The role of the PHB complex in mitochondrial biogenesis
Artal Sanz, M.

Citation for published version (APA):
Artal Sanz, M. (2003). The role of the PHB complex in mitochondrial biogenesis
General discussion
Contents

1. Outline 119

2. Model organisms 119

3. The PHB complex: A holdase/chaperone with a regulatory role in membrane protein quality control? 121
   3.1 PHB phenotypes and expression levels 122
   3.2 Genetic interactions 124

4. Can we learn from structure predictions? 127

5. Hypothesis for the function of the PHB complex 128

6. Concluding remarks and future prospects 131

7. References 132
1. Outline

This general discussion starts with a brief overview of the advantages that the model organisms used in this thesis offer for the study of mitochondrial biogenesis and function and in particular for the study of human mitochondrial disorders (Section 2). Subsequently, I will discuss the results presented in this thesis, together with results from other researchers, in view of the proposed role for the PHB complex as an additional component of the quality control system (Section 3). In Section 3.1 I will discuss phenotypes and expression levels reported for PHB proteins and in Section 3.2 genetic interactions between PHB genes and other mitochondrial genes described in yeast will be examined. In Section 4, I will briefly discuss the structural data presented in chapter 4, which might pave the way to a new working hypothesis and help to unravel the role of the PHB complex. In Section 5, I put forward an alternative hypothesis for the function of the PHB complex and recapitulate the data discussed in this thesis considering their contribution to each of the hypotheses presented. I will end this general discussion with concluding remarks and future prospects.

2. Model organisms

Despite advances in other systems, the facultative anaerobic yeast *Saccharomyces cerevisiae* is without doubt an irreplaceable model system for our understanding of mitochondrial biogenesis. One of the reasons is that yeast does not require respiration to survive, since it can rely on fermentation for ATP production. Therefore, only a few mitochondrial proteins are essential for cell viability. Since the publication in 1996 of the complete yeast genome sequence, the number of genes known to encode for mitochondrial proteins has increased from just fewer than 200 to the current 568 (as of August 2003; Yeast Proteome Database (YPD) [1]). The relative simplicity of generating knockouts by homologous recombination, the existence of both haploid and diploid yeast cells and the possibility to separate all four haploid cells from a meiotic event, makes *S. cerevisiae* a very useful organism for genetic manipulations. Yeast is unique in the sense that a comprehensive collection of yeast deletion mutants for ORFs longer than 100 codons is available [2] and has been successfully applied to identify mitochondrial proteins [3]. The relative simplicity of yeast and its well annotated genome allows for the application of new advanced technologies at a whole-genome level [3-7]. Moreover, newly discovered genes can easily be studied by classical genetics and biochemistry approaches. Because 30% to 40% of human disease-associated genes share significant sequence identity with yeast genes [8], yeast has proven to be a suitable simple organism to investigate the molecular basis of many human mitochondrial diseases ([9, 10] and references there in). However, mitochondria are much more complex in humans than in yeast, and the complexity of understanding nucleo-mitochondrial relationships and genotype-phenotype correlations in human disorders certainly increases when considering interdependent cells in different tissues and organs. Understanding this complexity hinges on the development of animal mitochondrial research.

The mouse, *Mus musculus* and the fruitfly, *Drosophila melanogaster*, are the oldest metazoan model systems used for genetic studies of conserved developmental and cellular process-
es, as well as for the identification and validation of human disease-associated genes. Gene knockouts and gene mutations have been successfully created in both systems. However, genetic manipulation in these organisms is time-consuming, and this hinders the analysis of many genes at a time. The nematode worm *Caenorhabditis elegans* offers several experimental advantages in this respect. With the adult hermaphrodite being 1 mm long and ~80 μm diameter, these animals can be grown as microorganisms on agar medium Petri dishes, where they are maintained on a diet of *Escherichia coli*. Their life cycle is short (3.5 days at 20°C) and each hermaphrodite produces ~300 larvae by self-fertilisation. It was the first metazoan to have had its genome completely sequenced [11] and it turned out that ~65% of the human disease genes have a counterpart in the worm [12]. Despite its apparent simplicity, there is a high degree of differentiation; these worms have muscle cells, neurons, gut, and an excretory system. The complete pattern of cell divisions that leads to its 959 somatic cells has been determined and cells can be recognised and identified due to the transparency of the animals’ body. Similarly, a wiring diagram of all 302 neurons has been elucidated. Moreover, *C. elegans* is amenable to molecular, genetic and biochemical analyses. Although targeted mutagenesis in *C. elegans* is still in its infancy, the recent development of RNA interference (RNAi) as a method for the study of gene function has tremendously increased the number of genes whose dysfunction has been associated with a given phenotype. Feeding on bacteria engineered to produce dsRNA is usually enough to produce a full or partial loss of function phenotype in the worm [13], and a library of bacteria producing dsRNA for most *C. elegans* genes is now available for genome-wide approaches [14]. This technique allows for temporal inactivation of thousands of genes at a time, since worms can be easily grown in microtitre plates of up to 384 wells. Combination of high-throughput RNAi with DNA microarrays and yeast two-hybrid analysis is proving to be a valuable global approach to understand gene expression and function [15, 16]. Still, RNAi has its disadvantages: usually the removal of the protein studied is partial, it does not work for all genes and the effectiveness can vary between genes, neuronal cells being less sensitive than other tissues. *C. elegans* is currently being used as a model system for the study of muscular dystrophy [17], of some neurological diseases [18] and peroxisome biogenesis disorders [19]. However, in contrast to other model systems such as *Drosophila* and mouse, the *C. elegans* potential value as genetic model for investigating mitochondrial biogenesis and human mitochondrial disorders is still unexplored.

In this thesis I have used *Saccharomyces cerevisiae* and *Caenorhabditis elegans* to study the role of the PHB complex in mitochondrial biogenesis. Many basic cellular activities are conserved from yeast to humans [9]. However, although PHB genes are highly conserved, their deletion in *S. cerevisiae* does not result in a clear observable phenotype, while they appear to be essential for invertebrate development ([20] and Chapter 5). A possible explanation for this is that redundant genes present in yeast make up for the lack of prohibitins. Alternatively, loss of function of prohibitins might have a stronger effect in the cellular metabolism of higher eukaryotes. Anyhow, this underscores the fact that model organisms complement each other, and all of them contribute to unravel the complexities of genotype-phenotype relations.
3. The PHB complex: A holdase/chaperone with a regulatory role in membrane protein quality control?

The mitochondrial respiratory chain (MRC) is the major source of ATP for eukaryotic cells under aerobic conditions. However, reactive oxygen species (ROS) are inevitable byproducts of oxidative metabolism. The respiratory chain is considered to be the main producer of cellular ROS and defects in oxidative phosphorylation (OXPHOS) result in increased ROS production. ROS can easily damage DNA, proteins and lipids, leading to MRC dysfunction [21]. Therefore, extremely complex and well-coordinated processes are required to precisely regulate the biogenesis of the OXPHOS system in response to the variable physiological circumstances of the cell. OXPHOS complexes need to be stoichiometrically assembled, with subunits originating from both the nuclear and the mitochondrial genome. Besides regulation at the gene expression level, an accurate control of the assembly process is needed. Mitochondrial-encoded proteins are highly hydrophobic core subunits of respiratory chain enzymes. On one hand, their localisation at the inner membrane makes them highly susceptible to ROS damage. On the other hand, unassembled hydrophobic proteins have a high potential of damaging the membrane. A damaged membrane can lead not only to increased ROS production, due to OXPHOS defects, but also to proton leakage and escape of ROS. Such a situation can dramatically compromise the metabolic capacity of the cell and possibly lead to apoptosis. In yeast, mitochondrial-encoded proteins are co-translationally inserted in the membrane with the help of the insertion machinery, Oxa1p and Mba1p, among others [22, 23]. However, until respiratory enzyme subunits attain their functional conformation and suitable partners become available for further assembly, these polypeptides represent a danger to the membrane.

The mitochondrial membrane-bound AAA proteases are responsible for the removal of potentially harmful unassembled polypeptides from the inner membrane [24]. Among these are polypeptides that are aberrant due to translational defects, damaged after synthesis or simply in excess. The sequence similarity between Phb1p and Phb2p proteins and the Escherichia coli complex HflC and HflK (∼20 % identity and 30 % similarity) was instrumental for the investigation into the role of the PHB complex in mitochondrial enzyme processing [25]. In bacteria, the AAA protease FtsH associates with the HflKC complex, which appears to modulate the activity of the protease [26]. Similarly, the PHB complex has been shown to associate with the mitochondrial m-AAA protease (Afg3/Rca1 complex) [25]. In the absence of the PHB complex, degradation of mitochondrial translation products by the m-AAA protease is accelerated, which was explained in terms of the action of the PHB complex as a negative regulator of the protease [25]. The synthetic lethality observed upon disruption of the PHB1 and PHB2 genes in yeast cells lacking subunits of the m-AAA protease, was taken as a genetic evidence for a functional interaction between the PHB complex and the m-AAA protease.

The results presented in chapter 3 prompted us to propose that the mitochondrial PHB complex functions as a membrane holdase/unfoldase for mitochondrial-encoded proteins. In chapters 2 and 3 we provide reasons to believe that the PHB complex has functions above and beyond the regulation of the m-AAA protease activity. First, the PHB complex is increased in two different yeast mutants (mss5Δ and shylΔ) that are defective in the synthesis of the Cox1p subunit and show decreased COX assembly [27, 28]. Such situations require the deg-
radation of unassembled subunits and therefore it seems contradictory to increase an inhibitor of the protease. Increasing a holdase/chaperone is consistent with a need to sequester excess unassembled subunits that can damage the integrity of the membrane. Second, the severe growth defect observed in the double deletion mutants, PHBI or PHB2 in combination with an m-AAA protease mutant, is better explained by a holdase/chaperone function, since deletion of an inhibitor should have no effect in the absence of the protease. The association of the PHB complex with the m-AAA protease could only be detected when very mild detergents were used and the complexes appeared to be autonomous since the disruption of either of the complexes did not destabilise the other [25]. Therefore, the physical association of the PHB complex with the m-AAA protease suggests rather a spatial organisation for the assembly of mitochondrial respiratory complexes than a regulatory role for the PHB complex. The PHB complex could interfere with membrane protein degradation by directly binding to substrate proteins. Similarly, the distantly related HflK and HflC are able to bind proteins that are substrates for the protease [26]. Nevertheless, understanding of the action of the PHB complex is still poor and experimental evidence for a regulatory function or for a cooperative action of the PHB complex linked to membrane protein degradation by AAA proteases is still lacking.

Between polypeptide insertion into the membrane and final assembly of complete respiratory chain complexes, the PHB complex could act at different levels. Newly synthesised polypeptides pass through different non-functional conformational stages until they attain the appropriate conformation in the membrane to interact with their partners. The PHB complex could act during protein folding, and deliver polypeptides that cannot be correctly folded to the AAA proteases for degradation, thus avoiding polypeptide damage and/or aggregation in the membrane. Another possibility is that the PHB complex could protect polypeptides that have already attained their proper membrane topology and await suitable partners for the assembly of functional complexes. In that case, the PHB complex will ensure that small pools of unassembled core respiratory enzymes subunits are readily available for assembly, when required, without harming the membrane.

3.1 PHB phenotypes and expression levels

Disruption of either one or both subunits of the PHB complex in S. cerevisiae results in a decreased replicative lifespan but does not result in any other observable growth phenotype under laboratory conditions [29, 30]. This shortening of the yeast replicative lifespan is accompanied by a defect in membrane potential, a lengthening of the cell division time and characteristic morphological changes of ageing cells [29]. This suggests that phb-null yeast cells undergo premature aging, plausibly due to a slight, but cumulative decline in cellular metabolic capacity [29]. Recent work by Piper and Bringloe [31] shows that prohibitins do not influence the chronological lifespan of non-dividing cells (G0-arrested), and that phb-null cells in stationary phase (G0-arrested) tend to lose respiratory capacity, which is associated with deletions of the mitochondrial genome ([rho-]) [31]. In contrast, others have reported that phb-null mutations do not increase the frequency of generation of [rho-] cells when compared to the parental strain [30, 32]. One possible explanation is that generation of [rho-] can only be detected in old, non-dividing phb-null cells and not in young phb-null mother cells. A similar situation is encountered regarding the mitochondrial morphology of phb-null yeast
cells. Piper and co-workers [33] report that old phb-null mother cells show defective mitochondrial segregation to the daughter cells and aberrant mitochondrial morphology. In contrast, other previous studies did not detect mitochondrial morphology defects in phb-null cells [30, 32]. Thus, aberrant mitochondrial morphology could only be observed in specifically sorted old mother cells that are at the end of their replicative lifespan [33].

The observed reduced replicative lifespan has been taken as an indication of increased oxidative stress and molecular damage in cells depleted of the PHB complex. If the PHB complex holds MRC subunits to assist with folding, incorrectly folded subunits might accumulate in its absence. Moreover, endogenous ROS can more easily damage subunits that are not protected by the PHB complex. These incorrectly folded or damaged subunits might not assemble properly into functional complexes, which may lead to proton and electron leakage. ROS production might increase, damaging DNA, lipids and proteins. This cumulative increase in ROS production and the progressive reduction of metabolic efficiency seems the most plausible explanation for the reduced replicative lifespan observed in yeast [29, 30]. Yet, several attempts have failed to show increased sensitivity to endogenous ROS or to exogenously added oxidative stress in phb-null yeast cells [29, 31]. Furthermore, the expression levels of prohibitins in human fibroblasts do not respond to exogenously added hydrogen peroxide [34]. Nevertheless, we find that reduction of the level of PHB proteins sensitises C. elegans to exogenously added oxidative stress (Chapter 5). A plausible explanation for this difference is that the PHB complex may prevent excess of ROS by acting as a chaperone/holdase, but it is not involved in ROS detoxification. phb-null yeast cells might respond to elevated levels of ROS by increasing the levels of enzymes involved in ROS detoxification to an extent sufficient to cope well with the increased level of ROS. However, oxidative damage to molecules will gradually accumulate, which may be responsible for the increased rate of ageing and reduced replicative lifespan observed in yeast [29, 30]. This might also in part explain the lack of a strong phenotype in yeast cells when compared to multicellular organisms. In contrast, reduction of PHB protein levels in C. elegans results in an increased sensitivity to exogenously added oxidative stress. It is conceivable that the increase in anti-oxidant enzymes might be just enough to deal with increased levels of ROS and pass through development. However, animals might be still seriously affected by the increased ROS level. Therefore, exogenously added oxidative stress completely overwhelms the worms and its anti-oxidant defence system. Obviously, measurements of endogenous ROS and levels of anti-oxidant enzymes in yeast and worms are required to substantiate this hypothesis. Recently, PHB proteins have been suggested to be in the proximity of ROS generation sites, and its unique and conserved tryptophan residue to be target for oxidation in respiring mitochondria of normal human heart tissues [35]. This might indicate that the PHB complex holds and targets oxidatively damaged subunits for degradation.

In Chapter 5 of this thesis I describe a strong loss-of-function phenotype for PHB gene inactivation in a multicellular organism. I show that, in C. elegans, prohibitins are necessary for viability and serve an essential role during organismal development. This is in strong contrast with the lack of an observable phenotype in yeast. The phenotypes observed suggest that PHB proteins are specifically required in embryonic and germline cells, thus in tissues that undergo cellular proliferation. This is probably because PHB deficiency is likely to manifest itself at moments of peak demands on mitochondrial biogenesis/function and therefore result in the
Similar to the lethality of the cells involved. Similarly, tissues that rely more heavily on mitochondrial function are more likely to be susceptible to the lack of prohibitins. Consistent with this, a strong effect on mitochondrial morphology was observed upon reduction of prohibitin expression in body wall muscle cells (Chapter 5). During post-embryonic development, muscle cells do not proliferate but grow in size. To meet the energy requirements of these cells, the number of mitochondria per cell should increase. When prohibitin protein levels are reduced in muscle cells, mitochondrial morphology and distribution are severely affected. This suggests that prohibitins play an important role in maintaining mitochondrial membrane integrity. This mitochondrial morphology defect could be due to a loss in membrane potential and/or to a deficiency in ATP production.

Changes in the expression levels of prohibitins further support a role for the PHB complex as holdase/chaperone. A holdase/unfoldase function can account for the requirement of the PHB complex during metabolic changes, specifically those that result in an imbalance between nuclear and mitochondrial encoded subunits. For instance, PHB expression increases in yeast cells during the diauxic shift, that is, when yeast cells switch from non-oxidative to oxidative metabolism [36]. Further, yeast mutant cells defective in the synthesis of the mitochondrial encoded Cox1p subunit (ms5Δ and shyΔ [27, 28]) show increased expression levels of the PHB complex ([37] and Chapter 3). Finally, inhibition of mitochondrial translation results in increased PHB expression in human cells and in C. elegans ([34] and Chapter 5). Extensive studies on the expression patterns of both PHB proteins in mammalian tissues and during murine development also support a role for the prohibitin proteins in regulating mitochondrial metabolism. PHB proteins are more expressed in cells that rely more on mitochondrial function, including neurons, muscle, heart, liver, renal tubules, adrenal cortex, brown adipocytes and pancreatic islet cells [34]. These tissues are often affected by mitochondrial disorders and they are particularly susceptible to mitochondrial dysfunction [38].

PHB proteins are more expressed in proliferating than in non-proliferating cells of the same type [34], and both PHB proteins are consistently highly expressed in neoplastic tissues [34, 39-44]. The high expression of prohibitins in tumour cells might be in part explained by the presence of conserved binding sites for the oncoprotein c-Myc in the promoters of both PHB genes [34]. c-Myc regulates cellular proliferation, replicative potential, growth, differentiation and is a potent activator of carcinogenesis [45]. The transcriptional activation of PHB by c-Myc might be necessary to coordinate mitochondrial proliferation to the proliferative status of the cells. Alternatively, as proposed in Chapter 2, the high expression of PHB proteins could act to reduce oxidative stress by acting as a chaperone, allowing the continued growth of the tumour cells.

3.2 Genetic interactions

Apart from decreased replicative lifespan, deletion of PHB1 or PHB2 genes in S. cerevisiae does not result in an observable growth phenotype, in either respiratory or fermentable carbon sources [29, 30]. However, phb-deletion in combination with deletions of several other mitochondrial proteins results in lethality or in a seriously impaired growth on fermentable carbon sources. A synthetic lethal interaction has been observed when PHB1 or PHB2 genes are disrupted in cells that lack subunits of the mitochondrial m-AAA protease (afg3Δ or
In contrast, disruption of PHBI or of PHB2 genes did not affect the growth of ymetΔ cells lacking the i-AAA protease on either fermentable or nonfermentable carbon sources [25]. The mitochondrial m-AAA protease is necessary for respiratory growth in S. cerevisiae but not for fermentative growth, while the i-AAA protease is not required for respiratory growth at the permissive temperature [46]. In the absence of prohibitins, authors observed accelerated degradation of the mitochondrial-encoded proteins Cox3p and Atp6p by m-AAA proteases, which was not observed for substrates of the i-AAA protease (Cox2p). Therefore, this synthetic lethal interaction was taken as a genetic evidence for a specific functional interaction of the PHB complex with the m-AAA protease [25].

In S. cerevisiae, disruption of PHB genes in combination with any of the three mitochondrial inheritance components of the mitochondrial outer membrane, MMM1, MDM10 and MDM12, also results in lethality [30]. All three proteins are required for inheritance of mitochondria to daughter cells and mmm1Δ, mmd10Δ and mmd12Δ show a similar phenotype of temperature-sensitive growth and enlarged spherical mitochondria [47-49]. Mmm1p, Mdm10p and Mdm12p are proposed to form a complex in the mitochondrial outer membrane and to mediate interaction with the yeast cytoskeleton [50]. mmm1Δ yeast cells show a dramatically disorganised mitochondrial inner membrane and Mmm1p is connected to mitochondrial nucleoids [51]. Further, mmm1Δ yeast cells are completely devoid of mtDNA [52]. Berger and Yaffe [30] showed that Phb1p and Phb2p are important for keeping mitochondrial morphology in cells that lack a functional mitochondrial genome ([rho-]). While PHBI PHB2 [rho-] contained typical tubular mitochondria, single or double phb- null [rho-] mutants had fragmented and disorganised mitochondria. The authors suggest that mtDNA or associated proteins may play a structural role that is revealed in the absence of prohibitins.

Recently, a synthetic lethal interaction of the PHB complex with the mitochondrial Phosphatidylethanolamine (PtdEtn) biosynthetic machinery has been reported in S. cerevisiae [32]. Phosphatidylserine decarboxylase 1 (Psdlp), located in the mitochondrial inner membrane, is responsible for the production of PtdEtn, a phospholipid essential for aerobic growth of yeast cells. Cells disrupted for PSD1 have a high tendency to form respiration deficient cells (petites) on glucose [53]. A screen for mutants that are synthetic lethal with a temperature-sensitive allele of PSD1 uncovered both PHBI and PHB2 [32]. phb1Δ/phb2Δ mutants have an increased level of PtdEtn. PtdEtn seems to compensate for the lack of prohibitins since in the absence of Psdlp the lack of PtdEtn results in lethality. Moreover PHB and PtdEtn share the function of stabilizing mitochondrial-encoded proteins. Mitochondrial-encoded proteins were more unstable in psdlΔ mutants than in phb1Δ/phb2Δ mutants and in the triple mutant only the soluble ribosomal protein Var1p could be detected. In fact, triple mutants had lost most of their mtDNA already at the permissive temperature, despite wild-type levels of mitochondrial PtdEtn [32]. This, together with the genetic interaction of PHB genes with MMM1, prompted authors to suggest that the PHB complex may also be involved in mtDNA stability. High local concentration of PtdEtn may determine the attachment site of mtDNA nucleoids. Prohibitins may be involved in the assembly of this domain and elevated levels of PtdEtn may overcome the absence of prohibitins. Further, triple mutants also had a very low membrane potential at the permissive temperature and their mitochondria appear fragmented and collapsed after shifting to the nonpermissive temperature. In Chapter 5 of this thesis
we show that the phenotypes elicited by the absence of prohibitins in *C. elegans* are seriously aggravated at higher temperatures. This might indicate a higher requirement for PHB proteins due to increased metabolic demands. Alternatively, temperature might affect the stability or turnover of mitochondrial respiratory complexes or the PHB complex. Therefore the mitochondrial morphology defects found in the *phblA/phb2A, psdlA* might be an indirect effect due to the increased temperature and not to the lack of PtdEtn. Similarly to what happens with *phblA/phb2A*, the *psdlA* mutant was synthetic slow with *afg3A* and *rcalA*, but not with *ymelA*. However, *psdlA* in combination with the mitochondrial morphology mutants *mmm1, mdm10* and *mdm12* did not result in lethality [32].

All the genes that, when mutated, show synthetic lethality in combination with *phb* mutations are genes somehow involved in processes related to the biogenesis, morphology or stability of the mitochondrial inner membrane. However, all these synthetic lethal interactions are insufficient to unravel the role of the PHB complex. In situations where mitochondria do not function properly because the integrity of the inner membrane is compromised, yeast cells might be unable to control the dysfunction without prohibitins. In that case, the synthetic lethal knockouts just substantiate the fact that the PHB complex is important for maintaining mitochondrial integrity, and do not necessarily provide any information about the function of the PHB complex. Could a holdase/chaperone function account for the severe growth defect observed in those mutants? In *afg3A/rcalA* mutants degradation is impaired and unwanted subunits might temporarily accumulate in the mitochondrial membrane [24]. Further, in addition to mitochondrial morphology defects, *mmm1A* and *mdm10A* mutants exhibit loss of mtDNA [51, 52], which may also result in the accumulation of unassembled subunits in the membrane. Finally, the absence of PtdEtn in *psdlA* cells also results in impaired assembly due to membrane instability and loss of mtDNA [32]. In such situations the PHB complex might be specifically required to hold MRC subunits that cannot be assembled into functional complexes, and in the absence of the PHB complex these cells become extremely sick.

Whether all these genetic interactions of prohibitins in yeast can be linked to membrane protein degradation in a cooperative action with the *m-AAA* protease awaits experimental evidence. It is worth mentioning that preliminary results indicate that *afg3A/rcalA* yeast cells have a decreased fraction of PtdEtn containing C16:0 moieties as a side chain, although the ratio between the different groups of phospholipids is not altered [54]. Interestingly, FtsH from *E. coli*, which is closely related to the mitochondrial *m-AAA* protease, is involved in the proteolytic control of lipid metabolism [55]. One possibility is that the *m-AAA* protease could affect membrane composition via the proteolytic regulation of one or more specific regulatory proteins. However, regulatory substrates for the *m-AAA* protease have not yet been identified. Moreover, the fact that PtdEtn mutants are synthetic lethal in combination with *m-AAA* protease mutants point to a cumulative defect in membrane stability. Membrane lipid composition is affected in the absence of both the *m-AAA* protease and the PHB complex. Therefore, further investigation in this field might shed new light on the complex relations between lipid composition and the stability of membrane proteins.
4. Can we learn from structure predictions?

Resolving the 3D-structure of proteins with unknown function can provide important clues to their role in vivo. Therefore, we attempted to obtain more detailed structural information on the PHB complex to elucidate its molecular mechanism of action. In Chapter 4, we derive a partial structure for the PHB complex. We generated amino acid distance information with the help of cross-links. Strikingly, only heterodimeric interactions were found, further supporting the strong co-dependence of both subunits. Secondary and tertiary structural predictions were made using several algorithms, and the spatial restraints of the cross-link data were used to validate possible protein folds. The 3D structure best fitting our experimental results was the four-helical bundle structure of the t-SNAREs syntaxin 1A and the yeast Ssolp. We should be extremely careful when modelling unknown structures with less than 30% homology to known solved structures, as is the case here. Moreover, validation of predicted structures with distance-restrictants from cross-linking lacks rigorous statistics. Nevertheless, and although it is known that proteins with the same fold can perform a variety of biochemical functions [56], it is still tempting to consider the fact that proteins with a similar fold might share some functional properties [57]. SNARE proteins are involved in membrane fusion and traffic, mediating vesicle docking [58-60]. Thus, if our structure prediction is correct, one could consider the possibility of the PHB complex having a role in membrane fusion. Some data might even support this idea. Two tryptophan residues are conserved and basic residues abound in the membrane proximal region of transmembrane SNAREs. Basic residues seem to facilitate interaction with negatively charged lipids [61] [62]. Similarly, a conserved tryptophan residue surrounded by basic residues can be found in the proximity of the transmembrane region of prohibitins (Figure 1A) although further away from the transmembrane region (25 to 33 amino acids) when compared to SNARE proteins. Moreover, prohibitins have been classified as members of the Band7.2b family of proteins. Members of this protein family share a conserved region that has been named the SPFH domain after its members Stomatins, Prohibitins, Flotillins and HflKC [63]. A domain within the SPFH domain, next to the predicted N-terminal transmembrane stretch present in this family, has been called the PHB domain [64]. This PHB domain of flotillin is sufficient to target a green fluorescent protein to the plasma membrane and probably constitutes a novel lipid recognition motif [64] (Figure 1B). Further, plasma membrane association is dependent on palmitoylation and requires a conserved cysteine residue within the domain [64]. A cysteine residue is present in prohibitins although not at a conserved position as compared to stomatins and flotillins. Human PHB1 protein contains a Cys within the domain but 9 amino acids apart from the conserved position. In yeast and C. elegans a Cys is present in the PHB2 protein, 32 and 63 residues apart from the conserved Cys of stomatins and flotillins. The bacterial HflK and HflC do not contain any cysteine. In addition, Phb1p was detected as a member of the Erp1p-associated complex by proteomic detection of multi-subunit complexes [65]. Erp1p involved in vesicle-mediated transport and membrane fusion [66, 67]. Although the specificity and biological relevance of this interaction might be dubious due to the localisation of Phb1p to mitochondria where it always forms a complex with Phb2p, it still might indicate a property of the protein to bind to membrane vesicles.
A

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB2_HUMAN</td>
<td>RGMGTALKLGGAGAVAYGVRESVFTVEGCHRRAIFNRIIGGVQDD</td>
</tr>
<tr>
<td>PHB2_yeast</td>
<td>RMAPAGLGGGGLGGALALFNNALFNVDGCHRRAIVTSRIGVSSR</td>
</tr>
<tr>
<td>PHB1_yeast</td>
<td>KLVDFVIKLPIGIAGIQYSMDVKGSRGETKDPRIINGVQQ</td>
</tr>
<tr>
<td>PHB1_HUMAN</td>
<td>KVFESICFKGLALAVGGSYNSALYNVDAGHRVAPIFDFRFGVQDI</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB2_HUMAN</td>
<td>TILABGLHHRIPFWQYPIIYDIRARPRKISSPTGS</td>
</tr>
<tr>
<td>PHB2_yeast</td>
<td>-IFNEGTHFIFPWLDTPIIYDVRAKPRNVASLTGT</td>
</tr>
<tr>
<td>PHB1_yeast</td>
<td>-VVGEGTHFILPVWOKAIYDVRKPSIATNTGT</td>
</tr>
<tr>
<td>PHB1_HUMAN</td>
<td>-VVGEGTHFILPVWOKPIIFDCRSPRNVPTIGS</td>
</tr>
<tr>
<td>STOM_HUMAN</td>
<td>G WillieSTFVTPPIWMCIIKEYFEAIIR-RLGRITQQ</td>
</tr>
<tr>
<td>FLT1_HUMAN</td>
<td>MFFTCGPNFAWVSFRCRSPPV</td>
</tr>
</tbody>
</table>

Figure 1. A. ClustalW alignment of the N-terminal region of yeast and human PHB proteins. Dark grey shading indicates basic residues present in the vicinity of the conserved tryptophan residue of prohibitins (bold). B. The so-called PHB domain [65] present in members of the SPFH family of proteins. ClustalW alignment of the N-terminal region of yeast and human PHB proteins with human Stomatin and Flotillin-1. Amino acids identical or chemically conserved between PHB and either Stomatin or Flotillin-1 are highlighted by black or dark grey shading, respectively. Groups of chemically conserved amino acids are: (V,L,M); (F,W,Y); (E,D); (Q,N); (S,T); (R,K); (A,G); H; P; C. Less conserved amino acids are highlighted by light grey shading. Groups of less chemically conserved amino acids are: (V,L,M,F,W,Y); (E,D,Q,N); (Q,K); (S,A,T); (F,Y,W,H). The conserved cysteine of Stomatins and Flotillins and the cysteine residue of human PHB1 are shown in bold letters. Putative transmembrane regions (predicted by TMHMM or TMPred algorithms) are underlined. Database accession numbers and the positions of the sequences shown are: Human PHB2 TrEMBL Q99623, residues 17-96; human PHB1 SwissProt P35232, residues 4-82; S. cerevisiae Phb1p SwissProt P40961, residues 6-84; S. cerevisiae Phb2p SwissProt P50085, residues 34-113; human Stomatin SwissProt P27105, residues 31-109; human Flotillin-1 SwissProt O75965, residues 1-56.

5. Hypothesis for the function of the PHB complex

Two main hypotheses have been presented in this general discussion. These are a role for the PHB complex in the biogenesis of OXPHOS as an additional component of the quality control
apparatus and a role in mitochondrial membrane biology (membrane integrity or membrane fusion). In a tentative way of recapitulating the data presented so far they are summarised in Table I in view of their contribution to each hypothesis.

### Table I

**OXPHOS biogenesis/assembly**

- Instability of mitochondrial-encoded subunits in phb-null yeast cells [25, 32].
- Stability of mitochondria-encoded subunits upon overexpression of the PHB complex (Chapter 3).
- Association of Cox2p and Cox3p with the PHB complex (Chapter 3).
- Sequence similarity with Hsp60 (Chapter 3).
- Association with the m-AAA protease.
- Reduced oxygen consumption rate in phb(RNAi) worms (Chapter 5).

**Membrane biology/stability/fusion**

- 3-D structural similarity to SNARE proteins (Chapter 4).
- SPFH membrane-binding domain. (PHB domain) [65].
- Altered phospholipid membrane composition in the absence of the PHB complex and the m-AAA protease [32, 55].
- Mitochondrial morphology defect in C. elegans body wall muscle cells upon phb(RNAi) (Chapter 5)*.
- Genetic interaction with mitochondrial inheritance components (MMM1, MDM10, MDM12) [30]*#.
- Genetic interaction with the Phosphatidylinethanolamine biosynthetic machinery [32]*#.
- Mitochondrial segregation and morphology defect in old phb-null yeast cells [33]*#.
- Mitochondrial morphology defect in phb-null [rho-] yeast cells [30]*#.

(*) These observations could also be explained by cumulative defects in the biogenesis of OXPHOS components. (#) In these cases yeast cells are known to be either [rho+] or [rho-]. The altered mitochondrial morphology could be due to increased defects of the OXPHOS system. Alternatively the PHB complex could have a role in the attachment of mitochondrial nucleoids to the inner membrane.

From the experimental data so far, the observed mitochondrial morphology defect in old phb-null [33] and [rho+] phb-null yeast cells [30], as well as the genetic interactions described in the previous section point to a role for prohibitins in mitochondrial membrane stability. However, the mitochondrial morphology defect observed in C. elegans body wall muscle cells upon reduction of PHB expression provides the strongest indication for a possible role of the PHB proteins in mitochondrial membrane fusion (Chapter 5).

How to explain the OXPHOS biogenesis/assembly hypothesis when considering a membrane biology function for the PHB complex or how to explain a membrane morphology defect when considering a chaperone/holdase function for the PHB complex? If the PHB complex
General discussion

is involved in membrane fusion, or in keeping the inner membrane in close proximity to the outer membrane, the lack of PHB could result in a defect on OXPHOS biogenesis and/or distribution. On the other hand, defects in OXPHOS biogenesis might be the immediate effect of the lack of PHB proteins and OXPHOS defects could lead to membrane morphology defects. These defects in mitochondrial morphology might be readily observable in highly oxidative organisms or tissues (Chapter 5) but not in yeast, where morphology defects only appear in old cells [33]. It is difficult to discern whether observed membrane defects are caused by mistakes in OXPHOS biogenesis or whether defects in OXPHOS biogenesis are caused by membrane alterations. It has been reported that defects in OXPHOS result in mitochondrial morphology defects. For instance, there is a link between cristae morphology and dimerisation of the mitochondrial ATP synthase [68], and C. elegans electron transport chain mutants show defects on mitochondrial morphology in body wall muscle cells [69] and in intestinal cells [70]. However, there is so far no direct experimental evidence that lack of prohibitins results in OXPHOS defects.

Ikonen and colleagues showed that prohibitins localise predominantly to the periphery of mitochondria upon immunogold labelling of human mitochondria [71], with a minor fraction of protein detected at the cristae. The localisation of most prohibitin to the inner boundary membrane, the part of the inner membrane that faces the outer membrane, might indicate a role in cristae formation or even in mediating connections between the inner and the outer mitochondrial membranes. It is known that cristae are dynamic structures and that the

Figure 2. Schematic representation of possible roles of the PHB complex in the mitochondrial inner membrane. Top left: A role in mitochondrial membrane fusion. The PHB complex is shown interacting with mitochondrial outer membrane proteins as part of a complex that might facilitate mitochondrial fusion. Alternatively, the PHB complex could help to keep the mitochondrial outer and inner membrane in close proximity without a direct interaction with outer membrane proteins. Top right: A role for the PHB complex in the biogenesis of OXPHOS complexes. The mitochondrial translocases TOM and TIM are depicted. Middle down: The localisation of the PHB complex in mitochondrial cristae could reflect a role in establishing communication sites within the two membranes of a crista, perhaps to facilitate the turnover of respiratory chain subunits.
number of cristae and their structure vary between cell types and depending on the physiological conditions of the cell. Considering that a fraction of PHB complex localises to mitochondrial cristae and the putative ability of the PHB domain to associate with membranes, another possibility is that the PHB complex could play role in maintaining both cristae in close proximity when required. Although extremely speculative at this stage, it might be worth keeping these alternative hypotheses in mind when further investigating the role of the PHB complex. The predominant localisation of the PHB complex in the inner boundary membrane is also in agreement with its proposed role as holdase in the process of OXPHOS complex assembly. Assembly of the respiratory chain and ATP synthase requires both proteins imported from the cytosol and mitochondrially synthesised subunits. While cristal membranes seem to be the principal site of oxidative phosphorylation [72], OXPHOS complexes are more likely to be assembled in the inner boundary membrane where mitochondrial- and nuclear-encoded subunits encounter each other (See Figure 2). Nevertheless, the prohibitin localisation reported by Ikonen and colleagues could be an artifact of the system used. They overexpressed prohibitin (PHB1) without simultaneously expressing BAP37(PHB 2), which is required for the stability of PHB1. Expression of prohibitin was allowed for a short period of time (6 h), which might not enable turnover to occur. Therefore, the detected signal could correspond to unassembled prohibitin and not to the functional PHB complex.

6. Concluding remarks and future prospects

As mentioned in Chapter 2 of this thesis, many different cellular functions have been attributed to both prohibitin proteins since Phblp was originally discovered as a potential tumour suppressor protein. More recent reports still suggest that prohibitin acts in the nucleus, where it represses E2F-mediated transcription [73, 74]. However, we believe that there is enough evidence that both prohibitin proteins function within mitochondria, and reports confirming this are rapidly accumulating [32, 75-78].

The results presented in Chapter 3 and other results from studies using S. cerevisiae as a model system have provided evidence for a role of the PHB complex in the assembly of the OXPHOS system and in membrane quality control [25]. However, the precise role of the PHB complex in the assembly process and how it relates to the m-AAA protease are still unresolved questions. Genetic interactions found in yeast point to a role for the PHB complex in mitochondrial inheritance [30], membrane stability or even in mitochondrial nucleoid attachment to the inner membrane [32]. Further investigations are required to ascertain whether the role of the PHB complex in these different cellular processes is linked in some way to membrane protein degradation by the quality control system.

Chapter 5 represents an important contribution to our understanding of the importance of the PHB complex in the development of higher organisms. We have for the first time addressed the effects of the loss of the PHB complex at different developmental stages of the multicellular organism C. elegans. In strong contrast to observations made in yeast, the results presented strongly suggest that a PHB null mutation will result in lethality in higher eukaryotes. From this we can predict that mutations in the human PHB genes are likely to result in a
mitochondrial disorder. In addition, our studies demonstrate that *C. elegans* serves as a useful model organism for the study of proteins involved in mitochondrial biogenesis and function.

Despite this progress, the understanding of the exact role of the PHB complex in mitochondrial biogenesis is still poor. On the structural level, still very little is known. The structural model presented in Chapter 4 is based on computed predictions and it is not refined to high resolution. Therefore, the structural similarity to t-SNARE proteins should be regarded very cautiously and further studies are certainly required to substantiate this finding. Moreover, the palisade-shaped macromolecular assembly model proposed is based on the observed molecular mass of the complex (= 1 MDa), the elongated structure of the unit-cell building block, the single helix membrane association, and the proposed function of the PHB complex as holdase/unfoldase capable of binding mitochondrial-encoded subunits. Experimental evidence for a barrel-like structure for the PHB complex where mitochondrial proteins can be held is still lacking. Furthermore, the unambiguous assignment of a holdase/chaperone function to the PHB complex certainly requires more information on the active site of the complex and on the mechanisms of binding and release of peptides.

In Chapter 2 we discuss the possible involvement of the PHB complex in cellular ageing and in degenerative disorders. Recently, altered expression of PHB1 has been correlated with a loss of mitochondrial function in the liver of knockout mice deficient in S-adenosylmethionine synthesis and in obese patients who are at risk for nonalcoholic steatohepatitis [77]. This emphasizes the importance of the PHB complex in keeping mitochondrial homeostasis, and the convenience of including prohibitins in microarrays designed to investigate mitochondrial dysfunctions.

There is obviously much to be understood about this highly evolutionarily conserved complex in the mitochondrial inner membrane. New ideas have been put forward, including a putative role in mitochondrial genome stability, in mitochondrial membrane morphology or even in mitochondrial membrane fusion. Assessing whether the PHB complex plays a direct role in these processes or whether these effects are related to its function as a holdase/chaperone or to protein degradation by the m-AAA protease calls for experimental testing. The lack of an observable phenotype in *S. cerevisiae* upon disruption of PHB proteins complicates the study of the function of these proteins in that organism. In this sense, *C. elegans* might serve as a better model system to investigate the role of prohibitins. Further, resolving the three-dimensional structure of the PHB complex will certainly help to define its role at the molecular level, and more efforts in this direction are clearly necessary.

### 7. References


54. de Jong, L. University of Amsterdam (2003).


