CMLS, Cell. Mol. Life Sci. 59 (2002) 143–155
1420-682X/02/010143-13 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing and degenerative disease

L. G. J. Nijtmans[a,*], M. Artal Sanz[a], L. A. Grivell[a] and P. J. Coates[b]

[a] Section for Molecular Biology, Swammerdam, Institute for Life Sciences, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam (The Netherlands), Fax +31 20 6685086, e-mail: nijtmans@science.uva.nl
[b] Department of Molecular and Cellular Pathology, University of Dundee, Dundee DD1 9SY (United Kingdom)

Received 21 May 2001; received after revision 2 July 2001; accepted 24 July 2001

Abstract. Although originally identified as putative negative regulators of the cell cycle, recent studies have demonstrated that the PHB proteins act as a chaperone in the assembly of subunits of mitochondrial respiratory chain complexes. The two PHB proteins, Phb1p and Phb2p, are located in the mitochondrial inner membrane where they form a large complex that represents a novel type of membrane-bound chaperone. On the basis of its native molecular weight, the PHB-complex should contain 12–14 copies of both Phb1p and Phb2p. The PHB complex binds directly to newly synthesised mitochondrial translation products and stabilises them against degradation by membrane-bound metalloproteases belonging to the family of mitochondrial triple-A proteins. Sequence homology assigns Phb1p and Phb2p to a family of proteins which also contains stomatins, HflKC, flotillins and plant defence proteins. However, to date only the bacterial HflKC proteins have been shown to possess a direct functional homology with the PHB complex. Previously assigned actions of the PHB proteins, including roles in tumour suppression, cell cycle regulation, immunoglobulin M receptor binding and apoptosis seem unlikely in view of any hard evidence in their support. Nevertheless, because the proteins are probably indirectly involved in ageing and cancer, we assess their possible role in these processes. Finally, we suggest that the original name for these proteins, the prohibitins, should be amended to reflect their roles as proteins that hold badly formed subunits, thereby keeping the nomenclature already in use but altering its meaning to reflect their true function more accurately.

Key words. Prohibitin; BAP37; Phb1p; Phb2p; chaperone; mitochondria; ageing.

Introduction

The study of the PHB proteins already spans more than 10 years and tells us an unusual story. The proteins display a remarkable degree of sequence conservation, and homologues have been discovered in a large variety of organisms including bacteria [1], plants [2], yeast [3], worms [4], flies [5] and humans [6]. Moreover, both proteins are ubiquitously and abundantly expressed [3]. Disrupting the PHB genes has effects ranging from decreased replicative lifespan in yeast [7] to a larval arrest phenotype in the fruitfly [5]. A number of diverse cellular functions have been attributed to these proteins. They include action as a tumour suppressor [6, 8], with a role

* Corresponding author.
Present address: Nijmegen Center for Mitochondrial and Metabolic Disorders, University Medical Center Nijmegen, Geert Grooteplein 10, PO Box 9101, 6500 HB Nijmegen (The Netherlands), Fax: + 31-24-3616428, e-mail: I.nijtmans@ckslkn.azn.nl

in cell cycle regulation [8]. Their putative association with the immunoglobulin (Ig)M receptor and oestrogen receptor has suggested a possible role in cellular signalling [4, 9], and binding to E2F proteins has been taken as an indication for a role in transcription regulation during the cell cycle [10]. A relation of the proteins with the induction of apoptosis has also been proposed [11]. Still, the role of the PHB proteins in all these processes remains unclear. The recent discovery of a chaperone function in the assembly process of mitochondrial respiratory chain complexes is a breakthrough in our understanding of the action of the proteins [12, 13]. This finding identifies the PHB proteins as members of a novel type of membrane-bound chaperone family. Intriguing questions arise. What is the structure of this membrane-bound chaperone and its orientation in the mitochondrial inner membrane? What is the working mechanism at the molecular level? How can we explain the diversity of its apparent cellular activities?

The localisation, structure and interactions of the proteins are outlined in the first paragraph of this review. The diversity of activities ascribed in early studies has made it difficult to form a coherent picture of their function. In particular, the early ideas about tumour suppression and cell cycle regulation, which are based mainly on indirect evidence, are not easy to reconcile with later data. In this review we critically examine the earlier ideas about the action of the PHB proteins and evaluate them in the light of later results. We will also analyse the evolutionary conservation, because there is sequence similarity of the PHB proteins with a large family of proteins containing also HflKC, stomatins, flotillins, HIR genes and mechanoreceptors [14, 15].

Several studies have implicated a role of for the PHB proteins in ageing [7, 16–19]. Given the significance of mitochondria in ageing [20], we assess their role in this process. Although to date no mutations in the PHB genes have been found that cause human disease, expression levels of the proteins implicate an indirect role in cancer. Moreover, defects in respiratory complex assembly can cause improper functioning of mitochondria, which in turn can lead to apoptosis and neurodegenerative disorders.

Nomenclature and chromosome localisation

The PHB family of proteins contains two members, which have been termed either prohibitin 1 (Phb1p) and prohibitin 2 (Phb2p), or BAP32 and BAP37. The human prohibitin 1 gene (h*PHB1*) is mapped to chromosome 17q21[6], and encodes a protein of 32 kDa [21]. Prohibitin 2, a 37-kDa protein with strong homology to Phb1p, was found by Terashima and colleagues to bind to the IgM receptor, as does Phb1p, and these authors named the two proteins B-cell-receptor complex-associated proteins (*BAP32* and *BAP37*) [4]. A BLAST search of the human genome places the *PHB2/BAP37* gene on chromosome 12p13 [22]. The name ‘prohibitin’ refers to a presumed function in the negative regulation of cell cycle progression, which is based on the method originally used to identify the *PHB1* gene [6]. However, in this review we conclude that this putative role of prohibitin is not correct, and B-cell-receptor association is also refuted (see below). For these reasons, both of the previously used names are misleading, and we propose to discard them. Moreover, it is not clear whether the generic term ‘prohibitin’ refers to Phb1p, Phb2p or the prohibitin complex. Therefore, we advocate the use of a terminology that better covers the function of the PHB proteins as a mitochondrial chaperone [13]. Because the name prohibitin is already well established in the literature, we propose to use the abbreviation PHB and give this the new meaning: ‘proteins that hold badly folded subunits’. So in summary we propose to use *hPHB1* for the human prohibitin gene, hPhb1p for the human prohibitin protein, *hPHB2* and hPhb2p for the BAP37 gene and protein, respectively. The complex that these two proteins form in the mitochondrial membrane becomes the PHB complex.

Cellular localisation, membrane topology and protein complex structure

Immunofluorescence and immunoelectron microscopy demonstrated that epitope-tagged rat Phb1p was located in the periphery of the mitochondria [23]. Also, in human cells it was demonstrated that hPhb1p and hPhb2p both locate to the mitochondria and display a physical association, as demonstrated by coimmunoprecipitation [7]. This result has subsequently been independently confirmed by native electrophoresis [13] (fig. 1).

The exact localisation and membrane orientation was investigated in more detail by several groups using yeast as a model [12, 24]. Berger and colleagues demonstrated that both yPhb1p and yPhb2p are associated with the mitochondrial inner membrane. Both proteins have one predicted transmembrane-spanning helix (TMpred) located near the N-terminus of the proteins, going from amino acid 10 to 30 for yPhb1p and from amino acid 36–54 for yPhb2p. These transmembrane-spanning helices are thought to anchor the proteins to the mitochondrial inner membrane [24]. Mammalian Phb1p with an epitope tag introduced into the N-terminus interferes with its localisation to the mitochondria [23], confirming that this region of the protein is important for its localisation.

A slight mobility difference between a C-terminally tagged Phb1p and an N-terminally tagged Phb1p lead to the suggestion that an N-terminal target signal is cleaved off after import [23]. Because cleavage of an N-terminal leader

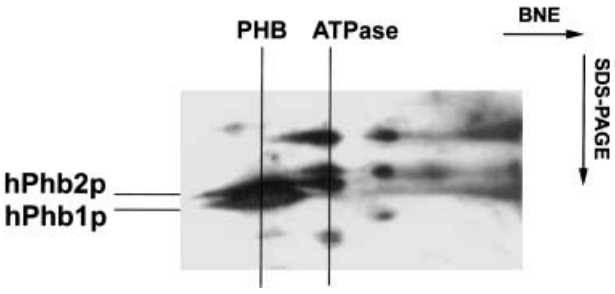

Figure 1. hPhb1p and hPhb2p form a high molecular weight complex in human mitochondria. Two-dimensional electrophoresis (BNE and SDS-PAGE) of mitochondria extracted from human fibroblasts. Intact protein complexes were separated in the first native dimension, and in the second dimension the individual components of the complexes are resolved by using denaturing conditions. The complexes were transferred to nitrocellulose and blots were incubated with antisera directed against Phb1p, Phb2p and human mitochondrial ATP synthase. Arrows indicate the directions of the first and second dimension. PHB indicates the PBH complex and ATPase the ATP-synthase complex, and hPhb1p and Phb2p indicate the prohibitin proteins.

would effectively truncate the putative membrane anchor, we investigated the N-terminus of the mature protein by protein sequencing and mass spectrometry [13]. The first indication that there is no cleavage of a mitochondrial import signal comes from the fact that amino acid sequencing revealed that both N-termini of yPhb1p and yPhb2p were blocked. Usually, cleavage of an import sequence gives a free N-terminus. More direct evidence comes from mass spectrometric data, which show that a tryptic digest of mature yPhb1p contains a peptide starting at Leu 7, and yPhb2p contains a peptide starting at Ser4 [13] (fig. 2).

Berger and colleagues performed protease protection experiments that suggested that the C-terminus of the Phb1p protein is located in the matrix, because when no detergent was used, the proteins were protected against degradation by trypsin [24]. However, Steglich and colleagues found that when mitoplasts were incubated with high concentrations of trypsin, yPhb1p and yPhb2p were both degraded [12]. Because high concentrations of trypsin had to be used and proteinase K was not able to degrade either of the proteins, they concluded that both yPhb1p and yPhb2p are likely to be tightly folded. These findings indicate that both proteins are anchored to the mitochondrial inner membrane and oriented towards the intermembrane space. Compatible with the putative tight folding and protease resistance were results from proteinase K-treated human mitochondria [19]. Chemical cross-linking experiments in combination with mass spectrometry revealed that a C-terminal residue of Phb1p cross-links to a C-terminal residue of Phb2p [our unpublished results], thereby excluding models in which the proteins are proposed to be located on opposite sides of the membrane (fig. 2).

Phb1p and Phb2p display interdependence, since Phb1p cannot be detected by Western blotting in a Phb2p disruption mutant and vice versa [24]. Because the messenger RNA (mRNA) levels were found to be normal, this interdependence is best explained in terms of mutual stabilisation of the two proteins. The mass of the complex

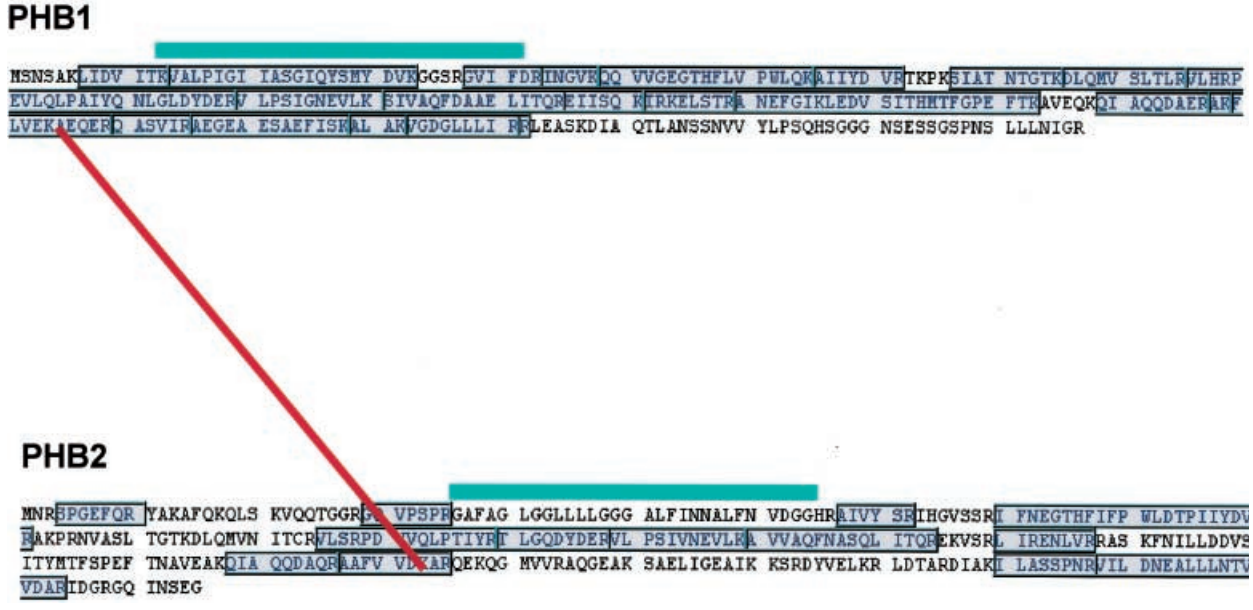

Figure 2. Phb1p and Phb2p do not contain a large cleavable mitochondrial import sequence and are localised in the same mitochondrial compartment. The yeast *PHB1* and *PHB2* genes are represented. The grey boxed parts are tryptic digests from the mature PHB complex that could be positively identified by mass spectrometry. The green bar indicates the predicted transmembrane helix, and the red line represents a chemical cross link (details see text and [13]).

was estimated by gel filtration experiments to be 2 MDa [12] and 1 MDa on the basis of native electrophoresis experiments [13] (fig.1). Neither method is particularly accurate for proteins of such a high molecular weight. Moreover, detergents that remain attached to the complex can influence both the retardation on a gel filtration column and the migration on a native gel, leading to an overestimation of the total molecular weight of the complex. Nevertheless, it is clear that Phb1p and Phb2p form a large, multimeric complex. Attempts to increase the amount of complex succeeded only when both Phb1p and Phb2p were overexpressed, leading to the suggestion that these proteins are the only components of the complex and other, if any, components are required only in non-limiting amounts [13]. Visualisation of the complex by a general protein staining of a gel revealed no other components in the complex [13]. Consistent with this finding, immunodepletion experiments of mammalian cells showed that all of the hPhb1p present in a cell is bound to hPhb2p, and vice versa [19].

Assuming a molecular mass of 1 MDa, the PHB complex should therefore contain between 12 and 14 molecules of both Phb1p and Phb2p. Based on the functional and sequence homology with HSP60 chaperones, it has been speculated that Phb1p and Phb2p form a similar barrel-shaped structure [13]. However, experimental data about the structure of the complex are lacking, and interesting questions remain: How are the molecules arranged in the complex? For instance, there could be alternating Phb1p and Phb2p molecules or there could be a ring of Phb1p and a ring of Phb2 molecules that are stacked on top of each other. How are the components folded, and how are they orientated in the membrane? It should be noted that the membrane-spanning domains are only computer predictions, and there is no hard experimental data yet for their existence in vivo. Phosphorylation of Phb1p has been suggested in two publications [17, 25]. When considering the HSP60 homology [13], ATP binding might be required for binding and release of substrates. Answers to these questions about the structure of the complex are required to obtain more insight into the mechanism of action of the PHB complex.

A mitochondrial chaperone for the stabilisation of proteins

The mitochondrial localisation of Phb1p and Phb2p makes it likely that the cellular function of these proteins lies also in this organelle. Using yeast as a model organism, several groups have gathered evidence for a mitochondrial function of the PHB complex [7, 12, 13, 24]. It was shown that disruption mutants of *PHB1*, *PHB2* and double-disruption mutants have a decreased replicative lifespan, which was accompanied by a decrease of mitochondrial membrane potential [7]. More evidence for a mitochondrial function was provided by Berger and Yaffe, who demonstrated that mutants disrupted for the *PHB1* and *PHB1* genes display synthetic lethality in combination with mutations in genes involved in mitochondrial morphology maintenance (Mmm1p) or mitochondrial DNA maintenance (Mdm10p and Mdm12p) [24]. Also a fusion protein of *PHB2-TetA* was able to rescue *MDM12 null* cells [24]. Although these results are in concordance with a mitochondrial function of the PHB complex, they still do not reveal the function of the proteins.

Sequence similarity between Phb1p and Phb2p and the bacterial complex HflC and HflK acted as a starting point for investigations into the role of the PHB complex in mitochondrial enzyme processing [12]. In bacteria, HlfC and HflK form a complex which is anchored to the plasma membrane and oriented towards the periplasmic space. This complex is associated with and negatively regulates the AAA protease FtsH (see below) [1]. Similarly it was shown that the PHB complex was associated with the mitochondrial mAAA protease (Afg3p/Rca1p) in yeast [12]. Moreover, it appeared that the PHB complex stabilised newly translated mitochondrial gene products, prompting the authors to suggest that the PHB complex is a negative regulator of the mAAA protease [12].

The increased stability of mitochondrial translation products was confirmed using a different assay [13]. Additionally, it was demonstrated by native electrophoresis and immunoprecipitation that the stabilised mitochondrial translation products, Cox2p and Cox3p, were associated with the PHB complex. At this point the full spectrum of substrates of the PHB complex is not known, and it is quite possible that proteins other than subunits of the respiratory chain can also bind. The direct interaction with the substrates Cox2p and Cox3p suggests a chaperone function for the PHB complex, instead of acting as a protease inhibitor. A small but significant sequence homology with HSP60, a soluble mitochondrial chaperone, supports this idea [13]. This suggests that there might be some evolutionary conservation of some aspect of mechanism or function between these chaperones, the one soluble, the other membrane associated. In this context, it is interesting to mention Tcm62p. Like Phb1p and Phb2p, this homologue of the Hsp60 family of proteins is a mitochondrial membrane-bound chaperone, and Tcm62p has been shown to be involved in the assembly of complex II. Tcm62p associates directly with subunits of complex II during the assembly process [26], giving a precedence for the notion that the PHB complex is also involved in respiratory chain complex assembly directly at the inner membrane.

Beside the direct interaction of substrates with the PHB complex, there are also genetic arguments that support the fact that the stabilisation of newly synthesised mito-

chondrial gene products by the PHB complex [12, 13] is a result of a chaperone activity rather than an inhibition of the mAAA protease. When *PHB1* or *PHB2* are disrupted in a yeast deletion mutant of the mAAA protease, this results in a severe growth defect which is not present in either of the single mutants [12]. Because the deletion of an inhibitor should have no effect in the absence of the protease, this observation is more likely to be explained by a chaperone activity. Furthermore, the PHB complex is increased in mutants that give rise to imbalances of subunits, such as a disruption of a COX1-translation activator (Mss51p) [13] or a COX assembly chaperone (Shy1p) (see below). This increase of chaperone/holdase activity to secure proper folding is consistent with a need to sequester excess unassembled subunits with potentially damaging effects on the integrity of the inner membrane. Although it has been found that an association between the PHB complex and the mAAA complex occurs, this interaction can only be demonstrated by use of mild detergents [12]. The PHB complex and mAAA protease are in fact autonomous complexes, because disruption of the genes encoding subunits of one does not destabilise the other. Nevertheless, the parallel with the bacterial HflKC and the protease FtsH, which also associate, is evident. The interaction of the PHB complex with the mAAA protease therefore suggests a spatial organisation for the assembly of the respiratory chain complexes (fig. 3). This is in line with earlier findings that degradation of proteins by the mAAA protease and the iAAA protease are linked to assembly processes [12]. The assembly of OXPHOS complexes is a complicated process, which brings together proteins encoded by the mitochondrial and nuclear genomes. Also, because these complexes are assembled in the inner mitochondrial membrane, mistakes might affect membrane integrity and cause membrane leakage. It is very likely that there are coordinated chaperones and proteases involved to control this process.

The size, composition and localisation of the human PHB complex is similar to the yeast homologue [13]. This makes it likely that also in mammals the PHB complex serves a similar function. Studies on the expression levels of prohibitin are indicative for a function in mitochondrial metabolism [19]. Moreover, this study clearly demonstrated that Phb1p and Phb2p are induced by metabolic stress caused by imbalances of mitochondrial and nuclear encoded proteins. Thiamphenicol treatment gives a twofold increase, and withdrawal gives a further increase again up to three times the control level. In contrast, other stresses such as heat shock, hydrogen peroxide, ultraviolet (UV) and X-radiation did not give a response [19].

The superfamily of Stomatin, Phb1p and Phb2p, flotillin, HflKC and HIR proteins

Homology searching with Psi-BLAST revealed that Phb1p and Phb2p can be categorised into a superfamily of proteins. A first report named the superfamily after its members, namely stomatin, prohibitins (Phb1p and Phb2p), flotilllin and HflKC (SPFH) [14]. A second paper grouped Phb1p and Phb2p in a family designated as PID because of the putative function of the family members in proliferation, ion channel regulation and death [15].

The PID family contains several proteins; PHB proteins (proliferation and cell cycle control), stomatins (ion-channel regulation) and the plant-defence-related genes (cell death) [15]. In this study it is demonstrated that in maize there are several genes that show homology with PHB proteins and stomatins. The authors tried to find a com-

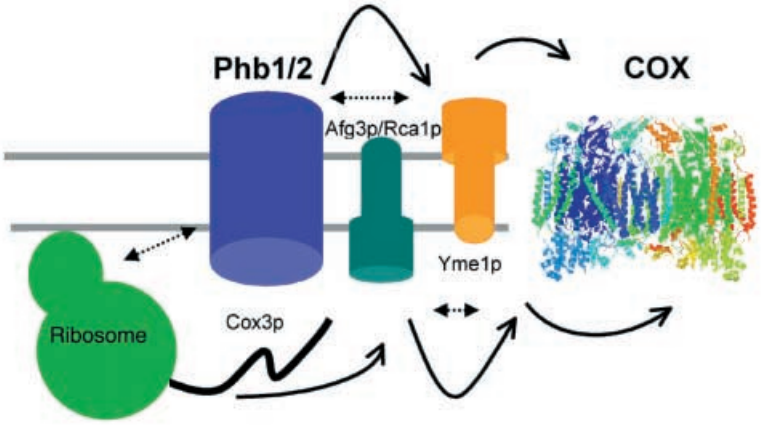

Figure 3. Schematic representation of the role of the PHB complex in the biogenesis of respiratory chain complexes. Mitochondrial translation takes place near the mitochondrial inner membrane. Translation products are use for respiratory chain complex assembly. When imbalances between mitochondrial and nuclear-encoded subunits occur, the mitochondrial translation products are bound to the PHB complex (Phb1/2), which acts as a holdase/unfoldase. In a next step the mitochondrial translation products are passed on to either the proteases (Afg3p/Rca1p or Yme1p) or to the complex, for instance cytochrome *c* oxidase (COX).

mon feature for these proteins and propose that they all play a role in ion-channel activity. We feel however that this common feature is too speculative to be accepted until direct experimental evidence has been obtained. Thus, although stomatins have been suggested to act as negative regulators of univalent cation permeability with a possible role in mechanotransduction, the function of stomatins is as yet unclear [15]. Second, the putative role of the PHB complex in regulation of mitochondrial membrane channels [3] is solely based on a proposal lacking experimental evidence. Finally, the exact role and mechanisms of action of HIR proteins in plant defence are still unknown. Moreover, despite the original proposal, recent data indicate that the PHB complex is not involved in cell proliferation and are not tumour suppressor proteins (see below). Therefore, the suggestion that HIR proteins might form a new class of plant tumour suppressor genes on the basis of homology with PHB proteins appears highly unlikely. We believe that the sequence similarity between the members of this superfamily points to an evolutionary related group of genes. Nevertheless, because no structure-function relationship of any of the members of this superfamily is known to date, the functional similarities of the family members remain speculative.

The description of the PID family of proteins is comparable to an earlier paper in which a Psi-BLAST search revealed sequence similarities between PHB proteins, stomatins, HflKC and flotillins, but did not include the plant defence genes [14]. The authors named the common domain of this group of genes SPFH domain (stomatin, PHB-proteins, flotillins and HflKC). Stomatin (also called erythrocyte protein band 7.2 or mammalian band 7 protein) is an integral membrane phosphoprotein of red blood cells thought to regulate cation conductance by interacting with other proteins of the junctional complex of the membrane skeleton. Stomatin is evolutionarily related to a large number of proteins. Structurally, all these proteins consist of a short N-terminal domain which is followed by a transmembrane region and a variable size (from 170 to 350 residues) C-terminal domain. The consensus for the stomatin family is: R-x(2)-{LIV}-{SAN} x(6)-{LIV}-D-x(2)-W-G-{LIV}-{KRH}-{LIV} x-{KR}-{LIV}-E-{LIV}-{KR} [15]. This consensus is not present in Phb1p and Phb2p. Flotillins are caveolae-associated integral membrane proteins with no known function [27]. The last family member is the *Escherichia coli* HflKC protein [1]. This protein has a remarkable conservation in function with the PHB complex which is discussed in more detail below. Besides the common SPFH domain, all family members are integral membrane proteins. The proposed common function of the SPFH family of proteins has been suggested to be regulation of targeted protein turnover. However, this proposal still requires experimental validation.

The PHB complex has functional similarities with HflKC

The bacterial HflKC proteins are related to PHB proteins, with HflKC and yPHB1 showing 20% similarity and 30% homology [12]. The bacterial HflKC family of proteins were found to play a role in the lysis decision after infection of bacteriophage λ. The CII gene of bacteriophage λ plays a role in the lysogenity decision and mutations in HflA resulted in a high frequence of lysogenisation (high frequency of lysogenisation) [28]. HflA appeared to be an operon of three genes, HflK, HflC and HflX [29]. Like *PBH1* and *PHB2*, HflK and HflC encode membrane proteins which form a complex with each other. An N-terminally located transmembrane segment anchors both proteins to the membrane and leaves the C-terminal domain exposed to the periplasmic space [30]. This situation is very comparable to the proposed PHB-complex topology in the mitochondrial inner membrane [12]. Analogous to Phb1p and Phb2p, HflK and HflC are also interdependent, because disrupting either one of the proteins causes the other one to become unstable [1].

Another parallel is that the PHB complex and HflKC protect membrane proteins against degradation and are associated with a membrane protease of the AAA family of proteases [1, 12]. HflKC was rediscovered because missense mutations in this gene antagonised degradation of the membrane protein SecY by the AAA protease FtsH [31]. However, when the HflKC genes were disrupted, this stabilisation of SecY disappeared, and instead there was an increased degradation [1]. It was shown both in vivo and in vitro that degradation of uncomplexed forms of SecY was inhibited when HflKC was present. Additionally the authors showed by coimmunoprecipitation and chemical cross-linking that HflKC was associated with FtsH. This led to the conclusion that HflKC is a negative regulator of FtsH. Because overexpressing FtsH suppressed the *hflK13* or *hflC9* mutation effect, the stabilising effect of the mutant genes was explained by suggesting that because of the mutation (*hflK13* and *hflC9*), HflKC does not dissociate from the FtsH complex; and therefore activation of the proteolytic activity of FtsH is not possible. Also, overexpression of the wild-type molecules of HflKC led to suppression of the mutant phenotype, and this was explained in terms of dilution of the mutant proteins [1].

HflKC and the PHB complex are both able to bind proteins which are substrates for the proteases [13, 32]. When YccA, a substrate of the FtsH protease, was mutated, it appeared to be resistant to proteolysis [32]. This mutant, which was named *yccA11*, also had a dominant-negative effect on SecY degradation by the FtsH protease because SecY was less prone to proteolysis. Cross-linking experiments and immunoprecipitations demonstrated

that the substrates could bind to both HflKC as well as to the FtsH protease. This led to the suggestion that because the mutant *yccA11* occupies the protease, SecY cannot enter the protease and is stabilised. Interestingly, this inhibitory action of *YccA11* was mediated by HflKC, since the deletion of *hflK-hflC* suppressed the *yccA11* phenotype. This modulation of FtsH-dependent protein degradation also occurred for the hydrophobic substrate subunit a of the ATP synthase and YccA, but not for the hydrophilic substrates CII and σ32. This led to the idea that there are two pathways for FtsH-dependent degradation, of which only one is regulated by the HflKC complex [32]. The experimental system in which hydrophobic membrane proteins are overexpressed put extra demand on the proteolytic system; thus coupling the mechanism of action of HflKC to the proteolytic system. In more physiological circumstances the role of the HflKC is possibly not linked so strongly to FtsH activity, and it is therefore quite possible that HflKC has a chaperone function similar to that proposed for the PHB complex.

PHB proteins and cancer

Shortly after cloning of the rat *PHB1* gene and the suggestion that Phb1p acts as a negative regulator of the cell cycle [8], the human *PHB1* homologue was cloned and shown to lie on chromosome 17q21, a region which was known to contain the then unidentified BRCA1 tumour suppressor gene involved in familial breast cancer [6, 21]. An early study found mutations in the human gene in a significant percentage of patients with sporadic breast cancer, suggesting that the BRCA1 locus might be the *PHB1* gene [6]. However, no evidence indicating loss of function of *PHB1* in those tumours has been presented, either then or subsequently. Moreover, genetic and cytogenetic studies quickly indicated that the two loci were separate, and that *PHB1* was not the gene involved in susceptibility to inherited breast cancer [33]. Indeed, further studies of familial breast cancer patients failed to confirm alterations in the *PHB1* gene [34, 35], and no mutations were found in bladder, ovarian, lung or liver tumours [35–38].

Consequently, the original hypothesis that Phb1p might be a tumour suppressor protein that acts to inhibit cell cycle progression has not been substantiated. Despite this, observations into the possible role of Phb1p as a tumour suppressor have continued. Interestingly, studies of various cell lines have indicated that the ability of microinjected *PHB1* mRNA to inhibit cell cycle activity is restricted to normal cells and cell lines of the so-called group B immortal complementation group [39–41]. This activity lies not in the protein-coding region of the mRNA (and is therefore not a function of the protein) but rather is a property of the 3′ untranslated region (UTR) of the mRNA [40, 41]. The mechanism by which the 3′ UTR of *PHB1* mRNA inhibits cell cycle entry is unexplained, although by analogy with other apparent examples of 3′ UTR-mediated proliferative effects [42], it is possible that *PHB1* mRNA acts as a 'riboregulator' [40, 41]. It is also interesting to note that the sequence of the *PHB1* 3′ UTR is distinct between group B and the other immortal complementation groups, suggesting that the inhibitory effect of the *PHB1* 3′ UTR may have been lost during the immortalization process of these cells [40, 41]. Most recently, a specific polymorphism in the *PHB1* 3′ UTR has been suggested to increase the risk of familial breast cancer [43]. However, no data have yet been produced to elucidate the mechanism by which such effects could be achieved, nor has it been shown that introduction of a 'wild-type' *PHB1* gene expressed at physiological levels can inhibit the growth of group B cells or reverse their immortalised phenotype.

More convincing data indicating a role for the PHB proteins in tumourigenesis have come from studies of the expression patterns of the proteins. A number of different studies have indicated that *PHB1* mRNA and/or protein are expressed at easily detectable levels in tumour cell lines [4, 36, 39–41, 44, 45], and high-level expression of *PHB2* has also been reported in transformed cell lines [4]. Many of these studies have also shown clearly that the levels of the proteins are in fact higher in transformed cells and tumours than in the normal cell counterpart, rather than expression being diminished in tumours as would be expected for a tumour suppressor protein. Most recently, an immunohistochemical study of primary human tumours showed a consistent overexpression of both Phb1p and Phb2p in neoplastic tissues from a wide range of anatomical sites [19].

The reason underlying the observed increase in expression of the PHB proteins is likely to be due at least in part to the presence of conserved binding sites for the myc oncoprotein in the promoters of the two genes [19]. Myc is highly expressed in tumours and is known to be involved in both cell cycle regulation and in cellular carbohydrate metabolism [46]. Tumours show abnormal metabolism, with a reliance on anaerobic glycolysis even in the presence of oxygen, the so-called Warburg effect, which may act to decrease the potential damaging effects of reactive oxygen species produced as an inevitable consequence of oxidative phosphorylation [46]. Myc expression induces the transcription of a variety of glycolytic enzymes and transporter molecules involved in glucose metabolism [47], and the induced expression of LDH-A by myc is sufficient to account for the Warburg effect [48]. Consequently, it is likely that transcriptional activation of the *PHB* genes by c-Myc in tumours is a necessary part of the coordinated response acting to reduce oxidative stress to the tumour cells and allow their continued growth even under situations of hypoxia which are common in vivo [46].

IgM binding receptor binding, E2F binding, MLK2, oestrogen-receptor binding?

Terashmina and colleagues [4] found three proteins of 32, 37 and 41 kDa that coprecipitate with surface IgM in murine B lymphocytes (IgM-BCR, for B-cell-receptor complex). They called these proteins BAP32 and BAP37 for BCR-associated proteins. Amino acid sequencing revealed that BAP32 and BAP37 were mPhb1p and mPhb2p respectively. They concluded that these proteins were noncovalently associated with the IgM antigen receptor complex in B lymphocytes. This association did not occur for a second receptor, IgD, which is expressed later in development in mature B cells. However, the mitochondrial localisation of Phb1p and Phb2p [7, 19, 23] indicates that the association observed with the IgM-BCR complex might be the result of an artefact of the system used, rather than indicating a true functional interaction. The coimmunoprecipitation experiment was done after lysis of the cells and was only successful with Triton X-100 and Nonidet P-40 but not with digitonin. This result is counterintuitive because digitonin is a relatively milder detergent compared to Nonidet P-40 and Triton X-100, and it is therefore expected to preserve noncovalent interactions at least as well as the harsher detergents. For instance, Steglich and coworkers showed that association of the yeast PHB complex with a mitochondrial protease could only occur when digitonin was used as a detergent and not when Triton X-100 was used [12]. A more plausible explanation for the results of Terashima et al. [4] would be that the Triton X-100 treatment, in contrast to the digitonin treatment, releases the PHB complex from the mitochondria and possibly also dissociates the complex, therefore allowing the proteins to bind to the solubilised IgM-BCR complex.

More recently, Wang and colleagues [10] postulated Phb1p as a negative regulator of the transcription factor E2F. The authors described a physical interaction of Phb1p with the E2F-transcription factor and with the retinoblastoma (Rb) tumour suppressor protein. E2F is a cell-cycle-regulated transcription factor that binds to both promoter DNA and the Rb protein. Rb inhibits E2F-mediated transcription. When Rb becomes hyperphosphorylated by activated CDKs, it dissociates from E2F, allowing E2F to activate transcription. A mutant Phb1p unable to bind to Rb failed to repress E2F activity and inhibit cell proliferation. The same authors found that Phb1p also associates with the signalling kinase c-Raf-1, which also interacts with Rb and reverses both Phb1p- and Rb-mediated repression of E2F activity. However, other upstream regulators of Rb function had no effect on Phb1p function. Serum stimulation of quiescent cells inactivated Rb and Phb1p with different kinetics and by different signaling pathways; whereas Rb inactivation was CDK dependent, the inactivation of Phb1p was not. Further, stimulation of IgM could specifically release Phb1p-mediated repression of E2F activity but had no effect on Rb-mediated repression. It has been reported that stimulation of IgM leads to an activation of Raf-1. The authors postulate that this inactivation might occur through the recruitment of Raf-1, although increased binding of Raf-1 to Phb1p was not detected. From that they conclude that Phb1p negatively regulates E2F activity, responding to extracellular signals that cannot target Rb-family members. All these data, however, are still far from providing insight on the true function of the PHB proteins, since all assays were performed in artificial situations where proteins are cotransfected and overexpressed.

An interaction of Phb1p with MLK2 (mixed lineage kinase 2) has also been described in the literature [49]. MLK2 is a mitogen-activated protein kinase like Raf-1 that functions in the p38/JNK-signalling pathway. In breast carcinoma cells, MLK2 was found to bind to Phb1p and to β-tubulin, a cytoskeletal protein. No subsequent data regarding the effect of MLK2 on Phb1p has yet been reported, so the functional significance of this proposed interaction is unknown.

A gene denoted as REA has been identified in a yeast two-hybrid screen as an oestrogen-receptor-binding protein, which represses estrogen-receptor-mediated transcriptional activation [9]. Sequencing of the REA gene identified it as Phb2p, suggesting that this protein is involved in steroid receptor function.

The significance of all these findings is hampered by the confirmed localisation of Phb1p to the mitochondrial inner membrane, where it forms a functional complex together with Phb2p [7, 12, 13, 19, 23, 24]. We believe that many of these proposed interactions with nonmitochondrial proteins are due to nonspecific association in the artificial systems employed (yeast two-hybrid, immunoprecipitation of harsh detergent lysates, nonphysiological overexpression of a single component of the PHB complex), and such artifacts show that, as monomers, Phb1p and Phb2p are 'sticky' proteins, able to bind a range of other proteins. Indeed, the ability to bind polypeptides is a necessary characteristic of chaperones. Furthermore, in a two-hybrid screen Phb1p could be found to bind to histone H2, Rb-binding protein1 and three small peptides produced by out-of-frame translation, indicating a relatively broad spectrum of binding activities in this artificial system [our unpublished data]. Presumably, the specificity of binding required for a chaperone/holdase function is achieved at least in part through the formation of the PHB complex containing both of the proteins. It is also highly likely that assembly of the PHB complex in a hydrophobic membrane environment will provide additional structural constraints on the proteins and contribute to the relatively narrow range of in vivo binding partners.

PHB proteins and the ageing phenotype

The potential for an involvement of Phb1p in the process of cellular ageing was recognised shortly after its original identification as a putative negative regulator of the cell cycle [16]. Despite more recent observations showing that Phb1p is unlikely to have a direct role in cell cycle control, the PHB complex has subsequently been implicated in the ageing process through a variety of different studies. Initially, studies of *PHB1* in human fibroblasts aged in vitro by continuous passage showed a marked decrease in mRNA levels in senescent cells compared with young subconfluent cells. Protein levels were also slightly lower in these senescent cells compared with the young subconfluent cells, although there was no direct correlation between mRNA and protein levels [16]. Further analysis of the expression of *hPHB1* mRNA and protein levels during cellular ageing indicated that neither mRNA nor protein levels change appreciably with in vitro age, but two-dimensional (2-D) electrophoresis indicated that young cells have an acidic form of Phb1p that is not present in the aged cells [17]. Other studies using hormone-treated ovarian cells suggested that the more acidic species represents a phosphorylated form of Phb1p [25]. Consequently, it appears that the ability to post-translationally phosphorylate Phb1p is lost during the process of cellular ageing, although the functional significance of this is not known. A more detailed immunofluorescence study of the expression of both PHB proteins during cellular ageing indicated that a subpopulation of senescent cells contain lower levels of Phb1p and Phb2p [19]. This heterogeneous decrease in protein levels was correlated with the heterogeneous decline in mitochondrial membrane potential seen during in vitro ageing of mammalian cells [19, 50]. It was also shown that chick embryonic fibroblasts show a similar heterogeneous decline in levels of both Phb1p and Phb2p during in vitro ageing, indicating that loss of the proteins is a conserved feature [19]. Further circumstantial evidence that PHB proteins are important for cellular ageing comes from observations of their expression in immortalised cells; all tumour cell lines express high levels of the PHB proteins, and primary human tumours from a variety of different anatomical sites also express high levels of the two proteins [19], suggesting that expression of *PHB1* and *PHB2* is associated with extended lifespan.

These observations in higher species show a correlation between expression of PHB genes and ageing, but do not indicate that these proteins influence ageing itself. Convincing data indicating a role for the PHB complex in the ageing process has come from the analysis of budding yeast. Although *Saccharomyces cerevisae* can grow continually in culture, the lifespan of individual cells is limited to a more or less defined number of population doublings. This replicative lifespan is measured by counting the number of times that individual 'mother' cells divide, and the daughter cells are easily recognised in budding yeast by their smaller size. It was found that deletion of either or both of the *PHB* genes shortened the replicative lifespan by about one-third [7, 24]. That this reduction in lifespan was caused by an acceleration of the ageing process is evident from analysis of the phenotypic characteristics of the cells. Old yeast cells show altered surface morphology and prolonged cell division times, and cells deleted for *PHB1* and *PHB2* showed a roughened cell surface and prolonged cell cycle times after fewer divisions than the wild-type counterparts, indicating clearly that the normal ageing process has been speeded up by deleting the PHB complex [7].

Thus, the levels of the PHB proteins are decreased during natural cellular ageing in cells from higher species, high-level expression may be required for lengthening of lifespan in tumour cells, and deletion of *PHB1* and *PHB2* accelerates the ageing process. The mechanism by which expression of the PHB genes influences ageing has not been fully elucidated at the present time. However, given the clear evidence linking the proteins to the regulation of mitochondrial function in both yeast and mammals, it must be postulated that they influence longevity through affects on mitochondrial metabolism. A key feature of the ageing process is the progressive deterioration in functional activity of cells and tissues. The free-radical theory of ageing states that ageing is essentially caused by the accumulation of cellular damage as a consequence of the production of reactive oxygen species [51]. These generally short-lived but harmful molecules are produced in large quantities during the process of aerobic respiration in mitochondria, and so mitochondria have been intensely investigated for their possible roles in the ageing process. It is clear that metabolic efficiency is a key determinant of lifespan in a wide variety of species from yeast to humans [20, 52]. There is also overwhelming evidence to show that ageing of higher species is associated with increasing levels of damage to mitochondrial DNA (mtDNA), at least in certain tissues [53, 54]. The damage can take the form of typical age-associated deletions, which often represent quite large parts of the mitochondrial genome, although other smaller deletions and mutations can also be observed. Such damage to mitochondrial DNA appears to be heterogeneous in ageing tissues [55]. Whether this damage to individual mtDNA genomes causes the cell to lose mitochondrial function has been a controversial area, although it is known that there are heterogeneous losses of mitochondrial enzyme activities in muscle and liver tissues in aged animals, whilst liver cells removed from old animals show a heterogeneous loss of mitochondrial membrane potential [56–58].

Considering the possible role of the PHB complex in ageing, it can therefore be hypothesised that a loss of function of the PHB complex would increase the rate of for-

mation of improperly processed respiratory enzyme complexes. This in turn would be expected to increase oxidative stress due to leakage of electrons as they are transferred along the respiratory chain. Such increased production of reactive oxygen species would be able to account fully for the accelerated rate of ageing seen in *PHB null* cells. Direct evidence to support such a proposal for the role of the PHB complexes in ageing has come from knockout yeast, which show a slight increase in content of reactive oxygen species [13]. In higher species, in vitro ageing is associated with increased oxidative stress [59], correlating with the observed loss of PHB function [19].

Consequently, the PHB complex, by playing a role in the assembly of complexes required for mitochondrial respiration, can be considered as protecting cells from the harmful effects of this essential process. Since the proteins appear to be lost in old cells, which show increased oxidative stress and mitochondrial dysfunction, restimulating the expression and/or activity of the PHB complex would be expected to delay or reverse the ageing process, and would represent an alternative to other approaches, such as the administration of antioxidant chemicals, enhanced expression of antioxidant enzymes, or caloric restriction, all of which have been shown to be effective in delaying ageing [20, 60–62].

The PHB complex and degenerative disorders

The currently available data strongly suggest a role for the PHB complex as a membrane-bound mitochondrial chaperone involved in the stability of newly mitochondrially encoded subunits of the respiratory chain. It is therefore likely that mutations in either of the *PHB* genes would result in a mitochondrial disorder phenotype. Mitochondrial disorders give a broad spectrum of diseases. The most common types of disorders that have been associated with mitochondrial dysfunction are myopathies and neuropathies. However, other disorders such as diabetes, hearing loss and optic neuropathy can also be caused by mitochondrial dysfunction [63, 64]. Additionally, recent data demonstrate that mutations in mtDNA can result in kidney failure [65].

The PHB-complex is implied to be a chaperone, which is capable of holding newly synthesised mitochondrial translation products in order to be assembled into respiratory chain complexes. Its role becomes even more important when imbalances of mitochondrially encoded subunits and nuclearly encoded subunits occur. Therefore, it may also be true that dysfunction of the PHB complex gives a phenotype only when found in combination with other disturbances in mitochondrial function. For instance, when mutations in the mtDNA occur, the PHB complex might be required to overcome problems arising from these mutations. An illustration for this is that HepG2 cells showed an increase of *PHB1* expression when treated with thiamphenicol, an inhibitor of mitochondrial respiration. In other words, mutations in one of the PHB proteins might amplify other mitochondrial defects [19]. In favour of this argument are the data showing that deletion of either *PHB* gene in combination with deletion mutants in the mitocondrial mAAA protease (see above) give a lethal phenotype for growth on glucose [12]. Also, in combination with *MDM10* and *MDM12*, deletion of *PHB1* or *PHB2* gives a lethal pheneotype [24]. In all these cases single deletions give no growth phenotype on glucose. Interestingly, in a disruption strain of *SHY1*, the yeast homologue of *SURF1* which is associated with Leigh syndrome, the amount of PHB complex is increased [66]. This suggests that increased *PHB* expression might be a good marker for mitochondrial dysfunction, especially in cases where this involves imbalances of respiratory chain subunits. We would therefore suggest that these proteins should be included in microarrays designed to detect mitochondrial disorders or to investigate mitochondrial dysfunction.

The PHB complex binds to mitochondrial AAA-metalloproteases [12] and plays a role in the stabilisation of respiratory chain subunits [13]. Interestingly, disease-causing mutations have recently been found in a gene which has strong homology with the yeast mitochondrial AAA proteases. These mutations caused hereditary spastic paraplegia, and the gene was called paraplegin [67]. The paraplegia patients showed ragged red fibres, a hallmark of mitochondrial dysfunction. However, muscle tissue is not the primarily affected tissue, and it is not clear how the mutations relate to the clinical phenotype. Three AAA-metalloproteases bound to the mitochondrial inner membrane have been described in yeast YME1, RCA1 and AFG3, and metazoan homologues have been described [68]. Although the molecular mechanism of this disease is still not known, these findings highlight the importance of proper handling of proteins of the mitochondrial inner membrane to maintain mitochondrial integrity. Defects in these processes, regulated by AAA proteases, the PHB complex and other chaperones might cause membrane leakage, reactive oxygen species production and possibly influence apoptosis.

Concluding remarks and prospects

In summary, there have been a number of recent publications that provide insight into the function of the PHB complex. Using yeast as a model system, evidence has been provided that the PHB complexes are chaperones that play a role in assembly of respiratory chain complexes and possibly ATP synthase. This knowledge is of great help in the interpretation of the role of PHB proteins

in the diverse cellular activities in which they are thought to play a role, such as in tumorigenesis and ageing. Despite this progress, understanding of the action of this protein complex is still poor, and many questions remain to be resolved.

On the structural level it has to be investigated whether Phb1p and Phb2p really form a barrel and how it sits in the mitochondrial inner membrane. Another interesting question is, What are the natural substrates of this complex? Are hydrophobic mitochondrially encoded proteins the only substrates of the complex, or are there also more hydrophilic and nuclear-encoded protein substrates that are processed by the complex? The finding that the PHB complex forms a mitochondrial chaperone with similarities to the GroEL chaperone system [13] also raises issues concerning the mechanistic function of the proteins: How does the substrate binding and release cycle in the PHB complex? Are ATP binding and dephosphorylation involved in this process to induce conformational changes allowing the proteins to bind and release?

Recent data on the physiological function of the PHB complex also leave questions to be resolved, particularly in terms of the relationships to tumour growth, ageing and degenerative diseases. Perhaps most fundamentally, in order to understand the PHB complex we need to know the precise role in the assembly process and how the PHB complex relates to the protease. Is the involvement really a question of quality control? If we compare the process of respiratory chain enzyme assembly with the process of traffic control, then a holdase/foldase function could be regarded as the regulation of cars merging from two lanes of traffic into a single lane. A role for the PHB complex in quality control, in contrast, would be comparable to the job of a recovery pickup truck that removes vehicles that have broken down due to overuse or even improper assembly at the factory, and are now blocking the efficient flow of traffic. It is clear that there is much to be understood about the role of the PHB complex, but now that a framework has been put in place for their functions, it has become possible to address these issues in a meaningful way.

1 Kihara A., Akiyama Y. and Ito K. (1996) 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:** 6122–6131

2 Snedden W. A. and Fromm H. (1997) Characterization of the plant homologue of prohibitin, a gene associated with antiproliferative activity in mammalian cells. Plant. Mol. Biol. **33:** 753–756

3 McClung J. K., Jupe E. R., Liu X. T. and Dell'Orco R. T. (1995) Prohibitin: potential role in senescence, development and tumor suppression. Exp. Gerontol. **30:** 99–124

4 Terashima M., Kim K. M., Adachi T., Nielsen P. J., Reth M., Kohler G. C. et al. (1994) The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. EMBO J. **13:** 3782–3792

5 Eveleth D. D. Jr and Marsh J. L. (1986) Sequence and expression of the Cc gene, a member of the dopa decarboxylase gene cluster of *Drosophila*: possible translational regulation. Nucleic Acids Res. **14:** 6169–6183

6 Sato T., Saito H., Swensen J., Olifant A., Wood C., Danner D. et al. (1992) The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer. Cancer Res. **52:** 1643–1646

7 Coates P. J., Jamieson D. J., Smart K., Prescott A. R. and Hall P. A. (1997) The prohibitin family of mitochondrial proteins regulate replicative lifespan. Curr. Biol. **7:** 607–610

8 Nuell M. J., Stewart D. A., Walker L., Friedman V., Wood C. M., Owens G. A. et al. (1991) Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. Mol. Cell Biol. **11:** 1372–1381

9 Montano M. M., Ekena K., Delage-Mourroux R., Chang W., Martini P. and Katzenellenbogen B. S. (1999) An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. Proc. Natl. Acad. Sci. USA **96:** 6947–6952

10 Wang S., Nath N., Adlam M. and Chellappan S. (1999) Prohibitin, a potential tumor suppressor, interacts with RB and regulates E2F function. Oncogene **18:** 3501–3510

11 Welburn S. C. and Murphy N. B. (1998) Prohibitin and RACK homologues are up-regulated in trypanosomes induced to undergo apoptosis and in naturally occurring terminally differentiated forms. Cell Death Differ. **5:** 615–622

12 Steglich G., Neupert W. and Langer T. (1999) Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. Mol. Cell Biol. **19:** 3435–3442

13 Nijtmans L. G., de Jong L., Artal Sanz M., Coates P. J., Berden J. A., Back J. W. et al. (2000) Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. EMBO J. **19:** 2444–2451

14 Tavernarakis N., Driscoll M. and Kyrpides N. C. (1999) The SPFH domain: implicated in regulating targeted protein turnover in stomatins and other membrane-associated proteins. Trends Biochem. Sci. **24:** 425–427

15 Nadimpalli R., Yalpani N., Johal G. S. and Simmons C. R. (2000) Prohibitins, stomatins and plant disease response genes compose a protein superfamily that controls cell proliferation, ion channel regulation and death [In Process Citation]. J. Biol. Chem. **275:** 29579–29586

16 McClung J. K., King R. L., Walker L. S., Danner D. B., Nuell M. J., Stewart C. A. et al. (1992) Expression of prohibitin, an antiproliferative protein. Exp. Gerontol. **27:** 413–417

17 Liu X. T., Stewart C. A., King R. L., Danner D. A., Dell'Orco R. T. and McClung J. K. (1994) Prohibitin expression during cellular senescence of human diploid fibroblasts. Biochem. Biophys. Res. Commun. **201:** 409–414

18 Dell'Orco R. T., McClung J. K., Jupe E. R. and Liu X. T. (1996) Prohibitin and the senescent phenotype. Exp. Gerontol. **31:** 245–252

19 Coates P. J., Nenutil R., McGregor A., Picksley S. M., Crouch D. H., Hall P. A. et al. (2001) Mammalian prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. Exp. Cell Res. **265:** 262–273

20 Sohal R. S. and Weindruch R. (1996) Oxidative stress, caloric restriction and aging. Science **273:** 59–63

21 White J. J., Ledbetter D. H., Eddy R. L. Jr, Shows T. B., Stewart D. A., Nuell M. J. et al. (1991) Assignment of the human prohibitin gene (PHB) to chromosome 17 and identification of a DNA polymorphism [published erratum appears in Genomics 1991 Nov; 11(3):782]. Genomics **11:** 228–230

22 Ansari-Lari M. A., Shen Y., Muzny D. M., Lee W. and Gibbs R. A. (1997) Large-scale sequencing in human chromosome 12p13: experimental and computational gene structure determination. Genome Res. **7:** 268–80

23 Ikonen E., Fiedler K., Parton R. G. and Simons K. (1995) Prohibitin, an antiproliferative protein, is localized to mitochondria. FEBS Lett. **358:** 273–277
24 Berger K. H. and Yaffe M. P. (1998) Prohibitin family members interact genetically with mitochondrial inheritance components in *Saccharomyces cerevisiae*. Mol. Cell Biol. **18:** 4043–4052
25 Thompson W. E., Sanbuissho A., Lee G. Y. and Anderson E. (1997) Steroidogenic acute regulatory (StAR) protein (p25) and prohibitin (p28) from cultured rat ovarian granulosa cells. J. Reprod. Fertil. **109:** 337–348
26 Dibrov E., Fu S. and Lemire B. D. (1998) The *Saccharomyces cerevisiae* TCM62 gene encodes a chaperone necessary for the assembly of the mitochondrial succinate dehydrogenase (complex II). J. Biol. Chem. **273:** 32042–32048
27 Volonte D., Galbiati F., Li S., Nishiyama K., Okamoto T. and Lisanti M. P. (1999) Flotillins/cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with caveolins in vivo. Characterization and epitope-mapping of a novel flotillin-1 monoclonal antibody probe. J. Biol. Chem. **274:** 12702–12709
28 Hoyt M. A., Knight D. M., Das A., Miller H. I. and Echols H. (1982) Control of phage lambda development by stability and synthesis of cII protein: role of the viral cIII and host hflA, himA and himD genes. Cell **31:** 565–573
29 Noble J. A., Innis M. A., Koonin E. V., Rudd K. E., Banuett F. and Herskowitz I. (1993) The *Escherichia coli* hflA locus encodes a putative GTP-binding protein and two membrane proteins, one of which contains a protease-like domain. Proc. Natl. Acad. Sci. USA **90:** 10866–10870
30 Kihara A. and Ito K. (1998) Translocation, folding, and stability of the HflKC complex with signal anchor topogenic sequences. J. Biol. Chem. **273:** 29770–29775
31 Kihara A., Akiyama Y. and Ito K. (1995) FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. Proc. Natl. Acad. Sci. USA **92:** 4532–4536
32 Kihara A., Akiyama Y. and Ito K. (1998) Different pathways for protein degradation by the FtsH/HflKC membrane-embedded protease complex: an implication from the interference by a mutant form of a new substrate protein, YccA. J. Mol. Biol. **279:** 175–188
33 Black D. M. and Solomon E. (1993) The search for the familial breast/ovarian cancer gene. Trends Genet. **9:** 22–26
34 Tokino T., Davidson N., Helzlsouer K., Zehnbouer B., Nakamura Y., Vogelstein B. et al. (1993) Absence of gremline prohibitin mutations in early onset breast cancer. Int. J. Oncol. **3:** 769–772
35 Sato T., Sakamoto T., Takita K., Saito H., Okui K. and Nakamura Y. (1993) The human prohibitin (PHB) gene family and its somatic mutations in human tumors. Genomics **17:** 762–764
36 Asamoto M. and Cohen S. M. (1994) Prohibitin gene is overexpressed but not mutated in rat bladder carcinomas and cell lines. Cancer Lett. **83:** 201–207
37 Cliby W., Sarkar G., Ritland S. R., Hartmann L., Podratz K. C. and Jenkins R. B. (1993) Absence of prohibitin gene mutations in human epithelial ovarian tumors. Gynecol. Oncol. **50:** 34–37
38 Tanno S., Fukuda I., Saito Y. and Ogawa K. (1997) Prohibitin expression is decreased in the regenerating liver but not in chemically induced hepatic tumors in rats. Jpn. J. Cancer Res. **88:** 1155–1164
39 Jupe E. R., Liu X. T., Kiehlbauch J. L., McClung J. K. and Dell'Orco R. T. (1995) Prohibitin antiproliferative activity and lack of heterozygosity in immortalized cell lines. Exp. Cell Res. **218:** 577–580
40 Jupe E. R., Liu X. T., Kiehlbauch J. L., McClung J. K. and Dell'Orco R. T. (1996) Prohibitin in breast cancer cell lines: loss of antiproliferative activity is linked to 3′ untranslated region mutations. Cell Growth Differ. **7:** 871–878
41 Jupe E. R., Liu X. T., Kiehlbauch J. L., McClung J. K. and Dell'Orco R. T. (1996) The 3′ untranslated region of prohibitin and cellular immortalization. Exp. Cell Res. **224:** 128–135
42 Rastinejad F. and Blau H. M. (1993) Genetic complementation reveals a novel regulatory role for 3′ untranslated regions in growth and differentiation. Cell **72:** 903–917
43 Jupe E. R., Badgett A. A., Neas B. R., Craft M. A., Mitchell D. S., Resta R. et al. (2001) Single nucleotide polymorphism in prohibitin 39 untranslated region and breast-cancer susceptibility. Lancet **357:** 1588–1589
44 Williams K., Chubb C., Huberman E. and Giometti C. S. (1998) Analysis of differential protein expression in normal and neoplastic human breast epithelial cell lines. Electrophoresis **19:** 333–343
45 Byrjalsen I., Mose Larsen P., Fey S. J., Nilas L., Larsen M. R. and Christiansen C. (1999) Two-dimensional gel analysis of human endometrial proteins: characterization of proteins with increased expression in hyperplasia and adenocarcinoma. Mol. Hum. Reprod. **5:** 748–756
46 Dang C. V. and Semenza G. L. (1999) Oncogenic alterations of metabolism. Trends Biochem. Sci. **24:** 68–72
47 Osthus R. C., Shim H., Kim S., Li Q., Reddy R., Mukherjee M. et al. (2000) Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J. Biol. Chem. **275:** 21797–21800
48 Shim H., Lewis B. C., Dolde C., Li Q., Wu C. S., Chun Y. S. et al. (1997) Myc target genes in neoplastic transformation. Curr. Top. Microbiol. Immunol. **224:** 181–190
49 Rasmussen R. K., Ji H., Eddes J. S., Moritz R. L., Reid G. E., Simpson R. J. et al. (1998) Two-dimensional electrophoretic analysis of mixed lineage kinase 2 N-terminal domain binding proteins. Electrophoresis **19:** 809–817
50 Martinez A. O., Over D., Armstrong L. S., Manzano L., Taylor R. and Chambers J. (1991) Separation of two subpopulations of old human fibroblasts by mitochondria (rhodamine 123) fluorescence. Growth Dev. Aging **55:** 185–191
51 Beckman K. B. and Ames B. N. (1998) The free radical theory of aging matures. Physiol. Rev. **78:** 547–581
52 Martin G. M., Austad S. N. and Johnson T. E. (1996) Genetic analysis of ageing: role of oxidative damage and environmental stresses. Nat. Genet. **13:** 25–34
53 Ozawa T. (1997) Genetic and functional changes in mitochondria associated with aging. Physiol. Rev. **77:** 425–464
54 Nagley P. and Wei Y. H. (1998) Ageing and mammalian mitochondrial genetics. Trends Genet. **14:** 513–517
55 Khrapko K., Bodyak N., Thilly W. G., Orsouw N. J. van, Zhang X., Coller H. A. et al. (1999) Cell-by-cell scanning of whole mitochondrial genomes in aged human heart reveals a significant fraction of myocytes with clonally expanded deletions. Nucleic Acids Res. **27:** 2434–2441
56 Muller-Hocker J., Schneiderbanger K., Stefani F. H. and Kadenbach B. (1992) Progressive loss of cytochrome c oxidase in the human extraocular muscles in ageing – a cytochemical-immunohistochemical study. Mutat. Res. **275:** 115–124
57 Muller-Hocker J., Aust D., Rohrbach H., Napiwotzky J., Reith A., Link T. A. et al. (1997) Defects of the respiratory chain in the normal human liver and in cirrhosis during aging. Hepatology **26:** 709–719
58 Hagen T. M., Yowe D. L., Bartholomew J. C., Wehr C. M., Do K. L., Park J. Y. et al. (1997) Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. Proc. Natl. Acad. Sci. USA **94:** 3064–3069
59 Chen Q., Fischer A., Reagan J. D., Yan L. J. and Ames B. N. (1995) Oxidative DNA damage and senescence of human diploid fibroblast cells. Proc. Natl. Acad. Sci. USA **92:** 4337–4341

60 Atamna H., Paler-Martinez A. and Ames B. N. (2000) N-t-butyl hydroxylamine, a hydrolysis product of alpha-phenyl-N-t-butyl nitrone, is more potent in delaying senescence in human lung fibroblasts. J. Biol. Chem. **275:** 6741–6748
61 Orr W. C. and Sohal R. S. (1994) Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. Science **263:** 1128–1130
62 Hagen T. M., Ingersoll R. T., Lykkesfeldt J., Liu J., Wehr C. M., Vinarsky V. et al. (1999) (R)-alpha-lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increased metabolic rate. FASEB J. **13:** 411–418
63 Lopez de Munain A. (1998) [Classification of mitochondrial diseases]. Rev. Neurol. **26 Suppl 1:** S9–14
64 Wallace D. C. (1999) Mitochondrial diseases in man and mouse. Science **283:** 1482–1488
65 Inoue K., Nakada K., Ogura A., Isobe K., Goto Y., Nonaka I. et al. (2000) Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. Nat. Genet. **26:** 176–181
66 Nijtmans L. G., Artal Sanz M., Bucko M., Farhoud M. H., Feenstra M., Hakkaart et al. (2001) Shy1p occurs in a high molecular weight complex and is required for efficient assembly of cytochrome *c* oxidase in yeast. FEBS Lett. **498:** 46–51
67 Casari G., De Fusco M., Ciarmatori S., Zeviani M., Mora M., Fernandez P. et al. (1998) Spastic paraplegia and OXPHOS impairment caused by mutations in paraplegin, a nuclear-encoded mitochondrial metalloprotease. Cell **93:** 973–983
68 Juhola M. K., Shah Z. H., Grivell L. A. and Jacobs H. T. (2000) The mitochondrial inner membrane AAA metalloprotease family in metazoans. FEBS Lett. **481:** 91–95