Elucidation of interactors of the pro-fusion proteins MFN1 and MFN2 sheds light on novel pathways regulating mitochondrial morphology in apoptosis

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Abstract
Large-scale proteomics analysis provide information on the proteomic signatures including interacting partners and post-translational modification. In the first part of the thesis, we identified a novel keratin-binding protein, Trichoplein (TpMs) that requires MFN2 to modulate mitochondrial shape and tethering. In the second part, we show that the pleiotropic mitogen activated protein (MAP) kinase phosphorylate the pro-fusion protein mitofusin (MFN) 1, switching its functions in apoptosis and mitochondrial fusion. A phosphoproteomic analysis revealed that MFN1 is phosphorylated at an atypical ERK1 site (T562) of its HR1 domain. This site proved essential to mediate the MFN1-dependent mitochondrial elongation and regulation of apoptosis by the MEK/ERK pathway. A T562 mutant mimicking constitutive phosphorylation of MFN1 triggered mitochondrial fragmentation, increased susceptibility to apoptosis, bound more avidly to the proapoptotic BCL-2 family member BAK and facilitated its activation. Our data identify a role for phosphorylation of MFN1 in mitochondrial shape and apoptosis.

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Elucidation of interactors of the pro-fusion proteins MFN1 and MFN2 sheds light on novel pathways regulating mitochondrial morphology in apoptosis

THÈSE
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"Elucidation of Interactors of the Pro-fusion Proteins MFN1 and MFN2 Sheds Light on Novel Pathways Regulating Mitochondrial Morphology in Apoptosis"

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1 Résumé
Les mitochondries sont des (Arnoult et al., 2005b) organelles très dynamiques essentielles à la fois pour la vie et la mort de la cellule. Elles produisent la quasi-totalité de l'ATP nécessaire pour la cellule (Danial et al., 2003), régulent la signalisation du Ca$^{2+}$ cytosolique (Rizzuto et al., 2000) et elles intègrent et amplifient différents stimuli apoptotiques (Green and Kroemer, 2004). Une telle versatilité fonctionnelle est rendue possible grâce à une morphologie et une ultrastructure complexes et dynamiques (Griparic and van der Bliek). Au niveau ultrastructuré, les cristae de la mitochondrie constituent un compartiment séparé connecté à un espace intermembranaire par des jonctions tubulaires étroites (Frey and Mannella, 2000). La nature dynamique des mitochondries leur permet de s'organiser en un réseau d'organelles qui fusionnent et se divisent de manière dynamique. La morphologie de la mitochondrie résulte de l'équilibre entre les processus de fission et de fusion, contrôlés par une famille de protéine ("mitochondria-shaping proteins") dont beaucoup sont des protéines apparentées à la dynamine, identifiées par criblage génétique chez les levures (Dimmer et al., 2002; Shaw and Nunnari, 2002). Les dynamines sont des mécano-enzymes ubiquitaires qui hydrolysent le GTP pour réguler les processus de fission, fusion, tabulation et élongation des membranes cellulaires (McNiven et al., 2000). Chez les mammifères, la fission mitochondriale est contrôlée par une dynamine cytosolique appelée DRP-1 (Smirnova et al., 2001) qui est recrutée vers les sites de fission où elle interagit avec un adaptateur récemment identifié, appelé Mff, au niveau de la membrane externe de la mitochondrie (Otera et al., 2010). La fusion est contrôlée par les mitofusine-1 (MFN-1) et -2 (MFN-2), deux larges GTPases de la membrane externe de la mitochondrie, orthologues de Fzo1p chez S. cerevisiae (Rapaport et al., 1998). OPA1, l’orthologue mammalien de Mgm1p chez S. cerevisiae, est la seule protéine dynamine-apparentée de la membrane interne mitochondriale (Olichon et al., 2002). Malgré la progression des connaissances sur les composants clés de la machinerie de la dynamique mitochondrial, notre compréhension de la régulation des différentes protéines de fusion est encore très épars.
Le but de ma thèse de PhD a donc été d'étudier les voies de signalisation et de régulation reposant sur les protéines de fusion et fission mitochondriales qui modulent la morphologie des mitochondries dans les cellules saine ou mourantes.
La fission des membranes mitochondriales par DRP1 est un processus complexe. La protéine DRP1 est relocalisée du cytosol vers la périphérie de la mitochondrie où elle se lie à son partenaire Mff, ce qui le stabilise sur la membrane externe de la mitochondrie.
Le mécanisme de fission nécessite ensuite l’oligomérisation de DRP1 en spirales tout autour de la mitochondrie, ce qui permet la fragmentation de cette organelle en deux fragments. En raison de cette complexité, il n’est pas surprenant que les modifications post-traductionnelles de DRP1 soient déterminantes pour réguler la morphologie de la mitochondrie (Santel and Frank, 2008). Alors que l’étude de la régulation fine de la fission mitochondriale a déjà été largement approfondie, la régulation de la fusion est beaucoup moins connue. Parmi les protéines de fusion, l’activité de la protéine OPA1 sur la membrane interne de la mitochondrie est régulée par son clivage post-traductionnel (Martinelli and Rugarli, 2010), tandis que la régulation des mitofusines localisées sur la membrane externe, à un endroit idéal pour la transduction des signaux cytosoliques vers la mitochondrie, est beaucoup moins bien caractérisée. L’immuno-purification à grande échelle des versions Flag-tag des protéines MFN1 et MFN2 dans les cellules MEF (mouse embryonic fibroblasts) suivi par une spectrométrie de masse LC-MS/MS a permis l’identification des protéines d’interaction et les sites de modifications post-traductionnelles. Une protéine appelée Trichoplein/mitostatin (TpMs), une protéine de fixation à la kératine qui co-localise partiellement avec les mitochondries et est fréquemment régulée dans les cancers épithéliaux, a été identifiée parmi les partenaires d’interaction de MFN2. Nous avons caractérisé sa fonction et montré que TpMs régule les contacts entre les mitochondries et le réticulum endoplasmique (RE) dépendamment de MFN2. Le fractionnement subcellulaire et les immuno-marquages ont montré que TpMs est présente à l’interface entre la mitochondrie et le RE. L’expression de TpMs entraîne la fragmentation des mitochondries et réduit les contacts avec le RE, tandis que la baisse d’expression de TpMs permet d’obtenir l’effet inverse. Sur le plan fonctionnel, la réduction des contacts mitochondries-RE par TpMs inhibe l’apoptose induite par des stimuli dépendants du calcium, qui nécessitent une juxtaposition RE-mitochondrie. Des preuves biochimiques et génétiques consolident le modèle selon lequel TpMs a besoin de MFN2 pour moduler la forme de la mitochondrie et le contact avec le RE (Cerqua et al., 2010). Ainsi, nos données ont contribué à identifier TpMs comme un nouveau régulateur de la juxtaposition mitochondrie-RE.

En analysant les modifications post-traductionnelles des mitofusines immuno-précipitées, nous avons identifié deux sites de phosphorylation pour MFN1 (T562 and T564), mais aucun pour MFN2. Nous avons créé des mutants non-phosphorylables simples ou doubles de MFN1 que nous avons purifié par chromatographie d’affinité en utilisant des phospho-colonnes qui lient spécifiquement les protéines phosphorylées. Cette approche
a permis d’identifier le site T562 comme un site majeur de phosphorylation qui repose sur une séquence consensus cible de ERK1. En effet, nous avons trouvé que ERK co-immunoprécipitait avec MFN1 mais pas avec MFN2. Etant donné que ERK1 interagit avec MFN1, nous avons décidé de regarder si des changements dans l’activité ou l’expression de ERK affectait la morphologie mitochondriale. L’expression d’un mutant de ERK constitutivement actif entraîne une fragmentation mitochondriale, tandis qu’une forme dominante-négative cause une élongation de cette organelle. Une approche génétique a confirmé que l’activation de ERK cause des changements de morphologie mitochondriale uniquement lorsque MFN1 est présent. L’expression d’un mutant non phosphorylable de MFN1 dans des cellules MEF Mfn1−/− a montré que l’effet de ERK1 sur la morphologie mitochondriale est dépendante des sites de phosphorylations identifiés par spectrométrie de masse. Puisque la voie des MAPK qui active ERK joue un rôle dans la régulation de l’apoptose, nous nous sommes demandé si la phosphorylation de MFN1 par ERK1 ne participait pas seulement au contrôle de la morphologie mitochondriale, mais également à la mort cellulaire. Nous avons montré que l’inhibition de ERK retardait l’apoptose induite par la staurosporine, H2O2 et l’étoposide, en corrélation avec une réduction du relargage du cytochrome c et une baisse de l’oligomérisation de Bak. Ces expériences nous ont amené à investiguer si le statut de phosphorylation de MFN1 par ERK1 était impliqué dans l’activation de Bak et la perméabilisation mitochondriale. En effet, Bak interagit avec les mitofusines et lors d’un stress cellulaire, bak se dissocie de MFN2 et s’associe principalement avec MFN1 (Brooks et al., 2007). Des expériences d’immunoprécipitation ont indiqué que MFN1 interagit avec Bak et que la force de cette interaction dépend du statut de phosphorylation de MFN1. Un mutant phospho-mimétique T562D de MFN1 interagit plus fortement, tandis qu’un mutant non phosphorylable interagit moins avec Bak. Sur le plan fonctionnel, l’oligomérisation de Bak après un stimuli apoptotique augmente dans les mitochondries exprimant MFN1 T562D. En conclusion, les données présentées dans cette thèse démontrent que la protéine pro-fusion MFN1 est régulée par la voie de signalisation MAPK par phosphorylation du résidu Thréonine 562. Cette phosphorylation de MFN1 affecte à la fois la morphologie de la mitochondrie et l’apoptose : lors de sa phosphorylation, MFN1 fixe plus fortement la protéine pro-apoptotique Bak, facilitant son oligomérisation, tandis que la mitochondrie se fragmente, ce qui suggère que la phosphorylation de MFN1 est moins efficace pour la fusion de ces organelles. Ainsi, nos données indiquent que la phosphorylation peut permettre de changer la fonction de MFN1 de pro-fusion à pro-apoptotique.
2 Summary

Mitochondria are very dynamic organelles essential for the life and death of the cell. They produce most of the cellular ATP required for the cell (Danial et al., 2003), regulate cytosolic Ca\(^{2+}\) signalling (Rizzuto et al., 2000), and integrate and amplify different apoptotic stimuli (Green and Kroemer, 2004). Such a functional versatility is possible due to a complex and dynamic ultrastructure and morphology (Griparic and van der Bliek). At the ultrastructural level, the mitochondrial cristae constitute a separate compartment connected to the thin intermembrane space by narrow tubular junctions (Frey and Mannella, 2000). The dynamic nature of mitochondria allows them to organize in a network of organelles that dynamically fuse and divide. Mitochondrial morphology results from the equilibrium between fusion and fission processes, controlled by a family of “mitochondria-shaping” proteins, many of which are dynamin-related proteins initially identified by genetic screens in budding yeast (Dimmer et al., 2002; Shaw and Nunnari, 2002). Dynamins are ubiquitous mechano-enzymes that hydrolyze GTP to regulate fusion, fission, tubulation and elongation of cellular membranes (McNiven et al., 2000). In mammals, mitochondrial fission is controlled by a cytosolic dynamin related protein DRP-1 (Smirnova et al., 2001) that translocates to sites of mitochondrial fragmentation where it binds to its newly identified adapter, Mff in the outer membrane (Otera et al., 2010). Fusion is controlled by mitofusin-1 (MFN1) and-2 (MFN2), two large GTPases of the outer mitochondrial membrane, orthologues of *S. cerevisiae* Fzo1p (Rapaport et al., 1998). OPA1, the mammalian homologue of *S. cerevisiae* Mgm1p, is the only dynamin-related protein of the inner mitochondrial membrane (Olichon et al., 2002). Despite our increasing knowledge on the core components of the mitochondria-shaping machinery, our understanding of the how the different pro-fusion shaping proteins are regulated is still scarce.

The aim of my PhD thesis has been therefore to study the signalling pathways and regulatory networks impinging on the mitochondrial-shaping proteins to modulate mitochondrial morphology in healthy and dying cells.

The fission of the mitochondrial membranes by DRP1 is a complex process. Cytosolic DRP1 translocates to the periphery of the mitochondria, where it binds to its partner Mff, which stabilizes it on the OMM. The process of fission further requires oligomerization of DRP1 into spirals around mitochondria that can efficiently break the mitochondria in two fragments. Given this complexity, it is no surprise that post-translational modifications in DRP1 are a key processes determining mitochondrial shape (Santel and Frank, 2008).
While the finely-tuned regulation of mitochondrial fission had been already appreciated, the regulation of fusion was much less clear. Among the fusion proteins, the activity of the inner mitochondrial membrane OPA1 was regulated by its post-translational cleavage, (Martinelli and Rugarli, 2010), whereas the regulation of the outer mitochondrial membrane (OMM) mitofusins, placed at an ideal location to transduce cytosolic signalling into mitochondrial shape changes, was much less characterized.

A large-scale immunopurification of Flag tagged MFN1 and MFN2 proteins from mouse embryonic fibroblasts (MEFs) followed by LC-MS/MS was useful to identify interacting proteins and sites of post-translational modification.

A protein called Trichoplein/mitostatin (TpMs), a keratin-binding protein that partly colocalizes with mitochondria and is often downregulated in epithelial cancers, was retrieved among the interactors of MFN2. We characterized its function and showed that TpMs regulates the tethering between mitochondria and endoplasmic reticulum (ER) in a MFN2-dependent manner. Subcellular fractionation and immunostaining show that TpMs is present at the interface between mitochondria and ER. The expression of TpMs lead to mitochondrial fragmentation and loosens tethering with ER, whereas its silencing has opposite effects. Functionally, the reduced tethering by TpMs inhibits apoptosis by Ca\(^{2+}\)-dependent stimuli that require ER-mitochondria juxtaposition. Biochemical and genetic evidence support a model in which TpMs requires MFN2 to modulate mitochondrial shape and tethering (Cerqua et al., 2010). Thus, our data contributed to identify TpMs as a new regulator of mitochondria-ER juxtaposition.

When we turned to the analysis of posttranslational modifications in the immunoprecipitated Mfn2s, we identified two sites of phosphorylation in MFN1 (T562 and T564), whereas no sites were retrieved in MFN2. To discriminate between the sites of phosphorylation, single and double phospho-mutants were created and subjected to purification by affinity chromatography using phosphocolumns that bind specifically phosphorylated proteins. This approach identified major site of phosphorylation in T562, which lies in a consensus ERK1 target sequence. In fact, we found that ERK co-immunoprecipitated with MFN1 but not MFN2.

Having established that ERK1 interacts with MFN1, we decided to investigate if changes in ERK activity or levels affected mitochondrial morphology. Expression of a constitutively active mutant of ERK caused mitochondrial fragmentation, whereas a dominant negative mutant caused elongation of the organelle. A genetic approach confirmed that the activation of ERK caused changes in mitochondrial morphology only when MFN1 was
present. Reconstitution of Mfn1−/− MEFs with non phosphorylable MFN1 mutants indicated that the effect of ERK1 on mitochondrial morphology was dependent on the site identified in the LC-MS/MS approach.

Since the mitogen activated protein kinase (MAPK) pathway that activates ERK plays a key role in the regulation of apoptosis, we wondered whether the phosphorylation of MFN1 by ERK1 participated not only in the control of mitochondrial morphology, but also of cell death. We found that inhibition of ERK delayed apoptosis by staurosporine, H2O2 and Etoposide, correlating with a reduction in cytochrome c release and with decreased levels of BAK oligomerization. These experiments prompted us to investigate if the status of ERK1 mediated phosphorylation of MFN1 is involved in BAK activation and BAK-mediated mitochondrial permeabilization. In fact, BAK interacts with the mitofusins and during cellular stress, BAK dissociates from MFN2 and it associates primarily with MFN1 (Brooks et al., 2007). Immunoprecipitation experiments indicated that MFN1 interacts with BAK and that the strength of the interaction depends on the phosphorylation state of MFN1: a phosphomimetic T562D mutant of MFN1 interacted more, whereas the non phosphorylable mutant T562A mutant interacted less with BAK. Functionally, BAK oligomerization following apoptotic stimuli was increased in mitochondria expressing MFN1T562D.

In conclusion, the data presented in this thesis demonstrate that pro-fusion protein MFN1 is regulated by the MAPK signalling pathway through the phosphorylation at its threonine 562 site. This phosphorylation of MFN1 affects both mitochondrial morphology and apoptosis: upon its phosphorylation, MFN1 binds more avidly to the pro-apoptotic protein BAK, facilitating its oligomerization, and mitochondria become fragmented, suggesting that the phosphorylated MFN1 is less efficient in fusing the organelles. Thus, our data indicate that phosphorylation can switch the function of MFN1 from pro-fusion to pro-apoptotic
3 Introduction

Mitochondria are one of the most important organelles of the cell. They convert most of the dietary energy and participate in the programmed cell death pathways. In addition, they are involved in many other processes, including Ca\textsuperscript{2+} homeostasis, cellular differentiation, control of cell cycle and growth, amplification of signaling cascades. These vital functions implicate mitochondria in many human disorders, including neurodegenerative and cancer.

The plethora of function carried out by mitochondria is matched with its elaborate and complex structure. Ultrastructurally, mitochondria can be subdivided into various components: mitochondria are surrounded by the Outer Mitochondrial membrane (OMM) and the Inner Mitochondrial Membrane (IMM). The IMM is further organized in distinct compartments, the peripheral inner membrane and the 	extit{cristae} that are separated from the peripheral inner membrane by narrow tubular junction. This complex ultrastructural pattern is more complicated by the fact that mitochondria display different shapes and sizes according to the need of the cells: they could appear as fragmented, puncta like structures, or linked in elongated network. The shape of mitochondria is determined by a set of “mitochondria-shaping proteins” that impinge on the equilibrium between fusion and fission processes.

3.1 Mitochondria

Mitochondria are crucial organelles taking part in many cellular pathways. They were described for the first time by the anatomist Rudolf Albrecht von Koelliker, who called them “sarcosomes”. They are regarded as the “power house” of the cells, due to their ability to produce most of the ATP required for the enoergonic cellular processes. However, in the mid 90’s mitochondria were found to be participating in Ca\textsuperscript{2+} signaling (Jouaville et al., 1995) and to amplify the apoptotic signals required for the programmed...
cell death, releasing cofactors that trigger the activation of effector caspases (Wang, 2001). Defects in any of these processes are catastrophic for the cells leading to several pathological conditions including cancer or neurodegenerative disease (Schapira, 1999). Mitochondria are the only organelles beside nucleus that possess their own DNA. In vertebrates, mitochondrial DNA (mtDNA) consists of a double stranded circular DNA molecule of about 16.5 kb. The mtDNA encodes 13 mRNAs of subunits of the respiratory chain as well as the tRNAs required for the synthesis of these proteins (Fernandez-Silva et al., 2003). Mitochondria indeed contain their own ribosomes where the translation of the mtDNA encoded proteins takes place. This feature of mitochondria supports the endosymbiotic theory, according to which mitochondria have an ancestral extracellular origin, deriving from primordial rickettsia-like intracellular bacteria that colonized the eukaryotic cell, eventually becoming essential for its life (Margulis, 1971).

3.1.1 Mitochondrial dynamics

Mitochondria are highly dynamic organelles whose location, size and distribution vary in different physiological conditions and in different cell types. The different mitochondrial morphologies can reflect the numerous mitochondrial functions. Mitochondria are the powerhouse of the cell, converting most of the energy into the ATP required for the endergonic processes, are involved in different metabolic pathways (Ernster and Schatz, 1981), control Ca$^{2+}$ signaling (Rizzuto et al., 2000) and finally they are core apoptotic components that amplify death signals (Green and Kroemer, 2004).

The complex ultrastructure and morphology of mitochondria depend on the equilibrium between fusion and fission processes regulated by “mitochondria-shaping” proteins. The majority of the mitochondria shaping proteins are dynamin-like large GTPases that are conserved from yeast to mammals (Dimmer and Scorrrano, 2006). In mammalian cells, the fusion of mitochondria is regulated by mitofusins (MFN) 1 and 2 (Fzo1 in yeast)
localized at the outer mitochondrial membrane (OMM) (Santel and Fuller, 2001) and Opa1 (Mgm1 in yeast) localized in the Inner mitochondrial membrane (IMM) (Cipolat et al., 2004). The division of mitochondria is regulated by DRP1 (Dynamin related protein 1, Dnm1 in yeast) (Smirnova et al., 2001), which translocates from the cytosol to mitochondria binding to its partner MFF (mitochondrial fission factor), located in the outer mitochondrial membrane (Otera et al., 2010). These proteins are not only responsible for the physiological cycles of mitochondrial fusion and fission, but also for the changes in mitochondrial shape that are often observed in several pathophysiological conditions. For example, mitochondrial shape must be adjusted during the formation of dendritic spines (Li et al., 2004), cellular migration (Campello et al., 2006) & during mitosis (Mitra et al., 2009) calling for complex mechanisms of fine regulation. Similarly, during apoptosis (Green and Kroemer, 2004) and programmed necrosis (Wang et al., 2012) mitochondria fragment and the cristae are remodeled (Scorrano et al., 2002). One established mechanism of regulation resides in the ability of the mitochondria shaping proteins to interact with each other and with numerous other proteins residing in and outside mitochondria. Another level of regulation is represented by their post-translational modification. Phosphorylation, ubiquitination, sumoylation and s-nitrosylation are being increasingly recognized as critical players in controlling mitochondria shaping proteins and therefore mitochondrial morphology. Here we will summarize the most recent findings on how posttranslational modifications impact on mitochondrial shape in healthy and dying cells.

3.1.2 Mitochondrial Fission

In mammalian cells, mitochondrial division is regulated by DRP1 (Smirnova et al., 2001), as testified by the highly interconnected mitochondrial network resulting from inhibition or downregulation of DRP1. DRP1 belongs to the dynamin family of GTPases which are
involved in tubulating and constricting membranes leading to their scission. Like classical
dynamins, DRP1 is found predominantly in the cytosol and structurally shows a GTPase
domain, a GTPase effector domain (GED) and a central domain. During fragmentation of
mitochondria, DRP1 translocates to mitochondria where it binds to MFF, a c-tail
anchored protein localized in the outer mitochondrial membrane recently characterized to
be the most prominent receptor for DRP1 in mammals (Otera et al., 2010). Fis1p acts as
the receptor for Dnm1 in yeast (Mozdy et al., 2000). DRP1 is recruited to mitochondria in
response to cellular and mitochondrial cues, where constriction of the membranes takes
place (Yoon et al., 2003). The translocation of DRP1 is regulated by phosphorylation and
dephosphorylation, whereas the stabilization at the OMM is enhanced by sumoylation.
We will further elaborate on the signals that control mitochondrial fission in greater detail
below. Mitochondrial fission is important to distribute equal numbers of mitochondria to
the two daughter cells during mitosis, as well as to clear old or damaged mitochondria
from the cell through a selective autophagic process known as mitophagy (Twig et al.,
2008). Excess fission leading to a completely fragmented mitochondrial network can in
principle lead to a heterogeneous population of organelles where mitochondrial DNA
distribution is non-uniform, reducing the capacity to generate ATP and increasing the
cellular susceptibility to apoptosis (Parone et al., 2008).

3.1.3 Mitochondrial Fusion

Fusion of OMM is carried out by two dynamin-related GTPases –mitofusins (MFN) 1 and
2 (Santel and Fuller, 2001). They display a very high degree of homology and accordingly
a similar structure composed of an N-terminal GTPase domain, two hydrophobic heptads
repeats (HR) flanking two transmembrane domains that insert them in the OMM.
Nevertheless, they are not functionally equivalent. The GTPase activity of MFN1 is
higher, but affinity towards GTP is lower compared to that of MFN2 (Ishihara et al., 2004).
Accordingly, MFN1 is specialized in mitochondria tethering by antiparallel interaction of HR2 of proteins from mitochondria in trans, whereas MFN2 has additional functions as a signaling molecule as well as a scaffold for multi-complex formation in tethering membranes (de Brito and Scorrano, 2008b). In addition, levels of MFN2 correlate with the oxidative metabolism of skeletal muscle (Bach et al., 2003) and the proliferation ability of vascular smooth muscle cells, by sequestering the proto-oncogene RAS (Chen et al., 2004). MFN2 also controls the shape of ER and tethers it to mitochondria (de Brito and Scorrano, 2008a). Finally, mutations in MFN2 are associated with Charcot-Marie-Tooth type Ila peripheral neuropathy (Zuchner et al., 2004). Fusion of the outer membrane is coordinated with that of the inner membrane, orchestrated by Optic atrophy 1 (OPA1), the only dynamin-like GTPase so far identified in the inner mitochondrial membrane, whose mutations are associated with dominant optic atrophy (DOA), the most common cause of inherited optic neuropathy (Alexander et al., 2000; Delettre et al., 2000). OPA1 exists in eight different splice variants, and its function appears to be tightly regulated by posttranscriptional mechanisms that include its proteolytic processing (Akepati et al., 2008). OPA1 requires MFN1 to mediate fusion (Cipolat et al., 2004), and it has an additional function fusing the inner mitochondrial membrane (Meeusen et al., 2006) along with maintaining of the cristae junction, which controls the release of the apoptotic factor cytochrome c (Frezza et al., 2006).

3.2 Regulation of mitochondrial dynamics

Mitochondria exist as different shape according to the need of the cell: accordingly, the continuous fusion and division occurring in cellular physiology determines the common morphology consisting of a mixture of long tubular mitochondria and puncta, short ones. However, cellular stress or different pathologies lead to remodelling of mitochondrial
network, often resulting in most of the mitochondria appearing as a single spherical structure (Martinou and Youle, 2011). In other cases, like cellular stress or nutrient deprivation, mitochondria tend to form a long interconnected network which is protective against mitochondrial degradation and activation of the mitochondrial cascade of apoptosis (Gomes et al., 2011; Tondera et al., 2009). Clearly, these different changes must result from the modulation of the mitochondria-shaping machinery that we have described above, to eventually determine shape and the size of the mitochondria. The appreciation of this integration of mitochondrial morphology into cellular signaling has promoted extensive research into the mechanism and the consequences of post-translational modifications of the mitochondria-shaping machinery. We will now analyze how signaling cascades and posttranslational modifications of mitochondria-shaping proteins affect mitochondrial morphology and physiology.

3.2.1 Post-translational modifications of DRP1

The fission of the mitochondrial membrane carried out by DRP1 is a complex process. Cytosolic DRP1 must translocate to the periphery of the mitochondria, where it can bind to its partner MFF, which stabilizes it on the OMM. The further process of fission requires oligomerization of the protein, forming a spiral around mitochondria that can efficiently break the mitochondria in two fragments. Given this complexity, it is no surprise that post-translational modifications in DRP1 are a key processes determining mitochondrial shape.

One of the key cellular processes during which mitochondrial shape must be regulated is cell cycle. First, during interphase in most cellular types, mitochondria appear long and tubular, clearly impeding the adequate redistribution of an even number of mitochondria in the two daughter cells during mitosis. Indeed, during the early mitotic stage
mitochondria are fragmented in puncta like structures. This fragmentation was found to be DRP1 dependent, and further analysis showed that DRP1 could be phosphorylated by cyclin dependent kinase 1 (Cdk1/cyclinB) (Taguchi et al., 2007). This process is mediated by the small RAS-like GTPase RALA and its effector RALBP1. Specifically, the mitotic kinase Aurora A phosphorylates Ser 194 of RALA, re-localizing it to the mitochondria, where it concentrates RALBP1 and DRP1. Furthermore, RALBP1 is associated with cyclin B–CDK1 kinase activity that leads to phosphorylation of DRP1 on Ser 616 (Kashatus et al., 2011).

A more widely studied and controversial site is the S637, which has been found to be phosphorylated by cyclic AMP-dependent protein kinase (PKA), and Ca2+/calmodulin-dependent protein kinase Iα (CaMKIα). Phosphorylation at this site has an opposing effect. CaMKIα phosphorylation increases DRP1 translocation to mitochondria inducing mitochondrial fission (Han et al., 2008). Conversely, phosphorylation of this site by PKA inhibits mitochondrial fission by two proposed mechanisms: reduction of the GTPase activity of DRP1 (Chang and Blackstone, 2007), or inhibition of its oligomerization and translocation to mitochondria, which is conversely stimulated by the dephosphorylation of this site, but not of the Ser 616, by the Ca2+-dependent phosphatase calcineurin (PP2A) (Cribbs and Strack, 2007; Cereghetti et al., 2008). Further insights into the mechanism of how these post-translational modifications modulate mitochondrial fission were prompted by the modeling of DRP1 on the recently solved structure of its homologue dynamin-1 (Ford et al., 2011). DRP1 possesses a variable domain (VD) between the two middle (MID) and GTP exchange domain (GED) that assemble in the stalk of the protein. All the post-translational modifications of DRP1 occur in the VD, which is ending with the highly conserved Ser 637. Once this residue is phosphorylated in the cytosol by PKA, the assembly of short DRP1 oligomers exposing the residue Arg 376, essential for binding to the mitochondrial receptor MFF, is not favored and DRP1 is retained in the cytosol.
Conversely, when the same residue is phosphorylated by mitochondrial anchored PKA, DRP1 is sequestered in large complexes that are not active (Strack and Cribbs, 2012). The different effects of cytosolic and mitochondrial PKA on translocation vs. activity offers a potential explanation for the contrasting reports on the effect of PKA on GTPase activity of DRP1 (Cribbs and Strack, 2007; Chang and Blackstone, 2007).

Recently, this site emerged as an important site for the regulation of mitochondrial function in autophagy and cell death. Upon induction of autophagy by starvation or other means that impinge on the master metabolic regulator mTOR, mitochondria elongate in response to PKA activation that phosphorylates DRP1 at Ser 637, keeping it in the cytosol and allowing unopposed mitochondrial fusion (Rambold et al., 2011; Gomes et al., 2011). Elongated mitochondria are protected from autophagic elimination and have a higher density of cristae, which favors oligomerization of ATPase to maintain ATP production and to allow survival of starving cells (Gomes et al., 2011).

The opposing effect of mitochondrial fragmentation in cellular models of Huntington’s Disease depends on the elevated amount of calcineurin, which dephosphorylates DRP1 at Ser 637, leading to increased translocation of DRP1 to mitochondria (Costa et al., 2010). Another example of mitochondrial morphology regulation through the phosphorylation at this site is the Siah2 dependent fragmentation. During hypoxia, Siah2 regulates the level of AKAP121, which is responsible for facilitating the inhibitory phosphorylation of DRP1 (Kim et al., 2011).

Excessive fission is also observed in other pathological conditions like Parkinson’s (PD) and Alzheimer’s disease (AD). In the case of AD, the toxic pathogenic Aβ peptide may be imported in human brain mitochondria inhibiting the respiratory chain. Nitric-oxide is produced in this process, inducing S-nitrosylation of the same VD of DRP1, which in turn causes excessive mitochondrial fission, synaptic loss, and neuronal damage (Cho et al., 2009). However, during myogenic differentiation, the elongation of the short myoblast
mitochondria into the extensively elongated network observed in myotubes appears to be a consequence of the inhibition of DRP1-dependent mitochondrial fission by NO (De et al., 2010). In addition, the role of S-nitrosylation on DRP1 activity in the context of AD has been challenged (Bossy et al., 2010), further confusing the interpretation of the effects of NO on mitochondrial fission.

The VD domain also contains all the 8 sites where DRP1 is sumoylated by the mitochondrial SUMO E3 ligase (MAPL) (Figueroa-Romero et al., 2009) to regulate its stability and therefore mitochondrial morphology (Braschi et al., 2009; Wasiak et al., 2007; Harder et al., 2004). DRP1 sumoylation is antagonistically regulated by the SUMO protease SENP5 (Zunino et al., 2007) that resides primarily within the nucleoli and during the G2/M phase translocates to the mitochondrial membrane to deSUMOylate DRP1 (Zunino et al., 2009). Antagonistically, DRP1 stability is also controlled by the ubiquitin proteasome system via its ubiquitination. MARCH-V was the first ubiquitin ligase found to ubiquitinate DRP-1 (Nakamura et al., 2006). MARCH-V might even play a general role in mitochondrial quality control, ubiquitinating damaged proteins of the outer membrane MOM (Yonashiro et al., 2006; Sugiura et al., 2011; Yonashiro et al., 2009).

The list of E3 ligases that influence DRP1 degradation include Parkin, whose familial PD mutations affect its ability to ubiquitinate DRP1 and therefore lead to its accumulation and mitochondrial fragmentation (Wang et al., 2011), and the APC/C^Cdh1 (anaphase-promoting complex/cyclosome and its coactivator Cdh1) E3 ubiquitin ligase complex that degrades DRP1 to allow mitochondrial elongation after release from mitotic arrest (Horn et al., 2011).

The list of post-translational modifications of DRP1 recently grew to include O-GlcNAcylation of Thr-585 and Thr-586. The inhibition of N-acetyl-glucosaminidase, leads to an increase in GTP-bound active DRP1, to its mitochondrial translocation and to mitochondrial fragmentation (Gawlowski et al., 2012). This modification occurs together
with the dephosphorylation of Ser637 upon exposure to high glucose and is detected in models of type II diabetes, suggesting that mitochondrial fission could participate in the pathogenesis of certain complications of this chronic disease.

3.2.2 Post-translational modifications of OPA1

The pro-fusion protein OPA1 which is localized in the inner mitochondria membrane and possesses a fusion independent role in maintaining the tight junctions of cristae (Frezza et al., 2006), is an example of how intricate is the regulation of mitochondrial morphology and ultrastructure. First, OPA1 exists in 8 different splice variants in H. sapiens and in 7 variants in M. musculus. In mammals, the splice variants differ for the region immediately downstream of the trans-membrane domain and therefore for their ability to be processed by the different proteases that have been recognized to process OPA1 to generate its mature forms (for the sake of clarity, we will call isoforms the splice variants of OPA1 and forms the different mature OPA1 proteins). Some attempts have been made to assign a biological function to each isoform, identifying in the presence of the exons 4b and 5b a requisite for participation in the control of cristae remodeling and apoptosis (Olichon et al., 2007). Such a role is likely to be related to the importance of this OPA1 region in its processing by the different proteases that have been identified to participate in the maturation of OPA1. The quest to understand how maturation of OPA1 was linked to the control of mitochondrial function and morphology started by the discovery that in yeast Mgm1, the homolog of OPA1, is processed by a mitochondrial rhomboid protease called Rbd1p or Pcp1p in a short, soluble and in a long, inner membrane integral form (Herlan et al., 2003; McQuibban et al., 2003) that are both required for the regulation of inner membrane structure and for mitochondrial fusion (DeVay et al., 2009; Meeusen et al.,
2006). While in S. cerevisiae this rhomboid protease is the only protease responsible for the posttranslational modification of Mgm1 and therefore for its alternative topology (Herlan et al., 2004), the picture in mammals is much more complicated and several proteases have been called upon to generate the mature forms of OPA1.

In mammals, several proteases process OPA1 at different sites, regulating the balance between long (L) - and short (S) -OPA1 forms. The matrix localised m-AAA proteases AFG3L1 and −2 and paraplegin and the intermembrane space i-AAA protease Yme1 participate in the constitutive cleavage of OPA1 at different sites. In addition to the constitutive cleavage that is required for the production of fusion-competent OPA1, OPA1 also undergoes processing by the peptidase OMA1 in response to cellular stress. This latter cleavage inactivates OPA1 and blocks mitochondrial fusion (for a comprehensive review, see (Martinelli and Rugarli, 2010)). Finally, and subsequently, the mammalian homologue of Pcp1p, called PARL, generates a pool of OPA1 soluble in the IMS (Herlan et al., 2004). This soluble OPA1 is crucial for the anti-apoptotic effects of PARL because it participates in the formation of the OPA1 oligomer that maintains the bottleneck configuration of the cristae and therefore the compartmentalization of cytochrome c. Genetic ablation of PARL reduce the levels of this soluble S-OPA1, widening the cristae junctions, accelerating cytochrome c release and eventually increasing the susceptibility to apoptosis (Cipolat et al., 2006).

In conclusion, the precise balance of proteolytic processing of OPA1 by a number of mitochondrial proteases is required for its pro-fusion and anti-apoptotic functions.
3.2.3 Post-translational modifications of Mitofusins

3.2.3.1 Ubiquitination

The first evidence that mitochondrial fusion could be regulated by the ubiquitin-proteasome pathway came from the studies in yeast. Levels of Fzo1p, the yeast orthologue of mammalian mitofusins, are regulated by the F-box protein Mdm30p through ubiquitination and proteasome dependent degradation (Escobar-Henriques et al., 2006). In mammals, MARCH-V was proposed to catalyze the ubiquitination of MFN1, since the depletion of MARCH-V increased levels of MFN1 and fusion (Park et al., 2010). An impulse to the research in this field came from the discovery that in Drosophila Mitofusins are the targets of the ubiquitin ligase Parkin, mutated in autosomal recessive juvenile forms of Parkinson disease. Loss of Drosophila Parkin causes an increase in MFN abundance in vivo and concomitant elongation of mitochondria and Parkin directly mediates the ubiquitination (Ziviani et al., 2010). Ubiquitinated dMfn is lost and the levels of dMfn1 stabilized in drosophila mutants lacking Pink or Parkin suggesting that dMfn is a direct substrate of Parkin in Drosophila (Poole et al., 2010). This study has been extended to mammalian system, where both MFN1 and MFN2 have been shown to be ubiquitinated by PARKIN in dopaminergic SH-SY5Y cells, causing mitochondrial fragmentation in a thought to be important for degradation of damaged mitochondria (Gegg et al., 2010). However, another report demonstrated that PARKIN ubiquitinates specifically MFN1 in the SH-SY5Y cells enhancing the turnover of MFN1 by proteasomal degradation (Glauser et al., 2011). PD-associated mutations impair the PARKIN-mediated ubiquitination of MFN1, which could explain why mitochondria appear elongated in mammalian model of Parkinson disease (Park et al., 2009).
3.2.3.2 Phosphorylation

The presence of a putative protein kinase A (PKA) phosphorylation site at Serine 442 (S442), which when mutated increased the inhibitory effects of MFN2 on proliferation of vascular smooth muscle cells (VSMCs) in culture, and neointimal hyperplasia and restenosis in the rat carotid artery balloon injury model, indicated MFN2 could be regulated directly by phosphorylation (Zhou et al., 2010). More recent finding reports direct phosphorylation of MFN2 at Serine 27 (S27) by stress-induced Jun N-terminal kinase (JNK), which becomes activated in response to genotoxic stresses including doxorubicin and other cellular stresses. Phosphorylation by JNK leads to recruitment of HECT domain ubiquitin ligase (E3) Huwe1, leading to enhanced ubiquitination and proteasomal degradation of MFN2 (Leboucher et al., 2012). This is the first direct phosphorylation reported for the mitofusins, and it should not be before long that additional signaling cascade phosphorylating MFN1 is discovered.

3.2.3.3 Cysteine oxidation

Mitochondrial hyperfusion has been shown to be the early stress response due to various cellular stress (Tondera et al., 2009; Gomes et al., 2011). New report suggests that oxidized glutathione (GSSG), the core cellular stress indicator, induces the formation of disulfide bonds between two MFN2 molecule forming oligomers, required for the mitochondrial fusion (Shutt et al., 2012).

3.3 Mitochondrial dynamics in cell death: apoptosis, autophagy and necroptosis.

In the recent years, the availability of molecular tools to explore the fate of mitochondrial shape during different forms of cell death has considerably increased our knowledge on
the mechanisms and the consequences of morphological changes in apoptosis, autophagy and more recently necroptosis. Here we will review the evidence assigning a role to the posttranscriptional modification cascades impinging on the mitochondria-shaping machinery in these forms of cell death.

Upon the induction of apoptosis, mitochondria release pro-apoptotic proteins including cytochrome c in a process controlled by the proteins of the BCL-2 family (Scorrano and Korsmeyer, 2003). Two morphological processes have been discovered to participate in the release of cytochrome c and other pro-apoptotic factors from mitochondria: DRP-1 dependent fragmentation (Frank et al., 2001) and OPA1-dependent remodeling of the cristae (Scorrano et al., 2002). Many excellent reviews overview the molecular mechanisms, the physiological consequences and the role in apoptosis of these two processes (see for example (Westermann, 2010)). Here we will briefly summarize the basic molecular mechanisms of these two processes and discuss how post-translational modifications can regulate them.

The process of cristae remodeling was originally discovered to participate in the amplification of cytochrome c release by allowing the majority of this molecule, normally stored in the cristae, to reach the intermembrane space from where it leaves mitochondria across selective pathways in the outer membrane. This process is regulated by OPA1 that, as described above, participates in the maintenance of cristae tightness. Many BH3-only molecules of the BCL-2 family target the OPA1-containing oligomers causing their disruption and cristae remodeling (Landes et al., 2010; Yamaguchi et al., 2008). In addition to cristae remodeling, DRP1-dependent mitochondrial fragmentation similarly amplifies the release of cytochrome c from mitochondria in the intrinsic pathway of apoptosis (Frank et al., 2001). To explain why DRP1 accumulation on mitochondria triggers the release of cytochrome c, two mechanisms have been proposed: DRP1-dependent mitochondrial fission can be associated with cristae remodeling, when
triggered by the BH-3 only molecule BIK (Germain et al., 2005) and by several intrinsic stimuli (Costa et al., 2010); alternatively, DRP1 stimulates tBid-induced BAX oligomerization and cytochrome c release by promoting tethering and hemifusion of membranes in vitro (Montessuit et al., 2010). In addition to DRP1 dependent mitochondrial fission, during apoptosis mitofusin-dependent mitochondrial fusion is inhibited (Karbowski et al., 2004), contributing to the fragmented phenotype observed in apoptotic cells.

An outstanding question in the field is how fragmentation and cristae remodeling are promoted during apoptosis. While in the latter case, OPA1 oligomers appear to be directly targeted by the BH3 only molecules of the BCL-2 family, fragmentation seems to be a more complex process that involves activation of DRP1 and inhibition of MFNs and both processes are likely to be regulated by post-translational modifications of the mitochondria-shaping proteins.

During the initial stages of apoptosis, Bax translocates to discrete foci on mitochondria, which subsequently become mitochondrial fission sites. Surprisingly, DRP1 and MFN2, but not other mitochondria-shaping proteins, colocalize with BAX in these foci (Karbowski et al., 2004). Once the foci are formed, Bax/BAK promote the sumoylation of DRP1, stabilizing it at the mitochondrial fission sites, stopping its normal cycling from cytosol to mitochondria, and ultimately initiating massive apoptotic fragmentation (Wasiak et al., 2007). These evidences clarify how DRP1 accumulates on apoptotic mitochondria, but do not indicate what triggers its translocation from the cytoplasm. One natural candidate is DRP1 dephosphorylation by calcineurin, implied in its mitochondrial translocation upon cytoplasmic Ca\(^{2+}\) rise. For example, a specific polypeptide (PPD1) from the immunophilin FKBP52 inhibits calcineurin activation, DRP1 translocation to mitochondria and fragmentation of the organelle ultimately delaying apoptosis by intrinsic stimuli (Cereghetti et al., 2010). Similarly, phosphorylation of DRP1 by PKA at the same residue
Dephosphorylated by calcineurin inhibits mitochondrial fission and increases resistance to apoptotic stimuli (Cribbs and Strack, 2007).

Dephosphorylation of DRP1-Ser637 is also implicated in programmed necrosis. Programmed necrosis is induced by tumor necrosis factor-α (TNFα), requires the activation of the kinase receptor interacting protein (RIP) 1 and 3, of the mixed lineage kinase domain-like (MLKL), and the mitochondrial protein phosphatase PGAM5 (Wang et al., 2012). Upon necrosis induction, the PGAM5 complex on the mitochondrial membrane activates DRP1 by dephosphorylating Ser637, leading to mitochondrial fragmentation, dysfunction and entry into programmed necrosis.

The opposing modification of Ser637 occurs during macroautophagy. Autophagy is a stereotypical response to limited nutrient availability that result in the degradation of cytoplasmic components and organelles that are cargoed to the lysosomes, where they are degraded into their elementary constituents and recycled to provide energy for the cellular needs. This metabolic importance of autophagy clashes with the possibility that mitochondria, the most efficient thermodynamic converters of the cell, are randomly targeted and degraded. Two mechanisms can be envisioned in order for mitochondria to be spared from autophagy: a sterological hindrance that makes these organelles incompatible with the size of the nascent autophagosomes, or the lack of “eat-me” signals on their surface. Converging evidence supports the first model: in response to starvation or to inhibition of the master sensor of nutrient availability mTOR (known triggers of autophagy), PKA is activated and phosphorylates Ser637 of DRP1, keeping it in the cytosol and leading to mitochondrial elongation as a consequence of unopposed fusion (Rambold et al., 2011; Gomes et al., 2011). The activation of PKA is triggered by the lack of aminoacids, rather than of glucose, and the process of DRP1-dependent mitochondrial elongation is reversible (Gomes et al., 2011). Functionally, mitochondrial elongation during autophagy not only preserves the organelle from autophagic degradation, but it
also increases their ATP output by increasing the cristae density and the dimerization of the ATP synthase (Gomes et al., 2011; Gomes and Scorrano, 2011).

While the posttranslational modifications of DRP1 are well established players in the course of apoptosis, necrosis and autophagy, our knowledge of how mitochondrial fusion is affected during cell death is scarcer. Inhibition of fusion is an established consequence of the induction of apoptosis and that it occurs around the time of BAX activation (Karbowski et al., 2004). However, the molecular determinants of this inhibition are not fully established. One possibility is that fusion is blocked as a consequence of mitochondrial outer membrane permeabilization and of the release of cytochrome c as well as of the soluble fraction of OPA1 (Arnoult et al., 2005b; Arnoult et al., 2005a). In addition, proteolytic cleavage of the long OPA1 forms (required for efficient fusion (DeVay et al., 2009)) occurs as a consequence of mitochondrial dysfunction (Duvezin-Caubet et al., 2006) and could also mediate the inhibition of mitochondrial fusion. However, as we said above, inhibition of fusion occurs earlier, before or around the activation of BAX, raising the question of its mechanism. For sure, proapoptotic BCL-2 family members like BAK and BAX can alter mitochondrial morphology by interacting with MFNs. Mitochondria appear fragmented in cells lacking BAK and BAX (Karbowski et al., 2006). In healthy cells, they promote the activity of MFN2 by facilitating its assembly. Conversely, during apoptosis, BAK dissociates from MFN2 and enhances the association with MFN1 (Brooks et al., 2007). These findings seem to suggest that the increased association of BAK with MFN1 could attenuates the rate of fusion during apoptosis. Alternatively, however, MFN1 could participate in the oligomerization of BAK and the outer membrane permeabilization. MFN1 could then shuttle between a form competent in fusion ad a second one that associates with BAK to promote outer membrane permeabilization. Conceivably, this switch could depend on intracellular signaling cascades that are activated during apoptosis and that could modify MFN1 to switch its function during cell death.
3.4 The MAPK pathway

Protein kinases play an important role in communicating signalling messages to the substrate through the means of phosphorylation. They are the crucial components of the signalling network that allows cells to function as an integral part of an organism. One of the most widely studied family of protein kinases, the mitogen-activated protein kinases (MAPKs, Figure 1) are well conserved in evolution and transmit signals from their regulated gene expression during normal cell proliferation, survival and differentiation. Due to its housekeeping activities in the physiological function of the cells, abnormal regulation of the MAPK pathways have been reported for a wide ranges of diseases including many cancers (Roberts and Der, 2007), obesity, diabetes (Hirosumi et al., 2002), polycystic kidney diseases (Omori et al., 2006) and neurodegenerative disease like Alzheimer's disease (Giovannini et al., 2008). This pathway is often activated in certain tumors by chromosomal translocations such as BCR-ABL, mutations in cytokine receptors such as Flt-3, Kit, Fms or overexpression of wild type or mutated receptors, e.g., EGFR (McCubrey et al., 2007). The RAF/MEK/ERK pathway also impinges on mitochondria apoptosis pathway by the post-translational phosphorylation of apoptotic regulatory molecules including BAD, BIM, MCL-1, caspase 9 and more controversially BCL-2. The importance of this pathway is also highlighted by the diverse effects on cell cycle progression, apoptosis and differentiation (Steelman et al., 2004). Mammalian cells possess four well characterized and widely studied MAPKs. These cascades are comprised of three protein kinases that act as a signaling relay controlled, in part, by protein phosphorylation: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK (Johnson and Lapadat, 2002). MAPK cascades function downstream of cell surface receptors and other cytoplasmic signaling proteins whose functions are deregulated in cancer and other human pathologic disorders. Of the four MAPK pathways
Figure 1: Mitogen-Activated Protein Kinase (MAPK) pathways

(Figure 1), we are going to introduce and focus on the extracellular signal-regulated kinase (ERK) MAPK pathway.

3.4.1 MAPK Hierarchy

3.4.1.1 RAS – an upstream activator

RAS is a small GTP-binding protein, with four isoform identified to date: Ha-RAS, N-RAS, Ki-RAS 4A and Ki-RAS 4B. RAS proteins show varying abilities to activate the RAF/MEK/ERK and PI3K/Akt cascades. RAS undergoes complex post-translational modification for its target to the different membranes. For RAS to be targeted to the cell membrane, it must be farnesylated by farnesyl transferase (Ha-, Ki-, and N-RAS) or geranylgeranylated by geranylgeranyl transferase (N- and Ki-RAS). RAS preferentially
undergoes farnesylation, however, in the presence of farnesylation inhibitors, N-RAS and Ki-RAS can undergo geranylgeranylation. Extracellular stimuli such as growth factors, cytokines, mitogens, hormones and oxidative or heat stress (Cobb and Goldsmith, 1995) trigger a signal by interacting with a multimolecular complex of receptors such as receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) or epidermal growth factor receptor (EGFR) (Mebratu and Tesfaigzi, 2009). These receptors transmit activating signals by recruiting SOS (son of sevenless), a RAS-activating guanine nucleotide exchange factor through the adaptor protein growth-factor-receptor-bound-2 (Grb 2) to stimulate RAS and convert GDP to GTP. The GTP bound active RAS can then recruit RAF to cell membrane.

3.4.1.2 RAFT and its complex activation process

RAF is a serine/threonine (S/T) kinase and is normally activated by a complex series of events including: (i) recruitment to the plasma membrane mediated by an interaction with RAS (Yan et al., 1998); (ii) dimerization of RAF proteins (Luo et al., 1996); (iii) phosphorylation/dephosphorylation on different domains (Fabian et al., 1993); (iv) disassociation from the RAF kinase inhibitory protein (RKIP) (Yeung et al., 1999) and (Dhillon et al., 2002) and (v) association with scaffolding complexes (e.g., kinase suppressor of RAS, (KSR) (Blalock et al., 1999;Chang et al., 2003;Lee and McCubrey, 2002). There are at least thirteen regulatory phosphorylation sites on RAF-1 (Steelman et al., 2004). The phosphorylation of inactivation site allows 14-3-3 to bind to RAF-1 and confer a inactive configuration. Protein phosphatase 2A (PP2A) dephosphorylation at these sites release 14-3-3, allowing the active phosphorylation site to be phosphorylated by Src family kinase and activating RAF-1 (Dhillon et al., 2002;Marais et al., 1997). Maximal
activation of RAF-1 and A-RAF requires both RAS and Src activity while B-RAF activation is Src-independent. Presence of many phosphorylation sites, which can both activate and inactivate RAF-1, makes it a complex post-translational regulation of this kinase. In addition, the presence of isoforms along with conservation of some residue but not others residue makes the activation of this kinase a complex process. As mentioned before, the scaffolding protein RKIP has been shown to inhibit RAF-1 activation and downstream signaling by binding to the active form (Yan et al., 1998).

The fine tuning in RAF/MEK/ERK kinase cascade is further demonstrated by the fact that many of the “functions” of RAF-1 persist in RAF-1 knock-out mice, and are likely maintained by endogenous B-RAF (Mercer and Pritchard, 2003). Interestingly, B-RAF may transduce its signal through RAF-1 and B-RAF can form heterodimers with RAF-1. Dimerization is one important component involved in RAF activation. (Garnett et al., 2005).

3.4.1.3 MEK and ERK activation

Mitogen-activated protein kinase/ERK kinase (MEK1) is a tyrosine (Y-) and S/T-dual specificity protein kinase. All three RAF family members are able to phosphorylate MEK on S residues in the catalytic domain and activate but with different potencies (Alessi et al., 1994). The main downstream target of MEK is ERK. In contrast, downstream ERK has multiple targets.

Extracellular-signal-regulated kinases 1, 2 (ERK), are S/T kinases and their activities are positively regulated by phosphorylation mediated by MEK1 and MEK2 on threonine and tyrosine residue with the recognition site being Thr-Glu-Tyr (TEY) (Kyriakis et al., 1992). In turn, ERKs have numerous downstream targets including transcription factors Ets-1, c-Jun and c-Myc. ERK can enter the nucleus to phosphorylate many transcription factors (Steelman et al., 2004). ERK2 has been positively associated with proliferation while
ERK1 may inhibit the effects of ERK2 in certain cells (Pouyssegur et al., 2002). Targeted deletion studies have identified the roles of ERK1 and ERK2 in the development of whole organisms (Nishimoto and Nishida, 2006). ERK2 and MEK1, rather than ERK1 and MEK2, are essential for embryonic development: ERK2- or MEK1-deficient mice show defects in development of the placenta, whereas ERK1- or MEK2-deficient mice are viable, fertile and normal in size (Belanger et al., 2003; Mazzucchelli et al., 2002; Pages et al., 1999).

Another well known activator of the RAF/MEK/ERK signaling pathways is represented by Reactive oxygen species (ROS). In some cases reactive oxygen intermediates act directly on growth receptors, such as the EGFR, in a ligand-independent fashion and induce the activation of RAS and ERK1/2 signaling. This type of responsiveness does not appear to be limited to the EGFR as ROS will also induce the ligand-independent activation of the platelet derived growth factor (PDGF) receptor and a subsequent increase in both RAS and ERK1/2 activity (Karlsson et al., 2004). Furthermore, ROS inhibit protein phosphatases (Whisler et al., 1995) and inhibition of phosphatase activity results in the activation of the ERK1/2 signaling pathways (Lee and Esselman, 2002). It should be noted, however, that the MEK1 and 2 inhibitors U0126 and PD98059 both block oxidative stress-induced ERK1/2 activation (Lee et al., 2006; Lee et al., 2005) indicating that activating actions of oxidative stress do not occur directly on ERK1/2 but instead are localized at upstream targets.

3.4.2 Erk 1 / 2 activation and cell proliferation

In physiological conditions, ERK is anchored in the cytoplasm by its association with MEK (Fukuda et al., 1997), the microtubule network (Reszka et al., 1995), or with phosphatases. Mitogens induce a biphasic activation of ERK1 and ERK2, with a rapid and strong burst of kinase activity peaking at 5–10 min followed by a second wave of
lower but sustained activity that persists throughout the G1 phase for up to 6 h (Kahan et al., 1992; Meloche et al., 1992; Meloche, 1995). Nuclear translocation of ERK1/2 occurs within 15 min of activation, persists during the entire G1 phase, and can be reversed upon removing the mitogenic stimulus. ERK1/2 activation must be sustained until late G1 for successful S-phase entry (Yamamoto et al., 2006) and ERK1/2 translocation to the nucleus is essential for G1 to S phase progression (Brunet et al., 1999), although it is nonetheless insufficient to drive cells into S phase (Cheng et al., 1998; Jones and Fangman, 1992; Jones and Kazlauskas, 2001; Treinies et al., 1999). ERK1/2 are rapidly inactivated at the G1/S transition (Meloche, 1995).

The transportation of phospho-ERK 1/2 to the nucleus happens by several methods. Firstly, integrin-mediated organization of the actin cytoskeleton is essential for the proper localization and translocation of activated ERK 1/2. Upon stimulation, ERK 1/2 becomes phosphorylated at threonine and tyrosine residues; the latter results in the dissociation of ERK 1/2 from MEK 1/2, leading to the translocation. The ERK 1/2 could directly interact with the nuclear pore complex for the internalization (Adachi et al., 1999; Khokhlatchev et al., 1998; Kondoh et al., 2005; Matsubayashi et al., 2001). The rapid and persistent nuclear transfer of ERK 1/2 during the entire G0-G1 is required for the function of these kinases in mediating the growth response (Lenormand et al., 1993). The translocated ERK 1/2 then phosphorylate the ternary complex factors Elk-1, Sap-1a and TIF-IA (Chen et al., 1992; Gille et al., 1994; Lenormand et al., 1993; Zhao et al., 2003). Phosphorylation of Elk-1 on the C-terminus (Marais et al., 1993) increases its affinity for the serum response factors and enhances transcription of growth related proteins, such as c-Fos (Marais et al., 1993; Whitmarsh et al., 1995). Once dephosphorylated in the nucleus, ERK 1/2 are rapidly exported out of the nucleus via an active mechanism that is mediated, at least in part, by MEK that enters the nucleus independently from ERK.
Other mechanisms by which ERK pathway regulates the cell proliferation includes dual mechanism comprising the post-translational modification and inactivation of a component of the cell death machinery and the increased transcription of pro-survival genes (Kolch, 2005). ERK 1/2 can regulate the gene expression of the FOXO transcription factors that activate multiple genes involved in tumor suppression including *Bim* and *FasL* for inducing apoptosis (Burgering and Kops, 2002; Finnberg and El Deiry, 2004) and *p27kip1* (Dijkers et al., 2000), *cyclin D* (Schmidt et al., 2002) for cell cycle regulation. The expression of FOXO3a is associated with the suppression of tumor progression, therefore inhibition of FOXO 3a promotes cell transformation, tumor progression and angiogenesis (Greer and Brunet, 2005; Potente et al., 2005). Phosphorylation of FOXO3a by ERK 1/2 increases FOXO3a-MDM2 interaction and enhances MDM2-dependent ubiquitin proteasome pathway. The involvement of ERK in inducing cell proliferation makes it a possible therapeutic target through the use of its specific inhibitors.

### 3.4.3 ERK 1/2 activation and cell death

It came as a big surprise that housekeeping cellular signalling pathway involved in regulating cell proliferation also can have an effect on cell death. Studies suggest that the balance among the intensity and duration of pro-versus anti-apoptotic signals transmitted by ERK 1/2 determines either the cell proliferation or death signals (Pearson et al., 2001). The role of ERK 1/2 in inducing cell death has mostly focused on two pathways: DNA damage induced cell death and IFNγ-induced cell death.

DNA damage-inducing agents, including etoposide, adriamycin and ionizing or ultraviolet irradiation activate ERK1/2 in a variety of cell lines (Tang et al., 2002b). The MEK1 inhibitor PD98059 prevents activation but not p53 stabilization, and ERK activation in response to DNA damage is not attenuated in *p53*−/− MEFs. Furthermore, ERK 1/2
activation in response to etoposide is abolished in ATM−/− MEFs suggesting that ERK activation takes place downstream of ATM and is independent of p53. The activation of RAF/MEK/ERK pathway may be decreased in some prostate cancer cell lines isolated from advanced prostate cancer patients by the deletion or inactivation of p53. After DNA damage, p53 may activate the PAC1, DUSP5 or other phosphatases which serve to fine tune the RAF/MEK/ERK cascade. In contrast, after growth factor stimulation, p53 may induce map kinase phosphatase (MKP1) or other phosphatases which alter activity of the RAF/MEK/ERK cascade (Wu, 2004). However, ERK could directly phosphorylate p53 on ser-15 (Pearson et al., 2000). However, it remains unclear how ERK, a proline-directed kinase, could phosphorylate Ser-15 of p53, because this residue is followed by Gln residue. The link could be through the activation of ATM kinase. DNA damage response pathway leads to the activation of ATM kinase, which could lead to ERK activation, consistent with the observation that ERK activation depends on ATM after DNA damage (Tang et al., 2002a).

The role of IFNγ in cell death remains debatable, since IFNγ may lead to cell death but through induction of many genes can also elicit an anti-proliferative and a proliferative state (Xiang et al., 2008). In oligodendroglial progenitor cells, ERK 1/2 blockage by U0126 prevented the cytotoxic effect of IFNγ (Horiuchi et al., 2006). Simultaneous activation of MEK-ERK and STAT pathways was proposed to account for the vulnerability of OP cells to IFNγ because IFNγ activates the STAT1 pathway in both oligodendroglial progenitor and mature oligodendrocytes (OD) but ERK1/2 is not activated in mature OD cells (Horiuchi et al., 2006). However, BCL-2 family protein BIK could be the link between ERK and IFNγ induced cell death. Screening of the BCL-2 family proteins identified BIK to be a specific mediator for IFNγ-induced death of airway epithelial cells. BIK directly interacts with activated ERK1/2 and sequesters it to the cytoplasm by blocking the translocation to the nucleus (Mebratu et al., 2008).
3.4.4 ERK and mitochondrial cell death

One of the first hints that the RAF/MEK/ERK pathway could be involved in the mitochondrial pathways of cell death was the discovery that ERK can phosphorylate S112 of BAD, contributing to its inactivation and subsequent sequestration by 14-3-3 proteins (Zha et al., 1996b). Similarly, phosphorylation of BIM results in its dissociation from BCL-2, BCL-XL, and MCL-1 leading to its ubiquitination and degradation. This leads to the inhibition of apoptosis through the binding of BAX by BCL-2, BCL-XL and MCL-1 (Harada et al., 2004a; Weston et al., 2003).

The BH-3 only protein BIM is phosphorylated on multiple sites by MAPK family and targeted for polyubiquitination and degradation via the ubiquitin-proteasome pathway (Hubner et al., 2008). Serum withdrawal leads to decreased ERK activation and consequent dephosphorylation and accumulation of BIM while ectopic expression of a constitutively active RAF-1 leads to phosphorylation and degradation of BIM (Ley et al., 2005). In addition, phosphorylation at Thr-112 of Bim decreases binding of BIM to the antiapoptotic protein BCL-2 and can increase cell survival.
3.5 Aim of the thesis

Considering that so little is known about the proteomic interaction and the post-translational modification in the “mitochondria-shaping proteins”, we set out to investigate the cellular signalling pathways that could impinge on mitochondrial fusion proteins.

i) What are the cellular signalling pathways that impinge on mitochondrial morphology?

ii) What are the post-translational modification undergoing in mitofusins that could have an impact in mitochondrial dynamics and cell death?
4 Results


**Trichoplein/mitostatin regulates endoplasmic reticulum-mitochondria juxtaposition.**


The close association between mitochondria and Endoplasmic Reticulum is important for various physiological function including mitochondrial energy and lipid metabolism to Ca$^{2+}$ signalling and cell death. The molecular basis of ER–mitochondria juxtaposition is starting to be unravelled, as researchers can now purify the mitochondria-associated membranes (MAMs)—patches of ER attached to the outer mitochondrial membrane (OMM; (Vance, 1990)). The ER Ca$^{2+}$ channel inositol triphosphate receptor (Szabadkai et al., 2006), enzymes of the lipid biosynthetic pathway (Stone and Vance, 2000), chaperons (Hayashi and Su, 2007; Szabadkai et al., 2006) and kinases (Simmen et al., 2005) are enriched in MAMs and these proteins can indirectly regulate juxtaposition between ER and mitochondria (Hayashi and Su, 2007; Simmen et al., 2005). The first structural tether identified between the two organelles was the mitochondria-shaping protein mitofusin 2 (MFN2), ablation of which in mammalian cells loosens the interaction between ER and mitochondria (de Brito and Scorrano, 2008a). Trichoplein/mitostatin (TpMs) shows weak homology to trichohyalin, plectin and myosin and was originally reported to bind to keratins 8 and 18 (Nishizawa et al., 2005), which are the major intermediate filaments in epithelial cells (Fuchs, 1996). In addition, the genomic locus of TpMs (12q24) is a hotspot of allele deletion in several cancers; it partly colocalizes with mitochondria and regulates apoptosis (Vecchione et al., 2009). In this report, the authors have described TpMs as a new MAM protein interacting with MFN2 to regulate mitochondrial morphology and tethering with the ER.

My contribution to this project was to verify the physical interaction between the TpMs and the MFN2. Following my successful large-scale immunopurification of Flag tagged as well as endogenous mitofusins, I was asked to to verify, if TpMs could co-immunoprecipitate with the MFN2. With the help of immunoprecipitation and the western blots, I was able to show the physical interaction between the MFN2 and TpMs, which is required for the effect of TpMs in regulating mitochondrial morphology and tethering with the ER.
Trichoplein/mitostatin regulates endoplasmic reticulum–mitochondria juxtaposition

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INTRODUCTION

Mitochondria are dynamic organelles; their location within the cytoplasm is controlled by the concerted action of organelle fusion/fission, movement along microtubuli and anchoring at defined cytoskeletal sites, including intermediate filaments (Anesti & Scorrano, 2006). This organization probably affects the juxtaposition between mitochondria and the endoplasmic reticulum (ER)—a structural feature that is required for mitochondrial lipid biosynthesis and delivery, for efficient mitochondrial Ca2+ uptake and for the amplification of apoptosis (Hayashi et al, 2009).

The molecular basis of ER–mitochondria juxtaposition is starting to be unravelled, as researchers can now purify the mitochondria-associated membranes (MAMs)—patches of ER attached to the outer mitochondrial membrane (OMM; Vance, 1990). The ER Ca2+ channel inositol triphosphate receptor (Szabadkai et al, 2006), enzymes of the lipid biosynthetic pathway (Stone & Vance, 2000), chaperones (Szabadkai et al, 2006; Hayashi & Su, 2007) and kinases (Simmen et al, 2005) are enriched in MAMs and these proteins can indirectly regulate juxtaposition between ER and mitochondria (Simmen et al, 2005; Hayashi & Su, 2007). The first structural tether identified between the two organelles was the mitochondria-shaping protein mitofusin 2 (Mfn2), ablation of which in mammalian cells loosens the interaction between ER and mitochondria (de Brito & Scorrano, 2008). Conversely, in yeast a multiprotein complex was retrieved (Kornmann et al, 2009).

Trichoplein/mitostatin (TpmS) shows weak homology to trichohyalin, plectin and myosin and was originally reported to bind to keratins 8 and 18 (Nishizawa et al, 2005), which are the major intermediate filaments in epithelial cells (Fuchs, 1996). In addition, the genomic locus of TpmS (12q24) is a hotspot of allele deletion in several cancers; it partly colocalizes with mitochondria and regulates apoptosis (Vecchione et al, 2009). These results have prompted a more detailed analysis of its biological function. In this study, we report that TpmS is a new MAM protein interacting with Mfn2 to regulate mitochondrial morphology and tethering with the ER.

RESULTS

TpmS localizes at the mitochondria–ER interface

Endogenous or expressed TpmS shows a punctuate pattern, partly overlapping with mitochondria (Nishizawa et al, 2005; Vecchione et al, 2009). As TpmS lacks a canonical mitochondrial targeting sequence or a cleor transmembrane domain for insertion into the mitochondrial membrane, we analysed the subcellular localization of a TpmS–green fluorescent protein (GFP) chimera. Visual inspection and a quantitative analysis showed that TpmS–GFP...
partly colocalized with mitochondria and ER, but not with autophagosomes or late endosomes—two other organelles with shapes that are reminiscent of the TpMs puncta (Fig 1A,B). TpMs was retrieved in a crude mitochondrial fraction from HeLa cells that also contains ER and MAMs as contaminants (Fig 1C). When these crude mitochondria were treated with proteinase K, TpMs was completely degraded, similarly to the OMM marker translocase of outer membrane 20 (TOM20). After alkaline carbonate extraction, TpMs was retrieved in the supernatant, similarly to the loosely attached Bax and actin, whereas the integral protein TOM20 remained in the membranous pellet (Fig 1C). Thus, TpMs is loosely attached to the OMM. To map the domain of TpMs required for its mitochondrial localization, we fused fragments of increasing length and the trichohyalin–plectin homology domain of TpMs to GFP. Live confocal imaging and subcellular fractionation revealed that only full-length TpMs was clearly localised to the mitochondria (supplementary Fig S1A,B online).
TpMs has a plectin homology domain, suggesting that the mitochondrial plectin (plectin 1b; Winter et al., 2008) could mediate its association with mitochondria. Efficient down-regulation of plectin 1b by small interfering RNA (siRNA; Fig 1D) reduced the mitochondrial levels of TpMs (Fig 1F), without affecting the total amount of TpMs (Fig 1E). Several biochemical approaches failed, however, to show a direct interaction between TpMs and plectin 1b (data not shown), raising the question of whether plectin 1b uses keratin as a ‘bridge’ to direct TpMs to the OMM. Immunoprecipitation confirmed an interaction between TpMs and keratins, and immunofluorescence showed that TpMs was present between mitochondria and keratins in HeLa cells. However, levels of TpMs did not affect the organization of keratins or other cytoskeletal components (supplementary Fig S2 online). In mouse embryonic fibroblasts (MEFs) that do not express keratins 8 or 18 (data not shown) the distribution of fragments of and full-length TpMs–GFP expressed was similar to that observed in HeLa (supplementary Fig S1C online). This excludes a role for keratin in the association of TpMs with mitochondria. The localization pattern of TpMs (Fig 1A,B) resembled that of the MAM component Mfn2 (de Brito & Scorrano, 2008). When we probed MAMs purified from mouse liver for TpMs and plectin 1b, these proteins were observed almost exclusively (Fig 1G,H). Immunofluorescence confirmed that expressed TpMs–V5 was localized at puncta overlapping with both ER and mitochondria (Fig 1I). In conclusion, TpMs is localized at the interface between mitochondria and ER.
Tpm5 regulates ER–mitochondria tethering

The subcellular location of TpMs prompted us to analyse its role in mitochondrial, ER morphology and tethering. Immunoblotting showed efficient siRNA-mediated downregulation and expression of TpMs (V5-tagged or untagged; supplementary Fig S3 online). Changes in the levels of TpMs affected mitochondrial morphology; less TpMs was associated with mitochondrial elongation, whereas overexpressed TpMs caused mitochondrial fragmentation (Fig 2A,B). The pro-fission effect of TpMs was replicated in MEFs, ruling out a role for keratin (supplementary Fig S1D online) and was not associated with increased levels of the pro-fission protein Drp1 on mitochondria (data not shown). Conversely, TpMs did not alter the ER structure, as shown by three-dimensional reconstructions of ER-targeted yellow fluorescent protein (de Brito & Scorrano, 2008; Fig 2C,D). A confocal semiquantitative assay of ER–mitochondria juxtaposition (de Brito & Scorrano, 2008) showed that tethering was reduced when we increased the levels of TpMs (Fig 2E,F). Accordingly, association of ER with mitochondria isolated from HeLa cells was also inversely proportional to levels of TpMs (Fig 2G). Finally, the phenotype could be reproduced in prostate LnCaP cells stably expressing TpMs and in MEFs (data not shown), thereby excluding the possibility that it was a consequence of transient transfection, or that it requires keratin. In conclusion, TpMs negatively regulates tethering of ER to mitochondria.

Tpm5 regulates ER–mitochondria tethering via Mfn2

The localization and function of TpMs suggested a possible interaction between it and Mfn2. Mfn2–GFP expressed in HeLa cells pulled down TpMs (Fig 3A) similar to endogenous Mfn2 in wild type, but not in Mfn2−/− MEFS (Fig 3B). We therefore analysed whether TpMs interacted genetically with Mfn2. TpMs was efficiently expressed in Mfn2−/− MEFS (Fig 3C), but did not further fragment mitochondria (Fig 3D,E) or inhibit mitochondrial fusion, as measured previously (Karbowiak et al., 2004; Fig 3F). Efficient silencing of Mfn2 in HeLa cells (Fig 3G) resulted in the expected fragmentation, whereas silencing of TpMs (Fig 3G) alone caused mitochondrial elongation (Fig 3H). When TpMs and Mfn2 were efficiently silenced together (Fig 3G), mitochondrial elongation was not detected (Fig 3H). We then checked whether the effect of TpMs on ER–mitochondria juxtaposition required Mfn2; the expression of TpMs–V5 in Mfn2−/− MEFS did not decrease the already limited tethering any further (Fig 3I,J). In HeLa cells, levels of juxtaposition were increased by silencing TpMs and decreased by ablation of Mfn2 alone or in combination with TpMs (Fig 3K,L). In conclusion, TpMs interacts physically and genetically with Mfn2 to regulate mitochondrial morphology and tethering with the ER.

Tpm5 selectively inhibits Ca2+-dependent cell death

To address whether TpMs affected ER–mitochondria communication, we tested whether it influenced the apoptotic response to death stimuli that require Ca2+ transfer between the two organelles (Scorrano et al., 2003). The expression of TpMs–V5 marginally sensitized HeLa cells to apoptosis by a panoply of intrinsic stimuli (Fig 4A–C,E), whereas it had no effect on the extrinsic stimuli tumour-necrosis-factor-related apoptosis-inducing ligand and tumour necrosis factor-α (Fig 4F). Conversely, it specifically conferred resistance to H2O2, which requires ER–mitochondria Ca2+ transfer to cause death (Scorrano et al., 2003) in HeLa cells (Fig 4D) and MEFS (supplementary Fig S4 online). When TpMs was silenced, death by H2O2, but not by the other stimuli tested, was accordingly increased (Fig 4G). As TpMs fragments mitochondria—a condition that also blunts death due to Ca2+-dependent stimuli (Szabadkai et al., 2004)—we tested whether an artificial tether that increases the juxtaposition between the organelles (Csordas et al., 2006) could revert the effect of TpMs on apoptosis by H2O2. Cells co-expressing TpMs and the artificial tether were no longer resistant to H2O2-induced death (Fig 4H). In conclusion, TpMs selectively inhibits death by stimuli that require Ca2+ transfer from ER to mitochondria.

DISCUSSION

In this study we identify TpMs as a new protein localized at the mitochondria–ER interface that regulates the interaction between the two organelles. TpMs was originally described as a keratin-binding protein (Nishizawa et al., 2005), but it partly localizes to mitochondria and is downregulated in several tumours (Vecchione et al., 2009). TpMs associates with the OMM, enriched at the points of juxtaposition between mitochondria and ER. The association of TpMs with mitochondria is reduced when plectin 1b, a mitochondrial isoform of the large cytolinker plectin (Winter et al., 2008), is downregulated. Accordingly, plectin 1b is also retrieved in MAMs. It is therefore tempting to speculate that MAMs are linked to the cytoskeleton.

Despite the physiological relevance of the interface between mitochondria and ER (Hayashi et al., 2009), only a few proteins have so far been retrieved at this site. Most of them are chaperones or have an active role in signalling, with the exception of Mfn2, which acts as a structural tether (de Brito & Scorrano, 2008). In this study, we expand this group to include a protein that can interact with the cytoskeleton. However, our data indicate that keratins are dispensable for the function of TpMs in mitochondrial shape and tethering to the ER. Conversely, biochemical and genetic data point to a model in which TpMs interacts with Mfn2 and requires it to modulate mitochondrial morphology and tethering.

TpMs is downregulated in several human tumours (Vecchione et al., 2009) and higher levels of this protein are associated with a slight increase in apoptosis. Why does TpMs sensitize the cell to death? High levels of TpMs do not cause mitochondrial depolarization or latent dysfunction (data not shown). It is possible that it effects could be related to mitochondrial fragmentation, associated with a greater tendency to apoptosis. However, TpMs protects against H2O2 that requires Ca2+ transfer from the ER to mitochondria. This indicates that the role of TpMs is as a negative modulator of ER–mitochondria juxtaposition and extends the evidence supporting the importance of inter-organelar juxtaposition in cell death (Csordas et al., 2006).

In conclusion, TpMs—a cytoskeleton-binding protein that is downregulated in cancer—modulates ER–mitochondria juxtaposition in an Mfn2-dependent manner. This indicates that the functions of Mfn2 can be modulated by the levels of its partners.

METHODS

Molecular biology. Mitochondrial red fluorescent protein (mtRFP), mitochondrial cyan fluorescent protein, ER-targeted red fluorescent protein, Mfn2–GFP and ER-targeted yellow fluorescent protein have been described previously (Cipolat et al., 2004; de Brito & Scorrano, 2008). pcDNA-DEST47-TpMs (TpMs–GFP) and pcDNA/V5-DEST-TpMs (TpMs–V5) were generated by standard
Fig 3 | Trichoplein/mitostatin physically and functionally interacts with mitofusin 2. (A) Proteins (300 μg) from pre-cleared lysates of HeLa cells transfected with Mfn2–GFP were immunoprecipitated as indicated and analysed by SDS–PAGE and immunoblotting. Input and supernatant are diluted 1:10. (B) Proteins (1 mg) from pre-cleared lysates of wt and Mfn2–/– MEFs were immunoprecipitated as indicated and analysed by SDS–PAGE and immunoblotting. Input and supernatant are diluted 1:10. (C) Proteins (20 μg) from Mfn2–/– MEFs 24 h after transfection with the indicated plasmids were analysed by SDS–PAGE and immunoblotting. (D) Representative confocal images of Mfn2–/– MEFs co-transfected for 24 h with mtRFP and the indicated plasmids. Scale bar, 20 μm. (E) Mean ± s.e. (n = 3) of morphometric analysis of mitochondrial shape from (D). (F) Mitochondrial fusion assay of Mfn2–/– MEFs co-transfected with mtRFP, mt-pAGFP and the indicated plasmids. Data are mean ± s.e. (n = 3). (G) HeLa cells were co-transfected with the indicated siRNA and after 48 h cells were lysed and protein (20 μg) was analysed by SDS–PAGE and immunoblotting using the indicated antibodies. (H) Mean ± s.e. (n = 3) of morphometric analysis of mitochondrial shape from HeLa cells transfected with the indicated siRNA and mtRFP. (I) Three-dimensional reconstructions of ER and mitochondria in Mfn2–/– MEFs co-transfected with mtRFP, ER-YFP and the indicated plasmids. Yellow, organelles are closer than about 270 nm. Scale bar, 20 μm. (J) Mean ± s.e. (n = 3) of interaction data from (G). (K) Three-dimensional reconstructions of ER and mitochondria in HeLa cells co-transfected with mtRFP, ER-YFP and the indicated siRNA. Yellow indicates that organelles are closer than around 270 nm. Scale bar, 20 μm. (L) Mean ± s.e. (n = 5) of interaction data from (K). ER, endoplasmic reticulum; ER-RFP, ER-targeted red fluorescent protein; EV, empty vector; GFP, green fluorescent protein; MEFs, mouse embryonic fibroblasts; Mfn2, mitofusin 2; mtRFP, matrix red fluorescent protein; pAGFP, green fluorescent protein; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; wt, wild type; YFP, yellow fluorescent protein.
Tritchoplein and ER-mitochondria interactions

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Fig 4 | Trichoplein/mitostatin protects cells from Ca\(^{2+}\)–dependent apoptosis. (A–D) Viability of HeLa cells transfected as indicated and treated for the indicated times with staurosporine (STS, 2 µM, A), etoposide (5 µM, B), thapsigargin (1 µM, C) and H\(_2\)O\(_2\) (1 mM, D). (E) Cell death of HeLa cells 48 h after transfection with the indicated and plasmids. (F) Cell death of HeLa cells transfected as indicated and treated with TNF-α (25 ng/ml plus 10 µg/ml cycloheximide) or TRAIL (50 ng/ml) for 16 h. In (A–F), data are represented as mean ± s.e. (n = 5). (G) Cell death of HeLa cells transfected as indicated and treated after 48 h with H\(_2\)O\(_2\) or STS for 6 h. Data are represented as mean ± s.e. (n = 3). (H) Cell death of HeLa cells co-transfected as indicated and treated for 24 h with H\(_2\)O\(_2\) for a duration of 4 h. Data are represented as mean ± s.e. (n = 6). EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; EV, empty vector; FAM, carboxyfluorescein; OMM, outer mitochondrial membrane; RFP, red fluorescent protein; tBID, truncated BID; TNF, tumour necrosis factor; TpMs, trichoplein/mitostatin; TRAIL, tumour-necrosis-factor-related apoptosis-inducing ligand; YFP, yellow fluorescent protein.

cloning. Yellow fluorescent protein-microtubule-associated protein 1 light chain 3 was provided by T. Yoshimori (Japan). The following siRNAs were used: TpMs, 5'–GGAGGUGAGGC GACCAAAATT-3'; plectin-1b, 5'–GGGGCAUCGGCAGGCAAAG-3'; actin, 5'–GAGCAAGATGAACGAGACCGTGTG-3'.

Fig 4 | Trichoplein/mitostatin protects cells from Ca\(^{2+}\)–dependent apoptosis. (A–D) Viability of HeLa cells transfected as indicated and treated for the indicated times with staurosporine (STS, 2 µM, A), etoposide (5 µM, B), thapsigargin (1 µM, C) and H\(_2\)O\(_2\) (1 mM, D). (E) Cell death of HeLa cells 48 h after transfection with the indicated and plasmids. (F) Cell death of HeLa cells transfected as indicated and treated with TNF-α (25 ng/ml plus 10 µg/ml cycloheximide) or TRAIL (50 ng/ml) for 16 h. In (A–F), data are represented as mean ± s.e. (n = 5). (G) Cell death of HeLa cells transfected as indicated and treated after 48 h with H\(_2\)O\(_2\) or STS for 6 h. Data are represented as mean ± s.e. (n = 3). (H) Cell death of HeLa cells co-transfected as indicated and treated for 24 h with H\(_2\)O\(_2\) for a duration of 4 h. Data are represented as mean ± s.e. (n = 6). EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; EV, empty vector; FAM, carboxyfluorescein; OMM, outer mitochondrial membrane; RFP, red fluorescent protein; tBID, truncated BID; TNF, tumour necrosis factor; TpMs, trichoplein/mitostatin; TRAIL, tumour-necrosis-factor-related apoptosis-inducing ligand; YFP, yellow fluorescent protein.

Real-time PCR. Total RNA was isolated from HeLa cells by using the QuickPrep™ Kit (Amersham Biosciences). RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen) and subsequent reverse transcriptase PCR amplification (Applied Biosystems 7000 Fast System) of a 200-bp fragment of the first exon of mouse plectin 1b was performed using the primers 5'-GAGCAAGATGAACGAGGACTGTTG-3' and 5'-TCCAGGGC AAATGCTATTTCGTC-3'. As a control, a fragment of mouse β-actin complementary DNA was amplified using the forward primers 5'-CTGGCTCCTAGCACCATAAATGAT-3' and reverse 5'-GGTGGACAGTGCCAGGAAAGATT-3'.

Cell culture. Simian vacuolating virus 40-transformed wild-type and Min2\(^{--}\) MEFs and HeLa cells were cultured and transfected as described previously (de Brito & Scorrano, 2008).

Imaging. Confocal imaging of live cells expressing fluorescent proteins was performed using a Nikon Eclipse TE300 inverted microscope equipped with a spinning-disk PerkinElmer UltraView LCI as described previously (de Brito & Scorrano, 2008). For immunofluorescence, HeLa cells transfected as indicated were processed, stained with rabbit anti-ciM6PR (serum 8738, 1:200), mouse anti-V5 (1:200, Invitrogen), goat anti-mouse or anti-rabbit fluorescent isothiocyanate- or tetramethyl rhodamine iso-thiocyanate-conjugated IgG (1:500; Invitrogen) and imaged as described previously (de Brito & Scorrano, 2008).

For mitochondrial fusion, 2 × 10\(^5\) cells—at 24 h after co-transfection with mRFP, mito-photoactivatable green fluorescent protein (pAGFP) and the indicated constructs—were placed on the
stage of a laser scanning microscope (TCS SP5, Leica). Using the LasAF software (Leica), regions of interest were manually defined and pAGFP was photoactivated in one z-plane using the 488-nm laser line (100%) with a ×63, 1.4 numerical aperture objective. Frames of pAGFP and mtRFP fluorescence were acquired at every minute using a 488-nm laser line. Mean pAGFP fluorescence in the photoactivated region was measured using Multi Measure plugin of ImageJ and normalized for mtRFP fluorescence (National Institutes of Health, Bethesda). In each experiment, 40 cells were analysed per condition.

**Morphometric and contact analysis.** Morphometric analysis of mitochondria and ER was performed with ImageJ 3.0 as described previously (de Brito & Scorrano, 2008). In each experiment, 120 cells were scored per condition. Analysis of mitochondria–ER interaction was performed as described previously (de Brito & Scorrano, 2008). In each experiment, 50 cells were scored per condition. The number of independent experiments is reported in the figure legend. Interaction analysis between the objects was performed using Manders’ colocalization coefficient (Manders et al, 1993).

**Biochemistry.** Whole-cell lysates were prepared by disrupting 10⁶ cells in lysis buffer (0.025 M Tris, pH 7.4, 0.025 M NaCl, 1% Triton X-100 (Sigma), 0.1% sodium dodecyl sulphate (Sigma) and 0.5 mM ethylene glycol tetraacetic acid) supplemented with complete protease-inhibitor mixture (Sigma). For immunoprecipitation, Protein-G was incubated with mouse GFP antibody (1:15, Invitrogen) or rabbit Mfn2 antibody (1:20; for 3 h at 4°C) and subsequently (overnight at 4°C) with the indicated antibodies prepared in lysis buffer.

Subcellular fractionation of cells and Percoll purification of MAMs were performed as described previously (Frezza et al, 2007; de Brito & Scorrano, 2008).

The following antibodies were used: GFP (1:200; Invitrogen), actin (1:30,000, Chemicon); MnSOD (1:8,000, Stressgen), TOM20 (1:4,000, Santa Cruz Biotechnology); leucine dehydrogenase (1:2,000, Rockland), V5 (1:1,000, Invitrogen), optic atrophy 1 (1:1,000, BD Biosciences), glucose regulatory protein 75 (1:1,000, Santa Cruz Biotechnology); fatty acyl-CoA ligase 4 (FACL4; 1:1,000, Santa Cruz Biotechnology); calnexin (1:1,000, Stressgen), TpMs (1:100; Vecchione et al, 2009) and Mfn2 (1:1,000, Abnova). Proteinase K assay and carbonate extraction were performed as described previously (Dimmer et al, 2008).

**Cell death.** Apoptosis was measured 24 h (unless noted) after transfection of 2 × 10⁵ cells with the indicated plasmids as described previously (Frezza et al, 2006). The number of independent experiments is reported in the figure legend.

**Supplementary information** is available at EMBO reports online (http://www.emboreports.org).

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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Trichoplein/Mitostatin regulates ER-mitochondria juxtaposition

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Supplementary online material
Supplementary online methods

Molecular Biology

Δ1-TpMs, Δ2-TpMs, Δ3-TpMs, Δ4-TpMs, Δ5-TpMs-GFP and TpMs-GFP were generated by amplifying the fragments of TpMs by PCR using the following primers: 5’-GGGGAATTCTACCATGGCGCTCCCGACGCT-3’ and 5’-GGGGGATCCTGCCTGTTTGGAGCTGCA GAT-3’ for Δ1-TpMs, 5’-AAAGGATCCCAAGTTCATGTGCAGGCCTCGAGCTCAG-3’ for Δ2-TpMs, 5’-AAAGGATCCCTCCATCTCTCGAGTTTTCTCGG-3’ for Δ3-TpMs, 5’-AAAGGATCCGCCTGTGCTCCAGCTCCT-3’ for Δ4-TpMs, 5’-AAAGGTACGGTTCAAAGCAATTTTTTGGATG-3’ for Δ5-TpMs-GFP and 5’-AAAGGTACCGTTCAAAGCAATTTTTTGGATG-3’ for TpMs-GFP. TpMs also was subcloned into pEGFP-N3, adding a codon stop to block the expression of the GFP, using the following primers: 5’-GGGGAATTCTACCATGGCGCTCCCGACGCT-3’ and 5’-AAAGGATCCCAAGTTCATGTGCAGGCCTCGAGCTCAG-3’

Cell culture

LnCap overexpressing stably TpMs-V5 (LnCaPB3A) were previously described (Vecchione et al., 2009). These cells were cultured in Roswell Park Memorial Istitute (RPMI, Invitrogen) supplemented
with 10% foetal bovine serum, 2 mM L-glutamine, non-essential amino acid 75 U/ml penicillin, 50 
µg/ml streptomycin and 200 µg/ml geneticin (G-418, GIBCO) at 37°C in a 5% CO₂ incubator.

MEFs were transfected using Trasfectin (Biorad) according to the manufacturer’s instructions. 
Transfection of HeLa cells and LnCapB3A with siRNAs and plasmid DNA was performed with 
Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. When indicated in the 
figure legend, HeLa cells were transfected with siRNA 24 hrs after seeding on glass coverslips and 
with mtRED/erYFP 48 hrs after plating.

HeLa cells stably overexpressing TpMs were generated by transfecting the cells with the plasmids 
pEGFP-N3 or TpMs-GFP in which GFP expression was blocked as described previously. Clones were 
selected by culturing cells in complete medium supplemented with 1 mg/mL G418 and single cell 
clones were generated by limiting dilution as previously described (Frezza et al., 2006).
References


Legends to supplementary figures

Fig. S1: Mitochondrial targeting and fragmentation by TpMs does not require keratins.

(A) HeLa cells were cotransfected with mtRFP and the indicated TpMs-GFP chimeras and after 24 hrs representative confocal images were acquired. Scale bar, 20 μm.

(B) HeLa cells were transfected with the indicated plasmids and after 24 hrs equal amounts (40 μg) of protein from mitochondrial, ER and cytosolic fractions were separated by SDS-PAGE and immunoblotted using the indicated antibodies. MnSOD, Mn-dependent superoxide dismutase; SERCA, sarco/endoplasmic reticulum Ca$^{2+}$ ATPase; LDH, lactate dehydrogenase.

(C) Subcellular fractions were prepared from MEFs transfected with the indicated constructs and equal amounts (40 μg) of protein from the indicated fractions were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

(D) Left: Representative confocal images of MEFs cotransfected with mtRFP and empty vector or TpMs-GFP. Scale bar, 20 μm. Right: Morphometric analysis of mitochondrial shape. 120 randomly selected images of mtRFP fluorescence were acquired, stored, and classified as described. Data represent mean ±SE of 3 independent experiments.

Fig. S2: TpMs interacts with keratins.

(A) Lysates from HeLa cells were prepared and equal amounts of protein (500 μg) dissolved in lysis buffer were immunoprecipitated with the indicated antibodies, separated by SDS-PAGE and immunoblotted using an anti-K8/K18 antibody. Input represents a 1:10 dilution of the total lysates.

(B) Pre-cleared lysates from HeLa cells were prepared and equal amounts of protein (500 μg) dissolved in CHAPS buffer were immunoprecipitated with the indicated antibodies (input represents a 1:10 dilution) and coprecipitated proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.
(C) Representative images of HeLa cells transfected as indicated. Cells were fixed and immunostained with anti-cytokeratin8/18 (K8/K18), rhodamine-conjugated phalloidin or anti-tubulin antibody. Scale bar, 20 µm.

(D) Representative confocal images of HeLa cells cotransfected with mtCFP (cyan) and TpMs-V5. Cells were fixed and immunostained with FITC-conjugated anti-K8/K18 (green) and TRITC-conjugated anti-V5 antibodies (red). The merged image is also shown. The boxed areas are magnified 9 folds. Scale bar, 20 µm. The arrow represents a group of mitochondria where the blue color is clearly distinguishable from violet color of the overlay of mitochondria and TpMs.

**Fig. S3 : Expression levels of TpMs.**

HeLa cells were transfected as indicated. After 24 h (A), or at the indicated times cells (B) cells were lysed and protein (20 µg) was analyzed by SDS-PAGE/immunoblotting.

(C) HeLa cells stably overexpressing empty vector or TpMs were lysed and equal amounts of protein (20 µg) were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(D) HeLa cells were transfected as indicated Twenty-four (plasmids) or 48 hrs (siRNA) after the indicated transfections cells were lysed and protein (20 µg) was analyzed by SDS-PAGE/immunoblotting.

**Fig. S4: MEFs overexpressing TpMs are protected from hydrogen peroxide induced cell death.**

(A) MEFs transfected as indicated were treated with 2 µm STS for 6 hrs or 5 µm etoposide for 48 hrs. Cell death was determined cytofluorimetrically as the percentage of YFP-positive, annexin-V-Alexa568-positive cells. Data represent mean ±SE of 3 independent experiments.
(B) MEFs were cotransfected with TpMs-V5 and pEGFP or tBID-GFP. After 48 hrs cell death was determined cytofluorimetrically as the percentage of GFP-positive, annexin-V-Alexa568-positive cells. Data represent mean ±SE of 3 independent experiments.

(C) MEFs transfected as indicated were treated with 1 mM H$_2$O$_2$. At the indicated times, viability was determined cytofluorimetrically as the percentage of YFP-positive, annexin-V-Alexa568-negative cells. Data represent mean ±SE of 3 independent experiments.
A

\[ \alpha\text{-}TpMs \quad \text{EV} \quad \text{TpmS-V5} \]

\[ \alpha\text{-}actin \]

B

\[ \begin{array}{c}
\text{siRNA TpMs} \\
24h \\
48h \\
72h \\
\end{array} \]

\[ \begin{array}{c}
\alpha\text{-}TpMs \\
\alpha\text{-}actin \\
\end{array} \]

C

\[ \begin{array}{c}
\text{EV} \\
\text{TpmS} \\
\end{array} \]

\[ \begin{array}{c}
\alpha\text{-}TpMs \\
\alpha\text{-}actin \\
\end{array} \]

D

\[ \begin{array}{c}
\alpha\text{-}TpMs \\
\alpha\text{-}K8/K18 \\
\alpha\text{-}actin \\
\text{EV} \\
\text{TpmS-V5} \\
\text{Scr} \\
\text{TpmS} \\
\text{siRNA} \\
24h \\
48h \\
\end{array} \]
Mitofusin 1 functions in mitochondrial morphology and apoptosis are regulated at an ERK phosphorylation site.

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**Running title:** Mitofusin 1 phosphorylation in morphology and apoptosis

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Abstract

Signaling cascades post-translationally modify mitochondria-shaping proteins to change mitochondrial morphology in response to cellular cues. Here we show that the pleiotropic mitogen activated protein (MAP) kinase phosphorylate the pro-fusion protein mitofusin (MFN) 1, switching its functions in apoptosis and mitochondrial fusion. A phosphoproteomic analysis revealed that MFN1 is phosphorylated at an atypical ERK1 site (T562) of its HR1 domain. This site proved essential to mediate the MFN1-dependent mitochondrial elongation and regulation of apoptosis by the MEK/ERK pathway. A T562 mutant mimicking constitutive phosphorylation of MFN1 triggered mitochondrial fragmentation, increased susceptibility to apoptosis, bound more avidly to the proapoptotic BCL-2 family member BAK and facilitated its activation. Our data identify a role for phosphorylation of MFN1 in mitochondrial shape and apoptosis.

Keywords: mitochondria/Mitofusin/phosphorylation/morphology/apoptosis/BAK
Introduction

Mitochondria are key organelles to sense and amplify apoptotic signals, releasing cytochrome c and other cofactors required for the activation of caspases and downstream cell death (Danial and Korsmeyer, 2004). The release of cytochrome c, which requires mitochondrial outer membrane permeabilization (MOMP) is tightly regulated by the Bcl-2 family of proteins and is associated with fragmentation of the organelle (Frank et al., 2001). The shape of the mitochondria is modulated by large dynamin-like GTPases: on the fission side, cytosol located dynamin related protein 1 (DRP1), which during fragmentation translocates to mitochondria, binding to its outer membrane (OMM) partner mitochondrial fission factor (MFF) (Otera et al., 2010) and constricts mitochondria. Profusion proteins display pleiotropic functions: the inner mitochondrial membrane (IMM) protein Optic Atrophy 1 (OPA1) not only promotes fusion (Cipolat et al., 2004), but it also regulates shape of the cristae (Olichon et al., 2003) and impinges on the cristae remodeling pathway to control the release of cytochrome c (Frezza et al., 2006).

Mammals possess two OMM Mitofusins (MFNs) that are both required for mitochondrial shape (Chen et al., 2003). However, while the function of MFN1 seems to be restricted to promoting fusion of the organelle together with OPA1 (Cipolat et al., 2004), MFN2 not only interact in trans with the mitofusins in the other mitochondria to tether the organelles in the fusion process (Koshiba et al., 2004), but it also tethers mitochondria with the endoplasmic reticulum (ER) (de Brito and Scorrano, 2008a).

The role of mitochondrial morphology in apoptosis is far from being understood. Several reports suggested that massive mitochondrial fragmentation occurs during apoptosis, but whether this is a causal event in the release of cytochrome c has been questioned by some authors [see for example (Westermann, 2010) for a review]. On the other hand, hyperfusion of mitochondria during cellular stress is unanimously found to be a strategy to prevent apoptosis and to allow survival of cells during deprivation of nutrients (Gomes
et al., 2011; Rambold et al., 2011b; Tondera et al., 2009b). Several models have been proposed as to the role of mitochondrial fragmentation during apoptosis. Fragmentation has been associated with mitochondrial hemifusion driven by the pro-fission molecule DRP1 that stimulates BAX accumulation on mitochondria (Montessuit et al., 2010) or with the remodeling of mitochondrial cristae (Germain et al., 2005) that is required for maximal cytochrome c release (Scorrano et al., 2002). Additionally, during apoptosis mitochondrial fusion has been found to be impaired (Karbowski et al., 2004) and indeed a direct relationship between the multidomain proapoptotic mitochondrial gatekeepers BAX and BAK and mitofusins has been identified (Karbowski et al., 2006). In healthy cells, they promote the activity of MFN2 by facilitating its assembly. Conversely, during apoptosis, BAK dissociates from MFN2 and associates with MFN1 (Brooks et al., 2007), albeit the functional role of this switch is not understood.

We reasoned that changes in mitochondrial fusion during apoptosis could be associated with posttranslational modification impinging on mitochondrial fusion and to verify this possibility we investigated if MFNs are posttranslationally modified. MFN1 was found to be phosphorylated at T562, an atypical ERK target site. This ERK MFN1 phosphorylation site modulates both mitochondrial morphology and apoptosis, influencing binding to and oligomerization of BAK in response to apoptotic stimuli.
Materials and Methods

Molecular biology

FLAG-MFN1 and FLAG-MFN2 plasmids were a generous gift of Dr. Yuka Eura (Kyushu University, Fukuoka, Japan). PBabe-MEK_{DN} and PBabe-MEK_{CA} were a generous gift from Prof. Chris Marshall (Institute of Cancer Research, London, UK). Amphotrophic viruses were generated by co-transfecting the HEK 293 packaging cell lines with the packaging vector pIK and the required pMIG constructs as previously described (Cheng et al., 2001). Viral supernatant were retrieved and used to transduce MEFs in the presence of 4μg/ml of Hexadimethrine Bromide (Sigma). Following an over-night transduction, cells were selected on 5 μg/ml puromycin. Details on the primers used to generate FLAG-MFN1 mutants by site directed mutagenesis can be found in the Supplemental Online Materials.

Cell Culture

Simian virus 40 (SV40)-transformed wild-type, Mfn1^{−/−} and Mfn2^{−/−} MEFs were from D. Chan (California Institute of Technology, USA) and cultured as described previously (de Brito and Scorrano, 2008a). Wild-type and Bax^{−/−}Bak^{−/−} MEFs were cultured as described previously (Scorrano et al., 2003). Transfection of MEFs with DNA was carried out using Transfectin (Biorad), according to the manufacturer’s instructions. When indicated, cells were transfected 24h after plating.

Analysis of cell death

MEFs treated as indicated were stained with propidium iodide (PI) and Annexin-V-FITC (Bender MedSystem). Where indicated, cells were cotransfected with pEGFP and the indicated vector. After 24 h cells were treated as described and stained with Annexin-V-PE (Bender MedSystem) according to the manufacturer's protocol. Cell death was
measured by flow cytometry (FACSCalibur) as the percentage of Annexin-V-positive events in the GFP-positive population and viability as the percentage of Annexin-V-negative, PI-negative cells for transfected and untransfected cells, respectively.

**Immunoprecipitation and phosphoprotein purification**

For immunoprecipitation experiments, cells were lysed in CPBS buffer (6 mM CHAPS in PBS, pH 7.4). Lysates were incubated with the indicated antibody (1:50, 14 h, and 4 °C) and the protein-antibody complex was precipitated by centrifugation after incubation with protein G-coated magnetic beads (Dynal, 2 h, 4 °C). The immunoprecipitated material was washed twice in CPBS and resuspended in SDS/PAGE loading buffer (NuPAGE), boiled, and loaded on 4–12% gels (NuPAGE). For phosphorylation studies, total cell lysates were loaded on a phosphoprotein binding column (Qiagen) as previously described (Cereghetti et al., 2008). Flow-through (unphosphorylated) and eluted (phosphorylated) proteins were collected and concentrated and 20 µg of proteins were separated by 4–12% SDS–PAGE.

**Mass spectrometric analysis**

Cell extracts (10 mg) from Mfn1−/− and Mfn2−/− MEFs transfected with Flag-MFN1 and Flag-MFN2 respectively were immunoprecipitated using anti-Flag antibody. The immunoprecipitates were separated by 4-12 % Tris–MOPS SDS–PAGE (NuPage, Invitrogen) and stained with Simply Blue Safe Stain (Invitrogen). Bands corresponding to Flag-MFN1 and Flag-MFN2 were excised and reduced with TCEP, alkylated with iodoacetamide and digested with trypsin or AspN. Details on liquid chromatography-mass spectrometry analysis can be found in Supplementary Online Material.
Biochemistry

Mitochondria were isolated by standard differential centrifugation in isolation buffer (IB) as described (Frezza et al., 2007). For BAK oligomerization assay, mitochondria (0.5 mg/ml) were incubated in experimental buffer (EB; 125 mM KCl, 10 mM Tris-MOPS [pH 7.4], 1 mM Pi, 5 mM glutamate, 2.5 mM malate, 10 μM EGTA-Tris [pH 7.4]), and treated as described at 25°C. At the indicated time, mitochondria were pelleted by centrifugation at 12000 × g at 4°C for 3 min and resuspended in the same volume of EB. For crosslinking, mitochondria were incubated with 10 mM (30 min, 37°C) BMH (Pierce) and the reaction was quenched with 20 mM β-mercaptoethanol (15 min, 25°C).

For immunoblotting, proteins were separated by 7% Tris–acetate, 3–8% Tris–acetate or 4–12% Tris–MOPS SDS–PAGE (NuPage, Invitrogen), transferred onto PVDF membranes (Millipore), probed using the indicated primary antibodies and isotype matched secondary antibodies conjugated to horseradish peroxidase (Amersham) and detected using ECL (Amersham). The following antibodies were employed: rabbit anti-ERK (1:1000, Cell Signalling); rabbit anti-pERK (1:1000, Cell Signalling), mouse anti-COX IV (1:1000, Molecular Probes); mouse anti-MFN2 (1:1000, Abnova); mouse anti-ACTIN (1:3000, Chemicon); chicken anti-MFN1 (1:500, Abcam); rabbit anti-BAK (1:1000, Millipore); mouse anti-FLAG (1:1000, Sigma). Densitometric quantification of western blot was performed using the Gel Pro analyser 4 software.

Immunostaining

For cytochrome c immunostaining, 2 × 10^4 MEFs of the indicated genotype grown on 13 mm round coverslips were transfected with as indicated and after 24 hours incubated as detailed in the text, fixed for 30 minutes at room temperature with 3.7% (V/V) ice-cold formaldehyde, permeabilized for 20 min with 0.01% (v/v) ice-cold Nonidet P-40, incubated for 15 min with a 0.5% solution of BSA. Cells were then sequentially incubated for 60
minutes at 37°C with a mouse monoclonal anti-cytochrome c antibody (Pharmingen, CA, clone 6h2.B4) and for 15 min at 37°C with a FITC-conjugated goat anti-mouse IgG.

For BAX translocation, cells were transfected with mtRFP, fixed in 2% paraformaldehyde for 15 min at room temperature and then washed five times with PBS. Samples were permeabilized in 0.5% CHAPS for 30 min, followed by incubation in blocking buffer (3% BSA in PBS) for 1 h at room temperature. Primary antibody (anti-BAX antibody 6A7; 5 µg/ml in blocking buffer) was added and incubated overnight at 4 °C, after which Alexa 488-conjugated goat anti-mouse secondary antibody (diluted 1:300 in blocking buffer) was added for 1 h at room temperature.

**Imaging**

For confocal microscopy imaging of live cells, 1.8×10^5 cells seeded onto 24-mm round glass coverslips transfected and treated as indicated were incubated in Hank’s Balanced Salt Solution (HBSS) supplemented with 10 mM HEPES and coverslips were placed on the stage of a Zeiss LSM 510 inverted microscope. Cells expressing mtYFP and mtRFP were excited using the 488 nm or 543 nm line of the Argon laser using a 63 × 1.4 NA Plan Apochromat objective (Zeiss).
Results

**MFN1 is phosphorylated at T562**

Post-translational modifications such as phosphorylation play a major role in relaying signaling pathways for different cellular function. In order to identify post-translational modifications in mitofusins, we transfected mouse embryonic fibroblasts with Flag-MFN1 or Flag-MFN2 and we separated by affinity chromatography phosphorylated and non-phosphorylated proteins on phosphocolumn, which specifically binds phosphorylated residues (Cereghetti *et al.*, 2008). Immunoblotting with Flag antibody indicated that both MFN1 and MFN2 avidly bound to the column (Figure 1A). We set forth to identify the site of phosphorylation in these proteins using mass spectrometry. Large-scale anti-Flag immuno-purification from *Mfn1*−/− and *Mfn2*−/− cells transfected with Flag-MFN1 and Flag-MFN2 was successfully carried out (Figure S1A). The bands corresponding to the MFNs were excised from the gel and analyzed by mass spectroscopy. 83 % of MFN1 sequence was covered whereas the coverage for MFN2 was only around 65 % (Figure S1B), possibly explaining why we could not detect any site of phosphorylation in MFN2. Conversely, two sites of phosphorylation were identified in MFN1 (Figure 1B) in T562 and T564 that lie in the HR1 region of MFN1 (Figure 1C). The sequence of this site, ASTPTAP resembles to some extent a canonical ERK site, Pro-Xaa-Ser/Thr-Pro, (Clarklewis *et al.*, 1991) and we therefore hypothesized that this could be an atypical ERK site. We therefore first addressed if MFN1 physically interacted with ERK by probing proteins co immunoprecipitating with Flag-MFN1 for ERK, which was retrieved in the co-immunoprecipitate (Figure 1D). To discriminate between the two potential sites of phosphorylation, we first analyzed them in silico. T562 is conserved throughout mammalian MFN1s (Figure 1F) and is lacking in MFN2 (Figure S1C) that did not display the same phosphorylation and that does not bind to ERK. We next generated single and double mutants mimicking phosphorylated (MFN1T562D, MFN1T564D and MFN1T562D,T564D)
or dephosphorylated MFN1 (MFN1<sup>T562A</sup>, MFN1<sup>T564A</sup> and MFN1<sup>T562A,T564A</sup>). Upon affinity purification of the expressed mutants on the phosphocolumn, we found that only MFN1<sup>T562A</sup> did not bind to the substrate, whereas all the T564 mutants showed comparable binding. Thus, these experiments suggest that the phosphorylated residue could be T562.

**ERK requires T562 of MFN1 to modulate mitochondrial morphology**

Prompted by these results, we next wished to verify if ERK affected mitochondrial morphology. We therefore expressed dominant negative and constitutively active mutants of the upstream MEK (Pages *et al.*, 1994) that as expected modulated levels of ERK phosphorylation (Figure S2). Expression of dominant negative MEK (MEK<sup>DN</sup>) led to mitochondrial elongation, whereas activation of ERK by constitutively active MEK (MEK<sup>CA</sup>) led to fragmentation. The ability of the MEK/ERK pathway to modulate mitochondrial morphology was further verified in cells lacking MEK1 and MEK2, where mitochondria appeared significantly elongated (Figure S3). A genetic analysis indicated that modulation of mitochondrial shape by MEK required MFN1, since it was abolished in *Mfn1<sup>-/-</sup>* but not in *Mfn2<sup>-/-</sup>* MEFs (Figure 2A). To understand whether ERK modulated mitochondrial morphology through the T562 residue of MFN1, we tested the effect of MEK<sup>DN</sup> and MEK<sup>CA</sup> in *Mfn1<sup>-/-</sup>* MEFs reconstituted with wt and mutated MFN1. As expected, in *Mfn1<sup>-/-</sup>* MEFs re-expressing wt MFN1, MEK<sup>DN</sup> triggered mitochondrial elongation while MEK<sup>CA</sup> caused fragmentation (Figure 2B). MEK<sup>DN</sup> and MEK<sup>CA</sup> were ineffective in *Mfn1<sup>-/-</sup>* MEFs reconstituted with MFN1<sup>T562A</sup> that display elongated mitochondria. The expression of MFN1<sup>T562D</sup> in *Mfn1<sup>-/-</sup>* MEFs conversely caused mitochondrial fragmentation and prevented the effect of MEK<sup>DN</sup> and MEK<sup>CA</sup>. This analysis shows that ERK modulates mitochondrial morphology by impinging on the T562 residue of MFN1.
Phosphorylation of Mfn1 by Erk modulates cell death

Both the MEK/ERK pathway and mitochondrial morphology have been widely implicated in the modulation of apoptosis (Green and Kroemer, 2004; McCubrey et al., 2007; Westermann, 2010). We therefore wondered whether the phosphorylation of MFN1 could represent the link between the two pathways and we therefore set out to test this possibility. Expression of MEK\textsuperscript{DN} delayed apoptosis induced by several mitochondria-dependent intrinsic stimuli (staurosporine, H\textsubscript{2}O\textsubscript{2} or Etoposide) in wt and \textit{Mfn2}\textsuperscript{−/−}, but not in \textit{Mfn1}\textsuperscript{−/−} MEFs (Figure 3A). The role of the MEK/ERK pathway in apoptosis was further confirmed in cells lacking \textit{Mek1} or \textit{Mek2}, where cell death was also significantly reduced (Figure S4). Conversely, increased apoptosis was measured in Wt and \textit{Mfn2}\textsuperscript{−/−}, but not \textit{Mfn1}\textsuperscript{−/−} cells expressing MEK\textsuperscript{CA} (Figure S5). Thus, inhibition of ERK causes a delay in apoptosis that depends on MFN1, a picture similar to the MFN1-dependent mitochondrial elongation caused by the inhibition of ERK.

To explore if ERK modulates apoptosis at the mitochondrial level, we measured if MEK\textsuperscript{DN} reduced the release of cytochrome c upon apoptotic stimuli. Cytochrome c release by H\textsubscript{2}O\textsubscript{2} was blunted by MEK\textsuperscript{DN} in Wt and \textit{Mfn2}\textsuperscript{−/−} cells, but not in \textit{Mfn1}\textsuperscript{−/−} MEFs (Figure 3C and 3D), further substantiating that ERK1 inhibits the mitochondrial pathway of apoptosis in a MFN1 dependent manner. Release of cytochrome c depends on the permeabilization of the outer mitochondrial membrane, controlled by the multidomain proapoptotics members of the Bcl-2 family BAX and BAK (Wei \textit{et al.}, 2001). We therefore evaluated if the MEK/ERK pathway affected BAX and BAK activation. Since expression of MEK\textsuperscript{DN} did not change H\textsubscript{2}O\textsubscript{2}-induced BAX activation in all the cell lines tested (Fig. 3D), we focused our attention on BAK. Mitochondria from cells treated with EGF, which activates MEK and increases ERK phosphorylation (Fig. S2), were more prone to BAK oligomerization in response to recombinant caspases-8 cleaved BID (cBID) (Fig. 3E,F). Conversely in \textit{Mek1}\textsuperscript{−/−} mitochondria BAK oligomerization in response to cBID was reduced (Fig. S6),
confirming a link between the MEK/ERK pathway and BAK activation. In conclusion, the MEK/ERK pathway regulates apoptosis in a MFN1 dependent manner and specifically impinges on BAK oligomerization.

**Interaction of BAK with MFN1 influences its oligomerization**

Our results indicate a role for the MEK/ERK pathway in the regulation of Mfn1-dependent apoptosis as well as in the control of BAK oligomerization. We therefore hypothesized that MFN1 could link the two processes. To verify this possibility, we started by testing if BAK coimmunoprecipitated with Wt and mutant Flag-tagged MFN1 expressed in Mfn1−/− MEFs. Interestingly, MFN1 interacted with BAK and the binding efficiency was influenced by the status of the T562 residue: the T to D mutant bound 2.4 times more BAK, whereas less BAK associated with MFN1T562A (Fig. 4A). Functionally, BAK activation in response to cBID was increased in mitochondria expressing MFN1T562D, whereas activation was not affected in MFN1T562A expressing mitochondria (Fig. 4B, C). The differences in BAK oligomerization correlated with the response to cell death: Mfn1−/− MEFs reconstituted with MFN1T562A were less susceptible to staurosporine and more importantly, they lost the modulation by the mutants of MEK. Similarly, Mfn1−/− MEFs reconstituted with Mfn1T562D were more susceptible to apoptosis and the expression of the MEK mutants had no effect on apoptosis (Fig. 4D). In conclusion, our results indicate that MEK modulates apoptosis by impinging on MFN1-dependent regulation of BAK oligomerization.
Discussion

Our results indicate that T562, lying in an atypical ERK site of MFN1, is phosphorylated. The status of this residue regulates mitochondrial morphology and apoptosis: phosho-mimetic mutants of T562 decrease mitochondrial length and favor mitochondrial apoptosis, by enhancing oligomerization of BAK.

The analysis of post-translational regulation of mitochondria-shaping protein has mostly focused on fission. Considering that cytosolic DRP1 needs signaling cues to translocate, form oligomers and constrict mitochondria, it is no wonder that DRP1 has been shown to be tightly regulated by post-translation modifications including phosphorylation at different sites and SUMOylation (reviewed in Pyakurel et al). Less is known on how mitochondrial fusion responds to cellular cues. IMM OPA1 sits at the interface between mitochondrial fusion and apoptosis, by regulating fusion (Cipolat et al., 2004) as well as cristae shape and remodeling (Frezza et al., 2006). OPA1 is posttranscriptionally cleaved by a number of proteases that produce short versions required for competent fusion and cristae maintenance (Meeusen et al., 1999). Conversely, post-translational modifications in mitofusins were hard to find: given their membrane localization, MFNs are difficult to immunopurify and therefore to analyze by mass spectrometry. However, significant efforts resulted in the identification of emerging post-translational modifications in mitofusins. As a matter of fact, mitofusins have been found to be ubiquitinated, even if the exact site of ubiquitination has not yet been identified. Interestingly, Mfn is a substrate of the E3 ubiquitin ligase Parkin, mutated in Parkinson disease (PD) (Ziviani et al., 2010) and Parkin mutations associated with genetic forms of PD compromise the ability to ubiquitinate Mfn (Glauser et al., 2011b), implying Mfns in the function of dopaminergic neurons and in pathogenesis of PD. Stress-induced phosphorylation by JNK in MFN2 has been shown to cause its degradation through the ubiquitin-proteasome pathway (Leboucher et al., 2012b). Our results indicate that also MFN1 is phosphorylated and place it downstream of the MEK/ERK pathway, suggesting that mitochondrial morphology is a previously
unidentified target of this pleiotropic regulatory signaling cascade (Shaul and Seger, 2007). Indeed, the localization of MFNs on the OMM critically places them to relay cellular cues to the mitochondria. In fact, the two HR and the GTPase domain of MFNs face the cytosol, exposing them to the cytosolic signaling cascades and making mitochondrial fusion an attractive mechanism to regulate organelle shape, localization and physiology in response to intra or extra-cellular stimuli (Campello and Scorrano, 2010).

MFN1 is specialized in mitochondria tethering by antiparallel interaction of HR2 of proteins from mitochondria in trans (Koshiba et al., 2004). The phosphorylation site identified here is located in the HR1 region and its mutation affect the pro-fusion ability of MFN1, suggesting a function for this portion of the molecule at a step of the fusion reaction different from tethering. Future experiments should be directed towards deciphering the exact mechanism by which phosphorylation at this site inhibits mitochondrial fusion.

While the posttranslational modifications of DRP1 are well established players in the course of apoptosis (Cribbs and Strack, 2007), necrosis (Wang et al., 2012b) and autophagy (Gomes et al., 2011) our knowledge of how mitochondrial fusion is affected during cell death is scarcer. Inhibition of fusion is an established consequence of the induction of apoptosis and it occurs around the time of BAX activation (Karbowski et al., 2004). However, how fusion is blocked is not fully understood: OMM permeabilization with the release of the soluble fraction of OPA1 (Arnoult et al., 2005a; Arnoult et al., 2005b) and the proteolytic cleavage of the long OPA1 forms (DeVay et al., 2009) as a consequence of mitochondrial dysfunction (Duvezin-Caubet et al., 2006) could explain the inhibition of mitochondrial fusion only during late apoptosis. However, the mechanism of the earlier inhibition of fusion, occurring before or around the activation of BAX, is unclear. Proapoptotic BCL-2 family members like BAK and BAX appear to participate in this process. Mitochondria are fragmented in cells lacking BAK and BAX, since they facilitate MFN2 assembly, promoting its activity (Karbowski et al., 2006). Conversely,
during apoptosis, BAK dissociates from MFN2 and enhances its association with MFN1 (Brooks et al., 2007). Our results suggest that the increased BAK-MFN1 association could depend on the phosphorylation status of the latter. In fact, the Mfn1\textsuperscript{T562D} mutant that mimics phosphorylation of MFN1 at the atypical ERK site binds more avidly to BAK, favoring its oligomerization, and causes mitochondrial fragmentation, perhaps by stealing free MFN1 from the fusion reaction. Similarly EGF, which activates ERK, increases BAK oligomerization, suggesting that MFN1 could be placed at the crossroad between extracellular signals and activation of the mitochondrial pathway of apoptosis.

During apoptosis, blockage of DRP1-dependent fragmentation is protective (Frank et al., 2001). In addition to DRP1, our data indicate a role for the ERK-mediated inhibition of fusion in mitochondrial fragmentation. During apoptosis induced by the intrinsic stimuli tested here, ERK is phosphorylated (not shown), as previously reported in the case of oxidative stress (Kodiha et al., 2009). However, the role of ERK activation during apoptosis remains unclear (Rasola et al., 2010). Our model indicates that the activation of MEK/ERK inhibits the pro-fusion function of MFN1 and triggers its association with BAK, facilitating its oligomerization and therefore cytochrome c release and cell death. Our data therefore contribute to place MFN1 in the core apoptotic pathway of OMM permeabilization. Whether phosphorylated MFN1 facilitates the dissociation of BAK from BCL-2, or it shifts the equilibrium of the BH3-only-BAK reaction towards their interaction remains to be clarified.

In conclusion, we have identified that the MEK/ERK housekeeping signaling cascade inhibits the pro-fusion and increases the pro-death activity of MFN1, facilitating its interaction with pro-apoptotic protein BAK. We speculate that our results could pave the way to the analysis of the role of mitochondrial fusion in the many types of cancer where the MEK/ERK pathway is constitutively upregulated (Dhillon et al., 2007).
Figure Legends

Figure 1: MFN1 is phosphorylated at T562

(A) Mfn1−/− or Mfn2−/− MEFs transfected with FLAG-MFN1 or FLAG-MFN2 respectively were subjected to phosphocolumn. The immunoblot against HSP60 acts as a negative control for phosphorylation.

(B) MS/MS spectrum of the tryptic phosphopeptide of MFN1 containing two sites of phosphorylation, T562 and T564. Spectrum highlighted with green circles accounts for the T562 phosphorylation whereas the spectrum highlighted with red circles accounts for the T564 phosphorylation.

(C) ClustalW alignment between the aminoacids sequence in mammals of a segment of MFN1 that is phosphorylated.

(D) FLAG-MFN1 was immunoprecipitated from Mfn1−/− MEFs either untransfected or transfected with FLAG-MFN1. The co-immunoprecipitation for ERK was checked by SDS-PAGE/immunoblotting.

(E) Mfn1−/− MEFs were transfected with either the Wt or phospho mutants of MFN1 and subjected to phosphocolumn. Immunoblot against p-ERK demonstrates the total amount of p-ERK available.

Figure 2: ERK requires T562 of MFN1 to modulate mitochondrial morphology

(B) Representative confocal micrographs of mitochondrial morphology in MEFs of the indicated genotype 24 h after transfection with mtYFP and indicated plasmids. Scale bar, 10 µm.

(C) Morphometric analysis of mitochondrial shape. Experiments were carried out as in A. Data represent mean ± s.e.m. of five independent experiments (n=100 cells per condition in each experiment)
(D) Representative confocal micrographs of mitochondrial morphology in Mfn1−/− MEFs 24 h after transfection with mtYFP, and the indicated plasmids. Scale bar, 10 μm.

(E) Morphometric analysis of mitochondrial shape. Experiments were carried out as in A. Data represent mean ± s.e.m. of five independent experiments (n=100 cells per condition in each experiment).

**Figure 3: Phosphorylation of MFN1 by ERK modulates cell death**

(A) MEFs of indicated genotypes were cotransfected with GFP and indicated plasmids and after 24 hr treated with 2 μM staurosporine (Top Panel) (mean ± SEM of 5 independent experiments), 1 mM H2O2 (Middle panel) (mean ± SEM of 5 independent experiments), or 2 μM etoposide (Bottom panel) (mean ± SEM of 5 independent experiments) for the indicated times.

(B) Representative images of subcellular cytochrome c distribution. MEFs of indicated genotypes were cotransfected with mtRFP and indicated plasmids and after 24 hr were either left untreated or treated for 30 min with 1 mM H2O2, fixed and immunostained for cytochrome c (green). Scale Bar, 10 μm.

(C) Localization index of cytochrome c. Experiments were performed as in (B). Data represent mean ± SEM of three independent experiments.

(D) Representative images of subcellular BAX distribution. Cells of the indicated genotypes were cotransfected with mtRFP and indicated plasmids and after 24 hr were either left untreated or treated for 30 min with 1 mM H2O2, fixed and immunostained for the translocation of BAX, with BAX-6A7 antibody. Scale Bar, 10 μm.

(E) Wt MEFs were left untreated or treated with 10 nM EGF. Isolated mitochondria were left untreated or treated with cBID for indicated times. 10 mM BMH was then added and after 30 min the crosslinking reaction was quenched (Wei et al., 2000) Equal amounts (30
μg) of mitochondrial proteins were analyzed by SDS-PAGE/immunoblotting using anti-BAK antibody. Asterisks: BAK multimers.

(F) Densitometric analysis of BAK oligomers normalized to the level of BAK monomers and loading control COX. Changes in the amount of BAK oligomers is represented as % of initial value. Data represent average plotted ± SEM of 3 independent experiments.

Figure 4: Interaction of BAK with MFN1 influences its oligomerization

(A) FLAG-MFN1 was immunoprecipitated from Mfn1−/− MEFs transfected with FLAG-MFN1 & the single phospho-mutants. The co-immunoprecipitation for BAK was checked by SDS-PAGE/immunoblotting.

(B) Isolated mitochondria from Mfn1−/− MEFs stably transfected with the Wt or the phospho-mutants of MFN1 were left untreated or treated with cBID for 30 min. DMSO or 10 mM BMH was then added and after 30 min the crosslinking reaction was quenched (Wei et al., 2000) Equal amounts (30 μg) of mitochondrial proteins were analyzed by SDS-PAGE/immunoblotting using anti-BAK antibody. Asterisks: BAK multimers.

(C) Densitometric analysis of BAK oligomers normalized to BAK monomers. Data represent average plotted ± SEM of 3 independent experiments.

(D) Mfn1−/− MEFs cotransfected with GFP and the indicated plasmids were treated with 2 μM staurosporine for 6 hours. Data represent average ± SEM of 4 independent experiments.
Mitofusin 1 functions in mitochondrial morphology and apoptosis are regulated at an ERK phosphorylation site.

Aswin Pyakurel\textsuperscript{1}, Daniel Hess\textsuperscript{2} and Luca Scorrano\textsuperscript{1,3}

Supplementary online material
Materials and methods

Molecular Biology

For the generation of the Mfn1 mutants, the following primers were used:

T562A (F: 5’- GTCCCTAGATCTTTAGCTTCAGCTCTACTGCTCCTTCTAACC-3’; R: GGTTAGAAGGAGCAGTGGAGCTGAAGCTAAGATCTAGGGAC-3’),
T562D (F: 5’- CCCTAGATCTTTAGCTTCAGACCCCTACTGCTCCTTCTAACC-3’; R: 5’- GGTTAGAAGGAGCAGTGGAGCTGAAGCTAAGATCTAGGG-3’),
T564A (F: 5’- ATCTTTAGCTTCAACTCCTACTGCTCCTTCTAACCAGC-3’; R: 5’- GCTGGGTTAGAAGGAGCAGTGGAGTGAAGCTAAGAT-3’),
T564D (F: 5’- TCTTTAGCTTCAACTCCTACTGCTCCTTCTAACCAGC-3’; R: 5’- GCTGGGTTAGAAGGAGCAGTGGAGTGAAGCTAAGA-3’),
T562AT564A (F: 5’- GTCCCTAGATCTTTAGCTTCAGCTCTACTGCTCCTTCTAACC-3’; R: 5’- GGTTAGAAGGAGCAGGAGCAGTGGAGCTGAAGATCTAGGGAC-3’),
T562DT564D (F: 5’- CCCTAGATCTTTAGCTTCACTCCTACTGCTCCTTCTAACC-3’; R: 5’- GGTTAGAAGGAGCAGCAGGAGGTGAAGCTAAGATCTAGGG-3’).

Liquid chromatography-mass spectrometry

Extracted peptides from gels were injected onto a reversed phase column for liquid chromatography-mass spectrometry (LC-MS) analysis in the information-dependent acquisition mode. Electrospray ionization LC-MS/MS was performed using a Magic C18 HPLC column (75 μm × 10 cm; Swiss BioAnalytics) with a 1200 Nano-HPLC system (Agilent Technologies) connected to a LTQ Orbitrap Velos (Thermo Scientific). The peptides were loaded onto a peptide captrap (Michrom BioResources) at a flow rate of 10 μl / min for 5 minutes. They were eluted at a flow rate of 400 nl / min with a linear gradient of 2 – 36 % acetonitrile in 0.1 % formic acid (in H2O) in 30 minutes. Information-
dependent acquisition analyses was done according to the manufacturer's recommendations, i.e. 1 survey scan at 60K resolution in the Orbitrap cell was followed by up to 20 product ion scans in the linear ion trap, and precursors were excluded for 15 s after their second occurrence. Individual MS/MS spectra, containing sequence information were compared with the program Mascot against the mammalian sub set of the protein sequence database Swiss-Prot 2010_09 (Perkins et al., 1999). Carboxyamidomethylation of cystein (+57.0245) was set as a fixed modification and phosphorylation of serine, threonine and tyrosine (+79.9663 Da), oxidation of methionine (+15.9949 Da), deamidation of asparagine and glutamine (+0.984016 Da) and Pyro-glu formation on N-terminal glutamine (-17.02655 Da) were set as variable. Parent tolerance was 3 PPM and fragment tolerance 0.8 Da. No enzyme specificity was used for the combined searches of the tryptic and AspN digested samples. The results were further analyzed with Scaffold and ScaffoldPTM (Proteome Software).
Legends to supplementary figure

Figure S1: LC-MS/MS identification of MFN1 phosphorylation site
(A) Large scale immuno-purification of FLAG-MFN1 and FLAG-MFN2 proteins from Mfn1\(^{-/}\) and Mfn2\(^{-/}\) MEFs respectively transfected with FLAG-MFN1 and FLAG-MFN2 plasmids. The purification was detected by immunoblotting using Flag antibody (Left panel), and simply blue safe stain (invitrogen; Right panel).
(B) LC-MS/MS coverage of the immune-purified FLAG-MFN1 and FLAG-MFN2. Yellow color highlights the detected peptides. Post-translational modifications are highlighted with the green color.
(C) ClustalW sequence alignment between the aminoacids sequence of MFN1 & MFN2, in the region where MFN1 was found to be phosphorylated.

Figure S2: Changes in the level of p-ERK
(A) Wt MEFs were transfected with the indicated plasmids. Equal amounts of proteins from total cell lysates (30 µg) were analyzed by SDS-PAGE/immunoblotting.
(B) Wt MEFs were left untreated or treated with 10 µM U0126 for 1 hour and 10 nM EGF for 10 min. Equal amounts of proteins from total cell lysates (30 µg) were analyzed by SDS-PAGE/immunoblotting.

Figure S3: Mitochondrial elongation in Mek1\(^{-/}\) and Mek2\(^{-/}\) MEFs.
(A) Representative confocal micrographs of mitochondrial morphology in MEFs of the indicated genotype 24 h after transfection with mtYFP. Scale Bar, 10 µm.
(B) Morphometric analysis of mitochondrial morphology. Experiments were carried out as in A. Data represent mean ± s.e.m. of five independent experiments (n=100 cells per condition in each experiment).

Figure S4: Delay in apoptosis in Mek1\(^{-/}\) and Mek2\(^{-/}\) MEFs.
MEFs of indicated genotypes were treated with 2 µM staurosporine (mean ± SEM of 5 independent experiments) for the indicated times.

Figure S5: Activation of ERK leads to cell death.
MEFs of indicated genotypes were cotransfected with GFP and indicated plasmids and after 24 hr treated with 2 µM staurosporine for 6 hours. Data represent mean ± SEM of 3 independent experiments.

Figure S6: Loss of MEK1 delays oligomerization of BAK.
Isolated mitochondria of the indicated genotypes were left untreated or treated with cBID for 30 min. DMSO or 10 mM BMH was then added and after 30 min the crosslinking reaction was quenched (Wei et al., 2000). Equal amounts (30 µg) of mitochondrial proteins were analyzed by SDS-PAGE/immunoblotting using anti-BAK antibody. Asterisks: BAK multimers.
**Fig. 1**

(A) **bound**

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Flag-MFN1
Flag-MFN2
\(\alpha\)-FLAG
\(\alpha\)-HSP60

(B) [Image of diagram]

(C) [Image of amino acid sequences for various Mfn1 isoforms]

(D) **Mfn1**

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\(\alpha\)-FLAG
\(\alpha\)-ERK

(E) **Mfn1**

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\(\alpha\)-FLAG
\(\alpha\)-pERK

[Image of Western blot results]
Fig. 2
Fig. S1
Fig. S2

A

\( \alpha \)-pERK

\( \alpha \)-ERK

\( \alpha \)-ACTIN

B

\( \alpha \)-pERK

\( \alpha \)-ERK

Fig. S2
**Fig. S3**

(A) Cells with elongated mitochondria (% of transfected cells)

(B) Bar graph showing the percentage of cells with elongated mitochondria in Wt, Mek1−/−, and Mek2−/− conditions.
Viability (% of Annexin V, PI cells)

Fig. S4
Cell Viability (% of Annexin V, GFP+ cells)

- **GFP**
- **GFP+MEK^{CA}**

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Fig. S5
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<td>cBID</td>
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*Fig. S6*
5 Discussion

In this thesis, we have carried out a large-scale proteomic studies in order to unravel the proteomic regulators and interactors of the pro-fusion proteins MFN1 & MFN2. This has led to the discovery of the proteomic interactor of the pro-fusion protein MFN2 & discovered novel signaling pathway impinging on the mitochondrial fusion protein, MFN1 regulating mitochondrial morphology and apoptosis. In the first part of the discussion, we are going to focus on the discovery of Trichoplein/mitostatin (TpMs), a novel interacting partner of MFN2, which regulates tethering between mitochondria and endoplasmic reticulum in a MFN2-dependent manner.

Large-scale proteomics is widely used technique to unravel the proteomic interactors as well as regulators using the powerful LC-MS/MS techniques. However, immune-purification of membrane proteins poses a greater challenge as compared to the cytosol or nucleus localized protein. The use of strong detergent to disrupt the membrane without losing the native complexes in order to co-immunopurify the protein interactors is a challenge. We were able to immuno-purify both MFN1 & MFN2 to a large amount required to carry out the proteomic analysis using LC-MS/MS (Figure S1A). This provided us the opportunity to detect any other proteins that could immunopurify with the MFN1 & MFN2. Whereas long lists of proteins were detected, one of the proteins which attracted our attention was TpMs, a new protein localized at the mitochondria–ER interface that regulates the interaction between the two organelles. TpMs was originally described as a keratinbinding protein (Nishizawa et al., 2005), but it partly localizes to mitochondria and is downregulated in several tumours (Vecchione et al., 2009). After the initial screening result, I went on to verify this interaction between MFN2 & TpMs by co-immunoprecipitation experiments. The detection of TpMs, when MFN2 was
immunoprecipitated showed us that these proteins actually interacted physically in a cellular level. My colleagues went on to characterise the role of TpMs, where they found that TpMs actually is enriched at the endoplasmic reticulum-mitochondria interface. Further analysis showed that TpMs alter the tethering between mitochondria and the ER, which is dependent on MFN2.

In the second part of the discussion we are going to discuss about the cellular signalling pathways impinging on the mitochondrial shaping proteins to regulate mitochondrial dynamics and apoptosis. We used a reverse proteomic approach in which we investigated the post-translational modification taking place in the pro-fusion proteins, MFN1 and MFN2. While no post-translational modification was detected in MFN2, two sites of phosphorylation were detected in MFN1. Actually, one of the site T562, resembled an atypical ERK site. In fact, ERK co-immunoprecipitated with MFN1 strengthening the idea that the site that we found could actually be an ERK site. Downregulating ERK caused significant elongation of mitochondria, whereas its upregulation caused fragmentation. The study of phospho-mutants in mitochondrial morphology showed that fragmentation caused by ERK phosphorylation could be prevented through the mutation of the phospho site. Downregulation or upregulation of ERK also had an effect on the cell death which could be prevented by the phospho specific mutants. The effect of MFN1 phosphorylation was found to be due to decreased ability of pro-apoptotic BAK to bind to MFN1 reducing the oligomerized form of BAK. We are going to discuss our results based on 5 key points:

i) Post-translational modification in mitochondria-shaping proteins

ii) Localization of mitofusins at the interface between cytosol and mitochondria

iii) Phosphorylation to inactivate protein GTPases

iv) Regulation of mitochondrial apoptosis through phosphorylation

v) BCL-2 family of proteins in mitochondria and the interaction with mitofusins
5.1 Post-translational modification in mitochondria shaping proteins

The mitochondrial fission is tightly regulated by numerous post-translational modification in DRP1 (Chang and Blackstone, 2010). Cytosolic localized DRP1 is maintained in the cytosol due to the phosphorylation of S637 by cyclic AMP dependent protein kinase (Cereghetti et al., 2008; Cribbs and Strack, 2007). Dephosphorylation at this site is required for the translocation of DRP1 to the mitochondria in order to excise the mitochondrial membrane (Cereghetti et al., 2008). DRP1 undergoes various other post-translational modification including sumoylation, s-nitrosylation and ubiquitination.

On the fusion side, extensive proteolytic processing of OPA1 allows it to control two very fundamental processes: mitochondrial fusion and the cristae remodelling (Frezza et al., 2006; Ishihara et al., 2006). Considering the bi-functional activity of OPA1, it is not a surprising fact. However, the regulations of mitofusins by the cellular signalling pathways were more elusive. Recent advances in the investigation of Parkinson Disease (PD) implicating mitofusins to be a substrate of Parkin brought them to the limelight. Although results vary over if both the mitofusins are the target of Parkin, most of them have found MFN1 to be the target (Glauser et al., 2011a; Poole et al., 2010). The presence of a putative protein kinase A (PKA) phosphorylation site at Serine 442 (S442), which when mutated increased the inhibitory effects of MFN2 on proliferation of vascular smooth muscle cells (VSMCs) in culture, indicated that MFN2 could be the probable target of signalling kinases (Zhou et al., 2010). More recent finding reports direct phosphorylation of MFN2 at Serine 27 (S27) by stress-induced Jun N-terminal kinase (JNK), which becomes activated in response to genotoxic stresses including doxorubicin and other cellular stresses (Leboucher et al., 2012a). This is the first direct phosphorylation revealed for the mitofusins.
In our investigation, we could not identify this site of phosphorylation. This could be because this site is phosphorylated specially during the stress. In our case, the large scale immunopurification was carried on the cell lysate without any treatment. However, two sites of phosphorylation were found in the HR1 region of MFN1. Through the specific phospho binding column, we narrowed down the actual site to T562. T564A mutant still bound to the column indicating that there was another site of phosphorylation. However, the binding of T562A or TT24AA mutant to the phosphocolumn was completely abolished, indicating T562 as the predominant sites of phosphorylation. At the very first glance, the site of phosphorylation (LASTPTA) resembled an ERk site. The consensus phosphorylation site for ERK is Pro-X-(Ser/Thr)-Pro, where X is a variable amino acid residue, but ideally not a Pro. Although, the sequence in MFN1 lacks the Pro at position 2, there have been descriptions of atypical ERK sites. For instance Ser 15 of p53 (PPLSQET) is described to be phosphorylated by ERK (Persons et al., 2000). Serine is followed by Glutamine instead of proline and therefore is an atypical ERK site. The fact that MFN1 could be phosphorylated by one of the most important cellular signalling pathways gave our investigation a big lift. Clustalw alignment of the MFN1 sequence in different species as well as comparison of MFN1 sequence to MFN2 highlighted our finding. For instance, T564 is a conserved residue in MFN2 as well, where as T562 is not. By phosphorylating T562, the residue is discriminated, and that is why we could not find the same site of phosphorylation in MFN2. Likewise, T562 is conserved among mammals.

HR2 region of MFN1 is responsible for the antiparaller interaction on two mitochondria (Koshiba et al., 2004). Considering this, the site of phosphorylation we have identified, could not prevent the trans-oligomerization of MFN1. We will discuss below what could be the mechanism of the inactivation of fusion caused by ERK phosphorylation on MFN1.
5.2 Mitofusins localize at the junction between cytosolic signalling cues and mitochondria

Upon dephosphorylation by calcineurin DRP1 translocates to mitochondria inducing the fragmentation of mitochondria (Cereghetti et al., 2008). However, the presence of DRP1 at the mitochondria is limited. On the other hand, mitofusins are located at the outer mitochondrial membrane (OMM), most of its domain facing the cytosol. This makes an ideal localization for them to relay the information from the cytosol localized signalling cues to the mitochondria. As we know that the shape of the mitochondria is a phenotypic expression for the cellular stress cues as well as metabolic cues, the mitochondria-shaping proteins has to integrate the cytosolic signalling pathways, which in turns integrate the extra-cellular stimuli, to maintain the shape and form of the mitochondria (Soubannier and McBride, 2009).

The ubiquitination of mitofusins leading to the destabilization of protein levels, thereby inhibiting fusion is already a well established fact in the field. In mammals, MARCH5 was proposed to catalyze the ubiquitination of MFN1, since the depletion of MARCH5 increased levels of MFN1 and fusion (Park et al., 2010). An impulse to the research in this field came from the discovery that in *Drosophila* Mitofusins are the targets of the ubiquitin ligase Parkin, mutated in autosomal recessive juvenile forms of Parkinson disease (Ziviani et al., 2010). In mammals either only MFN1 or both the mitofusins are target of this E3 ligase Parkin (Glauser et al., 2011a).

A recent study has given a new dimension to the roles of mitofusins in integrating signalling pathways to impact on the shape of the mitochondria and apoptosis. MFN2
was found to be phosphorylated at Serine 27 (S27) by stress-induced Jun N-terminal kinase (JNK), which becomes activated in response to genotoxic stresses including doxorubicin and other cellular stresses. Phosphorylation by JNK leads to recruitment of HECT domain ubiquitin ligase (E3) Huwe1, leading to enhanced ubiquitination and proteasomal degradation of MFN2 (Leboucher et al., 2012a).

The emergence of new evidence suggesting that mitofusins could be an indispensable participant in the signalling pathways could have a big impact to the mystery lying behind “how the shape of mitochondria are regulated”? In our investigation, we have found that ERK directly phosphorylates MFN1 at the T562 residue making an impact both in the mitochondrial morphology and apoptosis. The fine-tuned special and temporal activation of ERK can have different impacts. Depending on the length of activation and the place it could induce either cell proliferation or cell death as we introduced before (McCubrey et al., 2007). However, we have found that oxidative stress at an early time point (250 mM H2O2, 15 min) leads to the massive activation of ERK. The same stimuli lead to the massive fragmentation of mitochondria, initiating also the cellular death pathways (results not shown). So how does ERK execute the two closely related cellular functions of mitochondrial fragmentation and cell death? The evolution found the player to be MFN1. The phosphorylation of MFN1 by ERK leads to the fragmentation of mitochondria (by an unknown mechanism, which we will discuss later), and increased cell death also via MFN1. Earlier studies which showed that MFN1 has specific function in the fusion of mitochondria, where as MFN2 is the one sensing the cellular signalling cues, seems to be incomplete (de Brito and Scorrano, 2008c).
5.3 Phosphorylation to inactivate enzymes: a case for GTPases?

Phosphorylation is one of the most-important and widely studied post-translational modifications. The addition of a phosphate can change the activity, localization, interactions of an enzyme among many others. The inactivation of an enzyme due to phosphorylation is a widely recorded phenomenon in the literature. For instance, peroxiredoxin-1 (Prdx1), a cysteine containing, highly conserved enzyme that reduces $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$ is phosphorylated by serine / threonine specific kinases Mst1 & Mst2, and thereby inactivating Prdx (Rawat et al. 2013). There are other numerous examples of the inactivation of an enzyme due to the addition of a phosphate group. But what about GTPases? Can GTPase activity be reduced due to phosphorylation? The best example comes right away from a mitochondria-shaping protein GTPase. PKA phosphorylates DRP1 attenuating its GTPase activity and leading to decreased mitochondrial fragmentation (Chang and Blackstone, 2007). In the case of MFN1, the phosphorylation site similarly lies outside the GTPase domain, in the HR1 domain. HR domains are thought to be important in protein-protein interactions and accordingly, our results show that the phospho-mutants display altered interaction properties with the pro-apoptotic protein BAK. We will now discuss the implication of this alteration of interaction in mitochondrial morphology and apoptosis.

5.4 Mitochondrial apoptosis and regulation by phosphorylation

RAF/MEK/ERK pathway plays a regulatory role in mitochondrial pathway of apoptosis. One of the earliest described involvements is the phosphorylation of Bad on S112 by ERK, which contributes to its inactivation and subsequent sequestration by 14-3-3
proteins (Zha et al., 1996a). This allows BCL-2 to form homodimers and an anti-apoptotic response is generated. Since then several other BCL-2 family of proteins have been shown to be phosphorylated by this pathway. Phosphorylation of BIM results in its disassociation from BCL-2, BCL-Xₐ, and MCL-1 and BIM becomes ubiquitionated and targeted to the proteosome. This allows BCL-2, BCL-Xₐ and MCL-1 to bind BAX and prevent BAX activation and the formation of BAX:BAX homodimers preventing the release of cytchrome c (Harada et al., 2004b; Weston et al., 2003). BIM has been shown to be phosphorylated by AKT and JNK as well (Lei and Davis, 2003).

However, the phosphorylation of mitochondria-shaping proteins by this pathway has not been observed. In our investigation, ERK phosphorylates mitofusin during cellular stress inducing mitochondrial fragmentation as well as cell death. This comes in contrast to the phosphorylation in BAD and BIM which are anti-apoptotic. However, as we discussed above the outcome of the pathway really depends on the temporal and spatial activation (McCubrey et al., 2007). This could be an early event of ERK activation, whereas long-term activation could lead to the anti-apoptotic effect.

### 5.5 BCL-2 family at the mitochondria and the mitofusins

During apoptosis, mitochondria fragment and appear to be small puncta like structure (Martinou and Youle, 2006). DRP1 seems to be involved in the apoptotic fragmentation of mitochondria (Frank et al., 2001). The precise mechanism by which DRP1 is recruited to the mitochondria is still not clear. Under physiological condition, DRP1 cycles between cytosol and the outer mitochondrial membrane (OMM). The activation of BAX which induces BAX-BAK dependent SUMO modification is required for the stable association of DRP1 to the mitochondria (Wasiak et al., 2007). However, the link between the BAX
activation and SUMOylation of DRP1 is still missing. A link between mitochondrial fission and BAX activation was suggested when active BAX was found in discrete foci at mitochondrial fission sites (Karbowski et al., 2002). BAK is present throughout the organelles; but upon apoptotic stimuli aggregates at a focus at mitochondrial fission site. In many cases, these foci seem to be located in proximity to DRP1 activity (Frank et al., 2001; Ishihara et al., 2009; Montessuit et al., 2010). BAX foci were still seen when DRP1 was inhibited, thus delaying mitochondrial fragmentation (Karbowski et al., 2002). Some of these foci were located at constriction sites that the authors described as “aberrant mitochondrial scission attempts”. These observations calls for an additional hypothesis that DRP1 independent fragmentation of mitochondria takes place during apoptosis, as substantiated by experiments by the Youle group showing that fusion is inhibited in dying cells. Inhibition of fusion is an established consequence of the induction of apoptosis and that it occurs around the time of BAX activation (Karbowski et al., 2004).

However, the molecular determinants of this inhibition are not fully established. One possibility is that fusion is blocked as a consequence of mitochondrial outer membrane permeabilization and of the release of cytochrome c as well as of the soluble fraction of OPA1 (Arnoult et al., 2005a; Arnoult et al., 2005b). In addition, proteolytic cleavage of the long OPA1 forms (required for efficient fusion (DeVay et al., 2009) occurs as a consequence of mitochondrial dysfunction (Duvezin-Caubet et al., 2006) and could also mediate the inhibition of mitochondrial fusion. However, as we said above, inhibition of fusion occurs earlier, before or around the activation of BAX, raising the question of its mechanism. For sure, proapoptotic BCL-2 family members like BAK and BAX can alter mitochondrial morphology by interacting with MFNs. Mitochondria appear fragmented in cells lacking BAK and BAX (Karbowski et al., 2006). In healthy cells, they promote the activity of MFN2 by facilitating its assembly. Conversely, during apoptosis, BAK dissociates from MFN2 and enhances the association with MFN1 (Brooks et al., 2007).
These findings seem to suggest that the increased association of BAK with MFN1 could attenuates the rate of fusion during apoptosis. In our investigation, phospho-mimetic mutants MFN1\textsubscript{T562D} had an increased interaction of BAK, whereas, MFN1\textsubscript{T562A} attenuated this interaction. This interaction could well be the signal for the inhibition of the GTPase activity of MFN1, leading to the inhibition of mitochondrial fusion during apoptosis. But the inhibition of fusion by BAK association is just the first part of the story. The more interesting phenomenon is the facilitation by MFN1 for the apoptotic pathway to go ahead. The MFN1\textsubscript{T562D} mutant had increased level of oligomerization of BAK, whereas the decreased level of BAK oligomers in the MFN1\textsubscript{T562A} mutant. So this seems to be a two way process, where BAK facilitates the inhibition of fusion and MFN1 facilitates the apoptotic pathway.

To conclude, our investigation discovered a novel function of MFN1, switching its function from the mitochondrial fusion to mitochondrial apoptosis, mediated by one of the most important cellular signalling pathways. This investigation added a new dimension to the role of MFN1, which was thought to be primarily involved in the mitochondrial fusion, where as MFN2 was thought to be the protein responsible for sensing the cellular signalling cues (de Brito and Scorrano, 2008c). This work also added a new dimension in the control of mitochondrial dynamics by the BCL-2 family of member. Further work should be focused on exploring the mechanism by which MFN1 is inhibited by phosphorylation, and the precise mechanism of MFN1 in mitochondrial apoptotic pathway (Reszka et al., 1995).
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