Modulation of mitochondrial morphology through mFis1 splice variants in basal conditions and starving cells

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Abstract

Fis1 is an outer mitochondrial membrane protein that has been suggested to play a role in mitochondrial morphology and apoptosis, through its oligomerization and the recruitment of Drp1 to discrete structures on the mitochondria, leading to mitochondrial fragmentation. Fis1 also triggers autophagosome formation. However, the precise role of Fis1 in mitochondrial morphology and cell signaling have been challenged by the finding of new receptors for Drp1 and the lack of a clear mitochondrial shape phenotype in knock down models of Fis1. In order to clarify these questions, we have turned our attention to the existence of Fis1 splicing variants. Here we show that mouse Fis1 gene produces three alternative isoforms, mFis1.1, mFis1.2 and mFis1.3. Interestingly, we have found that the mRNA of these variants is differentially expressed in mouse tissues. In excitable tissues such as brain and heart, mFis1.1 and mFis1.2 variants are highly expressed. Additionally, over expression of the isoforms showed that the three isoforms where mitochondrially localized and that mFis1.3 was rapidly degraded, as indicated by its stabilization [...]
Modulation of mitochondrial morphology through mFis1 splice variants in basal conditions and starving cells.

Thèse

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DISCUSSION

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LIST OF ABBREVIATIONS.

AIF: apoptosis-inducing factor
ADP: adenosine diphosphate
Akap121: A kinase anchor protein 121
AMPK: 5' AMP-activated protein kinase
Apaf1: apoptotic protease activating factor 1
Atg7: Autophagy-related protein 7
ATP: adenosine triphosphate
Bad: Bcl-2-associated agonist of cell death
Bak: Bcl-2 homologous antagonist/killer
Bap31: B-cell receptor-associated protein 31
Bax: Bcl-2-associated X protein
Bcl-2: B-cell CLL/lymphoma 2
Bcl-xL: B-cell lymphoma-extra large or Bcl2 like 1 isoform 1
BH3-only protein: Bcl-2 homology domain-only protein
Bid: BH3 interacting domain death agonist
Bim: Bcl-2-like 11 (apoptosis facilitator)
BL6: C57 black/6 mouse strain
Bnip3: BCL2/adenovirus E1B 19 kDa protein–interacting protein 3
CI, II, III, IV, V: mitochondrial respiratory complex I, II, III, IV, V
C-terminal: carboxyl terminal
CAD: caspase-activated DNAse
cAMP: cyclic adenosine monophosphate
CCCP: Carbonyl cyanide m-chlorophenyl hydrazone
CoQ: Coenzyme Q

DMEM: Dulbecco 's modified Eagle's medium

DNA: deoxyribonucleic acid

Drp1: GTPase dynamin related protein

EM: electron microscopy

ER: endoplasmic reticulum

F0: ATP synthase, H+ transporting, mitochondrial F0 complex

F1: ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide

FACS: fluorescence-activated cell sorting

FAD: flavin adenine dinucleotide

FADH2: flavin adenine dinucleotide, reduced form.

FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

GABARAP: Gamma-aminobutyric acid receptor-associated protein

GTP: guanosine triphosphate

HBSS: Hank's Balanced Salt Solution

HE: Dihydroethidium

hFis1: human fission 1 protein

hnRNP C/K: heterogeneous nuclear ribonucleoprotein C/K

H2O2: hydrogen peroxide

IAP: inhibitor of apoptosis

IMM: inner mitochondrial membrane

IMS: Inter membrane space

KO: knock out

LC3: Microtubule-associated proteins 1A/1B light chain 3A

mΔΨ: mitochondrial membrane potential
MAM: mitochondrial-associated membranes
MEF: mouse embryonic fibroblast
mFis1: mouse fission 1 protein
mtDNA: mitochondrial DNA
mRNA: messenger RNA
mTOR: Mammalian target of rapamycin
Mcl1: Induced myeloid leukemia cell differentiation protein
Mff: mitochondrial fission factor
Mfn1/2: mitofusin 1 and mitofusin 2
MiD49/51: mitochondrial dynamics proteins of 49 and 51 KDa
mPTP: mitochondrial permeability transition pore
SOD2: manganese-containing superoxide dismutase 2
N-terminal: amino terminal
NADH: nicotidamine adenine dinucleotide, reduced form
NADPH: nicotidamine adenine dinucleotide phosphate, reduced form
NIX: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like
Noxa: Phorbol-12-myristate-13-acetate-induced protein 1
OMM: outer mitochondrial membrane
Opa1: optic atrophy 1
OXPHOS: oxidative phosphorylation
Parl: Presenilins-associated rhomboid-like protein
PFA: paraformaldehyde
PI: propidium iodide
Pink1: PTEN-induced putative kinase 1
PKA: protein kinase A
Puma: Bcl-2 binding component 3
PVDF: Polyvinylidene fluoride
qRT-PCR: quantitative retro-transcribed polymerase chain reaction
RCC: respiratory chain complex
RCS: respiratory chain supercomplex
RFP: red-fluorescent protein
RIPA: Radioimmunoprecipitation assay buffer
RNA: ribonucleic acid
ROS: reactive oxygen species
shRNA: short-hairpin RNA
Smac/Diablo: second mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein (IAP)-binding protein with low PI
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBC1D15: TBC1 domain family member 15
TBS: Tris-buffered saline buffer
TCA: tricarboxylic acid cycle
Tm: transmembrane
TMRM: Tetramethylrhodamine methyl ester
TPR: tetratricopeptide repeat
tRNA: transfer RNA
U2AF65: Splicing factor U2AF 65 kDa subunit
VDAC: Voltage-dependent anion channel
WT: wild type
YFP: yellow-fluorescent protein
Chapter 1

RESUME AND SUMMARY

1.1 Resume

Fis1 est une protéine de la membrane externe mitochondriale qui joue un rôle dans la morphologie mitochondriale en s’oligomérisant et en recrutant Drp1 au sein de structures distinctes sur la mitochondrie, aboutissant à la fragmentation mitochondriale. Fis1 joue également un rôle dans le déclenchement de l’apoptose et la formation des autophagosomes. Cependant, le rôle précis de Fis1 dans la morphologie mitochondriale et la signalisation cellulaire a été remis en cause par la découverte de nouveaux récepteurs de Drp1 et par l’absence d’un phénotype marqué sur la morphologie mitochondriale dans les modèles Knock Down de Fis1. Afin de clarifier ces questions, nous avons focalisé notre attention sur l’existence de variants d’épissage alternatif de Fis1. Nous avons montré que le gène murin codant pour Fis1 produit 3 isoformes de cette protéine suite à l’épissage alternatif de l’ARN prémessager pour obtenir les variants mFis1.1, mFis1.2 et mFis1.3. De façon surprenante, nous avons découvert que les ARNm de ces variants étaient exprimés différemment dans les tissus murins. Dans les tissus excitables tels que le cerveau ou le cœur, mFis1.1 et mFis.2 sont fortement exprimés. De plus, la surexpression de ces isoformes montre que les 3 variants sont localisés au niveau mitochondrial et que mFis1.3 est rapidement dégradé, comme le démontre sa stabilisation lors de l’inhibition du protéasome. D’autre part, alors que mFis1.1, l’orthologue murin de hFis1, et mFis1.3 induisent la fragmentation mitochondriale, mFis1.2 entraîne une élongation mitochondriale.
L’ablation génétique de Fis1 dans les cellules MEF induit une élongation mitochondriale, qui est corrélée avec une augmentation du $\Delta \Psi$, une baisse de la production de ROS et une respiration mitochondriale plus efficace. De plus, les cellules Fis1KO sont résistantes à l’apoptose, ce qui suggère que non seulement que mFis1 contribue à la fragmentation mitochondriale mais en plus mFis1 est aussi nécessaire à la mort cellulaire. En outre, en l’absence de sérum, la quantité d’ARNm de mFis1.2 est augmentée 6 fois, de manière PKA-dépendante.

L’inhibition de l’assemblage du spliceosome en dérégulant la sous-unité U2AF65 induit une réduction de l’expression de l’ARNm de mFis1.2 et inhibe l’élongation mitochondriale induite par l’absence de sérum, suggérant que la PKA agit en modulant le spliceosome pour induire l’augmentation de l’expression de mFis1.2. Enfin, nous avons montré que la régulation positive de mFis1.2 dans les cellules cultivées en l’absence de sérum était responsable de la fusion mitochondriale, puisque la dérégulation de mFis1.2 entraîne une diminution de l’élongation mitochondriale dans ces cellules. Dans ce travail de thèse nous montrons que le gène Fis1 possède des fonctions nouvelles et antagonistes de par l’utilisation d’un de ses variants, qui peuvent induire l’élongation mitochondriale durant l’autophagie de façon à favoriser la respiration dans des conditions de pénurie de nutriments.
1.2 Summary

Fis1 is an outer mitochondrial membrane protein that has been suggested to play a role in mitochondrial morphology and apoptosis, through its oligomerization and the recruitment of Drp1 to discrete structures on the mitochondria, leading to mitochondrial fragmentation. Fis1 also triggers autophagosome formation. However, the precise role of Fis1 in mitochondrial morphology and cell signaling have been challenged by the finding of new receptors for Drp1 and the lack of a clear mitochondrial shape phenotype in knock down models of Fis1. In order to clarify these questions, we have turned our attention to the existence of Fis1 splicing variants. Here we show that mouse Fis1 gene produces three alternative isoforms, mFis1.1, mFis1.2 and mFis1.3. Interestingly, we have found that the mRNA of these variants is differentially expressed in mouse tissues. In excitable tissues such as brain and heart, mFis1.1 and mFis1.2 variants are highly expressed. Additionally, over expression of the isoforms showed that the three isoforms where mitochondrially localized and that mFis1.3 was rapidly degraded, as indicated by it stabilization upon proteosomai inhibition. Interestingly, while mFis1.1, the mouse orthologue of hFis1, and mFis1.3 induced mitochondrial fragmentation, mFis1.2 triggered mitochondrial elongation. Ablation of Fis1 in MEF cells induces mitochondrial elongation, which correlated with an increase in mΔΨ, less ROS production and a more efficient mitochondrial respiration. Moreover, Fis1 KO cells were resistant to apoptosis, suggesting that, in basal conditions, mFis1 contribute to mitochondrial fragmentation and is required for cell death. On the other hand, during starvation, mFis1.2 mRNA was up regulated 6 folds in starving cells in a
PKA-dependent manner. Inhibition of the spliceosome assembly by down regulation of the spliceosome subunit U2AF65, induced reduction of mFis1.2 mRNA and inhibited starvation-mediated mitochondrial elongation, suggesting that PKA acts through spliceosome modulation to induce mFis1.2 up regulation. Finally, we show that mFis1.2 up regulation in starving cells was responsible for mitochondrial fusion, since knock down of mFis1.2 reduced mitochondrial elongation in starving cells. Hence, in this work we present a novel and opposite function for Fis1 gene, which through the use of one of its splice variants, induces mitochondrial elongation during starvation in order to optimize respiration in conditions of nutrient deprivation.
Chapter 2

INTRODUCTION

2.1 Mitochondrial Origins.

Mitochondria are key membrane-enclosed organelles in charge of energy production and cell growth and death, inside most of eukaryotic cells. Although mitochondria are known from as early as 1840, Richard Altman in 1890 clearly described these bodies as omnipresent, autonomous and vital for the cells metabolism (O’Rourke, 2010). Altman had the insight to compare these “bioblasts” with bacteria, remark that was taken as one of the arguments of Lynn Margulis to postulate the endosymbiotic theory (Margulis, 1981). This theory postulated that mitochondria were once bacteria that entered into an early eukaryotic cell to become the organelles they are today. Several hypotheses have tried to explain how this happened. One of them, the hydrogen hypothesis (Martin & Muller, 1998), puts the mitochondria in the center of the eukaryotic evolution by arguing that no proto-eukaryote was needed as a host. Instead, it postulates that a metabolic relationship occurred between a hydrogen-dependent methanogenic archean host and an hydrogen-producing and respiring α-proteobacteria. From this moment on and over hundreds of millions of years of evolution, the metabolic entanglement between the host and the proto-mitochondrion produced drastic changes to both partners. The early mitochondrion transferred most of its genes to the host nucleus (Gray et al, 1999) and acquired ways of export ATP and import the necessary proteins for its biogenesis (Andersson et al, 2003; Truscott et al, 2003). On the other hand, the
nascent eukaryotic cell not only received from the mitochondrion the means of using oxygen to generate ATP in an ever more oxidizing environment (Dyall et al, 2004) but, as well, it helped to organize the growth of multicellular metazoans by orchestrating the process of programmed cell death or apoptosis (Huettenbrenner et al, 2003). Nowadays, due to the their main involvement in energy production and cell death, mitochondria are in the center of cancer and neurodegeneration research.

2.2 Structure of the mitochondria.

The broad array of functions of mitochondria is matched by a complex structural organization. The term mitochondrion (mitos, thread and chondrion, granule) was coined by Benda in 1902 to describe the different shapes of mitochondria (Benda, 1898), which are found in a wide range of sizes and numbers inside the cells. The dynamic nature of mitochondria was confirmed in 1914 by Lewis who, equipped with an optic Zeiss microscope and Janus Green staining solution, was able to observe mitochondria in living cells from chick embryos. He observed that surprisingly mitochondria “were never at rest” and that “a single mitochondrion may bend back and forth” and they “can be seen to fuse together into rods or chains, and these elongate into threads” and these “may unite into a complicated network, which in turn may again break down into threads, rods, loops and rings” (Lewis & Lewis, 1914). Almost a half-century after, in 1953, with the help of the recently developed electronic microscopy, George E. Palade defined the internal structural of mitochondria (Palade, 1952). Mitochondria are organelles that have two membranes, the outer mitochondrial membrane (OMM)
and the inner mitochondrial membrane (IMM). These membranes define three regions inside the mitochondria. An inter membrane space (IMS) between the OMM and the IMM, a matrix delimited by the IMM; and the cristae, which are formed by invaginations of the IMM (Figure 1). Each region is very distinct from the others. The OMM possesses porin channels that allow the diffusion of small molecules such as pyruvate and ATP (Blachly-Dyson & Forte, 2001) and, importantly, is in the OMM where different stress signals converge in order to induce apoptosis (Gillies & Kuwana, 2014) (Figure 3). On the other hand, the IMM has a different lipid composition, with a higher proportion of the phospholipid cardiolipin, which makes this membrane impermeable to ions (Paradies et al, 2014) and helps to bend the IMM to form the invaginations called cristae (Acehan et al, 2011). Cristae are very important for cell metabolism since in their membrane the protein complexes for respiration and the ATP synthase are found (Figure 2). Hence, cristae structure is tightly regulated because changes in their shape affect cellular respiration and apoptosis (Mannella et al, 2013). Finally, the matrix is a highly dense region where mitochondria keep many enzymes involved in the citric acid cycle and their DNA (mtDNA), which encodes for 13 subunits of the respiratory complexes involved in oxidative phosphorylation, as well as 2 ribosomal RNAs genes (16S and 12S rRNAs) and 22 transfer RNAs concerned in the translation of the mitochondrial genome (Anderson et al, 1981).

All together, this variety of structures helps the mitochondria to achieve several and very dissimilar tasks, such as, oxidative respiration and cell death.
2.3 Mitochondrial respiration.

Mitochondria are the producers of most of the energy inside a cell through a process called oxidative phosphorylation. It couples the generation of a proton gradient resulting from the electron transfer along the respiratory chain, to ATP production by the ATP synthase. The process starts with the conversion of glucose into pyruvate through glycolysis in the cytosol, which is then transported into the mitochondrial matrix and converted into acetyl-coA by pyruvate dehydrogenase or from fatty acid degradation in the mitochondrial matrix through the beta-oxidation yielding acetyl-CoA, and reduced equivalent of nicotinamide adenine dinucleotide (NAD+) or Flavin adenine dinucleotide (FAD), NADH and FADH₂. Acetyl-CoA will fuel the Krebs cycle in order to produce more NADH and FADH₂ (Herzig et al, 2012). The energy released from these reactions is carried to the respiratory complexes embedded in the IMM by the electrons through the electron transport chain.
donor NADH and FADH$_2$ (Lunt & Vander Heiden, 2011). Once in the inner membrane, this electron flows along an array of protein complexes in a series of redox reactions, each complex has a higher redox potential than the previous one. The flow of electrons in this transport chain ends up when oxygen accepts the electron to form water.

The electron transport chain consists in four complexes arranged along the mitochondrial cristae. The classic random diffusion model stated that the complexes were separated entities connected by liposoluble factors such as Ubiquinone (Coenzyme Q or CoQ) and cytochrome c, which would diffuse in the IMM bilayer. According to this view, both Complex I (NADH:Ubiquinone oxidoreductase) and Complex II (succinate: Ubiquinone reductase), receive electrons from NADH and FADH$_2$, respectively, and transfer them to Ubiquinone. Complex III (Ubiquinol: cytochrome c reductase) receives the electrons from the CoQ and then, electrons flow to complex IV (cytochrome c oxidase) through cytochrome c. Finally, complex IV transfers the electrons to the final acceptor oxygen (Figure 2). Blue-native electrophoresis experiments have challenged this notion by showing functional interaction between complexes in native conditions (Schagger, 1995). Currently, a super complex model is postulated, where complexes associate in a dynamic way to regulate respiration (Genova et al, 2008; Lapuente-Brun et al, 2013). Regardless of the model of the respiratory chain organization, the seminal work of Peter Mitchell in 1961 (Mitchell, 1961) linked the electron transport chain to the production of ATP. During the electron flow along the respiratory chain, the energy from the electrons is used to translocate protons (H$^+$) from the matrix to the IMS. This generates an electrical and chemical gradient across the IMM. This potential energy is released by the
flow of protons back to the matrix through the F$_0$F$_1$ ATPase (ATP synthase or complex V), at the apex of the cristae as an oligomer (Strauss et al., 2008). The pumping of protons in favor of its electrochemical gradient induces the rotation of the F$_1$ subunit of the ATP synthase to generate ATP from ADP (Walker, 1998). Due to the oxidative nature of respiration, mitochondria are one of the major producers of reactive oxygen species (ROS), product that can trigger cell death. This, together with the presence of death factors inside mitochondria makes this organelle an important player in the process of programmed cell death or apoptosis.

Figure 2: Oxidative phosphorylation. The electrons (e$^-$) from NADH and FADH$_2$ pass along the four complexes of the respiratory chain (CI, CII, CIII and CIV) and the diffusible factors CoQ (Q) and cytochrome c (cyt c) (blue arrow), which induces the translocation of protons (H$^+$) from the matrix to the IMS where they accumulate. This electrochemical gradient is used by the ATPase to synthetize ATP from ADP (red arrows).
2.4 Apoptosis.

Programmed cell death by apoptosis is a process used by metazoans to regulate cellular homeostasis during development and throughout adulthood. John Kerr first described this process during the sixties, as the set of morphological changes that certain liver cells would suffer upon ischemic injury, such as the shrinking and rounding of cells, condensation of nucleus and loss of membrane integrity (Kerr et al, 1972). There are two apoptotic pathways, differed by the nature of the apoptotic stimulus. An extracellular stimulus induces extrinsic apoptosis and an intracellular stimulus triggers the intrinsic apoptosis (Green, 2000). Both pathways trigger the activation of a group of cysteine proteases, called caspases, which by cleaving proteins after aspartic residues induce the irreversible dismantling of cellular structures, such as the nuclear lamina where chromatin is organized and the cytoskeleton, or by activating DNase CAD that promotes DNA degradation (Thornberry & Lazebnik, 1998).

The intrinsic pathway is also known as the mitochondrial pathway, due to the relevance of this organelle in the process. All intrinsic stimuli converge into the permeabilization of the OMM, which in turns produces the release of apoptoticogenic factors Smac/Diablo (second mitochondria-derived activator of caspases/direct IAP-binding protein with low pl), AIF (Apoptotic Inducing Factor) and cytochrome c from the IMS to the cytosol. Once in the cytoplasm, cytochrome c binds APAF1 (Apoptotic Protease Activating Factor 1) to form a heptameric ring that recruits pro-caspase 9. This protein complex is called the apoptosome and is essential for apoptosis amplification and progression (Bao & Shi, 2007), because it activates caspase 9 which in turns activates the effector
caspases 3 and 7 to dismantle the cell (Wang, 2001) (Figure 3). Moreover the release of Smac is critical for the full activation of the effector caspases. In fact caspase 3 and 7 are kept in check by the inhibitor of apoptosis (IAP). Smac unleashes caspases from IAP inhibition allowing their full activation following apoptosis induction (Liu et al, 2000).

As any essential cellular process, apoptosis is tightly regulated. In particular, intrinsic apoptosis’ main regulators are the members of the BCL-2 family, which are divided into pro- and anti-apoptotic proteins. The pro-apoptotic BH3-only proteins (BID, BAD, BIM, PUMA and NOXA, among others) are sensor of cellular stress. For example, PUMA and NOXA are activated after irreversible DNA damage (Michalak et al, 2008); BIM is activated upon endoplasmic reticulum stress (Puthalakath et al, 2007) and BAD is activated by the lack of growth factors (Danial et al, 2003). Once activated, BH3-only proteins trigger the oligomerization of other BCL-2 pro-apoptotic members, BAX and BAK proteins, in the OMM, which leads to the above mentioned release of cytochrome c and other apoptogenic factors (Wei et al, 2001). This process is antagonized by the anti-apoptotic members of the BCL-2 family (BCL-2, BCL-XL, MCL-1, among others), which keep in check the activation of the pro-apoptotic members. The current model of the regulation of apoptosis states that the up- or down-regulation of the BCL-2 family members and the hierarchical interplay between pro- and anti-apoptotic proteins define the cell’s destiny upon stress (Figure 3).

In this model, the so-called “activator” BH3-only proteins (such as PUMA, BIM and BID) can trigger OMM permeabilization by directly inducing BAX and BAK oligomerization, but in normal condition their pro-apoptotic function is silenced by the direct interaction with the anti-apoptotic members of the BCL-2 family.
However, upon stressful conditions, the stimulation of “sensitizer” BH3-only proteins (such as NOXA and BAD) induces the displacement of the inhibitory interaction between anti-apoptotic proteins and the “activator” BH3-only proteins, freeing the latter to induce BAX and BK oligomerization (Kim et al, 2006; Youle & Strasser, 2008).

Apart from ER stress, DNA damage and lack of growth factors, cell death can occur upon mitochondrial dysfunction induced by reactive oxygen species (ROS) overload. In normal conditions, ROS is a second messenger that informs about the redox status of the mitochondria. ROS form by the leakage of electrons from the respiratory chain that react with oxygen (Muller et al, 2004). ROS levels are highly regulated by proteins such as superoxide dismutase 2 (SOD2) (Dobrowolny et al, 2008) and catalase (Chelikani et al, 2004). However, when in excess, ROS can induce severe changes into mitochondrial components. ROS produce oxidative damage to mtDNA, leading to mutations in the respiratory chain subunits that are encoded in the mtDNA (Croteau & Bohr, 1997). This, together with the ROS-dependent oxidative modifications suffered by the complexes of the respiratory chain, directly affects the enzymatic activity of the complexes (Cassina & Radi, 1996). Moreover, ROS induce cardiolipin oxidation, which in turns facilitates cytochrome c release (Ott et al, 2002). Finally, ROS and calcium overload trigger the opening of the permeability transition pore (mPTP) in the IMM, which is formed by voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT) and cyclophilin D (Zhivotovsky et al, 2009). The opening of this pore produces an influx of anions and water to the mitochondria that swells and disrupts the OMM and causes the expansion of the IMM, which induces a dissipation of the mitochondria membrane potential.
(ΔΨm), energetic dysfunction and the release of apoptogenic factors (Fulda et al, 2010) (Figure 3). However, repairing or discarding mechanisms can avoid cell death triggered by these dysfunctional mitochondria.

Figure 3: **Mitochondrial pathway of apoptosis.** Upper section of the mitochondrion is undergoing apoptosis through the bax/bak pore. In this case, cellular stress activates BH3-only proteins (BAD, NOXA, PUMA, BIM), which converge in the oligomerization of bax/bak in the OMM. This induces apoptogenic factor release such as AIF, Smac/Diablo and cytochrome c (cyt c). The latter binds to APAF-1 and caspase 9 to form the apoptosome, a cytosolic complex that activates effector caspasess in order to induce apoptosis. Lower section of the mitochondrion is suffering from ROS and/or calcium (Ca++) overload. This leads to the entry of water and solutes into the matrix by activation of the mPTP pore (composed by VDAC, cyclophilin D and ANT). The solute influx induces membrane potential loss and osmotic swelling which induces membrane permeabilization and the release of apoptogenic factors such as cytochrome c, AIF and Smac/Diablo.

2.5 Mitochondrial degradation.

Autophagy is the catabolic cellular process by which cellular components, like proteins and organelles, are selectively engulfed in double or multiple membrane vesicles, called autophagosomes, which fuse with the lysosome for
degradation (Cecconi & Levine, 2008). This process helps to restore nutrients under starvation but, as well, it works as a quality control system to discard defective mitochondria (mitophagy). The process of autophagy, also referred as macroautophagy, is triggered when a nutrient starvation condition depletes the cell from ATP. This results in an increase in cyclic AMP (cAMP) level, which activates AMP activated protein kinase (AMPK) that inhibits mTOR, the main repressor of autophagy. Upon stimulation of autophagy, a vesicle nucleation process takes place, which consist in the engulfment of proteins and organelles by membranes called phagophore. Later, the phagophore will elongate and enclose its content by fusing its membranes to form the autophagosome. The autophagosome will finally fuse with the lysosome to form the autophagolysosome. Inside the autophagolysosome, membranes and proteins will be degraded by lysosomal hydrolases. Finally, the recycled nutrients are poured into the cell through permeases (Mehrpour et al, 2010) (Figure 4, upper panel). In particular, mitophagy is activated upon starvation or glucagon treatment in hepatocytes (Elmore et al, 2001) or after treatment with the uncoupler CCCP (Aoki et al, 2011). All these stimuli trigger the opening of the mPTP, which finally, induces the loss of ΔΨm (Lemasters, 1999). Under physiological conditions, mitophagy takes place in the maturation of reticulocytes into red blood cells, model that has been used to unravel the molecular mechanism of this degradation process (Zhang et al, 2009a). In reticulocytes, the OMM-resident protein NIP3-like protein X (NIX, also known as BNIP3L) binds to LC3 and GABA receptor-associated protein (GABARAP) to induce the formation of the autophagosome (Novak et al, 2010), in addition to other proteins of the autophagic machinery, such as ULK1 and ATG7, which are
important in the vesicle nucleation and vesicle elongation, respectively (Kundu et al, 2008; Zhang et al, 2009b) (Figure 4, middle panel). Although mitochondria from NIX-deficient (NIX−/−) reticulocytes are spared from degradation, pathological conditions emulated by treatment with CCCP can trigger mitophagy (Sandoval et al, 2008). After CCCP treatment, mitochondria are depolarized, decreasing the degradation rate of the mitochondrial kinase PINK1 and inducing its accumulation in the OMM. Under this condition, PINK1 is free to phosphorylate and to help the docking onto the OMM of Parkin, an E3 ubiquitin ligase (Narendra et al, 2008), that ubiquitinates several targets to signal mitochondria to degradation by the recruitments of LC3 and GABARAP (Novak et al, 2010). The polyubiquitin-binding protein p62/SQSTM1 has also been implicated in Parkin-mediated mitophagy as the link between ubiquitinated aggregates and the autophagyc machinery, specifically to LC3 (Geisler et al, 2010). However, recent studies have challenged p62 role in mitophagy, albeit its clear role in ubiquitinated protein aggregation (Narendra et al, 2010) (Figure 4, lower panel).
2.6 Mitochondrial Dynamics.

The pioneering work of Lewis (Lewis & Lewis, 1914) depicted, as early as 1914, the never-resting nature of mitochondria. However, only during these last decades the mechanisms by which they fuse and divide are being elucidated.
More importantly, the notion of the relationship between mitochondrial morphology and function has grown stronger. Nowadays, the changes in mitochondrial shape and its consequences are in the center of cancer (Grandemange et al., 2009) and neurodegenerative diseases (Knott et al., 2008) research. Mitochondrial morphology is regulated by the so-called mitochondrial shaping proteins, a set of mostly mitochondrial-resident GTPases that help this organelle to modify its shape according to the physiological or pathological context. They can be divided into two groups, the pro-fusion and the pro-fission proteins.

There are 3 pro-fusion GTPase proteins, optic atrophy 1 (Opa1) in the IMM and mitofusins 1 and 2 (Mfn1 and Mfn2) in the OMM (Figure 5A). Opa1 is a GTPase anchored in the IMM but exposed to the IMS (Olichon et al., 2002) that helps to fuse the IMM (Figure 5B). Several mutations including missense and nonsense alterations, deletions and insertions in opa1 lead to dominant optic neuropathy in humans (Alexander et al., 2000), while mutations in the GTPase domain in mouse induces embryogenesis failure (Alavi et al., 2007). Regarding mitochondrial phenotype, its down-regulation induces mitochondrial fragmentation, loss of $\Delta\Psi$m and cristae disorganization (Olichon et al., 2003). Opa1 has 8 splice variants in human, which are susceptible of post-translational cleavage by the proteases Yme1L (Gripavic et al., 2007) and PARL (Cipolat et al., 2006). The cleavage produces soluble and membrane-bound isoforms, both of them required for proper mitochondrial fusion (DeVay et al., 2009).

Mfn1 and Mfn2 are responsible for OMM fusion (Figure 5B). They are inserted in the OMM through a transmembrane domain followed by two cytosolic heptad repeats (HR1 and HR2) domains, which are involved in the antiparallel
interaction between two mitofusins in juxtaposed mitochondria, and a GTPase domain in the N-terminus (Koshiba et al, 2004). Cells deficient for mfn1, mfn2 or both have fragmented mitochondria. Moreover, both mitofusins are indispensable for the proper embryonic development (Chen et al, 2003). However, due to different GTPase activities, the fragmentation phenotype is stronger in Mfn1 deficient cells than in Mfn2 (Ishihara et al, 2004). Mfn1, but not Mfn2, is necessary for Opal induced elongation of mitochondria (Cipolat et al, 2004). On the other hand, mutation in Mfn2 in humans produces the peripheral sensorimotor neuropathy Charcot-Marie-Tooth type IIa (CMTIIa) (Zuchner et al, 2004). Moreover, Mfn2 is involved in mitochondria-ER tethering (de Brito & Scorrano, 2008) and in the maintenance of glucose oxidation and mitochondrial functional integrity in muscle cells (Bach et al, 2003).

Mitochondrial fission is achieved by the cytosolic GTPase dynamin-related protein 1 (Drp1) and its putative receptors in the OMM, fission protein 1 (Fis1) and mitochondrial fission factor (Mff). Fission is modulated as well by the mitochondrial division proteins MiD49 and Mid51 (Figure 5A). Drp1 translocates to the OMM upon activation by several stress stimuli such as staurosporine (Frank et al, 2001) (Figure 5B). This activation depends on numerous post-translational modifications such as phosphorylation, ubiquitination, sumoylation and nitrosylation.

Drp1’s most robustly studied post-translational modification is phosphorylation. There are at least three different serines prone to receive a phosphate group: Ser616, Ser637 and Ser693. After mitochondrial dysfunction, the calcium-dependent phosphatase calcineurin is activated due to rising levels of calcium and dephosphorylates Drp1 at Ser637, which induces its translocation to the
OMM (Cereghetti et al, 2008). In fact, hyperactivation of Drp1 by calcineurin induces massive mitochondrial fragmentation, cristae malformation and apoptosis in a model of Huntington's disease (Costa et al, 2010). On the other hand, activation is inhibited through phosphorylation by protein kinase A (PKA) in a cyclic AMP (cAMP)-dependent manner, for example, upon starvation. Lack of nutrients activates PKA to induce the inhibition of Drp1 which leads to mitochondrial elongation by an unopposed fusion process that frees mitochondrial from degradation (Gomes & Scorrano, 2011). Likewise, the availability of the mitochondrial scaffolding protein AKAP121 can inhibit Drp1 activity and reduce apoptosis in cardiomyocytes upon hypoxia (Kim et al, 2011). Phosphorylation in Ser693 also inhibits fragmentation during apoptosis. This GSK3β-dependent phosphorylation does not inhibit Drp1 molecular interactions but rather impinges in Drp1 GTPase activity (Chou et al, 2012). Another phosphorylation that impinges in Drp1 activity is the one in Ser616. However, in opposition to phosphor-Ser637 and phosphor-Ser693, phospho-Ser616 produces mitochondrial fragmentation during mitosis via Cdk1/cyclin B (Taguchi et al, 2007) and protein kinase C (PKC) and it is thought to occur after Drp1 translocation onto the OMM since PKC requires diacylglycerol and calcium to be activated, two compounds found in high concentration in mitochondria (Huang et al, 2011).

Less clear is the relevance of other Drp1 post-translational modifications in its regulation. To this regard, ubiquitination seems to play a role through the mitochondrial E3 ubiquitin ligase MARCH5, which ubiquitinates Drp1 without affecting its stability, leading to mitochondrial fission, allegedly, by affecting Drp1 assembly at the sites where mitochondria divide (Karbowski et al, 2007;
Nakamura et al, 2006). Interestingly, MARCH5 is also supposed to ubiquitinate Mfn2 and Fis1, inducing their degradation, however, the actual mechanism by which MARCH5 acts on mitochondrial morphology remains controversial since some researchers have also observed degradation of Drp1 upon MARCH5 overexpression (Yonashiro et al, 2006). Likewise, the relevance of Drp1 nitrosylation in its activity is debatable, with data showing the importance of NO-dependent nitrosylation of Drp1 in promoting fission in Alzheimer's patients (Cho et al, 2009) and other data suggesting that this effect is due to the NO-dependent phosphorylation in Drp1's Ser616 (Bossy et al, 2010). Finally, sumoylation, a post-translational modification that consists in the covalent bond of sumo (small ubiquitin-like modifier) molecules, has also been involved in Drp1 regulation. In fact, two enzymes that are part of the sumoylation process, SUMO E3 ligase MAPL (mitochondrial-anchored protein ligase) and more recently the sumo protease SENP5 (SUMO-1/sentrin-specific peptidase 5) have been found to stabilize Drp1 and help to the fragmentation of the mitochondrial network in different cell lines (Braschi et al, 2009; Zunino et al, 2007).

Apart from the possible post-translational modifications that Drp1 may suffer, a key step in mitochondrial fission is the translocation and binding of Drp1 to one of its receptors. Fis1 is an OMM-resident protein, which was first discovered in yeast (Figure 5A). In this model, Fis1 is the only receptor of Drp1 and their interaction can be seen as a punctated colocalization on the mitochondria (Mozdy et al, 2000; Tieu et al, 2002). There, Caf4p (Griffin et al, 2005) and Mdv1p (Karren et al, 2005) proteins modulate their interaction. In mammals, Fis1, as its ortholog in yeast, has a cytosolic N-terminal region composed by a coiled coil domain followed by six alpha helices that form two tetratricopeptide-
repeat (TPR)-like domains used for protein-protein interaction, followed by a transmembrane (TM) domain to anchor to the OMM and a short IMS stretch at the C-terminus (Suzuki et al, 2003) (Figure 5A). Fis1’s IMS stretch and the TPR-like domain are necessary to achieve a proper mitochondrial fragmentations since the IMS stretch directs Fis1 to the OMM and the TPR-like domain is essential for its interaction with Drp1 (Serasinghe & Yoon, 2008; Yu et al, 2005). Interestingly, the coiled-coil domain has been connected to the regulation of fission by a steric effect on the interaction of this region with the TPR-like domain (Tooley et al, 2011).

Correlating with the pro-fission function of its yeast ortholog, hFis1 has been shown to also trigger mitochondrial fragmentation and enhance cytochrome c release and intrinsic apoptosis when overexpressed. In the same publication the importance of Drp1 to hFis1’s function became patent, since the mitochondrial fragmented pattern was rescued by overexpressing a dominant negative form of Drp1 (Yoon et al, 2003). In the same line, down-regulation of hFis1 induces a strong resistance to cell death (Lee et al, 2004). Moreover, hFis1 interacts with Drp1 in order to promote mitochondrial fragmentation (Kim et al, 2011; Yoon et al, 2003) and, interestingly, Fis1 also mediates mitochondrial fragmentation and apoptosis in an in vivo model of myocardial infarction (Wang et al, 2012) (Figure 5B). Human Fis1 is important in promoting extrinsic apoptosis, since it seems to interact with translocated Drp1 in lipid raft-like structures at the mitochondrial surface upon activation of the death receptors Fas and TRAIL (Ciarlo et al, 2010; Kaddour-Djebbar et al, 2010).

Despite that the function of hFis1, in terms of inducing mitochondrial fragmentation and apoptosis, correlates with the yeast model, there are no
orthologs of the adaptor proteins (Caf4p and Mdv1p) and no clear mechanism of action for mitochondrial fission in mammals (Zhao et al, 2013). In fact, during the last years, several data have challenged the precise role of mammalian Fis1 in mitochondrial fragmentation. In mammals Fis1 has a rather diffuse pattern in mitochondria, in contrast with Drp1 that has a punctate distribution when it translocate to the mitochondria (Smirnova et al, 2001). Moreover, its down-regulation has only a mild mitochondrial fusion phenotype (Otera et al, 2010). These controversial data on hFis1 have led researchers to look for other putative candidates for Drp1 receptor. Thus, over the last years, Mff, MiD49 and MiD51 have been involved in mitochondrial fission.

Mff was first discovered in a small interfering RNA (siRNA) screening aiming to find factors that would change mitochondrial shape in drosophila cells. In this study, siRNA against Mff produced inhibition of mitochondrial fragmentation, delayed cytochrome c release and inhibition of apoptosis; comparable to silencing Drp1 and Fis1 (Gandre-Babbe & van der Bliek, 2008). Later on, by studying the human ortholog, Mff was found to be located in the OMM where it had a punctated distribution and interacted with Drp1 to promote its recruitment to the OMM and induce mitochondrial fragmentation. Conversely, Mff silencing inhibited mitochondrial fission. Interestingly, MFF overexpression could induce mitochondrial fragmentation independently of Fis1, since Fis1 down-regulation did not influenced Mff effect on mitochondrial fission (Otera et al, 2010).

Another adaptor protein to Drp1 at the OMM is the mitochondrial dynamic protein of 51 KDa (MiD51), also known as mitochondrial elongation factor 1 (MIEF1) and its paralog MiD49. These proteins are found from vertebrates on
and, initially, the actual function of these factors was not totally understood with conflicting data showing opposite functions for the MiDs proteins. First, it was found that the MiDs had a punctated distribution on the mitochondrial surface and silencing of MiD49 and MiD51 expression reduced Drp1 recruitment to the mitochondria and induced mitochondrial elongation, connecting them with a pro-fission function; but, unexpectedly, their overexpression caused the same effect, namely mitochondrial elongation (Palmer et al, 2011). Another publication showed that the down-regulation of MiD51 induced mitochondrial fragmentation, supporting the idea of a pro-fusion function for MiD51 (Zhao et al, 2011). Recently, a different research group has confirmed the pro-fission role of MiD49 and MiD51, that could act independently of each other in order to promote Drp1 recruitment and mitochondrial fragmentation (Loson et al, 2013). In this last paper, by using null cells for Fis1 and Mff, the authors also showed that each of these OMM-resident proteins could act independently as receptors for Drp1 in the fission process, although the exact mechanism that leads to mitochondria division is still not clear (Loson et al, 2013). Clearly, these recent findings show that the mitochondrial fission process in mammals is far more complicated than the very well understood process in yeast. In fact, not only Mff and the MiDs have no ortholog in yeast, some of them (Drp1, Fis1 and Mff) have several spliced isoforms to add complexity to the process. The state of the art mechanism of mitochondrial fission suggests that each receptor could act independently and/or in sequence in order to achieve mitochondrial fragmentation and the preference for one or another might be orchestrated by the type of stimulus (Elgass et al, 2013; Zhao et al, 2013)(Figure 5C). In particular, Fis1 might be relevant in organizing mitochondrial fission upon
hypoxia (Wang et al, 2012) or it could induce fragmentation through a complete different mechanism than Drp1, as shown by a latest publication that presents the TBC1D15 protein, which has a domain found in the GTPase-activating proteins, as an interactor and effector of Fis1-mediated fission in a Drp1-independent manner (Onoue et al, 2013).

The increase in complexity in mammalian mitochondrial fission and the putative redundancy of having several Drp1 receptors may account for the appearance of new roles for these proteins. In particular, human Fis1 has been implicated in processes other than mitochondrial fission. First of all, Fis1-mediated apoptosis has been found to depend on mitochondrial dysfunction that requires the integrity of the short intermembrane space IMS stretch of the molecule, but not the domain required for binding to Drp1, suggesting that its role in apoptosis triggered by mitochondrial dysfunction is independent of its role in Drp1 recruitment and mitochondrial fragmentation (Alirol et al, 2006). In order to induce apoptosis, hFis1 requires not only the IMS region but as well a cue from the ER calcium-dependent gateway (Alirol et al, 2006). Remarkably, hFis1 can bind to the ER-resident protein Bap31 and bridges mitochondria and ER to form a complex with pro-caspase 8, which is activated to induce cell death (Iwasawa et al). In another aspect of cell biology, mitochondrial fragmentation is needed in order to segregate dysfunctional mitochondria with low membrane potential to proceed with the degradation through autophagy (mitophagy). As expected, the fission machinery plays a key role in this process since dominant negative Drp1 mutant and siRNA agains Fis1 inhibit mitophagy (Twig et al, 2008a), moreover, the over-expression of hFis1 increases autophagosomes formation and produces partial colocalization of these autophagosomes with mitochondria, suggesting
that hFis1 can, by itself, increase mitophagy (Gomes & Scorrano, 2008). In this publication it was found as well that, in order for Fis1 to promote autophagy, the same IMS stretch is required (Gomes & Scorrano, 2008). Supporting the participation of Fis1 in the removal of defective mitochondria through mitophagy, a recent paper showed that hFis1 mutation resulted in LC3 aggregates upon mitochondrial dysfunction. Interestingly, this effect was suppressed by mutation of Drp1 or Mff indicating that Fis1 acts downstream of Mff recruitment of Drp1 during the fission process (Shen et al, 2014).

Figure 5: Mitochondrial shaping proteins. Panel A shows a schematic representation of the domains of each member of the mitochondrial shaping proteins. Opa1 and Drp1 possess a GTPase, middle and GED domain which are found in dynamin proteins. Mitofusins (Mfn1 and Mfn2) miss the GED domain but instead they possess a coiled coil (CC) domain. Opa1 has a transmembrane (tms) and lipid binding domain to anchor to the IMM. Mitofusins only have the tms to bind to the OMM. On the other hand, apart from they tms domain, the receptors of Drp1 in the OMM, Fis1, Mff and MiD49/51, are very dissimilar between them and have different protein-protein interaction domains. Fis1 has two Tetratricopeptide repeats (TPR) domains, Mff posses short repeats and the MiDs are composed by a Mab-21 like domain. This dissimilarity of domains suggest a differential regulation and/or interaction with Drp1, showed in panel C. Finally panel B depicts the mitochondrial localization and function of the pro-fusion members of the mitochondrial shaping proteins. Opa1 is in charge of IMM fusion and the Mitofusins are localized in the OMM were they trigger their fusion. Mitochondrial fusion is achieved by the concerted function of Opa1 in the IMM and the Mitofusins in the OMM. On the other hand, mitochondrial fission in pane C occurs when, after dephosphorylation, Drp1 translocates to the OMM by binding to one or more of its receptors, Fis1, Mff and/or MiDs, which leads to its oligomerization and constriction around the mitochondrial to produce the fragmentation.
2.7 Mitochondrial dynamics in cell death.

Changes in mitochondrial morphology and ultrastructure have been linked to apoptosis due to early observation showing mitochondria fragmentation in dying neurons by growth factor deprivation (Martinou et al, 1999). In fact, Drp1 induces, along with mitochondrial fragmentation, cell death in a calcineurin-independent manner (Cribbs & Strack, 2007) and a dominant negative form of Drp1 delayed cytochrome c release and cell death (Frank et al, 2001). Moreover, the ablation of Drp1 produces longer mitochondria and inhibits the proper developmental apoptosis during the formation of the neural tube leading to mouse embryonic lethality (Wakabayashi et al, 2009). Likewise, Fis1-deficient cells are even more resistant to cell death (Lee et al, 2004) and, as discussed in the previous section, hFis1 has also linked to apoptosis independently of its fission function through the ER-gateway pathway (Alirol et al, 2006; Iwasawa et al). Interestingly, mitochondrial fragmentation increases ROS production (Yu et al, 2006) and is necessary for the release of cytochrome c, but not of Smac/Diablo (Estaquier & Arnoult, 2007). On the other hand, mitochondrial fusion gets inhibited during apoptosis (Karbowski et al, 2004). Actually, Mfn2 has a protective role during cell death in cerebellar granule neurons (Jahani-Asl et al, 2007) and Opa1-deficient cells are more sensitive to apoptosis stimuli, a process that needs Fis1 expression (Lee et al, 2004).

The connection between mitochondrial fragmentation and apoptosis is, nowadays, clear in neuronal (Leinninger et al, 2006; Yuan et al, 2007) and cardiomyocyte (Kim et al, 2011; Parra et al, 2008) models of cell death. However, the precise mechanism by which fragmentation induces cytochrome c release is
still debated. The first hints of a mechanism linking mitochondrial fragmentation, mitochondrial shaping proteins and apoptosis came from the study of the involvement of the Bcl-2 family in mitochondrial dynamics. Thus, the pro-apoptotic member of the Bcl-2 family Bax, was found to form clusters with Drp1 and Mfn2, suggesting a role for Bax in mitochondrial fragmentation preceding apoptosis (Karbowski et al, 2002). However, it has also been found that a canonical way to inhibit Bax, namely the expression of the anti-apoptotic bcl-2 family protein Bcl-xL blocks cytochrome c release without affecting mitochondrial morphology, advocating for the notion that mitochondrial fraction and apoptosis are two distinct and independent processes (Sheridan et al, 2008). To this respect, a recent paper has confirmed the interaction of Bax and Drp1 after its translocation to foci on the OMM, where it can, in fact, promote Bax oligomerization in cardiolipin-reach membranes that will turn into scission sites and induce cytochrome c release (Montessuit et al, 2010) (Figure 6). In this publication, the expression of mutant Drp1 indeed reduced apoptosis during short periods of apoptotic insults such as UV irradiation, but after longer incubation this protection was lost due to other unknown mechanism, conciliating both views by advocating for an early Drp1-dependent apoptosis followed by a later Drp1-independent apoptosis (Montessuit et al, 2010). Conversely, Bax and Bak can stabilize the Drp1 translocation to the OMM during cell death (Wasiak et al, 2007). In the case of the Mfn2-Bax interaction (Karbowski et al, 2002), Bax seems to be important for the mitochondrial distribution of Mfn2 in rather healthy cells (Karbowski et al, 2006).

Another aspect that relates mitochondrial-shaping proteins such as Opa1 and Drp1 with apoptosis is the remodeling of the cristae in the IMM. Being the site
where most of the cytochrome c resides, modulation of the cristae shape by opening of the cristae junction is also of the most relevance for apoptosis progression (Scorrano et al, 2002). Opa1 sites in the cristae junctions where the membrane-bound and soluble forms oligomerize in order to keep the junctions tight. This oligomerization is lost during apoptosis, with the consequent displacement of cytochrome c to the IMS and then to the cytosol (Cogliati et al, 2013; Frezza et al, 2006) (Figure 6). Interestingly, Drp1 can also induce cristae remodeling. In particular, the pro-apoptotic and ER-resident BH3-only protein BIK activates calcium waves from the ER, which in turn trigger cytochrome c release from mitochondria (Germain et al, 2002). The link between cause and effect seems to be Drp1, which as previously discussed, is activated by the calcium-dependent phosphatase calcineurin (Cereghetti et al, 2008). After translocation, Drp1 would induce cytochrome c movement to the IMS, by a yet unknown mechanism, since the expression of mutant Drp1 strongly inhibits this process (Germain et al, 2005).

Finally, during the last years, the ER has been found to play an important role in mitochondrial fission. Whether the ER needs the so-called mitochondrial shaping proteins to achieve this fission is still debated. As already mentioned, Bap31 can interact with Fis1, linking this pro-fission protein with an ER-resident protein such as Bap31, which is enriched in the mitochondrial associated membranes (MAMs), the contacts sites between mitochondria and ER (Iwasawa et al). However, certain reports have enriched the discussion by showing that ER could induce mitochondrial fission in early stages, by a mechanism independent of the mitochondria-shaping proteins (Friedman et al, 2011). Using high-resolution microscopy, the authors showed in living cells that ER surrounds mitochondria
in areas where fission will take place. Interestingly, this fission takes place even in cells with low levels of the pro-fission proteins Mff and Drp1 (Friedman et al, 2011) and it requires the ER-resident protein inverted formin 2 (INF2) to recruit actin filaments to help constrict mitochondria in MAM sites prior to Drp1 action (Korobova et al, 2013).

**Figure 6: Mitochondrial dynamics in cell death.** Upon apoptotic stimuli, the interaction of soluble and unsoluble forms of Opa1 at the Crista junction is lost. The disruption of these junctions leads to the remodeling of the cristae and the release of cytochrome c from the cristae to the IMS. Meanwhile, Drp1 translocation induces the bending of the OMM which helps for Bax/Bak oligomerization, allowing the release of cytochrome c to the cytoplasm. As previously described, this cytosolic cytochrome c will induce apoptosis through the formation of the apoptosome.
2.8 Mitochondrial dynamics in respiration.

Changes in mitochondrial cristae ultrastructure have been known to happen since the technology allowed the observation of sub-mitochondrial structures (e.g. electron and cryo-electron tomography). These structures can change according to external cues or special physiological states, suggesting from early studies that cristae shape is not random and would be, in fact, tightly regulated (Mannella, 2006). The regulation of cristae shape would be of a paramount importance, since the respiratory chain complexes (RCC) reside in these structures and, according to the supracomplex model, a dynamic organization of the RCS is needed in order to regulate respiration.

Recently, many progresses have been made regarding the molecular link between changes in mitochondrial morphology and its effects in respiration. In fact, the ATP synthase have been found to form oligomers in the apex of cristae. Interestingly, these dimers produced a curvature and helped to shape the cristae structure in order to generate proton-dense regions to enhance respiratory efficiency (Strauss et al, 2008). A different study in yeast, showed that mitochondria expressing mutated ATPase that were unable to dimerize, produced deformed cristae (Paumard et al, 2002). Conversely, it was found that cristae shape impinges in respiration process as well, since mitochondrial elongation upon starvation enhanced cristae density and favored ATP synthase dimerization, supporting with molecular basis the idea that mitochondrial shape and respiration are intertwined (Gomes et al, 2011). Finally, genetic proof of this relation arrived when in vitro and in vivo manipulation of cristae shape by modulating Opa1 levels showed to alter RCS assembly, which disturbed
respiratory efficiency and cellular growth (Cogliati et al, 2013) (Figure 7). These results were confirmed by a different group that showed that the modulation of respiration through Opa1 is regulated by the energy context of the cell and seems to be independent of Opa1’s role in mitochondrial fusion (Patten et al, 2014).

Figure 7: Mitochondrial dynamics in respiration. Cristae morphology and function are affected by the mitochondrial shaping proteins. (A) High respiration rates are induced by the pro-fusion proteins, because mitochondrial fusion produces a higher density of cristae which increases respiration. Moreover, ATPase oligomers at the apex of the cristae and Opa1 oligomers at the crista junctions favors a proper crista shape for the stabilization of the respiratory chain complexes (RCC) into super complexes which increases the respiration efficiency. (B) In contrast, dismantlement of the Opa1 oligomers at the crista junctions produces remodeling of the crista shape, which induces low respiration rate and impairs cell growth.
2.9 Mitochondrial dynamics and quality control.

As mentioned before, mitochondria have their own DNA, mtDNA that codes, mainly, for components of the RCS. This provides an efficient way to modify the RCS components according to the specific needs of each mitochondrion, but also places the mtDNA very close to the main source of ROS in the cell, the respiratory chain, which induces mutation to the mtDNA (Balaban et al, 2005). This ROS-mediated mtDNA heteroplasmicity can be detrimental for mitochondrial function. However, mitochondrial fusion helps diluting these mutations and induce complementation by fusing healthy mitochondria with mutated ones (Nakada et al, 2001). ROS can damage as well proteins and lipids in the mitochondria. These injuries can be mitigated by several mechanisms such as increasing the expression of chaperones (Nargund et al, 2012) or inducing degradation of damaged proteins by proteases (Baker et al, 2011) in the matrix or by triggering the ubiquitin-dependent degradation of OMM-resident proteins (Heo & Rutter, 2011).

As mentioned before, another quality control mechanism is the degradation of whole defective and depolarized mitochondria by mitophagy. Interestingly, mitophagy seems to be intimately connected with the cycles of fission and fusion. In fact, fission is needed to separate mitochondria with high and low ΔΨm in order to proceed with the degradation of the dysfunctional daughter, because dominant negative forms of Drp1 or knocking down Fis1 decrease mitophagy and mitochondria with oxidized proteins accumulate (Twig et al, 2008b). Conversely, upon mitochondrial damage, Mfn1 and Mfn2 get degraded in a Parkin-dependent manner to inhibit mitochondrial elongation, which favors
mitophagy (Tanaka et al, 2010) (Figure 8, top). On the other hand, a physiological inhibition of Drp1 by PKA during starvation induces rather mitochondrial elongation and confers mitophagy resistance and respiration efficiency (Gomes & Scorrano, 2011) (Figure 8, bottom).

Figure 8: Mitochondrial dynamics in mitochondrial degradation. Mitochondria have different shape according to the nature of the degradation stimulus. Dysfunctional mitochondria unevenly fragment in order for the damaged part to be degraded by mitophagy. Fragmentation is achieved by the proteosomal degradation of the pro-fusion Mitofusins by Parkin. On the other hand, upon starvation, mitochondria elongate to be spared from degradation and to increase the ATP production. This process is achieved by inhibition of the pro-fission protein Drp1 in a PKA-dependent manner.
2.10 Rationale and aims of the study.

The knowledge gathered over the last decades show the relevance of mitochondrial dynamics in every aspect of mitochondrial physiology and function. Opa1 is important for the maintenance of cristae structure and together with the fusion properties of mMfn1 and Mfn2 play a role in oxidative phosphorylation. Mitochondrial fission through Drp1 seems to be important for mitochondrial quality control and apoptosis. How this is achieved is not fully understood. In fact, the model of mitochondrial fragmentation proposed by studies in yeast has been shown to be different from the mammalian one. The proposed model where Drp1 oligomerize in the OMM by the interaction with Fis1 has been challenged with the discovery of new proteins such as Mff, which seems to be suitable for this interaction. Moreover, new functions for Fis1 have been proposed that include enhancement of autophagy, involvement in autophagosome formation and the induction of apoptosis through the interaction with the ER-resident protein bap31. Taken together, these data show that Fis1 is involved in different steps of mitochondrial dynamics and quality control. How Fis1 mediates its many functions in mitochondrial lifespan is still unclear. Interestingly, careful analysis of mouse Fis1 gene in Luca Scorrano’s laboratory has revealed the existence of three potentially translated splicing variants, which might explain the functional versatility of this protein. Therefore, the aim of my thesis work was to investigate the role of mouse Fis1 splice variants in mitochondrial morphology.
Chapter 3

RESULTS

3.1 An alternatively spliced mitochondrial Fission 1 variant participates in mitochondrial elongation during autophagy.

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Manuscript in preparation

The role of the outer mitochondrial membrane protein Fission 1 (Fis1) as a mitochondrial receptor for the pro-fission dynamin related protein 1 (Drp1) has been recently challenged and Fis1 has been conversely implied in mitophagy, but the molecular mechanisms governing Fis1 involvement in mitochondrial morphology and autophagy are unclear. Here we show that mouse Fis1 is alternatively spliced in variants with opposite effect on mitochondrial morphology. Fis1 variant 1 or 3 trigger fragmentation whereas variant 2 induces mitochondrial elongation. Upon starvation, Fis1 variant 2 expression is up-regulated in a protein kinase A-dependent manner and its specific knockdown inhibits autophagy associated mitochondrial elongation. Thus, Fis1 is alternatively spliced to modulate mitochondrial morphology during starvation.

I contributed with the planning and execution of experiments in this work. Some experiments required the help from the co-authors. Moreover, I contributed in the writing of the manuscript together with Denis Martinvalet.
An alternatively spliced mitochondrial Fission 1 variant participates in mitochondrial elongation during autophagy.

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Abstract

The role of the outer mitochondrial membrane protein Fission 1 (Fis1) as a mitochondrial receptor for the pro-fission dynamin related protein 1 (Drp1) has been recently challenged and Fis1 has been conversely implied in mitophagy, but the molecular mechanisms governing Fis1 involvement in mitochondrial morphology and autophagy are unclear. Here we show that mouse Fis1 is alternatively spliced in variants with opposite effect on mitochondrial morphology. Fis1 variant 1 or 3 trigger fragmentation whereas variant 2 induces mitochondrial elongation. Upon starvation, Fis1 variant 2 expression is up-regulated in a protein kinase A-dependent manner and its specific knockdown inhibits autophagy associated mitochondrial elongation. Thus, Fis1 is alternatively spliced to modulate mitochondrial morphology during autophagy.
INTRODUCTION

Mitochondria are crucial organelles for cellular energy conversion and metabolism (Attardi & Schatz, 1988; McBride et al, 2006; Saraste, 1999). Moreover, mounting evidence indicate that they regulated cell signaling, Ca^{2+} homeostasis, cell cycle regulation, differentiation, cell death and aging (Green & Kroemer, 2004; Kroemer et al, 2007; Rizzuto et al, 2000). The involvement in this plethora of cellular functions is matched by mitochondrial morphological and structural versatility. Mitochondria continuously adjust their morphology by fusion and fission events on which cellular cues or pathologic conditions impinge; moreover they intimately interact with other organelles such as the endoplasmic reticulum (ER) (Cereghetti et al, 2010; Cereghetti et al, 2008; Chan, 2006a; Dimmer & Scorrano, 2006; Frezza et al, 2006; Gomes et al, 2011; Hoppins et al, 2007; Ishihara et al, 2013; Liesa et al, 2009; Okamoto & Shaw, 2005; Westermann, 2010). A family of dynamin-related GTPases and their adaptor proteins regulates fusion and fission. Mitofusin (MFN) 1 and 2 on the outer membrane and Optic atrophy 1 (OPA1) on the inner membrane regulate fusion (Cereghetti et al, 2010; Cereghetti et al, 2008; Chan, 2006a; Chen et al, 2003; Dimmer & Scorrano, 2006; Eura et al, 2003; Frezza et al, 2006; Gomes et al, 2011), the latter being involved in maintenance of cristae shape and thereby in regulation of apoptosis and of mitochondrial respiration (Cipolat et al, 2006; Cogliati et al, 2013; Frezza et al, 2006). Mitochondrial fission requires the translocation of dynamin-related protein 1 (Drp1) from the cytosol to the mitochondria to induce fission (Smirnova et al, 1998; Westermann, 2010; Yoon et al, 2001). Cytosolic DRP1 is dephosphorylated by calcineurin to translocate to
mitochondrial outer membrane (MOM) (Cereghetti et al, 2008), while cyclic AMP-dependent protein kinase (PKA) phosphorylates DRP1 to inhibit fission (Chang & Blackstone, 2007; Cribbs & Strack, 2007). In yeast, the MOM protein Fis1 plays an essential role in the recruitment to the mitochondria of Dnm1, the yeast homolog of Drp1, (Hoppins et al, 2007; Mozdy et al, 2000; Okamoto & Shaw, 2005; Westermann, 2010). Fis1 has tetratricopeptide-repeat (TPR)-like domains at the N-terminus exposed to the cytoplasm, and a transmembrane (TM) domain at the C-terminus to anchor in the MOM (James et al, 2003; Jofuku et al, 2005; Yoon et al, 2003). In mammals, Drp1 uses as MOM receptors different proteins, including mitochondrial fission factor (Mff) and mitochondrial dynamics MiD51 and MiD49 (Gandre-Babbe & van der Bliek, 2008; Otera et al, 2010; Palmer et al, 2011; Zhao et al, 2011). Although forced human Fis1 expression induces mitochondrial fragmentation and its knockdown results in mitochondrial elongation, Fis1 seems dispensable for Drp1 docking (James et al, 2003; Jofuku et al, 2005; Otera et al, 2010; Young et al, 2003), but it interacts with TBC1D15 to regulate mitochondrial morphology in a Drp1-independent manner (Onoue et al, 2013). Moreover, Fis1 was also connected to autophagy induction (Gomes & Scorrano, 2008). In fact, Fis1 is important for the downstream removal of defective mitochondria through mitophagy since Fis1 mutation results in LC3/LGG-1 aggregates upon ROS-mediated mitochondrial stress (Shen et al, 2014). Interestingly, this effect was suppressed by mutation of Drp1 or Mff indicating that Fis1 acts downstream of Mff recruitment of Drp1 during the fission process (Shen et al, 2014). Taken together, it appears that Fis1 is involved in different aspects of mitochondrial morphology and quality control, but how one single molecule participates in many mitochondrial functions is still
unclear. Here we show that in addition to its canonical full length variant, mouse Fis1 is alternatively spliced in variants with opposite effects on mitochondrial shape in resting and starved cells.
RESULTS

Mouse Fis1 is alternatively spliced in variants with opposite effects on mitochondrial length.

Bioinformatics analysis of mouse Fis1 locus from the Ensembl Genome Browser showed three predicted splice variants that we named mFis1.1, mFis1.2 and mFis1.3 (Fig. 1A and B). Mouse Fis1.1 is homologous to human Fis1, while residues 4-10, part of the first α-helix, are missing from mFis1.2 and residues 7-56, encompassing half of the TPR motif, from mFis1.3. Analysis of messengers indicated that although mFis1.1 was expressed at high levels in all the tissues tested, mFis1.2 and mFis1.3 expression was restricted to tissues with high energy demand such as brain, heart, liver and muscle (Fig. 1C).

To address the subcellular localization of these isoforms, each N-terminal V5 tagged Fis1 variant was expressed in mouse embryonic fibroblasts (MEFs) together with dsRED targeted to mitochondria (mtRFP) or to the endoplasmic reticulum (ER-RFP). Confocal microscopy indicated that all three Fis1 variants strongly localized at mitochondria with a moderate colocalization with the ER (Fig. 2A and B). This subcellular distribution is in agreement with Fis1 being an outer mitochondrial membrane protein also reported to interact with ER-resident protein Bap31 (Iwasawa et al, 2011). MG132-mediated proteasome inhibition increased the expression of Fis1.3 suggesting that this variant is instable and most likely a byproduct of mouse Fis1 gene expression (Fig. 1E).

Interestingly, ectopic expression of either Fis1 variant 1 or 3 in the absence or presence of MG132 triggered mitochondrial shortening, whereas Fis1.2 overexpression caused an unexpected mitochondrial elongation (Fig. 2D and E).
Thus, *Fis1.2* at a major difference from *Fis1.1* or *Fis1.3* causes mitochondrial elongation.

**Fis1 variant 2 acts as a dominant negative.**

To further understand the mechanism by which *Fis1.2* triggers mitochondrial elongation, MEF expressing mtRFP were transfected with cDNA encoding *Fis1.1* alone or in the presence of increasing amount of cDNA coding for *Fis1.2* and the mitochondrial morphology assessed by confocal microscopy. As previously, overexpression of *Fis1.1* triggered a pronounced mitochondrial fragmentation, however the addition of *Fis1.2*, even at the smallest ratio significantly inhibited the *Fis1.1* triggered mitochondrial fragmentation (Fig. 3A and B). This result indicates that most likely *Fis1.2* acts as a dominant negative inhibitor of *Fis1.1*.

**Fis1 variant 2 is upregulated upon starvation.**

Given that *Fis1.2* is preferentially expressed in tissues characterized by high energy demand suggests that its expression can be modulated. Moreover, since increased *Fis1.2* levels are associated with mitochondrial elongation, we tested whether changes in *Fis1.2* level could play a role in mitochondrial elongation observed during macroautophagy (Gomes et al, 2011). Real time (RT)-PCR indicated that under starvation, *Fis1.2* mRNA levels were significantly increased, while they remained stable when mitochondria were depolarized using the uncoupler carbonyl cyanide chlorophenylhydrazone (CCCP) or when apoptosis was induced by staurosporine (Fig. 4A). Conversely, *Fis1.1* and *Fis1.3* mRNA levels did not change significantly under all the conditions tested (Fig. 4A). These results were further recapitulated by qRT-PCR using specific primers for *Fis1.1* and *Fis1.2* (Fig 4 B and C). Interestingly, the increase in *Fis1.2* levels was
reversible upon readdition of nutrient rich media as testified by qRT-PCR (Fig. 4D-F). Thus the expression of Fis1 variant 2 is regulated by extracellular cues.

**PKA controls mouse Fis1 variant 2 mRNA expression upon starvation.**

During starvation, cAMP rises, activating PKA to inhibit Drp1 via Ser637 phosphorylation and causes mitochondrial elongation (Cereghetti et al, 2008; Gomes et al, 2011). Since Fis1.2 mRNA expression is upregulated upon starvation, we tested whether PKA plays a role in its induction. The chemical PKA activator forskolin induced an up-regulation of variant 1 and 2 mRNAs that was inhibited by the PKA inhibitor H89 (Fig. 5A and B). However, under starvation, only the up regulation of Fis1.2 mRNA was significantly inhibited by H89 (Fig. 5C). Altogether, these results show that during starvation mouse Fis1.2 mRNA expression is regulated in a PKA-dependent manner.

We next asked how PKA modulates Fis1.2 expression level during starvation. PKA can modulate alternative splicing by acting on the splicing factor hnRNP K and U2AF65 (Cao et al, 2012; Naro & Sette, 2013) that compete for binding to PKA-responsive RNA element (KARRE), TCCCT and TCCT pyrimidine-rich 3’ splicing site at intron ends (Cao et al, 2012; Thisted et al, 2001). We found such motif at the 3’-end of the first intron of mouse Fis1 pre-mRNA, whose splicing dictates the production of mouse Fis1.1 over Fis1.2 (Fig. 1B). We therefore tested the effect of U2AF65 silencing on starvation-induced Fis1.2 upregulation. Upon efficient knockdown of U2AF65 (Fig. 5D), the starvation-induced upregulation of Fis1.2 mRNA was reduced (Fig. 5E) while expression of Fis1.1 mRNA was not affected (Fig. 5F). Together this indicates that during starvation PKA regulates Fis1.2 expression by impinging on the splicing factor U2AF65.
**Fis1 variant 2 is required for starvation-induced mitochondrial elongation.**

Since we could efficiently and specifically silence Fis1.2 expression (Fig. 5E and F), we investigated the functional significance of starvation-induced Fis1.2 upregulation. Silencing of Fis1.2 significantly inhibited starvation-induced mitochondrial elongation (Fig. 4G and H). Moreover, the indirect modulation of Fis1.2 expression through U2AF65 silencing also resulted in a significant reduction in starvation-induced mitochondrial elongation (Fig. 5G and H). These results indicate that during starvation PKA also acts on mitochondrial morphology by upregulating the profusion Fis1 variant 2.
Discussion

During macroautophagy, mitochondria elongate in a PKA, Drp1-dependent manner. Here we identify Fis1 alternative splicing as an additional target of PKA-induced autophagic mitochondrial elongation. Mouse Fis1 is alternatively spliced in three variants with opposite effects on mitochondrial length. Ectopic expression of Fis1 variant 1 and 3 triggers mitochondrial fragmentation while that of variant 2 is profusion. The fact that Expression of Fis1.1 is pro-fission in accordance to the reported effect of human Fis1 overexpression, whose mouse homologue is Fis1.1 (James et al, 2003). Interestingly, overexpression of mouse Fis1.3 that lacks residues 7-56, encompassing half of the Fis1’s TPR motif, still induces mitochondrial fragmentation. Human Fis1 requires the first 10 amino acid residues to cause mitochondrial fission (Yu et al, 2005). Mouse Fis1.3 possesses 6 of these 10 amino acids, narrowing down the Fis1 motif required for mitochondrial fission to its 6 first amino acids.

Unexpectedly, mouse Fis1.2 overexpression increases mitochondrial length. Yet, Fis1.2 is only missing residues 4-10, suggesting that the Fis1 motif required for fission can be narrowed to residues 4-6 at the N-terminal end of Fis1, in agreement with the prediction of Yu et al. (Yu et al, 2005). Fis1.2 is preferentially expressed in the brain, heart liver and muscle, tissues characterized for their high energy demand, suggesting that increased expression of this variant could maintain a developed mitochondrial network to meet the energy requirement of these organs. Interestingly, three fold less Fis1.2 cDNA was already enough to inhibit Fis1.1 overexpression-mediated mitochondrial fragmentation. These very exciting results suggested that a minority of Fis1.2 molecules could putatively counteract an excess of Fis1.1 suggesting that Fis1.1 might work as an oligomer.
Of note, starvation of cultured cells induces a 10 fold upregulation of Fis1.2 expression although the overall expression level of Fis1.1 remained higher than that of Fis1.2. Since, we found that a minority of Fis1.2 can counteract a threefold excess of Fis1.1 in co-transfection experiment, it is very likely that the endogenous lower level of Fis1.2 should be effective at inhibiting the endogenous excess of Fis1.1 and this even more efficiently after starvation had triggered the upregulation of the expression Fis1.2. This process rise the possibility of a fine tuning mechanism of Fis1.1 function in mitochondrial morphology through the regulation of Fis1.2 expression.

By increasing the pool of cAMP, starvation leads to the activation of PKA that triggers mitochondrial elongation by unopposed fusion following the inhibition of Drp1 (Gomes et al, 2011; Rambold et al, 2011). Here we found that PKA triggers the upregulation of Fis1.2 expression by regulating its alternative splicing (Cao et al, 2012; Naro & Sette, 2013). In fact, hnRNP K compete with U2AF65 for PKA-responsive RNA element (KARRE) that are TCCCT and TCCT pyrimidine-rich 3’ splicing site at intron ends. HnRNP K blocks the usage of this splicing site whereas U2AF65 favors it (Cao et al, 2012). We found 111 pyrimidine rich TCCCT and TCCT motifs in mouse Fis1 gene first intron (where the alternative splicing occurs), while this number is below 10 in all other 4 introns. A partial knockdown of U2AF65 reduced Fis1.2 induction upon starvation, consequently inhibiting starvation-induced mitochondrial elongation. This effect could be explained by the fact that hnRNP-C, another constitutive splicing factor, also competes with U2AF65 to bind to the pre-mRNA. It is possible that silencing U2AF65 favors the binding of hnRNP-C over that of hnRNP-K (Zarnack et al, 2013). Additional experiments will be necessary to
delineate the fine mechanisms by which PKA regulate alternative splicing of Fis1 exon1.

Interestingly, the direct silencing of Fis1.2 significantly reduced the elongation of the mitochondria triggered by starvation, suggesting that PKA not only acts on mitochondrial morphology by inhibition of Drp1 (Cereghetti et al, 2008; Gomes et al, 2011), but also through the upregulation of the profusion Fis1 variant 2 expression. Moreover, the fast induction of Fis1.2 upon starvation coupled with the fast repression of Fis1.2 when the cells sense nutrient-rich condition again, suggests that the alternative Fis1 splicing described here could represent a fast tunable mechanism to adjust mitochondrial morphology to cellular environment.

In conclusion, our results indicate that on the analysis of Fis1 profission function should take into consideration the divergent functions of its different variants on mitochondrial morphology. It is conceivable that also the predicted 4 splice variants of human Fis1 display a similar different effect on mitochondrial morphology.
MATERIAL AND METHODS

Antibodies and Reagents

Lipofectamine 2000, anti-V5 antibody, secondary anti-mouse antibodies Alexa488 or Alexa647, HBSS, Trizol, pcDNA3.1/nV5-DEST gateway system were from Invitrogen. PLVX shRNA2 plasmid was from Clontech. MG132, CCCP, H89, Forskolin were from Sigma. Protease inhibitors cocktail was from Roche. 10x TBS and PVDF membrane were from BioRad. ImProm-II Reverse Transcription System was from Promega. PuReTaq Ready-To-Go PCR Beads were from GE Healthcare and Power Sybr Green PCR master mix from Applied Bioscience.

Cell culture and transfection

Immortalized mouse embryonic fibroblasts (MEFs) from Fis1+/+ mice were cultured in DMEM supplemented with fetal bovine serum and non-essential amino acids (GIBCO) as previously described (Scorrano et al, 2003). Cells were co-transfected with Lipofectamine 2000 as indicated by the manufacturer with the Fis1 splice variants and different cellular fluorescent markers such as mitochondrial RFP (mtRFP) and endoplasmic reticulum RFP (ERRFP).

Molecular Biology.

Fis1 splicing variant cDNAs were amplified from WT MEFs mRNA samples and cloned into PCR8 plasmid with specific forward primers for variant 1 (5’-tggcccagtagac-3’), variant 2 (5’-acgccttctgtaacgc-3’) and variant 3 (5’-agtccttctgtaacgc-3’) together with a reverse primer common to the three isoforms (5’-cattgactgagac-3’). Then, they were cloned in frame to N-terminus V5 tag pcDNA3.1/nV5-DEST using the gateway system with specific
forward primers to delete the first codon (mFis1.1 5'- gaggccgtgctgaacgagct-3', mFis1.2 5'- ccagagacgaagctgcaag-3' and mFis1.3 5'- gatgccagacgaagctg-3') and a common reverse primer (5'-tcaggatttggaactggagaca-3'). All constructs were verified by sequencing. Then, from pcDNA3.1 plasmids, V5-tagged Fis1 variant 1 (5'- cgatgagcggccgacaccatgggtaagcctatccctaacctcttcggtctctgattct-3' and 5'- ctatgttttaattattcaggatttggaactggagaca-3') and HA-tagged mFis1.2 (5'- aaaaagcggccgacatctaccatacgactgccccagacagactcctccagacgaagc-3' and 5'- ctatgttttaattattcaggatttggaactggagaca-3') were also subcloned into pQCXI plasmid. All constructs were verified by sequencing. Fis1 variant 2 shRNA oligos forward strand

5'aaaaaaggatccgagacgaagctgcaaggaattttcaagagaaattccttcgatctccttttttgagccgcgaattcaaaaaa-3' and reverse strand 5'-aaaaaagatgccgcccacaggccacatgggtaagcctatccctaacctcttcggtctctgattctcggatcccaaaa -3' and U2AF65 shRNA oligos forward strand 5'-aaaaaaggatccgctcatgactatcagcattcttcgatctccttttttgagccgcgaattcaaaaa -3' and reverse strand 5'-aaaaaagatccgctcatgactatcagcattcttcgatctccttttttgagccgcgaattcaaaaa -3' were annealed, digested with BamHI and EcoRI and cloned into PLVX shRNA2 plasmid as described by the manufacturer. The knock down efficiency was measured by qRT-PCR with specific primers for Fis1 variant 2 (5'-gctgtcatgagaacatcctcg-3' and 5'-ggacacagaaccagctgcc-3') and U2AF65 (5'-atgtctggcctgacagactccttcgatctccttttttgagccgcgaattcaaaaa -3' and reverse strand 5'-aaaaaagatccgctcatgactatcagcattcttcgatctccttttttgagccgcgaattcaaaaa -3' were used to check for the specificity of the shRNA against Fis1 variant 2.
**Proteosomal Inhibition.**

MEFs transfected with V5-tagged Fis1 variants were treated for 2.5 and 5 hours with 10 µM of proteasome inhibitor MG132. To assess the effect of this stabilization in mitochondrial morphology, WT cells plated in coverslips were transfected with V5-tagged Fis1 variants together with mtRFP. After 24 hours, the cells were treated for 2.5 hours with 10 µM of MG132 and the mitochondrial phenotype analyzed by fluorescent microscopy.

**Imaging.**

Cells were plated onto 15 or 25-mm round glass coverslips and after treatment, were fixed with PFA 4% for 30 min at 4°C. Cells expressing the exogenous Fis1 variants were immune-labeled with monoclonal mouse anti-V5 antibody (1:200) for pCDNA3.1 V5-tagged mFis1.1, mFis1.2 and mFis1.3; and rat anti-HA (1:200) for pQCXI Ha-tagged mFis1.2, followed by incubation with the anti-mouse and anti-rat secondary antibodies Alexa488 or Alexa647 (1:1000). Images were acquired in a Zeiss LSM700 confocal microscope by exciting samples at 488nm, 555nm and 639nm and using a 63X objective. For mitochondrial length measurements in cells transfected with different proportions of mFis1.1 and mFis1.2, a total of 3 ug of DNA were transfected per well. The mitochondrial marker mtRFP remained constant at 1ug and then it was co-transfected together with and empty vector as a control, with 2ug of V5-mFis1.1 and with an increasing ratio of HA-mFis1.2/V5-mFis1.1 of 2ug total (ratio=0.3, 0.6, 1 or 2). In each experiment, 20 mitochondria per cell were measured in a total of 30 cells per condition. For colocalization experiments, z-stack images were collected each 0.37µm along the z axis, processed by volume rendering and analyzed with
ImageJ (NIH) using Mander’s coefficient. For mitochondrial length measurements, single plain images were convolved and analyzed with ImageJ.

**Immunoblotting.**

Cells after 24 hours of transfection and/or after treatment were collected and disrupted with RIPA buffer supplemented with protease-inhibitors. 20-50 µg of the extracted proteins were resolved on 12-15% SDS-PAGE and transferred to PVDF membrane, blocked in blocking buffer (TBS 0.05% Tween 20 with 5% milk) for half hour at room temperature then probed with the antibodies anti-V5 (1:1000 in blocking buffer) overnight at 4°C. Membranes were washed in TBS with 0.05% Tween20 before incubation with anti-mouse secondary antibodies (1:4000 dilution in blocking buffer). Finally, membranes were washed in TBS 0.05% Tween 20 and developed using homemade chemiluminescence reagents.

**mRNA analysis.**

To assess the mRNA levels of the Fis1 splicing variants in different mouse tissues, three BL6 mice were sacrificed and mRNA from forebrain, heart, spleen, liver and muscle was extracted with Trizol. To address the mRNA levels upon different cellular stressors, total mRNA from WT MEFs was extracted with Trizol according to the manufacturer’s protocol and was retro-transcribed with ImProm-II Reverse Transcription System. For semi-quantitative PCR, cDNA samples were amplified with PuReTaq Ready-To-Go PCR Beads with the primers used for cloning the Fis1 splicing variants mentioned above and β-actin primers (5’-acccacactgtgcccctactac-3’ and 5’-agccaagtccagcgagg-3’). qRT-PCR was performed in a StepOne Plus thermocycler (Applied Bioscience) using Power
Sybr Green PCR master mix and primers for Fis1 variant 1 (5′-tagttgagctttcaggg-3′ and 5′-ggacacgaaccagctgcc-3′), Fis1 variant 2 (5′-gcgtcatgagaacatcctcg-3′ and 5′-ggacacgaaccagctgcc-3′). As a control the same primers for β-actin were used.

To address the role of PKA in the Fis1 mRNA regulation, WT cells were pre-incubated or not for 30 minutes with 20 µM H89 and then treated with 50 µM Forskolin for 2, 4 and 6 hours.

To analyze the changes in Fis1 variant levels upon stress, WT MEF cells were incubated for 1 hour with 5µM CCCP, for 5 hours with 100µM etoposide or starved for 2.5 hours by culturing them in HBSS 10mM Hepes pH 7.4. For starvation kinetic experiments, cells were incubated with HBSS 10mM Hepes pH 7.4 for up to 22 hours and put back in complete medium for up to 22 hours.
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REFERENCES


BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point
FIGURE LEGENDS

Figure 1. Mouse Fis1 is alternatively spliced in three variants.
(A) Amino acid sequence multiple T-Coffee alignment of human Fis1 (hFis1) with the three mouse Fis1 variants (mFis1.1, mFis1.2 and mFis1.3). Mouse FIS1-V1 is homologous to human Fis1.
(B) Schematic of mouse Fis1 splicing.
(C) Mouse Fis1 variants are differentially expressed in brain, heart, spleen liver and skeletal muscle as detected by RT-PCR. 3 independent experiments are shown.

Figure 2. Mouse Fis1 variant 2 induces mitochondrial elongation.
(A) V5-tagged mouse Fis1 V1, V2 and V3 were individually co-transfected with either mitochondria- or ER-targeted RFP and Fis1 variant localization was analyze by immunostaining and cell imaging.
(B) Mean±SD of localization index calculated from experiments performed in (A).
(C) Fis1 variants are stabilized by proteasomal inhibition. Wild type MEF transfected as indicated were treated where indicated with MG132. Protein lysates were separated by SDS-PAGE and immunoblotted using the indicated antibodies. * p≤0.05, ** p≤0.01, *** p≤0.001 in a paired Student’s t test.
(D) Representative confocal images of MEFs co-transfected with mtRFP and the indicated plasmids. Where indicated, MG132 was added.
(E) Mean mitochondrial length ±SEM calculated in 3 independent experiments performed as in (D). * p≤0.05, ** p≤0.01, *** p≤0.001 in a 2 way ANOVA with a Tukey’s posttest.
**Figure 3.** Small amount of mFis1.2 inhibits excess of mFis1.1 mediated mitochondrial fragmentation. (A) Confocal images of wild type MEF expressing mtRFP marker together V5-tagged mFis1.1 (V1-V5) alone or with increasing among of HA-tagged mFis1.2 (V2-HA) as indicated by the Fis1.2:Fis1.1 ratio. Scale bar 10 μm. Images are representative of at least 3 independent experiments. (B) MEF treated as in (A) and the mitochondrial average length measured, means +/- SEM of at least three independent experiments. ** p≤0.01 and * p≤0.05.

**Figure 4.** Mouse *Fis1* variant 2 is upregulated during starvation.

(A) MEFs were treated as indicated and the mRNA expression level of *Fis1* variants was analyzed by RT-PCR. Data are representative of 3 independent experiments.

(B-C) MEFs were treated as in (A) and mRNA level of Variant 1 (B) and Variant 2 (C) was analyzed by qRT-PCR. Data are mean±SEM of 3 independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001 in a paired Student’s t test.

(D-E) MEFs were treated for up to 22 hours with HBSS and then supplemented with complete medium for up tp another 22 hours. mRNA level of Fis1 V1 (D) and V2 (E) was measured at the indicated times by qRT-PCR.

(F) Cells were treated as in D and C and the fold change in *Fis1-V1* and -V2 mRNA expression plotted over time. Data are mean±SEM of 3 independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001 in a 2 way ANOVA with Sidak’s posttest.

**Figure 5.** Starvation-induced mitochondrial elongation requires PKA-dependent alternative splicing of Fis1V2.
(A, B) MEFs were treated as indicated and Fis1.1 (A) and Fis1.2 (B) mRNA expression level was measured by qRT-PCR. Data are mean±SEM of 3 independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001 in a 2 way ANOVA with a Tukey’s posttest.

(C, D) MEFs were starved and treated for the indicated times and treated as indicated and Fis1.1 (C) and Fis1.2 (D) mRNA expression was followed by qRT-PCR. Data are mean±SEM of 3 independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001 in a 2 way ANOVA with a Tukey’s posttest.

(E-G) MEFs were stably transfected with shRNA against U2AF65 (shU2AF) (E and G) or against Fis1.2 (shV2) (F and G) and the mRNA expression level of U2AF65 (E), Fis1.2 (F), Fis1.1 (G) was followed by qRT-PCR. Data are mean±SEM of 4 independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001 in a 2 way ANOVA with Sidak’s posttest.

(H) Representative confocal images of MEFs stably expressing shV2 or shU2AF transfected with mtRFP and treated as indicated.

(I) Average mitochondrial length calculated in experiments performed as in (H). Data are mean±SEM of 4 independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001 in a 2 way ANOVA with a Sidak’s post test.
Zamorano et al Fig1
Zamorano et al. Fig 3
Zamorano et al Fig 4
3.2 Generation of a mouse model for the complete ablation of Fis1 splice variants produces embryonic lethality and protects against cell death at a cellular level.

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Different models have tried to address the role of the outer mitochondrial membrane protein Fission 1 (Fis1) in cell physiology. However, contradictory data have dimmed the relevance of Fis1 in mitochondrial morphology and cell death. Moreover, other receptors for the pro-fission dynamin related protein 1 (Drp1) have been found in mammals. By producing a complete KO mouse model for Fis1, here we highlight the importance of Fis1 during development, since its ablation induces embryonic lethality. Furthermore, at a cellular level, we show that the ablation of all the mouse Fis1 splice variants leads to increase in mitochondrial elongation, which can only be reverted after reconstitution with full-length Fis1 and not with the splice variant mFis1.2 that produces the opposite effect, mitochondrial elongation. The observed mitochondrial elongation observed in Fis1 KO cells correlates with a more efficient respiration and decreased ROS production. Finally, the ablation of Fis1 also provided resistance to apoptotic stimulus, repositioning Fis1 at the core of the mitochondrial fission and apoptosis process.

I contributed with the conception, planning and execution of the experiments presented in this work. Some experiments required the help from the co-authors. Finally, I participated in the writing of the manuscript together with Luca Scorrano and Denis Martinvalet.
Generation of a mouse model for the complete ablation of Fis1 splice variants produces embryonic lethality and protects against cell death at a cellular level.

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Abstract

The role of the outer mitochondrial membrane protein Fission 1 (Fis1) in cell physiology is controversial in part due to the different model used to address its function. Moreover, the characterization of other receptors for the pro-fission dynamin related protein 1 (Drp1) have challenged Fis1 in this role. In order to address the in vivo function of Fis1, we have generated a mouse deficient for all Fis1 variants. Highlighting the importance of Fis1 during development, its ablation induces embryonic lethality. Furthermore, at a cellular level, we show that this ablation leads to increase in mitochondrial elongation that can only be reverted after reconstitution with full-length Fis1 variant 1 and not with the splice variant mFis1 variant 2 that increases the mitochondrial length. The observed mitochondrial elongation observed in Fis1 KO cells is associated with a more efficient respiration and decreased ROS production. Finally,
the ablation of Fis1 also provided resistance to apoptotic stimulus, repositioning Fis1 at the core of the mitochondrial fission and apoptosis process.

INTRODUCTION

Mitochondria are highly dynamic organelles, which can undergo fusion and fission during cell division, differentiation or in order to cope with metabolic and energy demands of the cell (Chan, 2006b; Okamoto & Shaw, 2005). These processes are tightly regulated by a set of cytosolic and mitochondrial GTPases and mitochondrial receptors, the so-called mitochondrial shaping proteins (Scorrano, 2013). Fusion is coordinated by the inner-mitochondrial membrane (IMM) optic atrophy 1 (Opa1) (Cipolat et al, 2006; DeVay et al, 2009; Olichon et al, 2003) and the outer-mitochondrial membrane (OMM)-residents Mitofusin 1 and 2 (Mfn1 and Mfn2) (Ishihara et al, 2004; Koshiba et al, 2004). On the other hand, the pro-fission cytosolic dynamin-related protein 1 (Drp1) (Frank et al, 2001) and its OMM receptors mitochondrial fission protein (Fis1) (James et al, 2003; Wang et al, 2012; Yoon et al, 2003), mitochondrial fission factor (Mff) (Otera et al) and the mitochondrial division proteins (MiD49 and MiD51) (Palmer et al, 2011) orchestrate mitochondrial division. The proper balance between these two processes has been proven to be of great importance at the organism level since complete knockout mouse models for these proteins are embryonically lethal (Alavi et al, 2007; Chen et al, 2003; Ishihara et al, 2009; Wakabayashi et al, 2009). Alterations in mitochondrial dynamics have also been linked to pathological conditions such as neurodegenerative diseases such as
Alzheimer’s, Parkinson’s and Charcot Marie Tooth disease (Oettinghaus et al, 2012; Zuchner et al, 2004) and to cancer progression (Grandemange et al, 2009). In particular, mitochondrial fragmentation is activated after the dephosphorylation of the GTPase Drp1 resulting in its translocation to the OMM where it interacts with different receptors in order to induce the constriction of the mitochondria and induce its division (Cereghetti et al, 2008; Frank et al, 2001). In mammals, the first receptor to be found was human Fis1 (hFis1), the ortholog of the yeast form, which is the sole receptor of Drp1 and it interacts with Drp1 through its adaptor proteins Caf4p and Mdv1p (Griffin et al, 2005; Mozdy et al, 2000; Tieu & Nunnari, 2000). As its yeast counterpart, hFis1 has been found to interact with Drp1 and to induce mitochondrial fragmentation, cytochrome c release and cell death after overexpression (James et al, 2003; Yoon et al, 2003). However, in mammals, the exact role of Fis1 in mitochondrial dynamics remains obscure. Drp1 and Fis1 do not colocalize after the activation of mitochondrial fragmentation (Smirnova et al, 2001) and although its down-regulation has been shown to be decisive in the progression of cell death in an in vivo model of hypoxia (Kim et al, 2011), knocking down of Fis1 in cells only produce a mild fusion phenotype (Otera et al, 2010). Moreover, the discovery of new receptors for Drp1 in mammals, such as Mff and the MiDs has led to disregard Fis1 as a key player in mitochondrial fission. In particular, Mff and MiDs have been found not only to interact with Drp1 at the OMM, but also to induce fragmentation and cell death when overexpressed, whereas their silencing reduces Drp1 translocation and induces mitochondrial elongation (Otera et al, 2010; Palmer et al, 2011).
Meanwhile, hFis1 has been linked to functions other than mitochondrial fragmentation. hFis1 induces cell death in a calcium-dependent manner through the formation of a complex with the endoplasmic reticulum (ER)-resident protein Bap31 and pro-caspase 8, which helps the cleavage and activation of the latter to produce caspase8 (Alirol et al, 2006; Iwasawa et al). On the other hand, the over-expression of hFis1 increases autophagosomes formation and produces partial colocalization of these autophagosomes with mitochondria (Gomes & Scorrano, 2008). Furthermore, hFis1 mutation resulted in LC3 aggregates upon mitochondrial dysfunction suggesting a key role of Fis1 in the removal of defective mitochondrial through mitophagy (Shen et al, 2014).

Despite that the different Drp1 receptors and functions of Fis1 have induced confusion on the actual role of Fis1 in fragmentation, recent publications have helped to stress the relevance of Fis1 in mitochondrial fission. Thus, in an in vivo model of myocardial infarction, down-regulation of Fis1 induced resistance to mitochondrial fragmentation and apoptosis (Wang et al, 2012). Moreover, it has been found that the four receptors, Fis1, Mff, MiD49 and MiD51 are independently important for mitochondrial fission, by regulating the recruitment of Drp1 onto the OMM (Loson et al, 2013).

In summary, it appears that Fis1 is involved in different aspects of mitochondrial morphology and quality control, however how these functions are achieved is not well understood. Moreover, the physiological outcome of Fis1 functions has only been studied by silencing of Fis1 in specific tissues in adult mice, but the physiological role of Fis1 in development and in other tissues has not been addressed. Here we described the production of a new mouse model for the study of mouse Fis1 role in development and show that its complete ablation
leads to embryonic lethality. At a cellular level, we were also able to confirm the relevance of Fis1 in mammalian mitochondrial fission, mitochondrial function and apoptosis.

RESULTS

Generation of a Fis1 null mouse.

We aimed to study the physiological consequences of Fis1 ablation in a mouse model. Previous work in the laboratory of Luca Scorrano had given rise to a targeting vector for the conditional ablation of Fis1 in C57BL/6 mouse (Figure 1A). It is important to mention that the production of a conditional over a full knock out was intended, due to the fact that many full knockouts of mitochondrial shaping proteins are lethal (Alavi et al., 2007; Chen et al., 2003; Ishihara et al., 2009; Wakabayashi et al., 2009). The targeting vector was electroporated into ES cells and clones bearing the homologous recombination of the targeting vector into the Fis1 allele were sought (Figure 1B). The ES cell transfection with the targeting vector gave a total of 4 positive clones for homologous recombination. This was assessed by PCR for the 3’ end of the recombination and in the 5’ loxP site and by southern blot at the 5’ of the transgene (Figure 1C and D).

The 4 positive clones were then used to electroporate blastocysts out of which, chimeric mice were produced and then bred to generate the heterozygous transgenic mice Fis1<sup>flx/+</sup>. These mice were interbred to generate mice homozygous to the transgene Fis1<sup>flx/flx</sup>. The Fis1<sup>flx/flx</sup> mice were expected to be viable since the loss of Fis1 expression should take place only in the littermates from their crossing with CRE mice (floxed mouse in Figure 1B). The CRE
recombinase would excise the region between the loxP sites of the transgene (Figure 1B), which in turn would truncate the Fis1 protein. Moreover, at any stage, matting the Fis1^{flx/flx} or floxed mouse with a flipase mouse could remove the hygromycin cassette, used to positively select the ES clones (Figure 1B). However, after many breeding's, to our surprise only 4 Fis1^{flx/flx} pups were born out of a total of 40 newborns from matting heterozygous mice (Fis1^{flx/+}). This 10% of Fis1^{flx/flx} offspring was far from the expected mendelian ratio of 25% for a cross between heterozygous mice. Moreover, these Fis1^{flx/flx} pups died after 3 weeks. Nevertheless, we were able to extract embryonic fibroblast from 12.5 days Fis1^{flx/flx} embryos and generate immortalized MEFs. Sequencing analysis of Fis1^{flx/flx} MEF cells showed that Fis1 exons were present, as well the loxP sites and the hygromycin cassette. However, immunoblotting with anti-Fis1 antibody (Figure 2A) and qPCR with specific primers for Fis1 splice variants mFis1.1 and mFis1.2 (Figure 2B) did not show any expression of Fis1 full-length protein and very low expression of its mRNA (Figure 2B left panel). Due to the lack of an antibody against splice variant mFis1.2, only mRNA level was checked. To this regard, since in basal conditions the expression of mFis1.2 mRNA is low, we analyzed the expression levels upon mRNA induction by starvation, since we have found that starvation upregulate variant 2 mRNA expression level (Zamorano et al manuscript in preparation and Figure 4D). However, even upon starvation, mFis1.2 levels remained low in Fis1^{flx/flx} MEFs (Figure 2B right panel). All together, these results suggested that recombination of the targeting vector into the Fis1 allele impaired its expression producing, instead of a conditional, a constitutive Fis1 KO mouse. This hypomorphic phenotype induced an embryonic lethality of 90%. Although this issue posed major problems to assess the role of
Fis1 *in vivo*, we were able to use these KO Fis1 MEFs to assess the effects of Fis1 ablation in a cellular model.

**Fis1 KO cells present elongated mitochondria.**

We decided to use the Fis1^flx/flx^ MEFs as Fis1 null cells since they have no expression of mFis1. It is important to mention that mFis1 gene can give rise to three isoforms (mFis1.1, mFis1.2 and mFis1.3), out of which mFis1.1 and mFis1.2 are stable (Zamorano et al, 2015 manuscript in preparation). Neither mFis1.1 nor mFis1.2 was expressed in the Fis1^flx/flx^ MEF (Figure 2A and B). Assessment of mitochondrial morphology by confocal microscopy in WT and KO cells transfected with mtRFP showed that in agreement with previous report, the absence of Fis1 in our Fis1-null MEFs produced longer mitochondria compared to the WT cells (Figure 3A-B). Mitochondria and peroxisomes share the same Fis1 and Drp1 fission machinery (Schrader, 2006). Therefore, peroxisomal shape was analyzed by expressing peroxisomal-targeted RFP in Fis1 WT and KO cells. As expected, qualitative assessment of peroxisomal shape in cells showed that KO cells had more and longer peroxisomes than WT cells (Supplementary Figure 1). These results show that the ablation of Fis1 in MEF cells has a clear negative impact on the fission process, which leads to the expected mitochondrial and peroxisomal elongation.

**Fis1 ablation alters mitochondrial functionality and protects against cell death.**

Given the mitochondrial elongation observed in KO cells and the importance of mitochondrial dynamics in cell metabolism and apoptosis, we sought to
characterize the consequences of Fis1 ablation in cell’s life and death. In agreement with previous reports mitochondrial elongation also correlates with a higher mitochondrial membrane potential (mΔΨ) and less ROS production (Twig et al, 2008a). In fact, Fis1 KO cells not only have elongated mitochondria but also an increased mΔΨ (Figure 3C) and less ROS production compared to WT (Figure 3D). Mitochondrial respiration in intact cells is also affected by the ablation of Fis1. KO cells presented consistent and significant lower oxygen consumption than WT cells in basal conditions as well as after ATPase inhibition with oligomycin and after uncoupling respiration with FCCP (Figure 3E). All together these results strongly suggest a more efficient respiration in KO cells. Although the oxygen consumption was lower in KO cells, Fis1 ablation did not have a significant effect on cellular growth (Figure 3F), however, null cells for Fis1 were resistant to cell death by the apoptosis-inducing drug staurosporine (Figure 3G) indicating that the mitochondrial elongation observed in KO cells induces a more efficient respiration that may reduce ROS production correlating with resistance to apoptosis.

**Re-expression of Fis1 variant 1 reverts the elongated mitochondrial phenotype of Fis1 null cells**

As previously mentioned, mFis1 gene can give rise to three isoforms. Work in our laboratory has shown that this isoforms have opposite effects in mitochondrial morphology when overexpressed in a WT background. While the full-length ortholog of hFis1 in mouse, mFis1.1, induces mitochondrial fragmentation when overexpressed, mFis1.2 induces mitochondrial elongation. A different case is the third isoform, mFis1.3, which although induces
mitochondrial fragmentation when overexpressed, it is not stably expressed and is likely to be degraded in normal conditions (Zamorano et al 2015 manuscript in preparation). Since the Fis1 KO cells lack mFis1.1 and mFis1.2 isoforms, we decided to reconstitute them with each variant in order to address the role of each of them in mitochondrial morphology in a clean background for Fis1. As observed previously in Figure 3A and B, KO cells have elongated mitochondria. Interestingly, the observed shorter mitochondrial length in WT cells was only restored in KO cells upon mFis1.1 overexpression. On the other hand, reconstitution of KO cells with mFis1.2 further increased the length of Fis1-null mitochondria (Figure 4A and B), suggesting that mFis1.2-dependent mitochondrial elongation can also take place in the absence of mFis1.1 expression. Interestingly, mFis1.3 restitution in KO cells did not significantly changed mitochondrial morphology (Figure 4A and B), suggesting that mFis1.3-induced mitochondrial fragmentation requires the expression of mFis1.1. Previous data from our lab has also shown that mFis1.2 is up-regulated upon starvation in a PKA-dependent manner, which helps to elongate mitochondria during starvation (Zamorano et al 2015, manuscript in preparation). To this regard, KO Fis1 cells were able to elongate upon starvation, phenotype that was partially reverted when KO Fis1 cells were reconstituted with mFis1.1 (Supplementary Figure 2). However, the reconstitution with mFis1.2 did not significantly changed the mitochondrial length in comparison with the KO mitochondrial phenotype, suggesting that, in order to induce mitochondrial elongation during starvation, mFis1.2 might require mFis1.1 or the mitochondrial already reached their maximum length in such conditions (Supplementary Figure 2).
All together, these results show that Fis1 gene is important for mitochondrial morphology and function and in apoptosis. Moreover, the opposite effect of Fis1 splice variants in mitochondrial morphology suggests a role for these variants in the regulation of mitochondrial functions.

**DISCUSSION**

We have found compelling evidence supporting the importance of Fis1 gene at a developmental level and in mitochondrial morphology with consequences in mitochondrial function and cell death.

Although the way in which the KO model of Fis1 was achieved is not well understood, it is clear that the recombination between the transgene and the Fis1 locus was successful. Possible explanations for the lack of Fis1 expression could be a mutation in the promoter region, undetected mutations in the gene or the presence of the hygromycin cassette, which could produce a severe hypomorph phenotypic phenotype (Meyers et al, 1998). In any case, as other full KOs for mitochondrial shaping proteins, the correlation between the lack of Fis1 expression and the 90% of embryonic lethality of the Fis1^flx/flx mouse suggests that Fis1 ablation produces severe impairments to embryonic development. We obtained Fis1^flx/flx embryos up to the 13.5 day of embryonic development, placing the Fis1 ablation-dependent embryonic lethality later than for other genes involved in mitochondrial dynamics, around 11.5 days (Alavi et al, 2007; Chen et al, 2003; Ishihara et al, 2009; Wakabayashi et al, 2009). Due to the relevance of Fis1 in mitochondrial function and cell death presented in this work, deregulation of these processes as the cause of developmental failure in the Fis1^flx/flx is a plausible explanation. A careful histological analysis of the different
embryonic stages around the day 12.5 should give answers regarding the mechanisms that produce the lethality and the organs affected.

Recent studies using knockdown approaches have cast doubts about the pro-fission features of Fis1, showing only a mild mitochondrial elongation in cells with decreased levels of Fis1 (Otera et al, 2010). By using MEF cells lacking Fis1 we observed a clear phenotype in mitochondrial fission, since Fis1 KO cells had significantly longer mitochondria. This was indirectly confirmed by the fact that Fis1 KO cells had longer peroxisomes. These results have been confirmed by the work of Chan’s group showing similar mitochondrial phenotype in MEF cells lacking Fis1 gene (Loson et al, 2013).

Mitochondrial shape is intimately linked with mitochondrial function. Thus, we observed an elongated mitochondrial phenotype in Fis1 KO cells that correlated with an increase in mΔΨ, a decrease in ROS production and lower oxygen consumption rate. Taken together, these results suggest that the absence of the pro-fission protein Fis1 produced longer mitochondria, which, in turn, led to a more efficient respiration. Whether this efficiency has consequences in ATP production still has to be addressed. Nevertheless, several publications have shown that mitochondrial fusion enhances respiration efficiency and increases ATP production. For example, inhibition of mitochondrial fusion by ablation of the mitofusins creates defects in membrane potential and respiration (Chen et al, 2005) and manipulation of Opa1 also disturbs mitochondrial respiration and cell growth by disturbing the cristae shape (Cogliati et al, 2013). In our work, however WT and KO cells did not significantly differ in growth, maybe, due to the fact that mitochondrial elongation not only induces cellular growth but also, paradoxically, prolonged mitochondrial elongation can also induce cell
senescence (Mai et al, 2010) and mitochondrial fragmentation is necessary for cell division progression (Lee et al, 2014). Hence, the growth phenotype observed in the KO cells used in our study, although immortalization may be the result of the interplay between cell growth and senescence processes in cells bearing long mitochondria.

Mitochondrial morphology also plays a key role in the progression of cell death. In agreement with a study showing that decreased levels of Fis1 induced even higher resistance to apoptosis than Drp1 knock down (Lee et al, 2004), Fis1 KO cells were highly resistant to the incubation with staurosporine probably due to the fact that these cells are less prone to ROS production and have a higher membrane potential (Cassina & Radi, 1996; Croteau & Bohr, 1997; Fulda et al, 2010; Ott et al, 2002).

Finally, in this study we show that, in agreement with the literature (James et al, 2003; Stojanovski et al, 2004; Yoon et al, 2003), overexpression of Fis1 full length (mFis1.1) induces mitochondrial fragmentation in WT and restore mitochondrial length in reconstituted KO cells. We were also able to confirm previous data from our laboratory (Zamorano et al 2015, manuscript in preparation) showing that expression of mFis1.2 in pro-fusion not only in WT but also in KO cells. Furthermore, mFis1.3 induced fragmentation in WT cells, but interestingly, expression of mFis1.3 in KO cells did not induced fragmentation, suggesting that mFis1.3 requires Fis1.1 and/or mFis1.2 in order to induce fragmentation.

All together, these results show that Fis1 gene is important for mouse development and at a cellular level it impinges in mitochondrial morphology, and function as well as cell death.
MATERIAL AND METHODS

Antibodies and Reagents

Lipofectamine 2000, anti-V5 antibody, secondary anti-mouse antibodies Alexa488 or Alexa647, HBSS, Trizol, pcDNA3.1/nV5-DEST gateway system were bought from Invitrogen. TBS and PVDF membrane were from BioRad. ImProm-II Reverse Transcription System was from Promega. PuReTaq Ready-To-Go PCR Beads were from GE Healthcare and Power Sybr Green PCR master mix from Applied Bioscience.

Mouse generation

In order to generate we used a targeting vector for the conditional ablation of Fis1 in C57BL/6 mouse that was generated in Luca Scorrano's lab (Barbieri, 2007). It is important to mention that this experimental design was conceived before the discovery of the mouse Fis1 variants. Hence, when the targeting vector was designed, the first exon of the locus where mFis1.2 and mFis1.3 variants start was not considered. However, the loxP position in the targeting vector would not only produce a truncated mFis1.1 but also would produce a truncated form of mFis1.2 and mFis1.3. The targeting vector was sent to Polygene Company to continue with the production of the mouse model, where it was electroporated into C57Bl/6 ES cells. Positive clones were selected by Neomycin and the homologous recombination of the targeting vector into the Fis1 locus was checked by PCR at the 3’ end of the recombination and in the 5’ loxP site and by southern blot at the 5’ of the transgene (Figure 1C). First, the PCR to check recombination in the 3’ end using primers that landed in the transgene (5’-CAGCAGCCTCTGTTCCACATAC-3’) and WT genomic allele (5’-
TCCTGGGACACTCCCCATTG-3'), respectively, gave a total of 8 out of 384 positive clones, showed by the amplification of a 1.4Kb product (Figure 1D, upper panel).

To check the recombination in the 5' end, a probe was designed on genomic DNA by PCR (5'-CTTTCCAACACCCGATGC -3' and 5'-TACCCCTGCAGGCTGC-3'). This probe differentiates between the products of a digestion with the EcoRV enzyme. The WT allele, on EcoRV digested genomic DNA gives a size of >25 Kb, whereas the targeted allele should generate an additional signal at ~14.4 Kb.

Only 6 clones out of 8 were positive for the small product of 14.4 Kb from the transgene allele after digestion (Figure 1D, middle panel). Finally, the recombination of the 5' loxP site was also checked by PCR, using primers that flank this site (5'-TGAAAGCAGTGCGCTGCTCTG-3' and 5'-CTGACCTCCTGCCTCATTCTC-3'). Out of the 6 clones that were positive for recombination in the 5' and 3' ends, only 4 were also positive for the loxP site recombination (Figure 1D, lower panel). Moreover, karyotype and morphological analysis of the clones resulted normal. These clones were microinjected into blastocysts isolated from C57BL/6 donors and transfer into foster mothers. Viable chimeric offspring was then crossed and mice with germ-line transition were selected (Fis1+/flx mice). Fis1+/flx mice were crossed to generate Fis1 flx/flx mice and MEFs were isolated from embryos between days e12.5 to e13.5.

**Cell culture and transfection.**

Immortalized mouse embryonic fibroblasts (MEFs) from Fis1+/+ and Fis1 flx/flx mice were cultured in DMEM supplemented with fetal bovine serum and non-essential amino acids (GIBCO) as previously described (Scorrano et al, 2003). Cells were co-transfected with Lipofectamine 2000 as indicated by the
manufacturer with the mouse Fis1 splice variants and different cellular fluorescent markers such as mitochondrial RFP (mtRFP) and endoplasmic reticulum RFP (ERRFP).

**Molecular Biology.**

Mouse Fis1 splicing variant cDNAs were amplified from WT MEFs mRNA samples and cloned into PCR8 plasmid with specific forward primers for variant 1 (5’-tgtggccagtagagacctt-3’), variant 2 (5’-agcgtgcttctgtaaacgct-3’) and variant 3 (5’-agtgcctgtgaaacagctct-3’) together with a reverse primer common to the three isoforms (5’-caggatttggacttggagaca-3’). Then, they were cloned in frame to N-terminus V5 tag pcDNA3.1/nV5-DEST using the gateway system with specific forward primers to delete the first codon (mFis1.1 5’- gagcgtgctgaacgagct-3’, mFis1.2 5’- ccagagagagaactgcaag-3’ and mFis1.3 5’- gatgccagagacgaagctg-3’) and a common reverse primer (5’-tcaggattttgacttggagaca-3’). All constructs were verified by sequencing.

3.6 mRNA analysis.

To assess the mRNA levels of the Fis1 splicing variants in KO Fis1 MEF cells, mRNA was extracted with Trizol and retro-transcribed with ImProm-II Reverse Transcription System. qRT-PCR was performed in a StepOne Plus thermocycler (Applied Bioscience) using Power Sybr Green PCR master mix and primers for Fis1 variant 1 (5’- tagtgtgaggctttcagggg-3’ and 5’-ggacacagaaccagctgcc-3’), Fis1 variant 2 (5’- gctgtcatgagacatcctcg-3’ and 5’-ggacacagaaccagctgcc-3’). As a control we used β-actin primers (5’-acccacactgtgcccatctac-3’ and 5’-agccaatgccagcagg-3’).
**Immunoblotting.**

20-50 µg of the extracted proteins from WT and KO MEF cells were resolved on 12-15% SDS-PAGE and transferred to PVDF membrane, blocked in blocking buffer (TBS 0.05% Tween 20 with 5% milk) for half hour at room temperature then probed with the antibody anti-Fis1, overnight at 4°C at a 1:1000 dilution. Membranes were washed in TBS with 0.05% Tween20 before incubation with secondary antibodies (1:4000 dilution in blocking buffer). Finally, membranes were washed in TBS 0.05% Tween 20 and developed using homemade chemiluminescence reagents.

**Imaging.**

WT and KO cells for Fis1 were plated onto 15 or 25-mm round glass coverslips and after treatment, were fixed with PFA 4% for 30 min at 4°C. Cells expressing the exogenous Fis1 variants were immune-labelled with monoclonal anti-V5 antibody (1:200) and rat anti-HA antibody (1:200) followed by incubation with the anti-mouse or anti-rat secondary secondary antibodies Alexa488 or Alexa647 (1:1000), respectively. Mitochondria were labelled by transfecting cells with mitochondrial RFP (mtRFP) and peroxisomes were labelled by transfecting cells with peroxisome-GFP. Images were acquired in a Zeiss LSM700 confocal microscope by exciting samples at 488nm, 555nm and 639nm and using a 63X objective. For mitochondrial length measurements, single plain images were convolved and analysed with ImageJ. For measurement of mitochondrial length upon nutrient deprivation WT, KO and KO cells reconstituted with V5-tagged Fis1 variants were starved for 24 hours in HBSS 10mM Hepes pH 7.4
medium. In each experiment, 20 mitochondria per cell were measured in a total of 30 cells per condition.

**Mitochondrial functionality.**

Oxygen consumption rate, as a measurement of cellular respiration, was performed using the Seahorse XF24 analyser (Seahorse Bioscience). Respiration was measured under basal condition, and in the presence of 1µM ATP synthase inhibitor oligomycin, 0.75 µM uncoupler FCCP, 1 µM mitochondrial respiration complex I inhibitor Rotenone and 1µM mitochondrial respiration complex III inhibitor Antimycin A. WT and KO MEFs were plated the day before of experiment at a density of 20,000 cells. Cells were washed with unbuffered assay medium supplemented with 5.5 mM glucose, 1 mM pyruvate, 2 mM glutamine (same as their culture medium) and 1 hour in unbuffered assay medium before the experiment at 37°C without CO2. For ROS measurement, 2 x 10^5 WT and KO cells were immediately analysed after staining with dihydroethidium (HE) 5µM. For mΔΨ measurements, 2 x 10^5 WT and KO cells were plated per well in a 96-well plate, and then incubated for 15 min. at 37°C with tetramethylrhodamine methyl ester (TMRM) 500nM. In both experiments, stained cells were analysed by flow cytometry using Accuri cytometer (BD Bioscience).

**Cell death assay.**

WT and KO cells were plated in 24–well plates. The next day the cells were treated with 2µM staurosporine (Sigma) for 2, 4, 6 and 8 hours. Cells and medium were then collected and incubated with PI/Annexin-V as suggested by the manufacturer (eBioscience) and measured in an Accuri cytometer.
Statistical tests.

In each graph, except from the peroxisomal length experiment, data is represented as mean ± s.e.m. of at least 3 independent experiments. When comparing two sets of data, statistical significance was calculated by a two-tailed Student t-test. For the statistical analysis of more than two sets of data, significance was calculated by a 2-way ANOVA test, followed by a Tukey’s post-test. P values are indicated in the respective legends.

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FIGURE LEGENDS

Figure 1. Strategy for the development of a mouse model for Fis1 ablation.

(A) Map of the targeting vector used to generate a transgenic mouse bearing a cassette for the conditional ablation of Fis1 gene.

(B) Schematics of the targeting construct for the conditional ablation of Fis1. This model relies on the CRE and flipase (FRT) recombination system which allows the excision of genomic material when mice bearing these recombinases are mated with mice carrying the transgene (TV). Initially, after electroporation of the ES cells with the targeting vector into mouse oocytes, homologous recombination of the TV 5’ and 3’ arms with the Fis1 gene would produce a transgenic mouse heterozygous for the TV. Later on, transgenic mice are mated with CRE mice in order to truncate Fis1 gene (floxed mouse) and then with they could be mated with flipase mouse in order to remove the hygromycin (Hygrom.) cassette.

(C) Schematics depicting the two types of screening that were used to check targeting vector recombination, PCR (D upper and lower panels), southern blot (D middle panel).

(D) Recombination was checked by PCR at the 3’ end of the transgene (upper panel). The forward primer (3’F) lands in the transgene and the reverse primer (3’R) anneals to the genomic sequence of the Fis1 locus. The image represents part of the screening of 384 ES clones. 1 and 2 are two clones (of a total of 8 among the 384 clones) that are positive for the PCR product of 1.4Kb at the 3’ end (*). Southern blot was used to check for recombination, with a probe binding to the 5’ end of the targeted allele (5’probe) (middle panel). The positive clones for the PCR were digested with EcoRV to give rise to a 14.4Kb product for the
transgene and 30 Kb for the WT allele. Numbers on the top refer to the ES cells clones positive for the 3’ recombination that were tested with a probe at the 5’ end that gives rise to a 14.4 Kb product. From the 8 positive clones from (A), only 6 were positive for the 5’ end probe. WT genomic DNA is used as a control. The recombination of the 5’ loxP was also checked by PCR using primers flanking this site (lower panel). From the 8 positive clones in (B), only 5 were positive for the loxP site.

**Figure 2. Recombination of the targeting vector into Fis1 allele abolishes the expression of mFis1.1 and mFis1.2.**

(A) WB against Fis1 showing its expression in WT and Fis1^flx/flx (KO) cells. β-actin was used as loading control. Representative WB of three independent experiments.

(B) qPCR showing the mRNA levels of mFis1.1 and mFis1.2 in WT and Fis1^flx/flx (KO) cells in basal and starving conditions. Representative graph depicting means and standard deviation of three independent experiments.

**Figure 3. The ablation of mFis1 gene increases mitochondrial length, alters mitochondrial function and protects against apoptosis.**

(A) Confocal images of WT and KO MEF Fis1 cells transfected with mtRFP plasmids. Scale bar 10 μm.

(B) Quantification of mitochondrial length. Means and standard errors of 3 independent experiments are plotted. Significance was calculated by Student’s t-test. *** p ≤ 0.001.
(C) The fluorescence of WT and null cells for Fis1 (KO) was followed by flow cytometer after 500nM TMRM incubation. Significance was calculated by Student’s t-test. ** p≤0.01.

(D) WT and KO cells were incubated with 5μM HE and the fluorescence was recorded by flow cytometry. Significance was calculated by Student's t-test. ** p≤0.01.

(E) Oxygen consumption was followed in WT and KO cells by Seahorse analyzer during basal conditions and upon ATPase inhibition with 1μM oligomycin, uncoupling respiration with 0.75 μM of CCCP and upon inhibiting complex I (with 1 μM Rotenone ) and complex III (with 1μM Antimycin A). 3 independent experiments were performed in each case and significance was measured with a Student’s t-test. ** p≤0.01.

(F) Plot showing the average of 3 independent experiments where WT and null Fis1 (KO) MEF cells were plated and their number was counted at the mentioned time points.

(G) WT and KO cells where challenged with staurosporine 2 μg/mL and the percentage of cells negative for PI and Annexin-V was followed over time. Graphs show the average of 3 independent experiments. Significance was measured with a 2-way ANOVA and a Tukey’s post test. * p≤0.05, ** p≤0.01 and **** p≤0.0001.

**Figure 4. The ablation of mFis1 gene induces mitochondrial elongation.**

(A) Confocal images of WT and Fis1 KO MEF cells transfected with V5-mFis1 variants and with mtRFP plasmids. Only red channel is shown. Scale bar 10μm.

(B) Quantification of mitochondrial length. 3 independent experiments were
measured and ANOVA with a Tukey’s post-test was used to calculate statistical significance * p≤0.05, ** p≤0.01, *** p≤0.001 and **** p≤0.0001.
Figure 1

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Figure 2
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Figure 4
The ablation of mFis1 gene induces peroxisome elongation. Confocal images of WT and KO MEF cells transfected with peroxisomal-RFP. 3X zoom is shown in the right side. Scale bar 40μm. Lower panel shows the quantification of average peroxisomal length in 20 cells per condition.
**mFis1.1 and mFis1.2 modulate mitochondria elongation upon starvation.** (A) Confocal images of WT, Fis1 null MEFs (KO) and KO cells reconstituted with mFis1.1 and mFis1.2, together with a mRFP plasmid. 24 hours after transfection, cells were starved for 24 hours or left untreated. Only red channel is shown. Scale bar 20 μm. (B) Quantification of mitochondrial length. 3 independent experiments were measured and ANOVA with a Tukey's post test was used to calculate statistical significance * p<0.05, ** p<0.01, ***p<0.001.
Chapter 4

DISCUSSION

4.1 mFis1 splice variant expression.

The first hint of the importance of alternative splicing in mitochondrial shaping proteins was the discovery of eight isoforms coming from the human Opa1 gene (Delettre et al, 2001). The successive proteolytic cleavage of these variants forms soluble and membrane-bound Opa1 isoforms with distinct functions (Frezza et al, 2006; Ishihara et al, 2006). In mouse, there are 4 isoforms that have different tissue expression. Each isoform is differently susceptible to proteolytic cleavage, leading to different pools of Opa1 variants in each tissue, which could have different effects on mitochondrial shape (Akepati et al, 2008). Mouse Drp1 gene also suffers alternative splicing, which leads to the formation of isoforms with different tissue expression, with some variants being exclusively expressed in neurons (Uo et al, 2009). Likewise, careful analysis of mouse Fis1 gene in Ensembl database showed the existence of three mFis1 splice variants, namely, mFis1.1, mFis1.2 and mFis1.3. Moreover, we have found that the mRNA levels of these variants were differentially expressed in different tissues, being mFis1.1, the full length orthologue of hFis1, the one expressed in every tissue and the other variants, mFis1.2 and mFis1.3, mainly expressed in tissues with high energy demands, among them, brain and heart (Figure 1B, chapter 3.1). However, the protein level of overexpressed mFis1.3 was very low in comparison with the other isoforms and protein levels could only be stabilized after inhibiting the proteasome (Figure 2C, chapter 3.1). Moreover, in silico
analysis by the group of Blake Hill from the Medical College of Wisconsin shows that a relatively large hydrophobic surface in mFis1.3 protein is exposed, which would render the protein unstable (data not shown). These results, together with the impossibility to upregulate mFis1.3 mRNA by challenging WT cells with different stimuli (Figure 4A, chapter 3.1), would suggest that mFis1.3 is a byproduct of mFis1 gene. In order to address whether mFis1 variants, especially mFis1.3, are directly regulated by proteasome degradation, studies analyzing the ubiquitination status of the mFis1 variants are needed.

In agreement with the literature, the mouse Fis1 isoforms were evenly distributed in a diffused pattern along the mitochondria (James et al, 2003). This mitochondrial distribution was expected, since all mFis1 variants share the same C-terminal, comprising the IMS stretch and the transmembrane domains, regions which are necessary for the mitochondrial localization (Alirol et al, 2006; Yoon et al, 2003). Additionally, mFis1 variants partially colocalized with the ER (Figure 2A and 2B, chapter 3.1). This subcellular distribution is in agreement with Fis1 being an outer mitochondrial membrane protein also reported to interact with ER-resident protein Bap31 in specific mitochondria-ER contact sites called mitochondria-associated membranes, MAMs (Iwasawa et al, 2011). However, whether and how the subcellular or mitochondrial localization of these mFis1 variants might change according to the cellular context has yet to be studied. For example in mouse, Drp1 alternative splicing produces isoforms with microtubule and cytosolic localization, which has implications in the regulation of mitochondrial fragmentation (Strack et al, 2013).
4.2 mFis1 variants structure and mitochondrial shape.

Sequence and structural analysis of Fis1 protein have shown that, despite its small 17 KDa size, Fis1 has several distinct structural features. The first 16 amino acids of the cytosolic N-terminal region form a coiled-coil domain. The rest of the cytosolic domain of Fis1 is composed by 6 α-helices, out of which α2 to α5 form two TPR-like domains, important for protein-protein interaction, followed by a transmembrane domain and a short IMS stretch in the C-terminal. In mouse Fis1 gene, the differential exon usage in each isoform produces the full-length orthologue of hFs1, mFis1.1 and two other isoforms, which differ in the N-terminal region (Figure 1A and 1B, chapter 3.1).

Here we show that, in agreement with the literature (James et al, 2003; Stojanovski et al, 2004; Yoon et al, 2003), overexpression of mFis1.1 induces mitochondrial fragmentation in WT and in null cells reconstituted with Fis1. Interestingly, although it lacks part of the TPR-like domain, overexpression of mFis1.3 also induced mitochondrial fragmentation in WT cells. This result is in contradiction with the literature that clearly shows that an intact TPR-like domain is necessary for Fis1-dependent mitochondrial fission (Yu et al, 2005). However, overexpression of mFis1.3 in KO cells did not induced fragmentation, suggesting that mFis1.3 requires Fis1.1 and/or mFis1.2 in order to induce fragmentation (Figure 4A and 4B, chapter 3.2). On the other hand, mFis1.2 unexpectedly induced mitochondrial elongation when over-expressed in WT cells (Figure 4A and 4B, chapter 3.2). Moreover, the mitochondrial fragmentation induced by overexpression of mFis1.1 was abolished when this full-length variant was co-expressed with increasing amounts of mFis1.2 (Figure 3, chapter...
Even low amounts of mFis1.2 on top of mFis1.1 were sufficient to shift the initial fragmentation towards elongation, suggesting, at first, a dominant negative function for mFis1.2 against mFis1.1. However, the elongated phenotype was also observed in KO cells for Fis1 overexpressing mFis1.2 (Figure 4A and 4B, chapter 3.2), suggesting that Fis1.2-mediated elongation could also be induced independently of mFis1.1, maybe through interaction of mFis1.2 with another mitochondrial shaping protein, such as Drp1 or one of its receptors, Mff or the MiDs. mFis1.2 misses 7 of the 16 amino acids of the coiled-coil region, also called the inhibitory arm, because it supposes to interact with the TPR-like domain to inhibit oligomerization of Fis1 (Serasinghe & Yoon, 2008; Tooley et al, 2011; Yoon et al, 2003). In fact, overexpression of mutant hFis1 missing the coiled-coil domain shows more interaction with Drp1, more Fis1 homooligomers formation and an increase in mitochondrial fragmentation (Serasinghe & Yoon, 2008). This is in contradiction with the elongation phenotype observed when we overexpress mFis1.2 in WT cells. Despite the opposite mitochondrial phenotype of the coiled-coil mutant from Yoon’s laboratory (Serasinghe & Yoon, 2008) and mFis1.2, this mutant may give us hints on the mechanism by which mFis1.2 induces mitochondrial elongation. It may be possible that overexpression of mFis1.2 would indeed induce more Fis1 oligomers and induce more Drp1 interaction as the coiled-coil mutant. However, instead of inducing Drp1-mediated fragmentation, mFis1.2 could sequester Drp1 to inhibit fragmentation. This sequestration mechanism has been proposed as an explanation for the inhibition of fragmentation induced by the overexpression of the pro-fission protein MiD49/51 (Palmer et al, 2011). In order to test this hypothesis, the oligomerization of mFis1 variants and their interaction with
Drp1 must be assessed. In this line, we have shown that even low levels of mFsi1.2 are sufficient to produce a shift in mitochondrial morphology (Figure 3, chapter 3.1), suggesting that the stoichiometry of a putative hetero-oligomer between Fsi1.1 and mFis1.2 does not have to be 1:1 and the fine tuning of mitochondrial morphology by the Fis1 allele could be achieved by small changes in the expression of mFis1 variants. We are also currently working in collaboration with professor Blake Hill, from the Medical School of Wisconsin, to investigate the interaction of Drp1 with each variant and the effect of them in Drp1 GTP hydrolysis as a measure of Drp1 activity. Finally, it would be worth to address whether Fis1 variants could induce opposite mitochondrial shape phenotypes by differentially interacting with other fission factors, such as Mff and MiDs. Moreover, mFis1 variants could as well interact with TBC1D15, a recently discovered partner of Fis1 that induces mitochondrial fission independently of Drp1 (Onoue et al, 2013).

4.3 Modulation of mitochondrial shape by Fis1 gene and its effect on mitochondrial functionality.

Although early experiments of Fis1 overexpression in human cells clearly argued in favor of its role in mitochondrial fission (James et al, 2003; Yoon et al, 2003), more recent studies using knockdown approaches have cast doubts about the pro-fission features of Fis1 by showing only a mild mitochondrial elongation in cells with decreased levels of Fis1 (Otera et al, 2010). By using MEF cells lacking Fis1 we observed a clear phenotype in mitochondrial fission, since Fis1 KO cells had significantly longer mitochondria (Figure 3A and 3B, chapter 3.2). Fis1 is
also known to induce peroxisomal fission and, as expected, Fis1 KO cells had longer peroxisomes (Supplementary Figure 1, chapter 3.2). These results have been confirmed by the work of Chan’s group showing similar mitochondrial phenotype in MEF cells lacking Fis1 gene (Loson et al, 2013).

Mitochondrial shape is intimately linked with mitochondrial functionality. In fact, the elongation phenotype observed in Fis1 KO cells correlated with and increase in mΔΨ, a decrease in ROS production and lower oxygen consumption rate (Figure 3C, 3D and 3E, chapter 3.2). Taken together, these results suggest that longer mitochondria, induced by the absence of the pro-fission protein Fis1, leads to a more efficient respiration. Whether this efficiency has consequences in ATP production still has to be addressed. Supporting this notion, several publications have shown that mitochondrial fusion enhances respiration efficiency and increases ATP production. For example, inhibition of mitochondrial fusion by ablation of the mitofusins creates defects in membrane potential and respiration (Chen et al, 2005) and manipulation of Opa1 also disturbs mitochondrial respiration and cell growth by disturbing the cristae shape (Cogliati et al, 2013). However, although a slight increase in cell growth was observed in Fis1 KO cells, they did not significantly grow faster than WT cells (Figure 3F, chapter 3.2). It is worth mention that mitochondrial elongation not only induces cellular growth but, paradoxically, prolonged mitochondrial elongation can also induce cell senescence. In fact, low expression of Drp1 and Fis1 induces mitochondrial elongation and senescence in human endothelial cells (Mai et al, 2010). Moreover, mitochondrial fragmentation is necessary for cell division progression because the knock down of Fis1 induces cell cycle arrest and mitosis is only restored after the restitution of Fis1 (Lee et al, 2014). Hence,
the growth phenotype observed in the KO cells used in our study, although immortalized, may be the result of the interplay between cell growth and senescence processes in cells bearing long mitochondria. Thus, in order to clearly dissect the relevance of Fis1 variants in senescence, the use of primary cells is necessary.

Mitochondrial morphology also plays a key role in the progression of cell death. To this regard, Fis1 KO cells were highly resistant to the incubation with staurosporine, a drug that induces apoptosis by inhibiting protein kinases (Figure 3G, chapter 3.2). We also observed less ROS production and a high membrane potential in KO cells, which, according to the literature, may protect against cell death stimuli due to the effect of ROS and membrane potential loss in cell death progression (Cassina & Radi, 1996; Croteau & Bohr, 1997; Fulda et al, 2010; Ott et al, 2002). Moreover, the apoptosis resistance in KO cells is in agreement with a study showing that decreased levels of Fis1 induced even higher resistance to apoptosis than Drp1 knock down (Lee et al, 2004).

According to our data, it is very plausible that Fis1 might not be only relevant at cellular but as well at organism level. The correlation between the lack of Fis1 expression and the embryonic lethality of the Fis1\textsuperscript{flx/flx} mice suggests that, as other full KOs for mitochondrial shaping proteins, Fis1 ablation produces severe impairments to embryonic development. We were able to obtain Fis1\textsuperscript{flx/flx} embryos up to the 13.5 day of embryonic development, placing the putative embryonic lethality Fis1-dependent later than for other genes involved in mitochondrial dynamics, around 11.5 days (Alavi et al, 2007; Chen et al, 2003; Ishihara et al, 2009; Wakabayashi et al, 2009). Nevertheless, due to the relevance of Fis1 in mitochondrial functionality and cell death (Figures 3, chapter 3.2)
observed in this work, it is possible that deregulation of these processes might be the cause of developmental failure in the Fis1^{flx/flx}. A careful histological analysis of the different embryonic stages around the day 12.5 should give answers regarding the organs and mechanisms that produce the lethality. Of course, it is important to first find an explanation for the lack Fis1 expression in the Fis1^{flx/flx} mouse. To this regard, we have checked that the recombination between the transgene and the Fis1 locus was successful. Possible explanations could be a mutation in the promoter region, undetected mutations in the gene or the presence of the hygromycin cassette, which could produce an hypomorphic phenotype (Meyers et al, 1998).

4.4 PKA-dependent up regulation of mFis1.2 in starving cells.

Together with the observation of the elongation effect on mitochondrial morphology of the mFis1.2 variant (Figures 4, chapter 3.2), the most appealing result of this work was the finding of the physiological relevance of the up regulation of mFis1.2 upon starvation. In fact, mFis1.2 mRNA levels considerably and specifically increased after starvation (Figure 4C and 4E, chapter3.1). Moreover, this increase in mRNA levels upon starvation was sustained over time and only reverted after putting back the cells in normal medium, showing that this increased expression was tightly regulated (Figure 4E, chapter 3.1). During starvation, increased levels of cAMP activate PKA, which in turns inhibits Drp1 through the phosphorylation of S637. This produces mitochondrial elongation by unopposed mitochondrial fusion (Gomes & Scorrano, 2011). Remarkably, our study shows that PKA controls the up regulation of mFis1.2, which, as the
inhibition of Drp1, also induces mitochondrial elongation (Figure 5B and 5D, chapter 3.1). Additionally, genetically down regulation of mFis1.2 showed that this variant is partly responsible for the elongation phenotype of mitochondria in starving cells (Figure 5H and 5I, chapter 3.1).

PKA have been recently linked to the control of isoform expression by modulation of the spliceosome assembly during neuronal development (Cao et al, 2012). Alternative splicing requires the interplay of enhancing and silencing cis- and trans-acting elements in order for the spliceosome to assemble and produce splice variants. Cis-acting elements are special sequences in the pre-mRNA, which are recognized by the trans-acting factors such as the spliceosome subunits and modulators of its assembly like the heterogeneous nuclear ribonucleoproteins (hnRNPs). Particularly, in Cao’s publication, PKA-dependent activation of hnRNP K produced a competition between hnRNP K and the early spliceosome-assembly factor U2AF65 for binding to the PKA-responsive RNA element (KARRE) motif, a polypyrimidine-rich sequence at the 3' end of the introns. This competition for the KARRE site produced the absence of the downstream exon in the mature mRNA, showing that PKA can act as a master regulator of splice variant expression (Cao et al, 2012). To mFis1.2 respect, we found that this KARRE motif was repeatedly present along the first Fis1 intron (Figure 9). Hence, we hypothesized that the up regulation of hnRNP K or the down regulation of U2AF65 would induce a shift in favor of mFis1.2 expression. However, after genetically inhibit U2AF65 expression, we observed a decrease in mFis1.2 mRNA levels, which suggests that U2AF65 may have inhibitory effects on the formation of mFis1.2 (Figure 5F, chapter 3.1). In fact, it has been proposed that U2AF65 may induce the formation of aberrant isoforms when it binds to
polypyrimidine-rich sequences that are closer to the 5’ end of the intron (Zarnack et al, 2013), which happens to be the distribution of these motifs in the first intron of Fis1 gene. In this case, another ribonucleoprotein, hnRNP C, competes with U2AF65 for these motifs and inhibits the assembly of the spliceosome in incorrect regions. PKA-dependent activation of hnRNP C has not been studied, neither whether hnRNP K could compete for 5’ KARRE motifs. However, genetically inhibition of U2AF65 expression induced down regulation of mFis1.2 (Figure 5F, chapter 3.1), which in turn, produced the inhibition of starvation –dependent elongation of mitochondria (Figure 5H and 5I, chapter 3.1), suggesting that regulation of spliceosome assembly plays and important role in the PKA-dependent mitochondrial elongation mediated by mFis1.2 during starvation (Figure 9).

From the results obtained in this work, we hypothesize that, during starvation, PKA-dependent up regulation of the mRNA of mFis1.2 over mFis1.1 induces a shift in the proportion of this variants in favor of mFis1.2, which would lead to mitochondrial elongation by impinging in Fis1 and Drp1 interaction. This same mechanism of fine-tuning of mitochondrial morphology by modulation of Drp1/Fis1 interaction has been proposed in in vivo models of oxygen and nutrients deprivation (Kim et al, 2011). It has been proven that PKA-dependent inhibition of Drp1 induces mitochondrial elongation that inhibits mitophagy and enhances respiration efficiency (Gomes & Scorrano, 2011). As seen in the Fis1 KO cells, mitochondria elongation increases mΔΨ and enhances respiration efficiency, which would not only spare mitochondria from degradation but, as well, increase ATP production in a nutrient deprivation context.
Figure 9: Fis1 Alternative splicing might be governed by the competition of splicing factors U2AF65, hnRNP-K and hnRNP-C to the KARRE sites. Schematic draw depicting PKA-responsive RNA elements (KARRE), which are TCCCT and TCCT pyrimidine-rich 5’ and 3’ splicing sites at the first intron of the pre-mRNA of mouse Fis1 gene. U2AF65, an essential protein for the spliceosome assembly, can bind to the 5’ KARRE, which could induce mFis1.2 production; or U2AF65 could bind to the 3’ KARRE to induce mFis1.1 production. Ribonucleoproteins hnRNP-K or hnRNP-C compete with U2AF65 for the 5’ and 3’, respectively, in order to modulate exon usage.
Chapter 5

CONCLUSION AND PERSPECTIVES

5.1 Conclusion.

During the last decades, a vast amount of publications have unraveled the importance of mitochondrial dynamics during cells’s life and death. Nowadays, it is clear that the control of mitochondrial shape through the balance of the opposite processes of fusion and fission is essential in mitochondrial functions such as respiration and cell death. However, many aspects of the mechanisms underlying mitochondrial dynamics are yet unclear. In particular, mitochondrial fission was though to be a two-component system, with Fis1 being the receptor for the translocation of Drp1 to the OMM. The discovery of Mff and MiDs as receptors for Drp1 in mammals challenged the relevance of Fis1 in mitochondrial fission. In this project, we found compelling evidence supporting the importance of Fis1 gene in mitochondrial morphology with consequences in mitochondrial functionality and cell death. Additionally, we report the unexpected discovery that Fis1 is alternatively spliced in three variants expressed in several tissues. While two of these variants play a role in mitochondrial fission, increased mFis1.2 levels cause mitochondrial elongation. By mapping the relative expression of mFis1.2 in pathophysiological conditions, we found it increased during macroautophagy in a PKA-dependent manner to produce mitochondrial elongation. Hence, we have identifying a novel mechanism for mitochondrial morphology changes during autophagy that,
together with the already known mechanism of PKA-dependent inhibition of Drp1, helps to enhance respiration in conditions of nutrient deprivation.

5.2 Perspectives.

Great emphasis has been placed in the many post-translational modification that Drp1 suffers (phosphorylation, ubiquitination and sumoylation) and how they modulate mitochondrial fission. On the other hand, Drp1 receptors have been treated as invariable proteins, ready to interact with Drp1 whenever it is activated. The results presented in this work highlight the relevance of the modulation of Fis1 variant expression, especially of mFis1.2, in the regulation of mitochondrial morphology. Likewise, other fission factors may be regulated in a similar manner. For example, Mff also has splice variants that may have different expression according to internal or external cues as we showed for mouse Fis1. It is interesting to note that only a small deletion in the amino-terminus of mFsi1.2 induces an apposite effect on mitochondrial morphology, given to the coiled coil domain in this region a regulatory role in mitochondrial dynamics. However, whether the coiled coil arm is important in the interaction of Fis1 with itself or with other mitochondrial shaping proteins still has to be investigated. Additionally, human Fis1 gene is also able to produce four splice variants, some with differences in the coiled coil arm, however, nothing is known about their expression pattern and regulation nor about their role in mitochondrial morphology. Hence, it is possible that mitochondrial shape in human cells may also be regulated by Fis1 splice variants. To this regard, it is of paramount importance to direct studies in mouse and human cells aimed to address the
hierarchical interaction between the different Drp1 receptors and their splice variants in order to produce a specific mitochondrial shape in response to a given stimulus. Most likely, a detailed mapping of the mitochondrial fission transcriptome and interactome will contribute to a clearer view of how mitochondrial fragmentation is achieved.

Finally, mitochondria have a central role in cancer and neurodegenerative diseases and data in this work clearly show that mFis1 impinges not only in the efficiency of mitochondrial respiration but as well in the progression of cell death. Future work in animal models should clarify whether the modulation of mFis1 variants expression could have an impact in physiological processes with pathological consequences.
Chapter 6

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