UNIVERSIDADE DE COIMBRA

# The role of mitochondrial dynamics in autophagy

# A função da dinâmica mitocondrial durante a autofagia

Dissertation presented to the Faculty of Sciences and Technology, University of Coimbra in partial fulfilment of the requirements for obtaining a Doctoral degree in Molecular Biology.

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# Abbreviations

**ΔΨm** – mitochondrial membrane potential

**AFG3L1, AFG3L2** – ATPase family gene 3-like1/ 2

**AIF** – apoptosis-inducing factor

**AMBRA1** – activating molecule in BECN1-regulated autophagy

**AMPK** – AMP-activated protein kinase

**APAF1** - apoptotic peptidase activating factor 1

**Atg**/ **ATG** – autophagy-related gene

**ATG14L** – Atg14-like protein

**ATG16L** – Atg16-like protein

**ATP, ADP, AMP** – adenosine tri/ di/ monophosphate

**BAD** – BCL2-associated agonist of cell death

**BAK** – BCL2 antagonist killer 1

**BAX** – BCL2-associated X protein

**BCL2** – B-cell CLL/ lymphoma 2

**BECN1** – beclin 1

**BID** – BH3-interacting domain death agonist

**BIK** – BCL2-interacting killer

**BNIP3** – BCL2 and adenovirus E1B 19-kDa-interacting protein 3

**BNIP3L** – BCL2 and adenovirus E1B 19kDa interacting protein 3-like

**Caf4** - CCR4 associated factor

**CAMKIα –** calcium/calmodulin-dependent protein kinase I alpha

**cAMP** – cyclic AMP

**CCCP** – carbonyl cyanide m-chlorophenylhydrazone

**CDK1** – cyclin-dependent kinase 1

**CMA** – chaperone-mediated autophagy

**CMT2a, CMT4A** – Charcot-Marie-Tooth IIa / IVa

**CsA** – cyclosporine A

**Cvt** – cytoplasm to vacuole targeting

**DDP**/ **TIMM8a** – deafness dystonia protein/ translocase of inner mitochondrial membrane 8 homologue A

**DLP1** – dynamin-like protein 1

**DNA, mtDNA** – deoxyribonucleic acid, mitochondrial deoxyribonucleic acid

**Dnm1** – dynamin-related 1

**DOA** – dominant optic atrophy

**DRP1** – dynamin-related protein 1

**EM** – electron microscopy

**EPAC** – exchange protein directly activated by cAMP

**ER** – endoplasmic reticulum

**ERAD** – ER-associated degradation

**FCCP** - carbonylcyanide-p-trifluoromethoxy phenylhydrazone

**Fis1, FIS1, hFis1** – fission1, fission 1 homologue (*S. cerevisiae*), human Fis1

**FITC** – fluorescein-isothiocyanate

**Fmc1** - formation of mitochondrial complexes 1

**FOXO3** - forkhead box O3

**FRSK** – forskolin

**Fzo1** – fuzzy onions 1

**GABARAP** - gamma-aminobutyric acid receptor associated protein

**GATE16** – GABA(A) receptor-associated protein-like 2

**GDAP1** – ganglioside-induced differentiation-associated protein 1

**GED** – GTPase effector domain

**GTP** – guanosine triphosphate

**GTPase** – guanosine triphosphatase

**HBSS** – hanks balanced salt solution

**HDAC6** – histone deacetylase 6

**HSP70** – heat shock protein

**i-AAA** – inner membrane space AAA

**IBMX** – 3-isobutyl-1-methylxanthine

**IGF1** - insulin-like growth factor 1

**IM, IMM** – inner membrane, inner mitochondrial membrane

**IMS** – inter-membrane space

**JNK1** – c-Jun N-terminal kinase 1

**LC3** – light chain 3

**LETM1** - leucine zipper-EF-hand containing transmembrane protein 1

**LIR** – LC3-interating region

**LTG** – lysotracker green

**m-AAA** – matrix AAA

**MAPL** – mitochondrial-anchored protein ligase

**MAP1LC3** – microtubule-associated protein 1 light chain 3

**MAP4K3** – mitogen-activated protein kinase kinase kinase

**MARCH5** – membrane-associated ring finger (C3HC4) 5

**Marf** – mitochondrial assembly regulatory factor

**Mdm30** – mitochondrial distribution and morphology 30

**Mdm38** – mitochondrial distribution and morphology 38

**Mdv1** – mitochondrial division 1

**MEFs** – mouse embryonic fibroblasts

**MFF** – mitochondrial fission factor

**MFN1, MFN2** – mitofusin 1/ 2

**Mgm1** – mitochondrial genome maintenance 1

**MIB** – mitofusin-binding protein

**MitoPLD** – mitochondrial phospholipase D

**MnSOD** – manganese-containing superoxide dismutase

**MOMP** – mitochondrial outer membrane permeabilization

**mt-luciferase** – mitochondrially targeted luciferase

**mt-PAGFP** – mitochondrially targeted photoactivable green fluorescent protein

**MTP18** – mitochondrial protein 18 kDa

**mRFPI** – monomeric red fluorescent protein

**mtRFP** – mitochondrially targeted dsRED

**MTS** – mitochondrial targeting sequence

**mtYFP** – mitochondrially targeted yellow fluorescent protein

**MULAN** – mitochondrial E3 ubiquitin protein ligase 1

**NBR1** – neighbor of BRCA1 gene 1

**NO** – nitric oxide

**OMI/ HTRA2** – Omi stress-regulated endoprotease/ HtrA serine peptidase 2

**OM, OMM** – outer membrane, outer mitochondrial membrane

**OMMAD** – outer mitochondrial membrane-associated degradation

**OPA1** – optic atrophy 1

**PAS** – phagophore assembly site/ pre-autophagosome structure

**PARL** – presenilin associated, rhomboid-like

**Pcp1** – processing of cytochrome *c* peroxidase 1

**PE** – phosphatidylethanolamine

**PHB** – prohibitin

**PKA** – protein kinase A

**PKB** – protein kinase B

**PS** – phosphatidylserine

**PtdIns** – phosphatidylinositol

**PtdIns3K** – phosphatidylinositol 3-kinase

**PtdIns(3)P, PtdIns(4,5)$P_2$, PtdIns(3,4,5)$P_3$** – phosphatidylinositol (3)-phosphate/ (4,5)-diphosphate/ (3,4,5)-triphosphate

**PTP** – permeability transition pore

**RAB** – member RAS oncogene family

**RHEB** – Ras homologue enriched in brain

**RING** – really interesting new gene

**ROS** – reactive oxygen species

**RTG** – retrograde signalling pathway

**RUBICON** – RUN domain and cystein-rich domain containing, BECN1-interacting

**SENP5** – SUMO1/sentrin specific peptidase 5

**SLP2** – stomatin-like protein 2

**SMAC/ DIABLO** – second mitochondria-derived activator of caspase/ diablo homologue (*Drosophila*)

**SQSTM1** - sequestosome 1

**SUMO** – small ubiquitin-like modifier

**tBID** – truncated BID

**TM** – transmembrane

**TMRM** – tetramethyl rhodamine methyl ester

**Tor, TOR, mTOR** – target of rapamycin, mammalian TOR

**TORC1, TORC2, mTORC** – TOR complex 1/ 2, mammalian TORC

**TPR** – tetratricopeptide

**TP53INP2** – tumor protein 53-induce nuclear protein 2

**TSC1, TSC2** – tuberous sclerosis complex 1/ 2

**Ugo1** – ugo (japanese for fusion) 1

**ULK1** – unc-51-like kinase

**Uth1** – youth 1

**UVRAG** – ultraviolet irradiation resistance associated gene

**VMP1** – vacuole membrane protein 1

**Vps34, hVPS34** – vacuolar protein sorting 34, human VPS34

**WIPI1, WIPI2** – WD repeat domain, phosphoinositide interacting 1/ 2

**wortm** – wortmannin

**YFP-LC3** – yellow fluorescent protein-LC3

**YME1L** – YME1-like 1 (*S. cerevisiae*)

**3-MA** – 3-methyladenine

# Resumo

As mitocôndrias são organelos essenciais para a vida e morte das células. A maioria do ATP celular é produzido nas mitocôndrias e estes organelos são fundamentais para a regulação de vários processos celulares, como vias de sinalização mediadas por $Ca^{2+}$ e apoptose (Ernster and Schatz, 1981; Green and Kroemer, 2004; Rizzuto et al., 2000). A versatilidade funcional deste organelo é acompanhada pela sua complexidade morfológica (Bereiter-Hahn and Voth, 1994). Durante a vida celular, as mitocôndrias sofrem continuamente processos de fusão e fragmentação. Estas alterações morfológicas são reguladas por uma família de proteínas que controla a forma do organelo. Nos mamíferos, a fusão mitocondrial é controlada pelas MFN1 e MFN2 na membrana externa da mitocôndria e OPA1 na membrana interna. A fragmentação mitocondrial é regulada pela proteína citoplasmática DRP1, que se desloca para a membrana externa da mitocôndria, onde participa na reacção de fragmentação, e pela proteína FIS1, que funciona presumivelmente como o adaptador de DRP1 na membrana mitocondrial externa (Wasilewski and Scorrano, 2009). Um nível adicional de complexidade deste organelo consiste na ultra-estrutura da membrana interna. A membrana interna pode ser subdividida numa membrana que a delimita e em cristas, que representam compartimentos separados, ligados à membrana por junções tubulares estreitas (Frey and Mannella, 2000).

Durante a progressão da apoptose, observa-se uma dramática reestruturação das cristas mitocondriais (Scorrano et al., 2002) e a fragmentação do organelo (Frank et al., 2001). Alterações na forma das mitocôndrias regulam um número crescente de processos celulares, como a sinalização mediada por $Ca^{2+}$ (Szabadkai et al., 2004), a formação de dendrites (Li et al., 2004), a migração de linfócitos (Campello et al., 2006), o ciclo celular (Mitra et al., 2009) e a expectativa de vida de fungos (Scheckhuber et al., 2007). No entanto, o nosso conhecimento sobre as funções que este organelo desempenha e a sua morfologia durante a autofagia é escasso. O objectivo desta tese consistia em explorar a relação entre dinâmica mitocondrial e autofagia.

Na primeira parte desta tese, estudámos se a mitofagia pode ser desencadeada unicamente pela fragmentação mitocondrial ou se a disfunção do organelo, muitas vezes associada à fragmentação, é um pré-requisito para a sua degradação. Para tal, analisámos o efeito da sobre-expressão de FIS1 na indução de autofagia. FIS1 é uma proteína com duas funções, que regula independentemente a fragmentação mitocondrial e a apoptose, através de um efeito directo no funcionamento do organelo (Alirol et al., 2006). Formas mutadas desta proteína, que discriminam especificamente a fragmentação da disfunção

mitocondrial, permitiram-nos analisar se a mitofagia ou um processo mais generalizado de autofagia são induzidos por fragmentação e/ ou disfunção mitocondrial.

A sobre-expressão de FIS1 ou FIS1$^{\Delta\alpha1}$, que promove disfunção mitocondrial mas não leva à fragmentação do organelo, induziu autofagia. Pelo contrário, a sobre-expressão de FIS1$^{K148R}$, que causa fragmentação mitocondrial sem levar à disfunção do organelo, não induziu autofagia nas células. Em células que sobre-exprimiam FIS1 foram observadas mitocôndrias fragmentadas e disfuncionais circundadas por autofagosomas, sugerindo que, pelo menos algumas destas mitocôndrias, são degradadas por autofagia. É possível, portanto, concluir que é a disfunção mitocondrial, e não a fragmentação do organelo, que conduz à indução de autofagia (Gomes and Scorrano, 2008).

Na segunda parte desta tese, pretendíamos perceber se a morfologia mitocondrial é alterada durante a indução de autofagia e se a forma como as células respondem à autofagia é influenciada por alterações na morfologia mitocondrial. Surpreendentemente, a indução de autofagia por restrição nutritiva induziu o alongamento das mitocôndrias *in vitro* e *in vivo*. O alongamento mitocondrial é um evento essencial que determina o destino das células durante a autofagia. Nas células onde a autofagia é induzida, os níveis de cAMP aumentam rapidamente e levam à activação da PKA, que fosforila a proteína de fragmentação DRP1. Desta forma, DRP1 mantém-se no citoplasma, o que resulta no alongamento mitocondrial, por ausência de oposição à fusão do organelo. As mitocôndrias alongadas estão protegidas da degradação por autofagia, apresentam maior número de cristas onde a ATPase oligomeriza para manter a produção de ATP e permitir a sobrevivência celular. Pelo contrário, se o alongamento é bloqueado, as mitocôndrias tornam-se disfuncionais e "canibalizam" o ATP do citoplasma para manter o potencial de membrana, levando à morte celular (Gomes et al, *in press*).

Em conclusão, a autofagia é mais um processo celular regulado por alterações de morfologia e actividade mitocondrial.

# Abstract

Mitochondria are key organelles for life and death of the cell. They provide most of the cellular ATP and play crucial functions in different cellular processes, ranging from $Ca^{2+}$ signalling to apoptosis (Ernster and Schatz, 1981; Green and Kroemer, 2004; Rizzuto et al., 2000). Mitochondrial functional versatility is matched by their morphological diversity. During cell life, mitochondria continuously fuse and divide (Bereiter-Hahn and Voth, 1994). These events are regulated by a growing family of mitochondria-shaping proteins. In mammals, mitochondrial fusion is controlled by mitofusins (MFN) 1 and 2 in the outer mitochondrial membrane (OMM) and OPA1 in the inner mitochondrial membrane (IMM). Mitochondrial fission is regulated by DRP1, a cytosolic protein that has to translocate to the OMM to participate in the fission reaction and its presumable adaptor in the OMM, FIS1 (Wasilewski and Scorrano, 2009). Another level of complexity consists in the ultrastructure of the IMM. The IMM can be subdivided in an inner boundary membrane and in the cristae, that represent separate compartments, bound to the inner boundary by narrow tubular junctions (Frey and Mannella, 2000).

Remodelling of mitochondrial cristae (Scorrano et al., 2002) and fragmentation of the organelle network (Frank et al., 2001) participate in the progression of apoptosis. Moreover, changes in mitochondrial shape regulate a growing number of other cellular processes, such as calcium signalling (Szabadkai et al., 2004), formation of dendritic spines (Li et al., 2004), migration of lymphocytes (Campello et al., 2006), cell cycle (Mitra et al., 2009) and even lifespan in lower eukaryotes (Scheckhuber et al., 2007). However, our understanding of the role of mitochondria and of their morphology during autophagy is scarce. The aim of this thesis was to explore the relationship between mitochondrial dynamics and autophagy.

In the first part of this thesis, we addressed whether mitophagy could be triggered simply by mitochondrial fission or whether dysfunction, which is often associated with fragmentation of the organelle, was also required. In order to answer to these questions, we analyzed the effect of enforced FIS1 expression on autophagy. FIS1 is a bifunctional protein that independently regulates mitochondrial fission and apoptosis, through a direct effect on mitochondrial function (Alirol et al., 2006). The availability of mutants that could specifically dissociate mitochondrial fission or dysfunction allowed us to verify if mitophagy or a more generalized process of autophagy could be sustained by mitochondrial fission and/ or dysfunction .

Enforced expression of FIS1 or of $FIS1^{\Delta\alpha1}$, that prompts mitochondrial dysfunction but not fission, induced autophagy. In contrast, overexpression of $FIS1^{K148R}$ that causes mitochondrial fragmentation but not dysfunction did not induce autophagy. Additionally,

fragmented, dysfunctional mitochondria overexpressing FIS1 were found surrounded by autophagosomes, suggesting that at least some of these mitochondria are degraded by autophagy. Therefore, mitochondrial dysfunction rather than fragmentation triggers autophagy, suggesting that mitochondrial dysfunction can feedback to the autophagic machinery to activate it (Gomes and Scorrano, 2008).

In the second part of this thesis we aimed at understanding whether mitochondrial morphology changes during induction of autophagy and if the final outcome of autophagy was influenced by changes in mitochondrial morphology. Surprisingly, we found that during starvation mitochondria elongate both *in vitro* and *in vivo*. Mitochondrial elongation is a crucial event that determines cell fate during autophagy. In starving cells, a rapid increase in cAMP levels activates PKA that in turn phosphorylates the pro-fission molecule DRP1, keeping it in the cytosol and allowing unopposed mitochondrial fusion. Elongated mitochondria are protected from autophagic elimination, display denser cristae where ATPase can oligomerize to maintain ATP production and to allow survival of starving cells. On the contrary, if elongation is blocked, mitochondria become dysfunctional and "cannibalize" cytoplasmic ATP to maintain their membrane potential, precipitating cell death (Gomes et al, *in press*).

In summary, our findings exemplify a further cellular process, autophagy, that is regulated by changes in mitochondrial morphology and activity.

# Chapter 1

# Introduction

Eukaryotic cells are surrounded by a plasma membrane, which encloses the cytosol and various intracellular compartments also delimited by membranes, called organelles. The existence of organelles allows compartmentalization of biomolecules and, thus, functional specialization. For instance, the nucleus is the site of genetic codification; mitochondria produce most of the cellular ATP; protein synthesis and folding takes place in the endoplasmic reticulum (ER); the Golgi apparatus modifies proteins delivered from the ER and distributes them (as well as lipids) to their final cellular destination; and degradation of macromolecules and even of entire organelles takes place in the lysosome, by a process called autophagy.

Autophagy is a self-degradation process induced for example when nutrients are limited. During autophagy, an isolation membrane engulfs components of the cytosol, including entire organelles and expands, giving rise to autophagosome that ultimately fuses with lysosomes. Nowadays, it is clear that, under certain conditions, specific cargoes can be selectively removed by autophagy. In particular, autophagy is the prime mechanism to eliminate mitochondria, by a process named mitophagy.

Mitochondria participate in several processes ranging from ATP production to regulation of cell signalling or amplification of apoptosis. Mitochondrial functional versatility is matched by a complex morphological and structural variety. The availability of genetic tools allowed to explore the role of mitochondrial morphology in complex cellular processes. For example, remodelling of mitochondrial cristae and fragmentation of the organelle network participate in the progression of apoptosis.

In this thesis, the relationship between mitochondrial morphology/ structure and autophagy was explored, focusing on the role that changes in mitochondrial shape play during autophagy. The introduction will, therefore, address specifically our knowledge on mitochondrial shape and function, autophagy and, in particular, mitophagy molecular mechanisms.

## 1.1 Mitochondria

Mitochondria are cellular organelles delimited by two membranes, that embrace about one tenth of the cell's proteins. These organelles, on a weight basis proportion, are able to convert between 10 000 and 50 000 times more energy per second than the sun (Schatz, 2007).

Almost a century after the first descriptions of mitochondria, the acquirement of the ability to isolate them (CLAUDE, 1946) and the discovery that mitochondria contain a respiratory system and the enzymes of the tricarboxylic acid cycle and fatty acid oxidation (Kennedy and Lehninger, 1949) marked the start of the bioenergetics era. This period

culminated with Peter Mitchell's chemiosmotic theory (Mitchell and Moyle, 1965), that deserved him the Nobel Prize for chemistry in 1978. Thus, the free energy of respiration is used to pump protons from the matrix to the inter-membrane space (IMS), establishing an electrochemical gradient. Since the inner mitochondrial membrane (IMM) displays an extremely low passive permeability to protons, an electrochemical gradient ($\Delta\mu H+$) builds-up across the membrane. The electrochemical gradient is the sum of two components: the proton concentration difference and the electrical potential difference across the membrane. The estimated magnitude of the proton electrochemical gradient is about -220 mV (negative inside) and under physiological conditions most of the gradient is in the form of electrical potential difference. The proton gradient is converted in ATP, by the F1F0-ATP synthase. F1F0-ATP synthase couples the transport of the protons back to the matrix with the phosphorylation of ADP to ATP.

For two decades after the discovery by Peter Mitchell, the study of mitochondria suffered a considerable decline. These organelles were, however, able to surprise again the scientific community when their involvement in apoptosis was discovered (Kluck et al., 1997; Yang et al., 1997). From then on, these highly dynamic organelles have been implicated in the regulation of a great number of physiological processes, that keeps increasing.

## 1.2 Mitochondria ultrastructure

Pioneering work from Palade and Sjostrand revealed that mitochondria possess two membranes – an outer mitochondrial membrane (OMM) and a highly convoluted IMM, folded in a series of ridges that were named cristae by Palade (Palade, 1952; SJOSTRAND, 1953). Text books are based in Palade's model, also called the baffle model. According this model, cristae are invaginations of the IMM with broad openings to the IMS (Fig. 1A).

Technical improvements of electronic microscopy allowed researchers to better inspect mitochondrial ultra-structure. Applications of electron tomography to the study of mitochondria from several tissues highlighted that cristae are not just simple invaginations of the IMM, but they represent a distinct compartment that connects to the IMS by narrow tubular connections, called cristae junctions (Mannella et al., 1994; Perkins et al., 1997). Electron tomography revealed that the diameter of cristae junctions is about 28 nm and the average distance across the OMM and IMM is 20 nm (Fig.1B).

The discovery that cristae represent a distinct compartment suggests that diffusion between cristae and IMS is restricted, which obviously have important functional consequences. Cristae are defined as the site of oxidative phosphorylation (Gilkerson et al., 2003). Accordingly, they contain the proteins involved in oxidative phosphorylation, and ATP synthase dimers assemble at the cristae apices (Giraud et al., 2002; Strauss et al., 2008).

This highly defined compartmentalization suggests that cristae are special sub-structures that ensure optimal conditions for the ATP production, limiting the diffusion of metabolites such as protons or ADP during respiration (Demongeot et al., 2007). In turn, the cristae shape and density can be modified by the respiratory state of mitochondria (Hackenbrock, 1968; Hackenbrock et al., 1980). Additionally, the major part of cytochrome *c* is stored into the cristae (Scorrano et al., 2002). This corroborates the hypothesis that cristae are special compartments involved in respiration and with an important role during apoptosis, when cytochrome *c* must be released into the cytoplasm. The cristae shape is maintained by the cristae junctions that represent a functional barrier between the cristae space and the IMS (Fig. 1C,D).

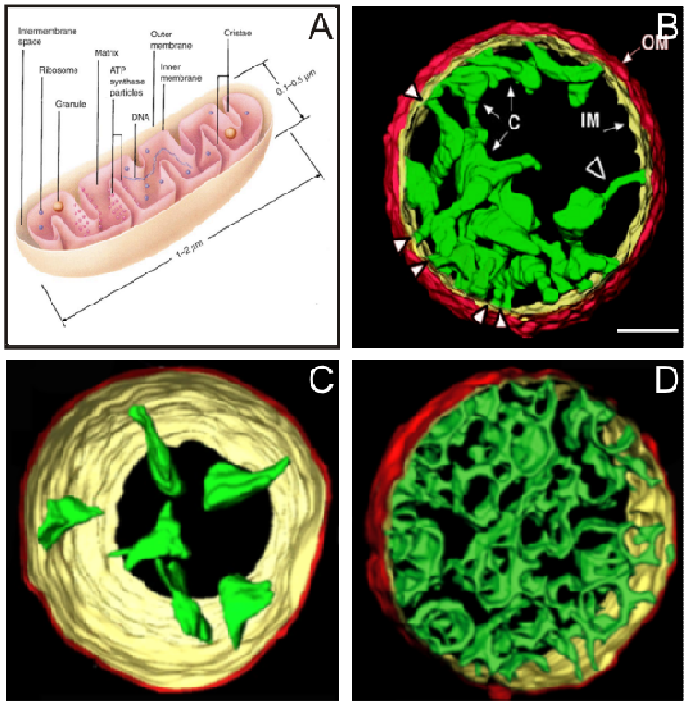


**Figure 1 – Mitochondrial ultrastructure. (A)** A text book-like representation of the baffle model adapted from (Frey and Mannella, 2000) **(B)** Three-dimensional reconstructions of isolated rat liver mitochondria obtained by high-voltage electron tomography. OM: outer membrane, IM: inner membrane, C: selected cristae; arrowheads point to narrow tubular regions that connect cristae to periphery and to each other. Bar, 0.4 µm. Adapted from (Frey and Mannella, 2000). **(C-D)** Representative surface-rendered views of electron microscopy tomography reconstructions of mitochondria before (C) and after (D) remodelling. The OM is depicted in red, the inner boundary membrane in yellow, and the cristae in green (Scorrano et al., 2002).

## 1.3 Mitochondrial shape and dynamics

Mitochondrial shape is very heterogeneous (Fig. 2), ranging from small spheres to interconnected tubules (Bereiter-Hahn and Voth, 1994). For example, the mitochondria of rat cardiac muscle and diaphragm skeletal muscle appear as isolated ellipses or tubules in embryonic stages but then reorganize into reticular networks in the adult (Bakeeva et al., 1978). The name of the organelle, “mitochondrion”, reflects their heterogeneous morphology, a combination of the Greek words for “thread” and “grain”.

During cell life, mitochondria undergo continuous cycles of fusion and fission. Real-time imaging reveals that individual mitochondrial tubules continually move back and forth along their long axes on radial tracks. Occasionally, two mitochondrial tubules encounter

each other and fuse (Bereiter-Hahn and Voth, 1994; Chen et al., 2003). On the other hand, these tubules can also undergo fission events, giving rise to two or more mitochondrial units. It was reported, in pancreatic β-cells, that fusion and fission events are paired. Fusion triggers fission, but fission has no effect on the following fusion event (Twig et al., 2008). In addition to complete fusion, a transient form of fusion was recently identified in which two mitochondria come into close apposition, exchange soluble IMS and matrix proteins, and separate, maintaining the original morphology. Transient fusion, called "kiss-and-run", supports mitochondrial motility and metabolism (Liu et al., 2009).

Being the mitochondria bordered by two membranes, any mechanism of fusion and fission must take into account that the coordinate fusion/ division of four lipid bilayers is required. Thus, mitochondrial fusion and fission are complicated processes that are controlled by a growing number of mitochondrial-shaping proteins.

Several members of the mitochondrial-shaping protein family, that include pro-fusion and pro-fission proteins, are dynamin-related proteins. Dynamins are ubiquitous mechano-enzymes that hydrolyze GTP to regulate fusion, fission, tubulation and elongation of cellular membranes (McNiven et al., 2000). The role of dynamins in controlling mitochondrial shape was initially identified by genetic screens in budding yeast, where deletion of specific genes resulted in gross alterations of the mitochondrial network, and ultimately in functional abnormalities including loss of mitochondrial DNA, growth defects and petite strains (Dimmer et al., 2002; Shaw and Nunnari, 2002).

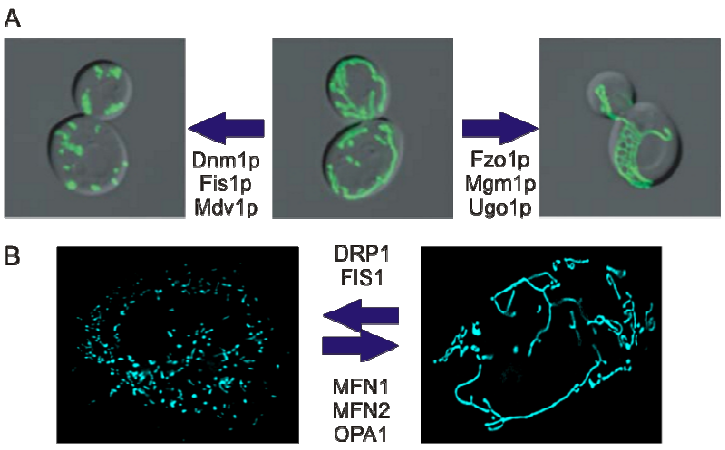


**Figure 2 – Fusion and fission of the mitochondrial network. (A)** Cartoon depicting the mitochondrial network in *S. cerevisiae* and the proteins regulating fusion and fission events. **(B)** Cartoon depicting mitochondrial morphology in mammals and mitochondria-shaping proteins.

### 1.3.1 Core mitochondrial-fusion proteins

#### 1.3.1.1 Fzo/ MFN1, 2

The first mitochondrial fusion protein identified was the *Drosophila melanogaster* Fuzzy onions 1 protein (Fzo1), a large transmembrane guanosine triphosphatase (GTPase)

required for the formation of the giant mitochondrial derivative during spermatogenesis (Hales and Fuller, 1997). The *Saccharomyces cerevisiae* orthologue of Fzo1 mediates mitochondrial fusion during mitotic growth and mating and is required for long-term maintenance of mitochondrial deoxyribonucleic acid (mtDNA) (Hermann et al., 1998). Two ubiquitous Fzo1 homologues were identified in mammals - Mitofusin (MFN) 1 and 2 (Eura et al., 2003; Rojo et al., 2002; Santel et al., 2003). Of note, besides Fzo1, *Drosophila melanogaster* possesses another mitofusin homologue, the mitochondrial assembly regulatory factor (Marf) which is expressed ubiquitously in males and females. Muscle tissue specific knock-down of Marf induces fragmentation and alteration of mitochondrial ultrastructure (Deng et al., 2008)

MFN1 and 2 present high (81%) identity, similar topologies and both reside in the OMM (Chen et al., 2003; Legros et al., 2002a; Rojo et al., 2002; Santel and Fuller, 2001; Santel et al., 2003). MFN1 and 2 possess a GTPase domain and a coiled coil domain located at the amino-terminus of the proteins, protruding towards the cytosol (Fig. 3). The two TM regions form a U-shaped membrane anchor, ending in a cytosolic, carboxy-terminal coiled coil motif (Koshiba et al., 2004; Rojo et al., 2002; Santel, 2006). The coiled coil is a widespread helical structural motif that functions as an oligomerization domain (Oakley and Hollenbeck, 2001). Two MFNs on opposing membranes can bind in *trans* to bridge mitochondria, maintaining a distance of 95 Å between the two membranes (Koshiba et al., 2004).

Despite their high homology, MFNs are not functionally redundant. First, MFN1 has a higher GTPase activity than MFN2, although its affinity for GTP is lower (Ishihara et al., 2004). In accordance, direct measurements of mitochondrial fusion rates in $Mfn1^{-/-}$ and $Mfn2^{-/-}$ cells showed that cells containing only MFN1 retain more fusion activity than those that contain only MFN2 (Chen et al., 2003). Finally, MFN1, but not MFN2, is essential for OPA1-dependent mitochondrial fusion in embryonic fibroblasts (Cipolat et al., 2004a). In compliance, our laboratory suggested a role beyond fusion for MFN2. MFN2 was the first molecule identified that tethers mitochondria to ER and, additionally, MFN2 regulates ER shape. MFN2 localizes not only on mitochondria, but it is highly enriched at the level of the mitochondria-ER interface and present (albeit to a lesser extent) at the ER. Genetic ablation of MFN2 disrupts the ER structure and loosens the ER-mitochondria interaction, thereby reducing mitochondrial $Ca^{2+}$ uptake dependent on the generation of $Ca^{2+}$ micro-domains between ER and mitochondria (de Brito and Scorrano, 2008a). In conclusion, MFN1 and 2 seem to play different roles in mitochondrial physiology, with MFN1 that (in cooperation with OPA1) exquisitely regulates mitochondrial fusion and MFN2 that plays a role in maintaining mitochondria-ER interactions, ultimately impacting on mitochondrial metabolism, apoptosis and even progression trough cell cycle.

#### 1.3.1.2 Mgm1/ Msp1/ OPA1

Optic atrophy 1 (OPA1) is a dynamin-related protein located in the IMM. Mitochondrial genome maintenance 1 (Mgm1), the yeast homologue of OPA1, has been identified in a genetic screen for nuclear genes required for the maintenance of mtDNA in the budding yeast *Saccharomyces cerevisiae* (Jones and Fangman, 1992). Years later, Pelloquin and colleagues isolated Msp1, the *Schizosaccharomyces pombe* orthologue (Pelloquin et al., 1998). The human gene *OPA1* was identified in 2000 by two independent groups (Alexander et al., 2000; Delettre et al., 2000). A more detailed analysis showed that Mgm1, Msp1 and OPA1 are localized in the IMS, tightly associated with the IMM (Guillou et al., 2005; Herlan et al., 2003; Olichon et al., 2002; Sesaki et al., 2003b; Wong et al., 2003). These proteins, albeit displaying just approximately 20% of sequence identity, exhibit a highly conserved secondary structure, consisting of two predicted coiled coils, one amino-terminal to the GTPase domain and the other at the carboxy-terminus. The carboxy-terminal coiled coil domain of OPA1 may function as a GTPase effector domain (GED). On its amino-terminal, OPA1 possesses a mitochondrial targeting sequence (MTS) that targets the protein to mitochondria. Studies in yeast show that the MTS of Mgm1 is cleaved by the mitochondrial processing peptidase upon import (Satoh et al., 2003) (Fig. 3).

The functional analysis of Mgm1 and Msp1 revealed that both proteins are required for the maintenance of fusion-competent mitochondria in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Mgm1 forms a complex together with Fzo1 which participates in the coordinated fusion of the IMM and OMM (Wong et al., 2003). The high degree of secondary structure conservation suggests that the function of OPA1 is conserved in mammals. But, it is less clear whether OPA1 plays a role in fission, rather than in fusion of mitochondria. Overexpression studies showed that high levels of OPA1 can drive fragmentation of the mitochondrial reticulum (Misaka et al., 2002; Olichon et al., 2003). However, overexpression of OPA1 or its downregulation by siRNA in mouse embryonic fibroblasts (MEFs) discloses a linear relationship between OPA1 levels and mitochondrial fusion (Cipolat et al., 2004a).

In addition to its role in mitochondrial fusion, OPA1 has been implicated in morphology and architecture of mitochondrial cristae. Mgm1/OPA1-depleted cells display disorganized cristae with irregular shape, some of which with large cristae junctions, suggesting that these proteins play a role in cristae maintenance (Griparic et al., 2004; Olichon et al., 2003; Sesaki et al., 2003b). A possible role of OPA1/Mgm1 in structuring cristae is consistent with its cristae localization, as confirmed by biochemical (Griparic et al., 2004; Olichon et al., 2003; Pelloquin et al., 1999; Wong et al., 2000) and immunogold staining (Vogel et al., 2006). Interestingly, Amutha and colleagues have reported that Mgm1p is required for oligomerization of ATP synthase (Amutha et al., 2004); this result causally

associates the cristae derangement typical of OPA1 depleted cells to an aberrant oligomerization of ATP synthase rather than establishing a direct effect of OPA1 on cristae morphology. In 2006, our laboratory demonstrated that OPA1 depleted cells show disorganized cristae and are more prone to apoptosis. Interestingly, OPA1 can regulate cytochrome *c* mobilization and apoptotic cristae remodelling independently of its pro-fusion activity; moreover, OPA1 organizes into high molecular weight complexes, that regulate cristae morphology and are targeted by BID during apoptosis. This correlates with the remodelling of the cristae that occurs during apoptosis (Frezza et al., 2006). The group of Nunnari proposed a similar model in yeast where Mgm1 was found to be required to tether and fuse mitochondrial inner membranes. Using a specific fusion assay, they observed an additional role of Mgm1 in IMM dynamics, specifically in the maintenance of cristae structures through Mgm1 interactions on opposing inner membranes (Meeusen et al., 2006). From these data, it is possible to conclude that OPA1, besides its role in IMM fusion, has a critical role in controlling cristae structure and, in particular, apoptotic cristae remodelling, that will be described in a separated chapter.

### 1.3.2 Core mitochondrial-fission proteins

#### 1.3.2.1 Dnm1/ DLP1/ DRP1

The core mitochondrial fission machinery in mammals is constituted by the cytoplasmic dynamin-related protein 1 (DRP1) (Smirnova et al., 2001) and the OMM protein, FIS1 (James et al., 2003).

Dnm1 in yeast, DRP1 in *Caenorhabditis elegans* and dynamin-like protein 1 (DLP1)/ DRP1 in mammals are homologues and present similarities with dynamin, a large GTPase that participates in membrane scission in multiple endocytic and secretory organelles (Praefcke and McMahon, 2004). Although DRP1 is cytosolic, a fraction of the protein is found in spots on mitochondria at sites of constriction (Labrousse et al., 1999; Smirnova et al., 2001). DRP1 contains a dynamin-like-central domain and a carboxy-terminal GTPase effector domain, in addition to its amino-terminal GTPase. Intra-molecular interactions between the GTPase and GED regions appear to be required for full GTPase and fission activities (Zhu et al., 2004) (Fig. 3). DRP1 can oligomerize *in vitro*, into ring-like structures and inter-molecular oligomerization is observed at membrane constriction sites. Given its similarities with dynamin, DRP1 was proposed to couple GTP hydrolysis with mitochondrial membrane constriction and fission (Hinshaw, 1999; Smirnova et al., 2001).

#### 1.3.2.2 Fis1/ FIS1

FIS1 is an outer membrane protein homogenously distributed on the surface of mitochondria (James et al., 2003). Its amino-terminal domain is exposed to the cytosol and forms a tetratricopeptide (TPR)-like domain, predicted to allow protein–protein interactions (Suzuki et al., 2003) (Fig. 3). The carboxy-terminal domain of FIS1 possesses a TM domain and a short stretch of amino acids facing the IMS. FIS1 overexpression induces mitochondrial fragmentation, although the protein does not possess any enzymatic activity. FIS1 probably recruits DRP1 to punctuate structures on mitochondria during mitochondrial fission, being therefore considered the limiting factor in the fission reaction. Accordingly, DRP1 and FIS1 seem to interact, as judged by crosslinking and co-immunoprecipitation (Yoon et al., 2003). However, whether FIS1 is absolutely needed for DRP1-dependent fission is not clear. Indeed, downregulation of FIS1 only partially diminishes DRP1 recruitment to mitochondria (Lee et al., 2004). In accordance, another OMM protein, MFF, has been recently reported to play a role in DRP1 recruitment to mitochondria, suggesting that FIS1 is not the only protein that performs this job (Otera et al., 2010).

FIS1 yeast orthologue displays the same structural properties and is called Fis1. During assembly of the yeast mitochondrial fission complex, Fis1 recruits Dnm1 to mitochondria. Although a direct physical interaction between Fis1 and Dnm1 has been reported (Wells et al., 2007), a third player is essential for mitochondrial fission in yeast – Mdv1 (Tieu and Nunnari, 2000) or its paralog Caf4 (Griffin et al., 2005). These proteins constitute adaptors, acting as scaffolds for the assembly of dynamins on membranes (Koirala et al., 2010). Orthologues of adaptor proteins in mammals are unknown.

Finally, as reported by our laboratory in 2006, FIS1 is a bifunctional protein that independently regulates mitochondrial fission and apoptosis, as will be described in a separate chapter (Alirol et al., 2006).

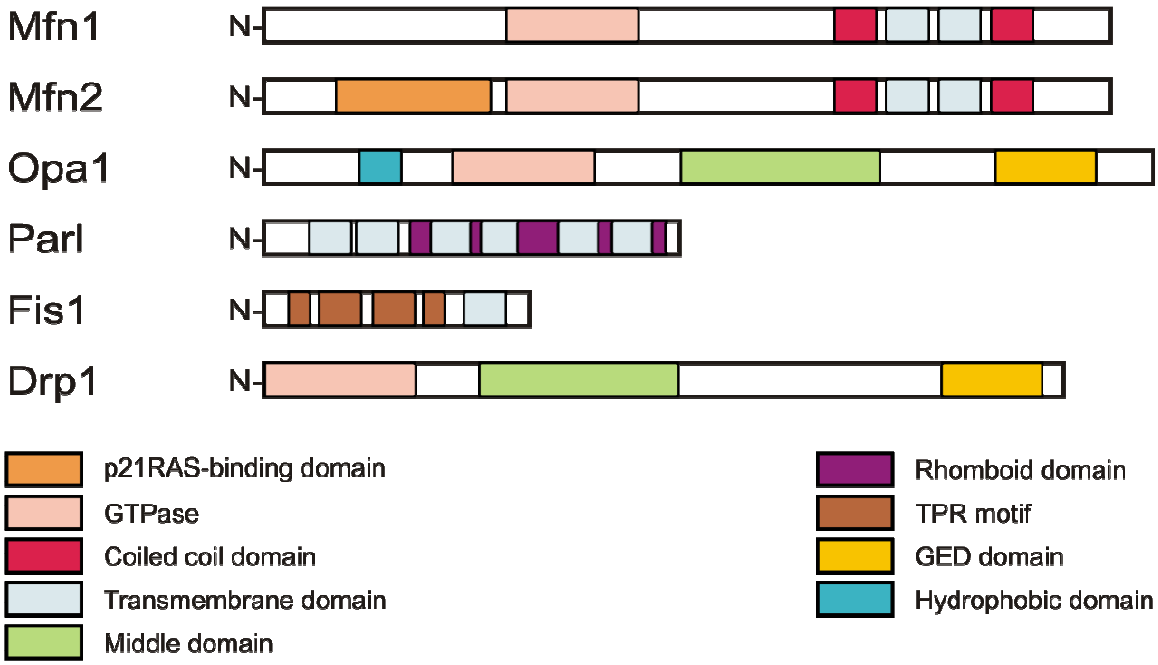


**Figure 3 – Mitochondrial morphology proteins in mammals and their domains.**

### 1.3.3 Other proteins involved in shaping mitochondria

Besides the canonical mitochondrial fission and fusion proteins, other molecules have been proposed to regulate mitochondrial shape.

#### 1.3.3.1 MTP18

The mitochondrial protein 18 kDa (MTP18), an intra-mitochondrial protein exposed to the IMS, was implicated in the control of fission in mammalian cells. Overexpression of MTP18 causes DRP1-dependent mitochondrial fragmentation and its downregulation leads to highly interconnected mitochondria. Additionally, knock-down of MTP18 increases susceptibility to apoptosis, constituting a rare example of increased susceptibility to apoptosis associated with a highly interconnected mitochondrial network. Interestingly, MTP18 is a transcriptional target of phosphatidylinositol 3-kinase (PtdIns3K), thus the protein may couple cellular cues to mitochondrial morphology and apoptosis (Tondera et al., 2004; Tondera et al., 2005).

#### 1.3.3.2 Endophilin B1

Endophilin B1 is a cytosolic protein implicated in regulating membrane curvature, that partially co-localizes with mitochondria. Endophilin B1 downregulation causes changes in mitochondrial shape and distribution. Moreover, silencing endophilin B1 leads to dissociation

of the OMM from the matrix, as well as, formation of OMM-bound tubules and vesicles. It was also reported that endophilin B1 translocates to the mitochondria during apoptosis (Karbowski et al., 2004b), where it participates in BAX activation (Takahashi et al., 2005).

#### 1.3.3.3 GDAP1

The ganglioside-induced differentiation-associated protein 1 (GDAP1), an OMM protein, mutated in Charcot–Marie–Tooth type 4A, has also been implicated in maintenance of the mitochondrial network (Pedrola et al., 2005). Overexpression of GDAP1 induces mitochondrial fragmentation, whereas GDAP1 downregulation by siRNA leads to tubular mitochondrial morphology (Niemann et al., 2005).

#### 1.3.3.4. MFF

Mitochondrial fission factor (MFF) is a novel component of the mammalian mitochondrial fission machinery. MFF is an integral protein of OMM. The hydrophobic carboxy-terminal segment probably serves as a membrane anchor, while the amino-terminal conserved repeats and the coiled coil domain are possibly involved in the interaction with other proteins. MFF knock-down inhibits mitochondrial fragmentation, leading to the appearance of an elongated mitochondrial network, similar to that obtained upon FIS1 or DRP1 silencing. MFF and FIS1 exist as two separate complexes, probably fulfilling different functions in the process of mitochondrial fragmentation (Gandre-Babbe and van der Bliek, 2008). Indeed, recently it was reported that MFF recruits DRP1 to the OMM, being MFF-dependent fission independent of FIS1(Otera et al., 2010).

#### 1.3.3.5. LETM1

Leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) is an inner-membrane protein, deleted in Wolff-Hirschhorn syndrome, homologue to the yeast regulator of mitochondrial morphology Mdm38 (Dimmer et al., 2002). Our group reported that downregulation of LETM1 leads to DRP1-independent fragmentation of the mitochondrial network (Dimmer et al., 2008).

### 1.3.4 Mitochondrial fusion and fission mechanisms

#### 1.3.4.1 Regulation of mitochondrial fusion

Mitochondrial fusion is thought to follow a similar mechanism from lower to higher eukaryotes. The mammalian orthologues of Fzo1, MFN1 and MFN2, are believed to dock

two juxtaposed mitochondria via their coiled coil domains. In the case of MFNs, two molecules on opposing membranes can bind in trans to bridge mitochondria, maintaining a distance of 95 Å between the two membranes (Koshiba et al., 2004). How is fusion of the OMM coordinated with that of IMM? In yeast, a multi-molecular complex of Mgm1, Ugo1 and Fzo1 apparently coordinates fusion of the two membranes (Sesaki and Jensen, 2004). On the other hand, a mammalian orthologue of Ugo1 has not been identified and it is unclear whether OPA1 and MFNs directly interact to promote mitochondrial fusion.

Studies in intact cells showed that, in higher eukaryotes, an intact potential is required for mitochondrial fusion, which conversely appears to be independent of a functional cytoskeleton, at least when considering a complete fusion event (Legros et al., 2002a; Mattenberger et al., 2003). Taking advantage of the yeast model, mitochondrial fusion has been recently recapitulated *in vitro*. This approach dissected the fusion process into two mechanistically distinct, resolvable steps: OMM fusion and IMM fusion. OMM fusion requires homotypic trans-interactions of the Fzo1, the proton gradient component of the inner membrane electrical potential, and low levels of GTP hydrolysis. Fusion of the IMM requires the electrical component of the inner membrane potential and high levels of GTP hydrolysis. However, time-lapse analysis of mitochondrial fusion in yeast and mammalian cells, *in vivo*, clearly shows that fusion of the OMM and IMM are temporally linked. These observations indicate that individual fusion machineries exist in each membrane, but they can communicate *in vivo*, resulting in coupled outer and inner membrane fusion (Meeusen et al., 2004).

1.3.4.1.1 MitoPLD

A member of the phospholipase D superfamily of lipid-modifying enzymes that is required for mitochondrial fusion was identified. MitoPLD localizes to the OMM and participates in mitochondrial fusion by hydrolyzing cardiolipin to generate phosphatidic acid. Phosphatidic acid facilitates vesicular fusion driven by specialized SNARE-complexes. Hence, a common mechanism between SNARE-mediated vesicle fusion and MFN-mediated mitochondrial fusion might exist (Choi et al., 2006).

1.3.4.1.2 MIB

Mitofusin-binding protein (MIB) regulates mitochondrial morphology via its interaction with MFN1 and, possibly, MFN2, with which MIB was shown to physically interact, although the biological significance of this interaction is not completely understood. MIB is partially localized in mitochondria, and is a member of the medium-chain dehydrogenase/reductase protein superfamily, displaying a conserved coenzyme binding domain essential for

interaction with MFN1. This interaction inhibits MFN1 function and causes mitochondrial fragmentation (Eura et al., 2006).

1.3.4.1.3 Degradation of Fzo1 / MFNs

Levels of Fzo1 as well as MFN1 and MFN2 are regulated by ubiquitination. During mating, yeast mitochondria fragment and Fzo1 levels decrease. Interestingly, elimination of Fzo1 depends on the proteasome and Fzo1 is ubiquitinated while still associated with the OMM (Neutzner and Youle, 2005). Also during vegetative growth, Mdm30 mediates ubiquitination of Fzo1 and its subsequent degradation by the proteasome (Cohen et al., 2008). Furthermore, identification of several membrane spanning RING domain E3 ubiquitin ligases in the OMM suggested the existence of an outer mitochondrial membrane-associated degradation (OMMAD) pathway, resembling the ER-associated degradation (ERAD) pathway in the ER (Neutzner et al., 2007).

Likewise, mammalian MFN1 and MFN2 expression levels have been reported to increase upon inhibition of the proteasome, suggesting its involvement in MFNs degradation (Karbowski et al., 2007). Indeed, recently, three independent groups reported that PARKIN, an E3-ubiquitin ligase, ubiquitinates MFN1, MFN2 (Gegg et al., 2010; Tanaka et al., 2010) and MARF, the *Drosophila* ortholog (Ziviani et al., 2010), targeting them to proteasomal degradation (Tanaka et al., 2010).

1.3.4.1.4 BAX and BAK in mitochondrial morphogenesis

The proapoptotic BCL2 family members BAX and BAK seem to play an additional role during life of the cell in controlling mitochondrial fusion. They are retrieved in a high-molecular weight complexes with MFN2 and their ablation reduces the rate of mitochondrial fusion. Consistent with a role for BAX and BAK in the regulation of mitofusins activity, the presence of BAX and BAK has been shown to alter the assembly, mobility and distribution of MFN2 complexes in healthy cells (Karbowski et al., 2006).

1.3.4.1.5 OPA1 processing

A further crucial level of control is exerted by the proteolytic cleavage of Mgm1/OPA1. In yeast, at steady state, Mgm1 exists in a long l-Mgm1 and a short s-Mgm1 form. s-Mgm1 results from the proteolytic cleavage of the long form by the rhomboid protease Pcp1 (Herlan et al., 2003; McQuibban et al., 2003; Sesaki et al., 2003a). Both l-Mgm1 and s-Mgm1 are necessary for mitochondrial fusion and deletion of Pcp1 results in loss of fusion activity (Herlan et al., 2003).

The regulation of OPA1 processing in mammalian cells is a matter of intense debate. In human cells, OPA1 is present in 8 alternatively spliced isoforms (Delettre et al., 2001), that are subjected to complex post-translational cleavage and have two described cleavage sites, S1 and S2, resulting in five bands on a western-blot. The two higher molecular weight bands represent proteins integrated into the IMM, whereas the three lower molecular weight bands lack the transmembrane domain and are localized in the IMS (Duvezin-Caubet et al., 2007).

Different proteases have been proposed to be involved in OPA1 processing. Mihara and colleagues reported that, in mammalian cells, the ATP-dependent matrix AAA (m-AAA) protease paraplegin, is involved in OPA1 processing. According to the authors, dissipation of membrane potential, paraplegin overexpression, or induction of apoptosis, stimulate OPA1 processing along with mitochondrial fragmentation (Ishihara et al., 2006).

Subsequently, Duvezin-Caubet and collaborators have reconstituted OPA1 processing in yeast. They demonstrated that homo-oligomeric m-AAA protease complexes composed of murine AFG3L1, AFG3L2, or human AFG3L2 subunits cleave OPA1 with higher efficiency than paraplegin-containing m-AAA proteases and OPA1 processing proceeded normally in murine cell lines lacking paraplegin (Duvezin-Caubet et al., 2007).

An ATP-independent peptidase in the IMM, OMA1, was recently identified. OMA1 mediates OPA1 processing if m-AAA proteases are absent or mitochondrial activity is impaired (Ehses et al., 2009).

Using *Opa1*$^{-/-}$ cells, it was shown that only Opa1 mRNA splice forms that generate a long form in addition to one or more short forms support substantial mitochondrial fusion activity. In this report, a new protease has been implicated in OPA1 processing - i-AAA protease YME1L (Song et al., 2007). The involvement of YME1L was confirmed later and YME1L-mediated processing of Opa1 was shown to be modulated by mitochondrial membrane potential (Guillery et al., 2008).

A role for prohibitins (PHB) (Merkwirth et al., 2008) and stomatin-like protein 2 (SLP2) (Tondera et al., 2009) in the regulation of OPA1 processing has been recently proposed. Ablation of *Phb2* causes selective loss of long forms of OPA1, resulting in fragmentation of the mitochondrial network and aberrant cristae morphogenesis (Merkwirth et al., 2008). Interestingly, the work of Tondera and colleagues reported that processing of OPA1 is crucial in stress-response conditions. Indeed, upon specific stressful conditions, the mitochondrial network elongates, “hyperfuse”, conferring cellular resistance to stress. The long form of OPA1 is necessary to promote the stress-induced hyperfusion and its maintenance during stress is ensured by SLP-2, a IMM protein with putative scaffolding function (Tondera et al., 2009).

OPA1 is probably a central molecular player linking mitochondrial dysfunction with changes in mitochondrial morphology/ structure. In principle, mitochondrial dysfunction and

depletion of mitochondrial ATP levels could lead to Opa1 processing, inhibition of mitochondrial fusion, and, therefore, to segregation of damaged mitochondria from the network of intact mitochondria (Duvezin-Caubet et al., 2006a; Baricault et al., 2007).

Finally, in our laboratory it has been demonstrated that PARL, the mammalian orthologue of Pcp1, is involved in OPA1 processing, resulting in the generation of a soluble form of OPA1 localized in the IMS (Cipolat et al., 2006). Regulation of OPA1 processing by PARL is not related to OPA1 function in fusion, but to its role in apoptosis (Frezza et al., 2006). Indeed, *Parl*$^{-/-}$ mitochondria undergo faster apoptotic cristae remodelling and cytochrome *c* release.

### 1.3.4.2 Regulation of mitochondrial fission

As discussed before, mitochondrial fission in mammalian cells seems to follow the same mechanism described in yeast. Like in yeast, it has been shown that DRP1 is recruited to spots on mitochondria and it seems that constriction of the membranes takes place via interaction with FIS1 (Yoon et al., 2003), or, possibly, MFF, according to a recent report (Otera et al., 2010). A growing number of studies is contributing to disclose the signals that control mitochondrial DRP1 recruitment and DRP1 turnover.

#### 1.3.4.2.1 DRP1 phosphorylation

DRP1 requires to be translocated to the mitochondria to drive mitochondrial fission, being its localization controlled by post-translational events. Rise of cytosolic $Ca^{2+}$, associated with mitochondrial depolarization, leads to DRP-1 dephosphorylation by calcineurin at serine 637 and concomitant translocation of DRP1 to mitochondria (Cereghetti et al., 2008), where it participates in fission. Conversely, cAMP-dependent protein kinase A (PKA) phosphorylates DRP1 at serine 637, restraining fission (Chang and Blackstone, 2007; Cribbs and Strack, 2007). Thus, DRP1 phosphorylation at serine 637 provides a mechanism for the integration of cAMP and $Ca^{2+}$ in the control of mitochondrial shape. Alternatively, DRP1 can be phosphorylated also at serine 637 by calcium/calmodulin-dependent protein kinase I alpha (CAMKI$\alpha$) (Han et al., 2008) or at serine 616 by cyclin-dependent kinase 1 (CDK1) (Taguchi et al., 2007). However, when phosphorylated by these two kinases, DRP1 drives mitochondrial fission. The fact that phosphorylation in a specific site by two different kinases have opposite effects in DRP1 function complicates the understanding of how a phosphorylation might affect DRP1 function. Probably, physiological effects of a modification may differ depending on the cellular context.

1.3.4.2.2 DRP1 SUMOylation

DRP1 interacts and is a substrate of the small ubiquitin-like modifier SUMO1 (Harder et al., 2004). SUMO family proteins covalently attach to other proteins as post-translational modifications. One of the roles attributed to SUMOylation consists in blocking ubiquitin attachment sites and, consequently, protecting its substrates from the proteasome (Johnson, 2004). The mitochondrial-anchored SUMO E3 ligase MAPL (mitochondrial-anchored protein ligase) has been reported to SUMOylate DRP1 (Braschi et al., 2009). Conversely, the SUMO protease SENP5 deSUMOylates DRP1. Silencing SENP5 stabilizes SUMOylated DRP1, triggering mitochondrial fragmentation (Zunino et al., 2007). Interestingly, BAX/BAK promote SUMOylation of DRP1 during apoptosis (Wasiak et al., 2007).

1.3.4.2.3 DRP1 ubiquitination

The OMM E3 ubiquitin ligase MARCH5, also known as MITOL, participates in the regulation of mitochondrial shape. MARCH5 was reported to ubiquitinate DRP1 and FIS1 (Nakamura et al., 2006; Yonashiro et al., 2006). Subsequently, Karbowski and colleagues reported that inhibition of MARCH5 increases elongation and interconnectivity of mitochondria and causes excessive recruitment of DRP1 to mitochondria. Thus, according to the authors, MARCH5 is involved in the regulation of DRP1 sub-cellular trafficking and correct assembly at the scission sites on mitochondria (Karbowski et al., 2007).

## 1.4 Apoptosis

The term apoptosis, also known as programmed cell death (PCD), was first coined by Currie and co-workers. Coming from the Greek, “apoptosis” is used to describe the “dropping off” or “falling off” of petals from flowers or leaves from trees, a necessary part of the life cycle (Kerr et al., 1972).

In multicellular organisms, apoptosis ensures the precise and orderly elimination of surplus or damaged cells. Apoptosis during embryonic development is essential for successful organogenesis and crafting of complex multicellular tissues, as elimination of the webbing between digits in humans and mice or the mammary tissue in males. During adulthood, apoptosis guarantees the maintenance of normal cellular homeostasis and immunity regulation. Activation or impairment of normal cell death levels in different tissues result in pathological conditions: insufficient apoptosis manifests as cancer or autoimmunity, while accelerated cell death is evident in acute and chronic degenerative diseases (Kerr et al., 1972; Kroemer and Zitvogel, 2007; Meier et al., 2000)

The fact that apoptosis is characterized by stereotyped morphological and biochemical changes suggests that a common intracellular pathway ultimately leads to cell

demise in a coordinated fashion, independently of the specific activating event. 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 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. In type I cells, caspase-8 rapidly cleaves caspase-3 and other caspases, which in turn cleave vital substrates in the cell, propagating this way the death signal. In type II cells, activated caspase-8 is not sufficient to directly activate other caspases and, thus, proper activation of the effector caspases depends on the mitochondrial amplification loop, that is activated by cleavage of BID by caspase-8 (Krammer, 2000; Wajant, 2002).

### 1.4.1 Mitochondria in apoptosis

Two main features characterize mitochondrial involvement in apoptosis: the release of effector proteins from the IMS and the initiation of a programme of mitochondrial dysfunction, that includes loss of mitochondrial membrane potential ($\Delta\Psi m$). Underlying molecular mechanisms and a putative crosstalk between these two events are still under investigation (Bernardi et al., 2001).

A reservoir of proteins involved in amplification of apoptosis localizes to the IMS of mitochondria. Among them, SMAC/ DIABLO and OMI/ HTRA2 enhance caspase activation through the neutralization of proteins that inhibit caspases (Du et al., 2000; Verhagen et al., 2000); Endonuclease G (Li et al., 2001) and apoptosis-inducing factor (AIF) (Susin et al., 1999) have been proposed to play a role in caspase-independent cell death. A central player in caspase activation released by mitochondria upon an apoptotic stimulus is cytochrome *c* (Liu et al., 1996). The OMM was shown to be impermeable to cytochrome *c* (Wojtczak et al., 1972); thus, in order to release cytochrome *c*, the OMM must change its permeability properties. This process is named mitochondrial outer membrane permeabilization (MOMP) and could explain the egress of cytochrome *c* from mitochondria. Although the precise mechanism of this event is still a matter of debate, it appears clear that BCL2 family proteins are critical regulators. In addition to MOMP, complete release of cytochrome *c* during apoptosis requires cristae remodeling, as will be explained bellow.

Once released, cytosolic cytochrome *c* in a complex together with APAF1, dATP and ADP forms the apoptosome, which recruits procaspase-9, facilitating its auto-activation. Caspase-9 activates downstream executioner caspases (Thornberry and Lazebnik, 1998; Zou et al., 1997) that cleave other intracellular substrates leading to the characteristic morphological changes in apoptosis such as chromatin condensation, nucloesomal DNA

fragmentation, nuclear membrane breakdown, externalization of phosphatidylserine (PS) and formation of apoptotic bodies (Hengartner, 2000).

#### 1.4.1.1 Mitochondrial morphology during apoptosis

Early during apoptosis, mammalian mitochondria co-ordinately fragment and undergo cristae remodelling, to achieve complete release of cytochrome *c* (Frank et al., 2001; Scorrano et al., 2002). Indeed, a growing number of evidences suggest that mitochondria-shaping proteins play a role in cell death. Dnm1 mediates mitochondrial fragmentation and apoptosis-like death in *Saccharomyces cerevisiae* (Fannjiang et al., 2004). Blocking DRP1 in *Caenorhabditis elegans* inhibits apoptotic mitochondrial fragmentation and results in the accumulation of supernumerary cells during development (Jagasia et al., 2005). In mammalian cells, inhibition of DRP1 prevents cytochrome *c* release, blocking apoptosis (Frank et al., 2001). Furthermore, also in mammalian cells, knock-down of DRP1 was reported to delay, but not completely prevent, apoptosis by two other groups (Estaquier and Arnoult, 2007; Parone et al., 2006). Inhibition of DRP1-mediated mitochondrial fission partially prevents cytochrome *c* release, but has no effect on the release of IMS proteins like SMAC/DIABLO, OMI/HTRA2, Adenylate Kinase and DDP/TIMM8a that can still mediate apoptosis. These data support the hypothesis that DRP1 plays a relevant function in regulating cytochrome *c* egress, probably by impacting on IMM ultrastructure.

Similarly, overexpression of FIS1 results in cytochrome *c* release and death (James et al., 2003) and its downregulation prevents apoptosis to a greater extent than DRP-1 silencing (Lee et al., 2004). Accordingly, in our laboratory, FIS1 was shown to regulate mitochondrial fission and cell death by genetically distinct pathways. A conservative single amino acid mutant in the short IMS stretch of FIS1 completely abolished mitochondrial dysfunction and cell death, without having any effect in mitochondrial fragmentation (Alirol et al., 2006).

Additionally, it was reported that early in the course of cell death, MFN1 dependent mitochondrial fusion is largely inhibited (Karbowski et al., 2004a) and combined overexpression of MFN1 and MFN2 protects from death by intrinsic stimuli like etoposide or BID (Sugioka et al., 2004). Also, OPA1 knock-down induces mitochondrial fragmentation, cytochrome *c* release and apoptosis (Lee et al., 2004).

These results seem to establish a relationship between fragmentation, blocking of fusion and apoptosis, but the picture is probably not so simple. Not always fission promotes apoptosis, for example overexpression of DRP-1 inhibits death by ceramide by interfering with the propagation of Ca2+ signals in cells primed to die (Szabadkai et al., 2004).

The exact mechanism by which mitochondrial fission promotes apoptosis is not entirely understood. It is possible that mitochondrial fission is not essential for cell death to occur, but it could contribute to a more rapid and complete release of cytochrome *c*. One hypothesis is that mitochondrial fission acts as positive feedback that results in MOMP amplification and complete release of cytochrome *c*.

#### 1.4.1.2 Mitochondrial ultrastructure during apoptosis

Most of cytochrome *c* is stored in the cristae. A turning point in the understanding of the mechanism by which cytochrome *c* is released during apoptosis was the discovery of mitochondrial cristae remodelling early in the course of apoptosis (Scorrano et al., 2002). Following several death stimuli, including the BH3-only proteins BID (Scorrano et al., 2002), BIK (Germain et al., 2005) BIM (Yamaguchi), Bnip3 (embo rep by belenguer) or after FAS pathway activation (Mootha et al., 2001), mitochondria remodel their internal structure: individual cristae fuse and cristae junctions widen, to allow cytochrome *c* mobilization from its intra-cristae compartment towards the IMS for subsequent release across the OMM.

As reported above, our laboratory reported a role for OPA1 in apoptotic cristae remodelling: cristae junctions are held together by OPA1 oligomers formed by integral inner membrane long forms and soluble PARL-processed short forms of OPA1. BID, Bim, Bik and Bnip3 all disrupt OPA1 oligomers and alter cristae morphology. Furthermore, overexpression of OPA1 impairs BID-induced cytochrome *c* release, whereas expression of an OPA1 mutant in the GTPase domain increases the width of cristae junctions. Interestingly, OPA1 efficiently protects cells lacking mitofusins, where fusion is completely abolished, indicating that OPA1 plays genetically and molecularly distinct functions in mitochondrial fusion and cristae remodelling during apoptosis (Frezza et al., 2006). Thus, OPA1 possibly works as a molecular link between mitochondrial network morphology, organelle dysfunction and inner mitochondrial membrane cristae structure.

## 1.5 Key role of mitochondrial dynamics in diverse cellular functions

Why do mitochondria continually fuse and divide? Fusion and fission events control the shape, length and number of mitochondria, ultimately determining mitochondrial morphology. Additionally, fusion and fission allow mitochondria to exchange lipid membranes and intra-mitochondrial content, probably crucial for the maintenance of a healthy mitochondrial population. Indeed, when mitochondrial fusion is abolished, a large fraction of the mitochondrial population loses nucleoids (Chen et al., 2007). Furthermore, mitochondrial shape affects the ability of cells to distribute mitochondria to specific sub-cellular locations.

Mitochondrial distribution is especially important in highly polarized cells, such as neurons. In fact mitochondrial distribution in dendrites is affected by fusion and fission. In hippocampal neurons, following neuronal stimulation, mitochondria accumulate at dendritic spines. Inhibition of fission causes mitochondrial elongation, decreases the abundance of dendritic mitochondria and reduces the density of dendritic spines. Conversely, increased mitochondrial fission facilitates the mobilization of dendritic mitochondria and leads to an increased spine number (Li et al., 2004). Likewise, mitochondrial dynamics appears to be important for proper mitochondrial re-distribution in lymphocytes during chemotaxis (Campello et al., 2006).

Mitochondria-shaping proteins are involved in a growing number of cellular functions, not limited to the ones referred above. As mentioned before, mitochondrial fission facilitates apoptosis by regulating the release of IMS proteins into the cytosol (Frank et al., 2001). Mitochondrial morphology plays also a role in $Ca^{2+}$ signalling. Mitochondria actively participate in the regulation of $Ca^{2+}$ signalling by taking up and releasing $Ca^{2+}$ in response to physiological, inositol triphosphate coupled agonists. This process relies on the relative position of mitochondria in the cytosol, as well as on their juxtaposition to the ER, required for the production of microdomains of high $Ca^{2+}$ concentration, essential for the activation of the low affinity mitochondrial $Ca^{2+}$ uniporter (Rizzuto et al., 2000). It is therefore conceivable that changes in mitochondrial shape influence mitochondrial participation in the $Ca^{2+}$ game. This hypothesis is substantiated by the finding that excessive fission by DRP1 blocks propagation of $Ca^{2+}$ waves (Szabadkai et al., 2004). In addition, changes in mitochondrial shape occur during cell cycle. During G1-S transition, mitochondria hyperfuse, forming a giant network. Manipulation of mitochondrial morphology results in deregulation of G1–S progression (Mitra et al., 2009). Finally, mitochondrial morphology was reported to impact in lifespan in lower eukaryotes. Deletion of the Dnm1 gene delays mitochondrial fragmentation and retards ageing in *Podospora anserina* and *Saccharomyces cerevisiae* (Scheckhuber et al., 2007).

In this thesis, the relationship between mitochondrial shape and autophagy is explored. In particular, we analysed the role mitochondrial morphology plays during selective degradation of mitochondria and after induction of macroautophagy. The next sections of the introduction are devoid to the molecular mechanisms, regulation and functions of autophagy.

## 1.6 Autophagy

The word “autophagy”, derived from the Greek meaning “self-eating”, was originally proposed by the Nobel Laureate Christian de Duve more than 40 years ago and was mainly based on ultrastructural changes observed in rat liver after injection with glucagon. Glucagon

results in an increase in cAMP levels, and, consequent, activation of PKA, that triggers autophagy in liver cells by a mechanism that is not yet fully understood (Deter and de, 1967).

Autophagy, a catabolic process conserved from lower to higher eukaryotes, is essential for recycling energy sources when cells have to deal with demanding conditions, as nutrient depletion and hypoxia, or during development. Cells increase the level of autophagy in response to other environmental stresses, such as oxidative stress, pathogen infection, radiation or anticancer drug treatment. Additionally, a basal, constitutive level of autophagy plays a key role in the quality control maintenance inside the cell, being essential for the degradation of superfluous or damaged/old organelles as well as long-lived proteins and protein aggregates.

Autophagy refers to any process of degradation of cytosolic components at the lysosome, embracing diverse pathways. Three autophagic pathways have been characterized based on the apparent differences observed in cargo delivery to the lysosome – macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Todde et al., 2009). Summarily, during macroautophagy, a phagophore forms in the cytosol and expands into a double-membrane, engulfing cytosolic components, including proteins, lipid droplets, ribosomes and organelles and giving rise to the autophagosome. The external membrane of the autophagosome fuses with the lysosomal membrane, being the inner vesicle together with its cargo degraded (Fig. 4). Resulting nutrients are recycled back to the cytosol, through membrane permeases (Mehrpour et al., 2010).

Microautophagy differs from macroautophagy since cytosolic components are directly sequestered by the lysosome through an invagination of the lysosomal membrane. Degradation of soluble proteins and entire organelles by microautophagy has been described in yeast, where a group of proteins involved in microautophagy have been identified, some of which are in common with macroautophagy (Uttenweiler and Mayer, 2008). On the other hand, our understanding of microautophagy in mammalian cells is poor.

CMA, a form of autophagy just described in mammals, mediates the translocation of soluble proteins from the cytosol to the lysosome, across a translocon-like complex in the lysosomal membrane. Proteins to be degraded by CMA are recognized by a chaperone of the HSP70 family in the cytosol. The complex chaperone-substrate binds to a receptor protein in the lysosomal membrane, that moves the substrate into the translocation complex. Once in the lysosomal lumen, the substrates are rapidly degraded (Kaushik et al., 2010).

As the major focus of this thesis is macroautophagy, autophagy will be hereafter used as a synonymous of macroautophagy.

## 1.7 Molecular mechanism of macroautophagy

Although autophagy was first described in mammalian cells, the understanding of the molecular mechanisms was largely based on studies in yeast. Thirty-five autophagy-related genes (*ATG*) in yeast have been so far identified, and, many of them present homologues in higher eukaryotes (Yang and Klionsky, 2009).

Atg proteins are organized in functional complexes that mediate the diverse steps of macroautophagy: induction/initiation, vesicle nucleation, cargo recognition and packaging, vesicle expansion and sealing, fusion with the lysosome, vesicle breakdown and recycling of the resulting macromolecules. Core Atg proteins, the subset of proteins required for autophagosome formation, are organized in four complexes: Atg1/ unc-51-like kinase (ULK) complex; class III phosphatidylinositol 3-kinase (PtdIns3K)/ Vps34 complex I; two ubiquitin-like protein conjugation systems, Atg5-Atg12 and Atg8/ light chain 3 (LC3); and, two transmembrane proteins, Atg9/ mATG9 and vacuole membrane protein 1 (VMP1).

The phagophore assembly site/ pre-autophagosome structure (PAS) constitutes the site of autophagosome formation, to which most of the core Atg proteins are recruited (Suzuki et al., 2001). According to the current view, the autophagosomal membrane originates from expansion and sealing of a small membrane cisterna called phagophore (Reggiori and Klionsky, 2005). While in yeast a single PAS is present per cell, higher eukaryotes display multiple points of ATG proteins co-localization (Yamada et al., 2005; Young et al., 2006), that constitute multiple sites of autophagosome formation and possibly correspond to multiple PAS.

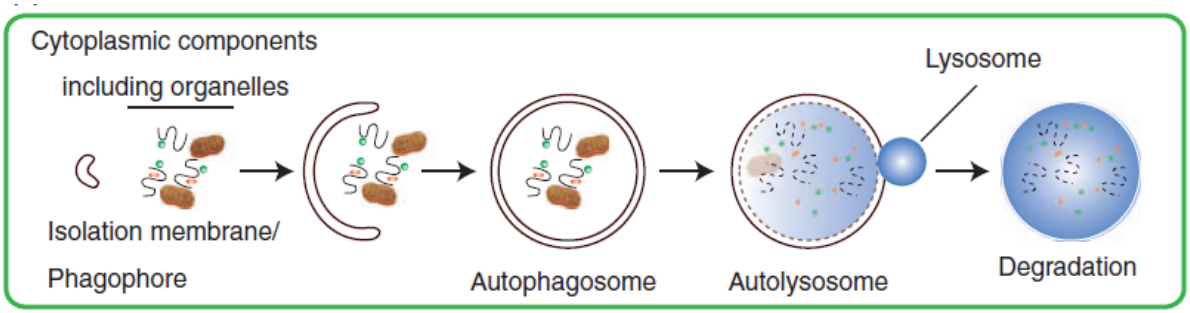


**Figure 4 – Schematic diagram of macroautophagy** (Komatsu and Ichimura, 2010).

### 1.7.1 ULK complexes

Downstream of the target of rapamycin (TOR) complex 1 (TORC1), the yeast serine/ threonine kinase Atg1 plays a crucial role in the induction of autophagy. A complex between Atg1, Atg13 and Atg17 is required for phagophore formation. Inactivation of TORC1 results in dephosphorylation of its target Atg13, that causes an increase in Atg1-Atg13-Atg17 complex formation and activation of autophagy (Kamada et al., 2000; Kamada et al., 2010). The proper autophagosome expansion requires a dynamic process of Atg proteins cycling.

Indeed, the Atg1 kinase activity was shown to be essential for the dissociation of Atg proteins from the PAS, but not for PAS recruitment of Atg8 or Atg17 (Cheong et al., 2008).

The ULK family of kinases is a family of Atg1 homologues that has been identified in mammals. Three proteins from this family have been shown to be involved in the regulation of autophagy - ULK1, ULK2 and ULK3 (Jung et al., 2009; Kundu et al., 2008; Young et al., 2009). Whether they can compensate for each other, or they present specific functions in the regulation of autophagy is not known. ULK-1 and ULK-2 appear in a large complex with mATG13 and FIP200 (an orthologue of yeast Atg17) (Jung et al., 2009). Association of TORC1 to this complex depends on the nutrient conditions. Under nutrient rich conditions, the complex contains TORC1, that phosphorylates ULKs and mATG13. Conversely, upon nutrient deprivation, TORC1 dissociates from the complex, leading to dephosphorylation of mATG13, ULK1 and ULK2 and, consequent, activation of ULK1 and ULK2. Activated ULK1 and ULK2 phosphorylate both mATG13 and FIP200 to promote autophagosome formation (Hara et al., 2008; Hosokawa et al., 2009). Although the functional significance of these phosphorylation events requires to be better characterized, phosphorylation of mATG13 in different sites by mTOR and ULKs seem to play an opposite effect in autophagy. Recently, another mATG13-interacting protein, found in complex with ULK1 and essential for autophagy, has been identified, ATG101. However, the role of ULK1-mATG13-ATG101 during autophagy needs to be clarified (Mercer et al., 2009). An additional mechanism of regulation of ULK1 activation was recently described by two independent groups – ULK1 is a direct target of AMP-activated protein kinase (AMPK) (Egan et al., 2011; Kim et al., 2011).

### 1.7.2 Class III phosphatidylinositol 3-kinase complex

Production of phosphatidylinositol (3)-phosphate (PtdIns(3)P) at the PAS is essential for the recruitment of the Atg proteins. In fact, blockage of PtdIns(3)P in yeast, flies, mammals or even plants by wortmannin or 3-methyladenine (3-MA) hampers autophagy progression (Blommaart et al., 1997; Eskelinen et al., 2002; Miller et al., 2010; Petiot et al., 2000; Takatsuka et al., 2004).

In yeast, there is just one PtdIns3K, the Vps34, that is part of two distinct complexes. Whereas complex I, formed by Vps34, Atg6, Vps15, and Atg14, is required for induction of autophagy, complex II participates in the vacuolar sorting of carboxypeptidase (Kihara et al., 2001).

In mammals, two classes of PtdIns3K complexes are involved in the regulation of autophagy – class I and class III. Class I PtdIns3K converts PtdIns(4,5)$P_2$ to PtdIns(3,4,5)$P_3$, at the plasma membrane, activating PKB and mTOR in response to agonist stimulation, and, thereby, inhibiting autophagy (Jacinto and Hall, 2003). Class III PtdIns3K (hVPS34) is

conserved from yeast to higher eukaryotes and catalyzes the formation of PtdIns(3)P from PtdIns. The interaction of beclin 1 (BECN1; Atg6 homologue) with hVPS34 promotes its catalytic activity, although how this interaction is regulated is poorly defined, progress has been made in the last years in identifying new players that interact with BECN1-hVPS34 complex (Furuya et al., 2005; Funderburk et al., 2010).

hVPS34-BECN1-p150 (Vps15 homologue) participate in the formation of different complexes, with diverse binding partners that include, ATG14L (Atg14-like protein, also named BARKOR), UVRAG (ultraviolet irradiation resistance associated gene) (an homologue of Vps38, that is part of yeast Vps34 complex II) and RUBICON (RUN domain and cystein-rich domain containing, BECN1-interacting) (Itakura et al., 2008; Liang et al., 2006; Matsunaga et al., 2009; Sun et al., 2008; Zhong et al., 2009).

ATG14L exists mainly in BECN1-ATG14L-hVPS34-VPS15 complex, that is crucial for autophagosome formation. Upon autophagy induction, ATG14L co-localizes with phagophore and autophagosomal markers independently of hVPS34 and BECN1 (Itakura et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009). Overexpression of ATG14L stimulates hVPS34 kinase activity, in a way that is dependent on the overexpression of BECN1. On the other hand, a decrease in hVPS34 activity occurs when ATG14L is knocked-down (Sun et al., 2008; Zhong et al., 2009). Depletion of ATG14L also leads to a decrease in ATG16L and LC3 puncta (Matsunaga et al., 2009), suggesting that ATG14L may play a role in recruiting PtdIns3K to the PAS and, consequently, in directing the ATG proteins to the phagophore.

UVRAG has been shown to participate in different complexes. It competes with ATG14L for BECN1, forming the BECN1-UVRAG-VPS34-VPS15 complex. Results from different groups disagree about the relevance of this complex for autophagosome formation (Liang et al., 2006; Itakura et al., 2008). UVRAG also interacts with endophilin B1, in a complex that has been shown to be required for autophagy (Takahashi et al., 2007), possibly playing a role in autophagosomal membrane bending (Takahashi et al., 2009). Additionally, UVRAG interacts with class C VPS/HOPS proteins, promoting fusion of autophagosomes with late endosomes and lysosomes. Finally, UVRAG-BECN1-hVPS34-p150 complex was reported to interact with RUBICON, which was found to negatively regulate autophagy (Matsunaga et al., 2009; Zhong et al., 2009).

Another interactor of BECN1 is AMBRA1 (activating molecule in BECN1-regulated autophagy), a positive regulator of BECN1-dependent autophagy (Fimia et al., 2007). Recently, it was reported that AMBRA1 tethers BECN1-VPS34 complex to the cytoskeleton under physiological conditions. When autophagy is induced, AMBRA1 is phosphorylated by ULK1, releases the autophagic core machinery from dynein in the cytoskeleton and re-localizes to the ER, facilitating autophagosome nucleation (Di et al., 2010).

BECN1 also interacts with BCL2, which disrupts BECN1-hVPS34 interaction, repressing autophagy (Maiuri et al., 2007; Pattingre et al., 2005). The endoplasmic reticulum (ER) pool of BCL2 (or BCL-XL) binds BECN1, through the BH3 domain in BECN1 under non-starving conditions (Maiuri et al., 2007). Upon induction of autophagy by starvation, BCL2 is phosphorylated by JNK1 and dissociates from BECN1, activating autophagy (Wei et al., 2008). Additionally, serum withdrawal triggers BAD activation (Zha et al., 1996), that competes with BECN1 for the BCL2 binding site, during starvation conditions (Maiuri et al., 2007).

As described above, hVPS34 activity, essential for autophagosome nucleation, is tightly controlled by a growing number of proteins, which, in some cases participate in further steps of autophagy. Production of PtdIns(3)P is believed to be crucial for the spatial organization of the core autophagic proteins at the phagophore. PtdIns(3)P binding proteins are recruited to the PAS, which in turn recruit other core autophagic proteins.

In yeast, Atg20 and Atg24 have been the first proteins described to bind PtdIns(3)P. This proteins, however, participate in the Cvt (cytoplasm to vacuole targeting) pathway, not in bulk autophagy (Nice et al., 2002). Similarly, proteins that participate in bulk autophagy have been described to bind PtdIns(3)P. Atg18-Atg2 complex is directed to the autophagic membranes through the interaction of Atg18 with PtdIns(3)P (Obara et al., 2008). Also, Atg21 binds PtdIns(3)P (Stromhaug et al., 2004).

It is assumed that PtdIns(3)P plays a similar function in mammals, but the effectors of PtdIns(3)P are poorly known. No homologues of Atg20 and 24 were identified in mammals. Atg18, on the other hand, has two homologues in mammals - WIPI1 and WIPI2 (Polson et al., 2010; Proikas-Cezanne et al., 2004), that are both effectors of PtdIns(3)P. WIPI2 is recruited to early autophagosomal vesicles together with ULK1, ATG16L and LC3, suggesting its involvement in early stages of autophagy (Polson et al., 2010).

### 1.7.3 Two ubiquitin-like protein conjugation systems

Two ubiquitin-like proteins, Atg12 and Atg8, and their respective conjugation systems regulate elongation and expansion of the forming vesicle. Both conjugation systems are conserved from yeast to human (Ohsumi, 2001).

Atg7 and Atg10, E1 and E2-like enzymes, respectively, promote the covalent linkage of Atg12 to an internal lysine residue of Atg5, in an ATP-dependent manner. Atg12-Atg5 conjugation occurs constitutively and it has been proposed to be irreversible, since an enzyme that cleaves the isopeptide bond is not known (Mizushima et al., 1998a). Atg5 further interacts with Atg16 non-covalently, forming a multimeric complex of 350 kDa through the homo-oligomerization of Atg16 (Kuma et al., 2002; Mizushima et al., 1999).

Atg12 conjugation system is conserved in higher eukaryotes (Mizushima et al., 1998b; Mizushima et al., 2002; Tanida et al., 2001). ATG16L (Atg16-like protein), the Atg16 homologue, mediates the formation of the 800 kDa ATG12-ATG5-ATG16L mammalian complex (Mizushima et al., 2003).

Atg8, in yeast, is cleaved at the carboxy-terminus by the cysteine protease Atg4 to generate the cytosolic Atg8 (Kirisako et al., 2000). Phosphatidylethanolamine (PE) is then conjugated to Atg8 in a reaction that requires Atg7 and the E2-like enzyme Atg3 (Ichimura et al., 2000). Atg8-PE attaches to both faces of the autophagosome double membrane. In opposite to Atg12-Atg5 conjugation, PE conjugation to Atg8 is reversible and Atg8-PE can be cleaved by Atg4. Like this, Atg8 is removed from the outer autophagosomal membrane before fusion with the lysosome, being recycled and used in other conjugation reactions (Kirisako et al., 2000).

Similarly to Atg12 conjugation system, Atg8 conjugation is conserved from yeast to higher eukaryotes. At least four homologues of Atg8 have been identified – MAP1LC3, GATE16, GABARAP and ATG8L, being LC3 the most abundant in autophagosomal membranes (Hemelaar et al., 2003; Kabeya et al., 2000; Tanida et al., 2002; Tanida et al., 2003; Tanida et al., 2006).

It has been proposed that the Atg16/ ATG16L complex acts as an E3-like enzyme during lipidation of Atg8/ LC3, indicating that the two conjugation systems are closely related (Fujita et al., 2008; Hanada et al., 2007). Indeed, Atg12-Atg5-Atg16 plays a crucial role in promoting Atg8 lipidation and correct localization (Fujita et al., 2008). Atg8/ LC3, on the other hand, appears to act as a scaffold component in membrane expansion, since the amount of Atg8 correlates with vesicle size (Xie et al., 2008). In selective types of autophagy, Atg8/ LC3 may also participate in cargo selection. For instances, in the Cvt pathway, Atg8 interacts with Atg19, the receptor for Ape1 precursor (Shintani et al., 2002). Likewise, in mammalian cells, LC3 interacts with p62, an ubiquitin-binding protein (Pankiv et al., 2007). It has also been recently reported that LC3/ GABARAP proteins bind NIX, promoting selective targeting of mitochondria to autophagy (Novak et al., 2010). Finally, Atg8-PE can mediate membrane tethering and hemifusion *in vitro* (Nakatogawa et al., 2007), although the physiological relevance of this finding is not known.

### 1.7.4 Transmembrane proteins in autophagy

Only two integral membrane proteins have been described to participate in autophagy. Atg9, in yeast, and its homologue protein mATG9 in mammals and VMP1, that, up to now, was just identified in mammalian cells.

The source of the lipid used in autophagosome formation and how lipids move to the PAS are among the most intriguing questions in the field. Yeast Atg9 was proposed to participate in the assembly process as a "membrane carrier" (He et al., 2006; Noda et al., 2000). Atg9 localizes to multiple punctuate structures in the cell, including the PAS. Atg9 cycles between vesicles and tubules, which are derived from the secretory pathway and are often adjacent to mitochondria, and the PAS (Reggiori et al., 2005; Mari et al., 2010). Thus, potentially, Atg9 shuttling could mediate membrane delivery to the forming autophagosome. Mammalian mATG9 localizes to in the *trans*-Golgi / late endosomes, in nutrient rich conditions. Upon starvation, mATG9 redistributes and co-localizes with GFP-LC3 positive structures (Young et al., 2006). The function of mATG9 still needs to be clarified.

Besides mATG9, mammalian cells have another integral membrane protein essential for autophagy – VMP1, that localizes to the plasma membrane and, upon induction of autophagy, also to LC3/ BECN1 positive structures. VMP1 is essential for autophagy in response to starvation or rapamycin (Ropolo et al., 2007). It has been suggested that VPM1 together with its interacting protein TP53INP2 (tumor protein 53-induce nuclear protein 2) may play a role in recruiting BECN1 together with other core autophagic proteins to the phagophore (Nowak et al., 2009).

### 1.7.5 Autophagosomal membrane origin

One of the most commonly asked questions in the autophagy field is: "Where does the autophagosome come from?" Although the molecular mechanisms of autophagy have started to be revealed in the last decade, studies on the origin of the autophagosomal membrane are just emerging and its source(s) remains an open question.

As one can easily predict, autophagosome formation must be strictly and spatiotemporally regulated. Studies in yeast and later in mammalian cells indicate that the autophagosome membrane forms through expansion of a membrane core, the phagophore, which origin is unknown (Mizushima et al., 2001; Noda et al., 2002; Seglen et al., 1990; Yang and Klionsky, 2009). As mentioned above, in yeast, a structure that precedes the autophagosome was identified, the PAS, that gives rise to the isolation membrane and subsequently to the autophagosome. Even if the function of the PAS is not completely clear, it was suggested that it may mediate nucleation and expansion of the phagophore, by recruiting the Atgs. Atg proteins depend on each other to be recruited to the PAS, but, in particular, Atg11 and Atg17, under nutrient rich and autophagy-inducing conditions respectively, seem to play a major role as scaffold proteins for the assembly of downstream Atgs at the PAS (Cheong et al., 2008; Kawamata et al., 2008; Suzuki et al., 2007). Moreover,

a dynamic cycling of Atg proteins was reported to be essential for proper autophagosome expansion (Cheong et al., 2008).

In mammals, as we mentioned before, a single PAS does not exist. Nevertheless, recent evidence indicate that multiple sites of Atg proteins recruitment could correspond to multiple PAS. Indeed, advances in the understanding of autophagy molecular mechanisms in mammals corroborate this hypothesis, given that homologues of crucial PAS components have been identified in mammalian cells and, similarly to yeast, they work as scaffold proteins for other Atgs (Hara et al., 2008; Ganley et al., 2009).

It is presently accepted that some Atgs recruited from other organelles to the PAS may supply lipids or membranes for autophagosome formation. But what are the compartments supplying the components for the formation of the autophagosome membrane?

A growing amount of results suggests that autophagosomes are not created from a single organelle or, at least, are not always created from the same organelle. In fact, recent studies reported the participation of diverse intracellular compartments in the formation of the autophagosome membrane – ER (endoplasmic reticulum) (Axe et al., 2008; Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009), mitochondria (Hailey et al., 2010), secretory pathway and Golgi (Itoh et al., 2008; Mari et al., 2010; Nishida et al., 2009; Zoppino et al., 2010), and even plasma membrane (Ravikumar et al., 2010).

ER was originally proposed to be the main source of autophagosome membranes (Arstila and Trump, 1968; Ueno et al., 1991), but this theory suffered from a lack of definitive evidences, as well as from contradictory results (Yamamoto et al., 1990). Recently, however, Axe and colleagues reported that in response to starvation a PtdIns(3)P-enriched compartment, termed omegasome due to its omega-like shape, forms in close proximity to the ER and Vps34-positive vesicles. This compartment is in dynamic equilibrium with the ER and is involved in autophagosome biogenesis, placing the ER again in the front of the discussion about autophagosome origin. Supporting the requirement of ER for autophagosome formation, it was later reported that ATG14L localization to the ER is essential to PtdIns(3)P production and, consequently, autophagy induction (Matsunaga et al., 2010). Additionally, electron tomography showed physical connections between ER and the forming autophagosome double membranes, that are encircled or cradled by two ER membranes (Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009).

Surprisingly, in a recent study from Hailey and colleagues the participation of the outer mitochondrial membrane in autophagosome biogenesis during starvation was reported. During starvation, PE is generated in mitochondria from PS, which is obtained from the ER. Lipids are then transferred from mitochondria to the forming autophagosome membranes (Hailey et al., 2010).

Besides ER and the mitochondria, Golgi and the secretory pathway have been proposed to be involved in autophagosome formation (Yamamoto et al., 1990). More recently, SAR1 and RAB1, two GTPases that regulate ER-Golgi traffic, have been reported to be required for autophagosome formation (Zoppino et al., 2010). RAB33B, a small GTPase resident in the *cis*-Golgi, interacts with ATG16L, modulating autophagosome formation through this interaction (Itoh et al., 2008). Furthermore, an alternative pathway of autophagy has been described (mentioned below), in which maturation of autophagosomes occurs through fusion of isolation membranes with membranes derived from the *trans*-Golgi and late endosomes, in a RAB9-dependent manner (Nishida et al., 2009). Also in yeast, the secretory pathway has been implicated in autophagosome formation. In a recent work from Mari and colleagues, Atg9 has been reported to be localized to a new compartment derived from the secretory pathway, that comprises clusters of vesicles and tubules. These clusters move *en bloc* to form the PAS next to the vacuole (Mari et al., 2010).

Lastly, the plasma membrane was also shown to contribute to ATG16L-positive autophagosome precursors, through clathrin-mediated endocytosis. Indeed, ATG16L interacts with clathrin and disruption of this interaction partially inhibits the formation of the PAS (Ravikumar et al., 2010).

The growing amount of data on the autophagosome origin suggests that multiple membrane sources may exist. It is reasonable to hypothesize that different intracellular compartments may contribute for the formation of the autophagosome depending on the stimuli that induce autophagy or its intensity. Alternatively, different organelles can contribute to the formation of one autophagosome, but these as well as other possibilities await experimental testing.

## 1.8 Regulation of autophagy

Autophagy occurs at basal levels in most cell types and its blockage can be prejudicial (Komatsu et al., 2007; Kuma et al., 2004). On the other hand, excessive autophagy may also be deleterious, emphasizing the requirement for a stringent regulation. Accordingly, several pathways participate in the regulation of autophagy. This chapter focus on the major regulator of autophagy from yeast to mammalian cells –Tor/ mTOR (Fig. 4).

TOR is an evolutionary conserved serine/ threonine kinase that plays a central role in the regulation of autophagy, sensing growth factors, nutrient signals and the energetic status of the cells. In yeast, Tor forms two complexes with distinct functions – TORC1 and TORC2 (Loewith et al., 2002), which are conserved in higher eukaryotes. The rapamycin-sensitive TORC1 complex is a negative regulator of autophagy. Nutrient deprivation inhibits TORC1, thus activating autophagy (Noda and Ohsumi, 1998).

Mammalian mTORC1 complex is also sensitive to rapamycin (Hara et al., 2002; Kim et al., 2002), that has been reported to induce autophagy under various settings. mTOR is under the control of different signalling pathways that regulate cell growth and autophagy in response to nutrient availability, growth factors and energy status.

RAGA family small GTPases (Sancak et al., 2008) together with MAP4K3 (mitogen-activated protein kinase kinase kinase) (Findlay et al., 2007) are responsible for sensing the amino acids availability (in particular branched amino acids like leucine). These two pathways act upstream of mTOR, activating the kinase in response to amino acids.

Growth factor signalling regulates mTORC1 through insulin/insulin-like growth factor 1 (IGF1) - PtdIns3K - PKB (protein kinase B)/ AKT pathway. In the presence of extracellular growth factors, this signalling pathway mediates activation of mTORC1 and consequent inhibition of autophagy. PKB activates mTOR though inhibition of TSC1/ TSC2 (tuberous sclerosis complex 1/ 2) and subsequent activation of the small GTPase RHEB (Manning et al., 2002; Inoki et al., 2002).

AMPK senses cellular energetic status upstream of mTOR. When the ATP/AMP ratio decreases, for instances under glucose deprivation or if mitochondria are dysfunctional, AMPK is activated, downstream of LKB1 kinase activation. AMPK acts as a negative regulator of mTOR, thus inducing autophagy (Corradetti et al., 2004; Shaw et al., 2004). Additionally, AMPK was recently reported to directly phosphorylate ULK1 (Egan et al., 2011; Kim et al., 2011), indicating that activation of autophagy by AMPK occurs via a dual mechanism.

Therefore, different signalling pathways act upstream of mTORC1 to regulate growth and autophagy. Additionally, a role for mTORC2 in autophagy regulation has been reported in muscle cells (Mammucari et al., 2007). mTORC2 depletion is required for inhibition of PKB (Sarbassov et al., 2005), that activates the transcription factor FOXO3 (forkhead box O3) to stimulate autophagy (Mammucari et al., 2007).

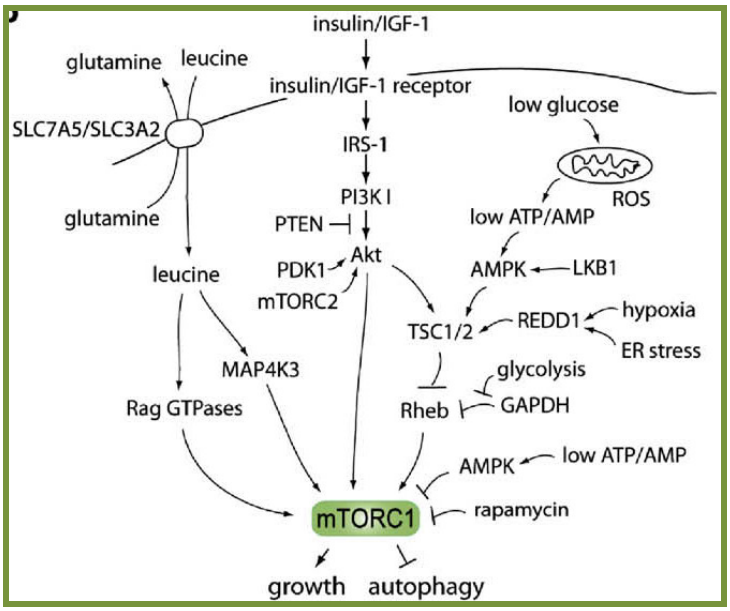


**Figure 5 - Signalling pathways upstream mTORC1 that regulate cell growth and autophagy** (Jung et al., 2010).

## 1.9 ATG5/ ATG7-independent pathway of autophagy

Surprisingly, Nishida and colleagues discovered an alternative form of autophagy, that is independent of ATG5 and ATG7, even if it is regulated by autophagic core proteins like BECN1 and ULK1. As mentioned above, maturation of autophagosomes during the alternative autophagic pathway occurs through fusion of isolation membranes with membranes derived from the *trans*-Golgi and late endosomes, in a RAB9-dependent manner. Alternative autophagy has been reported to be induced in response to cellular stress *in vitro* and during erythrocyte differentiation *in vivo* (Nishida et al., 2009).

Though both autophagic pathways lead to intracellular degradation of cellular components, they are possibly induced under different conditions and probably play different functions.

## 1.10 Physiological functions of autophagy

Autophagy is crucial for cell life. Indeed, autophagy is essential to maintain tissue homeostasis, due to its role in defending against metabolic stress, like, for instances, growth factor depletion, nutrient deprivation, hypoxia, ER stress and microbial infections (Levine and Kroemer, 2008; Mizushima et al., 2008).

In mammals, autophagy is indispensable for development and differentiation, at least at three different steps – pre-implantation development, survival to neonatal starvation and differentiation of erythrocytes, lymphocytes and adipocytes (Mizushima and Levine, 2010).

Indeed, mouse models knock-out for core *Atg* genes revealed embryonic or perinatal lethality (Cecconi and Levine, 2008).

A defect in autophagy can be retrieved in diverse pathological conditions. In particular, impairment of the autophagic activity has been reported in neurodegenerative diseases, like Parkinson's disease (Cuervo et al., 2004), Huntington's disease (Martinez-Vicente et al., 2010) and Alzheimer's disease (Yu et al., 2004). Decreased autophagic activity results in accumulation of aggregates formed by the mutated toxic proteins, a key feature of these diseases. Indeed, activation of autophagy was proven to be beneficial in animal models of Huntington's disease (Ravikumar et al., 2004). In addition, defective activation of the autophagic programme has been recently reported to be pathogenic also in congenital muscular dystrophies linked to collagen VI deficiency. Again, forced activation of autophagy improved the dystrophic phenotype (Grumati et al., 2010).

Autophagy plays also a crucial role in cancer progression. Several ATG core proteins – BECN1 (Qu et al., 2003; Yue et al., 2003), UVRAG (Liang et al., 2006), endophilin B1, (Takahashi et al., 2007) and ATG4C (Marino et al., 2007) - display tumour suppressor activity. An increase in DNA damage and genomic instability detected in cells with mono-allelic loss of *Becn1* or bi-allelic loss of *Atg5* may in part explain the tumour suppressor functions of autophagic proteins (Mathew et al., 2007). Paradoxically, autophagy may provide tumours with a tool to resist metabolic stress, for instances during chemotherapy. Indeed, during therapy, it may be useful to target autophagy (Carew et al., 2007).

In addition, autophagy plays an important role in innate and adaptive immunity, probably having a major role in degradation of pathogens, for subsequent antigen presentation (Levine and Deretic, 2007).

## 1.11 Selective autophagy

Initially, autophagy was believed to be a non-selective process, meaning that cytosolic components would be randomly surrounded by the autophagosome and broken down. Though de Duve in 1966 suggested that autophagy could be selective, data supporting it were lacking at the time (de and Wattiaux, 1966). Later on, it was observed in different studies that, under specific conditions, certain macromolecular components were preferentially delivered to the lysosome (Bolender and Weibel, 1973; Beaulaton and Lockshin, 1977; Veenhuis et al., 1983). Along the years, several examples of selective degradation have been revealed, as specific breakdown of aggregated proteins (Ravikumar et al., 2002), selective removal of superfluous or damaged organelles – like mitochondria (mitophagy) (Elmore et al., 2001), peroxisomes (pexophagy) (Tuttle et al., 1993) and endoplasmic reticulum (ER-phagy) (Bernales et al., 2006) - and specific degradation of invading bacteria (Zheng et al., 2009).

Molecular mechanisms of selective autophagy have just started to be unveiled. Generally, a receptor protein, that is part or binds the cargo to be engulfed, interacts also with the autophagosomal membrane protein Atg8/ LC3 and/ or an adaptor protein, as Atg11 in yeast, mediating the selective targeting of the cargo to the autophagosome. Receptor proteins for different cargoes have been revealed – for instances, Atg19, which acts as a receptor protein for the yeast Cvt pathway; Atg32, which functions as a receptor in yeast mitochondria; PpAtg30, in yeast peroxisomes; NIX, in mammalian mitochondria and p62, NBR1, two mammalian proteins that bind ubiquitinated substrates (Komatsu and Ichimura, 2010).

As one of the main focus of this thesis is the relationship between mitochondrial dynamics and mitophagy, the next chapters are dedicated to mitophagy.

## 1.12 Mitophagy

The term “mitophagy” was first introduced by Lemasters in 2005 (Lemasters, 2005), even if the first descriptions of mitochondria inside lysosomes date from about 40 years before. Engulfment of mitochondria together with other organelles by lysosomes in rat hepatocytes exposed to glucagon have been described in 1962 by Ashford and Porter (ASHFORD and PORTER, 1962). Moreover, back to 1977, Beaulaton and Lockshin described autophagy during metamorphosis in silkmoth muscles that targeted almost exclusively mitochondria, constituting the first mention to selective mitochondrial autophagy (Beaulaton and Lockshin, 1977).

As described in detail in the first chapters of the introduction, mitochondria are crucial organelles for energy production, regulation of cell signalling and amplification of apoptosis (Ernster and Schatz, 1981; Rizzuto et al., 2000; Green and Kroemer, 2004). Simultaneously, mitochondria are the major source of reactive oxygen species (ROS), that may cause oxidative damage to their own lipids, proteins and DNA (Scherz-Shouval and Elazar, 2010). Therefore, mitochondrial quality control is crucial for the fitness of the cell and mitophagy is believed to play a key role, being the prime mechanism to eliminate superfluous or damaged mitochondria (Kanki and Klionsky, 2010). The critical role that autophagy plays in the maintenance of a “healthy” cohort of mitochondria was showed both in yeast (Zhang et al., 2007) and mammals (Twig et al., 2008). Yeast strains with *ATG* (autophagy-related) genes deletions are unable to degrade mitochondria during stationary phase, present growth defects in a non-fermentable carbon source and accumulate dysfunctional mitochondria. In accordance, *ATG* mutants present lower oxygen consumption rates, decreased mitochondrial membrane potential and higher ROS levels (Zhang et al., 2007). Similarly,

mammalian cells deficient for ATG5 or treated with an inhibitor of autophagy – 3-MA – presented a reduction in maximal respiration (Twig et al., 2008).

In the last few years, mitophagy has been intensively studied. The accumulating evidences indicate that mitochondria can be selectively removed by autophagy and the signals that specifically target mitochondria to autophagy have started to be unravelled.

### 1.12.1 Induction of mitophagy in yeast

The first studies in *Saccharomyces cerevisiae* provided evidence that dysfunctional mitochondria are targeted to autophagy (Campbell and Thorsness, 1998; Priault et al., 2005). Priault and colleagues (Priault et al., 2005) found that a *FMC1* null mutant, that presents dysfunctional ATPase, display an increased mitophagy under anaerobic conditions. During anaerobiosis, the reversal of the ATPase is essential to maintain $\Delta\Psi m$, and, consequently, mitochondrial biogenesis. In *FMC1* null strain, under anaerobiosis, $\Delta\Psi m$ collapses since they cannot use glycolitic ATP to maintain $\Delta\Psi m$. The authors proposed that this defect targets mitochondria to autophagy. The idea that mitochondrial dysfunction leads to the removal of the organelle was further supported by Nowikovsky and colleagues (Nowikovsky et al., 2007). Shutting-off *MDM38* gene expression leads to loss of mitochondrial $K^+/H^+$ exchange activity, osmotic swelling, reduction of $\Delta\Psi m$, mitochondrial fragmentation, and, consequently, mitophagy. Even though mitochondrial dysfunction targets the organelle to autophagy, treatment of yeast with the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), that dissipates $\Delta\Psi m$, is not sufficient to induce mitophagy (Kissova et al., 2004; Kanki et al., 2009a), suggesting the requirement for an additional, yet unidentified factor (Kanki et al., 2010).

Induction of non-selective macroautophagy also leads to mitophagy in yeast (Tal et al., 2007; Kanki and Klionsky, 2008; Kanki et al., 2009a). Indeed, mitophagy can be induced by nitrogen starvation or the Tor kinase inhibitor rapamycin after pre-culturing yeast in a non-fermentable carbon source that directs mitochondrial proliferation. Nevertheless, macroautophagy and mitophagy are probably differently regulated: nitrogen-starvation in the presence of a non-fermentable carbon source induces macroautophagy, but under these conditions, that make mitochondria essential for energy production, mitophagy is not detected (Kanki and Klionsky, 2008).

Superfluous mitochondria in yeast are also removed by mitophagy. Mitochondrial removal is induced during stationary phase in a non-fermentable carbon source (Tal et al., 2007; Kanki and Klionsky, 2008; Okamoto et al., 2009), when energy requirements are reduced.

### 1.12.2 Molecular mechanism of mitophagy in yeast

Kissova and colleagues described in 2004 the first mitochondrial "eat me" signal in yeast. Uth1, an OMM protein, has been found to be essential for mitophagy induced by rapamycin or nitrogen starvation, although in its absence the autophagic machinery was fully functional. Lack of Uth1 blocks mitophagy and increases resistance to rapamycin and nitrogen starvation (Kissova et al., 2004). Few years later, in a screen for functional interactors of Atg1, the mitochondrial protein Aup1 was indentified. Aup1 was described as being required for efficient mitophagy during stationary phase. Under these conditions, mitophagy was suggested to play a pro-survival role, since Aup1 was found to be essential for cell viability (Tal et al., 2007). It has been recently suggested that Aup1 regulates mitophagy by controlling the retrograde signalling pathway (RTG) (Journo et al., 2009). Nevertheless, the function of both Uth1 and Aup1 in mitophagy has been challenged by Kanki and colleagues (Kanki et al., 2009b). In their hands, lack of these proteins doesn't block mitophagy, due to differences in the background of the strains used or to different detection methods.

Recently, in a genomic screen for yeast mutants defective in mitophagy, two mitochondrial proteins have been identified – Atg32 and Atg33 (Kanki et al., 2009a; Kanki et al., 2009b; Okamoto et al., 2009). Atg32 is an OMM protein, essential for mitophagy, but not for macroautophagy or other types of selective autophagy. Selective autophagy in yeast requires a receptor and an adaptor protein. Atg32 acts as a receptor protein that interacts with the adaptor protein Atg11, most likely to sequester mitochondria to the PAS (Kanki et al., 2009b; Okamoto et al., 2009). In addition, Atg32 possesses an evolutionary conserved motif (W/YXXL/I) critical for the interaction with Atg8, an ubiquitin-like protein essential for autophagosomal membranes growth. According to Okamoto and colleagues, the interaction between Atg32 and Atg8 is required for mitochondrial recruitment by the phagophore (Okamoto et al., 2009). Atg32 has been the first protein described to be required to mitophagy and interact with the autophagic machinery. Nonetheless, important questions remain open. What is the physiological significance of Atg32-induced mitophagy? In other words, what happens in the absence of Atg32? Surprisingly enough, having into account the mitochondrial defects detected in *ATG* deleted strains (Zhang et al., 2007), no differences were observed between wt and the strain lacking Atg32 when cell growth was analysed for 3 days in a non-fermentable carbon source or cell viability and ROS production under starvation conditions were examined. In addition, mitochondrial protein as well as mtDNA levels were similar in both strains, suggesting, according to the authors, that Atg32 plays a critical role in the removal of superfluous mitochondria. Conversely, Atg32 seems to be critical for mitophagy in *MDM38* depleted strain, where mitophagy has been proposed to take place in response to mitochondrial damage (Kanki et al., 2009b). But, how is the function of

Atg32 regulated? How are Atg32 levels and activity controlled in the cells? Okamoto and colleagues found that Atg32 levels increase in mid-log phase under respiratory conditions but then decrease through late to post-log phase, indicating that Atg32 is temporally up-regulated prior to mitophagy. Yet, the authors described moderate expression of Atg32 under other conditions, that do not lead to mitophagy, indicating that other factors must be involved in promoting mitophagy (Okamoto et al., 2009). So, although an essential player in mitophagy has been identified, the complete scenario of how mitophagy is regulated in yeast is still missing.

The screen of yeast mutants deficient in mitophagy revealed another mitochondrial protein involved in targeting the organelle to autophagy – Atg33 (Kanki et al., 2009a). Lack of Atg33, an outer mitochondrial membrane protein, blocks mitophagy to half of the level during nitrogen starvation and almost completely at stationary phase. Atg33 preponderant role during stationary phase lead the authors to suggest that Atg33 is probably required for the recruitment of aged mitochondria by the PAS. Again, however, functional studies to prove this hypothesis are lacking (Fig. 6A).

### 1.12.3 Induction of mitophagy in mammals

In opposite to yeast, where the role of mitochondrial depolarization in triggering mitophagy is still a matter of debate, in mammals the loss of $\Delta\Psi m$ prompts mitophagy. For example, mitochondria are selectively removed upon treatment with the uncoupler CCCP (Narendra et al., 2008; Sandoval et al., 2008). Lemasters and colleagues have long defended the idea that opening of the mitochondrial PTP triggers mitophagy (Lemasters et al., 1998). Starvation and treatment of hepatocytes with glucagon leads to PTP opening, depolarization and, consequent, mitochondrial removal. Although under these conditions few mitochondria seemed to be removed by autophagy, the authors suggested that mitochondrial damage precedes their elimination (Elmore et al., 2001). Additionally, photo-damaged mitochondria are selectively removed after mitochondrial depolarization (Rodriguez-Enriquez et al., 2006). In accordance with the idea that PTP opening triggers mitophagy, Xue and colleagues showed that when cells are treated with an apoptotic stimulus in the presence of caspase inhibitors and then returned to normal growing conditions, the entire cohort of mitochondria disappears (Xue et al., 2001).

Besides mitochondrial damage, mitochondria are removed by autophagy during differentiation of specific cells. For sure the best studied example is reticulocyte differentiation, where mitochondrial removal seems to play a crucial role. The studies on the molecular mechanism of mitophagy during erythrocyte development implicated the BH3-only protein NIX on the selective targeting of mitochondria to the autophagosome (see below for

details) (Schweers et al., 2007; Sandoval et al., 2008). Additionally, mitophagy has been reported to play a crucial role during T-lymphocytes development (Pua et al., 2009).

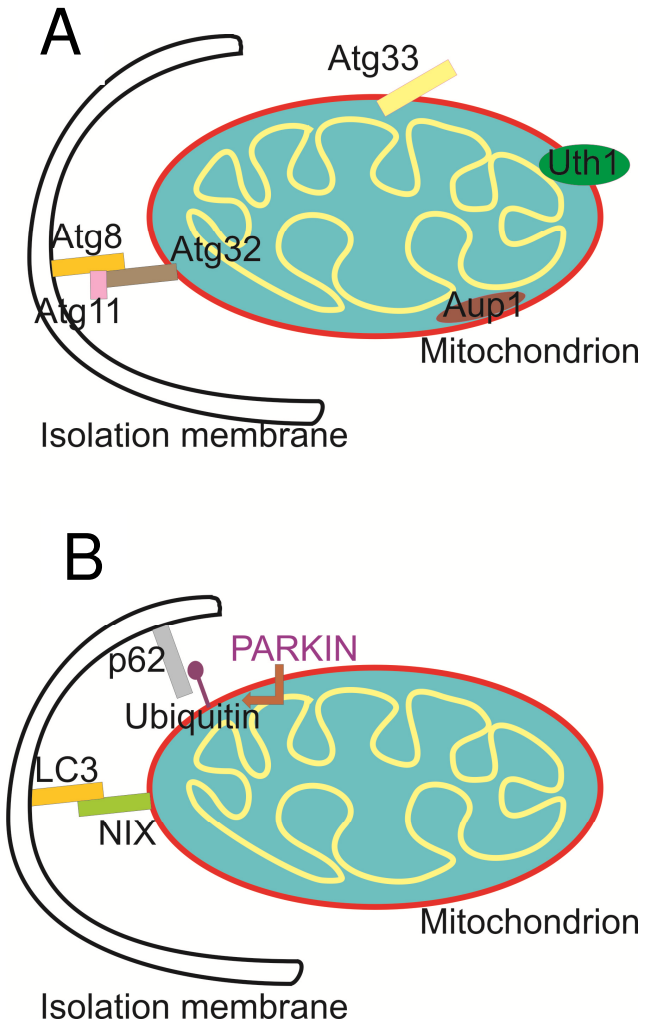


**Figure 6 – Signals that target mitochondria to autophagy. (A)** In yeast, four mitochondrial proteins have been reported to be important for targeting mitochondria to autophagy. Atg32 directs mitochondria to the autophagosome through its interaction with both Atg11 and Atg8. The mechanism by which Uth1, Aup1 and Atg33 target mitochondria to the autophagosome is not clear. **(B)** In mammals, during erythrocyte differentiation or upon mitochondrial depolarization, NIX targets mitochondria to the autophagosome through its interaction with LC3. Upon mitochondrial depolarization, PARKIN ubiquitinates proteins in the OMM. Whether ubiquitinated proteins target the mitochondria to the autophagosome by their interaction with p62 is still under debate.

### 1.12.4 Molecular mechanism of mitophagy in mammals

Mitochondrial clearance is a key step during reticulocyte maturation. This process likely occurs through autophagy since knock out of autophagy specific genes (ULK1 and ATG7) impairs mitochondrial removal (Kundu et al., 2008; Zhang et al., 2009). Recently, the BNIP3-like protein, BNIP3L, also known as NIX, has been described to be crucial for the complete removal of mitochondria during reticulocyte development (Schweers et al., 2007; Sandoval et al., 2008). NIX, a BH3-only member of the BCL2 family, is an OMM protein, whose expression increases during terminal stages of erythroid differentiation (Aerbajinai et al., 2003). *Nix*$^{-/-}$ mice display anaemia with reduced mature erythrocytes and compensatory expansion of erythroid precursors and, interestingly, *Nix*$^{-/-}$ red blood cells retain mitochondria (Schweers et al., 2007; Sandoval et al., 2008). NIX is essential for selective mitochondrial

removal, but not for induction of the autophagic core machinery. The mechanism by which NIX participates in mitochondrial clearance is still under debate and the different lines of evidence reported so far are not always consistent between them. Recently, NIX has been proposed to be a selective autophagy receptor for mitophagy (Novak et al., 2010), in analogy to Atg32 in yeast. Accordingly, NIX interacts with LC3 / GABARAP proteins (Atg8 homologues) (Schwarten et al., 2009; Novak et al., 2010), ubiquitin-like proteins essential for autophagosomal membranes growth, through a conserved WXXL motif, known as the LC3-interating region (LIR) (Novak et al., 2010). Novak and colleagues showed that ablation of the interaction between NIX and LC3/GABARAP proteins retards mitochondrial removal in mouse reticulocytes, although it does not completely abolishes it, meaning that other properties of NIX are important in the process, possibly independent of the classical core autophagic machinery, since ablation of ULK1, ATG7 and ATG5 impaired, but did not completely abolish mitochondrial clearance from maturating reticulocytes (Matsui et al., 2006; Kundu et al., 2008; Zhang et al., 2009). Additionally, NIX recruits GABARAP-L1 to damaged mitochondria, both in MEFs and HeLa cells. The recruitment is reduced by around 50% by a LIR region mutant of NIX (Novak et al., 2010). Conversely, according to Sandoval and colleagues mitochondrial depolarization can overcome NIX deficiency. In the absence of NIX, mitochondrial depolarization is inhibited, but artificial induction of depolarization can restore mitochondrial clearance in erythrocytes, putting NIX upstream of mitochondrial depolarization (Sandoval et al., 2008). Duration of treatments and the existence of signals other than NIX that target mitochondria to the autophagosomes may explain the observed discrepancies.

NIX was cloned from a human placenta cDNA library based on its homology to BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) (Matsushima et al., 1998). BNIP3 is also an OMM protein with a LIR region homologous to the one present in NIX. Likewise, it was reported that BNIP3 is required for hypoxia-induced mitophagy (Zhang et al., 2008) and, more recently, BNIP3 was shown to physically interact with LC3 (Rikka et al., 2010).

Several recent reports point for a crucial role of PARKIN in mediating mitophagy of damaged mitochondria. Pioneering work from Narendra and colleagues provided evidence that PARKIN, an E3 ubiquitin ligase, mutated in autosomal recessive forms of Parkinson's disease, translocates from the cytosol to dysfunctional mitochondria, after treating the cells with the uncoupler CCCP or paraquat, that increases complex I dependent ROS generation. After recruitment, PARKIN mediates selective engulfment of depolarized mitochondria by autophagosomes (Narendra et al., 2008). Later on, PINK1, a mitochondrial kinase, that also appears mutated in autosomal recessive forms of Parkinson's disease, was shown to be required for PARKIN recruitment to impaired mitochondria both in mammals (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010b; Vives-Bauza et al., 2010) and in *Drosophila melanogaster* (Ziviani et al., 2010). Ubiquitin has been proposed to act as a

signal for selective autophagy in mammalian cells. Different cargoes - protein aggregates, ribosomes, peroxisomes and pathogens - are ubiquitinated prior to autophagic removal (Kirkin et al., 2009b). Moreover, after fertilization, sperm mitochondria were reported to be ubiquitinated prior to degradation more than 10 years ago (Sutovsky et al., 1999). After translocation, PARKIN catalyzes poly-ubiquitination of depolarized mitochondria. Accordingly, the autophagy receptor p62/SQSTM1, that simultaneously bind ubiquitin and autophagy-specific ubiquitin-like modifiers (LC3/GABARAP proteins), has been implicated in PARKIN-mediated mitophagy. However, there is some disagreement about the precise role of p62. While according to two reports, p62 is indispensable for PARKIN-induced mitochondrial clearance (Geisler et al., 2010; Lee et al., 2010), according to other reports p62 is required for perinuclear clustering of depolarized mitochondria but not for mitophagy (Narendra et al., 2010a; Okatsu et al., 2010). A possible explanation for the differences observed may be functional redundancy, since lack of p62 could be compensated by another autophagy receptor that binds both ubiquitin and LC3/GABARAP proteins, NBR1 (Kirkin et al., 2009a). After ubiquitination and recruitment of p62 to depolarized mitochondria, mitochondria are transported along microtubules to the perinuclear region, where they form "mito-aggresome" structures (Lee et al., 2010; Okatsu et al., 2010; Vives-Bauza et al., 2010). Ubiquitinated mitochondria recruit not only p62 but also HDAC6, a ubiquitin binding protein deacetylase that mediates transport of damaged mitochondria, facilitating their clustering at the perinuclear region for subsequent clearance (Lee et al., 2010). Three independent groups reported PARKIN-dependent ubiquitination of MFN1 and MFN2, dynamin-like GTPases of the OMM that, as described before, mediate mitochondrial fusion (Gegg et al., 2010; Tanaka et al., 2010), and MARF, the *Drosophila* orthologue (Ziviani et al., 2010). Mitofusins, however, are not essential for PARKIN-dependent mitophagy, since this process was observed in *Mfn1*$^{-/-}$*Mfn2*$^{-/-}$ MEFs (Narendra et al., 2008). In addition, following recruitment of PARKIN to damaged mitochondria, mitofusins are lost prior to mitophagy (Gegg et al., 2010; Tanaka et al., 2010; Ziviani et al., 2010). Accordingly, Tanaka and colleagues showed that, after ubiquitination, mitofusins are degraded in a proteasome and p97-dependent manner (Tanaka et al., 2010). Hence, ubiquitination of mitofusins does not probably constitute a signal for mitophagy, but their removal by the proteasome can promote mitochondrial fragmentation, important for subsequent engulfment by the autophagosomes, as discussed below (Fig. 6B).

Although an incredible amount of information was revealed in the last two years about the mechanism of PARKIN-induced mitophagy, it is important to note that endogenous levels of PARKIN are not detectable in several cell types and the majority of the studies were made in cells overexpressing PARKIN. Even if the requirements of different cells are diverse, all of them need to get rid of damaged or surplus mitochondria. It would be interesting to

understand whether other E3 ubiquitin ligases that have been found associated with the mitochondrial outer membrane, like MARCH5/MITOL or MULAN (Germain, 2008), may be involved in inducing mitophagy.

PARKIN-induced autophagy has been studied in the context of mitochondrial damage. On the other hand, the role of NIX in mitophagy has been studied mainly during reticulocyte maturation. Interestingly, according to Novak and colleagues, the interaction between NIX and GABARAP-L1 plays an important role in mitophagy induced by the uncoupler CCCP, in cell types other than reticulocytes (Novak et al., 2010). Additionally, it has been reported that NIX promotes PARKIN translocation to depolarized mitochondria in MEF cells (Ding et al., 2010). Do NIX and PARKIN participate in the same pathway to induce mitophagy? Or do different proteins play a role in mitophagy under different conditions or in different cell types? Clarifying this open questions will be important for a better understanding of the mechanism of mitophagy in mammals.

Studies on the molecular mechanism of mitophagy in response to mitochondrial damage have been performed mainly after depolarizing mitochondria with an uncoupler. But how are dysfunctional mitochondria segregated from the network in order to be degraded in a living cell? The answer to this question came from an elegant study by Twig and colleagues, that followed mitochondrial dynamics in β-cells. According to this study, mitochondria undergo frequent cycles of fusion followed by fission. Often a fission event gives rise to uneven daughter mitochondria – one displaying higher and the other lower $\Delta\Psi m$. The probability of the mitochondrion with lower $\Delta\Psi m$ to fuse is reduced. This population of fragmented mitochondria with a decreased $\Delta\Psi m$ and lower levels of OPA1 was found to be removed by autophagy. Blocking fission, however, impaired mitophagy, resulting in the accumulation of dysfunctional mitochondria (Twig et al., 2008). As mentioned above, mitochondrial PARKIN ubiquitinates both MFN1 and MFN2, proteins critical for mitochondrial fusion. Ubiquitinated mitofusins are degraded by the proteasome, avoiding re-fusion of damaged mitochondria, and, consequently, facilitating its engulfment by the autophagosome (Tanaka et al., 2010).

Mitochondrial components are believed to freely mix through fusion of the mitochondrial network. Indeed mitochondrial mutations have been shown to spread across the network and exchange of mitochondrial network allows complementation of function (Sato et al., 2006). The work by Twig and colleagues, however, shows that mitochondrial fusion is not an unselective process – the probability of mitochondria with lower $\Delta\Psi m$ to fuse is reduced (Twig et al., 2008). Still, these two findings are not necessarily contradictory. Mutations of the mtDNA do not inevitably lead to a decrease in $\Delta\Psi m$. Additionally, the lag phase between fusion events is shorter than the half-time of respiratory complexes. Hence, it

is possible that more time would be needed for mitochondria to acquire a bioenergetic phenotype (Hyde et al., 2010). In this case, an inhibition of fusion would favour an improvement of mitochondrial quality control. Indeed, long-term overexpression of PARKIN, that impairs re-fusion of damaged mitochondria, promotes elimination of mitochondria with COXI mutations in heteroplasmatic cybrids (Suen et al., 2010).

# Chapter 2

# *Aims*

In this thesis we aimed at addressing the relationship between mitochondrial dynamics and mitophagy/ macroautophagy.

As described in the introduction, mitochondria are dynamic organelles that continuously fuse and fragment during cell life, therefore they can appear as short round-shaped or elongated organelles, with a major axis that can reach 5 μm, as observed in classical electron micrographs of isolated mitochondria (Cereghetti and Scorrano, 2006). On the other hand, autophagosomes are globular organelles with a diameter of about 1 μm (Komatsu and Ichimura, 2010), posing a sterical problem to mitochondrial engulfment by autophagosomes. Indeed, the co-existence of mitochondrial fission and mitophagy has been described under different situations. In response to nitric oxide (NO), for example, mitochondria from primary cortical neurons undergo fission. Under these conditions, mitochondria were retrieved inside autophagosomes (Barsoum et al., 2006). A similar connection was found in models of Alzheimer's disease, where mitochondrial fragmentation (Wang et al., 2008) is accompanied by an increase in the number of mitochondria found in the autophagosomes (Moreira et al., 2007). Furthermore, by the time we started the work presented in this thesis, some reports suggested that mitochondrial fragmentation was required and preceded fission. Arnoult and colleagues showed that induction of apoptosis triggers mitophagy that is preceded by mitochondrial fragmentation. Blocking mitochondrial fission with a dominant negative DRP1 also impaired mitophagy (Arnoult et al., 2005). Moreover, Parone and colleagues reported that preventing mitochondrial fission by DRP1 downregulation leads to mitochondrial dysfunction associated with an increase in autophagy levels. Nonetheless, mitophagy was not observed; even if mitochondria were damaged, they were probably too long to be engulfed by the autophagosome (Parone et al., 2008). Likewise, in yeast, mitophagy in *mdm38*Δ yeast is preceded by mitochondrial fragmentation. Preventing fission in *dnm1*Δ*mdm38*Δ blocks mitophagy (Nowikovsky et al., 2007). In conformity, later, *DNM1* was found in a genetic screen for yeast mutants defective in mitophagy, underlying the requirement of mitochondrial fission (Kanki et al., 2009a). Does this mean that mitochondrial fission is sufficient to trigger mitophagy? Or is mitochondrial dysfunction, which under many conditions is associated with the fragmentation of the organelle, also required? In order to answer these questions, we analyzed the effect of enforced FIS1 expression on autophagy. FIS1, as described above, is a bifunctional protein that independently regulates mitochondrial fission and apoptosis, through a direct effect on mitochondrial function (Alirol et al., 2006). The availability of mutants that specifically dissociate mitochondrial fission from dysfunction makes FIS1 a useful molecular tool to verify the relative role of sustained mitochondrial fission vs. that of dysfunction in triggering

mitophagy or a more generalized process of autophagy. The results obtained are described in chapter three of this thesis.

In addition, we aimed at understanding the role mitochondria play when bulk macroautophagy is induced, for instances by nutrient depletion or mTOR inhibition. Recent reports emphasize the idea that mitochondria are not just innocent bystanders during macroautophagy. Mitochondria might play a role in autophagosomal biogenesis, by providing membranes for the formation of the PAS (Hailey et al., 2010), in a process that depends on the tethering to the ER (de Brito and Scorrano, 2008b). Moreover, ROS, mainly produced by mitochondria during starvation, have been reported to play an essential role as regulators of autophagy (Scherz-Shouval et al., 2007). However, many questions on the morphology as well as on the functional role of mitochondria during autophagy remain open. Do they fragment? Are they randomly targeted to autophagosomes? Is the progression or the final outcome of autophagy influenced by changes in their morphology? As described in the introduction, mitochondria-shaping proteins are involved in a growing number of cellular functions. Here, we analyse the shape of mitochondria upon induction of autophagy, as well as the role that mitochondria play and how their function during autophagy is influenced by their morphology. The results obtained are described in chapter four of this thesis.

# Chapter 3

## *High levels of FIS1, a pro-fission mitochondrial protein, trigger autophagy.*

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## Abstract

Damaged mitochondria can be eliminated in a process of organelle autophagy, termed mitophagy. In most cells, the organization of mitochondria in a network could interfere with the selective elimination of damaged ones. In principle, fission of this network should precede mitophagy; but it is unclear whether it is *per se* a trigger of autophagy. The pro-fission mitochondrial protein FIS1 induced mitochondrial fragmentation and enhanced the formation of autophagosomes which could enclose mitochondria. These changes correlated with mitochondrial dysfunction rather than with fragmentation, as substantiated by FIS1 mutants with different effects on organelle shape and function. In conclusion, fission associated with mitochondrial dysfunction stimulates an increase in autophagy.

## Keywords

mitochondria; fission; FIS1; autophagy

## Introduction

Mitochondria are central organelles for the life and death of the cell. They provide most of the ATP required for endoergonic processes, participate in crucial biosynthetic pathways, shape $Ca^{2+}$ signalling and regulate cell death (Dimmer and Scorrano, 2006). Moreover, they are the only organelle with an autonomous DNA content and translation machinery, required for the *in organello* synthesis of some components of the respiratory chain. Mutations in mtDNA have been associated with a variety of maternally transmitted genetic diseases classically referred to as "mitochondrial diseases". In recent years, the number of diseases of genetic origin affecting mitochondria greatly increased. Several mutations in nuclear genes encoding for mitochondrial proteins have been associated with genetic diseases of previously unknown origin (DiMauro, 2004). Among these, dominant optic atrophy (DOA) (Alexander et al., 2000; Delettre et al., 2000), Charcot-Marie-Tooth IIa (CMT2a) (Zuchner et al., 2004) and Charcot-Marie-Tooth IVa (CMT4a) (Niemann et al., 2005) are caused by mutations in genes coding for "mitochondria-shaping" proteins.

The functional versatility of mitochondria is paralleled by their morphological complexity. In certain cell types mitochondria are organized in networks of interconnected organelles (Bereiter-Hahn and Voth, 1994). Ultrastructurally, the inner membrane (IM) can be further subdivided in an inner boundary membrane and in the cristae compartment, bag-like folds of the IM connected to it via narrow tubular junctions (Frey and Mannella, 2000). Mitochondria-shaping proteins impinge on the equilibrium between fusion and fission processes that ultimately determines the ultrastructural and cellular morphology of the organelle. Mitofusins (MFN) 1 and 2 are outer membrane proteins that control mitochondrial fusion in mammals (Legros et al., 2002b; Santel and Fuller, 2001; Chen et al., 2003). In the inner membrane, the only "core component" of the fusion/fission machinery identified so far is OPA1. OPA1 exists in eight different splice variants (Delettre et al., 2001), promotes fusion in a MFN1-dependent manner (Cipolat et al., 2004b; Meeusen et al., 2006), controls biogenesis of the cristae (Meeusen et al., 2006) and regulates the cristae remodelling pathway during apoptosis in a genetically distinct pathway regulated by the inner mitochondrial membrane rhomboid protease PARL (Cipolat et al., 2006; Frezza et al., 2006; Pellegrini et al., 2001). In mammalian cells, mitochondrial division is regulated by DRP1 and FIS1 (Smirnova et al., 2001; Labrousse et al., 1999; James et al., 2003; Bleazard et al., 1999). DRP1 is a cytosolic dynamin-related protein whose inhibition or downregulation result in a highly interconnected mitochondrial network. The same phenotype is caused by downregulation of FIS1 (Mozdy et al., 2000), a 16 kDa integral protein of the outer mitochondrial membrane, containing a single transmembrane domain and a tetratricopeptide repeat (TPR, involved in protein-protein interaction) domain facing the cytosol (Mozdy et al., 2000). Some evidence exists that FIS1 is the receptor on the outer membrane for DRP1, via

its TPR. DRP1 is recruited to mitochondria and constriction of the membranes takes place by direct or indirect interaction with FIS1 (Yoon et al., 2003).

Changes in mitochondrial shape appear to regulate crucial mitochondrial and cellular functions. During apoptosis mitochondria remodel their inner structure to allow the bulk of cytochrome *c* to be released from the cristae stores, a process called cristae remodelling (Scorrano et al., 2002). Moreover, in neurons as well as in model cell lines mitochondria undergo massive and reversible fragmentation prior to the release of cytochrome *c* (Frank et al., 2001; Martinou et al., 1999). Not only mitochondrial shape changes are important during death of the cell, but they appear also to influence crucial cellular functions, from $Ca^{2+}$ signalling (Szabadkai et al., 2004) to generation of reactive oxygen species (Yu et al., 2006), to neuronal plasticity (Li et al., 2004), to intermediate metabolism (Bach et al., 2003), to leukocyte dynamics (Campello et al., 2006), even to lifespan of the filamentous fungi (Scheckhuber et al., 2007).

A growing set of evidence is implying mitochondrial morphological changes in the course of another type of cell response, autophagy. During autophagy organelles and parts of cytoplasm are sequestered and subsequently delivered to lysosomes for hydrolysis (Levine and Klionsky, 2004). Autophagy is a constitutive process of all nucleated cells, but it can be activated by certain stimuli, like fasting and nutrient deprivation, when a burst in autophagy is important to generate amino acids, and ultimately fuel the tricarboxylic acids cycle to maintain ATP production. Autophagy has also a role in the removal of toxic protein aggregates and damaged or unneeded organelles. Changes in the levels of autophagy are capable of promoting cell injury, substantiating the requirement for a tightly regulated machinery (Levine and Yuan, 2005). The anatomy of autophagy allows such a precise control: an isolation membrane of unclear origin (probably endoplasmic reticulum) forms a cupshaped structure called phagophore that envelopes the autophagic target, becoming an autophagosome, and eventually fuses with lysosomes to form autolysosomes (Xie and Klionsky, 2007). In principle, the control can be exerted (i) upstream of phagophore formation, at the level of induction of autophagy; and (ii) at each step of membrane evolution. In yeast, a machinery of genetically conserved autophagy-related proteins regulate and participate in autophagy (Ohsumi and Mizushima, 2004). These Atg proteins include: (i) Atg1, Atg13, and Atg17, a serine–threonine kinase complex involved in autophagic induction; (ii) a Class III phosphatidylinositol-3-kinase (PtdIns3K) complex which functions in vesicle nucleation; (iii) Atg12 and Atg8, ubiquitin-like protein conjugating systems, involved in vesicle extension and completion together with Atg5, Atg7, Atg10 and Atg16 (Xie and Klionsky, 2007). LC3 is the mammalian orthologue of yeast Atg8. During autophagy, 22 amino acids are cleaved from the C-terminus of LC3, forming LC3-II that is lipidated to selectively localize to nascent and newly formed autophagosomes, making it a useful autophagosomal marker (Kabeya et al.,

2004; Klionsky et al., 2008). The activation of autophagy in mammalian cells is controlled by two classes of PtdIns3Ks with opposite effects: class I PtdIns3K, via its downstream effector mammalian Target Of Rapamycin (mTOR), blocks autophagy (Neufeld, 2003), while class III PtdIns3Ks, operating together with BECLIN, stimulates it (Pattingre et al., 2005).

Autophagy can be selective for certain organelles, as it was originally shown for peroxisomes (Farre and Subramani, 2004). Similarly, a considerable interest developed on the possibility that mitochondria undergo a process of selective elimination by autophagy, leading to the so-called mitophagy. The importance of mitochondria for the control of metabolism, production of reactive oxygen species and last but not least for the control of apoptosis suggests that mitophagy can be a crucial mechanism to regulate pivotal cellular functions. However, the existence of mitophagy per se, not to speak about its selective regulation, is still questioned (Mijaljica et al., 2007). It has been suggested that mitochondrial dysfunction dependent on the opening of the permeability transition pore, a non-selective large conductance inner mitochondrial membrane channel (Bernardi, 1999), is a trigger for autophagy when it does not result in cytochrome *c* release and apoptosis (Lemasters, 2005). However, there is a conceptual constraint in the development of mitophagy, as it should be preceded by the generation of individual organelles from the mitochondrial network observed in most cell types. This could be accomplished by a reduction in the levels of pro-fusion proteins, such as OPA1, in dysfunctional mitochondria that are targeted for mitophagy (Twig et al., 2008; Duvezin-Caubet et al., 2006b); or by the activation of the fission machinery. Interestingly, high levels of FIS1 are able to induce mitochondrial fission, release of cytochrome *c* and apoptosis (James et al., 2003). This cell death appears to be related to a direct effect of FIS1 on mitochondrial function, as substantiated by a genetic analysis of the requirements for FIS1-mediated apoptosis (Alirol et al., 2006). FIS1 can therefore be a useful molecular tool to verify the ability of sustained mitochondrial fission to trigger mitophagy or even a more generalized process of autophagy.

Here we analyzed the effect of enforced FIS1 expression on autophagy. Our data indicate that cells overexpressing FIS1 accumulate fragmented mitochondria and autophagic vesicles, where fragmented mitochondria are sometimes retrieved. Analysis of mutants of FIS1 suggests that stimulation of autophagy correlates with mitochondrial dysfunction rather than with fission of the organelle.

## Material and Methods

### Molecular Biology

peYFP-hLC3 (YFP-LC3) was kindly provided by Dr. M. Sandri (Venetian Institute of Molecular Medicine, Padua, Italy). Mitochondrially targeted dsRED (mtRFP) and pcDNA3.1Zeo(+)mRFPI (monomeric RFP) were kind gifts from M. Zaccolo (Venetian Institute of Molecular Medicine, Padua, Italy). Full length *hFis1*, the K148R mutant of hFis1 (*hFis1*$^{K148R}$) and Δ1-32 hFis1 (*hFis1*$^{\Delta\alpha1}$) (Alirol et al., 2006) were subcloned into the *EcoRI* site of pcDNA3.1Zeo(+). All constructs were confirmed by sequencing.

### Cell Culture and Transfection

SV40 transformed mouse embryonic fibroblasts (MEFs) from a mixed Sv129/CD1 background were cultured as described before (Scorrano et al., 2003). Cells were transfected using Transfectin (Biorad) following manufacturer's instructions. HeLa cells were grown in complete DMEM supplemented with 10% FBS. Transfection of HeLa cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfection efficiency reached 60% on average experiments.

### Imaging

For confocal imaging of MEFs, cells seeded onto 24-mm round glass coverslips, incubated in Hanks balanced salt solution (HBSS) supplemented with 10 mM Hepes were placed on the stage of a Nikon Eclipse TE300 inverted microscope equipped with a spinning-disk PerkinElmer Ultraview LCI confocal system, a piezoelectric *z*-axis motorized stage (Pifoc, Physik Instrumente, Germany), and a Orca ER 12-bit charge-coupled device camera (Hamamatsu Photonics, Japan). Cells expressing YFP-LC3 and mtRFP were excited using the 488 nm, and the 543 line of the HeNe laser (PerkinElmer) and images were acquired using a 60x 1.4 NA Plan Apo objective (Nikon).

HeLa cells grown on 13 mm round coverslips were transfected as indicated and, after 24h, fixed for 20 min at room temperature with 4% (w/V) ice-cold paraformaldehyde. Imaging was performed as described above.

For the analysis of mitochondrial incorporation by autophagosomes, confocal *z*-axis stacks of mtRFP and LC3-YFP fluorescence separated by 0.2 µm along the *z*-axis were acquired. 3D reconstruction and volume rendering of the stacks were performed with the appropriate plugins of ImageJ (National Institutes of Health, Bethesda).

**Induction and Quantification of Autophagy**

24 h after transfection, cells were blindly classified as autophagy negative cells (that present a predominantly diffuse YFP-LC3 fluorescence) or autophagy positive cells (cells with a punctuate YFP-LC3 pattern) (Klionsky et al., 2008).

For the induction of autophagy, cells were starved for 2h30min in a Hank's Balanced Salt Solution (HBSS, Invitrogen) supplemented with 10mM Hepes pH 7.4. 3-methyladenine (3-MA) was obtained from Sigma

**Immunoblotting**

Twenty-four hours after transfection, cells were harvested and disrupted in lysis buffer [1% (V/V) TritonX-100, 150 mM NaCl, 50 mM Tris, pH 7.4] in the presence of complete protease-inhibitor mixture (Sigma). Extracted proteins (25 µg) were separated by 4-12% SDS-PAGE (NuPAGE, Invitrogen) and transferred onto polyvinylidene difluoride (PVDF, BioRad). Membranes were probed using the following antibodies: monoclonal anti-LC3 (1:1000, MBL), anti-p62 (1:5000, Progen), anti-actin (1:5000, Chemicon).

**Analysis of lysotracker accumulation**

MEFs grown on 12-well plates were cotransfected with mRFPI and the indicated vector. After 24 hr cells were treated as described and incubated with 50 nM LysoTracker Green (LTG, Molecular Probes) DND-26 for 30 min at 37°C in the dark. Loaded cells were then washed free of excess LTG by centrifugation for 5 min at 200 g and resuspended in HBSS supplemented with 10mM Hepes pH 7.4

LTG accumulation was measured by flow cytometry (FACSCalibur, BD Biosciences) as the percentage of lysotracker-positive events in the RFP-positive population.

## Results

### Markers of autophagy in cells expressing FIS1.

In order to gain insights into the relationship between mitochondrial fission and autophagy, we took advantage of the pro-fission effect of FIS1 expression (Alirol et al., 2006). Non-tagged versions of wt and mutant FIS1 (Fig.1A) were produced by standard subcloning techniques and their expression resulted in comparable, several fold increase in the levels of endogenous FIS1, as judged by specific anti-FIS1 immunoblotting (Fig. 1B).

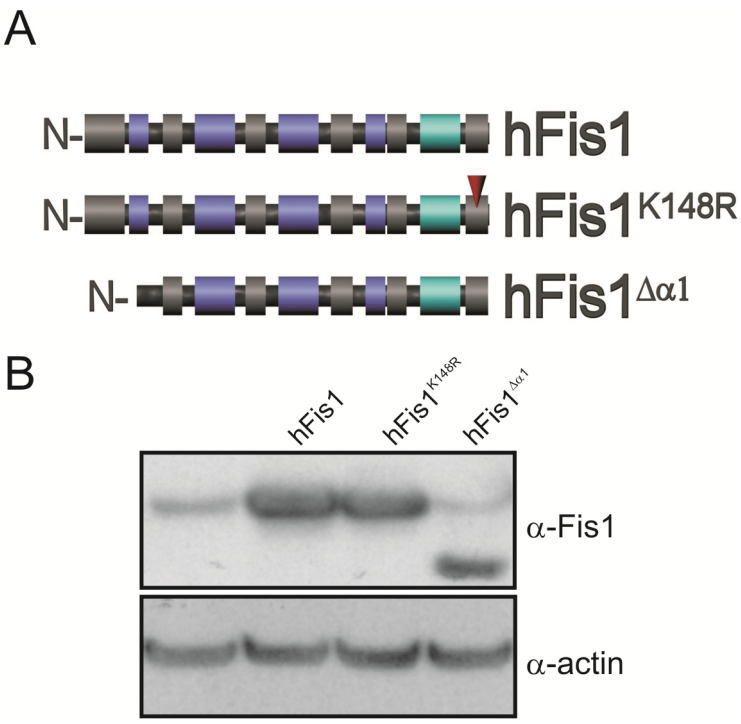


**Figura1 – Expression levels of the FIS1 mutants used in this study. (A)** Cartoon depicting the mutants used in this study. The gray blocks indicate the α-helices, the green one the transmembrane domain, the blue ones the tetratrichopeptide repeats. The red arrowhead shows the position of the point mutation. **(B)** immunoblot of wt and mutant FIS1 expression levels. MEFs were transfected with the indicated plasmid and after 24 hrs cells were harvested, lysed and equal amounts of proteins (25 µg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

Confocal microscopy of the mitochondrial marker mtRFP expressed in HeLa cells showed that as expected twenty four hours after co-transfection wt FIS1 induced fragmentation of the highly interconnected mitochondrial network observed in this cell line (Fig. 2A). It should be noted that in the case of untagged FIS1, extensive fragmentation is associated with perinuclear clustering, much less pronounced in cells overexpressing Myc-tagged FIS1 (James et al., 2003). When we analyzed the intracellular distribution of YFP-LC3 in the same cells, we surprisingly noticed that FIS1 induced the accumulation of this bona-fide marker of autophagy into punctuate, vesicular structures. On the contrary, HeLa cells that were not co-transfected with FIS1 displayed a reticular mitochondrial network and a faint, diffuse cytoplasmic YFP-LC3 distribution. Of note, the punctuate pattern of YFP-LC3 was similar to that observed in HeLa cells that underwent starvation, the prototypical inducer of autophagy (Fig. 2A), suggesting that co-expression of FIS1 causes a process similar to that of starvation. A quantitative, blind analysis of YFP-LC3 distribution showed that co-expression of hFis1

caused a ~50% increase in the punctuate autophagy-like pattern of this fluorescent marker (Fig. 2B).

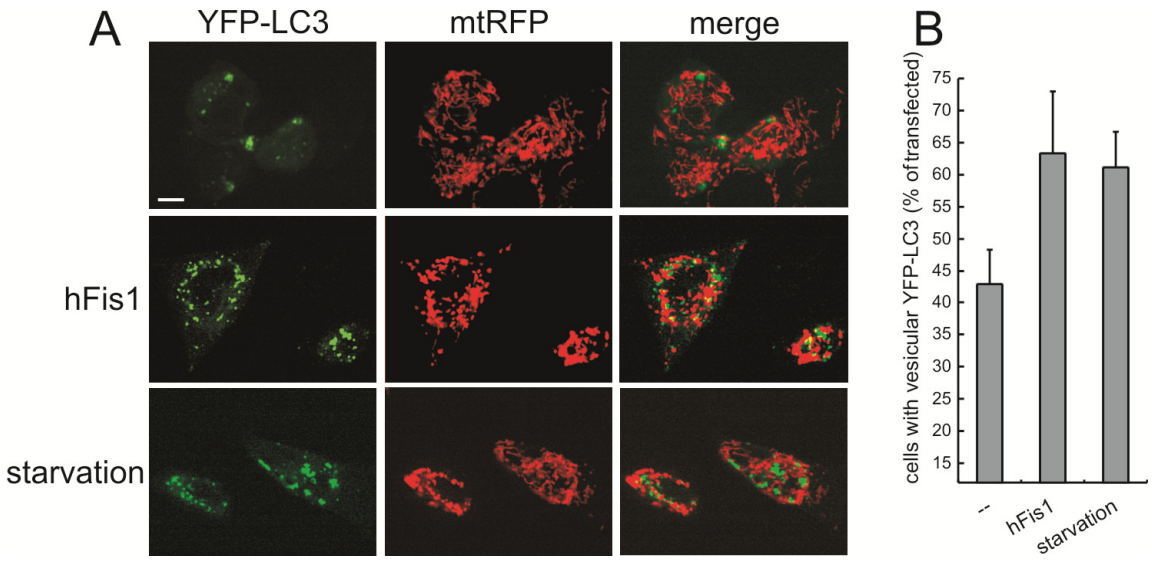


**Figure 2 – Accumulation of YFP-LC3 into vesicular structures in FIS1 expressing HeLa cells. (A)** Representative confocal images of YFP-LC3 and mtRFP fluorescence. HeLa cells grown on coverslips were co-transfected YFP-LC3, mtRFP and with empty plasmid or with FIS1. After 24 hrs cells were fixed and confocal images of YFP-LC3 and mtRFP fluorescence were acquired as described in Material and Methods. Merge indicates the superimposition of the single channel images. Where indicated (starvation), cells transfected with empty plasmid were incubated for 2.5 hrs in HBSS prior to fixation. Bar, 15 μm. **(B)** Quantitative analysis of YFP-LC3 vesicular distribution. Experiments were exactly as in (A). Data represent mean ± SE of 3 independent experiments in which 30-120 cells per condition were analyzed.

We wished to further confirm that expression of FIS1 changed the intracellular distribution of YFP-LC3 in a different cell line. To this end, we turned to mouse embryonic fibroblasts (MEFs), where prolonged (48 hrs), but not short-term (24 hrs) expression of FIS1 results in the activation of a program of mitochondrial dysfunction, cytochrome *c* release and cell death (Alirol et al., 2006). Co-expression of FIS1 with mtRFP showed extensive fragmentation of the mitochondrial network that was accompanied also in this case by the accumulation of YFP-LC3 in punctuate structures (Fig. 3A and quantification in 3B). Since changes in YFP-LC3 subcellular distribution cannot be used as sole marker of autophagy, we wished to verify if FIS1 expression caused LC3II accumulation as well as degradation of p62, two other well established indicators of the activation of autophagy. Consistently, FIS1 overexpression caused an accumulation of processed LC3II (Fig. 3C) as well as the degradation of p62 (Fig. 3D, note the densitometric analysis). It should be noted that transfection *per se* caused the appearance of a faint LC3II band in a probably unspecific cellular response to the lipidic transfection reagent (Kuma et al., 2007) (Fig. 3C).

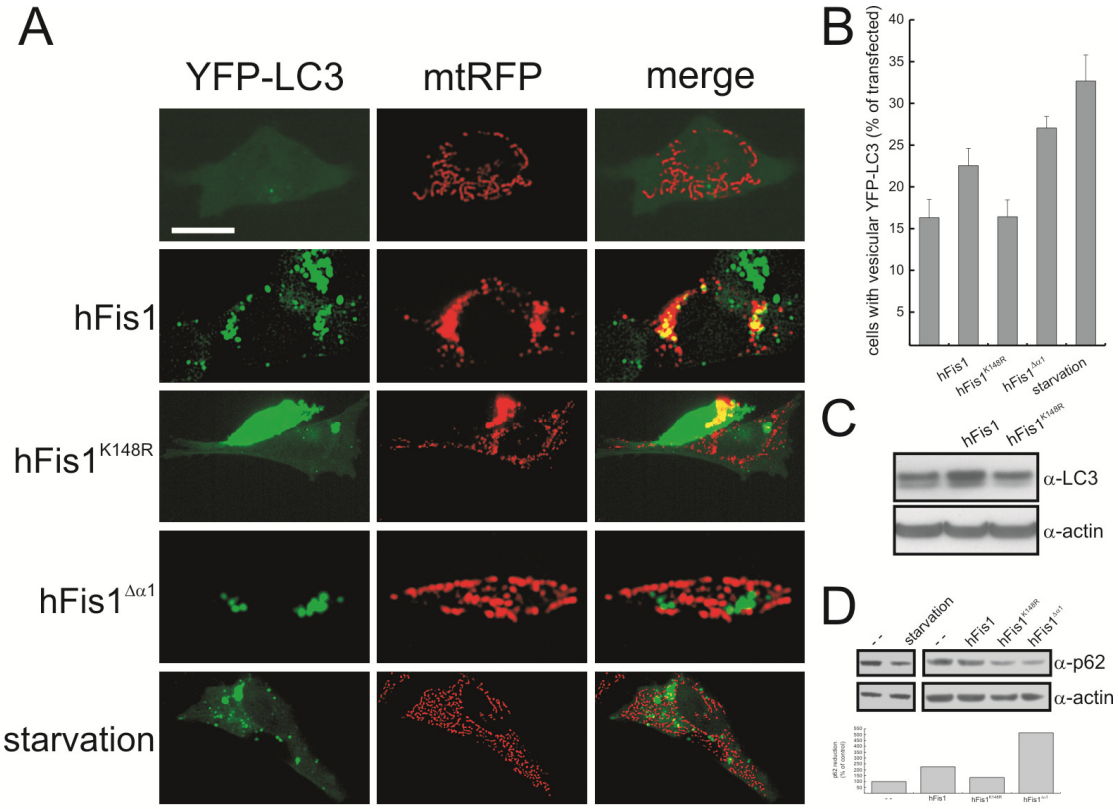


**Figure 3 – Effect of mutants of FIS1 on markers of autophagy. (A)** Representative confocal images of YFP-LC3 and mtRFP fluorescence. MEFs grown on coverslips were cotransfected YFP-LC3, mtRFP and with empty plasmid or with the indicated plasmid. After 24 hrs confocal images of YFP-LC3 and mtRFP fluorescence were acquired as described in Material and Methods. Where indicated (starvation), cells transfected with empty plasmid were incubated for 2.5 hrs in HBSS prior to acquisition. Bar, 25 μm. **(B)** Quantitative analysis of YFP-LC3 vesicular distribution. Experiments were exactly as in (A). Data represent mean ± SE of 5 independent experiments. For each condition, >100 cells were analyzed in each experiment. $p<0.01$ between control and FIS1, FIS1$^{\Delta\alpha1}$, and starvation in a paired Student's t test. **(C)** Processing of endogenous LC3. MEFs were transfected with the empty plasmid or with the indicated plasmid and after 24 hrs cells were harvested, lysed and equal amounts of proteins (25 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Note the accumulation of the lower MW, LC3II band in the FIS1 transfected sample. **(D)** Degradation of endogenous p62. Experiments were exactly as in (C). The bar graph shows densitometric analysis of p62 levels following normalization for actin.

Finally, we checked whether overexpression of FIS1 increased cellular staining with the lysosomal dye LysotrackerGreen (LTG), whose accumulation is proportional to lysosomal acidification and number and has been used to monitor activation of autophagy (Rodriguez-Enriquez et al., 2006). Exposure of MEFs to brief starvation caused in fact an increase in the labeling with LTG, which was completely sensitive to the inhibitor of autophagosome formation 3 methyladenine (3-MA) (Fig. 4A and B). A similar 3-MA-sensitive accumulation of LTG was observed in cells transfected with hFis1 (Fig. 4C).

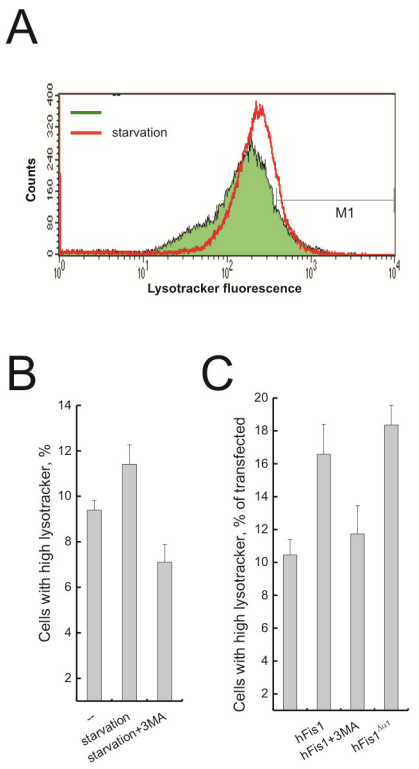


**Figure 4 – Lysotracker Green accumulation in FIS1 expressing cells. (A)** Representative histogram of cellular accumulation of LTG in response to starvation. Where indicated, $10^5$ MEFs were incubated in HBSS for 2.5 hrs and then loaded with LTG as described in Materials and Methods. Accumulation of LTG was evaluated by flow cytometry. **(B)** Quantitative analysis of LTG accumulation in response to starvation. Experiments were exactly as in (A) except that where indicated cells were pretreated with 10 mM 3MA. Data represent mean ± SE of 3 independent experiments. **(C)** Quantitative analysis of LTG accumulation in cells expressing FIS1. MEFs were transfected with mRFPI and empty or the indicated plasmid and after 24 hrs loaded with LTG as described in Materials and Methods. Accumulation of LTG was evaluated by flow cytometry in the mRFPI positive channel. Where indicated, cells were treated with 10 mM 3-MA 3 hrs before loading with LTG. Data represent mean ± SE of 3 independent experiments.

Taken together, our data indicate that overexpression of FIS1 causes mitochondrial fragmentation and accumulation of several markers of autophagy, before the activation of the cell death program and irrespective of the cell line tested.

## Induction of autophagy by FIS1 correlates with mitochondrial dysfunction rather than with fragmentation

We had recently developed and characterized a series of mutants of FIS1 with different effects on fusion/fission and dysfunction of mitochondria. A conservative mutation in the short stretch of amino acids of FIS ($FIS^{K148R}$) protruding in the intermembrane space retains the effect on mitochondrial fission but is unable to induce mitochondrial dysfunction, whereas a mutant in which the first α-helix had been ablated ($FIS^{\Delta\alpha1}$) acts as a dominant negative for mitochondrial fusion and causes extensive mitochondrial dysfunction (Alirol et al., 2006; Yu et al., 2005) (these mutants are depicted in the cartoon in Fig.1A). FIS1 mutants seemed a good tool to investigate the relationship between mitochondrial fragmentation and the accumulation of markers of autophagy. When we expressed $FIS1^{K148R}$ we noticed that it caused mitochondrial fission, yet it did not induce the accumulation of YFP-LC3 into vesicular structures (Fig. 3A and quantification in 3B). Similarly, $FIS1^{K148R}$ did not cause an accumulation of LC3II, or a significant reduction in the levels of p62 (Fig. 3C and D). On the other hand, the $FIS1^{\Delta\alpha1}$ mutant induced the appearance of large mitochondrial structures that

were accompanied by a significant increase in the number of YFP-LC3 positive vesicles, greater than that observed in cells expressing wt FIS1 (Fig. 3A and quantification in 3B). Along the same line, FIS1$^{\Delta\alpha1}$ caused a massive reduction in the levels of p62 (Fig. 3D) and an increased accumulation of LTG (Fig. 4C), both above the levels observed in the samples overexpressing wt FIS1. In conclusion, these mutants of FIS1 highlight that the appearance of markers of autophagy in cells seems to correlate better with mitochondrial dysfunction rather than with fragmentation *per se*.

## Mitochondria fragmented by FIS1 can be targeted to autophagosomes

It has been reported that fragmented and dysfunctional mitochondria are often targeted to autophagosomes (Rodriguez-Enriquez et al., 2006; Twig et al., 2008). Our model of FIS1 expression allowed us to verify the fate of these fragmented mitochondria, prior to the release of cytochrome *c* and the activation of the postmitochondrial apoptotic pathway. We therefore decided to verify whether YFP-LC3 positive autophagosomes were wrapping mitochondria fragmented by enforced FIS1 expression. To this end, we turned to an imaging approach in which we reconstructed and volume rendered confocal z-stacks of mtRFP and YFP-LC3 fluorescence images. This approach allowed us to generate images of the whole cellular volume, where the interrelationship between autophagosomes and mitochondria could be better evaluated.

Volume rendered composite mtRFP and YFP-LC3 images tilted along the y-axis showed that “yellow” dots appearing in non-stressed cells were due to the proximity between autophagosomes and mitochondria, but not to a “wrapping” of the latter by the former (Fig. 5, enlargement). On the other hand, in the case of FIS1 expressing cells, the fragmented mitochondria were really enclosed by the YFP-LC3 positive vesicles, as clearly visible in the magnified box in Figure 5. In general, we observed an increase in “false colocalization”, i.e. in mitochondrial targeting to autophagosomes, in 3D-reconstructed, volume rendered FIS1 expressing cells. A quantitative analysis of red-to-green colocalization using Manders’ coefficient (Manders et al., 1993) showed a 153% increase in targeting to autophagosomes (green) of FIS1 expressing mitochondria (red) as compared to the ones from empty-vector transfected cells. In conclusion, fragmented mitochondria expressing FIS1 can be engulfed by autophagosomes.

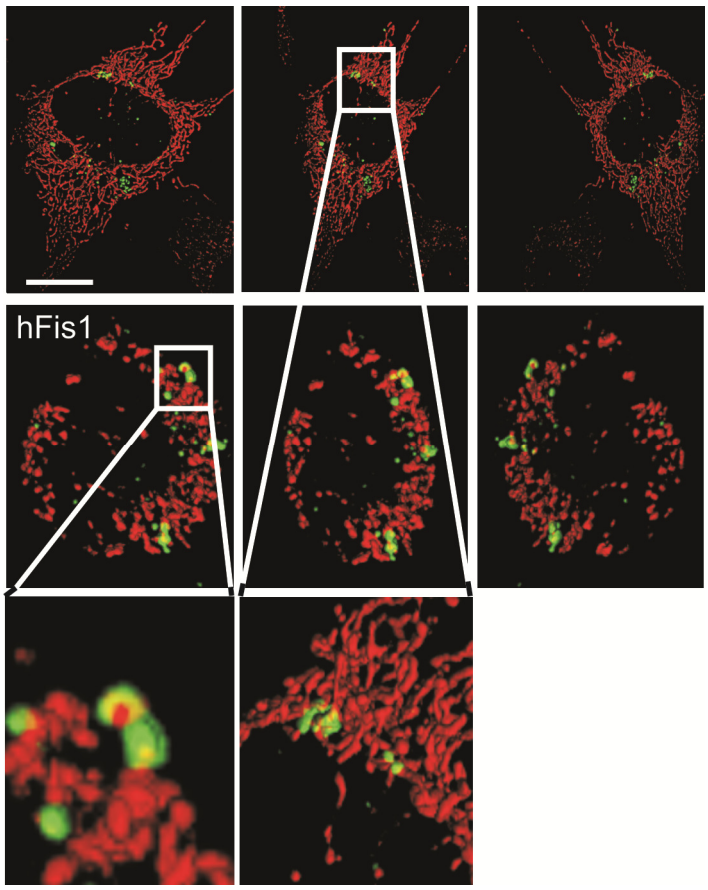


**Figure 5 – Mitochondria expressing FIS1 can be retrieved in autophagosomes.** MEFs grown on coverslips were cotransfected with YFP-LC3 and mtRFP and with empty plasmid or where indicated with FIS1. After 24 hrs confocal z-stacks of mtRFP and YFP-LC3 fluorescence were acquired, 3D reconstructed, volume rendered and merged. Red indicates mtRFP, green YFP-LC3. Bar, 25 μm. The boxed areas are magnified 3X.

## Discussion

Our current understanding of the relationship mitochondrial shape, mitophagy and autophagy in general is scarce. Here we used overexpression of a mitochondrial pro-fission protein in order to explore how mitochondrial morphology and function influences these processes. We found that excessive mitochondrial fission results in the accumulation of markers of autophagy and that mutants of FIS1 with limited effects on mitochondrial function are less potent in the induction of autophagy. The fragmented mitochondria induced by FIS1 expression can be targeted to autophagosomes, probably as a consequence of the exposure of an "eat-me" signal. In conclusion, our results indicate that mitochondrial dysfunction, rather than fragmentation per se, determines whether the cell induces a program of autophagy.

It is currently not understood whether mitochondrial fission is a determinant of autophagy. Evidence suggests that the opposite is true, *i.e.* that fragmentation is required for mitochondrial autophagy. This has been verified in neurons, where mitophagy can be induced by NO, causing accumulation of ROS production and mitochondrial fragmentation. Inhibition of mitochondrial fission or induction of mitochondrial fusion inhibits this NO-induced mitophagy (Barsoum et al., 2006). Similarly, autophagic degradation of yeast mitochondria observed in strains deficient in the inner membrane protein Mdm38p depends on fission (Nowikovsky et al., 2007). Since downregulation of LETM1, the human orthologue of Mdm38, causes fragmentation independently of the fission machinery (Dimmer et al., 2008), it is conceivable that in the mitophagy of Mdm38 deficient yeast mitochondria, Dnm1/Drp1 is involved at a different step than the fragmentation of the network. Finally, an elegant study by Shirihai and colleagues demonstrated that mitochondria targeted for autophagy undergo cycles of fusion followed by fission, sustained by a drop in the levels of the pro-fusion protein Opa1 (Twig et al., 2008). Here we report that expression of the pro-fission mitochondria shaping protein FIS1 results in the accumulation of several markers of autophagy. However, it appears that dysfunction, rather than fragmentation, is the determining event in the induction of autophagy. This was substantiated by the use of mutants of FIS1 that dissociate its ability to fragment mitochondria from its detrimental action on mitochondrial function (Alirol et al., 2006). The more toxic the mutant, the highest induction of autophagy was observed. It should be noted that the Δα1 mutant could form large proteic aggregates, which eventually could cause the accumulation of YFP-LC3 in a pathway independent of autophagocytosis (Kuma et al., 2007). Nevertheless, we observed the appearance of other markers of autophagy following expression of $FIS1^{\Delta\alpha1}$, suggesting that this mutant of FIS1 is indeed able to trigger autophagy more than the wild-type pro-fission protein. Thus, our results indicate that fragmentation per se is not sufficient to trigger autophagy. Moreover, they suggest that mitochondrial dysfunction can feedback to the machinery of autophagy to induce its activation.

In line with these observations, accumulating evidence suggests that mitochondrial dysfunction by itself can trigger mitophagy. Mitochondria-derived ROS may regulate ATG4, a cysteine protease essential in the autophagic pathway (Scherz-Shouval et al., 2007). Of note, mitochondrial dysfunction by FIS1 involves ROS formation, as substantiated by the inhibitory activity of the ROS scavenger N-acetylcysteine (Alirol et al., 2006). Another appealing possibility is that FIS1 expression, which converts mitochondria in sinks for ATP (Alirol et al., 2006), signal to AMP-activated protein kinase, a master regulator of autophagy (Liang et al., 2007; Hoyer-Hansen and Jaattela, 2007). Irrespective of the nature of this signal elicited by mitochondria, expression of FIS1 highlights the existence of yet another axis of retrograde mitochondrial signalling, in addition the so called mitochondrial stress response (Zhao et al., 2002), which is likely to involve regulators of mitochondrial dynamics such as PARL (Jeyaraju et al., 2006).

When we examined the fate of fragmented mitochondria, we found that only some of them were targeted to autophagosomes in a mitophagy process. While it is possible that we missed some mitophagic events, it should be kept in mind that it is similarly likely that not all mitochondria are targeted for mitophagy following massive fission and dysfunction. The recent study by Twig et al. substantiates indeed the requirement for a previous cycle of fusion for a mitochondrion to be targeted to autophagy (Twig et al., 2008). By combining our results with the ones of Twig et al., it is possible to at least partially explain why the fragmented, dysfunctional mitochondria bearing mtDNA mutations are not completely eliminated by autophagy (Duvezin-Caubet et al., 2006b).

In conclusion, the relationship between mitochondrial shape and mitophagy seems more complex than a straightforward equation fragmentation-autophagy. Future investigation is needed to address and identify the signals that emanate from the dysfunctional mitochondria and to verify whether induction of autophagy directly cross-talks with the machinery controlling mitochondrial morphology in a regulated manner.

## Acknowledgements

We thank C. Frezza of our laboratory for the artwork. L.C.G. is the recipient of a "Bolsa de Doutoramento" of the "Fundação para a Ciência e Tecnologia", Portugal. L.S. is a Senior Telethon Scientist of the Dulbecco-Telethon Institute and an EMBO Young Investigator. This research was supported by Telethon Italy, Compagnia di San Paolo Italy, United Mitochondrial Disease Fund USA, MDA USA, AIRC Italy, Fondazione Mariani ONLUS-Italy.

# Chapter 4

## *During autophagy mitochondria elongate, are spared from degradation and sustain cell viability.*

Lígia C. Gomes, Giulietta Di Benedetto and Luca Scorrano

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All the work presented in this chapter was performed by Lígia C. Gomes, except for Fig. 5d (Giulietta Di Benedetto)

## Abstract

A plethora of cellular processes, including apoptosis, depend on regulated changes in mitochondrial shape and ultrastructure. Scarce is our understanding of the role of mitochondria and of their morphology during autophagy, a bulk degradation and recycling process of eukaryotic cells' constituents. Here we show that mitochondrial morphology determines the cellular response to macroautophagy. When autophagy is triggered, mitochondria elongate in vitro and in vivo. Upon starvation cellular cAMP levels increase and protein kinase A (PKA) becomes activated. PKA in turn phosphorylates the pro-fission dynamin related protein 1 (DRP1) that is therefore retained in the cytoplasm, leading to unopposed mitochondrial fusion. Elongated mitochondria are spared from autophagic degradation, possess more cristae, increase dimerization and activity of ATP synthase, and maintain ATP production. When elongation is genetically or pharmacologically blocked, mitochondria conversely consume ATP, precipitating starvation-induced death. Thus, regulated changes in mitochondrial morphology determine the fate of the cell during autophagy.

## Introduction

Mitochondria are crucial organelles for energy production, regulation of cell signaling and amplification of apoptosis. This functional versatility is matched by their morphological and structural variety. During cell life, the mitochondrial network is continuously shaped by fission and fusion events (Bereiter-Hahn and Voth, 1994). The dynamin-related GTPases optic atrophy 1 (OPA1) of the inner mitochondrial membrane (Cipolat et al., 2004b), and mitofusins (MFN) 1 and 2 of the outer membrane (Santel and Fuller, 2001), regulate mitochondrial fusion in mammalian cells. Mitochondrial fission is controlled by the cytosolic dynamin related protein DRP1 (Smirnova et al., 2001). Translocation of DRP1 to mitochondria is an essential step in the fragmentation of the organelle and depends on dephosphorylation of the Ser637 residue, by calcineurin (Cereghetti et al., 2008). Conversely, phosphorylation of Ser637 by PKA promotes mitochondrial elongation (Cribbs and Strack, 2007; Chang and Blackstone, 2007). Once on mitochondria, DRP1 can be stabilized by SUMOylation (Harder et al., 2004) mediated by resident SUMO ligases such as MAPL (Braschi et al., 2009), elucidating how dynamic regulation of fission adapts mitochondrial morphology to the changing cellular needs.

The availability of genetic tools allowed to explore the role of mitochondrial morphology in complex cellular processes. For example, remodelling of mitochondrial cristae (Scorrano et al., 2002) and fragmentation of the organellar network (Martinou et al., 1999; Frank et al., 2001) participate in the progression of apoptosis. More recently, $Ca^{2+}$ signalling (Szabadkai et al., 2004), formation of dendritic spines (Li et al., 2004), migration of lymphocytes (Campello et al., 2006), cell cycle (Mitra et al., 2009), and even lifespan in lower eukaryotes (Scheckhuber et al., 2007) have been found to depend on regulated changes in mitochondrial morphology. Finally, mitophagy, a particular form of selective autophagy of mitochondria, requires fragmentation of the mitochondrial network to segregate the dysfunctional units to be removed (Twig et al., 2008; Gomes and Scorrano, 2008).

Autophagy is a self-degradation process induced for example when nutrients are limited (Klionsky and Emr, 2000). During autophagy, pre-autophagosomal structures engulf components of the cytosol, including entire organelles, giving rise to autophagosomes that ultimately fuse with lysosomes, where breakdown of cellular components takes place (Cecconi and Levine, 2008). For many years, autophagy has been regarded as an unselective process, but it is now clear that under certain conditions specific cargoes are selectively targeted to autophagy, including aggregated proteins (Ravikumar et al., 2002), invading bacteria (Zheng et al., 2009), as well as superfluous or damaged organelles like peroxisomes (Tuttle et al., 1993), endoplasmic reticulum (Bernales et al., 2006) and mitochondria (Elmore et al., 2001). Not only organelles can be selectively eliminated by autophagy, but they also participate in the different steps of macroautophagy, ranging from

the formation of the autophagosomal membrane (Tooze and Yoshimori, 2010), to the amplification of the process, in which mitochondria-derived reactive oxygen species seem to play a role (Scherz-Shouval et al., 2007). Mitochondria might also serve as docking sites for the formation of the autophagosomes (Hailey et al., 2010) in a process that depends on the tethering to the endoplasmic reticulum (de Brito and Scorrano, 2008b). However, many questions on the morphology as well as on the functional role of mitochondria during autophagy remain open. Do they fragment? Are they randomly targeted to autophagosomes? Is the progression or the final outcome of autophagy influenced by changes in their morphology? Here we show that mitochondria unexpectedly elongate during macroautophagy. Mitochondrial elongation is triggered by the PKA-mediated inhibition of the pro-fission protein DRP1 and is required to sustain cellular ATP levels and viability. Our results indicate that mitochondrial shape determines the cellular fate during macroautophagy.

## Material and Methods

### Molecular Biology

pEYFP-Mito (mtYFP), mito-dsRED (mtRFP), *Drp1-YFP*, *Drp1$^{S637A}$-YFP* were described (Cereghetti et al., 2008). mt-PAGFP (Karbowski and Youle, 2003) was from M. Karbowski (University of Maryland, Baltimore, MD, USA). EPACI-camps (Nikolaev et al., 2004) was from M. Lohse (University of Würzburg, Germany). Mt-luciferase (Jouaville et al., 1999) was from R. Rizzuto (University of Padova, Italy).

Two siRNA against the following target sequences from mouse *mTOR* were synthesized: 5'-GCGGAUGGCUCCUGACUAU-3' and 5'-CCAAGGUGCUACAGUACUA-3'. A siRNA against the following target sequence from human *mTOR* was synthesized: 5'-UAACAGGUUCGAGAUAAG-3'. The scrambled control was used at the same final concentration (Dharmacon). The siRNA against human *Opa1* targeted the 5'-GGACCUUAGUGAAUAUAAA-3' sequence (Ambion).

### Cell culture

SV40-transformed wt, *Mfn1$^{-/-}$*, *Mfn2$^{-/-}$* and *DMF$^{-/-}$* MEFs were from D. Chan and cultured as described (de Brito and Scorrano, 2008b). SV40-transformed *Opa1$^{-/-}$* and wt MEFs were a gift from C. Alexander and cultured as described (Song et al., 2007). SV40-transformed wt and *Drp1$^{-/-}$* MEFs were from K. Mihara and cultured as previously described (Ishihara et al., 2009). wt and *Bax$^{-/-}$Bak$^{-/-}$* MEFs were cultured as described (Scorrano et al., 2003). Transfection of MEFs with DNA was performed using Transfectin (Biorad), with siRNA using Oligofectamine (Invitrogen) according to manufacturer's instructions. When indicated, cells were transfected with siRNA 24 hrs after seeding on glass coverslips and with mtYFP 48hrs after plating. HeLa, HepG2 and C2C12 cells were kind gifts from C. Montecucco, A. Alberti and M. Sandri (University of Padova, Italy) and were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine, non-essential amino acids (0.89 g/l L-alanine, 1.32 g/l L-asparagine, 1.33 g/l L-aspartic acid, 1.47 g/l L-glutamic acid, 0.75 g/l glycine, 1.15 g/l L-proline, 1.05 g/l L-serine, Invitrogen), 75 U/ml penicillin, 50 µg/ml streptomycin (Invitrogen) at 37°C in a 5% $CO_2$ atmosphere. Transfection with siRNAs and DNA was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Primary hepatocytes were isolated as described (Danial et al., 2003) and cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 1nM insulin (Sigma), 2 mM L-glutamine, non-essential amino acids (Invitrogen), , 75 U/ml penicillin, 50 µg/ml streptomycin (Invitrogen) and Fungizone (Invitrogen) at 37°C in a 5% $CO_2$ atmosphere.

To trigger starvation cells were washed four times and then incubated in Hanks balanced salt solution (HBSS) supplemented with 10 mM Hepes pH 7.4, at 37°C for the indicated time.

**Fasting**

Animal studies were performed in compliance to local animal welfare regulations. CD1 mice were fasted for 12 hrs with free access to water.

**Imaging**

For confocal imaging of live cells, $1.8x10^5$ cells seeded onto 24-mm round glass coverslips transfected and treated as indicated were placed on the stage of a Nikon Eclipse TE300 inverted microscope equipped with a PerkinElmer Ultraview LCI confocal system, a piezoelectric z-axis motorized stage (Pifoc, Physik Instrumente, Germany), and a Orca ER 12-bit CCD camera (Hamamatsu Photonics, Japan). Cells expressing mtYFP or mtRFP were excited using the 488 nm or the 543 nm line of the HeNe laser (PerkinElmer) using a 60x 1.4 NA Plan Apo objective (Nikon).

For quantification of mitochondrial fusion rate, $2x10^5$ cells seeded onto 24-mm round glass coverslips were co-transfected with mtRFP and mt-pAGFP. After 24 hrs, cells were treated as indicated and placed on the stage of a laser scanning microscope (TCS SP5, Leica). Using the LasAF software (Leica), regions of interest (ROI) to be photoactivated were manually defined. To activate the pAGFP fluorescence, 1 z-plane was activated using 100% of the power of the 413 nm laser line with a 63X, 1.4NA objective. Frames were then acquired each min using the 488 nm and the 563 laser lines for 30 min. Standard deviation of the green fluorescence in the whole-cell was measured and normalized for the intensity of the mtRFP fluorescence using the Multi Measure plug-in of ImageJ (NIH, Bethesda).

For FRET imaging, $2x10^5$ MEFs seeded onto 24-mm round glass coverslips were transfected with EPACI-camps and after 24 hrs placed on a thermostated chamber at 37°C and maintained in complete medium on the stage of an Olympus inverted microscope equipped with a CellR imaging system and a beam-splitter optical device (Multispec Microimager; Optical Insights). Sequential images of the 545 nm fluorescence emission upon excitation at 430 and 480 nm were acquired every 1 s with a 40x, 1.4 NA objective (Olympus) using the CellR software and then processed using the multi measure plug-in of Image J (National Institutes of Health, Bethesda) following background subtraction and expressed as FRET 430/480 ratio.

Real-time imaging of mitochondrial membrane potential was performed on $1x10^5$ MEFs seeded onto 24-mm round glass coverslips and loaded with TMRM, using the

Olympus CellR imaging system. Imaging and analysis of TMRM fluorescence over mitochondrial regions of interest was performed as described (Scorrano et al., 2003).

**Immunofluorescence**

Primary hepatocytes were seeded onto 13-mm round glass coverslips coated with laminin. After 48 hrs cells were treated as indicated and fixed for 30 min at room temperature with 3.7% (w/V) formaldehyde, permeabilized for 20 min with ice-cold Nonidet P40 (GIBCO) and incubated with a rabbit anti-TOM20 (1:200, Santa Cruz Biotechnology). Staining was revealed with a goat anti-rabbit IgG conjugated to fluorescein-isothiocyanate (FITC) using Nikon Eclipse TE300 inverted confocal microscope and a 60x, 1.4 NA Plan Apo objective (Nikon). Stacks of 50 images separated by 0.2 µm along the z-axis were acquired. 3D reconstruction and volume rendering of the stacks were performed with the appropriate plug-in of ImageJ (National Institutes of Health, Bethesda).

**Electron microscopy**

MEFs of the indicated genotypes and treated as indicated were fixed with 1,25% (V/V) glutaraldehyde in 0.1 M Na-cacodylate pH 7.4 for 1 hr at room temperature. Muscle and liver specimens from mice treated as indicated were fixed in 2% formaldehyde, 2,5% (vol/vol) glutaraldehyde in 0.1 M Na-cacodylate pH 7.4 for 2 hr at room temperature and then overnight at 4°C. Electron microscopy was performed as described (Scorrano et al., 2002).

**Morphometric and colocalization Analysis**

Morphometric analysis of mitochondrial shape was performed as described[2]. For morphometric analysis of cristae biogenesis, mitochondria were randomly selected from coded samples and the number of cristae was counted and normalized for the surface of the organelle calculated by fitting a region of interest on the selected organelle using the Multi Measure plug-in of Image J (National Institutes of Health, Bethesda). Colocalization between autophagosomes and mitochondria was quantified using Manders' coefficient (de Brito and Scorrano, 2008b).

**Isolation of mitochondria**

Cells plated in 500 $cm^2$ plates were treated after 48 hrs as indicated and mitochondria were isolated as described (Frezza et al., 2007). Protein concentration was determined by Bradford.

**Cell lysis**

Cells ($10^6$) were harvested and disrupted in Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4) in the presence of complete protease-inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail 1 (Sigma). Protein concentration was determined by BCA assay (Pierce, Rockford, IL).

**Immunoprecipitation**

For immunoprecipitation, 150 µg of total cellular extract was dissolved in Triton X-100 lysis buffer and pre-cleared by centrifugation at 14000g. Fifty µl of dynabeads (Invitrogen) conjugated with protein-G were incubated with anti-DRP1 (1:50, BD Transduction) for 2 hrs at room temperature. Following 3 washes, cleared extracts were added and incubated o/n at 4°C. Beads were washed 3 times and boiled in NuPage loading buffer (Invitrogen).

**Immunoblotting**

The indicated amounts of proteins were separated by 4-12% Bis-Tris, 12% Bis-Tris or 7% Tris-acetate gels (NuPAGE, Invitrogen) and transferred onto polyvinylidene difluoride (PVDF, BioRad) membranes. The following antibodies were used: α-OPA1 (1:1000, BD Transduction), α-MFN1 (1:1000, Abcam), α-MFN2 (1:1000, Abcam), α-DRP1 (1:1000, BD Transduction), α-Fis1 (1:1000, Alexis), α-actin (1:30000, Chemicon), α-TOM20 (1:5000, Santa Cruz Biotechnology), α-phospho-Ser637-DRP1[5] (1:1000), α-phospho-CREB-Ser133 (1:1000, Cell Signalling), α-CREB (1:1000, Cell Signalling), α-β-tubulin (1:200, Santa Cruz Biotechnology), α-cyclophilin-D, α-MnSOD (1:5000, Stressgen), α-complex-II, subunit 70kDa (1:1000, MitoSciences), α-PMP70 (1:1000, Sigma), α-ATPase, α subunit (1:1000, MitoSciences), α-mTOR (1:1000, Cell Signalling), α-LC3 (1:200, Anaspec), α-p62 (1:5000, Progen).

**Blue Native-PAGE**

Cells (500 µg) were resuspended in 50 µL of native loading buffer (Invitrogen) containing 4% digitonin (Sigma) and protease-inhibitor cocktail (Sigma) After 30 min at 4°C the lysate was spun at 22000*g* for 30 min at 4°C. Four µL of native additive G250 5% (Invitrogen) was added to the supernatant and 30 µg of protein were loaded onto a 3-12% native gel (Invitrogen). Transfer of native gels was performed as described above.

**In-gel ATPase activity assay**

ATPase activity was measured according to (Alirol et al., 2006) and ATPase activity was followed for up to 4hrs at room temperature. Gels were washed in water to stop the reaction.

**ATP measurement**

Total ATP levels were measured by chemiluminescence using the ATP detection assay system ATPlite (PerkinElmer), according to manufacturer's protocol. ATP levels were normalized by total protein concentration.

To measure mitochondrial ATP levels during starvation, cells grown on 13 mm round glass coverslips at 50% confluence were transfected with mitochondrially targeted luciferase and after 24 hours luminescence was measured as described[35]. Basal luminescence was recorded in the absence of luciferin and then cells were perfused with 20 µM luciferin to record mitochondrial luminescence, which was then calibrated to the maximal luminescence emitted by the mt-luciferase by perfusing each sample with 10mM ATP in the presence of 100 µM digitonin.

**Flow cytometry**

For analysis of cell death, $4x10^4$ MEFs of the indicated genotype grown in 12-well plates were treated as indicated 24 hrs after seeding. When indicated, cells were harvested and stained with propidium iodide (PI) and annexin-V-FLUOS (BenderMedSystems) according to the manufacturer's protocol. Cell viability was measured by flow cytometry (FACSCalibur, BD Biosciences) as the percentage of annexin-V and PI negative events.

For evaluation of membrane potential, $4x10^4$ MEFs of the indicated genotype were grown in 12-well plates and indicated treatments (starvation) were started 36 hrs after seeding. At the indicated times, cells were harvested, washed with PBS, ressuspended in HBSS supplemented with 10 mM HEPES pH 7.4 and loaded with 20 nM TMRM (Molecular Probes) in the presence of 2 mg/ml cyclosporine H, a P-glycoprotein inhibitor for 30 min at 37°C. Cells were then analyzed by flow cytometry (FACSCalibur, BD Biosciences).

## Results

### Mitochondria elongate upon induction of autophagy

We assessed whether mitochondrial morphology is modified during autophagy. Confocal microscopy of wild type (wt) mouse embryonic fibroblasts (MEFs) expressing a mitochondrially targeted yellow fluorescent protein (mtYFP) showed that induction of autophagy by starvation led to an early elongation of mitochondria resulting in a network of highly interconnected organelles (Fig. 1a,b). Elongation was observed as soon as 1 hr after nutrient deprivation, was maintained for up to 48 hrs (not shown) and occurred in all the cell lines tested (mouse C2C12 myoblasts, human HeLa epithelial and HepG2 hepatocarcinoma cells) as well as in primary mouse hepatocytes (Fig. S1a). Inactivation of the mTOR metabolic sensor is another classical stimulus of autophagy. Efficient short RNA interference mediated knock down of mTOR also triggered mitochondrial elongation (Fig. 1c,d). The dilution rate of a mitochondrially targeted photoactivable green fluorescent protein (mt-PAGFP) is proportional to productive fusion events (Karbowski et al., 2004a). Dilution rate increased upon starvation in wt as well as *Mfn2*$^{-/-}$ cells (Fig. 1e-g and Movie 1). Accordingly, induction of autophagy led to mitochondrial elongation in *Mfn2*$^{-/-}$, but not in *Opa1*$^{-/-}$ (Song et al., 2007) and *Mfn1*$^{-/-}$*Mfn2*$^{-/-}$ (*DMF*$^{-/-}$) (Chen et al., 2005) MEFs, which lack the core components of the mitochondrial fusion machinery; and mitochondria remained elongated in *Drp1*$^{-/-}$ cells where fission is genetically impaired (Ishihara et al., 2009) (Fig.1a,b). BAX and BAK have been described to be necessary for fusion of mitochondria (Karbowski et al., 2006) and in their absence autophagy is enhanced as a default death mechanism (Shimizu et al., 2004). In response to starvation, the *Bax*$^{-/-}$*Bak*$^{-/-}$ punctiform mitochondria also elongated (Fig. S1c,d), indicating that BAX and BAK are not essential for elongation during induction of autophagy. In all the MEFs tested, autophagic flux measured in the presence of bafilomycin A1 was comparable, as judged by processing of LC3-I to LC3-II and by degradation of p62 (Fig. S2). In addition, mitochondrial elongation during starvation was still observed when proximal autophagic signalling was blocked by wortmannin (Fig. S3) and in cells lacking the key component of the autophagic machinery ATG5 (not shown). When autophagy was induced *in vivo* by fasting mice for 12 hrs, electron microscopy (EM) of muscle and liver revealed similar changes in mitochondrial morphology. Longitudinal sections of *tibialis anterior* from fasted mice showed that intermyofibrillar mitochondria were retrieved as one elongated organelle, no longer surrounded by glycogen granules; also in liver, perinuclear mitochondria were clearly elongated in fasted mice (Fig. 1h). In sum, mitochondrial elongation requires the core mitochondrial fusion machinery and is not dependent on autophagosome formation, which is conversely independent from mitochondrial elongation.

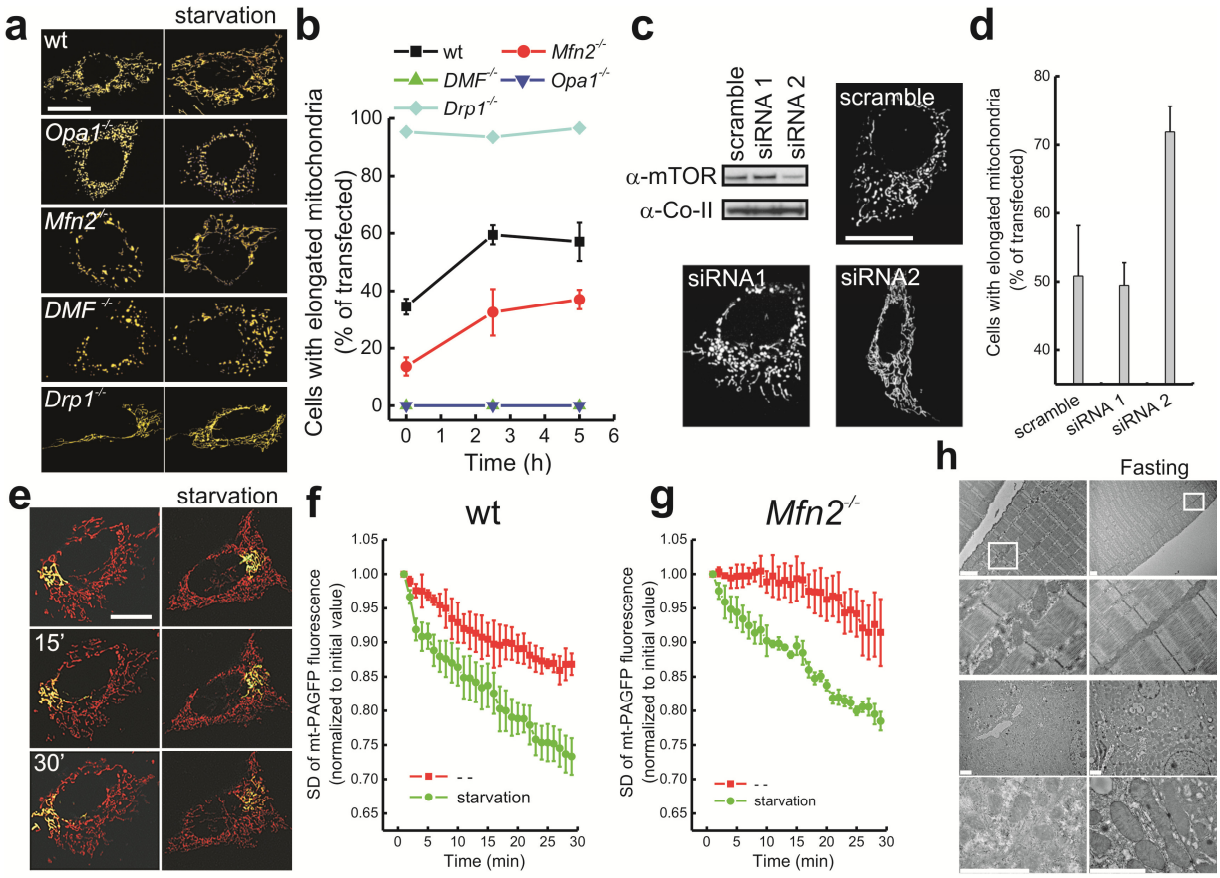


**Figure 1. Mitochondrial elongation in response to autophagy. (a)** Representative confocal images of mitochondrial morphology in MEFs of the indicated genotype 24 hrs following transfection with mtYFP. Where indicated, cells were starved for 2.5 hrs. Bar, 20 µm **(b)** Morphometric analysis of mitochondrial shape. Experiments were carried exactly as in (a). Data represent mean ± SEM of 3 independent experiments (n=100 cells per condition in each experiment). **(c)** Forty-eight hrs after transfection with the indicated siRNA MEFs were lysed and 25 µg of proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Representative images show mitochondrial morphology of MEFs transfected with the indicated siRNA and after 24 hrs with mtYFP. After further 24 hrs confocal images were acquired. Bar, 20 µm. **(d)** Morphometric analysis of mitochondrial shape. Experiments were carried exactly as in (c). Data represent mean ± SEM of 5 independent experiments (n=100 cells per condition in each experiment). **(e)** Representative images of mitochondrial fusion. MEFs were co-transfected with mt-PAGFP and mtRFP and after 24 hrs, mt-PAGFP was photoactivated in a region of interest (box) and cells were imaged by real time confocal microscopy. Where indicated, MEFs were starved for 2.5 hrs. Bar, 20 µm. See also Supplementary Movies 1-2. **(f-g)** Quantification of mitochondrial fusion in MEFs of the indicated genotype. Experiments were carried exactly as in (e). Data represent mean ± SEM of 4 independent experiments. **(h)** Representative electron micrographs of muscle (longitudinal sections) and liver from CD1 mice. Where indicated, mice were fasted for 12 hrs. Magnifications of boxed regions are presented below. Bar, 2 µm.

## Mitochondrial elongation during autophagy depends on DRP1 phosphorylation by PKA

Mitochondrial hyperfusion can be triggered by a panoply of stress stimuli and depends on changes in OPA1 forms (Tondera et al., 2009). In response to starvation, however, levels of total as well as of individual forms of OPA1 were stable, like those of MFN1, MFN2, and FIS1. The total amount of the fission protein DRP1 was slightly increased (Fig. 2a-d), but less DRP1 was associated with mitochondria in the course of starvation (Fig. 1e). This result suggests that during starvation mitochondrial fusion is left unopposed. The PKA/calcineurin couple regulates phosphorylation of the Ser637 residue of DRP1, its translocation to mitochondria and therefore the intensity of mitochondrial fission (Cereghetti et al., 2008). Notably, in the course of starvation phosphorylation of Ser637 of DRP1 was increased (Fig. 2f,g). Similarly, silencing of mTOR or its pharmacologic inhibition resulted in increased Ser637 phosphorylation (Fig. 2h,i). This phosphorylation could result from PKA activation or calcineurin inhibition: we therefore examined levels of the proximal PKA activator cAMP in response to starvation using a genetically encoded EPAC-based FRET probe for cAMP. Real time imaging reported a robust increase in cAMP levels in MEFs when switched from a nutrient-rich to the “starvation” medium (Fig. 3a,b). This increase resulted in the downstream activation of PKA, as measured by the phosphorylation of the PKA targets CREB and ATF1, sensitive to H89, a pharmacological inhibitor of PKA (Fig. 3c). Activation of PKA was detected in all the other cell lines (mouse C2C12, human HeLa and HepG2), where starvation induced mitochondrial elongation (Fig. S1b).

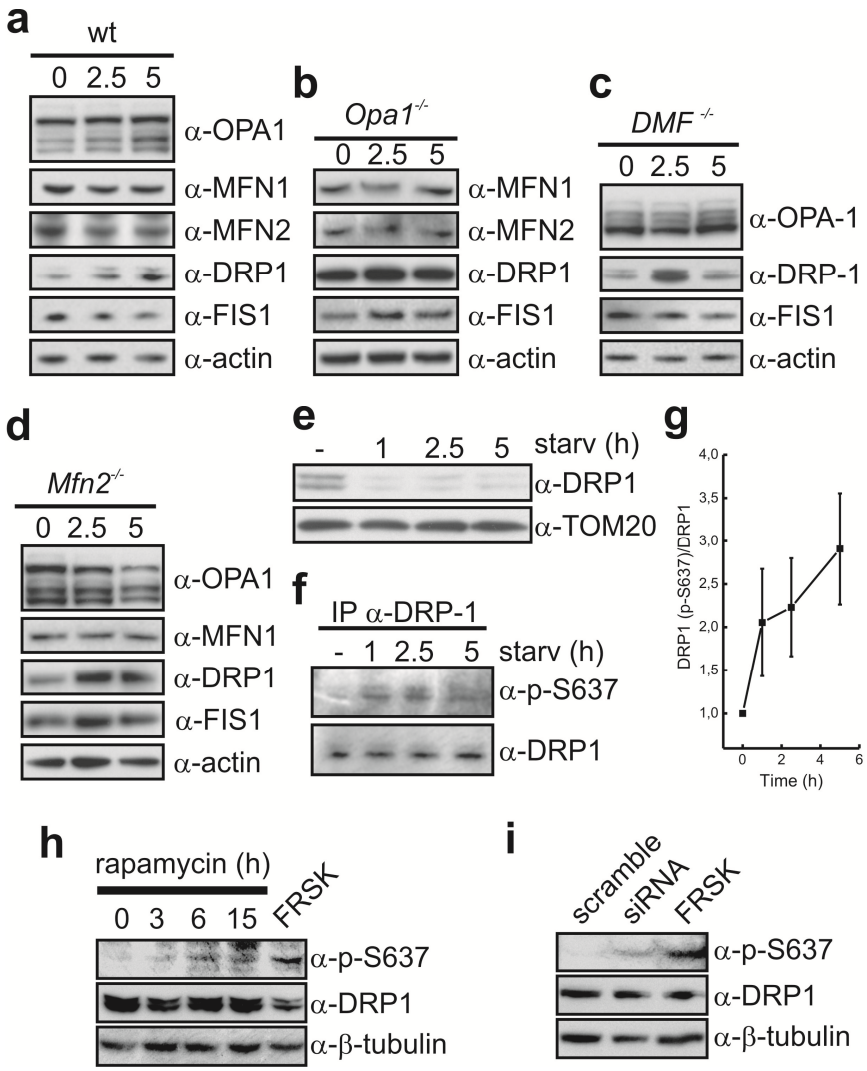


**Figure 2 – Increased phosphorylation of Ser637 of DRP1 during autophagy. (a-d)** Levels of mitochondria-shaping proteins during starvation. Twenty µg of proteins from MEFs of the indicated genotype were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Where indicated, cells were starved for the indicated times. **(e)** Association of DRP1 with mitochondria upon starvation. Mitochondria were isolated from MEFs starved for the indicated times and 25 µg of proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies. **(f)** Levels of Ser-637 phosphorylation of DRP1 during starvation. Equal amounts of cell lysates from wt MEFs starved for the indicated times were immunoprecipitated with the indicated antibody and the immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies. **(g)** Quantitative analysis of Ser-637 phosphorylation of DRP1 during starvation. Experiments were as in (F). Data are normalized to total levels of DRP1 and represent the mean ±SEM of 3 independent experiments. **(h)** MEFs were treated for the indicated times with 100 nM rapamycin or with 25 µM forskolin for 0.5 hrs, lysed and equal amounts (50 µg) of proteins were separated by SDS-PAGE and immunoblotted using the indicated antibodies. **(i)** HeLa cells were transfected for 2 days with the indicated siRNA or treated with 25 µM forskolin for 0.5 hrs and lysed. Equal amounts (50 µg) of proteins were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Uncropped images of all blots in this figure are shown in Supplementary Information, Fig. S8.

PKA activation was comparable in cells where mitochondria did (wt and *Mfn2*$^{-/-}$) and did not (*Opa1*$^{-/-}$ and *DMF*$^{-/-}$) elongate in response to starvation (Fig. 3c), indicating that activation does not depend on the mitochondrial morphology. The PKA inhibitor H89 blocked mitochondrial elongation during starvation (Fig. 3d,e), suggesting that PKA activation is conversely an essential step for mitochondrial elongation. Consistently, an increase in cAMP levels obtained pharmacologically led to mitochondrial elongation (Fig. S4). To test if the activation of PKA impinged on Drp1, we turned to a genetic approach. In MEFs expressing a Drp1-YFP chimera mutated in the PKA phosphorylation site (DRP1$^{S637A}$-YFP) (Cereghetti et al., 2008), mitochondria were unable to elongate during starvation, and inhibition of PKA had no effect on morphology (Fig. 3f,g). Finally, we reconstituted *Drp1*$^{-/-}$ MEFs with levels of DRP1-YFP or DRP1$^{S637A}$-YFP comparable to those of endogenous DRP1. In the reconstituted cells mitochondria reverted to short rods and starvation could induce H89-sensitive elongation only when MEFs were complemented with Drp1-YFP but not with DRP1$^{S637A}$-YFP (Fig. 3h,i). Thus, during starvation the activation of PKA impinges on DRP1 to trigger mitochondrial elongation.

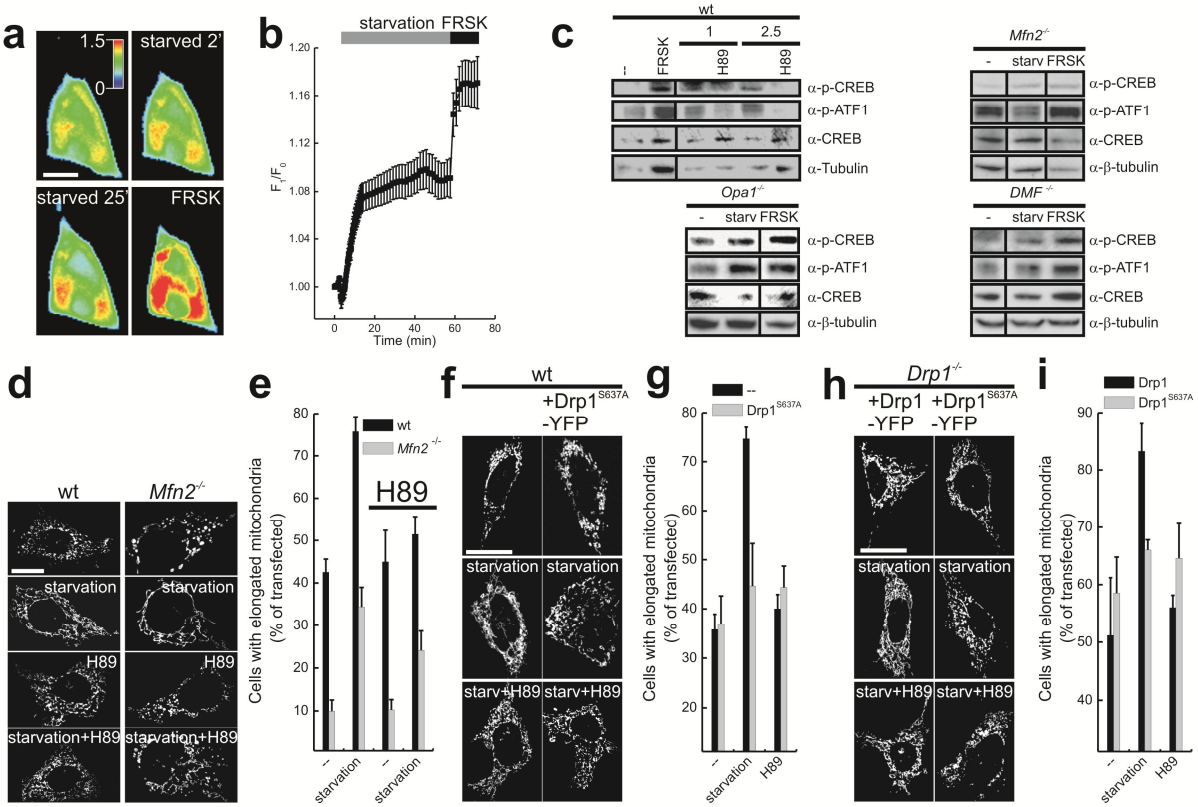


**Figure 3 – Mitochondrial elongation during starvation is mediated by the cAMP-PKA axis. (a)** Pseudocolor-coded images of EpacI-camps FRET from real time imaging of wt MEFs transfected with EpacI-camps. Where indicated, cells where perfused with the starvation solution for the indicated times or with 25 µM forskolin. Bar, 20 µm. See also Supplementary Movie 3. **(b)** Quantitative analysis of CFP/YFP FRET ratio. Experiments were as in

(a). Where indicated, cells were perfused with the starvation solution or with 25 µM forskolin. Data represent mean ± SEM of 13 independent experiments. **(c)** Fifty µg of lysates of MEFs of the indicated genotypes were analyzed by SDS-PAGE/ immunoblotting using the indicated antibodies. Where indicated, MEFs were starved, or treated with 25 µM forskolin (FRSK). Where indicated, 20 µM H89 was added during starvation. Uncropped images of all blots in this figure are shown in Supplementary Information, Fig. S8. **(d)** Representative images of the effect of H89 on mitochondrial morphology upon starvation. wt and *Mfn2*$^{-/-}$ MEFs were transfected with mtYFP, and after 24 hrs confocal images were acquired. Where indicated, cells were starved for 2.5 hrs and 20 µM H89 was added. Bar, 20 µm. **(e)** Morphometric analysis. Experiments were carried exactly as in (D). Data represent mean ± SEM of 5 independent experiments (n=100 cells per condition). **(f)** Starvation-induced mitochondrial elongation depends on Ser 637 of DRP1. Representative confocal images of mitochondrial morphology of wt MEFs co-transfected with mtRFP and the indicated plasmids. Twenty-four hrs after transfection, where indicated cells were starved for 2.5 hrs and imaged. Where indicated, 20 µM H89 was present during starvation. Bar, 20 µm. **(g)** Morphometric analysis of mitochondrial shape. Experiments were as in (f). Data represent mean ± SEM of 5 independent experiments (n= 50 cells per condition). **(h)** Representative confocal images of mitochondrial morphology of *Drp1*$^{-/-}$ MEFs co-transfected with mtRFP and the indicated plasmids. Twenty-four hrs after transfection, where indicated cells were starved for 2.5 hrs and imaged. Where indicated, 20 µM H89 was present during starvation. Bar, 20 µm. **(i)** Morphometric analysis of mitochondrial shape. Experiments were as in (h). Data represent mean ± SEM of 5 independent experiments (n= 50 cells per condition).

## Elongated mitochondria are spared from autophagic degradation and maintain ATP levels during starvation

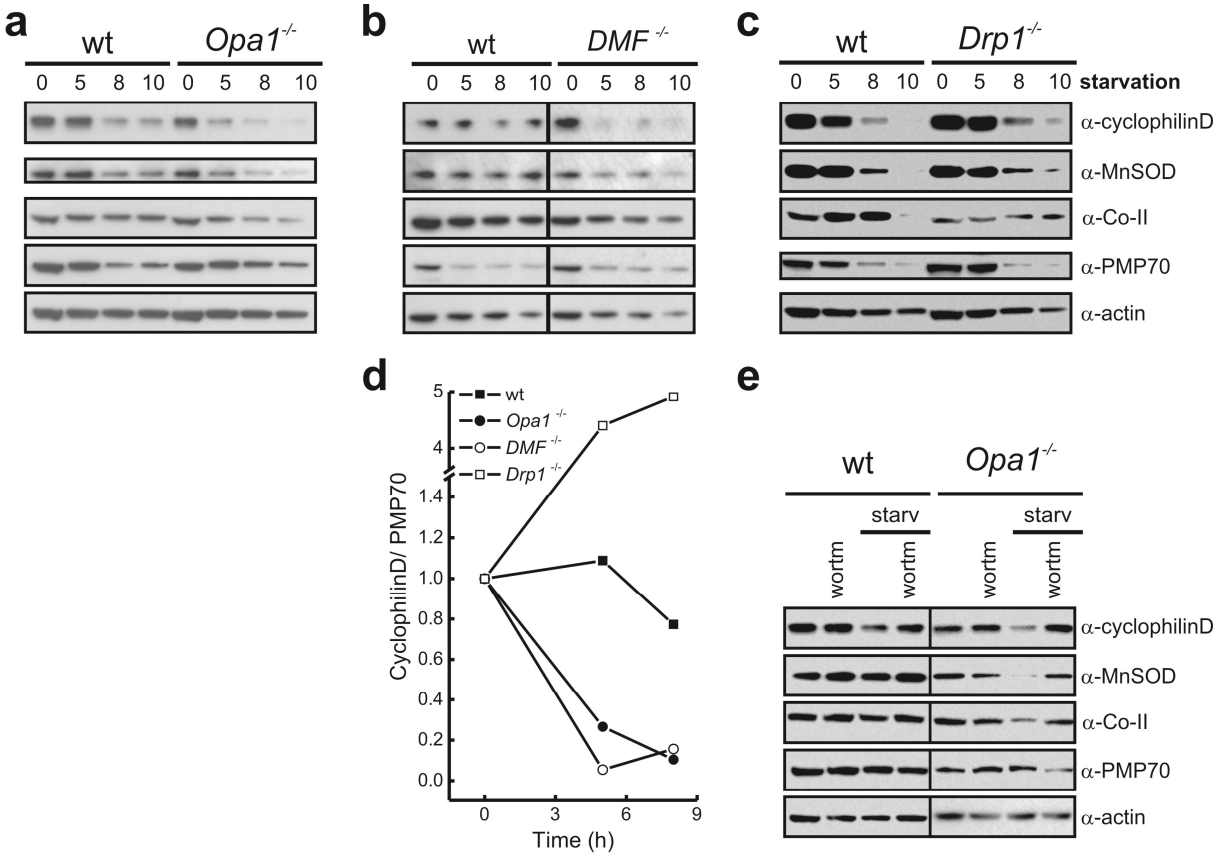

**Figure 4. Elongated mitochondria are spared from degradation during starvation (a-c)** MEFs of the indicated genotype were treated as indicated, counted and $2.7x10^5$ cells were lysed. Lysates were separated by SDS-PAGE and immunoblotted using the indicated antibodies. **(d)** Ratio between the densitometric levels of cyclophilin D and those of PMP70 in MEFs of the indicated genotype. One representative experiment of 5 independent repetitions carried as in (a-c) is shown. **(e)** MEFs of the indicated genotype starved for 5h were treated where indicated with 0.5 μM wortmannin (wortm). Lysates from $2.7x10^5$ cells were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Uncropped images of all blots in this figure are shown in Supplementary Information, Fig. S8.

Fragmentation of dysfunctional mitochondria precedes mitophagy, suggesting a role for mitochondrial elongation during autophagy in maintenance of the mitochondrial mass. Following starvation, mitochondrial proteins (cyclophilin D, MnSOD and a subunit of complex II) were lost more rapidly in *Opa1*$^{-/-}$ (Fig. 4a) and *DMF*$^{-/-}$ (Fig. 4b) cells that did not elongate mitochondria, while were retained in *Drp1*$^{-/-}$ cells (Fig. 4c,d) . These quantitative immunoblotting data were confirmed by a confocal analysis of colocalization (de Brito and Scorrano, 2008b) of mitochondria with YFP-LC3 labelled autophagosomes (Fig. S5). No differences were observed in the rate of degradation of proteins from other sub-cellular compartments (the peroxisomal marker PMP70 and the cytosolic protein actin). Furthermore, wortmannin that blocks autophagosome formation prevented mitochondrial elimination, supporting that mitochondria were degraded by autophagy (Fig. 4e). Increased degradation could be supported by dysfunction of *Opa1*$^{-/-}$ mitochondria during starvation. While during starvation the accumulation of the potentiometric fluorescent dye tetramethyl rhodamine methylester resulted higher in *Opa1*$^{-/-}$ cells (Fig. S6a), an assay for latent mitochondrial dysfunction revealed that mitochondria of starved *Opa1*$^{-/-}$ MEFs maintain their membrane potential by using the reversal of the ATPase[21] (Fig. 5a,b). Accordingly, total cellular ATP levels decreased in *Opa1*$^{-/-}$ and *DMF*$^{-/-}$ cells during starvation (Fig. 5c). Reduced mitochondrial ATP production contributed to these decreased total cellular ATP levels, while wild-type organelles where elongation occurs were able to sustain ATP output, as indicated by monitoring of mitochondrial ATP levels in situ using a genetically encoded luciferase probe targeted to the matrix of the organelle (Jouaville et al., 1999) (Fig. 5d). The free energy of the electrochemical potential is used by the mitochondrial ATPase to synthesize ATP (Rich, 2003). Although isolated ATPase is fully active as a monomer, the enzyme is ubiquitously found in more efficient dimeric and oligomeric forms (Strauss et al., 2008). Blue-native gel electrophoresis performed on whole cells allowed to inspect the ATP synthase organization and activity during starvation without isolating mitochondria (and therefore disrupting their morphology). The total levels of ATP synthase increased upon starvation as indicated by specific immunoblotting (Fig. S6b). In wt but not *Opa1*$^{-/-}$ or *DMF*$^{-/-}$ MEFs the ratio between the dimeric and the monomeric form of the ATPase was higher already in non

starved cells and increased during starvation, as judged by a specific in gel activity assay for ATPase activity (Fig. 6).

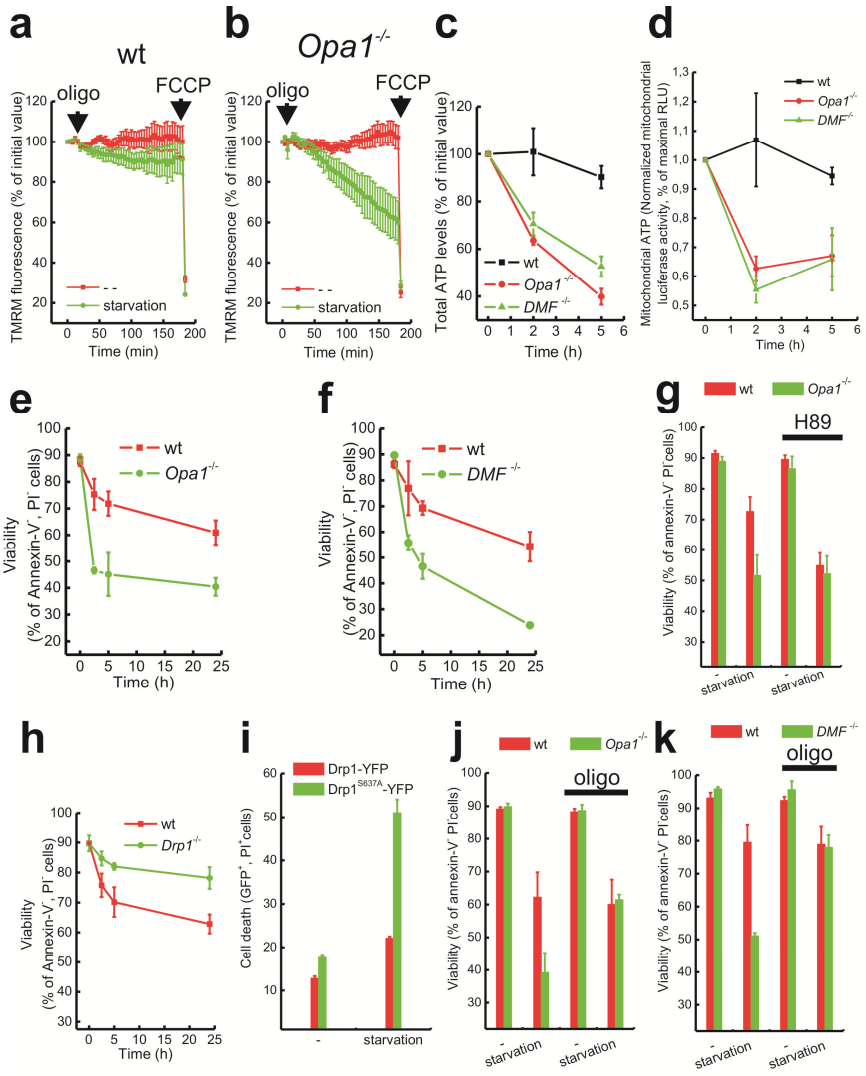


**Figure 5 – Mitochondrial elongation sustains cellular ATP production and viability during autophagy. (a,b)** Quantitative analysis of TMRM fluorescence changes over mitochondrial regions in MEFs of the indicated genotype. Where indicated, cells were starved for 5 hrs prior to TMRM loading. Where indicated (arrows), 2.5 µg/ml oligomycin and 2 µM FCCP were added. Data represent mean ± SEM of 7 independent experiments. **(c)** Total cellular ATP levels were measured in cells of the indicated genotype starved for the indicated times. Data represent mean ± SEM of 5 independent experiments. **(d)** Mitochondrial ATP measured in situ by mitochondrially targeted luciferase in cells of the indicated genotype starved for the indicated times. Data represent mean ± SEM of 5 independent experiments and are normalized to the initial value. **(e,f)** Cells of the indicated genotype were starved for the indicated times and viability was determined by flow cytometry. Data represent mean ± SEM of 5 independent experiments. **(g)** MEFs of the indicated genotype were starved for 2.5 hrs. Where indicated, cells were treated with 20 µM H89. Viability was determined by flow cytometry. Data represent mean ± SEM of 5 independent experiments. **(h)** Cells of the indicated genotype were starved for the indicated times and viability was determined by flow cytometry. Data represent mean ± SEM of 5 independent experiments. **(i)** *Drp1*$^{-/-}$ MEFs

were transfected with the indicated plasmids and after 24 hrs starved for 5 hrs where indicated. Viability was determined by flow cytometry. Data represent mean ± SEM of 4 independent experiments. **(j,k)** MEFs of the indicated genotype were starved for 5 hrs in the presence of 2.5µg/mL oligomycin where indicated and viability was determined cytofluorimetrically. Data represent mean ± SEM of 5 independent experiments.

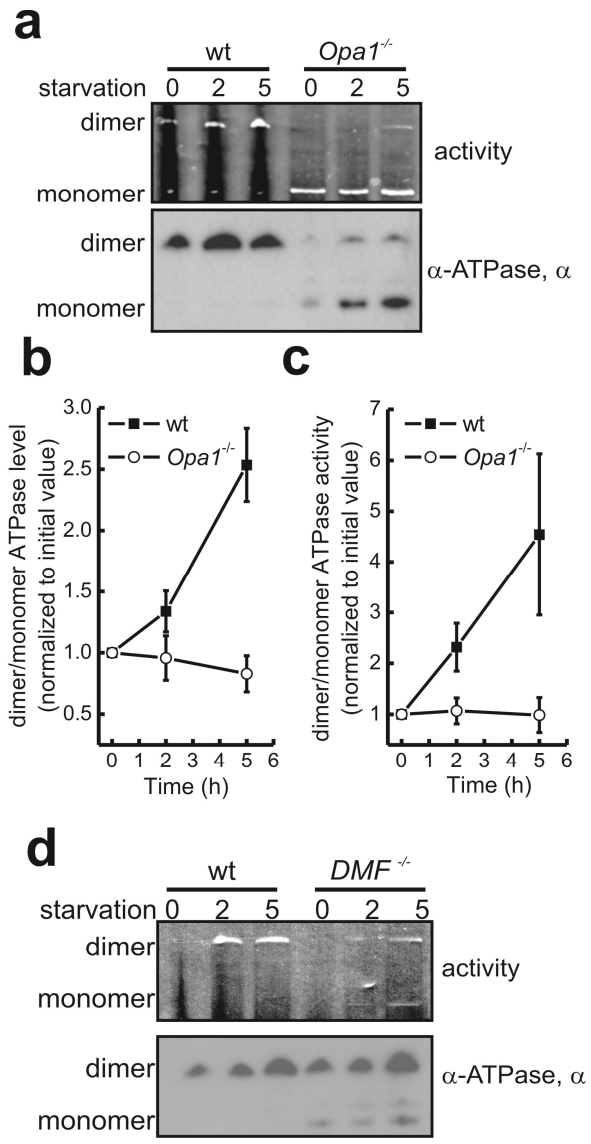


**Figure 6 – Mitochondrial elongation during starvation is associated with dimerization and activation of ATPase. (a)** Blue native electrophoresis analysis of ATPase dimerization and activity. Cells of the indicated genotype were treated as indicated and 500 µg of total cell extracts were solubilized with 4% digitonin and separated by BN-PAGE. ATPase activity was measured in gel (top) and ATPase levels were measured by immunoblotting for the indicated antibody (middle). **(b-c)** Quantitative analysis of levels (b) and activity (c) of the ratio between dimer and monomer of ATPase. Data represent mean ± SEM of 5 independent experiments carried as in (a). **(d)** Experiments were carried out as in (a), except that cells of the indicated genotype were used.

Dimerization of ATPase correlates with formation of cristae (Giraud et al., 2002; Strauss et al., 2008). However, the reverse can also be true, that increased cristae surface favors oligomerization of the ATPase. Electron microscopy and morphometric analysis showed that the number of cristae per unit of mitochondrial surface increased during starvation in wt and *Mfn2*$^{-/-}$ mitochondria that elongate, while it remained stable in *Opa1*$^{-/-}$ and *DMF*$^{-/-}$ mitochondria that do not (Fig. 7a-c). Thus, during starvation mitochondrial elongation correlates with increased cristae surface, oligomerization of the ATPase and maintenance of mitochondrial ATP production.

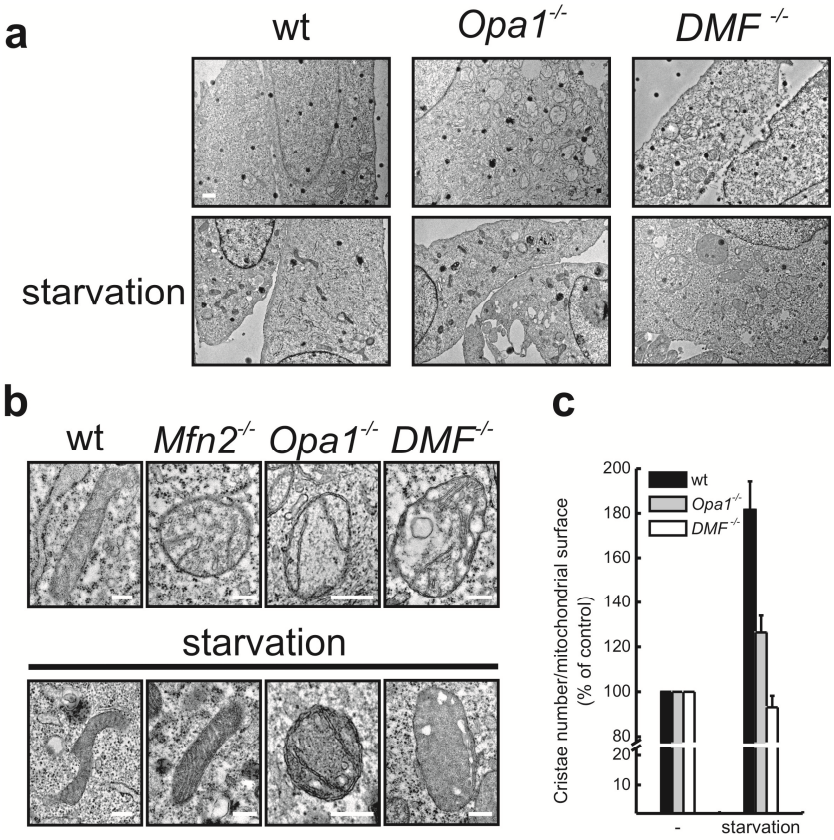


**Figure 7 – Density of cristae increases in mitochondria elongated during starvation. (a)** Representative electron micrographs of cells of the indicated genotype starved where indicated for 5h, fixed and processed for electron microscopy. Bar 2 µm. **(b)** Representative electron micrographs of randomly selected mitochondria from cells of the indicated genotype. Where indicated, cells were starved for 5h. Bar, 0.5 µm. **(c)** Morphometric analysis of cristae density in cells of the indicated genotype. Experiments were as in (a). The number of the cristae in randomly selected 50 mitochondria of the indicated genotype was normalized for the calculated surface of the organelle. Data represent mean ± SEM of 5 independent experiments.

## Mitochondrial elongation protects cells from death during starvation

What is the role of starvation-induced elongation in the cellular response to nutrient depletion? *Opa1*$^{-/-}$ and *DMF*$^{-/-}$ as well as cells where PKA was inhibited (Fig. 5e-g) died more rapidly, whereas *Drp1*$^{-/-}$ MEFs were less susceptible to starvation (Fig. 5h). The increased death by starvation of cells treated with H89 was phenocopied in *Drp1*$^{-/-}$ MEFs complemented with the mutant of Drp1 in the PKA site (Fig. 5i). Knock down of *OPA1* in HeLa cells also accelerated starvation-induced cell death (Fig. S7a,b). Death of *Opa1*$^{-/-}$ and *DMF*$^{-/-}$ MEFs was prevented by the ATPase inhibitor oligomycin (Fig. 5j,k), in accordance with the fact that mitochondria unable to elongate during starvation maintain their membrane potential by hydrolyzing and consuming cellular ATP (Fig. 5b,c). In conclusion, mitochondria unable to elongate during nutrient deprivation consume cellular ATP, leading to cell death.

## Discussion

We have demonstrated that during starvation mitochondria elongate and that this is a critical component of the cellular response to autophagy. In starving cells, a rapid increase in cAMP levels activates PKA that in turn phosphorylates the pro-fission molecule DRP1, keeping it in the cytosol and allowing unopposed mitochondrial fusion. Elongated mitochondria are protected from autophagic elimination, display denser cristae where ATPase can oligomerize to maintain ATP production and to allow survival of starving cells. On the contrary, if elongation is blocked mitochondria become dysfunctional and "cannibalize" cytoplasmic ATP to maintain their membrane potential, precipitating cell death (see model in Fig. 8).

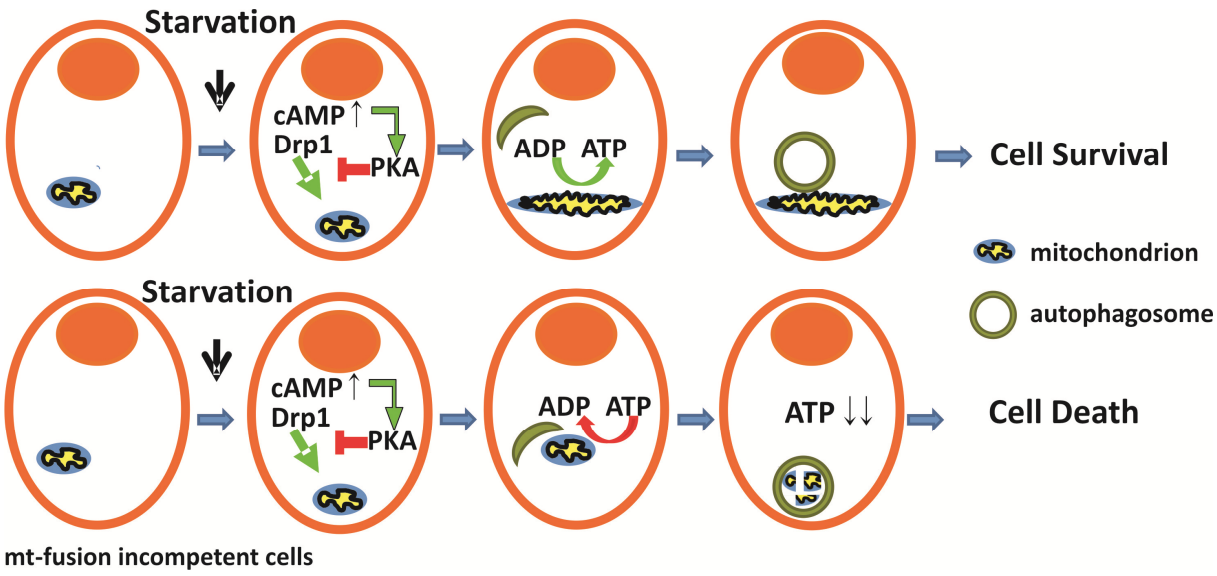


**Figure 8 – Mitochondrial elongation induced by PKA determines cell fate during starvation.** The cartoon depicts the cascade of mitochondrial elongation triggered during starvation and its role in determining cell fate. Upper row: mitochondrial elongation protects from organelle degradation and allows maintenance of ATP levels. Lower row: when mitochondrial elongation is impaired, mitochondria are degraded and the remaining organelles consume cellular ATP, precipitating cell death.

Macroautophagy triggered by nutrient deprivation degrades different constituents of the cell to allow their recycling. If mitochondria were also to be targeted to the autophagosome, their fragmentation should precede engulfment, as it occurs during selective mitochondrial autophagy (Twig et al., 2008). However, in response to stimuli that induce macroautophagy, mitochondria elongate as a consequence of Drp1 phosphorylation at Ser637 by PKA, triggered by an increase in cAMP levels. Interestingly, glucagon, the prototypical inducer of autophagy in liver, induces cAMP elevation in hepatocytes (Unger, 1985). Here we extend the importance of cAMP in autophagy by starvation in tissues other than liver. In yeast activation of PKA is important in pseudohyphal differentiation triggered by

nitrogen starvation (Pan and Heitman, 1999). Conversely, constitutive activation of PKA inhibits autophagosome formation (Budovskaya et al., 2004; Stephan et al., 2009) suggesting that the PKA loop elucidated here might be specific for higher eukaryotes. Along this line, the yeast orthologue of DRP1 lacks a conserved PKA phosphorylation site, suggesting that the metabolic response of yeast mitochondria during autophagy is differentially regulated. Elongation and phosphorylation of DRP1 occurs also in response to inactivation of the metabolic sensor mTOR, suggesting an interplay with PKA that is supported in different paradigms in mammals (Mavrakis et al., 2006) and in yeast (Slattery et al., 2008). Several mechanisms are responsible for the changes in mitochondrial shape triggered by external and internal cues: for example, in cells exposed to several stresses, a change in the relative levels of the forms of the pro-fusion OPA1 supports mitochondrial elongation (Tondera et al., 2009), while during apoptosis mitochondrial fragmentation is supported by the concerted action of inhibition of mitochondrial fusion (Karbowski and Youle, 2003), by the stimulation of DRP1 translocation to mitochondria (Cereghetti et al., 2010), where it is stabilized by a BAX, BAK dependent process of SUMOylation (Wasiak et al., 2007). Conversely, during starvation the forms of OPA1 remain stable, but mitochondrial levels of DRP1 are reduced. Multiple biochemical and genetic evidence support that the reduced levels are a consequence of its phosphorylation by activated PKA. Alternatively, another interesting possibility could be that during macroautophagy mitochondrial DRP1 is not stabilized by SUMOylation, being then ubiquitinated by resident mitochondrial ubiquitin ligases (Nakamura et al., 2006; Karbowski et al., 2007), and degraded in a proteasome-dependent fashion. Along this line, upon induction of mitophagy the ubiquitin ligase Parkin degrades components of the mitochondrial fusion machinery (Tanaka et al., 2010; Ziviani et al., 2010) and targets dysfunctional organelles to the autophagosome (Narendra et al., 2008). However, starvation induced mitochondrial elongation occurs also in HeLa cells where Parkin is not expressed (Narendra et al., 2008), further substantiating the key role of the cAMP-PKA-DRP1 loop in autophagy. Opposed to the “active” adaptation of mitochondrial morphology to the cellular cues described here, lies the pathological DRP1-mediated fragmentation by sustained activation of calcineurin like that observed in Huntington’s disease which results in increased susceptibility to apoptotic insults (Costa et al., 2010). These two extremes elucidate how mitochondrial morphology is extremely sensitive to, and plastically modulated by cellular inputs.

Why do mitochondria elongate during induction of autophagy? Elongated mitochondria are spared from autophagy, implying that the “targets” for degradation during macroautophagy might not be random. The limitation could be simply sterical –i.e. the elongated mitochondria cannot fit into the autophagosome. Alternatively, longer mitochondria might lack the signal that addresses them to the autophagosome. In this respect, it is

interesting to note that shorter mitochondria are not only less efficient in ATP production, but they also bear a latent dysfunction that could trigger the relocalization of Parkin on their surface (Narendra et al., 2008). Teleologically speaking, one could wonder why macroautophagy spares mitochondria. During nutrient restriction, cells try to maximize efficiency of energy conversion, a task perfectly performed by mitochondria (Brown, 1992) and that requires mitochondrial elongation, as we showed here. Elongated mitochondria display higher levels of dimers of the ATPase, associated with increased efficiency in ATP production (Strauss et al., 2008). Morphologically, this is mirrored by an increase in the number of cristae, the privileged compartments for ATP synthesis (Strauss et al., 2008), per mitochondrial surface. While OPA1 and cristae organization are directly linked (Frezza et al., 2006; Meeusen et al., 2006), it is less clear why *DMF*$^{-/-}$ MEFs are unable to respond to starvation with an increase in cristae biogenesis. However, OPA1 forms are altered in *DMF*$^{-/-}$ MEFs and knock down of DRP1 similarly alters processing of OPA1 (Mopert et al., 2009). Thus, it appears that processing of OPA1 (and hence biogenesis of the cristae) are exquisitely sensitive to changes in the fusion-fission equilibrium, ultimately impacting on the ability of the mitochondria to metabolically respond to the environment. In addition, mitochondria could be essential to provide membranes for the formation of the autophagosome (Hailey et al., 2010) and they should therefore be excluded from immediate degradation, in order to ensure the progression of the autophagic process.

During starvation, mitochondria unable to elongate are latently dysfunctional and they consume cytosolic ATP to sustain their membrane potential. The ensuing bioenergetic crisis due to ATP consumption causes starvation-induced cell death in *Opa1*$^{-/-}$ and *DMF*$^{-/-}$ MEFs. As expected, during starvation autophagy is essential to provide nutrients and its blockage accelerates death (Kuma et al., 2004), irrespective of whether mitochondrial elongation occurs or not. Whether the protective role of autophagy is a general feature of all forms of cell death remains a matter of intense debate. Our data conversely indicates that changes in mitochondrial morphology and function are an essential subroutine of the autophagic program: if a stereotypical response of the cell (mitochondrial elongation and activation) to limited nutrient supply is abolished, the ensuing mitochondrial dysfunction can lead to cell death. The unexpected role of mitochondrial elongation during starvation exemplifies a further cellular response regulated by these organelles.

## Acknowledgements

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## Authors’ contributions

LCG and LS conceived research, analyzed data and wrote the manuscript. LCG, GDB, LS performed experiments and analyzed data.

## Supplementary figures

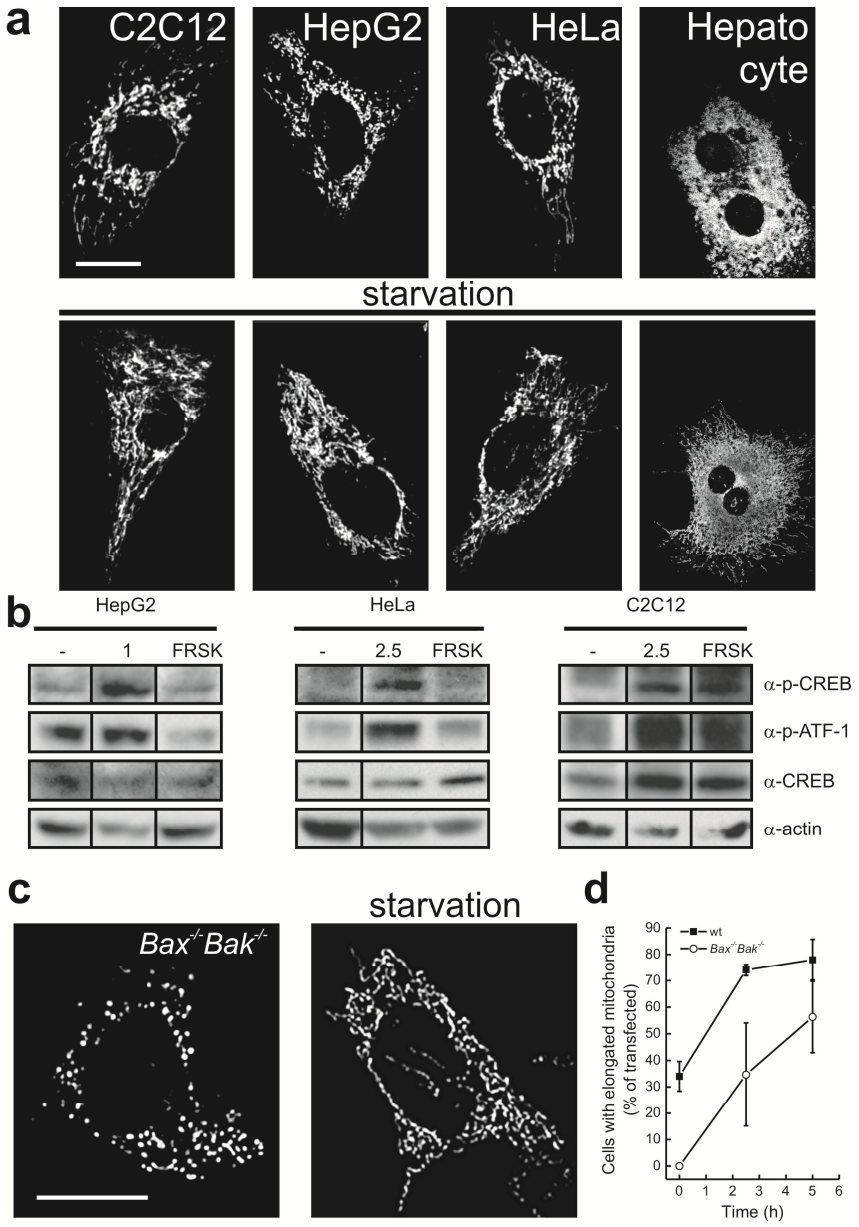


**Figure S1 – Starvation induces mitochondrial elongation and PKA activation in different cell lines. (a)** Representative images of mitochondrial morphology. C2C12, HeLa and HepG2 cells were transfected with mtYFP and after 24 hrs imaged. Primary hepatocytes were fixed and immunostained for TOM20. Where indicated, cells were starved for 2.5 hrs. Bar, 20 µm. **(b)** Fifty µg of proteins of the indicated cells were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Where indicated, cells were starved for 2.5 hrs or treated with 25 µM forskolin (FRSK) for 30 min. **(c)** Representative images of mitochondrial morphology in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. MEFs were transfected with mtYFP and imaged 24 hrs after by confocal microscopy. Where indicated, cells were starved. Bar, 20 µm. **(d)** Morphometric analysis of mitochondrial shape in *Bax*$^{-/-}$*Bak*$^{-/-}$ and the relative wt MEFs. Experiments were carried exactly as in (c). Data represent mean ± SEM of 5 independent experiments. In each experiment 50 cells were scored per condition.

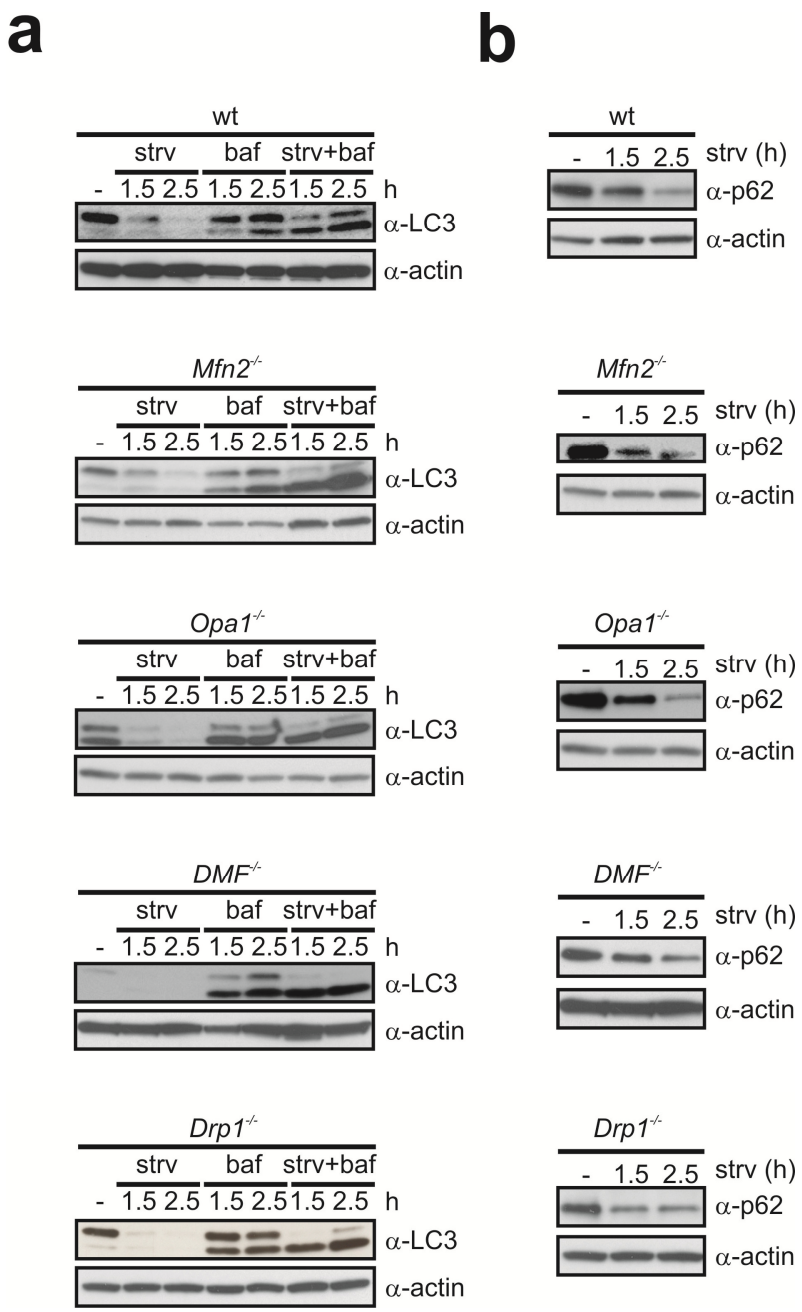


**Figure S2. Autophagic flux is not affected in fusion-deficient MEFs. (a-b)** Twenty µg of lysates of MEFs of the indicated genotypes were separated by SDS-PAGE and immunoblotted with the indicated antibodies. In (a) where indicated, MEFs were starved for the indicated periods and treated with 200 nM Bafilomycin A1 (baf). In (b) cells were starved for the indicated times.

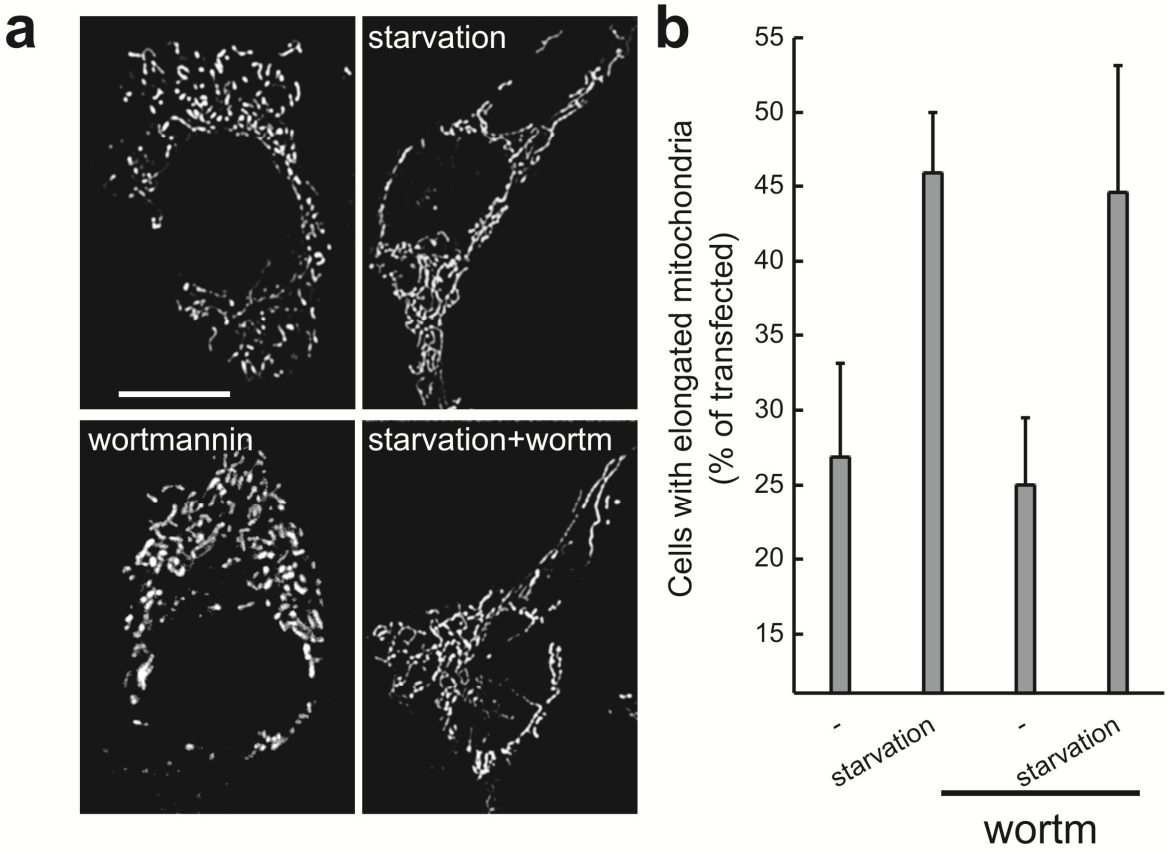


**Figure S3 – Blockage of autophagosome formation does not inhibit mitochondrial elongation in response to starvation. (a)** Representative images of mitochondrial morphology. MEFs were transfected with mtYFP and after 24 hrs confocal images were acquired. Where indicated, cells were starved for 2.5 hrs and treated with 0.5 μM wortmannin. Bar, 20 μm. **(b)** Morphometric analysis of mitochondrial shape. Experiments were carried exactly as in (a). Data represent mean ± SEM of 5 independent experiments. In each experiment 50 cells were scored per condition.

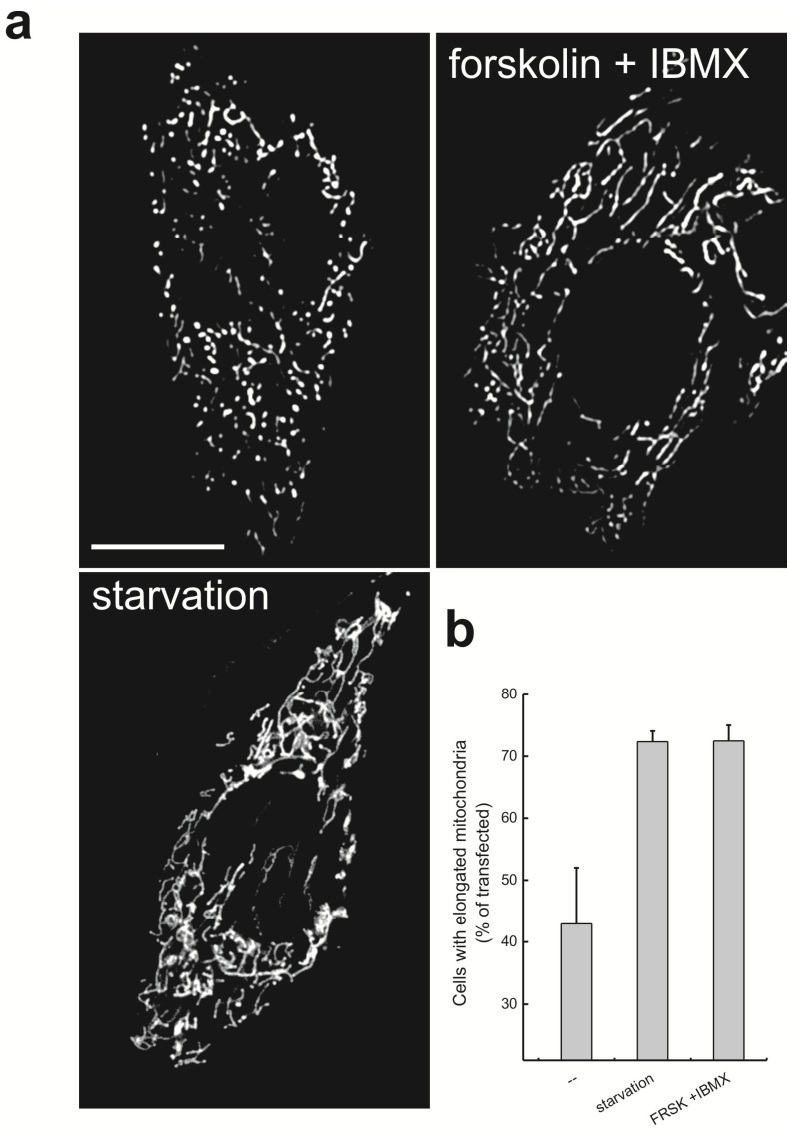


**Figure S4 – Pharmacological PKA activation leads to mitochondrial elongation. (a)** Representative images of mitochondrial morphology. MEFs were transfected with mtYFP and after 24 hrs imaged by confocal microscopy. Where indicated, cells were starved for 2.5 hrs or treated with forskolin (FRSK) plus IBMX for 30 min. Bar, 20 µm. **(b)** Morphometric analysis of mitochondrial shape. Experiments were carried exactly as in (a). Data represent mean ± SEM of 5 independent experiments. In each experiment 50 cells were scored per condition.

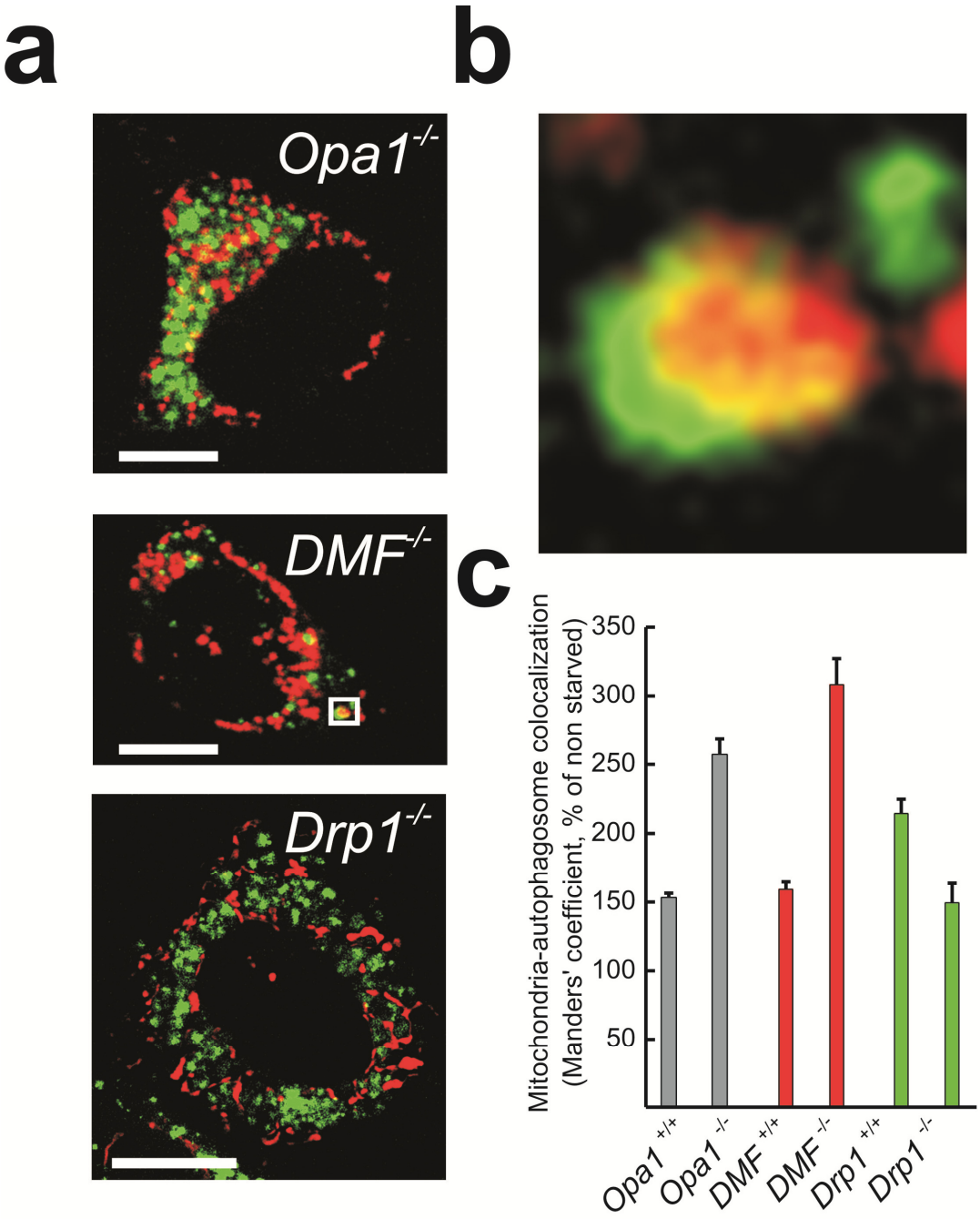


**Figure S5. Increased colocalization of mitochondria with autophagosomes upon starvation of fusion-incompetent MEFs. (a)** Representative images of mitochondria and autophagosomes. MEFs of the indicated genotype were starved 24 hrs after transfection with mtRFP and YFP-LC3 for 5 hrs in the presence of 200 nM Bafilomycin A1, fixed and imaged by confocal microscopy. Bars, 20 µm. **(b)** Magnification (10X) of the inset in (a) showing the engulfment of a fragmented mitochondrion by an LC3-positive autophagosome. **(c)** Quantitative analysis of mitochondria-autophagosome colocalization. Experiments were carried exactly as in (a). Data were normalized to the Manders' coefficient in non starved cells and they represent mean ± SEM of 5 independent experiments. In each experiment 50 cells were scored per condition.

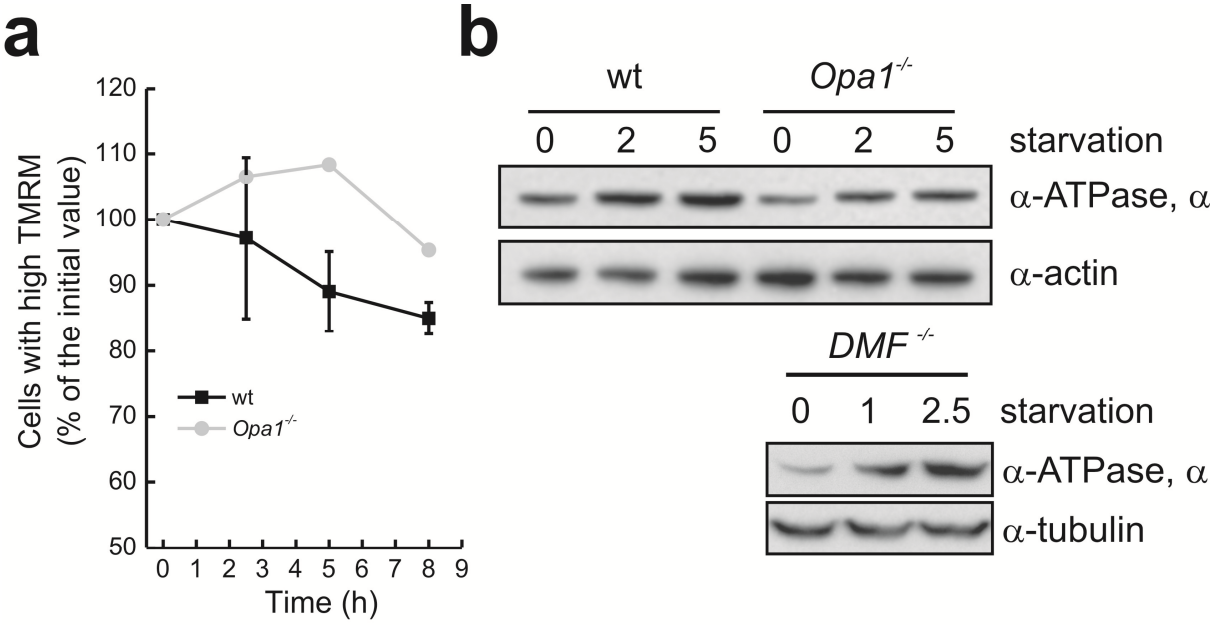


**Figure S6. Mitochondrial membrane potential and ATPase levels during starvation. (a)** Flow cytometric analysis of TMRM uptake during starvation. MEFs of the indicated genotype were starved for the indicated periods of time, harvested and stained with TMRM. Uptake of TMRM was determined by flow cytometry. Data represent the mean ± SEM of 5 independent experiments. **(b)** Mitochondrial ATPase levels during starvation. Twenty five µg of lysates of MEFs of the indicated genotypes were analyzed by SDS-PAGE/immunoblotting using the indicated antibodies. Where indicated, MEFs were starved for the indicated periods.

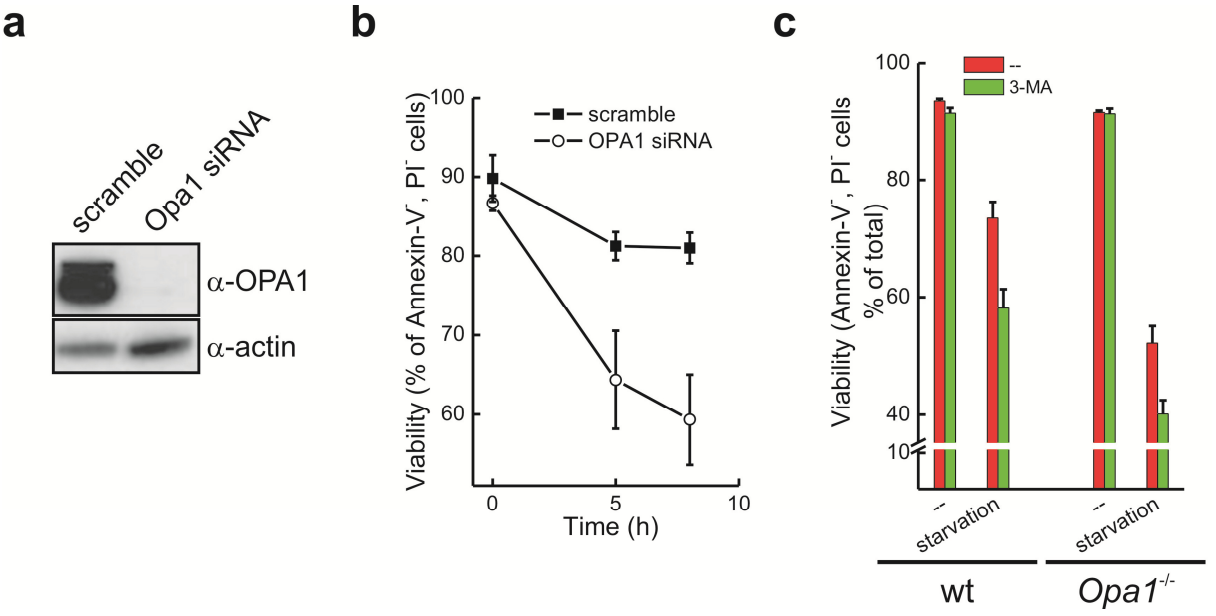


**Figure S7. Starvation-induced cell death is accelerated by downregulation of *OPA1* or by inhibition of autophagy. (a)** HeLa cells were transfected with the indicated siRNA and after 48 hrs lysed and 25 µg of proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies. **(b)** At the indicated times after starvation, cells were harvested and viability was determined by flow cytometry. Data represent mean ± SEM of 3 independent experiments. **(c)** Cells of the indicated genotype were starved for 5 hrs, harvested and viability was determined by flow cytometry. Where indicated, cells were treated with 10mM 3-MA. Data represent mean ± SEM of 7 independent experiments.

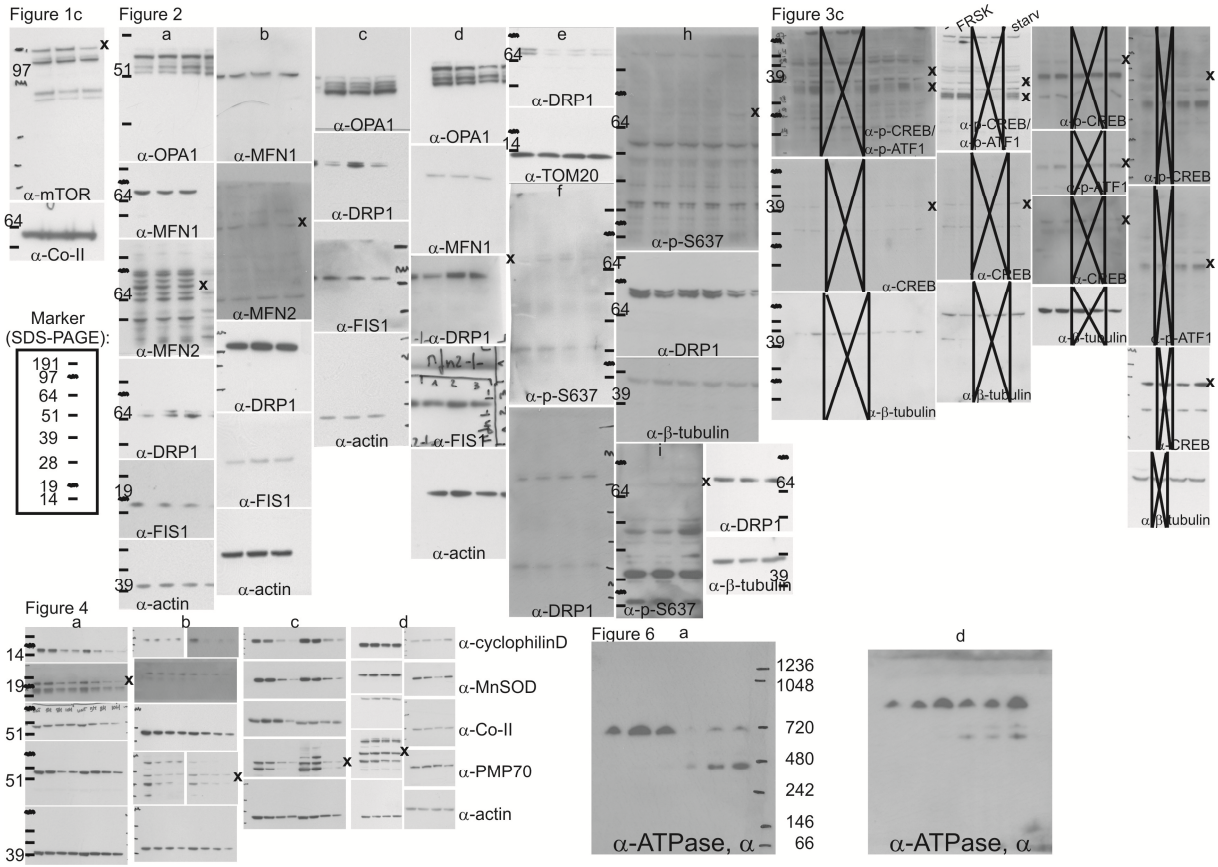


**Figure S8. Full scans of key Western blot data.** In many experiments, membranes were cut prior to probing each strip with a separate antibody. When unspecific bands are present, the band that corresponds to the molecular weight of the protein probed is indicated by an (x). Crossed lanes represent lanes that were cut out from the Western-blots in the main figures where this has been indicated by vertical bars. The orientation of the samples is the same as in the main figures except when specified.

## Supplementary online Movies (CD attached to the thesis)

**Movie 1. Real time imaging of mito-pAGFP diffusion in wt MEFs.** MEFs were cotransfected with mtRFP and mito-pAGFP. After 24 hrs mito-pAGFP was photoactivated in a ROI of fixed dimension as described and real time images of GFP and RFP fluorescence were acquired each min as described. The movie shows a composite image of GFP (green) and RFP (red) fluorescence.

**Movie 2. Real time imaging of mito-pAGFP diffusion in starved wt MEFs.** The experiment is exactly as in Movie S1, except that cells were starved for 2.5 hrs.

**Movie 3. Real time FRET imaging demonstrate an increase in cAMP levels during starvation.** MEFs transfected with Epac-Cl. After 24 hrs real time imaging of Epac-Cl FRET was performed as described. The movie shows the pseudocolor-coded FRET ratio of Epac-Cl (red, higher, blue, lower FRET). Where indicated, cells were perfused with the starvation medium or with Forskolin+IBMX.

# Chapter 5

# General Discussion and Conclusions

The work presented in this thesis contributes to elucidate the role that mitochondria play in the process of autophagy. Two major conclusions can be drawn: (i) mitochondrial dysfunction, rather than fragmentation of the mitochondrial network, participates in autophagy induction; (ii) remodelling of the mitochondrial shape determines cell fate during macroautophagy. Thus, the participation of mitochondria in the regulation of a further cellular response, autophagy, is illustrated in this thesis.

In the first part of this thesis, we analysed the effect of FIS1 overexpression in the induction of autophagy. FIS1, an OMM protein, participates in mitochondrial fission and dysfunction by two genetically distinct pathways, making it an useful model to distinguish between the effect of mitochondrial fragmentation versus dysfunction in autophagy induction. A conservative mutation in the short stretch of amino acids of FIS1 (FIS1$^{K148R}$) protruding the IMS abolishes mitochondrial dysfunction caused by FIS1, with no effect in mitochondrial fragmentation. Conversely, the deletion of the first $\alpha$-helix of FIS1 (FIS1$^{\Delta\alpha1}$) acts as a dominant-negative for mitochondrial fission, leading to extensive mitochondrial dysfunction (Alirol et al., 2006). Several markers of autophagy like accumulation of YFP-LC3-positive vesicles, lipidation of endogenous LC3, p62 degradation and lysotracker green accumulation (Klionsky et al., 2008), appear upon enforced expression of FIS1. Overexpression of FIS1$^{\Delta\alpha1}$ mutant also triggers autophagy. In contrast, overexpression of FIS1$^{K148R}$ does not induce autophagy, even if it caused mitochondrial fragmentation. Therefore, the results obtained indicate that mitochondrial dysfunction rather than fragmentation triggers autophagy and suggest that mitochondrial dysfunction can feedback to the autophagic machinery to activate it.

A function for mitochondria in the activation of autophagy has been previously proposed. Mitochondria-derived ROS play a key role in ATG4 regulation, a cysteine protease essential for autophagy (Scherz-Shouval et al., 2007). Interestingly, FIS1 overexpression involves ROS formation (Alirol et al., 2006). Additionally, upon enforced expression of FIS1, mitochondria present a latent dysfunction, consuming ATP to maintain mitochondrial membrane potential. Autophagy is triggered, for instances, upon activation of AMPK (Corradetti et al., 2004; Shaw et al., 2004). An appealing hypothesis would be that FIS1 overexpression triggers AMPK activation, through a decrease in ATP/ AMP ratio. Indeed, preliminary results (not shown in this thesis) indicate that FIS1 overexpression leads to the activation of AMPK in the cell models we used in our study. AMPK activation was also observed after overexpression of FIS1$^{\Delta\alpha1}$, but not hFis1$^{K148R}$, that accordingly does not induce mitochondrial dysfunction. Importantly, the results we obtained *in vitro*, were subsequently confirmed *in vivo*, in a collaboration with the group of M. Sandri. Enforced expression of the mitochondrial fission machinery caused muscle atrophy in adult mice, with

a concomitant augment of autophagy. The muscle atrophy programme induced by overexpression of FIS1 and DRP1 occurred through activation of AMPK, since inhibition of AMPK restored muscle size in myofibres (Romanello et al., 2010).

Induction of autophagy as an outcome of forced changes in the mitochondrial morphology was also reported by Parone and colleagues. Downregulation of DRP1 was shown to lead to an increase in ROS levels and a drop in cellular ATP with a concomitant increase of autophagy. Although mitochondria were dysfunctional upon DRP1 downregulation, they were not targeted to autophagy, probably due to a sterical constraint, since knock-down of DRP1 inhibits mitochondrial fission (Parone et al., 2008). Conversely, when we enforced FIS1 expression, some of the fragmented mitochondria were retrieved into autophagosomes. However, not all mitochondria were observed to be engulfed or to disappear, as was reported after treatment with CCCP (Narendra et al., 2008). It is possible that we missed some mitophagic events. Still, mitochondria targeting to the autophagosomes depends probably on the “eat me” signals exposed by mitochondria, that we did not analyse. Additionally, according to Twig and colleagues mitophagy is preceded by an uneven fission event that occurs following fusion (Twig et al., 2008). Our results together with the ones from Twig and colleagues could explain at least partially why dysfunctional mitochondria that present mtDNA mutations are not completely degraded by autophagy. In summary, mitochondrial dysfunction triggers autophagy, whereas mitochondrial fragmentation *per se* is not determinant for autophagy induction. Dysfunctional fragmented mitochondria are targeted to autophagy.

In the second part of this thesis, we examined the shape of mitochondria after induction of macroautophagy, by nutrient depletion or mTOR silencing. Surprisingly, we found that during starvation mitochondria elongate both *in vitro* and *in vivo*. Mitochondrial elongation is a crucial event that determines cell fate during autophagy. In starving cells, a rapid increase in cAMP levels activates PKA that in turn phosphorylates the pro-fission molecule DRP1, keeping it in the cytosol and allowing unopposed mitochondrial fusion. Elongated mitochondria are protected from autophagic elimination, display denser cristae where ATPase can oligomerize to maintain ATP production and to allow survival of starving cells. On the contrary, if elongation is blocked, mitochondria become dysfunctional and “cannibalize” cytoplasmic ATP to maintain their membrane potential, precipitating cell death.

During macroautophagy, intracellular components are degraded in order to recycle nutrients for *de novo* synthesis or energy production. Mitochondrial degradation is preceded by fission (Twig et al., 2008). However, upon induction of autophagy, we observed that mitochondria elongate. In accordance, mitochondrial enlargement was reported in rat hepatocytes (Miyazawa and Kametaka, 1990), as well as in Japanese monkey hepatocytes (Yang and Makita, 1998) in response to protein deprivation and fasting, respectively. We

showed that elongated mitochondria are spared from autophagy. This implies that macroautophagy is not a random process, and, that at least, mitochondria are initially protected from being degraded. Whether this is just due to a sterical limitation or whether elongated mitochondria lack the signals that target them to the autophagosome was not addressed. Interestingly, mitochondria that remain short during autophagy display a latent dysfunction, that could target them to the autophagosome.

Why does macroautophagy spare mitochondria? During nutrient limitation, the cells must maximize energy conversion, a task perfectly performed by mitochondria (Brown, 1992). We showed that elongation has a dual function in protecting mitochondria from being degraded and optimizing their efficiency in energy production. Elongated mitochondria display higher levels of dimers of the ATPase, associated with increased efficiency in ATP production. Morphologically, this is mirrored by an increase in the number of cristae, privileged compartments for ATP synthesis (Strauss et al., 2008). In line with our findings, Rossignol and colleagues reported that enforced usage of mitochondrial ATP synthase leads to mitochondrial elongation. Additionally, mitochondrial elongation in response to stress insults results in an increase in ATP production (Tondera et al., 2009). Alternatively, since upregulation of cAMP-PKA signalling increases mitochondrial activity, through phosphorylation of enzymes that participate in oxidative phosphorylation (Carlucci et al., 2008), the improvement of mitochondrial performance could be due to cAMP-PKA axis activation. However, PKA is activated in response to starvation both in cells in which mitochondria elongate during starvation and in cells unable to elongate mitochondria. Thus, differences observed in ATP production between these cells cannot be explained by differential activation of PKA.

Mitochondrial elongation and activation is a stereotypical response of the cell to nutrient deprivation. If this response is abolished, the consequent mitochondrial dysfunction can lead to cell death. It would be interesting to further analyse the physiological significance of mitochondrial elongation during nutrient depletion *in vivo*. Is this a stereotypical response of all tissues? How do individuals that present mutations in mitochondrial fusion proteins deal with neonatal starvation, for instances?

Opposed to the “active” adaptation of mitochondrial morphology in response to autophagy described in this thesis, is the pathological DRP1-mediated fragmentation in Huntington’s disease, that results in increased susceptibility to apoptosis. Increased basal activation of the phosphatase calcineurin causes DRP1 dephosphorylation, consequent translocation to mitochondria and organelle fragmentation. Fragmented mitochondria display cristae alterations. Correction of mitochondrial morphology and ultra-structure reduces susceptibility to apoptosis (Costa et al., 2010). In Huntington’s disease, however, these

mitochondria are probably not efficiently removed by autophagy, due to autophagosome defects in trapping cytosolic cargo (Martinez-Vicente et al., 2010).

In conclusion, in this thesis we describe a role for mitochondria during autophagy. Importantly, our findings exemplify a further cellular function that is regulated by mitochondria.

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