Functional characterization of prohibitins by conditional inactivation in the mouse

I n a u g u r a l - D i s s e r t a t i o n

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Carsten Merkwirth

aus Kassel

Köln, 2008

Berichterstatter:

Professor Dr. Thomas Langer

Professor Dr. Jens C. Brüning

Professor Dr. Ari Waisman

Tag der mündlichen Prüfung: 28. Mai 2008

Für meine Oma

"Wissenschaft ist wie Fussball –

Beide brauchen Kopf, Herz und Leidenschaft."

Jens Falta (Physiker)

Abstract

Prohibitins comprise an evolutionary conserved and ubiquitously expressed family of membrane proteins with poorly described functions. Large assemblies of PHB1 and PHB2 subunits are localized in the inner membrane of mitochondria, but various roles in other cellular compartments have also been proposed for both proteins. To determine physiological functions of mammalian prohibitins, a conditional mouse model for the analysis of the murine *Phb2* gene was established, which allows a tissue-restricted and time-controlled *Phb2* gene deletion. Mouse embryonic fibroblasts (MEFs) isolated from genetically modified $Phb2^{fl/fl}$ embryos were generated to define cellular activities of prohibitins. The presented experiments restrict the function of prohibitins to mitochondria and identify the processing of the dynamin-like GTPase OPA1, an essential component of the mitochondrial fusion machinery, as the central cellular process controlled by prohibitins. Cre-mediated deletion of *Phb2* in MEFs leads to the selective loss of long isoforms of OPA1. This results in fragmentation of the mitochondrial network accompanied by an aberrant cristae morphogenesis in prohibitin-deficient cells. Furthermore, loss of PHB2 is characterized by an impaired cellular proliferation and resistance towards apoptosis. Expression of a long OPA1 isoform in PHB2-deficient cells suppresses these defects identifying impaired OPA1 processing as the primary cellular defect in the absence of prohibitins. In conclusion, these results assign an essential function to prohibitins in the formation of mitochondrial cristae and suggest a coupling of cell proliferation to mitochondrial morphogenesis.

Table of Contents

1 Introduction

1.1 Organization of biological membranes

Cellular membranes represent essential barriers that define the boundary of individual cells (Engelman, 2005). Moreover, they compartmentalize organelles within a cell by segregating them from the cytosol thus providing permeability barriers that surround aqueous interiors and allow for the separation of ion and solute concentrations (Maxfield and Tabas, 2005). The biological membrane is a liquid-like structure representing a homogenous fluid lipid bilayer (Singer and Nicolson, 1972). This traditional view of a biological membrane has been extended over the last years to a more complex structure. Thousands of different lipids arranged with a multiplicity of membrane proteins give rise to a highly dynamic lipid-protein composite. The plasma membrane of eukaryotic cells, like other biological membranes, contains more lipid species than required for establishing a lipid bilayer. This diversity of lipids in eukaryotic membranes raised the question, whether specific lipids could serve to organize biological membranes into discrete domains with different properties (Karnovsky et al., 1982; Thompson and Tillack, 1985). Indeed, an asymmetric distribution of phospholipids was observed in the plasma membrane of erythrocytes suggesting a lateral heterogeneity of lipids and proteins in biological membranes (van Meer et al., 1980). This finding was supported by the discovery that glycospingolipids cluster in the Golgi apparatus before being sorted to the apical surface of polarized cells (van Meer et al., 1987). Based on these initial observations, a spatial organization of membranes into discrete microdomains has been proposed, which thereby provides the rationale for the compartmentalization of important biological processes including signal transduction pathways, apoptosis, cell adhesion and migration, synaptic transmission, organization of the cytoskeleton and membrane fusion during both exocytosis and endocytosis (Brown and London, 1998; Harris and Siu, 2002; Simons and Toomre, 2000; Tsui-Pierchala et al., 2002). The concept of lateral lipid assemblies within the plasma membrane finally lead to the hypothesis of lipid rafts, a particular type of microdomain in the plasma membrane (Simons and Ikonen, 1997).

1.2 Lipid microdomains

Lipid rafts were hypothesized to be lateral dynamic assemblies of lipids which constitute a non-random organization and partitioning of the plasma membrane into microdomains (Harder et al., 1998; Simons and Ikonen, 1997). In particular, these microdomains are enriched in cholesterol and sphingolipids and thought to provide a platform for the concentration of specific proteins and to spatially segregate molecules. Due to different biophysical properties of the enriched lipids, microdomains are present in the liquid-ordered phase separated from the surrounding lipid bilayer (Brown and London, 1998). The basic concept of lipid rafts is to facilitate specific protein-protein interactions by the selective exclusion or inclusion of proteins (Hancock, 2006). The lipid-based separation and fusion of such domains and their associated proteins would therefore provide a dynamic spatial and temporal regulation of signalling cascades according to the requirement of the cell (Simons and Toomre, 2000).

This concept was experimentally supported by the isolation of lipid microdomains based on their insolubility in cold non-ionic Triton X-100 detergent (Yu et al., 1973). These detergent-resistant membranes (DRM) were found to be enriched in both cholesterol and glycophosphatidylinositol (GPI)-anchored proteins and showed an altered density on sucrose-gradients (Brown and Rose, 1992; Varma and Mayor, 1998). On the basis of biophysical experiments, the size of lipid rafts was proposed to be in the range of 10 – 200 nm (Pralle et al., 2000). Small rafts tend to form larger platforms via protein-protein and protein-lipid interactions (Pike, 2006). This suggests that membrane microdomains contribute to the integrity of membranes by providing a scaffolding function. The association of the cytoskeleton with biological membranes has also been proposed to influence membrane organization (Janmey and Lindberg, 2004). In addition to the binding of membrane proximate cytoskeletal adaptors to membrane proteins, cytoskeletal proteins also interact with specific lipids which is likely to influence the organization of membrane microdomains through both protein and lipid anchorage points on the inner leaflet (Babiychuk and Draeger, 2000; Holowka et al., 2000; Vereb et al., 2003). These observations shed new light on possible regulatory mechanisms controlling the membrane lateral heterogeneity in the execution of important cellular functions like signal transduction and membrane trafficking (Simons and Toomre, 2000). In this context, rafts have been appreciated to serve as platforms allowing the recruitment of signalling molecules to defined patches. Most importantly, signalling by T cell receptors (Janes et al., 2000), B cell receptors (Cheng et al., 1999), growth factor receptors

(Waugh et al., 1999), interleukins and insulin (Mastick et al., 1995) were proposed to be associated with lipid rafts (Bromley et al., 2001; Paratcha and Ibanez, 2002). For individual receptors, rafts may form a concentrating platform, activated by ligand binding (Zajchowski and Robbins, 2002). It has been argued that the local restriction of signalling pathways in rafts would on the one hand allow activated receptors enhanced access to downstream effector molecules and, on the other hand, protect these signalling complexes from non-raft molecules that otherwise could negatively affect the transduction process (Simons and Toomre, 2000). In addition, it has been suggested that receptor clustering might in turn lead to the organization of smaller rafts into larger microdomains and hence increase the spatial concentration of signalling components (Anderson and Jacobson, 2002; Harris and Siu, 2002; Pike, 2003; Simons and Toomre, 2000; Subczynski and Kusumi, 2003). Interestingly, the partitioning in microdomains might also have a role in the transduction of signalling via lipids themselves or through lipid-modified proteins anchored to the membrane (Varma and Mayor, 1998). Among those, the GPI-modification of cell adhesion molecules like cadherins, NCAM120 and ephrins presumably drives sorting into special microdomains through their lipid anchorage (Bruckner et al., 1999; Doyle et al., 1998; Olive et al., 1995). Similarly, the Ras signalling cascade seems to be compartmentalized into membrane microdomains by the selective palmitoylation of the H-Ras isoform, thereby conferring a partition into lipid rafts (Hancock et al., 1990). An accumulation in lipid rafts has also been observed for lipid-modified Src kinases and G-subunits of heterotrimeric G proteins (Oh and Schnitzer, 2001; Resh, 1999). Phosphatidylinositol and its phosphorylated derivatives are associated with a wide variety of cellular functions including signalling (Berridge and Irvine, 1989), ion channel activation (Suh and Hille, 2005) and membrane trafficking (Simonsen et al., 2001). In addition to its role as a second messenger in signal transduction processes (Berridge and Irvine, 1984), phosphatidylinositol-4,5-bisphosphate (PIP_2) anchors proteins to the plasma membrane through pleckstrin homology (PH) domains and therefore potentially contributes to the organization of membrane microdomains (DiNitto et al., 2003; Lemmon, 2003). Recent evidence suggests that lipid micro-environments also contribute to the spatial segregation of components required for membrane docking and fusion at particular sites of the plasma membrane (Chamberlain et al., 2001; Ikonen, 2001; Lang et al., 2001). Moreover, PIP_2 links lipid microdomains to clathrin-mediated endocytosis and synaptic vesicle trafficking (Di Paolo et al., 2004; Honing et al., 2005; Wenk and De Camilli, 2004).

Although membrane lateral homogeneity is accepted as a requirement for the function of biological membranes and its incorporation into the lipid raft hypothesis confers specificity

to this broad concept, direct evidence for the existence of raft microdomains is still missing (Jacobson et al., 2007; Munro, 2003). On the one hand, this might be due to technical difficulties to prove the existence of lipid rafts. On the other hand, however, recent experiments have raised potential concerns about the lipid raft hypothesis (Munro, 2003). Critical points about the raft microdomains are mainly based on the fact that most of the evidence for their existence and function relies on indirect methods (Lai, 2003). Critics were corroborated further by an asymmetry in the lipid composition of the exo- and endoplasmic leaflets of cell membranes and a lack of evidence for the formation of lipid domains in the inner leaflet (Munro, 2003). Since the vast majority of findings concerning lipid rafts have been obtained for plasma membranes, the notion of lipid rafts as a generalized principle present in endoplasmic membranes of cell organelles has to be considered critically (Mukherjee and Maxfield, 2004). Notably, an emerging view favours the idea of a protein-assisted establishment of microdomains in biological membranes.

1.3 SPFH-domain containing proteins

Using stomatin sequences for bioinformatic analyses, a protein family associated with lipid microdomains was identified (Tavernarakis et al., 1999) which contains a domain within its central region bearing a high similarity to prohibitins, the flotillins/reggie-proteins and the bacterial plasma membrane proteins HflK and HflC (Figure 1). The conserved region was termed SPFH-domain (after Stomatin, Prohibitin, Flotillin and HflK/C) (Tavernarakis et al., 1999). The family of SPFH-domain containing proteins is also termed PHB (prohibitin) domain family or PID (after Proliferation, Ion and Death) (Morrow and Parton, 2005; Nadimpalli et al., 2000). Members of the SPFH protein family share a ~200 amino acid N-terminal core motif present in eukaryotes as well as archaea and prokaryotes suggesting an ancient origin (Rivera-Milla et al., 2006) (Figure 1). Although the alignment of prohibitins reveals a sequence similarity of 60% and an identity of 47%, the similarity of both proteins to mouse flotillin-2 is only 4 and 7%, respectively. Phylogenetic analyses of SPFH-domains from different proteins revealed only ambiguous relationships within this superfamily, indicating independent origins for the individual members and convergent evolution of the PHB domain (Rivera-Milla et al., 2006) (Figure 2). SPFH family members are integral or membrane-associated proteins present in various cellular membranes, including the plasma membrane (Lang et al., 1998; Snyers et al., 1999), Golgi (Glebov et al., 2006), endoplasmatic reticulum (Browman et al., 2006) and mitochondrial inner membrane (Ikonen et al., 1995)

exposing their SPFH-domain to a hydrophilic environment (Tavernarakis et al., 1999). Interestingly, various SPFH-domain-containing proteins are enriched in detergent-resistant membranes (DRM) suggesting an association with lipid microdomains in diverse membranes of cellular compartments (Browman et al., 2007; Langhorst et al., 2005). Subcellular targeting of SPFH members to plasma membrane microdomains occurs via different mechanisms. The SPFH-domain of stomatin homologues contains sequences driving the localization to the plasma membrane and lipid rafts (Salzer and Prohaska, 2001). Dependent on two hydrophobic regions within the SPFH-domain, flotillin-1/reggie-2 proteins are targeted to the plasma membrane (Liu et al., 2005) (Figure 1).

Lipid modification is an additional common mechanism for proper targeting of proteins to DRMs and lipid microdomains (Melkonian et al., 1999). Palmitoylation of several SPFH protein members has been shown to be involved in the sorting to plasma membrane microdomains. A cysteine residue (C43) in the SPFH-domain of flotillin-1/reggie-2 is modified by palmitoylation and functions together with hydrophobic regions in plasma membrane association (Morrow et al., 2002). Mutational exchange of this cysteine residue to alanine prevents the association of flotillin-1/reggie-2 with the plasma membrane (Morrow et al., 2002). Similarly, palmitoylation of a cysteine residue (C29) in stomatin targets the protein to lipid rafts suggesting a crucial role for palmitoylation in subcellular sorting (Snyers et al., 1999; Wang et al., 1991). Mutations in the SPFH-domain of podocin disrupt the proper localization to the plasma membrane and cause retention in the endoplasmic reticulum (Roselli et al., 2004). The sorting of endoplasmic reticulum- and mitochondria-localized SPFH proteins depends mostly on N-terminal regions. The N-termini of prohibitin-1 (PHB1) and prohibitin-2 (PHB2), erlins and stomatin are sufficient to target a heterologous protein to mitochondria (Kasashima et al., 2006; Tatsuta et al., 2005), to the endoplasmic reticulum (Browman et al., 2006) and to cytoplasmic vesicular structures, respectively (Umlauf et al., 2004). While a mitochondrial targeting signal is predicted for PHB2 (Kasashima et al., 2006; Tatsuta et al., 2005), specific sorting signals have not been identified in other SPFH-domain-containing proteins (Browman et al., 2007).

On a structural level, the SPFH-domain functions as a mediator of protein-protein interactions in stomatin proteins. The SPFH-domain of MEC-2, the *Caenorhabditis elegans* homologue of human stomatin, mediates interaction with MEC-4 and MEC-10 subunits of the degenerin channel in the plasma membrane of touch-sensory neurons (Huang et al., 1995). In this scenario, the SPFH-domain generates close proximity between the regulatory N- and C-

termini of MEC-2 and the degenerin channel subunits (Zhang et al., 2004). Moreover, biochemical data suggest a role of the SPFH-domain in self-oligomerization.

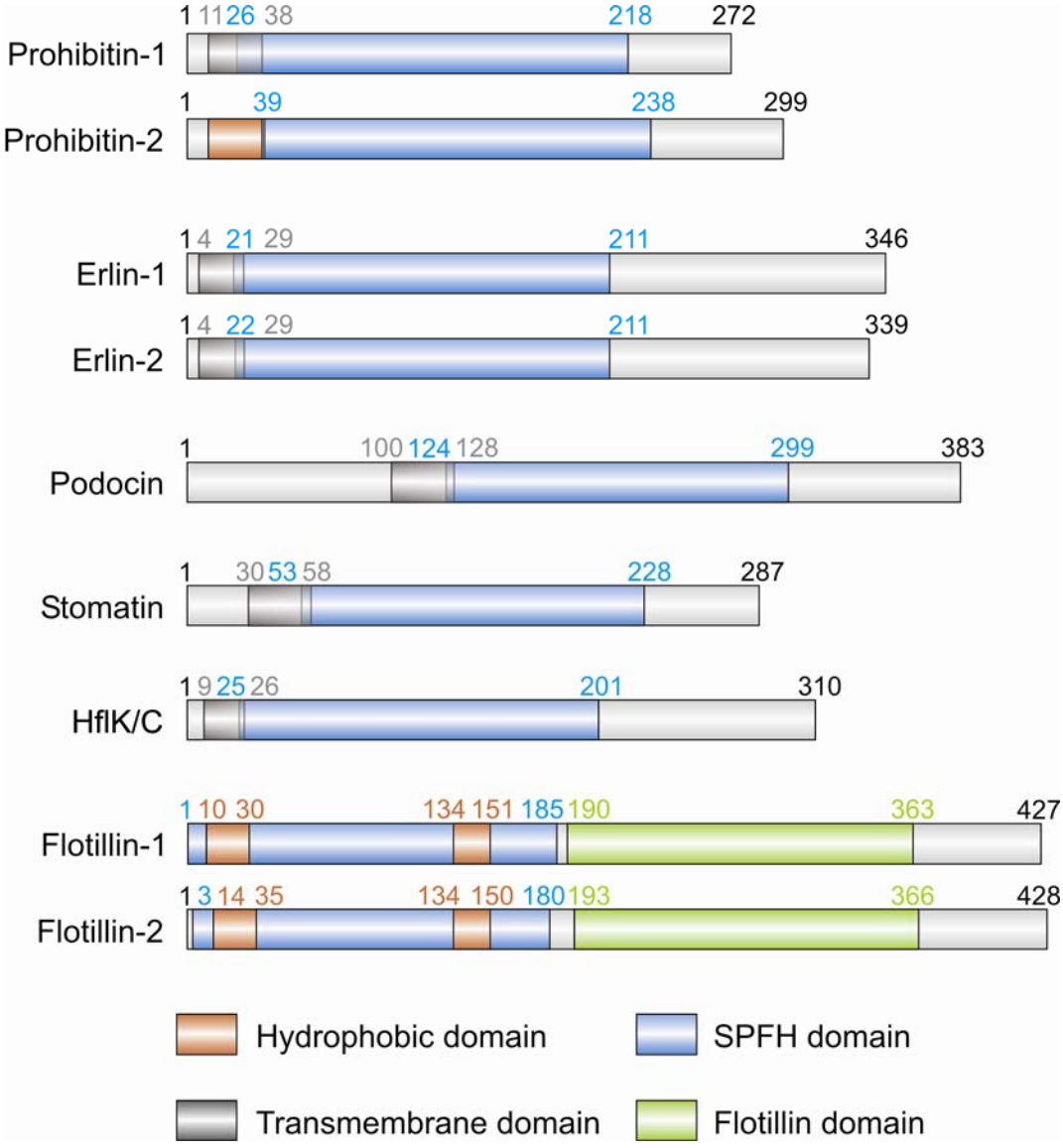

Figure 1. Domain structure of SPFH-domain containing proteins.

Schematic representation of the protein domain structure of mammalian prohibitins, erlins, stomatin, podocin, flotillins and bacterial HflK/C. Gray boxes indicate transmembrane domains; brown, hydrophobic domains; blue, SPFH-domains; green, flotillin domains. Numbers in corresponding colours indicate the amino acid residues marking the boundaries of domains. Modified from (Browman et al., 2007).

In human stomatin, the SPFH-domain is required for the correct positioning of the C-terminus for self-assembly which is disrupted in C-terminal truncated versions (Snyers et al., 1998). Genetic studies support the notion that homo-oligomerization of stomatins is required for their function (Gu et al., 1996; Tavernarakis and Driscoll, 1997). Recently obtained structural data from a archaebacterial stomatin orthologue further strengthen the findings on oligomerization (Yokoyama et al., 2008). In stomatin and podocin, the SPFH-domain forms a hairpin conformation which mediates the proper localization of N- and C-termini to the cytoplasm where interactions with accessory molecules take place (Roselli et al., 2002; Snyers et al., 1999). These findings suggest that the SPFH-domain also serves as a structural scaffold (Goodman et al., 2002; Zhang et al., 2004).

1.3.1 Stomatins and stomatin-like proteins

Stomatin is the most representative member of SPFH-domain proteins. The 31-kDa integral membrane protein belongs to the family of band 7.2b proteins and is widely expressed from prokaryotes to eukaryotes (Gallagher and Forget, 1995; Hiebl-Dirschmied et al., 1991). Human stomatin is highly expressed in erythrocytes and was originally thought to be associated with overhydrated hereditary stomatocytosis (OHSt), a form of haemolytic anaemia characterized by increased erythrocyte permeability to monovalent cations (Fricke et al., 2003). Erythrocytes isolated from OHSt patients lacked the stomatin protein (Stewart et al., 1993). However, further studies disproved an involvement of stomatin in the disease (Delaunay et al., 1999; Innes et al., 1999). Notably, stomatin-deficient mice show an apparently normal erythrocyte function (Zhu et al., 1999). Stomatin localizes predominantly to the plasma membrane and intracellular vesicles of the endocytic pathway (Snyers et al., 1999). In epithelial cells, stomatin has been shown to concentrate in plasma membrane protrusions and late endocytic compartments. At these subcellular localizations, stomatin is present in high-order oligomers consisting of 9-12 monomers (Snyers et al., 1998). At the plasma membrane, stomatin was detected in clusters in electron microscopy studies, where it colocalizes with the actin cytoskeleton (Snyers et al., 1997). These observations led to the notion that stomatin oligomers might serve a role in membrane compartmentalization by providing a microdomain scaffold (Snyers et al., 1998). Interestingly, stomatin has been found in lipid microdomains in numerous cell types (Foster et al., 2003; Garin et al., 2001) and was shown to be associated with lipid bodies (Umlauf et al., 2004). Further studies implied a role of stomatin in insulin-dependent glucose transport, since stomatin has been shown to interact

directly with the glucose transporter GLUT-1, thereby regulating its trafficking and transport activity (Zhang et al., 2001; Zhang et al., 1999). The analysis of stomatin-like proteins in various organisms suggested a role in ion channel regulation. The *C. elegans* homologue of stomatin, MEC-2, is exclusively expressed in specialized touch receptor neurons responsible for mechanosensation. Mutational loss of MEC-2 results in decreased touch sensitivity (Gillespie and Walker, 2001). The protein was shown to interact with and regulate the activity of members of the mechanosensitive degenerin-epithelial sodium channels (DEG-ENaC) (Ernstrom and Chalfie, 2002). The stomatin homologue exists in a multiprotein complex with the degenerin-type channel proteins MEC-4 and MEC-10 which is thought to link the MEC transduction channel to microtubules (Goodman et al., 2002; Huang et al., 1995). Functional conservation of this function is indicated by a recent report demonstrating that the mammalian stomatin homologue SLP-3 is also involved in mechanosensation in mice (Wetzel et al., 2007). In contrast to the ubiquitous expression of stomatin, SLP-3 expression is restricted to neuronal tissue. SLP-3-deficient mice exhibit a markedly reduced touch sensitivity due to loss of function of a subset of mechanoreceptors in the skin, acid-sensing ion channels (ASICs) (Wetzel et al., 2007). In a heterologous system, SLP-3 and stomatin were shown to interact with and modulate the activity of different ASICs (Price et al., 2004). These results suggest that SLP-3 might mediate touch sensitivity by regulating ASICs in mechanosensory axons in the skin similar to the regulation of MEC-4 and MEC-10 by MEC-2 (Wetzel et al., 2007).

Another specialized SPFH family member and mammalian stomatin homologue is podocin (NPHS2), a 42-kDa integral membrane protein which shows 47% identity to human stomatin (Figure 1). Podocin is exclusively expressed in podocytes, a subset of highly specialized kidney epithelial cells, which are involved in plasma ultrafiltration during primary urine formation (Roselli et al., 2002). The protein localizes to the slit diaphragm, a specialized intercellular junction in the mammalian kidney (Huber and Benzing, 2005; Roselli et al., 2002). Podocin was found in high order-oligomers that are constituents of detergent-insoluble microdomains in the plasma membrane of podocytes (Huber et al., 2003). In this respect, interactions of podocin with other microdomain-associated podocyte proteins, CD2-associated protein (CD2AP) and nephrin, have been reported (Schwarz et al., 2001). Podocin has been suggested to potentiate nephrin signalling by localizing nephrin to lipid raft domains (Huber et al., 2003). Inefficient nephrin signalling via the nephrin/CD2AP/podocin complex is thought to contribute to the development of podocyte dysfunction (Huber et al., 2001; Shih et al., 1999). Recently, the non-selective cation channel transient receptor potential canonical 6 (TRPC6) was identified as an additional component of the slit diaphragm protein complex.

Podocin was shown to interact with TRPC6 and to enhance its ion channel activity (Huber et al., 2006). Thus, podocin might be involved in mechanosensation at the kidney filtration barrier by regulating TRPC6 channel activity (Huber et al., 2007). Strikingly, mutations in podocin were associated with autosomal recessive steroid-resistant nephritic syndrome, a progressive disorder leading to end-stage renal disease (Boute et al., 2000; Fuchshuber et al., 1995). Interestingly, ion channel regulation by both MEC-2 and podocin is dependent on their ability to bind cholesterol (Huber et al., 2006). Thus, the feature of stomatin proteins to form large multimeric complexes in cellular membranes connected with their cholesterol binding ability could lead to localized changes in the membrane lipid composition and might provide the basis for a molecular understanding of their role in ion channel regulation. Ion channels might become activated by altered membrane properties caused by locally elevated cholesterol levels (Huber et al., 2006). The putative role in ion channel regulation and mechanosensation has only been shown for members of the stomatin family, suggesting that these functions might be unique to these proteins. However, the involvement of other SPFH-domain-containing proteins in ion-channel regulation during mechanosensation remains to be investigated (Browman et al., 2007).

In addition to SLP-1 and SLP-3, another protein of the stomatin family has been reported. Stomatin-like protein 2 (SLP-2) was initially discovered in erythrocytes (Wang and Morrow, 2000). Interestingly, more recent findings suggest a mitochondrial localization of SLP-2. SLP-2 forms a complex in the mitochondrial inner membrane and was shown to interact specifically with mitofusin-2 (Hajek et al., 2007), an essential dynamin-like GTPase in the outer membrane of mitochondria required for mitochondrial fusion (Santel and Fuller, 2001). These observations suggest that SLP-2 links the inner to the outer mitochondrial membrane and might regulate the activity of mitofusin-2 via a direct protein-protein interaction. However, the molecular mechanism of SLP-2 function and its putative contribution to inner membrane organization remain elusive and require to be addressed in further detail. Another striking observation is the interaction of SLP-2 with prohibitins in the mitochondrial inner membrane. RNAi-mediated depletion of SLP-2 affects the steady-state level of PHB1 and PHB2 suggesting a role for SLP-2 in the regulation of mitochondrial proteolysis (Da Cruz, 2008).

1.3.2 Flotillin/reggie proteins

Flotillin/reggie proteins are additional members of the SPFH-domain-containing protein family (Tavernarakis et al., 1999) (Figure 1). Reggie-1 and -2 are highly conserved proteins which were characterized initially in goldfish and rats as plasma membrane-associated proteins (Malaga-Trillo et al., 2002). In this context, reggie proteins were identified and named due to their upregulation during axonal regeneration of retinal ganglion cells upon optical nerve transsection (Lang et al., 1998; Schulte et al., 1997). The independent identification of reggie homologues in mouse and fruitfly led to the alternative name flotillin-1 and -2 (corresponding to reggie-2 and -1, respectively) which were associated with the floating lipid fraction isolated from murine lung tissue (Bickel et al., 1997; Galbiati et al., 1998). Mammalian flotillins are widely expressed in various tissues and cell types (Lang et al., 1998; Salzer and Prohaska, 2001; Solomon et al., 2002; Stuermer et al., 2001; Volonte et al., 1999). On a cellular level, flotillins are mainly localized at the plasma membrane and are considered lipid raft components due to their presence in detergent-resistant membranes (DRMs) (Stuermer et al., 2001). In addition, localizations of both flotillin-1 and -2 to endosomes, lipid droplets and phagosomes have been reported (Dermine et al., 2001; Gagescu et al., 2000; Morrow and Parton, 2005). Flotillin-1, but not flotillin-2, was also found to be associated with the trans-Golgi network (TGN) (Gkantiragas et al., 2001). Moreover, a cell-cycle dependent translocation of flotillin-1 to the nucleus has been observed (Santamaria et al., 2005).

Plasma membrane association of flotillins occurs in the absence of typical transmembrane domains suggesting alternative modes of membrane binding. Two conserved hydrophobic membrane-associating domains in the N-terminus of flotillins are considered to mediate the interaction with the inner leaflet of the plasma membrane (Morrow et al., 2002) (Figure 1). Post-translational modifications like palmitoylation and myristoylation of both flotillins may assist plasma membrane anchoring and targeting to lipid microdomains (Liu et al., 2005; Morrow et al., 2002; Neumann-Giesen et al., 2004). Both flotillin-1 and -2 purified from erythrocyte membrane fractions have been shown to form high-order homo-oligomers (Salzer and Prohaska, 2001). Moreover, hetero-oligomerization of flotillins into tetramer units, which depends on C-terminal coiled-coil domains, has been suggested (Neumann-Giesen et al., 2004; Solis et al., 2007). Whether hetero-oligomerization of mammalian flotillins is connected to a functional interdependence is discussed controversially. In contrast to a RNAi-mediated depletion of flotillin-2, which leads to a reduction in flotillin-1 protein

levels, the reciprocal experiments do not support this notion (Solis et al., 2007). Confocal and electron microscopy analyses revealed the presence of flotillin clusters at the plasma membrane, indicative of small, uniform microdomains with a diameter of 100 nm (Kokubo et al., 2003; Stuermer et al., 2001). A co-clustering of flotillins with various GPI-anchored cell surface proteins (Stuermer et al., 2001), cell adhesion molecules (Stuermer et al., 2004), intracellular signalling components (Rajendran et al., 2003; Slaughter et al., 2003) and the cytoskeleton (Langhorst et al., 2007) was observed suggesting that flotillins contribute structurally to the formation of membrane microdomains (Frick et al., 2007; Stuermer and Plattner, 2005). It has been further proposed that flotillin clusters form lipid raft-like membrane scaffolds with important roles in cell-cell adhesion and signal transduction (Langhorst et al., 2005; Simons and Toomre, 2000; Stuermer et al., 2001). It has recently been reported that mammalian flotillins are involved in the regulation of clathrin-independent endocytosis, supporting the idea that flotillin scaffolds provide functional dynamic microdomains (Frick et al., 2007; Glebov et al., 2006). Interestingly, a role for the secretion of signalling components has been demonstrated for *Drosophila melanogaster* flotillin-2 indicating a crucial role for intracellular trafficking and the generation of morphogen gradients during fruitfly development (Katanaev et al., 2008). A clinical relevance of flotillins is unclear, since natural mutations have not been discovered to date. However, increased flotillin-1 expression levels have been associated to type II diabetes (James et al., 2001) and neuropathological disorders such as Parkinson´s disease (Jacobowitz and Kallarakal, 2004) and Alzheimer´s disease (Girardot et al., 2003; Kokubo et al., 2000).

1.3.3 HflK/C proteins

The *Escherichia coli* HflK and HflC proteins are bacterial members of the SPFH protein family (Tavernarakis et al., 1999) (Figure 1). Both are transmembrane proteins consisting of approximately 310 amino acids encoded by the *hflA* (high frequency of lysogenization) operon that regulates the lysogenic decision during bacteriophage λ infection (Banuett and Herskowitz, 1987; Herskowitz and Hagen, 1980; Kihara et al., 1997). HflK and HflC proteins form a high molecular weight complex (HflK/C) which is anchored to the bacterial plasma membrane by N-terminal transmembrane segments and exposes C-terminal coiled-coil domains to the periplasmic side (Kihara et al., 1997; Kihara and Ito, 1998). Furthermore, the C-terminal domains are involved in hetero-oligomerization of the proteins (Briere and Dunn, 2006). HflK and HflC subunits exhibit functional interdependence which is

reflected by decreased stability of one subunit in the absence of the other (Banuett and Herskowitz, 1987). Notably, the *hflA* locus encodes an additional protein, FtsH, which is categorized as an ATP-dependent metalloprotease of the AAA family (ATPases associated with a variety of cellular activities) (Noble et al., 1993; Ogura and Wilkinson, 2001). The HflK/C complex interacts with and assembles into a larger complex with the membrane protease FtsH thereby modulating its proteolytic activity (Kihara et al., 1996; Saikawa et al., 2004). The FtsH protease itself controls the decision between lysogenic and lytic cycle growth during λ-phage infection by modulating the stability of the phage-derived cII protein (Banuett and Herskowitz, 1987). Additionally, the FtsH protease is involved in the degradation of a membrane protein translocase. In the absence of HflK/C, the non-assembled subunit SecY of the SecY-SecE translocase is rapidly degraded suggesting a negative regulation of the protease by the HflK/C complex (Kihara et al., 1996).

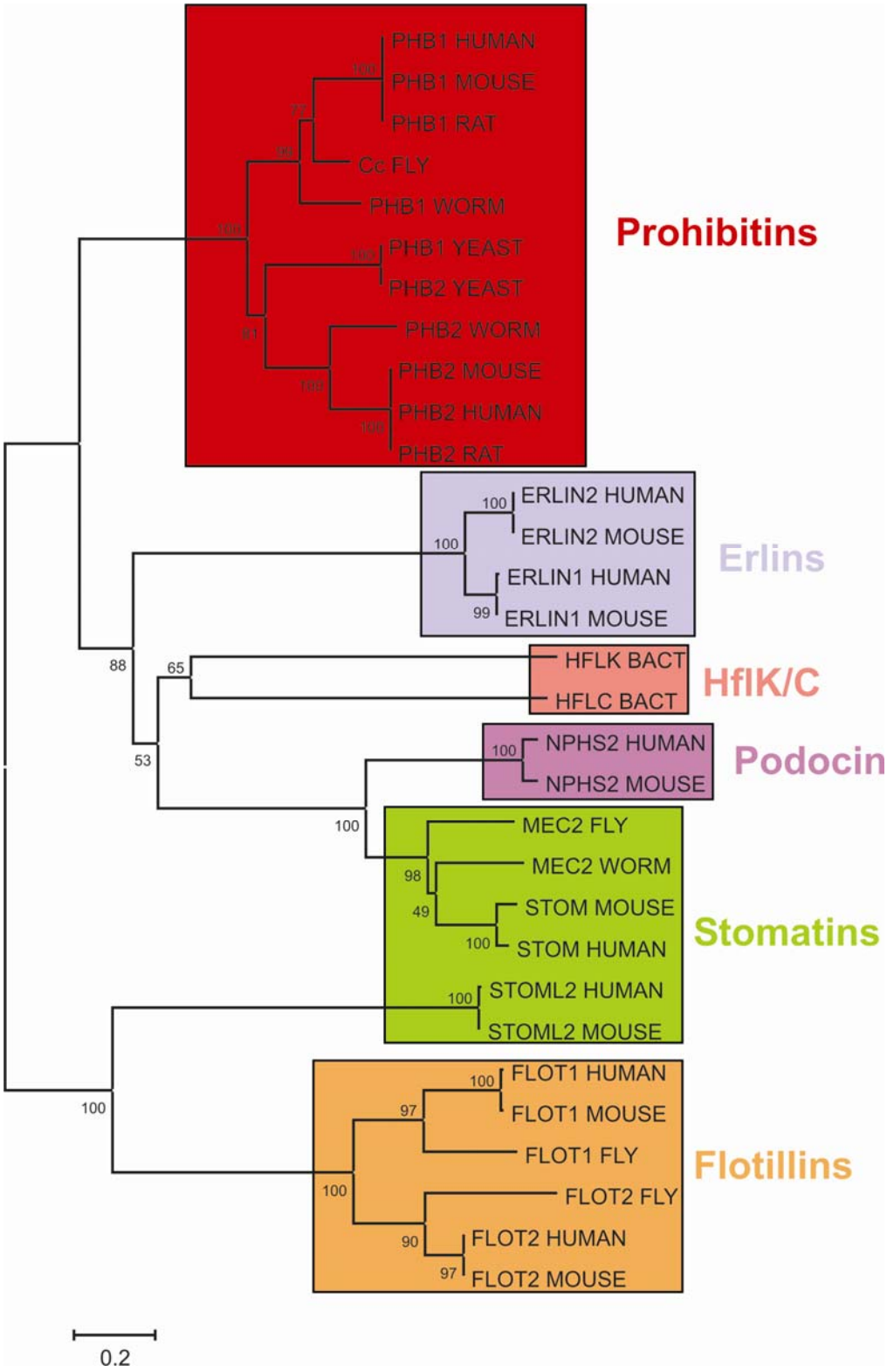

Figure 2. Evolutionary relationship among 31 SPFH-domain containing proteins.

Unrooted dendrogramm depicting the relationship of 31 representative SPFH-domain containing proteins inferred from the Neighbour-Joining method. The consensus tree is based on an amino acid sequence alignment. Protein clusters are coloured. Supporting bootstrap values are indicated at node positions. Phylogenetic analyses were conducted with MEGA4 software.

1.4 Prohibitins

1.4.1 Nomenclature of prohibitins

Prohibitins comprise a highly conserved and ubiquitously expressed protein family in eukaryotic cells (Figure 2). In accordance with their remarkable conservation and widespread distribution, prohibitins have been functionally associated with various cellular functions including cell cycle progression (McClung et al., 1989; Nuell et al., 1991), transcriptional regulation (Montano et al., 1999; Sun et al., 2004), cellular signalling (Terashima et al., 1994), cellular senescence (Coates et al., 1997; Coates et al., 2001; McClung et al., 1992), apoptosis (Fusaro et al., 2003; Vander Heiden et al., 2002), and mitochondrial biogenesis (Artal-Sanz et al., 2003; Berger and Yaffe, 1998; Nijtmans et al., 2000; Steglich et al., 1999). The family of prohibitin proteins consist of two homologous members, prohibitin-1 (PHB1) and prohibitin-2 (PHB2). Alternative names are B cell-receptor associated protein BAP32 and BAP37 (PHB1 and PHB2, respectively) (Terashima et al., 1994). PHB2 was also designated as prohibitone and REA (repressor of estrogen receptor activity) (Montano et al., 1999).

Prohibitin was initially discovered as an antiproliferative gene by differential hybridization to RNA isolated from regenerating rat liver (McClung et al., 1989). Subsequently, prohibitin has been proposed as a negative regulator of the cell cycle based on the observation that the microinjection of *Phb1* mRNA into mammalian cells blocks cell cycle progression (McClung et al., 1989) whereas injection of antisense oligonucleotides directed against *Phb1* mRNA stimulates cell proliferation (Nuell et al., 1991). Notably, the human *Phb1* gene was cloned and mapped to a region on chromosome 17 frequently mutated in familial breast cancer leading to the proposal that prohibitin could function as a tumour suppressor gene (Nuell et al., 1991; Sato et al., 1992; White et al., 1991). However, the interpretation of these results has been drawn into doubt, since subsequent studies revealed that these observations can be attributed solely to the 3´ untranslated region (3´UTR) of the prohibitin mRNA (Jupe et al., 1996; Manjeshwar et al., 2003).

1.4.2 Prohibitin distribution

Prohibitin homologues have been detected in various species from prokaryotic to eukaryotic organisms reflecting their widespread distribution and high degree of conservation

(Mishra et al., 2006; Nijtmans et al., 2002) (Figure 2). The bacterial proteins HflK and HflC are the prokaryotic homologues of prohibitins (Kihara et al., 1996). Two prohibitin members are present and extensively characterized in yeast (Berger and Yaffe, 1998). The *Drosophila melanogaster Cc* gene appears to be the PHB1 orthologue in flies and is required for normal larvae development (Eveleth and Marsh, 1986). Several prohibitin orthologues have also been identified in plants (De Diego et al., 2007; Snedden and Fromm, 1997). In mammals, the remarkable degree of sequence conservation is evident by the fact that amino acid sequences of rat and mouse prohibitin are identical and differ in only one amino acid from the human sequence. Moreover, yeast prohibitin-1 exhibits an identity of 52% to human PHB1 in its amino acid sequence. In multicellular organisms like *C. elegans* (Artal-Sanz et al., 2003) and mice (He et al., 2008; Park et al., 2005) prohibitins are also required for embryonic development suggesting an important function of these proteins which has been conserved throughout evolution.

1.4.3 Protein organization of prohibitins

Prohibitin-1 encodes a protein of 30 kDa, whereas prohibitin-2 gives rise to a 37 kDa protein. The PHB domain ranges from amino acids 26-187 in PHB1 and 39-201 in PHB2 (Rivera-Milla et al., 2006) (Figure 1).

The N-terminal domains of both PHB1 and PHB2 are involved in targeting the proteins to their intracellular localization and mediate membrane association (Berger and Yaffe, 1998). Both prohibitins harbour C-terminal coiled coil domains composed of antiparallel α-helices which are required for hetero-oligomerization (Tatsuta et al., 2005). Biochemical studies suggest complex formation of both PHB1 and PHB2 in various organisms (Coates et al., 1997; Steglich et al., 1999). Coimmunoprecipitation experiments verified an interaction between Phb1p and Phb2p in both yeast (Steglich et al., 1999) and mammalian cells (Coates et al., 1997). Moreover, a quantitative interaction of mammalian prohibitins was inferred from immunodepletion experiments (Coates et al., 2001). The homologous subunits are functionally interdependent in yeast, *C. elegans* and mammalian cells (Artal-Sanz et al., 2003; Berger and Yaffe, 1998; Kasashima et al., 2006). Hence, the genetic deletion of one prohibitin subunit results in the destabilization of the other, strongly suggesting that the prohibitin complex represents the physiologically active unit.

1.4.4 Cellular localization of prohibitins

The subcellular localization of prohibitins is still a matter of debate and controversial results have been obtained in mammalian cells. Although a mitochondrial localization of prohibitins has been widely accepted, PHB1 and PHB2 have also been identified in the nucleus, the cytosol and the plasma membrane in certain mammalian cell lines (Fusaro et al., 2003; Kasashima et al., 2006; Kurtev et al., 2004). In the nuclear compartment, subunits of prohibitins have been suggested to regulate cell cycle and apoptotic processes by interacting with retinoblastoma tumour suppressor protein, E2F transcription factors and p53 (Kasashima et al., 2006; Nuell et al., 1991; Wang et al., 2002a). It has been further proposed that prohibitins repress the transcriptional activity of estrogen receptors by interacting with histone deacetylases (Kurtev et al., 2004; Montano et al., 1999). Plasma membrane localized prohibitins are thought to modulate epithelial cell adhesion and migration by interacting with c-Raf (Rajalingam et al., 2005) or to function as cell-surface receptors (Kolonin et al., 2004; Mengwasser et al., 2004; Sharma and Quadri, 2004; Terashima et al., 1994). It should be noted, however, that many of these studies did not consider a mitochondrial localization of prohibitins or addressed only the function of either PHB1 or PHB2.

1.4.4.1 Complex assembly of prohibitins in mitochondria

The mitochondrial localization of prohibitins has been unambiguously demonstrated in the yeast *Saccharomyces cerevisiae.* In subcellular fractionation experiments, prohibitin subunits were found in association with the inner membrane (Berger and Yaffe, 1998). Multiple copies of both Phb1p and Phb2p assemble into a high molecular weight complex of 1.2 MDa in the inner membrane of mitochondria (Steglich et al., 1999). Interestingly, a prohibitin complex of similar size has also been detected in the mitochondrial inner membrane of *C. elegans* (Artal-Sanz et al., 2003) and mammalian cells (Nijtmans et al., 2000). The topology of the yeast prohibitin complex has been investigated by submitochondrial localization and protease treatment of mitoplasts (Steglich et al., 1999). Sodium carbonate treatment of isolated membrane fractions identified both Phb1p and Phb2p as integral components of the mitochondrial inner membrane (Berger and Yaffe, 1998). Furthermore, trypsin digestion of mitoplasts generated by osmotic disruption of the mitochondrial outer membrane revealed protease sensitivity of prohibitin subunits (Steglich et al., 1999). These findings suggests that the prohibitin complex is anchored to the inner

membrane via N-terminal transmembrane segments and exposes large C-terminal domains into the intermembrane space (IMS) of mitochondria (Steglich et al., 1999). It has been suggested that Phb1p and Phb2p are the only components of the complex since immunodepletion of one subunit results in the absence of the corresponding partner subunit (Coates et al., 2001). This is supported by overexpression studies in yeast demonstrating that only the simultaneous overexpression of both Phb1p and Phb2p leads to an increase in the PHB complex (Nijtmans et al., 2000). Mitochondrial targeting of prohibitins has been analysed by *in vitro* import studies in yeast (Tatsuta et al., 2005). These results suggest that the N-terminal domains of both Phb1p and Phb2p are required for mitochondrial import. Sorting of Phb1p to mitochondria is ensured by an unconventional presequence consisting of the first 28 amino acids. In contrast, Phb2p possesses a bipartite N-terminal presequence in amino acids 1-61 composed of the positively charged N-terminal domain followed by a hydrophobic transmembrane segment (Tatsuta et al., 2005). Neither Phb1p nor Phb2p are processed during mitochondrial import (Tatsuta et al., 2005). Crosslinking experiments revealed an association of newly imported Phb1p with the Tim8/Tim13 complex, a soluble, 70 kDa complex in the IMS that functions in the biogenesis of inner and outer membrane proteins (Curran et al., 2002; Davis et al., 2000; Hoppins and Nargang, 2004; Leuenberger et al., 1999; Paschen et al., 2000). The association with the Tim8/Tim13 complex may trap newly imported Phb1p in the IMS and facilitate its transfer to the inner membrane. The subsequent insertion into the inner membrane depends on the membrane potential and is mediated by the Tim23 translocase. In addition, crosslinking revealed that newly imported Phb1p assembles with Phb2p subunits into a subcomplex of ~120 kDa suggesting the presence of membrane-bound assembly intermediates during prohibitin complex formation (Tatsuta et al., 2005) (Figure 3).

Chemical crosslinking experiments and structural models predicted a ring-like assembly of alternating Phb1p and Phb2p subunits (Back et al., 2002). Supported by the finding that crosslinks appeared only within different prohibitin subunits (Back et al., 2002), it has been proposed that heterodimers composed of Phb1p and Phb2p are the building blocks of the prohibitin complex (Figure 3). The native molecular mass of the assembled prohibitin complex of ~ 1 MDa allowed the characterization by single particle electron microscopy. Large, ring-shaped complexes with an average diameter of ~ 200 Å were detected in images acquired from purified yeast prohibitin complexes (Tatsuta et al., 2005). These results indicate that prohibitins form large, ring-shaped complexes in the mitochondrial inner membrane

which are composed of ~ 16-20 alternating subunits of both Phb1p and Phb2p (Back et al., 2002; Tatsuta et al., 2005) (Figure 3).

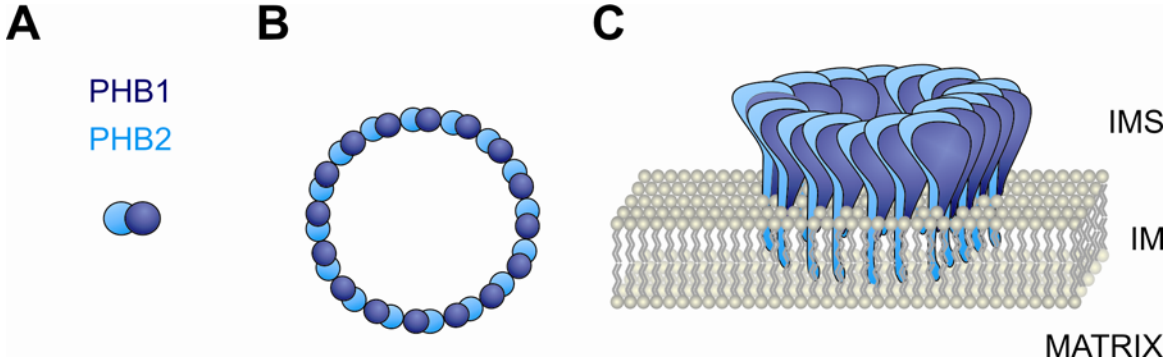

Figure 3. Complex assembly of prohibitin subunits in mitochondria.

Schematic representation of prohibitin subunits PHB1 and PHB2, the ring-shaped prohibitin complex and its topology in the mitochondrial inner membrane. **(A)** Dimeric assembly intermediates composed of PHB1 and PHB2 constitute the building block of the complex. **(B)** Circular prohibitin ring complex with alternating subunit composition. The average stoichiometry of the complex is speculative. **(C)** The prohibitin complex is anchored to the mitochondrial inner membrane via N-terminal transmembrane segments of PHB2. The tightly folded C-terminal coiled-coil domains are exposed to the intermembrane space (IMS). IM = inner membrane.

1.4.4.2 Regulatory roles of the mitochondrial prohibitin complex

To determine the physiological role of prohibitins *in vivo*, *PHB1* and *PHB2* have been extensively characterized using genetic approaches in the yeast *Saccharomyces cerevisiae*. Notably, the genetic ablation of either *PHB1* or *PHB2* does not result in apparent phenotypes (Berger and Yaffe, 1998). Neither single- nor double-mutant *phb1-null* and *phb2-null* cells exhibit growth defects on various carbon sources indicating respiratory competence in the absence of prohibitins. Moreover, mitochondrial morphology and distribution is not affected by the loss of prohibitins (Berger and Yaffe, 1998). Interestingly, prohibitins have been linked to ageing (Jazwinski, 1996; Kirchman et al., 2003). Several reports demonstrated reduced replicative lifespan, but unaffected chronological lifespan of prohibitin-deficient yeast cells (Berger and Yaffe, 1998; Coates et al., 1997; Piper and Bringloe, 2002). Furthermore, yeast lifespan was dramatically reduced in prohibitin-deficient yeast cells lacking the mitochondrial genome (*rho*^{0}) suggesting additive effects which are deleterious for survival (Berger and Yaffe, 1998; Kirchman et al., 2003). Remarkably, these phenotypes were suppressed by

genetic ablation of *RAS2* in these strains suggesting a cooperative function of prohibitins and Ras2p in yeast longevity (Kirchman et al., 2003).

Deletions of prohibitin genes are lethal in combination with a number of mutations indicating strong genetic interactions. For instance, prohibitins genetically interact with components required for mitochondrial inheritance in yeast (Berger and Yaffe, 1998). Genetic ablation of *PHB1* or *PHB2* combined with mutations in genes encoding the mitochondrial inheritance components Mdm12p, Mdm10p and Mmm1p results in synthetic lethality (Berger and Yaffe, 1998). It has been proposed that Mdm12p, Mdm10p and Mmm1p form a complex which links mitochondria to the actin cytoskeleton thereby ensuring mitochondrial movement and distribution (Boldogh et al., 1998; Boldogh et al., 2003). More recent findings suggested a new role for these components in the biogenesis of mitochondrial outer membrane proteins (Meisinger et al., 2007; Meisinger et al., 2004). Additionally, this complex is required for the assembly and stability of mitochondrial DNA nucleoids (Hanekamp et al., 2002; Hobbs et al., 2001), indicating a function as membrane-embedded machinery for simultaneous inheritance of mitochondria and mitochondrial DNA (mtDNA) (Boldogh et al., 2003). These genetic interactions suggest that prohibitins might function in the regulation of mitochondrial morphology and distribution (Berger and Yaffe, 1998). This is consistent with the finding that the genetic deletion of prohibitins in cells lacking the mitochondrial genome causes mitochondrial fragmentation and disorganization (Berger and Yaffe, 1998). Moreover, synthetic lethality of prohibitins has also been observed with mutations in the phosphatidylethanolamine biosynthetic pathway, pointing to a role of prohibitins in the phospholipid organization of mitochondrial membranes (Birner et al., 2003).

In addition, the simultaneous loss of the prohibitin complex with another integral component of the mitochondrial inner membrane, the *m*-AAA protease, strongly impairs cell growth (Steglich et al., 1999). The *m*-AAA protease, a conserved ATP-dependent metalloprotease belonging to the family of AAA^+ proteins (ATPases associated with a variety of cellular activities) (Ogura and Wilkinson, 2001), is composed of the homologous subunits Yta10p (Afg3p) and Yta12p (Rca1p) and is part of a protein quality control system in mitochondria (Arlt et al., 1996; Tatsuta and Langer, 2008). The *m*-AAA protease forms a high molecular mass complex of ~ 850 kDa in the mitochondrial inner membrane exposing catalytic subunits into the matrix (Arlt et al., 1996; Leonhard et al., 1996). Notably, the biochemical characterization revealed the presence of a high molecular mass supercomplex composed of both the prohibitin complex and the *m*-AAA protease (Steglich et al., 1999). This supercomplex eluted in fractions corresponding to a molecular mass of approximately 2

MDa in gelfiltration experiments (Steglich et al., 1999). Interestingly, the absence of Phb1p or Phb2p in mitochondria causes an accelerated proteolysis of nonassembled inner membrane proteins by the *m*-AAA protease suggesting a negative regulatory effect of prohibitins on the *m*-AAA protease activity (Steglich et al., 1999). A role of the prohibitin complex in the degradation of mitochondrial inner membrane proteins by the *m*-AAA protease is reminiscent of findings in prokaryotes. In *E. coli*, the activity of the homologous AAA protease FtsH was found to be negatively regulated by the HflK/C protein complex (Kihara et al., 1996; Kihara et al., 1997). These consistent findings suggest an evolutionary conservation of the regulatory mechanisms controlling AAA protease activities.

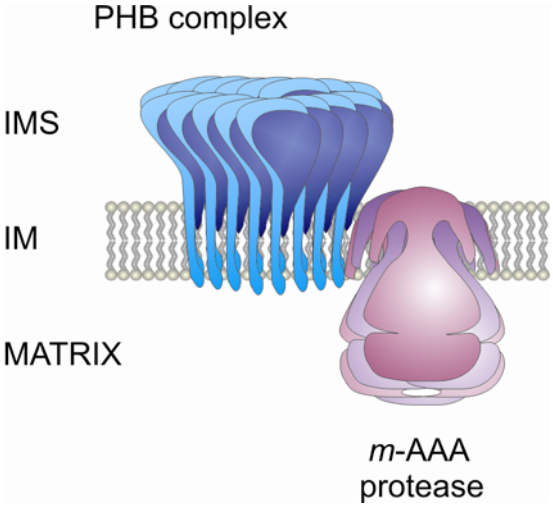

Figure 4. Supercomplex of prohibitins with the ATP-dependent *m*-AAA protease.

Schematic representation of supercomplex assembly of prohibitins (PHB) with the ATP-dependent *m*-AAA protease in the mitochondrial inner membrane. See text for details. IMS = intermembrane space, IM = inner membrane.

In further investigations, Nijtmans and coworkers proposed a chaperone-function for the yeast prohibitin complex. In the absence of prohibitins, mitochondrial translation products accumulated to a reduced amount as determined by ^{35}S-methionine pulse-chase labelling in isolated mitochondria (Nijtmans et al., 2000). Consistent with this finding, the overexpression of prohibitins results in a stabilization of these translation products. This observation was accompanied by decreased steady-state levels of the mitochondrially encoded proteins Cox2p and Cox3p in two-dimensional gel electrophoresis. Moreover, the authors could demonstrate a specific interaction of Cox2p and Cox3p with the prohibitin complex in coimmunoprecipitations (Nijtmans et al., 2000). These observations suggest that the

prohibitin complex interacts with and stabilizes newly synthesized mitochondrial translation products. Based on initial observations obtained from yeast, prohibitins might have a role in the regulation of mitochondrial morphology. An abnormal mitochondrial morphology was observed in the nematode *C. elegans* after RNAi-mediated depletion of prohibitins (Artal-Sanz et al., 2003). Indeed, these findings were corroborated in studies with mammalian cells. RNAi-mediated knockdown of either *Phb1* or *Phb2* in HeLa cells caused fragmentation of the mitochondrial network and spontaneous apoptosis (Kasashima et al., 2006). Recently, knockdown of *Phb1* in epithelial cells has been reported to induce cellular senescence associated with mitochondrial dysfunction (Schleicher et al., 2008). Interestingly, the authors provide the explanation that PHB1 deficiency causes an increased production of reactive oxygen species (ROS) through inhibition of complex I which leads to depolarization of the mitochondrial membrane potential and cellular senescence (Schleicher et al., 2008). Previously, PHB1 has also been suggested as a component of mitochondrial nucleoids, nucleoprotein complexes associated with mitochondrial DNA (mtDNA) (Bogenhagen et al., 2003). Proteins incorporated in these nucleoid complexes such as mitochondrial transcription factor A (TFAM) and mitochondrial single-strand DNA binding (mtSSB) protein regulate stability, packaging, replication, transcription and maintenance of mtDNA (Chen and Butow, 2005). A very recent study reports a role of prohibitin in the organization and maintenance of mtDNA (Kasashima et al., 2008). Remarkably, downregulation of PHB1 in HeLa cells affects the organization of mitochondrial nucleoids and the steady-state level of TFAM protein. These findings suggest that PHB1 maintains organization and copy number of mtDNA by regulating TFAM stability (Kasashima et al., 2008).

1.4.4.3 Regulation of gene expression by nuclear localized prohibitins

Despite the general acceptance of a predominant localization of prohibitins to mitochondria, several reports claim that either PHB1 or PHB2 also localize to the nuclear compartment (Fusaro et al., 2003; Gamble et al., 2004; Kurtev et al., 2004; Wang et al., 2002a). Nuclear localization of PHB1 was proposed based on colocalization with retinoblastoma protein (Rb), p53 and E2F transcription factors in various cell lines (Fusaro et al., 2003; Wang et al., 2002aa). The majority of human tumours, including breast and prostate cancer, are associated with mutations directly affecting Rb and p53, or components of pathways that regulate these proteins. The tumour suppressor protein Rb and its family

members have been demonstrated to modulate the G1-S phase transition during the mammalian cell cycle (Ewen, 1994). E2F transcription factors (E2F1-5) are the major downstream targets of Rb which exerts its growth suppressive effect through E2F inhibition (Weinberg, 1995). In accordance with a putative tumour suppressor function, PHB1 has been shown to interact directly with Rb and p53 to induce their antiproliferative and cell cycle-regulatory activities (Fusaro et al., 2003; Wang et al., 1999a). The association of PHB1 with retinoblastoma family members (Rb, p107 and p130) results in the inhibition of cell proliferation through repression of E2F transcription factors (Wang et al., 1999a). Consistently, a block in cell proliferation was observed after ectopic expression of PHB1 which inhibited E2F-mediated transcription (Wang et al., 1999b). Remarkably, PHB1 was also found to physically interact with E2F and to inhibit transcription from E2F-responsive promoters suggesting that prohibitin modulates E2F activity also in a Rb-independent manner (Wang et al., 1999b). This is supported by the observation that PHB1 and Rb bind to different regions of E2F (Wang et al., 1999b). Moreover, PHB1-mediated repression of E2F does not respond to signalling activity which reverses Rb-mediated repression (Wang et al., 1999b). Additional studies demonstrated that transcriptional repression by PHB1 involves the recruitment of the co-repressor N-CoR and chromatin-remodelling complexes like HDAC1 and Brg-1/Brm to promoter elements (Wang et al., 2002a; Wang et al., 2002b; Wang et al., 2004). Interestingly, the opposite effect on PHB1-mediated regulation of transcription was observed for p53. Fusaro and colleagues demonstrated a physical interaction of PHB1 and p53 required for the stimulation of transcription from p53-responsive promoters (Fusaro et al., 2003). The role of PHB1 as a putative modulator of gene transcription raised the intriguing question whether this function can also be attributed to PHB2, since compelling evidence approved the physiological relevance of a mammalian PHB1/2 complex (Coates et al., 2001).

Interestingly, independent studies revealed that PHB2 also acts as a transcriptional regulator in the nucleus (Montano et al., 1999; Sun et al., 2004). PHB2 was identified in a yeast two-hybrid screen as a novel regulator of the estrogen receptor (ER) and subsequently designated as Repressor of estrogen receptor activity (REA) (Montano et al., 1999). Expression studies demonstrated that PHB2 selectively represses transcription from ER-responsive promoters through direct binding of nuclear ER and recruitment of HDAC1 (Kurtev et al., 2004). Recent findings also suggest a functional interaction between ER signalling and PHB1-mediated repression of E2F (Wang et al., 2004). The repressive function in the estrogen signalling cascade has been substantiated for both PHB1 and PHB2 *in vivo* demonstrating the physiological relevance of this regulatory action (He et al., 2008; Mussi et

al., 2006; Park et al., 2005). An additional mode of repression was proposed that involves competitive binding of PHB2 and the transcriptional co-activator SRC-1 to the ligand-activated ER (Delage-Mourroux et al., 2000). It has been reported that, besides associating with the ER, PHB2 binds to additional members of the nuclear receptor family, like COUP-TFI and –TFII, suggesting a role as a general co-repressor (Kurtev et al., 2004). In accordance with these findings, PHB2 also interacted with the myogenic regulatory factors MyoD and MEF, thereby negatively regulating transcription from MyoD- and MEF2-dependent promoters, implying a role for PHB2 in myogenic differentiation (Sun et al., 2004).

Increasing data have emerged that propose a role in transcriptional regulation for both PHB1 and PHB2. Remarkably, both proteins act as transcriptional repressors and might interact with HDAC1 to execute their function. It remains unclear, however, whether both prohibitin proteins act as a complex to mediate transcriptional repression.

1.4.4.4 Functions of plasma membrane-localized prohibitins

Prohibitins have also been localized to the plasma membrane in several studies. An association of both PHB1 and PHB2 has been identified with the IgM isoform of the B-cell receptor (BCR) on the plasma membrane of B lymphocytes (Terashima et al., 1994). In this context, PHB1 and PHB2 were designated BAP32 and BAP37 (B-cell receptor associated protein), respectively. This association was assumed to influence BCR-mediated signal transduction and potentially regulate lymphocyte proliferation and differentiation after stimulation of either the IgM or the IgD antigen receptor (Terashima et al., 1994). More recent studies provided evidence for a receptor function of prohibitins at the plasma membrane (Kolonin et al., 2004; Sharma and Quadri, 2004). Kolonin and co-workers identified a peptide by *in vivo* phage display that was specifically targeted to the white fat vasculature in *ob/ob* mice. Surprisingly, this peptide was found to associate with PHB1 (Kolonin et al., 2004). The covalent attachment of a proapoptotic peptide to this peptide was selectively targeted to the adipose tissue vasculature and led to induction of apoptosis. A receptor function for membrane localized PHB1 was inferred by the selective binding of bead-coupled peptide to *in vitro* biotinylated PHB1 (Kolonin et al., 2004). Most recently, a receptor role for Phb1 was reinforced by the finding that a capsular polysaccharide (Vi), characterized as the virulence antigen of *Salmonella typhi,* interacts with the prohibitin complex on the surface of a human intestinal epithelial cell line (Sharma and Quadri, 2004).

In this study, a localization of PHB1 and PHB2 to the plasma membrane of this cell line was demonstrated by surface biotinylation of both proteins and immunofluorescence studies. Notably, both PHB1 and PHB2 were present in fractions enriched with lipid rafts after sucrose gradient centrifugation (Sharma and Quadri, 2004). Furthermore, both prohibitin homologues were identified in a novel assay that allows detection of tumour derived antigens (Mengwasser et al., 2004). For this purpose human colorectal tumour cells were subcutaneously injected into immuno-incompetent mice. Differential immunization identified PHB1 and PHB2 as serum-borne tumour antigens which were accessible to surface biotinylation and detected in whole cell ELISA assays. Immunofluorescence studies supported plasma membrane localization of PHB1 and PHB2 in colorectal cancer cells. These results suggested that prohibitins are partially membrane localized in colorectal tumour cells and might be released into the serum by secretion or shedding of the components (Mengwasser et al., 2004).

An unexpected role for plasma-membrane localized PHB1 in the activation of the Raf/MEK/ERK pathway has recently been demonstrated (Rajalingam et al., 2005). PHB1 directly interacts with C-Raf and mediates Ras-dependent displacement of 14-3-3 protein from C-Raf. PHB1 thereby facilitates plasma membrane localization of C-Raf and enhances its activation by promoting PP2A-mediated phosphorylation (Rajalingam et al., 2005). The investigators propose that PHB1 might act as plasma membrane scaffold that ensures Ras-Raf interaction *in vivo* (Rajalingam and Rudel, 2005). These observations suggest a function of PHB1 in the modulation of epithelial cell adhesion and migration, critical events in the development of malignant transformation.

1.5 Mitochondrial fusion

The highly dynamic morphology of mitochondria depends on the tissue, on the physiological condition of the cell and in particular on the functional status of the organelle (Detmer and Chan, 2007; Hoppins et al., 2007; McBride et al., 2006; Okamoto and Shaw, 2005). This dynamic behaviour is crucial for a number of cellular processes, such as apoptosis, the inheritance of mtDNA, defence against oxidative stress and development through spermatogenesis (Cereghetti and Scorrano, 2006; Chen and Chan, 2005; Chen and Butow, 2005; Hales and Fuller, 1997). Mitochondrial morphology is regulated by opposing but balanced fusion and fission events, which maintain the normal mitochondrial network

(Okamoto and Shaw, 2005). Loss of fusion results in mitochondrial fragmentation due to ongoing fission events. Conversely, loss of fission leads to the formation of elongated and highly interconnected mitochondria. The central components of the mitochondrial fusion and fission machineries have been identified, but the molecular mechanisms of both processes are not completely understood. Most proteins involved are conserved in yeast, flies, mice and humans, indicating that the fundamental mechanisms controlling mitochondrial dynamics have been maintained during evolution (Okamoto and Shaw, 2005).

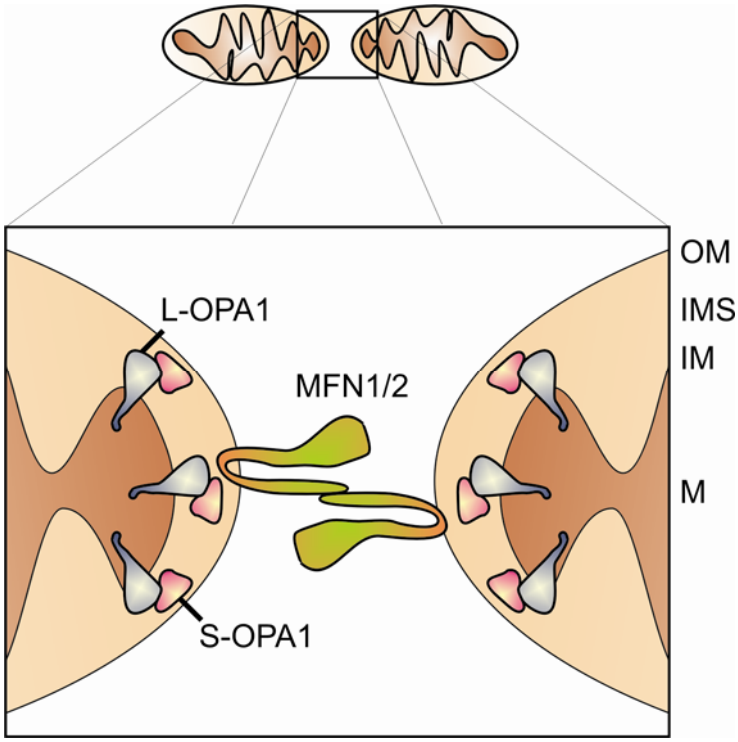

Figure 5. Fusion components of mammalian mitochondria.

Schematic representation of mitochondrial fusion in mammalian cells. Two mitochondria tether through coiled-coil domains of mitofusins (MFN1/2) that are anchored to the mitochondrial outer membrane (OM). L-OPA1 resides in the inner membrane whereas S-OPA1 localizes to the IMS. Both OPA1 isoforms participate in the fusion process. IMS = intermembrane space, IM = inner membrane; M = matrix. Modified from (Youle and Karbowski, 2005).

Mitochondrial fusion includes the coordinated fusion of the outer and the inner membranes of two organelles (Figure 5). Three proteins are essential for fusion in yeast and interact with each other to form a fusion complex: the two dynamin-related GTPases Fzo1p (Hales and Fuller, 1997) and Mgm1p (Sesaki et al., 2003; Wong et al., 2000) as well as Ugo1p (Sesaki and Jensen, 2001; Sesaki and Jensen, 2004). Fzo1p is integrated into the outer

membrane exposing its functional domains to the cytoplasm (Fritz et al., 2001; Hermann et al., 1998; Rapaport et al., 1998). Mgm1p, on the other hand, resides in the intermembrane space but is present in two forms, both of which are required for fusion (Herlan et al., 2003; Sesaki et al., 2003; Wong et al., 2000; Wong et al., 2003): the large (l-) isoform, which is integrated into the inner membrane, and the short (s-) isoform, which is peripherally associated with inner and/or outer membrane and generated by proteolytic processing of the l-form, as discussed below. The third protein, Ugo1p, is also embedded into the outer membrane and exposes its N-terminal domain to the cytoplasm and the C-terminal domain to the intermembrane space (Sesaki and Jensen, 2001). Ugo1p has been shown to bind to both Fzo1p and Mgm1p via its N- and C-terminal domains, respectively, thus linking the fusion components of the outer and inner membrane and possibly the fusion events of both membranes (Sesaki and Jensen, 2004). Outer and inner membrane fusion events are tightly coupled *in vivo*, but could be separated by the reconstitution of fusion *in vitro* (Meeusen et al., 2004). Furthermore, these *in vitro* studies revealed that Fzo1p is required for outer membrane fusion, whereas inner membrane fusion depends on Mgm1p (Meeusen et al., 2006; Meeusen et al., 2004). For both mitochondrial membranes, *trans* interactions of either Fzo1p (Griffin and Chan, 2006) or Mgm1p (Meeusen et al., 2006) mediate tethering of opposing membranes and subsequent fusion.

1.5.1 Mitofusins and Charcot-Marie-Tooth Disease

Components involved in mitochondrial fusion are highly conserved throughout evolution. In the mammalian system, mitofusin (MFN) 1 and 2 represent the homologues of Fzo1, whereas OPA1 is the homologue of Mgm1p (Alexander et al., 2000; Delettre et al., 2000; Rojo et al., 2002; Santel and Fuller, 2001). Interestingly, Ugo1p does not have an obvious counterpart in mammals. The localisation and topology of mitofusins and OPA1 in the outer membrane and intermembrane space, respectively, has been conserved in mammals (Griparic et al., 2004; Rojo et al., 2002). Biochemical data indicate that mitofusins can form both homo- and hetero-oligomeric complexes all competent for fusion (Chen et al., 2005; Chen et al., 2003; Eura et al., 2003). Importantly, mitofusins are required on adjacent mitochondria during the fusion process, implying that they form complexes in trans between apposing mitochondria (Meeusen et al., 2004). Structural data indicate that a heptad repeat region of MFN1 forms an antiparallel coiled coil that mediates tethering of mitochondria in trans during the fusion process (Koshiba et al., 2004) (Figure 5). The essential role of

mitofusins in mitochondrial fusion has been established through the generation of knockout mice and by knockdown experiments using RNA interference (RNAi). The absence of either MFN1 or MFN2 causes embryonic lethality in mice (Chen et al., 2003). Isolated mouse embryonic fibroblasts (MEF) exhibit greatly reduced levels of mitochondrial fusion leading to a highly fragmented mitochondrial population (Chen et al., 2005; Chen et al., 2003). Moreover, a dissipation of the membrane potential was observed in a small subset of mitochondria in both *Mfn1*- and *Mfn2*-deficient cells, resulting in a heterogenous cell population (Chen et al., 2005). Residual levels of mitochondrial fusion in single knockout cells might therefore prevent major cellular dysfunction, suggesting that homotypic interactions of MFNs are capable to maintain residual fusion activity (Chen et al., 2003). In contrast, cells devoid of both MFN1 and MFN2 completely lacked mitochondrial fusion and showed severe cellular defects, including reduced cell growth, widespread heterogeneity of mitochondrial membrane potential and decreased cellular respiration (Chen et al., 2005). The close interplay between the two mitofusins is further supported by complementation studies which highlight the functional importance of MFN1-MFN2 hetero-oligomeric complexes (Detmer and Chan, 2007). Taken together, these findings implicate similar roles for MFN1 and MFN2 in mitochondrial fusion and suggest a functional redundancy among these proteins. However, this conclusion has been challenged by several lines of evidence obtained from both *in vitro* cell culture work as well as genetic data *in vivo*. Notably, cells containing only MFN2 (*Mfn1*-null) appear to have less fusion activity compared to *Mfn2*-deficient cells (Chen et al., 2003). The analysis of kinetic properties additionally revealed differences in the GTPase activity of MFN1 and MFN2 (Ishihara et al., 2004). Furthermore, *Mfn1*, but not *Mfn2*, genetically interacts with OPA1 in the control of mitochondrial fusion (Cipolat et al., 2004). Differences were also observed in the analysis of mitofusin-deficient embryos *in vivo*. *Mfn2*-null embryos die in utero due to defects in the trophoblast giant cells in the placenta which is distinguishable from *Mfn1*-deficient embryos (Chen et al., 2003). These observations indicate that the role of mitofusins in mitochondrial fusion might not be completely redundant and suggest an additional regulatory role for MFN2.

Interestingly, mutations in *Mfn2* are causative for an autosomal dominant peripheral neuropathy, termed Charcot-Marie-Tooth type 2A (CMT2A), which is characterized by muscle weakness and sensory loss in the distal limbs (Züchner et al., 2004). The pathological symptoms are caused by the loss of motor axons due to neurodegeneration. The disease-causing mechanism of *Mfn2* mutations in CMT2A is unclear. However, it has recently been suggested that the disease might be the consequence of reduced axonal movement of

mitochondria (Baloh et al., 2007). Remarkably, the conditional ablation of *Mfn2* specifically in cerebellar purkinje cells leads to altered distribution and reduced movement of neuronal mitochondria (Chen et al., 2007). The clinical relevance of mutated *Mfn2* is further highlighted in a transgenic mouse model expressing a pathogenic $Mfn2^{T105M}$ allele specifically in motor neurons (Detmer et al., 2008). Transgenic mice develop clinical signs of CMT2A disease including a severe reduction of distal muscles, loss of motor axons and clustering of neuronal mitochondria (Detmer et al., 2008). These observations underscore the physiological importance of MFN2 in the control of mitochondrial fusion and neuronal survival (Chen and Chan, 2006).

1.5.2 OPA1 and dominant optic atrophy

Mammalian OPA1 has been originally identified by mutations causative for autosomal dominant optic atrophy (ADOA) type I, the most common form of inherited optic neuropathy (Alexander et al., 2000; Delettre et al., 2000). A degeneration of retinal ganglion cells leading to atrophy of the optic nerve is the underlying defect of this disease (Delettre et al., 2002). Thus, optic atrophy is also characterised by an intriguing tissue-specificity, although the OPA1 mRNA and protein are widely distributed in mammalian tissues including various brain areas (Aijaz et al., 2004; Alexander et al., 2000; Bette et al., 2005; Delettre et al., 2000; Misaka et al., 2002; Olichon et al., 2002).

OPA1 is a key player in regulating dynamic changes of mitochondrial morphology by promoting mitochondrial fusion (Cipolat et al., 2004; Olichon et al., 2003). Accordingly, downregulation of OPA1 results in fragmentation of the mitochondrial network owing to ongoing fission (Chen et al., 2005; Cipolat et al., 2004; Griparic et al., 2004; Olichon et al., 2003). In addition, cells depleted of OPA1 show an aberrant cristae structure (Griparic et al., 2004; Olichon et al., 2003), which is reminiscent of the role of yeast Mgm1p in maintenance of cristae morphology (Amutha et al., 2004; Meeusen et al., 2006; Sesaki et al., 2003). Further phenotypes that have been linked to loss of OPA1 are reduction of mitochondrial membrane potential, defects in mitochondrial respiration, accelerated release of cytochrome *c* and concomitantly increased propensity for apoptosis (Arnoult et al., 2005; Chen et al., 2005; Lee et al., 2004; Olichon et al., 2003). Thus, OPA1 seems to have additional functions in mitochondria which are independent from mitochondrial fusion. Indeed, evidence has been obtained for a direct role of OPA1 in the control of cristae remodelling during apoptosis and

release of cytochrome *c* which is sequestered in the intra-cristae regions (Cipolat et al., 2006; Frezza et al., 2006).

OPA1 is a dynamin-like GTPase which resides in the mitochondrial intermembrane space (Griparic et al., 2004; Olichon et al., 2002; Satoh et al., 2003) and features the following domain structure: an N-terminal mitochondrial targeting sequence is followed by one transmembrane segment (TM1) and two further hydrophobic stretches (TM2a and TM2b). A coiled-coil region (or heptad repeat) is located immediately in front of the GTPase domain containing the consensus GTP binding sites. The GTPase domain is followed by a middle domain and a C-terminal coiled-coil domain, known as the GTPase effector domain, which both are generally involved in oligomerisation and regulation of GTPase activity (Praefcke and McMahon, 2004). The mRNAs transcribed from the *OPA1* gene are subject to extensive alternative splicing, resulting in eight different splice variants (Delettre et al., 2001; Satoh et al., 2003). The alternative splicing involves the exons 4, 4b, and 5b which encode protein segments of the N-terminal region between the first transmembrane domain and the GTPase domain. Therefore, OPA1 splice variants differ in the presence or absence of the two additional hydrophobic stretches TM2a and TM2b. In addition, the different splice variants are not uniformly expressed in human tissues and appear to have distinct roles in mitochondria such as mitochondrial fusion and apoptosis (Delettre et al., 2001; Olichon et al., 2007). Several protein isoforms of OPA1 can be detected in human cells, e.g. five different isoforms are apparently present in HeLa cells (Ishihara et al., 2006; Olichon et al., 2003; Olichon et al., 2007), which have been designated L1 and L2 for the two larger isoforms and S3, S4, and S5 for the three smaller isoforms (Duvezin-Caubet et al., 2006). The large and small OPA1 isoforms differ in their membrane association. Whereas the L-isoforms are anchored to the inner membrane, S-isoforms are only peripherally attached to the membrane (Duvezin-Caubet et al., 2006).

Different OPA1 isoforms are generated by alternative splicing and/or proteolytic processing similar to yeast Mgm1p. Evidence for the involvement of proteolysis has recently been obtained by the observation that L-isoforms were converted into S-isoforms upon dissipation of the mitochondrial membrane potential (Duvezin-Caubet et al., 2006; Ishihara et al., 2006). This induced conversion was abolished in the presence of *o*-phenanthroline indicating proteolytic cleavage by metalloproteases. Moreover, this apparent OPA1 processing was accompanied by mitochondrial fragmentation, thus linking mitochondrial dysfunction to changes in mitochondrial morphology (Duvezin-Caubet et al., 2006; Ishihara et al., 2006). Large OPA1 isoforms are generated upon mitochondrial import by MPP (Ishihara

et al., 2006). However, the protease mediating further OPA1 cleavage has not yet been unambiguously identified. Due to the homology of Mgm1p and OPA1, the rhomboid protease PARL (presenilin-associated rhomboid-like) (Pellegrini et al., 2001), the mammalian homologue of yeast Pcp1p, has been implicated in OPA1 processing. PARL can functionally replace yeast Pcp1 and mediate processing of Ccp1p and Mgm1p (McQuibban et al., 2003). Furthermore, the analysis of *Parl*-deficient mice revealed a protective role of PARL against mitochondria-dependent apoptosis in a pathway which also depends on OPA1 (Cipolat et al., 2006). In support of proteolysis by PARL, it was found to interact with OPA1 and to be involved in the generation of small amounts of a soluble intermembrane space form of OPA1. Deletion of *Parl*, however, did not significantly impair the pattern of OPA1 isoforms (Cipolat et al., 2006). The mammalian *m*-AAA protease has also been linked to OPA1 processing, since overexpression of its subunit paraplegin in HeLa cells led to an increased accumulation of S-isoforms (Ishihara et al., 2006). Moreover, processing of human OPA1 by mammalian *m*-AAA proteases has been observed upon reconstitution in yeast (Duvezin-Caubet et al., 2007).

1.5.3 Regulation of mitochondrial dynamics

In yeast, two components of the mitochondrial fusion machinery, Fzo1p and Mgm1p, are under proteolytic control. In the case of Fzo1p, the maintenance of mitochondrial morphology depends on the tight control of its steady-state concentration, which is regulated by degradation of Fzo1p via two independent proteolytic pathways. On the one hand, Fzo1p turnover can be induced by cell cycle arrest with the mating factor alpha which leads to mitochondrial fragmentation (Neutzner and Youle, 2005). This induced degradation of Fzo1p is mediated by the ubiquitin-proteasome system (UPS) (Escobar-Henriques et al., 2006), the central proteolytic system in the cytosol of eukaryotic cells (Ciechanover, 2005). On the other hand, Fzo1p is degraded in vegetatively growing yeast cells in a constitutive manner which depends on the F-box protein Mdm30p (Escobar-Henriques et al., 2006). In the absence of Mdm30p, the steady-state concentration of Fzo1p is increased and yeast cells accumulate aggregated and fragmented mitochondria (Fritz et al., 2003). F-box proteins often assemble into Skp1-Cdc53-F-box (SCF) E3 ubiquitin ligase complexes which ensure ubiquitin-dependent degradation by the 26S proteasome (Petroski and Deshaies, 2005; Willems et al., 2004). However, Mdm30p-dependent turnover of Fzo1p does not involve SCF complexes or

the UPS, but rather proceeds along an alternative proteolytic pathway which remains to be identified (Escobar-Henriques et al., 2006).

In contrast to Fzo1p, Mgm1p is not subject to complete degradation but is proteolytically processed at its N-terminus yielding two isoforms within the mitochondrial intermembrane space: the large (l-) and the short (s-) isoform. Both Mgm1p isoforms are required for mitochondrial fusion and their balanced formation appears to be crucial for mitochondrial morphology (Herlan et al., 2003). According to the alternative topogenesis model, the ratio of both isoforms is dictated by the level of matrix ATP (Herlan et al., 2004). The Mgm1 protein contains two N-terminal transmembrane segments of which the first one serves as a stop-transfer signal during mitochondrial import of the pre-protein. At low ATP levels, the subsequent removal of the MTS by MPP and lateral membrane insertion result in l-Mgm1p, which is anchored to the inner membrane. At high ATP levels, however, Mgm1p can be pulled further into the matrix by the ATP-dependent mitochondrial import motor until the second hydrophobic segment reaches the inner membrane. Thereby, a second cleavage site within this segment gets accessible for the rhomboid protease Pcp1p in the inner membrane. Pcp1p-mediated cleavage generates s-Mgm1p (Herlan et al., 2003; McQuibban et al., 2003; Sesaki et al., 2003), which is peripherally associated with the inner and/or outer membrane. This mechanism may link the bioenergetic state of mitochondria and their morphology. Furthermore, it would allow separating damaged, non-functional mitochondria from the intact mitochondrial network by preventing fusion due to impaired formation of s-Mgm1p.

1.6 Objectives of the thesis

Prohibitins comprise a highly conserved and ubiquitously expressed protein family in eukaryotic cells which has been functionally associated with various cellular functions including cell cycle progression, transcriptional regulation, cellular signalling, cellular senescence, apoptosis, and mitochondrial biogenesis. Essential gene functions for prohibitins have been reported in various organisms including mice. However, the function of prohibitins on a molecular level remains unclear.

The objective of this thesis was to determine the physiological role of mammalian prohibitins. To provide the basis for a functional investigation of prohibitin gene function *in vivo*, conditional gene targeting of the murine *Phb2* gene was intended. This genetic *loss-of-function* approach will allow to determine the physiological role of mammalian prohibitins by Cre/*loxP*-mediated *Phb2* inactivation *in vivo* and *in vitro*. Thus, the isolation of conditional mouse embryonic fibroblasts (MEFs) from $Phb2^{fl/fl}$ embryos provides the opportunity to study cellular functions of prohibitins. Protein transduction of $Phb2^{fl/fl}$ MEFs with purified, cell permeable Cre recombinase will efficiently induce the genetic ablation of *Phb2*. The experimental examination of PHB2-deficient MEFs offers the possibility to define functional activities of PHB2 on a cellular level. On the basis of previously reported functions of prohibitins in mammals, the functional role of prohibitins in central cellular processes including cell proliferation, apoptosis, mitochondrial dynamics and maintenance of oxidative phosphorylation will be addressed. Furthermore, functional complementation assays will be developed to dissect the role of prohibitins localized to mitochondria compared to potentially other cellular localizations. Cre-mediated inactivation of *Phb2* in mice will aid to unravel the activity of prohibitins during mouse development and to investigate a potential tumour suppressor function *in vivo*.

The proposed experimental framework will add important information on physiological and cellular functions of prohibitins and will help to clarify the molecular role of prohibitins in mammals.

2 Material and Methods

2.1 Molecular Biology

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

2.1.1 Cloning procedures

2.1.1.1 Generation of the *Phb2* gene targeting vector

The targeting vector for the conditional allele of murine *Phb2* was generated by insertion of a 1.4 kb genomic fragment containing exons 3 and 4 in a *Xho*I site located between two *loxP* sites of the pRAPIDflirt vector (A. Bruehl and A. Waisman, unpublished). An upstream 2.2 kb and a 4.5 kb downstream fragment were used as homology arms and inserted into single *BamH*I and *Sal*I sites of pRAPIDflirt, respectively. Additionally, the targeting construct contained a *FRT*-flanked PGKneoR cassette and the Herpes Simplex Virus thymidine kinase gene (HSV-*tk*) for positive and negative selection, respectively. The final construct was linearized by *Swa*I-digestion prior to transfection.

2.1.1.2 Generation of stable *Phb2* expression plasmids

To generate *Phb2* expression plasmids, *Phb2* was PCR-amplified from C57BL/6 mouse liver cDNA and subcloned. Mutations were introduced using the QuikChange site-directed mutagenesis Kit (Stratagene). To obtain Cre-inducible expression plasmids allowing the generation of stable cell lines (pCAGs-STOP-IRES-EGFP), we inserted a chicken β-actin promoter (CAGs) into the *Pac*I site of pSTOP-IRES-EGFP (Sasaki et al., 2006). The *Phb2* wild type and mutant cDNAs were subcloned in a single *Asc*I site of pCAGs-STOP-IRES-EGFP. All plasmids were verified by DNA sequencing.

Table 1. List of plasmids used in this study.

Plasmid	Reference
pRAPIDflirt	Bruehl et al. unpublished
pRAPIDflirt-*Phb2*$^{flox(neo)}$	this study
pGEM-T easy-5´probe *Phb2*	this study
pGEM-T easy-3´probe *Phb2*	this study
pBS-neoR	Uyttersprot, unpublished
pSV40	(Benoist and Chambon, 1980)
pTriEx1-HTNC	(Peitz et al., 2002)
pGEM-T easy-mouse *Phb2 Asc*I	this study
pSTOP-IRES-EGFP	(Sasaki et al., 2006)
pCAGs-STOP-IRES-EGFP	Wunderlich, unpublished
pCAGs-STOP-PHB2-IRES-EGFP	this study
pCAGs-STOP-PHB2^{AARR}-IRES-EGFP	this study
pCAGs-STOP-PHB2^{RRAA}-IRES-EGFP	this study
pCAGs-STOP-PHB2^{AAAA}-IRES-EGFP	this study
pCAGs-STOP-PHB2^{NRBmut}-IRES-EGFP	this study
pCAGs-STOP-PHB2^{NLSmut}-IRES-EGFP	this study
p3xFLAG-CMV14	(Ishihara et al., 2006)
p3xFLAG-CMV14-rat OPA1 sp1 ΔS1	(Ishihara et al., 2006)
p3xFLAG-CMV14-rat AIF(1-95)-OPA1 sp7 (230-997)	(Ishihara et al., 2006)
pDsRed2-mito	Clontech
pEYFP-mito	Clontech

2.1.2 Competent cells and isolation of plasmid DNA

Competent *Escherichia coli* XL10Gold cells (Stratagene) were prepared as described (Inoue et al., 1990) and used in heat shock transformations of plasmid DNA. DNA ligation was performed with T4 DNA Ligase (NEB) according to the manufacturer´s instructions.

Plasmid DNA was isolated from transformed *Escherichia coli* DH5α bacteria with an alkaline lysis method (Birnboim, 1983) according to previously described protocols (Zhou et

al., 1990). Plasmid DNA of a higher purity was obtained with NucleoSpin™ columns (Machery & Nagel) following the supplier's instructions.

2.1.3 Isolation of genomic DNA from cells and tissues

Cells were lysed over night at 56°C in lysis buffer (10 mM Tris-HCl, pH 8; 10 mM EDTA; 150 mM NaCl; 0.2% (w/v) SDS; 400 µg/ml *Proteinase K*). Subsequently, DNA was precipitated from the solution by the addition of an equal volume of isopropanol. The DNA was pelleted by centrifugation, washed in 70% (v/v) EtOH and resuspended in TE-buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) supplemented with 50 µg/ml DNase-free RNase. DNA isolated from ES cell clones grown in 96-well tissue culture dishes was extracted and prepared as described (Pasparakis and Kollias, 1995).

Table 2. List of oligonucleotides used in cloning procedures.

Oligonu-cleotide	Description	Sequence
TL1969	5′-PHB2^{R11A,R17A}	5’-GAACTTGAAGGACTTAGCTGGGGCCCTGCCCG CCGGGCCTGCCGGCATGGGCACGGCGCTG-3’
TL1970	3′-PHB2^{R11A,R17A}	5’-CAGCGCCGTGCCCATGCCGGCAGGCCCGGCGG GCAGGGCCCCAGCTAAGTCCTTCAAGTTC-3’
TL1971	5′-PHB2^{L26A}	5’-CCTCGGGGCATGGGCACAGCGCTGAAGCTGGC TCTGGGGGCCGGGGCGGTGGCCTAC -3’
TL1972	3′-PHB2^{L26A}	5’-GTAGGCCACCGCCCCGGCCCCCAGAGCCAGCT TCAGCGCTGTGCCCATGCCCCGAGG-3’
TL1973	5′-PHB2^{R88N,K89Q}	5’-CTATGACATTCGGGCCAGACCTAACCAGATCTC CTCCCCCACAGGCTC-3’
TL1974	3′-PHB2^{R88N,K89Q}	5’-GAGCCTGTGGGGGAGGAGATCTGGTTAGGTCT GGCCCGAATGTCATAG-3’
TL2455	5’-*Asc*I-*Phb2*	5’-AAGGCGCGCCGCCACCATGGCCCAGAACTTGA AGGA-3’
TL2457	3’-*Asc*I-*Phb2*	5’-AAGGCGCGCCACTCATTTCTTACCCTTAATGA-3’
TL2847	5′-PHB2^{R48A,R54A}	5’-TCACCGTGGAAGGCGGTCATGCCGCCATCTTTT TTAATGCCATTGGTGGCGTGC-3’
TL2848	3′-PHB2^{R48A,R54A}	5’-CCTGCTGCACGCCACCAATGGCATTAAAAAAG ATGGCGGCATGACCGCCTTCC-3’

2.1.4 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was used for amplification of DNA fragments *in vitro* (Saiki et al., 1985). For cloning procedures, amplification of DNA fragments from genomic DNA or mouse cDNA was performed using the High Fidelity PCR Master Kit (Roche) according to the manufacturer´s instructions. Oligonucleotides used for the generation of plasmids are listed in Table 2. Reactions were performed in Thermocyclers (Biometra).

PCR reactions for genotyping of DNA isolated from MEFs and mouse tail biopsies were performed in a total volume of 50 µl containing 100 ng template DNA, 25 pmol of each primer, 250 µM dNTPs, 1.5 mM $MgCl_2$, 1.5 U *Taq* Polymerase, 10 mM Tris-HCl pH 8.3, 50 mM KCl. PCR started with an initial denaturation at 94°C for 5 min, followed by 35 repeating cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, elongation at 72°C for 1 min and a final extension step of 5 min at 72°C. Primers used for genotyping are listed in Table 3.

Table 3. List of oligonucleotides used for genotyping.

Oligonu-cleotide	Description	Sequence
TL1815	5´-Cre general 3	5'-TCCAATTTACTGACCGTACAC-3'
TL1816	3'-Cre general 7	5'-CATCAGCTACACCAGAGACGGAAATC-3'
TL1817	3'-Deleter Cre	5'-CGCATAACCAGTGAAACAGCAT-3'
TL1818	5'-Deleter Cre	5'-GAAAGTCGAGTAGGCGTGTACG-3'
TL1878	5'-Nestin-promoter	5'-CGCTTCCGCTGGGTCACTGTCG-3'
TL1879	3'-Nestin-Cre	5'-TCGTTGCATCGACCGGTAATGCAGGC-3'
TL1908	5'-*Phb2-exon2*	5'-ATCGTATTGGTGGCGTGCAGCA-3'
TL1909	3'-*Phb2 exon3*	5'-CGAGGTCTGGCCCGAATGTCA-3'
TL1910	3'-*Phb2 exon5*	5'-AGGGAGGCTTGGTTTGAGGGGA-3'
TL2650	5'-*ROSA26* promoter	5'-CAGGGTTTCCTTGATGATGTCA-3'
TL2651	3'-*NeoR-WSS*	5'-CGGACCGCTATCAGGACATA-3'
TL2653	3'-*Phb2* exon5	5'-GAGCTCTCTTCGGATCAACA-3'
TL3657	5´-FLPe Deleter	5'-GACAAGCGTTAGTAGGCACAT-3'
TL3658	3´-FLPe Deleter	5'-GAGAAGAACGGCATAGTGCGT -3'

2.1.5 RNA isolation and RT-PCR

RNA was isolated from MEFs using the RNeasy mini kit (Qiagen) according to the manufacturer´s instructions. cDNA synthesis was performed with the Transcriptor cDNA synthesis kit (Roche) following manufacturer´s guidelines. Oligonucleotides used in RT-PCR reactions are listed in Table 4.

Table 4. List of oligonucleotides used in RT-PCR reactions.

Oligonu-cleotide	Description	Sequence
TL1695	5'-*Phb1* exon2	5'-CTGGCGTTGGCAGTTGCAGGA-3'
TL1697	3'-*Phb1* exon5	5'-TGCCGCTCGCTCTGTGAGGT-3'
TL1698	5´-*Phb2* exon2	5'-GTGCAGCAGGACACGATCCT-3'
TL1700	3´-*Phb2* exon6	5'-CTCAGCCTGCACAATCTTCT-3'
TL1813	5'-*β-actin*	5'-CAGAAGGAGATTACTGCTCTGGCT-3'
TL1814	3'-*β-actin*	5'-AGGAGCCACCGATCCACACA-3'
TL2652	5´-CAGs exon1	5'-GCTCTGACTGACCGCGTTA-3'
TL2653	3´-*Phb2* exon5	5'-GAGCTCTCTTCGGATCAACA-3'
TL2950	5´-*EGFP*	5'-TCCGCCACAACATCGAGGA-3'
TL2951	3´-*EGFP*	5'-TCCGCCACAACATCGAGGA-3'
TL3307	3´-*Phb2*-UTR	5'-GCGAGGTCTGTAAGCTGGA-3'
TL3308	5´-*Phb2* exon4	5'-CAAGAGTGTGGTGGCCAAGT-3'
TL3434	5´-*Gapdh*	5'-ACCACAGTCCATGCCATCAC-3'
TL3435	3´-*Gapdh*	5'-TCCACCACCCTGTTGCTGTA-3'

2.1.6 DNA sequencing

Plasmids or DNA fragments were sequenced with the ABI Big Dye Terminator Sequencing Kit (Applied Biosystems) according to established methods (Sanger et al., 1977). The fluorescently labelled DNA fragments were analysed with an ABI Prism 3730 DNA analyser (Applied Biosystems).

2.1.7 Southern Blotting

10 µg of genomic DNA isolated from ES cells, MEFs or mouse tail biopsies were digested over night with 100 U of the appropriate restriction enzyme. Subsequently, DNA fragments were separated by agarose gel electrophoresis and transferred onto Hybond^{TM}-N+ (Amersham) nylon membranes by alkaline capillary transfer (Chomczynski and Qasba, 1984). Membranes were incubated at 80°C for 2 hours to fix the DNA, equilibrated in 2 x SSC (Sambrook et al., 1989) and then prehybridized for 10 hrs in hybridization solution (1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulfate, 50 mM Tris-HCl pH 7.5, 500 µg/ml sonicated salmon sperm DNA at 65°C.

The following probes were used:

Phb2 5' external probe (probe A): A 594 bp fragment, excised with *Eco*RI from pGEM-T easy-*Phb2* 5´probe.

Phb2 3' external probe (probe B): A 632 bp fragment, excised with *Eco*RI from pGEM-T easy-*Phb2* 3´probe.

neoR internal probe: A fragment of 600 bp, excised as a *Pst*I – *BamH*I fragment from pBS-neoR.

50 ng of probe DNA were radioactively labelled with 2.5 µC [$^{\alpha 32}$P]dCTP (Amersham) using the Ladderman^{TM} Labelling Kit (Takara) based on the principle of random primed oligolabelling (Feinberg and Vogelstein, 1984). Non-incorporated radiolabelled nucleotides were removed with MicroSpin^{TM} S-200HR columns (Amersham). Probes were denatured for 5 min in a boiling waterbath before addition to the hybridization solution. Hybridization was performed at 65°C for 10 h in a rotating cylinder (Hybaid). After hybridization, stringency washes were initially performed twice in 2 x SSC/0.1% (w/v) SDS followed by washes in 1 x SSC/0.1% (w/v) SDS and 0.5 x SSC/0.1% (w/v) SDS at 65°C under gentle agitation for 10 min. Radioactivity on the membranes was monitored with a Geiger-counter until a detection limit of 100 cps was reached. Afterwards, membranes were sealed in a plastic bag and exposed to X-ray films (BioMAX MS; Eastman Kodak) at –80°C. Films were developed in an automatic developer (Agfa). Alternatively, the filter were exposed at RT to a phosphoimager screen (Fuji) and analysed on a Bio-Imaging Analyser (Fuji Bas 1000; Fuji, Japan).

2.2 Cell Biology

2.2.1 ES cell culture

The conditional *Phb2* allele was generated with Bruce4 ES cells (Kontgen et al., 1993). Culturing and transfection of ES cells was performed as previously described (Pasparakis and Kollias, 1995; Torres and Kühn, 1997). ES cells were cultured in ES cell medium (Dulbecco´s Modified Eagle´s Medium (DMEM) (Invitrogen), 15% (v/v) fetal bovine serum (FBS) (PAA), 1 mM sodium pyruvate, 2 mM L-glutamine, 1 x non essential amino acids, 1:1000 diluted LIF supernatant, 0.1 mM β-mercaptoethanol) on a layer of mitotically inactivated embryonic feeder (EF) cells to maintain pluripotency. ES and EF cells were grown in tissue culture dishes (Falcon, Greiner) and kept at 37°C in a humidified atmosphere with 10% CO_2. EF cells were maintained in EF medium (DMEM, 10% (v/v) FBS (Invitrogen), 1 mM sodium pyruvate, 2 mM L-glutamine), passaged three times and mitotically inactivated by mitomycin-C treatment (10 µg/ml for 2 h) prior to seeding with ES cells. ES cell colonies were washed once with PBS and incubated with 0.05% (w/v) trypsin, 0.02% (w/v) EDTA in PBS (PAA) for 5 min at 37°C. The cell suspension was used for passaging, transfection or freezing. ES cells were frozen in ES cell medium containing 10% (v/v) DMSO at –80°C and transferred into liquid nitrogen for long term storage. For transfection, 1 x 10^7 ES cells were mixed with 40 µg of DNA in 800 µl transfection buffer (20 mM HEPES, pH 7; 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; 6 mM Glucose; 0.1 mM β-mercaptoethanol) and electroporated at RT (500 mF, 240V). Subsequently, transfected ES cells were transferred onto an embryonic feeder layer and after 48 hours selected with G418 (180 µg/ml, PAA). Selection against HSV-*tk* containing random integrants was performed five days after transfection by supplementing the medium with 2 mM gancyclovir (Cymeven, Syntex). At around day 10 after the transfection, double resistant colonies were picked and split into EF-containing 96-well tissue culture dishes for expansion.

2.2.2 MEF cell culture

Primary mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos derived from intercrosses of time-mated pregnant $Phb2^{fl/+}$ mice. Immortalization of early passage primary MEFs was achieved by SV40 transformation (Todaro and Green, 1965).

MEFs were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), 100 U/ml penicillin (PAA), 100 µg/ml streptomycin (PAA), 100 µM non-essential amino acids (PAA), 2 mM L-glutamine (PAA) and 1 mM sodium pyruvate (PAA). MEFs were cultured at 37°C in an atmosphere of 5% CO_2 and 90% humidity.

For the generation of stable cell lines, $1x10^7$ SV40-transformed *Phb2*$^{fl/fl}$ MEFs were transfected via electroporation with 40 µg of the pCAGs-STOP-IRES-EGFP plasmid containing different murine *Phb2* cDNAs. 24 hrs after transfection cells were selected with 300 µg/ml G418 (PAA) for 9 days. Single G418-resistant MEF clones were isolated and expanded for further analysis. Stable expression of the transgenes was examined by immunoblotting.

2.2.3 Cre protein transduction *in vitro*

Recombinant His-TAT-NLS-Cre (HTNC) fusion protein was expressed and purified as previously described (Peitz et al., 2002). Cre-recombinase was diluted in DMEM/PBS to a final concentration of 3-5 µM, sterile-filtered and applied to MEFs grown in cell culture dishes. Cells were incubated for 20 hrs, washed with PBS and supplemented with growth medium. The efficiency of recombination was assessed by *Phb2*-allele-specific PCR or Southern Blotting.

2.2.4 Assessment of cell proliferation

$1x10^6$ MEFs were transduced with Cre-recombinase and collected by trypsinization after 60 hrs. $1x10^4$ cells were seeded per well onto 96-well tissue culture plates and labelled for 12 hrs (1 µCi ^{3}H-thymidine/well). MEFs were harvested and spotted onto glass fiber filters. Incorporated ^{3}H-thymidine was determined with a microplate scintillation β-counter.

To determine cell growth of PHB2-deficient and control MEFs, $5x10^4$ cells were plated on 60 mm dishes and transduced with Cre-recombinase. Triplicates of cell samples were counted per time point.

2.2.5 Cell death analysis

For TUNEL staining, $1x10^5$ MEFs were grown on glass coverslips in 6-well plates, treated with Cre-recombinase when indicated and cultured for 72 hrs. Cells were fixed in 4% (w/v) *p*-formaldehyde for 10 min, washed in PBS and TUNEL staining was performed using the DeadEnd™ Fluorometric TUNEL system (Promega) according to the manufacturer´s instructions.

2.2.6 Flow cytometry

Mitochondrial membrane potential was measured by fluorescence-activated cell sorting after staining MEFs of the indicated genotypes with JC-1 or TMRM dyes (Molecular Probes) as recommended by the manufacturer.

To determine cell proliferation by flow cytometry**,** $5x10^5$ MEFs transduced with Cre-recombinase were labelled with 0,5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Asquith et al., 2006) and subjected to FACS analysis.

Samples were analysed on a FACSCalibur equipped with CellQuest software (Becton Dickinson).

2.2.7 Fluorescence microscopy

Mitochondrial morphology was examined by transfection of pDsRed2-mito or pEYFP-mito (Clontech) using GeneJuice transfection reagent (Merck Biosciences). $2x10^5$ MEFs were plated on coverslips and transfected twice with the indicated plasmids. Mitochondrial morphology was analysed using the DeltaVision microscope system equipped with Softworx software (Applied Precision). 25 stacks were acquired and subjected to deconvolution. Images were processed further using CorelDRAW™ 11 Graphics Suite software (Corel Corporation).

2.2.8 Transmission electron microscopy

$2x10^5$ MEFs were plated on glass coverslips (thickness 0.2 mm), transfected, Cre-transduced and flat embedded for transmission electron microscopy as follows. After 72 hrs cells were fixed in 0.1 M HEPES/KOH pH 7.2, 4 mM $CaCl_2$, 2.5% (v/v) glutaraldehyde for 4 hrs at RT. After three rinses with 0.1 M HEPES/KOH pH 7.2, 4 mM $CaCl_2$, cells were postfixed in 1% (v/v) osmium tetroxide for 45 min at 4°C, rinsed three times in distilled water and incubated in 1% (w/v) uranyl acetate for 1 hr at 4°C. Dehydration of the samples in a graduated ethanol series, infiltration with Epon and flat embedding was performed according to standard procedures. Ultrathin sections (40–70 nm) were cut and mounted on pioloform-coated copper grids (Plano, Wetzlar, Germany). Sections were stained with lead citrate and uranyl acetate and viewed with a Zeiss CEM 902 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at 80 kV. Micrographs were taken using EMS EM film (Maco, Stapelfeld, Germany). Three-dimensional reconstructions were prepared from scanned films using IMOD software, version 3.5.5 (Kremer et al., 1996).

2.2.9 Isolation of mitochondria from MEFs

Mitochondria of MEFs were isolated by differential centrifugation of fibroblast homogenates. After trypsinization cells were washed in PBS and resuspended in 5 ml isotonic homogenisation buffer (250 mM sucrose, 5 mM Tris-HCl pH 7.5, 0.1 mM PMSF). The cell suspension was homogenised by 35 strokes in a Dounce glass-homogeniser. Nuclei and unbroken cells were removed by centrifugation for 10 min at 1,200 *g* and 4°C. The supernatant was centrifuged for 20 min at 16,000 *g* and 4°C to collect the mitochondria. Subsequently, the mitochondrial pellet was washed and resuspended with homogenisation buffer supplemented with 1 mM EDTA (pH 8.0). Finally, the protein concentration of the suspension was determined using the Bradford assay according to manufacturer's instruction (Bio-Rad). Mitochondria were diluted, frozen as 100 µg aliquots in liquid nitrogen and stored at -80°C.

2.3 Biochemistry

2.3.1 Preparation of protein extracts

MEFs were harvested from tissue culture plates either by trypsinization or by cell scraping and lysed in protein cell lysis buffer (10 mM Tris-Cl pH 7.4, 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% (v/v) Triton X-100 supplemented with 20 µg/ml aprotinin, 2 mM sodium-orthovanadate, 1 mM PMSF and Complete Mini Protease inhibitor cocktail mix (Roche)) for 2 hrs at 1500 rpm in a Vibrax shaker. Protein concentrations were determined with a standard Bradford protein assay (BioRad).

2.3.2 Immunoblotting

50-200 µg of total protein were separated with 10-16% (w/v) SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose (Schleicher & Schuell) or PVDF membranes (Immobilon, Millipore). After protein transfer membranes were incubated for 30 min at RT in blocking solution [5% (w/v) milk powder in TBS buffer (10 mM Tris/HCl pH 7.4, 150 mM NaCl)]. This was followed by immunodecoration for 60-120 min with a specific antiserum (Table 5) diluted in 5% (w/v) milk powder in TBS buffer. Membranes were washed three times for 10 min with TBS supplemented with 0.05% (v/v) Triton X-100. To recognise bound antibodies, horseradish peroxidase-conjugated antibodies specific for immunoglobulins G of rabbit or mouse were employed in a dilution of 1:10,000 in 5% (w/v) milk powder in TBS. Membranes were incubated for 60 min and subsequently washed twice for 10 min with TBS. The bound peroxidase was detected after incubating the membranes with 2 ml of a 1:1 mixture of chemiluminescence reagents [solution 1: 10 ml 1 M Tris-HCl pH 8.5, 1 ml luminol (44 µg/ml in DMSO), 440 µl p-coumaric acid (15 mg/ml in DMSO), 88.56 ml H_2O; solution 2: 10 ml 1M Tris-HCl pH 8.5, 60 µl 30% (w/v) hydrogen peroxide, 89.94 ml H_20]. Subsequently, the membranes were exposed to light-sensitive X-ray films (Super RX, Fuji).

Table 5. Antibodies used in this study.

Antibody	Antigen	Dilution	Reference
α- BAP37	Recombinant C-terminus of human PHB2	1:500	BioLegend
α-prohibitin	Purified recombinant rat prohibitin	1:500	NeoMarkers
α-PHB1	peptide corresponding to amino acids 208-222 of mouse PHB1 (KAAIISAEGDSKAAE)	1:1000	this study
α-β-actin	Synthetic N-terminal peptide of β-actin (DDDIAALVIDNGSGK)	1:5000	Sigma, clone AC15
α-Bax	N-terminal peptide of human Bax	1:1000	Santa Cruz
α-Bcl2	N-terminal peptide of human Bcl2	1:200	Santa Cruz
α-Hax1	amino acids 10-148 of human HAX-1	1:500	BD Biosciences
α-p53	Recombinant human p53 protein	1:200	Santa Cruz
α-phospho-p53	Synthetic phospho-peptide surrounding amino acid Ser 15 of human p53	1:1000	Genetex
α-GFP	Purified recombinant GFP protein	1:1000	Abcam
α-OPA1	amino acids 708-830 of human OPA1	1:500	BD Biosciences
α-FLAG M2	synthetic FLAG-peptide DYKDDDDK	1:1000	Sigma
α-C I, 39 kDa	Purified 39 kDa subunit of human Complex I	1:1000	Molecular Probes
α-C II, 70 kDa	Purified 70 kDa subunit of human Complex II	1:1000	Molecular Probes
α-C III, Su IIa	Purified Core IIa subunit of human Complex III	1:1000	Molecular Probes
α-C V, Su α	Purified α subunit of bovine Complex V (F_1 complex)	1:1000	Molecular Probes
α-HSP60	Recombinant human HSP60	1:1000	Stressgen
α-TIM23	amino acids 5-126 of rat TIM23	1:1000	BD Biosciences
α-Aconitase	peptide corresponding to amino acids 767-780 of human Aconitase	1:1000	Ngo & Davies, unpublished
α-porin/VDAC	N-terminal peptide of human porin	1:1000	Calbiochem

2.3.3 Blue native polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE is a method to analyse native protein complexes of mitochondrial membranes (Reisinger and Eichacker, 2006; Schägger, 2001). It is based on the usage of an

anionic dye (Coomassie blue G-250), which is added after the solubilisation of mitochondria and binds to the surface of proteins. Thereby, a negative net charge is introduced into the protein complexes allowing an electrophoretic separation. A high resolution according to the mass of the protein complexes is achieved by the decreasing pore size of an acrylamide-gradient gel. The gradient gels used in this study were composed of a separation gel with a linear gradient of 3-13% polyacrylamide [3-13% (w/v) acrylamide, 0.09-0.4% (w/v) bisacrylamide, 0-20% (w/v) glycerol, 0.5 M ε-amino-*n*-caproic acid, 25 mM imidazole-HCl pH 7.0, 0.1% (w/v) APS, 0.1% (v/v) TEMED] and a stacking gel [3% (w/v) acrylamide, 0.09% (w/v) bisacrylamide, 0.5 M ε-amino-*n*-caproic acid, 25 mM imidazole-HCl pH 7.0, 0.1% (w/v) APS, 0.1% (v/v) TEMED]. Mitochondrial proteins (150 µg) isolated from yeast or human primary fibroblasts were solubilised by shaking (15 min; 1,400 rpm; 4°C) at a concentration of 5 mg/ml in 1% (w/v) digitonin, 30 mM Tris-HCl pH 7.4, 4 mM Mg-acetate, 5 mM ε-amino-*n*-caproic acid, 50 mM NaCl, 1 mM ATP. After a clarifying spin for 30 min at 125,000 *g* and 4°C, mitochondrial extracts were supplemented with 2 µl 50% (w/v) glycerol and 1 µl sample buffer [5% (w/v) Coomassie blue G-250 in 0.5 M ε-amino-*n*-caproic acid] and loaded onto the polyacrylamide-gradient gel. The electrophoretic separation was carried out in Mini-Protean-3-gel chambers (Bio-Rad) at 4°C [deep blue cathode buffer B: 50 mM Tricine, 7.5 mM imidazole, 0.02% (w/v) Coomassie blue G-250; anode buffer: 25 mM imidazole-HCl pH 7.0] using a constant voltage of 50 V and a current of 15 mA for ~30 min, followed by 300 V and 15 mA for ~30 min. Subsequently, the deep blue cathode buffer B was exchanged for a cathode buffer of identical composition but lacking Coomassie blue G-250 and the separation was continued at 300 V and 15 mA. Finally, the proteins were transferred from the gradient gel onto a PVDF membrane for 2 h at 200 mA. Thyroglobulin (669 kDa) and apoferritin (443 kDa) were used for calibration.

2.3.4 Respiratory chain function

Oxygen consumption studies of viable cells and spectrophotometric evaluation of respiratory chain and TCA cycle enzyme activities in frozen homogenates were performed as previously described (Rustin et al., 1994).

2.4 Mouse analysis

2.4.1 Animal Care

Care of all animal was within institutional animal care committee guidelines. All animal procedures were conducted in compliance with protocols and approved by local government authorities (Bezirksregierung Köln, Cologne, Germany) and were in accordance with NIH guidelines. Mice were housed in groups of 3 to 5 at 22–24°C using a 12 h light/dark cycle.

2.4.2 Mouse handling and breeding

General handling and breeding of mice was performed according to Hogan (Hogan et al., 1987) and Silver (Silver, 1995).

2.4.3 Mice

C57BL/6 and CB20 mouse strains were obtained from Charles River or Jackson Laboratories. Deleter-Cre (Schwenk et al., 1995), FLPe deleter mice (Rodriguez et al., 2000) and Nestin-Cre (Tronche et al., 1999) were maintained in the pathogen-free animal facility of the Institute for Genetics at the University of Cologne.

3 Results

3.1 Conditional gene targeting of the murine Phb2 gene

To investigate the function of PHB2 *in vivo*, conditional gene targeting of murine *Phb2* using Cre-*lox*P-mediated recombination was employed. The murine *Phb2* gene is located on chromosome 6 and composed of nine exons (Figure 6). We constructed a gene replacement vector that introduced a gene cassette consisting of the neomycin resistance gene (*Neo*) and a flanking *lox*P site upstream of exon 3 and a *lox*P site downstream of exon 4 into the endogenous *Phb2* gene (Figure 6). Deletion of the *lox*P-flanked exons 3 and 4 upon Cre-mediated recombination causes a frameshift mutation resulting in a stop of translation at amino acid 73 of PHB2.

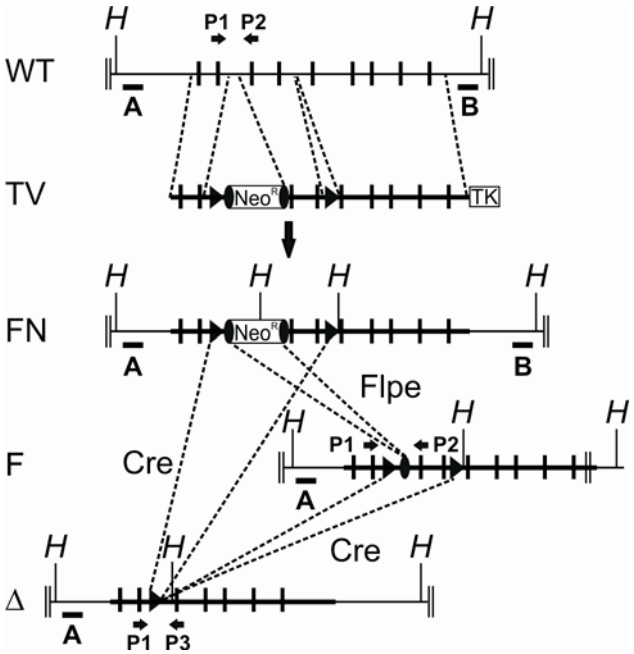

Figure 6. Conditional gene targeting of the murine *Phb2* locus.

Schematic representation of the wild type *Phb2* locus (WT), the targeting vector (TV), the targeted *Phb2*$^{fl(neo)/+}$ locus after homologous recombination (FN), the conditional *Phb2*$^{fl/fl}$ locus after Flpe-mediated recombination (F) and the knockout locus upon Cre-mediated recombination (Δ). Positive and negative selection markers (Neo^R and TK), exons (black bars), *FRT* and *loxP* sites (black ovals and black triangles, respectively), external probes (black boxes, A and B), locations of PCR primers (black arrows, P1-3), and relevant *Hind*III restriction sites (H) are indicated.

3.1.1 Homologous recombination of the targeting construct in murine ES cells

Bruce4 ES cells (Kontgen and Stewart, 1993) were electroporated with the *Swa*I-linearized targeting vector and selected with G418 and ganciclovir as previously described (Kuhn and Torres, 2002). 576 clones were isolated and analysed by Southern Blotting using external probes located upstream and downstream of the *Phb2* gene locus (Figure 6). Probe A was used to determine 5´integration of correctly targeted clones after *Hind*III digestion, resulting in a recombinant band of 7.8 kb besides the 13.8 kb wild type band (Figure 7). To determine co-integration of the 3´*lox*P site, DNA isolated from ES cell clones was *Hind*III-digested resulting in a recombinant band of 5.4 kb band which was detected after Southern Blotting with probe B (Figure 7A). Three homologous recombinant ES cell clones were identified. Single integration of the targeting construct in ES cells was verified with Southern Blotting using an internal probe (Figure 7B). Cre-transduction of ES cells harbouring the targeted $Phb2^{fl(neo)/+}$ allele demonstrated the conversion of the *loxP*-flanked into the deleted allele *in vitro* (Figure 7C).

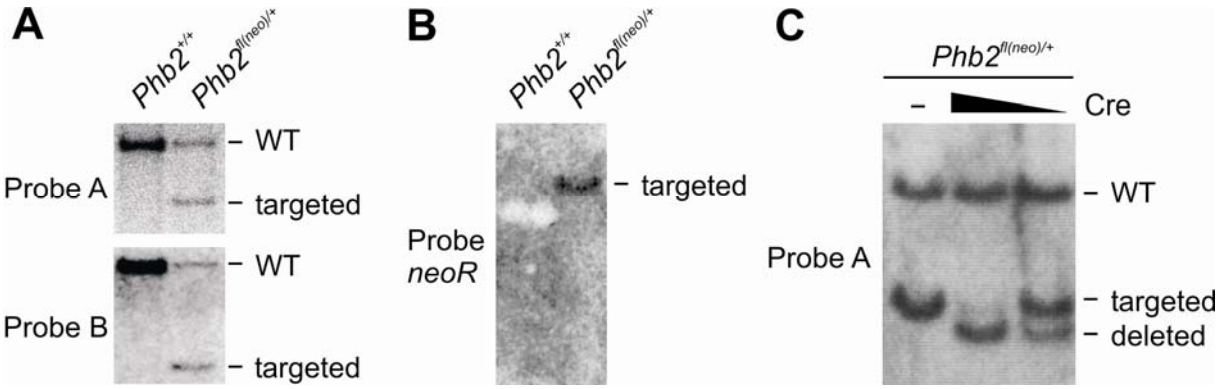

Figure 7. Homologous recombination of the targeting construct in murine ES cells.

(A) Southern blot analysis of wild type and ES cell clones harbouring the targeted $Phb2^{fl(neo)/+}$ allele after homologous recombination. Genomic DNA isolated from wild type (WT) and $Phb2^{fl(neo)/+}$ ES cells (targeted) was digested with *Hind*III, hybridized with an external 5´ probe (Probe A) and an external 3´ probe (Probe B), and analysed by autoradiography.
(B) Southern blot analysis as described in **(A)**. Blots were hybridized with the internal *neoR*-probe.
(C) Southern Blot analysis of genomic DNA isolated from targeted ES cells transduced with increasing concentrations (1.0, 2.0 µM) of purified Cre recombinase. Blots were hybridized with the 5´ external probe and analysed by autoradiography. The different alleles are indicated.

3.1.2 Germline transmission of the targeted *Phb2* allele

An ES cell clone harbouring the targeted *Phb2*$^{fl(neo)/+}$ allele was injected into CB20 blastocysts using standard procedures (Stewart, 1993). Several high-grade chimeras were obtained after implantation of injected blastocysts into foster mice. Male chimeras were backcrossed to C57BL/6 female mice and the resulting offspring was analysed for the presence of the targeted *Phb2*$^{fl(neo)/+}$ allele. Germline transmission was confirmed by Southern blot analysis (Figure 8).

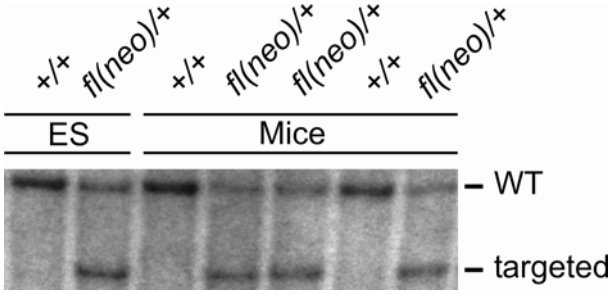

Figure 8. Germline transmission of the targeted *Phb2* allele in mice.

Southern blot analysis of germline transmitted offspring harbouring the targeted *Phb2*$^{fl(neo)/+}$ locus. Genomic DNA isolated from ES cells and tail biopsies were digested with *Hind*III, hybridized with an external 5´ probe and analysed by autoradiography.

3.1.3 Embryonic lethality of PHB2-deficient mice

Mice carrying the targeted *Phb2*$^{fl(neo)/+}$ allele were crossed to FLPe-deleter mice (Rodriguez et al., 2000) to remove the FRT-flanked neomycin resistance gene upon FLP/FRT-mediated recombination (Figure 6). Heterozygous *Phb2*$^{+/-}$ animals were generated upon breeding of *Phb2*$^{fl/+}$ mice with a *Cre* transgenic mouse strain allowing universal expression of Cre-recombinase in all tissues (Schwenk et al., 1995). Intercrossings of *Phb2*$^{+/-}$ mice revealed neither viable *Phb2*$^{-/-}$ offspring nor were homozygous mutant embryos identified before embryonic day 8.5 (Table 6). These findings are in agreement with observations using a conventional knockout strategy (Park et al., 2005) and suggest an essential function of PHB2 in early embryonic development.

Table 6. Genomic deletion of *Phb2* in mice causes embryonic lethality.

Embryonic lethality of PHB2-deficient mice. Analysis of progeny derived from intercrossings of *Phb2*$^{+/-}$ mice. Embryos from the indicated stages or newborn mice were genotyped by PCR using *Phb2* allele-specific primers. Expected numbers reflect the mendelian ratio. E, embryonic day.

		Genotypes		
Stage	Total	*Phb2*$^{+/+}$	*Phb2*$^{+/-}$	*Phb2*$^{-/-}$
E8.5	8	2	6	0
E10.5	15	4	11	0
Postnatal	77	29	48	0
Expected	100	25	50	25

3.1.4 Embryonic lethality of brain-specific PHB2-deficient mice

Conditional gene targeting is a valuable tool to investigate gene functions in a tissue-specific manner (Rajewsky et al., 1996). We reasoned that the generation of a mouse line carrying a tissue-specific deletion of *Phb2* in the brain might bypass embryonic lethality. To achieve this *Phb2*$^{fl/+}$ mice were crossed to a Nestin-Cre line which express the Cre-recombinase specifically in the brain during embryonic development (Tronche et al., 1999). The analysis of progeny by genotyping revealed the absence of viable offsprings (Table 7) suggesting that *Phb2* is required for brain development during embryogenesis.

Table 7. Brain-specific deletion of *Phb2* in mice causes embryonic lethality.

Brain-specific inactivation of murine *Phb2* causes embryonic lethality. Analysis of progeny derived from breedings of Nestin-Cre/*Phb2*$^{fl/+}$ males to *Phb2*$^{fl/fl}$ females. Newborn animals were genotyped by PCR for *Phb2* and the Nestin-Cre transgene. Expected numbers reflect the mendelian ratio. Nes-Cre refers to Nestin-Cre.

		Genotypes			
Stage	Total	*Phb2*$^{fl/+}$	*Phb2*$^{fl/fl}$	*Nes-Cre/ Phb2*$^{fl/+}$	*Nes-Cre/ Phb2*$^{fl/fl}$
Postnatal	142	34	58	40	0
Expected	142	33	33	33	33

3.2 Generation of in vitro systems for Phb2 ablation

3.2.1 Mouse embryonic fibroblasts

To define functional consequences of a deletion of *Phb2* on a cellular level, mouse embryonic fibroblasts (MEFs) were isolated from homozygous $Phb2^{fl/fl}$ and control embryos ($Phb2^{fl/+}$ and $Phb2^{+/+}$). To delete the *Phb2* gene upon Cre-mediated recombination *in vitro*, protein transduction of a recombinant Cre-recombinase was applied into cultured MEFs (Peitz et al., 2002). Thereby, the conditional *lox*P-flanked Phb2 allele is converted into the deleted allele after Cre protein transduction (Figure 9A) leading to efficient deletion of *Phb2 in vitro* (Figure 9B, C).

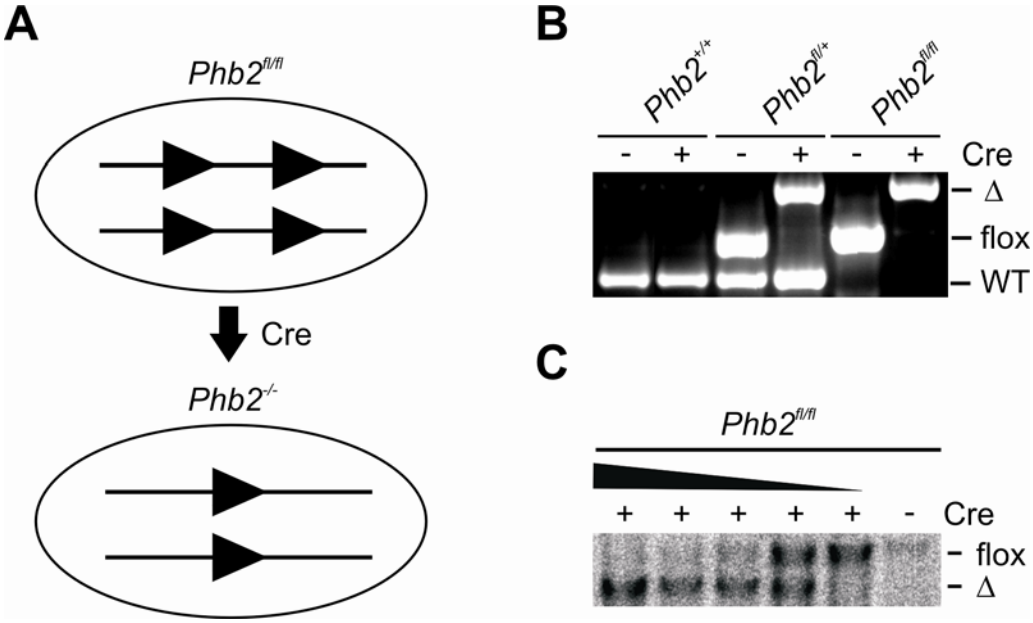

Figure 9. Establishment of Cre/*lox*P-mediated gene deletion *in vitro*.

(A) Schematic illustration of Cre-mediated recombination of the *lox*P-flanked *Phb2* allele. Black triangles indicate *lox*P sites. The black arrowhead indicates Cre protein transduction into MEFs.
(B) PCR analysis of DNA isolated from MEFs using *Phb2* allele-specific primers. Amplified DNA fragments for the WT, floxed (flox), and knockout (Δ) alleles are shown.
(C) Southern Blot analysis of DNA isolated from MEFs of the indicated genotype transduced with increasing concentrations (0.5, 1.0, 2.0, 3.0, 4.0 µM) of recombinant Cre recombinase. Genomic DNA isolated from MEFs was *Hind*III-digested, hybridized with the 5´ external probe and analysed by autoradiography. Conditional (flox) and the knockout (Δ) alleles are indicated.

To demonstrate the efficiency of the *Phb2* loss-of-function approach, MEFs after Cre protein transduction were examined for protein and transcript levels of endogenous prohibitins. Western blot analysis confirmed the absence of PHB2 in $Phb2^{-/-}$ cells (Figure 10A). Notably, depletion of PHB2 was accompanied by the loss of its assembly partner PHB1

(Figure 10A). RT-PCR experiments demonstrated that transcription of *Phb1* proceeds irrespective of the presence of *Phb2* in the cells (Figure 10B). Thus, similar to other organisms (Artal-Sanz et al., 2003; Berger and Yaffe, 1998), murine PHB1 and PHB2 are functionally interdependent, defining the assembled prohibitin complex as the functionally active unit.

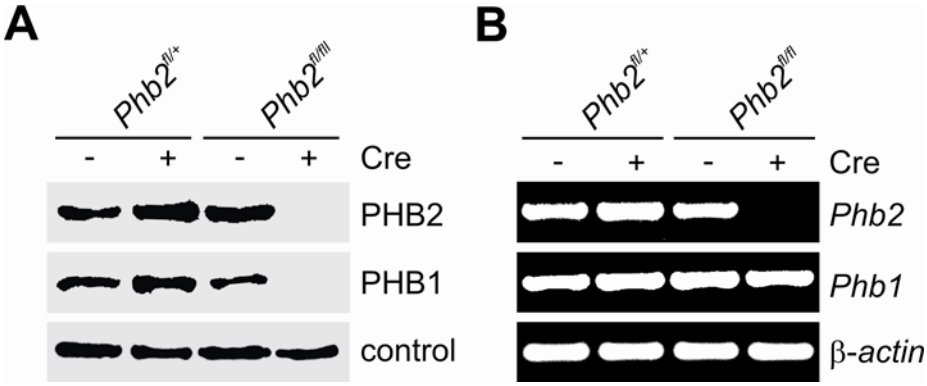

Figure 10. Functional interdependence of mammalian prohibitin subunits.

(A) Immunoblot analysis of total protein lysates. MEFs, transduced with Cre-recombinase when indicated, were lysed and analysed by immunoblotting using PHB1- and PHB2-specific antibodies. A crossreacting band was used as a loading control.
(B) RT-PCR analysis of *Phb1* and *Phb2* transcripts in *Phb2*-deficient and control MEFs. Transcripts of *β-actin* were used as control.

3.2.2 Complementation of PHB2 deficiency *in vitro*

To determine whether phenotypes associated with a genetic *Phb2* ablation can be solely attributed to the loss of PHB2, a cellular complementation system was developed. Stable *Phb2*$^{fl/fl}$ cell lines were established which harbour the *Phb2* cDNA downstream of a transcriptional stop-cassette with flanking *lox*P sites (Figure 11A). Expression of *Phb2* transgenes was induced by Cre transduction of these cells which results concomitantly in the deletion of genomic *Phb2*. Additionally, expression of the transgene was monitored by an IRES-EGFP reporter cassette which allowed ECMV-IRES-mediated expression of eGFP (Figure 11A). PCR analysis confirmed both Cre-mediated recombination of the endogenous *Phb2* allele and excision of the transcriptional STOP-cassette allowing expression of the *Phb2* transgene (Figure 11B). Immunoblotting of cell lysates from Cre-transduced stable *Phb2*$^{fl/fl}$*::Phb2* MEFs revealed expression of the *Phb2* transgene in the absence of endogenous PHB2 (Figure 11C). Notably, also the assembly partner PHB1 accumulated in cells

expressing the *Phb2* transgene in the absence of endogenous PHB2 supporting the notion that both proteins are functionally interdependent. Moreover, both PHB1 and PHB2 accumulated at similar levels as in *Phb2*$^{fl/fl}$ cells demonstrating the validity of the approach (Figure 11C).

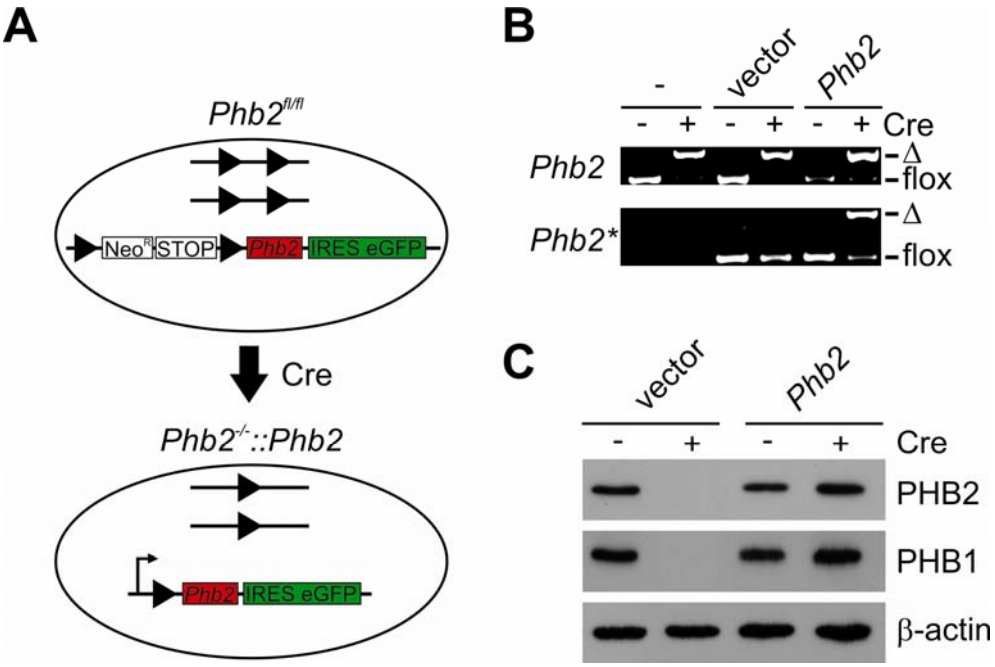

Figure 11. Stable *Phb2* expression system for complementation analysis.

(A) Cre-induced expression of *Phb2* in *Phb2*-deficient MEFs. *Phb2*$^{fl/fl}$ MEFs were stably transfected with the CAGs-NeoR-STOP-IRES-EGFP construct harbouring the *Phb2* cDNA (*Phb2*$^{fl/fl}$*::Phb2*). Cre-mediated recombination results in the inactivation of the endogenous, floxed *Phb2* allele and, simultaneously, in the removal of the *loxP*-flanked transcriptional STOP-cassette allowing expression of the *Phb2* transgene under control of the CAGs promoter. IRES-EGFP expression was used as a reporter.
(B) PCR analysis of DNA isolated from stable *Phb2*$^{fl/fl}$ MEFs transfected with the indicated constructs using allele-specific primers for the endogenous *Phb2* allele (*Phb2*) and the *Phb2* transgene (*Phb2**). Amplified DNA fragments for both *loxP*-flanked (flox) and deleted (Δ) alleles are indicated.
(C) Immunoblot analysis of total cell lysates. Stable transfected *Phb2*$^{fl/fl}$ MEFs of the indicated genotype were transduced with Cre-recombinase when indicated, lysed and analysed by immunoblotting using PHB1- and PHB2-specific antibodies. β-actin was used as a loading control.

3.3 Functional analysis of prohibitin-deficient MEFs

3.3.1 Loss of prohibitins does not lead to spontaneous apoptosis

Mammalian prohibitin proteins have previously been linked to apoptotic processes. Independent studies from several laboratories suggested an anti-apoptotic function of prohibitins (Fusaro et al., 2003; Fusaro et al., 2002; Kasashima et al., 2006; Vander Heiden et al., 2002). Recently, the RNAi-mediated depletion of PHB2 in HeLa cells was shown to induce apoptotic cell death (Kasashima et al., 2006). To investigate whether the deletion of *Phb2* in MEFs also leads to spontaneous apoptosis, $Phb2^{fl/fl}$ and control cells were transduced with purified Cre recombinase and apoptosis was assessed with fluorescent TUNEL staining. MEFs treated with recombinant DNaseI prior to TUNEL staining were used as a positive control. Notably, neither prohibitin-deficient nor Cre-transduced control MEFs exhibited TUNEL-positive nuclei. Thus, PHB2 depletion in MEFs does not result in spontaneous apoptosis (Figure 12).

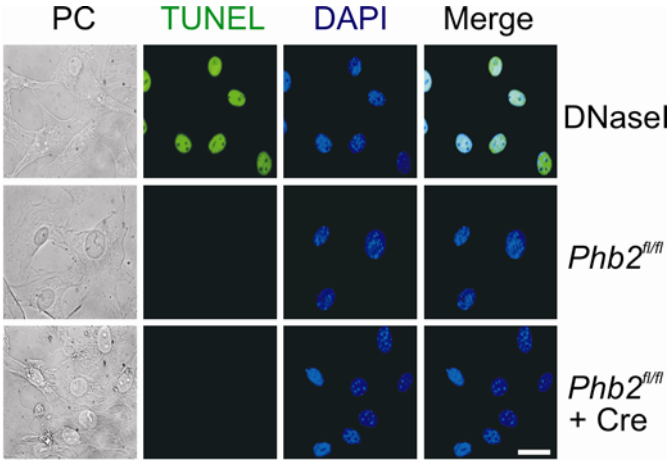

Figure 12. Absence of prohibitins in MEFs does not cause spontaneous apoptosis.

TUNEL staining of $Phb2^{fl/fl}$ MEFs treated with Cre-recombinase when indicated. DNaseI treated MEFs were analysed in parallel for control. Scale bar 10 µm.

To investigate whether the depletion of PHB2 in $Phb2^{-/-}$ cells leads to a differential expression of pro- and antiapoptotic proteins, cell lysates of Cre-transduced $Phb2^{fl/fl}$ and control MEFs were subjected to immunoblotting against several proteins involved in the regulation of programmed cell death. Bax and Bcl2 as well as the anti-apoptotic protein Hax1 accumulated at similar levels in wild type and PHB2-deficient cells. Additionally, protein

levels of p53 and the extent of phosphorylated p53 in *Phb2*$^{-/-}$ cells were indistinguishable from controls (Figure 13). Furthermore, immunoblotting of cell lysates for activated caspase-3 and cytochrome c immunofluorescence did not provide any evidence for spontaneous apoptosis (Sascha Dargazanli, personal communication). These results further substantiate that the loss of prohibitins in MEFs does not lead to spontaneous apoptosis.

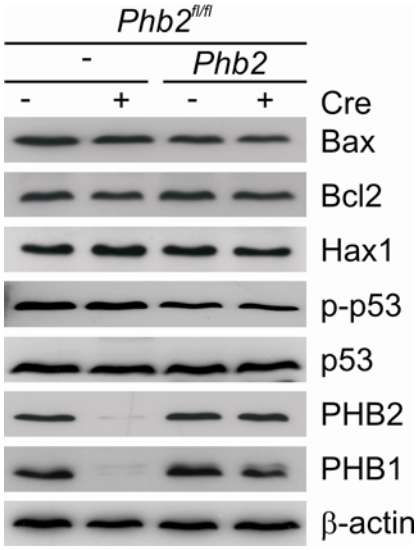

Figure 13. *Phb2* deletion does not affect steady state levels of pro- and anti-apoptotic proteins.
Immunoblot analysis of MEF lysates using the indicated antibodies. β-actin-specific antibodies were used as a loading control. p-p53, phosphorylated p53.

3.3.2 Cell proliferation arrest of prohibitin-deficient MEFs

PHB1 was originally identified through its ability to induce growth arrest in human fibroblasts suggesting a potential tumour suppressor protein function (Jupe et al., 1996; Nuell et al., 1991). Additionally, its homologue PHB2 was found to regulate transcription in response to estrogen receptor activation (Delage-Mourroux et al., 2000; Montano et al., 1999).

To assess the role of prohibitins in the control of cell growth, the effect of *Phb2* deletion on cell proliferation was examined by ^{3}H-thymidine incorporation in *Phb2*$^{fl/+}$ and *Phb2*$^{fl/fl}$ fibroblasts transduced with Cre-recombinase. Whereas Cre-transduction did not affect DNA labelling in wild type or heterozygous *Phb2*$^{fl/+}$ cells, incorporation of ^{3}H-thymidine was strongly impaired in cells lacking PHB2 (Figure 14A). In addition, cell growth of *Phb2*$^{-/-}$ and control cells was investigated by determining the total cell number of cultured MEFs over

time. Consistently, cell growth was impaired in *Phb2*$^{fl/fl}$ cells after Cre-transduction (*Phb2*$^{-/-}$) (Figure 14B) . Notably, expression of PHB2 in *Phb2*$^{-/-}$ cells restored cell proliferation (

Figure 14A) as well as cell growth (Figure 14B), demonstrating that these effects can be solely attributed to the loss of PHB2.

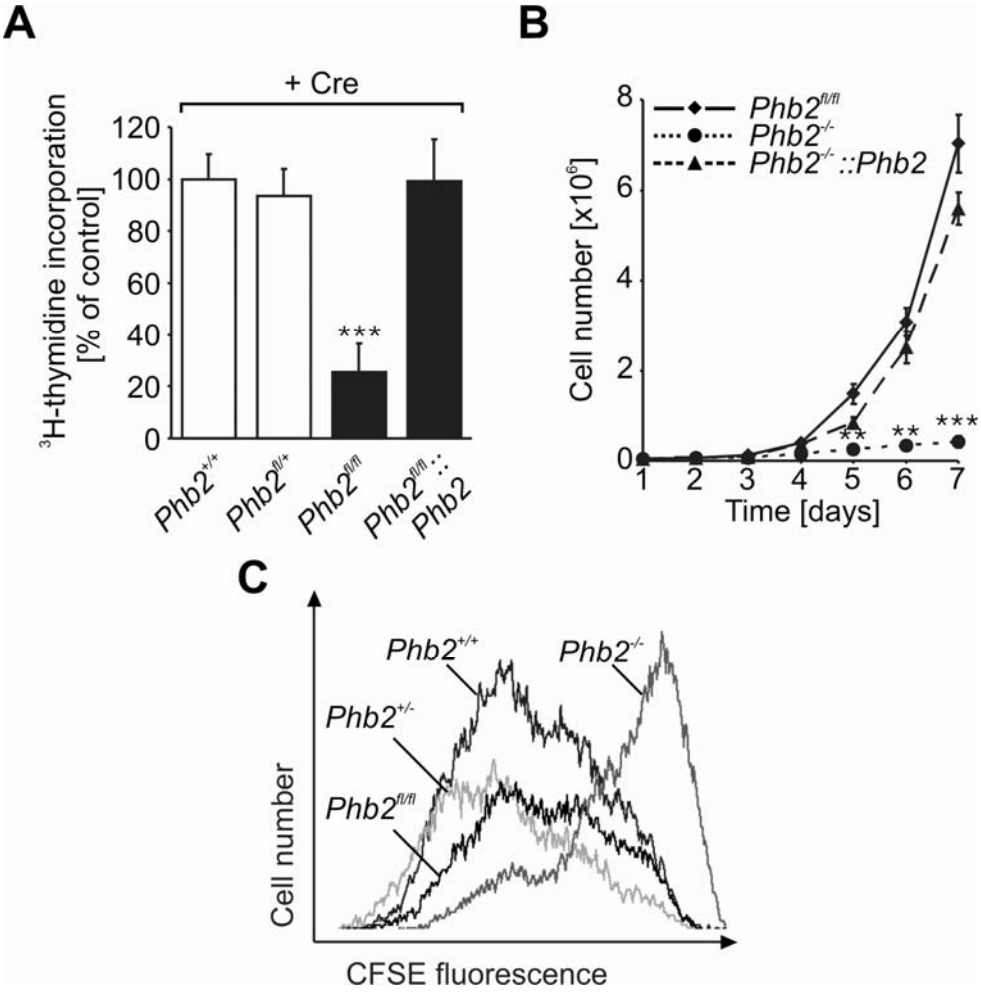

Figure 14. Defective cell proliferation in prohibitin-deficient MEFs.

(A) Incorporation of ^{3}H-thymidine in MEFs after Cre-transduction. Data represent mean ± standard deviations of three independent experiments. *** $\rho<0.001$
(B) Growth curves of PHB2-deficient and control MEFs. 5x10^4 cells were plated on 60 mm dishes and Cre-transduced when indicated. Triplicates of cell samples were counted. ** $\rho< 0.01$, *** $\rho< 0.001$.
(C) Flow cytometric analysis of CFSE-labelled MEFs. Cells were stained with CFSE and analysed by FACS. A lower number of *Phb2*$^{fl/fl}$ and *Phb2*$^{fl/+}$ cells was examined.

These results were further substantiated by *in vivo* cell labelling using the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE). When cells divide after CFSE uptake, CFSE is apportioned equally between daughter cells, resulting in decreased fluorescence (Asquith et al., 2006). Consistent with ^{3}H-thymidine labelling and cell growth

analysis, CFSE fluorescence remained high in *Phb2*$^{-/-}$ fibroblasts but not in *Phb2*$^{+/-}$ cells (Figure 14C). In summary, these results indicate that deletion of murine *Phb2* blocks cell proliferation independently of p53 and without inducing spontaneous apoptosis.

3.3.3 Cell proliferation depends on mitochondrially targeted PHB2

Given the pleiotropic functions that have been suggested for prohibitins in different cellular compartments, it was examined whether cellular defects of *Phb2*$^{-/-}$ cells are caused by the loss of PHB2 within mitochondria. To functionally investigate mitochondrial targeting, conserved arginine residues in the N-terminal mitochondrial targeting sequence of PHB2 (Kasashima et al., 2006) (Figure 15A) were replaced with alanines. To analyse mitochondrial targeting *in vivo*, PHB2 variants were transiently expressed as EGFP fusion proteins in *Phb2*$^{fl/fl}$ cells. This analysis revealed mitochondrial localization of PHB2^{AARR}-EGFP and PHB2^{RRAA}-EGFP, while PHB2^{AAAA}-EGFP accumulated in the cytosol indicating defective mitochondrial targeting of this variant (Sascha Dargazanli, personal communication).

To examine the activity of the PHB2 variants, we established stable *Phb2*$^{fl/fl}$ cell lines allowing the expression of PHB2^{AARR}, PHB2^{RRAA} and PHB2^{AAAA} transgenes upon Cre-mediated recombination (Figure 15A; see Figure 11). Cell proliferation of PHB2-deficient MEFs was restored by transgenic expression of PHB2^{AARR} or PHB2^{RRAA} (Figure 15B) which accumulated at similar levels as PHB2 in *Phb2*$^{fl/fl}$ cells (Figure 15C). In contrast, expression of the cytosolic variant PHB2^{AAAA} did not support cell proliferation (Figure 15B). Although the PHB2^{AAAA} transgene was expressed (Figure 15D), neither mitochondrial PHB1 nor cytosolic PHB2^{AAAA} accumulated stably in these cells indicating degradation of both proteins (Figure 15C). These results reveal a striking correlation between mitochondrial targeting and the maintenance of cell proliferation pointing to a crucial role of PHB2 within mitochondria.

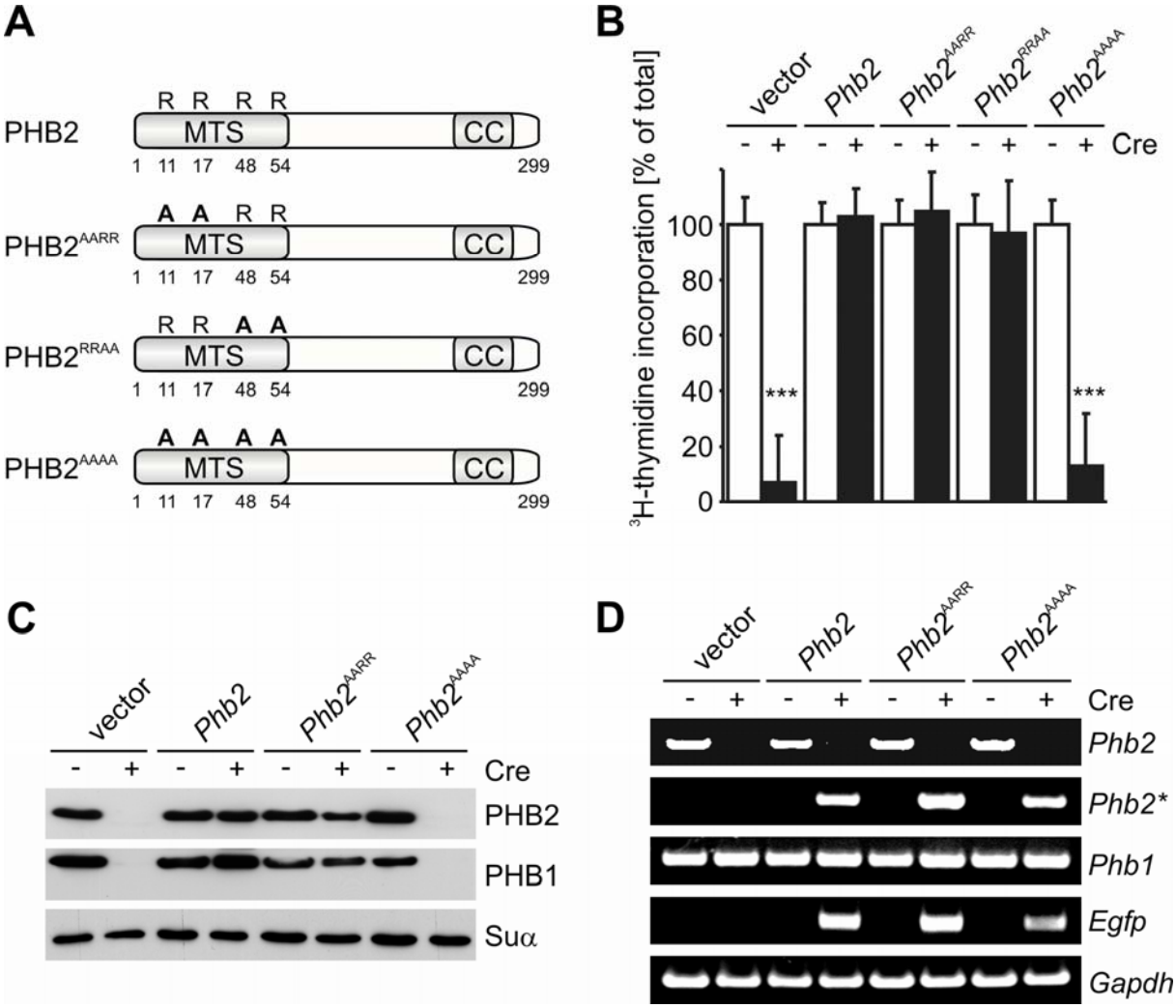

Figure 15. Cell proliferation depends on mitochondrial targeting of PHB2.

(A) Schematic representation of PHB2 variants mutated in the mitochondrial targeting signal. Highly conserved arginine residues in the N-terminus of PHB2 were replaced by alanine at positions 11, 17, 48, and 54 of PHB2. The full-length PHB2 protein comprises 299 amino acids. MTS = mitochondrial targeting signal; CC = coiled-coil.
(B) Proliferation of stable cell lines expressing PHB2 variants monitored by ^{3}H-thymidine incorporation. Stable cell lines expressing PHB2 mutants and IRES-EGFP were established as described above. Data represent ± standard deviation of three independent experiments. *** $\rho < 0.001$.
(C) Immunoblot analysis of MEF cell lines expressing PHB2 or mutant variants thereof. Cell extracts were analysed by SDS-PAGE and immunoblotting using PHB1- and PHB2-specific antibodies. The α-subunit of the F_1-particle of complex V (Suα) was used as a loading control.
(D) RT-PCR analysis of *Phb1* and *Phb2* transcripts in various MEF cell lines. *Phb2* transcripts derived from the genomic locus (*Phb2*) and the transgene (*Phb2**) were amplified using allele-specific primer pairs. Transcripts of *Gapdh* were used as control.

3.3.4 Mutations in predicted nuclear localization signals and receptor boxes of PHB2 do not interfere with cell proliferation

To further investigate whether the impaired cell proliferation of *Phb2*$^{-/-}$ cells was caused by non-mitochondrially localized PHB2, a mutational analysis of predicted nuclear motifs in PHB2 was performed. Namely, a putative nuclear-localization signal and a nuclear receptor box motif were identified and characterized previously (Delage-Mourroux et al., 2000; Montano et al., 1999) (Figure 16A).

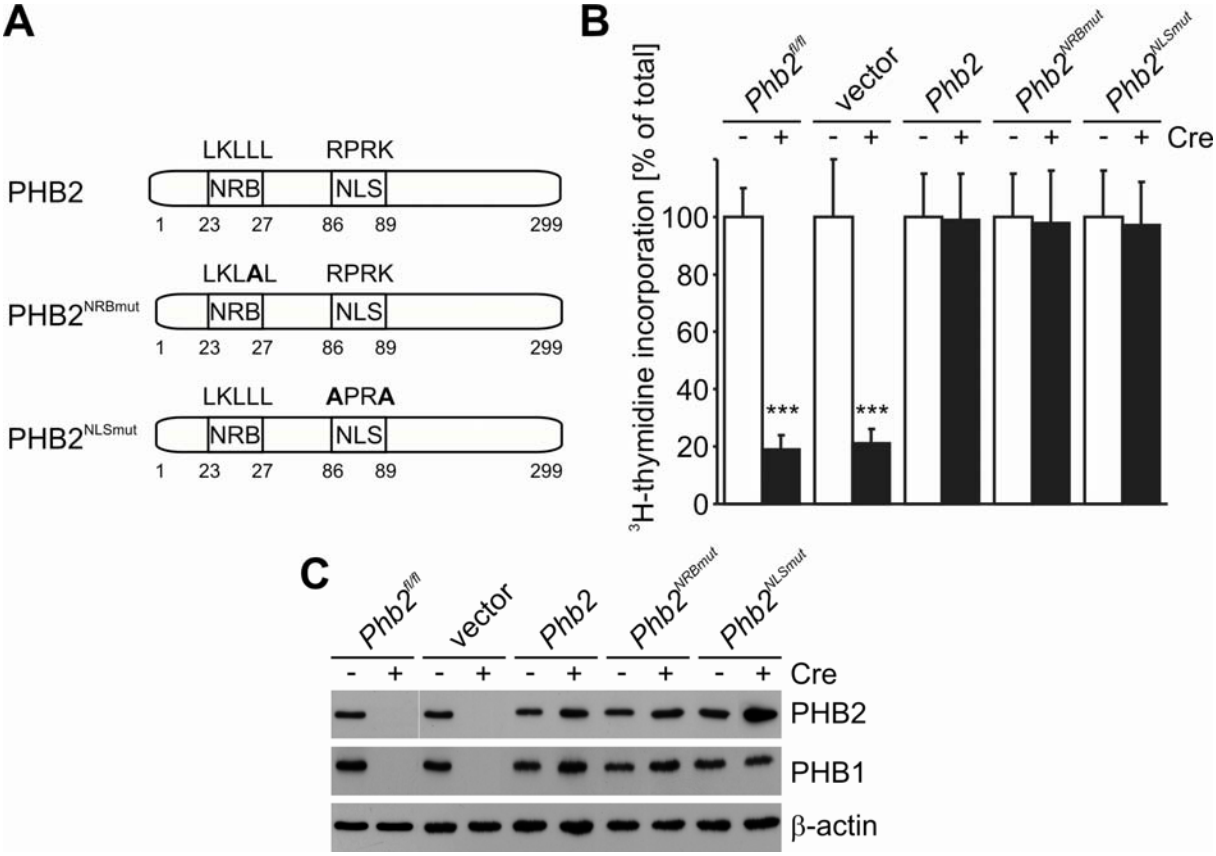

Figure 16. PHB2 variants mutated in putative nuclear localization motifs maintain cell proliferation.

(A) Schematic representation of murine PHB2 wild type and mutant variants. Mutated amino acid residues in a predicted nuclear localisation signal (NLS) and a nuclear receptor box motif (NRB) are shown in bold.

(B) Proliferation of stable cell lines expressing PHB2 variants upon Cre-mediated recombination monitored by ^{3}H-thymidine incorporation. PHB2 mutants are described in (A). Data represent ± standard deviation of three independent experiments. *** $p<0.001$

(C) Immunoblot analysis of cell lines expressing PHB2 mutants. Cell lysates were analysed by SDS-PAGE and immunoblotting using PHB1- and PHB2-specific antibodies. β-actin was used as a loading control.

To examine the activity of these PHB2 variants, a complementation approach was carried out. Stable *Phb2*$^{fl/fl}$ cell lines were established allowing the expression of PHB2^{NLSmut} and PHB2^{NRBmut} transgenes upon Cre-mediated recombination (Figure 16A; see Figure 11A). Cell proliferation of PHB2-deficient MEFs was restored by transgenic expression of both PHB2^{NLSmut} and PHB2^{NRBmut} transgenes (Figure 16B) which accumulated at levels comparable to the wild type protein in *Phb2*$^{fl/fl}$ cells (Figure 16C). These results exclude a role of these motifs in the regulation of cell proliferation by PHB2 and further strengthen the link between the mitochondrial localization of PHB2 and maintenance of cell proliferation.

3.3.5 Mitochondrial fragmentation in prohibitin-deficient MEFs

To examine the morphology of PHB2-deficient mitochondria, we expressed mitochondria-targeted red fluorescent protein in *Phb2*$^{+/+}$, *Phb2*$^{fl/+}$, *Phb2*$^{fl/fl}$, and in *Phb2*$^{fl/fl}$ MEFs complemented with *Phb2*. Ablation of *Phb2* by Cre-transduction had severe effects on mitochondrial morphology and led to fragmentation of mitochondria in >90% of *Phb2*$^{-/-}$ cells (Figure 17A, B). Fragmented mitochondria were not observed in *Phb2*$^{+/+}$ or *Phb2*$^{fl/+}$ cells excluding deleterious effects of Cre-recombinase on mitochondrial morphology (Figure 17A, B). Cre-mediated expression of *Phb2* in *Phb2*$^{-/-}$ cells restored the tubular morphology of mitochondria (Figure 17A, B) indicating that tubulation of mitochondria depends on PHB2.

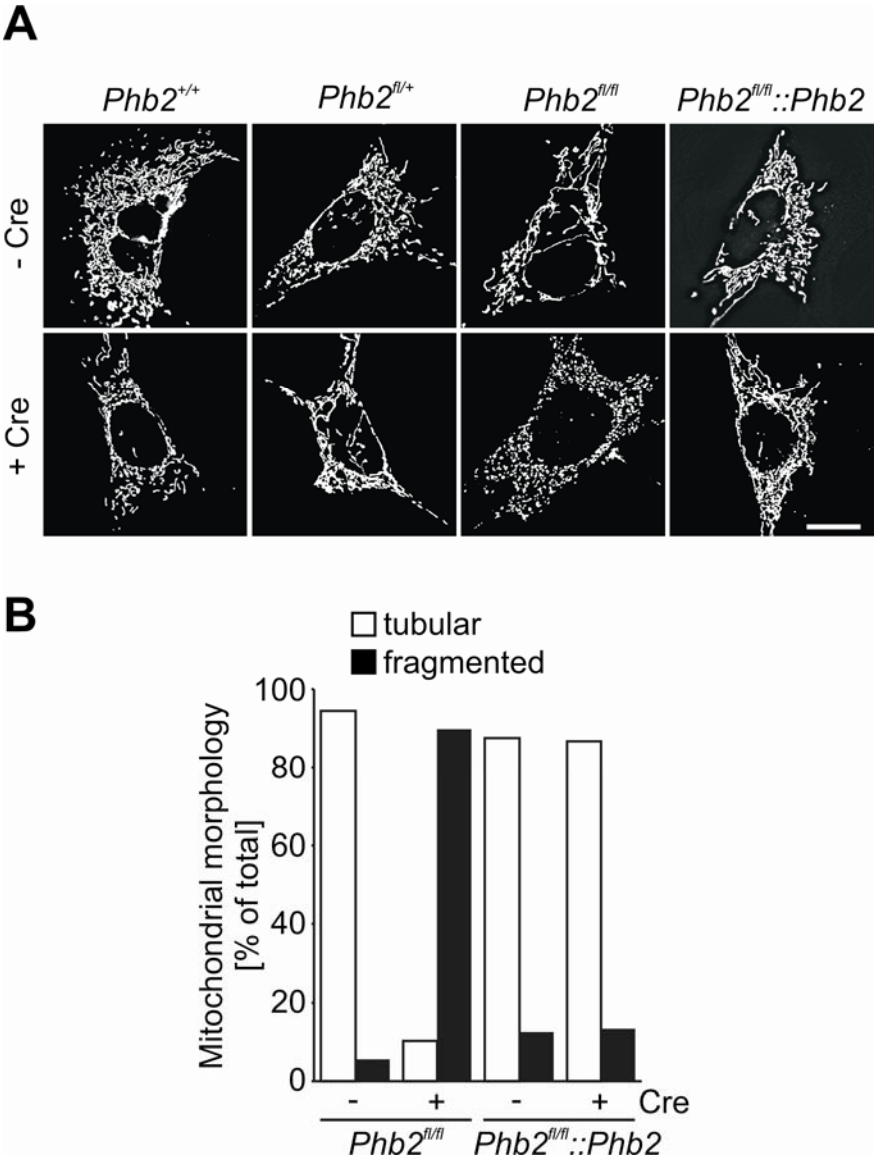

Figure 17. Loss of prohibitins lead to mitochondrial fragmentation.

(A) Fragmentation of mitochondria in prohibitin-deficient MEFs. Cell lines were transfected with mito-DsRed, treated with Cre-recombinase when indicated and analysed after 72 hrs by fluorescence microscopy. Scale bar 10 µm.
(B) Quantification of mitochondrial morphology in control (*Phb2*$^{fl/fl}$*::Phb2*) and prohibitin-deficient MEFs. Cells containing tubular (white bars) or fragmented mitochondria (black bars) were classified. >200 cells were scored per experiment.

3.3.6 Defective cristae morphogenesis in PHB2-deficient mitochondria

The ultrastructure of mitochondria in Cre-transduced *Phb2*$^{fl/+}$, *Phb2*$^{fl/fl}$ and *Phb2*$^{fl/fl}$*::Phb2* MEFs was examined by electron microscopy (Figure 18A, B). The presence of *lox*P-flanked *Phb2* or the deletion of *Phb2* in heterozygous *Phb2*$^{fl/+}$ cells did not affect the ultrastructure of mitochondria (Figure 18A, a-b). However, a large fraction of mitochondria in *Phb2*$^{-/-}$ cells harboured defective cristae (Figure 18A, d-g). Lamellar cristae were either

almost completely lost or balloon-like, vesicular structures were detected within mitochondria (Figure 18C, d-g). This effect was largely reversed upon expression of PHB2 in *Phb2*$^{-/-}$ cells (Figure 18A, c, B).

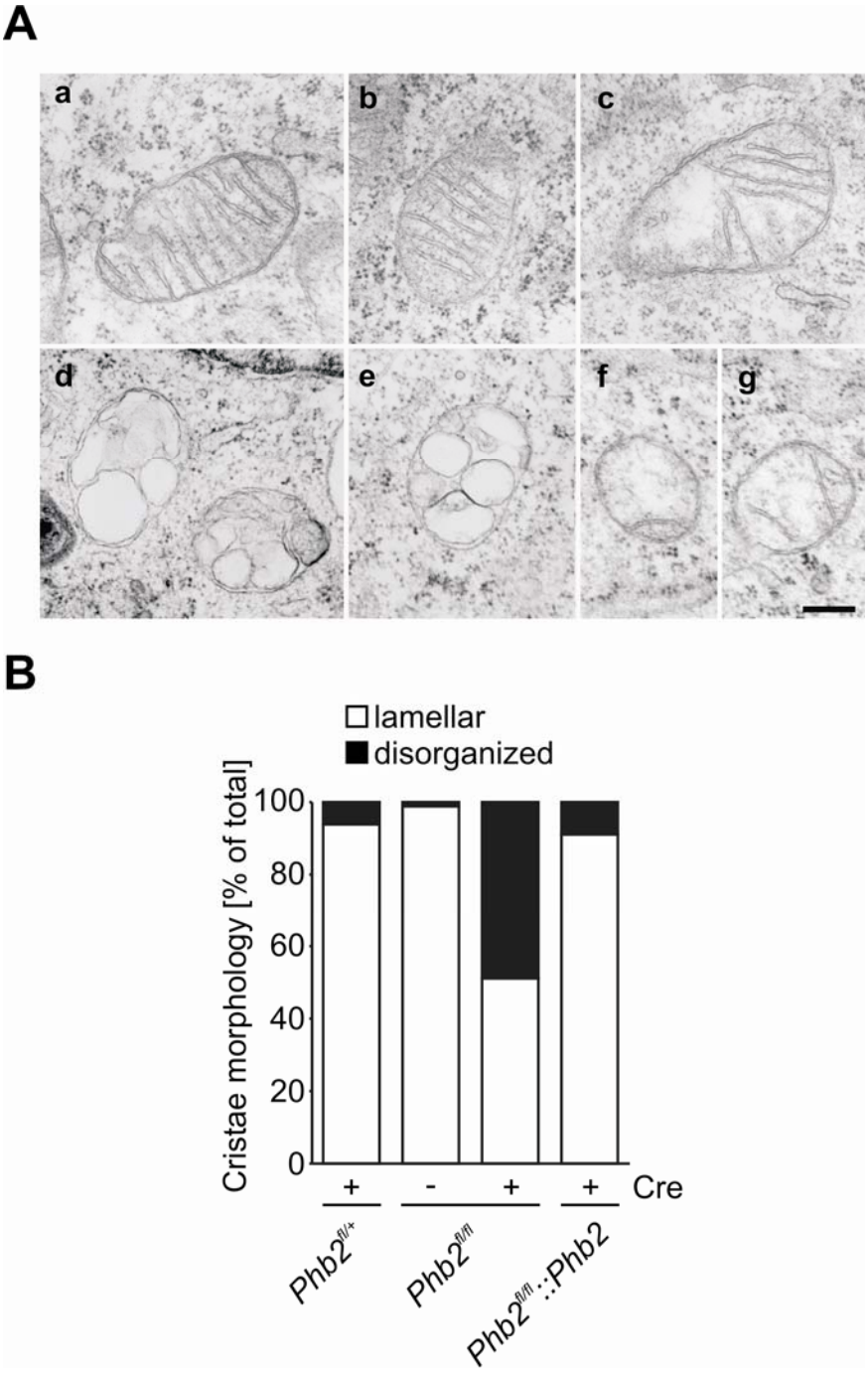

Figure 18. Aberrant cristae morphogenesis in the absence of prohibitins.

(A) Defective mitochondrial ultrastructure in *Phb2*$^{-/-}$ cells. Representative transmission electron micrographs of mitochondria in the following cell lines are shown: (a) *Phb2*$^{fl/fl}$, (b) *Phb2*$^{fl/+}$ +Cre, (c) *Phb2*$^{fl/fl}$*::Phb2* +Cre, (d-g) *Phb2*$^{fl/fl}$ +Cre. Scale bar 500 nm.
(B) Quantification of cristae morphology in *Phb2*$^{fl/fl}$, *Phb2*$^{fl/fl}$*::Phb2*, and *Phb2*$^{fl/fl}$ MEFs transduced with Cre-recombinase when indicated. ~50% of cells with disorganized cristae morphology contain vesicular cristae structures. ~100 sections of individual cells were scored per experiment.

To obtain high-resolution images of the three-dimensional organization of mitochondria, we analysed serial ultrathin sections of *Phb2*$^{fl/fl}$ and *Phb2*$^{-/-}$ cells by transmission electron microscopy (Figure 19A, B). Three-dimensional models generated from these images revealed the presence of regular lamellar cristae in *Phb2*$^{fl/fl}$ cells (Figure 19A). In contrast, morphologically distinct vesicular structures were observed to accumulate within mitochondria of PHB2-deficient cells (Figure 19B). Taken together, the ultrastructural analysis indicates that prohibitins are required for the formation of lamellar mitochondrial cristae.

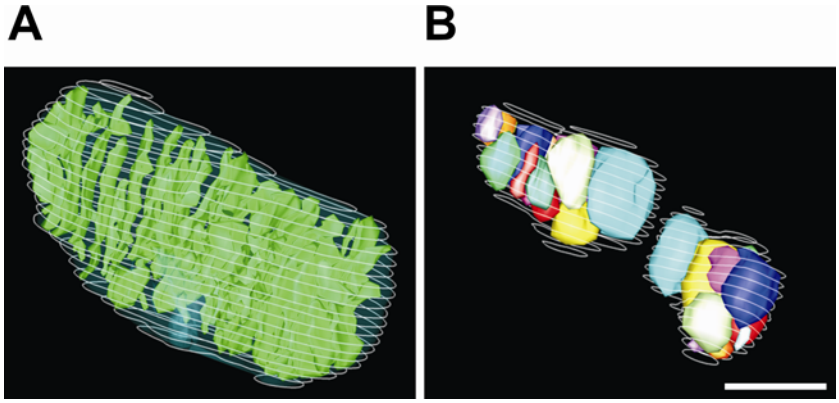

Figure 19. Vesicular-shaped cristae in prohibitin-deficient mitochondria.

Three dimensional reconstructions from 25 serial TEM sections (40 - 70 nm thickness) of *Phb2*$^{fl/fl}$ cells (**A**) transduced with Cre-recombinase (**B**). Scale bar 500 nm.

3.4 Prohibitins control OPA1 cleavage in mammalian mitochondria

3.4.1 Impaired OPA1 processing in prohibitin-deficient MEFs

The dynamin-like GTPase OPA1 is required for both the maintenance of normal cristae in the inner membrane and cristae remodelling during mitochondria-mediated apoptosis (Frezza et al., 2006; Griparic et al., 2004; Olichon et al., 2003). It is therefore conceivable that deletion of *Phb2* affects OPA1 function. Expression of eight OPA1 splice variants and proteolytic processing leads to the formation of at least five different isoforms of OPA1, two long forms designated L1 and L2, which can be proteolytically converted to three short forms, designated S3-S5 (Duvezin-Caubet et al., 2007; Griparic et al., 2007; Ishihara et al., 2006; Olichon et al., 2007; Song et al., 2007). Immunoblotting of *Phb2*$^{-/-}$ and *Phb2*$^{-/-}$*::Phb2* cells with OPA1-specific antibodies revealed drastic alterations in the pattern of OPA1 isoforms accumulating in the absence of PHB2 (Figure 20). While the long forms L1 and L2 and the short form S4 were absent or hardly detectable, S3 and, more pronounced, S5 accumulated in cells lacking PHB2 (Figure 20). These alterations were reversed in *Phb2*$^{-/-}$ cells complemented by PHB2 (Figure 20).

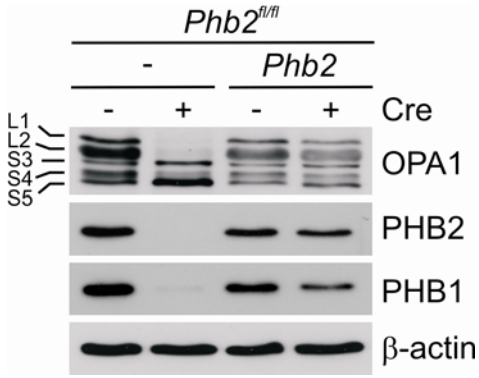

Figure 20. Selective loss of long OPA1 isoforms in prohibitin-deficient MEFs.

Immunoblot analysis of *Phb2*$^{fl/fl}$ and *Phb2*$^{fl/fl}$*::Phb2* cells transduced with Cre-recombinase. Cell lysates (50 µg total protein) were analysed by SDS-PAGE and immunoblotting using OPA1-, PHB1-, PHB2- and, for control, β-actin-specific antibodies.

3.4.2 Maintenance of respiratory activities in PHB2-deficient mitochondria

Mitochondrial dysfunction and the dissipation of the membrane potential across the inner membrane can induce OPA1 processing and mitochondrial fragmentation (Baricault et al., 2007; Duvezin-Caubet et al., 2006; Guillery et al., 2007; Ishihara et al., 2006) and may cause the accumulation of S-OPA1 in the absence of PHB2.

To determine whether prohibitin deficiency causes defects in mitochondrial oxidative phosphorylation (OXPHOS), a comprehensive analysis of the function and assembly of the respiratory chain was performed. Prohibitin-deficient and, as control, heterozygous *Phb2*$^{+/-}$ cells were generated by Cre-mediated deletion of *Phb2* in *Phb2*$^{fl/fl}$ and in *Phb2*$^{fl/+}$ MEFs, respectively (Figure 21A). First, the mitochondrial membrane potential was assessed in PHB2-deficient and control cells by JC-1 staining and fluorescence-activated cell sorting (Figure 21B). JC-1 is a cationic dye that indicates mitochondrial polarization by shifting its fluorescence emission from green (~525 nm) to red (~590 nm) due the potential-dependent formation of red fluorescent J-aggregates (Reers et al., 1995). Comparable intensities of red fluorescence were detected in both Cre-transduced *Phb2*$^{fl/fl}$ and control MEFs. Dissipation of the electrochemical potential with the proton ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used for control. This result indicated that the membrane potential was maintained in PHB2-deficient cells (Figure 21B).

To investigate the oxidative phosphorylation system on a functional level, cellular oxygen consumption and enzymatic activities of respiratory chain complexes in the inner membrane were determined in cells lacking prohibitins. Neither respiration driven by various substrates nor OXPHOS and TCA cycle enzyme activities were grossly affected in *Phb2*$^{-/-}$ cells (Figure 21C, D) which is consistent with the maintenance of a mitochondrial membrane potential. Thus, the accelerated processing of OPA1 in PHB2-deficient cells is not caused by an impaired membrane potential or respiratory activity.

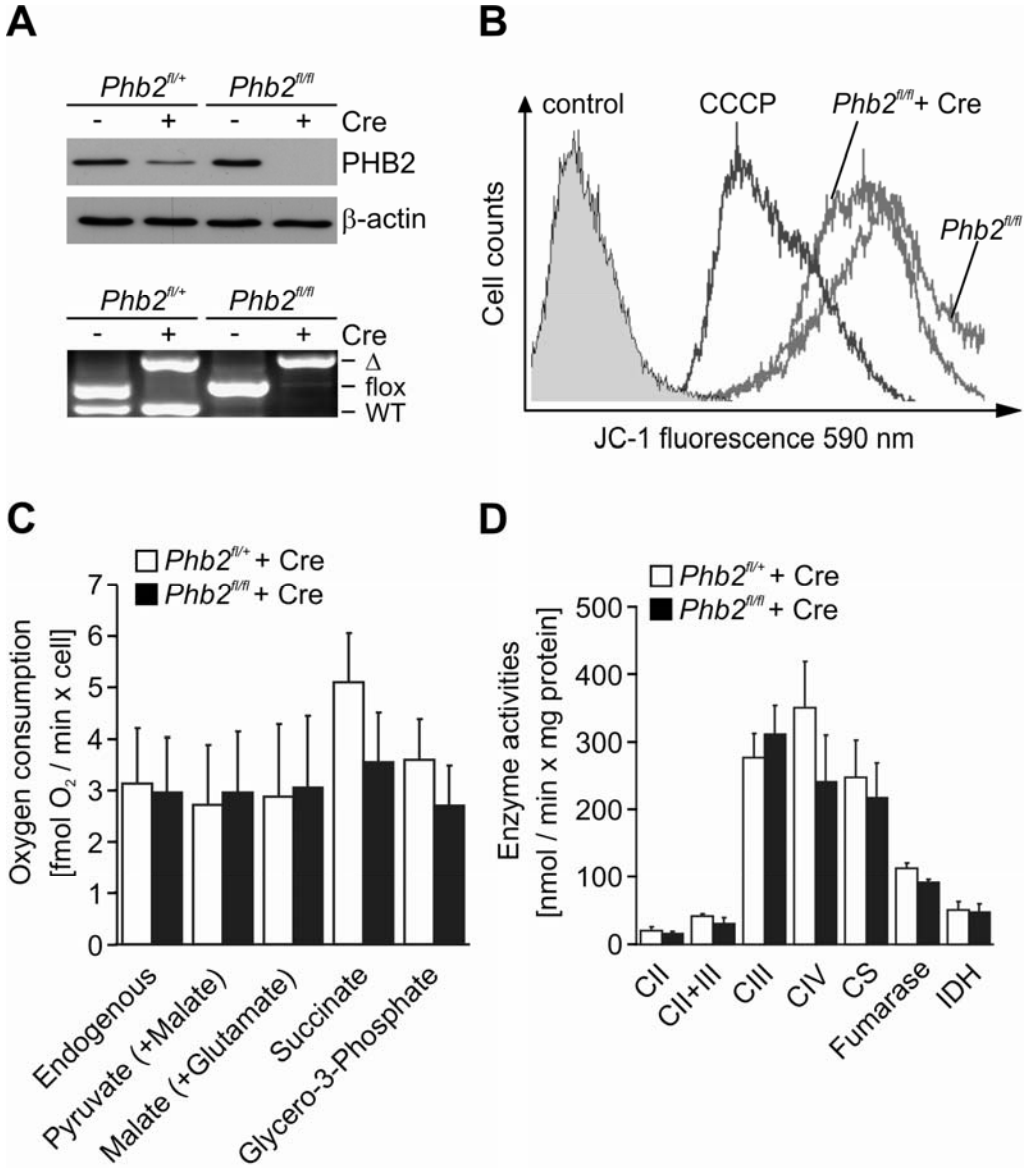

Figure 21. Maintenance of mitochondrial membrane potential and respiratory activities in prohibitin-deficient cells.

(A) Monitoring *Phb2* deletion *in vitro* by immunoblot and PCR analysis. MEFs used for oxygraphic and spectrophotometric analysis of respiratory activities were analysed by immunoblotting (upper panel) and PCR (lower panel). β-actin was used as a loading control.
(B) Maintenance of mitochondrial membrane potential in PHB2-deficient MEFs. Cell lines indicated were stained with the fluorescent dye JC-1 and analysed by flow cytometry at 590 nm. Unstained cells and cells after dissipation of the membrane potential with CCCP were used as controls.
(C) Oxygen consumption in permeabilized control (n=5; white bars) and *Phb2*$^{-/-}$ MEFs (n=3; black bars) after Cre-mediated inactivation of *Phb2* under conditions of substrate-driven respiration. Error bars represent ± standard deviations.
(D) Relative activities of respiratory chain and TCA cycle enzymes in control (n=5, white bars) and *Phb2*$^{-/-}$ MEFs (n=4, black bars) after Cre-mediated inactivation of *Phb2*. CII, succinate quinone dichlorophenol indophenol reductase; CII+CIII, succinate cytochrome c reductase; CIII, decylubiquinol cytochrome c reductase; CIV, cytochrome c oxidase; CS, citrate synthase; IDH, isocitrate dehydrogenase. Error bars represent ± standard deviation.

An enhanced OPA1 processing was previously associated with a decrease in mitochondrial ATP levels (Baricault et al., 2007). To directly address whether accelerated OPA1 processing in prohibitin-deficient MEFs was caused by ATP depletion, the cellular ATP content of Cre-transduced *Phb2*$^{fl/fl}$ cells was examined. Comparable levels of cellular ATP were detected in *Phb2*$^{-/-}$ compared to control cells (Figure 22). These results support the findings of a functional respiratory chain and suggest that accelerated OPA1 processing is not caused by a decreased amount of cellular ATP. However, the content of mitochondrial ATP could not be assessed using this method.

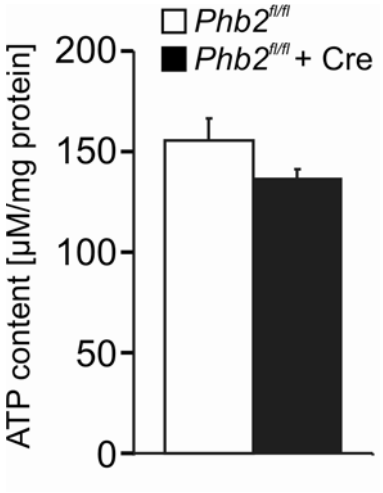

Figure 22. Maintenance of cellular ATP levels in prohibitin-deficient cells.

ATP content of PHB2-deficient and control MEFs. Cells were Cre-transduced when indicated and total ATP was determined using a bioluminescent ATP assay. Data represent mean ± standard deviation of three independent experiments.

To extend the analysis on the mitochondrial OXPHOS, the steady-state level of proteins involved in oxidative phosphorylation and the assembly status of respiratory chain components were examined. These experiments were performed to additionally investigate a proposed function of prohibitins in the assembly of the respiratory chain (Nijtmans et al., 2000). Mitochondria isolated from Cre-transduced *Phb2*$^{fl/fl}$ and control cells were analysed by SDS-PAGE and immunoblotting using antibodies against subunits of OXPHOS complexes I, II, III and V (Figure 23A). The steady-state level of neither respiratory chain subunits nor the matrix proteins HSP60 and Aconitase were affected in the absence of PHB2. Additionally, TIM23 and VDAC immunoblotting was performed to control the steady-state level of functionally unrelated proteins in the mitochondrial inner and outer membrane, respectively (Figure 23A).

To assess the assembly status of OXPHOS complexes, mitochondria isolated from Cre-transduced *Phb2*$^{fl/fl}$ and control cells were solubilized either in Triton X-100 or digitonin and subjected to blue-native gel electrophoresis (BN-PAGE) (Figure 23B,C) (Reisinger and Eichacker, 2006; Schägger, 2001). Coomassie-staining revealed an equal abundance of complexes I, III, IV and V in control and *Phb2*$^{-/-}$ mitochondria (Figure 23B). Moreover, the assembly of respiratory chain supercomplexes was not affected in mitochondria lacking prohibitins (Figure 23B). Taken together, the results indicate that prohibitins are not involved in function and assembly of OXPHOS complexes in mitochondria.

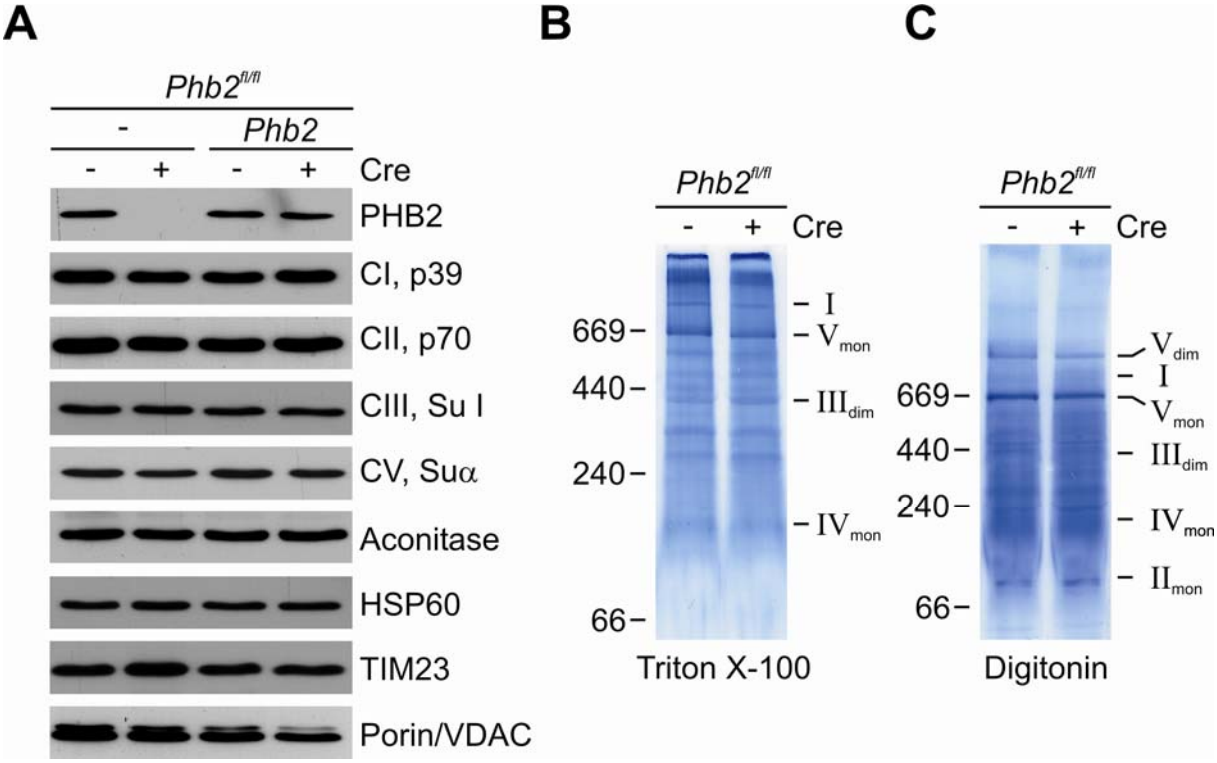

Figure 23. Correct assembly of respiratory chain complexes in prohibitin-deficient mitochondria.

(A) Steady-state levels of respiratory chain complex subunits are unaffected in the absence of prohibitins. Immunoblot analyses of mitochondria isolated from prohibitin-deficient MEFs. Mitochondria were isolated from *Phb2*$^{fl/fl}$ and *Phb2*$^{fl/fl}$*::Phb2* cell lines, which were subjected to Cre-transduction when indicated. Extracts (50 µg mitochondrial protein) were analysed by immunoblotting using the indicated antibodies.

(B) Presence of native respiratory chain complexes in prohibitin-deficient mitochondria. Blue-native gel analyses of mitochondria isolated from prohibitin-deficient and control MEFs. Mitochondria were solubilised in Triton X-100 and subjected to BN-PAGE (100 µg mitochondrial protein). Gels were stained with Coomassie Brilliant Blue. Thyroglobulin (669 kDa), apoferritin (443 kDa), ADH (240 kDa) and BSA (66 kDa) were used for calibration.

(C) Presence of native respiratory chain supercomplexes in prohibitin-deficient mitochondria. Blue-native gel analyses of mitochondria isolated from prohibitin-deficient and control MEFs. Mitochondria were solubilised in digitonin and subjected to BN-PAGE (100 µg mitochondrial protein). Gels were stained with Commassie Brilliant Blue. Calibration was used as in (B).

3.4.3 Expression of a long OPA1 isoform restores mitochondrial morphology in prohibitin-deficient MEFs

The selective loss of L-OPA1 in PHB2-deficient cells may explain both the accumulation of fragmented mitochondria and the disturbed cristae morphogenesis. To functionally complement for the loss of L-OPA1, a FLAG-tagged isoform of OPA1 that cannot be cleaved due to a deletion of amino acid residues flanking the processing site (L-OPA1$^{\Delta}$) (Ishihara et al., 2006) was transiently expressed in *Phb2*$^{fl/fl}$ cells. In parallel, expression of a FLAG-tagged hybrid protein composed of the short form of OPA1 (amino acids 230-997) fused to the mitochondrial targeting sequence of AIF (amino acids 1-95) (S-OPA1) was facilitated in *Phb2*$^{fl/fl}$ cells (Figure 24A). *Phb2* was deleted by Cre-transduction and the expression of the OPA1 variants was monitored by immunoblot analysis (Figure 24B). A long isoform of OPA1 was detected in cells upon transfection of L-OPA1$^{\Delta}$ which was absent in mock- or empty vector-transfected PHB2-deficient control cells (Figure 24B).

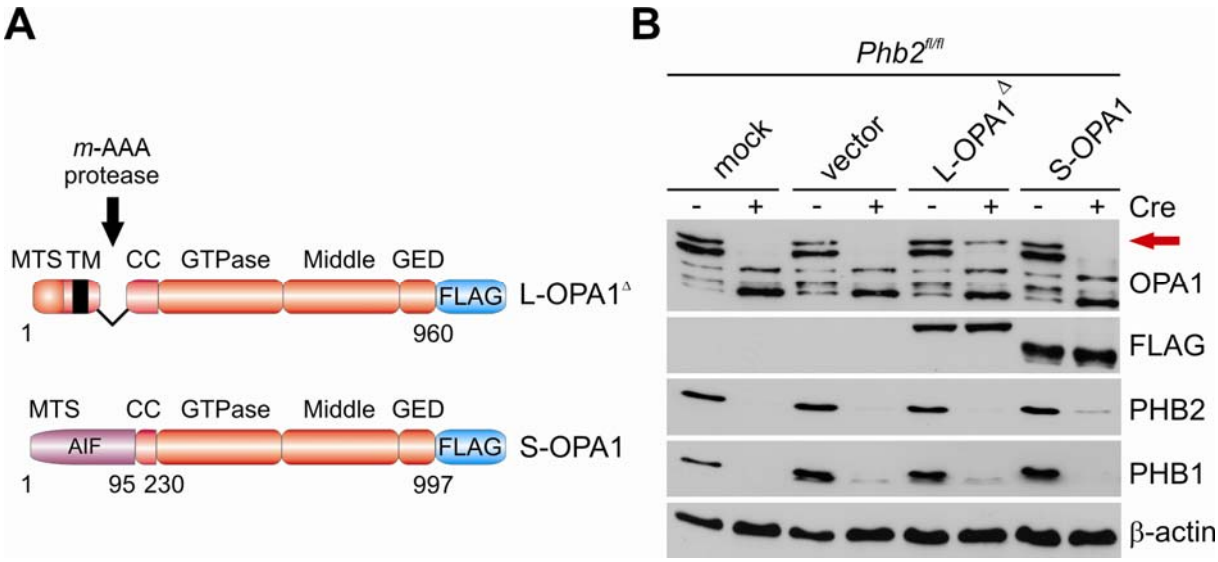

Figure 24. Transient expression of L- and S-OPA1 variants in prohibitin-deficient MEFs.

(A) Schematic representation of OPA1 isoforms used for transient expression in MEFs. The domain structure of a long OPA1 isoform lacking the putative *m*-AAA protease cleavage site (L-OPA1$^{\Delta}$, upper panel) and a short OPA1 isoform (S-OPA1, lower panel) are depicted. L-OPA1$^{\Delta}$ is based on rat splice variant 1, the S-OPA1 isoform contains amino acids 230-997 of rat splice variant 7 fused to amino acids 1-95 of rat AIF (Ishihara et al., 2006). The amino acid positions are indicated. The arrow indicates the putative *m*-AAA protease cleavage site. Both variants are FLAG-tagged. MTS, mitochondrial targeting sequence; TM, transmembrane domain; CC, coiled-coil domain; GTPase, GTPase domain; Middle, middle domain; GED, GTPase effector domain.
(B) Immunoblot analysis of *Phb2*$^{fl/fl}$ MEFs transfected with plasmids and transduced with Cre-recombinase as indicated. Endogenous OPA1 isoforms and transfected FLAG-tagged OPA1 variants were detected with OPA1- and FLAG-specific antibodies, respectively. The red arrow indicates the presence of the L-OPA1$^{\Delta}$ in PHB2-depleted MEFs after transfection. β-actin was used as a loading control.

After co-expression of mitochondria-targeted red fluorescent protein with L- and S-OPA1 in *Phb2*$^{-/-}$ cells, the morphology of mitochondria was assessed by fluorescence microscopy. While ~90% of PHB2-deficient cells contained fragmented mitochondria, expression of L-OPA1$^{\Delta}$ restored tubular mitochondria in ~50% of the cells (Figure 25A, B). Expression of S-OPA1, on the other hand, did not significantly affect the morphology of mitochondria (Figure 25A, B). These results suggest that the loss of L-OPA1 causes mitochondrial morphogenesis defects in the absence of prohibitins.

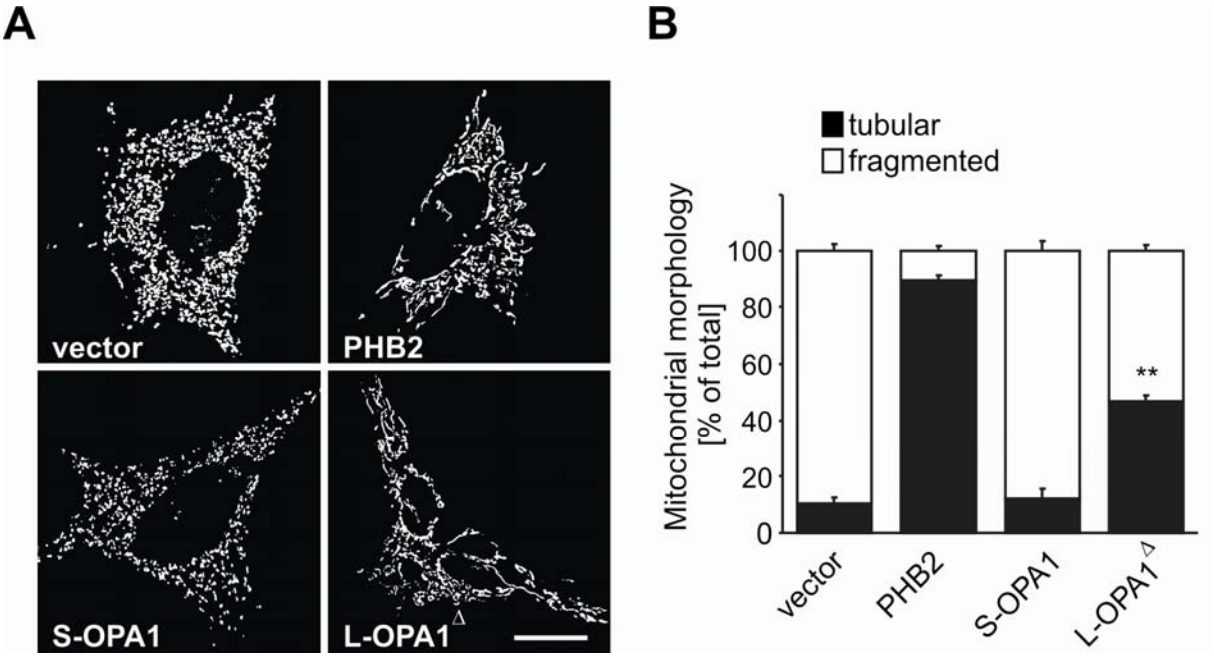

Figure 25. Expression of L-OPA1$^{\Delta}$ restores mitochondrial morphology in *Phb2*$^{-/-}$ cells.

(A) Restoration of tubular mitochondria in prohibitin-deficient MEFs upon expression of L-OPA1$^{\Delta}$. *Phb2*$^{fl/fl}$ cells complemented with PHB2, when indicated, were transfected with mito-DsRed and the indicated plasmids, treated with Cre-recombinase and analysed after 72 hrs by fluorescence microscopy. Scale bar 10 µm.
(B) Quantification of mitochondrial morphology in prohibitin-deficient MEFs transfected with mito-DsRed and OPA1 variants. Cells containing tubular (black bars) or fragmented mitochondria (white bars) were classified. >200 cells were scored in three independent experiments. ** $\rho<0.01$. Error bars indicate ± standard deviations.

3.4.4 L-OPA1$^{\Delta}$ expression restores cristae morphogenesis in *Phb2*$^{-/-}$ cells

To examine whether the absence of long OPA1 isoforms causes the aberrant cristae morphogenesis of PHB2-deficient mitochondria, the ultrastructure of mitochondria was examined by electron microscopy of *Phb2*$^{-/-}$ cells which expressed PHB2, S-OPA1, or L-OPA1$^{\Delta}$. Expression of PHB2 restored normal cristae morphology in >90% of *Phb2*$^{-/-}$ cells (Figure 26A, B). Strikingly, mitochondrial cristae were also maintained in ~55% of *Phb2*$^{-/-}$ cells expressing L-OPA1$^{\Delta}$ (Figure 26A, B). In contrast, aberrant mitochondria lacking cristae and containing vesiculated inner membrane structures accumulated in *Phb2*$^{-/-}$ MEFs regardless of the presence of S-OPA1 in these cells (Figure 26A, B). These findings suggest that L-OPA1$^{\Delta}$ is sufficient to maintain tubular mitochondria and normal cristae in the absence of PHB2. It should be noted that expression of L-OPA1$^{\Delta}$ appears to substitute for the loss of both L1- and L2-isoforms indicating functional redundancy. Taken together, the aberrant cristae morphogenesis in PHB2-deficient mitochondria is solely caused by the absence of long OPA1 isoforms.

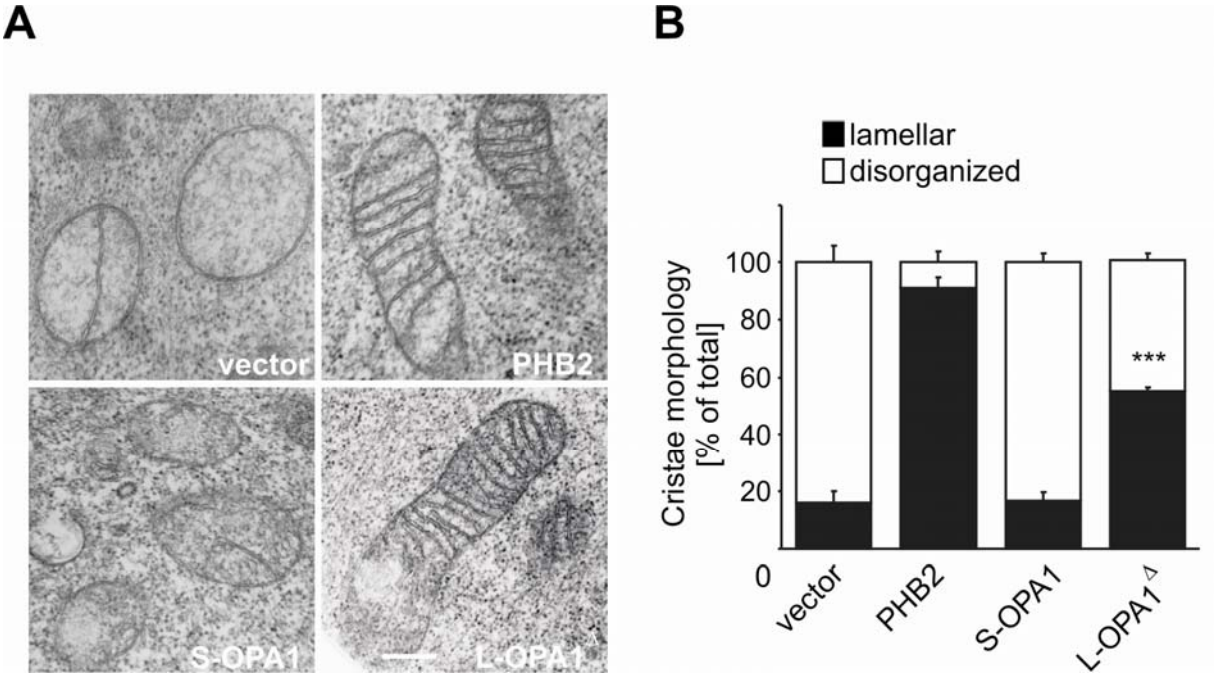

Figure 26. Restoration of cristae morphogenesis upon expression of L-OPA1$^{\Delta}$ in *Phb2*$^{-/-}$ cells.

(A) Restoration of cristae morphology in *Phb2*$^{-/-}$ cells upon expression of L-OPA1$^{\Delta}$. MEFs were transfected with the indicated plasmids and mitochondrial morphology was assessed by transmission electron microscopy. Scale bar 500 nm.
(B) Quantification of deficiencies in cristae morphology in *Phb2*$^{-/-}$ cells upon expression of S- and L-OPA1$^{\Delta}$. ~100 sections of individual cells were scored per experiment. *** $\rho<0.001$. Error bars indicate standard deviations.

3.4.5 Partial restoration of cell proliferation of *Phb2*$^{-/-}$ cells upon expression of L-OPA1$^{\Delta}$

The complementation analysis identifies the control of OPA1 cleavage as the central function of prohibitins within mitochondria. As cell proliferation depends on mitochondria-targeted PHB2, it is conceivable that the proliferation defect of PHB2-deficient cells is a direct consequence of the impaired processing of OPA1 within mitochondria as well. To assess a complementary role of L-OPA1 in the absence of prohibitins, ^{3}H-thymidine DNA labelling experiments in *Phb2*$^{-/-}$ cells transiently expressing S-OPA1 and L-OPA1$^{\Delta}$ were performed (Figure 27). While the presence of S-OPA1 did not improve proliferation of *Phb2*$^{-/-}$ cells, we observed a partial but statistically significant restoration of cell proliferation upon expression of L-OPA1$^{\Delta}$ (Figure 27). These results indicate that the absence of L-OPA1 and defects in mitochondrial morphology deteriorate the proliferation of PHB2-deficient cells.

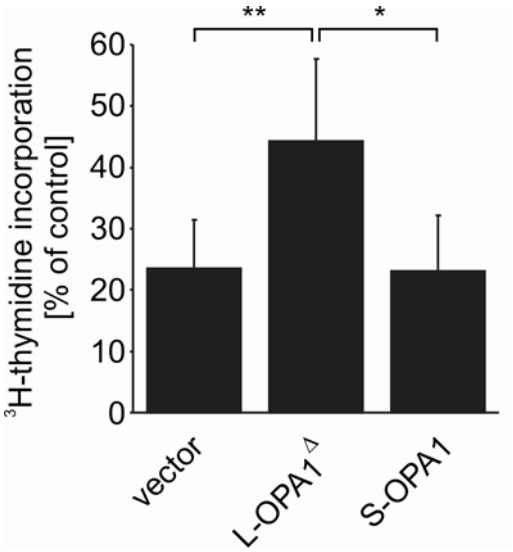

Figure 27. Expression of L-OPA1$^{\Delta}$ promotes cell proliferation of *Phb2*$^{-/-}$ cells.

Cell proliferation of prohibitin-deficient MEFs was monitored after transfection of S- and L-OPA1$^{\Delta}$ by determining the incorporation of ^{3}H-thymidine into DNA. Data represent mean ± standard deviation of four independent experiments. * $\rho<0.05$; ** $\rho< 0.01$.

In conclusion, these results restrict the function of prohibitins to mitochondria and identify the processing of the dynamin-like GTPase OPA1 as the central cellular process controlled by prohibitins. Thus, impaired OPA1 processing is conducive to the cellular defects observed in the absence of prohibitins.

4 Discussion

The remarkable evolutionary conservation and abundant expression of prohibitin genes throughout development as well as under physiological conditions in the adult organism are strong arguments for the essentiality of their gene function. Over the last two decades, a tremendous amount of research has focussed on these genes and established a role for prohibitins in a large number of cellular and developmental processes. It is therefore surprising that, despite the extensive scientific analyses conducted in a variety of organisms, the exact molecular role of prohibitins remains unresolved.

In this study, conditional gene targeting of the murine *Phb2* gene was employed to define cellular activities of mammalian prohibitins in mouse embryonic fibroblasts (MEFs), revealing novel roles for mammalian prohibitins in the processing of the dynamin-like GTPase OPA1 in the mitochondrial inner membrane (Merkwirth et al., 2008). PHB2 ensures cell proliferation, acts anti-apoptotic, and maintains normal cristae morphology. As PHB1 and PHB2 are functionally interdependent, cellular defects observed in $Phb2^{-/-}$ cells can be attributed to the loss of the assembled prohibitin complex. Complementation assays restrict its function to mitochondria and identify the processing of the dynamin-like GTPase OPA1 in the inner membrane as the central cellular process controlled by prohibitins.

4.1 Mitochondria-localized prohibitins are indispensable for cell proliferation

Cellular functions of PHB2 were examined in mouse embryonic fibroblasts isolated from conditional $Phb2^{fl/fl}$ embryos transduced with a cell-permeable, recombinant Cre recombinase (Peitz et al., 2002). The efficiency of this approach is reflected in the selective loss of both *Phb2* transcript and immunodetectable PHB2 protein, demonstrating specificity of the *Phb2 loss-of-function*. In contrast to gene knockdown approaches based on RNA interference (RNAi), this methods involves irreversible recombination of genomic DNA (Sauer and Henderson, 1988). Moreover, non-specificity and off-target effects observed in RNAi experiments can be excluded (Svoboda, 2007). Furthermore, the conditional cell culture system allowed functional complementation analyses by the selective, Cre-mediated expression of *Phb2* transgenes concomitant with a Cre-mediated ablation of endogenous

Phb2. Strikingly, functional interdependence of prohibitin subunits has been demonstrated with this system suggesting that the complex composed of assembled PHB1 and PHB2 subunits functions as the physiologically active unit. This finding is consistent with previous observations in yeast (Berger and Yaffe, 1998) and mammalian cells (Coates et al., 2001; Kasashima et al., 2006) and allows the conclusion that cellular defects observed in PHB2-deficient MEFs can be attributed to the loss of the assembled prohibitin complex.

Intriguingly, our studies demonstrate that the prohibitin complex is essential for cellular proliferation which is in contrast to previous reports claiming an anti-proliferative function (McClung et al., 1989; Nuell et al., 1991). The proposed function of PHB1 in the inhibition of cell cycle progression was initially based on *Phb1* mRNA injection experiments. However, this effect was later attributed to its 3´UTR (Jupe et al., 1996; Manjeshwar et al., 2003). On the other hand, interactions of PHB1 with Rb, E2F and p53 were identified in overexpression studies (Fusaro et al., 2003; Wang et al., 2002a; Wang et al., 1999a). These findings lead to a proposed tumour suppressor function and supported a role for prohibitins in cell cycle control and transcriptional regulation. Interestingly, however, prohibitin-deficient MEFs generated in this study neither reveal a specific block in cell cycle progression, nor an impaired phosphorylation of Rb and p53 proteins. These observations indicate that the impaired cell proliferation of prohibitin-deficient MEFs occurs independently of Rb- or p53-mediated cell cycle regulation. Thus, a proposed tumour-suppressor function of PHB1 appears unlikely considering the presented results.

Due to reported interactions with essential cell cycle regulators, a nuclear localization of prohibitins has been proposed. Nevertheless, the complementation experiments used in this study did not reveal evidence for functional nuclear-localized PHB2. Restoration of cell proliferation in prohibitin-deficient MEFs, which express PHB2 variants carrying mutations in a putative nuclear localization signal (NLS), as well as in a nuclear receptor box (NRB) occurred to a similar extent as in the wild type variant. Thus, a function of PHB2 in the nucleus seems unlikely. Rather, our results restrict the function of prohibitins to mitochondria. Stable expression of mitochondrial targeting mutants in prohibitin-deficient MEFs reveal a striking correlation between mitochondrial targeting and the maintenance of cell proliferation. Importantly, the proliferation of *Phb2*$^{-/-}$ cells is only restored upon expression of mitochondrially targeted PHB2, assigning a crucial role for cell proliferation to mitochondria-localized prohibitins. Remarkably, the expression of L-OPA1 did not only restore mitochondrial morphogenesis in *Phb2*$^{-/-}$ cells and their resistance towards apoptosis, but also

promoted the proliferation of prohibitin-deficient MEFs. These findings directly link growth deficiencies of *Phb2*$^{-/-}$ cells to defects in mitochondrial morphogenesis.

This raises the intriguing question: how do mitochondria affect the proliferative capacity of a cell? It is well established that functional mitochondria are required for all cell processes due to common energetic requirements (Saraste, 1999). Growth and cell cycle progression depend on intracellular energy supplies and calcium signalling (Duchen, 2000). In this context, mitochondria are the central organelles of both processes since they provide ATP through oxidative phosphorylation and regulate calcium influx via membrane-potential dependent calcium buffering (Gilabert and Parekh, 2000; Hoth et al., 1997). Mitochondrial signalling cascades have therefore been implicated in the control of cell proliferation (Rustin, 2002). Indeed, a recent report considered that mitochondrial dysfunction caused by mutations in the Krebs cycle enzyme fumarate hydratase could lead to aberrant cell proliferation (Tomlinson et al., 2002). Additional findings put emphasis on mitochondria as a relevant signalling platform for cell-cycle progression. Mainly, conditions of low energy caused by an impaired oxidative phosphorylation (OXPHOS) have been associated with defective cell cycle progression (Mandal et al., 2005). Mechanistically, activation of the AMPK pathway triggered by ATP depletion has been implicated in the inhibition of cell proliferation. In this scenario, activated AMPK promotes phosphorylation of p53 which subsequently leads to the loss of cyclin E and cell-cycle arrest at the G1 to S phase transition (Jones et al., 2005). These data defined a novel low-energy cell-cycle checkpoint that monitors the metabolic activity of mitochondria before committing to another round of cell division. However, in the absence of prohibitins cellular ATP levels and respiration were not grossly affected. Thus, the relevant trigger for AMPK activation is not given in prohibitin-deficient MEFs indicating that mitochondria control cell proliferation independent of their function in cellular energy metabolism. Rather, the presented data suggest a novel mode of proliferation controlled by the modulation of mitochondrial morphogenesis which is underscored by the partial restoration of cell proliferation by OPA1 expression in *Phb2*$^{-/-}$ cells. Nevertheless, it is tempting to assume that control of cell proliferation by organelle morphogenesis depends on specific signalling pathways involving mitochondria-to-nucleus communication (Figure 28). In this context, reactive oxygen species (ROS) and calcium ions have been implicated in the modulation of cellular signalling routes (Balaban et al., 2005; Clapham, 2007). Thus, the experimental identification of signalling mediators controlling this path of communication is a great importance. A very recent report is potentially relevant for OPA1-mediated mitochondrial morphogenesis and ROS signalling *in vivo*. Notably, an accumulation of ROS has been

observed in *D. melanogaster* harbouring a tissue-specific disruption of OPA1 in the eye. Interestingly, antioxidant treatment as well as overexpression of superoxide dismutase reversed ROS accumulation, suggesting a role for ROS in OPA1-deficiency and in the pathogenesis of optic atrophy (Yarosh et al., 2008). Further studies are required to provide more conclusive insights into this mechanism.

4.2 Prohibitins are required for mitochondrial cristae morphogenesis

Cristae are the main region of respiratory chain activity in the inner membrane of mitochondria (Gilkerson et al., 2003; Vogel et al., 2006). Disturbances of mitochondrial cristae morphology have been described for a number of inner membrane proteins involved in oxidative phosphorylation. Lack of non-essential subunits within the ATP synthase in yeast leads to the loss of cristae concomitant with the appearance of ring structures of the inner membrane within mitochondria (Arselin et al., 2004; Bornhovd et al., 2006; Goyon et al., 2008; Paumard et al., 2002). Similarly, loss of the inner membrane-anchored protein mitofilin leads to concentric sheets of inner membrane ring structures (John et al., 2005). Consistent with the localization of respiratory chain complexes to cristae, it is not surprising that aberrations in cristae morphology are accompanied by alterations in metabolism. The molecular details governing the contribution of the mentioned proteins to the inner membrane architecture are unknown, but these findings illustrate the fact that cristae do not form spontaneously and are created by active, regulated processes.

As demonstrated in this study, prohibitins are central to the regulation of mitochondrial cristae formation by affecting OPA1 (Figure 28). The fragmented mitochondria harbouring highly disorganized and swollen cristae in prohibitin-deficient cells strikingly resemble mitochondria that were observed upon downregulation of OPA1 (Griparic et al., 2004; Olichon et al., 2003). In fact, RNAi-mediated depletion of OPA1 in mammalian cells causes mitochondrial fragmentation and ultrastructural changes of mitochondrial cristae (Griparic et al., 2004; Olichon et al., 2003). The yeast orthologue of OPA1, Mgm1p, has been shown to mediate fusion of the inner membrane suggesting that this activity might be conserved for OPA1 (Meeusen et al., 2006). Although direct evidence for a specific inner membrane fusion activity of OPA1 is missing, several indications support this idea. The overexpression of OPA1 promotes mitochondrial fusion which depends genetically on the

outer membrane mitofusin-1 (MFN1) suggesting a coupling of inner and outer membrane fusion (Cipolat et al., 2004). In contrast, other reports indicated that outer and inner membrane fusion might be separate events (Malka et al., 2005; Meeusen and Nunnari, 2005). Recent evidence for a role of OPA1 in mitochondrial fusion was obtained through the dissection of specific OPA1 isoforms by complementation assays in OPA1-deficient cells (Song et al., 2007). Both, long (L) and short (S) isoforms of OPA1 are required for mitochondrial fusion in this model system which is consistent with previous observations. Long and short isoforms of Mgm1p are generated by proteolytic processing and both required for fusion activity (Herlan et al., 2003; Sesaki et al., 2003). Experiments in prohibitin-deficient mitochondria ascribe cristae morphogenesis defects to the selective loss of L-OPA1 isoforms and are therefore consistent with recent findings demonstrating the requirement of both L- and S-OPA1 for mitochondrial fusion (Song et al., 2007).

Considering the extensive morphogenesis defect of mitochondrial cristae observed in prohibitin-deficient mitochondria, the maintenance of respiratory activity and mitochondrial membrane potential is quite astonishing. In contrast to *Opa1*$^{-/-}$ cells which lack all OPA1 isoforms and exhibit compromised respiration, only individual OPA1 isoforms are absent in prohibitin-deficient cells. One could envision that the accumulation of S-OPA1 isoforms in the absence of prohibitins might protect the ultrastructurally altered mitochondria from respiratory dysfunction by a yet unknown mechanism. Alternatively, the maintenance of oxidative phosphorylation in prohibitin-deficient mitochondria could be due to compensatory effects of enhanced mitochondrial metabolism or increased expression of nuclear-encoded mitochondrial proteins. However, experimental support is currently scarce and requires more *in vitro* and *in vivo* experiments.

4.3 Prohibitins control OPA1 cleavage in the mitochondrial inner membrane

OPA1 cleavage correlates with the energy status of mitochondria and is induced upon apoptosis (Duvezin-Caubet et al., 2006; Baricoult et al. 2007). However, deletion of *Phb2* did not affect the mitochondrial membrane potential or respiratory activity, nor do PHB2-deficient cells undergo apoptosis in the absence of stimuli, indicating that OPA1 cleavage is under direct control by prohibitins. As deficiencies of *Phb2*$^{-/-}$ cells can be rescued by L-OPA1, a role for prohibitins in the biogenesis or folding of OPA1 appears unlikely. Rather,

the loss of L-OPA1 and accumulation of S-OPA1 isoforms in *Phb2*$^{-/-}$ cells indicates that L-OPA1 isoforms are destabilized and processing is facilitated in the absence of PHB2. This is reminiscent of findings in yeast where deletion of prohibitin genes results in an accelerated proteolysis of non-assembled inner membrane proteins by the *m*-AAA protease (Steglich et al., 1999). Large assemblies of prohibitins and *m*-AAA proteases are present in the mitochondrial inner membrane (Steglich et al., 1999). Moreover, reconstitution experiments in yeast revealed that various mammalian *m*-AAA protease isoenzymes are able to cleave OPA1 (Duvezin-Caubet et al., 2007). Notably, several peptidases appear to regulate OPA1 cleavage at different sites (Cipolat et al., 2006; Duvezin-Caubet et al., 2007; Griparic et al., 2007; Ishihara et al., 2006; Song et al., 2007). In contrast to other OPA1 isoforms, S4 appears to be generated by the *i*-AAA protease Yme1L (Griparic et al., 2007; Song et al., 2007). It accumulates at decreased levels absent in *Phb2*$^{-/-}$ cells suggesting that prohibitins regulate the cleavage of OPA1 by various peptidases.

4.4 Anti-apoptotic function of prohibitins

Depletion of prohibitins in MEFs resulted in impaired cell proliferation without causing spontaneous cell death. A participation of prohibitins in programmed cell death has previously been investigated in several studies. An anti-apoptotic activity of PHB1 has been demonstrated upon growth factor withdrawal in mammalian cells (Vander Heiden et al., 2002). In addition, the overexpression of PHB1 was shown to protect against camptothecin-induced apoptosis (Fusaro et al., 2002). In contrast to the presented results in this study, however, the cell death-inhibitory function of PHB1 was assigned to a putative interaction with p53 (Fusaro et al., 2003). More recently, it was shown that HeLa cells undergo spontaneous, caspase-dependent apoptosis upon downregulation of PHB2, an effect that was attributed to the loss of the anti-apoptotic mitochondrial protein Hax1 (Kasashima et al., 2006). Strikingly, the investigation of prohibitin-deficient cells in this study did not provide any evidence for spontaneous apoptosis. Moreover, the anti-apoptotic protein Hax1 accumulated at similar levels in wild type and PHB2-deficient cells suggesting a different pathway of apoptosis induction in prohibitin-deficient cells.

The results presented in this study assigned the increased susceptibility of *Phb2*$^{-/-}$ cells towards apoptosis to the absence of long OPA1 isoforms. To facilitate cytochrome *c* release from the intermembrane space, mitochondrial cristae are restructured at early stages of

apoptosis (Scorrano et al., 2002), a process controlled by OPA1 (Frezza et al., 2006). A current model suggests that a complex containing L- and S-OPA1 controls mitochondrial cristae junctions and prevents the redistribution of cytochrome *c* from the cristal lumen to the peripheral intermembrane space (Frezza et al., 2006) (Figure 28). Accordingly, the loss of L-OPA1 in *Phb2*$^{-/-}$ cells might facilitate cytochrome *c* release from intracristal compartments. However, alternative models are also conceivable. A recent tomographic analysis of mitochondrial transformation during apoptosis suggests that cristae remodelling is not required for efficient cytochrome *c* release (Sun et al., 2007). Energized mitochondria containing vesicular intramitochondrial structures have been observed in HeLa cells shortly after induction of apoptosis and cytochrome *c* release (Sun et al., 2007). This is highly reminiscent of *Phb2*$^{-/-}$ cells which are fully energized and not apoptotic but more susceptible towards apoptotic stimuli. It therefore appears that preexisting ultrastructural mitochondrial alterations in *Phb2*$^{-/-}$ cells cause the facilitated progression of the apoptotic programme after stimulation, which is required to trigger cytochrome *c* release. Consistently, expression of L-OPA1 substitutes for the absence of prohibitins and protects prohibitin-deficient cells against apoptosis, demonstrating that PHB2 exerts its anti-apoptotic function via OPA1. It remains to be established, however, how an altered cristae morphology due to loss of L-OPA1 triggers an increased sensitivity of mitochondria to outer membrane permeabilization. Notably, we did not observe an anti-apoptotic effect of S-OPA1 that has been described previously (Cipolat et al., 2006). This apparent discrepancy could be explained by the absence of L-OPA1 in *Phb2*$^{-/-}$ cells used in this study, while S-OPA1 was overexpressed in wild type fibroblasts in previous experiments (Cipolat et al., 2006). It therefore appears likely that S-OPA1 exerts its anti-apoptotic effect only in complex with L-OPA1.

4.5 Prohibitins are essential for embryonic development

Manipulation of the mouse genome using homologous recombination in embryonic stem cells has been recognised as an extraordinary valuable technique for the analysis of mammalian gene function (Mak, 2007). However, conventional gene targeting results in the loss of gene function in all cells during pre- and postnatal development. Importantly, previous studies demonstrated that a conventional inactivation of prohibitin genes in multicellular organisms like mouse and *C. elegans* results in embryonic lethality indicating essential functions during organismal development (Artal-Sanz et al., 2003; Park et al., 2005). To

circumvent embryonic lethality in mice, the *Phb2* gene was inactivated using a conditional gene targeting strategy (Rajewsky et al., 1996). For this purpose, *loxP* sites flanking exons 3 and 4 were introduced into the endogenous *Phb2* gene. Viability of homozygous conditional $Phb2^{fl/fl}$ mice demonstrates that the inserted *loxP* sites do not interfere with regular splicing and expression of the *Phb2* transcript. Genetic deletion of *Phb2 in vivo* was achieved by crossing conditional $Phb2^{fl/fl}$ mice to Deleter-Cre mice ubiquitously expressing the Cre-recombinase in the mouse including the germline (Schwenk et al., 1995). Intercrossings of heterozygous $Phb2^{+/-}$ mice revealed the absence of viable homozygous knockout animals. Moreover, neither *Phb2*-deficient embryos were identified before embryonic day (E) 8.5 of development indicating embryonic lethality at an early stage of development. These findings are consistent with previous observations using conventional gene targeting strategies for either PHB2 (Park et al., 2005) or PHB1 (He et al., 2008). To determine a specific role of PHB2 in tissue development, conditional $Phb2^{fl/fl}$ mice were crossed to Nestin-Cre mice expressing the Cre-recombinase specifically in the brain (Tronche et al., 1999). Similarly, viable mutant offsprings were not detected suggesting an essential role for PHB2 in the developing central nervous system. It should be noted, that the early timepoint of lethality in PHB2-deficient embryos prevented a careful investigation of the underlying reason.

Similar to the role of PHB2 in early embryonic development, mutational loss of mitochondrial morphology components has been described to cause embryonic lethal phenotypes. Mice lacking either MFN1 or MFN2, large GTPases in the mitochondrial outer membrane essential for fusion, die in midgestation around E10.5 (Chen et al., 2003). The independent generation of two OPA1-deficient mouse models revealed a need for OPA1 in embryonic development, although differences in the timepoint of lethality have been reported (Alavi et al., 2007; Davies et al., 2007). Notably, genetic disruption of the Drosophila homologue of OPA1 also causes embryonic lethality (Yarosh et al., 2008). These genetic data suggest an essential requirement of mitochondrial fusion for embryonic development. In accordance with this, impaired mitochondrial dynamics might be a causative factor for the embryonic lethality of *Phb2*-deficient mice. However, other reasons are not mutually exclusive. Defective cell proliferation and loss of anti-apoptotic activity in the absence of PHB2, as evidenced in prohibitin-deficient MEFs, might account for early embryonic death *in vivo*. Consistent with this, cell proliferation is essential for embryogenesis demonstrated by a large number of studies using genetically modified mice (Ciemerych and Sicinski, 2005). Furthermore, a requirement in early embryogenesis has been demonstrated for several cell death regulators (Ranger et al., 2001). Mice deficient in cytochrome *c* (Li et al., 2000),

apoptosis-inducing factor (AIF) (Joza et al., 2001) or the Bcl2 family member MCL1 (Rinkenberger et al., 2000) exhibit similar stages of lethality during embryonic development. Although the physiological role of prohibitins in cell proliferation and apoptosis *in vivo* has not yet been adequately addressed yet, it is tempting to speculate that the combined impairment of these essential processes might contribute to the early developmental defects in PHB2-deficient mice. Clearly, further experiments are required to dissect the role of prohibitins in cell proliferation, apoptotic resistance and mitochondrial dynamics *in vivo*.

A supercomplex of prohibitins and the *m*-AAA protease has been demonstrated in both yeast (Steglich et al., 1999) and mammalian mitochondria (M. Metodiev & T. Langer, unpublished observation) suggesting functional conservation of this interaction. Notably, the phenotype of prohibitin-deficient mice is in marked contrast to mice lacking paraplegin or AFG3L2, subunits of the mammalian *m*-AAA protease. Whereas paraplegin-deficient mice are viable and survive to adulthood, mutagenesis of Afg3l2 results in postnatal lethality at day P16 (Ferreirinha et al., 2004; Maltecca et al., 2008). These findings indicate different physiological requirements for prohibitins and *m*-AAA proteases *in vivo*. Moreover, prohibitins appear to play more decisive roles in mammalian mitochondria besides a putative regulation of the *m*-AAA protease.

4.6 Prohibitins may serve as scaffolds in the organization of the mitochondrial inner membrane

Maintenance of inner membrane topology is an essential requirement for the energy status and the dynamic nature of mitochondria. The large surface area of the protein-rich inner membrane requires an intimate organization into numerous cristae (Mannella, 2006). Recent evidence suggest that mitochondrial inner membrane remodelling contributes to various processes, ranging from respiration to calcium homeostasis and cytochrome *c* release during apoptosis (Heath-Engel and Shore, 2006). Considering the versatility and importance of these processes for cellular survival, a tight regulation of mitochondrial membrane dynamics is highly conceivable.

Prohibitins regulate mitochondrial dynamics and apoptotic resistance by controlling OPA1 stability. How do prohibitins affect the processing of OPA1 within mitochondria?

It is proposed here that prohibitins serve as scaffolds in the inner membrane and define the spatial organization of components, which control the stability and processing of OPA1 and coordinate membrane fusion. Ring-like prohibitin complexes may sequester OPA1 and recruit *m*-AAA proteases to membrane domains involved in the fusion process. In a hypothetical scenario, the prohibitin complex might provide the structural basis for the temporal assembly of a "mitochondrial fusion complex" allowing the interaction of OPA1 in the inner membrane with mitofusins in the outer membrane. Interestingly, both genetic and biochemical interactions of the fusion components OPA1 and MFN1 support this idea (Cipolat et al., 2004; Guillery et al., 2007). Considering the sequence similarity of prohibitins to lipid raft associated proteins (Langhorst et al., 2005; Morrow and Parton, 2005; Tavernarakis et al., 1999), prohibitins may also promote the lateral segregation of membrane lipids resulting in the formation of lipid microdomains. Surrounding membrane lipids are likely to affect the vectorial membrane dislocation of OPA1 or the proteolytic activity of *m*-AAA proteases. Moreover, increasing evidence points to an important role of lipids and lipid microdomains in various cellular fusion events (Altmann and Westermann, 2005; Boukh-Viner et al., 2005; Choi et al., 2006; Fratti et al., 2004) and apoptotic processes (Choi et al., 2007). Interestingly, recent observations suggest an impact of the mitochondria-specific phospholipid cardiolipin on membrane organization and dynamics (Kim et al., 2004). Cardiolipin interacts with several mitochondrial proteins including cytochrome *c* and provides its association with the inner membrane (Tuominen et al., 2002). Moreover, cardiolipin has been implicated in the higher order organization of respiratory chain complexes (Robinson, 1993; Zhang et al., 2002) and is required for tBid recruitment during mitochondrial apoptosis (Gonzalvez et al., 2005; Lutter et al., 2000). Strikingly, a defective mitochondrial cristae morphogenesis has been observed in cardiolipin-deficient human cells suggesting that inner membrane remodelling might be a cardiolipin-mediated process (Choi et al., 2007). Interestingly, cardiolipin-deficient cells display an increased sensitivity towards apoptotic stimuli but retain respiratory chain activity, a striking similarity to prohibitin-deficient cells (Choi et al., 2007; Merkwirth et al., 2008). Furthermore, *in vitro* studies demonstrated that cardiolipin and other lipids contribute to the insertion and oligomerization of the outer membrane proteins Bid and Bax (Kuwana et al., 2002; Lucken-Ardjomande et al., 2008). These and other observations suggest an active redistribution of cardiolipin from the inner membrane to specific locations in the outer membrane of mitochondria. In this context, prohibitins could serve as local platforms for the accumulation of "lipid hot spots" in the inner membrane that contribute to the active distribution of lipids.

The presented work shed new light on the regulation of inner membrane topology and provides a technical framework for further experiments to unravel the molecular basis of mitochondrial membrane morphogenesis. The identification of prohibitins as regulators of mitochondrial cristae morphology now paves the way for a detailed understanding of the role of spatial membrane organization for the morphogenesis and function of mitochondria.

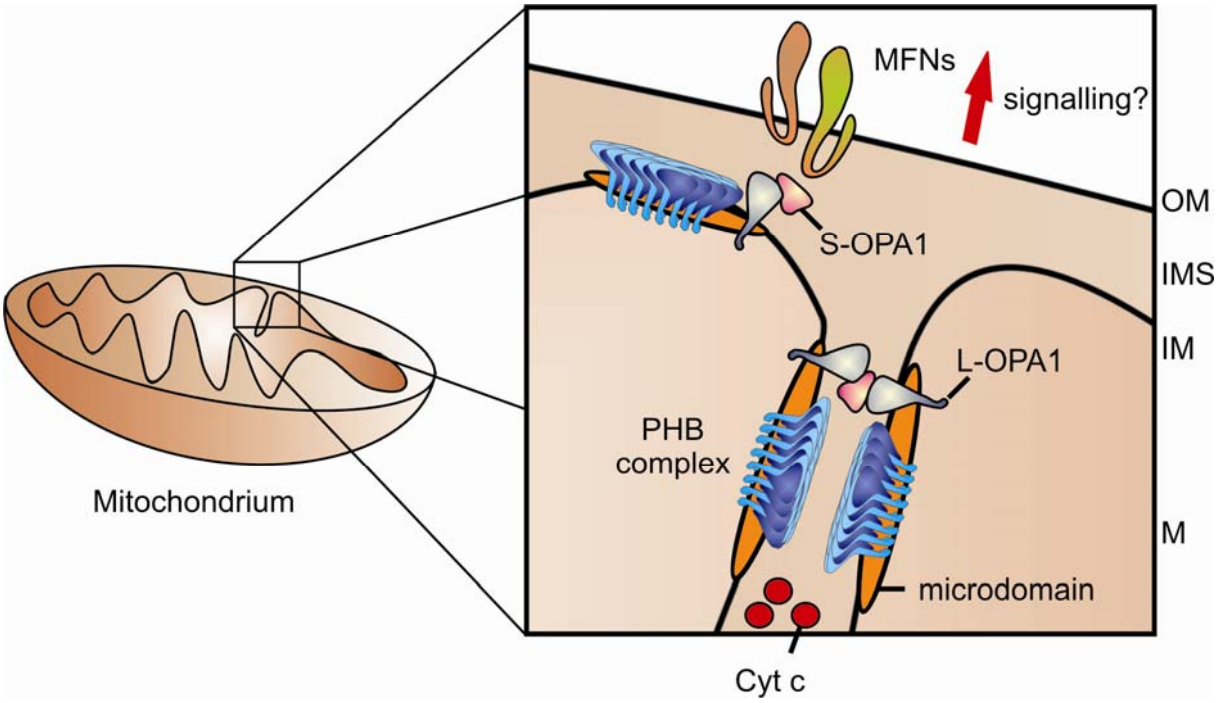

Figure 28. Model for the proposed functions of prohibitins within mitochondria.

The mitochondrial prohibitin complex determines cristae morphology and protects against apoptosis by the stabilization of L-OPA1. According to a current model, complexes of L- and S-OPA1 control mitochondrial cristae junctions and prevent the redistribution of cytochrome *c* from the cristal lumen to the peripheral intermembrane space (Frezza et al., 2006). Prohibitins might also regulate mitochondrial fusion by protecting L-OPA1 isoforms which, together with mitofusins, are required for mitochondrial membrane fusion. Cristae morphogenesis might also control cell proliferation by a yet unknown signalling pathway (red arrow). PHB = prohibitin; MFNs = mitofusins; Cyt c = Cytochrome *c*; OM = outer membrane; IMS = intermembrane space; IM = inner membrane; M = matrix.

4.7 Perspectives

The presented data support previous findings on the essential role of prohibitins for mouse development. Furthermore, the study provides a mechanistic explanation for the function of prohibitins in mitochondrial morphogenesis, apoptotic resistance and cell proliferation. Future experiments will focus on putative signalling events which are involved in the control of cell proliferation by organelle morphogenesis. Another interesting aspect to

investigate is the maintenance of respiratory activities in prohibitin-deficient MEFs. More detailed work will address a possible role of S-OPA1 isoforms in the regulation of oxidative phosphorylation.

Additional studies will concentrate on the analysis of prohibitin deficiency *in vivo*. With the help of tissue-specific PHB2 mutant mice, functional consequences of a PHB2 depletion on cell proliferation and apoptosis will be investigated *in vivo*. Inducible inactivation of prohibitins in adult mice will further support this experimental strategy. Several essential components controlling mitochondrial fusion are mutated in neurodegenerative disorders. With regard to the genetic link between prohibitins and OPA1 uncovered in this study, it is required to determine the activity of prohibitins in neurons. The neuron-specific ablation of prohibitins will be useful to elucidate a role of prohibitins in the pathogenesis of neurodegeneration

5 Summary

Prohibitins comprise an evolutionary conserved and ubiquitously expressed family of membrane proteins implicated in a large variety of cellular processes. Large assemblies of PHB1 and PHB2 subunits are localized in the inner membrane of mitochondria, but various roles in other cellular compartments have also been proposed for both proteins. However, the function of prohibitins on the molecular level remains unclear.

To investigate the physiological role of mammalian prohibitins, a mouse strain for the conditional, Cre/*loxP*-mediated inactivation of *Phb2* was generated. Ubiquitous and brain-restricted deletion of *Phb2 in vivo* caused embryonic lethality indicating an essential role of *Phb2* in mammalian development. To determine cellular functions of PHB2, a cell culture system was established allowing to define functional consequences of a *Phb2* deletion. Mouse embryonic fibroblasts (MEFs) isolated from $Phb2^{fl/fl}$ embryos were transduced with cell-permeable Cre-recombinase to inactivate *Phb2*. Functional interdependence of prohibitin subunits was observed in MEFs after PHB2 depletion, illustrating the physiological relevance of the assembled prohibitin complex. The absence of prohibitins in MEFs leads to impaired cell proliferation and increased sensitivity towards apoptotic stimuli. This is accompanied by fragmentation of the mitochondrial network and defective morphogenesis of mitochondrial cristae. Complementation experiments attribute these defects to the loss of mitochondria-localized prohibitins indicating an essential requirement of the mitochondrial prohibitin complex in these processes. Loss of prohibitins affects the proteolytic cleavage of OPA1, a dynamin-like GTPase in the inner membrane essential for mitochondrial fusion, leading to the selective loss of long isoforms of OPA1. The specific expression of a long OPA1 isoform in prohibitin-deficient MEFs restores cristae morphogenesis, apoptotic resistance and partially cell proliferation, identifying impaired OPA1 processing as the primary cause for the cellular defects in the absence of prohibitins.

These results identify a novel roles for mitochondrial prohibitins in cell proliferation and cristae formation by the proteolytic modulation of OPA1, suggesting a molecular interplay between cell growth and organelle morphogenesis.

6 Zusammenfassung

Prohibitine bilden eine hochkonservierte und ubiquitär exprimierte Familie von Membranproteinen, die mit einer Vielzahl zellulärer Prozesse in Verbindung gebracht wurden. PHB1- und PHB2-Untereinheiten bilden große Komplexe in der inneren Membran von Mitochondrien, allerdings wurden zusätzliche Funktionen für beide Proteine auch in anderen Zellkompartimenten vorgeschlagen. Die zugrunde liegenden Funktionsmechanismen beider Proteine auf molekularer Ebene sind jedoch unbekannt. Die Erstellung eines konditionalen Mausstammes für die Cre/*loxP*-vermittelte Inaktivierung des *Phb2*-Gens ermöglicht die Untersuchung der physiologischer Funktionen von Prohibitinen in Säugern. Die Inaktivierung des Phb2-Gens führte, sowohl im gesamten Organismus als auch gewebespezifisch im Gehirn der Maus, zu embryonaler Lethalität. Diese Befunde deuten auf eine entscheidende Rolle für Phb2 in der embryonalen Entwicklung der Maus hin. Um zelluläre Funktionen von PHB2 zu bestimmen, wurde ein Zellkultursystem entwickelt, welches die Identifizierung funktioneller Konsequenzen einer PHB2-Defizienz ermöglicht. Embryonale Mausfibroblasten wurden aus $Phb2^{fl/fl}$ Embryonen isoliert und mit zellgängiger Cre-Rekombinase transduziert, um das *Phb2*-Gens zu inaktivieren. Der simultane Verlust von PHB1 und PHB2 in Cre-behandelten $Phb2^{fl/fl}$-Zellen legte eine funktionelle Abhängigkeit beider Untereinheiten nahe, welche auf die entscheidende physiologische Bedeutung des Prohibitin-Komplexes hindeutet. Die Abwesenheit des Prohibitin-Komplexes in Mausfibroblasten führte zur Beeinträchtigung des Zellwachstums und zu einer erhöhten Anfälligkeit gegenüber Apoptose-auslösenden Stimuli. Des Weiteren wurde eine Fragmentierung des mitochondrialen Netzwerkes sowie die fehlerhafte Ausbildung von Cristaestrukturen beobachtet. Komplementationsstudien führten diese Effekte auf die mitochondriale Lokalisierung von Prohibitinen zurück. Prohibitin-Defizienz beeinträchtigte zusätzlich die Prozessierung von OPA1, einer dynamin-verwandten Fusions-GTPase in der mitochondrialen Innenmembran, welches zum Verlust von langen Isoformen führte. Die gezielte Expression einer langen OPA1-Isoform in PHB2-defizienten Zellen konnte sowohl die mitochondriale Innenmembranstruktur und Apoptoseresistenz als auch den Zellwachstumsdefekt wiederherstellen. Die Ergebnisse dieser Untersuchungen identifizierten eine neue Funktion von Prohibitinen in der Regulation von Zellwachstum und Cristaeformation und lassen daher eine molekulare Verbindung zwischen Zellproliferation and mitochondrialer Formgebung schliessen.

7 References

Aijaz, S., Erskine, L., Jeffery, G., Bhattacharya, S. S., and Votruba, M. (2004). Developmental expression profile of the optic atrophy gene product: OPA1 is not localized exclusively in the mammalian retinal ganglion cell layer. Invest Ophthalmol Vis Sci *45*, 1667-1673.

Alavi, M. V., Bette, S., Schimpf, S., Schuettauf, F., Schraermeyer, U., Wehrl, H. F., Ruttiger, L., Beck, S. C., Tonagel, F., Pichler, B. J.*, et al.* (2007). A splice site mutation in the murine Opa1 gene features pathology of autosomal dominant optic atrophy. Brain *130*, 1029-1042.

Alexander, C., Votruba, M., Pesch, U. E., Thiselton, D. L., Mayer, S., Moore, A., Rodriguez, M., Kellner, U., Leo-Kottler, B., Auburger, G.*, et al.* (2000). *OPA1*, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nat Genet *26*, 211-215.

Altmann, K., and Westermann, B. (2005). Role of essential genes in mitochondrial morphogenesis in Saccharomyces cerevisiae. Mol Biol Cell *16*, 5410-5417.

Amutha, B., Gordon, D. M., Gu, Y., and Pain, D. (2004). A novel role of Mgm1p, a dynamin-related GTPase, in ATP synthase assembly and cristae formation/maintenance. Biochem J *381*, 19-23.

Anderson, R. G., and Jacobson, K. (2002). A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. Science *296*, 1821-1825.

Arlt, H., Tauer, R., Feldmann, H., Neupert, W., and Langer, T. (1996). The YTA10-12-complex, an AAA protease with chaperone-like activity in the inner membrane of mitochondria. Cell *85*, 875-885.

Arnoult, D., Grodet, A., Lee, Y. J., Estaquier, J., and Blackstone, C. (2005). Release of OPA1 during apoptosis participates in the rapid and complete release of cytochrome *c* and subsequent mitochondrial fragmentation. J Biol Chem *280*, 35742-35750.

Arselin, G., Vaillier, J., Salin, B., Schaeffer, J., Giraud, M. F., Dautant, A., Brethes, D., and Velours, J. (2004). The modulation in subunits e and g amounts of yeast ATP synthase modifies mitochondrial cristae morphology. J Biol Chem *279*, 40392-40329.

Artal-Sanz, M., Tsang, W. Y., Willems, E. M., Grivell, L. A., Lemire, B. D., van der Spek, H., Nijtmans, L. G., and Sanz, M. A. (2003). The mitochondrial prohibitin complex is essential for embryonic viability and germline function in *Caenorhabditis elegans*. J Biol Chem *278*, 32091-32099.

Asquith, B., Debacq, C., Florins, A., Gillet, N., Sanchez-Alcaraz, T., Mosley, A., and Willems, L. (2006). Quantifying lymphocyte kinetics in vivo using carboxyfluorescein diacetate succinimidyl ester (CFSE). Proc R Soc B *273*, 1165-1171.

Babiychuk, E. B., and Draeger, A. (2000). Annexins in cell membrane dynamics. Ca(2+)-regulated association of lipid microdomains. J Cell Biol *150*, 1113-1124.

Back, J. W., Sanz, M. A., De Jong, L., De Koning, L. J., Nijtmans, L. G., De Koster, C. G., Grivell, L. A., Van Der Spek, H., and Muijsers, A. O. (2002). A structure for the yeast prohibitin complex: Structure prediction and evidence from chemical crosslinking and mass spectrometry. Protein Sci *11*, 2471-2478.

Balaban, R. S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. Cell *120*, 483-495.

Baloh, R. H., Schmidt, R. E., Pestronk, A., and Milbrandt, J. (2007). Altered axonal mitochondrial transport in the pathogenesis of Charcot-Marie-Tooth disease from mitofusin 2 mutations. J Neurosci *27*, 422-430.

Banuett, F., and Herskowitz, I. (1987). Identification of polypeptides encoded by an Escherichia coli locus (hflA) that governs the lysis-lysogeny decision of bacteriophage lambda. J Bacteriol *169*, 4076-4085.

Baricault, L., Segui, B., Guegand, L., Olichon, A., Valette, A., Larminat, F., and Lenaers, G. (2007). OPA1 cleavage depends on decreased mitochondrial ATP level and bivalent metals. Exp Cell Res *313*, 3800-3808.

Benoist, C., and Chambon, P. (1980). Deletions covering the putative promoter region of early mRNAs of simian virus 40 do not abolish T-antigen expression. Proc Natl Acad Sci U S A *77*, 3865-3869.

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.

Berridge, M. J., and Irvine, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature *312*, 315-321.

Berridge, M. J., and Irvine, R. F. (1989). Inositol phosphates and cell signalling. Nature *341*, 197-205.

Bette, S., Schlaszus, H., Wissinger, B., Meyermann, R., and Mittelbronn, M. (2005). OPA1, associated with autosomal dominant optic atrophy, is widely expressed in the human brain. Acta Neuropathol (Berl) *109*, 393-399.

Bickel, P. E., Scherer, P. E., Schnitzer, J. E., Oh, P., Lisanti, M. P., and Lodish, H. F. (1997). Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. J Biol Chem *272*, 13793-13802.

Birner, R., Nebauer, R., Schneiter, R., and Daum, G. (2003). Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine biosynthetic machinery with the prohibitin complex of *Saccharomyces cerevisiae*. Mol Biol Cell *14*, 370-383.

Bogenhagen, D. F., Wang, Y., Shen, E. L., and Kobayashi, R. (2003). Protein components of mitochondrial DNA nucleoids in higher eukaryotes. Mol Cell Proteomics *2*, 1205-1216.

Boldogh, I., Vojtov, N., Karmon, S., and Pon, L. A. (1998). Interaction between mitochondria and the actin cytoskeleton in budding yeast requires two integral mitochondrial outer membrane proteins, Mmm1p and Mdm10p. J Cell Biol *141*, 1371-1381.

Boldogh, I. R., Nowakowski, D. W., Yang, H. C., Chung, H., Karmon, S., Royes, P., and Pon, L. A. (2003). A protein complex containing Mdm10p, Mdm12p, and Mmm1p links mitochondrial membranes and DNA to the cytoskeleton-based segregation machinery. Mol Biol Cell *14*, 4618-4627.

Bornhovd, C., Vogel, F., Neupert, W., and Reichert, A. S. (2006). Mitochondrial membrane potential is dependent on the oligomeric state of F_1F_O-ATP synthase supracomplexes. J Biol Chem *281*, 13990-13998.

Boukh-Viner, T., Guo, T., Alexandrian, A., Cerracchio, A., Gregg, C., Haile, S., Kyskan, R., Milijevic, S., Oren, D., Solomon, J.*, et al.* (2005). Dynamic ergosterol- and ceramide-

rich domains in the peroxisomal membrane serve as an organizing platform for peroxisome fusion. J Cell Biol *168*, 761-773.

Boute, N., Gribouval, O., Roselli, S., Benessy, F., Lee, H., Fuchshuber, A., Dahan, K., Gubler, M. C., Niaudet, P., and Antignac, C. (2000). NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat Genet *24*, 349-354.

Briere, L. K., and Dunn, S. D. (2006). The periplasmic domains of Escherichia coli HflKC oligomerize through right-handed coiled-coil interactions. Biochemistry *45*, 8607-8616.

Bromley, S. K., Burack, W. R., Johnson, K. G., Somersalo, K., Sims, T. N., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., and Dustin, M. L. (2001). The immunological synapse. Annu Rev Immunol *19*, 375-396.

Browman, D. T., Hoegg, M. B., and Robbins, S. M. (2007). The SPFH domain-containing proteins: more than lipid raft markers. Trends Cell Biol *17*, 394-402.

Browman, D. T., Resek, M. E., Zajchowski, L. D., and Robbins, S. M. (2006). Erlin-1 and erlin-2 are novel members of the prohibitin family of proteins that define lipid-raft-like domains of the ER. J Cell Sci *119*, 3149-3160.

Brown, D. A., and London, E. (1998). Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol *14*, 111-136.

Brown, D. A., and Rose, J. K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell *68*, 533-544.

Bruckner, K., Pablo Labrador, J., Scheiffele, P., Herb, A., Seeburg, P. H., and Klein, R. (1999). EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains. Neuron *22*, 511-524.

Cereghetti, G. M., and Scorrano, L. (2006). The many shapes of mitochondrial death. Oncogene *25*, 4717-4724.

Chamberlain, L. H., Burgoyne, R. D., and Gould, G. W. (2001). SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis. Proc Natl Acad Sci U S A *98*, 5619-5624.

Chen, H., and Chan, D. C. (2005). Emerging functions of mammalian mitochondrial fusion and fission. Hum Mol Genet *14 Spec No. 2*, R283-289.

Chen, H., and Chan, D. C. (2006). Critical dependence of neurons on mitochondrial dynamics. Curr Opin Cell Biol *18*, 453-459.

Chen, H., Chomyn, A., and Chan, D. C. (2005). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J Biol Chem *280*, 26185-26192.

Chen, H., Detmer, S. A., Ewald, A. J., Griffin, E. E., Fraser, S. E., and Chan, D. C. (2003). Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J Cell Biol *160*, 189-200.

Chen, H., McCaffery, J. M., and Chan, D. C. (2007). Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell *130*, 548-562.

Chen, X. J., and Butow, R. A. (2005). The organization and inheritance of the mitochondrial genome. Nat Rev Genet *6*, 815-825.

Cheng, P. C., Dykstra, M. L., Mitchell, R. N., and Pierce, S. K. (1999). A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. J Exp Med *190*, 1549-1560.

Choi, S. Y., Gonzalvez, F., Jenkins, G. M., Slomianny, C., Chretien, D., Arnoult, D., Petit, P. X., and Frohman, M. A. (2007). Cardiolipin deficiency releases cytochrome c from the inner mitochondrial membrane and accelerates stimuli-elicited apoptosis. Cell Death Diff *14*, 597-606.

Choi, S. Y., Huang, P., Jenkins, G. M., Chan, D. C., Schiller, J., and Frohman, M. A. (2006). A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. Nat Cell Biol *8*, 1255-1262.

Ciechanover, A. (2005). Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol Cell Biol *6*, 79-87.

Ciemerych, M. A., and Sicinski, P. (2005). Cell cycle in mouse development. Oncogene *24*, 2877-2898.

Cipolat, S., Martins de Brito, O., Dal Zilio, B., and Scorrano, L. (2004). OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc Natl Acad Sci U S A *101*, 15927-15932.

Cipolat, S., Rudka, T., Hartmann, D., Costa, V., Serneels, L., Craessaerts, K., Metzger, K., Frezza, C., Annaert, W., D'Adamio, L*., et al.* (2006). Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. Cell *126*, 163-175.

Clapham, D. E. (2007). Calcium signaling. Cell *131*, 1047-1058.

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.

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

Curran, S. P., Leuenberger, D., Schmidt, E., and Koehler, C. M. (2002). The role of the Tim8p-Tim13p complex in a conserved import pathway for mitochondrial polytopic inner membrane proteins. J Cell Biol *158*, 1017-1027.

Da Cruz, S., Parone, P., Gonzalo, P., Bienvenut, W.V., Tondera, D., Jourdain, A., Quadroni, M. and Martinou, J.-C. (2008). SLP2 interacts with prohibitins in the mitochondrial inner membrane and contributes to their stability. Biochim Biophys Acta *in press*.

Davies, V. J., Hollins, A. J., Piechota, M. J., Yip, W., Davies, J. R., White, K. E., Nicols, P. P., Boulton, M. E., and Votruba, M. (2007). Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function. Hum Mol Genet *16*, 1307-1318.

Davis, A. J., Sepuri, N. B., Holder, J., Johnson, A. E., and Jensen, R. E. (2000). Two intermembrane space TIM complexes interact with different domains of Tim23p during its import into mitochondria. J Cell Biol *150*, 1271-1282.

De Diego, J. G., David Rodriguez, F., Rodriguez Lorenzo, J. L., and Cervantes, E. (2007). The prohibitin genes in Arabidopsis thaliana: expression in seeds, hormonal regulation and possible role in cell cycle control during seed germination. J Plant Physiol *164*, 371-373.

Delage-Mourroux, R., Martini, P. G., Choi, I., Kraichely, D. M., Hoeksema, J., and Katzenellenbogen, B. S. (2000). Analysis of estrogen receptor interaction with a

repressor of estrogen receptor activity (REA) and the regulation of estrogen receptor transcriptional activity by REA. J Biol Chem *275*, 35848-35856.

Delaunay, J., Stewart, G., and Iolascon, A. (1999). Hereditary dehydrated and overhydrated stomatocytosis: recent advances. Curr Opin Hematol *6*, 110-114.

Delettre, C., Griffoin, J. M., Kaplan, J., Dollfus, H., Lorenz, B., Faivre, L., Lenaers, G., Belenguer, P., and Hamel, C. P. (2001). Mutation spectrum and splicing variants in the *OPA1* gene. Hum Genet *109*, 584-591.

Delettre, C., Lenaers, G., Griffoin, J. M., Gigarel, N., Lorenzo, C., Belenguer, P., Pelloquin, L., Grosgeorge, J., Turc-Carel, C., Perret, E.*, et al.* (2000). Nuclear gene *OPA1*, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat Genet *26*, 207-210.

Delettre, C., Lenaers, G., Pelloquin, L., Belenguer, P., and Hamel, C. P. (2002). OPA1 (Kjer type) dominant optic atrophy: a novel mitochondrial disease. Mol Genet Metab *75*, 97-107.

Dermine, J. F., Duclos, S., Garin, J., St-Louis, F., Rea, S., Parton, R. G., and Desjardins, M. (2001). Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. J Biol Chem *276*, 18507-18512.

Detmer, S. A., and Chan, D. C. (2007). Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. J Cell Biol *176*, 405-414.

Detmer, S. A., and Chan, D. C. (2007). Functions and dysfunctions of mitochondrial dynamics. Nat Rev Mol Cell Biol *8*, 870-879.

Detmer, S. A., Vande Velde, C., Cleveland, D. W., and Chan, D. C. (2008). Hindlimb gait defects due to motor axon loss and reduced distal muscles in a transgenic mouse model of Charcot-Marie-Tooth type 2A. Hum Mol Genet *17*, 367-375.

Di Paolo, G., Moskowitz, H. S., Gipson, K., Wenk, M. R., Voronov, S., Obayashi, M., Flavell, R., Fitzsimonds, R. M., Ryan, T. A., and De Camilli, P. (2004). Impaired PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking. Nature *431*, 415-422.

DiNitto, J. P., Cronin, T. C., and Lambright, D. G. (2003). Membrane recognition and targeting by lipid-binding domains. Sci STKE *2003*, re16.

Doyle, D. D., Goings, G. E., Upshaw-Earley, J., Page, E., Ranscht, B., and Palfrey, H. C. (1998). T-cadherin is a major glycophosphoinositol-anchored protein associated with noncaveolar detergent-insoluble domains of the cardiac sarcolemma. J Biol Chem *273*, 6937-6943.

Duchen, M. R. (2000). Mitochondria and calcium: from cell signalling to cell death. J Physiol *529 Pt 1*, 57-68.

Duvezin-Caubet, S., Jagasia, R., Wagener, J., Hofmann, S., Trifunovic, A., Hansson, A., Chomyn, A., Bauer, M. F., Attardi, G., Larsson, N. G.*, et al.* (2006). Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. J Biol Chem *281*, 37972-37979.

Duvezin-Caubet, S., Koppen, M., Wagener, J., Zick, M., Israel, L., Bernacchia, A., Jagasia, R., Rugarli, E. I., Imhof, A., Neupert, W.*, et al.* (2007). Processing of OPA1 reconstituted in yeast depends on the subunit composition of the *m*-AAA protease. in press.

Engelman, D. M. (2005). Membranes are more mosaic than fluid. Nature *438*, 578-580.

Ernstrom, G. G., and Chalfie, M. (2002). Genetics of sensory mechanotransduction. Annu Rev Genet *36*, 411-453.

Escobar-Henriques, M., Westermann, B., and Langer, T. (2006). Regulation of mitochondrial fusion by the F-box protein Mdm30 involves proteasome-independent turnover of Fzo1. J Cell Biol *173*, 645-650.

Eura, Y., Ishihara, N., Yokota, S., and Mihara, K. (2003). Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. J Biochem (Tokyo) *134*, 333-344.

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.

Ewen, M. E. (1994). The cell cycle and the retinoblastoma protein family. Cancer Metastasis Rev *13*, 45-66.

Ferreirinha, F., Quattrini, A., Priozzi, M., Valsecchi, V., Dina, G., Broccoli, V., Auricchio, A., Piemonte, F., Tozzi, G., Gaeta, L.*, et al.* (2004). Axonal degeneration in paraplegin-deficient mice is associated with abnormal mitochondria and impairment of axonal transport. J Clin Invest *113*, 231-242.

Foster, L. J., De Hoog, C. L., and Mann, M. (2003). Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. Proc Natl Acad Sci U S A *100*, 5813-5818.

Fratti, R. A., Jun, Y., Merz, A. J., Margolis, N., and Wickner, W. (2004). Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles. J Cell Biol *167*, 10987-11098.

Frezza, C., Cipolat, S., Martins de Brito, O., Micaroni, M., Beznoussenko, G. V., Rudka, T., Bartoli, D., Polishuck, R. S., Danial, N. N., De Strooper, B., and Scorrano, L. (2006). OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell *126*, 177-189.

Frick, M., Bright, N. A., Riento, K., Bray, A., Merrified, C., and Nichols, B. J. (2007). Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. Curr Biol *17*, 1151-1156.

Fricke, B., Argent, A. C., Chetty, M. C., Pizzey, A. R., Turner, E. J., Ho, M. M., Iolascon, A., von During, M., and Stewart, G. W. (2003). The "stomatin" gene and protein in overhydrated hereditary stomatocytosis. Blood *102*, 2268-2277.

Fritz, S., Rapaport, D., Klanner, E., Neupert, W., and Westermann, B. (2001). Connection of the mitochondrial outer and inner membranes by Fzo1 is critical for organellar fusion. J Cell Biol *152*, 683-692.

Fritz, S., Weinbach, N., and Westermann, B. (2003). Mdm30 is an F-box protein required for maintenance of fusion-competent mitochondria in yeast. Mol Biol Cell *14*, 2303-2313.

Fuchshuber, A., Jean, G., Gribouval, O., Gubler, M. C., Broyer, M., Beckmann, J. S., Niaudet, P., and Antignac, C. (1995). Mapping a gene (SRN1) to chromosome 1q25-q31 in idiopathic nephrotic syndrome confirms a distinct entity of autosomal recessive nephrosis. Hum Mol Genet *4*, 2155-2158.

Fusaro, G., Dasgupta, P., Rastogi, S., Joshi, B., and Chellappan, S. (2003). Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. J Biol Chem *278*, 47853-47861.

Fusaro, G., Wang, S., and Chellappan, S. (2002). Differential regulation of Rb family proteins and prohibitin during camptothecin-induced apoptosis. Oncogene *21*, 4539-4548.

Gagescu, R., Demaurex, N., Parton, R. G., Hunziker, W., Huber, L. A., and Gruenberg, J. (2000). The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. Mol Biol Cell *11*, 2775-2791.

Galbiati, F., Volonte, D., Goltz, J. S., Steele, Z., Sen, J., Jurcsak, J., Stein, D., Stevens, L., and Lisanti, M. P. (1998). Identification, sequence and developmental expression of invertebrate flotillins from Drosophila melanogaster. Gene *210*, 229-237.

Gallagher, P. G., and Forget, B. G. (1995). Structure, organization, and expression of the human band 7.2b gene, a candidate gene for hereditary hydrocytosis. J Biol Chem *270*, 26358-26363.

Gamble, S. C., Odontiadis, M., Waxman, J., Westbrook, J. A., Dunn, M. J., Wait, R., Lam, E. W., and Bevan, C. L. (2004). Androgens target prohibitin to regulate proliferation of prostate cancer cells. Oncogene *23*, 2996-3004.

Garin, J., Diez, R., Kieffer, S., Dermine, J. F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., and Desjardins, M. (2001). The phagosome proteome: insight into phagosome functions. J Cell Biol *152*, 165-180.

Gilabert, J. A., and Parekh, A. B. (2000). Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca(2+) current I(CRAC). Embo J *19*, 6401-6407.

Gilkerson, R. W., Selker, J. M., and Capaldi, R. A. (2003). The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. FEBS Lett *546*, 355-358.

Gillespie, P. G., and Walker, R. G. (2001). Molecular basis of mechanosensory transduction. Nature *413*, 194-202.

Girardot, N., Allinquant, B., Langui, D., Laquerriere, A., Dubois, B., Hauw, J. J., and Duyckaerts, C. (2003). Accumulation of flotillin-1 in tangle-bearing neurones of Alzheimer's disease. Neuropathol Appl Neurobiol *29*, 451-461.

Gkantiragas, I., Brugger, B., Stuven, E., Kaloyanova, D., Li, X. Y., Lohr, K., Lottspeich, F., Wieland, F. T., and Helms, J. B. (2001). Sphingomyelin-enriched microdomains at the Golgi complex. Mol Biol Cell *12*, 1819-1833.

Glebov, O. O., Bright, N. A., and Nichols, B. J. (2006). Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol *8*, 46-54.

Gonzalvez, F., Pariselli, F., Dupaigne, P., Budihardjo, I., Lutter, M., Antonsson, B., Diolez, P., Manon, S., Martinou, J. C., Goubern, M*., et al.* (2005). tBid interaction with cardiolipin primarily orchestrates mitochondrial dysfunctions and subsequently activates Bax and Bak. Cell Death Differ *12*, 614-626.

Goodman, M. B., Ernstrom, G. G., Chelur, D. S., O'Hagan, R., Yao, C. A., and Chalfie, M. (2002). MEC-2 regulates C. elegans DEG/ENaC channels needed for mechanosensation. Nature *415*, 1039-1042.

Goyon, V., Fronzes, R., Salin, B., di Rago, J. P., Velours, J., and Brethes, D. (2008). Yeast cells depleted in Atp14p fail to assemble Atp6p within the ATP synthase and exhibit altered mitochondrial cristae morphology. J Biol Chem.

Griffin, E. E., and Chan, D. C. (2006). Domain interactions within Fzo1 oligomers are essential for mitochondrial fusion. J Biol Chem *281*, 16599-16606.

Griparic, L., Kanazawa, T., and van der Bliek, A. M. (2007). Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. J Cell Biol *178*, 757-764.

Griparic, L., van der Wel, N. N., Orozco, I. J., Peters, P. J., and van der Bliek, A. M. (2004). Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria. J Biol Chem *279*, 18792-18798.

Gu, G., Caldwell, G. A., and Chalfie, M. (1996). Genetic interactions affecting touch sensitivity in Caenorhabditis elegans. Proc Natl Acad Sci U S A *93*, 6577-6582.

Guillery, O., Malka, F., Landes, T., Guillou, E., Blackstone, C., Lombes, A., Belenguer, P., Arnoult, D., and Rojo, M. (2007). Metalloprotease-mediated OPA1 processing is modulated by the mitochondrial membrane potential. Biol Cell.

Hajek, P., Chomyn, A., and Attardi, G. (2007). Identification of a novel mitochondrial complex containing mitofusin 2 and stomatin-like protein 2. J Biol Chem *282*, 5670-5681.

Hales, K. G., and Fuller, M. T. (1997). Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. Cell *90*, 121-129.

Hancock, J. F. (2006). Lipid rafts: contentious only from simplistic standpoints. Nat Rev Mol Cell Biol *7*, 456-462.

Hancock, J. F., Paterson, H., and Marshall, C. J. (1990). A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. Cell *63*, 133-139.

Hanekamp, T., Thorsness, M. K., Rebbapragada, I., Fisher, E. M., Seebart, C., Darland, M. R., Coxbill, J. A., Updike, D. L., and Thorsness, P. E. (2002). Maintenance of mitochondrial morphology is linked to maintenance of the mitochondrial genome in Saccharomyces cerevisiae. Genetics *162*, 1147-1156.

Harder, T., Scheiffele, P., Verkade, P., and Simons, K. (1998). Lipid domain structure of the plasma membrane revealed by patching of membrane components. J Cell Biol *141*, 929-942.

Harris, T. J., and Siu, C. H. (2002). Reciprocal raft-receptor interactions and the assembly of adhesion complexes. Bioessays *24*, 996-1003.

He, B., Feng, Q., Mukherjee, A., Lonard, D. M., Demayo, F. J., Katzenellenbogen, B. S., Lydon, J. P., and O'Malley, B. W. (2008). A repressive role for prohibitin in estrogen signaling. Mol Endocrinol *22*, 344-360.

Heath-Engel, H. M., and Shore, G. C. (2006). Mitochondrial membrane dynamics, cristae remodelling and apoptosis. Biochim Biophys Acta *1763*, 549-560.

Herlan, M., Bornhovd, C., Hell, K., Neupert, W., and Reichert, A. S. (2004). Alternative topogenesis of Mgm1 and mitochondrial morphology depend on ATP and a functional import motor. J Cell Biol *165*, 167-173.

Herlan, M., Vogel, F., Bornhövd, C., Neupert, W., and Reichert, A. S. (2003). Processing of Mgm1 by the rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA. J Biol Chem *278*, 27781-27788.

Hermann, G. J., Thatcher, J. W., Mills, J. P., Hales, K. G., Fuller, M. T., Nunnari, J., and Shaw, J. M. (1998). Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. J Cell Biol *143*, 359-373.

Herskowitz, I., and Hagen, D. (1980). The lysis-lysogeny decision of phage lambda: explicit programming and responsiveness. Annu Rev Genet *14*, 399-445.

Hiebl-Dirschmied, C. M., Adolf, G. R., and Prohaska, R. (1991). Isolation and partial characterization of the human erythrocyte band 7 integral membrane protein. Biochim Biophys Acta *1065*, 195-202.

Hobbs, A. E., Srinivasan, M., McCaffery, J. M., and Jensen, R. E. (2001). Mmm1p, a mitochondrial outer membrane protein, is connected to mitochondrial DNA (mtDNA) nucleoids and required for mtDNA stability. J Cell Biol *152*, 401-410.

Holowka, D., Sheets, E. D., and Baird, B. (2000). Interactions between Fc(epsilon)RI and lipid raft components are regulated by the actin cytoskeleton. J Cell Sci *113 (Pt 6)*, 1009-1019.

Honing, S., Ricotta, D., Krauss, M., Spate, K., Spolaore, B., Motley, A., Robinson, M., Robinson, C., Haucke, V., and Owen, D. J. (2005). Phosphatidylinositol-(4,5)-bisphosphate regulates sorting signal recognition by the clathrin-associated adaptor complex AP2. Mol Cell *18*, 519-531.

Hoppins, S., Lackner, L., and Nunnari, J. (2007). The machines that divide and fuse mitochondria. Annu Rev Biochem *76*, 33.31-33.30.

Hoppins, S. C., and Nargang, F. E. (2004). The Tim8-Tim13 complex of Neurospora crassa functions in the assembly of proteins into both mitochondrial membranes. J Biol Chem *279*, 12396-12405.

Hoth, M., Fanger, C. M., and Lewis, R. S. (1997). Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. J Cell Biol *137*, 633-648.

Huang, M., Gu, G., Ferguson, E. L., and Chalfie, M. (1995). A stomatin-like protein necessary for mechanosensation in C. elegans. Nature *378*, 292-295.

Huber, T. B., and Benzing, T. (2005). The slit diaphragm: a signaling platform to regulate podocyte function. Curr Opin Nephrol Hypertens *14*, 211-216.

Huber, T. B., Kottgen, M., Schilling, B., Walz, G., and Benzing, T. (2001). Interaction with podocin facilitates nephrin signaling. J Biol Chem *276*, 41543-41546.

Huber, T. B., Schermer, B., and Benzing, T. (2007). Podocin organizes ion channel-lipid supercomplexes: implications for mechanosensation at the slit diaphragm. Nephron Exp Nephrol *106*, e27-31.

Huber, T. B., Schermer, B., Muller, R. U., Hohne, M., Bartram, M., Calixto, A., Hagmann, H., Reinhardt, C., Koos, F., Kunzelmann, K.*, et al.* (2006). Podocin and MEC-2 bind cholesterol to regulate the activity of associated ion channels. Proc Natl Acad Sci U S A *103*, 17079-17086.

Huber, T. B., Simons, M., Hartleben, B., Sernetz, L., Schmidts, M., Gundlach, E., Saleem, M. A., Walz, G., and Benzing, T. (2003). Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. Hum Mol Genet *12*, 3397-3405.

Ikonen, E. (2001). Roles of lipid rafts in membrane transport. Curr Opin Cell Biol *13*, 470-477.

Ikonen, E., Fiedler, K., Parton, R. G., and Simons, K. (1995). Prohibitin, an antiproliferative protein, is localized to mitochondria. FEBS Lett *358*, 273-277.

Innes, D. S., Sinard, J. H., Gilligan, D. M., Snyder, L. M., Gallagher, P. G., and Morrow, J. S. (1999). Exclusion of the stomatin, alpha-adducin and beta-adducin loci in a large kindred with dehydrated hereditary stomatocytosis. Am J Hematol *60*, 72-74.

Ishihara, N., Eura, Y., and Mihara, K. (2004). Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J Cell Sci *117*, 6535-6546.

Ishihara, N., Fujita, Y., Oka, T., and Mihara, K. (2006). Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO J *25*, 2966-2977.

Jacobowitz, D. M., and Kallarakal, A. T. (2004). Flotillin-1 in the substantia nigra of the Parkinson brain and a predominant localization in catecholaminergic nerves in the rat brain. Neurotox Res *6*, 245-257.

Jacobson, K., Mouritsen, O. G., and Anderson, R. G. (2007). Lipid rafts: at a crossroad between cell biology and physics. Nat Cell Biol *9*, 7-14.

James, D. J., Cairns, F., Salt, I. P., Murphy, G. J., Dominiczak, A. F., Connell, J. M., and Gould, G. W. (2001). Skeletal muscle of stroke-prone spontaneously hypertensive rats exhibits reduced insulin-stimulated glucose transport and elevated levels of caveolin and flotillin. Diabetes *50*, 2148-2156.

Janes, P. W., Ley, S. C., Magee, A. I., and Kabouridis, P. S. (2000). The role of lipid rafts in T cell antigen receptor (TCR) signalling. Semin Immunol *12*, 23-34.

Janmey, P. A., and Lindberg, U. (2004). Cytoskeletal regulation: rich in lipids. Nat Rev Mol Cell Biol *5*, 658-666.

Jazwinski, S. M. (1996). Longevity, Genes, and Aging. Science *273*, 54-59.

John, G. B., Shang, Y., Li, L., REnken, C., Mannella, C. A., Selker, J. M. L., Rangell, L., Bennett, M. J., and Zha, J. (2005). The mitochondrial inner membrane protein mitofilin controls cristae morphology. Mol Biol Cell *16*, 1543-1554.

Jones, R. G., Plas, D. R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M. J., and Thompson, C. B. (2005). AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol Cell *18*, 283-293.

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L.*, et al.* (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature *410*, 549-554.

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.

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.

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.

Karnovsky, M. J., Kleinfeld, A. M., Hoover, R. L., and Klausner, R. D. (1982). The concept of lipid domains in membranes. J Cell Biol *94*, 1-6.

Kasashima, K., Ohta, E., Kagawa, Y., and Endo, H. (2006). Mitochondrial functions and estrogen receptor-dependent nuclear translocation of pleiotropic human prohibitin 2. J Biol Chem *281*, 36401-36410.

Kasashima, K., Sumitani, M., Satoh, M., and Endo, H. (2008). Human prohibitin 1 maintains the organization and stability of the mitochondrial nucleoids. Exp Cell Res.

Katanaev, V. L., Solis, G. P., Hausmann, G., Buestorf, S., Katanayeva, N., Schrock, Y., Stuermer, C. A., and Basler, K. (2008). Reggie-1/flotillin-2 promotes secretion of the long-range signalling forms of Wingless and Hedgehog in Drosophila. Embo J *27*, 509-521.

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.

Kihara, A., Akiyama, Y., and Ito, K. (1997). Host regulation of lysogenic decision in bacteriophage λ: Transmembrane modulation of FtsH (HflB), the cII degrading protease, by HflKC (HflA). Proc Natl Acad Sci USA *94*, 5544-5549.

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.

Kim, T. H., Zhao, Y., Ding, W. X., Shin, J. N., He, X., Seo, Y. W., Chen, J., Rabinowich, H., Amoscato, A. A., and Yin, X. M. (2004). Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome C release. Mol Biol Cell *15*, 3061-3072.

Kirchman, P. A., Miceli, M. V., West, R. L., Jiang, J. C., Kim, S., and Jazwinski, S. M. (2003). Prohibitins and Ras2 protein cooperate in the maintenance of mitochondrial function during yeast aging. Acta Biochim Pol *50*, 1039-1056.

Kokubo, H., Helms, J. B., Ohno-Iwashita, Y., Shimada, Y., Horikoshi, Y., and Yamaguchi, H. (2003). Ultrastructural localization of flotillin-1 to cholesterol-rich membrane microdomains, rafts, in rat brain tissue. Brain Res *965*, 83-90.

Kokubo, H., Lemere, C. A., and Yamaguchi, H. (2000). Localization of flotillins in human brain and their accumulation with the progression of Alzheimer's disease pathology. Neurosci Lett *290*, 93-96.

Kolonin, M. G., Saha, P. K., Chan, L., Pasqualini, R., and Arap, W. (2004). Reversal of obesity by targeted ablation of adipose tissue. Nat Med *10*, 625-632.

Kontgen, F., and Stewart, C. L. (1993). Simple screening procedure to detect gene targeting events in embryonic stem cells. Methods Enzymol *225*, 878-890.

Kontgen, F., Suss, G., Stewart, C., Steinmetz, M., and Bluethmann, H. (1993). Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. Int Immunol *5*, 957-964.

Koshiba, T., Detmer, S. A., Kaiser, J. T., Chen, H., McCaffery, J. M., and Chan, D. C. (2004). Structural basis of mitochondrial tethering by mitofusin complexes. Science *305*, 858-862.

Kremer, J. R., Mastronarde, D. N., and McIntosh, J. R. (1996). Computer visualization of three-dimensional image data using IMOD. J Struct Biol *116*, 71-76.

Kuhn, R., and Torres, R. M. (2002). Cre/loxP recombination system and gene targeting. Methods Mol Biol *180*, 175-204.

Kurtev, V., Margueron, R., Kroboth, K., Ogris, E., Cavailles, V., and Seiser, C. (2004). Transcriptional regulation by the repressor of estrogen receptor activity via recruitment of histone deacetylases. J Biol Chem *279*, 24834-24843.

Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R., Green, D. R., and Newmeyer, D. D. (2002). Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell *111*, 331-342.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lai, E. C. (2003). Lipid rafts make for slippery platforms. J Cell Biol *162*, 365-370.

Lang, D. M., Lommel, S., Jung, M., Ankerhold, R., Petrausch, B., Laessing, U., Wiechers, M. F., Plattner, H., and Stuermer, C. A. (1998). Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. J Neurobiol *37*, 502-523.

Lang, T., Bruns, D., Wenzel, D., Riedel, D., Holroyd, P., Thiele, C., and Jahn, R. (2001). SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. Embo J *20*, 2202-2213.

Langhorst, M. F., Reuter, A., and Stuermer, C. A. (2005). Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. Cell Mol Life Sci *62*, 2228-2240.

Langhorst, M. F., Solis, G. P., Hannbeck, S., Plattner, H., and Stuermer, C. A. (2007). Linking membrane microdomains to the cytoskeleton: regulation of the lateral mobility of reggie-1/flotillin-2 by interaction with actin. FEBS Lett *581*, 4697-4703.

Lee, Y. J., Jeong, S. Y., Karbowski, M., Smith, C. L., and Youle, R. J. (2004). Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell *15*, 5001-5011.

Lemmon, M. A. (2003). Phosphoinositide recognition domains. Traffic *4*, 201-213.

Leonhard, K., Herrmann, J. M., Stuart, R. A., Mannhaupt, G., Neupert, W., and Langer, T. (1996). AAA proteases with catalytic sites on opposite membrane surfaces comprise a proteolytic system for the ATP-dependent degradation of inner membrane proteins in mitochondria. EMBO J *15*, 4218-4229.

Leuenberger, D., Bally, N. A., Schatz, G., and Koehler, C. M. (1999). Different import pathways through the mitochondrial intermembrane space for inner membrane proteins. EMBO J *18*, 4816-4822.

Li, K., Li, Y., Shelton, J. M., Richardson, J. A., Spencer, E., Chen, Z. J., Wang, X., and Williams, R. S. (2000). Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. Cell *101*, 389-399.

Liu, J., Deyoung, S. M., Zhang, M., Dold, L. H., and Saltiel, A. R. (2005). The stomatin/prohibitin/flotillin/HflK/C domain of flotillin-1 contains distinct sequences that direct plasma membrane localization and protein interactions in 3T3-L1 adipocytes. J Biol Chem *280*, 16125-16134.

Lucken-Ardjomande, S., Montessuit, S., and Martinou, J. C. (2008). Contributions to Bax insertion and oligomerization of lipids of the mitochondrial outer membrane. Cell Death Differ.

Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X., and Wang, X. (2000). Cardiolipin provides specificity for targeting of tBid to mitochondria. Nat Cell Biol *2*, 754-761.

Mak, T. W. (2007). Gene targeting in embryonic stem cells scores a knockout in Stockholm. Cell *131*, 1027-1031.

Malaga-Trillo, E., Laessing, U., Lang, D. M., Meyer, A., and Stuermer, C. A. (2002). Evolution of Duplicated reggie Genes in Zebrafish and Goldfish. J Mol Evol *54*, 235-245.

Malka, F., Guillery, O., Cifuentes-Diaz, C., Guillou, E., Belenguer, P., Lombes, A., and Rojo, M. (2005). Separate fusion of outer and inner mitochondrial membranes. EMBO Rep *6*, 853-859.

Maltecca, F., Aghaie, A., Schroeder, D., Cassina, L., Taylor, B., Phillips, S., Malaguti, M., Previtali, S., Guénet, J., Quattrini, A.*, et al.* (2008). The mitochondrial protease AFG3L2 is essential for axonal development. J Neurosci.

Mandal, S., Guptan, P., Owusu-Ansah, E., and Banerjee, U. (2005). Mitochondrial regulation of cell cycle progression during development as revealed by the tenured mutation in Drosophila. Dev Cell *9*, 843-854.

Manjeshwar, S., Branam, D. E., Lerner, M. R., Brackett, D. J., and Jupe, E. R. (2003). Tumor suppression by the prohibitin gene 3'untranslated region RNA in human breast cancer. Cancer Res *63*, 5251-5256.

Mannella, C. A. (2006). Structure and dynamics of the mitochondrial inner membrane cristae. Biochim Biophys Acta *1763*, 542-548.

Mastick, C. C., Brady, M. J., and Saltiel, A. R. (1995). Insulin stimulates the tyrosine phosphorylation of caveolin. J Cell Biol *129*, 1523-1531.

Maxfield, F. R., and Tabas, I. (2005). Role of cholesterol and lipid organization in disease. Nature *438*, 612-621.

McBride, H. M., Neuspiel, M., and Wasiak, S. (2006). Mitochondria: more than just a powerhouse. Curr Biol *16*, R551-560.

McClung, J. K., Danner, D. B., Stewart, D. A., Smith, J. R., Schneider, E. L., Lumpkin, C. K., Dell´Orco, R. T., and Nuell, M. J. (1989). Isolation of a cDNA that hybrid selects antiproliferative mRNA from rat liver. Biochem Biophys Res Com *164*, 1316-1322.

McClung, J. K., King, R. L., Walker, L. S., Danner, D. B., Nuell, M. J., Stewart, C. A., and Dell'Orco, R. T. (1992). Expression of prohibitin, an antiproliferative protein. Exp Gerontol *27*, 413-417.

McQuibban, G. A., Saurya, S., and Freeman, M. (2003). Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. Nature *423*, 537-541.

Meeusen, S., DeVay, R., Block, J., Cassidy-Stone, A., Wayson, S., McCaffery, J. M., and Nunnari, J. (2006). Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. Cell *127*, 383-395.

Meeusen, S., McCaffery, J. M., and Nunnari, J. (2004). Mitochondrial fusion intermediates revealed in vitro. Science *305*, 1747-1752.

Meeusen, S. L., and Nunnari, J. (2005). How mitochondria fuse. Curr Opin Cell Biol *17*, 389-394.

Meisinger, C., Pfannschmidt, S., Rissler, M., Milenkovic, D., Becker, T., Stojanovski, D., Youngman, M. J., Jensen, R. E., Chacinska, A., Guiard, B.*, et al.* (2007). The

morphology proteins Mdm12/Mmm1 function in the major beta-barrel assembly pathway of mitochondria. Embo J *26*, 2229-2239.

Meisinger, C., Rissler, M., Chacinska, A., Szklarz, L. K., Milenkovic, D., Kozjak, V., Schonfisch, B., Lohaus, C., Meyer, H. E., Yaffe, M. P.*, et al.* (2004). The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. Dev Cell *7*, 61-71.

Melkonian, K. A., Ostermeyer, A. G., Chen, J. Z., Roth, M. G., and Brown, D. A. (1999). Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. J Biol Chem *274*, 3910-3917.

Mengwasser, J., Piau, A., Schlag, P., and Sleeman, J. P. (2004). Differential immunization identifies PHB1/PHB2 as blood-borne tumor antigens. Oncogene *23*, 7430-7435.

Merkwirth, C., Dargazanli, S., Tatsuta, T., Geimer, S., Lower, B., Wunderlich, F. T., von Kleist-Retzow, J. C., Waisman, A., Westermann, B., and Langer, T. (2008). Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. Genes Dev *22*, 476-488.

Misaka, T., Miyashita, T., and Kubo, Y. (2002). Primary structure of a dynamin-related mouse mitochondrial GTPase and its distribution in brain, subcellular localization, and effect on mitochondrial morphology. J Biol Chem *277*, 15834-15842.

Mishra, S., Murphy, L. C., and Murphy, L. J. (2006). The prohibitins: emerging roles in diverse functions. J Cell Mol Med *10*, 353-363.

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.

Morrow, I. C., and Parton, R. G. (2005). Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. Traffic *6*, 725-740.

Morrow, I. C., Rea, S., Martin, S., Prior, I. A., Prohaska, R., Hancock, J. F., James, D. E., and Parton, R. G. (2002). Flotillin-1/reggie-2 traffics to surface raft domains via a novel golgi-independent pathway. Identification of a novel membrane targeting domain and a role for palmitoylation. J Biol Chem *277*, 48834-48841.

Mukherjee, S., and Maxfield, F. R. (2004). Membrane domains. Annu Rev Cell Dev Biol *20*, 839-866.

Munro, S. (2003). Lipid rafts: elusive or illusive? Cell *115*, 377-388.

Mussi, P., Liao, L., Park, S. E., Ciana, P., Maggi, A., Katzenellenbogen, B. S., Xu, J., and O'Malley, B. W. (2006). Haploinsufficiency of the corepressor of estrogen receptor activity (REA) enhances estrogen receptor function in the mammary gland. Proc Natl Acad Sci U S A *103*, 16716-16721.

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. J Biol Chem *275*, 29579-29586.

Neumann-Giesen, C., Falkenbach, B., Beicht, P., Claasen, S., Luers, G., Stuermer, C. A., Herzog, V., and Tikkanen, R. (2004). Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression. Biochem J *378*, 509-518.

Neutzner, A., and Youle, R. J. (2005). Instability of the mitofusin Fzo1 regulates mitochondrial morphology during the mating response of the yeast *Saccharomyces cerevisiae*. J Biol Chem *280*, 18598-18603.

Nijtmans, L. G. J., Artal Sanz, M., Grivell, L. A., and Coates, P. J. (2002). The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing and degenerative disease. Cell Mol Life Sci *59*, 143-155.

Nijtmans, L. G. J., de Jong, L., Sanz, M. A., Coates, P. J., Berden, J. A., Back, J. W., Muijsers, A. O., Van der Speck, H., and Grivell, L. A. (2000). Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. EMBO J *19*, 2444-2451.

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 U S A *90*, 10866-10870.

Nuell, M. J., Stewart, D. A., Walker, L., Friedman, V., Wood, C. M., Owens, G. A., Smith, J. R., Schneider, E. L., Dell´Arco, R., Lumpkin, C. K.*, et al.* (1991). Prohibitin, an evolutionary conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. Mol Cell Biol *11*, 1372-1381.

Ogura, T., and Wilkinson, A. J. (2001). AAA+ superfamily of ATPases: common structure-diverse function. Genes to Cells *6*, 575-597.

Oh, P., and Schnitzer, J. E. (2001). Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default. Mol Biol Cell *12*, 685-698.

Okamoto, K., and Shaw, J. M. (2005). Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu Rev Genet *39*, 503-536.

Olichon, A., Baricault, L., Gas, N., Guillou, E., Valette, A., Belenguer, P., and Lenaers, G. (2003). Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome *c* release and apoptosis. J Biol Chem *278*, 7743-7746.

Olichon, A., Elachouri, G., Baricault, L., Delettre, C., Belenguer, P., and Lenaers, G. (2007). OPA1 alternate splicing uncouples an evolutionary conserved function in mitochondrial fusion from a vertebrate restricted function in apoptosis. Cell Death Differ *14*, 682-692.

Olichon, A., Emorine, L. J., Descoins, E., Pelloquin, L., Brichese, L., Gas, N., Guillou, E., Delettre, C., Valette, A., Hamel, C. P.*, et al.* (2002). The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. FEBS Lett *523*, 171-176.

Olive, S., Dubois, C., Schachner, M., and Rougon, G. (1995). The F3 neuronal glycosylphosphatidylinositol-linked molecule is localized to glycolipid-enriched membrane subdomains and interacts with L1 and fyn kinase in cerebellum. J Neurochem *65*, 2307-2317.

Paratcha, G., and Ibanez, C. F. (2002). Lipid rafts and the control of neurotrophic factor signaling in the nervous system: variations on a theme. Curr Opin Neurobiol *12*, 542-549.

Park, S.-E., Xu, J., Frolova, A., Liao, L., O´Malley, B. W., and Katzenellenbogen, B. S. (2005). Genetic deletion of the repressor of estrogen receptor activity (REA) enhances the response to estrogen in target tissues *in vivo*. Mol Cell Biol *25*, 1989-1999.

Paschen, S. A., Rothbauer, U., Kaldi, K., Bauer, M. F., Neupert, W., and Brunner, M. (2000). The role of the TIM8-13 complex in the import of Tim23 into mitochondria. EMBO J *19*, 6392-6400.

Pasparakis, M., and Kollias, G. (1995). Production of cytokine transgenic and knockout mice (Oxford: Oxford University Press).

Paumard, P., Vaillier, J., Coulary, B., Schaeffer, J., Soubannier, V., Mueller, D. M., Brethes, D., di Rago, J. P., and Velours, J. (2002). The ATP synthase is involved in generating mitochondrial cristae morphology. EMBO J *21*, 221-230.

Peitz, M., Pfannkuche, K., Rajewsky, K., and Edenhofer, F. (2002). Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. Proc Natl Acad Sci USA *99*, 4489-4494.

Pellegrini, L., Passer, B. J., Canelles, M., Lefterov, I., Ganjei, J. K., Fowlkes, B. J., Koonin, E. V., and D'Adamio, L. (2001). PAMP and PARL, two novel putative metalloproteases interacting with the COOH-terminus of Presenilin-1 and -2. J Alzheimers Dis *3*, 181-190.

Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol *6*, 9-20.

Pike, L. J. (2003). Lipid rafts: bringing order to chaos. J Lipid Res *44*, 655-667.

Pike, L. J. (2006). Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. J Lipid Res *47*, 1597-1598.

Piper, P. W., and Bringloe, D. (2002). Loss of prohibitins, though it shortens the replicative life span of yeast cells undergoing division, does not shorten the chronological life span of G0-arrested cells. Mech Ageing Dev *123*, 287-295.

Praefcke, G. J., and McMahon, H. T. (2004). The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol *5*, 133-147.

Pralle, A., Keller, P., Florin, E. L., Simons, K., and Horber, J. K. (2000). Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. J Cell Biol *148*, 997-1008.

Price, M. P., Thompson, R. J., Eshcol, J. O., Wemmie, J. A., and Benson, C. J. (2004). Stomatin modulates gating of acid-sensing ion channels. J Biol Chem *279*, 53886-53891.

Rajalingam, K., and Rudel, T. (2005). Ras-Raf signaling needs prohibitin. Cell Cycle *4*, 1503-1505.

Rajalingam, K., Wunder, C., Brinkmann, V., Churin, Y., Hekman, M., Sievers, C., Rapp, U. R., and Rudel, T. (2005). Prohibitin is required for Ras-induced Raf-MEK-ERK activation and epithelial cell migration. Nat Cell Biol *7*, 837-843.

Rajendran, L., Masilamani, M., Solomon, S., Tikkanen, R., Stuermer, C. A., Plattner, H., and Illges, H. (2003). Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells. Proc Natl Acad Sci U S A *100*, 8241-8246.

Rajewsky, K., Gu, H., Kuhn, R., Betz, U. A., Muller, W., Roes, J., and Schwenk, F. (1996). Conditional gene targeting. J Clin Invest *98*, 600-603.

Ranger, A. M., Malynn, B. A., and Korsmeyer, S. J. (2001). Mouse models of cell death. Nat Genet *28*, 113-118.

Rapaport, D., Brunner, M., Neupert, W., and Westermann, B. (1998). Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae. J Biol Chem *273*, 20150-20155.

Reers, M., Smiley, S. T., Mottola-Hartshorn, C., Chen, A., Lin, M., and Chen, L. B. (1995). Mitochondrial membrane potential monitored by JC-1 dye. Methods Enzymol *260*, 406-417.

Reisinger, V., and Eichacker, L. A. (2006). Analysis of Membrane Protein Complexes by Blue Native PAGE. Proteomics *6 Suppl 2*, 6-15.

Resh, M. D. (1999). Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta *1451*, 1-16.

Rinkenberger, J. L., Horning, S., Klocke, B., Roth, K., and Korsmeyer, S. J. (2000). Mcl-1 deficiency results in peri-implantation embryonic lethality. Genes Dev *14*, 23-27.

Rivera-Milla, E., Stuermer, C. A., and Malaga-Trillo, E. (2006). Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain. Cell Mol Life Sci *63*, 343-357.

Robinson, N. C. (1993). Functional binding of cardiolipin to cytochrome c oxidase. J Bioenerg Biomembr *25*, 153-163.

Rodriguez, C. I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A. F., and Dymecki, S. M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat Genet *25*, 139-140.

Rojo, M., Legros, F., Chateau, D., and Lombes, A. (2002). Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. J Cell Sci *115*, 1663-1674.

Roselli, S., Gribouval, O., Boute, N., Sich, M., Benessy, F., Attie, T., Gubler, M. C., and Antignac, C. (2002). Podocin localizes in the kidney to the slit diaphragm area. Am J Pathol *160*, 131-139.

Roselli, S., Moutkine, I., Gribouval, O., Benmerah, A., and Antignac, C. (2004). Plasma membrane targeting of podocin through the classical exocytic pathway: effect of NPHS2 mutations. Traffic *5*, 37-44.

Rustin, P. (2002). Mitochondria, from cell death to proliferation. Nat Genet *30*, 352-353.

Rustin, P., Chretien, D., Bourgeron, T., Gerard, B., Rotig, A., Saudubray, J. M., and Munnich, A. (1994). Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta *228*, 35-51.

Saikawa, N., Akiyama, Y., and Ito, K. (2004). FtsH exists as an exceptionally large complex containing HflKC in the plasma membrane of *Escherichia coli*. J Struct Biol *146*, 123-129.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science *230*, 1350-1354.

Salzer, U., and Prohaska, R. (2001). Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts. Blood *97*, 1141-1143.

Sambrook, J., and Russell, D. (2001). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Santamaria, A., Castellanos, E., Gomez, V., Benedit, P., Renau-Piqueras, J., Morote, J., Reventos, J., Thomson, T. M., and Paciucci, R. (2005). PTOV1 enables the nuclear translocation and mitogenic activity of flotillin-1, a major protein of lipid rafts. Mol Cell Biol *25*, 1900-1911.

Santel, A., and Fuller, M. T. (2001). Control of mitochondrial morphology by a human mitofusin. J Cell Sci *114*, 867-874.

Saraste, M. (1999). Oxidative phosphorylation at the fin de siecle. Science *283*, 1488-1493.

Sasaki, Y., Derudder, E., Hobeika, E., Pelanda, R., Reth, M., Rajewsky, K., and Schmidt-Supprian, M. (2006). Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. Immunity *24*, 729-739.

Sato, T., Saito, H., Swensen, J., Olifant, A., Wood, C., Danner, D., Sakamoto, T., Takita, K., Kasumi, F., Miki, Y., and et al. (1992). The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer. Cancer Res *52*, 1643-1646.

Satoh, M., Hamamoto, T., Seo, N., Kagawa, Y., and Endo, H. (2003). Differential sublocalization of the dynamin-related protein OPA1 isoforms in mitochondria. Biochem Biophys Res Commun *300*, 482-493.

Sauer, B., and Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A *85*, 5166-5170.

Schägger, H. (2001). Blue-native gels to isolated protein complexes from mitochondria. Methods Cell Biol *65*, 231-244.

Schleicher, M., Shepherd, B. R., Suarez, Y., Fernandez-Hernando, C., Yu, J., Pan, Y., Acevedo, L. M., Shadel, G. S., and Sessa, W. C. (2008). Prohibitin-1 maintains the angiogenic capacity of endothelial cells by regulating mitochondrial function and senescence. J Cell Biol *180*, 101-112.

Schulte, T., Paschke, K. A., Laessing, U., Lottspeich, F., and Stuermer, C. A. (1997). Reggie-1 and reggie-2, two cell surface proteins expressed by retinal ganglion cells during axon regeneration. Development *124*, 577-587.

Schwarz, K., Simons, M., Reiser, J., Saleem, M. A., Faul, C., Kriz, W., Shaw, A. S., Holzman, L. B., and Mundel, P. (2001). Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. J Clin Invest *108*, 1621-1629.

Schwenk, F., Baron, U., and Rajewsky, K. (1995). A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res *23*, 5080-5081.

Scorrano, L., Ashiya, M., Buttle, K., Weiler, S., Oakes, S. A., Mannella, C. A., and Korsmeyer, S. J. (2002). A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell *2*, 55-67.

Sesaki, H., and Jensen, R. E. (2001). *UGO1* encodes an outer membrane protein required for mitochondrial fusion. J Cell Biol *152*, 1123-1134.

Sesaki, H., and Jensen, R. E. (2004). Ugo1p links the Fzo1p and Mgm1p GTPases for mitochondrial fusion. J Biol Chem *279*, 28298-28303.

Sesaki, H., Southard, S. M., Hobbs, A. E., and Jensen, R. E. (2003). Cells lacking Pcp1p/Ugo2p, a rhomboid-like protease required for Mgm1p processing, lose mtDNA and mitochondrial structure in a Dnm1p-dependent manner, but remain competent for mitochondrial fusion. Biochem Biophys Res Commun *308*, 276-283.

Sesaki, H., Southard, S. M., Yaffe, M. P., and Jensen, R. E. (2003). Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. Mol Biol Cell *14*, 2342-2356.

Sharma, A., and Quadri, A. (2004). Vi polysaccharide of *Salmonella typhi* targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses. Proc Natl Acad Sci USA *101*, 17492-17497.

Shih, N. Y., Li, J., Karpitskii, V., Nguyen, A., Dustin, M. L., Kanagawa, O., Miner, J. H., and Shaw, A. S. (1999). Congenital nephrotic syndrome in mice lacking CD2-associated protein. Science *286*, 312-315.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569-572.

Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. Nat Rev Mol Cell Biol *1*, 31-39.

Simonsen, A., Wurmser, A. E., Emr, S. D., and Stenmark, H. (2001). The role of phosphoinositides in membrane transport. Curr Opin Cell Biol *13*, 485-492.

Singer, S. J., and Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. Science *175*, 720-731.

Slaughter, N., Laux, I., Tu, X., Whitelegge, J., Zhu, X., Effros, R., Bickel, P., and Nel, A. (2003). The flotillins are integral membrane proteins in lipid rafts that contain TCR-associated signaling components: implications for T-cell activation. Clin Immunol *108*, 138-151.

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.

Snyers, L., Thines-Sempoux, D., and Prohaska, R. (1997). Colocalization of stomatin (band 7.2b) and actin microfilaments in UAC epithelial cells. Eur J Cell Biol *73*, 281-285.

Snyers, L., Umlauf, E., and Prohaska, R. (1998). Oligomeric nature of the integral membrane protein stomatin. J Biol Chem *273*, 17221-17226.

Snyers, L., Umlauf, E., and Prohaska, R. (1999). Association of stomatin with lipid-protein complexes in the plasma membrane and the endocytic compartment. Eur J Cell Biol *78*, 802-812.

Snyers, L., Umlauf, E., and Prohaska, R. (1999). Cysteine 29 is the major palmitoylation site on stomatin. FEBS Lett *449*, 101-104.

Solis, G. P., Hoegg, M., Munderloh, C., Schrock, Y., Malaga-Trillo, E., Rivera-Milla, E., and Stuermer, C. A. (2007). Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. Biochem J *403*, 313-322.

Solomon, S., Masilamani, M., Rajendran, L., Bastmeyer, M., Stuermer, C. A., and Illges, H. (2002). The lipid raft microdomain-associated protein reggie-1/flotillin-2 is expressed in human B cells and localized at the plasma membrane and centrosome in PBMCs. Immunobiology *205*, 108-119.

Song, Z., Chen, H., Fiket, M., Alexander, C., and Chan, D. C. (2007). OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. J Cell Biol *178*, 749-755.

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.

Stewart, C. L. (1993). Production of chimeras between embryonic stem cells and embryos. Methods Enzymol *225*, 823-855.

Stewart, G. W., Argent, A. C., and Dash, B. C. (1993). Stomatin: a putative cation transport regulator in the red cell membrane. Biochim Biophys Acta *1225*, 15-25.

Stuermer, C. A., Lang, D. M., Kirsch, F., Wiechers, M., Deininger, S. O., and Plattner, H. (2001). Glycosylphosphatidyl inositol-anchored proteins and fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and -2. Mol Biol Cell *12*, 3031-3045.

Stuermer, C. A., Langhorst, M. F., Wiechers, M. F., Legler, D. F., Von Hanwehr, S. H., Guse, A. H., and Plattner, H. (2004). PrPc capping in T cells promotes its association with the lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction. Faseb J *18*, 1731-1733.

Stuermer, C. A., and Plattner, H. (2005). The 'lipid raft' microdomain proteins reggie-1 and reggie-2 (flotillins) are scaffolds for protein interaction and signalling. Biochem Soc Symp, 109-118.

Subczynski, W. K., and Kusumi, A. (2003). Dynamics of raft molecules in the cell and artificial membranes: approaches by pulse EPR spin labeling and single molecule optical microscopy. Biochim Biophys Acta *1610*, 231-243.

Suh, B. C., and Hille, B. (2005). Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. Curr Opin Neurobiol *15*, 370-378.

Sun, L., Liu, L., Yang, X. J., and Wu, Z. (2004). Akt binds prohibitin 2 and relieves its repression of MyoD and muscle differentiation. J Cell Sci *117*, 3021-3029.

Sun, M. G., Williams, J., Munoz-Pinedo, C., Perkins, G. A., Brown, J. M., Ellisman, M. H., Green, D. R., and Frey, T. G. (2007). Correlated three-dimensional light and electron microscopy reveals transformation of mitochondria during apoptosis. Nat Cell Biol *9*, 1057-1065.

Svoboda, P. (2007). Off-targeting and other non-specific effects of RNAi experiments in mammalian cells. Curr Opin Mol Ther *9*, 248-257.

Tatsuta, T., and Langer, T. (2008). Quality control of mitochondria: protection against neurodegeneration and ageing. Embo J *27*, 306-314.

Tatsuta, T., Model, K., and Langer, T. (2005). Formation of membrane-bound ring complexes by prohibitins in mitochondria. Mol Biol Cell *16*, 248-259.

Tavernarakis, N., and Driscoll, M. (1997). Molecular modeling of mechanotransduction in the nematode Caenorhabditis elegans. Annu Rev Physiol *59*, 659-689.

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.

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

Thompson, T. E., and Tillack, T. W. (1985). Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells. Annu Rev Biophys Biophys Chem *14*, 361-386.

Todaro, G. J., and Green, H. (1965). Successive Transformations Of An Established Cell Line By Polyoma Virus And Sv40. Science *147*, 513-514.

Tomlinson, I. P., Alam, N. A., Rowan, A. J., Barclay, E., Jaeger, E. E., Kelsell, D., Leigh, I., Gorman, P., Lamlum, H., Rahman, S.*, et al.* (2002). Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nat Genet *30*, 406-410.

Torres, R. M., and Kühn, R. (1997). Laboratory Protocols for Conditional Gene Targeting.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., Schütz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat Genet *23,* 99-103.

Tsui-Pierchala, B. A., Encinas, M., Milbrandt, J., and Johnson, E. M., Jr. (2002). Lipid rafts in neuronal signaling and function. Trends Neurosci *25*, 412-417.

Tuominen, E. K., Wallace, C. J., and Kinnunen, P. K. (2002). Phospholipid-cytochrome c interaction: evidence for the extended lipid anchorage. J Biol Chem *277*, 8822-8826.

Umlauf, E., Csaszar, E., Moertelmaier, M., Schuetz, G. J., Parton, R. G., and Prohaska, R. (2004). Association of stomatin with lipid bodies. J Biol Chem *279*, 23699-23709.

van Meer, G., Poorthuis, B. J., Wirtz, K. W., Op den Kamp, J. A., and van Deenen, L. L. (1980). Transbilayer distribution and mobility of phosphatidylcholine in intact erythrocyte membranes. A study with phosphatidylcholine exchange protein. Eur J Biochem *103*, 283-288.

van Meer, G., Stelzer, E. H., Wijnaendts-van-Resandt, R. W., and Simons, K. (1987). Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. J Cell Biol *105*, 1623-1635.

Vander Heiden, M. G., Choy, J. S., VanderWeele, D. J., Brace, J. L., Harris, M. H., Bauer, D. E., Prange, B., Kron, S. J., Thompson, C. B., and Rudin, C. M. (2002). Bcl-x(L) complements Saccharomyces cerevisiae genes that facilitate the switch from glycolytic to oxidative metabolism. J Biol Chem *277*, 44870-44876.

Varma, R., and Mayor, S. (1998). GPI-anchored proteins are organized in submicron domains at the cell surface. Nature *394*, 798-801.

Vereb, G., Szollosi, J., Matko, J., Nagy, P., Farkas, T., Vigh, L., Matyus, L., Waldmann, T. A., and Damjanovich, S. (2003). Dynamic, yet structured: The cell membrane three decades after the Singer-Nicolson model. Proc Natl Acad Sci U S A *100*, 8053-8058.

Vogel, F., Bornhovd, C., Neupert, W., and Reichert, A. S. (2006). Dynamic subcompartmentalization of the mitochondrial inner membrane. J Cell Biol *175*, 237-247.

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.

Wang, D., Mentzer, W. C., Cameron, T., and Johnson, R. M. (1991). Purification of band 7.2b, a 31-kDa integral phosphoprotein absent in hereditary stomatocytosis. J Biol Chem *266*, 17826-17831.

Wang, S., Fusaro, G., Padmanabhan, J., and Chellappan, S. P. (2002a). Prohibitin co-localizes with Rb in the nucleus and recruits N-CoR and HDAC1 for transcriptional repression. Oncogene *21*, 8388-8396.

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

Wang, S., Nath, N., Fusaro, G., and Chellappan, S. (1999b). Rb and prohibitin target distinct regions of E2F1 for repression and respond to different upstream signals. Mol Cell Biol *19*, 7447-7460.

Wang, S., Zhang, B., and Faller, D. V. (2002b). Prohibitin requires Brg-1 and Brm for the repression of E2F and cell growth. Embo J *21*, 3019-3028.

Wang, S., Zhang, B., and Faller, D. V. (2004). BRG1/BRM and prohibitin are required for growth suppression by estrogen antagonists. Embo J *23*, 2293-2303.

Wang, Y., and Morrow, J. S. (2000). Identification and characterization of human SLP-2, a novel homologue of stomatin (band 7.2b) present in erythrocytes and other tissues. J Biol Chem *275*, 8062-8071.

Waugh, M. G., Lawson, D., and Hsuan, J. J. (1999). Epidermal growth factor receptor activation is localized within low-buoyant density, non-caveolar membrane domains. Biochem J *337 (Pt 3)*, 591-597.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Wenk, M. R., and De Camilli, P. (2004). Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals. Proc Natl Acad Sci U S A *101*, 8262-8269.

Wetzel, C., Hu, J., Riethmacher, D., Benckendorff, A., Harder, L., Eilers, A., Moshourab, R., Kozlenkov, A., Labuz, D., Caspani, O.*, et al.* (2007). A stomatin-domain protein essential for touch sensation in the mouse. Nature *445*, 206-209.

White, J. J., Ledbetter, D. H., Eddy, R. L., Jr., Shows, T. B., Stewart, D. A., Nuell, M. J., Friedman, V., Wood, C. M., Owens, G. A., McClung, J. K., and et al. (1991). Assignment of the human prohibitin gene (PHB) to chromosome 17 and identification of a DNA polymorphism. Genomics *11*, 228-230.

Willems, A. R., Schwab, M., and Tyers, M. (2004). A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. Biochim Biophys Acta *1695*, 133-170.

Wong, E. D., Wagner, J. A., Gorsich, S. W., McCaffery, J. M., Shaw, J. M., and Nunnari, J. (2000). The dynamin-related GTPase, Mgm1p, is an intermembrane space protein required for maintenance of fusion competent mitochondria. J Cell Biol *151*, 341-352.

Wong, E. D., Wagner, J. A., Scott, S. V., Okreglak, V., Holewinske, T. J., Cassidy-Stone, A., and Nunnari, J. (2003). The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion. J Cell Biol *160*, 303-311.

Yarosh, W., Monserrate, J., Tong, J. J., Tse, S., Le, P. K., Nguyen, K., Brachmann, C. B., Wallace, D. C., and Huang, T. (2008). The molecular mechanisms of OPA1-mediated optic atrophy in Drosophila model and prospects for antioxidant treatment. PLoS Genet *4*, e6.

Yokoyama, H., Fujii, S., and Matsui, I. (2008). Crystal structure of a core domain of stomatin from Pyrococcus horikoshii Illustrates a novel trimeric and coiled-coil fold. J Mol Biol *376*, 868-878.

Youle, R. J., and Karbowski, M. (2005). Mitochondrial fission in apoptosis. Nat Rev Mol Cell Biol *6*, 657-663.

Yu, J., Fischman, D. A., and Steck, T. L. (1973). Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. J Supramol Struct *1*, 233-248.

Zajchowski, L. D., and Robbins, S. M. (2002). Lipid rafts and little caves. Compartmentalized signalling in membrane microdomains. Eur J Biochem *269*, 737-752.

Zhang, J. Z., Abbud, W., Prohaska, R., and Ismail-Beigi, F. (2001). Overexpression of stomatin depresses GLUT-1 glucose transporter activity. Am J Physiol Cell Physiol *280*, C1277-1283.

Zhang, J. Z., Hayashi, H., Ebina, Y., Prohaska, R., and Ismail-Beigi, F. (1999). Association of stomatin (band 7.2b) with Glut1 glucose transporter. Arch Biochem Biophys *372*, 173-178.

Zhang, M., Mileykovskaya, E., and Dowhan, W. (2002). Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. J Biol Chem *277*, 43553-43556.

Zhang, S., Arnadottir, J., Keller, C., Caldwell, G. A., Yao, C. A., and Chalfie, M. (2004). MEC-2 is recruited to the putative mechanosensory complex in C. elegans touch receptor neurons through its stomatin-like domain. Curr Biol *14*, 1888-1896.

Zhu, Y., Paszty, C., Turetsky, T., Tsai, S., Kuypers, F. A., Lee, G., Cooper, P., Gallagher, P. G., Stevens, M. E., Rubin, E.*, et al.* (1999). Stomatocytosis is absent in "stomatin"-deficient murine red blood cells. Blood *93*, 2404-2410.

Züchner, S., Mersiyanova, I. V., Muglia, M., Bissar-Tadmouri, N., Rochelle, J., Dadali, E. L., Zappia, M., Nelis, E., Patitucci, A., Senderek, J.*, et al.* (2004). Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat Genet *36*, 449-451.

8 Figure index

9 Table index

10 List of Abbreviations

AAA	ATPases associated with a variety of cellular activities
ADOA	autosomal dominant optic atrophy
ADP	adenosine diphosphate
APS	ammoniumperoxo disulfate
ATP	adenosine triphosphate
bp	base pairs
BN	blue native
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cDNA	complementary DNA
CMT2A	Charcot-Marie-Tooth type 2A
DMSO	dimethyl sulfoxide
C-terminal	carboxyterminal
C-terminus	carboxy terminus
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
ES	embryonic stem
Fig.	Figure
g	standard gravity
GTP	guanosine triphosphate
h	hour(s)
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic-acid
HSP	hereditary spastic paraplegia
K	potassium
kb	kilobase pairs
KCl	potassium chloride
kDa	kilodalton
KOH	potassium hydroxide
m	meter
M	molar (mole per liter)
mA	milliampere
MDa	megadalton
MEF	mouse embryonic fibroblast
µg	microgram
µl	microliter
mg	milligram
ml	milliliter
Mg	magnesium
min	minute(s)
mM	millimolar
MPP	mitochondrial processing peptidase
mRNA	messenger RNA
mtDNA	mitochondrial DNA
MTS	mitochondrial targeting sequence
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide (reduced form)
NaOH	sodium hydroxide

NP-40	Nonidet P-40
N-terminal	aminoterminal
N-terminus	amino terminus
OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rounds per minute
RT	room temperature
s	second(s)
SCF	Skp1-Cdc53-F-box
SDS	sodium dodecyl sulfate
SPG	spastic paraplegia gene
Tab.	Table
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TIM	translocase of the inner membrane
TM	transmembrane domain
TOM	translocase of the outer membrane
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiole
U	unit(s)
UPS	ubiquitin-proteasome system
v/v	volume per volume
V	volt
WT	wild-type
w/v	weight per volume

11 Appendix

Teilpublikationen im Rahmen dieser Arbeit:

Merkwirth C, Dargazanli S, Tatsuta T, Geimer S, Löwer B, Wunderlich FT, von Kleist-Retzow JC, Waisman A, Westermann B, and Langer T (2008).

"Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria."

Genes & Development, 22(4):476-488, 2008

Abstract:

Prohibitins comprise an evolutionary conserved and ubiquitously expressed family of membrane proteins with poorly described functions. Large assemblies of PHB1 and PHB2 subunits are localized in the inner membrane of mitochondria, but various roles in other cellular compartments have also been proposed for both proteins. Here, we used conditional gene targeting of murine *Phb2* to define cellular activities of prohibitins. Our experiments restrict the function of prohibitins to mitochondria and identify the processing of the dynamin-like GTPase OPA1, an essential component of the mitochondrial fusion machinery, as the central cellular process controlled by prohibitins. Deletion of *Phb2* leads to the selective loss of long isoforms of OPA1. This results in an aberrant cristae morphogenesis and an impaired cellular proliferation and resistance towards apoptosis. Expression of a long OPA1 isoform in PHB2-deficient cells suppresses these defects identifying impaired OPA1 processing as the primary cellular defect in the absence of prohibitins. Our results therefore assign an essential function for the formation of mitochondrial cristae to prohibitins and suggest a coupling of cell proliferation to mitochondrial morphogenesis.

Acknowledgement

I sincerely thank my supervisor Prof. Dr. Thomas Langer for the opportunity to work on this interesting and challenging project. I deeply appreciate your continuous support and endless motivation in a great atmosphere. I would like to express my special gratitude for all that I have learned in your laboratory over the last years.

I would like to thank Prof. Dr. Jens Brüning, Prof. Dr. Siegfried Roth, Prof. Dr. Ari Waisman and Dr. Matthias Cramer for agreeing to form my thesis committee.

Thanks to Jens for continuous comments and discussions on the project as well as technical support with mouse strains, reagents and great parties. I would like to thank Ari for labspace and help in the generation of conditional *Phb2* mice.

Special thanks to my friend Dr. Thomas “Wundi” Wunderlich for permanent help, great support and suggestions in all areas of research.

I would like to thank all former and present members of the Langer Lab for help with experiments, stimulating discussions, instructions and beer. I am especially grateful to Sascha Dargazanli for his support and friendship. Many thanks to Fabian Anton, Dr. Isabel Arnold, Steffen Augustin, Florian Bonn, Phat Vinh Dip, Sarah Ehses, Tanja Engmann, Dr. Mafalda Escobar-Henriques Dias, Dr. Martin Graef, Dr. Brigitte Kisters-Woike, Dr. Metodi Dimitrov Metodiev, Dr. Mark Nolden, Christof Osman, Joanna Majczak, Ines Raschke, Susanne Scheffler, Dr. Takashi Tatsuta, Daniela Tils, Claudia Wilmes. Special thanks to Gudrun “Guzi” Zimmer for excellent technical assistance. I would like to thank Natze Hövelmeyer and Riki Frommer for help with mouse analysis and the time in the Weyertal Lab.

Many thanks to Christoph Göttlinger for FACS analysis and Brüning´s for technical help, especially to André Kleinridders, Brigitte Hampel, Gaby Spohn and Jan Mauer. I am very grateful to Linda Koch for critically reading my thesis.

I particularly appreciate the help from my collaborating colleagues PD Dr. Jürgen-Christoph von Kleist-Retzow, Dr. Stefan Geimer, Prof. Dr. Benedikt Westermann, Beatrix Löwer, Paola Martinelli and Prof. Dr. Elena Rugarli.

Finally, I am forever indebted to Bine and to my family, Christa and Martin Merkwirth, my grandparents Elisabeth and Wilhelm Dietrich, for all their love and support, patience and encouragement.

Eidesstattliche Erklärung

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

Köln, im März 2008 Carsten Merkwirth

Teilpublikationen im Rahmen dieser Arbeit:

Merkwirth, C., Dargazanli, S., Tatsuta, T., Geimer, S., Lower, B., Wunderlich, F. T., von Kleist-Retzow, J. C., Waisman, A., Westermann, B., and Langer, T. (2008). Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. Genes Dev *22*, 476-488.

Lebenslauf

Vor- und Zuname:	Carsten Merkwirth
Geburtstag:	24.06.1977
Geburtsort:	Kassel
Staatsangehörigkeit:	deutsch

Schulausbildung

1983 – 1987	Grundschule, Bad Emstal-Balhorn
1987 – 1989	Elbetalschule, Naumburg
1989 – 1993	Christine-Brückner-Schule, Bad Emstal
1993 – 1996	Georg-Christoph-Lichtenberg-Gymnasium, Kassel
Juni 1996	Abitur

Zivildienst

August 1996 – September 1997	Arbeiter-Samariter-Bund, Bad Emstal

Hochschulausbildung

Oktober 1997 – September 2003	Studium der Biologie an der Universität zu Köln
August 2002 – September 2003	Diplomarbeit am Institut für Genetik bei Prof. Dr. Thomas Langer mit dem Thema: „Conditional Gene Targeting of the *Phb2* Gene in the Mouse“
September 2003 – Mai 2008	Doktorarbeit am Institut für Genetik der Universität zu Köln bei Prof. Dr. Thomas Langer; Dissertation zum Thema: „Functional characterization of prohibitins by conditional inactivation in the mouse“

Publikationen

Hovelmeyer, N., Wunderlich, F. T., Massoumi, R., Jakobsen, C. G., Song, J., Worns, M. A., **Merkwirth, C.**, Kovalenko, A., Aumailley, M., Strand, D., et al. (2007). Regulation of B cell homeostasis and activation by the tumour suppressor gene CYLD. J Exp Med *204*, 2615-2627.

Merkwirth, C., Dargazanli, S., Tatsuta, T., Geimer, S., Lower, B., Wunderlich, F. T., von Kleist-Retzow, J. C., Waisman, A., Westermann, B., and Langer, T. (2008). Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. Genes Dev *22*, 476-488.