Università degli Studi di Padova

Dipartimento di Biologia

SCUOLA DI DOTTORATO DI RICERCA IN: BIOSCIENZE E BIOTECNOLOGIE

INDIRIZZO: BIOCHIMICA E BIOFISICA

CICLO XXVIII

Exploring the role of FIS1 in mitochondrial (patho)physiology

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1 Feb 2016

Section I - Summary

1. Summary

Mitochondria are key players in a plethora of (patho)physiological processes and show a striking morphological and structural versatility, which is ensured by inter-organellar fusion and fission processes mediated by a group of dynamin-related ATPases and their adaptor proteins, the so-called mitochondrial shaping proteins (Friedman and Nunnari, 2014; Hoppins et al., 2007a). Mitochondrial fission requires the translocation of cytosolic dynamin-related protein 1 (DRP1) to the mitochondria and its interaction with FIS1, MFF and MiD49/50, its putative receptors.

FIS1 is a tail-anchored protein evenly distributed on the outer mitochondrial membrane and composed by a C-terminus transmembrane (TM) domain and two cytosolic tetratricopeptide repeat (TRP) motifs (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003). FIS1 has been proved to mediate mitochondrial-ER tethering, ER-gated apoptosis, autophagy and ischemia/hypoxia-induced fragmentation (Alirol et al., 2006; Iwasawa et al., 2011a; Kim et al., 2011; Shen et al., 2014; Yamano et al., 2014). However, the discovery of MFF and MiD49/50, along with the finding that both DRP1 recruitment and mitochondrial fragmentation take place also in two models of *Fis1 in vitro* ablation, led to partially discharge FIS1 involvement in such events (Loson et al., 2013; Otera et al., 2010; Palmer et al., 2013). Besides the mechanistic aspects of FIS1-mediated fragmentation, its (patho)physiological relevance is still far from been clarified, and no human pathology has been directly linked to a mutation in *Fis1* gene so far.

In order to place FIS1 functions into a physiological context and to further define the relevance of mitochondrial fission *in vivo*, we decided to generate a hypomorphic *Fis1* mouse model ($Fis1^{hyp}$ allele) that could be turned into a conditional ablation system ($Fis1^{fl}$ allele). The powerful genetic tool we generated circumvents many of the potential pitfalls of the most commonly used gene targeting approaches.

We showed that *Fis1* hypomorphism causes a perinatal pleiotropic lethal phenotype, comprising severe progressive muscular atrophy, heart ischemia and compromised vessel integrity. The concomitant muscular atrophy and heart ischemia would suggest (cardio)myopathy and/or neuromuscular defects, whereas extensive blood clots in the thorax, the presence of blood in the lungs and in the intestine and the altered microcircular bed in the retina seem to suggest defects in the maintenance of vessel

integrity and/or increased vessel permeability and consequent blood leakage. Remarkably, although reduced, also heterozygous mice display lethality at a later stage and show defects comparable to their *Fis1*$^{hyp/hyp}$ littermates, thus suggesting a dose-dependent relationship between phenotype severity and extent of *Fis1* gene ablation.

In vitro, we observed mitochondrial elongation and upregulation of the fission machinery as a consequence of *Fis1* hypomorphism.

Furthermore, we showed that expression of both human and mouse *Fis1* genes is controlled by multiple splicing. In particular, an exon-skipping event of conserved exon 2 dictates the preferential expression of variant 2 versus the canonical variant 1 in both species. Remarkably, a non-canonical START codon seems to be responsible for transcription of human variant 2 (*hFis1*.2). The resulting protein, although lacking only the first 18 amino acids, triggers mitochondrial elongation when overexpressed in mouse. 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.

2. Riassunto

I mitocondri svolgono una funzione chiave in una serie di processi (pato)fisiologici e presentano una sorprendente versatilità morfologica e strutturale, assicurata da processi di fusione e fissione mitocondriali. Quest'ultimi sono mediati da un gruppo di ATPasi simili alla dinamina e dalle loro proteine adattatrici, dette mitochondrial shaping proteins (Friedman and Nunnari, 2014; Hoppins et al., 2007a). La fissione mitocondriale richiede la traslocazione di dynamin-related protein 1 (DRP1) dal citosol ai mitocondri e la sua interazione con FIS1, MFF e MiD49/50, i suoi recettori putativi.

In particolare, FIS1 è una proteina legata alla membrana mitocondriale via l'N-terminale, distribuita in maniera regolare nella membrana e composta da un dominio transmembrana C-terminale (TM) e due motivi a ripetute tetratricopeptidiche (TRP) citosolici (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003). E' stato dimostrato che FIS1 media il collegamento tra mitocondri e reticolo endoplasmatico (ER), l'apoptosi via ER, l'autofagia, e la frammentazione mitocondriale indotta da ischemia/ipossia (Alirol et al., 2006; Iwasawa et al., 2011a; Kim et al., 2011; Shen et al., 2014; Yamano et al., 2014). Tuttavia, la scoperta di MFF e MiD49/50, insieme ai risultati sperimentali che sia il reclutamento di DRP1 che la frammentazione mitocondriale si svolgono anche in due modelli di ablazione di *Fis1 in vitro*, hanno portato in parte a smentire il coinvolgimento di FIS1 in tali eventi (Loson et al., 2013; Otera et al., 2010; Palmer et al., 2013). Oltre agli aspetti meccanicistici della frammentazione mediata da FIS1, la sua rilevanza (pato)fisologica è tutt'altro che chiara e fino ad ora nessuna patologia umana è stata legata direttamente a mutazioni nel gene *Fis1*.

Per collocare le funzioni di FIS1 in un contesto fisiologico e meglio definire l'importanza della fissione mitocondriale *in vivo* abbiamo deciso di generare un modello murino ipomorfico di *Fis1* (allele Fis^{hyp}) che possa essere convertito in un sistema per l'ablazione condizionale di Fis1 (allele $Fis1^{fl}$). Il potente strumento genetico da noi generato evita molti dei potenziali problemi caratteristici degli approcci più comunemente usati per intervenire su un gene.

Abbiamo scoperto che l'ipomorfismo di *Fis1* causa un fenotipo pleiotropico con letalità perinatale che comprende una grave atrofia muscolare progressiva, ischemia cardiaca e danni all'integrità dei vasi sanguigni. L'atrofia muscolare in concomitanza con ischemia

cardiaca parrebbe suggerire una (cardio)miopatia e/o difetti neuromuscolari, mentre gli estesi coauguli nel torace, la presenza di sangue nei polmoni e nell'intestino, e la microcircolazione alterata nella retina paiono suggerire difetti nel mantenimento dell'integrità dei vasi sanguigni e/o un aumento della permeabilità dei vasi sanguigni con conseguenti emorragie.

Anche topi eterozigoti mostrano letalità, benché in misura ridotta ed ad uno stadio più tardo, e hanno difetti comparabili a quelli esibiti dai topi *Fis1*$^{hyp/hyp}$, suggerendo così una relazione dose-dipendente tra severità del fenotipo ed entità dell'ablazione del gene *Fis1*. Per verificare tale ipotesi è necessaria una misura della produzione residuale di mRNA di *Fis1* via 5'RACE seguita da una correlazione tra i livelli residuali di mRNA di *Fis1* in topi Fis$^{hyp/+}$ e severità del fenotipo.

In conseguenza dell'ipomorfismo di *Fis1* abbiamo osservato *in vitro* un'elongazione dei mitocondri e una regolazione su livelli più alti delle proteine coinvolte nella fissione mitocondriale.

Un altro risultato è stato la dimostrazione che l'espressione dei geni *Fis1* umano e murino è controllata da splicing multiplo. In particolare, un evento di salto di esone dell'esone 2, conservato in entrambe le specie, determina l'espressione preferenziale della variante 2 rispetto alla variante canonica 1 in entrambe le specie. E' notevole che un codone START non canonico sembri essere responsabile della trascrizione della variante umana 2 (*hFis1*.2). La proteina risultante, benché manchi solo dei primi 18 amminoacidi, induce elongazione dei mitocondri se overespressa in topo. In caso di starvation, l'espressione della variante 2 di *Fis1* viene regolata su livelli più alti in maniera dipendente da protein kinase A e il suo knockdown specifico inibisce l'elongazione mitocondriale indotta da autofagia: ne deriva che *Fis1* è soggetto ad uno splicing alternativo per modulare la morfologia mitocondriale durante l'autofagia

Section II - Introduction

1 Mitochondrial biogenesis and metabolism

Evolutionary, mitochondria derive from the endosymbiosis of an α-proteobacterium by the progenitor of contemporary eukaryotes approximately 1.5–2 billion years ago. This peculiar origin left the organelle with a sophisticated double membrane architecture, a genome, a complex protein import and sorting machinery to its numerous suborganellar compartments and specific bioenergetic functions.

1.1 Mitochondrial ultrastructure

In the 1950s, Palade with his pioneering work described mitochondria as organelles possessing two very different membranes: an outer membrane (OMM) and a highly convoluted inner membrane (IMM). The latter was depicted to fold in *cristae* with broad openings to the intermembrane space (IMS) and protruding across the matrix (Palade, 1952) (Figure 1A and 1B). Some years later, Hackenbrock demonstrated that the structure of the IMM is linked to the metabolic state of the organelle and provided the very first evidence of mitochondrial *cristae* shape (Hackenbrock, 1966) (for details see 2.1).

In the nineties, application of the electron microscopic tomography (EMT) allowed 3D reconstruction of the mitochondrial ultrastructure of thick sections both from isolated mitochondria and *in situ*, showing that the classical Palade's model was far from exhaustive. Indeed, the IMM is actually composed by two functional and structural different units: the inner boundary membrane (IBM) and the *cristae* membrane. The IBM is in continuous and close apposition to the OMM, forming a kind of organelle envelope. On the other hand, *cristae* are pleomorphic structures connected by narrow, usually multiple, tubular *cristae* junctions to the IBM and to each other (Frey and Mannella, 2000; Mannella, 2006a, b) (Figure 1C and 1D).

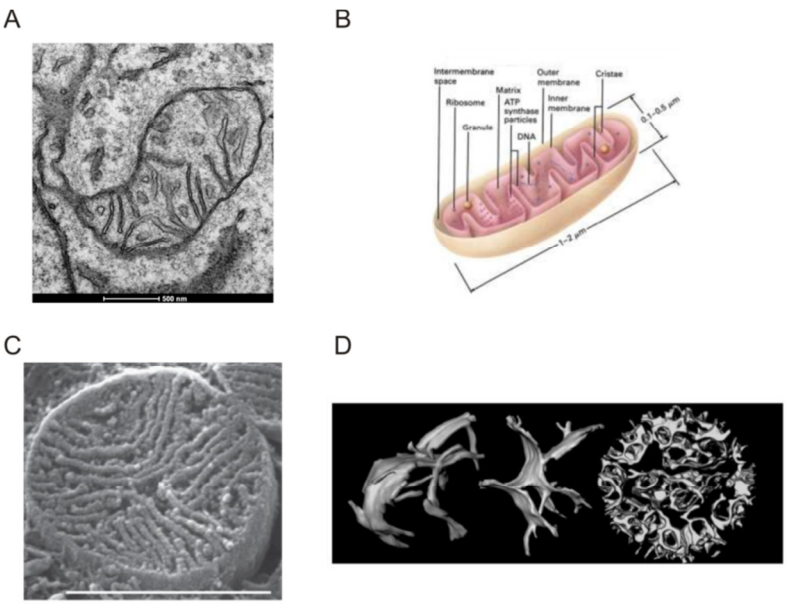

Figure 1 Mitochondrial ultrastructure. (A) Electron microscopy of mitochondria from wildtype MEFs . (B) Palade's model predicting inner membrane convolution into wide *cristae* . (C) Scanning EM images of lamellar mitochondria *cristae* from rat ventricular myocytes. Scale bars: 1 µm. Adapted from Hoppel et al., 2009. (D) Left and middle: Cristae in intact, frozen-hydrated rat liver mitochondria. The larger compartments appear to be formed by fusion of tubular membranes. Right: Inner membrane of a mouse liver mitochondrion after treatment with the pro-apoptotic protein t-Bid. Curvature of the cristal membranes is reversed and the intracristal space essentially forms one continuous compartment. Adapted from Mannella, 2008 .

1.2 Mitochondrial DNA

As mentioned above, mitochondria have their own genome. In vertebrates, mitochondrial DNA (mtDNA) consists of a double stranded covalently closed circular DNA molecule of about 16.5 kb. mtDNA harbors 13 genes coding for proteins that are part of the oxidative phosphorylation system, two rRNAs and 22 tRNAs. mtDNA is organized in nucleoids which appear as discrete *punctae* distributed throughout the mitochondrial network (Alam et al., 2003; Legros et al., 2004). Nucleoids are composed by mtDNA molecules and an array of proteins which comprises the mitochondrial transcription factor A (mTFA or TFAM), the mitochondrial single-strand binding protein (mtSSB), the DNA polymerase POLG, the mtRNA polymerase (POLRMT), several helicases, a number of mitochondrial ribosomal proteins, and the Lon and ClpX

proteases, both of which are involved in mitochondrial quality control (Bogenhagen, 2012; Bogenhagen et al., 2008). The core components of the nucleoids exert both structural and functional roles. For instance, POLG, mtSSB, and the DNA helicase Twinkle are the minimal factors necessary for mitochondrial replication. Conversely, POLRMT, along with and mitochondrial transcription factor B (mtTFB) are the minimal factors necessary for mitochondrial transcription.

Among all the core components of the nucleoid TFAM plays a key role. Indeed, TFAM is the main packaging factor and is responsible for the actual bending of mtDNA into its characteristic U-turn (Kukat and Larsson, 2013). Additionally, TFAM is necessary for transcription (Garstka et al., 2003) and initiation of replication by regulating protein binding at the D-loop (Ghivizzani et al., 1994). The role of TFAM in mtDNA copy number is controversial: data reported in literature suggest that mtDNA copy number depends on TFAM levels (Ekstrand et al., 2004; Kanki et al., 2004); other lines of evidences suggest, however, that TFAM is dispensable for mtDNA copy number regulation (Maniura-Weber et al., 2004; Noack et al., 2006).

Nucleoid size and number vary in response to physiological conditions and the maintenance of the mtDNA integrity is important for keeping proper cellular functions. Indeed, mtDNA mutations, deletions and copy number alterations are associated with several pathological conditions.

Classically, mtDNA replication was thought to be linked to cell cycle and to display slow turnover rate in postmitotic cells (Wang et al., 1997) . Additionally, mtDNA replication is known to be regulated by factors involved in cell proliferation (Trinei et al., 2006). However, rapid and random mtDNA turnover was reported also in quiescent cells (Kai et al., 2006), suggesting that mtDNA replication is instrumental for replacing damaged mtDNA molecules.

As discussed in *3. Mitochondrial dynamics: functional implications*, mitochondria are continuously undergoing fission and fusion events. Since long, mitochondrial fragmentation (Parone et al., 2008), along with selective removal of damaged organelles through mitophagy (Wei et al., 2015), have been believed to be somehow implicated in regulating mtDNA abundance and removal of damaged mtDNA molecules. Ishihara and coworkers recently provided *in vivo* evidences: *Drp1* ablation in the heart and in primary cultured cardiomyocytes results in severe mtDNA nucleoid clustering and leads to mosaic deficiency of mitochondrial respiration with subsequent immature myofibril

assembly and defective cardiomyocyte hypertrophy (Ishihara et al., 2015).

The link between mitochondria-shaping proteins and mitochondrial membranes and mtDNA is further substantiated by the growing findings supporting tethering of mtDNA to the IMM. Indeed, Twinkle helicase was reported to mediate transient association between the nucleoids and the IMM in order to regulate mtDNA replication (Rajala et al., 2014). Additionally, prohibitins, known regulators of *cristae* morphology, were reported to peripherally associate with nucleoids (He et al., 2012), whereas mitofilin, a component of the MICOS complex involved in tethering the IBM to the OMM, was recently reported to be essential for nucleoid organization (Li et al., 2015).

Recently, mtDNA was shown to be transferred between cells *in vitro* (Berridge et al., 2015). Indeed, a growing number of publications report mtDNA transfer in co-culture systems of different cancer cells and non-tumoral cell lines, such as mesenchymal and endothelial cells and fibroblasts (Lou et al., 2012; Pasquier et al., 2013). Additionally, mitochondrial transfer was described also from stem/progenitor cells to differentiated cells (Cho et al., 2012; Lin et al., 2015; Spees et al., 2006; Vallabhaneni et al., 2012). mtDNA receiving cells were reported to rescue their metabolic phenotype(Islam et al., 2012) and/or to display chemoresistance (Pasquier et al., 2013). Mechanistically, mtDNA transfer seems to be mediated by membrane-bound cell-bridging structures named tunneling nanotubes (Bukoreshtliev et al., 2009; Liu et al., 2014; Lou et al., 2012; Vallabhaneni et al., 2012; Wang and Gerdes, 2015). Interestingly, Miro, a protein known to mediate mitochondrial movement along microtubules, has been implicated in nanotube-mediated mitochondrial transfer (Ahmad et al., 2014). Recently mtDNA transfer was described also *in vivo* (Tan et al., 2015).

1.3 Mitochondrial protein import

As mentioned, a key step during mitochondrial evolution was the development of a protein import and sorting machinery. Indeed, nuclear-encoded proteins are translated on cytosolic ribosomes and precursors need to be imported and sorted into the

numerous mitochondrial subcompartments. Similarly, mtDNA-encoded proteins need to be integrated into the IMM from the matrix side. Depending on their origin, their targeting sequence and their final destination, mitochondrial protein precursors follow distinct importing and sorting routes (Bohnert et al., 2012) (Figure 2).

The translocase of the outer membrane (TOM) complex is responsible for the import of the majority of nuclear-encoded proteins. The TOM complex is composed by a central pore-forming subunit and a number of ancillary subunits, which recognize different targeting signals and bind to heat shock protein 70 (Hsp70) chaperons that deliver hydrophobic precursors to the complex (Wu and Sha, 2006). Although it has been proposed that the TOM complex can release specific precursors directly into the OM, the majority of mitochondrial proteins require additional insertion machineries (Bohnert et al., 2012).

The mechanism mediating insertion into the OMM of signal-anchored proteins has been only partially revealed. Indeed, whereas the biogenesis of *N*-terminal targeted proteins (with transmembrane domain at the *N*-terminus) seem to be mediated by the mitochondrial import machinery (MIM) complex, the mechanisms for *C*-terminal targeted proteins (TM at the *C*-terminus) is still elusive. Remarkably, the lipid composition seems to play a role (Krumpe et al., 2012). Soluble targeting factors and quality control mechanisms may be involved (Borgese and Fasana, 2011).

Conversely, precursors of β-barrel proteins are fully imported through the TOM complex to the IMS and transferred to the sorting and assembling machinery (SAM) (Bohnert et al., 2012). The mechanism of β-barrel protein folding and insertion in the OMM has been debated; recent studies demonstrated that the folding is completed before membrane insertion (Qiu et al., 2013). Similarly, the shuttling of the precursors from TOM to SAM complex has been elusive since the recent discovery and characterization of the mitochondrial contact site and *cristae* organization system (MICOS). Indeed TOM and SAM complexes appear to be highly organized and bridged together by MICOS (Bohnert et al., 2012; Zerbes et al., 2012a) and to form transient supercomplexes that cooperate with the small Tim chaperons of the IMS (Qiu et al., 2013). After crossing the OMM, proteins targeted to IMM or to the matrix are sorted to the TIM23 complex. The import through the TIM23 complex seems to depend on the membrane potential ($\Delta\Psi_m$) across the IMM. Additionally, translocation of large hydrophobic protein requires the presequence translocase-associated import motor (PAM) complex. The PAM complex is

also required for the release of proteins into the matrix (Bohnert et al., 2012).

Interestingly, in human, TIM21, a subunit of the TIM23 complex, has been shown to mediate the shuttling of subunits of the respiratory complexes from the TIM23 complex to the mitochondrial translation regulation assembly intermediate of cytochrome c oxidase (MITRAC) complex (Aiyar et al., 2014).

Finally, mtDNA-encoded subunits of the respiratory complexes require insertion into the IMM through the oxidase assembly (OXA) machinery (Bonnefoy et al., 2009).

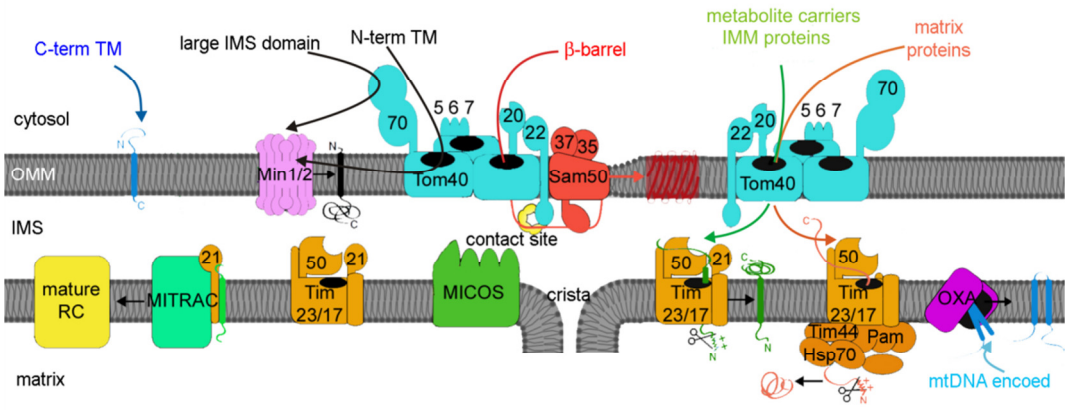

Figure 2 Mitochondrial protein import. Depending on their origin, their targeting sequence and their final destination, mitochondrial protein precursors follow distinct importing and sorting routes. Signal-anchored proteins bearing *N*-terminal targeting sequence are imported through the TOM complex. Import of β-barrel proteins targeted to the OM is mediated by the TOM-SAM-MICOS complex. Metabolite carriers directed toward the inner membrane are imported through the TOM-TIM complex and their insertion into the IM is mediated by the TIM complex. Matrix proteins are imported through the TOM-TIM pathway, their release in the matrix is then mediated by PAM. Proteins encoded by the mitochondrial genome are inserted into the inner membrane by OXA machinery.

1.4 Mitochondrial metabolism

Mitochondria are at the core of the cellular metabolism: they provide most of the energy to the cell by coupling complete oxidation of metabolites to ATP production. Mitochondria also participate to the biosynthesis of fatty acids, amino acids, ketone-bodies and heme. Such metabolic complexity is achieved by the fine coupling of three

key processes: the tricarboxylic acid (TCA) (Figure 3) cycle fuels the electron transport chain (ETC) to support concomitant oxidative phosphorylation (OXPHOS) (Figure 4).

1.4.1 The Krebs cycle and its amphibolic nature

The tricarboxylic acid (TCA) cycle, also known as Krebs cycle or citric acid cycle, leads to the complete oxidation of nutrient-derived carbons to CO_2 with concomitant production of three NADH, one $FADH_2$ and one ATP molecules per cycle (Figure 3).

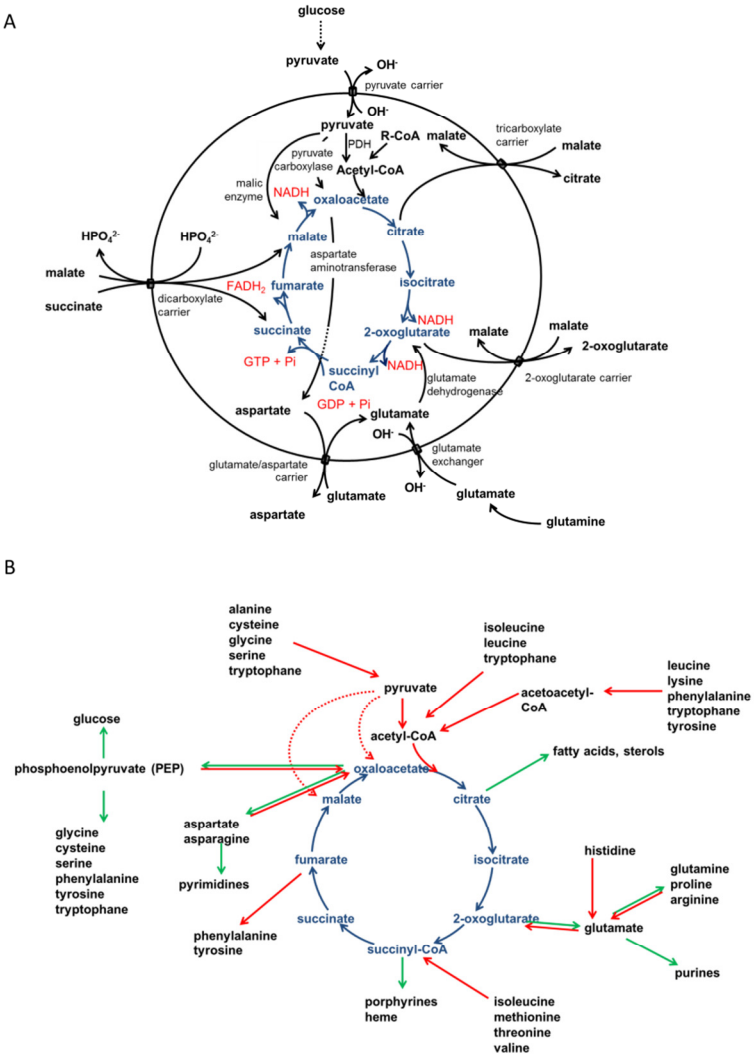

Figure 3 Mitochondrial metabolic pathways. (A) TCA cycle and metabolite carries. (B) TCA cycle, anaplerotic reactions (red arrows) and biosynthetic pathway (green arrows).

Acetyl-Coenyzme A (acetyl-CoA) is the entry of carbons into the TCA cycle. Indeed, glycolysis-derived pyruvate can be transported into the mitochondrial matrix by the pyruvate carrier, oxidized and combined with coenzyme A, forming acetyl-CoA. This reaction is carried out by the pyruvate dehydrogenase complex (PDH), whose activity is tightly regulated by counteracting kinases and phosphatases. On the other hand, in fat catabolism, triglycerides are hydrolyzed into fatty acids, which are further broken down through mitochondrial β-oxidation leading to acetyl-CoA molecules entering the TCA cycle. Conversely, carbon skeletons derived from amino acids enter the TCA through its intermediates, thus working as anaplerotic metabolites. Indeed, given that all intermediates of the cycle are regenerated at each turn, addition or withdrawal of any of these intermediates has respectively a filling up (anaplerotic) or a withdrawing (cataplerotic) effect (Figure 3).

The amphibolic nature of the TCA cycle is assured by the dicarboxylate carrier, the tricarboxylate carrier, the pyruvate carrier and the 2-oxoglutarate carrier which complessively shuttle TCA intermediates between the organelle and the cytosol (Figure 3).

1.4.2 Oxidative phosphorylation

Along the respiratory chain, electrons carried by NADH and $FADH_2$ are transferred in a stepwise manner from lower to higher reduction potential multiprotein complexes, allowing a progressive release of the free energy and its storage as electrochemical potential. The electron transfer along the respiratory chain is used to pump protons across the inner membrane. In turn, the proton pumping by the oxidative complexes creates both an electrical ($\Delta\Psi_m$) and a chemical potential, the proton concentration gradient (ΔpH). The chemiosmotic theory postulates that the electrochemical potential is the actual source of energy for ATP production (Mitchell, 1961) This process is known as oxidative phosphorylation (OXPHOS) (Figure 4).

1.4.2.1 Electron transport, proton gradient generation and ATP production

The components of the electron-transport chain (ETC) are well characterized multiprotein complexes.

Complex I, also called NADH-CoQ oxidoreductase or NADH dehydrogenase, represents the major entry point for electrons deriving from oxidation of organic molecules into the respiratory chain. Complex I oxidizes NADH to NAD^+ and transfers electrons to coenzyme Q (CoQ); the free energy released is used for the transport of four protons to the intermembrane space. Complex I is the largest complex of the ETC and it is codified both by mitochondrial and nuclear DNA.

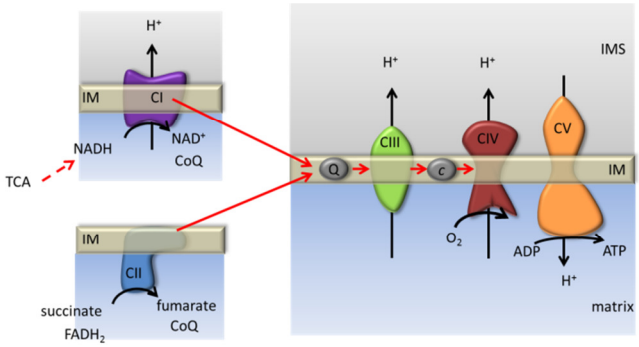

Figure 4 Components of the electron transport chain and oxidative phosphorylation system. Electrons transferred along the respiratory chain are used to create an electrochemical potential by pumping protons across the inner membrane. The electrochemical potential is then used by the ATPase to produce ATP. Complex I (CI) collects electrons through NADH from the TCA cycle, whereas complex II (CII) derives electrons from $FADH_2$. Both CI and CII transfer electron to Coenzyme Q (Q). Complex III, through the Q cycle, transfers electrons to cytochrome c, which shuttles them to complex IV (CIV). Red arrows indicate electron transfer steps.

Complex II, the succinate dehydrogenase complex, oxidizes carbons from succinate and transfer electrons through $FADH_2$ to CoQ. Complex II is composed by a peripheral membrane sector, responsible for the oxidation of succinate to fumarate in the Krebs cycle, and an integral sector anchoring the enzyme to the inner membrane. Complex II is the only component of the ETC not pumping protons into the inter membrane space and the only complex entirely codified by nuclear genome.

Complex III, also known as cytochrome bc1 complex, collects $CoQH_2$ -carried electrons from complex I and complex II and, through the Q cycle, transfers them to cytochrome c

while pumping four protons into the intermembrane space. Complex III is a dimeric complex whose monomers are composed by 11 subunits, several redox-active moieties and a dimer of Fe_2S_2. Only the cytochrome b subunit is codified by the mtDNA.

Complex IV, cytochrome c oxidase, transfers electrons from cytochrome c to O_2, the final electron acceptor. This reaction is coupled with the pumping of two protons. Complex IV is codified both by nuclear and mitochondrial DNA.

ATP synthase (Boyer, 1999; Schnitzer, 2001) is composed by two main building blocks, F_1 and F_0, both formed by interconnected stator and rotor subunits. This interconnection is responsible for the transmission of the movement through the entire complex in such a way that the energy of the proton electrochemical gradient is converted first to mechanical and then to chemical energy. As a general model, the backflow of protons along their gradient through the F_0 rotor and their final release into the matrix compartment is supposed to induce a rotation of the c ring, whose movement is transferred by the F_1 rotor to the F_1 stator (Dimroth, 2000; Rastogi and Girvin, 1999; Sambongi et al., 1999). The relative movement of the rotor to the stator may induce conformational changes which are responsible for binding and catalysis (Boyer et al., 1993). When the membrane potential decreases, the ATP synthase can also hydrolyze ATP to ADP and functions as an ATPase. This functional switch is controlled by the IF1 subunits (Cabezon et al., 2003).

1.4.2.2 Respiratory chain supercomplexes

Initially, respiratory complexes were thought to be single units freely diffusing in the IMM and electron transport was thought to occur during random collisions. This model, usually called fluid model, was mainly based on the observation that the complexes could be purified as single units preserving their enzymatic activity (Hackenbrock and Chazotte, 1986). However, during the last twenty years, BlueNative PAGE (BNGE) studies conducted in yeast demonstrated that the respiratory complexes actually organize into supercomplexes. Further studies in patient-derived cells and murine models of mtDNA mutations revealed that different complexes play different roles in the assembly of the supercomplexes and in the stability of other complexes. For instance, complexes III and IV are essential for the stabilization of complex I and when they are mutated or not assembled the assembly of supercomplexes is impaired. Conversely, the absence of

complex I affects supercomplexes formation, but does not impair the formation of the other complexes (Acin-Perez et al., 2004). However, the existence of supercomplexes *in situ* has been challenged in the last few years.

Enriquez and co-workers demonstrated that RCS are not artifacts induced by the detergent used during permeabilization of the mitochondrial membranes. On the contrary, RCS are functional units who retain in-gel their activity upon BNGE isolation and whose assembly is consequent on the assembly of the single complexes, as indicated by pulse-chase experiments of radiolabeled mtDNA-encoded proteins (Acin-Perez et al., 2008). Therefore, Enriquez and co-workers proposed a so-called plasticity model. Accordingly, the complexes are single units which assemble into dynamic functional structures in response to the energetic needs of the cell. Although the molecular mechanism and the regulatory factors of the RCS assembly are still largely unknown, several evidences suggest that this organization might regulate the electron transport, allowing channeling of electrons, increasing the stability of the single complexes and modifying the IMM topology (Boekema and Braun, 2007).

1.5 Transcription factors and signaling pathways controlling mitochondrial biogenesis and metabolism

Mitochondrial content and respiratory capacity vary according to specific energy demands and mitochondrial biogenesis is modulated in response to physiological conditions. For instance, muscle exercise training, brown fat adaptive thermogenesis and neuronal activity are all well-known inducers of mitochondrial biogenesis. Mitochondrial biogenesis is also regulated during development: massive mtDNA amplification occurs during oogenesis to allow mtDNA distribution among the cells and during the blastocyst stage, when embryonic mtDNA replication is initiated. Finally, proliferation of defective mitochondria has been associated with ragged red fibers

formation in muscles. Since mitochondria are semiautonomous organelles and mitochondrial functions relay on nuclear encoded genes, mitochondrial biogenesis depend on a fine cross-talk between the organelle and the nucleus.

The seminal work conducted by Scarpulla and co-workers on the rat cytochrome *c* promoter (Evans and Scarpulla, 1989) opened the field of the transcriptional regulation of mitochondrial biogenesis. Since then, a number of DNA-binding transcription factors have been characterized. During the last decade, however, it has been emerging that the coordinated expression of nuclear genes involved in different mitochondrial functions is regulated by a family of coactivators. Such coactivators integrate cellular and extracellular cues and implement the complex transcriptional program controlling respiration, β-oxidation, Krebs cycle, mtDNA replication, transcription and translation, protein import and respiratory subunit assembly in an integrated and coordinated fashion.

1.5.1 DNA-binding transcription factors

The list of DNA-binding factors described so far include nuclear respiratory factor NRF-1 and NRF-2, the peroxisome proliferator-activated receptor proteins (PARPs), the estrogen-related receptors (ERRs), stimulatory protein 1 (Sp1) and Ying Yang 1 (YY1) (Scarpulla, 2008, 2011; Scarpulla et al., 2012) (Figure 5).

1.5.1.1 Nuclear respiratory factor NRF1/2

The first transcription factor implicated in mitochondrial biogenesis was respiratory factor NRF1, initially shown to regulate cytochrome *c* expression (Evans and Scarpulla, 1989). NRF-1 binds DNA as a homodimer and functions as a positive regulator of transcription. Conversely, NRF-2 (GABP in mouse) was identified as a regulator of cytochrome oxidase subunit IV (COXIV) and acts as a heterotetramer (Virbasius et al., 1993). *In silico*, both NRF-1 and NRF-2 sites have been identified in many genes coding for components of the respiratory chain and mitochondrial ribosomal proteins (Scarpulla, 2002). *In vitro*, NRF-1 and NRF-2 were proven to regulate the expression of TFAM (Virbasius and Scarpulla, 1994), TFB (Gleyzer et al., 2005) and TOM20 (Blesa et al., 2007).

1.5.1.2 Peroxisome proliferator-activated receptor proteins (PPARs)

The peroxisome proliferator-activated receptor proteins (PPARs) are members of the nuclear hormone receptor superfamily. Upon binding with their cognate ligands, PPARs form heterodimers with 9-*cis* retinoic acid-activated receptors (RXRs) and bind to DNA. All PPARs have been shown to regulate different aspects of lipid metabolism (Fan and Evans, 2015; Madrazo and Kelly, 2008).

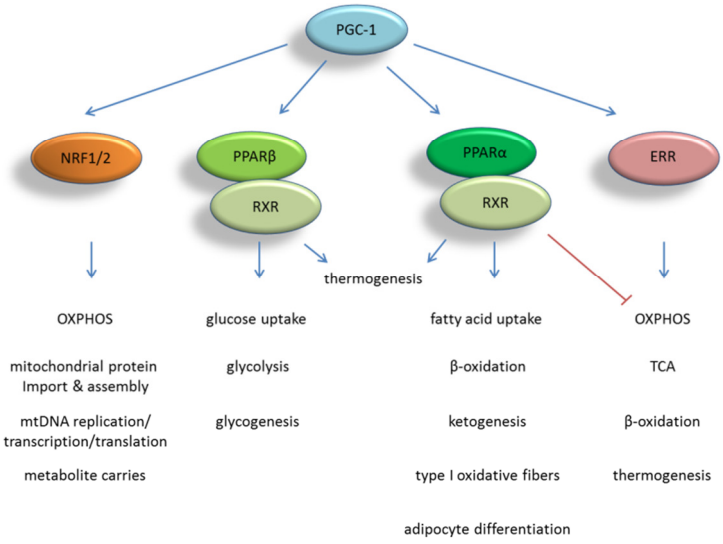

Figure 5 DNA-binding transcriptor factors and coactivator controlling mitochondrial biogenesis The coactivators members of the PGC family regulate the DNA-binding factors, control their specificity and integrate cellular and extracellular cues. As a result, a restricted number of ubiquitous factors regulate many mitochondrial functions and mediate many metabolic and developmental processes.

In particular, several *in vivo* studies elucidated the key role of PPARα in promoting expression of genes involved in fatty acids used in different tissues. In the heart PPARα promotes fatty acid uptake and β-oxidation with concomitant repression of glucose import, glycolysis and oxidative phosphorylation (Finck et al., 2002; Oka et al., 2011), possibly through suppression of oxidative genes regulated by the estrogen-related receptors (ERRs) (Oka et al., 2011). Similarly, in muscles PPARα induces upregulation of fatty acid metabolism and β-oxidation (Finck et al., 2005) and promotes conversion of muscle fibers to type I oxidative fibers (Wang et al., 2004). Finally, in the liver PPARα regulates fatty acid uptake, β-oxidation, and ketogenesis, and mediates liver response to fasting conditions (Aoyama et al., 1998; Kersten et al., 1999; Leone et al., 1999).

Conversely, PPARβ/δ has been implicated in regulating glucose utilization. PPARβ/δ overexpression in the heart induces upregulation of glucose uptake and glycolysis (Burkart et al., 2007). Similarly, in muscles it increases the coupling of glycolysis to complete glucose oxidation by promoting, in conjunction with AMPK, LDHA expression, thus favoring conversion of lactate to pyruvate (Gan et al., 2011). Finally, in liver PPARβ/δ seems to mediate hepatic glycogenesis and lipogenesis (Liu et al., 2011; Sanderson et al., 2010).

Both PPARα and PPARβ/δ are reported to regulate thermogenesis in brown adipose tissue (BAT) by regulating the expression of the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) and uncoupling protein 1 (UCP1) both in basal and cold conditions (Barbera et al., 2001; Hondares et al., 2011; Lee et al., 2011). Additionally, PPARα mediates expression of adipocyte differentiation genes (Goto et al., 2011).

1.5.1.3 Estrogen related receptors (ERRs)

The estrogen related receptors (ERRs) are also members of the nuclear receptor superfamily and have been shown to regulate Krebs cycle and respiration genes. The ERR family is composed by three members: ERRα, ERRβ and ERRγ. *In silico* analysis revealed that ERRα and ERRγ share many target genes (Fan and Evans, 2015).

In vitro and *in vivo* studies demonstrated that both ERRα and ERRγ mediate expression of genes involved in mitochondrial oxidative metabolism in the heart and ERRα/ERRγ-mediated gene expression is essential for proper cardiac functions both under normal and stress conditions (Alaynick et al., 2007; Huss et al., 2007; Huss et al., 2004; LaBarge et al., 2014; Rangwala et al., 2010). Additionally, ERRα is also reported to be required for lipogenesis, both under normal and high-fat diet conditions, by controlling genes involved in lipid and energy metabolism (Luo et al., 2003). Furthermore, ERRα mediates thermogenesis by controlling genes involved in oxidative phosphorylation and mitochondrial biogenesis (Villena et al., 2007).

1.5.1.4 Stimulatory protein 1 (Sp1) and Ying Yang 1 (YY1)

Stimulatory protein 1 (Sp1) mediates activation and repression of cytochrome c1 (Li et al., 1996b) and adenine nucleotide translocase 2 (Li et al., 1996). Similarly, Ying Yang 1

(YY1) can stimulate and repress subunits of the cytochrome oxidase(Basu et al., 1997; Seelan and Grossman, 1997).

1.5.1.5 ATF/CREB family

Both activating transcription factor (ATF) and cAMP response element binding protein (CREB) are reported to control cytochrome *c* expression in response to serum (Evans and Scarpulla, 1989; Gopalakrishnan and Scarpulla, 1994; Herzig et al., 2000; Vercauteren et al., 2006).

1.5.2 Coactivators

The coactivators of mitochondrial biogenesis are members of the peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) family. They bind to the DNA-binding transcriptor factors to regulate their functions. The PGC-1 family is composed by the two closely related PGC-1α and PCG-1β members and by the more distal PGC-1-related coactivator (PRC) (Scarpulla, 2008; Scarpulla et al., 2012; Villena, 2015).

1.5.2.1 Peroxisome proliferator-activated receptor-γ coactivator-1α and -1β (PGC-1α/β)

Both PGC-1α and PCG-1β are induced in muscles during exercise (Baar et al., 2002; Russell et al., 2005). Classically, PGC-1α was reported to play a key role in mitochondrial biogenesis under normal, exercise and ageing conditions (Handschin et al., 2007a; Handschin et al., 2007b; Kamei et al., 2003; Lin et al., 2002), to induce conversion of type II fibers toward type I fibers (Lin et al., 2002), to mediate neuromuscular junction maintenance (Handschin et al., 2007b; Wenz et al., 2009), to mediate exercise-induced muscle angiogenesis (Chinsomboon et al., 2009) and to mediate caloric restriction-induced mitochondrial gene expression and biogenesis (Finley et al., 2012). More recently, however, the pivotal role of PGC-1α in muscles has been challenged. Indeed, PGC-1α was shown to be dispensable for exercise-induced mitochondrial biogenesis in skeletal muscles (Rowe et al., 2012), in muscle fiber type conversion (Geng et al., 2010; Zechner et al., 2010) and in muscle insulin sensitivity (Zechner et al., 2010).

In liver both PGC-1α and PCG-1β regulate the expression of mitochondrial genes. In addition, PGC-1α controls the expression of gluconeogenesis genes in response to fasting (Yoon et al., 2001a), whereas PCG-1β regulates lipid metabolism and the synthesis of very-low density lipoproteins (VLDL) in response to feeding (Chambers et al., 2012; Chambers et al., 2013; Lin et al., 2005; Wolfrum and Stoffel, 2006). Remarkably, both fasting and feeding conditions in liver are reported to modulate PGC-1α transcriptional activity through counteracting pathways.

In brown adipose tissue, PGC-1α and PGC-1β seem to regulate different processes. PGC-1α expression is strongly induced by adrenergic stimulation upon cold exposure and PGC-1α was shown to positively regulate thermogenesis through UCP pathway. However, PGC-1α seems to be dispensable for BAT functions under normal temperature conditions and expression of genes of the oxidative phosphorylation, tricarboxylic acid cycle or β-oxidation (Kleiner et al., 2012; Uldry et al., 2006). Conversely, PGC-1β appears to be more important for the preservation of basal mitochondrial function in BAT and ablation of PGC-1β results in a noticeable decrease in the expression of mitochondrial genes, which is not observed in mice lacking PGC-1α only (Enguix et al., 2013; Lelliott et al., 2006; Sonoda et al., 2007). Remarkably, PGC-1β has no effect on the UCP thermogenic program in BAT, but the mitochondrial dysfunctions caused by its ablation seem to be responsible for the cold intolerance exhibited by PGC-1β knockout mice (Sonoda et al., 2007).

1.5.2.2 PGC-1 –related coactivator (PRC)

PGC-1-related coactivator (PRC) has some structural similarity with PGC-1α and it is rapidly induced upon serum treatment and in proliferating cells. Under such conditions PCR is reported to bind to CREB and NRF1 and to promote cytochrome *c* expression in relation to cell cycle progression (Vercauteren et al., 2006).

1.5.3 Signaling pathways

As mentioned, mitochondrial biogenesis is tightly controlled to meet cellular energy demand in response to cell growth, development or (patho)physiological conditions.

Inside the cell the cAMP/PKA/Ca^{2+}/AMPK and the PI3K/Akt/mTOR pathways are two main signaling pathways responsible for energy sensing and they have been implicated in modulating mitochondrial biogenesis and cellular metabolism at many different stages (Figure 6).

The cAMP/PKA/Ca^{2+}/AMPK pathway is activated by high energy demand conditions, such as fasting, muscle exercise and cold exposure. For instance, under fasting conditions, catecholamine and glucagon induce cAMP increase, PKA activation and CREB phosphorylation in the liver. In turn, CREB promotes mitochondrial biogenesis and gluconeogenesis both in a direct and indirect fashion. Indeed, CREB directly drives expression of mitochondrial genes, such as cytochrome *c* and COXIV, by binding to CRE elements on the promoters of mitochondrial genes (Gopalakrishnan and Scarpulla, 1994); additionally it promotes PGC-1α transcription (Herzig et al., 2001; Yoon et al., 2001a). Similarly, in BAT, sympathetic production of norepinephrine induces cAMP increase through β_3 adrenergic receptors and subsequent CREB and ATF2 activation, leading to thermogenesis through PGC-1α and UCP expression (Cao et al., 2004).

Under fasting conditions, PGC-1α activity is promoted also by SIRT1. In the liver both starvation and pyruvate levels induce NAD^+-dependent activation of SIRT1, which in turn deacetylates PGC-1α, regulating its transcriptional activity. Remarkably, SIRT1 is reported to be essential for PGC-1α-mediated gluconeogenesis, hepatic glucose output and repression of glycolysis. The action of SIRT1 on PGC-1α target genes is therefore quite intriguing: on one side SIRT1-mediated deacetylation induces gluconeogenic genes, on the other it promotes repression of glycolytic genes. Interestingly, respiratory genes transcribed by PGC-1α, such as cytochrome *c* and ATP synthase, are unaffected. The dual action of SIRT1 on PGC-1α-transcribed genes seem to be mediated by a selective recruitment of a different set of coactivators and corepressors on PGC-1α-driven genes (Rodgers et al., 2005). Interestingly, SIRT1-mediated deacetylation in muscles is reported to induce a switch from glycolysis to fatty acid β-oxidation in response to low glucose and the action of SIRT1 on fatty acid genes is counteracted by GCN5-mediated acetylation of PCG-1α (Gerhart-Hines et al., 2007). As a consequence of acetylation, PGC-1α becomes transcriptionally inactive and relocalizes to nuclear foci (Lerin et al., 2006). Interestingly, SIRT1 deacetylation is reported to regulate also PGC-1β activity (Kelly et al., 2009).

As mentioned, the cAMP/PKA/Ca^{2+}/AMPK pathway is exploited also by signals

associated with muscle exercise. For instance, Ca^{2+} rises in muscles activate AMPK, which in turn phosphorylates PGC-1α, resulting in induction of mitochondrial genes. Phosphorylated PGC-1α then promotes its own expression in a positive feedback loop (Jager et al., 2007). Additionally, Ca^{2+} rises induce PGC-1α expression through CREB and MEF2. Indeed, both transcription factors are stimulated by CamKIV and calcineurin A (CaN) respectively and promote PCG-1α expression in conjunction with PCG-1α itself (Handschin et al., 2003). PGC-1α expression is stimulated also through ATF2-mediated transcription upon p38 MAPK activation (Akimoto et al., 2005). Moreover, phosphorylation of PGC-1α by p38 MAPK prevents binding and repression of PGC-1α by p160MBP (Fan et al., 2004; Knutti et al., 2001; Puigserver et al., 2001). Finally, in skeletal muscles, SIRT1 controls also PGC-1α expression by binding to PGC-1α promoter and stimulating PGC-1α binding to its own promoter, thus generating a positive autoregulatory loop (Amat et al., 2009).

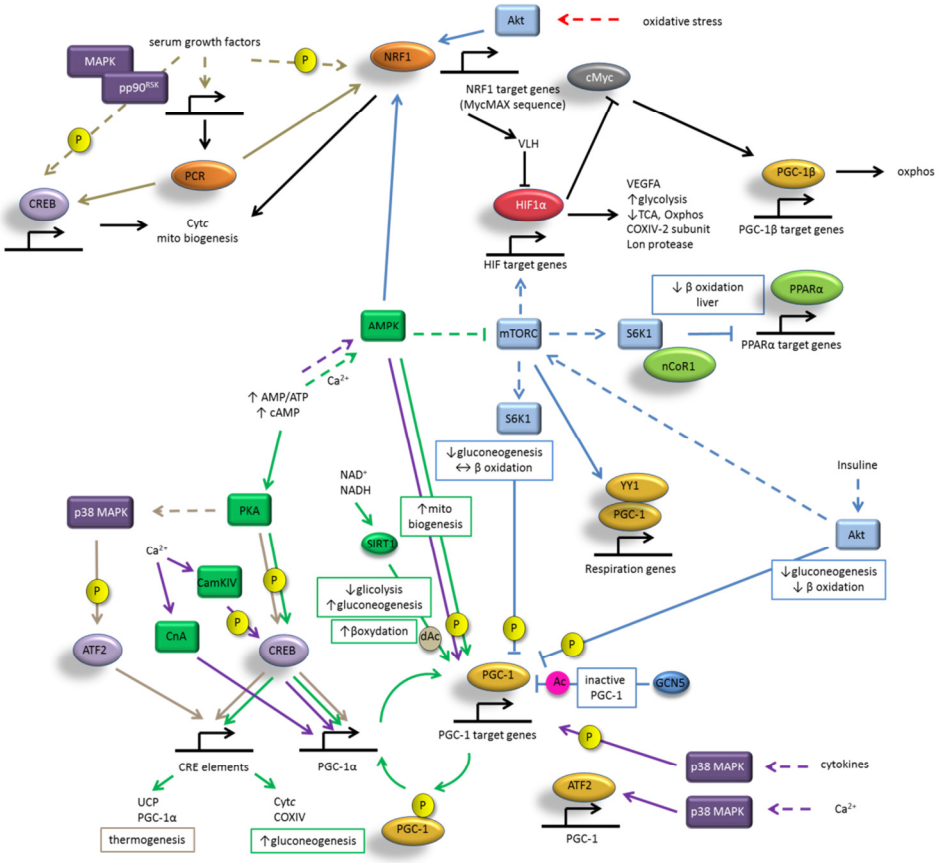

Figure 6 Signaling pathways controlling mitochondrial biogenesis and metabolism. Transcription factors are represented in round-like shape, whereas kinases/phosphatases in rectangular shape. Different colours represent different pathways. Green is used for pathways associated with low energy, blue for glucose/high metabolite levels, purple for pathways reported in muscles, brawn for serum/growth factors, red for

oxidative stress and black for cell cycle/proliferation/cancer.

A Ca^{2+}/AMPK-dependent mechanism seems to be responsible also for NRF-1/2 induction during muscles exercise (Baar et al., 2002; Bergeron et al., 2001; Ojuka et al., 2003).

Ca^{2+} transients might play a role also in mitochondrial biogenesis in neurons. Indeed, both NRFs are induced by electrical stimulation (Yang et al., 2006; Zhang and Wong-Riley, 2000) and their nuclear translocation promotes the expression of NMDA and AMPA glutamate receptors along with respiratory genes (Dhar et al., 2009; Dhar and Wong-Riley, 2009; Priya et al., 2014). PGC-1α activity in neurons is regulated by ubiquitin-dependent proteasomal degradation. When phosphorylated by GSK3β and p38 MAPK, PGC-1α is bound by SCF(Cdc4), an E3 ubiquitin ligase that promotes PGC-1α ubiquitylation and proteasomal degradation. Remarkably, under conditions of oxidative stress in neurons, Cdc4 levels are decreased, leading to an increase in PGC-1α protein and PGC-1α-dependent transcription (Andrade et al., 1998; Olson et al., 2008). Interestingly, such pathway is reported to selectively degrade only the nuclear PGC-1α, without affecting the cytosolic pool (Chang et al., 2010b; Trausch-Azar et al., 2010).

As mentioned above, the PI3K/Akt/mTOR pathway signals glucose levels through insulin in the liver. It is known that activated PI3K/Akt enhances glycolysis (Elstrom et al., 2004). Indeed, Akt2/PKB-β phosphorylation of PCG-1α reduces PGC-1α ability to induce gluconeogenesis and β-oxidation genes (Li et al., 2007). Additionally, Akt pathway seems to trigger NRF-1 under oxidative conditions and this seems to be mediated by Akt-dependent direct phosphorylation of NRF1 (Piantadosi and Suliman, 2006).

mTOR, the downstream target of Akt, regulates many aspects of mitochondrial biogenesis and metabolism. In liver and cultured hepatocytes, upon feeding, S6 kinase 1 (S6K1), a downstream target of mTOR, mediates phosphorylation of PGC-1α, thus attenuating PGC-1α ability to activate gluconeogenesis genes. Interestingly, by interfering with the ability of PGC-1α to bind to HNF4α, a transcription factor required for gluconeogenesis, S6K1-mediated phosphorylation blunts only the gluconeogenesis, without impinging on the expression of mitochondrial and β-oxidation genes which are driven by PGC-1α/ERRα (Lustig et al., 2011). mTOR/S6K2 is also responsible for suppressing PPARα activity: S6K2 induces recruitment of nuclear receptor corepressor 1 (NCoR1), a PPARα corepressor, thus repressing ketogenesis in the liver (Kim et al., 2012; Sengupta et al., 2010). Additionally, in skeletal muscles and cells, mTOR is reported to

control mitochondrial oxidative function, through modulation of YY1-PGC-1α interaction (Cunningham et al., 2007). Finally, mTOR is a known positive regulator of HIF1α pathway (Wouters and Koritzinsky, 2008). The overall output of HIF1α activation is glycolysis increase and concomitant decrease of the TCA cycle. Moreover, HIF1α has been shown to induce a switch in the components of the cytochrome *c* oxidase (COX) complex, which is ultimately responsible for O_2 consumption. Indeed, HIF1α simultaneously induces expression of COX4-2 subunit and of Lon protease. The latter targets COX4-1 subunit for degradation, leading to optimization of COX activity and overall more effective respiration with less ROS production (Fukuda et al., 2007).

Finally, mitogenic signals impinge on mitochondrial biogenesis. For instance, in proliferating cells NRF-1 is phosphorylated and this enhances its DNA binding and *trans*-activity (Gugneja and Scarpulla, 1997). Remarkably, serum-induced cell cycle entry promotes PRC transcription and phosphorylation of CREB and NRF1. This results in activation of PRC/CREB- and PRC/NRF1- driven transcription of respiratory genes and of mitochondrial biogenesis (Vercauteren et al., 2006).

Moreover, c-Myc stimulates overall increase in respiration and promotes mitochondrial biogenesis (Li et al., 2005). c-Myc can regulate the expression NRF-1 through noncanonical Myc:MAX sites and upregulate a subgroup of NRF-1 target genes involved in respiration including cytochrome *c*, COX5b, and COX6A1; c-Myc might thus play a role in the control of the oxidative phosphorylation. In parallel, c-Myc seems to sentitize cells to apoptosis after serum depletion. Interestingly, overexpression of a dominant negative form of NRF1 rescues the cell death induced by c-Myc without affecting c-Myc-induced proliferation (Morrish et al., 2003). Additionally, c-Myc activates PGC-1β (Zhang et al., 2007).

Interestingly, HIF1α negatively regulates mitochondrial biogenesis and O_2 consumption in renal carcinoma cells lacking the von Hippel-Lindau tumor suppressor (VHL) by inhibiting c-Myc activity. On one side, HIF-1α binds to and activates transcription of the MXI1 gene, which encodes a repressor of c-Myc transcriptional activity; on the other, HIF-1 promotes MXI-1-independent, proteasome-dependent degradation of c-Myc (Zhang et al., 2007).

1.6 When numbers matters: mitochondrial DNA and gliomagenesis

As discussed, mitochondrial biogenesis and mtDNA play a crucial role in tumorigenesis.

Cell Death and Differentiation (2013) 20, 1601–1602
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Editorial

When numbers matters: mitochondrial DNA and gliomagenesis

E Barbieri[1] and L Scorrano*[1,2]

Cell Death and Differentiation (2013) **20**, 1601–1602; doi:10.1038/cdd.2013.156

Besides being crucial for cellular bioenergetics, mitochondria are key players in many pathophysiological processes: from cell death to autophagy, immunity.[1] In cancer, not only they regulate the evasion from apoptosis that characterizes most tumor cells, but they are also crucial for the metabolic switch from oxidative to glycolytic.[2] Among all other organelles, mitochondria are peculiar because they harbor their own DNA (mtDNA), whose replication is tightly controlled and concerted with the biogenesis of the vast majority of mitochondrial proteins that are encoded by the nuclear DNA. It is therefore no surprise that mtDNA copy number deregulation alters mitochondrial and cellular functions. For instance, mtDNA mutations and copy number expansions and depletions are common features of cancer cells. Remarkably, mtDNA copy number has been reported to be increased in some tumors and decreased in other. Additionally, mtDNA-depleted cancer cells grow at a lower rate and are more resistant to chemotherapeutic agents compared to their parental cell lines.[3] Therefore, although many evidences suggest mtDNA copy number variations in tumorigenesis, their role in tumor formation and growth is still intensively debated.

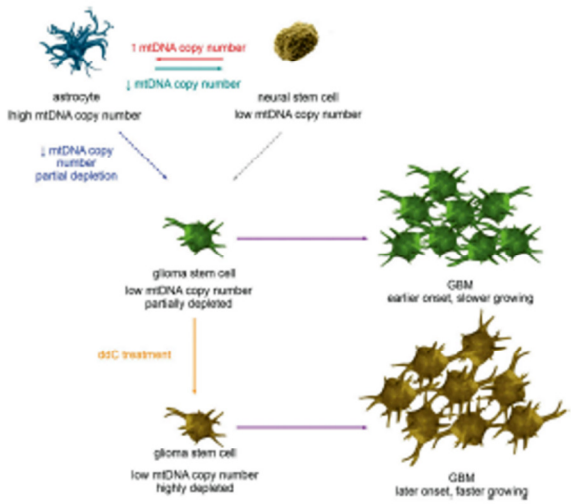

Figure 1 During differentiation, neural stem cells expand their mtDNA copy number in order to sustain mitochondrial biogenesis. Glioma stem cells, which might derive from adult neural stem cells or dedifferentiating astrocytes, have a reduced stem potential compared to neural stem cells possibly due to an incomplete nuclear reprogramming and a partial reduction of mtDNA copy number. Partial mtDNA depletion in GBM cells gives rise to cancer with later onset and subsequent faster growth compared to their 100% mtDNA GBM counterparts

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In this issue of *CDD*, Dickinson *et al.*[9] investigate the role of mtDNA copy number in gliomagenesis and further explore their intriguing hypothesis of the 'mtDNA set point'.[4–6] Accordingly to their previous publications, after fertilization, glycolytic embryonic stem cells (ESCs) progressively reduce their mtDNA copy number until they reach the so-called 'mtDNA set point', the minimal mtDNA content per cell that ensures adequate mtDNA replenishing after cell division and that sets the point for the subsequent proper expansion of mtDNA pool in differentiating cells. This mtDNA expansion would sustain proper mitochondrial biogenesis and the switch from glycolysis to oxidative metabolism occurring during differentiation.

Dickinson and colleagues assess the effect of mtDNA copy number on stemness and differentiation potential of glioblastoma multiforme (GBM) cell lines. They demonstrate that defective mtDNA copy number reduces astrocytic differentiation of GBM cells compared to human neural stem cells (hNSCs). In line with their 'mtDNA set point' theory, they suggest that GBM cells are less 'stem' and unable to regulate their mtDNA content tightly in order to ensure proper differentiation. Additionally, they show that a minimal mtDNA copy number is required to maintain GBM cells in the stem-like state and that mtDNA depletion impacts on stemness. Remarkably, they demonstrate that different levels of mtDNA depletion regulate the expression of stemness *versus* differentiation genes. Moreover, under basal condition only partially mtDNA-depleted GBM cells can recover their mtDNA copy number, whereas differentiation can promote effective mtDNA replenishment even in seriously mtDNA-depleted GBM cells.

Dickinson *et al.*[9] go on to asses the impact of mtDNA copy number on tumorigenesis *in situ* by implanting mtDNA-depleted GBM cells into nude mice. This approach shows that unexpectedly, frequency of tumor formation is inversely proportional to the degree of mtDNA ablation: the lower the ablation degree, the higher the frequency of tumor formation. This work demonstrates a link between mtDNA copy number and tumor formation and sheds light on the apparently contradictory data reported in literature. In particular, the authors show a relationship between mtDNA depletion and stemness/differentiation and between mtDNA depletion and cancer growth rate. Their results suggest that different degree of mtDNA depletion might differentially impinge on tumor development and that the mtDNA levels might be modulated during tumorigenesis. Additionally, the nuclear background, different among different glioma cells, and cancer cells in general, might influence how mtDNA depletion affects tumorigenesis. For instance, p53 and Ras, two almost invariantly mutated gene in cancer, have been reported to regulate mtDNA replication and mitochondrial biogenesis[7,8] and their status might interplay, or determine, the mtDNA changes reported here. Furthermore, the link between differentiation/stemness stage and mtDNA levels suggests that even within the same tumor type mtDNA depletion could differentially affect tumor growth, depending on its developmental stage and grade.

How the expansion of mtDNA copy number during differentiation promotes mitochondrial biogenesis and hence mitochondrial oxydative phosphorylation which sustains differentiation, is unclear. Depletion of mtDNA levels in GBM cells, instead of increasing their stemness, disrupts their stem-like state and induce an anomalous differentiation. This suggests that precise mtDNA content is required to maintain stemness. Additionally, differentiation might not only rely solely on oxidative phosphorylation, which is depressed in mtDNA-depleted cells, but also on other mitochondrial functions such as, for example, morphology. However and surprisingly, Dickinson *et al.*[9] conclude that mtDNA depletion does not alter OPA1 processing and mitochondrial morphology. Further experiments are required to more precisely link mtDNA depletion-induced loss of stemness to other key mitochondrial functions, like regulation of calcium signaling, of apoptosis, or of cell motility.

In conclusion, the work by Dickinson *et al.*[9] further develops the intriguing hypothesis that the 'mtDNA set point' is a check point for stemness, proper differentiation and tumorigenesis. Their work opens the possibility that somehow mtDNA content controls tumorigenesis (see Figure 1).

Conflict of Interest

The authors declare no conflict of interest.

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2. Mitochondrial shaping proteins: structures, functions and regulation

Mitochondria exhibit a great variety of shapes, from spherical organelles to branched tubular networks, depending on the organism, cell type and metabolic state. Additionally, they are highly dynamic structures, continuously undergoing both inter- and intra- organelle cycles of fission and fusion. These processes are mediated by a group of so-called mitochondria-shaping proteins. Mitochondria-shaping proteins are usually classified in pro-fusion and pro-fission proteins. Additionally, a growing number of proteins have been implicated in controlling *cristae* shape in the last ten years.

2.1 Pro-fusion and cristae remodeling proteins

Mitochondrial fusion is mediated by two classes of GTPases: Mitofusin 1 and 2 (MFN1/2) (Fzo in yeast) on the OM, and OPA1 (Mgm1p in yeast) on the IM. Additionally, OPA1/Mgm1p regulates *cristae* shape and are tightly controlled by proteolytic cleavage. It is now emerging that besides these classical players, other factors are crucial in shaping the *cristae* and in maintaining mitochondrial fusion. These include Prohibitins, thought to organize functional microdomains in the IM, Mitofilin and the MICOS complex docking the IM to the OM at the contact sides, and the ATPase, forming oligomers at the apex of the *cristae*. Finally, also lipid composition, in particular cardiolipin, is emerging as key player in mitochondrial fusion.

The fact that mitochondria are double-membrane organelles renders their fusion a complex process: when two organelles fuse, four membranes need to be handled. The molecular mechanism leading to complete fusion of two organelles has not been fully clarified yet. However, different mutant yeast strains have been instrumental in deciphering some of the molecular events underlying fusion; the majority of these steps has been validated also in mammalian systems (Escobar-Henriques and Anton, 2013). A general model predicts that MFNs, after oligomerization in *cis*, undergo oligomerization in *trans*, thus mediating inter-organellar docking. This step requires GTP hydrolysis. Subsequently, ubiquitin-dependent degradation of MFNs/Fzo seems to be required for

complete OM fusion in yeast. Remarkably, the mammalian homologue of the ubiquitin ligase mediating this step has not been identified yet. In a next distinct step, IM fusion takes place. An oligomerization in *cis*- followed by an in *trans*- GTP-dependent oligomerization of OPA1/Mgm1p seems to be responsible for IM fusion. This step requires adequate balance of the different OPA1/Mgm1p isoforms produced by proteolytic processing (Escobar-Henriques and Anton, 2013). In yeast, coordination between outer and inner membrane fusion seems to be mediated by Ugo1 located on the OM. Ugo1 is reported to interact with the MICOS complex and simultaneously both with Fzo through its N-terminal cytosolic domain and with Mgm1p through its *C*-terminal IMS domain. No mammalian homologue for Ugo1 has been reported so far (Escobar-Henriques and Anton, 2013). The entire fusion process seems to be prevented by altered or complete proteolysis of OPA1/Mgm1p, reorganization in the BAX/BAK-MFNs complexes during apoptosis and by MFNs ubiquitination during mitophagy. The latter two events inhibit docking by impinging on MFNs (Brooks et al., 2007; Chen and Dorn, 2013).

In the following paragraphs, the proteins involved in mitochondrial fusion and *cristae* shaping and their regulation are discussed in detail.

2.1.1 Fzo / Mitofusin-1/2

The first mediator of mitochondrial fusion identified was the *D. melanogaster* Fuzzy onion (Fzo1), a large transmembrane GTPase located in the OMM, mediating the formation of giant mitochondria during fly spermatogenesis (Hales and Fuller, 1997). One year later, the *S. cerevisiae* orthologue of Fzo1 was reported to mediate mitochondrial fusion events during mitotic growth and mating (Hermann et al., 1998; Rapaport et al., 1998). In mammals, two homologues, named Mitofusin 1 and 2 (MFN1/2) (Santel and Fuller, 2001), were reported in 2001.

MFN1 and -2 display high similarity: they both possess an *N*-terminal GTPase domain, two transmembrane domains spanning the OM and two coiled coil motifs crucial for protein–protein interaction (Chen et al., 2003b; Rojo et al., 2002; Santel et al., 2003; Santel and Fuller, 2001). Remarkably, MFN2 possesses also a p21ras-binding domain at its *N*-terminal, which is not present in MFN1, and a proline-rich domain poorly

conserved in MFN1 (Chen et al., 2004).

Despite their high similarity, MFNs seem to have distinct roles in mitochondrial morphology. For instance, MFN1 has a higher GTPase activity than MFN2, although its affinity for GTP is lower (Ishihara et al., 2004). In agreement with this, MFN1 exhibits a higher capacity to induce fusion (Ishihara et al., 2004) and *Mfn2*-/- cells display higher mitochondrial fusion rates compared to *Mfn1*-/- cells (Chen et al., 2003b). Moreover, OPA1 was shown to specifically require MFN1 to induce mitochondrial fusion (Cipolat et al., 2004). The differential role of MFNs in mitochondrial shaping is further substantiated by the finding that *Mfn1*-/- mice die in midgestation, whereas *Mfn2*-/- embryos display deficient placentation (Chen et al., 2003b).

Pro-apoptotic BAX and BAK are known to facilitate MFN2 assembly on mitochondrial surface, thus promoting its activity and mitochondrial fusion under non-apoptotic conditions (Hoppins et al., 2011b; Karbowski et al., 2006). In contrast, during apoptosis, BAK dissociates from MFN2 and associates with MFN1 (Brooks et al., 2007). Interestingly, our laboratory has shown that ERK-mediated phosphorylation of MFN1 might be responsible for this switch. Indeed, under stress condition, ERK phosphorylates MFN1, thus stimulating BAK oligomerization and preventing MFN1-mediated docking of mitochondria and subsequent mitochondrial fusion (Pyakurel et al., 2015).

Both MFNs are reported to be phosphorylated by PINK and subsequently ubiquitinated by Parkin, thus triggering selective elimination of damaged mitochondria by autophagy (Chen and Dorn, 2013; Gegg et al., 2010; Tanaka et al., 2010; Ziviani et al., 2010). Parkin-mediated ubiquitination of MFN2 is reportedly also stimulated by phosphorylation by c-Jun *N*-terminal kinase (JUNK) (Leboucher et al., 2012).

Our lab demonstrated that MFN2 is located also on ER, from where, through interaction with MFN1/MFN2 on the outer mitochondrial membrane, it tethers the two organelles. The regulation of this juxtaposition is crucial for the mitochondrial Ca^{2+} uptake upon Ca^{2+} release from the ER stores (de Brito and Scorrano, 2008). Interestingly, MFN2-mediated ER-mitochondria tethering is controlled by ubiquitination of MFN2 by MITOL/MARCH5 (Sugiura et al., 2013), an ubiquitin ligase implicated in the regulation of many aspects of mitochondrial dynamics and transport (Nagashima et al., 2014). Although the model predicting a MFN2-mediated tether between mitochondria and ER has been recently challenged (Filadi et al., 2015), it is widely accepted that MFN2 plays a key role in ER and Ca^{2+} physiology and in the Ca^{2+}crosstalk between the two organelles. Indeed, *Mfn2*

ablation induces ER stress (Debattisti et al., 2014; Munoz et al., 2013; Ngoh et al., 2012; Sebastian et al., 2012). Remarkably, in flies lacking *Marf* (a *Drosophila* homologue of *Mfn2*) the functional and developmental defects are reduced by attenuation of the ER stress (Debattisti et al., 2014). Conversely, MFN2 overexpression kills cancer cells by inducing mitochondrial Ca^{2+} uptake from the ER (Wang et al., 2015).

Interestingly, MFN2 has been linked also to mitochondrial transport along microtubules through interaction with the Miro/Milton complex and this has been suggested as a possible molecular mechanism of the axonal degeneration observed in Charcot-Marie-Tooth type 2A (Baloh et al., 2007; Misko et al., 2010; Misko et al., 2012). Indeed, in 2004 *Mfn2* was described as the causative gene of Charcot-Marie-Tooth type 2A (CMT2A) syndrome, one of the most common inherited neurodegenerative disorders, characterized by loss of sensory and motor axons resulting in subsequent neuron degeneration (Lawson et al., 2005).

2.1.2 Mgm1p / OPA1

Mitochondrial genome maintenance 1 (Mgm1p) was initially identified in the nineties in yeast (Jones and Fangman, 1992), but only in 2000 Optic Atrophy 1 (OPA1) was described in humans (Alexander et al., 2000; Delettre et al., 2000). Since then, the number of publications regarding OPA1 has been dramatically growing and OPA1 is nowadays considered a key player in mitochondrial fusion and *cristae* remodeling.

2.1.2.1 OPA1 structure

Both Mgm1p and OPA1 are located in the IM and are tightly associated with the IMS (Cortopassi and Wong, 1999; Sesaki et al., 2003b; Wong et al., 2003). Mgm1p and OPA1 display a highly conserved secondary structure, consisting of an *N*-terminal mitochondrial leader sequence, a central GTPase domain conserved across all dynamins and two predicted coiled coil domains, one *N*-terminal to the GTPase domain and the other at the *C*-terminus. The *C*-terminal coiled coil domain may function as a GTPase effector domain (GED) (Figure 7A). The high degree of secondary structure conservation suggests functional conservation through evolution.

In human, OPA1 is ubiquitously expressed, with the highest levels in retina, brain, testis, heart and muscle (Alexander et al., 2000). The human *Opa1* gene is composed of 30 coding exons spanning more than 90 kb of genomic DNA on chromosome 3q28-q29 (Alexander et al., 2000). The alternative splicing of exons 4, 4b and 5b results into eight different splicing variants which, at the protein level, differ for the presence/absence of a region located between the mitochondrial leader sequence and the GTPase domain (Delettre et al., 2001). This region carries the sites responsible for OPA1 proteolytic cleavage (Ishihara et al., 2006) (Figure 7A).

2.1.2.2 OPA1 functions

In yeast Mgm1p was initially described as to be required for maintenance of mtDNA and of fusion-competent mitochondria (Wong et al., 2003). The involvement of OPA1 in fusion of mammalian mitochondria was initially challenged (Misaka et al., 2002; Olichon et al., 2002). However, we now know that excessive OPA1 overexpression exerts a toxic effect on the organelle, resulting in mitochondrial fragmentation (Cipolat et al., 2004; Cogliati et al., 2013). *In vitro* mild OPA1 overexpression enhances fusion, whereas OPA1 downregulation by siRNA represses it (Cipolat et al., 2004). These effects are mirrored by the mouse models for *Opa1* ablation and overexpression recently generated in our lab (Cogliati et al., 2013). Remarkably, both OPA1 and Mgm1p require MFN1 and Fzo1p respectively to induce fusion (Cipolat et al., 2004; Wong et al., 2003).

Besides its role in mediating mitochondrial fusion, striking evidence supports a role of OPA1 also in regulating *cristae* morphology. Initial hints came from the *in situ* EM observation of altered *cristae* structure and enlarged *cristae* junctions in Mgm1/OPA1-depleted mitochondria (Griparic et al., 2004; Olichon et al., 2003; Sesaki et al., 2003b). This intriguing hypothesis was further supported by the *cristae* localization of OPA1/Mgm1, (Griparic et al., 2004; Olichon et al., 2003; Pelloquin et al., 1999; Vogel et al., 2006; Wong et al., 2000), and by the observation that *cristae* derangement reported *in situ* in Mgm1-ablated cells was resulting in defective ATP synthase oligomerization (Amutha et al., 2004). In 2006, our laboratory provided compelling evidence for OPA1-mediated *cristae* remodeling. Indeed, through biochemical and genetic approaches, we demonstrated that OPA1 regulates *cristae* morphology through self-aggregation into high molecular weight complexes (Frezza et al., 2006a). These complexes assemble at the *cristae* junction and are targeted during apoptosis, leading to *cristae* remodeling and

cytochrome *c* release (Cipolat et al., 2006; Frezza et al., 2006a). Remarkably, Nunnari and co-workers proposed a similar model in yeast, where Mgm1 was found to be required for tethering and fusion of the mitochondrial inner membranes and for the maintenance of the *cristae* structures trough self-aggregation (Meeusen et al., 2006)

Moreover, our laboratory demonstrated that OPA1-dependent *cristae* remodeling has functional implications beyond apoptosis. Indeed, genetic and apoptotic manipulations of the OPA1-*cristae* pathway affect the assembly and the activity of respiratory chain supercomplexes both *in vitro* and *in vivo*, impacting on the efficiency of mitochondria-dependent cell growth (Cogliati et al., 2013). Recently, our laboratory further substantiated the role played by OPA1 in mediating both apoptotic and metabolic *cristae* remodeling *in vivo* (Civiletto et al., 2015; Varanita et al., 2015).

2.1.2.3 OPA1 proteolytic processing

A key step in regulating OPA1 protein stability and function is its complex proteolytic cleavage, which results in short and long forms. The proteolytic processing in association with the alternative splicing increases the variety of OPA1 splices inside the cell. Indeed, in mammalian cells OPA1 displays a typical 8-band western blot pattern corresponding to two long (L-OPA1) and three short (S-OPA1) isoforms (Ishihara et al., 2006; Song et al., 2007).

In yeast, while being imported in a TIM23-dependent manner, Mgm1 is laterally inserted as type 1 transmembrane protein in the IM and its *N*-terminal targeting sequence is removed by the matrix processing peptidase (MPP). This produces the mature long form l-Mgm1p. Subsequently, matrix ATP levels drive further import of l-Mgm1, thus enabling the mitochondrial rhomboid protease Pcp1 to cleave a hydrophobic stretch that serves as IM anchor. As a consequence, the short soluble IMS isoform s-Mgm1 is generated (Herlan et al., 2004; Herlan et al., 2003; McQuibban et al., 2003; Sesaki et al., 2003a) (Figure 7B). As described in yeast, also in mammals MPP mediates removal of the mitochondrial leading sequence (Ishihara et al., 2006). However, OPA1 proteolytic processing in mammals involves additional complex, not fully deciphered steps (Figure 7C).

During the last ten years, a number of laboratories have been intensively seeking for the proteases responsible for the constitutive and the stress-induced cleavage of OPA1 in mammals. Such a great interest is easily explainable when taking into account that OPA1 proteolytic processing is appearing as a key process in the regulation of the protein stability and function and it has been implicated in different (patho)physiological conditions. The field is further complicated by the lack/low level of functional conservation in mammals of the proteases identified in lower eukaryotes. For instance, involvement of PARL, the mammalian homologue of the yeast Pcp1, in OPA1 processing is controversial. Indeed, our laboratory reported PARL as controlling *cristae* remodeling and cytochrome *c* release during apoptosis by impinging on OPA1 processing (Cipolat et al., 2006); however direct proof of PARL cleavage is still missing and both overexpression and downregulation of PARL in mammalian cells have no significant effect on OPA1 processing (Cipolat et al., 2006; Ishihara et al., 2006).

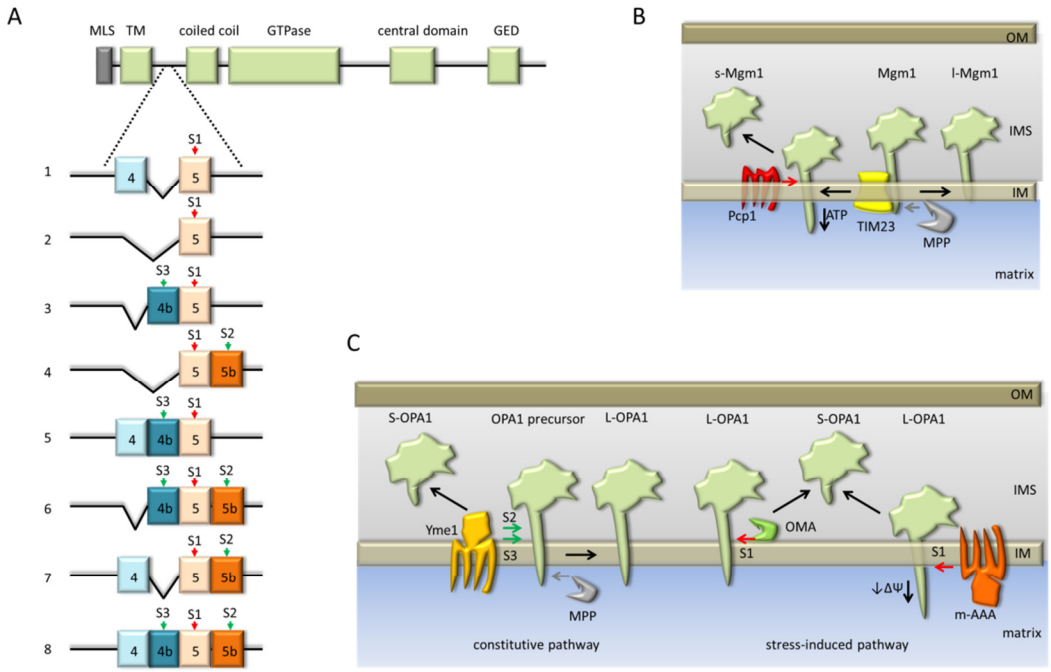

Figure 7 OPA1 structure and proteolytic processing. (A) Although OPA1 structure is conserved through evolution, in human eight splicing variants arise from the alternative splice of exons 4, ab, 5 and 5b. Exon 4b and 5b carry respectively S3 and S2, mediating constitutive OPA1 cleavage by the iAAA protease Yme1. Exon 5 carries site S1, involved in stress-induced cleavage by OMA1 and mAAA proteases. (B) Schematic representation of Mgm1p processing in yeast. (C) Constitutive and inducible pathways mediating OPA1 processing in mammals.

Nowadays, it is quite accepted that OPA1 displays two cleavage pathways. Splicing variants carrying exon 4b or 5b display S3 or S2 sites respectively and are constitutively cleaved producing S-OPA1. Conversely, splicing variants that do not carry exon 4b or 5b are normally left uncleaved (L-OPA1), but are processed at the S1 site upon stress stimuli (McBride and Soubannier, 2010) (Figure 7). Both S1 and S2 sites were initially identified by pulse-and-chase experiments and N-terminal sequencing of the polypeptides retrieved from cells overexpressing Flag-OPA1 by Mihara and co-workers (Ishihara et al., 2006). Evidence for a third site (S3) in exon 4b was provided by van der Blieck and co-workers, but its precise location and nucleotide composition has not been described yet (Griparic et al., 2007; Head et al., 2009).

In 2007 the laboratories of Chan and van der Bliek identified Yme1L as responsible for constitutive cleavage at S2/S3 site (Griparic et al., 2007; Song et al., 2007). Yme1L belongs to the i-AAA protease family. The i-AAA proteases are ATP-dependent proteolytic machines located in the IM and exposing their catalytic site toward the IMS. The finding that stress conditions, such as mitochondrial depolarization and apoptosis, increase cleavage at S1 site without affecting cleavage at the S2/S3 site (Griparic et al., 2007; Song et al., 2007) provided evidence that the Yme1 pathway is constitutively active and is not involved in apoptosis. Accordingly, Yme1 knock-down prevents the constitutive cleavage of a subsets of OPA1 isoforms, but has no impact on OPA1 cleavage during apoptosis (Griparic et al., 2007). However, Rojo and co-workers challenged this model (Guillery et al., 2008). Interestingly, mitochondrial fusion, albeit reduced, takes place also in Yme1-depleted cells, possibly through a compensatory S1-mediated cleavage (Griparic et al., 2007).

The cleavage at the S1 site seems to be mediated by mAAA proteases, which differ from iAAA in the orientation of their catalytic domain toward the matrix. mAAA proteases can exist both as hetero-oligomeric (paraplegin/Afg3l1 or paraplegin/Afg3l2) and homo-oligomeric (Afg3l1 or Afg3l2 only) complexes (Koppen and Langer, 2007). The first evidence that OPA1 processing is mediated by mAAA proteases was provided by Mihara and coworkers, who identified paraplegin as the responsible for cleavage at S1 (Ishihara et al., 2006). Later, Reichert and Langer questioned the role of paraplegin and provided evidence for a direct cleavage by the AFG3L1 and AFG3L2 subunits (Duvezin-Caubet et al., 2007; Ehses et al., 2009). Remarkably, although some constitutive cleavage might

occur at low level, processing at the S1 site is mainly triggered by mitochondrial depolarization, apoptosis, low ATP levels and PTP opening (Baricault et al., 2007; Duvezin-Caubet et al., 2006; Ehses et al., 2009; Griparic et al., 2007; Ishihara et al., 2006). Moreover, the fact that mAAA proteases display their catalytic site toward the matrix implies that depolarization induces a further translocation of OPA1 across the IM, so that the S1 site gets exposed to the matrix and hence becomes available for mAAA-mediated processing (Ishihara et al., 2006).

Additionally, in 2009 two independent studies by the laboratories of van der Bliek and Langer respectively, identified the zinc metalloprotease OMA1 as to be responsible for cleavage at the S1 site (Ehses et al., 2009; Head et al., 2009). OMA1 is constitutively active and mediates cleavage at S1 site also under basal conditions, as suggested by the accumulation of both L- and S-OPA1 forms in *Oma1* depleted cells (Baker et al., 2014). However, OMA1 proteolytic activity is strongly enhanced in response to various stress stimuli such as mitochondrial depolarization, heat shock and reactive oxygen species (Baker et al., 2014; Ehses et al., 2009; Head et al., 2009). In line with this, OMA1 siRNA inhibits inducible cleavage, helps to retain fusion competence, and slows the onset of apoptosis (Head et al., 2009). The stress-induced activation of OMA1 is mediated by an *N*-terminal stress-sensor domain that modulates OMA1 activity through autocatalytic degradation, resulting in complete OMA1 turnover. This ensures the reversibility of the response to stress stimuli and allows OPA1-mediated mitochondrial fusion to resume upon alleviation of stress (Baker et al., 2014; Zhang et al., 2014). Interestingly, loss or downregulation of AFG3L2/1 unexpectedly decreases the stability of L-OPA1 isoforms and induces L-OPA1 processing by OMA1 (Ehses et al., 2009), suggesting that OMA1 can compensate for absence of the mAAA pathway.

Further, OMA1 autocatalytic activation seems to be modulated by Prohibitin PHB2, (Zhang et al., 2014), thus suggesting that the previously reported loss of L-OPA1 in *Phb2* ablated cells (Merkwirth et al., 2008) might actually be mediated by OMA1 (Zhang et al., 2014).

2.1.2.4 Autosomal dominant optic atrophy (ADOA)

Heterozygous mutations of *OPA1* cause autosomal dominant optic atrophy (ADOA), the most common form of inherited optic neuropathy, with an estimated prevalence of 1:50000 (Alexander et al., 2000; Delettre et al., 2000). ADOA is a specific disease of the

retina characterized by retinal ganglion cells (RGC) degeneration, followed by demyelination and ascending atrophy of the optic nerve (Kjer et al., 1996). ADOA usually begins before 10 years of age, with a large variability in the severity (Carelli et al., 2004; Delettre et al., 2000).

Very little is known on the pathogenesis of ADOA; the lack of pain and inflammation during the development of the disease suggest that apoptosis may play a key role in the loss of RGC. More than 117 different pathogenic mutations in *OPA1* have been described; the vast majority results in a truncated protein or affect the GTPase domain (Ferre et al., 2005). How *OPA1* mutations cause the clinical symptoms of ADOA remains to be clarified. Non-neuronal cells from patients with ADOA can have fragmented or normal mitochondria (Delettre et al., 2000; Olichon et al., 2007). In addition, *OPA1* mutations have been associated with reduced ATP production and reduced mtDNA content. This led to the hypothesis that OPA1 might play a role in regulating mtDNA stability and copy number. (Amati-Bonneau et al., 2008; Kim et al., 2005; Lodi et al., 2004).

2.1.3 Prohibitins

Prohibitins 1 and 2 (PHB1/2) are located on the inner mitochondrial membrane. Both PHBs are composed of an *N*-terminal transmembrane hydrophobic α-helix, which serves as unconventional targeting sequence and is not cleaved upon mitochondrial import, an evolutionarily conserved PHB domain that is common to several other scaffold proteins (also called SPFH domain) and is important for lipid raft associations and protein–protein interactions and a *C*-terminal coiled-coil domain mediating interaction between PHBs (Figure 8A). Biochemical approaches along with single-particle electron microscopy revealed that multiple PHBs assemble in an alternate fashion in the IM, thus forming a ring-like structure with an outer diameter of about 200-250 Å (Back et al., 2002; Tatsuta et al., 2005). Remarkably, PHB interaction is essential for their stability, as demonstrated by the finding that the absence of either PHB results in the degradation of the other (Kasashima et al., 2006; Merkwirth et al., 2008).

PHBs have been associated with many aspect of mitochondrial biology, ranging from

mtDNA maintenance (Kasashima et al., 2006) to respiratory chain assembly (Schleicher et al., 2008) and mitochondrial morphology (Artal-Sanz et al., 2003; Kasashima et al., 2006; Merkwirth et al., 2008).

Phb2-depleted MEFs display mitochondrial fragmentation and *cristae* derangement, possibly as a consequence of altered OPA1 cleavage (Merkwirth et al., 2008). The effect exerted by PHB on OPA1 cleavage can be easily explained when taking in account that PHBs are known to interact with mAAA proteases (Steglich et al., 1999) and that OMA1 autocatalytic activation seems to be modulated by PHB2 (Zhang et al., 2014).

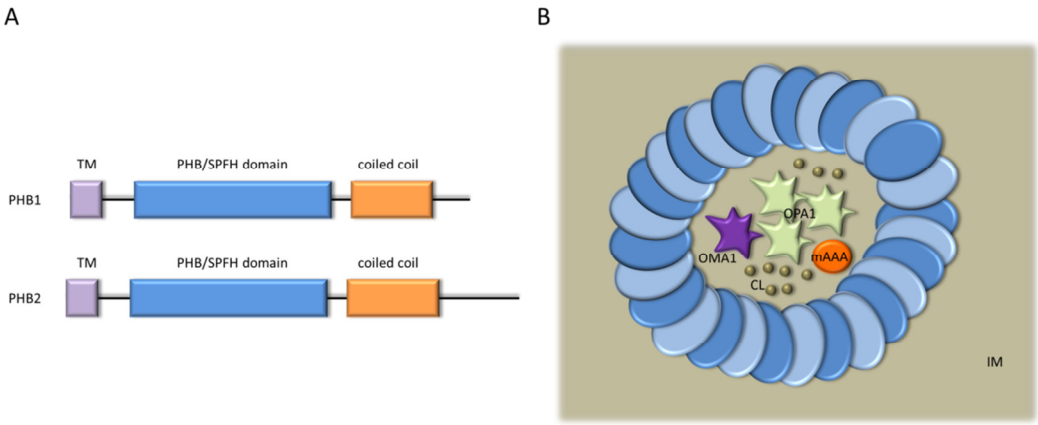

Figure 8 Prohibitin structures and assembly.

A wide screen for synthetic lethality in *Phb*-depleted yeast cells identified 35 genes that are essential for cell survival in the absence of prohibitins (Osman et al., 2009a). The majority of these genes encode for mitochondrial proteins, including genes that control the assembly of respiratory complexes and β-barrel proteins, mitochondrial morphology, as well as enzymes that mediate the terminal steps in phosphatidylethanolamine (PE) and cardiolipin (CL) biosynthesis (Richter-Dennerlein et al., 2014). An unifying model proposes that, through protein-protein interaction and by modulating CL and PE biosynthesis, PHBs might contribute to the formation of specific functional lipid/protein microdomains in the inner mitochondrial membrane(Osman et al., 2009b). In this scenario PHBs might regulate OPA1 proteolytic cleavage by regulating mAAA/OMA1 proteases activity and/or orchestrating OPA1-mAAA/OMA1 interaction in specific microdomains (Figure 8B).

2.1.4 Mitofilin/Mic60 and the MICOS complex

Mitofilin (also known as HMP, IMMT, MINOS2, Mic60), as its yeast homologue formation of *cristae* junctions 1 (Fcj1), is located in the IM and is preferentially enriched at *cristae* junctions (Gieffers et al., 1997; Odgren et al., 1996; Rabl et al., 2009). Mitofilin possesses an *N*-terminal mitochondrial targeting sequence, followed by a transmembrane segment and a large domain exposed to the intermembrane space. The IMS domain consists of two subdomains: an extended putative coiled-coil region and a short *C*-terminal mitofilin signature domain (Korner et al., 2012; Rabl et al., 2009; von der et al., 2011; Zerbes et al., 2012a) (Figure 9). Depletion of Mitofilin in human cells, worms and yeast leads to an extension of the inner membrane surface, a massive loss of *cristae* junctions and to abnormal *cristae* structures disconnected from the inner boundary membrane (Alkhaja et al., 2012; Hoppins et al., 2011a; John et al., 2005; Korner et al., 2012; Mun et al., 2010; Rabl et al., 2009; von der et al., 2011).

Mitofilin is now widely accepted to be a component of a large heterooligomeric protein complex of the IM conserved from yeast to humans that plays crucial roles in the maintenance of *cristae* junctions, inner membrane architecture and formation of contact sites to the OM. Several names have been used by different research groups to describe the complex, including mitochondrial contact site (MICOS) complex, mitochondrial inner membrane organizing system (MINOS), mitochondrial organizing structure (MitOS), Mitofilin complex, or Fcj1 complex (Pfanner et al., 2014; Zerbes et al., 2012b).

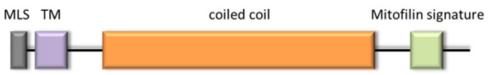

Figure 9 Mitofilin domains

Mutants of Mitofilin/Fcj1 as well as of other MICOS/MINOS/MitOS subunits show a strikingly altered inner membrane architecture. *Cristae* junctions are lost, *cristae* membranes appear as large membrane stacks, the respiratory activity is reduced, and mitochondrial DNA nucleoids are altered (Pfanner et al., 2014; Zerbes et al., 2012b). The MICOS complex is reported to interact with a variety of outer membrane proteins, such

as channel proteins and components of protein translocases and mitochondrial fusion machines, and defects impair the biogenesis of mitochondrial proteins (Pfanner et al., 2014; Zerbes et al., 2012b). The MICOS complex thus plays crucial roles in mitochondrial architecture, dynamics, and biogenesis.

2.1.6 ATP synthase

The mitochondrial F_1F_0-ATP synthase complex is known to form oligomers at the apex of mitochondrial *cristae* and this apical oligomers have been implicated in the curvature of the IM and in *cristae* morphology (Dudkina et al., 2005; Minauro-Sanmiguel et al., 2005). For instance, mutations in subunits e and g, required for dimerization and subsequent oligomerization, result in concentric onion-like *cristae* (Bornhovd et al., 2006; Paumard et al., 2002). Additionally, IF1, the inhibitor factor of reversal F_1F_0-ATP synthase, is reported to increase *cristae* when overexpressed (Campanella et al., 2008). In general, F_1F_0-ATP synthase dimers at the *cristae* apex could force a strong local curvature, thus shaping the *cristae*. Nevertheless, the precise role of ATP synthase in regulating *cristae* curvature is still a matter of debate: in particular, it is still unknown whether the ultrastructural anomalies observed when ATPase assembly is impaired are a direct consequence of defective assembly or are second effects due to impaired mitochondrial respiration.

2.2 Pro-fission proteins

Mitochondrial fragmentation is mediated by dynamin related protein 1 (DRP1), a cytosolic GTPase which, upon different stimuli, translocates to mitochondria and induces fission in a GTPase-dependent manner. Dnm1p/DRP1 shares many structural similarity with classical dynamins (Figure 10), but lacks the pleckstrin homology (PH) domain which, in dynamins, mediates interaction with lipids. This observation led to the idea that DRP1 recruitment on mitochondrial membranes requires interaction with specific

organellar receptors. Indeed, a growing number of integral OM proteins is emerging as mediators of DRP1 mitochondrial docking. These include FIS1, MFF, Mid49 and MiD50. Remarkably, their different roles in DRP1 recruitment and mitochondrial fragmentation have not been fully established yet and, in some cases, contradictory data have been reported. Additionally, besides the involvement of specific mitochondrial adaptors, DRP1 recruitment seems to depend also on the lipid composition of the mitochondrial membranes. Indeed, cardiolipin has been shown to promote DRP1 interaction with the lipid bilayer, both *in vitro* and in reconstructed lipid vesicles, and to undergo a reorganization from a lamellar bilayer to a non-bilayer configuration concomitant with DRP1 self-assembly (Bustillo-Zabalbeitia et al., 2014; Francy et al., 2015; Stepanyants et al., 2015).

A growing number of post-translational modifications, such as phosphorylation, ubiquitination and SUMOylation, has been reported to regulate DRP1 mitochondrial translocation and activity. Moreover, ER and the actin cytoskeleton have been also recently implicated in marking perspective fission sites, assisting DRP1 mitochondrial recruitment and regulating DRP1 function, in a process called ER-associated mitochondrial division (ERMD).

Finally, it should be stressed that the contradictory and sometime puzzling data regarding not only DRP1 mitochondrial adaptors, but also DRP1 activity modulation (especially upon phosphorylation), could, at least in part, be explained by the existence of many splicing variants for the majority of the factors implicated in mitochondrial fragmentation in vertebrates and by the evolutionary differences and divergences existing between yeast and mammals. Indeed, whereas DRP1 and FIS1 are somehow conserved in yeast (Dnm1p and Fis1p), MFF and MiDs are present only in vertebrates, whereas Caf4p and Mvd1p have no vertebrate homologue.

In the following paragraphs, the proteins involved in mitochondrial fragmentation are discussed in detail.

2.2.1 Dnm1p / Dlp1 / DRP1

Dynamin-related protein 1 (Dnm1p) was initially identified as involved in endocytosis in

yeast (Gammie et al., 1995) and subsequently re-discovered in a genetic screen for yeast mutants with defective mitochondrial morphology. Indeed, in Dnm1p mutants mitochondrial networks appear to be collapsed on one side of the cell, forming an elongated mass of mitochondrial membranes (Hermann et al., 1997; Otsuga et al., 1998). The mammalian homologue dynamin-related protein 1 (DRP1) was initially described as capable of tubulating cytosolic membranes, in particular ER (Pitts et al., 1999; Yoon et al., 1998; Yoon et al., 2001b). Concomitantly, DRP1 was recognized as capable of specifically mediating mitochondrial fragmentation and, when mutated or depleted, to induce mitochondrial perinuclear clustering as reported in yeast (Smirnova et al., 2001; Smirnova et al., 1998b).

2.2.1.1 DRP1 structure

Dnm1p/DRP1 contains GTPase, middle, and GTPase effector (GED) domains, all conserved across evolution and found in classical dynamins. However, Dnm1p/DRP1 lacks the pleckstrin homology (PH) and carboxy-terminal proline-rich domains (PRD) commonly found in dynamins, but instead carries a unique sequence called variable domain (VD) (Figure 10A). Remarkably, the GED domain not only stimulates the GTPase activity *per se*, but also promotes self-assembly (Zhang et al., 2011; Zhu et al., 2004). On the other side, the variable domain is thought to exert auto-inhibitory functions and to prevent interaction with MFF, DRP1 self-assembly and mitochondrial recruitment (Clinton et al., 2015; Strack and Cribbs, 2012).

Human *Drp1* gene is composed by 31 exons and gives rise to eight differentially spliced variants produced by the different combinations of exons 3, 16 and 17 (Strack et al., 2013; Uo et al., 2009; Yoon et al., 1998) (Figure 10A). The resulting proteins carry different functional regions and are emerging as having distinct molecular properties and functional roles. Indeed, exon 3 codes for a short amino acid sequence named insert A (InsA), mapping inside the GTPase domain, whereas both exon 16 and exon 17 code for a region called insert B (InsB), mapping inside the VD domain (Macdonald et al., 2015; Strack et al., 2013; Yoon et al., 1998) (Figure 10A). Remarkably, the splicing variants carrying exon 3/InsA seem to be neuronal specific, whereas the other variants have a broader expression spectrum (Uo et al., 2009). The presence of InsA rather than InsB differentially impinges on the geometry of DRP1 oligomers, with the InsA promoting and InsB restricting DRP1 self-assembly (Macdonald et al., 2016).

Based on the crystal structure of Dynamin, DRP1 is predicted to exist as a T-shaped dimer or tetramer containing a head (GTPase domain), a leg (VD), and a stalk (middle and GED domains). GTP hydrolysis is thought to induce a rearrangement of head and stalk which generates the force ultimately resulting in membrane constriction.

Under normal conditions, DRP1 is mainly cytosolic and is reported to exist as polymeric species which, through a dimeric intermediate, equilibrates with mitochondria-located polymeric DRP1. The equilibrium between the two pools is reportedly mediated by MFF (Macdonald et al., 2014). Furthermore, since MFF functions as an allosteric effector of DRP1 by alleviating the constraints imposed by the A- and B-inserts, there seems to be a isoform-specific requirement of MFF for DRP1-mediated fragmentation (Macdonald et al., 2016).

2.2.1.2 DRP1 functions

So far, DRP1 is the main player known to mediate mitochondrial fragmentation under basal conditions. DRP1 is also known to mediate mitochondrial fragmentation occurring during apoptosis (Frank et al., 2001). Under these circumstances, DRP1 translocation seems to depend on BAX/BAK induction (Frank et al., 2001) and/or Ca^{2+} release from the ER in a process that relies on BAP31 (Breckenridge et al., 2003). Interestingly, FIS1 has been implicated in BAP31 activation during apoptosis (Iwasawa et al., 2011a). However, the precise role of mitochondrial fragmentation during apoptosis is still matter of intense debate. This topic is discussed further in 3.2. Moreover, DRP1 has been implicated also in peroxisome division (Li and Gould, 2003) and in mitophagy as discussed in 3.4.

Drp1 ablation in mice is lethal around midgestation and causes several developmental abnormalities, particularly in the forebrain. Similarly, neural specific ablation causes death short after birth as a consequence of brain hypoplasia with apoptosis and developmental defects (Ishihara et al., 2009; Wakabayashi et al., 2009). Furthermore, ablation of *Drp1* in adult mouse forebrain results in progressive neuronal alterations of mitochondrial morphology impacting on hippocampal synaptic transmission and memory function (Oettinghaus et al., 2015). Interestingly, a dominant-negative middle domain mutation (A395D) in DRP1 has been reported in a lethal disorder with

microcephaly, abnormal brain development, optic atrophy and hypoplasia (Waterham et al., 2007). It is suggested that the mutation impairs higher order assembly of DRP1, leading to decreased fission, elongated mitochondria, and altered cellular distribution of mitochondria (Chang et al., 2010a).

2.2.1.3 DRP1 regulation by phosphorylation

The major phosphorylation site in DRP1 is a conserved serine residue in the GED domain: Ser^{637} in hDRP1 isoform 1, corresponding to Ser^{600} in mDRP1 isoform b, and Ser^{565} in rat (Figure 10B). Phosphorylation at this site is controlled by the counteracting couple of protein kinase A (PKA)/kinase anchor protein (AKAP) and Calcineurin (CaN). These kinases and phosphatases respectively inhibit and activate DRP1 functions (Cereghetti et al., 2008a; Chang and Blackstone, 2007a). Mechanistically, Phospho-Ser^{637} was initially suggested to prevent the interaction between the GED and the GTPase domain and to prevent DRP1 mitochondrial recruitment (Chang and Blackstone, 2007a). However, Strack and co-workers demonstrated that DRP1 phosphorylation inhibits the disassembly step after GTP hydrolysis, accumulating large, slowly recycling DRP1 oligomers at the OM. Consequently, unopposed fusion promotes mitochondrial elongation (Merrill et al., 2011). In neurons, CaN has been implicated in triggering mitochondrial fragmentation and neuronal cell death in response to oxygen-glucose deprivation (Slupe et al., 2013), whereas the counteracting action of PP2A/Bβ2 and AKAP/PKA was shown to regulate dendrite and synapse development (Dickey and Strack, 2011). Interestingly, PKA is activated by fasting conditions and promotes mitochondrial elongation, thus sustaining cell survival (Gomes et al., 2011b), and inhibition of AKAP/PKA-mediated phosphorylation is reported to induce fragmentation and mediate the cellular response to hypoxia (Kim et al., 2011). Additionally, the GSK3β/GSKIP couple is reported to inactivate DRP1, thus favoring mitochondrial elongation in response to stress, either by direct phosphorylation of DRP1 on a distinct Ser residue in the GED domain (Chou et al., 2012), or by bridging together PKA and DRP1 (Loh et al., 2015).

Recently, the physiological relevance of CaN-mediated dephosphorylation was confirmed *in vivo*. Calcineurin ablation from skeletal muscles enhances DRP1 hyperphosphorylation, increases mitochondrial elongation and respiration, and protects

from high-fat diet-induced obesity, possibly as a consequence of the hyperfused mitochondrial network (Pfluger et al., 2015).

However, some reports demonstrate that phosphorylation on Ser^{637} has a stimulating effect on DRP1. In hippocampal neurons, Ca^{2+}/calmodulin-dependent protein kinase 1α (CaMK1α) phosphorylates and activates DRP1 in response to Ca^{2+} influx from voltage-dependent channels (Han et al., 2008). Additionally, the serine/threonine kinase ROCK1 phosphorylates and activates DRP1 in response to hyperglycemic conditions (Wang et al., 2012). Curiously, Strack ad co-workers, who recently showed a stimulatory effect of CaN-mediated dephosphorylation, initially also reported a stimulatory effect of PKA-mediated phosphorylation in neurons in response to Ca^{2+} levels and sympathetic tones (Cribbs and Strack, 2007a).

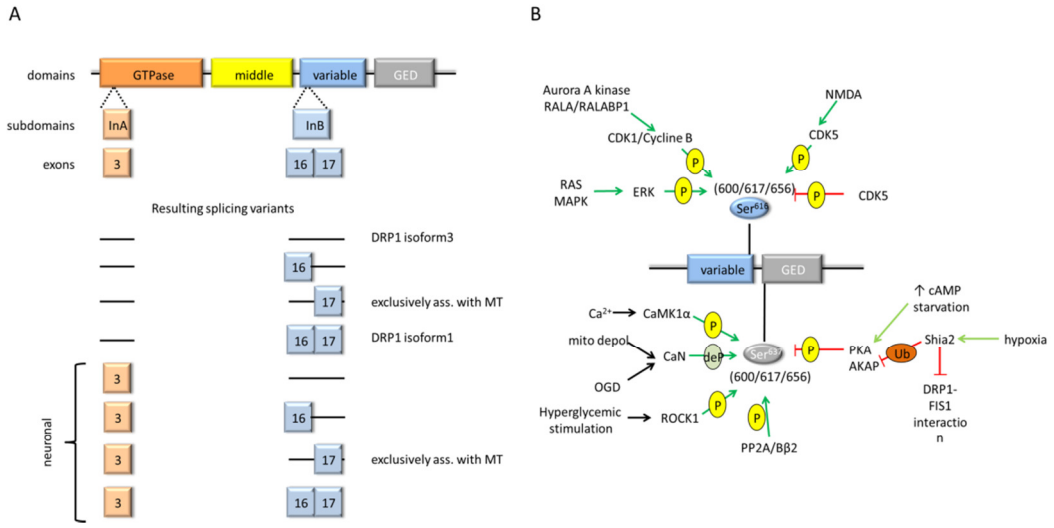

Figure 10 DRP1 protein structure and phosphorylation. (A) DRP1 domains and exon splicing coding for InsA versus InsB. (B) regulation of DRP1 by phosphorylation.

Another important phosphorylation site regulating DRP1 is Ser^{616} in humans (Ser^{585} in mouse): phosphorylation of this residue promotes DRP1 mitochondrial translocation and pro-fission activity (Figure 10B). In particular, Ser^{616} is targeted by CDK1/Cycline B at early mitosis (Kashatus et al., 2011; Taguchi et al., 2007). Interestingly, two recent independent studies demonstrated that ERK-mediated phosphorylation of DRP1 Ser^{616} is required for RAS/MAPK-driven transformation and tumor growth (Kashatus et al., 2015; Serasinghe et al., 2015). However, some contradictory results are reported also

regarding phosphorylation at Ser^{616}. Indeed, CDK5-mediated phosphorylation at this residue is reported both to inhibit (Cho et al., 2014) and to activate DRP1 function (Jahani-Asl et al., 2015).

2.2.1.4 DRP1 regulation by ubiquitination

DRP1 ubiquitination is mediated by MARCH5/MITOL and Parkin, but the two pathways seem to have different physiological meanings.

MARCH5/MITOL is an ubiquitin ligase of the OM and controls DRP1 stability, thus impinging on mitochondrial dynamics (Karbowski et al., 2007; Nakamura et al., 2006; Yonashiro et al., 2006). However, the precise role of MARCH5/MITOL has not been clarified yet. MARCH5-mediated ubiquitination is reported to target DRP1 (and FIS1) for degradation, as suggested by the finding that the expression of a dominant negative mutant of DRP1 prevents mitochondrial fragmentation induced by MARCH5/MITOL depletion, or by a MARCH5/MITOL mutant lacking ubiquitin ligase activity (Karbowski et al., 2007; Nakamura et al., 2006; Yonashiro et al., 2006). Curiously, Youle and co-workers, after having reported a MARCH5/MITOL-mediated degradation of DRP1 and FIS1 (Karbowski et al., 2007), reported that inhibition of MARCH5/MITOL increases elongation and interconnectivity of mitochondria along with induction of cellular senescence, possibly by blocking DRP1 activity and/or promoting accumulation of MFN1 on mitochondrial membranes (Park et al., 2010).

DRP1 is ubiquitinated also by Parkin in a PINK dependent manner (Narendra et al., 2010; Wang et al., 2011). However, this ubiquitination is used to signal dysfunctional mitochondria and activate mitophagy, rather than mediate DRP1 degradation/stabilization on mitochondrial membranes (Chan et al., 2011; Matsuda et al., 2010; Narendra et al., 2008).

2.2.1.5 DRP1 regulation by SUMOylation

DRP1 is also post-translationally modified by the small ubiquitin-like modifier (SUMO). Indeed, DRP1 is the substrate of the mitochondrial-anchored SUMO E3 ligase MAPL (Braschi et al., 2009). McBride and co-workers showed that DRP1 SUMOylation is increased during apoptosis in a BAX/BAK dependent fashion (Wasiak et al., 2007) and that it stabilizes ER-mitochondrial contact sites (Prudent et al., 2015). Indeed SUMOylated DRP1 seems to act as a hotspot for mitochondrial constriction, Ca^{2+} flux,

cristae remodeling and cytochrome *c* release during apoptosis (Prudent et al., 2015). SENP5, a SUMO protease, can desumoylate DRP1, thus counteracting MAPL-mediated fragmentation and regulating DRP1-dependent mitochondrial fragmentation during mitosis (Zunino et al., 2009; Zunino et al., 2007).

2.2.1.6 DRP1 regulation by other subcellular structures and compartments

Intriguingly, two recent papers propose a role for ER in determining the position of the perspective fission sites and in regulating DRP1 activity in a process called ER-associated mitochondrial division (ERMD) (Arasaki et al., 2015; Friedman et al., 2011). Indeed, ER tubules were shown to wrap around mitochondria at prospective fission sites, mediating constriction of the mitochondrial membranes and reducing the mitochondrial diameter prior to DRP1 recruitment. Interestingly, ER constriction mainly occurs at positions of MFF and DRP1 foci, although both proteins seem to be dispensable for this process (Friedman et al., 2011). This intriguing model has been recently put forward by the finding that the SNARE protein syntaxin 17 (Syn17) promotes mitochondrial fission at ER-mitochondria contact sites. Indeed, Syn17 seems to specifically interact with MFF-bound DRP1 in a way that depends on DRP1 GTPase activity and to promote DRP1-mediated fragmentation. This action occurs both by regulating DRP1 localization and by preventing DRP1 inhibitory phosphorylation by the AKAP Rab32 kinase. Remarkably, starvation induces redistribution of Syn17 at the mito-ER contacts accompanied by a switch in its binding from DRP1 to ATG14L. This makes DRP1 susceptible to phosphorylation and hence contributes to halt mitochondrial fragmentation during starvation (Arasaki et al., 2015).

In the ERMD process a key role is played by actin filaments. A general model predicts that at perspective fission sites, where ER and mitochondria interact, actin filaments are nucleated and elongate with the ER-bound inverted formin 2 (INF2), known to accelerated actin (de)polymerization. This step tethers the actin polymers to the ER membranes. Subsequently, Myosin II is recruited to the fission site and, by acting on actin filaments, causes deformation of the network, resulting in constriction of both the surrounding ER and the underlying mitochondrial membranes. After that, DRP1 binds and oligomerizes at the pre-constriction site, and, through GTP hydrolysis, increases

mitochondrial constriction. Finally, the actual membrane fission process occurs and components of the fission complex disassemble (De Vos et al., 2005; Hatch et al., 2014; Ji et al., 2015; Korobova et al., 2014; Korobova et al., 2013).

Interestingly, DRP1 splicing variants carrying exon 17 have been show to interact specifically with microtubules in a cell cycle CDK1/5 dependent manner (Strack et al., 2013) and cytoskeletal rearrangements by the Src kinases require Rab11a-mediated DRP1-dependent redistribution of mitochondria in the cytosol, resulting in final cytoskeletal polarization (Landry et al., 2014).

2.2.2 Fis1p / FIS1

Fis1p/FIS1 is a 17-kDa integral protein of the outer mitochondrial membrane (James et al., 2003). Its N-terminal domain is exposed to the cytoplasm and forms a tetratricopeptide (TPR)-like fold (Suzuki et al., 2003). The *C*-terminal domain of FIS1 possesses a predicted TM domain and a short stretch of amino acids facing the IMS. FIS1 has been implicated in mitochondrial fragmentation, apoptosis and autophagy. However, recent publications challenge the role of FIS1 in DRP1 recruitment and mitochondrial fragmentation. FIS1 is discussed in detail in Section 4.

2.2.3 MFF

MFF was initially discovered by high-throughput screening of a Drosophila RNAi library for mitochondrial morphology. The silencing of the gene Tango11 induced perinuclear clustering, as reported for DRP1 depletion. The orthologue protein in human was named mitochondrial fission factor (MFF) (Gandre-Babbe and van der Bliek, 2008). MFF is anchored to the OM through a *C*-terminal transmembrane domain, whereas the bulk of the protein is exposed to the cytosol. *Mff* gene in human codes for at least nine different splice variants, but their function is unknown (Gandre-Babbe and van der Bliek, 2008). Remarkably, depletion of MFF by siRNA induces mitochondrial fusion, whereas overexpression induces fragmentation (Gandre-Babbe and van der Bliek, 2008).

The role of MFF in mitochondrial fragmentation was further substantiated by Youle and co-workers, who demonstrated that MFF displays a punctate distribution on the mitochondrial membrane independently of DRP1 and FIS1, and that MFF and DRP1 colocalize at these foci. The interdependence of DRP1 and MFF is further demonstrated by the finding that mitochondrial fragmentation upon MFF overexpression is abolished in *Drp1*$^{-/-}$ MEFs. Moreover MFF knock-down prevents CCCP-induced mitochondrial fragmentation and rescues OPA1 knock-down-induced fragmentation (Otera et al., 2010).

Recently, a *Mff* mouse gene trap (*Mff*^{gt}) was characterized. *Mff*^{gt} mice die at 13 weeks, as a result of severe cardiomyopathy leading to heart failure. Hearts from mutant mice show reduced mitochondrial density and respiratory chain activity along with increased mitophagy. Remarkably, concomitant deletion of *Mfn1* completely rescues heart dysfunctions, life span, and respiratory chain function (Chen et al., 2015). However, how abolishing mitochondrial fragmentation could lead to mitophagy is left unexplained. In addition, the phenotype is drastically different from that of *Drp1* deficient mice, dying at mid gestation, indicating that *Mff* ablation does not phenocopy that of the master fission protein *Drp1*. Thus, *Mff* is not the only receptor for *Drp1* on mitochondria, or *Drp1* has additional functions.

2.2.4 MiD49/MIEF2, MiD51/MIEF1

In 2011, two independent reports described mitochondrial elongation factor 1 (MIEF1) / mitochondrial dynamics protein (MiD51) and MIEF2/MiD49 as implicated in mitochondrial fragmentation and DRP1 recruitment (Palmer et al., 2011; Zhao et al., 2011). In both cases MiDs overexpression resulted in mitochondrial fusion and unexpectedly increase of DRP1 mitochondrial localization even in absence of FIS1 and MFF. However, while one report described that MiDs downregulation results in mitochondrial fragmentation (Zhao et al., 2011), the other reported mitochondrial fusion upon MiDs downregulation (Palmer et al., 2011). Two independent publications by the groups of Chan and Ryan, respectively, help to better clarify the apparent contradictory results (Loson et al., 2013; Palmer et al., 2013). As a general model, the mitochondrial phenotype associated with MiDs overexpression strongly depends on the

expression level, at high expression levels MiDs induce fusion whereas at lower expression levels mitochondrial fragmentation is still taking place (Palmer et al., 2011). MiDs display a punctate distribution pattern on mitochondria and these foci usually correspond to perspective fission sites (Palmer et al., 2011). Overexpression of MiDs induces mitochondrial DRP1 recruitment (Loson et al., 2013; Palmer et al., 2013; Palmer et al., 2011) and concomitant increase of DRP1 Phopsho-Ser^{636} which, as discussed in Section 2.2.1.3, is known to be fission incompetent (Loson et al., 2013). Indeed, MiDs seem to preferentially interact DRP1 Phopsho-Ser^{636} and to promote its mitochondrial recruitment, thus inducing the unexpected elongated mitochondrial phenotype (Loson et al., 2013). Therefore, MiDs might recruit DRP1 and maintain it in an inactive status until adequate cellular signal trigger their activation (Loson et al., 2013; Palmer et al., 2013; Palmer et al., 2011). MiD49 forms high molecular weight oligomers, whereas MiD51 is largely present as a dimer, and its oligomerization is not required for mitochondrial localization and interaction with DRP1 (Liu et al., 2013). Furthermore, MiD49 is reported to stimulate DRP1 assembly and reduce DRP1 polymer diameter (Koirala et al., 2013). On the other hand, MiD51 contains a nucleotidyl-transferase domain that binds ADP with high affinity. Although this domain is not required for DRP1 binding, addition of ADP to purified MiD51 and DRP1 is essential for DRP1 assembly into spirals and GTP hydrolysis (Loson et al., 2014; Richter et al., 2014). Such a nucleotidyl transferase domain seems not to be present in MiD49 (Loson et al., 2015).

2.2.5 Endophilin B1

Endophilin B1, also known as BAX-interacting factor 1 (Bif-1) or SH3GLB1, is a member of the endophilin family of fatty acid acyl transferases that participates in endocytosis. Endophilin B is primarily present in the cytosol and only a small fraction localizes to mitochondria in a dynamic cycling between the two compartments. Endophilin B1 downregulation by siRNA leads to changes in mitochondrial shape, as well as to the formation of OM-bound vesicles. Therefore, endophilin B1 seems to participate in the control of OM morphology by altering the curvature of the membrane. Whether this is a direct effect, or it requires the recruitment of other proteins, remains to be elucidated (Karbowski et al., 2004b). More recently, endophilin B1 has been implicated in autophagy, where it seems to regulate membrane trafficking (Takahashi et al., 2009)

2.2.6 Mitochondrial protein 18kDa (MTP18)

Mitochondrial protein 18kDa (MTP18) is located in the IMS. Overexpression of MTP18 leads to mitochondrial fragmentation, whereas downregulation induces mitochondrial elongation. Interestingly, MTP18 is a downstream effector of phosphatidyl-inositol 3-kinase (PI3K) signaling and could regulate mitochondrial morphology in response to PI3K activation (Tondera et al., 2005; Tondera et al., 2004).

2.2.7 Ganglioside-induced differentiation-associated protein 1 (GDAP1)

Ganglioside-induced differentiation-associated protein 1 (GDAP1) is located in the OM. GDAP1 overexpression induces mitochondrial fragmentation without inducing apoptosis, whereas its downregulation results in mitochondrial elongation. Interestingly, MFNs and dominant negative $DRP1^{K38A}$ can counterbalance the GDAP1-dependent fission (Niemann et al., 2005). GDAP1 is mutated in Charcot-Marie-Tooth disease (CMT), and a growing number of pathological mutations have been described as preventing GDAP1 mitochondrial localization and/or function (Huber et al., 2013; Kabzinska et al., 2011; Niemann et al., 2005).

2.2.8 Mitochondrial targeting GxxxG motif protein (MTGM)

Mitochondrial targeting GxxxG motif protein (MTGM) is characterized by an unusual tetrad of the GxxxG motif in the transmembrane segment. Overexpression of MTGM results in mitochondrial fragmentation and release of mitochondrial Smac/Diablo to the cytosol with no effect on apoptosis. MTGM-induced mitochondrial fission can be blocked by a dominant negative $DRP1^{K38A}$. Overexpression of MTGM also results in inhibition of cell proliferation at S phase, whereas knock-down induces mitochondrial elongation, cell proliferation and inhibition of cell death induced by apoptotic stimuli (Zhao et al., 2009).

3. Mitochondrial dynamics: functional implications

Mitochondria form a highly dynamic reticulum, continuously redistributing in the cytoplasm. The plasticity of the mitochondrial (ultra)structure has important consequences on mitochondrial and cellular physiology and function. Fusion and fission control the shape, length and number of mitochondria. Fusion and fission allow mitochondria to exchange lipid membranes and intramitochondrial content and such exchange is probably crucial for maintaining the health of a mitochondrial population. Furthermore, the shape of mitochondria affects the ability of cells to distribute their mitochondria to specific subcellular locations. This function is especially important in highly polarized cells, such as neurons. In complex, mitochondrial dynamics plays a crucial role in the mitochondria-ER interaction and in regulation of Ca^{2+} signaling, in apoptosis and during autophagy.

3.1 Mitochondria, ER and Ca^{2+}

Inside the cell, mitochondria and ER are in close proximity. In mammals, these regions of close interaction are usually called mitochondrial associated ER membranes (MAMs). Structurally, MAMs are a subdomain of the ER with localized functional specifications. Mito-ER contacts are dynamic structures that have been implicated in a number of processes which include lipid transfer, mitochondrial fragmentation (see 2.2.1.6), apoptosis (see 3.2.1 and 4.2.2), autophagy (see 4.2.3) and Ca^{2+} homeostasis (Naon and Scorrano, 2014).

As described in section 2.1.1, our lab reported that one major tether in mito-ER contacts in MFN2. However, a growing number of proteins is emerging as enriched or specifically localized at the mito-ER contacts and to be involved in the tethering between the two organelles (Naon and Scorrano, 2014). Among the others, FIS1 has been proposed to mediate the tether and trigger caspase 8 activation during apoptosis (discussed in 4.2.2).

As said above, mitochondria-ER proximity is required for the production of microdomains of high [Ca^{2+}], essential for the activation of the low affinity mitochondrial Ca^{2+} uniporter (MC) (Rizzuto et al., 2012; Rizzuto et al., 2009). Interestingly, Ca^{2+} release from the ER is mediated by the inositol 1,4,5 trisphosphate IP3-sensitive receptor (IP_3R), whose activity is regulated by IP3, Ca^{2+} itself and members of the BCL2 family. Remarkably, the Ca^{2+} dependence of IP_3R allows mitochondrial-mediated control of ER Ca^{2+} release. Indeed, in the absence of mitochondria, the initial increase of cytosolic Ca^{2+} levels favors the opening of the IP_3R and Ca^{2+} release from the ER. Conversely, sustained IP_3R activity leads to the build-up of a local high cytosolic Ca^{2+} concentration that feeds-back on the IP_3R inhibiting its activity and blocking further Ca^{2+} release. By taking up Ca^{2+}, mitochondria can locally buffer the initial cytosolic Ca^{2+} increase thus sustaining prolonged release (Rizzuto et al., 2012).

Mitochondria can take up Ca^{2+} also from extracellular medium through the plasma membrane. The mitochondrial Ca^{2+} uptake through the plasma membrane is mainly responsible for the mitochondria-mediated capacitative Ca^{2+} entry (CCE), a pathway triggered by cellular Ca^{2+} depletion during which cells takeup Ca^{2+} from the outside (Putney, 1990). CCE is inhibited by high cytosolic Ca^{2+} concentrations and mitochondria act as local buffers by preventing Ca^{2+}-dependent inactivation of the plasma membrane channels responsible for Ca^{2+} influx (Malli et al., 2003).

Furthermore, Ca^{2+} waves influence mitochondrial dynamics (Breckenridge et al., 2003; Germain et al., 2005), while mitochondrial shaping proteins modulate Ca^{2+} transients (Frieden et al., 2004; Szabadkai et al., 2004), as discussed in 3.2.1 and 4.2.2.

3.2 Mitochondrial shaping under apoptosis

Apoptosis is a form of programmed cell death essential for embryonic development, tissue homeostasis and regulation of immunity in multicellular organisms. Defects in apoptosis are related to excessive cell proliferation, cancer, neurodegeneration and autoimmunity. Depending on the cell type and the death stimulus, two main apoptotic pathways are activated: the intrinsic pathway, which is triggered by intracellular

apoptotic signals and is almost always mediated by mitochondria, and the extrinsic pathway, which is triggered by activation of specific death receptors at the cell surface, and leads to pro-caspase 8 recruitment and activation. Caspase 8 can cleave other caspases (type I cells), or generate truncated Bid (tBid), which in turn triggers the mitochondrial amplification loop (type II cells).

During apoptosis, Ca^{2+} is released from ER and taken up by mitochondria. The locally induced Ca^{2+} wave can propagate within the mitochondrial reticulum and ultimately triggers mitochondrial apoptosis. Additionally, BCL2-related proteins integrate different *stimuli* and regulate mitochondrial permeabilization (Ferri and Kroemer, 2001; Kroemer et al., 2007b). The relative levels and activities of pro- and anti-apoptotic members of the family are responsible for life versus death decision. Pro-apoptotic BH3-only members (Bid, Bik, Bad, Noxa, Puma) are thought to counteract the anti-apoptotic members and to act as apoptotic-signal sensors which can subsequently activate multidomain pro-apoptotic members (BAX and BAK). For example, tBID migrates to the mitochondrial surface, where it triggers BAK and BAX activation and oligomerization and eventually cytochrome *c* release (Adams and Cory, 2001).

Upon mitochondrial permeabilization, cytochrome *c* is released into the cytosol together with a plethora of pro-apoptotic factors, such as Smac/DABLO and Omi/HtrA2, AIF and endonuclease G, therefore mitochondrial outer membrane permeabilization (MOMP) is generally considered as the point of non-return in the apoptotic cascade (Kroemer et al., 2007b). A key step for MOMP to occur is the oligomerization of the proapoptotic BAX/BAK. Irrespective of the exact mechanism by which active BAX/BAK leads to MOMP and release of cytochrome *c*, it appears clear that they act as essential gateway controllers, regulating the critical step of mitochondrial engagement in apoptosis.

However, recent data indicate that additional steps are required to ensure complete release of cytochrome *c*: this includes remodeling of the mitochondrial *cristae* and fragmentation of the mitochondrial network.

3.2.1 Mitochondrial fragmentation

The mitochondrial fragmentation that occurs during apoptosis seems to be mediated by the fission machinery. Indeed, expression of a DN DRP1^{K38A} mutant (Frank et al., 2001) or of RNA*i* against DRP1/FIS1 (Lee et al., 2004a), are sufficient to maintain a normal mitochondrial morphology during apoptosis. Early during apoptosis DRP1 translocates to mitochondria upon ER Ca^{2+} release (Breckenridge et al., 2003) and/or BAX/BAK activation (Frank et al., 2001). Although well established, the relationship between BAX/BAK activation and DRP1 recruitment is not fully elucidated yet. Indeed, whereas Youle and coworkers proposed that DRP1 recruitment, fission activation and fusion inhibition are required and preceed cytochrome *c* upon BAX/BAK activation (Karbowski et al., 2004a; Karbowski et al., 2002; Neuspiel et al., 2005), Blackstone and co-workers demonstrated that DRP1 recruitment requires BAX/BAK-mediated MOMP and release of apoptogenic factors in the cytosol (Arnoult et al., 2005b). On the other hand the ER Ca^{2+} release responsible for DRP1 recruitment seems to depends on BIK (Germain et al., 2005) and/or BAP31 cleavage (Breckenridge et al., 2002; Breckenridge et al., 2003).

The role of mitochondrial morphology in cell death is not completely understood and it has been questioned whether mitochondrial fragmentation is causal for cytochrome *c* release or whether it is an epiphenomenon of the apoptotic cascade. Indeed, inhibition of mitochondrial fragmentation has been reported to only delay and not completely block apoptosis progression (Parone et al., 2006) and *Drp1*$^{-/-}$ cells can still undergo MOMP at the same rate of their wild-type counterparts but display delayed cytochrome *c* release and caspase activation (Ishihara et al., 2009). Conversely, peptide-mediated DRP1 inhibition increases the clonogenic potential of cells treated with apoptotic stimuli (Cereghetti et al., 2010).

Therefore, it has been proposed that mitochondrial fragmentation could act as a positive feedback mechanism to amplify BAX/BAK activation and could also be involved in the remodeling of the cristae and therefore amplify the release of cytochrome *c*. The amount of cytochrome *c* that is initially released, before fission activation would be sufficient to trigger the formation of the apoptosome and caspase activation, in most of cases. Therefore, inhibiting mitochondrial fission would only slow down the kinetics of

cell death, whereas it would seriously impinging on apoptotic progression in a limited number of cases.

It has also been suggested that mitochondrial fragmentation is caused by inhibition of the fusion machinery. Indeed, the mitochondrial fusion machinery and pro-apoptotic BAX/BAX were shown to be functionally linked: in healthy cells BAX/BAK complex with MFNs to facilitate MFN2 oligomerization (Karbowski et al., 2006), conversely, during apoptosis, BAK dissociates from MFN2 and associates with MFN1 preventing mitochondrial fusion (Brooks et al., 2007). Furthermore, a DN mutant of MFN2 prevents BAX activation and cytochrome *c* release (Neuspiel et al., 2005). Remarkably, our laboratory demonstrated that MFNs and BAX/BAK interaction is regulated by extracellular signals in course of apoptosis (Pyakurel et al., 2015). Indeed ERK-mediated phosphorylation of MFN1 stimulates its interaction with BAK and promotes BAK oligomerization thus enhancing mitochondrial permabilization and cytochrome *c* release (Pyakurel et al., 2015). Additionally, during apoptosis OPA1 is cleaved and released (see 3.2.2) and this might also contribute to mitochondrial fragmentation.

Recently, DRP1 recruitment has been suggested to stabilize mito-ER contacts during apoptosis. Indeed, activation of apoptosis triggers MAPL/MUL1-dependent SUMOylation of DRP1. Once SUMOylated, DRP1 stabilizes ER/mitochondrial contact sites that act as hotspots for mitochondrial constriction, Ca^{2+} flux, *cristae* remodeling, and cytochrome *c* release (Prudent et al., 2015).

3.2.2 Mitochondrial cristae remodeling

During apoptosis, the kinetic of cytochrome *c* release is extremely rapid (Goldstein et al., 2000), however cytochrome *c* is not freely soluble in the IMS but mainly bound to cardiolipin and the 85% resides within the *cristae* (Bernardi and Azzone, 1981). Therefore, the initial observation of its detachment from cardolipin (Ott et al., 2002), possibly as consequence of cariolipin peroxidation by ROS which are usually produced during apoptosis (Nomura et al., 2000) was not enough to account for the fast release.

A turning point was the demonstration that during apoptosis mitochondria remodel their internal structure: individual *cristae* fuse and *cristae* junctions widen to allow cytochrome *c* mobilization from its intra-*cristae* compartment toward the IMS for its

subsequent release across the OM (Scorrano et al., 2002). This process was named *cristae* remodeling and accounts for the complete release of cytochrome *c* (Scorrano et al., 2002).

Besides the mitochondrial fragmentation discussed in 3.2.1, soon it become clear that OPA1 is cleaved and released during apoptosis and that this is required for cytochrome *c* mobilization from *the cristae* (Arnoult et al., 2005a). Our laboratory demonstrated that OPA1 forms high molecular weight oligomers that comprise both the inner membrane integral (L-OPA1) and the soluble intermembrane space (S-OPA1) forms of OPA1. These complexes are targeted and disrupted by BID during apoptosis and these events correlate with the *cristae* remodeling and the cytochrome *c* release occurring during cell death. In line with this, *Opa1* depleted MEFs show disorganized *cristae* and are more prone to apoptosis (Frezza et al., 2006a). Despite, the relative contribution and requirement of L-OPA1 versus S-OPA1 isoforms in maintaining a balanced mitochondrial network (OPA1 processing is discussed in detail in 2.1.2.3), the production of the soluble S-OPA1 seems to be instrumental for apoptotic *cristae* remodeling (Cipolat et al., 2006).

3.3 Mitochondrial shape and mitophagy

The word autophagy, derived from the Greek and meaning “self-eating”, was originally proposed by Christian de Duve more than 40 years ago to describe a catabolic process conserved from lower to higher eukaryotes. Autophagy is essential for recycling energy sources when cells deal with challenging conditions, such as nutrient depletion or hypoxia, or during development. Additionally, autophagy plays a key role in cellular quality control processes, being essential for the degradation of superfluous or damaged organelles and oxidized proteins. Initially, autophagy was believed to be a non-selective process. We now know that autophagy is a specific process and that selectivity in cargo targeting to the autophagosome is mediated by autophagy receptors. The latter simultaneously interact with specific cargoes and with proteins conjugated to the autophagosomal membranes.

The term mitophagy was introduced by Lemasters in 2005 (Lemasters, 2005) and

indicates the selective removal of damaged mitochondria.

3.3.1 Pink/PARKIN activation and selective removal of damaged mitochondria

Mitophagy seems to be initiated in a PINK/Parkin dependent manner. In healthy mitochondria, PINK1 is partially translocated across the mitochondrial membranes though the TOM complex, exposing its N-terminal mitochondrial targeting sequence (MTS) to the matrix in a manner dependent on the mitochondrial membrane potential ($\Delta\Psi_m$). The MTS is then removed by the mitochondria processing peptidase (MPP). Subsequently, the TM segment is stalled in the inner membrane, and processed by the intramembrane serine protease PARL. The double-cleaved form is eventually released back to the cytosol, and quickly degraded. As a result, Parkin is kept as an inactive state in the cytosol. On the other hand, in case of dysfunctional mitochondrial PINK1 cannot reach to the matrix due to $\Delta\Psi_m$ dissipation, and instead interacts with the TOM. This leads to PINK autophosphorylation and ultimately leads to Parkin activation (Campello et al., 2014; Nardin et al., 2015).

Parkin is a ubiquitin E3 ligase that catalyzes poly-ubiquitination of several substrates, calling into play the autophagy receptor p62/SQSTM1 that simultaneously binds ubiquitin and autophagy-specific ubiquitin-like modifiers (LC3/GABARAP proteins). Moreover, AMBRA1, a component of the core autophagic machinery, interacts with PARKIN to mediate mitochondrial clearance. Finally, depolarized mitochondria are transported along microtubules to the perinuclear region, where they form mito-aggresome structures and get wrapped by the autophagosomes membranes and get graded (Campello et al., 2014; Gomes and Scorrano, 2012).

3.3.2 Mitochondrial fragmentation and mitochondrial clearance

Accumulating evidence emphasizes the requirement of mitochondrial fragmentation prior to mitophagy. Conceptually, this is not surprising given that an organelle that is going to be engulfed and degraded by the autophagosome needs to fit into this forming

structure. However, the role of mitochondrial fragmentation is not fully understood.

Mitochondrial fragmentation is known to occur in association with mitophagy. When mitochondrial fission is chronically blocked and mitochondria become dysfunctional, levels of autophagy increase, but mitophagy is not observed, suggesting that even if mitochondria were damaged, they were probably too long to be engulfed by the autophagosome (Arnoult et al., 2005b; Parone et al., 2008). However, we reported that over-expression of a mutant of FIS1 that induces mitochondrial fragmentation, but not dysfunction, was not associated with increased autophagy and mitophagy, thus dissociating mitochondrial fragmentation *per se* from mitophagy (Gomes and Scorrano, 2008).

Shirihai and colleagues provided evidence for a selection process that filters healthy from unhealthy mitochondria. In pancreatic β-cells, mitochondria undergo frequent cycles of fusion followed by fission. Often a fission event gives rise to uneven daughter mitochondria in respect to their membrane potential: one displays high ΔΨm, the other low ΔΨm and has a reduced probability to fuse. This population of fragmented mitochondria with decreased ΔΨm and lower levels of OPA1 (that is degraded in depolarized mitochondria) is removed by autophagy. Blocking fission, however, impairs mitophagy, resulting in the accumulation of dysfunctional mitochondria (Twig et al., 2008).

In conclusion, mitophagy requires efficient fission that helps segregating the bad organelles and prepares them to fit into the autophagosomes. However, fission *per se* is not the trigger of mitophagy, for which a concomitant dysfunction of the organelle, or other yet unclear signals, are required.

3.4 Mitochondria in macroautophagy

Starvation is a known trigger of macroautophagy; during this process mitochondria do not fragment but fuse instead. Nowadays, hyperfusion represents a recognized strategy to allow survival during nutrient deprivation and cellular stress.

Stress-induced mitochondrial hyperfusion (SIMH) is independent of MFN2, BAX/BAK, and prohibitins, but requires L-OPA1, MFN1, and the mitochondrial inner membrane Stomatin-like protein 2 (SLP-2). SLP-2 is required for maintenance of L-OPA1 and subsequent mitochondrial hyperfusion. Moreover SLP-2 promotes optimal ATP production (Tondera et al., 2009). Similarly, our group and Rambold and co-workers independently reported that starvation (as well as mTOR inhibition) -induced PKA activation is responsible for DRP1 inactivation, thus leading to unopposed fusion. The resulting elongated mitochondria are spared from autophagic degradation, possess more *cristae* and display increased ATP synthase activity, thus sustaining cell survival (Gomes et al., 2011b; Rambold et al., 2011b).

Remarkably, mitochondria hyperfusion might be a temporary response that cells adopt to survive short periods of stress. For instance, when hyperfusion is triggered by apoptotic stimuli, such as UV or actinomycin D, it precedes mitochondrial fission (Tondera et al., 2009).

4. FIS1: what for? A close look to a puzzling protein

Fission 1 (FIS1) was initially discovered as a key protein regulating mitochondrial morphology and for long it has been considered as the organelle receptor for DRP1. However, this role has been recently challenged, and FIS1 seems to play a more marginal role in the fission process. Nevertheless, FIS1 has been implicated in apoptosis, mitophagy and mitochondrial fragmentation in response to hypoxia/ischemia. In the following paragraphs, FIS1 protein structures and putative functions are discussed in detail.

4.1 FIS1 structure and functional domains

FIS1 is a tail-anchored protein evenly distributed on the outer mitochondrial membrane and its structure is well conserved in yeast, human, mouse and rat (James et al., 2003; Stojanovski et al., 2003; Yoon et al., 2003).

The C-terminus of FIS1 displays the typical features found in many mitochondrial transmembrane (TM) domains, where a stretch of about 30 hydrophobic amino acids are sandwiched between positively charged arginine and lysine residues, while the last few *C*-terminal amino acids protrude into the IMS forming a very short tail (James et al., 2003; Stojanovski et al., 2004). The *N*-terminus, on the other hand, is exposed to the cytosol and carries six α-helices connected by short loops folding in hairpin-like structures forming two tetratricopeptide repeat (TRP) motifs, corresponding respectively to the α2-turn-α3 and to the α4-turn-α5 hairpins (Suzuki et al., 2003; Suzuki et al., 2005) (Figure 11). The tetratricopeptide repeat (TPR) is a degenerated 34-aa repeat, often arranged in tandem arrays with a consensus at positions 4 (W/L/F), 7 (L/I/M), 8 (G/A/S), 11 (Y/L/F), 20 (A/S/E), 24 (F/Y/L), 27 (A/S/L), and 32 (P/K/E). A single TPR motif is composed by two antiparallel α-helices packaged with a characteristic 24°-angle that generates an amphipathic channel. Both yeast Fis1p and human FIS1 fold in a very similar TRP motif, which forms a hydrophobic pocket, edged by two additional α-helices, one *C*-terminal and one *N*-terminal to the motif. However, the two proteins

display important differences at their *N*-termini. Indeed, whereas human FIS1 carries a short stretch of largely unstructured amino acids *N*-terminal to α-helix 1, yeast Fis1p carries a longer *N*-terminal domain, which folds in a flexible *N*-terminal end (Met^{1}-Phe^{6}) followed by a more rigid portion (Trp^{7}-Tyr^{18}). Interestingly, in yeast the *N*-terminal region is folded back into the hydrophobic pocket of the TRP motif and is required to induce mitochondrial fragmentation and Mdv1p recruitment in reconstituted yeast systems. It was also found out that the *N*-terminal region might undergo conformational change upon binding and exert auto-inhibitory functions (Picton et al., 2009; Suzuki et al., 2005; Tooley et al., 2011; Zhang and Chan, 2007). On the other side, the human FIS1 *N*-terminal portion is shorter, largely unstructured and lacks the residues mediating the hydrophobic interactions with the TRP motif (Suzuki et al., 2005). These structural discrepancies might explain the inability of hFIS1 to complement the phenotype of ΔFis1p yeast cells (Stojanovski et al., 2004).

```
Fis1p           MTKVDFWPTLKDAYEPLYPQQLEILRQQVVSEGG-PTATIQSRFNYAWGL
hFIS1           MEAVL--------NELVSVEDLLKFEKKFQSEKAAGSVSKSTQFEYAWCL
mFIS1           MEAVL--------NELVSVEDLKNFERKFQSEQAAGSVSKSTQFEYAWCL
                *  *          * :  ::*  :.::. ** .  :.: .::*:*** *

Fis1p           IKSTDVNDERLGVKILTDIY-KEAESRRRECLYYLTIGCYKLGEYSMAKR
hFIS1           VRSKYNDDIRKGIVLLEELLPKGSKEEQRDYVFYLAVGNYRLKEYEKALK
mFIS1           VRSKYNEDIRRGIVLLEELLPKGSKEEQRDYVFYLAVGNYRLKEYEKALK
                ::*.  :* * *: :* ::  * ::..:*: ::**::* *:* **. * :

Fis1p           YVDILFEHERNNKQVGALKSMVEDKIQKETLKGVVVAGGVLAGAVAVAS-
hFIS1           YVRGLLQTEPQNNQAKELERLIDKAMKKDGLVGMAIVGGMALGVAGLAGL
mFIS1           YVRGLLQTEPQNNQAKELERLIDKAMKKDGLVGMAIVGGMALGVAGLAGL
                **  *:: * :*:*.  *: :::. ::*: * *:.:.**:  *...:*.

Fis1p           --FFLRNKRR
hFIS1           IGLAVSKSKS
mFIS1           IGLAVSKSKS
                  : : :.:
```

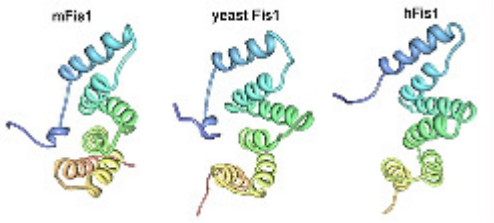

Figure 11 FIS1 sequence and structure. Upper: T-Coffee alignment of yeast, human and mouse FIS1 proteins. Lower: NMR-derived structures. PDB accession number: 1PC2 (hFIS1), 1IYG (mFIS1), 1YM8M (fis1p).

A regulatory role similar to that described for the yeast *N*-terminal domain has been proposed in human for the first 30 amino acids, corresponding to the flexible N-terminal domain plus α-helix 1 (Yu et al., 2005b). Indeed, deletion of these residues prevents induction of mitochondrial fragmentation upon overexpression of mutant hFIS1, causing appearance of swollen mitochondria, and increases the usually weak DRP1-hFIS1 binding, thus stimulating DRP1 recruitment on mitochondrial membranes (Yu et al., 2005b). The swollen mitochondria might result in part from a mitochondrial permeability transition caused by an excessive retention of DRP1 on mitochondrial membranes (Yu et al., 2005b). Conversely, the TRP motif is required for DRP1 binding and mitochondrial recruitment (Yu et al., 2005b).

The transmembrane domain and the tail are necessary and sufficient for targeting of FIS1 to mitochondria: their ablation causes cytosolic mislocalization, while a fusion between GFP and the TM domain of FIS1 has a mitochondrial localization. Additionally, when the basic amino acids in the tail are mutated, hFIS1 is mistargeted to the ER (Stojanovski et al., 2004; Yoon et al., 2003).

Interestingly, both human and rat FIS1 are reported to form oligomers on the mitochondrial membranes, and oligomerization correlates with their ability to induce DRP1 translocation and mitochondrial fragmentation, as demonstrated by mutants that disrupt the oligomers and are unable to induce fission (Jofuku et al., 2005; Serasinghe and Yoon, 2008). However, different portions of human and rat FIS1 have been shown to mediate the oligomeric assembly. In particular, whereas in human deletion of the first 30 *N*-terminal amino acids greatly increases oligomer formation and DRP1 recruitment (Serasinghe and Yoon, 2008), in rat deletion of the same *N*-terminal portion decreases oligomer formation (Jofuku et al., 2005), suggesting once again that FIS1 functions might not be fully conserved through evolution.

FIS1 is also involved in apoptosis and it has been shown that hFIS1 is a bifunctional protein which independently regulates fission and apoptosis: its first helix mediates fission, while the tail mediates apoptosis (Alirol et al., 2006).

4.2 An overview of the processes in which FIS1 has been implicated

FIS1 has been implicated in mitochondrial fragmentation, apoptosis, autophagy, Ca^{2+} homeostasis, cell senescence and cell cycle. In the following sections each of these aspects is discussed in detail.

4.2.1 Mitochondrial fragmentation and DRP1 recruitment

Initially, FIS1 levels on the OMM were reported to regulate mitochondrial fission: overexpression was shown to induce fragmentation (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003), while silencing to induce hyperfused mitochondria projecting out from the nucleus (Stojanovski et al., 2004). Moreover, FIS1 and DRP1 were reported to act in the same genetic pathway: mitochondrial fragmentation and perinuclear clustering induced by hFIS1 overexpression could be recovered by a DN $DRP1^{K38A}$ (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003). Furthermore, the physical interaction between FIS1 and DRP1 was reported to be weak, but detectable by co-immunoprecipitation and FRET experiments (Yoon et al., 2003). Additionally, the fact that DRP1 overexpression was reported to induce a modest mitochondrial fragmentation when compared to FIS1 overexpression led to the hypothesis that FIS1 was the rate-limiting step in the fission process (Yoon et al., 2003). The different distribution of FIS1 and DRP1 on mitochondrial surface, with FIS1 distributed evenly (James et al., 2003; Suzuki et al., 2003) and DRP1 in foci (Smirnova et al., 2001), was explained by postulating a third unknown protein, or a conformational change in a subset of FIS1 molecules at perspective fission sites, leading to DRP1 recruitment to specific spots (Yu et al., 2005b). Possibly, this would have also explained the finding that, whereas overexpression of FIS1 mutants lacking α-helix 1 was increasing DRP1 mitochondrial localization (Yu et al., 2005b), overexpression of wild-type FIS1 was not perturbing DRP1 cytosolic distribution (Lee et al., 2004b). However, the lack of mammalian homologues for Mdv1p (Karren et al., 2005; Naylor et al., 2005; Tieu et al., 2002) and Caf4p (Griffin et al., 2005; Schauss et al., 2006), both known mediators of Fis1p-Dnm1p interaction, was further complicating the subject. We now know that Mdv1p and Caf4p are exclusively yeast proteins and that the mammalian

system might have evolved specific sets of pro-fission proteins: indeed, both MFF and MiDs are vertebrate specific.

The discovery of MFF in 2010 and, one year later, of MiDs (discussed in detail in section 2.2.3 and 2.2.4, respectively), which have been all implicated in DRP1 recruitment and mitochondrial fragmentation, challenged the role of FIS1.

In 2010, Youle and Mihara and co-workers showed that downregulation of MFF by siRNA promotes mitochondrial elongation and reduces DRP1 mitochondrial localization, whereas FIS1 downregulation by an array of *siRNA* and *shRNA* had minor/no effect on mitochondrial morphology and DRP1 localization in HeLa (Otera et al., 2010). Similarly, MFF overexpression is a more powerful inducer of mitochondrial fragmentation than FIS1 overexpression, and overexpressed MFF is able to cause mitochondrial fragmentation in $Fis1^{siRNA}$ HeLa but not in $Drp1^{-/-}$ MEFs (Otera et al., 2010). Furthermore, they reported that downregulation of FIS1 has no impact on CCCP-induced mitochondrial fragmentation and actinomycin D-triggered cytochrome *c* release. The authors further generated two *Fis1* cKO alleles in human colon carcinoma HTC116 cell lines and observed no significant alteration in mitochondrial morphology, or in the expression levels of other mitochondrial shaping proteins. Consequently, they concluded that FIS1 is dispensable for mitochondrial fragmentation, suggesting that FIS1 role, if any, might be either regulatory downstream of MFF or different than mediating DRP1 recruitment (Otera et al., 2010).

On the other hand, the characterization of a different genetic model for *Fis1* ablation by Chan and co-workers in 2013 lead to different results (Loson et al., 2013). By using a commercially available *Fis1* gene trap allele ($Fis1^{gt}$), Chan and co-workers observed mitochondrial elongation and interconnection in $Fis1^{gt/gt}$ MEFs. Remarkably, the phenotype was less severe than that observed in *Mff* null MEFs and reintroduction of either FIS1 or MFF in null MEFs could efficiently restore mitochondrial morphology. Similarly, DRP1 mitochondrial localization was altered to a much greater extent in *Mff* null MEFs when compared to *Fis1* null cells. Remarkably, in the latter cells mitochondrial fragmentation upon several stress stimuli was reduced but still occurring, in contrast to what observed in *Mff* null MEFs. Furthermore, MEFs ablated for both factors display a more dramatic phenotype than the single null cell lines. Therefore, the authors conclude

that, although FIS1 might have a minor role in mitochondrial fragmentation, it exerts different, but partially overlapping functions compared to MFF (Loson et al., 2013).

4.2.2 Apoptosis, ER and Ca^{2+}

As already described, involvement and role of mitochondrial fragmentation in apoptosis are still matter of intense debate. As for DRP1, also FIS1 overexpression can lead to release of apoptogenic factors from mitochondria (James et al., 2003), while its downregulation can inhibit cell death (Lee et al., 2004a). However, as for DRP1, FIS1 downregulation might not be sufficient to prevent apoptosis, but only to delay it (Parone et al., 2006). Despite this controversy, FIS1 is emerging as an important factor in regulating ER-gated apoptotic signals and seems to have a role in apoptosis beyond influencing mitochondrial morphology.

By undertaking a genetic approach and treating MEFs with different apoptotic stimuli, our laboratory demonstrated that FIS1-mediated apoptosis is preceded by latent mitochondrial dysfunctions which depend on adequate $[Ca^{2+}]_{ER}$ and on Ca^{2+} release from the ER, leading to PTP and ultimately to cell death (Alirol et al., 2006). The link between ER and FIS1-mediated apoptosis was further substantiated and completed by Grimm and co-workers, who demonstrated that FIS1 and BAP31 bridge mitochondria and ER together, forming a signaling complex (ARCosome). Under apoptotic conditions, this complex constitutes a platform for pro-caspase 8 auto-activation, p20BAP31 production and subsequent Ca^{2+} release from the ER (Iwasawa et al., 2011a). Indeed, caspase-mediated cleavage of BAP31 generates an ER-attached fragment, p20BAP31, which is known to mediate rapid Ca^{2+} transmission from ER to mitochondria through the inositol-1,4,5-trisphosphate receptor (IP3R) (Breckenridge et al., 2002; Breckenridge et al., 2003; Rizzuto et al., 2012; Rizzuto et al., 2009). Remarkably, Grimm and co-workers demonstrated that signaling through the ARCosome complex is specifically initiated by FIS1, thus placing it downstream of apoptotic stimuli impinging on mitochondria.

Therefore, by putting together the findings from our laboratory, the paper from Grimm and co-workers and more general knowledge about the consequences of Ca^{2+} release from ER in the course of apoptosis, a general model can be envisioned: FIS1 mediated-apoptosis, rather than directly impinging on mitochondria, progresses from

the mitochondria to the ER, where it triggers caspase 8 activation and Ca^{2+} release; Ca^{2+} then feeds-back to mitochondria by triggering PTP, *cristae* remodeling and DRP1 recruitment.

Furthermore, both the work from our laboratory and from Grimm and co-workers demonstrate how a pro-fission protein can actually trigger apoptosis without requiring mitochondrial fragmentation. In our hands, a FIS1 mutant unable to fragment the reticulum retains the ability to induce apoptosis, and fragmentation and apoptosis are mediated by two independent domains, the first α-helix and the IMS tail respectively (Alirol et al., 2006). Similarly, Iwasawa et al. demonstrate that treatment with caspase inhibitors prevents signaling through the ARCosome, whereas this has no effect on FIS1-mediated fragmentation and that, conversely, upon treatment with apoptotic agents mitochondrial fragmentation appears after BAP31 activation (Iwasawa et al., 2011a).

Additionally, this model provides an explanation for the previous observation that BCL-XL is able to prevent FIS1-mediated apoptosis without impinging on its pro-fission activity (James et al., 2003). Indeed, it is known that BCL-XL can interact and counteract both BAP31 activation (Breckenridge et al., 2002) and Ca^{2+} release through the IP3R (Distelhorst and Bootman, 2011).

Altogether, this provides a mechanistic explanation for what previously observed by Demaurerex and co-workers (Frieden et al., 2004): the FIS1-BAP31 tether might maintain mito-ER contact despite the remodeling underwent by the mitochondrial reticulum upon FIS1 overexpression, thus ensuring normal mitochondrial Ca^{2+} uptake from the ER in FIS1-overexpressing cells (Frieden et al., 2004). Moreover, the small reduction in capacitative Ca^{2+} entry (CCE) observed upon FIS1 overexpression (Frieden et al*.,* 2004) can be viewed as a compensatory mechanism to protect cells with high FIS1 levels, since CCE its known to dictate Ca^{2+} refilling of the ER and to be inhibited by intracellular Ca^{2+} increase (Jaconi et al., 1997).

However, and not surprisingly, regardless of the source of Ca^{2+}, FIS1 overexpression decreases the speed of Ca^{2+} propagation (Frieden et al., 2004), as reported for DRP1 (Germain et al., 2005). Once again, this demonstrates the requirement for an integral mitochondrial reticulum for Ca^{2+} propagation (Frieden et al., 2004).

4.2.3 Mitophagy

A number of pieces of evidence links FIS1 to mitophagy and suggests that FIS1-DRP1 interaction might be important in mitochondrial disposal through autophagy.

The first piece of evidence emerged in 2008, when our lab demonstrated that FIS1 overexpression leads to accumulation of autophagic markers (Gomes and Scorrano, 2008). Remarkably, by using FIS1 mutants that dissociate its ability to fragment mitochondria from its detrimental action on mitochondrial functions, we could demonstrate that mitochondrial dysfunctions, rather than fragmentation *per se*, are responsible for autophagy induction (Gomes and Scorrano, 2008).

Another piece of evidence comes from three papers published more recently by Mihara and Ishihara (Onoue et al., 2013) and Youle and van der Bliek (Shen et al., 2014; Yamano et al., 2014), which, put together, might provide a mechanistic model for FIS1-mediated mitophagy.

In 2013 Mihara and Ishihara demonstrated that FIS1 acts as a mitochondrial receptor for TBC1D15 (Onoue et al., 2013). TBC1D15 is a GTPase-activating protein (GAP) which blunts Rab membrane trafficking activity by stimulating its GTPase activity. Indeed, FIS1 seems to be responsible for TBC1D15 mitochondrial recruitment through its TRP motif and this interaction is important for a balanced mitochondria morphology, as demonstrated by the mitochondria elongation caused by TCB1D15 RNA*i* (Onoue et al., 2013). However, how a GAP could play a role in mitochondria fragmentation was left unsolved.

Youle and van der Bliek and co-workers in 2014 reported that *Fis1*$^{-/-}$ cells display LC3 cleavage under basal conditions and large LC3 aggregates upon mitophagy induction (Shen et al., 2014). Since the formation of these aggregates depends on Parkin, but Parkin clearance, Parkin substrate degradation and p62 degradation are unaltered in *Fis1*$^{-/-}$ cells compared to their wildtype counterparts, even in the presence of bafilomycin, Shen et al. concluded that *Fis1* ablation causes a slowing down of a late stage of mitophagy before lysosome fusion, thus resulting in a temporary accumulation of LC3 aggregates. Interestingly, overexpression of DN DRP1^{A38K} suppresses the formation of LC3 aggregates, and, upon autophagy induction, DRP1 switches its binding

partner on mitochondria from MFF to FIS1, as demonstrated by coimmunoprecipitation. Remarkably, ER proteins, such as BAP31 and calnexin, also enter in this complex, and DRP1 phosphorylated at Ser^{600} (corresponding to Ser^{616} in hDRP1 variant 1) is specifically recruited. However, ablation of FIS1 does not prevent interaction of DRP1 with ER proteins in course of autophagy (Shen et al., 2014).

The same laboratories reported that also *TBC1D15*$^{-/-}$ cells display defective mitophagy and large LC3 aggregate accumulation similar to what they previously observed in *Fis1*$^{-/-}$ cells (Yamano et al., 2014). Remarkably, they did not report any significant morphological alterations in the mitochondrial network, in opposition to what Onouse et al. previously described (Onoue et al., 2013). In *TBC1D15*$^{-/-}$ cells LC3 aggregates appear as long interconnected structures edging mitochondria and partially co-localizing with microtubules (Yamano et al., 2014). By exploiting a mutational approach combined with coimmunoprecipitation, the authors demonstrated that, in course of autophagy, FIS1 recruits TBC1D15 or its homologue TBC1D17 as homo/heterodimer(Yamano et al., 2014). In turn, TBC1D15 can interact with Atg8-family proteins, thus bridging together the dysfunctional mitochondrion and the nascent autophagosome membrane. The authors identified Rab7, previously involved in the biogenesis of the autophagosome, as the target for TBC1D17 GAP activity during autophagosome formation. Indeed, siRNA for Rab7 completely suppresses the abnormal LC3 accumulation in both *TBC1D15*$^{-/-}$ and *Fis1*$^{-/-}$ cells, and reintroduction of a catalytic inactive TBC1D17 in *TBC1D15*$^{-/-}$ cells fails to rescue abnormal LC3 aggregates (Yamano et al., 2014).

Altogether, a general model can be envisioned. Upon mitophagy induction, DRP1 switches its binding partners and forms a complex with FIS1 and ER proteins. This step seems to be regulated by DRP1 phosphorylation on $Ser^{600/616}$. Simultaneously, FIS1 recruits TBC1D15/17 which exerts a double action. By binding with Atg8-related proteins, it allows docking of the mitochondrion to the isolation membrane. In parallel, through its GAP activity, TBC1D15 inhibits Rab7, thus preventing excessive autophagosome growth and leading to autophagosome wrapping around the mitochondrion. The fact that DRP1 forms a complex with FIS1 and ER proteins goes along with the idea that ER might mark the perspective fission sites and regulate DRP1 activity, as discussed in 2.2.1.6.

Another paper supporting an involvement of FIS1 in mitophagy was published by Piacentini and co-workers and proposes a model where translutaminase 2 (TG2) interacts both with FIS1 and DRP1 to prevent mitophagy (Rossin et al., 2015). TG2 is a Ca^{2+}-dependent transaminase that, by catalyzing the formation of an isopeptide bond involving the side chains of glutamine and lysine residues, causes protein crosslinking. Ablation of TG2 causes accumulation of LC3 and of ubiquitinated proteins aggregates. Piacentini and co-workers recently showed that TG2 interacts *in vitro* both with FIS1 and DRP1 and that this interaction decreases during autophagy, as suggested by time-point crosslinking experiments on CCCP-treated MEFs. Additionally, TG2 ablation leads to defective autophagy, as suggested by the accumulation of mitochondria and PINK/Parkin mitochondrial substrates in TG2 KO MEFs upon CCCP treatment. Therefore, they proposed a model in which TG2 usually interferes with DRP1 binding to FIS1, thus preventing mitochondrial fragmentation and autophagy, whereas ablation of TG2 allows DRP1-FIS1 interaction leading to mitochondrial fragmentation and defective autophagy (Rossin et al., 2015). Why and how then this FIS1-DRP1 interaction in the absence of TG2 leads to impaired mitophagy and not just to sustained mitochondrial clearance still needs to be clarified.

Additionally, FIS1 was shown to be a target of DJ1/PARK7 (Zhang et al., 2012), a chaperone-like protein mutated in familiar forms of Parkinson and implicated in regulating mitochondrial morphology and autophagy in a PINK/Parkin-independent fashion. Indeed, FIS1 protein levels are decreased both in cells overexpressing DJ1 and in the *substantia nigra* of DJ1 transgenic mice. It seems the DJ1, through activation of AKT, leads to RING-finger protein 5 (RNF5, also known as RMA1)-mediated ubiquitination of FIS1 thus promoting its proteasomal degradation. Interestingly, AKT seems to promote RNF5 mitochondrial localization, rather than phosphorylating it (Zhang et al., 2012). The functional implication of this ubiquitination are still to be defined. For instance, it is not known whether this pathway simply regulates FIS1 levels inside the cells or mediates removal of dysfunctional mitochondria through autophagy, as described for PINK/Parkin mediated ubiquitination of DRP1 .

4.2.4 Hypoxia/ischemia

In 2011 Kim et al. provided evidence for the physiological relevance of FIS1-DRP1 interaction in modulating mitochondrial fragmentation in response to hypoxia/ischemia both *in vitro* and *in vivo(Kim et al., 2011)*. Mechanistically, hypoxia induces mitochondrial fragmentation by promoting the ubiquitin-dependent degradation of A-kinase anchoring protein 121 (AKAP121). Under normal conditions, AKAP121 promotes PKA-mediated inhibitory phosphorylation of DRP1 and prevents DRP1-FIS1 interaction. Under hypoxia, Siah2-mediated degradation of AKAP121 relieves this inhibitory effect and ultimately leads to mitochondrial fragmentation (Kim et al., 2011). Consequently, mitochondrial fragmentation under hypoxia is prevented in *Siah* null MEFs. Similarly, down regulation of Siah or DRP1 in cardiomyocites reduces mitochondrial fragmentation and protects from ischemia-induced cell death. These effects are paralleled *in vivo* by a reduction of the infarct size and cell death in a mouse model of cardiac infarct ablated for *Siah* when compared to wild type mice (Kim et al., 2011).

4.2.5 Cellular senescence and cell cycle

As demonstrated for DRP1, FIS1 might impinge on cell cycle progression through regulation of mitochondrial fragmentation. Indeed, Cho and co-workers demonstrated that FIS1 donwregulation causes cell cycle arrest (Lee et al., 2014) and cellular senescence (Lee et al., 2007).

Cells treated with $Fis1^{shRNA}$ fail to enter mitosis and display cell cycle arrest at the G2/M transition, as well as downregulation, at the mRNA level, of key regulators of cell cycle progression such as cyclin A and B, CDK1, polo-like kinase (PLK1) and aurora kinase A. Interestingly, cell cycle can be resumed by overexpression of PLK1 or FoxM1, which are both known transcriptional regulators of the cell cycle (Lee et al., 2014). Consequently, arrested cells might undergo cell senescence, as FIS1 downregulation increases β-galactosidase activity (Lee et al., 2007). The authors hypothesized that reactive oxygen species (ROS), whose production is increased upon FIS1 downregulation (Lee et al., 2007; Lee et al., 2014), act as the retrograde mediators of the cellular response to reduced FIS1 levels, possibly through DNA damage and activation of the G2/M checkpoint (Lee et al., 2014).

4.3 FIS1: what next?

More than ten years after its discovery, FIS1 still remains an enigmatic protein and its function as mitochondria shaper remains elusive. The low degree of sequence identity and structure conservation between yeast and mammals, along with the evolution of additional receptors for DRP1, makes it difficult, if not impossible, to deduce FIS1 functions in mammals on the basis of what observed in lower eukaryotes. The existence of several splicing variants in mammals both for DRP1 and MFF further complicate this scenario.

So far, the two mammalian models of *Fis1* ablation led to different conclusions. On one side, Otera et al. generated two *Fis1*$^{-/-}$ alleles in HTC116 cells. These cells display no obvious mitochondrial alteration, but defective mitophagy. On the other side, by using a commercially available *Fis1* gene trap allele, Loson et al. generated *Fis1*$^{gt/gt}$ MEFs, which display reduced mitochondrial elongation when compared to *Mff*$^{-/-}$ MEFs. Taken together, the effect of *Fis1* ablation might be influenced by the cell type and might not recapitulate an *in vivo* physiological situation, as in the case of a mouse model. Furthermore, the strategy used to ablate *Fis1* gene might also play a key role. For instance, a closer look at Otera at al., 2010 reveals that the complex targeting strategy adopted actually does not alter much the *Fis1* genomic *locus*, leaving the majority of the exons unperturbed and impinging mainly on exon 2 and its two flanking introns. Accordingly, 5'RACE PCR in their *Fis1*$^{-/-}$ HTC116 cell lines could detect some mRNAs resulting from the alternative splicing of the exons left in the genomic *locus*. The authors declare these mRNAs non-functional, without, however, verifying this assumption. One might therefore conclude that the lack of phenotype reported by Otera and co-workers could actually be due to uncomplete ablation of FIS1 protein, which, through the existence of some splicing variants expressed at low levels and/or not detectable by western blot using the common antibodies, could still exert some pro-fission functions.

The fact that FIS1 can recover mitochondrial morphology in *Mff*$^{-/-}$ MEFs (Loson et al., 2013) would suggest that it actually has a role in mitochondrial shaping. Remarkably, it has been proposed that FIS1 is required in response to specific stimuli. Kim et al., 2011

proposes FIS1-mediated fragmentation as specifically induced by hypoxia/ischemia. On the other hand, both Loson et al., 2013 and Shen et al., 2014 report that *Fis1*$^{-/-}$ MEFs fragment to an extend comparable to wild-type MEFs when treated with CCCP. Conversely, Shan et al., 2014 reports that antimycin A, PMA and staurosporin increase DRP1 binding to FIS1. It remains to be explained how CCCP and antimycin A, both inducing mitophagy, actually differentially impinge on FIS1.

An intriguing hypothesis is that rather than participating to specific fission events, FIS1 might be the last step of a process leading to mitochondrial fragmentation both under basal and induced conditions. Given its role in tethering mitochondrial and ER, the role of ER in marking perspective fusion sites and in regulating DRP1 pro-fission activity, and given that DRP1 seems to exists in an equilibrium between cytosol and mitochondria, it is tempting to speculate that MFF might dock DRP1 in equilibrium with the cytosolic pool and then, before fission starts, MFF might provide FIS1 with DRP1 oligomers already *in situ*. This would explain why FIS1-DRP1 interaction is not easily detected under basal condition, and why *Fis1*$^{-/-}$ cells have a milder mitochondrial phenotype compared to *Mff*$^{-/-}$ cells. Indeed, preassembled DRP1 oligomers might directly bind lipids (cardiolipin) or be assisted by other factors (both from mitochondria and ER) when FIS1 is ablated.

Furthermore, it would be interesting to verify the existence of *Fis1* splicing variants and assess their functions. Indeed, the presence of different functional domains in DRP1 splicing variants might underline some splicing-specific requirements which might be fulfilled by different proteins (MFF versus FIS1), or by different splicing variants of the same protein.

Besides the mechanistic aspect of FIS1-mediated fragmentation, its (patho)physiological relevance is still far from been clarified. No human pathology so far has been directly linked to a mutation in *Fis1* gene and FIS1-mediated mito-ER tether has not been investigated further.

In summary, this calls for a systematic analysis of FIS1 function *in vivo*. To this end, the generation of a suited model for the conditional ablation of *Fis1* is highly required. Only the combination of *in vivo* (mouse model) and *in vitro* (cell lines) models will help to clarify FIS1 functions.

Section III - Results

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

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 the expression of both human and mouse *Fis1* genes is controlled by multiple splicing. In particular, an exon skipping event of conserved exon 2 dictates the preferential expression of variant 2 versus the canonical variant 1 in both species. Remarkably, a non-canonical START codon seems to be responsible for transcription of human variant 2 (*hFis1*.2). The resulting protein, although lacking only the first 18 amino acids triggers mitochondrial elongation when overexpressed in mouse. Upon starvation, *mFis1* 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 and Schatz, 1988; McBride et al., 2006a; Saraste, 1999). Moreover, mounting evidence indicate that they regulate cell signaling, Ca^{2+} homeostasis, cell cycle regulation, differentiation, cell death and aging (Green and Kroemer, 2004; Kroemer et al., 2007a; 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., 2008b; Chan, 2006; Dimmer and Scorrano, 2006; Frezza et al., 2006b; Gomes et al., 2011a; Hoppins et al., 2007b; Ishihara et al., 2013; Liesa et al., 2009; Okamoto and 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., 2008b; Chan, 2006; Chen et al., 2003a; Dimmer and Scorrano, 2006; Eura et al., 2003b; Frezza et al., 2006b; Gomes et al., 2011a), 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., 2006b). Mitochondrial fission requires the translocation of dynamin-related protein 1 (DRP1) from the cytosol to the mitochondria to induce fission (Smirnova et al., 1998a; Westermann, 2010; Yoon et al., 2001b). Cytosolic DRP1 is dephosphorylated by calcineurin to translocate to mitochondrial outer membrane (MOM) (Cereghetti et al., 2008b), while cyclic AMP-dependent protein kinase (PKA) phosphorylates DRP1 to

inhibit fission (Chang and Blackstone, 2007b; Cribbs and Strack, 2007b). Classically, mitochondrial Fission 1 (FIS1) was believed to be required for DRP1 docking on mitochondrial membranes. FIS1 is a tail-anchored protein evenly distributed on the outer mitochondrial membrane and composed by a C-terminus transmembrane (TM) domain and two cytosolic tetratricopeptide repeat (TRP) motifs (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003). FIS1 has been proved to mediate mitochondrial-ER tethering, ER-gated apoptosis, autophagy and ischemia/hypoxia-induced fragmentation (Alirol et al., 2006; Iwasawa et al., 2011a; Kim et al., 2011; Shen et al., 2014; Yamano et al., 2014). However, the discovery of MFF and MiD49/50 along with the finding that both DRP1 recruitment and mitochondrial fragmentation take place also in two models of *Fis1 in vitro* ablation led to partially discharge FIS1 involvement in mitochondrial fission (Loson et al., 2013; Otera et al., 2010; Palmer et al., 2013). Furthermore, the existence of yeast-specific DRP1 adaptors, such as Mdv1p and Caf4p (Griffin et al., 2005; Karren et al., 2005; Naylor et al., 2005; Schauss et al., 2006; Tieu et al., 2002) and the evolution of vertebrate specific proteins, such as MiD49/50, makes it difficult, if not impossible, to deduce FIS1 functions in mammals on the basis of what observed in lower eukaryotes.

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, both human and mouse *Fis1* genes undergo multiple splicing resulting in the production of variants with opposite effects on mitochondrial shape in resting and starved cells.

RESULTS

Fis1 gene is alternatively spliced both in mouse and in human

Since splicing variants are reported both for DRP1 and MFF, and, at least for DRP1, they are described to exert distinct functions (Macdonald et al., 2016; Strack et al., 2013; Uo et al., 2009; Yoon et al., 1998), we speculated on *Fis1* splicing variants. Indeed, we hypothesized that the existence of numerous splicing variants for MFF and DRP1 might have rendered the fission process at least partially splicing-specific, possibly in a tissue/time-dependent manner. These considerations prompted us to investigate for *Fis1* alternative splicing. Bioinformatic analysis revealed that *Fis1* genomic *locus* is composed by seven exons in mouse and nine in human and that a number of splicing variants are actually annotated. In order to verify their existence, we designed and performed nested multiple PCRs on cDNA exacts from MEFs and human cell lines by using a common reverse primer annealing in the last exon (Ex7RV for mouse and EX9RV for human) and exon-specific forward primers (Figure 1A and 1B). Remarkably, in order to unveil possible tissue-specific variations, we included in our analysis two different human cell lines, breast cancer MDA-MB-231 and HeLa. Indeed, besides the canonical cDNAs, which we named *mFis1.1* and *hFis1.1*, all primers successfully amplified cDNA stretches of different sizes (Figure 1A and 1B). Interestingly, the band pattern was conserved in both the human cell lines analyzed. Based on the dimensions of the amplified cDNA fragments and the exons predicted sizes, along with sequencing of some of the amplicons, we could identify the splicing events responsible for the production of the amplified fragments. Remarkably, we could establish that no alternative splicing was occurring at the 3' of *Fis1* gene. Indeed, exons 5-6-7 in mouse and 7-8-9 in human, known to code for the TM domain and to be conserved between the two species, were always spliced together. On the other hand, exons 1, 2, 3 and 4, known to be conserved

between mouse and human (Figure 1C), were alternatively spliced, producing a similar pattern in the two species (Figure 1A and 1B). Interestingly, exons 2 and 3 are known to code for helixes α1, α2, α3 and α4 and the observation of alternative splicing between these exons and previously unreported exon 1 suggested us the intriguing possibility of splicing-specific functions in the N-terminal cytosolic αhelixes.

A *Fis1* variant 2, which lacks the N-teminal arm is produced by exon skipping in mouse and human and by a non-canonical START codon in human.

Two of the mouse splicing variants that we identified as *mFis1.2* and *mFis1.3* were also reported in Ensamble (ENSMUST00000019198 for *mFis1.1* and ENSMUST00000111094 for *mFis1.2*) and UCSC Genome Browser (uc009abf.2 for *mFis1.1* and uc012eeu.1 for *mFis1.2*) databases as protein coding. Moreover, our PCR analysis suggested the existence of human counterparts, which we named *hFis1.2* (AK311665 and BG473763 in UCSC Database) and *hFis1.3* (AK310969 in UCSC database) (Figure 2A). To our surprise, however, these variants were poorly annotated in the databases, with no information with respect to the coding sequence and the START/STOP codon. Therefore, we decided to scan the hypothetical mRNA sequences for open reading frames. Since only the first exon is variant-specific, while the rest of the mRNAs is shared, we assumed that the STOP codon might have been conserved too. Therefore, we aligned putative variant mRNAs with *hFis1.1* and by, walking backward along the mRNAs from the STOP codon, we identified a putative START codon. Next, we verify this *a posteriori* reading frame by *in silico* translation. However, to our surprise, only frame +3 of *hFis1.2* could be translated: all the other frames for both variants were abortive due to the presence of multiple STOP codons. Therefore, we hypothesized that *hFis1.2* might be translated

from a non-ATG codon. Indeed, non-canonical START codons are reported to be responsible for the translation of a growing number of alternative reading frames, in some cases produced by alternative splicing, in other cases within the main open reading frame itself (Haimov et al., 2015). In the case of *hFis1.2* a TGA codon emerged as responsible for translation (Figure 2A).

As we expected, the proteins codified by the splicing variants share a great degree of sequence identity and differ only for the first 18 amino acids which in the principal variants mFIS1.1 and hFIS1.1 compose the N-terminal arm and the first half of helix α1 (Figure 2B). Remarkably, whereas in yeast the N-terminal arm folds back into the hydrophobic pocket of the TRP motif, in mouse and human the N-terminal arm is shorter, flexible and lacks the residues mediating the interaction with the TRP motif, and, along with helix α1, is reported to be required for FIS1-mediated fragmentation (Picton et al., 2009; Suzuki et al., 2005; Tooley et al., 2011). Both human and mouse variants 2, although to a different extend, seem to lack the N-terminal arm and part of helix α1, whereas mouse variant 3 is devoid also of helixes α2 and α3 both folding into the first TRP repeat (Figure 2B).

In order to verify that the predicted variants are actually translated into proteins, and in order to unveil possible tissue specific-expression pattern, we performed qPCR on total RNA extracts from different mouse tissues and compared the relative expression levels of variant 2 versus the principal isoform. We could observe that, although at very low levels, *mFis1.2* was expressed in all the tissues, but was significantly enriched in the brain. Next, we performed western blot analysis on tissue extracts and, indeed, we could confirm the presence of a band of the expected molecular weight in the liver and in the brain. Remarkably, mFis1.2 appeared to be highly expressed in the brain, thus confirming at the protein level what we observed at the mRNA level. Remarkably in the

muscles, appearance of a lower molecular weight band suggested that also *mFis1.3* could be expressed in that tissue.

Mouse *Fis1* variants exert opposite effects on mitochondrial length.

To address the subcellular localization of the mouse isoforms, each N-terminal V5 tagged mFIS1 variant was expressed in MEFs together with dsRED targeted to mitochondria (mtRFP) or to the endoplasmic reticulum (ER-RFP). Confocal microscopy indicated that all three mFIS1 variants strongly localized at mitochondria with a moderate colocalization with the ER (Fig. 3A and B). This subcellular distribution is in agreement with mFIS1 being an outer mitochondrial membrane protein and to interact with ER-resident protein Bap31 (Iwasawa et al., 2011b). MG132-mediated proteasome inhibition increased the expression of mFIS1.3 suggesting that this variant is instable and most likely a byproduct of *mFis1* gene expression (Fig. 3E). Interestingly, ectopic expression of either *mFis1*.1 or or *mFis1.3* in the absence or presence of MG132 triggered mitochondrial shortening, whereas *mFis1.2* overexpression caused an unexpected mitochondrial elongation (Fig. 3D and E). Thus, *mFis1.2* at a major difference from *mFis1.1* or *mFis1.3* causes mitochondrial elongation.

***mFis1* variant 2 is upregulated upon starvation.**

Since increased *Fis1.2* levels are associated with mitochondrial elongation, we tested whether changes in *mFis1.2* level could play a role in mitochondrial elongation observed during macroautophagy (Gomes et al., 2011a). Semiquantitative real time PCR indicated that under starvation, m*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, *mFis1.1* and *mFis1.3* mRNA levels did not change significantly under all the conditions tested (Fig. 4A). These results were further recapitulated by qPCR using specific primers for *mFis1.1* and *mFis1.2* (Fig 4 B and C). Interestingly, the increase in *mFis1.2* levels was reversible upon readdition of nutrient rich media as testified by qPCR (Fig. 4D-F). Thus the expression of *mFis1.2* 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* Ser^{637} phosphorylation and causes mitochondrial elongation (Cereghetti et al., 2008b; Gomes et al., 2011a). Since *mFis1.2* mRNA expression is upregulated upon starvation, we tested whether PKA plays a role in *Fis1* induction. The chemical PKA activator forskolin induced an up-regulation of both 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 *mFis1.2* 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 *mFis1.2* expression level during starvation. PKA can modulate alternative splicing by acting on the splicing factor hnRNP K and U2AF65 (Cao et al., 2012; Naro and 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 *mFis1.1* over *mFis1.2* (Fig. 5B). We therefore tested the effect of U2AF65 silencing on starvation-induced *mFis1.2* upregulation. Upon efficient knockdown of U2AF65 (Fig. 4D), the starvation-

induced upregulation of *mFis1.2* mRNA was reduced (Fig. 4E) while expression of *mFis1.1* mRNA was not affected (Fig. 5F). Together this indicates that during starvation PKA regulates *mFis1.2* expression by impinging on the splicing factor U2AF65.

***mFis1* variant 2 is required for starvation-induced mitochondrial elongation.**

Since we could efficiently and specifically silence *mFis1.2* expression (Fig. 5E and F), we investigated the functional significance of starvation-induced *mFis1.2* upregulation. Silencing of *mFis1.2* significantly inhibited starvation-induced mitochondrial elongation (Fig. 5G and H). Moreover, the indirect modulation of *mFis1.2* expression through U2AF65 silencing also resulted in a significant reduction in starvation-induced mitochondrial elongation (Fig. 5H and H). These results indicate that during starvation PKA also acts on mitochondrial morphology by upregulating the profusion *mFis1* variant.

DISCUSSION

During macroautophagy, mitochondria elongate in a PKA, DRP1-dependent manner. Here we identify mouse FIS1 alternative splicing as an additional target of PKA-induced autophagic mitochondrial elongation. *mFis1* is alternatively spliced in three variants with opposite effects on mitochondrial length. Ectopic expression of *mFis1* variant 1 and 3 triggers mitochondrial fragmentation while that of variant 2 is profusion. The fact that expression of *mFis1.1* is pro-fission is in accordance to the reported effect of human Fis1 overexpression . Interestingly, overexpression of mouse *mFis1.3* that lacks half of the TPR motif, still induces mitochondrial fragmentation. 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., 2005a). *mFis1.2* is preferentially expressed in the brain, suggesting that increased expression of this variant could maintain a developed mitochondrial network to meet specific energy requirements.

Of note, starvation of cultured cells induces a 10 fold upregulation of *mFis1.2* 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., 2011a; Rambold et al., 2011a). Here we found that PKA triggers the upregulation of FIS1.2 expression by regulating its alternative splicing (Cao et al., 2012; Naro and 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 *mFis1.2* 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 *mFis1* exon 1.

Interestingly, the direct silencing of *mFis1.2* 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., 2008b; Gomes et al., 2011a), but also through the upregulation of the profusion *mFis1* variant 2 expression. Moreover, the fast induction of *mFis1.2* upon starvation coupled with the fast repression of *mFis1.2* when the cells sense nutrient-rich condition again, suggests that the alternative *mFis1* splicing described here could represent a fast tunable mechanism to adjust mitochondrial morphology to cellular environment.

The fact mFIS1.2, with such peculiar and specific functions compared to its principal isoform, has past unobserved it is most likely due to the reduced difference in molecular weight with regard to the canonical protein. Indeed, as we experienced, standard western blot techniques fail in detecting endogenous mFIS1.2.

The splicing event responsible for the production of mouse variant 2 seems to be conserved also in human. Indeed we identified a putative hFis1.2 counterpart that is produced by alternative splicing of conserved exon 1 versus conserved exon 2 and that seems to share the same structural properties (e.g. absence of N-terminal arm and part of the helix 1α) with the counterparts mouse variant *mFis1.2*. Remarkably, the human

variant seems to be produced by an alternative starting codon. Further characterization of human variant 2 will be required.

In conclusion, our results indicate that on the analysis of FIS1 pro-fission function should take into consideration the divergent functions of its different variants on mitochondrial morphology. It is conceivable that also human variant 2 might display a similar effect on mitochondrial morphology and might be regulated in a PKA-dependent manner thus participating in the cellular response to macroautophagy.

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.

In order to assess *Fis1* splicing, total RNA was extracted with Trizol from the indicated cell lines and retrotranscribed. An array of primers annealing in the different exons of both mouse and human *Fis1* were used to amplified by standard PCR the cDNA produced as described. The complete list of primers used is supplied in Table 1 in Supplementary Materials. *mFis1* splicing variants were amplified from WT MEFs mRNA

samples and cloned in frame to N-terminus V5 tag pcDNA3.1/nV5-DEST using the gateway system with specific forward primers for variant 1 (5'-tgtggcccagtagagacctt-3'), variant 2 (5'-agcgtgctttctgtaacgcct-3') and variant 3 (5'-agtgcctgtagaaaacagctct-3') together with a reverse primer common to the three isoforms (5'-caggatttggacttggagaca-3'). All constructs were verified by sequencing. Fis1 variant 2 shRNA oligos forward strand 5'aaaaaaggatccgagacgaagctgcaaggaattttcaagagaaattccttgcagcttcgtctctttttgcggccgcgaattcaaaaaa-3' and reverse strand 5'aaaaaagaattcgcggccgcaaaaaagagacgaagctgcaaggaatttctcttgaaaattccttgcagcttcgtctcggatccaaaaaa-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.

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 uM 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 anti-V5 antibody (1:200), followed by incubation with

the anti-mouse 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 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.

For detection of endogenous mFIS1 variants 30 µg of tissue lysates were load on home-made 14% Tris-tricine gel acrylamide, as described in Schägger, Nat Protocols 2006.
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 *mFis1* splicing variants in different mouse tissues, three BL6 mice were sacrificed and total RNA 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'-acccacactgtgcccatctac-3' and 5'-agccaagtccagacgcagg-3'). qPCR was performed in a StepOne Plus thermocycler (Applied Bioscience) using Power Sybr Green PCR master mix and primers for *mFis1.1* (5'- tagtgtgaggctttcagggg-3' and 5'-ggacacagaaccagctgcc-3'), *mFis1. 2* (5'-gctgtcatgagaacatcctcg-3' and 5'-ggacacagaaccagctgcc-3'). As a control the same primers for β-actin were used.

To address the role of PKA in the *mFis1* 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 *mFis1* 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.

ACKNOWLEDGEMENTS

LS is a Senior Scientist of the Dulbecco-Telethon Institute. Supported by Telethon-Italy GGP12162, ERC FP7-282280, FP7 CIG PCIG13-GA-2013-618697, Italian Ministry of Research FIRB RBAP11Z3YA_005, Swiss National Foundation 31-118171 and CRSII3_132396 (to LS) and by Swiss National Foundation Ambizione SNSF PZ00P3_126710/1 (to DM).

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FIGURE LEGENDS

Figure 1. *Fis1* gene is alternatively spliced both in mouse and in human.

(A) PCR analysis of *mFis1* splicing. PCRs on cDNA extracted from wildtype MEFs performed with the primers depicted on the left. Schematic of *mFis1* exons and the amplicons retrieved by PCR.

(B) PCR analysis of *hFis1* splicing. PCRs on cDNA extracted from wildtype MEFs performed with the primers depicted on the left. Schematic of *hFis1* exons and the amplicons retrieved by PCR.

(B) Schematic of mouse and human *Fis1* gene alignment. The exons conserved and used by the principal isoforms are indicated in blue.

Figure 2. FIS1.2 lacking the N-terminal arm is produced by exon skipping and non canonical START codon.

(A) Schematic of mouse and human *Fis1* splicing alignment. The exons used be the single variants are depicted in blue (upper panel). Nucleotide sequence of the alternative starting codon GAA (in blue) and its flanking region. The canonical starting codon is indicated in italics (lower panel).

(B) Multiple T-Coffee alignment of predicted human and mouse variants. Amino acids conserved at the N-terminal are indicated in blue, whereas variant-specific amino acid are indicated in red. Black boxes indicate αhelixes, whereas orange box indicates the TM domain.

(C) qPCR analysis of the relative abundance of *mFis1.2* versus *mFis1.1* in the indicated organs. Data are mean±SE, n=3.

(D) Representative image of western blot analysis of total extracts from the indicated organs.

Figure 3. Mouse Fis1 variant 2 induces mitochondrial elongation.

(A) V5-tagged mouse FIS1 variants 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. Proteinlysates were separated by SDS-PAGE and immunoblotted using the indicated antibodies. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ 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). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ in a 2 way ANOVA with a Tukey's posttest.

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 *mFis1.1* (B) and *mFis1.2* (C) was analyzed by qRT-PCR. Data are mean±SEM of 3 independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ 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 *mFis1.1* (D) and *mFis1.2* (E)

was measured at the indicated times by qRT-PCR.

(F) Cells were treated as in D and C and the fold change in *mFis1.1* and *mFis1.2* 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 *mFis1.1* (A) and *mFis1.2* (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 *mFis1.1* (C) and *mFis1.2* (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 ($U2AF^{shRNA}$) (E and G) or against *mFis1.2* ($mFis1.2^{shRNA}$) (F and G) and the mRNA expression level of U2AF65 (E), *mFis1.2* (F), *mFis1.1* (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 $mFis1.2^{shRNA}$ or $U2AF^{shRNA}$ 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.

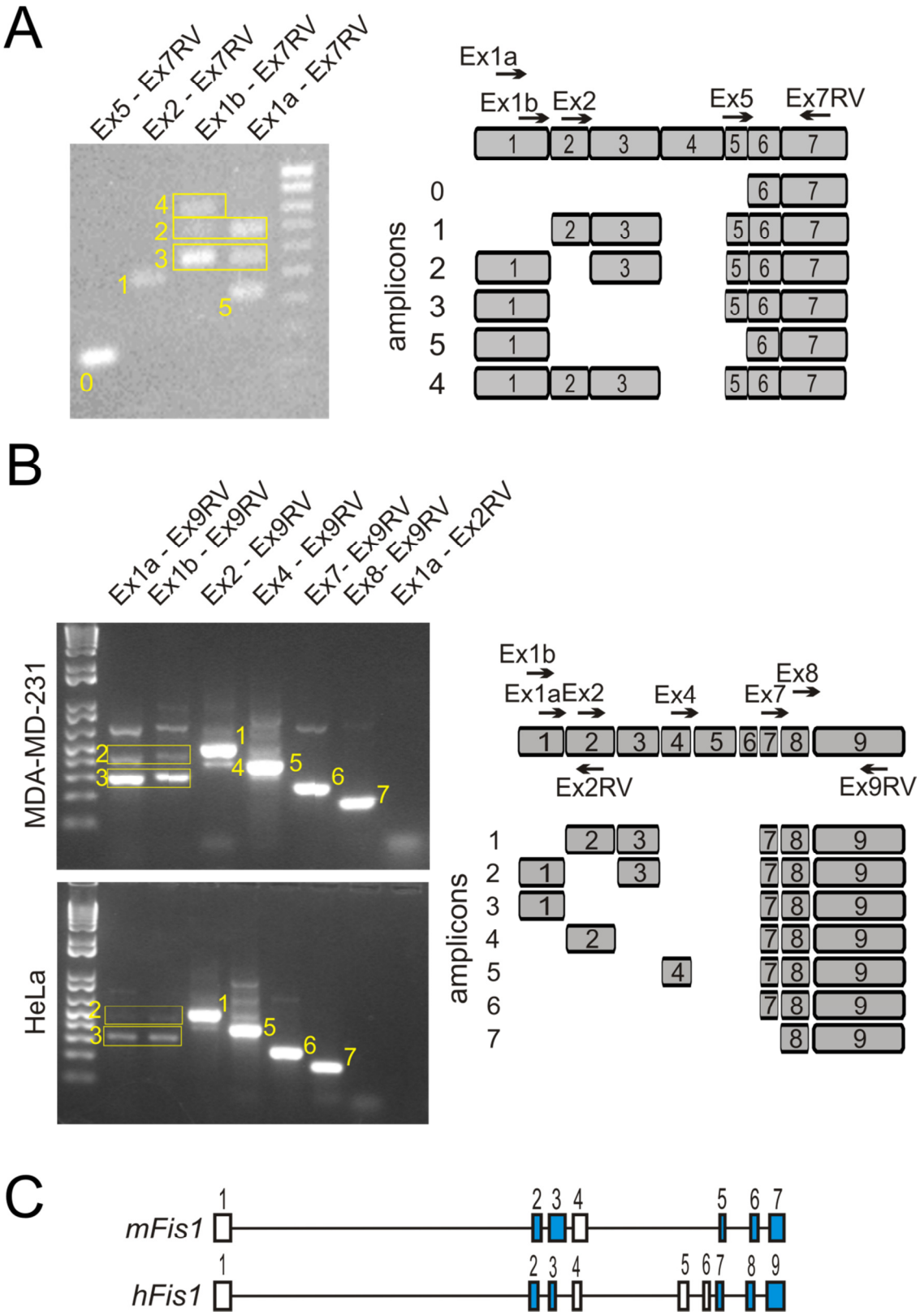
A
Ex5 - Ex7RV
Ex2 - Ex7RV
Ex1b - Ex7RV
Ex1a - Ex7RV
Ex1a
Ex1b
Ex2
Ex5
Ex7RV
amplicons
B
Ex1a - Ex9RV
Ex1b - Ex9RV
Ex2 - Ex9RV
Ex4 - Ex9RV
Ex7 - Ex9RV
Ex8 - Ex9RV
Ex1a - Ex2RV
MDA-MD-231
HeLa
Ex1b
Ex1a
Ex2
Ex4
Ex7
Ex8
Ex2RV
Ex9RV
amplicons
C
mFis1
hFis1

Figure 1

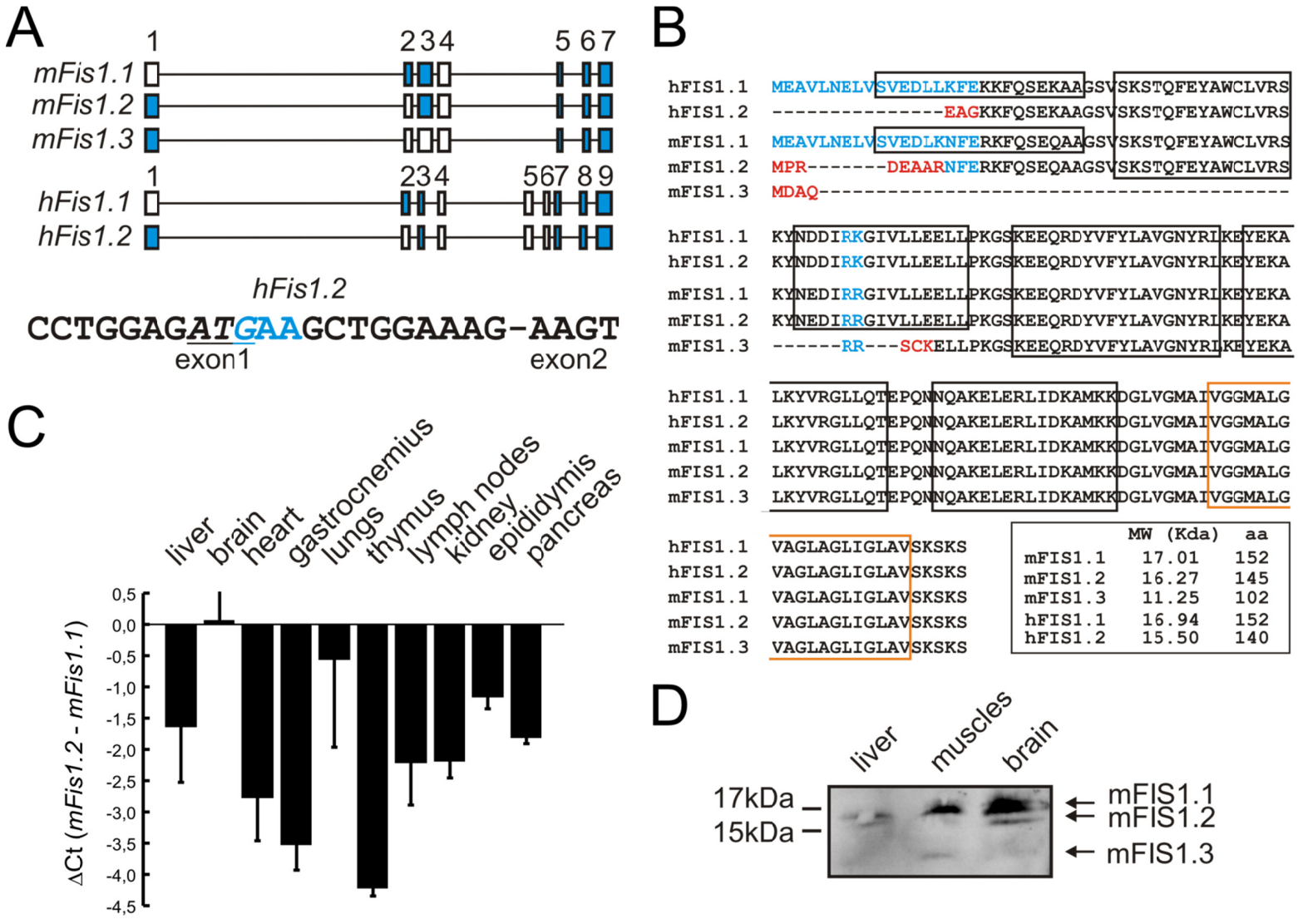
A
1
2 3 4
5 6 7
mFis1.1
mFis1.2
mFis1.3
1
2 3 4
5 6 7 8 9
hFis1.1
hFis1.2
hFis1.2
CCTGGAGATGAAGCTGGAAAG-AAGT
exon1
exon2
B
hFIS1.1 MEAVLNELVSVEDLLKFEKKFQSEKAAGSVSKSTQFEYAWCLVRS
hFIS1.2 ----------EAGKKFQSEKAAGSVSKSTQFEYAWCLVRS
mFIS1.1 MEAVLNELVSVEDLKNFERKFQSEQAAGSVSKSTQFEYAWCLVRS
mFIS1.2 MPR-------DEAARNFERKFQSEQAAGSVSKSTQFEYAWCLVRS
mFIS1.3 MDAQ----------
hFIS1.1 KYNDDIRKGIVLLEELLPKGSKEEQRDYVFYLAVGNYRIKEYEKA
hFIS1.2 KYNDDIRKGIVLLEELLPKGSKEEQRDYVFYLAVGNYRIKEYEKA
mFIS1.1 KYNEDIRRGIVLLEELLPKGSKEEQRDYVFYLAVGNYRIKEYEKA
mFIS1.2 KYNEDIRRGIVLLEELLPKGSKEEQRDYVFYLAVGNYRIKEYEKA
mFIS1.3 ------RR---SCKELLPKGSKEEQRDYVFYLAVGNYRIKEYEKA
hFIS1.1 LKYVRGLLQTEPQNNQAKELERLIDKAMKKDGLVGMAIVGGMALG
hFIS1.2 LKYVRGLLQTEPQNNQAKELERLIDKAMKKDGLVGMAIVGGMALG
mFIS1.1 LKYVRGLLQTEPQNNQAKELERLIDKAMKKDGLVGMAIVGGMALG
mFIS1.2 LKYVRGLLQTEPQNNQAKELERLIDKAMKKDGLVGMAIVGGMALG
mFIS1.3 LKYVRGLLQTEPQNNQAKELERLIDKAMKKDGLVGMAIVGGMALG
hFIS1.1 VAGLAGLIGLAVSKSKS
hFIS1.2 VAGLAGLIGLAVSKSKS
mFIS1.1 VAGLAGLIGLAVSKSKS
mFIS1.2 VAGLAGLIGLAVSKSKS
mFIS1.3 VAGLAGLIGLAVSKSKS
MW (Kda) aa
mFIS1.1 17.01 152
mFIS1.2 16.27 145
mFIS1.3 11.25 102
hFIS1.1 16.94 152
hFIS1.2 15.50 140
C
liver
brain
heart
gastrocnemius
lungs
thymus
lymph nodes
kidney
epididymis
pancreas
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
-3,0
-3,5
-4,0
-4,5
ΔCt (mFis1.2 - mFis1.1)
D
liver
muscles
brain
17kDa
15kDa
mFIS1.1
mFIS1.2
mFIS1.3

Figure 2

A
mtRFP
anti -V5
merge
ER-RFP
anti -V5
merge
mFIS1.1
mFIS1.2
mFIS1.3
B
mFIS1.1
mFIS1.2
mFIS1.3
localization index (a.u.)
1,0
0,8
0,6
0,4
0,2
0,0
mitochondria
ER
C
Time (h)
mFIS1.1
0 2.5 5
mFIS1.2
0 2.5 5
mFIS1.3
0 2.5 5
V5
β-actin
Protein level (a.u.)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
***
mFIS1.1
mFIS1.2
mFIS1.3
0h
2.5h
5h
D
MG132
control
mFIS1.1
mFIS1.2
mFIS1.3
E
mitochondrial length (μm)
5
4
3
2
1
0
Mock
mFIS1.1
mFIS1.2
mFIS1.3
*
***
-/-
MG132

Figure 3

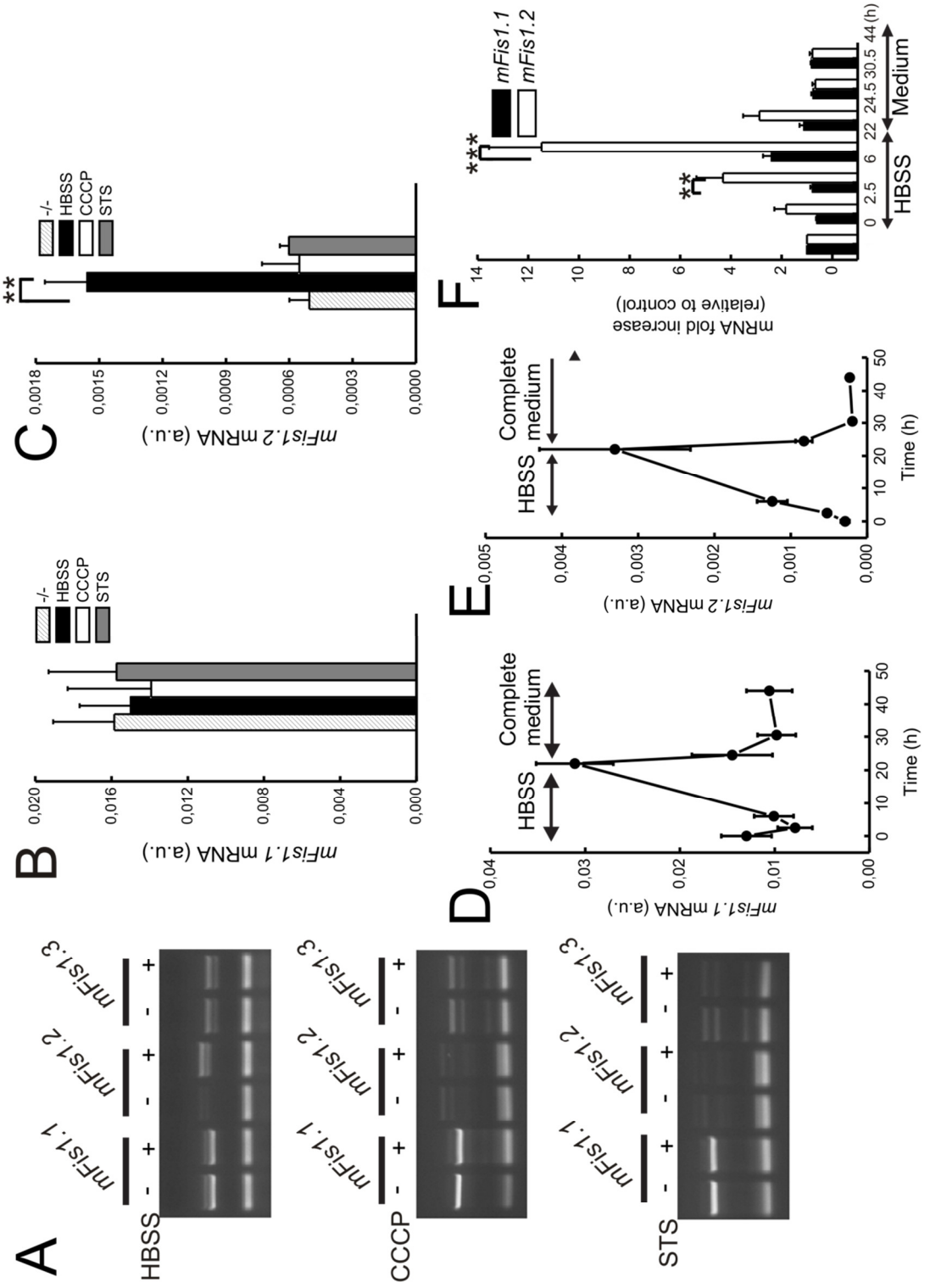
A
HBSS
CCCP
STS
mFis1.1
mFis1.2
mFis1.3
B
mFis1.1 mRNA (a.u.)
-/-
HBSS
CCCP
STS
C
mFis1.2 mRNA (a.u.)
**
D
mFis1.1 mRNA (a.u.)
HBSS
Complete medium
Time (h)
E
mFis1.2 mRNA (a.u.)
HBSS
Complete medium
Time (h)
F
mRNA fold increase (relative to control)
mFis1.1
mFis1.2
**
***
HBSS
Medium

Figure 4

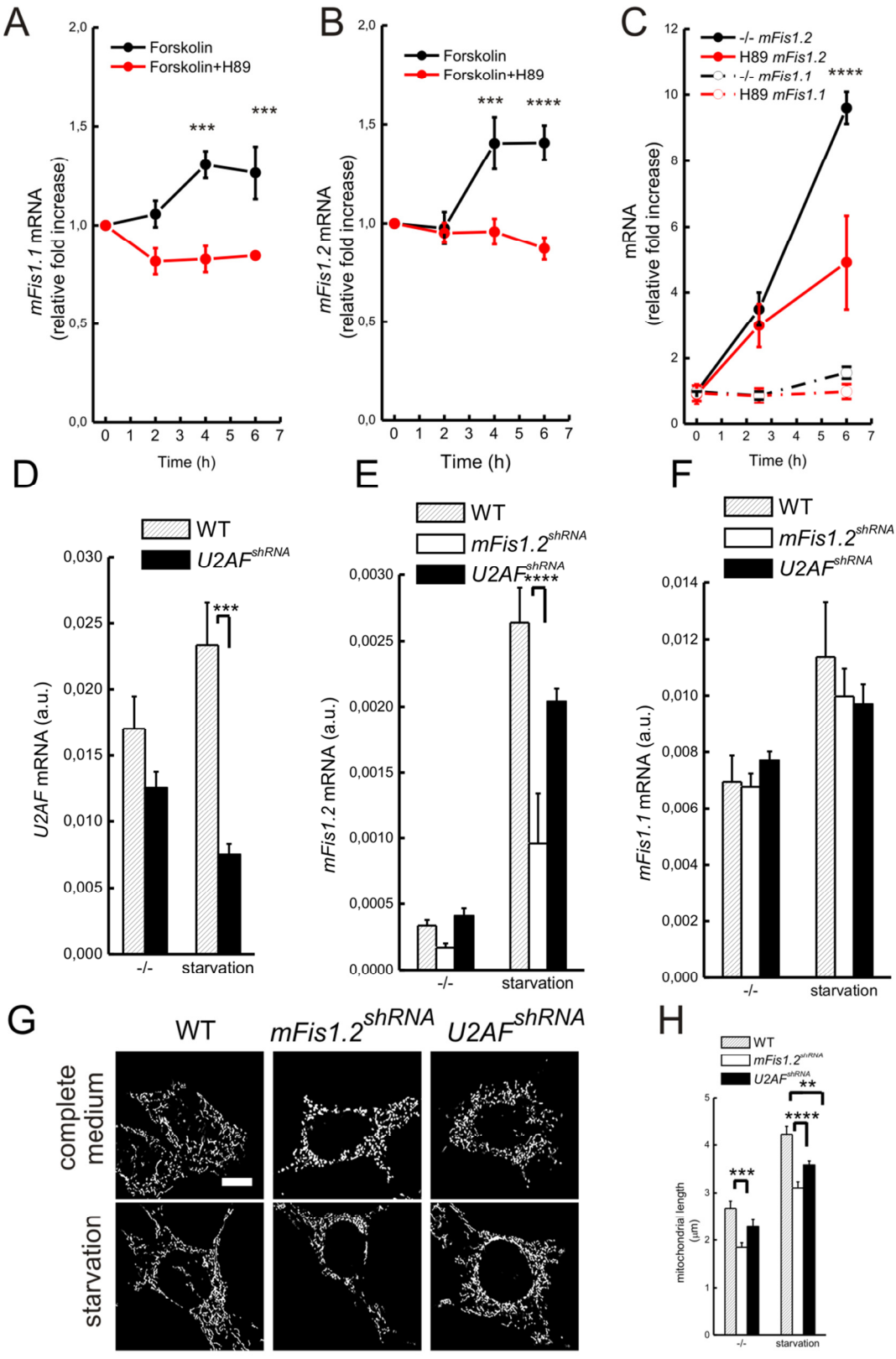
A
Forskolin
Forskolin+H89
mFis1.1 mRNA (relative fold increase)
Time (h)
B
Forskolin
Forskolin+H89
mFis1.2 mRNA (relative fold increase)
Time (h)
C
-/- mFis1.2
H89 mFis1.2
-/- mFis1.1
H89 mFis1.1
mRNA (relative fold increase)
Time (h)
D
WT
U2AF shRNA
U2AF mRNA (a.u.)
-/-
starvation
E
WT
mFis1.2 shRNA
U2AF shRNA
mFis1.2 mRNA (a.u.)
-/-
starvation
F
WT
mFis1.2 shRNA
U2AF shRNA
mFis1.1 mRNA (a.u.)
-/-
starvation
G
WT
mFis1.2 shRNA
U2AF shRNA
complete medium
starvation
H
WT
mFis1.2 shRNA
U2AF shRNA
mitochondrial length (µm)
-/-
starvation

Figure 5

SUPPLEMENTARY MATERIAL

Table 1. Primers used to amplify human *Fis1* cDNA

Primer name	Sequence (5' – 3')
Exon1_1FW	TGCCTGGAGATGAAGCTGGAA
Exon1_2FW	TGGGTGCCTGGAGATGAAGC
Exon2_FW	ATGGAGACTGTGGCACAGTAG
Exon2_RV	ACAGTAGACTGTAGTGTGAGG
Exon4_FW	AGTCTGAGAAGGCAGCAGGC
Exon7_FW	AGGAACAGCGGGATTACGTC
Exon8_FW	AAAGTACGTCCGCGGGTTGC
Exon9_RV	TCAGGATTTGGACTTGGACAC

Table 2. Primers used to amplify mouse *Fis1* cDNA

Primer name	Sequence (5' – 3')
Exon1_1FW	AGCGTGCTTTCTGTAACGCCT
Exon1_2FW	AGTGCCTGTAGAAAACAGCTCT
Exon2_FW	CTACTGGACCATCGAGACTGT
Exon5_FW	CAAAGAGGAACAGCGGGACTA
Exon7_RV	CAGGATTTCGACTTGGAGACA

2. Mouse Fis1 hypomorphism causes a dose-dependent multiorgan lethal phenotype.

Mouse *Fis1* hypomorphism causes a dose-dependent multiorgan lethal phenotype.

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ABSTRACT

Mitochondrial fission is essential for key cellular quality control mechanism including mitophagy and its genetic inhibition by ablating the master fission protein Dynamin related protein 1 (Drp1) is detrimental, leading to neurodegeneration and cardiac insufficiency. To investigate the role of Fission (FIS)1 a putative Drp1 receptor whose role in mitochondrial dynamics is controversial, we generated a hypomorphic *Fis1* mouse model that, given the reduction but not complete absence of FIS1, circumvents developmental lethality. Constitutive reduction in FIS1 levels results in a pleiotropic dose-dependent phenotype of defective growth, progressive muscular atrophy, focal heart ischemia and disseminated hemorrhages, eventually leading to death at weaning. Since the generated hypomorphic *Fis1* allele can be converted into an inducible floxed allele by breeding with a Flipper mouse, this versatile genetic tool will be instrumental in studying the consequences of constitutive hypomorphism in the affected organs without the need of further gene editing.

INTRODUCTION

In the last three decades, the functional role of mitochondria has been expanded and from cellular energy converters and metabolic reactors they turned into key players in a plethora of (patho)physiological processes, ranging from cell death and autophagy to Ca^{2+} homeostasis and cell signaling, and from differentiation, to immunity, aging and cancer (Corrado et al., 2012; Frezza and Gottlieb, 2008; Kasahara and Scorrano, 2014; McBride et al., 2006b; Rizzuto et al., 2000). These pleiotropic functions are fulfilled by a striking morphological and structural versatility. Mitochondria structural plasticity is ensured by inter-organellar fusion and fission processes mediated by a group of dynamin-related GTPases and their adaptor proteins, the so-called mitochondrial shaping proteins (Friedman and Nunnari, 2014; Hoppins et al., 2007a). Mitofusin (MFN) 1 and 2 on the outer membrane and Optic atrophy 1 (OPA1) on the inner membrane mediate mitochondria juxtaposition and subsequent fusion (Chen et al., 2003b; Cipolat et al., 2004; Eura et al., 2003a). Furthermore, OPA1 plays a key role in regulation of mitochondrial cristae impacting on mitochondrial apoptosis and metabolism (Cipolat et al., 2006; Civiletto et al., 2015; Cogliati et al., 2013; Frezza et al., 2006a; Varanita et al., 2015). On the other side, mitochondrial fission requires the translocation of cytosolic dynamin-related protein 1 (DRP1) to the mitochondria (Cereghetti et al., 2008a; Cribbs and Strack, 2007a; Frank et al., 2001; Smirnova et al., 2001; Yoon et al., 2001b) and its interaction with FIS1, MFF and MiD49/50, its putative receptors (Gandre-Babbe and van der Bliek, 2008; James et al., 2003; Mozdy et al., 2000; Otera et al., 2010; Palmer et al., 2011; Yoon et al., 2003; Zhao et al., 2011).

The physiological relevance of mitochondria dynamics is testified by the finding that mutations in mitochondria shaping genes are associated with severe diseases. For

instance, mutations in *MFN2* and *OPA1* cause Charcot-Marie-Tooth neuropathy type 2A (Zuchner et al., 2004) and dominant optic atrophy (Alexander et al., 2000; Delettre et al., 2000). Additionally, a mutation in *Drp1* causes neurological dysfunctions and neonatal lethality (Chang et al., 2010a; Waterham et al., 2007), and *Mff* mutations have been associated to neuromuscular defects (Shamseldin et al., 2012) and early-onset Leigh-like encephalopathy (Koch et al., 2016). Furthermore, mouse models for *Mfns*, *Drp1* and *Opa1* ablation are embryonically lethal (Alavi et al., 2007; Chen et al., 2003b; Davies et al., 2007; Ishihara et al., 2009; Wakabayashi et al., 2009). Conversely, *Mff* null mice develop a lethal dilated cardiomyopathy at 13 weeks of age (Chen et al., 2015). However, although *in vitro* studies are dissecting the molecular mechanisms behind mitochondrial shape and its cellular regulation, our understanding of the physiological *in vivo* significance of mitochondrial dynamics is far from comprehensive. For instance, mitochondrial fragmentation has been classically believed to be essential for neuronal polarity and synapse maintenance. This idea was mainly based on the phenotype of *Drp1*$^{-/-}$ embryos (Ishihara et al., 2009; Wakabayashi et al., 2009) and on the thought that long polarized cells, such as neurons, might relay on fission to ensure adequate mitochondrial trafficking and redistribution between the soma and the synapses, as demonstrated by neuronal specific *Drp1* ablation models (Oettinghaus et al., 2015; Shields et al., 2015). More recently, both *Mff* gene trap and *Drp1* muscle- and cardiomyocyte- specific ablation models were reported to develop lethal dilated cardiomyopathy thus showing the importance of mitochondrial fragmentation for heart function (Chen et al., 2015; Ikeda et al., 2015; Ishihara et al., 2015; Kageyama et al., 2014; Song et al., 2015a; Song et al., 2015b). However, it is also emerging that mitochondrial fragmentation *per se* is not sufficient but that a balance between fission

and fusion is rather required in the heart (Chen et al., 2011; Song et al., 2015b; Wai et al., 2015). Furthermore, the fact that whole body *Mff* ablation does not phenocopy *Drp* null embryos highlights the possibility that *in vivo* fission might be a tissue-specific, complex, highly regulated process, as the existence of a plethora of DRP1 receptors seems to suggest. Indeed, FIS1, MFF and MiD49/50 have been reported to have *in vitro* partially overlapping functions and to be somehow dispensable for DRP1 recruitment and mitochondrial fragmentation, but their exact roles remain elusive (Loson et al., 2013; Otera et al., 2010; Palmer et al., 2013).

In particular, FIS1 is a tail-anchored protein evenly distributed on the outer mitochondrial membrane and composed by a C-terminus transmembrane (TM) domain and two cytosolic tetratricopeptide repeat (TRP) motifs (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003). FIS1 has been proved to mediate mitochondrial-ER tethering, ER-gated apoptosis, autophagy and ischemia/hypoxia-induced fragmentation (Alirol et al., 2006; Iwasawa et al., 2011a; Kim et al., 2011; Shen et al., 2014; Yamano et al., 2014). However, the discovery of MFF and MiD49/50 along with the finding that both DRP1 recruitment and mitochondrial fragmentation take place also in two models of *Fis1 in vitro* ablation led to partially discharge FIS1 involvement in such events (Loson et al., 2013; Otera et al., 2010; Palmer et al., 2013). Besides the mechanistic aspect of FIS1-mediated fragmentation, its (patho)physiological relevance is still far from been clarified, and no human pathology so far has been directly linked to a mutation in *FIS1* gene. Furthermore, the existence of yeast-specific DRP1 adaptors, such as Mdv1p and Caf4p (Griffin et al., 2005; Karren et al., 2005; Naylor et al., 2005; Schauss et al., 2006; Tieu et al., 2002) and the evolution of vertebrate specific proteins, such as MiD49/50, makes it

difficult, if not impossible, to deduce FIS1 functions in mammals on the basis of what observed in lower eukaryotes.

In order to place FIS1 functions into a physiological context and to further define the relevance of mitochondrial fission *in vivo*, we decided to generate a hypomorphic *Fis1* mouse model that could be turned into a conditional ablation system. Here we report that hypomorphic *Fis1* induces a pleiotropic lethal dose-dependent phenotype of defective growth, progressive muscular defects, heart ischemia and blood vessel integrity alteration.

RESULTS

Generation of hypomorphic and floxed *Fis1* mice.

To assess the physiological role of FIS1 and in order to overcome the possible lethality induced by its constitutive ablation, we decided to generate a versatile mouse model that could be used both for constitutive reduced expression (hypomorphic $Fis1^{hyp}$ allele) and selective ablation (floxed $Fis1^{fl}$ allele). This implied that the element(s) responsible for attenuating *Fis1* expression in the hypomorphic allele should be removed without perturbing the gene structure in the floxed allele, thus allowing reconstitution of normal expression and subsequent selective ablation. To this end, we introduced a hygromycin B resistance cassette under the control of the phosphoglycerate kinase (PGK) promoter flanked by two FRT sites immediately after the stop codon; additionally, we provided our transgene with two *LoxP* sites placed after exon 3 and in between the STOP codon and the hygromycin cassette (Figure 1A). Since the 3'UTR controls mRNA translation efficiency, localization, and stability, and contains sequences required for poly(A) maturation, we reasoned that adding a relatively long stretch of nucleotides, in our case a 2kb hygromycin cassette, in between the STOP codon and the *Fis1* 3'UTR might have altered, and possibly reduced, the stability of the relatively small *Fis1* mRNA, which counts less than 800 bp. Alternatively, the transcription terminator sequence and/or the poly(A) region in the hygromycin resistance gene might have stropped transcription and RNA processing before the endogenous *Fis1* 3'UTR thus impinging on *Fis1* expression. Indeed, western blot analysis of liver extracts confirmed reduction but not complete ablation of FIS1 protein levels (Figure 1B). However, to our surprise, we could detect a certain variability in the levels of residual FIS1 protein among $Fis1^{hyp/hyp}$ and $Fis1^{hyp/+}$ mice compared to wild type littermates (Figure 1B). For instance, in $Fis1^{hyp/+}$ mice FIS1 protein

levels ranged from similar to wild type to strongly reduced. Similarly, the amount of detectable residual protein in *Fis1*$^{hyp/hyp}$ mice was variable (Figure 1B). This unexpected variability could be explained by hypothesizing that the destabilizing action exerted by the hygromycin cassette is not constant thus resulting in a variability of residual/functional *Fis1* mRNA produced by the *Fis1*^{hyp} allele.

Breeding of *Fis1*$^{hyp/+}$ mice with Flipper mice (*Fis1*$^{+/+}$ *Flp*$^{+/-}$) led to the excision of the hygromycin cassette as confirmed by mouse tail PCR on F1 littermates (Figure 1C). The resulting allele is an inducible floxed *Fis1* (*Fis1*^{fl}) allele. Indeed, *Fis1*$^{fl/+}$*Flp*$^{+/-}$ mice are viable and fertile and do not show the defects observed in *Fis1*$^{hyp/+}$ mice but are indistinguishable from wild type mice (not shown). After Cre-mediated recombination, only exons 1, 2 and 3 are expected to remain in the genomic *locus* and the hypothetical protein will be devoid both of the transmembrane domain, known to be required for FIS1 mitochondrial localization and function, and of half of the TRP domain, which is thought to mediate DRP1 interaction (Figure 1D). Remarkably, ablation is also predicted for all *Fis1* splicing variants (Zamorano, Barbieri et al. submitted) (Figure 1D).

Hypomorphic *Fis1* expression induces perinatal lethality.

Intercross progeny of *Fis1*$^{hyp/+}$ mice comprised only 15% *Fis1* hypomorphic mice suggesting developmental lethality. Born *Fis1*$^{hyp/hyp}$ mice died soon after birth at p12-p16 (Figure 2A). The fact that a fraction of *Fis1*$^{hyp/hyp}$ mice escaped *in utero* lethality suggested that hypomorphic mice might die late during development. In line with this, pregnant *Fis1*$^{hyp/+}$ females had a normal belly size and shape. Strikingly, the fact that *Fis1* hypomorphic allele was not circumverting developmental lethality, as usually expected

by hypomorphic alleles, suggests indispensable physiological functions for FIS1. Interestingly a 35% lethality was observed also in *Fis1*$^{hyp/+}$ mice around p16-p30 (Figure 2A).

Phenotypic heterogeneity ranging from altered gait to progressive muscular atrophy suggests hypomorphic variability.

In order to unveil the cause of death in *Fis1*$^{hyp/hyp}$ and *Fis1*$^{hyp/+}$ mice, we decided to monitor *Fis1*$^{hyp/+}$ intercross progeny daily. We observed that, although the initial growth rate was similar among littermates, *Fis1* hypomorphic mice invariably lose weight few days before death (around 15% less compared to their littermates) (Figure 2B and 2C). Additionally, *Fis1*$^{hyp/hyp}$ mice displayed a wide range of gait defects, which, in the majority of cases, appeared when mice started to walk. Usually, barely detectable side-to-side wobbling with posterior paws pointing to the side of the body (p5-p10) were ultimately leading to kyphosis (p10-p15) (Figure 2D). At later stages, then, *Fis1* hypomorphic mice showed reduced mobility, wheeze, seizure and irregular heart beating. In complex, this suggested us that *Fis1* hypomorphic mice might suffer of progressive (neuro)muscular defects.

On the other hand, *Fis1*$^{hyp/+}$ mice displayed a heterogeneous phenotype with different degrees of severity but somehow recalling what observed in their hypomorphic littermates. Based on their growth rate, gait defects and survival, we decided to group heterozygous mice into three cohorts. *Fis1*$^{hyp/+\ low}$ mice showed gait defects and kyphosis (not shown) and died at p16-p30 (Figure 2A). *Fis1*$^{hyp/+\ low}$ showed the same detrimental defects observed in Fis1$^{hyp/hyp}$ mice, though at later stage. The *Fis1*$^{hyp/+\ low}$ cohort

comprises the 35% of the *Fis1*$^{hyp/+}$ mice analyzed so far. We also observed a second phenotypically distinct cohort of mice which we named *Fis1*$^{hyp/+\ med}$. These mice lived up to 4wk, yet they seemed to suffer a severe growth defect, being smaller in seize with round-like shape body, longer tail and a characteristic "jumping" gait when compared to healthy *Fis1*$^{hyp/+}$ littermates (Figure 2E). *Fis1*$^{hyp/+\ med}$ mice comprised the 10% of the *Fis1*$^{hyp/+}$ mice analyzed so far. At the moment *Fis1*$^{hyp/+\ med}$ mice aged up till 4wk, but we can't exclude death at later stage. Finally, *Fis1*$^{hyp/+high}$ mice showed no evident gait defects, were viable and fertile and have been used to maintain *Fis1* colony.

In complex, *Fis1*$^{hyp/+}$ and *Fis1*$^{hyp/hyp}$ phenotypes appeared as a *continuum* from highly severe to barely detectable defects. This finding prompted us to two considerations. On one side, hypomorphic alleles are generally expected to display a dose-dependent phenotype; in line with this heterozygous mice generally show a milder phenotype compared to *Fis1*$^{hyp/hyp}$ mice. On the other hand, the phenotypic heterogeneity observed in heterozygous mice along with the individual variability in terms of FIS1 protein levels observed among *Fis1*$^{hyp/+}$ littermates (Figure 1C) temped us to speculate that *Fis1*$^{hyp/+}$ mice might differ in terms of hypomorphism degree. Similarly, the *Fis1*$^{hyp/hyp}$ mice that died *in utero* might have had the lowest *Fis1* residual expression.

Hypomorphic *Fis1* causes a pleiotropic phenotype comprising muscular defects, heart ischemia and compromised vessel integrity.

In line with the severe defects observed before death, visual inspection of dead and euthanized *Fis1*$^{hyp/hyp}$ mice revealed that *Fis1* hypomorphic hearts displayed extensive ischemia/necrosis, possibly as a consequence of myocardial infarct. (Figure 3A and 3B).

Remarkably, *Fis1*$^{hyp/hyp}$ mice showed blood effusion in the thorax and/or in the lungs (Figure 3A). Moreover, in some mice, massive blood clots were found in the pericardial space, suggestive of cardiac tamponade perhaps as a consequence of myocardial rupture after acute myocardial infarction (Figure 3B). In few cases, extensive blood clots were observed on the visceral pleura (Figure 3B). We reasoned that the breathing problems observed before death might have been caused by the presence of blood in the lungs. Given that lung blood vessels are sensible to blood pressure, the presence of blood in the lungs could be due either to heart dysfunctions or to increased blood pressure resulting in partial vessel leakage, as for instance in case of thrombosis. To rule out this possibility, we decided to inspect retina blood vessels. Surprisingly, we could detect blood also in retina from *Fis1*$^{hyp/hyp}$ mice and to a lower extend also in *Fis1*$^{hyp/+}$ mice when compared to their wildtype littermates. A further analysis of isolectin B4-stained flat mount retinas revealed a reduced vessels branching but a similar radial expansion of the vascular plexus (Figure 3D). Furthermore, the skull vessels of *Fis1*$^{hyp/hyp}$ mice appeared as a diffused red staining (Figure 3C) and in some animals blood was found in the intestine (not shown). Taken together, this suggested us an alteration in the microcirculation and an increased permeabilization. However, we could not establish whether this was due to defects in the maintenance of the vessel integrity rather than to increased blood pressure (e.g. thrombosis) and/or heart failure (e.g. ischemia). Dissection of posterior paws also confirmed muscular defects in *Fis1*$^{hyp/hyp}$ mice (Figure 3E). On the other hand, liver, brain and thymus did not seem to be macroscopically affected in *Fis1*$^{hyp/hyp}$ mice (Figure 3F).

In order to better understand the heterogeneity observed among Fis1$^{hyp/+}$ mice, we decided to dissect also heterozygous mice. Not surprisingly, dead *Fis1*$^{hyp/+\ low}$ mice were

barely distinguishable from their *Fis1*$^{hyp/hyp}$ littermates, showing similar levels of heart ischemia, blood in the lungs and muscular atrophy (Figure 3A). Next, we decided to euthanize also healthy *Fis1*$^{hyp/+\ high}$ mice in order to verify whether they presented differences compared to the other *Fis1*$^{hyp/+}$ mice. To our surprise, also *Fis1*$^{hyp/+\ high}$ mice displayed heart ischemia, although they were not showing any overt clinical sign of myocardial infarction.

In conclusion, this finding suggests that *Fis1* ablation-caused lethality might be due by myocardial dysfunctions along with anomalies in blood pressure and/or vessels integrity. Furthermore, our observations seemed to confirm the hypothesis of a variability in *Fis1* hypomorphism and its dose dependent effect.

Cells ablated for *Fis1* display elongated mitochondria and upregulation of the fission machinery.

In order to assess the cellular consequence of *Fis1* ablation, we isolated tail fibroblasts from neonatal mice (p7) and immortalized them by using SV40 Large T antigen. Live imaging revealed that mitochondrial network was highly interconnected in immortalized *Fis1*$^{hyp/hyp}$ fibroblasts with longer mitochondrial tubules when compared to their wildtype counterparts, suggesting an impaired mitochondrial fragmentation (Fig. 4A) despite the compensatory increase in DRP1 and MFF levels identified by western blotting (Fig. 4B). Therefore, reduction in FIS1 levels upregulates the fission machinery possibly to maintain a certain level of mitochondrial fragmentation. However, confocal analysis revealed that *Fis1* hypomorphic mitochondria were more elongated and interconnected when compared to wildtype mitochondria. This would suggest that DRP1 and MFF

upregulation is not sufficient to maintain a fusion/fission balance. Taken together, FIS1 and DRP1/MFF might act in the same genetic pathway of mitochondrial fragmentation.

DISCUSSION

Although initially discovered as a key protein regulating mitochondrial morphology and, for long, considered as the organelle receptor for DRP1 (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003), FIS1 role in mitochondrial fragmentation has been recently challenged (Loson et al., 2013; Otera et al., 2010; Palmer et al., 2013). Nevertheless, FIS1 has been implicated in apoptosis, mitophagy and mitochondrial fragmentation in response to hypoxia/ischemia (Alirol et al., 2006; Iwasawa et al., 2011a; Kim et al., 2011; Shen et al., 2014; Yamano et al., 2014). However, its physiological role is far from being established. Here we report a perinatal pleiotropic lethal phenotype caused by a *Fis1* hypomorphic allele. After-birth lethality is preceded by weight loss and is associated with severe progressive muscular atrophy, heart ischemia and compromised vessel integrity. The concomitant muscular atrophy and heart ischemia would suggest (cardio)myopathy and/or neuromuscular defects. However, the extensive blood clots in the thorax, the presence of blood in the lungs and in the intestine and the altered microcircular bed in the retina seems to suggest also defects in the maintenance of vessels integrity and/or increased vessel permeability and consequent blood leakage. Furthermore, the body weight loss preceding death seems not to reconcile with a sudden death due, for instance, to heart failure, and the presence of milk in the stomach seems to exclude sucking problems due to muscles impairment. Analysis of heart and muscle functionality, along with assessment of integrity of the microcirculation in the retina will help in clarifying the observed phenotype.

Remarkably, although reduced, also heterozygous mice display lethality at a later stage (p25-p30) and show defects comparable to their $Fis1^{hyp/hyp}$ littermates. In complex, this

points out a dose dependent relationship between phenotype severity and extend of *Fis1* gene ablation. The fact that only the 35% of heterozygous mice die could be explained by a different degree of FIS1 expression by the $Fis1^{hyp}$ allele rather than by compensatory mechanisms. Indeed, the hygromycin cassette in the transgene we generated is supposed to cause *Fis1* ablation by impinging on mRNA production/stability, leading to a variable extent of *Fis1* ablation. To verify this hypothesis, assessment of residual *Fis1* mRNA production by 5'RACE is required. Subsequently, it would be interesting to correlate residual *Fis1* expression in $Fis1^{hyp/+}$ mice with phenotype severity. The generated transgene we can be converted into a floxed allele by breeding with Flipper mice ($Flp^{+/-}$). This versatile genetic tool will be instrumental in verifying the consequences of constitutive ablation in the more affected organs without the need of further transgene generation and subsequent gene targeting.

In vitro, we observed mitochondrial elongation and upregulation of the fission machinery as a consequence of *Fis1* hypomorphism. The two mammalian models of *in vitro Fis1* ablation so far described display no obvious phenotype (Loson et al., 2013; Otera et al., 2010). This can be explained by the fact that the consequences of *in vitro Fis1* ablation might be influenced by the cell type and might not recapitulate an *in vivo* physiological situation, as in the case of a mouse model. Furthermore, also the gene targeting strategy adopted might play a role. On one side, constitutive ablation might activate compensatory mechanisms thus concealing or attenuating the primary effects induced by the ablation *per se*. To this regard, our hypomorphic allele retains a low FIS1 expression thus reducing potential compensation mechanisms. Therefore, the mild phenotype observed by Chan and co-workers could be explained by *in vitro* compensatory mechanisms. On the other hand, a gene targeting strategy based on exon

excision from the genomic *locus* should consider the pitfall of splicing variants and prevent splicing to occur among the exons left in the *locus*, as it seems the case of the strategy adopted by Youle and Mihara and co-workers. Indeed in this case the removal of exon 2 (corresponding to exon 3 in our system) might not be sufficient to prevent alternative splicing. Consequently, unbalanced or altered splicing could produce protein variants which retain or acquire new functions thus leading to incorrect conclusions. To this regard, our transgene impinges on the 3'UTR which, as we demonstrated in Zamorano et al. (in preparation) is shared among all the splicing variants. Strikingly, our findings bring back FIS1 on the stage of mitochondrial dynamics as a key, jet not fully understood, player. It would be interesting to assess DRP1 translocation and phosphorylation status.

In conclusion, the powerful genetic tool we generated circumvents many of the potential pitfalls of the most commonly used gene targeting approaches and our preliminary results suggest that FIS1 exerts both *in vivo* and *in vitro* key roles thus bringing back FIS1 in the spotlight of the mitochondrial morphology field. Further investigation is required to completely characterized *Fis1* hypomorphic phenotype and to unveil the molecular mechanisms leading to *Fis1* depletion-mediated defects and death.

MATERIAL AND METHODS

Generation of *Fis1* hypomorphic mice and cells.

Fis1 genomic sequence was derived from the C75BL/6J RP24-456B4 BAC clone from the BACPAC Resources Center (AC147987 at NCBI). Digestion of the BAC with EcoRI enabled to retrieve a 7.8 kb *Fis1* fragment covering exons 2-7 (Figure 1A). The fragment was cloned into EcoRI-linearized pUC-18 (Figure S1A). Given the big size of the retrieved *Fis1* fragment, *Fis1* gene was splitted into a 5' and a 3' portion with respect to the *LoxP* site. On one side digestion with XbaI produced the 3' fragment that was subcloned into pBluescript II SK. An XhoI restriction site was introduced by site-directed mutagenesis after the STOP codon using forward primer 5'-GTCCAAATCC**TGA***ctcg*AGCCTCACCTGC-3', reverse primer 5'- GCAGGTGAG**GCT***cgagt*CAGGATTTGGAC-3' and the QuickChange® II XL Site-Directed Mutagenesis Kit (Agilent technology, 200521). On the other side, XbaI SphI double digestion enabled to eliminate the 3' fragment and to add the *LoxP* site inside intron 3. Afterwards, the 5' and the 3' fragments were joined together. Finally, the hygromoycin B resistance gene, driven by the phosphoglycerate kinase (PGK) promoter flanked by two FRT sites and one *LoxP* site was cloned into the XhoI site and the resultant targeting vector was fully sequenced (Figure S1A).

The targeting vector was linearized with PciI and electroporated into C57Bl/6 ES cells. Hygromycin resistant clones were screened for homologous recombination both at the 5' and the 3' of the construct. Recombination at the 3' was verified by primer 3'homFR (5'-CAGCAGCCTCTGTTCCACATAC-3') annealing inside the transgene and primer 3'homRV (5'-TCCTGGGCACACTCCCATTG-3') annealing in the genomic locus (Figure S1B). The primers were expected to produce a 1.4 kb fragment (Figure S1C, upper panel). Recombination

at the 5' was verified by southern blotting using EcoRV digested genomic DNA in combination with a 565 bp probe annealing in the genomic locus up stream of *Fis1* (Figure S1B). The probe was generated by PCR on genomic DNA using primers 5'homFR (5'-CTTTCCAACACCCGGTAGCG-3') and 5'homRV (5'-TACCCTTGCAGCTTCGTCTC-3'). Digestion of genomic DNA with EcoRV was expected to produce a >25 kb for the wild type allele plus an additional 14.4 kb fragment for the targeted allele (Figure S1C, central panel). Finally, recombination at the distal 5' *LoxP* site was verified by PCR using primer couple loxPhomFW (5'-TGAAAGCACTGGCCGTCCTCTG-3') and loxPhomRV (5'-CTGACCTTCCTGCCTCTATCTC-3') (Figure S1B) expected to produce a 266 bp fragment for the wild type allele and a 330 bp fragment for the targeted allele (Figure S1C, lower panel).

Out of the initial 384 hygromycin-resistant clones, 4 clones resulted to be positive for homologous recombination of the targeting vector. All clones had good morphology and karyotype and were injected into blastocyst isolated from C57BL/6 donors and transferred into foster mothers. Viable chimeric offspring was then crossed and mice with germ-line transmission were selected (*Fis1*$^{+/hyp}$ mice). Genotyping was performed using primer couple 5'LoxPFW (5'-ctgtatagagagtaagctggaa-3') and 5'LoxPRV (5'-ctcaaatttggtcaggagtpg-3'), expected to produce a 304 bp fragment for the transgene allele and a 240 bp for the wild type allele.

Breeding of *Fis1*$^{+/hyp}$ *Flp*$^{-/-}$ mice with Flipper *Fis1*$^{+/+}$ *Flp*$^{+/-}$ mice allowed the removal of the hygromycin B resistance cassette and generation of the *Fis1*^{fl} allele. Excision of the hygromycin cassette was verified using primer couple Ex7FW (5'-tggccatchyptghypggcat-3') and FRTRV (5'-tcagatctctcgacggtatcg-3') producing an expected amplicon of 293 bp

and primer couple HygFW (5'-ctcccactcatgatctataga-3') and 3'RV (5'-tctcaccctachypcatcahyp-3') producing an expected amplicon of 721 bp.

For the generation of fibroblasts, neonatal mice (p5) were sacrified and their tails were sliced. The slices were let adhere on 10cm cell culture dishes and supplemented with complete DMEM medium. After 3-4 days, fibroblasts started to migrate outside the tail slices and medium was carefully changed. Once fibroblast colonies were formed around the tail slices, tail pieces were removed and cells were passed. Subsequently, fibroblasts were transfected with SV40 large T antigen previously described (Cogliati et al., 2013) and selected for replication potential by 6 1:10 serial passages.

Mouse retinal blood vessels.

To analyze retinal blood vessels in the mouse retina, eyes were fixed in 4% paraformaldehyde stained with Isolectin B4 and flat-mounted as previously described (Bajou et al., 2014).

Cell culture and transfection

Immortalized fibroblasts from *Fis1*$^{+/+}$, *Fis1*$^{+/hyp}$ and *Fis1*$^{hyp/hyp}$ mice were cultured in DMEM supplemented with 5% fetal bovine serum and non-essential amino acids (GIBCO). Transfection of MEFs with DNA was performed using Transfectin (Biorad) according to the manufacturer's instructions. Cells were transfected 24 hr after plating at 60-70% confluency.

Immunoblotting

For immunoblotting, total cell lysates were prepared in RIPA buffer supplemented with Protease Inhibitor Cocktail (Sigma), whereas tissues were homogenized. 30 μg proteins were separated by AnyKD gel (Biorad), transferred onto PVDF membranes (Millipore) and probed using the indicated primary antibodies. Isotype-matched secondary antibodies were conjugated to horseradish peroxidase (Amersham) and detected using ECL (Amersham). The following antibodies were employed: rabbit anti-FIS1 (1:500, Alexis), mouse anti-DRP1 (1:1000 BD Bioscience), rabbit anti-MFF (1:1000, Proteintech), mouse anti-ACTIN (1:20,000, Chemicon).

Imaging

For confocal microscopy imaging of live cells, 1.5×10^5 cells were seeded onto 24-mm round glass coverslips transfected as indicated. Cells were incubated in Hank's Balanced Salt Solution (HBSS) (Gibco) supplemented with 10 mM HEPES and coverslips were placed on the stage of a Leica SP5 inverted microscope. Cells expressing mtRFP were excited using the 543 nm line of the Argon laser using a 63c × objective.

Acknowledgements

LS is a Senior Scientist of the Dulbecco-Telethon Institute. Supported by Telethon-Italy.

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FIGURE LEGENDS

Figure 1. Generation of hypomorphic and floxed *Fis1* mice.

(A) Schematic drawing of *Fis1* genomic locus, targeting vector and the different alleles resulting after homologous recombination (hypomorphic allele, $Fis1^{hyp}$), after Flippase-mediated hygromycin cassette excision (floxed allele, $Fis1^{fl}$) and after Cre-mediated recombination (null allele, $Fis1^{-}$). Position of the primers used for genotyping (LoxPFR and LoxPRV, red arrows) and for assessment of the removal of the hygromycin cassette (Ex7FW-FRTRV green arrows and HygFW-3'RV gray arrows) are indicated.

(B) Representative image for genotyping (up) and western blot analysis of liver extracts (down) of intercross progeny of $Fis1^{hyp/+}$ mice.

(C) Representative image for genotyping of progeny of $Fis1^{hyp/+}$ crossed with $Fis1^{+/+}$ $Flp^{+/-}$ mice. The colured arrows refer to the primer couples used for genotyping and depicted in panel A.

(D) Amino acid sequence multiple T-Coffee alignment of FIS1 splicing variants. In red the amino acidic composition of the putative abortive proteins that might be produced by the $Fis1^{-}$ allele.

Figure 2. Dose dependent effects of hypomorphic *Fis1*.

(A) Kaplan-Meier curve showing the survival probability within the first month of life of *Fis1* mice. n=4 for $Fis1^{+/+}$, n=26 for $Fis1^{hyp/+}$, n=17 for $Fis1^{hyp/hyp}$.

(B) Representative images of $Fis1^{hyp/hyp}$ mouse dead at p13 and a wild type littermate sacrified as a control.

(C) Body weight of the mice of the indicated genotype measured up to p30.

(D) Representative images of a $Fis1^{hyp/hyp}$ mouse and a wild type littermate at p8 (up), and of the same hypomorphic mouse at p12 (down) showing the progression of the gait defects and of the kyphosis. Arrows indicate the appearance of the initial symptoms in the posterior paws.

(E) Representative images of one $Fis1^{hyp/+\ high}$ mouse and one $Fis1^{hyp/+\ med}$ littermate at p29.

Figure 3. Hypomorphic *Fis1* causes multiple detrimental defects.

(A) Representative images of sacrified littermates of the indicated genotypes.

(B) Representative images of two hypomorfic littermates showing blood clot (white arrow), extensive ischemia/necrosis in the heart and blood in the lungs.

(C) dorsal view of the skull of a hypomorphic mouse dead at p13. Arrows indicate the compromised blood vessels.

(D) Representative images of isolectin B4 flat mount retinas from mice of the indicated genotypes.

(E) Representative images of posterior paws from a *Fis1*$^{hyp/hyp}$ mouse and healthy heterozygous mouse.

(F) Representative images of heart, lungs, brain and liver from sacrified mice of the indicated genotypes.

Figure 4. Cells ablated for Fis1 display elongated mitochondria and upregulation of the fission machinery.

(A) Western blot analyisis of total cell extracts from cells fo the indicated genotypes.

(B) Representative confocal live images of mitochondrial network in mtRFP-transfected cells of the indicated genotypes.

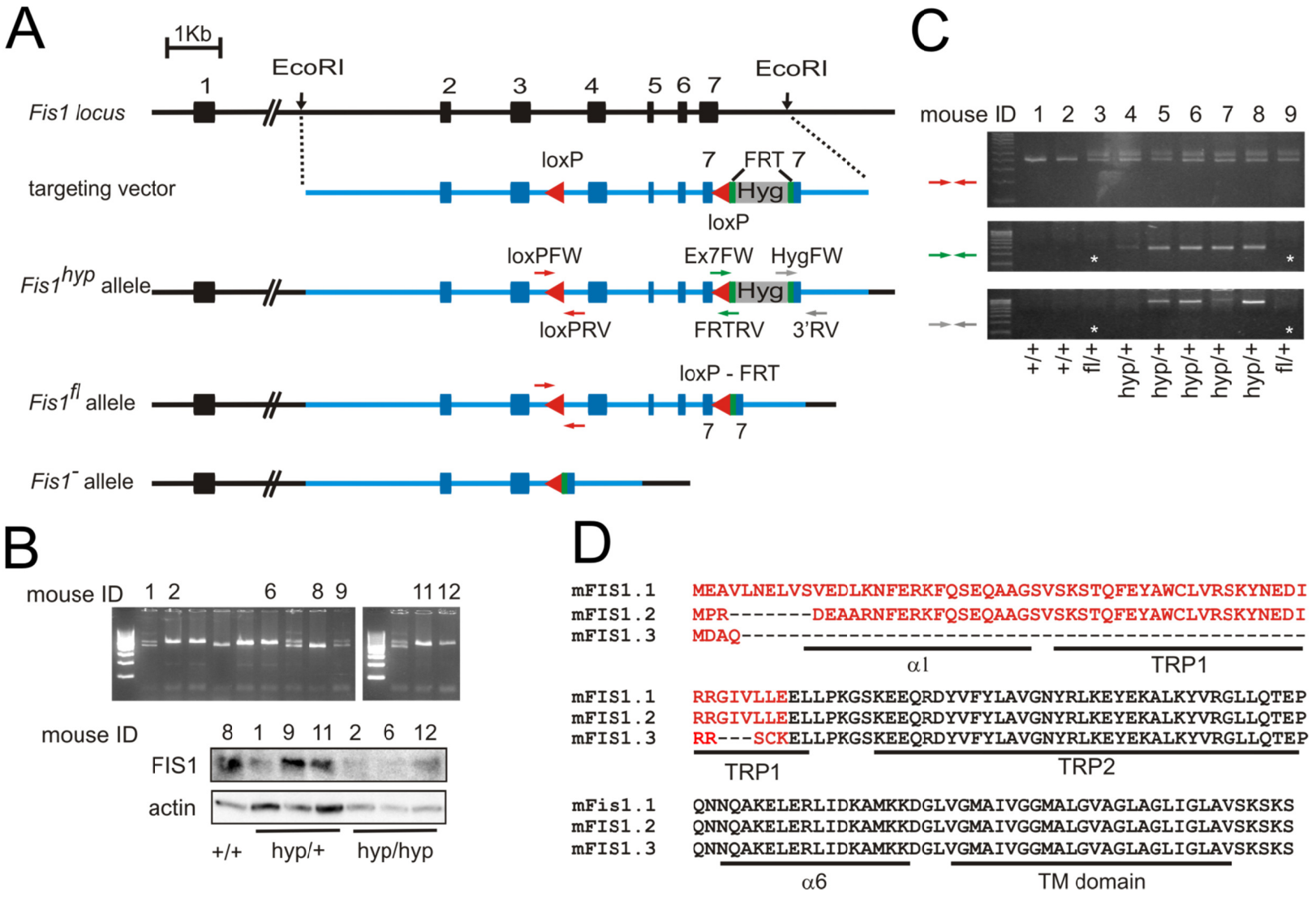
A
1Kb
EcoRI
EcoRI
1
2
3
4
5 6 7
Fis1 locus
loxP
7 FRT 7
targeting vector
Hyg
loxP
loxPFW
Ex7FW HygFW
Fis1hyp allele
Hyg
loxPRV
FRTRV
3'RV
loxP - FRT
Fis1fl allele
7 7
Fis1- allele
B
mouse ID 1 2 6 8 9 11 12
mouse ID 8 1 9 11 2 6 12
FIS1
actin
+/+ hyp/+ hyp/hyp
C
mouse ID 1 2 3 4 5 6 7 8 9
+/+ +/+ fl/+ hyp/+ hyp/+ hyp/+ hyp/+ hyp/+ fl/+
D
mFIS1.1 MEAVLNELVSVEDLKNFERKFQSEQAAGSVSKSTQFEYAWCLVRSKYNEDI
mFIS1.2 MPR-------DEAARNFERKFQSEQAAGSVSKSTQFEYAWCLVRSKYNEDI
mFIS1.3 MDAQ----------
α1
TRP1
mFIS1.1 RRGIVLLEELLPKGSKEEQRDYVFYLAVGNYRLKEYEKALKYVRGLLQTEP
mFIS1.2 RRGIVLLEELLPKGSKEEQRDYVFYLAVGNYRLKEYEKALKYVRGLLQTEP
mFIS1.3 RR---SCKELLPKGSKEEQRDYVFYLAVGNYRLKEYEKALKYVRGLLQTEP
TRP1
TRP2
mFis1.1 QNNQAKELERLIDKAMKKDGLVGMAIVGGMALGVAGLAGLIGLAVSKSKS
mFIS1.2 QNNQAKELERLIDKAMKKDGLVGMAIVGGMALGVAGLAGLIGLAVSKSKS
mFIS1.3 QNNQAKELERLIDKAMKKDGLVGMAIVGGMALGVAGLAGLIGLAVSKSKS
α6
TM domain

Figure 1

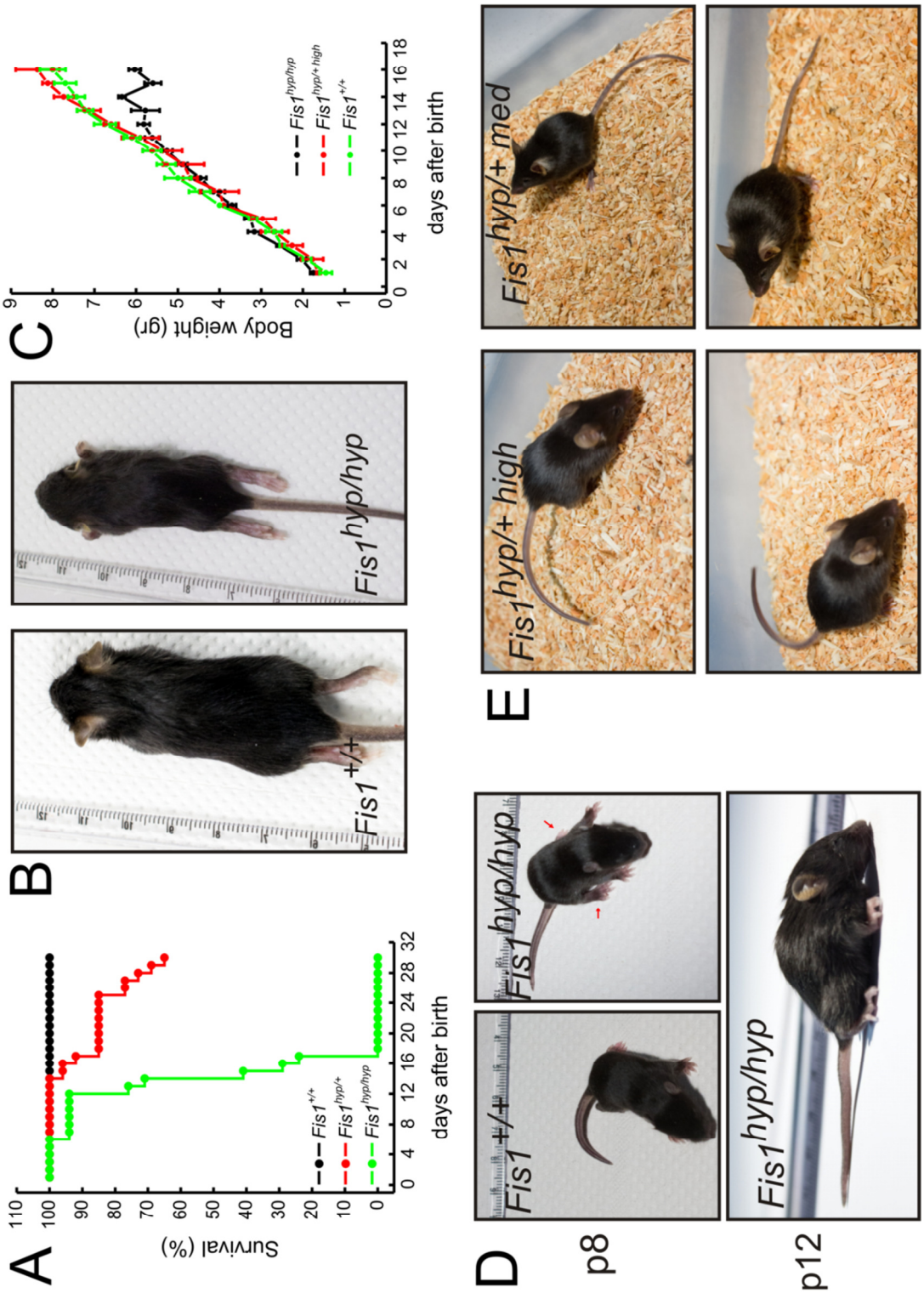
A
Survival (%)
days after birth
Fis1+/+
Fis1hyp/+
Fis1hyp/hyp
B
Fis1+/+
Fis1hyp/hyp
C
Body weight (gr)
days after birth
Fis1hyp/hyp
Fis1hyp/+ high
Fis1+/+
D
p8
p12
Fis1+/+
Fis1hyp/hyp
Fis1hyp/hyp
E
Fis1hyp/+ high
Fis1hyp/+ med

Figure 2

A
Fis1+/+
Fis1hyp/hyp
Fis1hyp/+ high
B
Fis1hyp/+ high
C
Fis1hyp/hyp
D
Fis1+/+
Fis1hyp/hyp
E
Fis1hyp/hyp
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E
Fis1+/+
Fis1hyp/hyp
Fis1hyp/+ high
heart
lungs
brain
liver

Figure 3

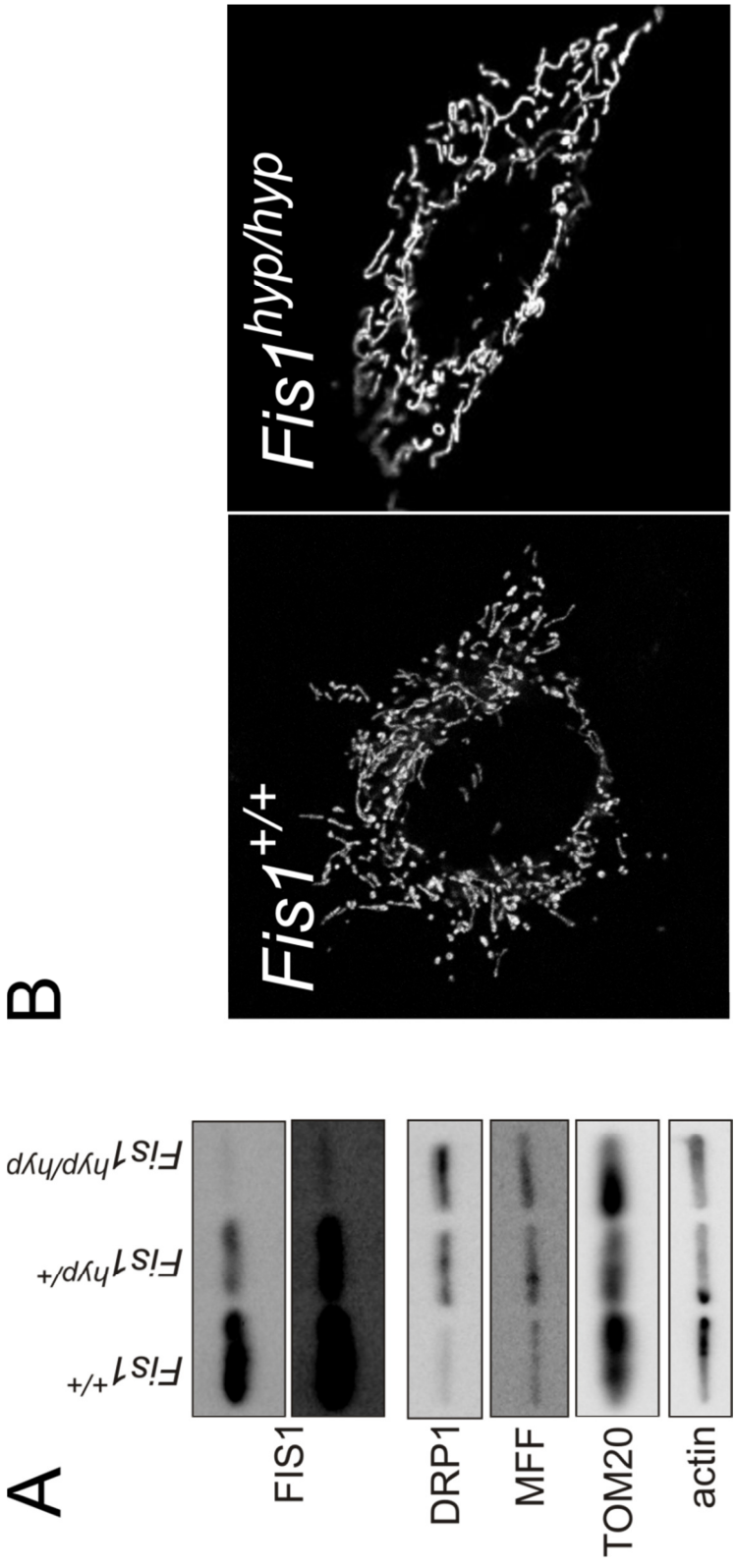
B
Fis1hyp/hyp
Fis1+/+
A
Fis1+/+
Fis1hyp/+
Fis1hyp/hyp
FIS1
DRP1
MFF
TOM20
actin

Figure 4

SUPPLEMENTARY FIGURE LEGENDS

Figure S1 Strategy adopted to generate the targeting vector and to screen hygromycin-resistant colonies for homologous recombination.

(A) Schematic of the cloning of the targeting vector. For details, refer to Materials and methods.

(B) Schematic of *Fis1* genomic locus after homologous recombination. Positions in the genomic locus of the primers used to verify homologous recombination at the 3' end of the targeting vector (3'homFW and 3'homRV) and of the *LoxP* site (loxPhomFW and loxPhomRV) and the dimensions of the expected amplicons are indicated. EcoRV restriction and annealing region of the radioactive probe used to verify homologous recombination at the 5' of the targeting vector are also indicated. For details, see Materials and methods.

(C) Representative images of clone screening performed following the strategy depicted in (B).

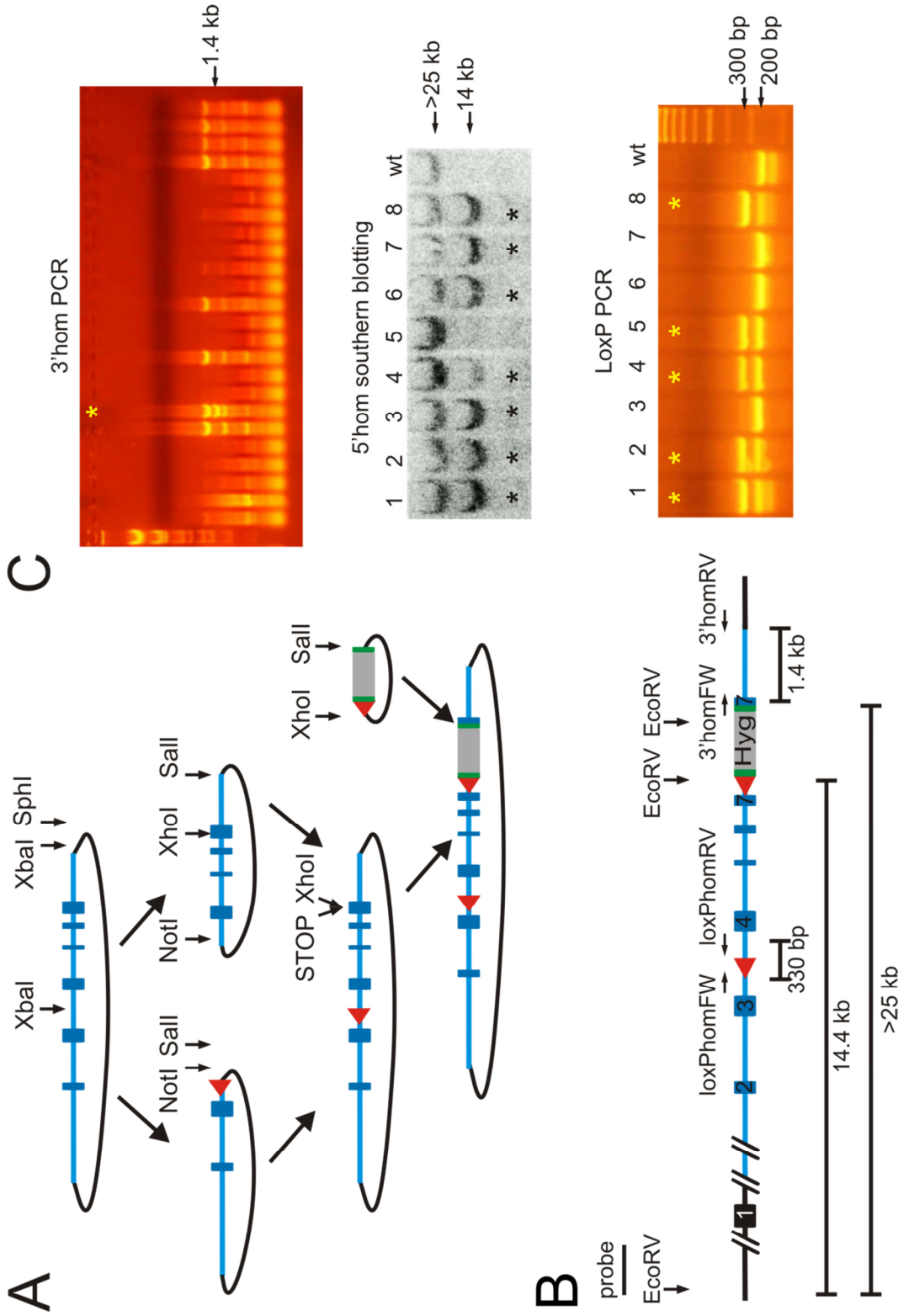
A
XbaI
XbaI SphI
NotI
NotI SalI
XhoI
SalI
STOP XhoI
XhoI SalI
B
probe
EcoRV
EcoRV EcoRV
loxPhomFW
loxPhomRV
3'homFW
3'homRV
Hyg
330 bp
1.4 kb
14.4 kb
>25 kb
C
3'hom PCR
1.4 kb
5'hom southern blotting
>25 kb
14 kb
1 2 3 4 5 6 7 8 wt
LoxP PCR
300 bp
200 bp

Figure S1

Section IV - General conclusions

In this thesis work we generated and started to characterize genetic tools for the assessment of the (patho)physiological role of FIS1.

In particular, we reported the generation of a mouse model that can be used both for constitutive reduced expression (hypomorphic *Fis1*^{hyp} allele) and selective ablation (floxed *Fis1*^{fl} allele). The powerful genetic tool we generated circumvents many of the potential pitfalls of the most commonly used gene targeting approaches. On one side, our hypomorphic allele retains a low FIS1 expression level, thus partially circumventing developmental lethality and concomitantly reducing potential compensation mechanisms due to constitutive ablation. On the other hand, our transgene impinges on the 3'UTR which, as we demonstrated, is shared among all the splicing variants, thus ensuring real *Fis1* depletion.

We showed that *Fis1* hypomorphism causes a perinatal pleiotropic lethal phenotype, comprising severe progressive muscular atrophy, heart ischemia and compromised vessel integrity. The concomitant muscular atrophy and heart ischemia would suggest (cardio)myopathy and/or neuromuscular defects, whereas extensive blood clots in the thorax, the presence of blood in the lungs and in the intestine and the altered microcircular bed in the retina seem to suggest defects in the maintenance of vessel integrity and/or increased vessel permeability and consequent blood leakage. Analysis of heart and muscle functionality, along with assessment of integrity of microcirculation in the retina, will help in clarifying the observed phenotype. Remarkably, also heterozygous mice display lethality at a later stage, although reduced, and show defects comparable to their *Fis1*$^{hyp/hyp}$ littermates, thus suggesting a dose-dependent relationship between phenotype severity and extent of *Fis1* gene ablation. To verify this hypothesis, assessment of residual *Fis1* mRNA production by 5'RACE is required. Subsequently, it would be interesting to correlate residual *Fis1* expression in *Fis1*$^{hyp/+}$ mice with phenotype severity.

Remarkably, the transgene that we generated can be converted into a floxed allele by breeding with Flipper mice (*Flp*$^{+/-}$). This versatile genetic tool will be instrumental in verifying the consequences of constitutive ablation in the more affected organs without the need of further transgene generation and subsequent gene targeting.

In vitro, we observed mitochondrial elongation and upregulation of the fission machinery as a consequence of *Fis1* hypomorphism.

Furthermore, we showed that expression of both human and mouse *Fis1* genes is controlled by multiple splicing. In particular, an exon-skipping event of conserved exon 2 dictates the preferential expression of variant 2 versus the canonical variant 1 in both species. Remarkably, a non-canonical START codon seems to be responsible for transcription of human variant 2 (*hFis1*.2). The resulting protein, although lacking only the first 18 amino acids, triggers mitochondrial elongation when overexpressed in mouse. 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.

In conclusion, our preliminary results suggest that FIS1 exerts both *in vivo* and *in vitro* key roles, thus bringing back forgotten FIS1 in the spotlight of the mitochondrial shaping field. Further investigation is required to completely characterize *Fis1* hypomorphic phenotype and to unveil the molecular mechanisms leading to *Fis1* depletion-mediated defects and death.

Section V - Complete Reference list

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Acknowledgement

Apparently, this one is the very page everybody is expecting and everybody will read first. So I decided to put it at the end: whoever will try to find it, will quickly leaf through the entire thesis.
I do believe that people that I want to thank are smart and sensitive enough that do not need to have their names down on paper to know that I'm deeply thankful to them. Real feelings are the heaviest and non-erasable thing ever. These are the things that will stay: the science and the private life intermingled feelings and memories about this long experience, the many flavors of all the people I've met, the places I lived, the things I did. It was more than a PhD, it was a huge life experience that screwed me up, changed me completely and made me know the incredible human nature, both through myself and through people around me. The friends I've made will be friends for life, although, maybe, we will not keep in touch.
Luca, thanks for the second chance you gave me. Thank you for the freedom you left me. I hope I can say that I've learnt from my mistakes.
Thanks to all the people of the Lab, past and present. Thanks to the founding members of the Lab in Geneva, nice memories and happy time. Thanks to the Swiss *enclave* (almost disappeared now) in Padova. Thanks to more recent members. I've learnt something from each of you.
Veronica, thank you for the nice time back in Geneva and our dinners in Basel, where I cook and you eat. Aswin, we shared a big part of Scorrano Lab's history. You have been the best PhD-mate I could imagine. Claudia, I will never forget all the moments spent together. Camilla, thanks for your molecular biology-point of view. Sowmya, thanks for you India- Buddhist- point of view. Atsuko, thanks for bringing me back a small sweet memory of Geneva-old-time. It made me feel home. Steph, you know what I think.
All this started in Belgium some years ago. Thanks to Bart and Lutgarde for making me understand, from the very first day, that science if tough. Thanks to all De Strooper's Lab members. Special thanks to Vanessa, Alexandra, Laura, Tomek. Thanks to Sara who joined for a while and shared with me the painful experience of doing cloning all day long…
Thanks to Daniela for our week ends in Bruxelles.
Grazie alla mia famiglia.
Grazie Piero.