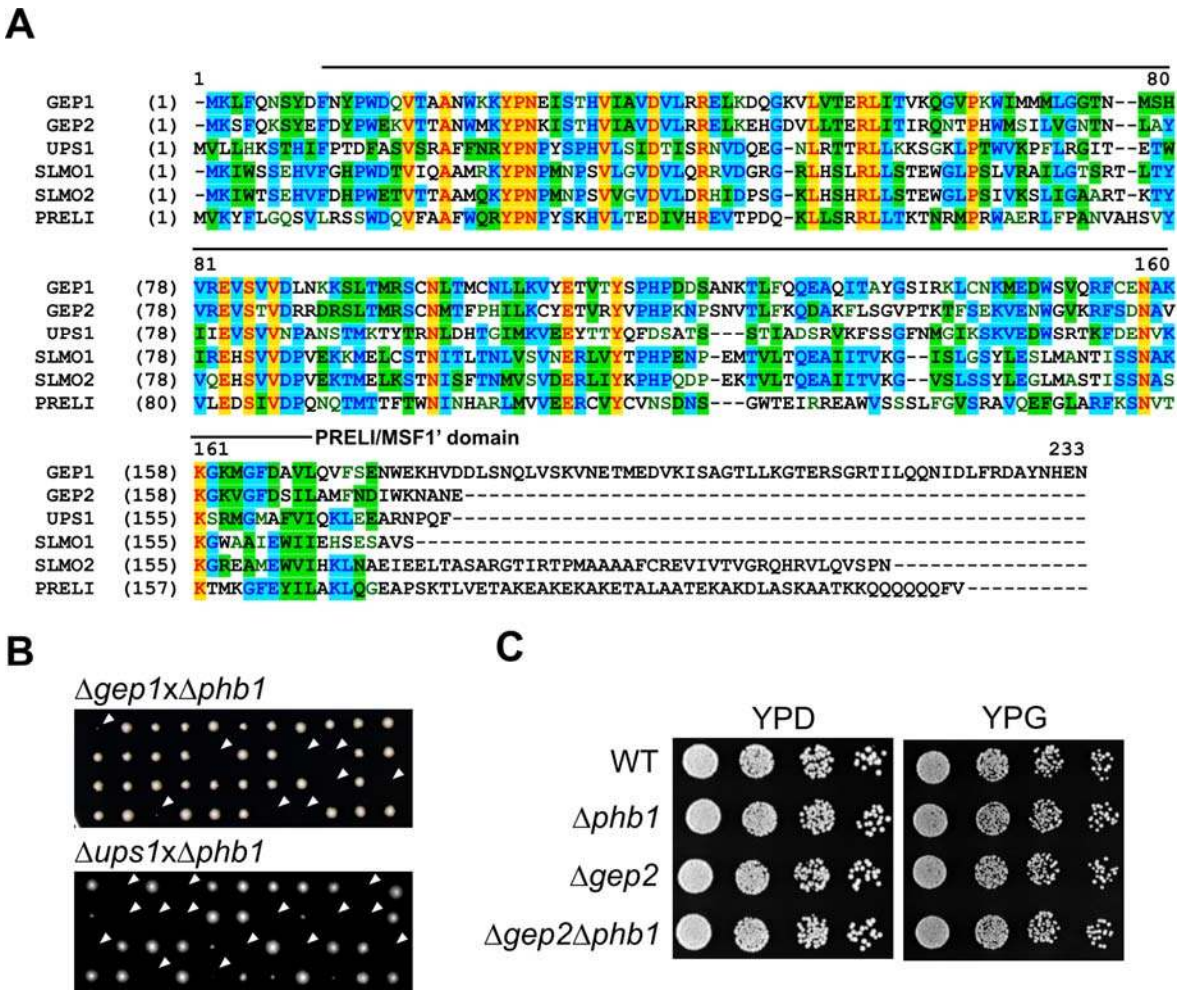

Figure 17 Gep1 and Ups1, members of the PRELI/MSF1' family, genetically interact with prohibitins **A** Gep1-like proteins are conserved in humans. Multiple sequence alignment (Score matrix: Blosum62) of Gep1, Gep2, Ups1 and human homologs. The conserved, but uncharacterized PRELI/MSF1' domain is depicted. **B** Gep1 and Ups1 are essential in prohibitin-deficient cells. Tetrads derived from diploid strains heterozygous for *GEP1* and *PHB1* or *UPS1* and *PHB1* deletions were dissected. Genotypes were inferred from the distribution of markers linked to the deletions. Inviable *Δgep1Δphb1* or *Δups1Δphb1* double mutant haploid spore clones are indicated by arrowheads. **C** *GEP2* and *PHB1* do not interact genetically. Serial dilutions of indicated strains were spotted on YPD or YPG plates that were incubated at 30°C for two or four days, respectively.

combination with other domains. A function for this domain, however, has so far not been described.

No viable double mutants were retrieved when the synthetic lethality was confirmed by tetrad dissection of diploid cells heterozygous for deletions of *PHB1* and *GEP1* or *PHB1*

and *UPS1* (**Figure 17 B**). This is especially remarkable for the *Δgep1Δphb1* double mutant as neither the *Δgep1* nor the *Δphb1* single mutants display drastic phenotypes on fermentable or non-fermentable carbon sources, pointing to roles of both proteins in functionally closely related processes essential for mitochondrial integrity. The PRELI/MSF1' protein family contains another yeast protein, encoded by the open reading frame *YDR185c*, which is to ~57% identical with Gep1 but is not essential in prohibitin-deficient cells (**Figure 17 C**). For reasons that are explained in a later section, this gene was termed *GEP2*. Gep1 and Gep2 have not been studied in any detail to date and await their assignment to a functional process. Ups1, in contrast, has recently evoked some interest because it was implicated in the regulation of processing of Mgm1, a GTPase present in the intermembrane space of mitochondria (Sesaki et al., 2006). Its precise function in this process, however, remained elusive.

4.9 Mitochondrial inner membrane integrity depends on Gep1 and prohibitins

To examine the molecular basis of the genetic interaction between *GEP1* and *PHB1*, we developed an approach that allowed us to follow the fate of *Δgep1* cells, upon downregulation of prohibitins. Therefore, we generated *Δphb1* or *Δgep1Δphb1* strains expressing Phb1 from a doxycycline-repressible promoter ([*PHB1*]). First, cell growth was determined on media containing glucose or galactose as the carbon source in the presence or absence of doxycycline. Downregulation of Phb1 expression on doxycycline-containing medium led to a dramatic growth defect in Gep1-deficient cells, but had no effect in cells with a functional *GEP1* gene (**Figure 18 A**). This observation is in accordance with the identified genetic interaction between *PHB1* and *GEP1*. Analysis of mitochondrial proteins upon downregulation of Phb1 in *Δgep1Δphb1*[*PHB1*] cells revealed that specifically proteins resident in the inner mitochondrial membrane (Cox2, Yme1 and Tim23) or the matrix (Aco1) were depleted from mitochondria after prolonged incubation of the cells in the presence of doxycycline (**Figure 18 B**). Strikingly, steady-state levels of three different outer membrane marker proteins (Tom40, Tom70 and Por1) remained unchanged even after 36 hours of doxycycline treatment. We further investigated the membrane potential, which is maintained across the inner

mitochondrial membrane and is required for import of proteins into the matrix and the inner mitochondrial membrane. Already after 12 hours of doxycycline treatment of *⊿gep1⊿phb1*[*Phb1*] cells, the membrane potential was completely dissipated in isolated mitochondria (**Figure 18 C**). In line with previous observations, depletion of Phb1 in

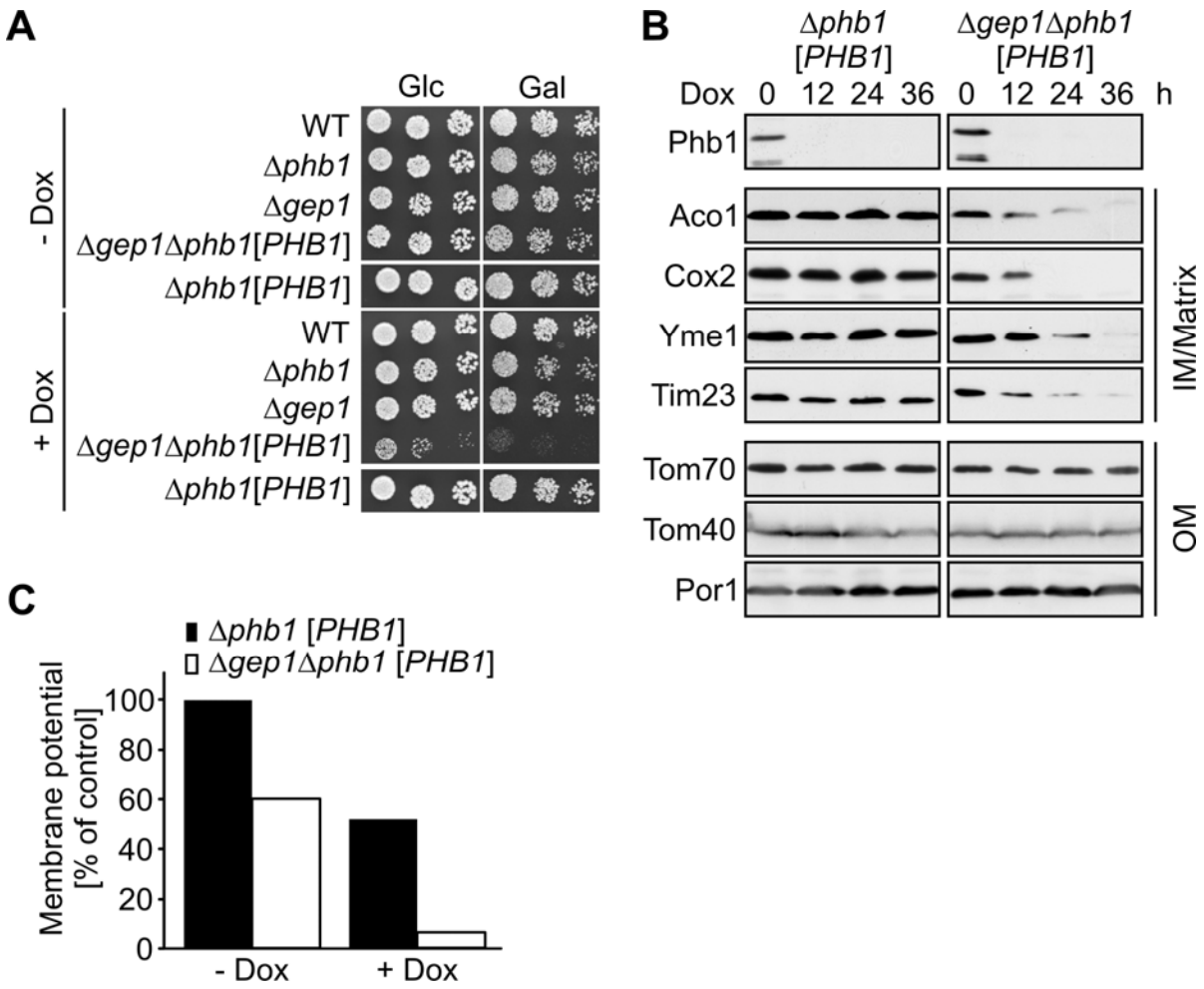

Figure 18 Phb1 is required for maintenance of inner membrane integrity in the absence of Gep1 **A** Synthetic lethal interaction of *GEP1* with *PHB1*. Fivefold dilutions of identical cell amounts were spotted on glucose or galactose containing YP plates, where indicated doxycycline (Dox) was supplemented. [*PHB1*] indicates the presence of a vector allowing doxycycline repressible expression of *PHB1* **B** Steady-state levels of mitochondrial inner membrane proteins decrease upon depletion of Phb1 in *⊿gep1⊿phb1*[*PHB1*] cells. Mitochondria were isolated from strains grown in the presence of doxycycline in YPD for different time periods. Mitochondrial proteins were analyzed by SDS-PAGE and Western Blot **C** The membrane potential is depleted in mitochondria lacking Gep1 and Phb1. Membrane potential was assessed in mitochondria isolated from strains grown for 12 h in YPD in the presence or absence of doxycycline by the potential-sensitive dye 3,3'-dipropylthiadicarbocyanine iodide ($DiDC_3(5)$).

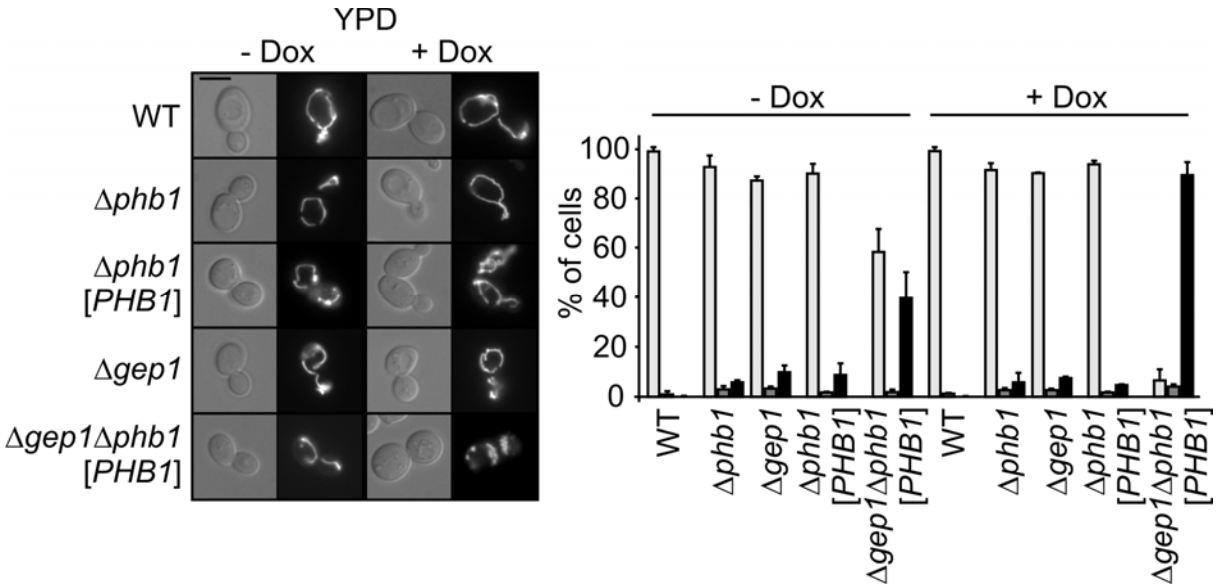

Figure 19 Aberrant mitochondrial morphology in cells lacking Gep1 and Phb1. Wild-type (WT), Δ*phb1*, Δ*gep1*, Δ*phb1*[*PHB1*] and Δ*gep1*Δ*phb1*[*PHB1*] cells expressing mitochondria-targeted GFP or DsRed were grown to log phase in YPD medium in the presence or absence of doxycycline (DOX) and analyzed by DIC (left) and fluorescence (right) microscopy. Bar, 5 µm. The bar plot indicates the percentage of wild-type-like (light grey), fragmented (dark grey) and ball shaped (black) mitochondria. n≥100. Data represent mean values ± s. d. of three independent experiments. (Microscopic analysis was performed in collaboration with Jonathan Rodenfels)

otherwise wild-type cells already resulted in a decreased membrane potential (Coates et al., 1997).

To assess mitochondrial morphology in cells lacking Gep1 and prohibitins, we expressed mitochondrially targeted GFP variants in wild-type, Δ*gep1*, Δ*phb1* or Δ*gep1* and Δ*gep1*Δ*phb1* cells harbouring [*PHB1*]. An inspection of these cells by fluorescence microscopy revealed the accumulation of ball-shaped and clustered mitochondria in Δ*gep1*Δ*phb1*[*PHB1*] cells upon downregulation of Phb1 (**Figure 19**). In the absence of doxycycline, mitochondrial morphology was slightly impaired in these cells reflecting most likely a deleterious effect of Phb1 overexpression. Notably, the aberrant morphology of mitochondria was not a consequence of cell death, as the majority of the cells (>85%) examined in these experiments were still viable (data not shown). Deletion of *PHB1* or *GEP1* did not interfere with the formation of tubular mitochondria on glucose-containing medium (**Figure 19**).

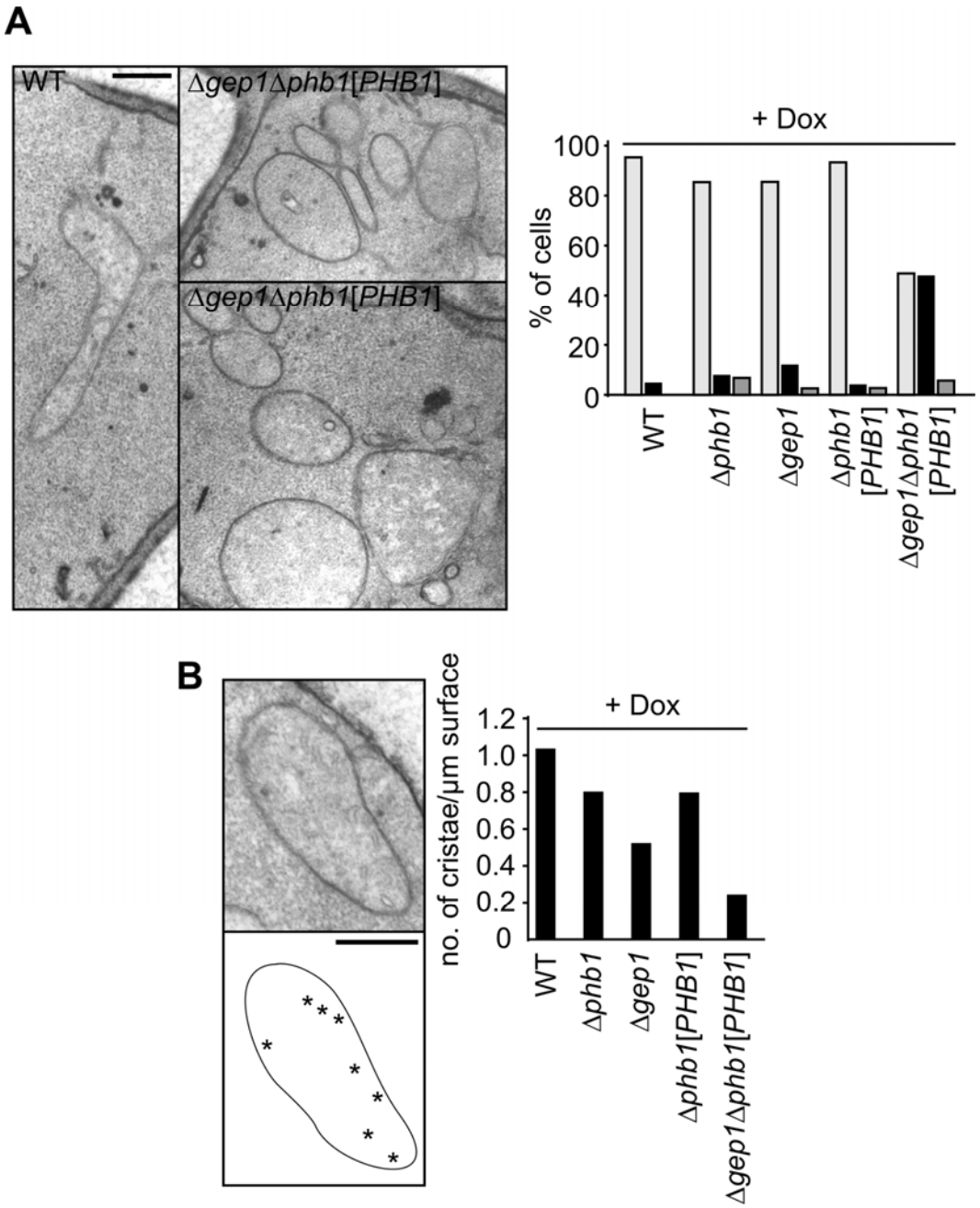

Figure 20 Mitochondrial ultrastructure is compromised in cells lacking Gep1 and Phb1 **A** Wild-type, Δ*phb1* and Δ*gep1* or Δ*phb1*[*PHB1*] and Δ*gep1*Δ*phb1*[*PHB1*] cells depleted of prohibitin were grown to log phase in YPD medium containing doxycycline (DOX) and analyzed by transmission electron microscopy (bar, 500 nm). The bar plot indicates the percentage of wild-type-like (light grey) or clustered mitochondria (black), and other mitochondrial phenotypes (dark grey), n=100. **B** The cristae/contour ratio was determined as described in the method section. A characteristic mitochondrion containing 8 cristae marked with asterisks is shown in the left panel (bar, 500 nm), the quantification in the right panel. n≥100. (Microscopic analysis was performed in collaboration with Jonathan Rodenfels)

The analysis of mitochondrial ultrastructure by transmission electron microscopy revealed aggregation of mitochondria in ~40% of Δ*gep1*Δ*phb1*[*PHB1*] cells upon downregulation of Phb1 but not in wild-type, *Δphb1*, *Δgep1* or Δ*phb1*[*PHB1*] cells depleted of Phb1 (**Figure 20 A**). The surface of cristae membranes was carefully quantified and related to the surface of the mitochondrial contours in semithin sections (**Figure 20 B**). The surface of cristal membranes decreased only slightly in Δ*phb1*[*PHB1*] cells depleted of prohibitins. A more pronounced effect was observed in *Δgep1*, where cristal membranes decreased by ~50%. Δ*gep1*Δ*phb1*[*PHB1*] cells exhibited a dramatic decrease of cristal membranes by ~80% upon downregulation of Phb1 (**Figure 20 B**), demonstrating that Gep1 and Phb1 concomitantly affect mitochondrial cristae morphogenesis. The described findings provide an explanation for the synthetic lethal interaction of *GEP1* and *PHB1* and reveal essential functions of both genes for the integrity of the inner membrane that also affects mitochondrial morphology and ultrastructure.

If cells were grown on glycerol-containing medium, ~80% of Δ*gep1* and ~60% of Δ*phb1* cells contained an at least partially fragmented mitochondrial network indicating that both proteins are crucial for mitochondrial morphology under conditions of increased mitochondrial demand (**Figure 21 A**). Inner membrane fusion and the formation of cristae depend on the dynamin-like GTPase Mgm1 (Meeusen et al., 2006), which undergoes proteolytic processing by the rhomboid protease Pcp1 in the inner membrane. Cleavage results in the accumulation of two isoforms, l- and s-Mgm1, that functionally cooperate during inner membrane fusion. Interestingly, deletion of *GEP1* impaired significantly the formation of s-Mgm1 in cells grown under respiring conditions (**Figure 21 B**). These findings identify Gep1 as a novel modulator of Mgm1 processing in the inner membrane and suggest that the unbalanced accumulation of l- and s-Mgm1 causes deficiencies in cristae morphogenesis in Δ*gep1* mitochondria.

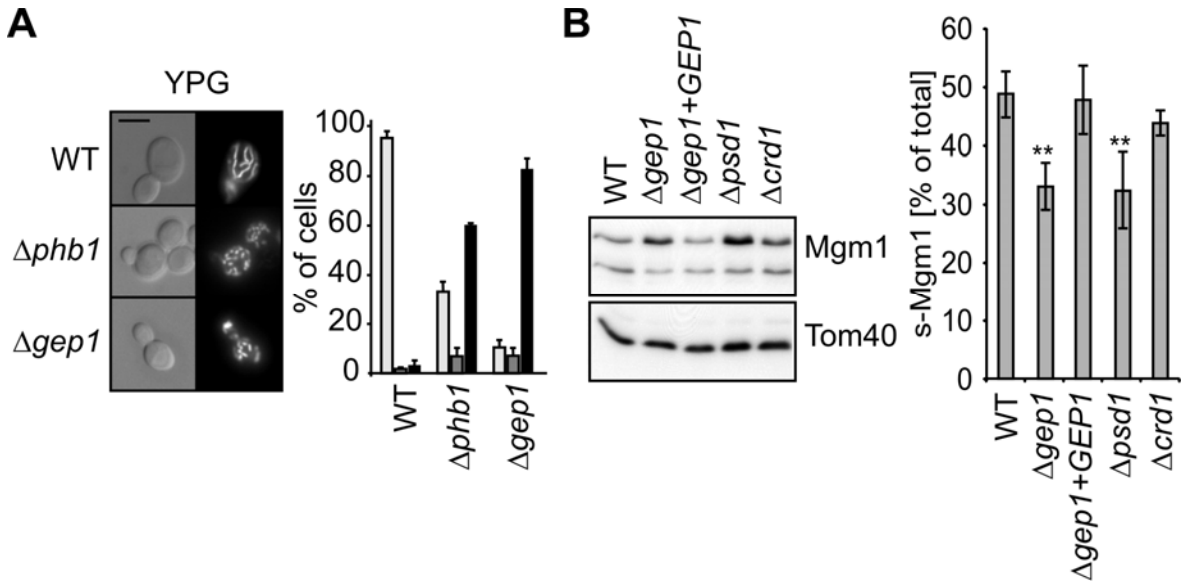

Figure 21 Mitochondrial morphology and Mgm1 processing is compromised in cells lacking Gep1 **A** Wild-type (WT), Δ*phb1* and Δ*gep1* cells expressing mitochondrially targeted GFP were grown to log phase in YPG medium and analyzed as in figure 19 A. Bar, 5 µm. The bar plot indicates the percentage of wild-type-like (light grey), fragmented (dark grey) and short tubular, partially fragmented (black) mitochondria. **B** Extracts of the indicated cells grown in YPG were analyzed by SDS-PAGE and immunoblotting using Mgm1- and Tom40-specific antisera (see 4.11 for explanation of *Δ*psd1 and *Δcrd1* results). A quantification of the immunoblots is shown in the right panel. The percentage of s-Mgm1 was calculated from the ratio s-Mgm1/(s-Mgm1+l-Mgm1), values are mean ± s. d., *p<0.05, **p<0.01, (n≥4). (Microscopic analysis was performed in collaboration with Jonathan Rodenfels; Analysis of Mgm1 processing was performed by Christoph Potting)

4.10 Gep1-like proteins are localized to the intermembrane space of mitochondria

We further examined the localization of the PRELI/MSF1' proteins Gep1, Gep2 and Ups1 in mitochondria. Whereas Ups1 has been localized to the intermembrane space of mitochondria in a report specifically analyzing Ups1 (Sesaki et al., 2006), Gep1 and Gep2 have been localized to mitochondria only by large scale studies that did not address the submitochondrial localization (Huh et al., 2003; Kumar et al., 2002). Due to the lack of specific antibodies recognizing Gep1 or Gep2, we genomically fused a triple HA- or a ninefold myc-tag to the C-termini of *GEP1* or *GEP2*, respectively. The tag did not interfere with Gep1 function, as deletion of *PHB1* in a strain with genomically tagged *GEP1* did not lead to synthetic lethality (data not shown). Similarly, C-terminal tagging of Gep2 did not compromise its function (see **Figure 23**). Isolated mitochondria from

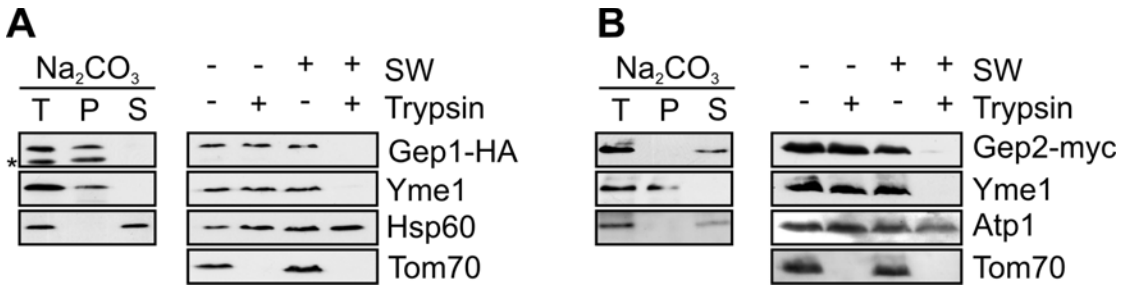

Figure 22 Gep1 and Ups1 are localized to the intermembrane space of mitochondria Mitochondria isolated from a strain expressing Gep1-HA (**A)** or Gep2-myc (**B**) were treated with sodium carbonate (pH 11.5) (T) and subjected to ultracentrifugation to obtain soluble (S) and insoluble (P) fractions, which were analyzed by SDS-PAGE and immunoblotting. For submitochondrial localization mitochondria were incubated with trypsin with or without hypotonic disruption (SW) of the outer membrane. Samples were subjected to SDS-PAGE followed by immunoblotting.

strains expressing the tagged Gep1 or Gep2 variants were treated with trypsin under isotonic conditions that preserve the integrity of the outer membrane or hypotonic conditions that result in its disruption. As expected, the matrix proteins $F_1\alpha$ or Hsp60 were not degraded under any condition, whereas Tom70, an outer membrane protein exposed to the cytosol, was sensitive to trypsin treatment even without swelling of the outer membrane (**Figure 22**). Protease treatment led to degradation of the intermembrane space protein Yme1 only when the outer membrane was disrupted. The same behavior was observed in these experiments for Gep1-HA or Gep2-Myc, indicating that both proteins are localized to the intermembrane space, the same compartment where also Ups1 has been localized (Sesaki et al., 2006). Analysis of the amino acid sequences of Gep1, Gep2 or Ups1 with a variety of transmembrane prediction programs did not provide any evidence for membrane-spanning segments in these proteins. Membrane association of these proteins was experimentally analyzed by sodium carbonate (pH 11.5) extraction of isolated mitochondria. Surprisingly, Gep1 was recovered from the pellet fraction in these experiments together with the integral membrane protein Yme1, while Hsp60 was released into the soluble fraction (**Figure 22**). Gep2 displayed an extraction pattern identical to the soluble protein $F_1\alpha$ in analogous experiments. Thus, all three PRELI/MSF1' proteins in yeast are components of the intermembrane space of mitochondria. They differ in respect to their alkaline extraction profile, which suggests a tighter membrane association for Gep1 compared to

Gep2 and Ups1 (Sesaki et al., 2006). It has to be stated though that all experiments were performed with tagged protein variants and an influence of the tag on the extraction profile cannot be excluded.

4.11 Gep1 is required for normal mitochondrial phosphatidylethanolamine levels

We used a genetic approach to functionally characterize Gep1 in further experiments. A high-copy suppressor screen was performed, in which we searched for genes whose overexpression would restore growth of the otherwise inviable *Δgep1Δphb1* strain. A haploid double mutant *Δgep1Δphb1* strain was generated that was kept viable by the presence of a plasmid allowing the expression of Phb1 ([*PHB1*]). This strain was transformed with a high-copy expression library and several suppressing clones were isolated on medium counterselecting against cells harbouring the *PHB1* expression plasmid. One very potent suppressor was the above introduced *GEP2*, which therefore genetically interacts with prohibitins as well. The identification of *GEP2* in the high-copy suppressor screen provides evidence for overlapping functions of the members within the PRELI/MSF1' protein family (**Figure 23 A**).

Strikingly, overexpression of the phosphatidylserine synthase Cho1 promoted growth of the *Δgep1Δphb1* strain close to wild-type levels, linking the defective process in the double mutants to phospholipid metabolism (**Figure 23 A**). Cho1 acts in the endoplasmic reticulum, where it synthesizes the precursor phospholipid PS that can be converted to PE by the phosphatidylserine decarboxylase Psd1 in mitochondria. Interestingly, *PSD1* is among the genes required for growth of prohibitin-deficient cells (**Figure 5 A**), pointing to a complex network of genetic interactions between prohibitins, *GEP1* and the cellular phospholipid metabolism.

We therefore determined the mitochondrial phospholipid profile of *Δgep1* cells by TLC. For the analysis of mitochondrial phospholipids, highly purified mitochondria were isolated that did not contain significant contaminations of other cellular organelles still detected in conventional mitochondrial isolations (**Figure 23 C**). Whereas most

phospholipids accumulated to wild-type levels irrespective of the presence of Gep1, PE appeared to be significantly reduced in these mitochondria (**Figure 23 B**). This suggested that Cho1, identified in the high-copy suppressor screen, rescued growth of the *⊿gep1⊿phb1* double mutant by restoring the PE defect of the single *GEP1* deletion, most likely by providing an increased amount of the PE precursor lipid PS. TLC analysis of mitochondrial lipids from *⊿gep1* strains overexpressing Cho1, Gep2 or Ups1 revealed

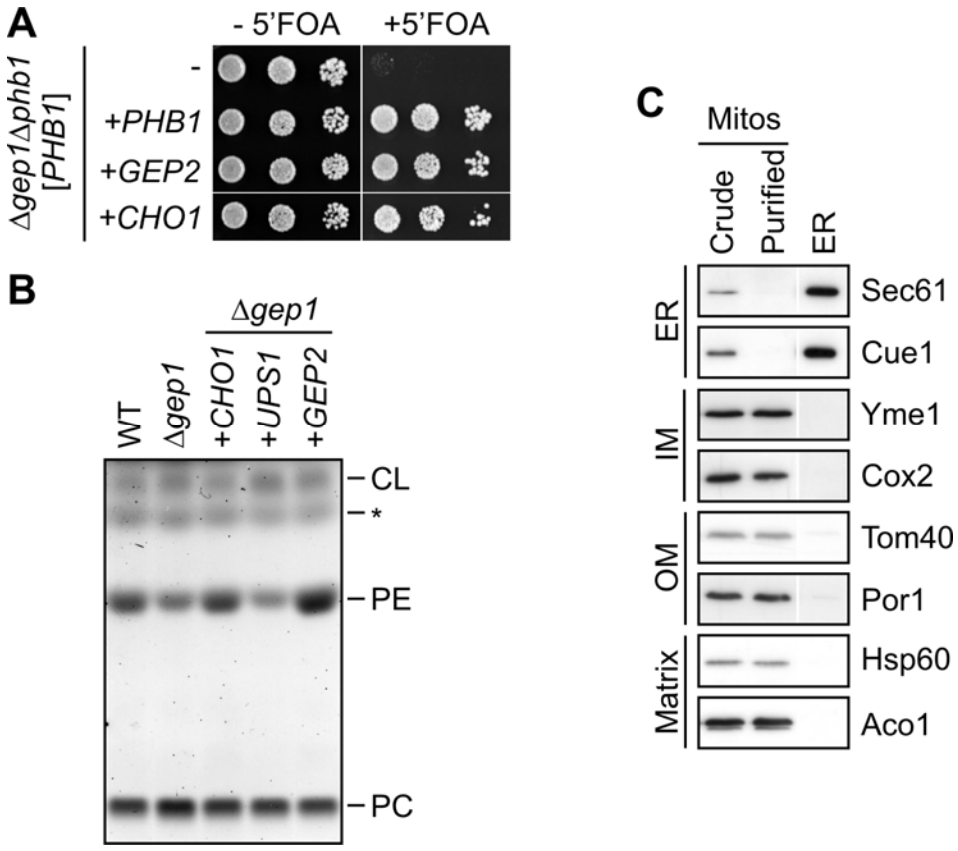

Figure 23 Gep1 is required for normal mitochondrial PE synthesis **A** Overexpression of Gep2 or Cho1 suppresses the lethality of *⊿gep1⊿phb1* cells. Serial dilutions of *⊿gep1⊿phb1*[*PHB1*] cells overexpressing Phb1, Gep2 or Cho1 were spotted on media with or without 5'FOA (5-fluoroorotic acid), which counterselects against cells harbouring the [*PHB1*] expression plasmid **B** Cho1 and Gep2 overexpression restore PE levels in *⊿gep1* cells. Lipids extracted from isolated mitochondria were separated by TLC. Phospholipid species were identified by the comparable migration of synthetic phospholipids. (* unidentified lipid species) **C** Exemplary purification of mitochondria for lipid analysis. Crude mitochondria were further purified by a sucrose gradient (see material and methods, 3.8.1). Cellular fractions were compared by SDS-PAGE and immunoblotting with antisera directed against marker proteins from mitochondria or the endoplasmic reticulum (Sec61, Cue1). IM – inner mitochondrial membrane, OM – outer mitochondrial membrane, ER – endoplasmic reticulum

that indeed Cho1 was capable of restoring normal PE levels in *Δgep1* mitochondria (**Figure 23 B**). Furthermore, the phospholipid profile was restored upon overexpression of Gep2, which is in contrast to Ups1 overexpression that did not lead to a significant increase of reduced PE levels in *Δgep1* cells. The latter observation is in line with the weak suppressive competence of Ups1 overexpression on the synthetic lethality of *Δgep1Δphb1* cells (data not shown). In conclusion, *GEP1* is essential for maintaining wild-type PE levels in mitochondria. However, PE levels can be maintained in the absence of Gep1, if the related Gep2 or Cho1 are overexpressed.

The examination of PE biosynthesis in yeast is complicated by the fact that besides Psd1 another PS-decarboxylase is present in the Golgi apparatus, termed Psd2 (Trotter and Voelker, 1995). Cells lacking both decarboxylases are unable to grow in the absence of externally added ethanolamine, due to the absence of PE biosynthesis (Robl et al., 2001). To discriminate between roles for Gep1 in the PE-biosynthesis pathways, we examined the growth of cells with deletions of *GEP1*, *PSD1*, *PSD2* or combinations of *GEP1* deletion with either one of the PS-decarboxylase gene deletions. If Gep1 is required for mitochondrial PE synthesis and therefore the Psd1-branch of PE biosynthesis, *GEP1* should also genetically interact with *PSD2*. Conversely, *GEP1* should be required for cell survival in *Δpsd1* cells if it is required for PE biosynthesis via the Psd2-branch. The results of the growth tests affirmed the first scenario (**Figure 24 A**). Under conditions of increased mitochondrial demand, specifically on a non-fermentable carbon source and elevated temperatures, the double mutant lacking Gep1 and Psd2 failed to grow, whereas no genetic interaction was observed for the *Δgep1Δpsd1* double mutant under any growth condition.

Another known genetic interaction allowed us to further support a role for Gep1 in mitochondrial PE maintenance. As described above, components of the PE biosynthesis pathway are required in cells with drastically reduced CL biosynthesis. We therefore tested whether Gep1 is required for growth of cells either lacking the CL-synthase Crd1 or Gep4, which was identified to be essential for normal CL levels in the present study. Strikingly, tetrad dissection of diploid cells heterozygous for *GEP1* and

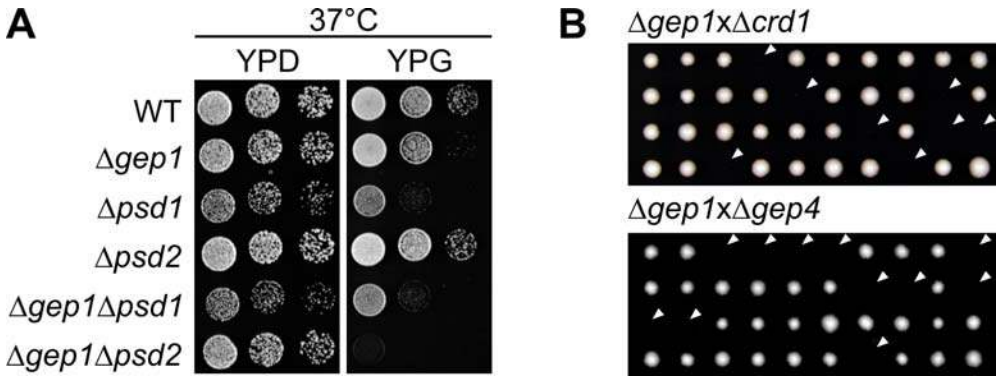

Figure 24 Gep1 is required for the mitochondrial PE biosynthesis pathway **A** *GEP1* and *PSD2* interact genetically. Serial dilutions of indicated strains were spotted on YPD or YPG plates and incubated at 37°C. **B** Synthetic lethal interaction of *GEP1* and *CRD1* or *GEP4*. A diploid strain heterozygous for *GEP1* and *CRD1* or *GEP4* deletions was subjected to sporulation and tetrad dissection. Genotypes were inferred from the distribution of markers linked to the deletions. Arrowheads indicate inviable double mutant progeny.

CRD1 or *GEP1* and *GEP4* deletions did not return any viable haploid double mutant progeny (**Figure 24 B**). Thus, overwhelming genetic support for a role of Gep1 in allocation of PE via the mitochondrial biosynthesis route is provided.

These findings raise the possibility that an altered phospholipid composition in the inner membrane impairs Mgm1 cleavage and causes an altered cristae morphogenesis in the absence of Gep1 (**Figure 20**, **Figure 21**). We therefore assessed processing of Mgm1 in mitochondria lacking Psd1 or Crd1 (**Figure 21 B**). Deletion of *PSD1*, as that of *GEP1*, impaired the formation of s-Mgm1, while Mgm1 cleavage was not affected in the absence of *CRD1* under these conditions. We conclude that the phospholipid composition, in particular the PE content, is crucial for efficient Mgm1 processing by rhomboid in the inner membrane.

4.12 Gep1 function is evolutionary conserved

To examine whether the function of Gep1 in mitochondrial PE biosynthesis is evolutionary conserved, we heterologously expressed C-terminally myc-tagged human Gep1 homologues in yeast and tested their ability to maintain growth of *Δgep1Δphb1* cells or to restore PE levels in mitochondria lacking Gep1. Whereas previous

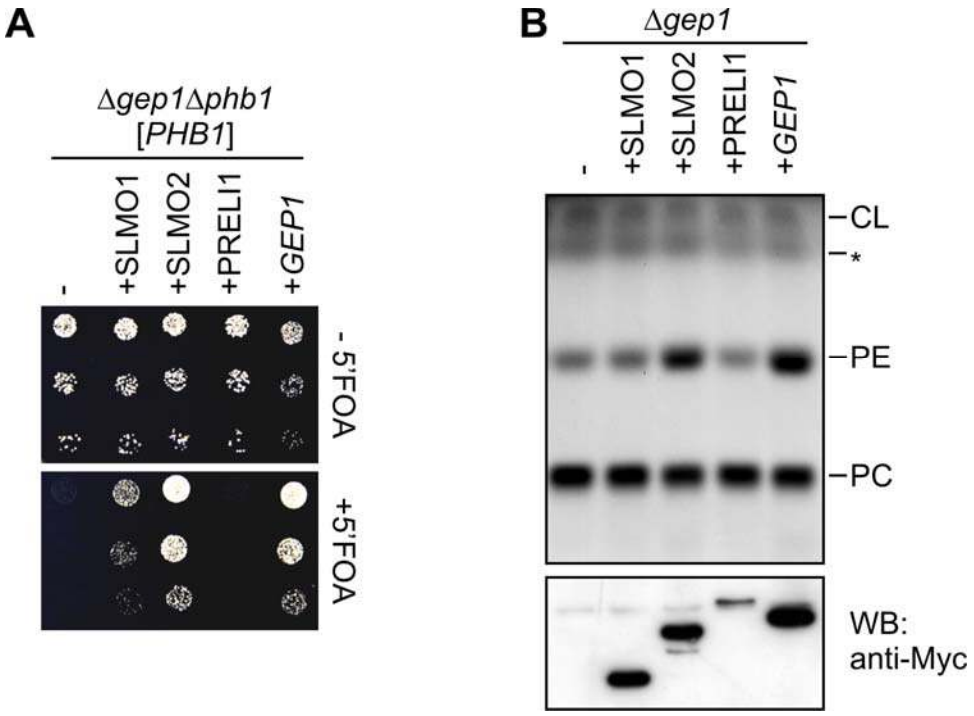

Figure 25 SLMO2 is the functional orthologue of Gep1 **A** Expression of human SLMO2 suppresses the lethality of *Δgep1Δphb1* cells. Serial dilutions of *Δgep1Δphb1*[*PHB1*] cells expressing C-terminally myc-tagged SLMO1, SLMO2 or PRELI were spotted on media with or without 5'FOA, which counterselects against cells harbouring the [*PHB1*] expression plasmid. **B** SLMO2 expression restores PE levels in *Δgep1* mitochondria. TLC analysis of lipids extracted from mitochondria isolated from *Δgep1* cells expressing C-terminally myc-tagged SLMO1, SLMO2 or PRELI. Phospholipid species were identified by the comparable migration of synthetic phospholipids (* unidentified lipid species). Isolated mitochondria were subjected to SDS-PAGE and Western blot analysis (WB). Expression of human homologs was monitored with anti-myc antiserum.

complementation studies in yeast have identified PRELI as the functional orthologue of Ups1 (Sesaki et al., 2006), SLMO1 and SLMO2 remain largely uncharacterized. Inactivation of homologues genes in *Drosophila* causes developmental defects of the central nervous system and locomotion deficits (Carhan et al., 2004; Dee and Moffat, 2005; Reeve et al., 2007). SLMO2 restored growth of *Δgep1Δphb1* cells to a similar extent as Gep1 (**Figure 25 A**). SLMO1 only weakly allowed cell survival of the double mutant and PRELI had no effect at all. The conclusion that SLMO2 is the functional orthologue of *GEP1* was further substantiated by the ability of SLMO2 to rescue the PE reduction in *Δgep1* cells (**Figure 25 B**). Neither SLMO1 nor PRELI were able to confer this complementation upon *Δgep1* cells.

4.13 Gep1 is required for the stability of mitochondrially synthesized phosphatidylethanolamine

Our genetic analyses have clearly pointed to a role of Gep1 for mitochondrial PE biosynthesis. Many steps that lead to the maintenance of normal mitochondrial steady-state PE levels are not well understood. PS generated in the endoplasmic reticulum needs to be transferred to the mitochondrial outer membrane, from where it has to be shuttled to the inner membrane, where Psd1 can catalyze its conversion to PE. Finally, the turnover of generated PE and the export of mitochondrial PE need to be controlled. Theoretically, proteins facilitating or controlling each of these steps directly or indirectly could lead to altered steady-state levels of PE. The localization of Gep1 in the intermembrane space does not exclude any of these roles. To identify the step in which Gep1 acts, we first determined whether the precursor lipid PS reaches mitochondria. To this aim, we made use of our observation that PS accumulates in mitochondria lacking Psd1. Highly purified mitochondria isolated from wild-type, *Δgep1*, *Δpsd1* or

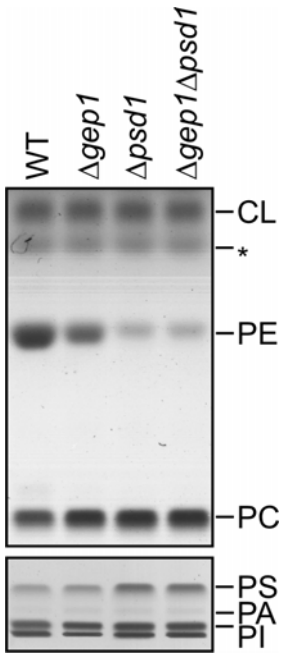

Figure 26 PS import into mitochondria is unaffected in the absence of Gep1. Lipids were extracted from highly purified mitochondria from indicated strains. Lipids were subjected to TLC analysis (Developing solvent, upper TLC: $CHCl_3/CH_3OH/25\%\ NH_3$ 50/50/3 (v/v/v), developing solvent, lower TLC: $CHCl_3/CH_3OH/25\%\ NH_3$ 65/35/5 (v/v/v)) (* unidentified lipid species).

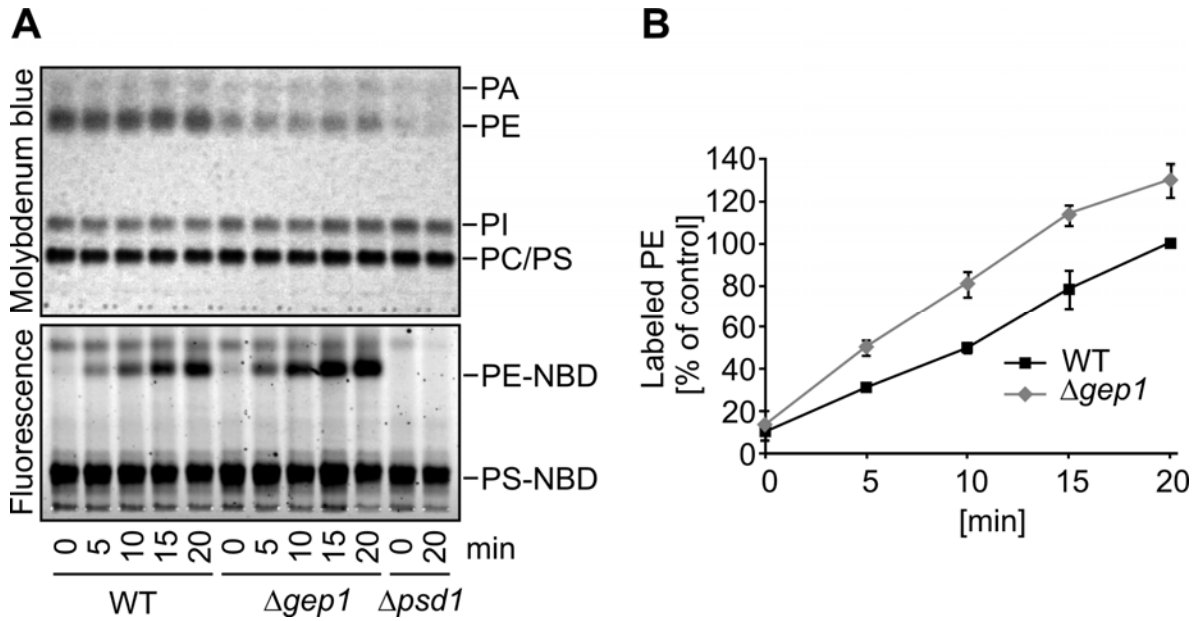

Figure 27 Psd1 activity is not decreased in *⊿gep1* mitochondria **A** WT or *⊿gep1* mitochondrial fractions were incubated with PS-NBD for indicated time periods. Lipids were isolated and separated by TLC. Fluorescence was detected (Typhoon) (**A**, lower panel) and quantified (ImageQuant) (**B**). Mean values ± s. d. were calculated as percentage of WT labeling after 20 min. Equal loading was monitored by molybdenum blue staining of the TLC plate (**A**, upper panel) (n=3).

⊿gep1⊿psd1 cells were subjected to lipid extraction and TLC analysis. PS levels in these mitochondria were identical comparing wild-type and *⊿gep1* or *⊿psd1* and *⊿gep1⊿psd1* mitochondrial phospholipids (**Figure 26**). Thus, the transport of PS from the endoplasmic reticulum to mitochondria appears to be unaffected by the absence of Gep1.

As Gep1 might directly regulate Psd1 or affect its assembly in the inner mitochondrial membrane, we developed an assay to monitor the conversion of PS by Psd1. After osmotic disruption of the outer membrane, mitoplasts were incubated with fluorescently labeled PS (NBD-PS), which is converted to NBD-PE. NBD-PE did not accumulate in mitochondria lacking Psd1, demonstrating that PE is formed in a Psd1-dependent manner (**Figure 27**). The synthesis of PE was not affected by the absence of Gep1. We rather observed a slightly but significantly increased rate of PE synthesis in *⊿gep1* mitochondria, which may indicate an alleviated product inhibition of Psd1 in these mitochondria. In conclusion, Gep1 is not important for normal Psd1 activity. No

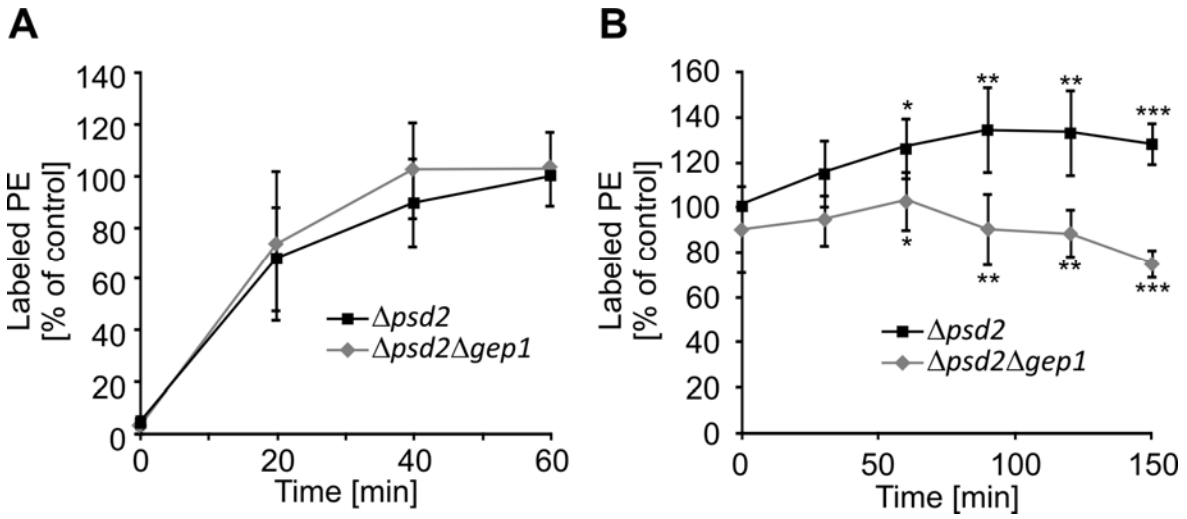

Figure 28 Mitochondrial PE stability is decreased in cells lacking Gep1. **A** *Δpsd2* and *Δpsd2Δgep1* cells were incubated with ^{3}H-serine for 0, 20, 40 or 60 min. Crude mitochondrial isolations from these samples were subjected to lipid extraction. Phospholipids were separated by TLC, PE was recovered from the TLC plate and radioactivity was determined by liquid scintillation counting. Mean values ± s. d. were calculated as percentage of *Δpsd2* labeling after 60 min. (n=5). **B** PE stability is decreased in the absence of Gep1. *Δpsd2* and *Δpsd2Δgep1* cells were incubated with ^{3}H-serine for 10 min, cells were pelleted and the supernatant was removed. Cells were incubated further for indicated time periods in the presence of excess unlabeled serine. Labeled PE was quantified as described in (**A**). Values were calculated as percentage of *Δpsd2* labeling at 0 min. *p<0.05, **p<0.01, ***p<0.001 (Student's unpaired *t* test) (n=4).

differences in these experiments were observed, when mitochondria were incubated in isotonic buffer to preserve the integrity of the outer membrane, indicating that transport of NBD-PS from the outer membrane to the inner membrane is as well unaffected by the absence of Gep1 (data not shown).

The results so far indicated that Gep1 is dispensable for mitochondrial PE synthesis. This conclusion was substantiated by pulse labeling experiments *in vivo*, which were carried out in a *Δpsd2* strain background to exclude masking effects of non-mitochondrial PE synthesis. *Δpsd2* and *Δgep1Δpsd2* cells were incubated with ^{3}H-serine and its incorporation into PE was monitored over different time periods. Synthesis rates of PE were not significantly altered in cells lacking Gep1 (**Figure 28 A**). In contrast to this, the stability of PE was significantly affected in the absence of Gep1 in pulse-chase experiments. In these experiments, cells were labeled for 10 min with ^{3}H-serine and were then further incubated in the presence of unlabeled serine to monitor

the turnover of ^{3}H-serine labeled phospholipids. While PE accumulated in *Δpsd2* cells and remained almost entirely stable over the whole chase period, PE amounts clearly decreased in *Δgep1Δpsd2* cells, suggesting that PE is either degraded or converted to PC more rapidly in cells lacking Gep1 (**Figure 28 B**). Thus, it appears that Gep1 is required to regulate PE turnover rather than to facilitate its synthesis.

4.14 Lipid-specific functions of Gep1-like proteins for phosphatidylethanolamine and cardiolipin

The finding that Gep1 is required for normal mitochondrial PE levels is the first evidence for the function of the so far uncharacterized PRELI/MSF1' proteins. The presence of two further proteins in yeast that share a high similarity with Gep1 suggests that these proteins might have overlapping or similar functions in mitochondrial phospholipid metabolism. To test this hypothesis, we generated strains lacking *GEP1*, *GEP2*, *UPS1* or all possible combinations of deletions of these three genes and first examined the growth of the strains. As reported, a strain with the single *UPS1* deletion exhibited a clear growth defect on fermentable carbon sources while *GEP1* or *GEP2* deletion strains grew like the wild-type strain (**Figure 29 A**). To our surprise, the growth defect associated with loss of Ups1 was rescued by the deletion of *GEP1*. Remarkably, deletion of *GEP2,* which shares 55% sequence identity with Gep1 on the amino acid level, did not restore growth of *Δups1* cells. An additional *GEP1* deletion in a *Δgep2Δups1* strain again restored wild-type like growth.

Since our data provide evidence for a role of Gep1 in regulating mitochondrial phospholipid composition, we determined the phospholipid profile of highly purified mitochondria from these strains by TLC and mass spectrometry. In contrast to *Δgep1* mitochondria, loss of Gep2 or Ups1 did not result in any reduction of mitochondrial PE levels, regardless of the presence of Gep1 (**Figure 29 B, C**). Our analysis revealed, however, a crucial role of Ups1 for formation of CL. CL was ~7-fold decreased in *Δups1* mitochondria but remained unaffected in the absence of either Gep1 or Gep2. Strikingly, CL levels in *Δups1* mitochondria were reverted to wild-type amounts upon deletion of *GEP1* in *Δups1* or *Δgep2Δups1* strains, which is consistent with the restoration of the

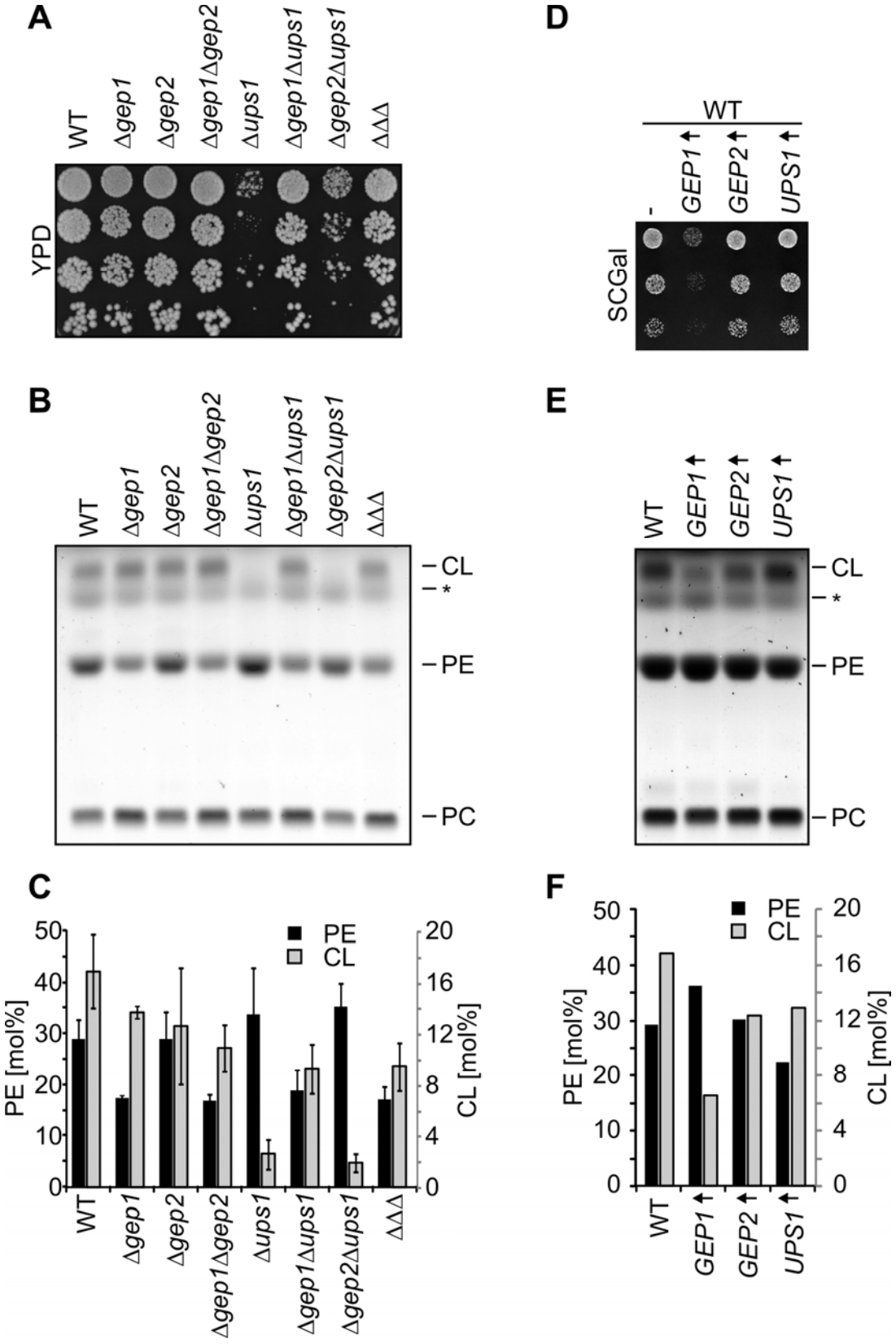
A
WT
Δgep1
Δgep2
Δgep1Δgep2
Δups1
Δgep1Δups1
Δgep2Δups1
ΔΔΔ
YPD
D
WT
-
GEP1↑
GEP2↑
UPS1↑
SCGal
B
CL
*
PE
PC
E
C
PE
CL
PE [mol%]
CL [mol%]
F

Figure 29 Gep1 and Ups1 regulate mitochondrial lipid composition **A** Growth test of *GEP1*, *GEP2* and *UPS1* deletion strains Fivefold dilutions were spotted on a YPD plate that was incubated at 30°C (ΔΔΔ – *Δgep1Δgep2Δups1*) **B+C** Deletion of *GEP1* restores CL levels in *Δups1* cells. Lipids extracted from isolated mitochondria were analyzed by TLC (* unidentified lipid species) (**B**) and mass spectrometry (**C**). Values in (**C**) are mean values ± s. d. from at least two independent mitochondrial isolations, each of them was analyzed in duplicate **D** Overexpression of *GEP1* severely impairs growth of yeast cells. Genes were expressed from high-copy plasmids under the control of the strong Gal-promoter. Fivefold dilutions were spotted on a SCGal plate that was incubated at 30°C. **E+F** Gep1 and Ups1 overexpression mimic *Δups1* and *Δgep1* phenotypes, respectively. Lipids extracted from mitochondria isolated from strains grown on galactose, where the endogenous promoter was replaced genomically by the strong Gal-promoter, were analyzed by TLC (* unidentified lipid species) (**E**) or mass spectrometry (**F**). Values in (**F**) represent mean values from single mitochondrial isolations which were analyzed in duplicate. (Mass spectrometric analyses were performed in collaboration with Mathias Haag and Britta Brügger)

growth defect (**Figure 29 A**). PE levels, on the other hand, remained reduced in *Δgep1Δups1* cells, demonstrating that the deletion of *UPS1* does not alleviate the requirement of Gep1 for the accumulation of PE. Consistent with the growth analysis, *GEP2* deletion did not restore CL levels in *Δups1* cells (**Figure 29 B, C**). These findings therefore define lipid-specific activities of Gep1-like proteins for the synthesis of CL and PE and at the same time point to common steps in the regulation of both phospholipids within mitochondria.

To further characterize the functional network of Gep1-like proteins in the control of mitochondrial PE and CL, we generated yeast strains overexpressing Gep1, Gep2 or Ups1 from a strong galactose-inducible promoter. Overexpression of Gep1 strongly compromised growth of yeast cells on galactose-containing media, whereas increased levels of Gep2 or Ups1 did not result in an apparent growth phenotype (**Figure 29 D**). When we monitored mitochondrial phospholipid composition in the strains overexpressing Gep1-like proteins by TLC and mass spectrometry, we obtained results that to some extent mirrored results from the analysis of the single deletion strains. Overexpression of Gep1 led to reduced levels of CL, similar to mitochondria lacking Ups1. Conversely overexpression of Ups1 resulted in a slight reduction of PE, similar to mitochondria devoid of Gep1 (**Figure 29 E, F**). Other phospholipid species were unchanged in these strains. Furthermore, overexpression of Gep2 did not have any influence on phospholipid composition of mitochondrial membranes.

These experiments reveal a crucial role for Gep1-like proteins in the regulation of mitochondrial PE and CL. Ups1 is required for CL accumulation in mitochondria and Gep1 is essential for the maintenance of wild-type PE levels. Strikingly, both pathways are interdependent: Whereas deletion of *GEP1* leads to a reduction of PE levels, overexpression of Gep1 impairs accumulation of normal CL levels. This observation points to a competition between Gep1 and Ups1 which is consistent with the observed restoration of CL upon deletion of *GEP1* in *Δups1* cells.

4.15 Survival of prohibitin-deficient cells depends on the lipid composition of mitochondrial membranes

The genetic interaction of prohibitins with henceforth five genes (*GEP1, UPS1, GEP4, PSD1 and CRD1)* required for a defined mitochondrial phospholipid composition suggests an essential role of prohibitins in cells with an altered mitochondrial phospholipid profile. Accordingly, deletion of other genes identified to genetically interact with prohibitins might also affect PE or CL levels in mitochondrial membranes. We therefore isolated highly purified mitochondria from various deletion strains lacking *PHB1* or genes required in the absence of prohibitins, extracted lipids and quantified PE and CL species by mass spectrometry. Among the 23 examined strains, only six strains (*Δgep7*, *Δgep8*, *Δyme1*, *Δoxa1*, *Δatp23* and *Δatp10*) displayed an unaltered phospholipid profile (**Figure 30**). We observed a previously undetected reduction of the CL levels in *Δphb1* cells, whereas PE accumulated at slightly increased levels. Deletion of *PSD1* or *CRD1* led to the expected reduction in PE or CL content of mitochondrial membranes, respectively. Strikingly, deletion of a large number of genes genetically interacting with prohibitins led to a significant reduction in PE and/or CL levels (**Figure 30**). PE amounts appeared to be decreased in mitochondria lacking Mdm35, Gep1, Gep3 and Cox6, while CL levels were specifically reduced in mitochondria lacking Ups1, Mdm34, Mdm32, Gep4, Gep5 or Gep6. In five other strains, a more profound effect on the mitochondrial lipid composition was revealed. Loss of Mmm1, Mdm31, Yta10 and Yta12 or Mdm10 resulted in a reduction of CL and PE in mitochondrial membranes. Our findings link the function of these genes to mitochondrial phospholipid

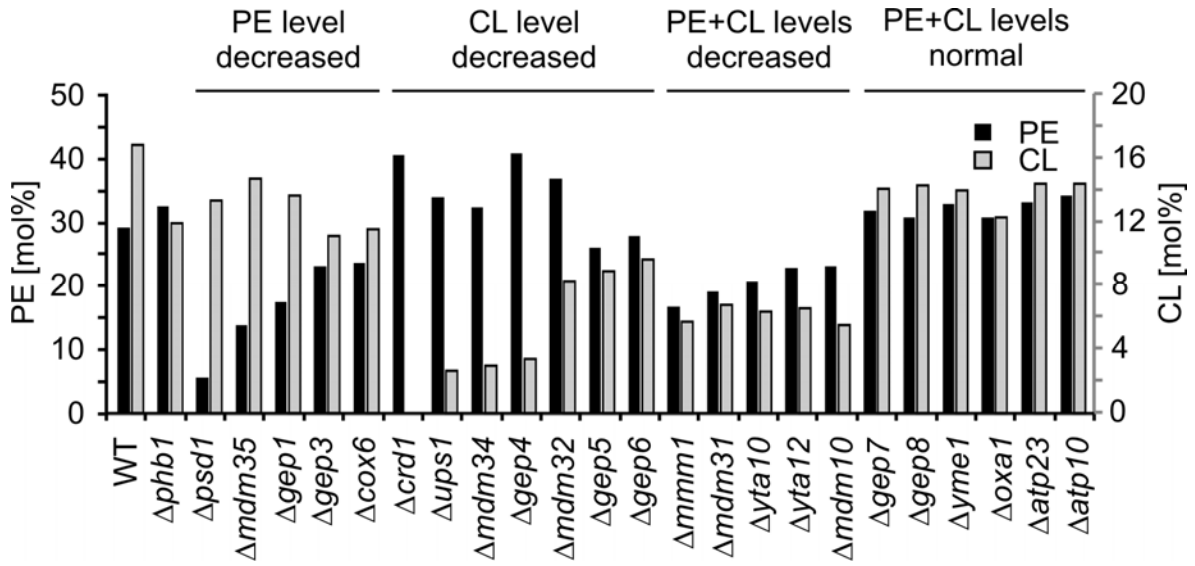

Figure 30 Mitochondrial lipid composition is important for survival of *Δphb1* cells. Lipid analysis of a collection of deletion strains synthetically lethal with prohibitins. Lipids were extracted from isolated mitochondria and analyzed by mass spectrometry. Values represent mean values from single mitochondrial isolations, which were analyzed in duplicate. Strains were arbitrarily grouped into strains with decreased PE, decreased CL, decreased PE and CL or unaffected lipid levels. (Mass spectrometric analyses were performed in collaboration with Mathias Haag and Britta Brügger)

metabolism and further substantiate an essential role of prohibitins in cells with an altered mitochondrial phospholipid profile.

5 Discussion

The remarkably high conservation of prohibitins and the severe phenotypes associated with prohibitin deletion in higher eukaryotes suggest an involvement of prohibitins in a cellular process of utmost importance. Deletion of prohibitins in yeast, however, results in a very mild phenotype that affects the replicative life-span of yeast cells. It is assumed that redundant processes are able to fulfill the roles of prohibitins in their absence. In the present thesis, the genetic interactome of prohibitin has been defined by a comprehensive genetic approach, with the aim to uncover these redundant processes.

5.1 Maintenance of mitochondrial PE and CL levels is essential in prohibitin-deficient cells

Integration of prohibitins into a genetic network by various genetic experiments has yielded overwhelming support for a very close functional relationship between prohibitins and components required for maintenance of the mitochondrial phospholipid composition (**Figure 31 A**). This points to roles of prohibitins associated with mitochondrial membranes. The phospholipids PE and CL appear to be of particular importance in this respect as many of the genetic interactors are required for normal steady-state levels of these two lipids. Crd1 and Psd1 are enzymes required for the last steps in the synthesis of CL and PE. Besides these, several other genes have been identified to affect mitochondrial PE and CL content that have previously not been linked to this process (**Figure 30**). The roles of Gep1, Ups1 and Gep4 in this process were studied in more detail and new functions for these so far uncharacterized proteins can be proposed (see below).

PE and CL are related phospholipids that are required for optimal activity of mitochondrial enzymes and mitochondrial activities (Joshi et al., 2008; Voelker, 2004). Moreover, alterations in the CL profile are associated with many pathophysiological states (Chicco and Sparagna, 2007; Joshi et al., 2008). PE and CL share the tendency

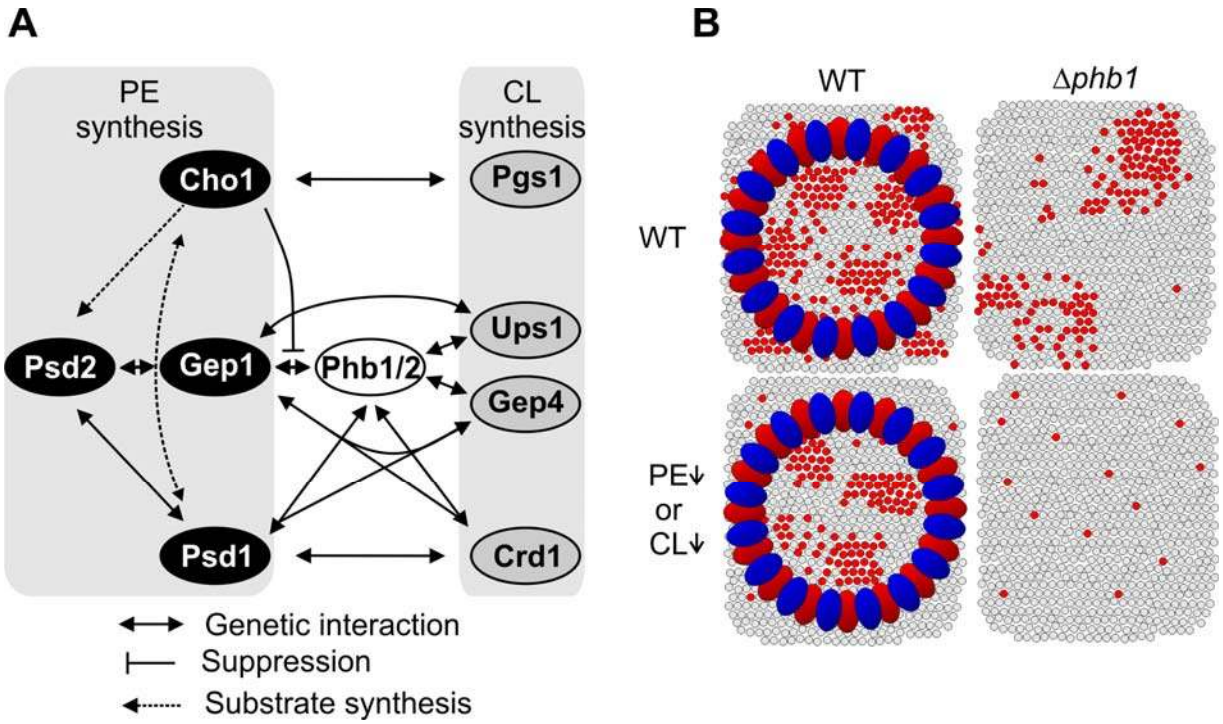

Figure 31 A Genetic interaction of prohibitins with PE and CL biosynthetic pathways. Genetic interactions between *CHO1* and *PGS1* and between *PSD1* and *CRD1* have been described previously (Gohil et al., 2005; Janitor et al., 1996) **B** Hypothetical model for the role of prohibitins as membrane organizers. The maintenance of putative functional membrane domains containing CL and PE (red dots) depends on prohibitin ring complexes or a high level of CL and PE in the inner membrane.

to form non-bilayer formations, termed hexagonal phases, which are discussed to play roles in membrane contact zones during fusion and fission processes or in transmembrane movements of proteins (Cullis and de Kruijff, 1979; Dowhan, 1997; Schlame et al., 2000; van den Brink-van der Laan et al., 2004). The functional importance of non-bilayer forming lipids is highlighted by the observation that yeast or *E. coli* cells with dramatically decreased levels of PE and CL are inviable (Gohil et al., 2005; Rietveld et al., 1993) (**Figure 31 A**). Moreover, both PE and CL tend to aggregate due to their biochemical properties and this leads to the formation of defined lipid clusters that have been observed in bacteria (Kawai et al., 2004; Matsumoto et al., 2006; Nishibori et al., 2005). It was proposed that these clusters play important roles in bacterial cell division and sporulation. PE or CL patches might also serve as platforms for the recruitment of soluble proteins that bind to the head groups or proteins with transmembrane segments that preferentially localize in these clusters (Matsumoto et al.,

2006). It is conceivable that a similar scenario holds true for the inner membrane and that a lateral segregation of PE and CL in the inner membrane is crucial for mitochondrial processes that rely on these non-bilayer forming lipids, like fusion and fission, enzymatic activities or membrane protein integration events.

5.2 Prohibitins as membrane scaffolds in the inner mitochondrial membrane

Prohibitins display a sequence similarity to a group of distantly related membrane proteins that are found in prokaryotes and eukaryotes and belong to the SPFH-family of proteins (see 1.7). Members of this family form large assemblies in membranes. Although roles of these proteins have yet to be established, the current available data suggest scaffolding functions for the lateral separation of microdomains within the plasma membrane. In line with this, several proteins of the SPFH-family have been demonstrated to associate with lipid rafts and to directly bind lipids. Similar to other proteins of the SPFH-family, prohibitins form large multimeric complexes. A scaffolding function of prohibitins is currently less evident and lipid rafts have yet not been isolated from mitochondria. The strong genetic interactions of prohibitins with components required for the maintenance of normal levels of non-bilayer forming lipids, however, strongly suggest a model for prohibitin function in promoting lipid phase partitioning (**Figure 31 B**). Under wild-type conditions, where PE and CL are synthesized normally and levels are not limiting, the function of prohibitins is dispensable and an asymmetric lipid distribution can be maintained due to the biochemical properties of different phospholipids. However, if PE or CL levels are decreased, prohibitin function becomes essential and PE or CL patches can only be formed by the help of the prohibitin complex that enables concentration of PE and CL within the inner membrane. Absence of prohibitins and decreased levels of non-bilayer forming lipids would accordingly lead to a loss of lipid microdomains within the inner membrane and this would in turn be detrimental for processes that depend on domains enriched in non-bilayer forming lipids. The breakdown of inner membrane integrity in the absence of prohibitins and reduced PE amounts (in *Δgep1Δphb1*[*PHB1*] cells upon downregulation of prohibitin)

suggests that such processes are essential for mitochondrial function and biogenesis (**Figure 18**).

In addition to their role in lipid partitioning, it is conceivable that prohibitins also serve functions as protein scaffolds for the recruitment of certain proteins to microdomains. Of special interest in this respect is the physical interaction of the prohibitin complex with the *m*-AAA protease (Steglich et al., 1999), which may rely on the presence of a certain lipid composition for the degradation or processing of special substrates. A sequestration of the *m*-AAA protease to microdomains would additionally allow the regulation of degradation or processing of substrates by the protease, by preventing access of substrates to these domains. This consideration is in line with the increased turnover of substrate proteins in the absence of prohibitins in yeast (Steglich et al., 1999).

In the current thesis the morphology of cristae was identified as a process that critically depends on PE levels in mitochondria. Decreased PE levels compromise the processing of the dynamin-like GTPase Mgm1 (**Figure 21**), which is required for membrane fusion and cristae formation (Meeusen et al., 2006). Under conditions of low PE levels, prohibitin function could, according to the considerations described above, be essential to ensure processing of Mgm1 above a certain level to prevent breakdown of mitochondrial morphology. Notably, the processing of the mammalian Mgm1-homologue OPA1 was recently identified as the central process controlled by prohibitins in murine fibroblasts (Merkwirth et al., 2008), indicating that the same processes might depend on prohibitin function in evolutionary distant organisms. Therefore, phenotypic differences associated with the loss of prohibitins in yeast and mammals likely reflect differences in the phospholipid profile of mitochondrial membranes.

Although the absence of CL did not inhibit Mgm1 processing (**Figure 21**), a role of CL for proteolytic cleavage under certain growth conditions in yeast or in other organisms cannot be ruled out. Variations of the PE content of the inner membrane may mask the dependence of Mgm1 processing on CL. Accordingly, differences in the relative content of PE and CL may explain why the loss of Ups1 was observed previously to inhibit

Mgm1 processing (Sesaki et al., 2006). It is therefore an attractive possibility that an impaired processing of the mammalian Mgm1-homologue OPA1 causes the disturbed formation of mitochondrial cristae, which was observed in lymphoblasts of Barth syndrome patients or yeast cells lacking the CL transacylase tafazzin (Acehan et al., 2007; Claypool et al., 2008).

5.3 Gep4, a putative phosphatase regulating CL biosynthesis

The enzymes required for the synthesis of all different phospholipid species in yeast are all known with one exception. Although an enzyme activity for dephosphorylation of phosphatidylglycerolphosphate (PGP) has been characterized in yeast cell extracts, the protein responsible for the catalysis of this step has yet not been identified (Kelly and Greenberg, 1990). PGP is an intermediate in the biosynthesis pathway of CL that is synthesized by the enzyme Pgs1. Absence of the enzyme mediating the dephosphorylation of PGP would consequently lead to a severe reduction of cardiolipin within mitochondria. Strikingly, the gene *GEP4*, which was identified among the genes synthetically lethal with prohibitins, encodes a mitochondrial protein (Kumar et al., 2002) that belongs to the superfamily of hydrolases and displays an inverted DXDX(T/V) motif that is conserved in homologous proteins (**Figure 14**). This motif is present in diverse phosphotransferases and it was demonstrated that the aspartate residues serve as intermediate phosphoryl acceptors (Collet et al., 1998). In conjunction with our findings that show the absence of detectable CL levels in a yeast strain lacking Gep4 or expressing Gep4 variants with mutations in the phosphotransferase motif (**Figure 16**), it is an attractive hypothesis that Gep4 might represent the long sought for dephosphorylase that mediates the synthesis of PG, which in turn is converted to CL by Crd1 (**Figure 32 A**).

In alternative models, Gep4 could be involved in a different process that directly or indirectly affects mitochondrial CL synthesis. The presence of a motif in Gep4, which is characteristic for phosphotransferases, raises the possibility that Gep4 might act in a signaling cascade that involves phosphorylation events. Interestingly, a report demonstrated that the PGP synthase Pgs1 is inhibited by reversible phosphorylation

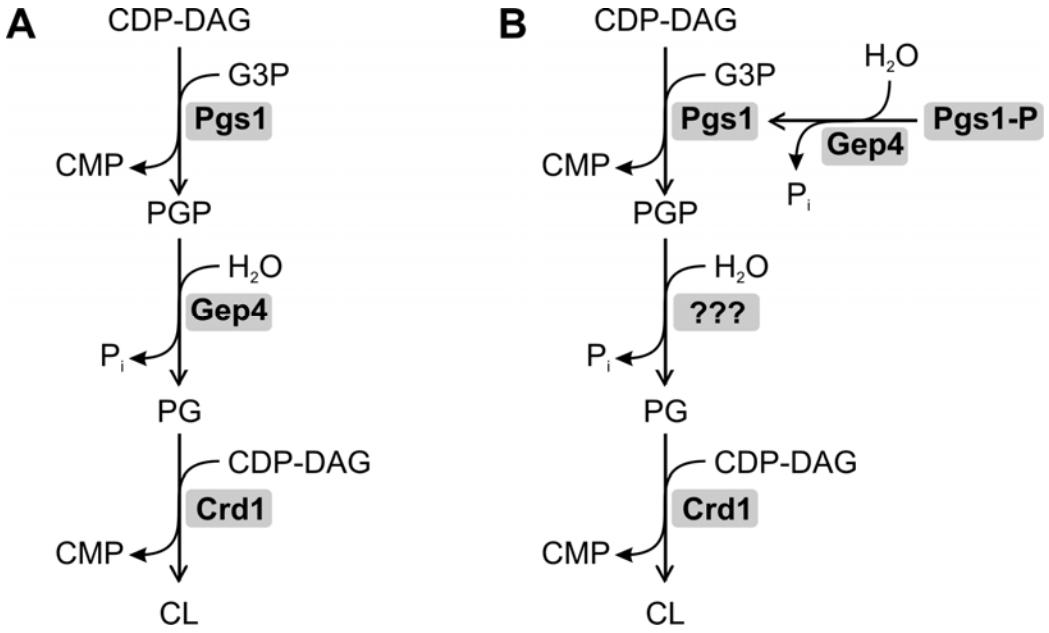

Figure 32 Hypothetical roles for Gep4. **A** Gep4, as the PGP-phosphatase. **B** Gep4, as a phosphatase required for dephosphorylation of an inactive phosphorylated form of Pgs.1

(He and Greenberg, 2004). According to this, Gep4 could be required for dephosphorylation of Pgs1 to activate its enzymatic activity (**Figure 32 B**). In the absence of Gep4, Pgs1 would thus be inactive due to an irreversible phosphorylation, which would lead to a decrease in CL levels, owing to an absence of PGP synthesis.

According to both models, predictions can be made regarding the phospholipid spectrum of *Δgep4* mitochondria. PG should be absent in these mitochondria in both scenarios. A way to distinguish between both possibilities is the determination of the PGP levels in *Δgep4* mitochondria, because PGP should accumulate when Gep4 mediates its dephosphorylation. However, attempts to identify and determine PGP levels in wild-type and *Δgep4* mitochondria have failed so far.

5.4 Gep1-like proteins are novel regulators of mitochondrial phospholipid composition

Whereas most enzymes required for synthesis of all phospholipids have been identified in *S. cerevisiae*, great gaps exist in our understanding of lipid transport processes that

are required to maintain sufficient mitochondrial phospholipid supply. It is currently unclear how lipids traffic from the endoplasmic reticulum to mitochondria and how they traverse the intermembrane space. Furthermore, regulatory mechanisms that determine the phospholipid composition of mitochondrial membranes are currently not known.

Our findings demonstrate that Gep1 and Ups1, two members of the three protein encompassing group of Gep1-like proteins in yeast, are essential to maintain normal mitochondrial PE and CL levels, respectively. The observation that absence of all three Gep1-like proteins only modestly affects mitochondrial phospholipid composition makes it unlikely that these proteins directly mediate crucial steps in biosynthesis of any phospholipid (**Figure 29**). These proteins might rather function as regulators to determine mitochondrial phospholipid composition. The decrease of PE amounts in *Δgep1* mitochondria is caused by a reduced stability of newly synthesized PE, whereas biosynthesis rates are unaffected (**Figure 28**). Stability of mitochondrial PE is governed by two processes that are equally poorly understood. On one hand, PE is most probably subject to turnover by specific lipases that have not been identified yet. On the other hand, mitochondria massively contribute to the cellular phospholipid composition by exporting PE, which can be converted to PC or triacylglycerols in the endoplasmic reticulum (Daum et al., 2007; Voelker, 2004). Consistently, Gep1 might regulate degradation of PE by a lipase or export of mitochondrially synthesized PE. In line with an increased export of PE in the absence of Gep1 is the observation of an increased number of lipid particles that were identified in electron microscopic analyses in *Δgep1* cells (unpublished observation). A deregulated lipid export from mitochondria could lead to an overload of the endoplasmic reticulum. Phospholipids could in response be converted into triacylglycerols, which would be stored in lipid particles. Massive export of PE might also lead to increased synthesis of PC in the MAM compartment. Interestingly, mitochondrial PC levels appear slightly elevated in the absence of Gep1 (**Figure 29**), further supporting a deregulated export of PE in *Δgep1* mitochondria.

The role of Ups1 in ensuring normal CL levels is currently less clear and further experiments are required to examine, whether Ups1 affects CL biosynthesis or

degradation. Nevertheless, it became evident that mitochondrial levels of PE and CL are regulated coordinately by Gep1 and Ups1. Ups1 is essential for the accumulation of CL, while mitochondrial PE is decreased in the absence of Gep1. Strikingly, CL is reduced upon overexpression of Gep1 and PE is slightly reduced upon overexpression of Ups1 (**Figure 29**). This suggests a competition between Gep1 and Ups1. Consistently, deletion of *GEP1* in *Δups1* cells restores mitochondrial CL levels.

At least three different simplified models can be proposed that are in line with the bulk of the experimental data. In an "antiport model", the intermembrane space proteins Gep1 and Ups1 might control the antiport of PE and PA between the MAM compartment and the inner membrane via a yet unidentified structure that might be of proteinaceous or lipid origin (**Figure 33 A**). In its ground-state this "antiporter" would export PE and import PA, which is required for CL synthesis (**Figure 3**). In line with the experimental data, Gep1 could be an inhibiting factor of this "antiporter". The role of *UPS1* would be to compete with Gep1 for binding to the antiporter and to relieve the transport block that would put it into its ground-state again. Absence of Ups1 would accordingly result in an inactive antiporter, owing to a constant binding of Gep1 that would lead to a decrease of mitochondrial PA levels and eventually decreased CL biosynthesis (**Figure 3**). Overexpression of either one of both proteins would result in a competition for the regulation of this process that would mimic the deletion phenotype of the respective other subunit. It has to be stated that no increase of CL was observed in the absence of Gep1 that might be expected in this model, due to an increased import of PA (**Figure 33 A**). Many factors, however, determine mitochondrial phospholipid composition and it is possible that, for example, the cardiolipin synthase in the inner membrane is inhibited by CL and CL amounts can, therefore, not be elevated above a certain level.

Similar to the "antiport model", it can be envisioned that Gep1 and Ups1 compete, respectively, for the activation of the export of PE and CL that is mediated by the same unknown structure. In such an "export model", absence of Gep1 would lead to increased export of PE, that is stimulated by Ups1 and absence of Ups1 would result in elevated CL export, due to activation by Gep1 (**Figure 33 B)**. The finding that only PE

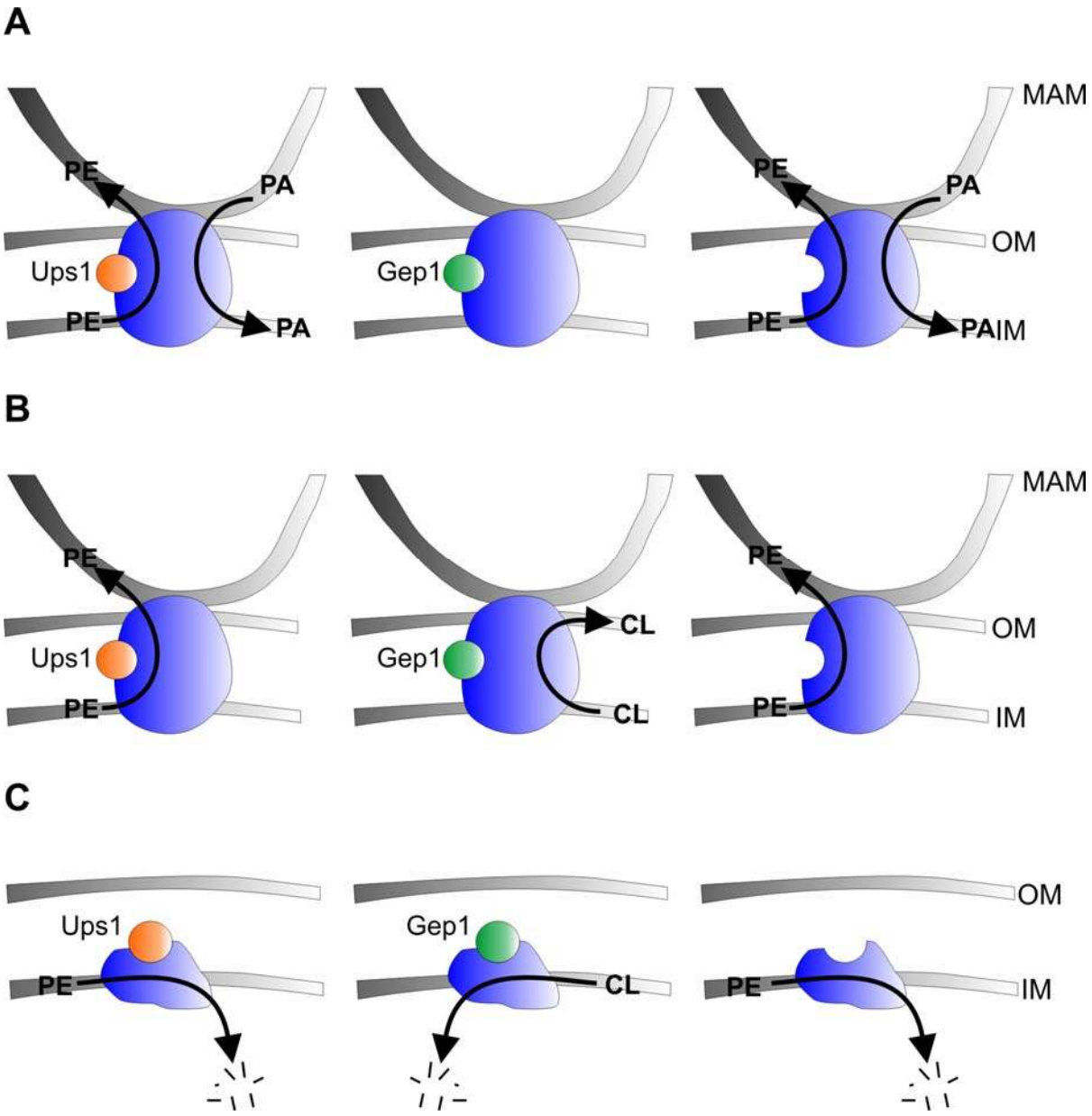

Figure 33 Hypothetic models for the function of Gep1 and Ups1. **A** "Antiporter model." **B** "Export model" **C** "Lipase model", the lipase could as well be localized in the outer membrane. MAM – mitochondrial associated membranes, OM – outer mitochondrial membrane, IM – inner mitochondrial membrane

levels are affected in the absence of both proteins would imply that PE is mainly exported when this process is not regulated, which might be explained by the higher steady-state levels of PE compared to CL. Interestingly, an role for export of CL form the inner to the outer membrane has recently been suggested to be important for mitochondrial fusion (Choi et al., 2006b). In this report, the authors propose that CL in

the outer membrane is converted to phosphatidic acid, a fusiogenic lipid, by a phospholipase (MitoPLD). Therefore, export of CL to the outer membrane, hypothetically stimulated by Gep1, would result in its cleavage and eventually reduced CL amounts in mitochondria. It has to be noted, though, that MitoPLD still awaits its identification in yeast.

In an alternative "lipase model", Gep1 and Ups1 exert their regulatory role on a lipase, which could be localized in the outer or the inner membrane. The lipase in its ground-state would degrade PE and binding of Gep1 could shift the specificity of the lipase towards CL (**Figure 33 C**). The function of Ups1 would, similar to the "antiporter model", be a competitive binding to displace Gep1, to restore the ground-state of the lipase. The assumption that the role of Ups1 lies solely in competing with Gep1 for the binding partner in the antiport and lipase models is consistent with the presented experiments, which show that deletion of *UPS1* only has an effect as long as Gep1 is present.

Intriguingly, competition of Gep1 and Ups1 in the regulation of either a lipid transporter or a lipase would allow an adjustment of non-bilayer forming phospholipid ratios in mitochondria, simply by modulating protein amounts or availability of Gep1 and Ups1. For example, a lower expression level of Ups1 under fermentative growth conditions would lead to a decrease in CL levels that has been reported previously (Claypool et al., 2008; Jakovcic et al., 1971). Although these models are currently hypothetic, they offer first explanations for the function of Gep1 and Ups1 and specific questions can now be addressed. It will be a challenging but fascinating task for future studies to further clarify the roles of Gep1 and Ups1 in regulating mitochondrial phospholipid composition. Of particular interest will be, to examine to what cues the lipid composition is altered and how this is translated by Gep1 and Ups1. Furthermore, the function of Gep2, the third protein in yeast among the Gep1-like proteins, remains to be elucidated. Moreover, the identification of Gep1-like proteins as regulators of CL and PE and their functional conservation in humans (Sesaki et al., 2006) (**Figure 17**) now allows examining directly the role of an altered phospholipid composition for mitochondrial activities and their dysfunction in disease.

Several other genes identified in the screen for genes essential in the absence of prohibitins have been identified to have an altered mitochondrial lipid composition. Interestingly, these genes encode proteins with diverse functions in the outer membrane (Mmm1, Mdm10, Mdm34), the intermembrane space (Mdm35), the inner membrane (Mdm31, Mdm32) or the matrix (Yta10, Yta12). It is tempting to speculate that these proteins exert roles at all possible steps that are required for mitochondrial phospholipid supply. The Mdm-proteins, for example, have been implicated in the process of β-barrel assembly in the outer membrane and maintenance of mitochondrial morphology. It is conceivable that the Mdm-proteins might function in phospholipid transport processes and that a defect in this process would lead to altered phospholipid composition of the outer membrane that in turn could impact on β-barrel assembly and mitochondrial morphology maintenance. Similarly, it is possible that a compromised β-barrel assembly in the outer membrane leads to a secondary effect in mitochondrial phospholipid composition. In summary it appears that the conducted genetic array has not only yielded genes essential in prohibitin-deficient cells but also genes required for normal mitochondrial phospholipid composition.

5.5 Prohibitins and the assembly of F_O-particle of the F_1F_O-ATP synthase

The maintenance of mitochondrial phospholipid composition has been identified as the major process essential in the absence of prohibitins and the strongest genetic interactions can be found in this class. The fidelity of another process, namely the assembly of the F_O-particle of the F_1F_O-ATP synthase, however, appears to be of high importance in prohibitin-deficient cells as well. The discovery of the functional relationship between prohibitins and F_O-assembly is largely due to the identification of the novel processing peptidase and chaperone for F_O-assembly, Atp23. Besides Atp23, two more proteins, namely Atp10 and Oxa1, are required in this process and genetically interact with prohibitins (Jia et al., 2007; Tzagoloff et al., 2004). Our studies did not reveal crucial roles of these proteins in ensuring normal mitochondrial phospholipid composition (**Figure 30**), which suggests that facilitating assembly of the F_O-particle is the function of these proteins that is required in the absence of prohibitins. It has to be

mentioned that a recent study reported decreased PE levels in a strain lacking Oxa1 (Nebauer et al., 2007). These analyses, though, were performed on cells grown on glucose medium that is in contrast to our experiments which therefore might explain this discrepancy. Nevertheless, Oxa1 might be required to maintain mitochondrial PE levels (on glucose medium) and assembly of the F_1F_O-ATP synthase.

What might be the reason for the synthetic genetic interaction of prohibitins with factors required for F_1F_O-ATP synthase assembly? Cell growth is not impaired by the loss of the F_1F_O-ATP synthase activity per se in prohibitin-deficient cells, because we did not observe a genetic interaction of prohibitins with genes encoding for subunits of the F_1-ATPase or many other subunits of the ATP synthase complex. This includes also the accessory subunit e, which is only present in dimeric F_1F_O-ATP synthase complexes and whose loss leads to aberrant cristae formation (Arselin et al., 2004). It therefore appears that an impaired mitochondrial ultrastructure is not the basis of the genetic interaction of prohibitins with Atp23. One possibility is that the accumulation of a specific assembly-intermediate during assembly of the F_1F_O-ATP synthase, present in mitochondria lacking Atp10, Atp23 or Oxa1, is hazardous for cell growth in the absence of prohibitins. A partially or uncontrolled assembled F_O-moiety may for instance allow proton-leakage through the membrane and thereby dissipate the inner membrane potential. Prohibitins have been proposed to exert chaperone function and thereby might mask the deleterious effect of these assembly-intermediates. However, we did not obtain evidence for a direct interaction of the prohibitin complex with non-assembled inner membrane proteins. In the light of the above stated considerations about a membrane organizing function of prohibitins, it is conceivable that the accumulation of hydrophobic F_O-assembly-intermediates in the inner membrane is deleterious for the lateral organization of membrane domains and essential processes that depend on their presence. According to this, prohibitins would be required for the formation of these microdomains in the presence of hazardous assembly-intermediates. Alternatively, prohibitins could be involved in the sequestration of such assembly-intermediates into functional domains required for the deposition of these intermediates.

Increasing evidence suggest a higher order organization of respiratory chain complexes that ensures efficient metabolite channelling and maintains a high membrane potential (Bornhovd et al., 2006; Everard-Gigot et al., 2005). Both oligomeric ATP synthase as well as ring-like prohibitin complexes are proposed to exert scaffolding functions and may define functional microdomains in the inner membrane (Bornhovd et al., 2006). Their loss in the absence of both complexes together with the accumulation of deleterious F_O-assembly-intermediates may therefore promote the breakdown of the membrane potential across the inner membrane and inhibit cell growth. Our findings that prohibitins are not required in the absence of subunit e of the ATP synthase, which is responsible for the formation of dimeric ATP synthase and therefore higher order complexes, however, does support this hypothesis.

5.6 Atp23, a novel processing peptidase and chaperone for the F_1F_O-ATP synthase

The results provided in this thesis identify Atp23 as a novel processing peptidase in the mitochondrial intermembrane space that exerts chaperone function during the assembly of the F_1F_O-ATP synthase. Atp23 mediates the maturation of newly synthesized Atp6 and, independent of its proteolytic activity, promotes the subsequent assembly of Atp6 into the membrane-embedded F_O-particle (**Figure 11**). The assembly of mitochondrially encoded Atp6 is thus under the control of two substrate-specific chaperones, Atp10 and Atp23, acting on the matrix and the intermembrane space side of the inner membrane, respectively.

Atp6 is synthesized within mitochondria as a precursor protein, from which 10 N-terminal amino acids are cleaved off (Michon et al., 1988). Maturation of Atp6 was found to depend on Atp23 and on the integrity of the consensus metal binding site, HEXXH, characteristic of metallopeptidases. Together with the observed physical interaction of Atp23 with newly synthesized Atp6 (Osman et al., 2007), this strongly suggests that Atp23 serves as a metal-dependent processing peptidase for Atp6, which removes N-terminal amino acids when they become accessible in the intermembrane space (**Figure 34**). Atp23 therefore represents the second processing peptidase in this

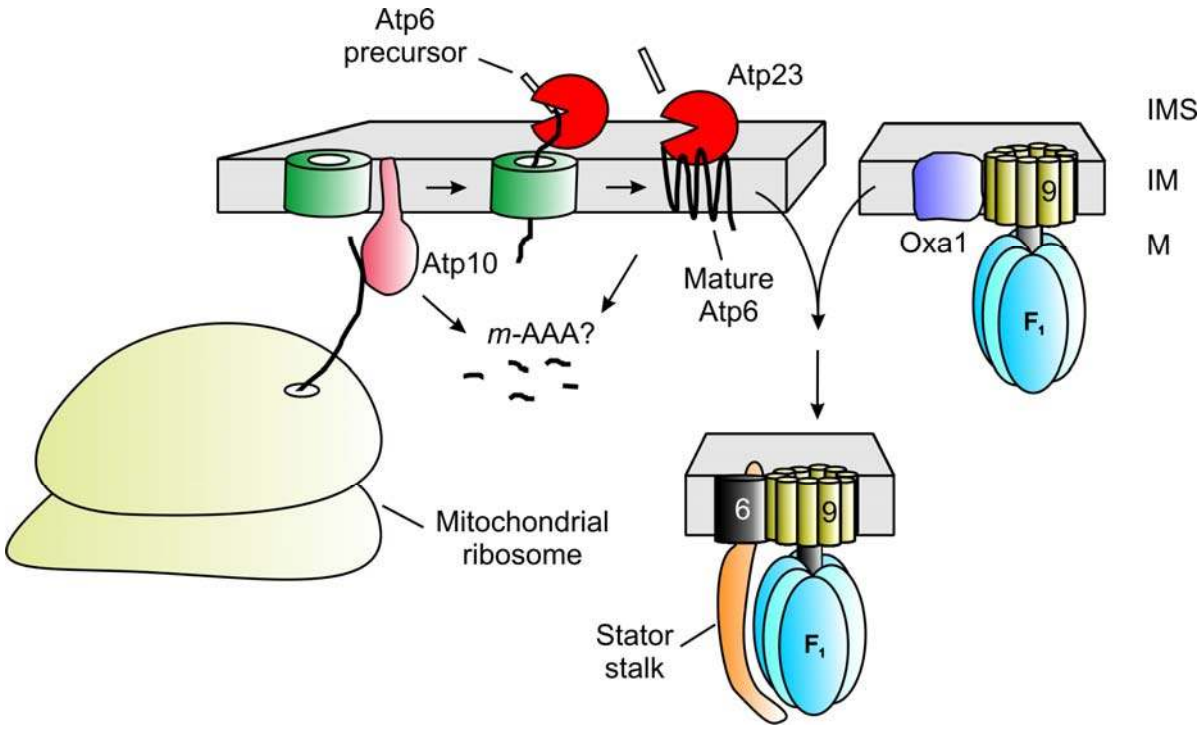

Figure 34 Model of Atp6 assembly into F_1F_O-ATP synthase complexes. See text for details. The translocase mediating membrane insertion of Atp6 has not been identified. 6 – Atp6, 9 - Atp9; *m*-AAA - *m*-AAA protease.

compartment besides the IMP protease, which mediates maturation of several nuclearly encoded proteins and of mitochondrially encoded Cox2 (Gakh et al., 2002). It remains to be determined whether other substrates of Atp23 exist. Notably, the human homologue of Atp6 lacks the N-terminal extension, which is cleaved off from the newly synthesized yeast protein. Nevertheless, Atp23 including the consensus metal binding site is conserved from yeast to man, pointing to further substrate proteins present in mammalian mitochondria.

A dual activity of Atp23 was unraveled by the mutational analysis of its proteolytic center. Cells harbouring proteolytically inactive Atp23 were deficient in the maturation of Atp6 but were able to grow on non-fermentable carbon sources and contained fully assembled F_1F_O-ATP synthase. These findings demonstrate that the formation of a functionally active ATP synthase complex does not require processing of Atp6, but the presence of Atp23 in the intermembrane space. Atp23 directly interacts with mature

Atp6 and mediates its assembly with Atp9, constituting the proton-translocating channel that is maintained in an assembly-competent state by Oxa1 in the inner membrane (Jia et al., 2007) (**Figure 34**). Assembly of Atp6 also depends on another substrate-specific chaperone, Atp10 (Tzagoloff et al., 2004). Consistently, similar-sized intermediate complexes were detected in Atp10- and Atp23-deficient mitochondria. Given the membrane topology of Atp10, exposing a domain to the matrix space (Ackermann and Tzagoloff, 1990), it is conceivable that newly synthesized Atp6 initially interacts with Atp10 before it is bound by Atp23 in the intermembrane space (**Figure 34**).

How these substrate-specific chaperones ensure the assembly of the F_O-moiety remains to be established. A detailed analysis is made difficult by the increased turnover of Atp6 in the absence of Atp23 or Atp10, which thus are at the interface between assembly and proteolytic processes. Atp23 may be directly involved in the insertion of newly synthesized Atp6 into the inner membrane by trapping the N-terminal tail of Atp6 in the intermembrane space. Alternatively, Atp23 may stabilize membrane-inserted Atp6 in an assembly-competent conformation. According to this scenario, the protective effect against proteolysis would simply result from the efficient assembly of Atp6 into F_O-complexes. This is reminiscent of Cox20, which is required for the maturation by the IMP protease of Cox2, the second mitochondrial-encoded protein synthesized as a pre-protein (Hell et al., 2000). Evidence for Cox20-induced conformational changes in newly synthesized Cox2 was recently obtained analyzing the turnover of non-assembled Cox2 (Graef et al., 2007). However, although a role of Cox20 for the assembly of mature Cox2 remained speculative, the findings presented in this thesis, demonstrate a chaperone function of Atp23 during the assembly of F_1F_O-ATP synthase, independent of Atp6 maturation. Finally, it cannot be excluded that the main function of Atp23 is to protect newly synthesized Atp6 against proteolysis and thereby increase the time frame available for Atp6 assembly. Accordingly, inhibition of Atp6 proteolysis should alleviate the requirement of Atp23 for the assembly of the F_1F_O-ATP synthase. A direct examination of this possibility, however, awaits the identification of proteases involved in the turnover of Atp6. Impairment of *m*-AAA protease activity in *Δatp23* mitochondria did not result in the stabilization of the precursor form of Atp6

suggesting the involvement of additional proteases in its proteolytic breakdown (unpublished observations).

6 Zusammenfassung

Prohibitine bilden eine hochkonservierte und ubiquitär exprimierte Familie von Membranproteinen mit essentiellen Funktionen für die Entwicklung höherer Eukaryoten. In der inneren mitochondrialen Membran bilden Prohibitine große Ringkomplexe und regulieren die Dynamik und wichtige Funktionen von Mitochondrien. Außerdem wurden Funktionen von Prohibitinen in zellulären Signalvorgängen an der Plasmamembran und in der transkriptionellen Regulation im Zellkern vorgeschlagen. Der molekulare Mechanismus ist bei all diesen Funktionen allerdings noch nicht verstanden. Im Gegensatz zu höheren Eukaryoten sind Hefezellen in Abwesenheit von Prohibitinen lebensfähig und weisen lediglich eine reduzierte replikative Lebensspanne auf.

Ein genetischer Ansatz wurde gewählt, um die Funktion der Prohibitine in Hefe zu untersuchen und redundante Prozesse zu identifizieren, welche die Funktion der Prohibitine in deren Abwesenheit ersetzen können. „Synthetisch genetische Arrays“ wurden verwendet, die zur Identifizierung von synthetisch letalen Interaktionen mit Prohibitinen führen sollten. Dieser Ansatz zeigte 35 Gene auf, die für das Überleben von Hefezellen essentiell sind, welche kein Prohibitin besitzen. Die Assemblierung des F_O-Sektors der F_1F_O-ATP Synthase wurde als äußerst wichtiger Prozess in prohibitin-defizienten Zellen identifiziert. Es konnte gezeigt werden, dass das Protein Atp23 eine neue Prozessierungspeptidase darstellt und eine duale Funktion bei der Reifung der mitochondrial kodierten Untereinheit Atp6 und deren Assemblierung in die funktionelle F_1F_O-ATP Synthase ausübt. Für ~50% der Gene, die in prohibitin-defizienten Zellen benötigt werden, darunter auch die stärksten genetischen Interaktionen, konnte erstmalig gezeigt werden, dass diese für die Aufrechterhaltung der mitochondrialen Phospholipidzusammensetzung benötigt werden. Weitere Ergebnisse deuten darauf hin, dass zwei Proteine einer konservierten Proteinfamilie, Gep1 und Ups1, die Mengen der nicht-doppelschicht-bildenden Phospholipide Cardiolipin und Phosphatidyl-ethanolamin in Mitochondrien regulieren. Zusätzlich wurde eine potentielle Phosphatase

identifiziert, welche möglicherweise das letzte, noch fehlende Enzym des Cardiolipin-Synthesewegs in Hefe darstellt.

Das in dieser Arbeit definierte genetische Interaktom der Prohibitine weist auf eine Funktion der Prohibitine bei der Stabilisierung von mitochondrialen Membrandomänen hin, welche Proteine und nicht-doppelschicht-bildende Phospholipide beinhalten. Diese Funktion ist dann essentiell, wenn die Mengen an Cardiolipin und Phosphatidyl-ethanolamin in Mitochondrien reduziert sind. Die Abwesenheit von Prohibitinen, kombiniert mit einer Reduktion nicht-doppelschicht-bildender Phospholipide, führt dazu, dass essentielle Prozesse, welche auf diese Mikrodomänen angewiesen sind, nicht mehr ablaufen können.

7 References

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8 List of abbreviations

AAA	-	ATPases associated with a variety of cellular activities
ADP	-	adenosine diphosphate
ATP	-	adenosine triphosphate
BN	-	blue native
CL	-	cardiolipin
C-terminal	-	carboxyterminal
C-terminus	-	carboxy terminus
CDP	-	cytosine diphosphate
DAG	-	diacylglycerol
DNA	-	deoxyribonucleic acid
DRM	-	detergent resistant membranes
EDTA	-	ethylene diamine tetraacetic acid
ER	-	endoplasmic reticulum
GA	-	Golgi apparatus
GTP	-	guanosine triphosphate
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic-acid
IM	-	inner mitochondrial membrane
IMS	-	intermembrane space
kDa	-	kilodalton
MAM	-	mitochondrial associated membranes
mRNA	-	messenger RNA
mtDNA	-	mitochondrial DNA
N-terminal	-	aminoterminal
N-terminus	-	amino terminus
OD_{600}	-	optical density at a wavelength of 600 nanometer
OM	-	outer mitochondrial membrane
PA	-	phosphatidic acid
PAGE	-	polyacrylamide gel electrophoresis
PC	-	phosphatidylcholine
PCR	-	polymerase chain reaction
PE	-	phosphatidylethanolamine
PG	-	phosphatidylglycerol
PGP	-	phosphatidylglycerolphosphate
PI	-	phosphatidylinositol
PMSF	-	phenylmethylsulphonyl fluoride
PS	-	phosphatidylserine
PVDF	-	polyvinylidene fluoride
rpm	-	rounds per minute
RT	-	room temperature
s.d.	-	standard deviation
SDS	-	sodium dodecyl sulfate
TIM	-	translocase of the inner membrane

TLC	-	thin layer chromatography
TM	-	transmembrane domain
TOM	-	translocase of the outer membrane
WB	-	Western blot
WT	-	wild-type
YP	-	yeast extract-peptone
YPD	-	yeast extract-peptone with glucose
YPG	-	yeast extract-peptone with glycerol

9 Appendix

Osman, C., Wilmes, C., Tatsuta, T., and Langer, T. (2007).

"Prohibitins interact genetically with Atp23, a novel processing peptidase and chaperone for the F_1F_O-ATP synthase"

Mol Biol Cell *18*, 627-635

Abstract

The generation of cellular energy depends on the coordinated assembly of nuclear and mitochondrial-encoded proteins into multisubunit respiratory chain complexes in the inner membrane of mitochondria. Here, we describe the identification of a conserved metallopeptidase present in the intermembrane space, termed Atp23, which exerts dual activities during the biogenesis of the F_1F_O-ATP synthase. On one hand, Atp23 serves as a processing peptidase and mediates the maturation of the mitochondrial-encoded F_O-subunit Atp6 after its insertion into the inner membrane. On the other hand and independent of its proteolytic activity, Atp23 promotes the association of mature Atp6 with Atp9 oligomers. This assembly step is thus under the control of two substrate-specific chaperones, Atp10 and Atp23, which act on opposite sides of the inner membrane. Strikingly, both *ATP10* and *ATP23* were found to genetically interact with prohibitins, which build up large, ring-like assemblies with a proposed scaffolding function in the inner membrane. Our results therefore characterize not only a novel processing peptidase with chaperone activity in the mitochondrial intermembrane space but also link the function of prohibitins to the F_1F_O-ATP synthase complex.

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11 Eidesstattliche Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Thomas Langer betreut worden.

Köln, im Oktober 2008 Christof Osman

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12 Lebenslauf

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