GIULIA CISBANI

PARL et HAX1 dans la régulation de l'activité mitochondriale

Mémoire présenté
à la Faculté des études supérieures de l'Université Laval
dans le cadre du programme de maîtrise en neurobiologie
pour l'obtention du grade de maître es sciences (M.Sc.)

FACULTÉ DE MÉDECINE
UNIVERSITÉ LAVAL
QUÉBEC
2010

Résumé

Hax1 joue un rôle important dans les syndromes d'immunodéficience et l'apoptose. Une étude récente suggère que Hax1, une protéine membre de la famille Bcl-2, inhibe l'apoptose dans les neurones et les lymphocytes, via un mécanisme impliquant sa liaison avec PARL, la protéase rhomboïde de la membrane mitochondriale, ce qui active par protéolyse la sérine protéase Omi/HtrA2 et élimine Hax1 actif. Ce modèle indique que la sensibilité des cellules aux stimuli pro-apoptotiques est contrôlée par le complexe PARL/Hax1 de l'espace intermembranaire de la mitochondrie. D'une manière plus globale, les protéines membres de la famille Bcl-2 pourraient contrôler la perméabilité de la membrane mitochondriale externe à partir de l'intérieur de la mitochondrie. De plus, ce modèle définit une nouvelle voie anti-apoptotique de PARL, indépendante de Opa1. Dans la présente étude, nous montrons que, *in vivo*, l'activité de Hax1 ne peut pas être couplée à PARL, car les deux protéines sont dans des compartiments cellulaires différents, et leur interaction *in vitro* est un artéfact. Par une analyse de séquence et de prédiction de structure secondaire, nous montrons aussi que Hax1 n'est pas membre de la famille Bcl-2, en raison de l'absence des modules d'homologie de Bcl-2. Ces résultats indiquent la présence de fonctions et de mécanismes différents de Hax1 dans l'apoptose, et ouvrent de nouvelles questions sur la capacité de PARL de réguler, en plus de l'apoptose, le stress mitochondrial via une voie Omi/HtrA2 dépendante.

Abstract

Hax1 has an important role in immunodeficiency syndromes and apoptosis. A recent report proposed that the Bcl-2-family-related protein, Hax1, suppresses apoptosis in lymphocytes and neurons through a mechanism that involves its association to the inner mitochondrial membrane rhomboid protease PARL, to proteolytically activate the serine protease Omi/HtrA2 and eliminate active Bax. This model implies that the control of cell-type sensitivity to pro-apoptotic stimuli is governed by the PARL/Hax1 complex in the mitochondria inter-membrane space and, more generally, that Bcl-2-family-related proteins can control mitochondrial outer-membrane permeabilization from inside the mitochondrion. Further, it defines a novel, anti-apoptotic Opa1-independent pathway for PARL. In this study, we present evidence that, *in vivo,* the activity of Hax1 cannot be mechanistically coupled to PARL because the two proteins are confined in distinct cellular compartments and their interaction *in vitro* is an artifact. We also show by sequence analysis and secondary structure prediction that Hax1 is extremely unlikely to be a Bcl-2-family-related protein because it lacks Bcl-2 homology modules. This result indicates a different function and mechanism of Hax1 action in apoptosis and re-opens the question of whether mammalian PARL, in addition to apoptosis, regulates mitochondrial stress response through Omi/HtrA2 processing.

Avant-propos 1

Authors contributions in the accompanying review article:
Calcium regulation of mitochondria motility and morphology
Jeyaraju DV, Cisbani G, Pellegrini L. Biochim.Biophys Acta 2009 Nov; 1787(11):1363-73. Epub 2008 Dec 24. Review

Giulia Cisbani
Discussed and reviewed the manuscript

Danny Jeyaraju
Discussed and wrote the manuscript

Dr. Luca Pellegrini
Designed and wrote the manuscript

Avant-propos 2

Authors contributions in the accompanying paper entitled
Hax1 lacks BH modules and is peripherally associated to heavy membranes: implications for Omi/HtrA2 and PARL activity in the regulation of mitochondrial stress and apoptosis. Jeyaraju DV, Cisbani G, De Brito OM, Koonin EV, Pellegrini L. Cell Death Differ. 2009 Dec: 16(12): 1622-9. Epub 2009 Aug 14.

Giulia Cisbani
Site directed mutagenesis, competent cells preparation, plasmid DNA preparation, cloning, PCR screening, preparative western blots

Danny Jeyaraju
Performed the experiments in Figs. 2, 3, 4, 5, 6; designed experiments; analyzed data,

wrote the manuscript; characterized anti-Hax rabbit antibody; developed all the mutants and constructs used in Figs. 4b and 4c

Dr. Olga Martins De Brito

Contributed reagents and protocol for the preparation of Fig. 3.

Dr. Eugene V Koonin

Contributed data for the preparation of Fig. 1.

Dr. Luca Pellegrini

Conceived, initiated, designed and directed the study; wrote the manuscript.

The important things in life cannot be achieved easily, but with constancy, dedication and believing in us. (L.B.)

Acknowledgments

First of all, I have to thank my supervisor, Dr Luca Pellegrini, for giving me the chance to come to Quebec city and join his lab. A big thank also goes to him for believing in me and supporting me. Thanks also for his advices, teaching and for the opportunity to be in a highly professional working environment. I will not forget all his fundamental help in the first stages of my settling here in Quebec.

A big thank to Danny Jeyaraju. He was a very good lab mate, helping and advising me at any time in lab life. He has been also a good friend to me and a great climbing mate.

I want also to thank Katalyn Toth for being very available and helpful.

I also have to thank my friends of the research center, CRULRG.
A special thank to Lynda, a very good friend, always close to me in every moment. Thanks for listening to me and for being with me in this adventure and helping through hard times.
Sincere thanks to Francesco and Charalampos for their friendship and for giving me moments of fun and supporting me.
I can not forget the other people I met here in Quebec and without whom my life would be much more boring: Vincent, Lucine, Camille, the new arrived Sophia, and all the other people I had the opportunity to meet here for being so nice and helpful, and for trying to break the linguistic barrier.

I have to cherish my friends in Italy. They supported me in my decisions, without leaving me alone.
First of all Luca who has been always the supporter number one at any given time, respecting all my decisions.
Serena, Roby, Tina who have been with me in good and bad moments in the last few years and have always been with me, demonstrating that friendship can win over big distances.

And last but not least, my biggest thank is for my family. Their love and their support are the most important and valuable thing in my life.

Table des matières

Liste des tableaux

Liste des figures

Abbreviations

PARL - Presenilin-Associated Rhomboid-Like protein

MAMP - Mature Mitochondrial Protein

PACT - PARL C-Terminal product

Mfn 1- Mitofusin-1

OPA1 - Optic Atrophy-1

Drp-1- Dynamin related protein- 1

Fis1 - Mitochondrial fission protein -1

ATP - Adenosine Triphosphate

GTP - Guanosine Triphosphate

ROS - Reactive Oxygen Species

IMM - Internal Mitochondria Membrane

Apaf1 - Apoptotic Protease Activating Factor -1

HtrA2 - High Temperature Requirement protein A2

Bcl-2 - B-cell Lymphoma Protein- 2

CARD - Caspase-Associated Recruitment Domain

DIABLO - Direct IAP Binding Protein with low pI

SMAC - Second Mitochondria-derived Activator of Caspase

MOMP - mitochondrial outer membrane permeabilization

IMS - Intermembrane Space

HAX1- HS-1Associated protein X-1

HS-1 - Hematopoietic linage cell-specific protein-1

BCR- B cells Receptor

BH - Bcl-2 Homology

PEST- Proline, Glutamic acid, Serine, Threonine

PLN - Phospholamban

IAPs - Inhibitor of Apoptosis Proteins

MAMs - Mitochondria Associated Membrane

OMM - Outer Mitochondrial Membrane

ER - Endoplasmatic Reticulum

Chapter 1

1. Introduction

The last years have been witness of an increased interest in mitochondria, a fundamental intracellular organelle, with pivotal function in the cells, such as maintenance of cellular supplies and vital role for several regulatory pathways, such as stress induced or developmental cell death, calcium buffering and signaling[1]. This multitasking property of the mitochondria is supported by the highly regulated compartmentalization of the organelle characterized by a double membrane and the external one separates the mitochondrial matrix content from the exterior cytosol. These unusual cellular organelles cannot be considered as individual organelles of defined size and nature but a dynamic network characterized by high motility and dynamism. In fact their morphology is deeply controlled by the balance between the mechanism of fission and fusion with each other Their shape changes according to cellular activity, nutritional status and developmental programs they have to accomplish in the cells[2]. The dynamism of this organelle is very important for the process of apoptosis, a form of programmed cell death. During their life cells are continuously exposed to several stimuli that could have positive or harmful properties, and they could lead towards syrvival or death of the cells[3].

1.1 Apoptotic mechanism

The cellular process called apoptosis involves a preprogrammed set of signal transduction pathways that leads to cell death. It is a very important event involved in a plethora of physiological processes such as embryogenesis, post embryonic development and adult tissue homeostasis and its deregulation determines numerous human diseases [4, 5] .This

process is highly conserved from *Caenorhabditis elegans* to humans and differs from necrosis which is characterized by loss of membrane integrity and cell homeostasis[6]. Apoptosis occurs through highly ordered and regulated events in which the cell is an active participant of its own demise. This has been the bases for designing novel therapies against cellular targets known to activate or repress apoptotic pathway [7]. The hallmark of the apoptotic pathway is caspase activation and the perturbation of mitochondrial membrane modulated by Bcl-2 family members in response to apoptotic signals[8].

Caspases are a set of cysteine proteases highly conserved through evolution and activated specifically during apoptosis. In living cells they are present as inactive zymogens that become activated through intracellular caspase cascade[9]. In vertebrates caspase dependent apoptosis occurs via two main pathways: extrinsic and intrinsic ones. The extrinsic pathway is mediated by the binding of an extracellular signal such as the tumor necrosis factor family members, to a transmembrane receptor[10]. The intrinsic pathway, also known as the mitochondrial pathway, is turned on by stress signals such as DNA damage, hypoxia and loss of survival signals. It is closely linked to the permeabilization of the outer mitochondrial membrane by pro-apoptotic members of the Bcl-2 family[110] and to the release of the protein content from the intermembrane space, including cytochrome C, Smac/Diablo and Omi/HtrA2, although we have little knowledge regarding these two latter proteins[12]. Several proteins initiate or regulate caspase activation, including cytochrome C. The release of cytochrome C is a crucial step in the apoptotic pathway. The protein binds to apoptotic-protease-activating- factor-1 (Apaf1) in the cytosol. Apaf-1 consists in three functional domains: the N-terminal caspase recruitment domain (CARD) which icludes the middle nucleotide –binding and oligomerization domain, and the C-terminal regulatory region composed of 13 WD-40 repeats[9, 13]. The regulatory region keeps Apaf1 inactive, but when cytochrome C binds to it, Apaf1 becomes activated in the presence of ATP or dATP. Seven individual complexes of Apaf1/cytochrome C oligomerize into a wheel-like heptamer, called apoptosome[8]. The apoptosome subsequently recruits pro-caspase 9 through CARD, resulting in the activation of caspase 9[12]. Once caspase 9 is activated, it triggers a cascade of effector caspase activation and proteolysis, leading eventually to cell death. During the perforation of mitochondria not only is cytochrome C released but also

Smac/Diablo and Omi/HtrA2, which indirectly promote caspase activation by antagonizing the inhibitor apoptosis proteins (IAPs).

Prior or simultaneously to cytochrome C release and upstream to caspase activation, mitochondria undergo fission, fragmenting themselves into smaller organelles. The block of fission causes the delay of cell death, linking the morphological apparatus of this organelle to the cell death program. The knowledge about the proteins that constitute the apoptotic pathway and the definition of molecular structure of the apoptosome complex might represent new potential drug targets for molecular therapy[14]. Moreover the discovery of new proteins involved in the apoptosis is a crucial contribution to a better understanding of these fascinating mechanisms (Fig.1). Recently few proteins were highlighted for their role in apoptosis, more specifically the protein PARL, which has been well studied and characterized by the group headed by Dr Pellegrini[15], giving important information to understand the protein and, HAX1 which is a subject of great interest since it appears to be ubiquitously expressed[16].

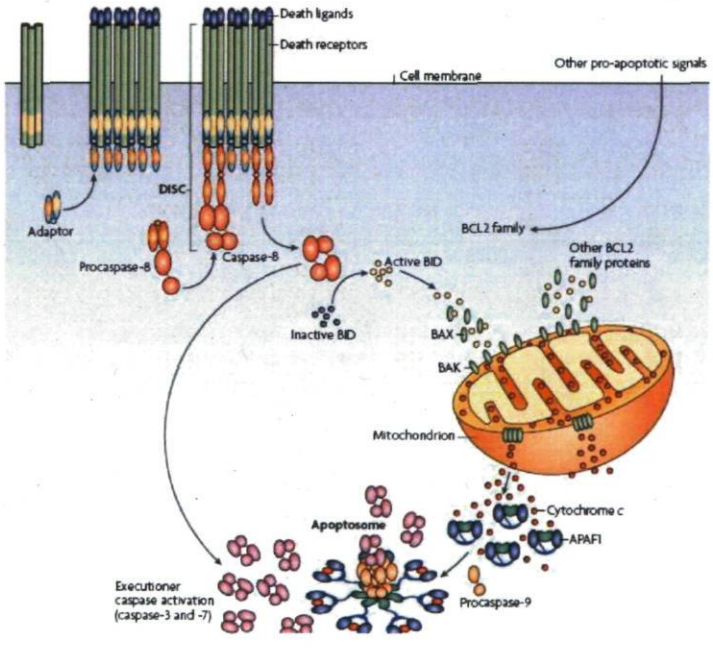


Figure 1 Extrinsic versus intrinsic apoptotic pathway[10].

1.2 Bcl-2 protein family

Bcl-2 family protein governs the mitochondrial dependent apoptotic pathway. The ratio between pro and anti apoptotic proteins dictates the resistance or sensitivity of the cells to apoptotic stimuli such as calcium hypoxia, radiation, and growth factor deprivation. The alteration of the balance between these two classes of proteins could lead to cell death. Overexpression of the anti-apoptotic protein BAX in solid tumors is responsible for the resistance to chemiotherapy and to apoptotic radiation[17]. The Bcl-2 family, which influence the integrity of the mitochondrial outer membrane, regulates the mitochondrial apoptotic pathway through complex interactions. Bcl-2 is a large family of proteins, including more than 30 members, with high degree of homology although they can have different roles, being pro-apoptotic or anti-apoptotic. The two groups of proteins differ from each other for the number of Bcl homology (BH) domain. Pro-apoptotic proteins, like Bcl-2, Bcl-xL, present four BH domains while anti-apoptotic proteins show either three BH domains, like BAX and BAK, or only the third domain and they are called BH3-only proteins. This last group is the most represented and it includes Bim, Bad, Bid, Noxa and Puma [3].

Among those proteins, BAX and BAK are of particular interest because they mediate the permeabilization of the mitochondria outer membrane (MOM), forming pores in the membrane subsequently to their oligomerization. This mechanism, still not fully understood, leads to the release of cytochrome c and other proteins. Concomitantly with the permeabilization of the outer membrane, mitochondria fragmentation occurs, and is characterized by the complete opening of the cristae junctions allowing the release of cytochrome c [3].

1.3 Mitochondria dynamics

Mitochondria are extremely dynamics organelles. Their dynamic organization is supported by the fact that they can exist in different shapes and numbers depending on the cell type and the energetic demands. Within a single cell they can assume three different shapes, rod-like, tubular and spherical, which are interconverting and in a dynamic equilibrium, undergoing continuously processes of fusion and fission (Fig.2)[18]. Aberrations of this equilibrium and alterations of mitochondria function can lead to a range of inherited and

acquired diseases including neurodegenerative diseases and they can be implicated in aging processes[19].

Mitochondria are constituted of an inner and an outer membrane: the first almost entirely permeable, the second permeable only to ions and small molecules. Maintenance of membrane integrity is fundamental to cellular and mitochondria functions[20]. The physical location of mitochondria in the cells, which is regulated by the cytoskeleton and is associated to proteins is extremely important, in fact mitochondria are concentrated in regions with higher energetic demands. Their distribution is also affected by their dynamics changes thus the balance between fusion and fission is essential to guarantee the correct functioning of the cells. Neurons are cells with higher energy demands than any other cells and they rely on the integrity of these organelles for the correct functioning of the brain. Abnormalities in mitochondria dynamics have been documented in several neurodegenerative disease such as Alzheimer disease (AD)[20].

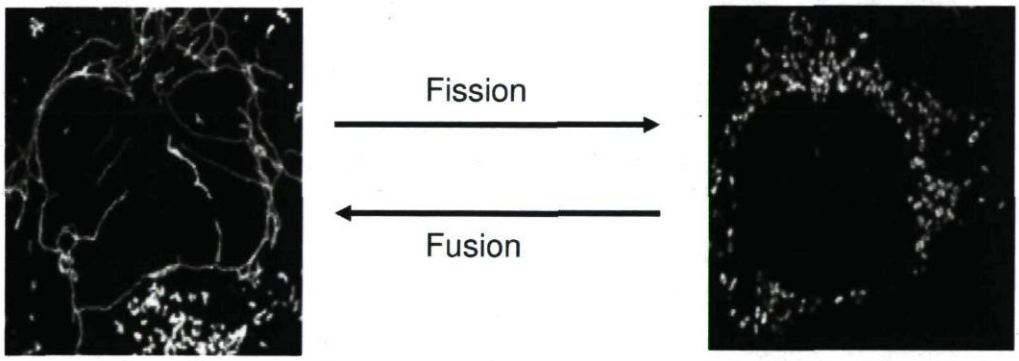


Figure 2 Equilibrium between the process of fusion and fission. The mitochondria present a tubular network (left) and a rod-like shape (right)

1.4 Mitochondria remodelling

The internal structure and cytosolic organization of such complex organelles are orchestrated by a growing number of conserved 'mitochondria-shaping' proteins. These include mitochondrial proteins such as the large dynamin-related GTPases Mitofusin (Mfn)

1 and 2, Optic Atrophy 1 (Opa1), which controls fusion; as well as the cytosolic dynamin-related protein 1 (Drp1) and its receptor on the outer mitochondrial membrane Fis1, which indeed controls fission (Fig.3) [21, 22]. These proteins influence not only the shape of mitochondria, but also the function of the organelle and eventually integrated cellular signalling cascades, including apoptosis. Ablation of fusion proteins promotes cell death. How the changes in mitochondrial morphology are related to apoptotic pathway is still not fully understood even if the fission process is associated with cristae remodeling[23]. In neurons, imbalance of mitochondrial fission and fusion can influence neuronal physiology, such as synaptic transmission and plasticity, and can also affect neuronal survival and lead to neurodegenerative disease[24]. By example Opa1 does not have only a pro-fusion property but also control cristae remodeling. Active Opa1 requires the inner mitochondrial membrane protease PARL to be efficiently assembled into oligomers and guarantee the structural integrity of cristae preventing cytochrome c release [22, 23, 25, 26]. Mutations in Opa1 cause dominant optic atrophy, the most common form of autosomal inherited optic neuropathy, which leads to the loss of retinal ganglion cells and a progressive loss of vision in children[27].

Therefore mitochondrial dysfunction in cell death is characterized by a loss of membrane potential, respiratory defects, increase in ROS production, changes in ATP levels and release of apoptogenic factors, including cytochrome C. Emerging evidence also suggests that changes in mitochondria morphology seem to be a crucial process in apoptosis so it could represent a valid drug target to modulate apoptosis in pathological conditions [14, 23].

During apoptosis, mitochondria undergoes fragmentation and one of the causes that trigger this morphological change is calcium[28]. Calcium is a ubiquitous second messenger involved in several physiological processes within the cell and in the extracellular matrix through interactions with proteins with different affinities[29]. Calcium concentration in the cytosol varies dynamically under the tight control of homeostatic mechanisms and mitochondria play a pivotal role in regulating apoptotic mechanisms triggering fission. Increase in calcium in the cytoplasm induces calcineurin activation and dephosphorylation of the dynamin Drp1 promoting mitochondria fragmentation, increasing the vulnerability of the organelle to apoptotic stimuli[30, 31]. Drp1 is recruited to the mitochondrial surface at

potential fission sites and GTP hydrolysis provides the energy for fission. However there is no evidence that calcium is involved in mitochondria fusion.

Calcium homeostasis has also a critical role in cell death signals. In fact, it has been observed that regulatory proteins in apoptosis, the protein of Bcl- 2 family, are localized in organelles deeply involved in calcium signaling (mitochondria and ER)[32]. However, calcium is not only important in cell death but also in several other mitochondrial functions (elaboration in details in chapter 3)[1]

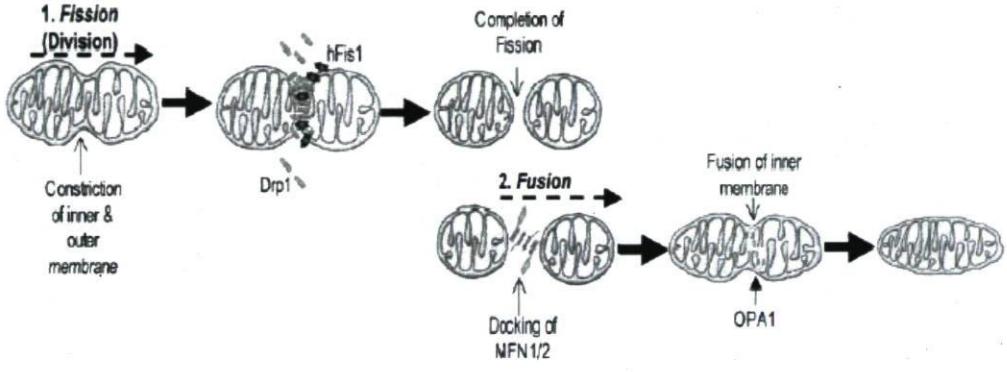


Figure 3 Schematic representation of the mitochondria fission and fusion processes occurring in mitochondria and of the "mitochondria-shaping" proteins [33].

1.5 PARL and HAX1

The mechanisms of cell death and mitochondria remodeling are highly regulated by several proteins interacting among them. The interest of the lab was mainly address to two proteins, PARL and HAX1.

1.5.1 Presenilin-associated-rhomboid-like PARL

PARL (Presenilin-Associated Rhomboid-Like) belongs to the eukaryotic subfamily of rhomboid protease. PARL was identified in a two-hybrid screen for proteins that interact

with Alzheimer's presenilin protein [34]. PARL is a mitochondrial polytopic membrane protein located in the inner mitochondrial membrane (IMM). It is homolog to the rhomboid protein PCP1P in *S. cerevisiae*. Studies in *S. cerevisiae* demonstrated that the rhomboid protein PCP1P is involved in the regulation of different mitochondrial activities through cleavage of Mgm1p, a dynamin-like GTPase found in the intermembrane space and which have a fundamental role in mitochondrial membrane fusion. PARL can rescue the phenotype of Pcp1p lacking strain indicating that the role of this protein in mitochondrial protein processing is conserved. PARL is also implicated in functional and morphological maintaining of normal mitochondria. The ablation of PCP1P in yeast determines a change in mitochondrial morphology, but intriguingly, the ablation of PARL in cells does not affect the morphology and function of the organelle but increase apoptosis in situ[25]. Thus this result raises the question whether PARL is really involved in mitochondria morphology. Despite the fact that are structurally and functionally conserved, the amino terminal domain of PARL and PCP1P is not conserved [15, 35]. PARL amino terminal domain, Pβ, spans the first 100 amino acids and is vertebrate specific and highly conserved in mammals. This conservation indicates that Pβ underwent strong purifying evolutionary selection, explained only by functional constraints. The amino terminal domain undergoes two consecutive cleavages: the constitutive α-cleavage ($_{52}$Gly↓Phe$_{53}$) cleaves the signal sequence, resulting in MAMP (Mature Mitochondrial PARL). Thus α-cleavage is associated to PARL import into the mitochondria. Following the first cleavage, β-cleavage occurs in position 77-78 ($_{78}$Ser↓Ala$_{79}$) resulting in PACT (PARL C terminus) and in the release of a 25 amino-acid long peptide called Pβ (Fig.4 and 5)[32]. Whereas α-cleavage occurs in all animal orthologs of PARL, β-cleavage is unique to mammals and requires high sequence conservation. β-cleavage is blocked if any of the residues surrounding the cleavage site are mutated [22,32]. PARL is an intra-membrane protease and it is known to be involved in the cleavage of Opa1, to guarantee the integrity of mitochondrial cristae, avoiding the release of cytochrome C [25].

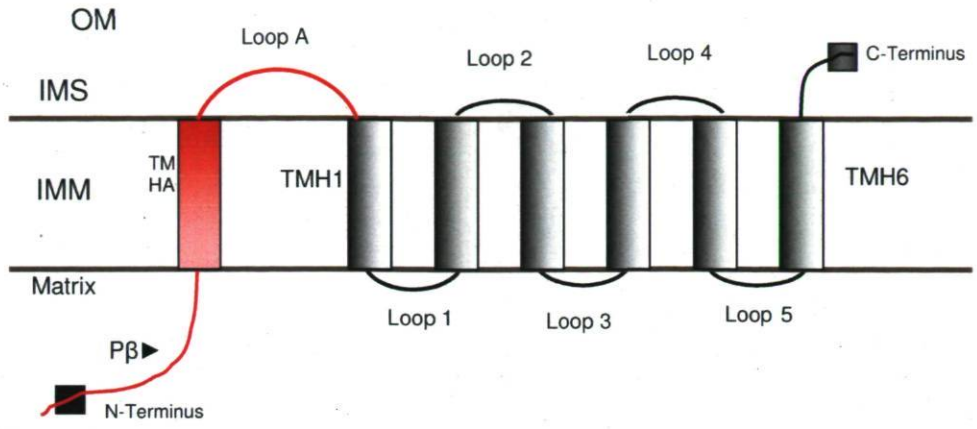
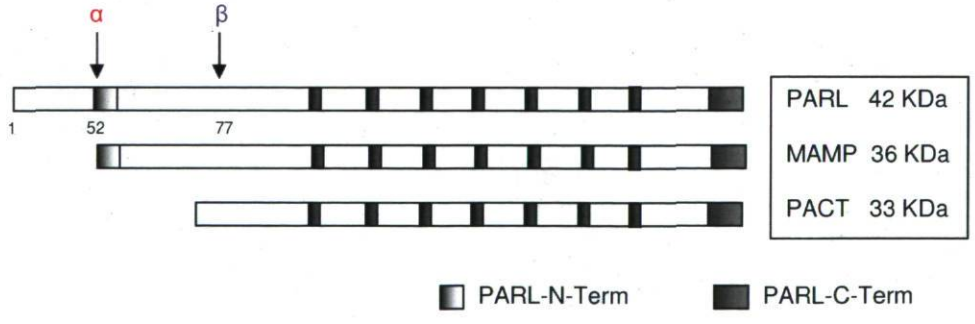


Figure 4 Scheme summarizing the topology of PARL. Differently from bacterial and archeal rhomboids which have 6 transmembrane helices (TMH), eukaryotic rhomboids present 7 TMH, which can be attached to the core either at the amino terminal or at the carboxyl terminal. In PARL the extra-TMH (in red) is present at the N-terminal and it shows an extra TMH and Loop called TMHA and LoopA. Pβ is protruding towards the matrix (IMM: inner membrane; IMS: intramembrane space; OM: outer membrane; TMH: transmembrane helix).

A



B

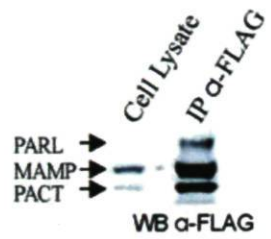


Figure 5 A) Schematic representation of α- and β- cleaved forms of PARL, MAMP and PACT.

B) Western blot showing the three forms of PARL migrating according to their molecular weight[32].

1.5.2 HAX1: description of the protein and its structure

Hax1-1 was first identified by Suzuki *et al.* in 1997 and was described as an interacting protein of HS-1 by the yeast two-hybrid system (HS-1 associated protein X-1). HS-1 is a hematopoietic specific molecule that is rapidly tyrosine phosphorylated by tyrosine variety of non-receptor tyrosine kinases upon stimulation of lymphocytes and has been proposed to be involved in B cell signal transduction. Thus the interaction of HAX1 with HS1 suggests it plays a role in BCR (B cell receptor) induced apoptosis[36, 37]. HAX1 is a 35 KDa protein constituted by 279 amino acids and its mRNA is ubiquitously expressed in the cells (predominantly in the endoplasmatic reticulum, cytoplasm, and mitochondria) and this suggests an essential role for the protein in intracellular signaling[36, 38]. Alternative splicing of HAX1 gene occurs in mouse, rat and human, giving five different isoforms of the protein[39]. Sequence analysis of HAX1 suggests that this protein presents some similarities to Bcl-2 family proteins: it shows two BH like domains (BH1 and BH2 domain) and although the BH3 domain is absent, it has about 100 amino acids with low similarity to Nip3, a Bcl-2 interacting protein with pro-apoptotic activity. Moreover it shows a putative hydrophobic transmembrane domain at residues 261 to 273. However these similarities are not well supported by bioinformatic analysis and the presence of a putative transmembrane domain is questionable[40]. Hax1 also contains a PEST sequence in position 104-117 which

suggests a rapid regulation and degradation of the protein. Moreover the protein presents some additionally interesting characteristics: an acid box at residues 30-41, with unknown functions and some protein binding regions in the C-terminal recently identified (Fig. 6)[40]. Chao *et al.*, also reported the existence of "HAX1-1 homology domains" (HD) that binds to HtrA2. However these regions seem to overlap with the previously described BH domains or with the C-terminal region of the protein which is involved in the binding with numerous other factors[16, 40, 41]. Along with the observations of similarities with Bcl-2 family members and with the support of *in vitro* and *in vivo* studies, it was proposed that Hax1 is involved in promoting cell survival [40-42].

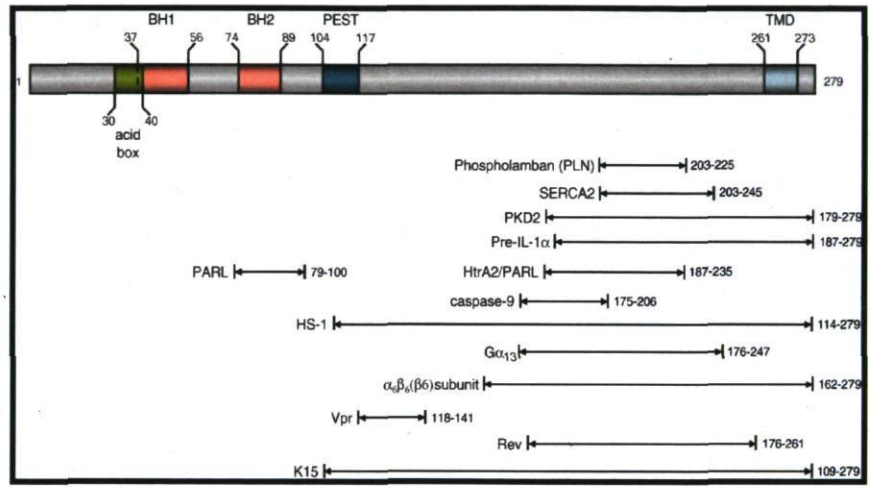


Figure 6 Schematic representation of HAX1. There are also indicated regions of binding with other cellular and viral proteins[3].

1.5.3 HAX1: a multifunctional protein

HAX1 has been shown as a ubiquitously expressed protein indicating it may have other functional roles besides anti-apoptotic functions. Recent studies demonstrated that HAX1 is a multifunctional protein interacting with several other proteins. HAX1 is well known as an anti-apoptotic protein, interacting with apoptosis related proteins and it is involved in both death receptor and mitochondria-mediated apoptotic pathway, however from previous

studies it appears to have a regulatory role, although no mechanisms were proposed[33, 43]. It has been shown to interact of with proteins involved in the permeabilization of the mitochondria and elements of mitochondrial mega-channel but it also interacts with viral proteins and cytoskeleton elements. However but the biological significance of this interaction remains unclear[43].

So far the clearest role of HAX1 is in the pathogenesis of severe congenital neutropenia, also known as Kostmann disease. This disease is an autosomal recessive disease and represents the primary immunodeficiency syndrome associated to apoptosis in myeloid cells. It has been reported[44] that HAX1 protects myeloid cells from apoptosis, thus mutations in HAX1 gene could lead to a higher degree of cell death of myeloid precursor in patients affected by neutropenia[45]. Mutations in HAX1 in the context of congenital nutropenia can cause neurological symptoms according to the nature of the mutations; in fact HAX1 presents two isoforms, isoform I and isoform II. The impairment of both the isoform could lead to neurological dysfunction in patients affected[45, 46]. The neurological problems could be explained by a possible anti-apoptotic role of HAX1 even in the brain, protecting from neurodegeneration[45].

Another interesting role of HAX1 is its interaction with phospholamban (PLN), a transmembrane protein of the cardiac sarcoplasmic reticulum and a pivotal regulator of Ca^{2+} cycling and determinant of β-andrenergic stimulation in the heart. The interaction between HAX1 and PLN seems to be modulated by the concentration of Ca^{2+} and the phosphorylation state of PLN. Moreover, the anti apoptotic function of HAX1 appears to be enhanced by the presence of PLN, thus the interaction HAX1/PLN is important in cardiac survival [42]. HAX1 is also involved in mechanisms crucial for carcinogenesis such as cell migration. In addition, it is significantly overexpressed in tumoral cells and analysis of the HAX1 isoforms revealed a tumor specific variations in the pattern of alternative splicing[40,43]. The varies roles of HAX1 can also depend on the cell types expressing the proteins. HAX1 has been shown to have different roles and interacting with various proteins in different cellular contexts. HAX1 also has RNA-binding properties but the significance of this function is still not well understood[40].

1.5.4 HAX1 as an anti-apoptotic molecule

Several studies have reported the anti-apoptotic role of HAX1 within cells even if the mechanism is not fully understood[33]. Overexpression of HAX1 makes the cells more resistant to apoptotic stimuli and to the loss of the membrane potential. It was hypothesized that the ability to block the collapse of the membrane potential during apoptosis could mediate the anti-apoptotic function of the protein. This could mean that the cleavage of HAX1 by Omi leads to the depolarization of the membrane during apoptosis followed by the release of mitochondrial proteins into the cytoplasm[33].

The anti-apoptotic function of HAX1 was observed in psoriatic skin; a chronic inflammatory disease characterized by increased proliferation and altered differentiation of keratinocytes caused by a combination of genetic, immunological and environmental factors. In psoriasis, an up-regulation of HAX1 was observed, compared to weak Hax1 expression in healthy and non lesioned skin and a diminished susceptibility to apoptosis[47]. *In vivo* studies have also showed that homozygous deletions of the HAX1 in mice results in increased apoptosis of neurons of the striatum and cerebellum, as well as postnatal lethality due to loss of motor coordination and function. Mutations of HAX1 cause neutrophils apoptosis, and result in neurotropenia, and in some cases, in neurological impairment[16]. The anti-apoptotic function of HAX1 is reported to occur in different cellular compartments: in the cytosol, on the endoplasmatic reticulum (ER) and in mitochondria, suggesting different scenarios for HAX1 role (Fig.7).

The first scenario reported by Han *et al.*, 2006 showed the anti-apoptotic function of Hax1 by its direct interaction with the cytosolic caspase-9 and inhibiting caspase-9 activation and processing, suggesting not only the anti-apoptotic function of HAX1 but also a new mechanism for the regulation of caspase-9 itself[48]. The interaction between the two proteins has been demonstrated via a yeast-two-hybrid screening of adult human heart cDNA and moreover overexpression oh HAX1 confers 30% more protection from apoptosis compared to the control cells. Suppression of the caspase-9 pathway is likely to be protective in cardiac myocytes[45]. The second scenario proposed is the interaction of HAX1 with PLN and SERCA2 (sarcoplasmic reticulum Ca^{2+} ATPase). These two proteins have an important role in modulating calcium homeostasis. The modulation of the calcium content in the ER

can affect the cell sensitivity to apoptosis. Deregulation of calcium could lead to mitochondria overload and cell death via caspase-dependent or independent pathway.

Recently, a new scenario has been suggested where HAX1 is localized in the mitochondria interacting with Htr2A/Omi and PARL. Omi is a mitochondrial serine protease, which has a pro-apoptotic function. Upon induction of apoptosis, it is released into the cytosol and participates in caspase-dependent apoptosis by binding and degrading inhibitors of apoptosis proteins (IAPs)[33, 49]. It is not only a pro-apoptotic protein but it is also involved in maintaining the mitochondrial homeostasis, so it might have a chaperone like activity. However the mechanism is still unclear. The proteolytic activity of Omi is essential both for its pro-apoptotic and chaperone like activity thus, via screening Cilenti *et al.*, have shown that HAX1 can be degraded by Omi when cells are treated with apoptotic stimuli, both *in vitro* and *in vivo*. They hypothesized that HAX1 controls mitochondrial membrane potential, therefore the cleavage of this protein by Omi would allow the depolarization of the mitochondria and the release of the pro-apoptotic proteins into the cytoplasm[33]. Chao *et al.*, 2008 showed that HAX1 not only interacts with HtrA2 but also with PARL. They proposed a novel model in which HAX1 forms a complex with PARL to process HtrA2/Omi. According to this model, HAX1 is associated with PARL in the inner mitochondrial membrane, and when unprocessed HtrA2 is imported into the mitochondria, it then associates with the complex and as a consequence is cleaved by PARL. The mature protein is then released in the inter-membrane space and plays an anti-apoptotic role. This result is contradictory with previous studies where a pro-apoptotic role was demonstrated for HtrA2. Co-expression of Hax1 with increased amount of PARL, results in an increased amount of processed HtrA2, whereas the co-expression of proteolitically inactive PARL (S277G) does not produce the same outcome. Moreover the processing of HtrA2 is reduced in PARL null mutants compared to the HAX1 null mutants.

The results obtained by these groups are contradictory to the former published data but also, since the data we obtained did not confirm the interaction of the endogenous protein, they represented the starting point of our questions and experiments.

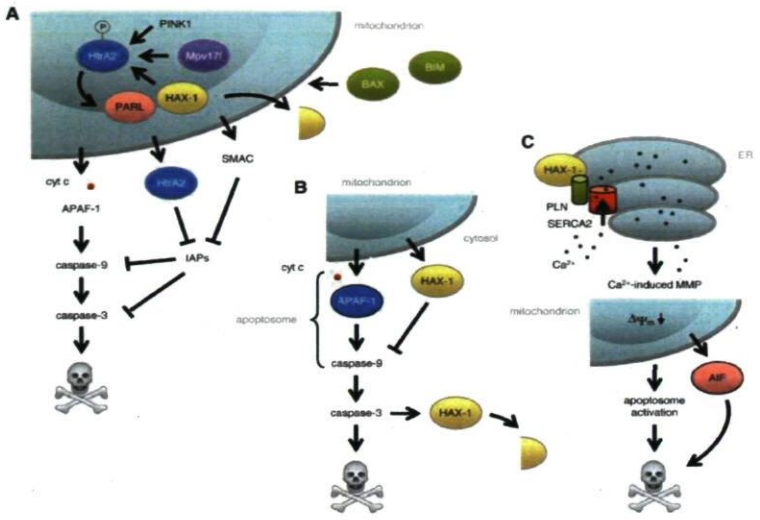


Figure 7 HAX1 and apoptosis signaling. Schematic representation of the anti-apoptotic function of HAX1 in three putative scenarios: in mitochondria, in the cytosol, and on the endoplasmic reticulum[38].

2. Participation to the project

My thesis is constituted by two main part: the first part is a manuscript published in Cell death and Differentation (impact factor 7.5) and the second part is a review article published in Biochimica et Biophysica Acta (impact factor 4.8).

During my thesis I participated to the discussion related to the two manuscripts and read several paper concerning the subject.

I also perform technical support performing several site directed mutagenesis both on PARL and HAX1 and PCR to clone the two proteins in the vector pBIND and pACT used for the two-hybrid essay. I also contributed doing some preparative western blot and preparative mitochondria extraction following the protocol indicated in the material and methods. The published paper that represents the main core of my thesis is completed by a supplementaty chapter with all the techniques used during my training in the lab.

Chapter 2

2.1 Results: Hax1 lacks BH modules and is peripherally associated to heavy membranes: implications for Omi/HtrA2 and PARL activity in the regulation of mitochondrial stress and apoptosis

D V Jeyaraju[1], G Cisbani[1], O M De Brito[2], E V Koonin[3] and L Pellegrini[1,4]

[1]Mitochondria Biology Laboratory, CRULRG, UniversitéLaval, Quebec, QC, Canada

[2]Molecular Neuropathobiology Laboratory, Cancer Research UK, London, UK

[3]National Center for Biotechnology Information, NLM, NIH, Bethesda, MD, USA

[4]Department of Cell Biology, Medical Biochemistry and Pathology, Faculty of Medicine, Université Laval, Quebec, QC, Canada

Correspondence: Luca Pellegrini,

Mitochondria Biology Laboratory, CRULRG, Room F-6500,

2601 Ch. De la Canardiere, Quebec, QC, Canada G1J 2G3.

Tel: +141 866 35000 ext 6879; Fax: +141 866 35000;

E-mail: Luca.Pellegrini@crulrg.ulaval.ca

Keywords:

Parl, Hax1, rhomboids, mitochondrial stress, apoptosis, neurodegenerative disease

Abbreviations:

MOMP, mitochondrial outer-membrane permeabilization; IMS, mitochondrial intermembrane space; IMM, inner mitochondrial membrane.

Acknowledgements

This study was supported by a grant from the Canadian Institutes of Health Research to LP (MOP-82718). DVJ is a CIHR Banting and Best Canada Graduate Scholar. LP is a FRSQ Chercheur-Boursier Senior scholar.

Abstract

Hax1 has an important role in immunodeficiency syndromes and apoptosis. A recent report (Chao *et al.*, *Nature*, 2008) proposed that the Bcl-2-family-related protein, Hax1, suppresses apoptosis in lymphocytes and neurons through a mechanism that involves its association to the inner mitochondrial membrane rhomboid protease PARL, to proteolytically activate the serine protease Omi/HtrA2 and eliminate active Bax. This model implies that the control of cell-type sensitivity to pro-apoptotic stimuli is governed by the PARL/Hax1 complex in the mitochondria intermembrane space and, more generally, that Bcl-2-family-related proteins can control mitochondrial outer-membrane permeabilization from inside the mitochondrion. Further, it defines a novel, anti-apoptotic Opa1-independent pathway for PARL. In this study, we present evidence that, *in vivo,* the activity of Hax1 cannot be mechanistically coupled to PARL because the two proteins are confined in distinct cellular compartments and their interaction *in vitro* is an artifact. We also show by sequence analysis and secondary structure prediction that Hax1 is extremely unlikely to be a Bcl-2-family-related protein because it lacks Bcl-2 homology modules. These results indicate a different function and mechanism of Hax1 in apoptosis and re-

opens the question of whether mammalian PARL, in addition to apoptosis, regulates mitochondrial stress response through Omi/HtrA2 processing.

Résumé

Hax1 joue un rôle important dans les syndromes d'immunodéficience et l'apoptose. Une étude récente suggère que Hax1, une protéine membre de la famille Bcl-2, inhibe l'apoptose dans les neurones et les lymphocytes, via un mécanisme impliquant sa liaison avec PARL, la protéase rhomboïde de la membrane mitochondriale, ce qui active par protéolyse la sérine protéase Omi/HtrA2 et élimine Hax1 actif. Ce modèle indique que la sensibilité des cellules aux stimuli pro-apoptotiques est contrôlée par le complexe PARL/Hax1 de l'espace intermembranaire de la mitochondrie. D'une manière plus globale, les protéines membres de la famille Bcl-2 pourraient contrôler la perméabilité de la membrane mitochondriale externe à partir de l'intérieur de la mitochondrie. De plus, ce modèle définit une nouvelle voie anti-apoptotique de PARL, indépendante de Opa1. Dans la présente étude, nous montrons que, *in vivo*, l'activité de Hax1 ne peut pas être couplée à PARL, car les deux protéines sont dans des compartiments cellulaires différents, et leur interaction *in vitro* est un artéfact. Par une analyse de séquence et de prédiction de structure secondaire, nous montrons aussi que Hax1 n'est pas membre de la famille Bcl-2, en raison de l'absence des modules d'homologie de Bcl-2. Ces résultats indiquent la présence de fonctions et de mécanismes différents de Hax1 dans l'apoptose, et ouvrent de nouvelles questions sur la capacité de PARL de réguler, en plus de l'apoptose, le stress mitochondrial via une voie Omi/HtrA2 dépendante.

2.1 Introduction

Mitochondria are key players in central cellular processes, such as ATP production, Ca^{2+} signaling and apoptosis. Mitochondrial involvement in apoptosis has been thoroughly documented during the last decade. Its two main features include the release of proteins from the mitochondrial intermembrane space (IMS) and the initiation of a program of dysfunction that includes the loss of the proton electrochemical gradient across the inner mitochondrial membrane (IMM).[1] These two cascades of events seem to be mediated by the crosstalk of several molecular mechanisms that are still not fully characterized.[2] Nevertheless, the general consensus is that during apoptosis, mitochondria release cytochrome c and other proteins that cooperate to execute programmed cell death.[3, 4, 5]

The function of mitochondria in the regulation and amplification of the apoptotic cascade is regulated by members of the Bcl-2 protein family.[6, 7] These are cytosolic proteins that, under steady state conditions, are mainly peripherally associated to heavy membranes,[8] and that share a limited structural similarity with Bcl-2 in the so called Bcl-2 homology (BH) module. Bcl-2-family-related proteins participate in the same process of regulation of apoptosis and are classified as pro- and anti-apoptotic, depending on their effect on programmed cell death. Pro-apoptotic members are further subdivided into 'multidomain' ones that share the BH1, BH2 and BH3 modules, and the 'BH3-only' proteins that only share the BH3 module. The pro-apoptotic BH3-only proteins 'sense' the death stimuli and transduce them to mitochondria, where they activate the 'multidomain' pro-apoptotic proteins Bax and Bak, ultimately resulting in mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release. With respect to the anti-apoptotic Bcl-2 family members, two models of activity have been proposed and are the subject of intense investigations. In the first model, these proteins are endogenous inhibitors of the multidomain pro-apoptotic proteins, requiring to be antagonized by 'BH3-only' molecules; in the second model, the anti-apoptotic Bcl-2 family proteins act by sequestering BH3-only proteins, a possibility strongly supported by recent, elegant studies.[9] However, a recent report puzzled these two models by proposing that Hax1, a purported Bcl-2-family-related protein of the mitochondrial IMS, mediates the élimination of active Bax, thereby introducing the concept that anti-apoptotic Bcl-2 proteins can antagonize MOMP also from inside the mitochondria through the activation of a proteolytic cascade.[10]

To ensure the complete release of cytochrome c, the architecture of the mitochondrial reticulum and the ultrastructure of the organelle changes in the early stages of apoptosis.[11, 12, 13, 14] A combination of electron tomography and physiological measurements identified a pathway of cristae remodeling characterized by the widening of the narrow tubular junction and by the fusion of individual cristae. These morphological changes support the mobilization of cytochrome c from the cristae to the IMS, and eventually to the cytosol.[15] Mechanistically, the mitochondrial rhomboid protease PARL and the dynamin-related GTPase Opa1, two proteins of the IMM, participate in the control of the shape and structure of the cristae and of the cristae junctions.[16, 17] Under steady state conditions, active Opa1 can prevent the widening of the cristae junctions by forming an oligomer that functions as a molecular staple between the adjacent membranes of the cristae. This high molecular weight complex contains both an IMM-bound form of Opa1 and an IMS-soluble one. Generation of the latter form requires the rhomboid protease PARL, whose ablation is lethal in adult mice owing to a pathology caused by excessive apoptosis in multiple tissues.[16, 17] A similar phenotype was recently observed in mice lacking Hax1, which also displayed reduced levels of a cleaved form of Omi/HtrA2,[10] a serine protease of the IMS implicated in oxidative stress and apoptosis.[18] These observations and the finding that Hax1 could be co-immunoprecipitated with PARL suggested a model in which Hax1 presents Omi/HtrA2 to PARL, to generate a form of Omi/HtrA2 that may proteolytically antagonize from the IMS active Bax during MOMP,[10] thereby defining a novel anti-apoptotic Opa1-independent pathway for PARL.[16, 17, 19]

Our study shows that Hax1 is not a bona fide Bcl-2-family-related protein because it does not share any sequence similarity with BH modules, and its confidently predicted secondary structure is incompatible with the presence of BH modules. We further show that, in vivo, the activity of Hax1 cannot be mechanistically coupled to PARL because the two proteins are confined in different cellular compartments, and their interaction in vitro is an artifact. Hax1 has a key role in autosomal recessive severe congenital neutropenia,[20] a primary immunodeficiency syndrome associated with increased apoptosis in myeloid cells; therefore, correcting the mechanism of Hax1 activity remains an outstanding question, which has to be addressed to decipher the molecular pathways that link mitochondrial stress response to apoptosis.

2.2 Results

2.2.1 Hax1 lacks BH modules

Hax1 was initially proposed to be a Bcl-2 family protein on the basis of purported structural similarities to Bcl-2 family members, including the presence of BH1- and BH2-like modules and a C-terminal transmembrane domain.[21] Recent studies have embraced this notion,[10] contributing to consolidate Hax1 as a member of this important family of proteins. However, our sequence analysis and structure prediction do not support the presence of any BH modules in Hax1. Indeed, the purported BH1 and BH2 modules, located at position 37–56 and 74–89, respectively, are not recognized by conserved protein domain search, even with the most relaxed threshold. Further, multiple secondary structure predictions show with full consistency that the regions of Hax1, which were previously aligned with the BH1 and BH2 modules, are largely disordered, whereas the bona fide BH1 and BH2 modules are stable hairpins formed by hydrophobic α-helices.[22, 23] In addition, the corresponding regions of the Hax1 sequence are not well conserved even in closely related animals, such as mammals, and show no sequence conservation at all in more distantly related species (Figure 1), which would be incompatible with the key roles of these regions in the function of Hax1 in apoptosis as a Bcl-2 protein. Our analysis shows instead that Hax1 is an α/β-protein that contains a strongly predicted and relatively well-conserved, in animals, three-strand β-sheet near the C-terminus (Figure 1), a structural element absent in Bcl-2 proteins. Interestingly, Hax1 also contains a conspicuous pattern of three universally conserved aspartates embedded in a predicted disordered loop, which is suggestive of functionally important metal (possibly, calcium)-binding residues. None of these structural elements are present in any of the Bcl-2 proteins. We conclude that it is exceedingly unlikely that Hax1 is a member of the Bcl-2 family.

2.2.2 Hax1 is not an integral membrane protein

At the C-terminus of Hax1, there is a strongly predicted and conserved C-terminal α-helix, which has been purported to constitute the transmembrane domain that anchors Hax1 to both mitochondrial membranes.[10] Multiple methods of transmembrane region prediction, as well as visual inspection of Hax1 sequence for long hydrophobic stretches, indicate that

Hax1 does not contain such transmembrane domain, either near the C-terminus or anywhere within the protein sequence (Figure 8). To span the lipid bilayer, an α-helix must be composed by a minimum of about 20 amino acids, mainly hydrophobic.[24] The purported transmembrane domain of Hax1 consists in an α-helix of 16 amino acids, 4 of which are charged and 1 is polar (RPPALDDAFSILDLFL), which could not form a transmembrane domain capable of anchoring Hax1 within a lipid bilayer. This analysis is consistent with our experimental data from alkaline and high salt extraction of heavy membranes prepared from HeLa cells, which showed that endogenous Hax1 is peripherally associated, but not integrated, to these membranes (Figure 9). We conclude that Hax1 is not an integral membrane protein and suggest that the conserved C-terminal domain might be functionally important in coordinating Hax1 interaction with other proteins.

2.2.3 Hax1 is not localized inside the mitochondria

A recent report claimed that endogenous Hax1 is localized on the inner and on the outer mitochondrial membrane, where it is exposed to the IMS.[10] However, such dual membrane localization and protein topology are at odd with those of every other known bona fide mitochondrial protein, as well as with the known mechanisms of protein import in the organelle. Hax1 does not contain cysteine residues required for IMS protein import through the MIA pathway,[25] or any predictable mitochondrial import peptide. Further, recent extensive proteomic studies do not list Hax1 in the compendium of mitochondrial proteins.[26, 27] Consistent with these findings, our data show that heavy membranes contain Hax1, but that the protein is absent in highly purified, intact mouse liver mitochondria preparations (Figure 10). We conclude that Hax1 is not imported inside the mitochondria, although it might be peripherally associated to the organelle.

2.2.4 PARL association to Hax1 is unspecific

PARL is a 7-transmembrane domain-containing protein of the IMM.[28, 29] Topological studies have shown that the N-terminus of the protease is exposed to the matrix and the C-terminus to the IMS[29] (Figure 11a). PARL contains three loops exposed to the IMS: one large Loop-A that is functionally dispensable for PARL proteolytic activity (Figure 4a and b)[29] and seems to coordinate the interaction with OPA1,[17] and two very small loops that are

a part of the rhomboid domain.[28, 30] Recently, co-immunoprecipitation studies were used to propose a mechanism in which Hax1 interaction with PARL allows presentation of the serine protease Omi/HtrA2 to the rhomboid protease to generate a cleaved active form of Omi/HtrA2 in the IMS.[10] As this model is incompatible with the notion that Hax1 is not targeted to the IMS (Figure 3; Pagliarini et al.[26] and Yamada et al.[27]), we investigated the unspecific nature of the reported PARL/Hax1 interaction[10] by co-transfecting HEK293 cells with constructs expressing wild-type Hax1 and mutant forms of PARL that lack mitochondrial import. Data showed that Hax1 could be co-immunoprecipitated with PARL irrespective of the fact that the latter was neither properly targeted nor folded in the IMM (data not shown), suggesting that Hax1 association is not mediated by IMS domain(s) of PARL but, rather, by unspecific hydrophobic interaction between the two misfolded proteins. To address this possibility, we deleted the only two IMS domains of PARL that could potentially bind to Hax1 and confirmed their dispensability for co-immunoprecipitating Hax1 (Figure 11b). Further, we excluded their role as bona fide IMS Hax1-binding domain by means of a mammalian two-hybrid system (Figure 4c). To further validate the unspecific nature of the PARL/Hax1 association, we tested whether Hax1 co-immunoprecipitation with PARL occurs after cellular lysis by mixing (i) lysates of cells that were independently transfected with constructs expressing PARL and Hax1 (Figure 12a) and (ii) lysates of heavy membranes isolated from Parl–/– and Hax1–/– MEFs (Figure 12b). In both cases, PARL/Hax1 association could be recreated, supporting the conclusion that the interaction between these two proteins is an in vitro artifact that occurs post lysis through unspecific hydrophobic interactions

2.2.5 PARL activity does not require Hax1

Although some types of proteases require substrate presentation by an accessory protein, rhomboids do not seem to need one.[31, 32] The recently proposed Hax1/PARL model assigns a PARL substrate-presenting function to Hax1.[10, 33] However, this seems to be an unlikely possibility because loss of Hax1 function only partially reduces the expression of a cleaved form of Omi/HtrA2.[10] Further, genetic ablation of Hax1 compromises neither Opa1 cleavage[10] nor PARL cleavage (Figure 6), an N-terminal processing that requires PARL activity supplied in trans.[34] Together with the non-IMS localization of Hax1 (Figure 3;

Pagliarini et al.[26] and Yamada et al.[27]), these evidences indicate that the protein is not a PARL substrate-presenting protein and that PARL rhomboid activity does not require Hax1.

2.3 Discussion

This study arises from a recent report that addressed the role of Hax1 in mediating the processing of the mitochondrial stress-related Omi/HtrA2 protease to allow the survival of lymphocytes and neurons.[10] In this study, cell-type sensitivity to pro-apoptotic stimuli was proposed to be governed by the formation of a complex between Hax1, a purported Bcl-2 family-related protein, and PARL, a mitochondrial rhomboid protease implicated in apoptosis and mitochondria dynamics regulation.[17, 29] Mechanistically, Hax1/PARL association in the IMS was reported to allow the recruitment and presentation of Omi/HtrA2 to PARL,[10, 33] to generate a cleaved active form of Omi/HtrA2 that, in turn, could antagonize MOMP from the IMS through proteolytic elimination of active Bax,[10] a concept that, however, is at odd with the notion that active Bax does not expose to the IMS, a cleavable domain.[6]

For Hax1 to recruit and present Omi/HtrA2 to PARL, the protein must be localized within the IMS. However, although we observed an association of Hax1 with the heavy membrane fraction, we did not detect Hax1 in Percoll-purified mouse liver mitochondria (Figure 3), a finding consistent with the absence of Hax1 from the mitochondrial proteome,[26, 27] as well as with the lack of a predictable mitochondrial targeting peptide or amino acid signature in animal orthologs of this protein (Figure 1 and data not shown). The association of Hax1 with heavy membranes is peripheral, as it was removed by alkaline and high salt extraction (Figure 2). Thus, contrary to previous reports,[10, 35] we found that endogenous Hax1 is neither resident inside the mitochondria nor anchored to the membranes of the organelle. Although our findings cannot exclude the possibility that a subtle amount of Hax1 could be targeted inside the mitochondria, they nevertheless indicate that the loss of the heavy membrane-associated form of Hax1 is likely responsible for the lethal phenotype displayed by Hax1−/− mice, as well as for the reduced levels of processed Omi/HtrA2.[10]

In this study, we tested the specificity of the interaction between Hax1 and PARL, a highly hydrophobic protein of the IMM.[28, 29] Consistent with previous reports,[10] we also observed that Hax1 can be co-immunoprecipitated with PARL, but not vice versa (not shown). However, the specificity of this association is dubious at best. Mixing lysates of cells that were independently transfected with constructs expressing PARL-Flag-CT and Hax1-HA-CT reconstituted PARL/Hax1 interaction (Figure 12a); similarly, the interaction of endogenous PARL and Hax1 could be recreated by mixing lysates of heavy membranes isolated from Parl−/− and Hax1−/− MEFs (Figure 12b). Furthermore, we could not identify any domain within PARL that could mediate interaction to Hax1 (Figure 4). Together, these results suggest that Hax1 does not specifically bind PARL and thus it cannot present a substrate to PARL.[10, 33] This conclusion is consistent with the observation that genetic ablation of Hax1 does not fully abolish Omi/HtrA2 processing,[10] it is not required for the cleavage of OPA1[10] and it does not affect PARL β-cleavage (Figure 13), which is self-regulated.[34]

Whether mammalian PARL cleaves Omi/HtrA2, a mitochondrial stress response serine protease,[18, 36] remains to be shown, but in the light of the data presented here, a direct role of Hax1 in this processing can be ruled out. Given the peripheral association of Hax1 to heavy membranes as well as its possible calcium-binding capacity (Figure 8) and its interaction with Phospholamban,[37] a sarcoplasmic reticulum protein and a key regulator of Ca^{2+} homeostasis, the reduced level of processed Omi/HtrA2 observed in Hax1−/− cells could be explained by the defects in calcium signaling and homeostasis.[38, 39, 40, 41, 42, 43] Accordingly, the phenotype of the Hax1−/− mouse could be explained by multiorganellar failure due to defective calcium homeostasis. Thus, our study re

opens the question of whether and how the organelle governs PARL activity to regulate mitochondrial stress, a process that can trigger mitochondrial dysfunctions that are central to the etiology of cancer[44, 45, 46] and multiple neurodegenerative disorders, including Parkinson's disease.[47, 48] Our study showed that Hax1 is not an IMS protein containing BH modules, thereby ruling out a model in which Bcl-2 proteins mediate the elimination of active Bax from inside the organelle during MOMP. Identification of Bcl-2 family-related proteins is a challenging task because some members of this family show no conserved

sequence motifs (at least, not statistically significant ones)[49] and are recognized solely on the basis of structural similarity to bona fide Bcl-2-family proteins.[23] However, Hax1 does not meet even the most liberal criteria for the presence of BH modules. First, the secondary structure of Hax1 that we confidently and consistently predicted with three computational methods is incompatible with the presence of such modules and, second, the sequences of Hax1 that were previously aligned with BH1 and BH2 domains are very poorly conserved during the evolution of Hax1, an observation that effectively rules out a key role of these regions in Hax1 function and that is in agreement with reports recently published by other groups.[50] In conclusion, although it could be formally argued that only the crystal structure of Hax1 can put the hypothesis that Hax1 is a member of the Bcl-2 family to final rest, the analysis reported here makes this possibility exceedingly unlikely.

2.4 Materials and Methods

2.4.1 Computational analysis of protein sequences

Hax1 orthologs from all the sequenced animal genomes were detected by searching the Genpept database using the PSI-BLAST program,[51] and a multiple alignment of a selected set of diverse sequences was constructed using the T-Coffee program.[52] Secondary structure (SS) prediction was performed using the program PSIPRED,[53] JPRED[54] and PredictProtein (PRPROT).[55] The search for conserved protein domains was performed using the RPS-BLAST program and the Conserved Domain Database.[56, 57] Mitochondrial import prediction was done using the MitoProt II program.[58] Transmembrane domains were predicted using PredictProtein,[55] as well as TMHMM[59] and TMPred.[60]

2.3.2 Mitochondria purification

Mitochondria were purified as described.[41] Briefly, mouse livers were washed once with PBS, suspended in isolation buffer (200mM sucrose, 1mM EGTA-Tris and 10mM Tris-MOPS, pH 7.4), and then disrupted by dounce homogenization on ice. The homogenate was spun at 800 × g for 10min at 4°C; the supernatant was recovered and further centrifuged for 10min at 8000 × g at 4°C. The resulting pellet (mitochondrial fraction) was

collected, whereas the supernatant was further spun for 30 min at 100000 × g at 4°C. The resulting pellet (light membrane fraction) and supernatant (cytosolic fraction) were spun again at 100000 × g to further purify the fractions. The mitochondrial fraction was purified further by centrifuging twice at 8000 × g for 10 min at 4°C. The obtained pellet was purified by centrifugation at 95000 × g for 30 min on a 30% Percoll gradient in isolation buffer. The mitochondrial layer was washed free of Percoll and resuspended in isolation buffer. Subcellular fractions were also obtained by differential centrifugation from mouse liver and MEFs as described.[61] Protein concentration was determined and the indicated amounts of protein were separated by SDS-PAGE and immunoblotted.

2.4.3 Alkaline extraction

Heavy membranes prepared from HeLa cells were diluted to a final concentration of 1 mg/ml in 20 mM HEPES/KOH (pH 7.4) (hyposmotic buffer). After the addition of an equal volume of freshly prepared 0.2 M sodium carbonate (pH 11.5), samples were incubated for 30 min at 4°C. The membrane and soluble fractions were separated by ultracentrifugation at 100000 × g for 30 min at 4°C.

2.4.4 Cell culture and transfection

HEK 293T and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell types used in this study were maintained under standard cell culture conditions. Cells were transfected at 40% confluence with FuGENE 6 (Roche, Mannheim, Germany). Hax1−/− MEFs and brains were kindly provided by Dr. JN Ihle; Parl−/− MEFs by B De Strooper.

2.4.5 Cloning and mutagenesis

The pcDNA3 vector was used to express the human PARL and mouse Hax1 protein in mammalian cells. Mutants were obtained by site-directed mutagenesis (Clontech, Mountain

View, CA, USA). For the mammalian two-hybrid assay, the Checkmate System and vectors (pBind, pACT, pG5luc) were used (Promega, Madison, WI, USA). The identity of every construct and mutant was confirmed by DNA sequencing.

2.4.6 Co-immunoprecipitations

Transfected HeLa cells were lysed in STEN buffer; heavy membranes in CHAPS buffer. Immunoprecipitations were conducted as described.[10, 29]

2.4.7 Antibodies

Rabbit anti-PARL;[29, 34] rabbit anti-MnSOD (Stressgen, Ann Arbor, MI, USA; 1:1000); mouse anti-UQCRC2 (clone 13G12, Molecular Probes, CA, USA; 1:3000); mouse anti-HA (Roche); mouse anti-Flag M2 (Sigma, St Louis, MO, USA); mouse anti-actin (clone C4, Cedarlane, Hornby, ON, Canada; 1:1000); mouse anti-cytochrome c (clone 7H8.2C12, BD Pharmingen, Mississauga, ON, Canada; 1:1000); mouse anti-Hax1 (clone-52, BD Biosciences, Mississauga, ON, Canada; 1:250) and rabbit anti-calnexin (Stressgen, Ann Arbor, MI, USA; 1:4000). The polyclonal rabbit anti-Hax1 antibody was raised against peptides encompassing amino acid 126–142 and 201–217 of mouse Hax1 (1:500).

References

1. Henry-Mowatt J, Dive C, Martinou JC, James D. Role of mitochondrial membrane permeabilization in apoptosis and cancer. Oncogene 2004; 23: 2850–2860.

2. Bernardi P, Petronilli V, Di Lisa F, Forte M. A mitochondrial perspective on cell death. Trends Biochem Sci 2001; 26: 112–117.

3. Martinou JC, Youle RJ. Which came first, the cytochrome c release or the mitochondrial fission? Cell Death Differ 2006; 13: 1291–1295.

4. Jourdain A, Martinou JC. Mitochondrial outer-membrane permeabilization and remodelling in apoptosis. Int J Biochem Cell Biol 2009, doi:10.1016/j.biocel.2009.05.001.

5. Scorrano L. Opening the doors to cytochrome c: changes in mitochondrial shape and apoptosis. Int J Biochem Cell Biol 2009, doi:10.1016/j.biocel.2009.04.016.

6. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008; 9: 47–59.

7. Aravind L, Dixit VM, Koonin EV. Apoptotic molecular machinery: vastly increased complexity in vertebrates revealed by genome comparisons. Science 2001; 291: 1279–1284.

8. Germain M, Shore GC. Cellular distribution of Bcl-2 family proteins. Sci STKE 2003; 2003: pe10.

9. Lovell JF, Billen LP, Bindner S, Shamas-Din A, Fradin C, Leber B et al. Membrane binding

by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. Cell 2008; 135: 1074–1084.

10. Chao JR, Parganas E, Boyd K, Hong CY, Opferman JT, Ihle JN. Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons. Nature 2008; 452:98–102.

11. Martinou I, Desagher S, Eskes R, Antonsson B, Andre´ E, Fakan S et al. The release of cytochrome c from mitochondria during apoptosis of NGF-deprived sympathetic neurons is a reversible event. J Cell Biol 1999; 144: 883–889.

12. Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F et al. The role

of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 2001; 1: 515–525.

13. James DI, Martinou JC. Mitochondrial dynamics and apoptosis: a painful separation. Dev Cell 2008; 15: 341–343.

14. Herzig S, Martinou JC. Mitochondrial dynamics: to be in good shape to survive. Curr Mol Med 2008; 8: 131–137.

15. Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA et al. A distinct pathway

remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell 2002; 2: 55–67.

16. Frezza C, Cipolat S, Martins de Brito O, Micaroni M, Beznoussenko GV, Rudka T et al.

OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell 2006; 126: 177–189.

17. Cipolat S, Rudka T, Hartmann D, Costa V, Serneels L, Craessaerts K et al. Mitochondrial

rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. Cell 2006; 126: 163–175.

18. Vande Walle L, Lamkanfi M, Vandenabeele P. The mitochondrial serine protease HtrA2/ Omi: an overview. Cell Death Differ 2008; 15: 453–460.

19. Pellegrini L, Scorrano L. A cut short to death: Parl and Opa1 in the regulation of mitochondrial morphology and apoptosis. Cell Death Differ 2007; 14: 1275–1284.

20. Klein C, Grudzien M, Appaswamy G, Germeshausen M, Sandrock I, Scha¨ ffer AA et al. HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). Nat Genet 2007; 39: 86–92.

21. Sharp TV, Wang HW, Koumi A, Hollyman D, Endo Y, Ye H et al. K15 protein of Kaposi's sarcoma-associated herpesvirus is latently expressed and binds to HAX-1, a protein with anti-apoptotic function. J Virol 2002; 76: 802–816.

22. Feng Y, Zhang L, Hu T, Shen X, Ding J, Chen K et al. A conserved hydrophobic core at Bcl-x(L) mediates its structural stability and binding affinity with BH3-domain peptide of pro-apoptotic protein. Arch Biochem Biophys 2009; 484: 46–54.

23. Petros AM, Olejniczak ET, Fesik SW. Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta 2004; 1644: 83–94.

24. White SH, von heijne G. Transmembrane helices before, during, and after insertion. Curr Opin Struct Biol 2005; 15: 378–386.

25. Stojanovski D, Mu¨ ller JM, Milenkovic D, Guiard B, Pfanner N, Chacinska A. The MIA system for protein import into the mitochondrial intermembrane space. Biochim Biophys Acta 2008; 1783: 610–617.

26. Pagliarini DJ, Calvo SE, Chang B, Sheth SA, Vafai SB, Ong SE et al. A mitochondrial protein compendium elucidates complex I disease biology. Cell 2008; 134: 112–123.

27. Yamada A, Yamamoto T, Yamazaki N, Yamashita K, Kataoka M, Nagata T et al. Differential permeabilization effects of Ca2+ and valinomycin on the inner and outer mitochondrial membranes as revealed by proteomics analysis of proteins released fromnmitochondria. Mol Cell Proteomics 2009; 8: 1265–1277.

28. Koonin EV, Makarova KS, Rogozin IB, Davidovic L, Letellier MC, Pellegrini L. The rhomboids: a nearly ubiquitous family of intramembrane serine proteases that probably evolved by multiple ancient horizontal gene transfers. Genome Biol 2003; 4: R19.

29. Jeyaraju DV, Xu L, Letellier MC, Bandaru S, Zunino R, Berg EA *et al.* Phosphorylation and cleavage of presenilin-associated rhomboid-like protein (PARL) promotes changes in mitochondrial morphology. Proc Natl Acad Sci USA 2006; 103: 18562–18567.

30. Lemberg MK, Freeman M. Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. Genome Res 2007; 17: 1634–1646.

31. Urban S, Wolfe MS. Reconstitution of intramembrane proteolysis *in vitro* reveals that pure rhomboid is sufficient for catalysis and specificity. Proc Natl Acad Sci USA 2005; 102: 1883–1888.

32. Urban S. Rhomboid proteins: conserved membrane proteases with divergent biological functions. Genes Dev 2006; 20: 3054–3068.

33. Freeman M. Rhomboid proteases and their biological functions. Annu Rev Genet 2008; 42: 191–210.

34. Sík A, Passer BJ, Koonin EV, Pellegrini L. Self-regulated cleavage of the mitochondrial intramembrane-cleaving protease PARL yields Pbeta, a nuclear-targeted peptide. J Biol Chem 2004; 279: 15323–15329.

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

36. Moisoi N, Klupsch K, Fedele V, East P, Sharma S, Renton A *et al.* Mitochondrial dysfunction triggered by loss of HtrA2 results in the activation of a brain-specific transcriptional stress response. Cell Death Differ 2009; 16: 449–464.

37. Vafiadaki E, Sanoudou D, Arvanitis DA, Catino DH, Kranias EG, Kontrogianni-Konstantopoulos A. Phospholamban interacts with HAX-1, a mitochondrial protein with anti-apoptotic function. J Mol Biol 2007; 367: 65–79.

38. Giacomello M, Drago I, Pizzo P, Pozzan T. Mitochondrial Ca2+ as a key regulator of cell life and death. Cell Death Differ 2007; 14: 1267–1274.

39. Pizzo P, Pozzan T. Mitochondria-endoplasmic reticulum choreography: structure and signaling dynamics. Trends Cell Biol 2007; 17: 511–517.

40. Szabadkai G, Simoni AM, Bianchi K, De Stefani D, Leo S, Wieckowski MR *et al.* Mitochondrial dynamics and Ca^{2+} signaling. Biochim Biophys Acta 2006; 1763: 442–449.

41. de Brito OM, Scorrano L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature 2008; 456: 605–610.

42. Rimessi A, Giorgi C, Pinton P, Rizzuto R. The versatility of mitochondrial calcium signals: from stimulation of cell metabolism to induction of cell death. Biochim Biophys Acta 2008; 1777: 808–816.

43. Jeyaraju DV, Cisbani G, Pellegrini L. Calcium regulation of mitochondria motility and morphology. Biochim Biophys Acta 2008, doi:10.1016/j.bbabio.2008.12.005.

44. Gottlieb E, Tomlinson IP. Mitochondrial tumour suppressors: a genetic and biochemical update. Nat Rev Cancer 2005; 5: 857–866.

45. King A, Selak MA, Gottlieb E. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. Oncogene 2006; 25: 4675–4682.

46. Frezza C, Gottlieb E. Mitochondria in cancer: not just innocent bystanders. Semin Cancer Biol 2009; 19: 4–11.

47. Schapira AH. Mitochondrial dysfunction in Parkinson's disease. Cell Death Differ 2007; 14: 1261–1266.

48. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 2008; 183: 795–803.

49. Koonin EV, Aravind L. Origin and evolution of eukaryotic apoptosis: the bacterial connection. Cell Death Differ 2002; 9: 394–404.

50. Fadeel B, Grzybowska E. HAX-1: a multifunctional protein with emerging roles in human disease. Biochim Biophys Acta 2009, doi:10.1016/j.bbagen.2009.06.004.

51. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997; 25: 3389–3402.

52. Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol 2000; 302: 205–217.

53. McGuffin LJ, Bryson K, Jones DT. The PSIPRED protein structure prediction server. Bioinformatics 2000; 16: 404–405.

54. Cole C, Barber JD, Barton GJ. The Jpred 3 secondary structure prediction server. Nucleic Acids Res 2008; 36: W197–W201.

55. Rost B, Yachdav G, Liu J. The PredictProtein server. Nucleic Acids Res 2004; 32: W321–W326.

56. Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S *et al.* CDD: a curated Entrez database of conserved domain alignments. Nucleic Acids Res 2003; 31: 383–387.

57. Marchler-Bauer A, Bryant SH. CD-Search: protein domain annotations on the fly. Nucleic Acids Res 2004; 32: W327–W331.

58. Claros MG, Vincens P. Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 1996; 241: 779–786.

59. Sonnhammer EL, von Heijne G, Krogh A. A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol 1998; 6: 175–182.

60. Hofmann K, Stoffel W. TMbase – A database of membrane spanning proteins segments. Biol Chem Hoppe-Seyler 1993; 374: 166.

61. Frezza C, Cipolat S, Scorrano L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat Protoc 2007; 2: 287–295.

Figure 8 Multiple alignment of selected Hax1 sequences from diverse animals.

Hax1 does not contain BH1 or BH2 modules, or a transmembrane domain. The numbers between aligned blocks indicate poorly conserved sequence segments that are not shown. Secondary structure (SS) predicted with three methods is shown above the alignment. c, random coil (disordered structure); h, α-helix; e, β-strand (extended conformation). Amino acid residues that are conserved in all aligned sequences are shown in bold type, and the three invariant aspartates that comprise a putative metal-binding site are shown by reverse

shading. The positions of purported BH1 and BH2 modules are shown above the respective regions of the alignment using the alignment from Sharp *et al.* [21] for BH1, and arbitrarily centering the alignment on a conserved hydrophobic residue for BH2. 'SS_BH' denotes the consensus secondary structure motifs of BH1 and BH2 modules derived from multiple crystal and NMR structures of Bcl-2-family proteins.[23] The position of the transmembrane domain (TMD) predicted in Sharp *et al.*,[21] but not in our analysis, is indicated in the C-terminal block of the alignment.

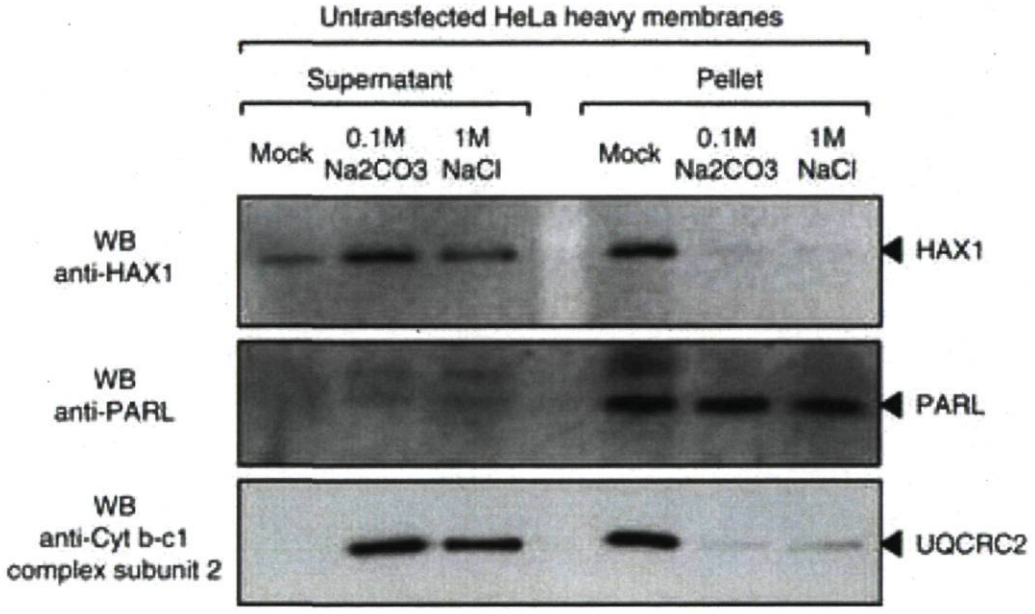


Figure 9 Hax1 is not an integral membrane protein.

Alkaline extraction of heavy membranes isolated from HeLa cells (200 g); whereas membrane-associated proteins and proteins associated to membrane-bound proteins (e.g. UQCRC2) are solubilized in the supernatant, integral membrane proteins like PARL are recovered in the membrane pellet. Lack of Hax1 integration in heavy membranes is consistent with our computational analysis (Figure 1), which does not predict any potential transmembrane domain.

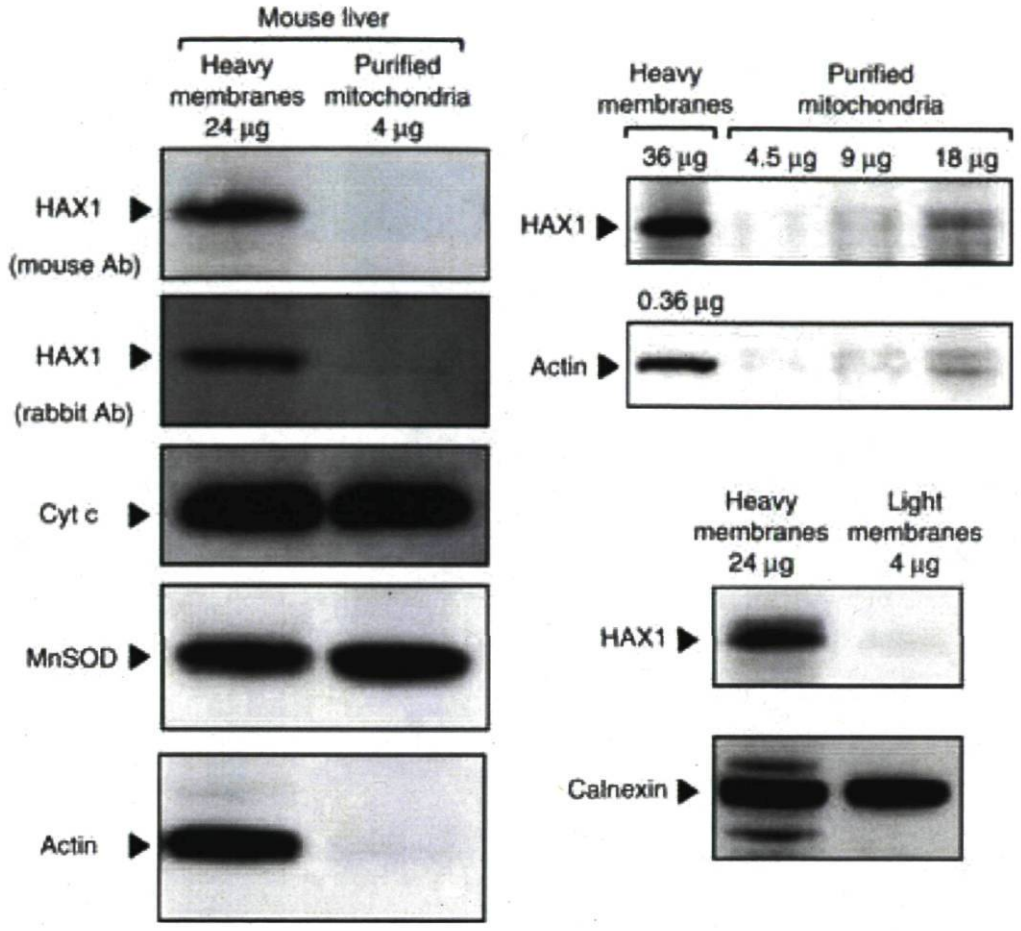
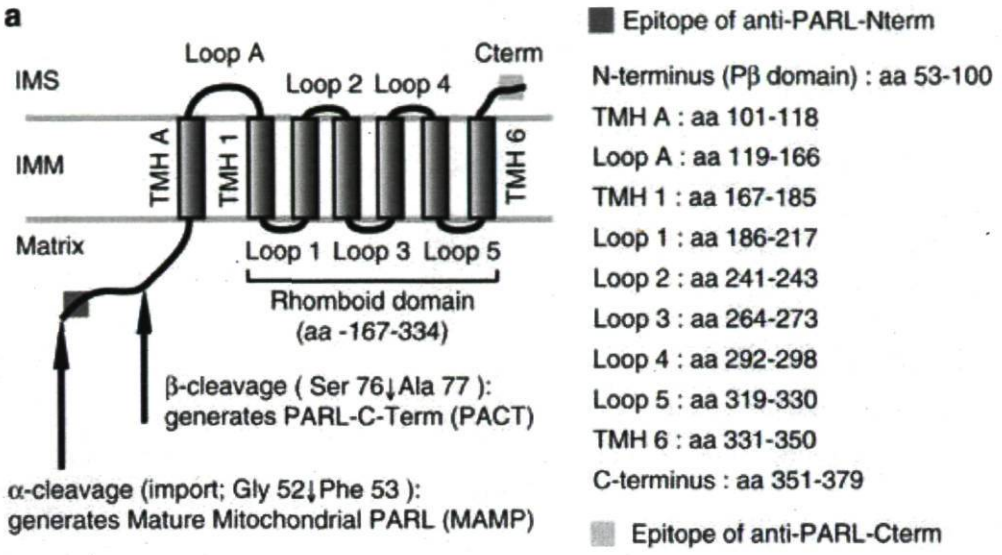
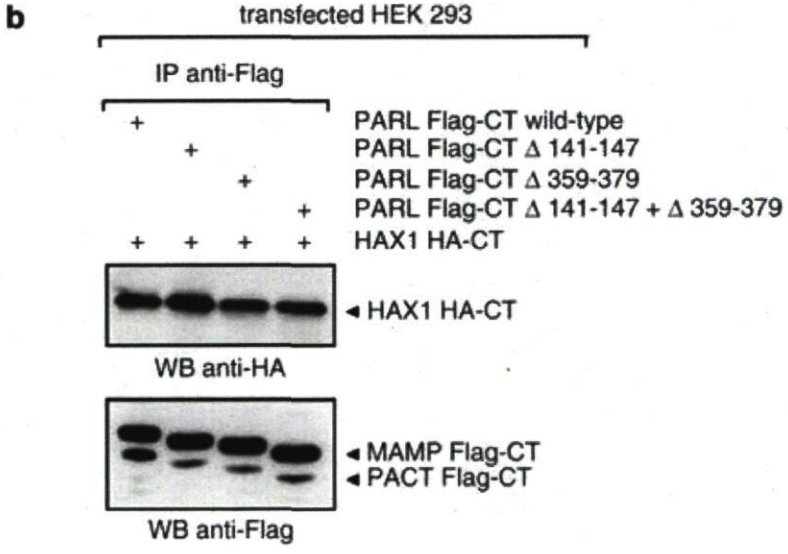


Figure10 Hax1 is not imported in mitochondria but is peripherally associated to heavy membranes. Immunoblot analysis of Percoll-purified fractions of mouse livers. In this preparation, lack of cross-contaminating organelles was assayed by electron microscopy (not shown); inner and outer mitochondrial membrane integrity was tested for presence of diffusible proteins of the IMS (cytochrome c) and mitochondrial matrix (MnSOD); purity from membranes-associated cytosolic proteins by the absence of actin. The left panel shows that, on normalization for cytochrome c and MnSOD, endogenous Hax1 is detected in heavy membranes but not purified mitochondria, a finding consistent with lack of Hax1 in the human and murine mitochondrial proteome[26]


a
Loop A
Cterm
IMS
Loop 2 Loop 4
IMM
TMH A
TMH 1
TMH 6
Matrix
Loop 1 Loop 3 Loop 5
Rhomboid domain
(aa -167-334)
β-cleavage (Ser 76↓Ala 77):
generates PARL-C-Term (PACT)
α-cleavage (import; Gly 52↓Phe 53):
generates Mature Mitochondrial PARL (MAMP)
Epitope of anti-PARL-Nterm
N-terminus (Pβ domain) : aa 53-100
TMH A : aa 101-118
Loop A : aa 119-166
TMH 1 : aa 167-185
Loop 1 : aa 186-217
Loop 2 : aa 241-243
Loop 3 : aa 264-273
Loop 4 : aa 292-298
Loop 5 : aa 319-330
TMH 6 : aa 331-350
C-terminus : aa 351-379
Epitope of anti-PARL-Cterm


b
transfected HEK 293
IP anti-Flag
PARL Flag-CT wild-type
PARL Flag-CT Δ 141-147
PARL Flag-CT Δ 359-379
PARL Flag-CT Δ 141-147 + Δ 359-379
HAX1 HA-CT
◂ HAX1 HA-CT
WB anti-HA
◂ MAMP Flag-CT
◂ PACT Flag-CT
WB anti-Flag

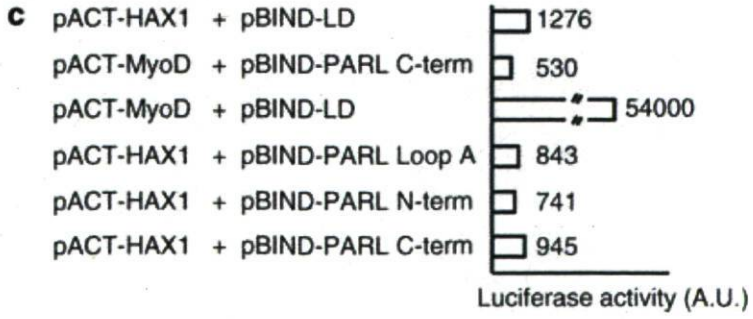

c
pACT-HAX1 + pBIND-LD
1276
pACT-MyoD + pBIND-PARL C-term
530
pACT-MyoD + pBIND-LD
54000
pACT-HAX1 + pBIND-PARL Loop A
843
pACT-HAX1 + pBIND-PARL N-term
741
pACT-HAX1 + pBIND-PARL C-term
945
Luciferase activity (A.U.)

Figure 11 PARL does not have a domain that coordinates interaction to Hax1 in the IMS. (**a**) Schematic representation of the topology and domain composition of PARL.[28, 30] (**b**) Co-immunoprecipitation of Hax1 with mutant forms of PARL in which the IMS domains (loop-A and C-terminus) have been deleted. Note that PACT formation in indicates that the mutant proteins are correctly imported and folded in the inner membrane. (**c**) Mammalian two-hybrid assays fails to identify an IMS domain in PARL that could mediate interaction to Hax1. For a positive control reaction, pBIND-LD and pACT-MyoD control vectors are cotransfected along with pG5luc. Values, expressed as arbitrary units (A.U.), indicate mean of three independent experiments done in triplicates.

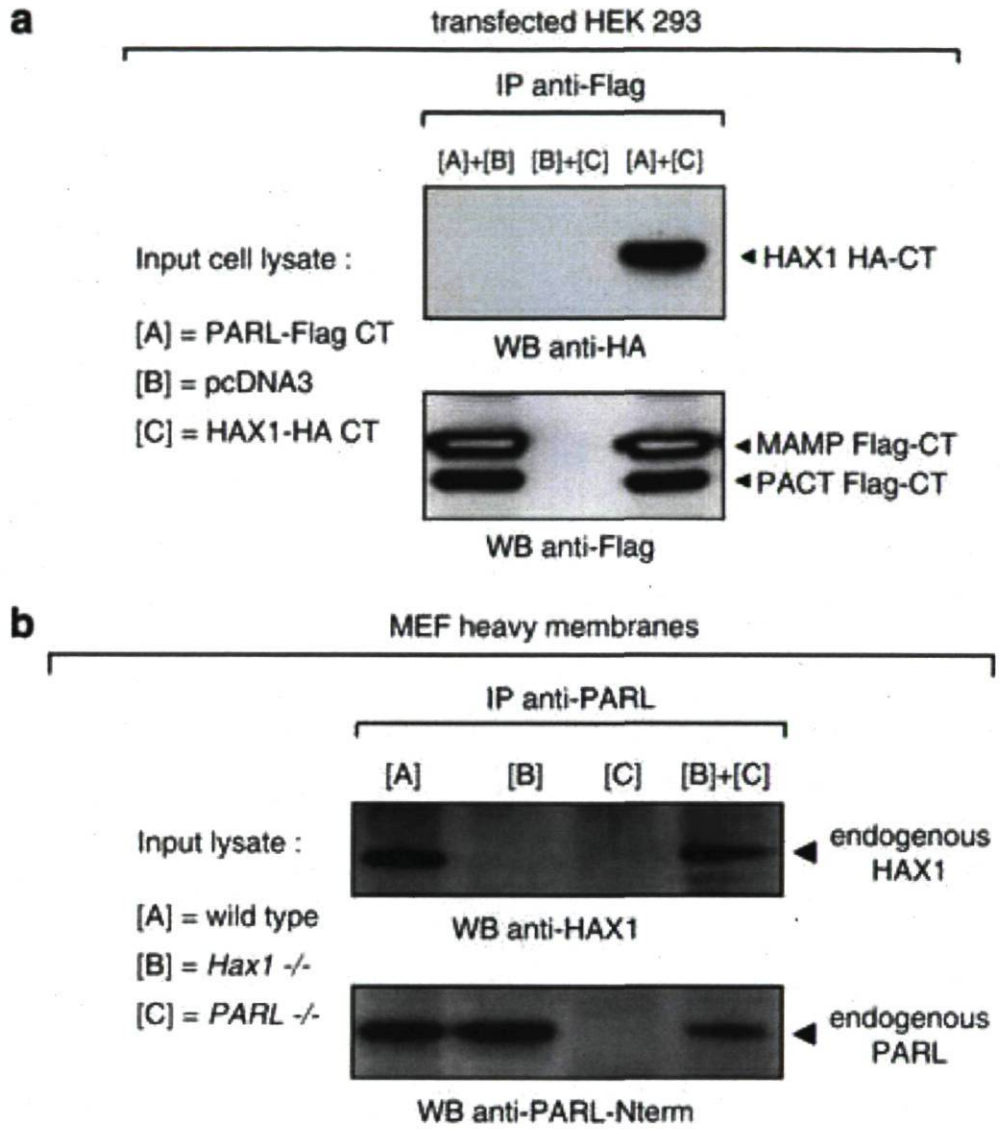


Figure 12 Hax1 binding to PARL is unspecific

(**a**) *In vitro* reconstitution of PARL/Hax1 complex by mixing lysates of HEK293 cells transfected with a construct expressing PARL-Flag-CT or Hax-HA-CT. (**b**) *In vitro* reconstitution of PARL/Hax1 complex by mixing lysates of heavy membranes (150 g of proteins per genotype used) isolated from MEFs +/+, *Hax1* -/- or *Parl* -/-.

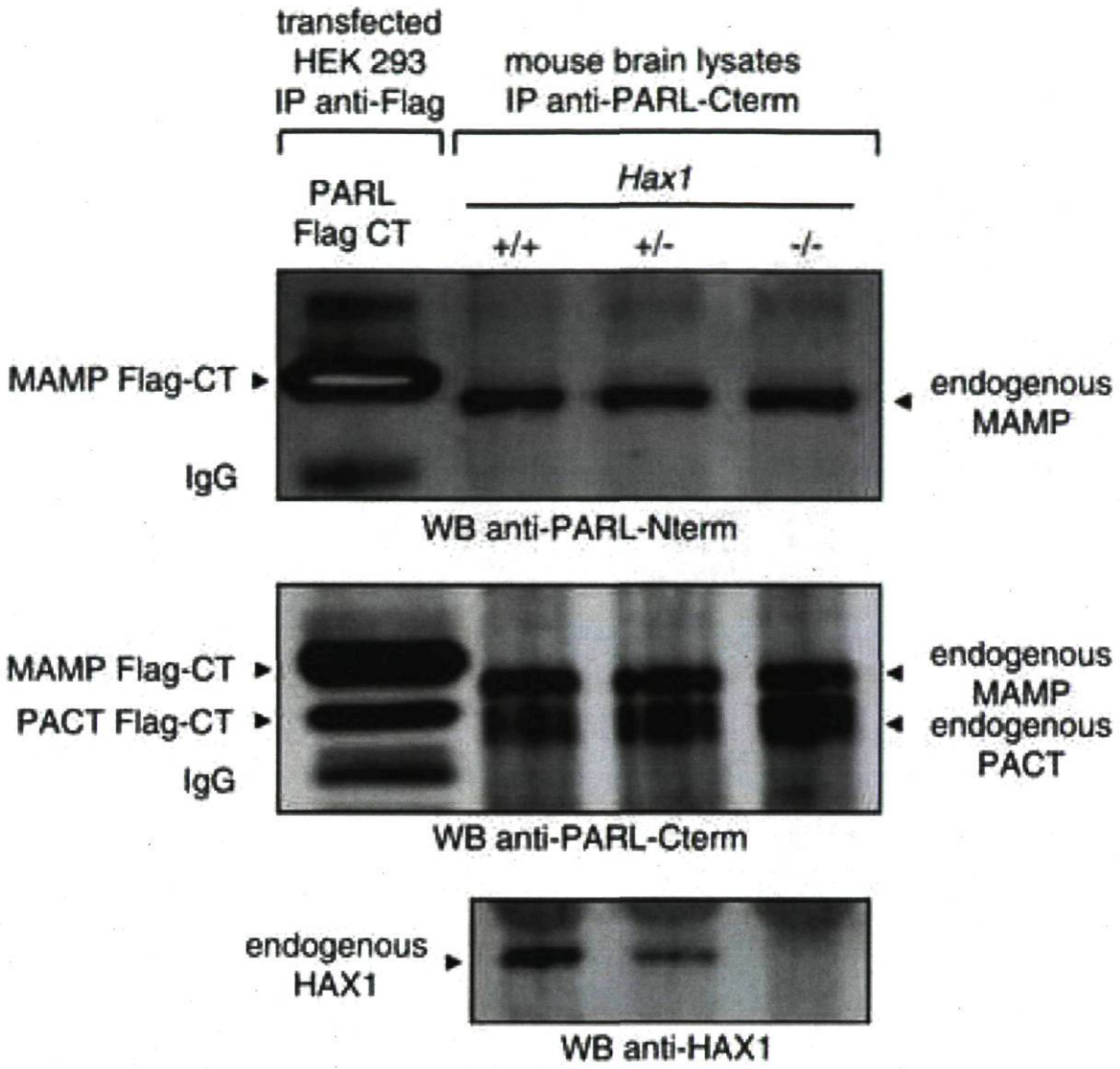


Figure 13 PARL proteolytic activity does not require Hax1.

The upper panel shows that the level of expression of the mitochondrial mature form of PARL, MAMP, is not altered in any of the indicated genotypes. The middle panel indicates that genetic ablation of *Hax1* does not impair the generation of PACT, a shorter form of MAMP that requires PARL activity supplied *in trans*.[34] The lower panel shows the expression levels of Hax1 in mouse *Hax1* +/+, +/- and -/- brain lysates.

Chapter 3

3. Appendix l to chapter 2: material and methods

3.1 DNA preparation

Plasmid DNA preparation was done by miniprep or PEGprep. Miniprep allows us to purify small quantity of DNA (5-10μg) from small volumes (1-2 ml) of bacterial culture in about 30 minutes. This DNA can be used for transformation and cloning purpose.
PEGprep allow us to obtain transfection plasmid in large quantities (~5mg) from 400ml of culture.

3.1.1 DNA miniprep

Plasmid minipreps were done using the High Pure Plasmid isolation kit (Roche).
This procedure is used to extract plasmid DNA from bacterial cell suspensions and is based on the alkaline lysis procedure. This method takes advantage of the fact that plasmids are supercoiled. The difference in topology allows for selective precipitation of the chromosomal DNA and cellular proteins from plasmids and RNA molecules. The cells are lysed under alkaline conditions, which denature both nucleic acid and proteins, and when the solutions is neutralized by addition of potassium acetate, chromosomal DNA and proteins precipitate because it is impossible for them to renature correctly owing to their large size. Plasmids on the other hand, renature and stay in solution, effectively separating them from chromosomal DNA and proteins. The process does not require DNA precipitation, organic solvents extractions, or extensive handling of the DNA.

Protocol: Grow bacterial cultures overnight in a rotatory shaker at 200rpm at 37°C in Miller LB medium (10g/L Tryptone, 5g/L Yeast extract and 0.5g/L NaCl) with the required antibiotic based on the vector used. Centrifuge the cells at 13000 rpm for 2 minutes. Resuspend the pellet in 250 µl of suspension buffer with RNaseA followed by the addition of 250 µl of Lysis buffer. Invert the tube 2 or 3 times to facilitate even lysis of the cells in suspension. Then add 350 µl of neutralizing buffer followed by gentle inversion of the tube. Place the tube in ice for 10 minutes followed by centrifugation for 10 minutes at 13000rpm. Remove the supernatant carefully and transfer to the purification column followed by a minute of centrifugation. Discard the flow trough and add 750 µl of wash buffer followed by two centrifugations where the flow trough has to be discarded in both. Purified plasmid DNA can be eluted by adding 50 µl of elution buffer (TE: 10Mm Tris, 1mM EDTA) followed by centrifugation at maximal speed for one minute.

3.1.2 DNA maxiprep for cell transfection (PEGprep)

Protocol: Incubate a single bacterial colony in 400ml of Miller LB broth with the appropriate antibody at 37°C in a rotatory shaker at 135 rpm overnight. Centrifuge at 4000 rpm at 4°C in a swing bucket rotor for 15 minutes. Resuspend the pellet in 4ml of Solution I (50mM glucose, 25Mm Tris-HCl pH 7.6, 10mM EDTA), transfer to a corning tube and add 5 µl of RNaseA (10mg/ml, Fermentas). Add 8 ml of Solution II (0.2M NaOH and 1%SDS). Invert gently 2-3 times until the solution is limpid. Add 4 ml of Solution III (3M KAc, 11%v/v glacial acetic acid). Invert gently and place in ice for 10 minutes. Centrifuge for 15 minutes at 4°C, 14000rpm and if the supernatant is still turbid, transfer to a new tube and recentrifuge. Transfer the clear supernatant to a new tube and note the volume. Add 0.6 volumes of Isopropanol followed by inversion of the tube and incubation at -20°C for one hour. Centrifuge at 14000rpm at 4°C for 15minutes. Discard the supernatant and air dry for 5 minutes. Resuspend the pellet in 600 µl of TE and transfer to an eppendorf tube and add 3 µl of RNaseA and centrifuge for 30 minutes full speed. Transfer the supernatant to a new eppendorf tube and note the volume. Add an equal volume of PEG (20%PEG and 2.5NaCl) and invert the tube few times. Incubate in ice for 30 minutes.

Centrifuge at 14000 rpm for 10 minutes and resuspend the pellet in 500 µl of TE. Incubate at 37 °C for 30 minutes. Add one volume of Phenol: Chloroform: Isoamyl alcohol 25:24:1 saturated with 10mM Tris pH 8.0 and 1Mm EDTA, (Sigma), vortex for 10 seconds and centrifuge for 5 minutes at 13200 rpm. Collect the upper layer and note the quantity. Repeat the above phenol/chloroform step once more with the collected upper layer. Collect the upper layer again and add an equal volume of chloroform (Sigma), vortex for 30 seconds, and centrifuge at 13200 rpm for 5 minutes and collect the supernatant. Add 0.1 volume of Na Acetate pH 5.3 followed by 3 volumes of Ethanol 100% and invert the tubes a few times. Centrifuge at full speed for 10 minutes and discard the supernatant. Add 600 µl of ethanol 70% and centrifuge for 10 minutes at 13200 rpm and discard the supernatant. Resuspend the pellet in 1ml TE. Quantify the DNA by measuring the optical density at 260nm.

3.2 Mutagenesis

Mutagenesis was performed based on the oligonucleotide base site-directed mutagenesis methodology. In this strategy, two primers are used. A mutagenic primer that introduces the desired mutation and a selection primer containing a mutation in the recognition sequence for a denatured double-stranded plasmid under conditions favoring the formation of hybrids between the primers and the DNA template. After standard elongation, ligation and a primary selection by restriction digest, the mixture of mutated and unmutated plasmids is transformed into a mutS *E.coli* strain defective in mismatch repair. Transformants are pooled, and plasmid DNA is prepared from the mixed bacterial population. The isolated DNA is then subjected to a second selective restriction enzyme digestion. Since the mutated DNA lacks the restriction enzyme recognition site, it is resistant to digestion. The parental DNA, however, is sensitive to digestion and will be linearized, rendering it at least 100 less efficient in transformation of bacterial cells. A final transformation with the selectively digested DNA results in highly efficient and specific recovery of the desired mutated plasmid.

This strategy has some advantages:

- Does not require single-stranded vectors or specialized double-stranded plasmids
- Does not require viral transductions

- Does not require subcloning
- Results in mutation efficiency if 70-90%

In addition, the use of T4 DNA polymerase instead of PCR reduces the risk of introducing spurious mutations (Figure 14)

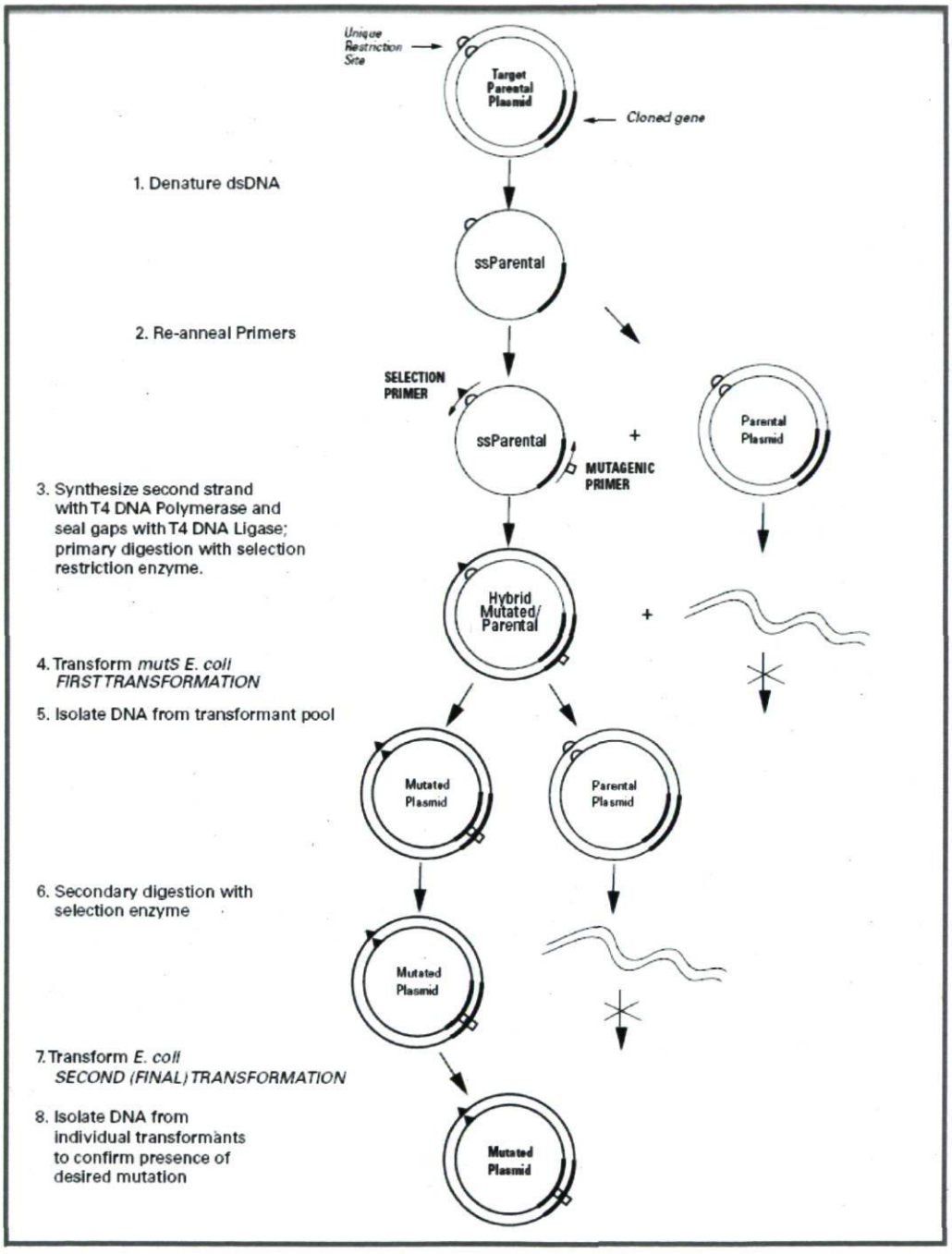


Figure 14 Schematic representation of the mutagenesis protocol (Clontech)

3.2.1 Primer design

Selection primer: the function of the selection primer is to eliminate the original unique restriction enzyme site. The selection primer can be designed by incorporating one or more base pair changes within the targeted unique restriction site. Since restriction enzymes recognize an exact DNA sequence, one or more changes within the recognition sequence should be sufficient to abolish the restriction digestion. If the selection site is located within a gene, avoid using a selection primer that will introduce a change that could interfere with the expression of that gene (by causing a reading frame shift or premature termination codon).

Mutagenic primer: the function of the mutagenic primer is to introduce the desired mutation.

Important to remember:

1. The mutagenic and the selection primers must anneal to the same strand of the plasmid
2. The distance between the selection primer and the mutagenic primer is not critical. If possible, however, design the selection and mutagenic primers so that they will be evenly spaced after annealing to the DNA template; this will allow the DNA polymerase to extend both primers an equivalent distance. In rare cases, where the unique restriction site and the targeted mutagenic site are very close to each other, one single primer can be designed to introduce both mutations simultaneously
3. In most cases, 10 nucleotides of uninterrupted matched sequences on both ends of the primer flanking the mismatch site, should give a sufficient annealing stability, provided that the GC content of the primer is greater than 50%. If the GC content is less than 50%, the lengths of the primer arms should be extended accordingly. The mismatch bases should be placed in the center of the primer sequence. For optimum primer annealing, the oligonucleotides should start and end with a G or C. The annealing strength of the mutagenic primer should always equal or greater than that of the selection primer.

4. There can be more than one base mismatch in the mutagenic primer. However, the success rate for targeted mutagenesis of a plasmid is optimized when there are only one or few mismatches.

3.2.2 Primer phosphorylation

The primers must be phosphorylated at the 5' end, so that they can be ligated to 3' end of the newly synthesized strand.

Protocol: Resuspend primers in water at a concentration of 1 µg/ µl. For each primer prepare a reaction mix of 20 µl (final volume) composed of 2 µl of 10mM ATP (Fermentas), 2 µl of 10X PNK buffer A (500mM Tris-HCl pH 7.6, 100mM $MgCl_2$, 50mM DTT, 1mM Sperimidine, 1mM EDTA), 1 µl of primer and, 1 µl if T4 Polynucleotide Kinase (PNK). Incubate at 37ºC for 30 minutes and then inactivate PNK at 75ºC for 10 minutes. Briefly spin the tubes after this step.

3.2.3 Plasmid denaturation and primers annealing

Protocol: To a final volume of 30 µl, add 1 µl of the phosphorylated selection primer, 1 µl of the phosphorylated mutagenic primer, 1 µl of the DNA template (concentration 100ng/ µl), 3 µl of Buffer Tango 10X (Fermentas, 330mM Tris-Acetate pH 7.9, 100mM magnesium acetate, 66mM potassium acetate, 1mg/ml BSA). Incubate at 100ºC for 3 minutes and immediately place in ice for 5 minutes.

3.2.4 Synthesis of the mutant DNA strand and restriction digestion selection

Protocol: To each tube of the primer/plasmid annealed mix, add 1 µl of T4 DNA Polymerase (10U/ µl, Fermentas), 1 µl of T4 DNA Ligase (10U/ µl, Fermentas), 1 µl of dNTPs and ATP mix (2.5mM dNTP and 1mM ATP). Mix well and centrifuge briefly. Incubate at 37ºC for 60 minutes. Stop the reaction by heating at 75ºC for 10 minutes to inactivate the enzymes.

After allowing cooling, add 1 µl of the required restriction enzyme to digest the unmodified plasmids and incubate at 37° for 60 minutes.

3.2.5 Transformation into MutS cells

The purpose of this step is to amplify the mutated strand (as well as the parental strand) in chemical competent cells mutS (repair deficient) *E.coli* strain (BMH 71-18 MutS; Clontech or Promega).

Protocol: Preheat a water bath to 42°C. Add 2 µl of the digested plasmid DNA digestion to 50 µl of mutS cells and incubate on ice for 10 minutes. Heat-shock cells at 42° C for 40 seconds and incubate in ice for 2 minutes and add 500 µl of LB media. Incubate at 37° for 45 minutes and add to a falcon tube containing 5ml of LB media with required antibiotic. Shake overnight at 37° at 200 rpm.

3.2.6 Isolation of the mixed plasmid pool by miniprep

Protocol: Do a DNA miniprep from 2ml of *E.coli* mutS cells

3.2.7 Selection of the mutant plasmid

This procedure selects for the mutant plasmid by complete digestion of parental (unmutated) plasmids.

Protocol: Digest 15 µl of the miniprep done above (~1 µg) in 20 µl final volume; transform 2 µl in 50 µl of competent *E.coli* K12 cells, then plate1/10 of the transformed cells in LB agar media containing the appropriate antibiotic.

3.2.8 Mutants screening

Mutants are identified by sequencing. In some cases the mutagenic primer can be designed in such a way that along with mutation, a restriction site can be eliminated or introduced. In such cases, restriction digestion should be performed and potential mutants analyzed by agarose gel electrophoresis.

3.3 DNA sequencing

Plasmid sequencing was done using the sequencing service from Université Laval. Chromatogram files were analyzed using Chromas Pro from Technelysium Ltd.

3.4 Cloning

Linearized and dephosphorylated vector using the appropriate restriction enzyme and CIAP (Fermentas) were ligated to the phosphorylated PCR product and positive clones were identified by PCR screening.
PCR product obtained using KOD hot start DNA polymerase (Novagen), following manufacturer's instructions.

3.5 E.coli strains used

- BMH 71-18 *mut*S is a mismatch repair minus strain of *E. coli.* Use of this strain prevents repair of the newly synthesized unmethylated strand leading to high mutation efficiencies. **Genotype:** *thi, sup*E, Δ (*lac-pro*AB), [*mut*S::Tn10, [F´, *pro*AB, *laq*I^qZΔM15 (Promega).
- NovaBlue Singles Competent Cells. **Genotype**: *endA1 hsdR17* (r_{K12}^- m_{K12}^+) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA*$^+$*B*$^+$ *lacI*^{q}Z*DM15*::Tn*10* (Tet^R) (EMDbiosciences)

3.6 Vectors

The mammalian expression vector pcDNA3 was used in this study.
The vector pACT and pBIND (Promega) are two mammalian expression vectors for two-hybrid assay to detect protein-protein interactions. The two vectors were used to study the interaction between HAX1 and PARL.

3.7 Immunoprecipitation

Immunoprecipitation is used to analyze target antigen from a complex mixtures of proteins using a specific antibody immobilized either covalently or non-covalently on a solid-phase matrix to allow separation by low speed centrifugation. Usually antibodies are bound non-

covalently to Protein A- or Protein G- agarose (from *Current protocols in Molecular Biology*)

Protocol: In an eppendorf tube mix 40 µl of Protein A-agarose bead slurry (Roche) with antibody anti HAX1 and anti-PARL (dilution 1:100). Let rotate overnight at 4°C in a tube rotator. Wash the resin with non covalently attached the antibody three times in 1X STEN-NaCl (50mM Tris pH 7.6, 500mM NaCl, 2mM EDTA, 0.2% NP40). Add 15 µl of the slurry to 800 µl of cell lysate; allow mixing by gentle rotation at room temperature for three hours.

Wash the beads twice with 1X STEN-NaCl and once with 1X STEN- SDS (50mM Tris pH 7.6, 500mM NaCl, 2mM EDTA, 0.2% NP40, 0.1% SDS), leaving at the end the beads in 50 µl of buffer (Fig. 9).

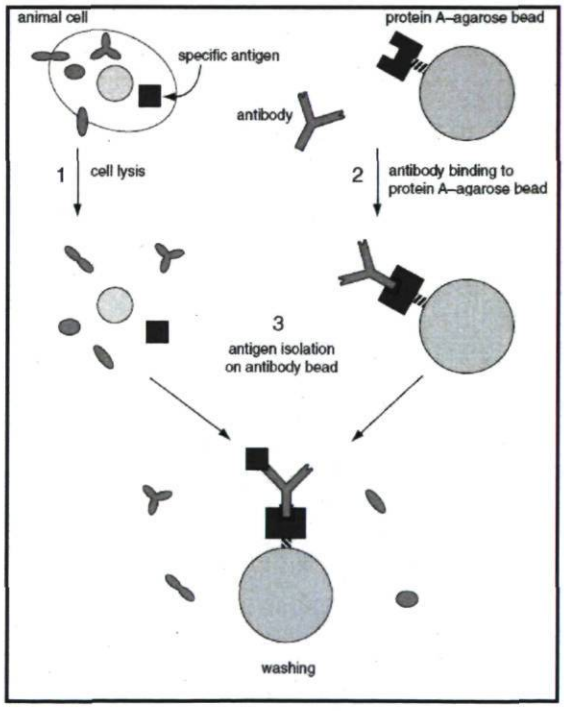


Figure 15 Schematic representation of the stages of Immunoprecipitation protocol (from Current protocols in Molecular Biology)

3.8 Crosslinking of antibody to Protein-A agarose beads

Covalent coupling of antibodies was performed using the SeizeX immunoprecipitation kit from Pierce. This Kit offers an improvement over the classical method of immunoprecipitation (IP) by immobilizing the antibody to the Protein A gel using the cross-linker DSS (disuccinimidyl suberate). This procedure results in a permanent affinity support with a properly oriented antibody. The crude sample is then incubated with the immobilized antibody to form the immune complex. The affinity support is washed by centrifugation using a Spin Cup Column and the remaining antigen is dissociated from the antibody using an elution buffer. The primary antibody does not contaminate the final antigen preparation and the immobilized antibody support is preserved for future IPs. Depending on the stability of the immobilized antibody, the prepared affinity support may be used 2-10 times, thus conserving precious antibody.

Since the antibody concentration in the antisera may vary, covalent coupling should be tested using various antisera dilutions, so that the optimal chemical crosslinking efficiency can be obtained for immunoprecipitation.

Protocol: Equilibrate the Immobilized Protein A and reagents to room temperature. Add 500 ml of ultrapure water to the dry-blend buffer. To store excess buffer, add a preservative such as 0.02% sodium azide and store at 4°C. Gently swirl the bottle of ImmunoPure Immobilized Protein A to obtain an even suspension. Add 0.4 ml of the Immobilized Protein A (50% slurry) into one of Spin Cup Columns and place inside a microcentrifuge tube and centrifuge (note: all centrifugations in this procedure should be performed at 4000 rpm and at room temperature for 1 minute). Discard flow-through and replace spin cup into the tube. Wash gel by adding 0.4 ml of Binding/Wash Buffer to the spin cup containing the gel. Cap tube and resuspend gel by inverting with gentle shaking. Centrifuge tube and discard flow-through. Repeat this step once. Place spin cup into a new microcentrifuge tube and apply 50-500 µg of purified antibody prepared in 0.3-0.4 ml of Binding/Wash Buffer. Cap tube and place it on a rocker for at least 15 minutes to allow the antibody to bind to the gel. Centrifuge the tube. Place spin cup into another microcentrifuge tube and add 0.5 ml of Binding/Wash Buffer. Invert tube 5-10 times. Centrifuge tube and discard flow-through.

Repeat this step two additional times using the same collection tube. Transfer the spin cup into a new microcentrifuge tube and add 0.4 ml of Binding/Wash Buffer.

Puncture the foil covering of a single tube of No-Weigh™ DSS with a pipette tip and add 80 µl of DMSO or DMF. Use the pipette to thoroughly mix the solution until the DSS is dissolved. Add 25 µl of the DSS solution to the spin cup containing the bound antibody support. The tube containing the resuspended DSS can be discarded from the strip by pushing tube from the bottom, away from the strip.

(Note: because DSS is a hydrophobic molecule, a microprecipitate may form when it is added to the aqueous medium, which results in a cloudy appearance. Nevertheless, the reaction will proceed efficiently and the microprecipitate may disappear during conjugation).

Place cap on the microcentrifuge tube and gently mix for 30-60 minutes in a tube rotetor. Centrifuge tube and discard flow-through. Add 500 µl of Elution Buffer to the spin cup. Cap tube and invert it 10 times. Centrifuge tube and discard flow-through. Place the spin cup back into the microcentrifuge tube. (Note: The pH of the elution buffer is 2.8, so it should be used with care since excessive time in the elution buffer was observed to disrupt antibody activity). Repeat four additional times to quench the reaction and to remove excess DSS and uncoupled antibody. Place the spin cup in a new microcentrifuge tube and wash two times with 500 µl of Binding/Wash Buffer.

3.9 Migration of proteins in gel electrophoresis

Protocol: Prepare 1X SDS Running Buffer by adding 35 ml 20X NuPAGE (Invitrogen) MES or MOPS SDS running buffer to 665ml of deionized water. Prepare the electrophoresis setup and install the precast 2-12% Bis Tris gel and add the running buffer.

Add the appropriate amount of NuPAGE LDS 4X buffer (Invitrogen) and DTT (1M, 10X) and incubate at 85° C for 4 minutes. During the incubation period, remove the comb from the gel and wash the pocket with running buffer.

Add the appropriate amount of sample (~12 µl) per well. Add 3 µl of Seeblue Plus Prestained Ladder (Invitrogen) to one of the wells (usually the first and the last). Attach electrical supply and run at 200 Volts until the bleu dye front reaches the bottom of the gel.

3.10 Western blotting

Western Blotting is a technique that uses antibodies to probe for a specific protein in a sample bound to a membrane. After separating the protein sample via electrophoresis, the separated protein are transferred to a nitrocellulose or PVDF membrane, making an exact replica of the original protein gel separation on the membrane, to render the protein accessible to the antibody.

Protocol: Prepare one liter of 1X NuPAGE Transfer Buffer (Invitrogen) by adding 50 ml of 20X NuPAGE transfer Buffer and 100 ml of methanol to 850 ml of deionized water. Prepare transfer membranes by moistening in methanol and then immediately adding transfer buffer. Soak briefly the blotting paper in 1X NuPAGE Transfer Buffer.

The gel and membrane sandwich is then arranged by positioning in the proper apparatus 2 blotting paper soaked in the transfer buffer, then the gel. On top of the gel place the transfer membrane removing any trapped air bubbles. Then place other two blotting papers on top of membrane. One the sandwich is ready place it in the specialized blotting apparatus, with the gel closest to the cathode, so the proteins can be electrophoretically transferred from the gel to the membrane.

Perform transfer for nitrocellulose or PVDF membrane using 100C constant fro 1 hour.

Remove the cassette and rinse the membrane with PBST (137mM NaCl, 27mM KCl, 43mM Na_2HPO_4, 14mM KH2PO4 and 1% Tween-20). Wash the membrane three times. Proceed with blocking the membrane with 5% milk solution made in PBST. Add the required antibody at its optimal dilution in 4ml of milk 5% and incubate. The following table gives the dilution and concentration of the various antibodies used:

Antibody	Dilution
Rabbit anti-PARL[15]	1:1000
Rabbit anti-MnSOD	1:1000
Mouse anti-UQCRC2	1:3000
Mouse anti-HA	1:1000
Mouse anti-Flag M2	1:1000
Mouse anti-actin	1:1000
Mouse anti-Cytochrome C	1:1000
Mouse anti-Hax1	1:250
Rabbit anti-calnexin	1:4000

Table 1 List of antibody used for western blot and of their respective dilutions.

After the incubation with primary antibody, wash the membrane three times and add HRP conjugated secondary antibody (1:2000, Chemicon).
Incubate at room temperature for 1 hour and wash thrice with PBST.
Reveal blotted proteins by chemioluminescence with Super Signal West Femto (Pierce) using the Versadoc imaging station (Biorad).

3.11 Mitochondria isolation

Mitochondria isolation is performed according the protocol: Frezza C., Cipolat S., Scorrano L. "Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts", *Nature Protocol*, 2007, Vol.2, N.2
These protocols represent a valuable starting point to obtain pure mitochondria from tissues and cells. Isolated mitochondria can then be used to study the function of the organelle, response to apoptotic stimuli, characteristics of cytochrome c release, protein import and many other aspects of mitochondrial biology and pathophysiology that require a source of pure and functional organelles.

mouse livers were washed once with PBS, suspended in isolation buffer (200mM sucrose, 1mM EGTA-Tris and 10mM Tris-MOPS, pH 7.4), and then disrupted by dounce

homogenization on ice. The homogenate was spun at 800 × *g* for 10min at 4°C; the supernatant was recovered and further centrifuged for 10min at 8000 × *g* at 4°C. The resulting pellet (mitochondrial fraction) was collected, whereas the supernatant was further spun for 30min at 100000 × *g* at 4°C. The resulting pellet (light membrane fraction) and supernatant (cytosolic fraction) were spun again at 100000 × *g* to further purify the fractions. The mitochondrial fraction was purified further by centrifuging twice at 8000 × *g* for 10min at 4°C. The obtained pellet was purified by centrifugation at 95000 × *g* for 30min on a 30% Percoll gradient in isolation buffer. The mitochondrial layer was washed free of Percoll and resuspended in isolation buffer. Subcellular fractions were also obtained by differential centrifugation from mouse liver and MEFs (Frezza et al 2007). Protein concentration was determined and the indicated amounts of protein were separated by SDS-PAGE and immunoblotted.

3.12 Alkaline extraction

Heavy membranes prepared from HeLa cells were diluted to a final concentration of 1 mg/ml in 20 mM HEPES/KOH (pH 7.4) (hyposmotic buffer). After the addition of an equal volume of freshly prepared 0.2 M sodium carbonate (pH 11.5), samples were incubated for 30 min at 4°C. The membrane and soluble fractions were separated by ultracentrifugation at 100 000 × *g* for 30 min at 4°C (debrito et al)

3.13 Mammalian cell culture and transfection

HEK 293, HeLa and Cos-7 cells were obtained from American Type Culture Collection (ATCC). DMEM (Dulbecco's Modified Eagle Medium, Invitrogen) was used for HEK 293 and Cos-7 cells while MEM (Minimum Essential Medium, Sigma) was used for HeLa cells. In all cases; the media was supplemented with 10% heat-inactivated fetal bovine serum, 2mM glutamine, 10μg/μl.1 penicillin and 10 μg/μl streptomycin. Keep cells in culture by growing them in Petri dishes. When the cells are 100% confluent, remove and

discard culture medium. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove ail traces of serum that contains trypsin inhibitor. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid dumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. Add appropriate aliquots of the cell suspension to new culture vessels. Incubate cultures at 37°C. *Sub cultivation ratio:* A sub cultivation ratio of 1:2 to 1:4 is recommended *Medium renewal:* Every 2 to 3 days.

For transfection, grow cells in 6 well plates until they reach 50% of confluence. Per every well of cells to transfect, add 1 ug of DNA (PEG prep) to 100 µl of serum free media and add 3 µl of Fugene 6 (Roche Applied Science). Incubate for 30 minutes and add the mix to the cells. To check transfection efficiency, every experiment included a control transfection with a plasmid expressing GFP (pEGFP-Nl, Clontech).

3.13 Cell harvesting

Protocol: When transfected cells reach 90-100% of confluence (24-48 hours after transfection, depending on the cell type), add 800 ul of RIPA (65 mM tris base, 0.15 M NaCl, 1% NP40, 0.25% sodium deoxycholate, lmM EDTA pH 7.4) to each well. Centrifuge cell lysate at 14000 rpm at 4°C for 30 minutes. Collect the supernatant and continue with immunoprecipitation or freeze at -30°C for further use.

3.14 Computational analysis

Hax1 orthologs from all sequenced animal genomes were detected by searching the Genpept database using the Position specific interactive BALST (PSI-BLAST) program and multiple alignement of a selected set of diverse sequence was constructed with T-Coffee program.

PSI-BLAST allows to construct automatically a position specific scoring matrix, PSSM) from a multiple alignment of the highest scoring hits in an initial BLAST search. Calculating position-specific scores for each position in the alignment generates the PSSM. Highly conserved positions

receive high scores and weakly conserved positions receive scores near zero. The profile is used to perform a second (etc.) BLAST search and the results of each "iteration" used to refine the profile. This iterative searching strategy results in increased sensitivity.

T-Coffee is a multiple sequence alignment program. Multiple sequence alignment programs are meant to align a set of sequences previously gathered using other programs such as blast. The main characteristic of T-Coffee is that it will allow you to combine results obtained with several alignment methods. T-Coffee will combine all that information and produce a new multiple sequence having the best agreement whith all these methods.

Secondary structure was predicted with the program PSIRED, JPRED and PredictionProtein (PRPROT) which are secondary structure prediction server. The search for conserved protein domain was run with RPS-BLAST. Reverse psi-blast, or rpsblast, is a program that searches a query protein sequence or a protein sequence against a database of position specific scoring matrices (PSSMs, profiles, or more commonly known as conserved domains) to identify the ones the query is simlar to. This rpogram is the opposite of PSI-BLAST.

Mitonchondrial import prediction was using MitoProt II program which the N-terminal protein region that can support a Mitochondrial Targeting Sequence and the cleavage site.

Transmembrane domains were predicted by using PredicProtein, TMHMM, TMred.

Chapter 4

4.1 Calcium regulation of mitochondria motility and morphology

Danny V. Jeyaraju, Giulia Cisbani and Luca Pellegrini

Centre de Recherche Université Laval Robert Giffard 2601, Chemin de la Canardière, Québec, QC, Canada

Keywords: Mitochondria; Calcium; Motility; Membrane dynamic; Fusion; Fission; Signaling; Cristae; Apoptosis; Metabolism; Neurodegeneration; Neurological syndrome; Rhomboid; Parl; Opa1; Drp1; PKA; Calcineurin; CaMK

Abbreviations: IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; IMS, intermembrane space

Acknowledgments

This work is supported by a grant from the Canadian Institute of Health Research (CIHR MOP-82718). D.V.J. is a CIHR Banting and Best Canada Graduate Scholar. L.P. is a FRSQ Chercheur-Boursier Senior scholar.

Abstract

n the Fifties, electron microscopy studies on neuronal cells showed that mitochondria typically cluster at synaptic terminals, thereby introducing the concept that proper mitochondria trafficking and partitioning inside the cell could provide functional support to the execution of key physiological processes. Today, the notion that a central event in the life of every eukaryotic cell is to configure, maintain, and reorganize the mitochondrial network at sites of high energy demand in response to environmental and cellular cues is well established, and the challenge ahead is to define the underlying molecular mechanisms and regulatory pathways. Recent pioneering studies have further contributed to place mitochondria at the center of the cell biology by showing that the machinery governing remodeling of mitochondria shape and structure regulates the functional output of the organelle as the powerhouse of the cell, the gateway to programmed cell death, and the platform for Ca(2+) signaling. Thus, a raising issue is to identify the cues integrating mitochondria trafficking and dynamics into cell physiology and metabolism. Given the versatile function of calcium as a second messenger and of the role of mitochondria as a major calcium store, evidences are emerging linking Ca(2+) transients to the modulation of mitochondrial activities. This review focuses on calcium as a switch controlling mitochondria motility and morphology in steady state, stressed, and pathological conditions.

Résumé

Dans les années 1950, les études de microscopie électronique sur cellules neuronales ont montré que les mitochondries se regroupent typiquement au niveau des terminaisons synaptiques, introduisant ainsi le concept que le trafic et la répartition appropriés de mitochondries dans la cellule pourraient fournir un support fonctionnel en vue de l'exécution de processus physiologiques clés. Aujourd'hui, la notion voulant qu'un événement central dans la vie de chaque cellule eucaryote permette de configurer, de maintenir et de réorganiser le réseau mitochondrial aux sites de haute demande d'énergie en réponse à des répliques environnementales et cellulaires est bien établie, et le défi avancé est de définir les mécanismes moléculaires sous-jacents et les voies régulatrices. Des études

novatrices récentes ont de loin contribué à placer les mitochondries au centre de la biologie cellulaire en montrant que le remodelage de la machinerie régissant la forme et la structure des mitochondries régule la production fonctionnelle de l'organelle comme la centrale énergétique de la cellule, la passerelle pour la mort cellulaire programmée et la plate-forme pour la signalisation calcique Ca2+. Ainsi, une question soulevée est d'identifier les répliques intégrant le trafic et les dynamiques mitochondriaux dans la physiologie et le métabolisme cellulaires. Étant donné la fonction polyvalente du calcium comme second messager et le rôle des mitochondries comme une réserve majeure de calcium, des preuves apparaissent liant les transits calciques Ca2+ à la modulation des activités mitochondriales. Cette revue se focalise sur le calcium comme commutateur contrôlant la motilité et la morphologie mitochondriales à l'état stable, en condition de stress et pathologiques.

4.1 Introduction

In any given eukaryotic cell, the molecular machineries governing the trafficking and shape of the organelle determine the architecture of the mitochondrial network. Its design reflects the physiological state of the cell in steady state, stressed, and pathological conditions because the mechanisms controlling mitochondrial dynamics also regulate the function of the organelle as the powerhouse of the cell, the gateway to programmed cell death, and the platform for Ca^{2+} signaling. In this process, calcium is emerging as a relay preposed to the intracellular distribution of the organelle at sites of high energy demand as well as a molecular switch for a number of mitochondrial membrane remodeling proteins. This review focuses on the aspects of mitochondria motility and dynamics that are directly or indirectly related to calcium, and it is intended to complement recent authoritative reviews on mitochondrial transport 1, 2, 3, 4 and 5, dynamics 6 and 7, apoptosis 8, 9, 10 and 11, control of neuronal activities 12, and link to cell signaling 13. We apologize in advance for omitting to quote every publication that has contributed to make emerge such exciting fields.

4.2 Distributing, positioning, anchoring, and regulating mitochondria activity at sites of high energy demand through calcium

The first observations describing differential positioning of mitochondria in cultured cells were reported nearly a century ago 14. Forty years later, in the Fifties, electron microscopy studies showed that mitochondria typically cluster at synaptic terminals 15, eliminating the concept that the architecture of the mitochondrial network is the outcome of a random distribution process of the organelle, and introducing the possibility that proper mitochondria trafficking and partitioning inside the cell could provide functional support to the execution of key physiological processes 16, 17 and 18. Today, the notion that a central event in the life of every eukaryotic cell is to configure, maintain and reorganize the mitochondrial network at sites of high energy demand in response to environmental and physiological cues is well established 1, and the challenge ahead is to define the underlying molecular mechanisms and regulatory pathways.

4.3 Mitochondria anterograde and retrograde transport: raison d'être and calcium regulation

In vivo studies using time-lapse microscopy of fluorescently labelled mitochondria have shown that a sub-population of the organelle remains stationary, while another commutes, often pausing between movements. In polarized cells like budding yeast and neurons, two widely used models in mitochondria trafficking studies, the organelle movements along the major axis of the cell are prominent, and occur both away from the nucleus (anterograde transport) and toward the center of the cell and nucleus (retrograde transport). In *Drosophila* motor neuron axons, anterograde and retrograde movement velocities averaged 0.26 and 0.45 mm/s, respectively 19. It should be noted, however, that observations of run length, pausing frequency, average velocity, and persistence of direction can significantly differ, possibly reflecting the diverse physiological states of the cell as well as tissue and experimental approaches used 2 and 20. Interestingly, moving mitochondria that switch direction of movement are not observed 19, suggesting that individual organelle reprogramming is required to alternate between anterograde and retrograde trafficking.

Several pioneering laboratories have investigated the raison d'être of mitochondria bidirectional transport and found it to be linked to maintaining bioenergetically competent organelles at sites of high energy demand. In neurons, among the moving mitochondrial sub-population 90% of the organelles with high membrane potential move anterogradely and accumulate in the growth cone, where metabolic activities are intense. Conversely, 81% of mitochondria showing low membrane potential are transported in the opposite direction, toward the cell body 21, suggesting a role of retrograde transport in eliminating energetically compromised organelles. An alternative possibility would be that retrograde transport actively uncouples membrane potential, thereby working as an energy switch; however, this is unlikely because arresting mitochondria motility does not correlate to an overall increase in respiration. Therefore, while anterograde transport generates the network of stationary active organelles required at sites of high metabolic activities, retrograde motility maintains it by removing depolarized and damaged organelles. In line with this notion, elegant imaging and computational studies from the Sheetz's group have shown that, in the axons of neurons isolated from chicken embryos, stationary mitochondria originate from a fast moving, anterogradely transported pool of organelles 21. In these

cells, mitochondria are evenly distributed, a pattern that originates because the organelles are preferentially dispensed in the middle of the gaps existing between stationary mitochondria 21, suggesting that transport at these "drop site" must be inhibited. If so, a gradient of signaling factors such as Ca^{2+}, ADP, or a small mitochondrial G-protein like Rab32 22, could play a role in mitochondria trafficking arrest. In this respect, inhibition of mitochondrial motility has been shown to correlate with altered Na^{+}, K^{+} or Ca^{2+} ionic balance, suggesting the possibility that calcium could be part of the mechanism that arrests mitochondrial trafficking 21 and 23. Consistent with this concept, in neuronal axons, using a micropipette to locally manipulate ion balance and composition, iso-osmotic replacement of NaCl with mannitol, which does not change overall metabolic activity, increases intracellular Ca^{2+} levels and decreases anterograde and retrograde trafficking 21, 24 and 25. Further, in hippocampal rat neurons mitochondria motility is suppressed by calcium influx through L-type voltage-gated calcium channels and NMDA receptors. Conversely, reduced calcium-dependent synaptic activities increase overall mitochondrial trafficking 26.

Mitochondrial oxidative phosphorylation uncoupling agents like CCCP also induce calcium transients and inhibit mitochondrial transport. However, this seems to be a non-specific effect, independent of mitochondrial depolarization and calcium release 27 because other uncouplers, such as DNP, FCCP and PCP, and complex III inhibitors like Antimycin A, increase or have no effect on mitochondrial transport 21, 27 and 28. Since CCCP is one of the most effective uncouplers 29 and can cause mitochondria to become consumers of ATP 25, its effect might occur because it lowers local ATP levels such that transport cannot be sustained 21. More direct evidence of calcium participation to the organelle trafficking can be inferred from the identification of Miro, a mitochondrial outer membrane protein whose function in anterograde transport depends on intact EF-hand calcium binding domains 30 and, by implication, cytosolic calcium transients (discussed below).

4.4 Stationary mitochondria: regulating local power supply through calcium

Anterograde transport distributes functional organelles to sites of intense metabolic activities which, in neurons, include active synapses, nodes of Ranvier, myelination boundaries, axonal branches, and growth cones 1 and 31. Mitochondria cluster at these locations, and their density changes in response to physiological cues. In rat hippocampal

cultured neurons, the organelle dynamically redistributes into dendritic protrusions in response to synaptic excitation and their number increases during synaptogenesis and spine formation 26. Conversely, preventing mitochondria to access synapses has profound deleterious effects on neuronal activity and synaptic plasticity 26 and 32. In neurons, the pools of stationary mitochondria located at synaptic and dendritic terminals likely support bioenergetic and calcium buffering requirements for dendritic development and synaptic plasticity, two essential processes known to be regulated through Ca^{2+}-triggered transcriptional programs that drive the synthesis of the effector molecules required for long-term changes in neuronal function 33. The mechanisms controlling mitochondrial docking within axons has just started to emerge. A recent study from the laboratory of Zu-Hang Sheng reported a role for axon-targeted syntaphilin (SNPH) in mitochondrial docking through its interaction with microtubules. Axonal mitochondria that contain exogenously or endogenously expressed SNPH lose mobility. Deletion of the mouse *snph* gene results in a substantially higher proportion of axonal mitochondria in the mobile state and reduces the density of mitochondria in axons, a phenotype that is fully rescued by reintroducing the *snph* gene into the mutant neurons. The *snph* mutant neurons exhibit enhanced short-term facilitation during prolonged stimulation, probably by affecting calcium signaling at presynaptic boutons 34.

The cellular strategy of crowding mitochondria to supply chemical energy at sites of intense metabolic activities is intrinsically limited by the typically modest amount of space available at sites like synaptic terminals, spines, and lamellipodia of the growth cone. Consistent with this concept, recent groundbreaking studies from Verburg and Hollenbeck have shown that the cell can also concentrate mitochondrial bioenergetic function by locally increasing respiration of mitochondria located at these sites. Using beads covalently coupled to Nerve Growth Factor (NGF) and Semaphorin to provide localized stimuli, this group showed that these survival and guidance cues could differentially affect mitochondria membrane potential. In areas of intense metabolic activities, organelles immediately adjacent to the site of NGF or Semaphorin stimulation showed a 40–50% increase in membrane potential, whereas mitochondria 10–50 μm away showed a smaller effect that declined to insignificance beyond 50 μm 35. Inhibitors of PI3 kinase and MAP kinase abolished this effect, implicating these signal-transducing molecules in the upregulation of

mitochondrial membrane potential and, by implication, ATP synthesis. Importantly, because simultaneous inhibition of these two kinases does not produce an additive effect, their activity likely converges on one pathway before reaching an effector molecule on the mitochondria 35. The identity of this pathway is unknown. However, calcium is known to activate MAPK via L-type voltage-gated calcium ion channel (L-VSCC) 36. In this system, Ca^{2+} influx is sensed by calmodulin (CaM), which is bound to the L-VSCC carboxyl terminus situated at the mouth of the channel. Activated calmodulin initiates a cascade of events that leads to the activation of the Ras-MAPK pathway which, ultimately, is central to calcium-dependent gene transcription and control of synapse development and function 33, 37 and 38. Thus, dendritic development and synaptic plasticity could be linked to energetic metabolism through local Ca^{2+}-dependent activation of MAPK signaling that boost membrane potential of the stationary resident mitochondria.

4.5 Mechanisms of mitochondria transport in yeast and higher eukaryotes

The first clues revealing the *modus operandi* of mitochondrial transport came from the observation that, in neurons, the organelle is associated to central components of the trafficking machinery, microtubules 39, 40 and 41. Subsequent *in vivo* imaging studies investigating the velocity of mitochondria translocation provided further support to the notion that the organelle moves using these intracellular motor proteins. However, the mechanisms of mitochondria trafficking are significantly different in yeast, animals, and plants, perhaps reflecting the diverse functional specialization that the organelle acquired during evolution. As such, the identification of the regulatory mechanisms underpinning mitochondria motility in the major genetic systems are still the object of intense exploration 42 and exciting debates 2 and 43. We here give an overview of the molecular processes implicated in mitochondria distribution, which have recently been authoritatively described in detail by others 2 and 3.

In the budding yeast *Saccharomyces cerevisiae*, mitochondria predominantly interact with the actin cytoskeleton and use actin tracks for both anterograde and retrograde movements during cell division. Several lines of evidence support this notion. First, mutations in genes encoding actin or factors involved in actin filament dynamics lead to aberrant mitochondrial distribution and morphology 44, 45, 46, 47, 48, 49 and 50. Second, treatment

of wild-type cells with actin filament-depolymerizing drugs produces defects in mitochondrial shape and transport 51. Third, isolated mitochondria bind, in an ATP-sensitive manner, to actin filaments *in vitro* 45, 46 and 51, and colocalize with and move along actin cables *in vivo* 44 and 45. Currently, the model for retrograde transport in yeast supports a system where mitochondria bind to actin cables undergoing retrograde flow and use the associated forces for passive transport toward the mother cell tip 3 and 52. Similar mechanisms are used for retrograde movement of endosomes in budding yeast and appear conserved 3. However, it should be noted that in the fission yeast *Schizosaccharomyces pombe*, mitochondria distribution depends on microtubules 50. In this organism, mitochondria colocalize with microtubules during interphase 53 and 54, and certain mutations in tubulin genes cause dramatic changes in mitochondrial distribution, with mitochondria becoming aggregated and asymmetrically positioned 54. Thus, additional studies of the molecular basis for mitochondrial distribution are necessary to extend our understanding of this process in lower eukaryotes.

Studies in *Drosophila* and in the mammalian system have shown that mitochondria movement and distribution depend on the microtubule motor as well as on the actin cytoskeleton of the cell (reviewed in 1, 2, 3, 55, 56 and 57). Retrograde transport is mediated by the minus end-directed microtubule motors, dyneins 19, 58 and 59. Elegant studies using *in vivo* imaging and loss-of-function mutants have shown that kinesin-1 is also critical for dynein-mediated retrograde movement 19, indicating that the two transport machineries are functionally coupled, and explaining why microtubule-depolymerizing agents, such as nocodazole, halt mitochondria motility altogether. In higher eukaryotes the mechanisms of mitochondria motility thus in part recapitulate those described in the budding and fission yeast. Nonetheless, the mechanisms of mitochondrial transport in higher eukaryotes are unique in few ways. For instance, in fission yeast mitochondria do not translocate along microtubules as they do in higher eukaryotic cells. Rather, the organelle binds to microtubules, and the polymerization and depolymerization of microtubules drive an extension and retraction, respectively, of mitochondrial tubules during interphase 50. A possible reason for evolving new mechanisms of mitochondria trafficking in higher eukaryotes is to allow precise long-range distribution in cell types that, from nematodes to vertebrates, increased their longitudinal axis by few orders of magnitude

(e.g. myocytes and neurons); or, in plants, to orchestrate light-dependent organelle positioning and orientation (reviewed in 60).

4.6 Calcium and the regulation of the mitochondria transport machinery

Kinesins (KIFs) are the molecular motors conveying cargos along microtubules (for a recent review and nomenclature changes see 61 and 62). In neurons, few members of this protein superfamily, including KIF5B, KIF5C (kinesin-1 family) and KIF1Bα (kinesin-3 family), transport mitochondria anterogradely 23, 62, 63 and 64. KIF5s also transport lysosomes 65 and tubulin oligomers 66 and 67 and kinesin-1-mediated disruption of mitochondria motility impairs vesicular transport 68. Further, the carboxyl-terminal region of KIF1Bα selectively interact with the PDZ domains of PSD-95, PSD-97, and S-SCAM, members of a family of synaptic vesicle-associated scaffolding proteins 69. Taken together these data indicate that mitochondria do not utilize exclusive motor proteins to move inside the cell.

The regulated bidirectional transport to which mitochondria are subjected implies that this organelle has unique binding partners for the motor proteins that transport them. Consistent with this possibility, few mitochondrial proteins have been shown to anchor the organelle to the microtubule motor, including KBP, Syntabulin, Milton and Miro 68, 70, 71 and 72. Syntabulin links mitochondria to motor protein KIF5B and participates in anterograde trafficking 71. Knockdown of syntabulin expression with targeted small interfering RNA or interference with the syntabulin–kinesin interaction reduces mitochondrial density within axonal processes by impairing anterograde movement of the organelle 71. Similarly, without Milton, mitochondria accumulate in neuronal cell bodies but not at synaptic terminals and axons. Further, transfected into HEK 293T cells, Milton induces a redistribution of mitochondria within the cell 72, supporting a role in organelle distribution in all cell types. It should be noted, however, that Milton does not appear to be conserved in yeast, perhaps reflecting the primary dependence of yeast mitochondria on the actin cytoskeleton rather than microtubules. In *Drosophila*, Milton and Miro directly interact with each other, to facilitate the recruitment of Milton to the mitochondria. Thus, these proteins are likely to form an essential protein complex that links kinesin-1 to mitochondria for anterograde transport 73. Consistent with this possibility, Miro ablation in *Drosophila*

also disrupts axonal and dendritic mitochondrial distribution, causing defective locomotion in the fly and premature death 68.

Miro (mitochondrial Rho) belongs to a family of highly conserved eukaryotic proteins required for mitochondrial distribution and morphology 30 and 74. Whereas mammals have two Miro family members, Miro-1 and Miro-2 74, yeast has only one, Gem1p 30. Their structural organization is similar and includes two cytosolic GTPase domains separated by a linker region containing a pair of calcium-binding EF-hand folds and a carboxyl-terminal transmembrane domain for anchoring to the mitochondrial outer membrane 30, 74 and 75. In Gem1p, the CaM-like EF-hand domain consists of two α-helices that flank a 12 residue loop 30. In CaM-like EF-hand proteins, carboxylated side chains in positions 1, 3, 5, 9, and 12 of the loop act as electron donors for calcium coordination 76. This differs from a second class of EF-hand motifs, where calcium is coordinated via backbone oxygen atoms. Thus, Miro proteins appear to have bona fide CaM calcium-binding domains, which are known to govern structural changes and protein activity by coordinated binding to Ca^{2+} 76. Consistent with this notion, substitutions of conserved residues required for calcium coordination in this domain impair Gem1p activity 30. Like other calcium binding proteins 77, mutagenesis of both EF-hands in Gem1p produces a stronger phenotype than mutagenesis of either domain alone 30. Given the presence of two conserved GTPase domains, it appears likely that the EF-hands control their cycle of GTP hydrolysis. An alternative possibility would be a regulation of protein interaction with other Miro-binding partners. While this review was in press, Xinnan Wang and Thomas Schwarz (Harvard University) published a study in Cell showing that kinesin is present on all axonal mitochondria, including those that are stationary or moving retrograde. In this study the authors showed that the EF-hand motifs of Miro mediate Ca^{2+}-dependent arrest of mitochondria and elucidated the regulatory mechanism. Rather than dissociating kinesin-1 from mitochondria, Ca^{2+}-binding permits Miro to interact directly with the motor domain of kinesin-1, preventing motor/microtubule interactions. Thus, kinesin-1 switches from an active state in which it is bound to Miro only via Milton, to an inactive state in which direct binding to Miro prevents its interaction with microtubules. Disrupting Ca^{2+}-dependent regulation diminished neuronal resistance to excitotoxicity.

A number of studies have shown that mitochondrial movement requires signal-transducing proteins, including cyclin-dependent kinase 5 (CDK5), protein phosphatase-1 (PP1), and glycogen synthase kinase 3 beta (GSK3β) (reviewed in 13), but their specific role in this process remains to be elucidated. Future exploration of the impact of these signaling pathways in regulating the molecular mechanisms that achieve specificity, directionality, and temporal control of mitochondrial trafficking in response to cellular cues is necessary as transport systems are known to be vulnerable to genetic and/or environmental insults and often result in human neurological or neurodegenerative diseases (discussed below).

An early event in T_h cell activation, which is central to the adaptive immune response, consists in the formation of an immunological synapse (IS) with an antigen presenting cell (the name IS was chosen in recognition of the structural and functional ways in which it resembles the synapses of the nervous system 78). At IS, sustained calcium flowing into the T_h cell turns on transcription factors that ultimately activate the lymphocyte. Calcium enters in the IS through voltage-gated ion channels located in the plasma membrane of the IS. Therefore, when calcium levels rise in the IS, extracellular calcium influx in this structure stops. Thus, effective T_h cell activation requires that calcium IS channels stay open long enough to generate calcium transients capable to trigger downstream effector signaling cascades. Pioneering studies from the Hoth group have shown that when a T_h cell engages an antigen presenting cell, mitochondria rapidly move and position 200 nm from the IS. Such close proximity allow the organelle to soak up some of the Ca^{2+} influx in the IS, which in turn facilitates a larger and more sustained Ca^{2+}-transient in the IS and activation of the NFAT, AP1, and NF-kB pathways 79. T_h cell mitochondria require microtubule tracks for their positioning in the IS. However, actin was also necessary in this process, perhaps reflecting the fact that motor complexes might be differently organized in non polarized cells 79. Thus, mitochondrial re-localization during IS formation introduces the paradigm that mitochondria motility controls the Ca^{2+}-dependent activation of transcription factors.

Lymphocytes are able to sense extracellular directional chemoattractant gradients and to respond with asymmetric changes in cell morphology (polarization) and mobility (chemotaxis). Cell polarization and chemotaxis depend on the signaling of seven-

transmembrane receptors coupled to heterotrimeric Gi proteins (G protein-coupled receptors). To achieve directed movement, cells organize and maintain spatial and functional asymmetry with a defined anterior (leading edge) and posterior (uropod). Elegant studies from the Viola group reported that mitochondria are transported to the uropod along microtubules during lymphocyte migration in a process requiring Gi protein signaling and mitochondrial fission. Classically, one of the initial events in chemoattractant receptor signaling is the physical association of heterotrimeric Gi proteins to the receptor, leading to the inhibition of adenylate cyclase and intracellular calcium mobilization. However, no inhibition of lymphocyte migration was observed in a Ca^{2+}-free medium, and no specific inhibition was observed when intracellular calcium was buffered. Further, DT40 (B lymphocyte cell line) triple inositol 1,4,5-trisphosphate receptor-knockout cells migrated as efficiently as wild-type cells, indicating that calcium signaling does not play a critical role during lymphocyte migration. However, by interfering with the expression of mitochondria-shaping proteins that regulate the dynamics of the organelles, it was shown that fusion–fission of the organelle constrains lymphocyte polarization and migration, suggesting that accumulation of mitochondria at the uropod of a migrating cell is required to regulate the cell motor of migrating lymphocytes in a calcium-independent manner 80.

4.7 Mitochondria motility and diseases

The energetic metabolism of the central nervous system (CNS) is unique because its rate is extraordinarily high and constant. Under resting conditions, CNS activities absorb 20% of the oxygen consumed by the human body, a staggering amount when considering that the CNS accounts for only 2% of the body weight. Mitochondria produce over 95% of ATP utilized by the brain, which is mostly (50–60%) used to maintain Na^+, K^+ and Ca^{2+} ion gradients across the plasma membrane (reviewed in 12). Recent studies are beginning to indicate that mitochondria, in addition to serve as the powerhouse of the cell 81 and as crucial amplifiers of death signals 82, 83, 84 and 85, may also function as a unique and essential signaling platform (reviewed in 13). It is not surprising, therefore, that mitochondrial dysfunctions impact neuronal survival and functions, and are central to the etiopathogenesis of a plethora of neurodegenerative diseases (reviewed in 9) and neurological disorders (reviewed in 86). This includes severe behavioral syndromes whose

underpinnings are still largely unknown, like schizophrenia, bipolar disorder, and autism, and that recent studies have related to defective mechanisms of mitochondrial calcium uptake and energetic homeostasis 87, 88 and 89.

Aberrant mitochondrial transport has been recognized to be implicated in the development of major pathologies of the CNS, including Alzheimer's (AD), Parkinson's, Huntington's (HD), Lou Gehrig's, and amyotrophic lateral sclerosis (ALS). However, given the broad impact of organelle positioning on cell physiology and survival 68, 72 and 73, the actual contribution of defective mitochondrial transport in the progression of these diseases remains unclear and is likely indirect. Consistent with this notion, axonal transport defects caused by mutations in a number of kinesin and dynein motor proteins have been implicated in neurodegeneration 90, 91 and 92.

In HD, the pathological relevance of disturbed mitochondrial transport is illustrated by the extensive axonal clogging that are caused by HD mutant forms of huntingtin 93, 94 and 95, suggesting that steric blockade of mitochondria transport at these clogged sites could be an etiological agent of the disease. However, this concept should be accepted with caution as other findings challenge it. For instance, *in vivo* analysis of "organelle clogs" caused by kinesin and dynein mutations showed that mitochondria could still move vigorously within and pass through the clogs, raising the possibility that axonal jamming may instead reflect sites of autophagocytosis of senescent mitochondria that are stranded in axons by retrograde transport failure, possibly a protective process aimed at suppressing cell death signals and neurodegeneration 19. Nonetheless, the notion that sustained altered distribution of mitochondria can be sensed by the cell and trigger an apoptotic elimination process is supported by recent findings showing that buildup of mitochondria in neurites and dysregulation of mitochondrial motor proteins caused by an AD-associated mutant form of ubiquitin is associated with activation of both the mitochondrial stress and p53 cell death pathways, thereby supporting the hypothesis that neuritic clogging of mitochondria triggers a cascade of events characterized by local activation of mitochondrial stress followed by global cell death 96.

In ALS, motor nerve specimens from affected patients show decreased retrograde axonal transport of organelles, including mitochondria 97 and 98. However, since reduced

retrograde transport of cargos such as neurotrophic factors, mitochondria, and membrane vesicles could damage motor neurons by multiple mechanisms, at this time the pathogenic contribution of impaired mitochondria retrograde transport remains speculative 99. Interestingly, hereditary spastic paraplegia (HSP) caused by mutations in a subunit of the m-AAA protease paraplegin has also been associated to impaired mitochondria axonal transport 100. Paraplegin presides to protein quality control of the organelle, suggesting that HSP-related defects in mitochondrial transport and axonal degeneration may result from the accumulation of non-degraded, misfolded inner membrane proteins or impaired regulatory steps during mitochondrial biogenesis, or both 101. Thus, the failure to regulate central mitochondrial activities can phenotypically converge in compromised organelle motility and contribute to the pathological development of diverse neurodegenerative diseases.

4.8 Regulating mitochondrial membrane fusion and fission through calcium

The eukaryotic cell evolved between two and three billion years ago through the acquisition of an alpha-proteobacteria endosymbiont, which later became the mitochondria. This ancestry resulted in the formation of a double-layered organelle that is organized in four distinct subcompartments: the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM), and the matrix. The IMM can be further subdivided in an inner boundary membrane and in the cristae compartment, bag-like folds of the IMM connected to it via narrow tubular junctions which are known to change structure depending on the energetic state of the organelle 102. Mitochondria morphology can assume spherical, rod-like, or tubular shapes, and depends on membrane-fusion and -fission activities. The equilibrium between these two opposing actions determines the shape of the mitochondrial network in steady state. Typically, suppression of membrane fusion or dominance of membrane fission causes mitochondria to fragment into short rods or spheres; conversely, disruption of membranes fission or prevalence of fusion generates interconnected tubules. In certain cell types mitochondria are mainly elongated, while in others they are fragmented. However, the mitochondrial architecture is typically dynamic, as it reflects the physiological state of the cell in steady state, stressed, and pathological conditions. Importantly, the functional versatility of the organelle depends on maintaining a

highly dynamic mitochondrial reticulum, a paradigm demonstrated by the fact that loss of membrane fusion or fission is lethal and that a number of human diseases are caused by disruption of these activities (reviewed in 9). Pioneering studies in *S. cerevisiae* have shown that mitochondria fusion and fission are governed by a small but growing set of conserved proteins (reviewed in 103). Here, we will discuss recent findings supporting calcium participation in cellular control of these "mitochondria shaping" proteins.

4.9 Calcium and the mechanisms of mitochondria fission

A central effector molecule in mitochondria fission is the mammalian dynamin-related protein 1 (Drp1 104), Dnm1 in *S. cerevisiae* 105. Drp1 and Dnm1 are large cytosolic GTPases that, upon fission triggering, are recruited to the mitochondrial outer membrane at punctate foci. At these sites, the dynamin-like protein self-assembles into a functional scission complex consisting of oligomers that form ring-like structures that, through a GTP-dependent mechanism, constrict the organelle, thereby providing the force required to produce a local membrane scission event. *In vitro*, Dnm1 forms spirals whose internal diameter matches that of mitochondrial constriction sites (111 nm 106). The mechanism underpinning the initial recruitment of Drp1 at punctate locations of the mitochondrial OMM is a rate-limiting step of mitochondria fission 106. Thus, Drp1 inhibition, or its down-regulation, results in an overall dominant effect of membrane fusion activities, causing mitochondria to be fused and interconnected.

A similar phenotype is caused by down-regulation of another central component of the mitochondrial fission program, the mammalian Fission protein 1 (hFis1 107), Fis1p in *S. cerevisiae* 108. hFis1 and Fis1p are two 16 kDa conserved integral proteins of the OMM, containing a single transmembrane domain and a tetratricopeptide repeat domain facing the cytosol (TPR, involved in protein–protein interaction 107). Fis1p is required for yeast Dnm1 recruitment and oligomerization on the OMM 108. Fis1p engages Dnm1 through one of two adaptor proteins, Mdv1 or Caf4 108, 109 and 110. However, homologs of these yeast proteins have not been identified in mammals, and in this system hFis1 is not essential for Drp1 recruitment 111 and 112, suggesting that during metazoan evolution the mechanism of Drp1 activation on the OMM might have lost the requirement for adaptor proteins. Nonetheless, cross-linking studies and fluorescence resonance energy transfer

experiments indicate that hFis1 is a Drp1 receptor on the OMM as these proteins bind to each other via hFis1 TRP domain 113. However, it should be noted that in mammalian cells actin filaments and microtubules also appear to function in recruiting Drp1 to the organelle, thereby providing support to alternative mechanisms of Drp1 activation 114 and 115. The central role of Drp1 and hFis1 in mitochondrial fission is compellingly supported by the notion that their level of expression is proteolytically controlled by the opposing actions of sumoylation and ubiquitination 116, 117 and 118. Recently, calcium-dependent regulation of sumoylation activities have been reported 119 and 120, and examples of calcium regulation of ubiquitin ligases exist 121 and 122. Evidence for the involvement of calcium signaling in sumoylation and ubiquitination of mitochondrial proteins is lacking, but their potential impact on mitochondrial dynamics warrants future investigations in this area.

Endophilin B1, a member of the endophilin family of fatty acid acyl transferases that participate in endocytosis, is another molecule implicated in mitochondrial fission 123. Endophilin B1 cycles between the cytosol and the OMM, where it binds through the concave face of a highly conserved banana-shaped fold (BAR domain) that senses the curvature of the lipid bilayer and facilitates membrane pinching during fission 124. Downregulation of Endophilin B1 results in the formation of a net of long tubules of OMM enveloping several distinct inner mitochondrial membrane-bound matrix compartments, indicating IMM scission continuing in the absence of OMM scission. The mechanism of Endophilin B1 in OMM fission is still elusive, although it might be similar to that of Endophilin-1, a key regulator of clathrin-mediated synaptic vesicle endocytosis. During endocytosis, Endophilin-1 builds complexes with Dynamin I, the fission GTPase responsible for severing the neck of the nascent endocytic vesicle 125. Endophilin-1 interacts directly with voltage-gated Ca^{2+} channels, and the formation of this complex is Ca^{2+} dependent 126. In hippocampal neurons, expression of a dominant-negative Endophilin-1 mutant that constitutively binds to Ca^{2+} channels inhibits endocytosis. The primary structural determinant implicated in this process resides within amino acid residues 200–219 of Endophilin-1. This motif is conserved among Endophilin-1 isoforms, but not in Endophilin B1. However, this small motif is not a Ca^{2+}-binding domain per se but, rather, a structural regulatory determinant for Endophilin-1 Ca^{2+}-regulated activity. Therefore, sequence conservation is not required, and Endophilin B1 regulation through Ca^{2+} is

conceivable. Consistent with this possibility, recent studies have linked Endophilin B1 to Ca^{2+}-regulated autophagy. Endophilin B1 binds Beclin-1, a Bcl-2-interacting protein with autophagy-promoting activity 127, 128, 129 and 130, and colocalizes within punctate foci with autophagy related proteins during nutrient depletion 131. Autophagy is an evolutionarily conserved lysosomal pathway involved in the starvation-dependent turnover of cellular macromolecules and organelles. This process is potently induced by a Beclin-1-dependent rise in free cytosolic calcium 132 and 133, suggesting that further investigation of Endophilin B1 regulation through calcium might help elucidating how mitochondria fission intersects this cellular pathway.

MTP18 (mitochondria protein, 18 kDa) is an intermembrane space protein anchored to the IMM. Knockdown experiments showed that MTP18 is essential for cell viability as it causes cytochrome *c* release and sensitizes cells to proapoptotic stimuli. Reduction of MTP18 also impairs mitochondrial morphology, resulting in the formation of a highly interconnected mitochondrial reticulum. Conversely, overexpression of MTP18 induces Drp1-dependent mitochondria fragmentation, suggesting a functional role of MTP18 in maintaining the integrity of the mitochondrial reticulum 134 and 135. Given its localization at the interface between the IMM and OMM, it has been hypothesized that MTP18 could control the concerted fission of the two mitochondrial membranes 13. MTP18 is also a downstream effector of phosphatidylinositol 3 kinase (PI3K) signaling pathway, which plays a crucial role in cell growth and survival by controlling the magnitude of Ca^{2+} influx from voltage-gated calcium channels opening upon membrane depolarization 136. Thus, this protein could modulate mitochondrial dynamics in response to physiological changes in intracellular Ca^{2+} concentrations.

GDAP1 (ganglioside-induced differentiation associated protein 1) is a mammalian mitochondrial outer membrane protein that is mutated in Charcot–Marie–Tooth disease type 4A (CMT-4A) 137 and 138. Whereas its overexpression causes fragmentation of mitochondria with no effect on cell death, down-regulation of GDAP1 or the expression of certain patient mutant forms which are truncated and no longer localize to mitochondria tend to elongate the organelle indicating that GDAP1 activity impact the mitochondrial network architecture by promoting mitochondrial fission 137, 138 and 139. The available

information about the biology of GDAP1 is scarce. Some hints about the molecular function of this protein have been provided by bioinformatic analyses 140. These studies showed that GDAP1 and related proteins in invertebrates and vertebrates contain characteristic glutathione S-transferase domains (GST). Based on its particular domain features, GDAP1 was proposed as the founder of a new GST family. Members of this family are characterized by an enlarged interdomain located between the GST-N and GST-C domains, and contain COOH-terminal hydrophobic helices that could serve as transmembrane domains. It has been speculated that GDAP1 could be part of a mechanism regulating mitochondrial fission by acting as a sensor of the mitochondrial redox (NAD^+/NADH) state 138, a process that, in turn, depends on cytosolic Ca^{2+} oscillations 141. All together, these data suggest the possibility that GDAP, rather than a bona fide fission protein, could be part of the molecular mechanism integrating fission into cell physiology through calcium.

4.10 Calcium and the mechanisms of mitochondria fusion

In mammals, the central components of the mitochondrial fusion machinery include mitofusin-1 (Mfn1), mitofusin-2 (Mfn2), optic atrophy 1 (Opa1), and presenilin-associated rhomboid-like (Parl) (for recent reviews see 6, 9, 10, 13, 142 and 143). Whereas deletion of Mfn1, Mfn2, and Opa1 impairs embryonic development in the mouse at different stages 144, 145 and 146, ablation of Parl is lethal from the fourth postnatal week due to cachectic death 147. Unlike the fission protein Drp1, the regulation of the proteins governing the fusion program of the organelle has not yet been directly linked to calcium. Further, mitochondrial fusion reaction *in vitro* does not require presence of Ca^{2+} 148 and 149.

Mitofusins possess a cytosolic N-terminal GTPase domain, two TM domains spanning the OMM and two cytosolic C-terminal domains that are crucial for protein–protein interaction 150 and 151. To promote fusion, Mfn1 docks two juxtaposed mitochondria via its second coiled coil domain, forming homo- and hetero-oligomeric complexes 152. The role of the two mitofusins in fusion seems to be different. Mfn1 has higher GTPase activity and induces fusion more efficiently than Mfn2 153. Mfn2 is also localized to the ER 154, and a role of this protein in the formation of the juxtaposition between endoplasmic reticulum (ER) and mitochondria that form the structural basis for interorganellar Ca^{2+} signaling has

been proposed and recently elegantly demonstrated by the group of Luca Scorrano 155 and 156.

Opa1, mutated in dominant optic atrophy, the most common cause of inherited optic neuropathy, promotes fusion from the inside of the organelle as it is anchored to the IMM, with the GTPase domain facing the IMS 148 and 157. Opa1 and Mfn1 are on the same genetic pathway as Opa1 requires Mfn1, but not Mfn2, to mediate fusion 157. Opa1 exists in eight different splice variants 158 and its function in cristae morphogenesis and apoptosis 147 and 159 is proteolytically regulated by the mitochondrial rhomboid protease Parl 147 and by members of the mitochondrial AAA metalloprotease family via a prohibitin pathway 160, 161, 162, 163, 164 and 165.

Mammalian Parl proptotypes a eukaryotic subfamily of the rhomboid protease family (PARL subfamily) 166. Its members are mitochondrial and possess, as a unique structural signature, a transmembrane helix (TMH) located upstream of the six TMHs that form a catalytically active rhomboid domain 166, 167, 168, 169, 170, 171, 172, 173 and 174. Although initially described as a molecule implicated in PS1 biology, a possibility now no longer valid, Parl discovery first introduced the concept that rhomboids are intramembrane-cleaving proteases 175, a notion subsequently validated in rhomboid proteins from bacteria to mammals(reviewed in 176). Three lines of evidence indicate that calcium is unlikely implicated in the regulation of members of the PARL family: (i) rhomboid activity *in vitro* does not require calcium 177; (ii) crystallographic data of the rhomboid domain do not show any possible structural or functional role for this element 167, 168, 169 and 170; (iii) non-mitochondrial rhomboid with EF-hand calcium-binding domains have been described in *Drosophila* and humans (Rhomboid-2 and -4 166), but no calcium-related domains are present in members of the PARL subfamily.

Parl is embedded in the IMM, with the N-terminus, termed Pβ domain 178, exposed to the matrix, and the C-terminus to the IMS 179. The Pβ domain emerged during vertebrate evolution and it is strongly conserved in mammals as 58 of its 62 residues are invariant, and there are no insertions or deletions 178. This indicates that this domain was subjected to strong purifying selection, which can only be explained by functional constraints because in unconstrained sequences evolving neutrally, very few, if any, invariant residues would be

expected to survive the 100 Ma of evolution separating mammalian orders 180 and 181. Parl N-terminal Pβ domain is constitutively phosphorylated at Ser-65, Thr-69, and Ser-70 179. Functionally, this hyper-phosphorylation blocks a proximal cleavage event (Ser-77 ↓ Ala-78; β-cleavage 178) that induces mitochondria fragmentation 179 through a block of mitochondrial fusion (Pellegrini and McBride, unpublished observations). The identity of the protease and of the kinase/phosphatase switch that executes β-cleavage is unknown, thereby leaving open the possibility that the β-cleavage-dependent role of Parl in mitochondria dynamics could be ultimately regulated through calcium.

4.11 Calcium as a molecule integrating mitochondrial dynamics to cell physiology

The overall configuration of the mitochondrial architecture is determined by the integration of organellar and cytosolic cues. This is essential to coordinate, for instance, mitochondria shape remodeling during cell cycle. In HeLa cells, the interconnected mitochondrial network observed in interphase becomes fragmented in the prophase, and the organelle stays organized in small spherical units during metaphase and anaphase, possibly to secure minimal interference with mitotic spindle assembly and proper stochastic mitochondria distribution into the daughter cells; restoration of filamentous mitochondria begins in the late mitotic phase, to be completed in the daughter cells 182. Mitochondria fragmentation at the prophase depends on the activation of the pro-fission molecule Drp1 through phosphorylation of its Ser-585 residue by Cdk1/cyclin B, a universal cell cycle kinase required for mitotic/meiotic cell cycle entry. Cdk1/cyclin B activity needs to be eliminated for mitotic/meiotic exit. This process is triggered by sustained cytosolic Ca^{2+} concentration rise 183, 184 and 185, suggesting that Drp1 activity during cell cycle is regulated by calcium transients. However, the Drp1 phosphorylation consensus motif (PASPQK) is conserved in mammals and, to a less extent, in *Caenorhabditis elegans* (KTSPQE), but not in yeast, consistent with the notion that in *S. cerevisiae* the filamentous mitochondrial network is maintained throughout mitosis 103. Thus, the possible role of calcium in the regulation of Drp1-mediated mitochondrial fission during cell cycle would be a process acquired during metazoan evolution.

A step forward in our understanding of calcium as a cellular cue governing mitochondrial shape remodeling came with the discovery from the Strack and Blackstone labs that Drp1-

dependent mitochondrial fragmentation is controlled by phosphorylation at a conserved site by Cdk1 and protein kinase A (PKA) 186 and 187. These groups showed that phosphorylation of the Ser residue in the consensus sequence $RKLS_{637}ARE$ of Drp1, which is conserved in all splice variants of the human, rat, and mouse protein, inhibits fission activity. Conversely, dephosphorylation of Ser-637 by the Ca^{2+}-dependent phosphatase calcineurin promotes mitochondrial fission and is involved in the propagation of apoptosis 186. Recent studies from the Scorrano's group have shown that inducers of mitochondrial depolarization also trigger Ca^{2+}, calcineurin-dependent dephosphorylation of Drp1 on Ser-637, driving its translocation to mitochondria and causing fission of the organelle 188. Calcineurin is a heterodimeric protein composed of a catalytic subunit (CnA) that binds calmodulin and a regulatory subunit (CnB) that binds Ca^{2+}. It is involved in the transduction of Ca^{2+}-mediated signals in diverse cellular programs, including proliferation, death, and vesicles secretion. Calcineurin is activated in a Ca^{2+}-calmodulin dependent fashion 189. *In vitro* assays using mutant *Drp1* carrying the phosphomimetic Ser → Asp substitution (S637D), suggest that phosphorylation of this residue impairs intramolecular association of Drp1 into large fission-related complexes, and attenuates the dynamin's GTPase activity 187. However, this latter observation is controversial 186 and inconsistent with the recent finding that mutant *Drp1* S637D is retained in the cytosol 188. It has been proposed that the opposing effects of PKA and calcineurin on the same site serve as a switch to translate signals associated with Ca^{2+} changes of different strength, duration, and tone into different mitochondrial morphologies. During physiological (i.e., agonist evoked) Ca^{2+} signaling, activation of PKA can prevail over calcineurin mediated dephosphorylation of Drp1. Moreover, compartmentalization of both Ca^{2+} and cAMP signals could play a role in the local regulation of mitochondrial shape. Conversely, long lasting Ca^{2+} plateaus in the cytosol linked to full activation of calcineurin and to generalized fragmentation could for example account for the apoptotic mitochondrial fission 188.

Data recently reported from the Matsushita group show a different scenario of how Drp1 activity and mitochondrial fission could be regulated through calcium transients-dependent phosphorylation of this protein. In hippocampal neurons isolated from rat embryos and grown in culture for 9–12 days, treatment with 45 mM K^+ for 15 min produce an immediate and partially reversible arrest in mitochondria motility. The organelle then fragments and

looses its cristae organization without, however, inducing apoptosis. This stimulation triggers a rapid increase in intracellular Ca^{2+}, possibly through N and P/Q types Ca^{2+} channels (VDCC). VDCC-associated Ca^{2+} signaling stimulates phosphorylation of Drp1 at Ser-637 via activation of Ca^{2+}/calmodulin-dependent protein kinase I (CaMKIα) (note that in this study Ser-637 corresponds to Ser-600, due to the rat Drp1 splice variant used). In neurons and HeLa cells, phosphorylation of Ser-637 activates Drp1 translocation to the mitochondria, thereby inducing fission of the organelle. *In vitro* studies show that Ser-637 phosphorylation of wild-type Drp1 by CaMKIα substantially increases the amount of Drp1 pulled down by GST-hFis1, suggesting that phosphorylation increases Drp1 binding affinity for hFis1 and, by implication, its recruitment to the mitochondrial surface. The molecular and cellular bases explaining the reason why in this study Drp1 phosphorylation activates fission are unclear, particularly because CaMKIα, PKA, Cdk1, and calcineurin are all widely expressed proteins, and no cell type-specific mechanisms can thus be envisaged. It has been suggested that phosphorylation of Drp1 at Ser-637, although necessary for regulation of mitochondrial morphology, is not sufficient on its own and that activation of CaMKIα has additional roles in regulation of Drp1 interaction with mitochondria 190. Additional investigations on whether Ca^{2+}- and cAMP-dependent signaling pathways can act synergistically to regulate mitochondrial dynamics are thus required.

The endoplasmic reticulum can elicit proapoptotic signaling that results in transmission of Ca^{2+} to the mitochondria, which in turn activates Drp1 recruitment to the OMM to drive mitochondrial fragmentation and apoptosis. An additional role for Drp1 in this process consists in opening the cristae, where the major stores of cytochrome *c* reside, suggesting the possibility that Drp1 participation in this pathway may require still undiscovered postranslational modifications of the GTPase protein 191.

4.12 Conclusions

The contribution of calcium in the regulation and execution of mitochondria motility and morphology in steady state and pathological conditions has just started to emerge. Being mostly indirect, the actual function of calcium in these processes might appear minor. However, there are convincing evidences suggesting that this second messenger might play

a much bigger role in orchestrating these activities, thereby warranting future research in this field.

References

1 P.J. Hollenbeck and W.M. Saxton, The axonal transport of mitochondria, *J. Cell. Sci.* **118** (2005), pp. 5411–5419.

2 R.L. Frederick and J.M. Shaw, Moving mitochondria: establishing distribution of an essential organelle, *Traffic* **8** (2007), pp. 1668–1675.

3 I.R. Boldogh, L.A. Pon, M.P. Sheetz and K.J. De Vos, Cell-free assays for mitochondria–cytoskeleton interactions, *Methods Cell Biol.* **80** (2007), pp. 683–706.

4 G. Hajnóczky, M. Saotome, G. Csordás, D. Weaver and M. Yi, Calcium signalling and mitochondrial motility, *Novartis Found. Symp.* **287** (2007), pp. 105–117 discussion 117–21.

5 D.G. Nicholls, Forty years of Mitchell's proton circuit: from little grey books to little grey cells, *Biochim. Biophys. Acta* **1777** (2008), pp. 550–556.

6 S. Hoppins and J. Nunnari, The molecular mechanism of mitochondrial fusion, *Biochim. Biophys. Acta* **1793** (1) (2008), pp. 20–26.

7 S. Merz, M. Hammermeister, K. Altmann, M. Dürr and B. Westermann, Molecular machinery of mitochondrial dynamics in yeast, *Biol. Chem.* **388** (2007), pp. 917–926.

8 J.C. Martinou and R.J. Youle, Which came first, the cytochrome *c* release or the mitochondrial fission?, *Cell Death Differ.* **13** (2006), pp. 1291–1295.

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

10 S.B. Berman, F.J. Pineda and J.M. Hardwick, Mitochondrial fission and fusion dynamics: the long and short of it, *Cell Death Differ.* **15** (2008), pp. 1147–1152.

11 D.F. Suen, K.L. Norris and R.J. Youle, Mitochondrial dynamics and apoptosis, *Genes Dev.* **22** (2008), pp. 1577–1590.

12 O. Kann and R. Kovács, Mitochondria and neuronal activity, *Am. J. Physiol., Cell Physiol.* **292** (2007), pp. C641–C657.

13 V. Soubannier and H.M. McBride, Positioning mitochondrial plasticity within cellular signaling cascades, *Biochim. Biophys. Acta* **1793** (1) (2008), pp. 154–170.

14 M.R. Lewis and W.H. Lewis, Mitochondria in tissue culture, *Science* **39** (1914), pp. 330–333.

15 S.L. Palay, Synapses in the central nervous system, *J. Biophys. Biochem. Cytol.* **2** (1956), pp. 193–202.

16 K.Y. Chan and A.H. Bunt, An association between mitochondria and microtubules in synaptosomes and axon terminals of cerebral cortex, *J. Neurocytol.* **7** (1978), pp. 137–143.

17 M.H. Heggeness, M. Simon and S.J. Singer, Association of mitochondria with microtubules in cultured cells, *Proc. Natl. Acad. Sci. U. S. A.* **75** (1978), pp. 3863–3866.

18 D.S. Smith, U. Järlfors and M.L. Cayer, Structural cross-bridges between microtubules and mitochondria in central axons of an insect (*Periplaneta americana*), *J. Cell. Sci.* **27** (1977), pp. 255–272.

19 A.D. Pilling, D. Horiuchi, C.M. Lively and W.M. Saxton, Kinesin-1 and dynein are the primary motors for fast transport of mitochondria in *Drosophila* motor axons, *Mol. Biol. Cell* **17** (2006), pp. 2057–2068.

20 A.A. Gerencser and D.G. Nicholls, Measurement of instantaneous velocity vectors of organelle transport: mitochondrial transport and bioenergetics in hippocampal neurons, *Biophys. J.* **95** (2008), pp. 3079–3099.

21 K.E. Miller and M.P. Sheetz, Axonal mitochondrial transport and potential are correlated, *J. Cell. Sci.* **117** (2004), pp. 2791–2804.

22 N.M. Alto, J. Soderling and J.D. Scott, Rab32 is an A-kinase anchoring protein and participates in mitochondrial dynamics, *J. Cell Biol.* **158** (2002), pp. 659–668.

23 Y. Kanai, Y. Okada, Y. Tanaka, A. Harada, S. Terada and N. Hirokawa, KIF5C, a novel neuronal kinesin enriched in motor neurons, *J. Neurosci.* **20** (2000), pp. 6374–6384.

24 A.J. Borgdorff, G.G. Somjen and W.J. Wadman, Two mechanisms that raise free intracellular calcium in rat hippocampal neurons during hypoosmotic and low NaCl treatment, *J. Neurophysiol.* **83** (2000), pp. 81–89.

25 D.G. Nicholls and S.L. Budd, Mitochondria and neuronal survival, *Physiol. Rev.* **80** (2000), pp. 315–360.

26 Z. Li, K. Okamoto, Y. Hayashi and M. Sheng, The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses, *Cell* **119** (2004), pp. 873–887.

27 P.J. Hollenbeck, D. Bray and R.J. Adams, Effects of the uncoupling agents FCCP and CCCP on the saltatory movements of cytoplasmic organelles, *Cell Biol. Int. Rep.* **9** (1985), pp. 193–199.

28 J. Bereiter-Hahn and M. Vöth, Ionic control of locomotion and shape of epithelial cells: II. Role of monovalent cations, *Cell Motil. Cytoskelet.* **10** (1988), pp. 528–536.

29 J. Cunarro and M.W. Weiner, Mechanism of action of agents which uncouple oxidative phosphorylation: direct correlation between proton-carrying and respiratory-releasing properties using rat liver mitochondria, *Biochim. Biophys. Acta* **387** (1975), pp. 234–240.

30 R.L. Frederick, J.M. McCaffery, K.W. Cunningham, K. Okamoto and J.M. Shaw, Yeast Miro GTPase, Gem1p, regulates mitochondrial morphology via a novel pathway, *J. Cell Biol.* **167** (2004), pp. 87–98.

31 M. Amiri and P.J. Hollenbeck, Mitochondrial biogenesis in the axons of vertebrate peripheral neurons, *Dev. Neurobiol.* **68** (2008), pp. 1348–1361.

32 P. Verstreken, C.V. Ly, K.J. Venken, T.W. Koh, Y. Zhou and H.J. Bellen, Synaptic mitochondria are critical for mobilization of reserve pool vesicles at *Drosophila* neuromuscular junctions, *Neuron* **47** (2005), pp. 365–378.

33 S. Cohen and M.E. Greenberg, Communication between the synapse and nucleus in neuronal development, plasticity, and disease, *Annu. Rev. Cell Dev. Biol.* (24) (2008), pp. 183–209.

34 J.S. Kang, J.H. Tian, P.Y. Pan, P. Zald, C. Li, C. Deng and Z.H. Sheng, Docking of axonal mitochondria by syntaphilin controls their mobility and affects short-term facilitation, *Cell* **132** (2008), pp. 137–148.

35 J. Verburg and P.J. Hollenbeck, Mitochondrial membrane potential in axons increases with local nerve growth factor or semaphorin signaling, *J. Neurosci.* **28** (2008), pp. 8306–8315.

36 R.E. Dolmetsch, U. Pajvani, K. Fife, J.M. Spotts and M.E. Greenberg, Signaling to the nucleus by an L-type calcium channel–calmodulin complex through the MAP kinase pathway, *Science* **294** (2001), pp. 333–339.

37 A.E. West, W.G. Chen, M.B. Dalva, R.E. Dolmetsch, J.M. Kornhauser, A.J. Shaywitz, M.A. Takasu, X. Tao and M.E. Greenberg, Calcium regulation of neuronal gene expression, *Proc. Natl. Acad. Sci. U. S. A.* **98** (2001), pp. 11024–11031.

38 P.L. Greer and M.E. Greenberg, From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function, *Neuron* **59** (2008), pp. 846–860.

39 J.R. La Fountain, An association between microtubules and aligned mitochondria in Nephrotoma spermatocytes, *Exp. Cell Res.* **71** (1972), pp. 325–328.

40 C.S. Raine, B. Ghetti and M.L. Shelanski, On the association between microtubules and mitochondria within axons, *Brain Res.* **34** (1971), pp. 389–393.

41 F. Grainger and D.W. James, Mitochondrial extensions associated with microtubules in outgrowing processes from chick spinal cord in vitro, *J. Cell. Sci.* **4** (1969), pp. 729–737.

42 K. Altmann, M. Frank, D. Neumann, S. Jakobs and B. Westermann, The class V myosin motor protein, Myo2, plays a major role in mitochondrial motility in *Saccharomyces cerevisiae*, *J. Cell Biol.* **181** (2008), pp. 119–130.

43 R.R. Valiathan and L.S. Weisman, Pushing for answers: is myosin V directly involved in moving mitochondria?, *J. Cell Biol.* **181** (2008), pp. 15–18.

44 D.G. Drubin, H.D. Jones and K.F. Wertman, Actin structure and function: roles in mitochondrial organization and morphogenesis in budding yeast and identification of the phalloidin-binding site, *Mol. Biol. Cell* **4** (1993), pp. 1277–1294.

45 D.A. Lazzarino, I. Boldogh, M.G. Smith, J. Rosand and L.A. Pon, Yeast mitochondria contain ATP-sensitive, reversible actin-binding activity, *Mol. Biol. Cell* **5** (1994), pp. 807–818.

46 V.R. Simon, T.C. Swayne and L.A. Pon, Actin-dependent mitochondrial motility in mitotic yeast and cell-free systems: identification of a motor activity on the mitochondrial surface, *J. Cell Biol.* **130** (1995), pp. 345–354.

47 G.J. Hermann, E.J. King and J.M. Shaw, The yeast gene, MDM20, is necessary for mitochondrial inheritance and organization of the actin cytoskeleton, *J. Cell Biol.* **137** (1997), pp. 141–153.

48 V.R. Simon, S.L. Karmon and L.A. Pon, Mitochondrial inheritance: cell cycle and actin cable dependence of polarized mitochondrial movements in *Saccharomyces cerevisiae*, *Cell Motil. Cytoskelet.* **37** (1997), pp. 199–210.

49 H.C. Yang, A. Palazzo, T.C. Swayne and L.A. Pon, A retention mechanism for distribution of mitochondria during cell division in budding yeast, *Curr. Biol.* **9** (1999), pp. 1111–1114.

50 M.P. Yaffe, N. Stuurman and R.D. Vale, Mitochondrial positioning in fission yeast is driven by association with dynamic microtubules and mitotic spindle poles, *Proc. Natl. Acad. Sci. U. S. A.* **100** (2003), pp. 11424–11428.

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

52 K.L. Fehrenbacher, H.C. Yang, A.C. Gay, T.M. Huckaba and L.A. Pon, Live cell imaging of mitochondrial movement along actin cables in budding yeast, *Curr. Biol.* **14** (2004), pp. 1996–2004.

53 T. Kanbe, I. Kobayashi and K. Tanaka, Dynamics of cytoplasmic organelles in the cell cycle of the fission yeast *Schizosaccharomyces pombe*: three-dimensional reconstruction from serial sections, *J. Cell. Sci.* **94** (Pt. 4) (1989), pp. 647–656.

54 M.P. Yaffe, D. Harata, F. Verde, M. Eddison, T. Toda and P. Nurse, Microtubules mediate mitochondrial distribution in fission yeast, *Proc. Natl. Acad. Sci. U. S. A.* **93** (1996), pp. 11664–11668.

55 D.T. Chang and I.J. Reynolds, Differences in mitochondrial movement and morphology in young and mature primary cortical neurons in culture, *Neuroscience* **141** (2006), pp. 727–736.

56 K. De Vos, F. Severin, F. Van Herreweghe, K. Vancompernolle, V. Goossens, A. Hyman and J. Grooten, Tumor necrosis factor induces hyperphosphorylation of kinesin light chain and inhibits kinesin-mediated transport of mitochondria, *J. Cell Biol.* **149** (2000), pp. 1207–1214.

57 A.J. Pereira, B. Dalby, R.J. Stewart, S.J. Doxsey and L.S. Goldstein, Mitochondrial association of a plus end-directed microtubule motor expressed during mitosis in *Drosophila*, *J. Cell Biol.* **136** (1997), pp. 1081–1090.

58 L.A. Ligon and O. Steward, Role of microtubules and actin filaments in the movement of mitochondria in the axons and dendrites of cultured hippocampal neurons, *J. Comp. Neurol.* **427** (2000), pp. 351–361.

59 D. Horiuchi, R.V. Barkus, A.D. Pilling, A. Gassman and W.M. Saxton, APLIP1, a kinesin binding JIP-1/JNK scaffold protein, influences the axonal transport of both vesicles and mitochondria in *Drosophila*, *Curr. Biol.* **15** (2005), pp. 2137–2141.

60 S.S. von Braun and E. Schleiff, Movement of endosymbiotic organelles, *Curr. Protein Pept. Sci.* **8** (2007), pp. 426–438.

61 N. Hirokawa and Y. Noda, Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics, *Physiol. Rev.* **88** (2008), pp. 1089–1118.

62 N. Hirokawa and R. Takemura, Molecular motors and mechanisms of directional transport in neurons, *Nat. Rev., Neurosci.* **6** (2005), pp. 201–214.

63 M. Nangaku, R. Sato-Yoshitake, Y. Okada, Y. Noda, R. Takemura, H. Yamazaki and N. Hirokawa, KIF1B, a novel microtubule plus end-directed monomeric motor protein for transport of mitochondria, *Cell* **79** (1994), pp. 1209–1220.

64 Y. Tanaka, Y. Kanai, Y. Okada, S. Nonaka, S. Takeda, A. Harada and N. Hirokawa, Targeted disruption of mouse conventional kinesin heavy chain, kif5B, results in abnormal perinuclear clustering of mitochondria, *Cell* **93** (1998), pp. 1147–1158.

65 T. Nakata and N. Hirokawa, Point mutation of adenosine triphosphate-binding motif generated rigor kinesin that selectively blocks anterograde lysosome membrane transport, *J. Cell Biol.* **131** (1995), pp. 1039–1053.

66 S. Takeda, H. Yamazaki, D.H. Seog, Y. Kanai, S. Terada and N. Hirokawa, Kinesin superfamily protein 3 (KIF3) motor transports fodrin-associating vesicles important for neurite building, *J. Cell Biol.* **148** (2000), pp. 1255–1265.

67 T. Nakata and N. Hirokawa, Neuronal polarity and the kinesin superfamily proteins, *Sci. STKE* **2007** (2007), p. pe6.

68 X. Guo, G.T. Macleod, A. Wellington, F. Hu, S. Panchumarthi, M. Schoenfield, L. Marin, M.P. Charlton, H.L. Atwood and K.E. Zinsmaier, The GTPase dMiro is required for axonal transport of mitochondria to *Drosophila* synapses, *Neuron* **47** (2005), pp. 379–393.

69 H. Mok, H. Shin, S. Kim, J.R. Lee, J. Yoon and E. Kim, Association of the kinesin superfamily motor protein KIF1Balpha with postsynaptic density-95 (PSD-95), synapse-associated protein-97, and synaptic scaffolding molecule PSD-95/discs large/zona occludens-1 proteins, *J. Neurosci.* **22** (2002), pp. 5253–5258.

70 M.J. Wozniak, M. Melzer, C. Dorner, H.U. Haring and R. Lammers, The novel protein KBP regulates mitochondria localization by interaction with a kinesin-like protein, *BMC Cell Biol.* **6** (2005), p. 35.

71 Q. Cai, C. Gerwin and Z.H. Sheng, Syntabulin-mediated anterograde transport of mitochondria along neuronal processes, *J. Cell Biol.* **170** (2005), pp. 959–969.

72 R.S. Stowers, L.J. Megeath, J. Górska-Andrzejak, I.A. Meinertzhagen and T.L. Schwarz, Axonal transport of mitochondria to synapses depends on Milton, a novel *Drosophila* protein, *Neuron* **36** (2002), pp. 1063–1077.

73 E.E. Glater, L.J. Megeath, R.S. Stowers and T.L. Schwarz, Axonal transport of mitochondria requires Milton to recruit kinesin heavy chain and is light chain independent, *J. Cell Biol.* **173** (2006), pp. 545–557.

74 A. Fransson, A. Ruusala and P. Aspenström, Atypical Rho GTPases have roles in mitochondrial homeostasis and apoptosis, *J. Biol. Chem.* **278** (2003), pp. 6495–6502.

75 A.M. Wolff, J.G. Petersen, T. Nilsson-Tillgren and N. Din, The open reading frame YAL048c affects the secretion of proteinase A in *S. cerevisiae*, *Yeast* **15** (1999), pp. 427–434.

76 A. Lewit-Bentley and S. Réty, EF-hand calcium-binding proteins, *Curr. Opin. Struct. Biol.* **10** (2000), pp. 637–643.

77 V. Janssens, J. Jordens, I. Stevens, C. Van Hoof, E. Martens, H. De Smedt, Y. Engelborghs, E. Waelkens and J. Goris, Identification and functional analysis of two Ca^{2+}-binding EF-hand motifs in the B″/PR72 subunit of protein phosphatase 2A, *J. Biol. Chem.* **278** (2003), pp. 10697–10706.

78 C.R. Monks, B.A. Freiberg, H. Kupfer, N. Sciaky and A. Kupfer, Three-dimensional segregation of supramolecular activation clusters in T cells, *Nature* **395** (1998), pp. 82–86.

79 A. Quintana, C. Schwindling, A.S. Wenning, U. Becherer, J. Rettig, E.C. Schwarz and M. Hoth, T cell activation requires mitochondrial translocation to the immunological synapse, *Proc. Natl. Acad. Sci. U. S. A.* **104** (2007), pp. 14418–14423.

80 S. Campello, R.A. Lacalle, M. Bettella, S. Mañes, L. Scorrano and A. Viola, Orchestration of lymphocyte chemotaxis by mitochondrial dynamics, *J. Exp. Med.* **203** (2006), pp. 2879–2886.

81 H.M. McBride, M. Neuspiel and S. Wasiak, Mitochondria: more than just a powerhouse, *Curr. Biol.* **16** (2006), pp. R551–R560.

82 C. Garrido, L. Galluzzi, M. Brunet, P.E. Puig, C. Didelot and G. Kroemer, Mechanisms of cytochrome *c* release from mitochondria, *Cell Death Differ.* **13** (2006), pp. 1423–1433.

83 L. Galluzzi, M.C. Maiuri, I. Vitale, H. Zischka, M. Castedo, L. Zitvogel and G. Kroemer, Cell death modalities: classification and pathophysiological implications, *Cell Death Differ.* **14** (2007), pp. 1237–1243.

84 M. Giacomello, I. Drago, P. Pizzo and T. Pozzan, Mitochondrial Ca^{2+} as a key regulator of cell life and death, *Cell Death Differ.* **14** (2007), pp. 1267–1274.

85 A. Oberst, C. Bender and D.R. Green, Living with death: the evolution of the mitochondrial pathway of apoptosis in animals, *Cell Death Differ.* **15** (2008), pp. 1139–1146.

86 L. Shao, M.V. Martin, S.J. Watson, A. Schatzberg, H. Akil, R.M. Myers, E.G. Jones, W.E. Bunney and M.P. Vawter, Mitochondrial involvement in psychiatric disorders, *Ann. Med.* **40** (2008), pp. 281–295.

87 I.S. Yoon, P.P. Li, K.P. Siu, J.L. Kennedy, R.G. Cooke, S.V. Parikh and J.J. Warsh, Altered IMPA2 gene expression and calcium homeostasis in bipolar disorder, *Mol. Psychiatry***6** (2001), pp. 678–683.

88 M. Kubota, T. Kasahara, T. Nakamura, M. Ishiwata, T. Miyauchi and T. Kato, Abnormal Ca^{2+} dynamics in transgenic mice with neuron-specific mitochondrial DNA defects, *J. Neurosci.* **26** (2006), pp. 12314–12324.

89 L. Palmieri, V. Papaleo, V. Porcelli, P. Scarcia, L. Gaita, R. Sacco, J. Hager, F. Rousseau, P. Curatolo, B. Manzi, R. Militerni, C. Bravaccio, S. Trillo, C. Schneider, R. Melmed, M. Elia, C. Lenti, M. Saccani, T. Pascucci, S. Puglisi-Allegra, K.L. Reichelt and A.M. Persico, Altered calcium homeostasis in autism-spectrum disorders: evidence from biochemical and genetic studies of the mitochondrial aspartate/glutamate carrier AGC1, *Mol. Psychiatry* **14** (2008).

90 B.H. LaMonte, K.E. Wallace, B.A. Holloway, S.S. Shelly, J. Ascaño, M. Tokito, T. Van Winkle, D.S. Howland and E.L. Holzbaur, Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration, *Neuron* **34** (2002), pp. 715–727.

91 E. Reid, M. Kloos, A. Ashley-Koch, L. Hughes, S. Bevan, I.K. Svenson, F.L. Graham, P.C. Gaskell, A. Dearlove, M.A. Pericak-Vance, D.C. Rubinsztein and D.A. Marchuk, A kinesin heavy chain (KIF5A) mutation in hereditary spastic paraplegia (SPG10), *Am. J. Hum. Genet.* **71** (2002), pp. 1189–1194.

92 I. Puls, C. Jonnakuty, B.H. LaMonte, E.L. Holzbaur, M. Tokito, E. Mann, M.K. Floeter, K. Bidus, D. Drayna, S.J. Oh, R.H. Brown, C.L. Ludlow and K.H. Fischbeck, Mutant dynactin in motor neuron disease, *Nat. Genet.* **33** (2003), pp. 455–456.

93 S. Gunawardena, L.S. Her, R.G. Brusch, R.A. Laymon, I.R. Niesman, B. Gordesky-Gold, L. Sintasath, N.M. Bonini and L.S. Goldstein, Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in *Drosophila*, *Neuron* **40** (2003), pp. 25–40.

94 D.T. Chang, G.L. Rintoul, S. Pandipati and I.J. Reynolds, Mutant huntingtin aggregates impair mitochondrial movement and trafficking in cortical neurons, *Neurobiol. Dis.* **22** (2006), pp. 388–400.

95 E. Trushina, R.B. Dyer, J.D. Badger, D. Ure, L. Eide, D.D. Tran, B.T. Vrieze, V. Legendre-Guillemin, P.S. McPherson, B.S. Mandavilli, B. Van Houten, S. Zeitlin, M. McNiven, R. Aebersold, M. Hayden, J.E. Parisi, E. Seeberg, I. Dragatsis, K. Doyle, A. Bender, C. Chacko and C.T. McMurray, Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro, *Mol. Cell. Biol.* **24** (2004), pp. 8195–8209.

96 Z. Tan, X. Sun, F.S. Hou, H.W. Oh, L.G. Hilgenberg, E.M. Hol, F.W. van Leeuwen, M.A. Smith, D.K. O'Dowd and S.S. Schreiber, Mutant ubiquitin found in Alzheimer's disease causes neuritic beading of mitochondria in association with neuronal degeneration, *Cell Death Differ.* **14** (2007), pp. 1721–1732.

97 A.C. Breuer, M.P. Lynn, M.B. Atkinson, S.M. Chou, A.J. Wilbourn, K.E. Marks, J.E. Culver and E.J. Fleegler, Fast axonal transport in amyotrophic lateral sclerosis: an intra-axonal organelle traffic analysis, *Neurology* **37** (1987), pp. 738–748.

98 A.C. Breuer and M.B. Atkinson, Fast axonal transport alterations in amyotrophic lateral sclerosis (ALS) and in parathyroid hormone (PTH)-treated axons, *Cell Motil. Cytoskelet.* **10** (1988), pp. 321–330.

99 A.L. Ström, J. Gal, P. Shi, E.J. Kasarskis, L.J. Hayward and H. Zhu, Retrograde axonal transport and motor neuron disease, *J. Neurochem.* **106** (2008), pp. 495–505.

100 F. Ferreirinha, A. Quattrini, M. Pirozzi, V. Valsecchi, G. Dina, V. Broccoli, A. Auricchio, F. Piemonte, G. Tozzi, L. Gaeta, G. Casari, A. Ballabio and E.I. Rugarli, Axonal degeneration in paraplegin-deficient mice is associated with abnormal mitochondria and impairment of axonal transport, *J. Clin. Invest.* **113** (2004), pp. 231–242.

101 T. Tatsuta and T. Langer, Quality control of mitochondria: protection against neurodegeneration and ageing, *EMBO J.* **27** (2008), pp. 306–314.

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

103 S. Hoppins, L. Lackner and J. Nunnari, The machines that divide and fuse mitochondria, *Annu. Rev. Biochem.* **76** (2007), pp. 751–780.

104 E. Smirnova, L. Griparic, D.L. Shurland and A.M. van der Bliek, Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells, *Mol. Biol. Cell* **12** (2001), pp. 2245–2256.

105 W. Bleazard, J.M. McCaffery, E.J. King, S. Bale, A. Mozdy, Q. Tieu, J. Nunnari and J.M. Shaw, The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast, *Nat. Cell Biol.* **1** (1999), pp. 298–304.

106 E. Ingerman, E.M. Perkins, M. Marino, J.A. Mears, J.M. McCaffery, J.E. Hinshaw and J. Nunnari, Dnm1 forms spirals that are structurally tailored to fit mitochondria, *J. Cell Biol.* **170** (2005), pp. 1021–1027.

107 D.I. James, P.A. Parone, Y. Mattenberger and J.C. Martinou, hFis1, a novel component of the mammalian mitochondrial fission machinery, *J. Biol. Chem.* **278** (2003), pp. 36373–36379.

108 A.D. Mozdy, J.M. McCaffery and J.M. Shaw, Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p, *J. Cell Biol.* **151** (2000), pp. 367–380.

109 Q. Tieu and J. Nunnari, Mdv1p is a WD repeat protein that interacts with the dynamin-related GTPase, Dnm1p, to trigger mitochondrial division, *J. Cell Biol.* **151** (2000), pp. 353–366.

110 E.E. Griffin, J. Graumann and D.C. Chan, The WD40 protein Caf4p is a component of the mitochondrial fission machinery and recruits Dnm1p to mitochondria, *J. Cell Biol.* **170** (2005), pp. 237–248.

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

112 S. Wasiak, R. Zunino and H.M. McBride, Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death, *J. Cell. Biol.* **177** (2007), pp. 439–450.

113 Y. Yoon, E.W. Krueger, B.J. Oswald and M.A. McNiven, The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1, *Mol. Cell. Biol.* **23** (2003), pp. 5409–5420.

114 A. Varadi, L.I. Johnson-Cadwell, V. Cirulli, Y. Yoon, V.J. Allan and G.A. Rutter, Cytoplasmic dynein regulates the subcellular distribution of mitochondria by controlling the recruitment of the fission factor dynamin-related protein-1, *J. Cell. Sci.* **117** (2004), pp. 4389–4400.

115 K.J. De Vos, V.J. Allan, A.J. Grierson and M.P. Sheetz, Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission, *Curr. Biol.* **15** (2005), pp. 678–683.

116 Z. Harder, R. Zunino and H. McBride, Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission, *Curr. Biol.* **14** (2004), pp. 340–345.

117 R. Yonashiro, S. Ishido, S. Kyo, T. Fukuda, E. Goto, Y. Matsuki, M. Ohmura-Hoshino, K. Sada, H. Hotta, H. Yamamura, R. Inatome and S. Yanagi, A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics, *EMBO J.* **25** (2006), pp. 3618–3626.

118 N. Nakamura, Y. Kimura, M. Tokuda, S. Honda and S. Hirose, MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology, *EMBO Rep.* **7** (2006), pp. 1019–1022.

119 A. Shalizi, B. Gaudillière, Z. Yuan, J. Stegmüller, T. Shirogane, Q. Ge, Y. Tan, B. Schulman, J.W. Harper and A. Bonni, A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation, *Science* **311** (2006), pp. 1012–1017.

120 A.F. Deyrieux, G. Rosas-Acosta, M.A. Ozbun and V.G. Wilson, Sumoylation dynamics during keratinocyte differentiation, *J. Cell. Sci.* **120** (2007), pp. 125–136.

121 Y. Huang, J. Niwa, G. Sobue and G.E. Breitwieser, Calcium-sensing receptor ubiquitination and degradation mediated by the E3 ubiquitin ligase dorfin, *J. Biol. Chem.* **281** (2006), pp. 11610–11617.

122 P.E. Ryan, G.C. Davies, M.M. Nau and S. Lipkowitz, Regulating the regulator: negative regulation of Cbl ubiquitin ligases, *Trends Biochem. Sci.* **31** (2006), pp. 79–88.

123 M. Karbowski, S.Y. Jeong and R.J. Youle, Endophilin B1 is required for the maintenance of mitochondrial morphology, *J. Cell Biol.* **166** (2004), pp. 1027–1039.

124 J.C. Dawson, J.A. Legg and L.M. Machesky, Bar domain proteins: a role in tubulation, scission and actin assembly in clathrin-mediated endocytosis, *Trends Cell Biol.* **16** (2006), pp. 493–498.

125 A. Schmidt, M. Wolde, C. Thiele, W. Fest, H. Kratzin, A.V. Podtelejnikov, W. Witke, W.B. Huttner and H.D. Söling, Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid, *Nature* **401** (1999), pp. 133–141.

126 Y. Chen, L. Deng, Y. Maeno-Hikichi, M. Lai, S. Chang, G. Chen and J.F. Zhang, Formation of an endophilin-Ca^{2+} channel complex is critical for clathrin-mediated synaptic vesicle endocytosis, *Cell* **115** (2003), pp. 37–48.

127 X.H. Liang, S. Jackson, M. Seaman, K. Brown, B. Kempkes, H. Hibshoosh and B. Levine, Induction of autophagy and inhibition of tumorigenesis by beclin 1, *Nature* **402** (1999), pp. 672–676.

128 M.C. Maiuri, E. Tasdemir, A. Criollo, E. Morselli, J.M. Vicencio, R. Carnuccio and G. Kroemer, Control of autophagy by oncogenes and tumor suppressor genes, *Cell Death Differ.* **16** (1) (2008), pp. 87–93.

129 F. Scarlatti, R. Granata, A.J. Meijer and P. Codogno, Does autophagy have a license to kill mammalian cells?, *Cell Death Differ.* **16** (1) (2008), pp. 12–20.

130 B. Levine, S. Sinha and G. Kroemer, Bcl-2 family members: dual regulators of apoptosis and autophagy, *Autophagy* **4** (2008), pp. 600–606.

131 Y. Takahashi, D. Coppola, N. Matsushita, H.D. Cualing, M. Sun, Y. Sato, C. Liang, J.U. Jung, J.Q. Cheng, J.J. Mul, W.J. Pledger and H.G. Wang, Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis, *Nat. Cell Biol.* **9** (2007), pp. 1142–1151. 132 M. Høyer-Hansen, L. Bastholm, P. Szyniarowski, M. Campanella, G. Szabadkai, T. Farkas, K. Bianchi, N. Fehrenbacher, F. Elling, R. Rizzuto, I.S. Mathiasen

and M. Jäättelä, Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2, *Mol. Cell* **25** (2007), pp. 193–205.

133 A. Criollo, M.C. Maiuri, E. Tasdemir, I. Vitale, A.A. Fiebig, D. Andrews, J. Molgó, J. Díaz, S. Lavandero, F. Harper, G. Pierron, D. di Stefano, R. Rizzuto, G. Szabadkai and G. Kroemer, Regulation of autophagy by the inositol trisphosphate receptor, *Cell Death Differ.* **14** (2007), pp. 1029–1039.

134 D. Tondera, A. Santel, R. Schwarzer, S. Dames, K. Giese, A. Klippel and J. Kaufmann, Knockdown of MTP18, a novel phosphatidylinositol 3-kinase-dependent protein, affects mitochondrial morphology and induces apoptosis, *J. Biol. Chem.* **279** (2004), pp. 31544–31555. 135 D. Tondera, F. Czauderna, K. Paulick, R. Schwarzer, J. Kaufmann and A. Santel, The mitochondrial protein MTP18 contributes to mitochondrial fission in mammalian cells, *J. Cell. Sci.* **118** (2005), pp. 3049–3059.

136 P. Viard, A.J. Butcher, G. Halet, A. Davies, B. Nürnberg, F. Heblich and A.C. Dolphin, PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane, *Nat. Neurosci.* **7** (2004), pp. 939–946.

137 L. Pedrola, A. Espert, X. Wu, R. Claramunt, M.E. Shy and F. Palau, GDAP1, the protein causing Charcot–Marie–Tooth disease type 4A, is expressed in neurons and is associated with mitochondria, *Hum. Mol. Genet.* **14** (2005), pp. 1087–1094.

138 A. Niemann, M. Ruegg, V. La Padula, A. Schenone and U. Suter, Ganglioside-induced differentiation associated protein 1 is a regulator of the mitochondrial network: new implications for Charcot–Marie–Tooth disease, *J. Cell Biol.* **170** (2005), pp. 1067–1078.

139 L. Pedrola, A. Espert, T. Valdés-Sánchez, M. Sánchez-Piris, E.E. Sirkowski, S.S. Scherer, I. Fariñas and F. Palau, Cell expression of GDAP1 in the nervous system and pathogenesis of Charcot–Marie–Tooth type 4A disease, *J. Cell. Mol. Med.* **12** (2008), pp. 679–689.

140 A. Marco, A. Cuesta, L. Pedrola, F. Palau and I. Marín, Evolutionary and structural analyses of GDAP1, involved in Charcot–Marie–Tooth disease, characterize a novel class of glutathione transferase-related genes, *Mol. Biol. Evol.* **21** (2004), pp. 176–187.

141 L.D. Robb-Gaspers, P. Burnett, G.A. Rutter, R.M. Denton, R. Rizzuto and A.P. Thomas, Integrating cytosolic calcium signals into mitochondrial metabolic responses, *EMBO J.* **17** (1998), pp. 4987–5000.

142 K.L. Cerveny, Y. Tamura, Z. Zhang, R.E. Jensen and H. Sesaki, Regulation of mitochondrial fusion and division, *Trends Cell Biol.* **17** (2007), pp. 563–569.

143 L. Pellegrini and L. Scorrano, A cut short to death: Parl and Opa1 in the regulation of mitochondrial morphology and apoptosis, *Cell Death Differ.* **14** (2007), pp. 1275–1284.

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

145 M.V. Alavi, S. Bette, S. Schimpf, F. Schuettauf, U. Schraermeyer, H.F. Wehrl, L. Ruttiger, S.C. Beck, F. Tonagel, B.J. Pichler, M. Knipper, T. Peters, J. Laufs and B. Wissinger, A splice site mutation in the murine Opa1 gene features pathology of autosomal dominant optic atrophy, *Brain* **130** (2007), pp. 1029–1042.

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

147 S. Cipolat, T. Rudka, D. Hartmann, V. Costa, L. Serneels, K. Craessaerts, K. Metzger, C. Frezza, W. Annaert, L. D, Adamio, C. Derks, T. Dejaegere, L. Pellegrini, R. D, Hooge, L. Scorrano and B. De Strooper, Mitochondrial rhomboid PARL regulates cytochrome *c* release during apoptosis via OPA1-dependent cristae remodeling, *Cell* **126** (2006), pp. 163–175.

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

149 E. Ingerman, S. Meeusen, R. Devay and J. Nunnari, In vitro assays for mitochondrial fusion and division, *Methods Cell Biol.* **80** (2007), pp. 707–720.

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

151 F. Legros, A. Lombès, P. Frachon and M. Rojo, Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins, *Mol. Biol. Cell* **13** (2002), pp. 4343–4354.

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

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

154 D. Bach, S. Pich, F.X. Soriano, N. Vega, B. Baumgartner, J. Oriola, J.R. Daugaard, J. Lloberas, M. Camps, J.R. Zierath, R. Rabasa-Lhoret, H. Wallberg-Henriksson, M. Laville, M. Palacín, H. Vidal, F. Rivera, M. Brand and A. Zorzano, Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity, *J. Biol. Chem.* **278** (2003), pp. 17190–17197.

155 L. Scorrano, Multiple functions of mitochondria-shaping proteins, *Novartis Found. Symp.* **287** (2007), pp. 47–55 discussion 55–9.

156 O.M. de Brito and L. Scorrano, Mitofusin 2 tethers endoplasmic reticulum to mitochondria, *Nature* **456** (2008), pp. 605–610.

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

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

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

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

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

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

163 S. Duvezin-Caubet, M. Koppen, J. Wagener, M. Zick, L. Israel, A. Bernacchia, R. Jagasia, E.I. Rugarli, A. Imhof, W. Neupert, T. Langer and A.S. Reichert, OPA1 processing reconstituted in yeast depends on the subunit composition of the m-AAA protease in mitochondria, *Mol. Biol. Cell* **18** (2007), pp. 3582–3590.

164 O. Guillery, F. Malka, T. Landes, E. Guillou, C. Blackstone, A. Lombès, P. Belenguer, D. Arnoult and M. Rojo, Metalloprotease-mediated OPA1 processing is modulated by the mitochondrial membrane potential, *Biol. Cell* **100** (2008), pp. 315–325.

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

166 E.V. Koonin, K.S. Makarova, I.B. Rogozin, L. Davidovic, M.C. Letellier and L. Pellegrini, The rhomboids: a nearly ubiquitous family of intramembrane serine proteases that probably evolved by multiple ancient horizontal gene transfers, *Genome Biol.* **4** (2003), p. R19.

167 Y. Wang, Y. Zhang and Y. Ha, Crystal structure of a rhomboid family intramembrane protease, *Nature* **444** (2006), pp. 179–180.

168 Z. Wu, N. Yan, L. Feng, A. Oberstein, H. Yan, R.P. Baker, L. Gu, P.D. Jeffrey, S. Urban and Y. Shi, Structural analysis of a rhomboid family intramembrane protease reveals a gating mechanism for substrate entry, *Nat. Struct. Mol. Biol.* **13** (2006), pp. 1084–1091.

169 A. Ben-Shem, D. Fass and E. Bibi, Structural basis for intramembrane proteolysis by rhomboid serine proteases, *Proc. Natl. Acad. Sci. U. S. A.* **104** (2007), pp. 462–466.

170 M.J. Lemieux, S.J. Fischer, M.M. Cherney, K.S. Bateman and M.N. James, The crystal structure of the rhomboid peptidase from *Haemophilus influenzae* provides insight into intramembrane proteolysis, *Proc. Natl. Acad. Sci. U. S. A.* **104** (2007), pp. 750–754.

171 R.P. Baker, K. Young, L. Feng, Y. Shi and S. Urban, Enzymatic analysis of a rhomboid intramembrane protease implicates transmembrane helix 5 as the lateral substrate gate, *Proc. Natl. Acad. Sci. U. S. A.* **104** (2007), pp. 8257–8262.

172 Y. Wang and Y. Ha, Open-cap conformation of intramembrane protease GlpG, *Proc. Natl. Acad. Sci. U. S. A.* **104** (2007), pp. 2098–2102.

173 S. Maegawa, K. Koide, K. Ito and Y. Akiyama, The intramembrane active site of GlpG, an *E. coli* rhomboid protease, is accessible to water and hydrolyses an extramembrane peptide bond of substrates, *Mol. Microbiol.* **64** (2007), pp. 435–447.

174 A. Del Rio, K. Dutta, J. Chavez, I. Ubarretxena-Belandia and R. Ghose, Solution structure and dynamics of the N-terminal cytosolic domain of rhomboid intramembrane protease from *Pseudomonas aeruginosa*: insights into a functional role in intramembrane proteolysis, *J. Mol. Biol.* **365** (2007), pp. 109–122.

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

176 S. Urban and M.S. Wolfe, Reconstitution of intramembrane proteolysis in vitro reveals that pure rhomboid is sufficient for catalysis and specificity, *Proc. Natl. Acad. Sci. U. S. A.* **102** (2005), pp. 1883–1888.

177 S. Urban, Rhomboid proteins: conserved membrane proteases with divergent biological functions, *Genes Dev.* **20** (2006), pp. 3054–3068.

178 A. Sík, B.J. Passer, E.V. Koonin and L. Pellegrini, Self-regulated cleavage of the mitochondrial intramembrane-cleaving protease PARL yields Pbeta, a nuclear-targeted peptide, *J. Biol. Chem.* **279** (2004), pp. 15323–15329.

179 D.V. Jeyaraju, L. Xu, M.C. Letellier, S. Bandaru, R. Zunino, E.A. Berg, H.M. McBride and L. Pellegrini, Phosphorylation and cleavage of presenilin-associated rhomboid-like protein (PARL) promotes changes in mitochondrial morphology, *Proc. Natl. Acad. Sci. U. S. A.* **103** (2006), pp. 18562–18567.

180 G.A. Bazykin, F.A. Kondrashov, A.Y. Ogurtsov, S. Sunyaev and A.S. Kondrashov, Positive selection at sites of multiple amino acid replacements since rat–mouse divergence, *Nature* **429** (2004), pp. 558–562.

181 A.Y. Ogurtsov, S. Sunyaev and A.S. Kondrashov, Indel-based evolutionary distance and mouse–human divergence, *Genome Res.* **14** (2004), pp. 1610–1616.

182 N. Taguchi, N. Ishihara, A. Jofuku, T. Oka and K. Mihara, Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission, *J. Biol. Chem.* **282** (2007), pp. 11521–11529.

183 V.L. Nixon, M. Levasseur, A. McDougall and K.T. Jones, Ca(2+) oscillations promote APC/C-dependent cyclin B1 degradation during metaphase arrest and completion of meiosis in fertilizing mouse eggs, *Curr. Biol.* **12** (2002), pp. 746–750.

184 J.R. Hutchins, D. Dikovskaya and P.R. Clarke, Regulation of Cdc2/cyclin B activation in *Xenopus* egg extracts via inhibitory phosphorylation of Cdc25C phosphatase by Ca(2+)/calmodulin-dependent protein corrected kinase II, *Mol. Biol. Cell* **14** (2003), pp. 4003–4014.

185 L.A. Hyslop, V.L. Nixon, M. Levasseur, F. Chapman, K. Chiba, A. McDougall, J.P. Venables, D.J. Elliott and K.T. Jones, Ca(2+)-promoted cyclin B1 degradation in mouse oocytes requires the establishment of a metaphase arrest, *Dev. Biol.* **269** (2004), pp. 206–219.

186 J.T. Cribbs and S. Strack, Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death, *EMBO Rep.* **8** (2007), pp. 939–944.

187 C.R. Chang and C. Blackstone, Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology, *J. Biol. Chem.* **282** (2007), pp. 21583–21587.

188 G.M. Cereghetti, A. Stangherlin, O. Martins de Brito, C.R. Chang, C. Blackstone, P. Bernardi and L. Scorrano, Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria, *Proc. Natl. Acad. Sci. U. S. A.* **105** (2008), pp. 15803–15808.

189 S.H. Snyder, M.M. Lai and P.E. Burnett, Immunophilins in the nervous system, *Neuron* **21** (1998), pp. 283–294.

190 X.J. Han, Y.F. Lu, S.A. Li, T. Kaitsuka, Y. Sato, K. Tomizawa, A.C. Nairn, K. Takei, H. Matsui and M. Matsushita, CaM kinase I alpha-induced phosphorylation of Drp1 regulates mitochondrial morphology, *J. Cell Biol.* **182** (2008), pp. 573–585.

191 M. Germain, J.P. Mathai, H.M. McBride and G.C. Shore, Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis, *EMBO J.* **24** (2005), pp. 1546–1556.

Chapter 5

5. Discussion

This work arises from a recent paper [16] where the authors reported the anti-apoptotic role of HAX1 in neurons and in lymphocytes. HAX1 has been identified as a Bcl-2 family related protein and localized on the inner and outer membrane of mitochondria and exposed to the intermembrane space and to a lesser extent in the endoplasmatic reticulum. However, our bioinformatic analysis, the secondary structure of HAX1 does not meet the requirements for being a Bcl-2 family related protein, a mitochondrially imported protein, or a membrane anchored protein. Firstly, the secondary structure of HAX1, which we predicted with three computational methods, does not show the presence of BH modules. Secondly, the lack of conservation of these proposed modules across species does not show the presence of a function associated with these domains to qualify as BH modules, as sequence conservation reflects function. Moreover, for a protein to be a membrane anchored protein, it has to have a stretch of 20 hydrophobic amino acids forming an α-helix which is not observed either with three computational methods of secondary structure prediction.

5.1 HAX1 is not a member of the Bcl-2 family

The protein has been classified as a Bcl-2 family protein because of some similarities with the proteins of this family. It shows similarities to BH like domains (BH1 and BH2 domains), although it does not show any BH3 domain and it has about 100 amino acids with low similarity to Nip3, a Bcl-2 interacting protein with pro-apoptotic activity. It has also been reported to be composed of a putative hydrophobic transmembrane domain, which is not strongly supported by bioinformatic analysis[40]. Our study sequence analysis

did not support the presence of any BH domain in the proteins, in fact the two domains BH1 and BH2 located at position 37-56 and 74-89 respectively, can not be classified as conserved protein domain even with the most permissive threshold. An other interesting aspect that came out from the sequence analysis is the fact that the sequences corresponding to the BH1 and BH2 domains in HAX1 are extremely disordered while the BH1 and BH2 domains are stable hairpins constituted by hydrophobic α-helices[50].

However our analysis show the presence of a structural element, the three-strand β sheet near the C-terminal, which is instead well predicted and conserved and allowing to classify it as a α/β protein. These proteins are constituted by the repeat ion of β-α-β super secondary units, such that the outer layer of the structure is composed of alpha helices packing against a central core of parallel β sheets. HAX1 also contains three-conserved aspartate (DXDXD motif) in a predicted loop which could be involved in the binding of metals, such as calcium. Neither the three-strand β sheet element and the aspartate residues are not presented in the Bcl-2 protein family, suggesting that this protein is not part a member of the Bcl-2 family.

5.2 HAX1 is not localized in the mitochondria

Mitochondria are extremely complex organelles, constituted by a double membrane. Mitochondrial proteins encoded by the cell genome must be imported into the organelles and for this purpose they present target sequence and they are recognized by receptors on the surface of the mitochondria and then sorted into the outer or inner membrane, to the intramembrane space or to the matrix [51].

HAX1 was reported to be localized in the inner and outer membrane[16] of the mitochondria but this is an odd statement because it doesn't reflect any other mitochondrial protein localization. Moreover, it does not present any predictable mitochondrial import peptide or any cysteine residues necessary for the import in the mitochondria through the Mirochondria Intramembrane space Assembly (MIA) system. It is well known that many intramembrane mitochondrial proteins presents conserved cysteine residues necessary for the binding to cofactor, metals and the formation of disulfide bonds essential for the protein

structure and function [52]. Additionally, mitochondrial dysfunction could cause several diseases thus it is very important to identify and classify mitochondrial and how they integrate into mitochondrial pathways. Recent proteomic studies aiming to study mitochondrial proteome did not include HAX1 in the list of mitochondrial proteins[53]. Further, it has been reported that HAX1 contain a highly conserved α-helix at the C-terminal, which should constitute the transmembrane domain anchoring the protein to both the mitochondrial membrane[16, 36]. However, we could not identify any transmembrane domain through the use of methods to predict transmembrane domain and hydrophobic stretches. The predicted α-helix is constituted of 16 amino acid, 4 of these are charged and 1 is polar. This data is not consistent with what has been reported about membrane-spanning α-helix. In fact the α-helix must be 20 amino acid-long to span the hydrocarbon core of the lipid bilayer [54]. Thus, the predicted sequence in HAX1 is not long enough to anchor the protein to the membrane. However this domain is highly conserved and it could be responsible of the interaction with other proteins.

All these elements lead to the conclusion that HAX1 is not localized in the mitochondria. All the bioinformatic analyses are supported by *in vitro* experiments that show that HAX1 is peripherally associated to heavy membrane. Mitochondria were extracted from mouse liver using a Percoll-gradient and we performed immunoblot analysis on the heavy membrane and ultra-pure mitochondria fractions. To assess the purity of the mitochondria, we checked the absence of membrane-associated protein such as actin and tubulin. The absence of actin and the presence of diffusible proteins which are cytochrome c localized in the IMS and protein MnSOD (Manganese Superoxide Dismutase) presented in the mitochondrial matrix allow defining the purity of the mitochondria and the integrity of the membrane. Additionally, we also determined the integrity of the organelles and the lack of cross-contaminating organelles or cellular debris, by electron microscopy. Normalizing for MnSOD and cytochrome c, endogenous HAX1 is present only on the heavy membrane fraction and not in the purified mitochondria. This data is also confirmed using two different anti-HAX1 antibodies, one from mouse and the other one from rabbit.

This result is consistent with our bioinformatic analysis but also with the lack of HAX1 in the murine and human mitochondrial proteome as reported by Pagliarini *et al.*, 2008. We also demonstrated that the association to heavy membrane is only peripheral: we generated

membrane (pellet) and IMS (supernatant) from mitochondria fraction by high salt washes which is a technique that allow the dissociation of weakly bound proteins from the membrane. The immunoblot shows that HAX1 is present only in the supernatant but not in the pellet. This result is consistent with our computational analysis, which does not predict any transmembrane domain. Based on these data, we decided to experimentally investigate the validity of the interaction between HAX1 and PARL, a highly hydrophobic protein in the IMM.

5.3 HAX and PARL interaction

Mitochondria are very dynamic organelles constituted by two membranes, an inner and an outer membrane creating two compartments, an intramembrane space and the matrix space. However, the inner membrane also folds creating a third compartment, formed by the juxtaposition of the membrane folds. The intercristae space avoids the diffusion of proteins, such as cytochrome c maintaining this conformation. The protein Opa1 plays a pivotal role. Opa1 is present in the Inner membrane but it also has a soluble form and the processing of the protein is regulated by the intramembrane serine protease PARL.

PARL is a member of the rhomboid family of proteases and has the characteristic of catalyzing intermembrane cleavage of its substrate. In the past, we have shown that PARL, a mitochondrial inner membrane anchored protein, undergoes a self-regulated N-terminal cleavage in the mitochondrial matrix, which is governed by a triple phosphorylation. In our collaborative studies, we have also shown that PARL regulates apoptosis by sequestering cytochrome c within the mitochondrial cristae by processing OPA1 which staples the opening of the cristae, thereby regulating the release of cytochrome c[15, 22, 25]. PARL presents seven transmembrane domains with N-terminus of the protease exposed to the IMS and the C-terminus to the matrix[15, 22]. It also has 3 loops exposed to the IMS: 2 loops are part of the rhomboid core and a bigger loop, called loopA, which is important for its proteolytic activity[15, 22, 25].

The recent study of Chao *et al.*, highlighted a very interesting point concerning the interaction of the anti-apoptotic protein HAX1 with the mitochondrial protein PARL. The study reported that HAX1 interacts with PARL in the mitochondrial intermembrane space and it is essential for the cleavage of Opa1. However our analysis shows that this is an

impossible interaction based on the subcellular localization of HAX1, according to our bioinformatic analysis. So we undertook to address this point using classical biochemical approaches.

First of all, we wanted to address the specificity of the binding between HAX1 and PARL. For this purpose, we co-transfected HEK293 cells with constructs carrying wild type HAX1 and mutant forms of PARL missing the mitochondrial import sequence. Although PARL is not target to the mitochondria and cannot fold properly, the two proteins can be co-precipitated, indicating a non specific binding, due to unspecific hydrophobic interactions. To confirm this result, the two IMS domains of PARL, which are the only domains that potentially, could bind HAX1, have been deleted. We also perform a mammalian two-hybrid assay, which is a very strong method to validate the interaction between two proteins. Even with this method we could not identify a domain in PARL that could mediate the interaction with HAX1. The last demonstration of the unspecific binding was to mix HEK293 cell lysates independently transfected with constructs expressing PARL or HAX1 and MEF cell lysates of Hax1-/- and Parl-/-. The interaction between HAX1 and PARL is recreated in both cases demonstrating that is only an artifact. Finally, we demonstrated that the proteolytic activity of PARL does not require HAX1. In fact, we observed that the endogenous level of expression of the mature form of PARL is not altered in the brain lysates from mice Hax +/+, +/-, -/-. This result is consistent with the fact that PARL β-cleavage which self regulated and occurs in *trans* [35]. Thus, our results show that HAX1 is not a Blc2 protein and cannot be localized in the mitochondria, and thereby cannot interact with PARL in the inner membrane.

Chapter 6

6. Conclusions and future perspectives

The core of my thesis is composed by two published manuscripts: one manuscript published in Cell Death and Differentiation where we highlighted the interaction of HAX1 with the mitochondrial protein PARL; the second one is a review article on the role of calcium in mitochondria motility and morphology. The main paper is also integrated with supplementary materials and methods that I used along my internship in the laboratory of Dr. Pellegrini.

Many intracellular organelles are involved in calcium homeostasis and an important mechanism to remove cytosolic ion is the mitochondrial calcium uniporter system. Even the endoplasmatic reticulum has a pivotal role in calcium homeostasis acting as a source of calcium and via SERCA pump can partecipate to the recovery of resting intracellular calcium after neuronal activation. SERCA pumps are the main regulator of calcium homeostasis and contractility in the skeletal and cardiac muscle[55]. Phospholamban (PLN) is involved in calcium homeostasis and contractility in the heart. At low calcium concentration, dephosphorylated PLN interacts with SERCA, inhibiting its affinity to calcium reversibly. Upon phosphoryaltion of PLN, it looses affinity for SERCA enhancing the calcium transport[42]. It has been reported that PLN has affinity also for HAX1. This could represent a new mechanism to regulate PLN activity. Modulation of the ER content of Ca^{2+} could affect the apoptosis sensitivity of the cells; deregulation of ER Ca^{2+} uptake may cause mitochondrial overload and dissipation of the mitochondrial membrane potential (MMP), with activation of caspase-dependent and -independent pathways of apoptosis downstream of the mitochondria[40]. In light of the interaction of HAX1 with PLN, we could explain the participation of HAX1 in calcium binding since an accurate sequence analysis

of the protein shows the presence of the DXDXD motif which is associated to metal binding domains (Figure 16).

FGGVLESDARSESPQPAPDWGSQRPFHRFDDVWPMDPHPRTREDNDLDSQVSQEG
LGPVLQPQPKSYFKS

Figure16 HAX1 sequence showing the putative DXDXD motif.

A DXDXD motif is a conserved pattern identified in many calcium-binding proteins including calmodulin, and the acidic residues have a role in coordinating the calcium and also other metals like magnesium[56]. The bioinformatic analysis of HAX1 suggested a new role for the protein that we would like to investigate. Calcium transport into and out of the mitochondria can modulate and regulate many cellular processes, even leading to cell death[56]. Therefore the introduction of a new calcium binding protein, with anti-apoptotic function, could introduce a novel mechanism of regulation of calcium trafficking between the two organelles and a new grade of regulation of cell death mechanism.

Another interesting element that came out from our study is that endogenous HAX1 is not present in the mitochondria and in the ER allowing concluding that HAX1 might be present in the periphery of the mitochondria. This result represents a contradiction with the previous data obtained overexpressing the protein[16]. Therefore, our work represents a shift in the paradigm of the protein within the cells. One of the aspects to pursue is where HAX1 is located within the mitochondria and if the protein is really multifunctional. Since the protein was found in the crude heavy membrane but not in the hyperpure membranes, meaning it is not present either in mitochondria nor in ER, we hypothesize that the protein could be present in the mitochondria associated membrane (MAM), which is the area of contact between the ER and mitochondria. ER and mitochondria form contacts on 20% of mitochondrial surface[57] that support the communication between the two organelles, which is fundamental for calcium homeostasis, lipid biosynthesis and therefore for the regulation of mitochondrial metabolism and apoptosis [57, 58]. ER and mitochondria communicates through MAMs, which are contiguous ER membranes in close contacts with MOM and which are thought to represent the site of phospholipids exchange between the two organelles [58, 59]. Morphological evidences of close juxtapositions between ER

(sarcoplasmic reticulum) and mitochondria have emerged by electron microscopy in fixed samples of several cell types. Since the observed interaction could be due to artefacts, quickly frozen samples were analyzed and interestingly it was noticed that appositions between the two organelles are visible and more frequent than in the fixed cells [59]. The interaction between the two organelles makes them functionally related: mitochondria and ER control the calcium signaling which is crucial for proper functioning of the cells.
Moreover it would be interesting to understand the different expression patterns of the protein in various tissues because in diseases associated with the mutated HAX1, clinical manifestations are associated only with principal organs such as bone marrow and brain. For the localization of the endogenous protein within the cells it would be useful to perform immunofluorescence. Unfortunately to date there is no good antibody against HAX1 which could serve this purpose, neither the commercial one or the one produced in our laboratory, thus it would be necessary to raise a new antibody agaist the protein.

6.1 HAX1 and its involvement in apoptosis

An increasing number of studies highlighted the role of PARL as a multifunctional protein involved in many signaling pathways and cellular processes. It has been attibuted a role in apoptosis due to its similarity to Bcl-2 family; in fact it has observed an increase of apoptosis in myeloid cells in patients affected by autosomal recessive severe congenital neutropenia where HAX1 is mutated. However, our data show that HAX1 is not a Bcl-2 like protein since it does not present BH domains and that it can not bind with PARL. So one of the points to address is the understanding of the actual role of this protein in the cells by mutating HAX1 and by knocking it out. Correcting the mechanism of action of HAX1 would be an outstanding result, that could make light on the anti-apoptotic role of the protein in the different models suggested; in fact three different scenarios of action has been proposed for HAX1 as already described in the introduction.

6.2 PARL

Our work also reopens the question on how the organelle regulates PARL activity in response to stress. The identification of the phosphatase and kinase involved in the reversible phosphorylation of PARL and the protease that cleaves PARL would contribute to a better understanding of the pathway implicated in the regulation of mitochondria remodeling but also of the reversible phosphorylation in the organelle. Reversible phosphorylation is emerging as fundamental signaling means in mitochondrial regulation since the recent literature reveals that more than 60 mitochondrial proteins undergo phosphorylation although the effects of this modification are not fully clear and phosphatase and kinase involved have not been well characterized[60]. A well-known example is Pyruvate dehydorgenase complex (PDC) in the matrix, which is subjected to continuous phosphorylation (inactivation) - dephosphorylation (activation)[61].

Understanding the mechanism of reversible phosphorylation and so the identification of the phospatases and kinases involved, is becoming more compelling since the recent discovery that mutations in these mitochondrial proteins are linked to human diseases, such as Parkinson disease and diabetes[62, 63]. Moreover, the identification of these proteins would reveal the pathways implicated in sensing and transducing the signals that regulate mitochondrial shape.

7. References

1. Jeyaraju, D.V., *et al.* (2008) Calcium regulation of mitochondria motility and morphology. *Biochim Biophys Acta*

2. Yaffe, M.P. (2003) The cutting edge of mitochondrial fusion. *Nat Cell Biol* 5, 497-499

3. Jourdain, A., and Martinou, J.C. (2009) Mitochondrial outer-membrane permeabilization and remodelling in apoptosis. *Int J Biochem Cell Biol* 41, 1884-1889

4. Thomenius, M.J., and Distelhorst, C.W. (2003) Bcl-2 on the endoplasmic reticulum: protecting the mitochondria from a distance. *J Cell Sci* 116, 4493-4499

5. Galluzzi, L., *et al.* (2009) Targeting post-mitochondrial effectors of apoptosis for neuroprotection. *Biochim Biophys Acta* 1787, 402-413

6. Thomenius, M.J., *et al.* (2003) Bcl-2 on the endoplasmic reticulum regulates Bax activity by binding to BH3-only proteins. *J Biol Chem* 278, 6243-6250

7. Shaw, J., and Kirshenbaum, L.A. (2006) HAX-1 represses postmitochondrial caspase-9 activation and cell death during hypoxia-reoxygenation. *Circ Res* 99, 336-338

8. Bruick, R.K. (2000) Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc Natl Acad Sci U S A* 97, 9082-9087

9. Zou, H., *et al.* (1999) An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 274, 11549-11556

10. Ow, Y.P., *et al.* (2008) Cytochrome c: functions beyond respiration. *Nat Rev Mol Cell Biol* 9, 532-542

11. Fulda, S., and Debatin, K.M. (2006) Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* 25, 4798-4811

12. Broughton, B.R., *et al.* (2009) Apoptotic mechanisms after cerebral ischemia. *Stroke* 40, e331-339

13. Malladi, S., *et al.* (2009) The Apaf-1*procaspase-9 apoptosome complex functions as a proteolytic-based molecular timer. *Embo J* 28, 1916-1925

14. D'Amelio, M., *et al.* (2008) The apoptosome: emerging insights and new potential targets for drug design. *Pharm Res* 25, 740-751

15. Jeyaraju, D.V., *et al.* (2006) Phosphorylation and cleavage of presenilin-associated rhomboid-like protein (PARL) promotes changes in mitochondrial morphology. *Proc Natl Acad Sci U S A* 103, 18562-18567

16. Chao, J.R., *et al.* (2008) Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons. *Nature* 452, 98-102

17. Reed, J.C. (2000) Mechanisms of apoptosis. *Am J Pathol* 157, 1415-1430

18. Bernard, D.G., *et al.* (2009) An allelic mutant series of ATM3 reveals its key role in the biogenesis of cytosolic iron-sulfur proteins in Arabidopsis. *Plant Physiol* 151, 590-602

19. Ryan, M.T., and Hoogenraad, N.J. (2007) Mitochondrial-nuclear communications. *Annu Rev Biochem* 76, 701-722

20. Bonda, D.J., *et al.* Mitochondrial dynamics in Alzheimer's disease: opportunities for future treatment strategies. *Drugs Aging* 27, 181-192

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

22. Pellegrini, L., and Scorrano, L. (2007) A cut short to death: Parl and Opa1 in the regulation of mitochondrial morphology and apoptosis. *Cell Death Differ* 14, 1275-1284

23. Hausenloy, D.J., and Scorrano, L. (2007) Targeting cell death. *Clin Pharmacol Ther* 82, 370-373

24. Lu, B. (2009) Mitochondrial dynamics and neurodegeneration. *Curr Neurol Neurosci Rep* 9, 212-219

25. Cipolat, S., and Scorrano, L. (2006) To fuse and to protect. A novel role for CED-9 in mitochondrial morphology reveals an ancient function. *Cell Death Differ* 13, 1833-1834

26. Frezza, C., *et al.* (2006) OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* 126, 177-189

27. Bossy-Wetzel, E., *et al.* (2003) Mitochondrial fission in apoptosis, neurodegeneration and aging. *Curr Opin Cell Biol* 15, 706-716

28. Scorrano, L. (2003) Divide et impera: Ca2+ signals, mitochondrial fission and sensitization to apoptosis. *Cell Death Differ* 10, 1287-1289

29. Wilkins, A.L., *et al.* (2002) Metal-binding studies for a de novo designed calcium-binding protein. *Protein Eng* 15, 571-574

30. Jahani-Asl, A., and Slack, R.S. (2007) The phosphorylation state of Drp1 determines cell fate. *EMBO Rep* 8, 912-913

31. Scorrano, L. (2009) Opening the doors to cytochrome c: Changes in mitochondrial shape and apoptosis. *Int J Biochem Cell Biol*

32. Pinton, P., *et al.* (2008) Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. *Oncogene* 27, 6407-6418

33. Cilenti, L., *et al.* (2004) Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death. *J Biol Chem* 279, 50295-50301

34. Pellegrini, L., *et al.* (2001) PAMP and PARL, two novel putative metalloproteases interacting with the COOH-terminus of Presenilin-1 and -2. *J Alzheimers Dis* 3, 181-190

35. Sik, A., *et al.* (2004) Self-regulated cleavage of the mitochondrial intramembrane-cleaving protease PARL yields Pbeta, a nuclear-targeted peptide. *J Biol Chem* 279, 15323-15329

36. Sharp, T.V., *et al.* (2002) K15 protein of Kaposi's sarcoma-associated herpesvirus is latently expressed and binds to HAX-1, a protein with anti-apoptotic function. *J Virol* 76, 802-816

37. Suzuki, Y., *et al.* (1997) HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of Src family tyrosine kinases. *J Immunol* 158, 2736-2744

38. Yin, H., *et al.* (2001) Evidence that HAX-1 is an interleukin-1 alpha N-terminal binding protein. *Cytokine* 15, 122-137

39. Fadeel, B., and Grzybowska, E. (2009) HAX-1: a multifunctional protein with emerging roles in human disease. *Biochim Biophys Acta* 1790, 1139-1148

40. Fadeel, B., *et al.* (2008) Big wheel keeps on turning: apoptosome regulation and its role in chemoresistance. *Cell Death Differ* 15, 443-452

41. Fadeel, B., and Grzybowska, E. (2009) HAX-1: A multifunctional protein with emerging roles in human disease. *Biochim Biophys Acta*

42. Vafiadaki, E., *et al.* (2007) Phospholamban interacts with HAX-1, a mitochondrial protein with anti-apoptotic function. *J Mol Biol* 367, 65-79

43. Trebinska, A., *et al.* HAX-1 overexpression, splicing and cellular localization in tumors. *BMC Cancer* 10, 76

44. Klein, C., *et al.* (2007) HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). *Nat Genet* 39, 86-92

45. Carlsson, G., *et al.* (2008) Central nervous system involvement in severe congenital neutropenia: neurological and neuropsychological abnormalities associated with specific HAX1 mutations. *J Intern Med* 264, 388-400

46. Ishikawa, N., and Kobayashi, M. (2009) [Neurological findings in severe congenital neutropenia with HAX1 mutations. *No To Hattatsu* 41, 415-419

47. Mirmohammadsadegh, A., *et al.* (2003) HAX-1, identified by differential display reverse transcription polymerase chain reaction, is overexpressed in lesional psoriasis. *J Invest Dermatol* 120, 1045-1051

48. Han, Y., *et al.* (2006) Overexpression of HAX-1 protects cardiac myocytes from apoptosis through caspase-9 inhibition. *Circ Res* 99, 415-423

49. Cilenti, L., *et al.* (2005) Omi/HtrA2 protease mediates cisplatin-induced cell death in renal cells. *Am J Physiol Renal Physiol* 288, F371-379

50. Petros, A.M., *et al.* (2004) Structural biology of the Bcl-2 family of proteins. *Biochim Biophys Acta* 1644, 83-94

51. Hildenbeutel, M., *et al.* (2008) New insights into the mechanism of precursor protein insertion into the mitochondrial membranes. *Int Rev Cell Mol Biol* 268, 147-190

52. Stojanovski, D., *et al.* (2008) The MIA system for protein import into the mitochondrial intermembrane space. *Biochim Biophys Acta* 1783, 610-617

53. Pagliarini, D.J., *et al.* (2008) A mitochondrial protein compendium elucidates complex I disease biology. *Cell* 134, 112-123

54. White, S.H., and von Heijne, G. (2005) Transmembrane helices before, during, and after insertion. *Curr Opin Struct Biol* 15, 378-386

55. Toescu, E.C., and Verkhratsky, A. (2007) Role of calcium in normal aging and neurodegeneration. *Aging Cell* 6, 265

56. Bist, P., and Rao, D.N. (2003) Identification and mutational analysis of Mg2+ binding site in EcoP15I DNA methyltransferase: involvement in target base eversion. *J Biol Chem* 278, 41837-41848

57. Simmen, T., *et al.* (2005) PACS-2 controls endoplasmic reticulum-mitochondria communication and Bid-mediated apoptosis. *Embo J* 24, 717-729

58. de Brito, O.M., and Scorrano, L. (2009) Mitofusin-2 regulates mitochondrial and endoplasmic reticulum morphology and tethering: the role of Ras. *Mitochondrion* 9, 222-226

59. Giorgi, C., *et al.* (2009) Structural and functional link between the mitochondrial network and the endoplasmic reticulum. *Int J Biochem Cell Biol* 41, 1817-1827

60. Pagliarini, D.J., and Dixon, J.E. (2006) Mitochondrial modulation: reversible phosphorylation takes center stage? *Trends Biochem Sci* 31, 26-34

61. Holness, M.J., *et al.* (2003) Diabetogenic impact of long-chain omega-3 fatty acids on pancreatic beta-cell function and the regulation of endogenous glucose production. *Endocrinology* 144, 3958-3968

62. Pagliarini, D.J., *et al.* (2005) Involvement of a mitochondrial phosphatase in the regulation of ATP production and insulin secretion in pancreatic beta cells. *Mol Cell* 19, 197-207

63. Valente, E.M., *et al.* (2004) PINK1 mutations are associated with sporadic early-onset parkinsonism. *Ann Neurol* 56, 336-341