

## Prohibitins in human diseases: diagnostic and therapeutic applications

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### ABSTRACT

Prohibitins are chaperone proteins highly conserved among eukaryotes. Many different functions have been attributed to Prohibitins depending on their tissue expression and sub-cellular localization. Prohibitins play a major role in mitochondrial physiology, regulating the stability and the processing of both respiratory chain complex subunits and OPA1, a mitochondrial fusion regulating protein. Prohibitins are also involved in the control of proliferation, apoptosis, transcription and signal transduction by interacting with important regulators of these processes located either in mitochondria or in other subcellular sites, such as nucleus, cytosol, plasma membrane. Here, I will review recent experimental data linking the impairment of the different functions of Prohibitins with the onset of important human pathologies, such as cancer, chronic inflammation and drug-induced toxicity. These data highlight how Prohibitins could represent promising candidate targets for the development of novel therapeutic approaches.

**Keywords:** *Prohibitin, Mitochondria, Cancer, Chronic inflammation, Drug toxicity.*

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### INTRODUCTION

Defects in mitochondria function have been linked to a wide range of human pathologies (1,2). Mitochondria play crucial roles in energy production by housing catabolic and intermediary metabolic pathways, such as beta oxidation and Krebs cycle, dedicated to the production of high-energy molecules which are then employed by the respiratory chain machinery to produce adenosine triphosphate (ATP) (1,2). In addition, mitochondria play important roles in apoptosis and cell signaling. For example, the so-called “intrinsic” pathway of the apoptotic process requires the release from mitochondria of pro-apoptotic proteins, such as Cytochrome C, Smac/Diablo, Omi, Endonuclease G and AIF (3).

Furthermore, the crosstalk between mitochondria and endoplasmic reticulum is crucial in regulating cytosolic levels of calcium, an important signalling molecule controlling life-death decisions following different external stimuli and/or stress insults (4).

In contrast to other cytoplasmic organelles, a unique feature of mitochondria is the presence of its own genetic material that encodes for a subset of the mitochondrial proteins (1,2). Mitochondria replicate their DNA and divide by binary fission (5). Moreover, mitochondria are able to fuse with other mitochondria in response to different signals, such as energy needs, and this fusion is essential for normal mitochondrial morphology and function (5). A direct link between mitochondria dysfunction and human pathologies has been formally established by the identification of mutations in the mitochondrial DNA of patients

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with genetically-inherited myopathies, cardiopathies and neuropathies (1,2). On the other hand, most of the mitochondrial resident proteins are encoded by nuclear genes; protein import complexes, located in the outer and inner membranes (TOM and TIM complexes), are responsible for the correct translocation of these proteins to mitochondria (6,7). Proper folding of mitochondrial-imported proteins and their assembly into macromolecular complexes is then assisted by various chaperones, which work in cooperation with specific proteolytic enzymes in order to degrade improperly folded or supernumerary imported proteins (8). Notably, the impairment of the mitochondrial folding apparatus has been also associated with the onset of human diseases (9,10). Among the mitochondrial chaperones, a highly particular role is played by the Prohibitin proteins. Indeed, these chaperones have been shown to be essential for mitochondrial physiology and, at the same time, to play crucial roles in other cellular processes which do not involve Prohibitin mitochondrial function (11). Here, I will review the experimental evidence linking the different proposed functions of Prohibitins and the onset of specific human disorders.

## Structure and localization of Prohibitins

Prohibitins (PHBs) are evolutionary-conserved proteins (11). In humans there are two genes encoding for sequence-related proteins, PHB1 (BAP32, often simply termed Prohibitin) and PHB2 (BAP37, REA). These proteins show sequence similarity to lipid raft-associated proteins of the SPFH family (for Stomatin/Prohibitin/Flotillin/HflK) such as stomatins, flotillins (also known as reggies) and Erlins (for endoplasmic reticulum lipid raft protein) (12).

PHB1 and PHB2 have a predominant mitochondrial localization where they associate with the inner membrane (13). However, these proteins have been also detected in the cytosol, the plasma membrane and the nucleus, so suggesting different functions of these proteins depending on their cellular locations (11).

PHBs are composed of an amino-terminal hydrophobic region responsible for membrane binding, a central domain, called PHB domain, characteristic of all SPFH family proteins and a coiled-coil region at the carboxy-terminal, crucial for the assembly of PHBs into complexes (14). In mitochondria, PHBs are assembled into a ring-like structure with 16–20 alternating PHB1 and PHB2 subunits in the inner membrane (14).

Translocation of PHB1 into mitochondria is mediated, at least in yeast, by its association with Tim8/13 import complexes in the intermembrane space (15), followed by its insertion into the inner membrane via the TIM23 translocase, where it assembles with PHB2 subunits to form the large ring complexes (15). In line with the reported nuclear localization, both PHBs also have nuclear localization/export signals (16,17). However, in the nucleus as well as in the other non-mitochondrial sites, it is not clear whether PHBs form ring-like structures similar to those described in the mitochondria.

## Mitochondrial functions of Prohibitins

Different targets have been proposed for the chaperone activity of PHBs in mitochondria. An important example is represented by the interaction of PHBs with the m-AAA proteases (18). m-AAA proteases are capable of degrading non-assembled proteins, such as respiratory chain subunits, in the inner membrane and act together with ATP-binding cassette (ABC) transporter that exports proteolytic-derived peptides from the matrix to the inter-membrane space (19). PHBs

are able to interfere with m-AAA protease activity by binding to the non-assembled proteins and preventing their proteolysis (18). In line with this hypothesis, a reduction of PHB level leads to a hyperactivity of m-AAA proteases and a consequent decrease of a set of mitochondrial proteins (18). Conversely, increased levels of PHB1 and PHB2 result in the protection of non-native polypeptides from degradation (20). It is interesting to note that the stability of PHBs is also regulated by the interaction with other members of the SPFH family, such as the stomatin protein SLP-2. In fact, depleting HeLa cells of SLP-2 leads to increased proteolysis of PHBs and of subunits of the respiratory chain complexes I and IV (21).

Further insights into the function of PHBs derived from the analysis of PHB2 knock-out cells. In these cells, PHB1 is also undetectable, a clear indication that the stability of the PHB proteins are dependent on their reciprocal interaction. PHB2 <sup>-/-</sup> mitochondria show high levels of fragmentation associated with disorganized and swollen cristae (22). In contrast to the previous hypothesis, these cells display no major defects in the respiratory chain activity, but show an increased susceptibility to apoptotic insults, this being associated with an increased release of Cytochrome C. Interestingly, these defects have been linked at molecular level to a dysregulated processing of OPA1, a dynamin-like GTPase protein involved in the control of mitochondrial membrane fusion (19,22). Mitochondria express five different isoforms of OPA1: two long forms designated L1 and L2, which can be proteolytically converted to three short forms, designated S3–S5 (19,22). OPA1 has been proposed to be a substrate of m-AAA proteases (23) In PHB2 mutant cells, the long isoforms of OPA1 are drastically reduced while some of the short ones are increased (22). Notably, the expression of a non-cleavable form of OPA1 is sufficient to rescue the defects

observed in PHB2 null cells (22). These results support a major role of PHBs in controlling protein processing in mitochondria.

Another interesting target of PHBs is the anti-apoptotic Hax-1. PHB2 is able to bind Hax-1 in association with the ANT2 and VDAC2 proteins (17). Notably, Hax-1 stability is dependent on PHB2 expression, suggesting that PHBs could regulate cell survival by directly interacting with the apoptotic pathway (17).

Other mitochondrial functions besides the regulation of protein stability have been proposed for PHBs. For example, PHB1 has been shown to inhibit the activity of the pyruvate carboxylase (24), a mitochondrial enzyme involved in replenishing tricarboxylic acid cycle intermediates. Recently, it has been reported that PHBs is also essential for the maintenance of the mitochondrial nucleoid organization and regulation of mtDNA copy number (25).

## Non-mitochondrial function of Prohibitins

Many experimental data support a role of PHBs in the nucleus (26). PHB1 has been found to interact with different transcription factors or cofactors regulating proliferation and apoptosis, such as E2F1, p53 and retinoblastoma protein (27,28). In most of the reported data, PHB1 negatively regulates the transcriptional activity of E2F1 and it has been proposed that this interaction accounts for the anti-proliferative properties of PHB1 (26). The PHB transcriptional repression is mediated by the recruitment to E2F responsive promoters of histone deacetylase, such as HDAC1, and additional co-repressors like NCoR and proteins of the SWI-SNF complex (29,30,31). Conversely, PHB1 is able to stimulate p53 transcriptional activity by enhancing its recruitment to promoters (28).

Another class of transcriptional factors regulated by PHBs is represented by nuclear

receptors. In particular, PHBs bind to and repress the activity of estrogen and androgen receptors (32,33,34). Also in this case, HDACs and SWI-SNF complex proteins have been shown to be implicated in PHB-mediated repression (35,36,37). Importantly, a role of PHBs in regulating estrogen receptor activity has been confirmed in mice with heterozygotic deletion of the PHB2 gene. In fact, these mice show both increased epithelial proliferation and enhanced gene expression in response to estrogen stimulation (38).

Beside mitochondrial and nuclear roles, other functions have been assigned to PHBs. For example, PHB1 is able to interact with cytosolic c-Raf kinase (39). This interaction is required for the binding of c-Raf with the activated c-Ras and, in turn, regulates c-Raf-mediated epithelial cell adhesion and migration (39). PHBs have also been localized on the plasma membrane in some cell types. PHBs bind to the IgM antigen receptor on the surface of B lymphocytes (40), whereas in intestinal epithelial cells PHB is a membrane receptor for the Vi capsular polysaccharide of *Salmonella typhi*, the causative agent of typhoid fever in humans (41). Moreover, PHBs are expressed on the surface of vascular cells of the white adipose tissue, as shown by the binding and the internalization of synthetic PHB-interacting peptides with pro-apoptotic activity (42). Finally, both PHB1 and PHB2 have been detected in the circulating blood, most likely released from the cells entrapped in lipid droplets (43,44). A proposed function of extracellular PHBs is the enhancement of complement activation via its binding to the complement component C3 (45).

### **Role of Prohibitins in tumorigenesis**

A large number of scientific reports documented that PHBs expression is altered in different pathological conditions (26). Notably,

many of these publications described an up-regulation of PHBs in a wide range of tumors, such as breast, prostate, ovarian, lung, bladder, thyroid and gastric cancer (46,47,48,49,50,51,52,53). Moreover, causative links between mutations in the PHB1 gene and tumor progression have been proposed (54), including single-nucleotide polymorphisms located at 3' untranslated region of the PHB1 mRNA, a region known to influence cell growth independently of PHB1 protein synthesis (55,56). However, the examination of a large number of human tumors of various origins has revealed that these mutations are uncommon and mainly restricted to breast cancers (54,57,58). Interestingly, a recent report proposes that microRNA-27 acts as an oncogene by targeting PHB1 in gastric adenocarcinoma, uncovering novel potential relationships between PHBs and tumorigenesis (59).

The increase of PHBs is usually interpreted as one of the attempts of the cell to counteract uncontrolled proliferation by up-regulating anti-proliferative genes. An alternative explanation of the increased expression of PHBs in tumor cells is based on the identification of the oncoprotein c-Myc binding sites in the PHB1 promoter (60,61). In normal proliferating cells, the regulation of PHB1 levels by c-Myc may represent a mechanism to balance cell cycle progression and energy need. In tumors, c-Myc is often up-regulated and this could explain why PHB1 is over-expressed. Increased PHB1 levels could play a positive rather than negative role in the growth and survival of transformed cells by reducing mitochondrial ROS production and inhibiting apoptosis.

### **Role of Prohibitins in response to the oxidative stress**

A reduction of PHB levels has been also described in different pathological conditions. For

example, PHB1 has been found to be down-regulated in human and animal models of inflammatory bowel disease, such as Crohn's disease (62). Notably, high levels of PHB1 expression, achieved by a transgenic approach, protect PHB mice from oxidative stress induced by ulcerative colitis, highlighting a major role of PHB in cellular defense against oxidant injury (63). The protective functions of PHB against oxidative stress have been also reported in cardiomyocytes, where it has been associated with the anti-apoptotic properties of PHB1 (64). An interesting relationship between PHB expression levels, oxidative stress and senescence has been recently described. Knockdown of PHB1 in endothelial cells increases mitochondrial production of reactive oxygen species via inhibition of complex I, which induces Akt hyper-activation, stress fiber formation, loss of migratory capacity and, eventually, cellular senescence (65).

Further evidence for a role of PHBs in controlling oxidative stress comes from our recent analysis of mitochondrial alterations induced by the antiretroviral therapy used to control HIV infection (unpublished results). Highly active anti-retroviral therapy (HAART), a combination of inhibitors of the HIV reverse transcriptase (NRTI and NNRTI) and HIV protease (PI), has significantly improved the life expectancy of HIV patients (66). However, HAART produces major side effects such as neuropathy, myopathy, cardiomyopathy, myelopoiesis, pancreatitis, hepatic steatosis, lactic acidosis and lipodystrophy (67). Mitochondrial damage is considered one of the main causes of HAART's long-term toxicities (68). Proposed mechanisms of HAART toxicity include the inhibition of DNA polymerase- $\gamma$  by the NRTI backbone of the therapy, together with the inhibition of endogenous nucleotide kinases, the impairment of oxidative phosphorylation, the generation of ROS, changes in expression of uncoupling proteins, and mutation in mitochondrial and nuclear DNA (69). We have

found that a decrease of PHB1 is one of the earliest alterations detectable following *in vitro* treatment of a wide range of cells with NRTI, suggesting that a decrease of PHB1 could have a role in causing the side-effects associated with the drug treatment (unpublished results). In line with this hypothesis, we found that PHB1 over-expression protects cells from the mitochondrial oxidative stress induced by NRTI (unpublished results).

## Conclusions and Perspectives

The activity of chaperone proteins is critical for many cellular functions because they are able to control the folding and the expression levels of a large set of target proteins. PHB proteins represent a peculiar family of chaperones that combine specific lipid- and protein-binding properties. Many different functions have been attributed to PHBs depending on their tissue expression and sub-cellular localization. Although many aspects of the PHB function remain to be elucidated, there is increasing evidence for an important involvement of PHBs in human pathology. PHB expression is altered in different pathological conditions, making PHB proteins good candidates as diagnostic markers. Even more relevantly, it has been shown that experimental modulation of PHB expression could be beneficial in some pathological conditions, such as chronic inflammation and drug toxicity. Based on these considerations, the development of therapeutic agents to modulate the activity of PHBs may represent an important challenge for the drug-research industry in the near future.

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