Biochimica et Biophysica Acta 1777 (2008) 860–866

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

# High levels of Fis1, a pro-fission mitochondrial protein, trigger autophagy

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ARTICLE INFO

*Article history:*
Received 1 February 2008
Received in revised form 20 May 2008
Accepted 21 May 2008
Available online 26 May 2008

*Keywords:*
Mitochondria
Fission
Fis1
Autophagy

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.



## 1. 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 [16]. 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 [14]. Among these, dominant optic atrophy (DOA) [1,13], Charcot-Marie-Tooth IIa (CMT2a) [60] and Charcot-Marie-Tooth IVa (CMT4a) [41] 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 [5]. 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 [20]. 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 [9,29,47]. 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 [12], promotes fusion in a Mfn1-dependent manner [10,37], controls biogenesis of the cristae [37] and regulates the cristae remodelling pathway during apoptosis in a genetically distinct pathway regulated by the inner mitochondrial membrane rhomboid protease Parl [11,21,45]. In mammalian cells, mitochondrial division is regulated by Drp1 and Fis1 [7,23,28,52]. Drp1 is a cytosolic dynamin-related protein whose inhibition or downregulation results in a highly interconnected mitochondrial network. The same phenotype is caused by downregulation of Fis1 [39], 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 [39]. 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 [56].

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 [50]. Moreover, in neurons as well as in model cell lines mitochondria undergo massive and reversible fragmentation prior to the release of cytochrome *c* [19,36]. 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 [53] to generation of reactive oxygen species [58], to

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doi:10.1016/j.bbabio.2008.05.442

neuronal plasticity [33], to intermediate metabolism [3], to leukocyte dynamics [8], even to lifespan of the filamentous fungi [48].

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 [31]. 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 [32]. 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 autophagosomes, and eventually fuses with lysosomes to form autolysosomes [55]. 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 regulates and participates in autophagy [43]. These Atg proteINS include: (i) Atg1, Atg13, and Atg17, a serine–threonine kinase complex involved in autophagic induction; (ii) a Class III phosphatidylcholine-3–kinase (PI3K) 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 [55]. 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 [25,26]. The activation of autophagy in mammalian cells is controlled by two classes of PI3Ks with opposite effects: class I PI3K, via its downstream effector mammalian Target Of Rapamycin (mTOR), blocks autophagy [40], while class III PI3Ks, operating together with Beclin, stimulates it [44].

Autophagy can be selective for certain organelles, as it was originally shown for peroxisomes [18]. 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 [38]. 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 [6], is a trigger for autophagy when it does not result in cytochrome *c* release and apoptosis [30]. 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 [17,54]; 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 [23]. 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 [2]. 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.

## 2. Materials and methods

### 2.1. 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$^{\Delta\alpha1}$) [2] were subcloned into the EcoRI site of pcDNA3.1Zeo(+). All constructs were confirmed by sequencing.

### 2.2. Cell culture and transfection

SV40 transformed mouse embryonic fibroblasts (MEFs) from a mixed Sv129/CD1 background were cultured as described before [51]. Cells were transfected using Transfectin (Biorad) following the 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.

### 2.3. 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 60× 1.4 NA Plan Apo objective (Nikon).

HeLa cells grown on 13 mm round coverslips were transfected as indicated and, after 24 h, 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).

### 2.4. Induction and quantification of autophagy

Twenty-four hours 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) [26].

For the induction of autophagy, cells were starved for 2 h30 min in a Hank's balanced salt solution (HBSS, Invitrogen) supplemented with 10 mM Hepes pH 7.4. 3-methyl adenine (3MA) was obtained from Sigma.

### 2.5. 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).

### 2.6. Analysis of lysotracker accumulation

MEFs grown on 12-well plates were co-transfected with mRFPI and the indicated vector. After 24 h 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 10 mM 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.

## 3. Results

### 3.1. Markers of autophagy in cells expressing hFis1

In order to gain INSights into the relationship between mitochondrial fission and autophagy, we took advantage of the pro-fission effect of hFis1 expression [2]. Non-tagged versions of wt and mutant

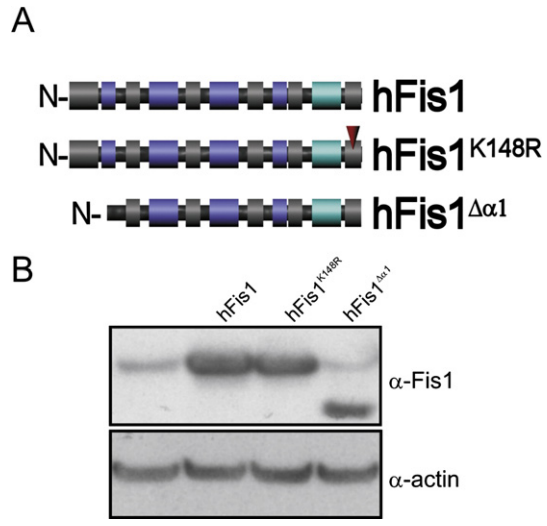


**Fig. 1.** Expression levels of the hFis1 mutants used in this study. (A) Cartoon depicting the mutants used in this study. The gray blocks indicate the α-helices, the green one the transmembrane domain, the blue ones the tetratricopeptide repeats. The red arrowhead shows the position of the point mutation. (B) Immunoblot of wt and mutant hFis1 expression levels. MEFs were transfected with the indicated plasmid and after 24 h cells were harvested, lysed and equal amounts of proteins (25 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

hFis1 (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). Confocal microscopy of the mitochondrial marker mtRFP expressed in HeLa cells showed that as expected 24 h after co-transfection wt hFis1 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 hFis1, extensive fragmentation is associated with perinuclear clustering, much less pronounced in cells overexpressing Myc-tagged hFis1 [23]. When we analyzed the intracellular distribution of YFP-LC3 in the same cells, we surprisingly noticed that hFis1 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 hFis1 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 hFis1 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).

We wished to further confirm that expression of hFis1 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 h), but not short-term (24 h) expression of hFis1 results in the activation of a program of mitochondrial dysfunction, cytochrome *c* release and cell death [2]. Co-expression of hFis1 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 B). Since changes in YFP-LC3 subcellular distribution cannot be used as sole marker of autophagy, we wished to verify if hFis1 expression caused LC3II accumulation as well as degradation of p62, two other well established indicators of the activation of autophagy. Consistently, hFis1 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 [27] (Fig. 3C). Finally, we checked whether overexpression of hFis1 increased cellular staining with the lysosomal dye Lysotracker Green (LTG), whose accumulation is proportional to lysosomal acidification and number and has been used to monitor activation of autophagy [46]. 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 (3MA) (Fig. 4A and B). A similar 3MA-sensitive accumulation of LTG was observed in cells transfected with hFis1 (Fig. 4C). Taken together, our data indicate that overexpression of hFis1 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.

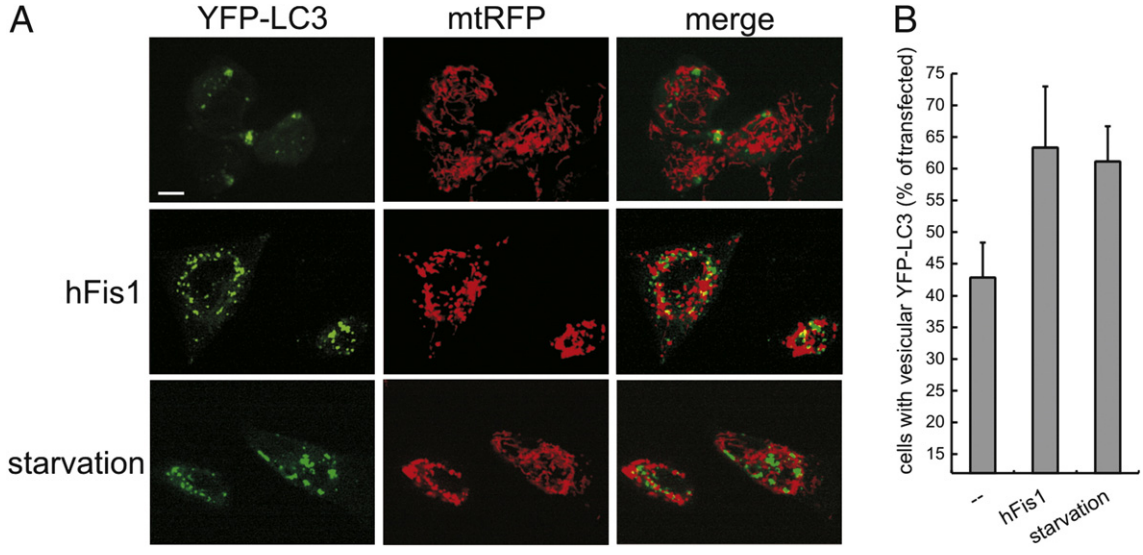


**Fig. 2.** Accumulation of YFP-LC3 into vesicular structures in hFis1 expressing HeLa cells. (A) Representative confocal images of YFP-LC3 and mtRFP fluorescence. HeLa cells grown on coverslips were co-transfected YFP-LC3, mtRFP and with empty plasmid or with hFis1. After 24 h cells were fixed and confocal images of YFP-LC3 and mtRFP fluorescence were acquired as described in Materials and methods. Merge indicates the superimposition of the single channel images. Where indicated (starvation), cells transfected with empty plasmid were incubated for 2.5 h 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.

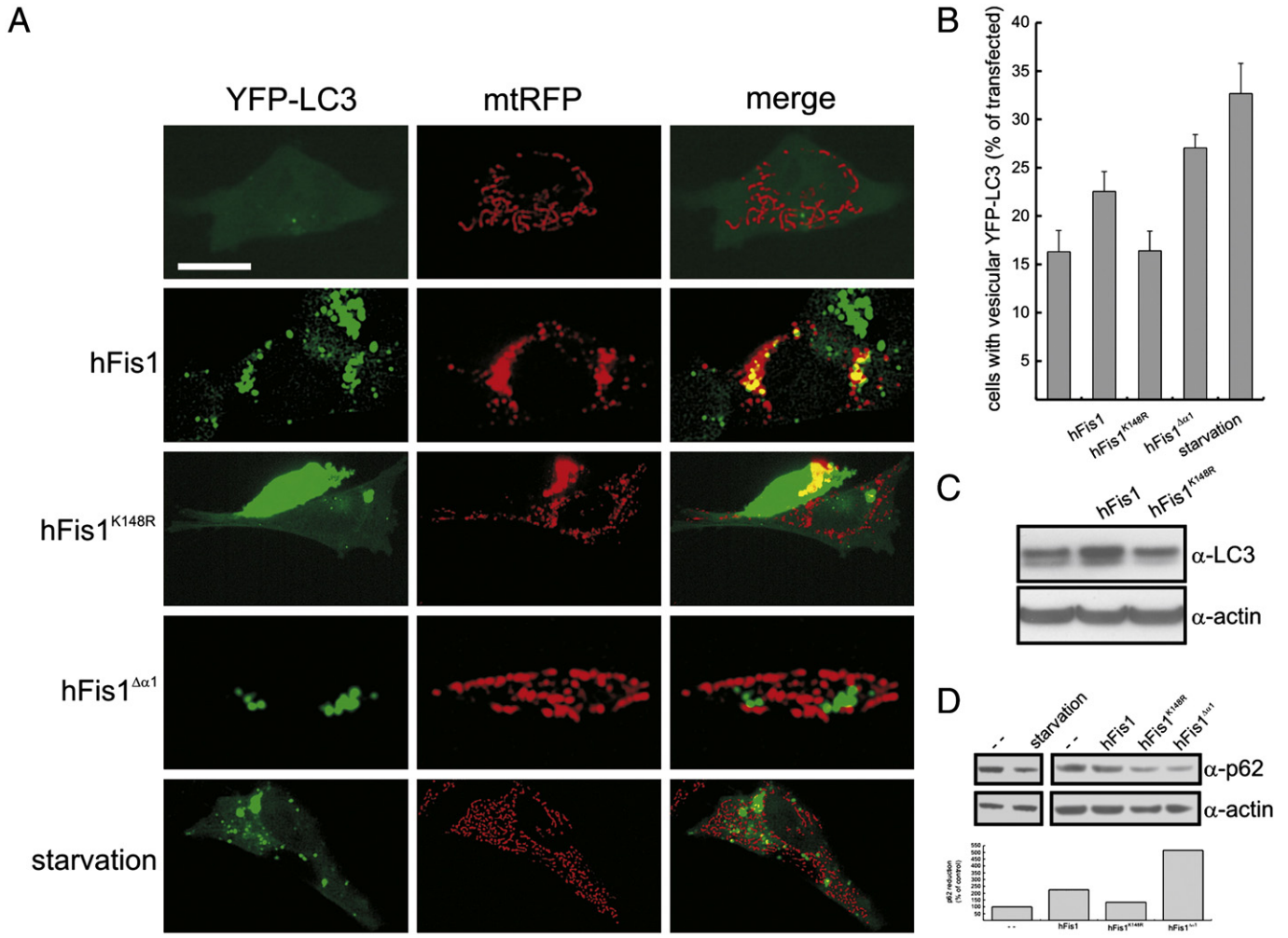


**Fig. 3.** Effect of mutants of hFis1 on markers of autophagy. (A) Representative confocal images of YFP-LC3 and mtRFP fluorescence. MEFs grown on coverslips were co-transfected YFP-LC3, mtRFP and with empty plasmid or with the indicated plasmid. After 24 h confocal images of YFP-LC3 and mtRFP fluorescence were acquired as described in Materials and methods. Where indicated (starvation), cells transfected with empty plasmid were incubated for 2.5 h 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 hFis1, hFis1$^{\Delta\alpha1}$, 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 h 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 hFis1 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.

### *3.2. Induction of autophagy by hFis1 correlates with mitochondrial dysfunction rather than with fragmentation*

We had recently developed and characterized a series of mutants of hFis1 with different effects on fusion/fission and dysfunction of mitochondria. A conservative mutation in the short stretch of amino acids of hFis1 (hFis1$^{K148R}$) 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 (hFis1$^{\Delta\alpha1}$) acts as a dominant negative for mitochondrial fusion and causes extensive mitochondrial dysfunction [2,57] (these mutants are depicted in the cartoon in Fig. 1A). hFis1 mutants seemed a good tool to investigate the relationship between mitochondrial fragmentation and the accumulation of markers of autophagy. When we expressed hFis1$^{K148R}$ 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 B). Similarly, hFis1$^{K148R}$ 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 hFis1$^{\Delta\alpha1}$ 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 hFis1 (Fig. 3A and quantification in B). Along the same line, hFis1$^{\Delta\alpha1}$ 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 hFis1. In conclusion, these mutants of hFis1 highlight that the appearance of markers of autophagy in cells seems to correlate better with mitochondrial dysfunction rather than with fragmentation *per se*.

### *3.3. Mitochondria fragmented by hFis1 can be targeted to autophagosomes*

It has been reported that fragmented and dysfunctional mitochondria are often targeted to autophagosomes [46,54]. Our model of hFis1 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 hFis1 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.

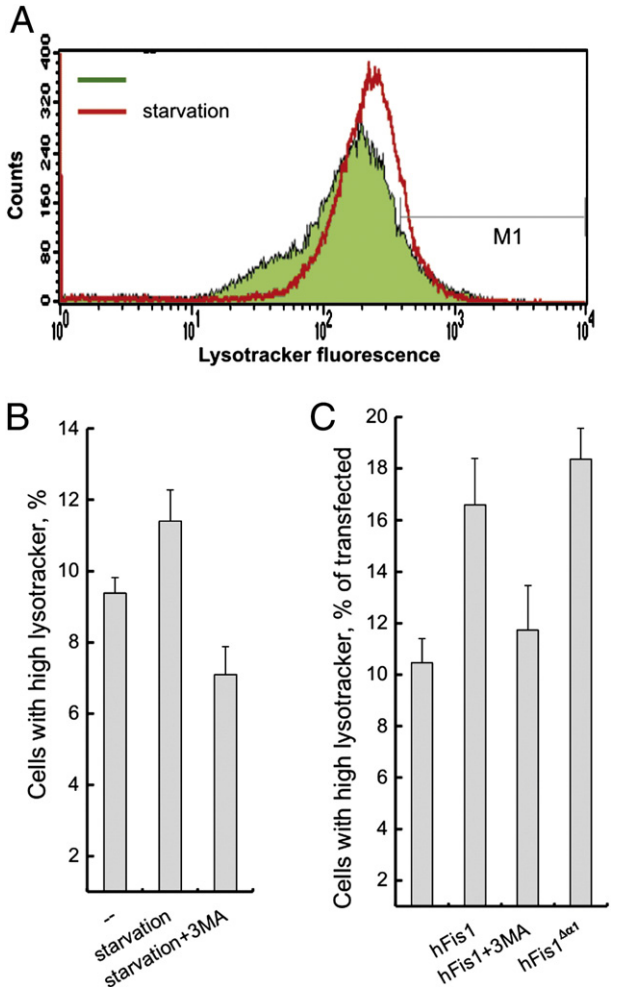


**Fig. 4.** Lysotracker Green accumulation in hFis1 expressing cells. (A) Representative histogram of cellular accumulation of LTG in response to starvation. Where indicated, $10^5$ MEFs were incubated in HBSS for 2.5 h and then loaded with LTG as described in Materials and methods. Accumulation of LTG 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 hFis1. MEFs were transfected with mRFPI and empty or the indicated plasmid and after 24 h 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 3MA 3 h before loading with LTG. Data represent mean ± SE of 3 independent experiments.

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 hFis1 expressing cells, the fragmented mitochondria were really enclosed by the YFP-LC3 positive vesicles, as clearly visible in the magnified box in Fig. 5. In general, we observed an increase in "false colocalization", *i.e.* in mitochondrial targeting to autophagosomes, in 3D-reconstructed, volume rendered hFis1 expressing cells. A quantitative analysis of red-to-green colocalization using Manders' coefficient [35] showed a 153% increase in targeting to autophagosomes (green) of hFis1 expressing mitochondria (red) as compared to the ones from empty-vector transfected cells. In conclusion, fragmented mitochondria expressing hFis1 can be engulfed by autophagosomes.

## 4. Discussion

Our current understanding of the relationship mitochondrial shape, mitophagy and autophagy in general is scarce. Here we used over-expression 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 hFis1 with limited effects on mitochondrial function are less potent in the induction of autophagy. The fragmented mitochondria induced by hFis1 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 [4]. Similarly, autophagic degradation of yeast mitochondria observed in strains deficient in the inner membrane protein Mdm38p depends on fission [42]. Since downregulation of Letm1, the human orthologue of Mdm38p, causes fragmentation independently of the fission machinery [15], it is conceivable that in the mitophagy of Mdm38p deficient yeast mitochondria, Dnm1p/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

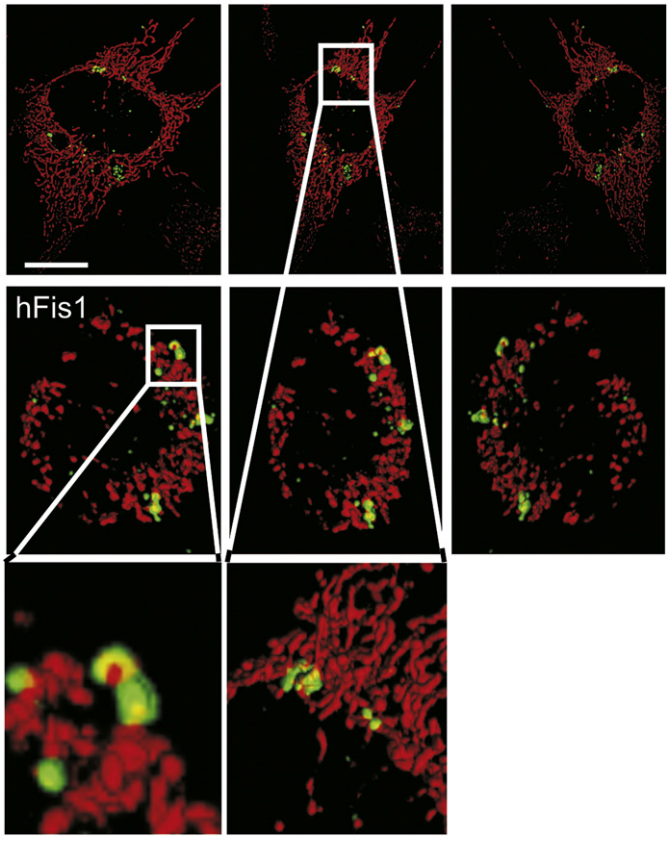


**Fig. 5.** Mitochondria expressing hFis1 can be retrieved in autophagosomes. MEFs grown on coverslips were co-transfected with YFP-LC3 and mtRFP and with empty plasmid or where indicated with hFis1. After 24 h confocal z-stacks of mtRFP and YFP-LC3 fluorescence were acquired, 3D reconstructed, volume rendered and merged. Red indicates mtRFP, green YFP-LC3. Bar, 25 μm. The boxed areas are magnified 3×.

undergo cycles of fusion followed by fission, sustained by a drop in the levels of the pro-fusion protein Opa1 [54]. Here we report that expression of the pro-fission mitochondria-shaping protein hFis1 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 hFis1 that dissociate its ability to fragment mitochondria from its detrimental action on mitochondrial function [2]. The more toxic the mutant, the highest induction of autophagy was observed. It should be noted that the $\Delta\alpha1$ mutant could form large proteic aggregates, which eventually could cause the accumulation of YFP-LC3 in a pathway independent of autophagocytosis [27]. 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 triggers mitophagy. Mitochondria-derived ROS may regulate Atg4, a cysteine protease essential in the autophagic pathway [49]. Of note, mitochondrial dysfunction by hFis1 involves ROS formation, as substantiated by the inhibitory activity of the ROS scavenger *N*-acetylcysteine [2]. Another appealing possibility is that hFis1 expression, which converts mitochondria in sinks for ATP [2], signal to AMP-activated protein kinase, a master regulator of autophagy [22,34]. Irrespective of the nature of this signal elicited by mitochondria, expression of hFis1 highlights the existence of yet another axis of retrograde mitochondrial signalling, in addition to the so-called mitochondrial stress response [59], which is likely to involve regulators of mitochondrial dynamics such as PARL [24].

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 [54]. 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 [17].

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.

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