hFis1, a novel component of the mammalian mitochondrial fission machinery.

Dominic I James, Philippe A Parone, Yves Mattenberger and Jean-Claude Martinou

Department of Cell Biology, University of Geneva, Quai Ernest-Ansermet 30, Geneva, Switzerland.

1 Correspondence should be addressed to Jean-Claude Martinou, Department of Cell Biology, University of Geneva, Quai Ernest-Ansermet 30, 1211 Geneva, Switzerland
Tel: +41 22 702 64 43. Fax: +41 22 702 64 42.
Email: jean-claude.martinou@cellbio.unige.ch

Running title: hFis1 induces fragmentation and apoptosis
Summary

The balance between the fission and fusion mechanisms regulate the morphology of mitochondria. In this study we have identified a mammalian protein that we call hFis1, which is the orthologue of the yeast Fis1p known to participate in yeast mitochondrial division. hFis1, when over-expressed in various cell types, localised to the outer mitochondrial membrane and induced mitochondrial fission. This event was inhibited by a dominant negative mutant of Drp1 (Drp1(K38A)), a major component of the fission apparatus. Fragmentation of the mitochondrial network by hFis1 was followed by the release of cytochrome c and ultimately apoptosis. Bcl-xL was able to block cytochrome c release and apoptosis but failed to prevent mitochondrial fragmentation. Our studies show that hFis1 is part of the mammalian fission machinery and in addition they suggest that regulation of the fission processes might be involved in apoptotic mechanisms.

Key words: hFis1, Drp1, mitochondria, division, apoptosis, cytochrome c, Bcl-x
Introduction

Mitochondria play a key role in many cellular processes ranging from apoptosis\(^1\) to ageing\(^2\) and it is possible that the processes that determine their morphology also modulate their function\(^3,4\). The morphology of mitochondria is regulated by the controlled action of fusion and fission mechanisms which give rise to, in many cases, a branched tubular network extending throughout the cell\(^5\).

In Saccharomyces cerevisiae, the fission of the outer mitochondrial membrane (OMM) is regulated by three proteins: Dnm1p, Fis1p and Mdv1p\(^6,7\); whilst Mdm33 is involved in the fission of the inner mitochondrial membrane\(^8\). Deletion of any of these genes abrogates the fission mechanism and fusion continues unabated leading to a fused mitochondrial network. Dnm1p is a dynamin-related GTPase that assembles on the fission points and is thought to form a circular structure that ‘pinches’ or constricts the outer membrane. The constriction and complete fission of the OMM requires Mdv1p which is thought to act as an adaptor molecule between Dnm1p and Fis1p, the latter being an integral outer mitochondrial membrane protein. In the absence of Fis1p, Dnm1p can no longer associate with the OMM and is distributed in a diminished number of punctate spots in the cytosol. Further delineation of this pathway has shown that Fis1p not only allows initial assembly of Dnm1p but also the final scission process which is dependent on the presence of Mdv1p.

The mechanisms of mitochondrial fission in other eukaryotic species are less clearly understood. Orthologues of Dnm1p have been characterized in C. elegans (DRP1\(^9\)) and mammals (Drp1\(^10\)) but they are different in their functionality. Expression of DRP1 in C.elegans promotes mitochondrial fission, but expression of mammalian Drp1 has no
effect on the morphology of mitochondria. In addition Drp1 has recently been implicated in the division of peroxisomes. Furthermore, the regulation of mitochondrial morphology is a key factor in the process of apoptosis. Exposure of cells to apoptotic inducers leads to fragmentation of their mitochondria and the release of apoptogenic proteins from the intermembrane space. Recently it was shown that expression of a mutant Drp1 (Drp1K38A) blocked the fragmentation of mitochondria induced by either treatment with the kinase inhibitor staurosporine, or expression of Bax, the pro-apoptotic Bcl-2 family member. Importantly, expression of Drp1K38A reduced the staurosporine-induced release of cytochrome c and inhibited apoptosis.

As yeast Dnm1p requires Fis1p for complete fission of the mitochondria we identified and cloned the human orthologue of Fis1p (hFis1) and studied its function in mammalian mitochondrial morphology.
Materials and methods

Isolation and expression of hFis1

The human homologue of Fis1 (NP057152) was shown to be CGI-135-like (BC009428) after performing a Fasta 3.3 homology search (EBI, Hinxton, UK). Oligos derived from the 5 prime and 3 prime UTR of CGI-135-like were used to amplify, by PCR (Pwo or Tgo polymerase-Roche, Rotkreuz, Switzerland), CGI-135-like from a human liver library (Serono, Geneva, Switzerland). This template was used in a further PCR reactions to add His (MAHHHHHHH) or HA (MQDLPGNDNSTAGL) tags 5 prime to the ORF of hFis1 lacking the initial methionine, or a deletion mutant which lacked the last 28 amino acids (TM). The PCR products were ligated into pCI (Promega, Madison, USA) and all positive clones sequenced. YFP/CFP/GFP-hFis1 was generated by PCR with the appropriate oligonucleotides to add restriction enzyme sites to the ORF or ORF-TM of hFis1, which was then subcloned into pECFP-CI, pEGFP-CI or pEYFP-CI or pEGFP-N1 (Clontech, Palo Alto, CA). Expression plasmids for HA-Drp1 and HA-Drp1 K38A were obtained from A van der Bliek and subcloned using PCR into pCI with an alternative HA tag (as above) or as YFP/CFP fusions. Recombinant hFis1 protein was generated by subcloning His-hFis1 TM into pTYB1 (New England Biolabs, Beverly, MA). The resultant plasmid was transformed into E.coli BL21 cells for production of the recombinant protein. The intein-His-hFis1 fusion protein was purified over a chitin column, cleaved by DTT, further purified by affinity to Nickel resin (Qiagen, Basel, Switzerland) and subsequently dialysed against 30% glycerol, 25 mM Hepes, 1 mM...
DTT. Immunisations and antisera collection were performed by Sigma-Genosys (Pampisford, UK).

Cell Culture and reagents
HEK 293, Cos-7 and HeLa cells were cultured in DMEM+ 10% Fetal bovine serum, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin and 2 mM Glutamine and maintained in 5% CO₂ at 37°C. Tissue culture plates were obtained from Nunc (Roskilde, Denmark) and all other cell culture reagents were obtained from Sigma (Buchs, Switzerland). The mitochondrial specific dye Mitotracker Red CMXRos, was obtained from Molecular Probes (Eugene, OR) and the caspase inhibitor zVAD was from Enzyme Systems Products (Livermore, CA). Fluorescent images were visualized using a Zeiss Axiovert 135TV with a 100x objective and images captured using a CCD camera (photometric CE200A) with IP Lab software. Alternatively, for confocal microscopy, images were visualised using a Zeiss inverted 200M microscope with a scanhead QLC100 Nipkow disk (Yokogawa) equipped with a 3 lines Argon Laser 457/488/514 (Laser Physics) with AOTF control of laser lines (Visitech). Pictures were acquired with a Coolsnap HQ Camera (Roper Scientific). Image acquisition and analysis was performed with Metamorph/Metafluor 4.1.2 software (Universal Imaging). The resultant images were processed using Adobe Photoshop 6.

Subcellular fractionation
HeLa cells transfected with His-hFis1 for 24 h (or untransfected for endogenous hFis1) were fractionated as described previously 14. 100 µg of the mitochondrial fraction was incubated in 200 µl of MB buffer with 20 µg of proteinase K at room temp for the
indicated times, subsequently pelleted at 10,000 g and lysed in 50 µl 1x SDS loading buffer and separated by SDS/PAGE for analysis by western blotting. The anti-prohibitin antibody was from Neo Markers (Fremont, CA), anti-human Bcl-xL from Chemicon (Tenecula, CA) and anti-His (H15) from Santa Cruz (Santa Cruz, CA).

Live cell fluorescence

Cos-7 or HeLa cells were plated at 2.5x10^5 cells/ 3 cm glass bottomed dish (Mattek, USA) 6 h prior to transfection. Cells were then transfected using Fugene (Roche) with a 1:1 ratio of target DNA to YFP-mito (Clontech) and a total of 1 µg DNA/ dish and observed after a minimum of 10 h post-transfection.

Luciferase Assay

Cos-7 cells in 3 cm dishes were transfected using Calcium phosphate with up to 2.0 µg of target DNA (pCI-GFP was used to make up any difference) together with 2.0 µg of pCI-Luc. All transfections were performed in triplicate. 16 h after transfection cells were washed once in TBS and cultured for a further 20 h. Cells were then washed once in PBS and lysed in 500 µl luciferase assay buffer (Promega) for 15 min at room temp. 10 µl of the extract was mixed with 50 µl of LDBII and immediately measured using a Turner TD-20e luminometer. All tranfections were performed in triplicate.

Immunocytochemistry

HeLa or Cos-7 cells were plated on glass coverslips at a confluency of 50%-80% in 6 well plates and maintained in culture medium for 2-3 hours before transfection with 5 µg of DNA using a standard calcium Phosphate transfection procedure. 16 h after
transfection the cells were washed in TBS for 10 minutes, followed by up to 20 h incubation in culture medium supplemented with 50 µM zVAD. Cells were fixed in 4% paraformaldehyde/PBS for 20 minutes at room temperature followed by PBS washes. The cells were then permeabilised with 0.1% saponin in PBS for 15 minutes at room temperature and after PBS washes were blocked for 1 hour at 4°C with PBS containing 0.1% saponin and 5% BSA (bovine serum albumin). The cells were then incubated with primary antibodies diluted in PBS 0.1% saponin 5% BSA for 2 hours at room temperature followed by washes in PBS 0.1% saponin. Immunoreactive proteins were visualised by incubating the cells with FITC coupled mouse secondary and Texas Red coupled rabbit secondary antibodies (Vector Labs, Burlingame, CA) in PBS containing 0.1% saponin for 1 hour at room temperature, followed by PBS washes. During the last PBS wash, the cells were co-stained with Hoechst 33258 (25 µg/ml) in order to visualise the nucleus. The coverslips were then mounted using Vectashield H-100 fluorescent mounting medium (Vector Labs). In order to visualise endogenous hFis1, untransfected cells, that had been plated on glass coverslips, were fixed for 5 minutes in 100% methanol and processed as above. For UV induced apoptosis, cells were plated on glass coverslips as described above and transfected, using Fugene (Roche) with 0.2 µg YFP-mito together with 1.0 µg of Bcl-xL or HA-Drp1(K38A) or pCI-Luciferase. 48 h after transfection 100 µM zVAD was added to the media and the cells irradiated at 90 mJ/cm² using a UV Stratalinker 2400 (Stratagene), cultured for a further 16 h and processed for immunofluorescence as above. To assay cell death, prior to UV irradiation zVAD was omitted from the culture medium. 16h following UV irradiation cells were incubated for 30 min with Hoechst 33258, as described above, and transfected cells scored for
apoptotic nuclei. Cells that had a diffuse, non-mitochondrial immunofluorescence using the cytochrome c antibody were scored as having released cytochrome c. Cells were scored as having a fragmented mitochondrial network when >50% of the mitochondria appeared punctate. The following primary antibodies were used: monoclonal anti-cytochrome C antibody (Pharmingen, Becton Dickinson, Germany), anti-His polyclonal antibodies (Santa Cruz), anti-mHsp70 (ABR, Golden, CO).

Immunoprecipitation

Cells were seeded in 14 cm dishes 2 h prior to transfection and transfected with a total of 20 µg DNA using standard calcium phosphate techniques. 16 h after transfection, cells were washed once with 1xTBS, cultured for a further 8-20 h, washed once with 1xPBS and lysed in 1 ml of immunoprecipitation buffer (10 mM Hepes, 143 mM KCl, 5mM MgCl₂, 1mM EGTA, 0.5% (v/v) IGEPAL CA 630 and supplemented with 1x proteinase inhibitor cocktail (Roche)) for 20 min on ice. The lysate was cleared by centrifugation at 10,000 g, incubated with anti-HA antibody (D. Picard, University of Geneva) for 16 h at 4°C and the immunocomplexes captured on 20 µl protein A/G beads (Pharmacia) for 3 h at 4°C. Beads were subsequently washed 4-5 times with 1 ml of lysis buffer and finally resuspended in 40 µl 1x loading buffer, separated by SDS PAGE, transferred to Nitrocellulose and probed with monoclonal anti-Bcl-xL (Pharmingen) or anti –HA antibody.
Results

The human homologue of yeast Fis1p

Recent advances made in yeast have shed more light on the mechanisms that serve to divide or fuse mitochondria\(^6\), and the possibility that these may play a part in the apoptotic demise of a cell\(^12\). Whilst yeast Fis1p has been shown to be localized in the outer membrane of yeast mitochondria and to be necessary for yeast mitochondrial fission\(^15\), the function of this protein in higher order eukaryotes is unknown. A search of the EMBL database confirmed the human homologue of yeast Fis1p as CGI-135-like (Acc no: Q9BTB3) which was similar to a transcript identified using comparative gene identification (CGI) of the human and\(^16\) C.elegans databases. CGI-135-like, which we call hFis1, is 30% homologous to Fis1p at the protein level and 23% at the nucleotide level. Further analysis revealed an orthologue in the mouse (Acc. No: Q9C092) which was 96% homologous to hFis1 and orthologues in C.elegans (Q19383) and D.melanogaster (AAL48886) which show 41% and 42% similarity to hFis1 respectively(Figure 1a). Interestingly there are two proteins in C. elegans (Q19383, Q20291) and two in plants (A.thaliana, Q94CK3, Q9M1J1) which are homologous to hFis1 but as yet, only one Fis1p homologue has been found in humans. Two regions of high similarity are apparent from the alignment: the region spanning amino acids (aa) 71-87 from hFis1 which contains no known motif and the region spanning aa 127-144 which Prosite (EBI, Hinxton, UK) identifies as a putative transmembrane domain. Prosite additionally identified a possible leucine zipper (aa 77-98), a coiled coil (aa108-120) and a TPR repeat which suggest that hFis1 preferentially interacts with proteins containing WD40 repeats and could aggregate in multi-protein complexes.
**hFis1 localises to the mitochondria**

A polyclonal antibody was raised against recombinant hFis1\x1f\x26TM and used to assess the subcellular localization of hFis1 in HeLa cells. The cells were found to display a typical mitochondrial staining which was confirmed by co-staining with the mitochondrial marker mHsp70 (Figure 1c). We also generated expression constructs (Figure 1b) to confirm the localization of hFis1, and tested the expression in other cell types including Cos-7 and HEK-293 cells (data not shown). Immunofluorescence performed on these cells expressing His-hFis1 confirmed a mitochondrial staining pattern (Figure 1d). In contrast, a mutant of hFis1 lacking the C-terminus (His-hFis1 \x26TM) was found to be cytosolic, indicating that the C-terminus is required for the mitochondrial localization. In order to define the submitochondrial localization of endogenous hFis1, mitochondria from HeLa cells were isolated and treated with proteinase K which digested the parts of the outer membrane proteins exposed to the cytosol. Incubation with proteinase K reduced endogenous hFis1 whereas it did not reduce the levels of prohibitin which is localized on the inner mitochondrial membrane, indicating that the outer mitochondrial membrane remained intact during the treatment with proteinase K (Figure 1e). We furthermore showed that the over-expressed His-tagged hFis1 was also sensitive to proteinase K digestion (Figure 1f). The proteolytic treatment significantly reduced the levels of another mitochondrial membrane protein: Bcl-x\_L. We therefore concluded that both endogenous and over-expressed hFis1 were localized to the outer mitochondrial membrane and that the presence of the N-terminal tag did not affect the localisation of hFis1.
**hFis1 promotes fragmentation of the mitochondrial network.**

During these studies we noticed that the morphology of mitochondria in cells overexpressing hFis1 was abnormal (Figure 1d) and therefore decided to assess the role of hFis1 in mitochondrial morphology. In order to clearly visualise the changes induced by hFis1 on mitochondrial morphology we fused hFis1 to the C-terminus of YFP. Figure 2 shows representative fluorescent images of YFP-hFis1 expressing cells. At early time points (approx 10 h, top panel) the mitochondrial network started to bud or fragment and some mitochondrial swelling was apparent. Later time points (middle panel) were typified by mitochondria which appear banded or striped and later still (approx 16h, bottom panel) the network had degenerated into the punctiform phenotype which appeared to be identical to the His-tagged hFis1 (Figure 1d and data not shown). We furthermore confirmed that the structures were part of a mitochondrial network by loading the cells with Mitotracker Red CMX Ros (data not shown). The deletion mutant of hFis1 (hFis1 TM) was fused to the N-terminus of GFP and its expression pattern showed that this mutant remained localized to the cytosol and did not affect mitochondrial morphology (data not shown). To rule out the possibility that insertion of a protein with a transmembrane domain into the outer membrane of mitochondria was sufficient to trigger fragmentation, we expressed a GFP protein fused to the transmembrane domain of Bax. This construct localized to mitochondria but did not induce fragmentation (data not shown). Therefore the effects seen with hFis1 appear to be specific.
**hFis1 induced fragmentation is inhibited by mutant Drp1**

Changes in mitochondrial morphology in mammals is known to involve Drp1 which can relocalise from the cytosol to the points of mitochondrial division. Expression of a dominant negative mutant of Drp1 (Drp1(K38A)) has also been shown to hinder the changes in mitochondrial morphology induced by apoptotic inducers such as staurosporine (STS). We therefore examined the possibility that Drp1(K38A) might prevent the fragmentation induced by hFis1. Drp1 and Drp1(K38A) were subcloned and expressed as HA or CFP fusions and subsequently co-transfected with YFP-hFis1 to assess their effect on hFis1 induced changes in mitochondrial morphology. Expression of CFP-Drp1 had no discernable effect on hFis1-induced fragmentation (Figure 3a). However, co-expression of CFP-Drp1(K38A) altered the morphology induced by hFis1 (Figure 3b), and the mitochondrial network appeared as a number of long filaments as well as cisternae-like structures. A quantitative analysis revealed that expression of Drp1(K38A) was able to reduce the number of hFis1-expressing cells with fragmented mitochondria by 31% and 24% at 24 and 36 h respectively as compared to cells transfected with hFis1 alone.

These results prompted us to investigate how the dominant negative of Drp1 inhibited hFis1 induced fragmentation. In yeast, it has been shown that in the absence of Fis1p, Dnm1p (the yeast orthologue of Drp1) is not recruited to mitochondria. Therefore we tested if Drp1(K38A) blocked the recruitment of Drp1 to the mitochondria. HeLa cells were co-transfected with hFis1, CFP-mito and YFP-Drp1 in the presence or absence of HA-Drp1(K38A). Thirty six hours after transfection the cells were fixed and examined using confocal microscopy. Figure 3c shows that Drp1 (shown in red) localized
principally to the ends of the mitochondria (shown in green). Expression of Drp1(K38A) dramatically altered the localization of Drp1, sequestering it into a number of (usually 3-5) discrete spots (Figure 3d). We also verified that expression of the dominant negative Drp1 was able to alter localization of Drp1 in the absence of transfected hFis1 using YFP-Drp1 and CFP-Drp1(K38A) (see supplemental data). In addition, western blot analysis of sodium carbonate treated mitochondria showed that Drp1(K38A) diminished the amount of Drp1 found strongly associated with the outer mitochondrial membrane (Figure 3e). Taken together, these data indicate that Drp1(K38A) prevented hFis1-induced mitochondrial fragmentation by sequestering Drp1, thereby preventing its localization to the mitochondria.

*Expression of HA-FisFL induced a caspase-dependent reduction in cell viability.*

Our data so far showed that tagged hFis1 was effective in promoting the fragmentation of mitochondria and that its localization to the mitochondrial membrane was necessary for its function. However we had noted that expression of hFis1 for more than 24 hours correlated with an increased number of detached cells. We therefore investigated if fragmentation of mitochondria by hFis1 could affect cell viability. We utilized a luciferase assay where a reduction in the translation of a transfected luciferase construct correlates with a decrease in cellular viability after co-transfection with a target gene. Co-transfection with increasing amounts of HA-hFis1 reduced the amount of luciferase in a dose-dependent manner (Figure 4a). As a positive control we used a plasmid encoding His-tagged Bax which has previously been shown to induce cell death and observed a pronounced decrease in luciferase activity (data not shown). The addition
of the pan-caspase inhibitor zVAD (100 µM) effectively blocked the reduction in the luciferase activity at all concentrations of HA-hFis1 transfected (Figure 4a and data not shown) indicating that a caspase-dependent process was leading to the changes in viability as assayed by luciferase activity.

**hFis1 induces cytochrome c release**

Mitochondria are considered as pivotal organelles in many apoptotic responses. Perturbation of the outer membrane often leads to the release of apoptogenic factors including cytochrome c. We therefore investigated if the expression of hFis1 led to cytochrome c release (Figure 4b). HeLa cells were transiently transfected with His-tagged hFis1 and were fixed and stained for His expression and cytochrome c. Expression of hFis1 promoted the release of cytochrome c as shown by the diffuse staining of the cells. Identical results were also obtained using YFP-hFis1. Expression of the TM mutant (His-hFis1 TM), which does not localize to mitochondria, did not alter the distribution of cytochrome c (Figure 4c). These results therefore suggested that misregulated expression of hFis1 was sufficient to promote the redistribution of cytochrome c from the mitochondria to the cytosol.

**Bcl-xL and Drp1(K38A) inhibit the cytochrome c release induced by hFis1**

Anti-apoptotic proteins of the Bcl-2 family, as well as Drp1(K38A) 12, are known to inhibit the apoptotic process at the mitochondrial membrane. We therefore tested if the co-expression of Bcl-xL and Drp1(K38A) could block the hFis1 induced cytochrome c release. Cells were transiently transfected with His-hFis1 and Bcl-xL or Drp1(K38A) and
stained for cytochrome c 36 h later (Figure 5a). While 50% of cells expressing hFis1 had released cytochrome c, only 10% of cells co-expressing Bcl-xL and hFis1 and 15% of cells expressing Drp1(K38A) displayed a diffuse cytosolic cytochrome c staining (Figure 5b). Interestingly, the mitochondria in all cells co-expressing Bcl-xL and hFis1 were punctiform (Figure 5a), whereas cells co-expressing Drp1(K38A) and hFis1 principally displayed elongated mitochondria.

*Bcl-xL but not Drp1(K38A) prevent hFis1-induced apoptosis*

We also investigated if Bcl-xL and Drp1(K38A) could also prevent apoptosis-induced by hFis1. We therefore co-transfected His-tagged hFis1 with Bcl-xL or Drp1(K38A) and luciferase (Figure 5c). Bcl-xL but not Drp1(K38A) was able to inhibit the reduction in luciferase activity induced by hFis1. To ensure that the changes in luciferase activity were mirrored by changes in cell viability, cells were incubated with Hoechst and apoptotic nuclei were counted. As shown in Figure 5c Bcl-xL significantly reduced the number of apoptotic nuclei induced by hFis1 expression whereas Drp1(K38A) had no effect.
Discussion

hFis1 triggers the fragmentation of mitochondria

In this study we have identified the human orthologue of Fis1p (hFis1) and show that its expression in cultured cells induces fragmentation of the mitochondrial network. The morphology of mitochondria resulting from the overexpression of hFis1 in mammalian cells is indicative of a role in mitochondrial fission and is consistent with previous data obtained in yeast \(^\text{18,15}\). Although this finding would argue that the mitochondrial division machinery is conserved during evolution, nevertheless some differences are notable between species. Over-expression of YFP-hFis1 in various mammalian cells (HEK, Cos-7, HeLa) clearly resulted in mitochondrial fragmentation (Figure 2a) whereas yeast cells that expressed GFP-Fis1p displayed no apparent fragmentation effect yet there was clear mitochondrial localization\(^\text{15}\). Moreover, overexpressed Drp1 did not lead to fragmentation of mammalian mitochondria (data not shown and \(^\text{10}\), as also shown in \textit{S. cerevisiae} (Dnm1p\(^\text{15}\)), whereas the orthologue in \textit{C. elegans} (DRP\(^\text{p}\)) localised to sites of mitochondrial division and promoted fission. Our data also showed that overexpressed Drp1 was insensitive to sodium carbonate treatment which suggested that Drp1 was strongly attached to the mitochondrial membrane. In our experiments, overexpressed hFis1 in the outer mitochondrial membrane was sufficient to trigger a complete mitochondrial fission. Although no increase in the amount of overexpressed Drp1 was observed it is possible that the conformation of Drp1 is altered upon hFis1 expression. One hypothesis is that high hFis1 levels overcome an endogenous inhibitor that would normally maintain the endogenous hFis1 in an inactive state and would impede the recruitment of the division apparatus. It is possible that in mammalian cells the signal that


triggers fission of the OMM, as in yeast\(^8\), initiates from the inner membrane fission apparatus. The inhibition of hFis1-induced fragmentation by Drp1(K38A) also places hFis1 within the mitochondrial fission apparatus, upstream of Drp1. However, we have been unable to co-immunoprecipitate endogenous Drp1 and hFis1 and therefore the mechanism by which hFis1 recruits Drp1 is still unclear. The fact that this inhibition was only partial (on average 30\%) suggests that Drp1(K38A) is unable to sequester all of the endogenous Drp1 from points of mitochondrial fission or that Drp1-independent fragmentation also occurred.

*Mitochondrial fragmentation and apoptosis*

A link between mitochondrial fission and apoptosis has been recently made by Frank et al. who reported that a mutant of Drp1, DrpK38A, was able to prevent apoptosis triggered by many stimuli. In addition, it was found that the pro-apoptotic Bcl-2 family member Bax colocalised with members of both the fusion (Mfn2) and fission (Drp1) apparatus\(^12,19\). Here we show that prolonged mis-regulation of the mitochondrial fission apparatus was lethal for mammalian cells. Over-expression of hFis1 rapidly fragmented the mitochondrial network (Figures 1d and 2) into clusters of punctate mitochondria that surrounded the nucleus. This striking change in morphology was accompanied 16 h later by a release of cytochrome c from the mitochondria and subsequent cell death. Overexpression of Bcl-x\(_L\) was able to inhibit cytochrome c release and cell death, although this failed to prevent mitochondrial fragmentation. This suggests that Bcl-x\(_L\) does not interfere with the fission machinery per se. However, over-expressed Bcl-x\(_L\) was found to co-immunoprecipitate with over-expressed hFis1 (see supplemental data) but
this interaction was not detected between the endogenous proteins. In contrast to Bcl-xL, Drp1K38A was able to prevent mitochondrial fission but not cell death. This suggested that the mechanism by which hFis1 triggered apoptosis did not involve proapoptotic Bcl-2 family members since Drp1K38A is known to inhibit many death stimuli involving these proteins\textsuperscript{12}. Indeed, we found that neither Bax nor Bak were activated in this process as no conformational change in the N-terminus of these proteins was detected using specific antibodies (data not shown). In addition, the cell death process appeared to be independent of the opening of the permeability transition pore (PTP) which Bcl-xL is known to prevent\textsuperscript{20}. Indeed, treatment of hFis1 expressing cells with inhibitors of the PTP, namely bongkrekic acid and cyclosporin A, did not reduce the amount of cell death (data not shown). Taken together these results suggest that Bcl-xL inhibit Fis1-induced apoptosis by a mechanism that is Bax, Bak and PTP independent. How prolonged expression of hFis1 triggers apoptosis requires further investigation.

These data are important in light of findings that have shown that a disease linked to mutations in the OPA1 gene, which encodes a protein involved in the fusion of the inner mitochondrial membrane\textsuperscript{22,23,24}, leads to degeneration of the retinal ganglion cells. Monocytes from patients with OPA1 mutations displayed an abnormal mitochondrial network, with clumps of punctate mitochondria\textsuperscript{21}, therefore suggesting that mutations in OPA1 lead to a more fragmented mitochondrial network. Our results suggest that the deregulated fission of mitochondria in the ganglion cells of the retina could be responsible for their degeneration through apoptosis.

The characterization of a second component of the mammalian mitochondrial fission machinery provides another piece of the jigsaw of this complex process. It will be
interesting to see, if like OPA1, defects in the regulation hFis1 are involved in human pathologies.

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Figure Legends

Figure 1 Isolation, expression and localization of hFis1

A) Comparison of the orthologs of yeast Fis1p from human (Q9BTP3), mouse (Q9CO92) C.elegans (Q19383) and drosophila (AAM68367). Sequences were aligned using the ClustalW tool (EBI, Hinxtor, UK). Solid box – region of high homology between species. Dashed box putative transmembrane domain. B) Schematic of the tagged hFis1 proteins used within this study. L= leucine zipper, C=coiled coil, TM= transmembrane domain. C) Localisation of endogenous hFis1 in HeLa cells. Double staining with anti-mHsp70 and anti-hFis1 antibodies on methanol fixed cells revealed a mitochondrial localization. D) Expression patterns of His-tagged hFis1 and hFis1 TM in fixed Cos-7 cells. hFis1 revealed by staining with anti-His antibody and mitochondria revealed by staining for the mitochondrial matrix protein mHsp70. E) Endogenous hFis1 from isolated HeLa cell mitochondria is localized on the outer mitochondrial membrane and diminished following treatment with proteinase K. R = recombinant His-tagged hFis1, I = untreated mitochondria, Pk = mitochondria treated with proteinase K. F) Transfected His tagged hFis1 is localized on the outer mitochondrial membrane and levels diminish with proteinase K treatment. Levels of Bcl-xL and prohibitin, localized on the outer and inner membranes respectively are shown as controls.

Figure 2. hFis1 induced changes in mitochondrial morphology.
A) Cos-7 cells transfected with YFP-hFis1 showing different time points after transfection: from early (approx. 10 h, top panel) to late (approx. 16 h, bottom panel). Inset in middle panel (approx. 14 h) shows the detail of the banded mitochondria.

Figure 3. Drp1 mutant inhibits the morphological changes induced by hFis1

Cos-7 cells expressing A) YFP-hFis1 and CFP-Drp1 or B) YFP-hFis1 and CFP-Drp1(K38A). C) HeLa cells expressing YFP-Drp1 (in red), CFP-mito (in green) and hFis1. Most of the Drp1 is localized to the tips of mitochondria. D) As in (C) but with co-expression of HA-Drp1(K38A). YFP-Drp1 relocalized from the tips of mitochondria to three discrete points that do not co-localize with mitochondria. E) Mitochondria were isolated from cells expressing the constructs as shown and treated with sodium carbonate prior to analysis by western blotting. Co-expression of His-Drp1(K38A) reduced the amount of HA-Drp1 tightly associated/bound to the outer mitochondrial membrane.

Figure 4. hFis1 induces cell death and cytochrome c release

A) HeLa cells transfected with luciferase together with His-tagged hFis1 or with luciferase together with a control plasmid (pCI-GFP) and assayed 36h after transfection. These data are representative of five independent experiments; data represent the mean luciferase values + standard deviation. HeLa cells transfected with His-tagged hFis1 (B) or His-tagged hFis1 TM (C) for 36 hours and probed with anti-His or anti-cytochrome c as shown.

Figure 5. Bcl-xL and Drp1(K38A) prevents hFis1 induced cytochrome c release
A) HeLa cells were transfected with His-tagged hFis1 and Bcl-xL or Drp1(K38A) and probed as in Figure 5. B) Transfected cells were scored for those that had released cytochrome c. The data are shown as the mean + standard deviation of three independent experiments. C) Cotransfection of Bcl-xL, but not Drp1(K38A), inhibits cell death induced by hFis1 (left panel). Luciferase values were normalized by taking the value of luciferase activity in the presence of zVAD as 100%. Transfected cells were also cultured in the absence of zVAD and labelled with Hoechst; apoptotic cells scored according to nuclear morphology (right panel).
Figure 1
Figure 1
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Figure 4
Figure 5
Figure 5
Supplemental Figure 1. Sequestration of Drp1 by Drp1(K38A) in live cells.

YFP-Drp1 and CFP-Drp1(K38A) were transfected singly or together in HeLa cells and visualized 24 hours after transfection. YFP-Drp1 exhibited a fine punctate fluorescence in the cytosol, whereas CFP-Drp1(K38A) fluorescence was limited to 3-5 large dots in the cytoplasm. When expressed together, YFP-Drp1 fluorescence colocalised with CFP-Drp1(K38A).

Supplemental Figure 2. Bcl-xL immunoprecipitated by hFis1.

Immunoprecipitation of Cos-7 cells 36 h after transfection with HA-tagged hFis1 in the presence or absence of His tagged Bcl-xL or His-tagged Bax. HA-tagged hFis1 immunoprecipitated Bcl-xL but not Bax.
Figure S1 (James et al)
Figure S2 (James et al)
hFis1, a novel component of the mammalian mitochondrial fission machinery
Dominic I. James, Philippe A. Parone, Yves Mattenberger and Jean-Claude Martinou

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hFis1, a novel component of the mammalian mitochondrial fission machinery.

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Page 36378: Fig. 5C was inadvertently omitted. Panel C is shown below.

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Wounding induces motility in sheets of corneal epithelial cells through loss of spatial constraints. Role of heparin-binding epidermal growth factor-like growth factor signaling.

Ethan R. Block, Abigail R. Matela, Nirmala SundarRaj, Erik R. Iszkula, and Jes K. Klarlund

Page 24309, Fig. 2A: This experiment was performed in SHEM medium, which contains EGF. In the absence of EGF, little cell-cell dissociation occurs after removal of the agarose droplets, although numerous lamellipodia are formed at the edges of the strands of cells.