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The role of the PHB complex in mitochondrial biogenesis

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Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins

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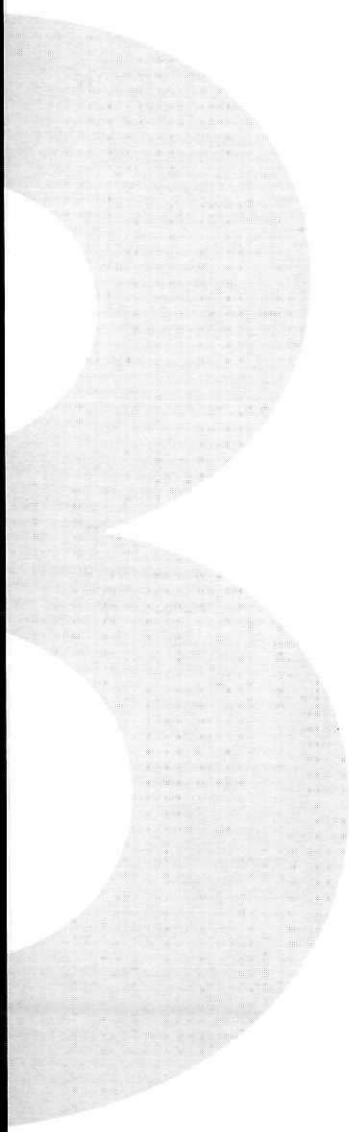
Abstract

Prohibitins are ubiquitous, abundant and evolutionarily strongly conserved proteins that play a role in important cellular processes. Using blue native electrophoresis we have demonstrated that human prohibitin and Bap37 together form a large complex in the mitochondrial inner membrane. This complex is similar in size to the yeast complex formed by the homologues Phb1p and Phb2p. In yeast, levels of this complex are increased on co-overexpression of both Phb1p and Phb2p, suggesting that these two proteins are the only components of the complex. Pulse–chase experiments with mitochondria isolated from *phb1/phb2*-null and *PHB1/2* overexpressing cells show that the Phb1/2 complex is able to stabilize newly synthesized mitochondrial translation products. This stabilization probably occurs through a direct interaction because association of mitochondrial translation products with the Phb1/2 complex could be demonstrated. The fact that Phb1/2 is a large multimeric complex, which provides protection of native peptides against proteolysis, suggests a functional homology with protein chaperones with respect to their ability to hold and prevent misfolding of newly synthesized proteins.

Introduction

Because prohibitin occurs in a wide range of organisms, is strongly conserved, abundant and ubiquitously expressed, it probably serves an important role in cell function. Nevertheless, the physiological function of prohibitin has so far been difficult to define. The first mammalian prohibitin gene was originally characterized as a tumour suppressor gene [1]. The protein appeared to be associated with anti- proliferative activity, thus explaining its tumour suppression characteristic. Prohibitin mRNA microinjected into HeLa cells [2] indeed causes growth arrest. However, this effect was subsequently found to be specifically attributable to the 3'-UTR of the prohibitin mRNA [3] and probably had nothing to do with the true action of prohibitin at the protein level. An important clue for cellular function was provided when it was demonstrated that prohibitin localizes to mitochondria [4]. Coates and co-workers [5] confirmed the mitochondrial localization of prohibitin in mammals, and demonstrated that prohibitin interacts with the structurally related protein, BAP37. This report additionally showed that prohibitins play a role in the determination of the replicative lifespan of yeast. Because it is widely accepted that mitochondria play a role in ageing, this finding indirectly suggests that prohibitins play a role in mitochondrial function.

The yeast homologues of prohibitin and BAP37, Phb1p and Phb2p, respectively, also interact [6]. When the gene for either was disrupted, the other protein was no longer detectable even though the corresponding mRNA was expressed. This interdependence indicates that Phb1p and Phb2p are unstable in the absence of their partner, implying that together they form a complex. A genetic interaction of Phb1p and Phb2p with mitochondrial inheritance components has been demonstrated. However, an explanation for these observed phenomena could not be found [6]. More indications about the cellular function of prohibitins have been provided by comparisons with their *Escherichia coli* homologues. It has been demonstrated that the *E.coli* homologues of prohibitin, HflKC, are associated with and negatively modulate the

protease FtsH [7]. Similarly, in yeast, an association of homologues of HflKC (the Phb1/2 complex) with the FtsH homologue (the Afg3p/Rca1p complex) has been demonstrated [8]. Also, an increased breakdown of mitochondrial translation products could be observed in a *Δphb1/phb2* strain.

Here we report that the human prohibitin and BAP37 form a high molecular weight complex very similar to the yeast Phb1/2 complex. We therefore continued by studying the yeast Phb1/2 complex to obtain more insight into the working mechanism of prohibitins. We provide evidence that the stabilization of mitochondrial translation products by the Phb1/2 complex does not result from a direct inhibition of the Afg3p/Rca1p protease complex, but results from a protection through direct binding of translation products to the Phb1/2 complex. This leads to the hypothesis that the Phb1/2 complex is a novel type of membrane-associated chaperone/holdase.

Results

Complex formation between prohibitin and BAP37

Prohibitin and BAP37, proteins in the mitochondrial inner membrane, have been shown to interact physically with each other [5]. To investigate the supramolecular status of this complex, two-dimensional (2D) electrophoresis was performed. A western blot of a 2D gel was probed with polyclonal antibodies against prohibitin, BAP37 and as a reference, cytochrome *c* oxidase (COX) (Figure 1A). That prohibitin and BAP37 are indeed in the same complex can be deduced from the fact that they have the same mobility in the first dimension. The molecular weight of this complex was estimated to be 1 MDa by using the mobility of the respiratory chain complexes as reference.

Because it has been suggested in the literature [6] that prohibitins in yeast genetically interact with mitochondrial inheritance components, we investigated whether the expression of this prohibitin complex was altered in cells without mitochondrial DNA (ρ^0 cells). Figure 1B shows that there is no difference between expression of the prohibitin complex in ρ^0 cells compared with control cells. Both antibodies against prohibitin and against BAP37 were able to detect the prohibitin complex, confirming that both proteins are part of the complex. An antibody against COX was used to verify the lack of expression of mitochondrial translation products in ρ^0 cells.

Phb1p and Phb2p form a complex in the mitochondrial inner membrane

To check whether the yeast homologues Phb1p and Phb2p form a complex comparable to that of the human prohibitins, similar experiments were performed using yeast mitochondria. Since no antibody against Phb2p was available we expressed Phb2-T7p in the wild-type W303 strain. After transfer to nitrocellulose membranes of the proteins in the resulting 2D electrophoresis gel, the blot was probed first with a monoclonal anti-T7 antibody and subsequently with anti-Phb1 polyclonal antibody. The result obtained shows clearly that the Phb1p and Phb2-T7p proteins migrate identically in the first dimension and form part of the same high molecular weight complex. In the second, denaturing dimension, both proteins run ac-
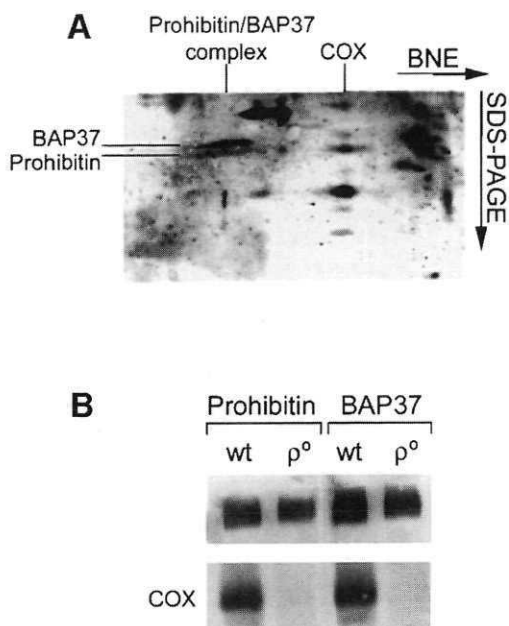
Fig. 1. Prohibitin and BAP 37 form a high molecular weight complex in human mitochondria. (**A**) Two-dimensional electrophoresis (BN and SDS–PAGE) of mitochondria extracted from human fibroblasts. The complexes were transferred to nitrocellulose and blots were incubated with antisera directed against prohibitin, BAP37 and human cytochrome c oxidase holoenzyme (COX). Arrows indicate the directions of the first and second dimension. (**B**) A western blot of a first dimension BN electrophoresis gel of mitochondria of B2.ρ^0 (ρ^0) and wild-type A549 (wt) lung carcinoma cells. The blots were probed with polyclonal antibodies against prohibitin, BAP37 and COX.

cording to their calculated molecular weight (Phb1p 32 kDa and Phb2p 34 kDa) (Figure 2A). Disruption of either the *PHB1* or *PHB2* gene results in a disappearance of the Phb1/2 complex, again emphasizing the interdependence of the proteins (results not shown). Overexpression of either *PHB1* or *PHB2* did not result in a significant increase of the Phb1/2 complex (results not shown). However, when both *PHB1* and *PHB2* were overexpressed, a clear increase of the Phb1/2 complex could be demonstrated (Figure 2B). This suggests either that Phb1p and Phb2p are the only two components of the complex or that other components, if any, are present in non-limiting amounts. In a 2D PAGE gel the Phb1/2 complex was visualized by general protein staining and no other components except Phb1p and Phb2p could be found at the same molecular weight in the first dimension (Figure 2C), yet another indication that Phb1p and Phb2p are the only components of the complex. Given a mol. wt of ~1 MDa, this would mean that the Phb1/2 complex contains between 12 and 16 copies each of Phb1p and Phb2p.

Phb1p and Phb2p are membrane-associated proteins [6] that are thought to be anchored to the membrane by a single transmembrane helix located close to the N-terminus of both proteins. Because most mitochondrial proteins contain an N-terminal leader peptide that is cleaved off upon import into the mitochondria [9], this would affect the putative membrane anchor of Phb1p and Phb2p. We therefore analysed the mature proteins Phb1p and Phb2p as incorporated in the complex. Spots from a 2D gel (e.g. Figure 2C) were either blotted onto PVDF membranes and submitted to micro sequencing, or cut out of the gel and analysed by mass spectrometry. Amino acid sequencing revealed that the N-termini of both Phb1p and Phb2p were blocked, in contrast to a protein from the same blot with a similar staining intensity (ATP synthase subunit α). However, both prohibitins were unambiguously identified from the mass spectra. For Phb1p, 26 peaks covering 77.7% of the sequence were found;
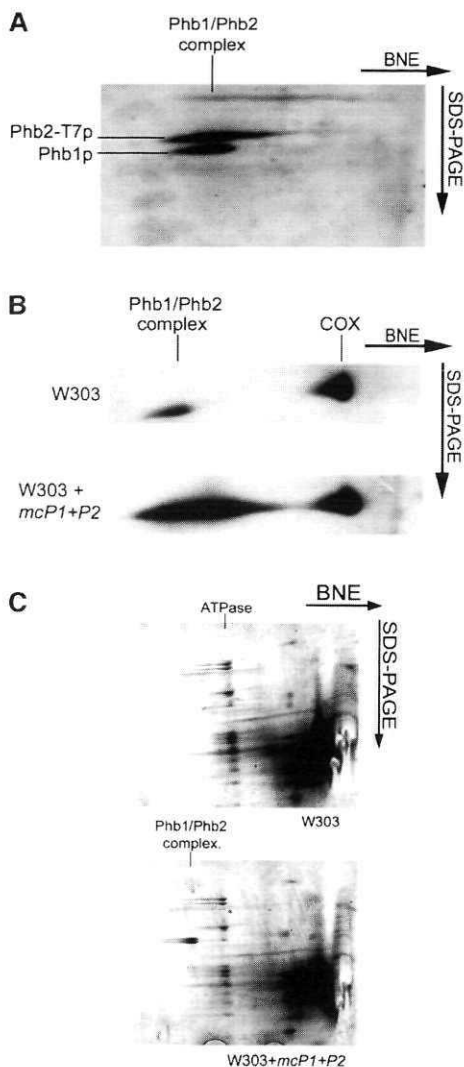
A

B

C

Fig. 2. Phb1p and Phb2p form a 1 MDa complex of which they are the only components. (**A**) 2D PAGE of mitochondrial membranes of a W303 Δ*phb2* strain transformed with the multicopy plasmid YEplac195-PHB2-T7-tag. After western blotting, the blots were incubated with a monoclonal antibody recognizing the T7-tag and subsequently with the polyclonal antibody against Phb1p. (**B**) A Phb1p polyclonal antibody was used to check the expression level of the Phb1/2 complex. The Phb1 signal was only increased when both *PHB1* and *PHB2* were overexpressed (W303+*mcP1+P2*). COX is indicated as a reference protein, which could be demonstrated by incubating with the Cox1p monoclonal antibody. (**C**) When overexpressed [see (B)], the Phb1/Phb2 complex could also be detected after Coomassie staining. For better comparison mitochondrial ATP synthase (ATPase) is indicated.

for Phb2p, 16 peaks covering 53.6% were identified. Experimental evidence is provided that the proteins are imported into the mitochondria without cleaving off a large N-terminal leader peptide, because from Phb1p a peptide starting at Leu7 and from Phb2p a peptide starting at Ser4 were positively identified by tandem mass spectrometry. Also, these findings mean that the predicted transmembrane domains for Phb1p (amino acids 10–30) and Phb2p (amino acids 36–54) are present in the mature protein as incorporated in the complex.

Stabilization of subunits II and III of COX occurs through direct association with the Phb1/2 complex

Steglich and co-workers found that the absence of the Phb1/2 complex resulted in a decreased stability of mitochondrially encoded translation products [8]. Similar results were obtained by pulse– chase experiments using mitochondria of a *PHB1/2* overexpressing strain and a phb1/2 disruptant strain (Figure 3). A possible mechanism for the decreased stability of mitochondrially encoded subunits in the Δ*phb1/2* strain might be that these subunits are protected through direct interaction with the Phb1/2 complex. In order to test this hypothesis we incubated 2D PAGE blots with antibodies against COX subunits. In the PHB1/2 overexpressing strain, but not in the Δphb1/2 strain, we observed an extra spot of Cox3p, which co-migrated
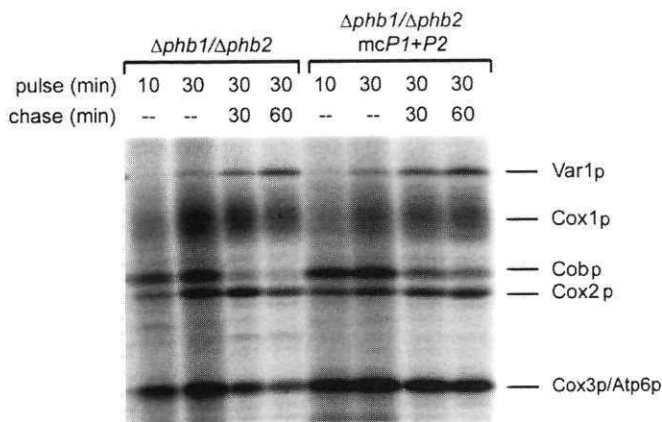
Fig. 3. The Phb1/2 complex stabilizes mitochondrial translation products. Proteins were pulse-labelled with [³⁵S]methionine in isolated mitochondria for 10 and 30 min. After addition of a large excess of un-labelled methionine, mitochondria were chased for 30 and 60 min, respectively and analysed on SDS–PAGE (see Materials and methods). Mitochondrially translated products were compared in a disruptant strain (*Δphb1/Δphb2*) and in a strain overexpressing the Phb1/2 complex (*Δphb1/Δphb2 mcP1+P2*). The mitochondrial translation products Var1p, Cox1p, Cobp and Cox2p are indicated. Cox3p and Atp6p were not separated using these electrophoresis conditions.

in the first dimension with the Phb1/2 complex (Figure 4A). Co-migration of Cox2p could also be observed (our unpublished data). The Cox3p antibody cross-reacts with multiple species that co-migrated with the COX complex. An explanation for this is that the COX complex did not fully dissociate in the second dimension. It is well documented that the very hydrophobic subunits are difficult to dissociate even in the presence of SDS [10]. The co-migration of Cox2p and Cox3p with Phb1p strongly suggests that these proteins associate with the Phb1/2 complex. Additional evidence for the direct binding of mitochondrial translation products to the Phb1/2 complex was provided by co-immunoprecipitation (Figure 5A). Mitochondria isolated from a *PHB1/2* overexpressing and a *Δphb1/2* strain were used as starting material for co-immunoprecipitation with anti-Phb1 polyclonal antibody. Western blot analysis of the immunoprecipitate shows that although the mitochondrial lysates of both strains contained similar amounts of mitochondrial protein (Figure 5A, lanes 1 and 2), Cox2p and Cox3p could only be co-precipitated with the Phb1 antibody in the *PHB1/2* overexpressing strain (Figure 5A, lanes 3 and 4). Figure 5B illustrates that indeed no Phb1p was present in the *phb1/2* disruptant strain and that Phb1p was immunoprecipitated from the *PHB1/2* overexpressing strain. The co-immunoprecipitation of Cox2p and Cox3p with Phb1p was specific because the high abundance, hydrophobic, mitochondrial ADP/ATP carrier protein (AAC), also an integral inner membrane protein, did not co-immunoprecipitate (Figure 5C). In order to obtain information about the binding and release of mitochondrial translation products from the Phb1/2 complex, a pulse–chase experiment of the Phb1/2 disruptant and overexpressing cells in the presence of cycloheximide (to block nuclear translation) was performed, followed by 2D PAGE analysis (Figure 4B). In the 40 min pulse labelling, labelled mitochondrial translation products were co-migrating with the Phb1/2 complex only in the *PHB1/2* overexpress-
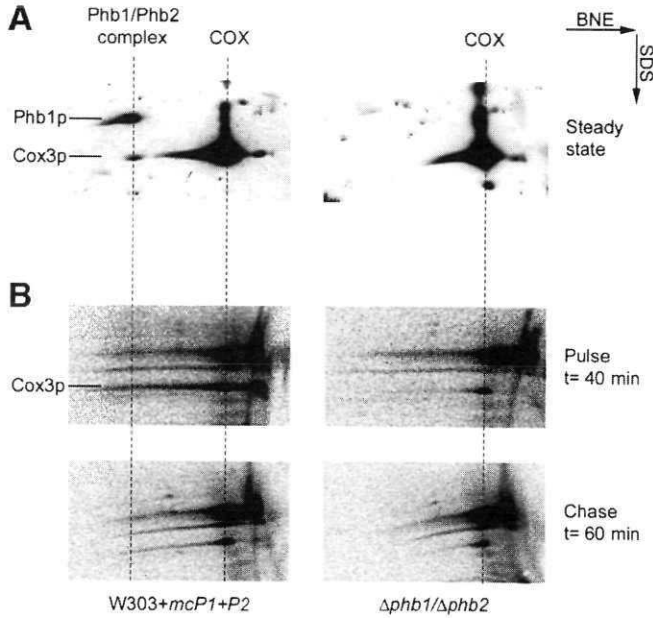
Fig. 4. COX subunits associate temporarily with the Phb1/2 complex. (**A**) A 2D gel of mitochondrial membranes of cells overexpressing the Phb1/2 complex (W303+*mcP1+P2*) and cells lacking this complex (*Δphb1/Δphb2*), which were blotted to nitrocellulose. Western blots were first incubated with the monoclonal antibody against Cox3p, and subsequently with the polyclonal antibody against Phb1p. (**B**) The same strains were used in a pulse–chase experiment (see Materials and methods). After 40 min pulse labelling, cells were chased for 60 min. Mitochondrial pellets were run on 2D PAGE as described before. Labelled proteins were detected using a PhosphorImager. Cox3p is indicated.
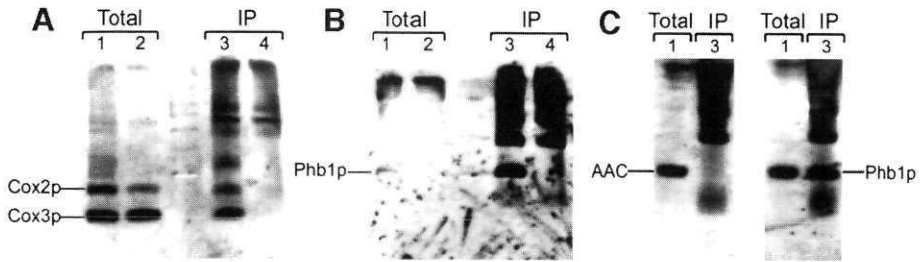
Fig. 5. Cox2p and Cox3p specifically co-immunoprecipitate with the Phb1/Phb2 complex. Mitochondria from W303+*mcP1+P2* (lanes 1 and 3) and *Δphb1/Δphb2* (lanes 2 and 4) were used for immunoprecipitation with the polyclonal antibody against Phb1p. Lanes 1 and 2 represent 1% of the total lysate and lanes 3 and 4 correspond to the immunoprecipitate. The same blot was first incubated with the monoclonal antibodies directed against Cox3p and Cox2p (**A**) and subsequently with the polyclonal antibody against Phb1p (**B**). To demonstrate the specificity of the co-immunoprecipitate, a separate blot of an SDS gel containing total lysate (1) and the immunoprecipitate (3) was incubated with a polyclonal antibody against the mitochondrial ADP/ATP carrier protein (AAC) [(**C**), left panel] and additionally with the Phb1p antibody [(**C**), right panel].

ing strain and not in the *Δphb1/2* strain, confirming the western blot results (Figure 4A). However, after a 60 min chase most of the label had disappeared from this position in the gel and most of the label co-migrated with the fully assembled complex (COX). We infer that newly synthesized proteins were transferred from the Phb1/2 complex to assembling COX or were broken down.

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PHB_YEAST    TKPKSIATNTGTKDLQMVSLTLRVLHRPEVLQLPAIYQNLGLDYDERVLPSICNEVLKSI
PHB_HUMAN    SRPRNVPVITGSKDLQNVNITLRILFRPVASQLPRIFTSIGEDYDERVLPSITTEILKSV
L2CC_DROME   SQPRNVPEITGSKDLQNVNITLRILYRPIPDQLPKIYTILGQDYDERVLPSIAP------
PHB2_YEAST   AKPRNVASLTGTKDLQMVNITCRVLSRPDVVQLPTIYRTLGQDYDERVLPSIVNEVLKAV
BAP37_HUMA   ARPRKISSPTGSKDLQMVNISLRVLSRPNAQELPSMYQRLGLDYEERVLPSIVNEVLKSV
AAC49691.1   TKPHTFSSISGTKDLQMVNLTLRVLSRPEVSRLPYIFQTLGLEYDEKVLPSICNEVLKAV
CH60_ECOLI   VLDKSFGAPTITKDGVSVAREIEL-----------------EDKFENMGAQMVKEV

PHB_YEAST    VAQFD--AAELITQREIISQKIRKELSTRANEFGIKLEDVSITHMTFGPEFTKAVEQKQI
PHB_HUMAN    VARFD--AGELITQRELVSRQVSDDLTERAATFGLILDDVSLTHLTFGKEFTEAVEAKQV
L2CC_DROME   --------------EMVSQRVSQELTVRAKQFGFILDDISLTHLTFGREFTLAVEMKQV
PHB2_YEAST   VAQFN--ASQLITQREKVSRLIRENLVRRASKFNILLDDVSITYMTFSPEFTNAVEAKQI
BAP37_HUMA   VAKFN--ASQLITQRAQVSLLIRRELTERAKDFSLILDDVAITELSFSREYTAAVEAKQV
AAC49691.1   VAQFN--ADQLLTERPHVSALVRESLITRAKDFNIVLDDVAITHLSYGVEFSRAVEQKQV
CH60_ECOLI   ASKANDAAGDGTTTATVLAQAIITE-------GLKAVAAGMNPMDLKRGIDKAVTA---

PHB_YEAST    AQQDAERAKFLVEKAEQERQASVIRAEGEAESAEFISKALAKVG-DGLLLIRRLEASKDI
PHB_HUMAN    AQQEAERARFVVEKAEQQKKAAIISAEGDSKAAELIANSLATAG-DGLIELRKLEAAEDI
L2CC_DROME   AQQEAEKARFVVEKAEQQKLASIISAEGD-AERACVGQVIARPE-TVWWSLRLIDRPRYR
PHB2_YEAST   AQQDAQRAAFVVDKARQEKGMVVRAQGEAKSAELIGEAIKKS--RDYVELKRLDTARDI
BAP37_HUMA   AQQEAQRAQFLVEKAKQEQRQKIVQAEGEAEAAKMLGEALSKN--PGYIKLRKIRAACNI
AAC49691.1   AQQEAERSKFVVMKADQERRAAVIRAEGESEAAQLISDATAKAG-MGLIELRRIEASREI
CH60_ECOLI   AVBELKALSVPCSDSKAIAQVGTISANSDETVGKLIAEAMDKVGKEGVITVEDGTGLQDE

PHB_YEAST    AQTLANSS-NVVYLPSQHSGGGNSESSGSPNSLLLNIGR-
PHB_HUMAN    AYQLSRSR-NITYLPAGQSVLLQLPQ-------------
L2CC_DROME   LTSYPRSR-GVAYLPSGQSHAAQSAIDHRAVAGCI-----
PHB2_YEAST   AKILASSP-NRVILDNEALLLNTVVDARIDGRGQINSEG-
BAP37_HUMA   SKTIATSQ-NPIYLTADNLVLNLQDESFTRGSDSLIKGKK
AAC49691.1   ASTLARSP-NVAYLPGGQSMLFALNR-------------
CH60_ECOLI   LDVVEGMQFDRGYLSPYF
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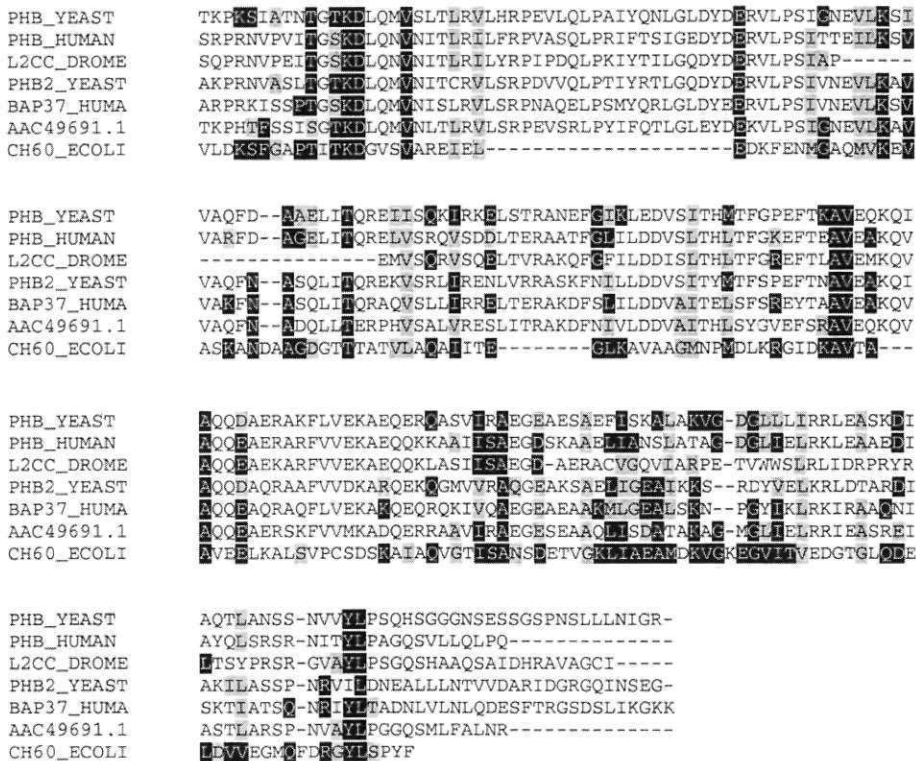
Fig. 6. Sequence conservation between prohibitins and part of *E.coli* GroEL. Representative members of the prohibitin family were aligned using ClustalW (version 1.4) using values of 10 and 0.05 as penalties for gap opening and extension, respectively. The aligned sequences were then compared with the region of the sequence identified by Psi-Blast in the CPN60 chaperone family as displaying similarity (E-value of $5e^{-50}$ after four iterations against the NCBI non-redundant protein database). Amino acids identical or chemically conserved between *E.coli* GroEL, as representative of the CPN60 family, and other sequences are highlighted by black and grey shading, respectively. Groups of chemically conserved amino acids are: (V,I,L,M); (F,W,Y); (E,D); (Q,N); (S,T); (R,K); (A,G); H; P; C. Database accession numbers and the positions of the sequences shown are: *S.cerevisiae* Phb1 SwissProt P40961, residues 73–287; human prohibitin SwissProt P35232, residues 71–272; *Drosophila melanogaster* prohibitin homologue SwissProt P24156, residues 13–203; *S.cerevisiae* Phb2 SwissProt P50085, residues 102–315; human B-cell-associated protein (BAP37) DDBJ/EMBL/GenBank AAF17231, residues 85–299; *Arabidopsis thaliana* prohibitin homologue, DDBJ/EMBL/GenBank AAC49691.1, residues 76–277; *E.coli* GroEL SwissProt P06139, residues 39–203.

Does Phb1/2 complex function as a membrane-bound chaperone?

Our findings suggest that the Phb1/2 complex somehow protects mitochondrial translation products from proteolytic breakdown. Both the observed size and the function of the Phb1/2 complex are reminiscent of hsp60-like chaperones, raising the possibility that the Phb1/2 complex protects newly synthesized mitochondrially encoded respiratory chain subunits from proteolysis by functioning as a novel, inner membrane-bound chaperone. Sequence database comparison of members of the prohibitin family shows that they are unrelated to any of the known protein chaperone families. However, use of the Psi-Blast algorithm, which is sensitive to weak, but often biologically relevant sequence similarities, shows that Phb1p and Phb2p are members of an extensive family of integral membrane proteins, whose principal other members include stomatin and mammalian band 7 proteins (Pfam PF01145; Prosite PDOC00977) [11]. Additionally, Psi-Blast detects a short, but statistically significant similarity to members of the hsp60 family (Figure 6) in a conserved region of this family that has been shown to exert influence on several aspects of hsp60 (GroEL) function, including the ATPase, binding of GroES and the binding/release of unfolded peptides [12].

Phb1/2 complex is increased when there is an imbalance of mitochondrially translated products

A holdase/unfoldase function of the Phb1/2 complex could imply that this complex becomes limiting in situations in which extreme imbalances of translation products occur. This may trigger increased levels of the complex, which in turn displays a higher degree of occupation by unassembled translation products. A situation of imbalance might occur when the respiratory activity of cells is changing. This is supported by the mRNA levels for both *PHB1* and *PHB2*, which are induced 2.5- and 2.6-fold at the diauxic shift, but return to low levels immediately afterwards [13]. Also, western blot analysis using a Phb1p antibody shows that the protein levels of Phb1p are increased upon metabolic stress (our unpublished data). Furthermore, we investigated the expression of the Phb1/2 complex in a yeast strain in which there is an imbalance in the mitochondrially encoded subunits because of a mutation in a translational activator of *COXI* (Mss51p) [14]. Figure 7 demonstrates that, consistent with our expectations, expression of the Phb1/2 complex is indeed increased when compared with the α and β subunits of the ATP synthase complex.
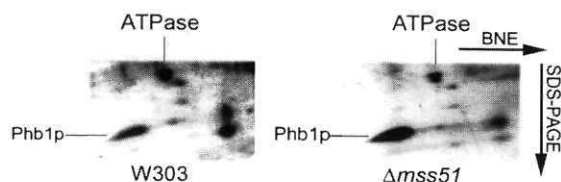

Fig. 7. Phb1/Phb2 complex levels are increased in respiratory chain deficient mutants. Cells lacking the *MSS51* gene (*Δmss51*) were compared with wild-type cells (W303). Mitochondrial membrane extracts were analysed by 2D (BN/SDS)–PAGE and the Phb1/Phb2 complex was detected with a polyclonal antibody against Phb1p. For better comparison, mitochondrial ATP synthase (ATPase) is indicated.

Discussion

Important cellular functions such as tumour suppression, cell cycle arrest and determination of replicative lifespan have been attributed to prohibitin, and although it has been shown that prohibitins influence the stability of mitochondrially translated proteins [8], the relationship of this activity to the cellular roles of this complex remains poorly understood.

The exact structure of the Phb1/2 complex has still to be resolved; however, the molecular size of the complex observed in 2D electrophoretic separations suggests that, like other members of the stomatin/band 7 protein family, it may contain 12–16 copies each of Phb1p and Phb2p [15]. Taken together with the observed association of the complex with newly synthesized mitochondrial translation products and sequence similarities with other chaperones, it is tempting to suggest that the complex has a barrel-like structure within which such products can be contained. As such, its action may parallel that of the mitochondrial inner membrane protein Tcm62p [16]. This member of the hsp60 family of chaperones appears to be specifically required for assembly of complex II. Like Phb1/2, it is unusual in being membrane associated, it forms a similar high molecular weight complex and has recently been demonstrated to bind subunits of complex II prior to their assembly.

The function most recently proposed for the prohibitins is as negative regulators of the Afg3p/Rca1p protease, since disruption of the prohibitin genes causes destabilization of mitochondrial polypeptides [8]. Several of our observations show that it is unlikely that a direct inhibition of the protease by Phb1/2 is the mechanism of this negative regulation. First, we found an increase in the level of the Phb1/2 complex upon disruption of a COX1 translation initiation factor. In this situation, due to decreased COX assembly, degradation of redundant subunits is required and activity of the protease should increase. To increase an inhibitor that leads to a decreased protease activity seems contradictory. In contrast, an imbalance in the production of subunits would be expected to increase the levels of a chaperone/holdase whose function is to ensure correct assembly. Secondly, a protease inhibitor function does not satisfactorily explain why deletion of the PHB1 and PHB2 genes in a background of disruptants of afg3 or rca1 causes a severe growth defect, since an inhibitor should have no effect in the absence of the protease. However, abolishing the activity of a chaperone/holdase that is linked to the protease ought to compound the effect of protease dysfunction. These observations strongly suggest that the prohibitins have functions above and beyond their putative role as protease inhibitors. Additionally, since the function of a protease inhibitor could be efficiently served by a single, small polypeptide, the sophisticated nature of the prohibitin complex with its multiple subunit structure suggests that they should have additional properties.

For the above reasons, we do not think that the prohibitins act mainly as inhibitors of the m-AAA protease in the inner mitochondrial membrane and our observations are more in keeping with a direct role in respiratory chain enzyme processing as a chaperone/holdase. One reason for supposing a role as protease inhibitors is the finding that the two complexes are physically associated. The association of the Phb1/2 complex with the Afg3p/Rca1p complex is detected only when very mild detergents are used, and the complexes appear to be autonomous because disruption of either of the complexes fails to destabilize the other [8]. In com-

bination with our evidence that the Phb1/2 complex directly binds polypeptides of the respiratory chain, this association of the Pbh1/2 and the Afg3p/Rca1p complex suggests a spatial organization for the assembly of respiratory chain enzymes. This model implies that the prohibitins are used to assist with polypeptide folding, and if a polypeptide cannot be folded correctly, it is passed directly to the protease for destruction, rather than being free for incorporation into a respiratory chain complex. Polypeptide subunits with reduced ability for correct folding could arise due to mutations in the mitochondrial genome, which occur naturally and accumulate during ageing, or as a result of direct oxidative damage to proteins, which also occurs naturally in the mitochondrial environment. The incorrect incorporation of a mutant or damaged polypeptide subunit into the respiratory chain would cause increased oxidative stress as well as compromising metabolic efficiency, and this would explain the effect of deletion of the prohibitin genes in shortening cellular lifespan [5, 6]. In support of this hypothesis, analysis of steady-state reactive oxygen species in prohibitin-null yeast with the fluorescent probe H2DCFDA shows that the deletion strains have a higher level of oxidative stress than the corresponding wild-type cells (our unpublished data). Thus, the observations imply that the prohibitins act as an additional component of the quality control system for assembly of the respiratory enzyme complexes, which itself includes the Afg3p/Rca1p protease [17].

The assembly of OXPHOS complexes is an intricate process and involves gene products from both the nuclear and the mitochondrial genome. Because in COX the subunits are assembled in stoichiometric amounts, nuclear- and mitochondrially-encoded subunits need to be provided in a one-to-one ratio. When a yeast cell switches from one state to another, e.g. stationary phase to log phase or aerobic versus anaerobic growth, there might be a temporary imbalance between nuclear and mitochondrial gene products. This is an undesirable situation because aggregation of unassembled, hydrophobic mitochondrial translation products present in the mitochondrial inner membrane may cause proton leakage. Since the Phb1/2 complex is induced transiently in such circumstances, it might also have a function as a holding complex, preventing these peptides from misfolding and thereby either causing damage to the membrane, or leading to the accumulation of incompletely assembled complexes that cause escape of reactive oxygen species.

Prohibitin disruption gives only a very mild phenotype in yeast, and is presumed to cause a reduction in lifespan due to a gradual decline in cellular metabolic capacity [5]. In contrast, disruption of the *Drosophila* homologue of prohibitin is lethal during embryonic development [18], suggesting a more rapid effect on cellular metabolism in higher species. It is likely that in man prohibitins serve an important role in ageing and mitochondrial function.

Materials and methods

Cell lines, strains and media

The standard cell culture medium in this study was Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 110 mg/l pyruvate, with 10% fetal bovine serum. Tissue culture reagents were purchased from Life Technologies (Bethesda, MD). The ρ^0 cells (a kind gift of Dr I.J. Holt, Dundee), derived from the lung carcinoma cell line A549, were supplemented with 50 µg/ml uridine. The absence of mitochondrial DNA from both these cell lines has been shown previously by Southern blotting and PCR [19].

The wild-type *Saccharomyces cerevisiae* strain used in this study is W303/1A (MATa, *ade2-2*, *his3-11,-15*; *leu2-3,-112*; *ura3-1*, *trp1-1*; *can1-100*) [20]. Using this strain, disruptions of either *PHB1* (W303/1A-Δ*phb1*) or *PHB2* (W303/1A-Δ*phb2*), or both (W303/1A-Δ*phb1* Δ*phb2*), were made.

Escherichia coli strain DH5α was used [21] and cells were grown in LB medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl) supplemented with ampicillin (100 μg/ml) when necessary for plasmid selection.

Cloning and epitope tagging of PHB1 and PHB2

PHB1 and *PHB2* genes were cloned individually and together into the multicopy shuttle vector YEplac195 [22]. The *PHB1* gene was isolated as a 1.9 kb *Msc*I fragment from the cosmid pEGH195 (kindly provided by FYSA Investigator Purnelle Bénédicté). The *PHB2* gene was obtained as a 2.4 kb *Sph*I–*Pst*I fragment from cosmid pEGH484 [23].

The 11-residue T7-tag (MASMTGGQQMG) was added at the C-terminus of Phb2 protein using overlapping PCR. Primers used were (epitope-encoding nucleotides are underlined): 5'-CTTCTAT GACCGGTGGTCAACAAATGGGTTAGATATTATCTTTAAGAATTGAG-3' (53mer) and 5'-GT TGACCACCGGTCATAGAAGCCATGCCTTCGCTATTTATTTGAC-3' (45mer). Oligonucleotide 5'-GAATTGAAAAGCTTAGATACAG-3' introduced a *Hind*III site (bold) for further cloning. These primers were designed based on the sequence in the yeast genomic database (SGD). Sequence analysis of *PHB2-T7* revealed a small discrepancy at the 3' end of the gene compared with the wild type. The extreme C-terminus of the fusion protein was changed to RVKKNSEG-tag-stop instead of RGQINSEG-tag-stop.

Preparation of mitochondrial fractions for blue native PAGE

Human fibroblasts. Cells from exponentially growing cultures were collected as described previously [24]. The cell pellet was resuspended in phosphate-buffered saline (PBS) and the cell concentration was determined by use of a counting chamber. Crude mitochondrial pellets were obtained by incubating 1 x 10^6 cells in 200 ml of PBS for 10 min on ice with 4 mg/ml digitonin. The samples were centrifuged for 1 min at 12 000 g and the pellet was washed with PBS. The mitochondrial pellets were stored at –70°C until further processing for 2D PAGE.

Yeast. Yeast cells were grown on 20 ml of YPGal medium (1% yeast extract, 1% peptone, 2% galactose) overnight to an OD_{600} of ~2.0. Cells were harvested at 1800 g and washed once in cold tap water. The pellet (± 0.2 mg wet weight) was resuspended in 0.65 M mannitol, 20 mM Tris pH 7.1 and 1 mM EDTA, and 0.4 ml of glass beads were added. This suspension was vortexed for 5 min at 4°C. After sedimentation of the glass beads, the lysate was transferred to another tube and the glass beads were washed once more. The pooled supernatants were centrifuged for 3 min at 4°C to remove the unbroken cells. Mitochondria were collected by centrifugation at 10 000 g for 15 min and the pellets were stored at –70°C.

Electrophoresis and western blotting

Standard SDS–PAGE was performed according to Laemmli [25] and 2D PAGE was carried out by the method of Schägger and von Jagow [26]. Following electrophoresis, proteins were blotted to nitrocellulose [27]. Immunoreactive material was visualized by chemiluminescence (ECL™; Amersham) according to the manufacturer's instructions.

Antibodies

Polyclonal antiserum to Phb1p was raised by repeated injections of a synthetic peptide (Chiron Mimotopes, Australia) representing the 15 amino acids at the C-terminus of Phb1p conjugated to keyhole limpet haemocyanin (Sigma) using glutaraldehyde. Prior to use, the serum was adsorbed against yeast acetone powder prepared from phb1-null cells, to remove antibodies that recognize

other yeast antigens. Human polyclonal antibodies against prohibitin and BAP37 were described previously [5]. The monoclonal antibody for the detection of T7-tag was obtained from Novagen. The monoclonal antibodies to subunits COX1, COX2, COX3 and COX4 were obtained from Molecular Probes.

Pulse-chase experiments

In mitochondria. Cells were grown in rich medium containing 2% galactose until mid-log phase and all further procedures were performed as described by McKee and Poyton [28]. Mitochondria were isolated with a few minor modifications: spheroplasts were immediately lysed (pottering) and the post-mitochondrial fraction was not used. Mitochondria were resuspended in 0.6 M mannitol to a final concentration of 2 mg/ml.

For efficient translation, isolated mitochondria were incubated in optimized protein-synthesizing medium D in the presence of 8 μl/ml TRAN^{35}S-LABELTM (1175 Ci/mmol; 10.5 mCi/ml; containing 70% L-[^{35}S]methionine and 15% L-[^{35}S]cysteine; ICN Biomedicals, Inc.). Labelling was allowed to continue for 30 min and 250 μl samples were taken at 10 and 30 min. An excess of cold methionine (final concentration 0.2 M) was added after 30 min to start the chase. Samples were taken at 30 and 60 min chase.

Samples were pelleted and the pellet was prepared for SDS–PAGE in LSB (2% SDS, 5% β-mercaptoethanol, 5.8% glycerol, 62.5 mM Tris–HCl pH 6.8, 100 μg/ml bromophenol blue). Gels were dried under vacuum and labelled proteins were quantified using a PhosphorImager (Molecular Dynamics, USA).

In cells. Cells from exponentially growing cultures in YPGal medium were collected by centrifugation and washed with PBS. The pellet was resuspended in labelling medium (40 mM potassium phosphate buffer pH 7.4, 0.3% glucose) to a concentration of 25 mg/ml (wet weight). To stop cytoplasmic translation, cells were pre-incubated for 10 min with cycloheximide (20 μg/ml). Pulse labelling was started by adding [^{35}S]methionine to a final concentration of 50 μCi/ml. After 30 min, cells were collected by centrifugation and washed twice. Cells were chased by adding unlabelled methionine (0.1 ml of 0.2 M to each sample). Mitochondrial fractions for blue native (BN) PAGE were prepared as described above.

Immunoprecipitation

Mitochondria were resuspended in 100 μl of PBS containing 2.5 mM phenylmethylsulfonyl fluoride (PMSF). Lauryl maltoside (final concentration 1.5%) was added to solubilize cells and to extract the membrane proteins. Samples were left on ice for 30 min and centrifuged at 12 000 g for 5 min at 4°C. Supernatants were adjusted to 1 ml with PBS containing 1 mg of bovine serum albumin and 2.5 mM PMSF. Incubation with antibodies coupled to protein A–Sepharose 4B was performed in a rotating wheel overnight at 4°C. The immunoprecipitates were eluted by incubating the beads in sample buffer (4% SDS, 20% glycerol in 10 mM Tris–HCl pH 6.8) at 37°C. After centrifugation, eluates were run on SDS–PAGE gels and blotted to nitrocellulose. Proteins were detected by immunostaining.

Mass spectrometry

In-gel digestion. Protein-containing gel slices were S-alkylated with iodoacetamide, digested with trypsin (Boehringer-Mannheim; sequencing grade) and extracted according to the protocol of Schevchenko et al. [29]. Only the peptides eluted with 20 mM NH$_4$HCO$_3$ were used for mass spectrometric analysis. These were collected and cleaned on ZipTip C18 (Millipore) micropipette tips according to the manufacturer's instructions, and subsequently eluted in 10 μl of 60% methanol, 1% formic acid.

MALDI-TOF mass spectrometry. The peptide solution (0.5 µl) was mixed with 0.5 µl of a 10 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma) solution in acetonitrile/ethanol (1:1 v/v). The mixture (0.5 µl) was spotted on the target and allowed to dry at room temperature. MALDI spectra were acquired on a Micromass TofSpec 2EC (Micromass, Wythenshawe, UK) equipped with a 2 GHz digitizer.

ESI-QTOF mass spectrometry. The solution (2 µl) was introduced into a nanospray capillary, and positive mode spectra were recorded on a Q-TOF mass spectrometer (Micromass, Wythenshawe, UK) equipped with a Z-spray source.

Miscellaneous

Escherichia coli transformations were carried out by electroporation with the *E.coli* pulser (Bio-Rad). *Saccharomyces cerevisiae* was transformed by using the one-step method [30]. DNA manipulations were performed using standard protocols. N-terminal amino acid sequencing was performed on a Procise 494A (Applied Biosystems).

Acknowledgements

References

1. McClung, J.K. et al. Isolation of a cDNA that hybrid selects antiproliferative mRNA from rat liver. *Biochem. Biophys. Res. Commun .***164**, 1316-22 (1989).

2. Roskams, A.J. et al. Cell cycle activity and expression of prohibitin mRNA. *J. Cell Physiol.* **157**, 289-95 (1993).

3. Jupe, E.R., Liu, X.T., Kiehlbauch, J.L., McClung, J.K. & Dell'Orco, R.T. Prohibitin in breast cancer cell lines: loss of antiproliferative activity is linked to 3' untranslated region mutations. *Cell Growth Differ.* **7**, 871-8 (1996).

4. Ikonen, E., Fiedler, K., Parton, R.G. & Simons, K. Prohibitin, an antiproliferative protein, is localized to mitochondria. *FEBS Lett.* **358**, 273-7 (1995).

5. Coates, P.J., Jamieson, D.J., Smart, K., Prescott, A.R. & Hall, P.A. The prohibitin family of mitochondrial proteins regulate replicative lifespan. *Curr. Biol.* **7**, 607-10 (1997).

6. Berger, K.H. & Yaffe, M.P. Prohibitin family members interact genetically with mitochondrial inheritance components in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **18**, 4043-52 (1998).

7. Kihara, A., Akiyama, Y. & Ito, K. A protease complex in the *Escherichia coli* plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. *EMBO J.* **15**, 6122-31 (1996).

8. Steglich, G., Neupert, W. & Langer, T. Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. *Mol. Cell. Biol.* **19**, 3435-42 (1999).

9. Neupert, W. Protein import into mitochondria. *Annu. Rev. Biochem.* **66**, 863-917 (1997).

10. Verheul, F.E., Draijer, J.W., Dentener, I.K. & Muijsers, A.O. Subunit stoichiometry of cytochrome c oxidase of bovine heart. *Eur. J. Biochem.* **119**, 401-8 (1981).

11. Tavernarakis, N., Driscoll, M. & Kyrpides, N.C. The SPFH domain: implicated in regulating targeted protein turnover in stomatins and other membrane-associated proteins. *Trends Biochem. Sci.* **24**, 425-7 (1999).

12. Fenton, W.A., Kashi, Y., Furtak, K. & Horwich, A.L. Residues in chaperonin GroEL required for polypeptide binding and release. *Nature* **371**, 614-9 (1994).

13. DeRisi, J.L., Iyer, V.R. & Brown, P.O. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278**, 680-6 (1997).

14. Decoster, E., Simon, M., Hatat, D. & Faye, G. The MSS51 gene product is required for the translation of the COX1 mRNA in yeast mitochondria. *Mol. Gen Genet.* **224**, 111-8 (1990).

15. Snyers, L., Umlauf, E. & Prohaska, R. Oligomeric nature of the integral membrane protein stomatin. *J. Biol. Chem.* **273**, 17221-6 (1998).

16. Dibrov, E., Fu, S. & Lemire, B.D. The *Saccharomyces cerevisiae* TCM62 gene encodes a chaperone necessary for the assembly of the mitochondrial succinate dehydrogenase (complex II). *J. Biol. Chem.* **273**, 32042-8 (1998).

17. Suzuki, C.K. et al. ATP-dependent proteases that also chaperone protein biogenesis. *Trends Biochem. Sci.* **22**, 118-23 (1997).

18. Eveleth, D.D., Jr. & Marsh, J.L. Sequence and expression of the Cc gene, a member of the dopa decarboxylase gene cluster of *Drosophila*: possible translational regulation. *Nucleic Acids Res.* **14**, 6169-83 (1986).

19. Bodnar, A.G., Cooper, J.M., Holt, I.J., Leonard, J.V. & Schapira, A.H. Nuclear complementation restores mtDNA levels in cultured cells from a patient with mtDNA depletion. *Am. J. Hum. Genet.* **53**, 663-9 (1993).

20. Muroff, I. & Tzagoloff, A. CBP7 codes for a co-factor required in conjunction with a mitochondrial maturase for splicing of its cognate intervening sequence. *EMBO J.* **9**, 2765-73 (1990).

21. Woodcock, D.M. et al. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.* **17**, 3469-78 (1989).

22. Gietz, R.D. & Sugino, A. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527-34 (1988).

23. van der Aart, Q.J., Kleine, K. & Steensma, H.Y. Sequence analysis of the 43 kb CRM1-YLM9-PET54-DIE2-SMI1-PHO81-YHB4-PFK1 region from the right arm of *Saccharomyces cerevisiae* chromosome VII. *Yeast* **12**, 385-90 (1996).

24. Klement, P., Nijtmans, L.G., Van den Bogert, C. & Houstek, J. Analysis of oxidative phosphorylation complexes in cultured human fibroblasts and amniocytes by blue-native-electrophoresis using mitoplasts isolated with the help of digitonin. *Anal. Biochem.* **231**, 218-24 (1995).

25. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5 (1970).

26. Schagger, H. & von Jagow, G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* **199**, 223-31 (1991).

27. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U S A* **76**, 4350-4 (1979).

28. McKee, E.E. & Poyton, R.O. Mitochondrial gene expression in *Saccharomyces cerevisiae*. I. Optimal conditions for protein synthesis in isolated mitochondria. *J. Biol. Chem.* **259**, 9320-31 (1984).

29. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850-8 (1996).

30. Chen, D.C., Yang, B.C. & Kuo, T.T. One-step transformation of yeast in stationary phase. *Curr. Genet.* **21**, 83-4 (1992).