Levels of human Fis1 at the mitochondrial outer membrane regulate mitochondrial morphology

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Accepted 12 December 2003
Journal of Cell Science 117, 1201-1210 Published by The Company of Biologists 2004
doi:10.1242/jcs.01058

Summary

Mitochondria undergo balanced fission and fusion events that enable their appropriate networking within the cell. In yeast, three factors have been identified that co-ordinate fission events at the mitochondrial outer membrane. Fis1p acts as the outer membrane receptor for recruitment of the dynamin member, Dnm1p and the WD40-repeat-containing protein Mdv1p. In mammals, the Dnm1p counterpart Drp1 has been characterized, but other components have not. Here, we report the characterization of human Fis1 (hFis1). hFis1 is inserted into the mitochondrial outer membrane via a C-terminal transmembrane domain that, along with a short basic segment, is essential for its targeting. Although expression of hFis1 does not complement the phenotype of yeast cells lacking Fis1p, overexpression of hFis1 in tissue culture cells nevertheless causes mitochondrial fragmentation and aggregation. This aggregation could be suppressed by expressing a dominant-negative Drp1 mutant (Drp1^{K38A}). Knockdown of hFis1 in COS-7 cells using RNA interference results in mitochondrial morphology defects with notable extensions in the length of mitochondrial tubules. These results indicate that the levels of hFis1 at the mitochondrial surface influences mitochondrial fission events and hence overall mitochondrial morphology within the cell.

Key words: Mitochondria, Protein import, Fis1, Organelle biogenesis

Introduction

Mitochondria are dynamic organelles that vary in copy number, size and position within any given cell type, often reflecting the energy requirements of the cell. The inheritance of mitochondria, maintenance of their characteristic shape and their positioning within the cell are mediated by their association with cytoskeletal elements as well as balanced, and opposing, fusion and fission events (Bereiter-Hahn and Voth, 1994; Yaffe, 1999; Shaw and Nunnari, 2002). Mitochondrial fusion and fission processes are vital for maintenance of organellar morphology and distribution, but also for cell proliferation and cell differentiation. The mechanisms underlying these processes are likely to be more complex for mitochondria because the breaking and mixing of four lipid bilayers is required, which presents distinct topological and energetic barriers.

Components of the mitochondrial fission and fusion machinery have been identified and characterized in both lower and higher eukaryotes, and particular progress has been made on the fission machinery of the outer membrane (McNiven et al., 2000; Yoon and McNiven, 2001; Shaw and Nunnari, 2002; Westermann and Prokisch, 2002). In *Saccharomyces cerevisiae*, mitochondrial fission was found to be dependent on the dynamin-related GTPase Dnm1p. Dnm1p assembles on the mitochondrial outer membrane, cycling on and off the mitochondria similar to the cycling of dynamin on and off the plasma membrane during endocytosis (Otsuga et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999).

Mutations in both Dnm1p and its functional orthologues, such as human (Smirnova et al., 2001) and *Caenorhabdtis elegans* (Labrousse et al., 1999) Dynamin-related protein (Drp1), were all shown to block mitochondrial fission, establishing a central role for the protein in the fission process. It has recently been found that mitochondrial fission and apoptosis are linked via the pro-apoptotic molecule Bax, which localizes to Drp1-containing puncta (Frank et al., 2001; Karbowski et al., 2002). This finding, as well as mitochondrial fragmentation being one of the early indicators of apoptosis (Desagher and Martinou, 2000), indicates that mitochondrial fission is involved in the process of programmed cell death.

Genetic approaches to identify Dnm1p partner proteins in yeast allowed the identification of two novel molecules, Mdv1p (mitochondrial division 1 protein, also termed Fis2p, Net2p or Gag3p) and Fis1p (Fission 1 protein, also termed Mdv2p or Gag2p) (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000). Although it is not clear how Dnm1p, Mdv1p and Fis1p behave at the molecular level to facilitate mitochondrial fission, all three are clearly required. Mutations in Mdv1p and Fis1p, like Dnm1p, block mitochondrial fission, giving rise to a highly interconnected mitochondrial network owing to the occurrence of fusion events (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Mdv1p is an 80 kDa, WD-40-repeat-containing, peripheral membrane protein that localizes with Dnm1p at punctate structures along the mitochondrial outer membrane. Fis1p, a 17-kDa integral membrane protein, is uniformly distributed along the mitochondrial outer membrane. It has been suggested that Fis1p regulates the targeting and assembly

of Dnm1p to the mitochondria early on in the fission path (Mozdy et al., 2000; Tieu and Nunnari, 2000), although recent genetic studies indicate that Fis1p also functions at a later step in the fission process with Mdv1p (Tieu et al., 2002).

Here, we report the characterization of human Fis1 (hFis1) and identify it as a mitochondrial outer membrane protein with its targeting dependent on the presence of a C-terminal basic segment. Although expression of hFis1 does not complement the phenotype in yeast *Δfis1* cells, we show that increases in expression of hFis1 in mammalian cells result in mitochondrial fragmentation and aggregation as a consequence of increased fission events. Conversely, depletion of hFis1 induces the formation of extensions and interconnections of mitochondrial tubules. These results indicate that hFis1 is a component of the fission machinery at the mitochondrial surface.

Materials and Methods

Cloning procedures

Standard methods were used for manipulation of DNA (Sambrook et al., 2001). The cDNA encoding hFis1 (accession number AAH03540) was obtained from the I.M.A.G.E. consortium (MRC geneservice, UK). For creation of fusions between green fluorescent protein and hFis1 (GFP-hFis1), GFP-hFis1$^{K149/151A}$, GFP-hFis1^{K149A} or GFP-hFis1^{K151A} and GFP-hFis1$^{\Delta 122\text{-}152}$, the hFis1 open reading frame was amplified using the common forward primer 5′-GAAAAGCTT-GAGGCCGTGCTGAACGAGCTG-3′. Reverse primers used for the creation of each construct were: 5′-GAAGGATCCTCAGGATTT-GGACTTGGACAC-3′ for GFP-hFis1; 5′-TTCGGATCCTCAGGA-CCCGGAGCCGGACACAGCAAGTCC-3′ for GFP-hFis1K149/151A; 5′-TTCGGATCCTCAGGATTTGGAGCCGGACACAGCAA-GTTC-3′ for GFP-hFis1K149A; 5′-TTCGGATCCTCAGGACCCG-GACTTGGACACAGCAAGTTC-3′ for GFP-hFis1K151A and 5′-GGCGGATCCTCATTTCTTCATGGCCTT-3′ for GFP-hFis1$^{\Delta 122\text{-}152}$. The DNA products were digested with *Hin*dIII and *Bam*HI and cloned into a modified form of the pE-GFP vector (Clontech) to give in-frame C-terminal fusions to GFP. For creation of GFP-hFis1$^{\Delta 1\text{-}121}$, the GFP-hFis1 plasmid was used as a template for inverse PCR using the forward primer 5′-GGGGGTACCAAGCTTGTACAGCTCGTCCA-TGCCGAG-3′ and reverse primer 5′-GGGGGTACCGGACTC-GTGGGCATGGCCATCGTGGGA-3′. For creation of p-hFis1, a construct permitting the expression of hFis1 alone in mammalian cells, the hFis1 ORF was amplified by PCR using the forward primer 5′-GCCAAGCTTATGGAGGCCGTGCTGAAC-3′ and the reverse primer 5′-GAAGGATCCTCAGGATTTGGACTTGGACAC-3′. The DNA obtained was cloned into pE-GFP at *Hin*dIII and *Bam*HI sites, but the inclusion of a hFis1 termination codon prevented the creation of a GFP fusion, permitting overexpression of hFis1 alone.

For yeast constructs, PCR was performed to generate an *Eco*RI-*Sac*I fragment encoding hFis1 (amino acids 2-152) fused to the C-terminus of GFP (for p-GFP-hFis1), an *Eco*RI-*Sal*I fragment encoding hFis1 (amino acids 2-119) fused at the C-terminus of GFP and a *Sal*I-*Sac*I fragment encoding yFis1 (amino acids 122-155) [for p-GFP-hFis1(N)-yFis1(TM)], an *Eco*RI-*Sac*I fragment encoding yFis1 (amino acids 1-155) (for p-yFis1), an *Eco*RI-*Sac*I fragment encoding hFis1 (amino acids 1-152) (for p-hFis1), and an *Eco*RI-*Sal*I fragment encoding hFis1 (amino acids 1-119) and a *Sal*I-*Sac*I fragment encoding yFis1 (amino acids 122-155) [for p-hFis1(N)-yFis1(TM)]. Those fragments were cloned into the *Eco*RI-*Sac*I sites of pYX142, a low-copy-number yeast expression vector with the strong constitutive promoter of triose phosphate isomerase (Novagen).

For RNA interference studies, two complementary oligonucleotides (sense, 5′-GATCCAGGCATCGTGCTGCTCGAGTTCAAGAGAC-TCGAGCAGCACGATGCCTTTTTTTTGGAAA-3′; antisense, 5′-A-GCTTTTCCAAAAAAAGGCATCGTGCTGCTCGAGTCTCTTGA-ACTCGAGCAGCACGATGCCTG-3′) were designed specific for hFis1 depletion using p*Silencer* vectors according to the manufacturer's instructions (Ambion). The oligonucleotides were, annealed and cloned into p*Silencer* 2.0-U6 and p*Silencer* 3.0-H1 at *Bam*HI and *Hin*dIII sites. All clones were verified by DNA sequencing.

Yeast complementation analysis

The strains used in this study were ADM551 (*MATa*, *ura3-52*, *leu2Δ1*, *trpΔ63*, *his3Δ200*) and ADM552 (*MATa*, *ura3-52*, *leu2Δ1*, *trpΔ63*, *his3Δ200*, *fis1::HIS3*) (Mozdy et al., 2000). Standard genetic methods were used for transformation (Burke et al., 2000). Yeast transformants were grown at 30°C on SC media (2% dextrose) without leucine and/or uracil for plasmid selection. To visualize mitochondria, cells were transformed with the plasmid pVT100U-mtGFP encoding a mitochondria-targeted GFP (Westermann and Neupert, 2000).

Cell culture, transfection and immunofluorescence

COS-7 cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL) containing 10% (v/v) foetal calf serum at 37°C under an atmosphere of 5% CO_2 and 95% air. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At appropriate time points after transfection, cells were incubated with 80 nM MitoTracker Red CMXRos (Molecular Probes) for 30 minutes. The medium was removed and cells were rinsed in PBS before mounting coverslips face down onto microscope slides. For RNA interference studies, p*Silencer* 2.0-U6-hFis1 and p*Silencer* 3.0-H1-hFis1 constructs were co-transfected into COS-7 cells with pE-pOTC-GFP at a 9:1 ratio, using Lipofectamine 2000 (Invitrogen). Expression of pOTC-GFP afforded visualization of mitochondria in transfected cells.

For immunofluorescence, cells were fixed by incubation with 4% (w/v) paraformaldehyde in PBS, pH 7.4, for 10 minutes at room temperature, and permeabilized by incubation with PBS containing 0.2% (v/v) Triton X-100. For nuclear staining, cells were incubated with 0.01 mg ml^{-1} Hoechst 33258 (Sigma) in PBS for 10 minutes at room temperature before incubation with primary antibody (affinity-purified anti-hFis1 polyclonal antibodies) in 3% (w/v) bovine serum albumin, 0.2% (v/v) Triton X-100 in PBS for 1 hour. The secondary antibody used was a goat anti-rabbit FITC conjugate (Sigma).

Microscopy

Yeast cells were visualized using a Zeiss Axioplan microscope (Carl Zeiss) equipped with differential interference contrast (DIC) optics, epifluorescence capabilities and a Zeiss Acroplan-Neofluar 100× (NA 1.3) objective. GFP fluorescence was detected using a filter set (BP 450-490, FT 510 and LP 520). Images were captured using a Hamamatsu C5810 colour chilled 3CCD camera (Hamamatsu Photonics) and then processed using Adobe Photoshop (Adobe Systems).

Mammalian cells were visualized using an Olympus BX-50 fluorescence microscope fitted with a SPOT RT 3CCD camera (Diagnostic Instruments) and processed using SPOT Advanced software (version 3.4). In other cases, a Leica TC5 SP2 confocal microscope was used and images were processed using Adobe Photoshop.

Antibodies

Antibodies specific for hFis1 were raised in rabbits using a recombinantly expressed hFis1 cytosolic domain as antigen. Antibodies were subsequently affinity purified using hFis1 coupled to cyanogen-bromide-activated Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia). Antibodies to

Tom40, Tom22 and mtHsp70 have been reported elsewhere (Johnston et al., 2002). Anti-cytochrome-*c* antibodies were obtained from P. Nagley (Monash University, Melbourne) and NDUFA9 antibodies from Molecular Probes.

Miscellaneous

Mitochondrial isolation, protease digestion and carbonate extraction were performed using standard methods (Ryan et al., 2001; Johnston et al., 2002). Tris-tricine sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Schagger and von Jagow, 1987). Western blotting was performed using a semi-dry transfer method (Harlow and Lane, 1999). Immunoreactive proteins from blots were detected on Hyperfilm ECL (Amersham Pharmacia) using horseradish-peroxidase-coupled secondary antibodies and SuperSignal West Pico chemiluminescent substrate (Pierce).

Results

A human cDNA with significant homology (22% identity, 31% similarity) to yeast Fis1p has been previously described (Mozdy et al., 2000). The human cDNA contains a 456 base-pair open reading frame encoding a protein of 152 amino acids with a predicted molecular mass of 16.9 kDa. An amino acid alignment of the human ORF with known Fis1 orthologues from yeast, along with putative Fis1 proteins from *C. elegans* (T16332 and T16053) and mouse (*Mus musculus* Accession No. XP_213746), revealed that they all display a predicted transmembrane domain at their C-terminal ends with a short (three to five amino acids), basic C-segment, and a substantial hydrophilic N-terminal domain (Fig. 1).

To determine the subcellular location of the putative Fis1 protein, we transiently expressed it as a GFP fusion protein (GFP-hFis1) in COS-7 cells. Fluorescence microscopy revealed that, at low expression levels, the GFP-hFis1 chimera, was targeted to mitochondria, like that of the control pOTC-GFP (Yano et al., 1997) (Fig. 2A). Counterstaining of cells expressing GFP-hFis1 with MitoTracker Red confirmed this result (Fig. 2A, right).

In yeast, Fis1p is found uniformly distributed along the mitochondrial outer membrane, whereas Dnm1p and its mammalian homologue are located in punctae that form mitochondrial scission sites (Otsuga et al., 1998; Mozdy et al., 2000; Smirnova et al., 2001). To determine the fluorescence pattern of GFP-hFis1, we performed high-resolution confocal microscopy of mitochondrial networks from live cells expressing either GFP-hFis1 or the matrix-targeted pOTC-GFP. Before visualization of mitochondria, cells were additionally incubated with MitoTracker Red. pOTC-GFP was observed within mitochondrial tubules and overlapped with the matrix located MitoTracker Red (Fig. 2B, top). Interestingly however, in the case of GFP-hFis1-transfected cells, GFP fluorescence was found at the periphery of the mitochondrial tubules. This fluorescence was clearly separate from the matrix staining of MitoTracker Red, suggesting that hFis1 was located in the mitochondrial outer membrane in a diffuse pattern.

In order to verify that hFis1 was indeed found in the mitochondrial outer membrane, polyclonal antibodies were raised against amino acids 2-121 of hFis1 and used in western blot analysis. hFis1 was detected as a protein of 17 kDa and found in mitochondrial outer membrane vesicles (Fig. 3A). As a positive control, antibodies to the outer membrane protein Tom40 were used, and antibodies to the matrix located mtHsp70 and inner membrane complex I subunit NDUFA9 were used as negative controls. hFis1 was found in mitochondria isolated from various rat organs, indicating that it is probably ubiquitously expressed (Fig. 3B). The varying levels of hFis1 observed might reflect different morphological states of mitochondria in these tissues. Alternatively, they might be due to maintaining balances in opposed fusion events, given that differences in the levels of Mitofusins are also present in different cell types (Eura et al., 2003). This remains to be investigated.

Immunofluorescence analysis of mitochondria using affinity-purified hFis1 antibodies also confirmed that hFis1 was found in a diffused pattern with no punctae observed, whereas staining with pre-immune serum failed to reveal any signal. The labelling pattern with hFis1 antibodies was similar to that observed using antibodies to the control mitochondrial form of Hsp70 (mtHsp70) (Fig. 3C).

hFis1 is predicted to be anchored in the mitochondrial outer membrane via a C-terminal hydrophobic domain, with its N-terminal domain exposed to the cytosol. To confirm this, mitochondria were treated with or without externally added protease before western analysis. As can be seen (Fig. 3D), protease digestion leads to loss of detectable signal for hFis1 like that of the outer membrane receptor Tom22, whereas the intermembrane-space-located cytochrome *c* and matrix-located mtHsp70 remained proteolytically protected. Because

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H. sapiens              1 ---MEAVLNELVSVEDLLKFEKKFQSEKAAG----SVSKSTQFEYAWCLVRTRYNDDIRK
M. musculus             1 ---MEAVLNELVSVEDLKNFERKFQSEQAAG----SVSKSTQFEYAWCLVRSKYNDDIRR
C. elegans (T16053)     1 --------------MTNKNAREQYMRQCARG----DPSAASTFAFAHAMIGSKNKLDVKE
C. elegans (T16332)     1 --------MEPESILDFHTEQEEILAARAR-----SVSRENQISLAIVLVGSEDRREIKE
S. cerevisiae           1 MTKVDFWPTLKDAYEPLYPQQLEILRQQVVSEGGPTATIQSRFNYAWGLIKSTDVNDERL
                                                                          *

H. sapiens             54 GIVLLEELLPKGS-KEEQRDYVFYLAVGNYRLKEYEKALKYVRGLLQTEPQNNQAKELER
M. musculus            54 GIVLLEELLPKGS-KEEQRDYVFYLAVGNYRLKEYEKALKYVRGLLQTEPQNNQAKELER
C. elegans (T16053)    43 GIVCLEKLLRDDEDRTSKRNYVYYLAVAHARIKQYDLALGYIDVLLDAEGDNQQAKTLKE
C. elegans (T16332)    48 GIEILEDVVSDTAHSEDSRVCVHYLALAHARLKNYDKSINLLNALLRTEPSNMQATELRR
S. cerevisiae          61 GVKILTDIYKEAE--SRRRECLYYLTIGCYKLGEYSMAKRYVDTLFEHERNNKQVGALKS
                             *  *            *  * *         *        *   *  * *  *

H. sapiens            113 LIDKAMKKDGLVGMAIVGGMALGVAGLAGLIGLAVSKSKS  152
M. musculus           113 LIDKAMKKDGLVGMAIVGGMALGVAGLAGLIGLAVSKSKS  152
C. elegans (T16053)   103 SIKSAMTHDGLIGAAIVGGGALALAGLVAIFSMSRK----  138
C. elegans (T16332)   108 AVEKKMKREGLLGLGLLG-GAVAVVGGLVIAGLAFRK---  143
S. cerevisiae         119 MVEDKIQKETLKGVVVAGGVLAGAVAVASFFLRNKRR---  155
                                  * *    *
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Fig. 1. Amino acid sequence alignment of Fis1 from different species. Residues in bold indicate conserved amino acids in three or more species. Asterisks (*) indicate residue conservation amongst all species. The predicted transmembrane domain of hFis1 is indicated (underlined).

the antibodies are directed to the N-terminal region of hFis1, we conclude that the N-terminal region of hFis1 faces the cytosol. In addition, hFis1, like the outer-membrane-located Tom40 (Suzuki et al., 2000) was not released from the mitochondrial membranes following carbonate treatment (Fig. 3E). By contrast, mtHsp70, a matrix protein that peripherally associates with the inner membrane, was found in the supernatant after carbonate treatment. Thus, we concluded that hFis1 is indeed an integral mitochondrial outer membrane protein.

The experimental evidence indicates that hFis1 is a tail-anchored protein. Although the mitochondrial targeting information for outer membrane proteins is not well known, evidence is mounting that tail-anchored proteins contain their targeting information within the hydrophobic membrane anchor as well as in flanking basic residues (Wattenberg and Lithgow, 2001; Horie et al., 2002; Kaufmann et al., 2003). In order to address the targeting of hFis1 to mitochondria, we analysed several GFP-hFis1 fusion constructs (Fig. 4A). When a GFP-reporter construct of hFis1 lacking both its predicted transmembrane domain and C-terminal segment (GFP-hFis1$^{\Delta 122–152}$) was expressed in cells, no mitochondrial targeting was observed, with most protein found in the cytosol and nucleus (Fig. 4B). By contrast, expression of GFP-hFis1$^{\Delta 1\text{-}121}$, which lacks the cytosolic domain of hFis1, still results in mitochondrial localization (Fig. 4C). hFis1 contains two lysine residues in the C-segment at positions 149 and 151. To address whether these residues are important for mitochondrial targeting, we first mutated both to alanine. Unlike for GFP-hFis1, the fluorescence pattern of GFP-Fis1$^{K149/151A}$ was clearly distinct from labelling of mitochondria with MitoTracker Red and the protein appeared to be misdirected to the endoplasmic reticulum (ER) (Fig. 4D). This was subsequently confirmed using ER Tracker (data not shown). Mutation of either of the lysine residues alone to alanine led to targeting of GFP-hFis1^{K149A} and GFP-hFis1^{K151A} to both mitochondria and ER (Fig. 4E,F). We conclude that the overall basic charge within the C-segment of hFis1 is crucial for mitochondrial targeting.

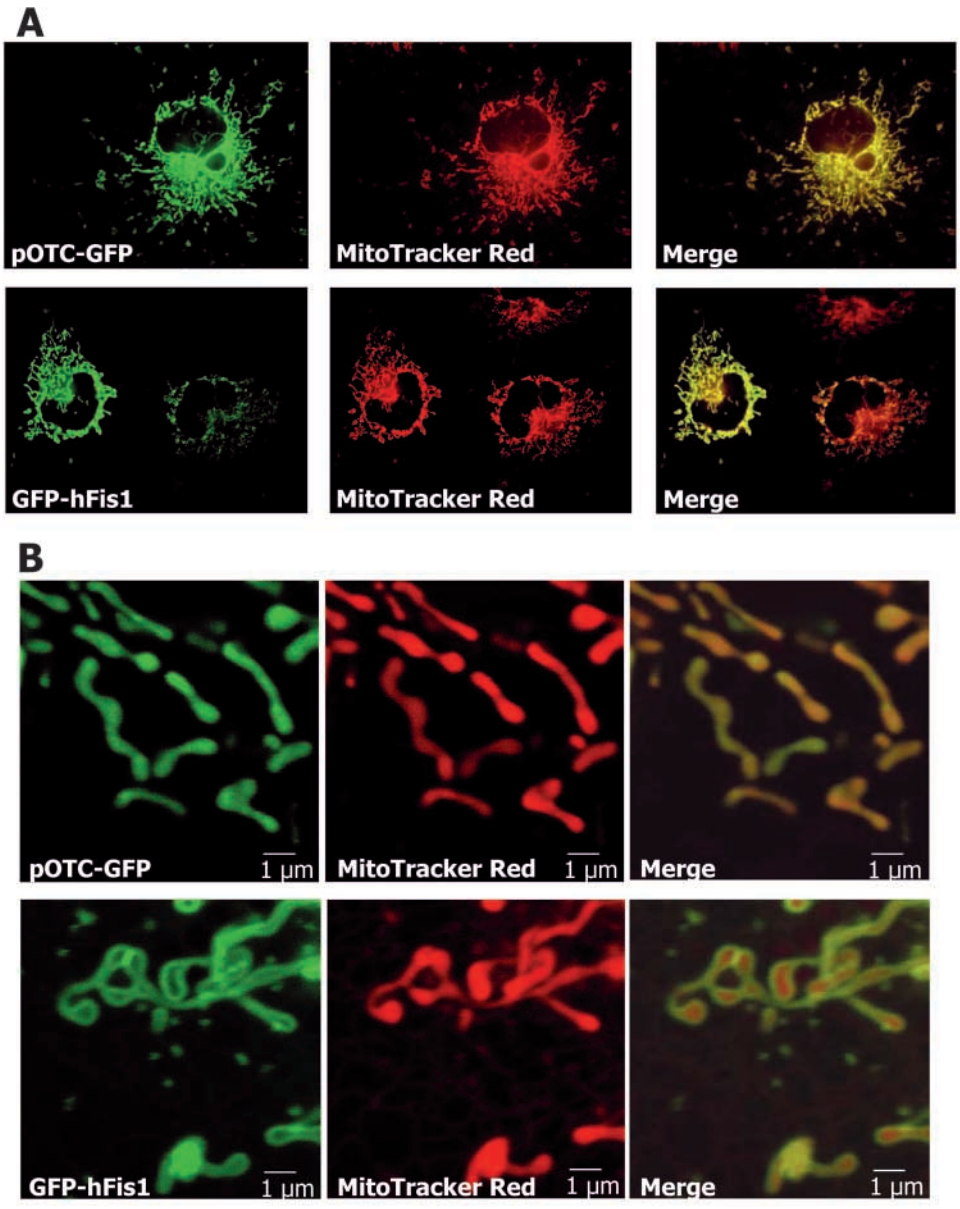

Fig. 2. GFP-hFis1 is targeted to the mitochondria. (A) COS-7 cells transfected with pOTC-GFP, a mitochondrially located protein, or GFP-hFis1 were counterstained with MitoTracker Red and analysed by fluorescence microscopy 24 hours after transfection. The far right panel depicts overlay images of the GFP and MitoTracker Red fluorescence. (B) High resolution confocal microscopy of mitochondrial tubules as seen in (A).

Fis1p is suggested to function in mitochondrial fission by recruiting cytosolic Dnm1p to the organelle. In yeast, *Δfis1* cells contain mitochondria that display highly interconnected tubules, typical of mitochondria undergoing continuous fusion without fission events. In this context, it was interesting that, when GFP-hFis1 was overexpressed in COS-7 cells, an increase in both mitochondrial fragmentation and aggregation around the nucleus were observed (Fig. 5A,B). This aggregation was not due to overexpression of hFis1 as a GFP fusion because the same phenotype was observed when cells overexpressed hFis1 alone (Fig. 5E), whereas little mitochondrial aggregation was observed when cells overexpressed hTom7 (Fig. 5C,E), a mitochondrial outer membrane protein involved in protein import (Johnston et al., 2002). This aggregation might be due to either fused mitochondria collapsing around the nucleus as a

result of impaired mitochondrial fission or, alternatively, mitochondrial fragmentation through increased fission events. In order to analyse these clumps in more detail, we performed confocal microscopy to visualize mitochondria in a single plane. As can be seen (Fig. 5D), the mitochondria appeared to be fragmented, with GFP-hFis1 still localized to the periphery of the mitochondria relative to matrix fluorescence of MitoTracker Red. Although overexpression of wild-type Drp1 with hFis1 did not result in changes in the amount of mitochondrial aggregation, expression of a Drp1 dominant-negative mutant ($Drp1^{K38A}$) (Smirnova et al., 1998) led to a significant reduction (Fig. 5F). We therefore conclude that not only are the mitochondrial aggregates observed probably due to increased fission events, but Drp1 and hFis1 function together in the same process.

Given the similarity between yeast and human Fis1 at the sequence and apparent functional level, we decided to determine whether they were functionally interchangeable in vivo. To test this, we examined the ability of hFis1 to complement the defective mitochondrial networking phenotype of yeast cells lacking Fis1p (Fig. 6). When hFis1 was expressed in *Δfis1* yeast cells, wild-type mitochondrial networking was not observed, indicating a lack of complementation (Fig. 6A). Analysis of the targeting of a GFP-hFis1 construct revealed that the fusion protein was not targeted to mitochondria in yeast but instead to the ER (Fig. 6B, top). We therefore made a chimeric fusion protein consisting of the cytosolic domain of hFis1 fused to the transmembrane domain and C-segment of yeast Fis1p [hFis1(N)-yFis1(TM)]. When expressed as a GFP fusion in yeast cells, this protein was targeted to mitochondria (Fig. 6B, middle). However, when we expressed the hFis1(N)-yFis1(TM) chimera in *Δfis1* cells, aberrant mitochondrial morphology was still observed (Fig. 6B, bottom). We conclude that neither hFis1 nor a yeast mitochondrion-targeted variant can complement the mutant phenotype observed in *Δfis1* yeast cells.

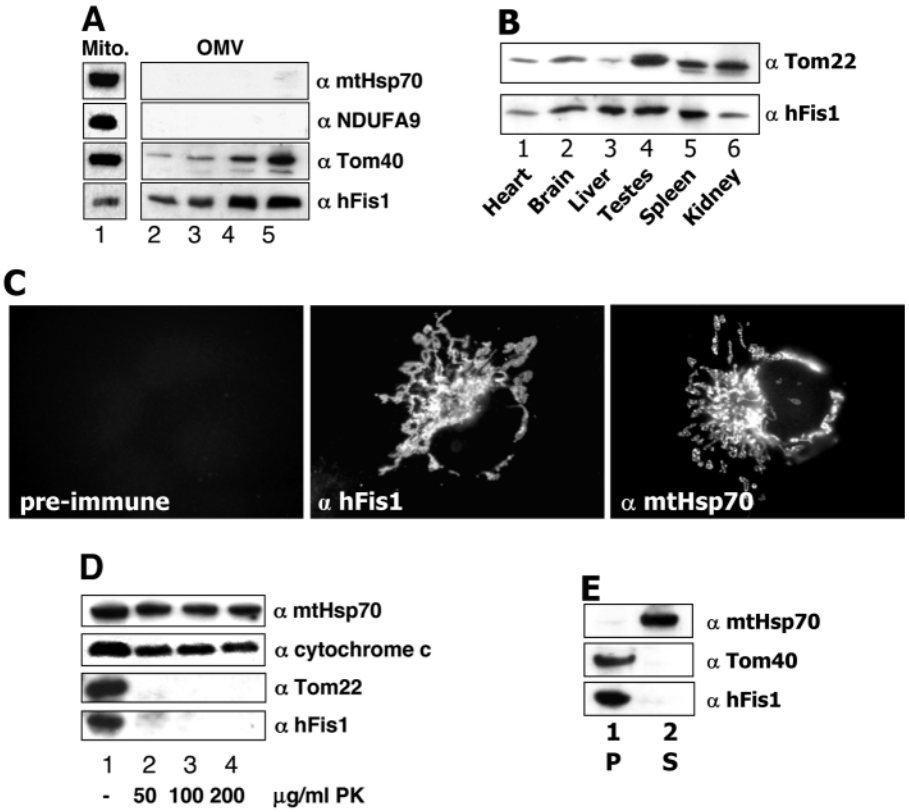

Fig. 3. Characterization of hFis1. (A) hFis1 is located on the mitochondrial outer membrane. Samples of mitochondria (lane 1) and mitochondrial outer membrane vesicles (OMV; increased loading, lanes 2-5) were subjected to SDS-PAGE and western blot analysis using antibodies against the matrix located mtHsp70, the inner membrane complex I subunit NDUFA9, the outer membrane marker Tom40 and hFis1. (B) hFis1 is ubiquitously expressed. Isolated mitochondria from heart, brain, liver, testes, spleen and kidney rat tissues were subjected to SDS-PAGE and western blot analysis using Tom22 and hFis1 antibodies. (C) hFis1 displays a diffuse distribution along the mitochondrial surface. COS-7 cells were subjected to immunofluorescence assays using pre-immune serum, affinity-purified anti-hFis1 antibodies or mtHsp70 antibodies. (D) hFis1 is a mitochondrial protein with its N-terminus exposed to the cytosol. Isolated HeLa cell mitochondria (50 μg) were treated with or without externally added protease as indicated. Mitochondria were reisolated and subjected to SDS-PAGE and western blot analysis using mtHsp70, cytochrome *c*, Tom22 and hFis1 antibodies. (E) hFis1 is an integral membrane protein. HeLa cell mitochondria (100 μg) were extracted with 100 mM sodium carbonate, pH 11.5. After centrifugation at 18,000 ***g***, the membrane pellet fraction (P; lane 1), and soluble supernatant fraction (S; lane 2) were subjected to SDS-PAGE and western analysis using antibodies directed against hFis1, mtHsp70 or Tom40.

To confirm the involvement of hFis1 in mitochondrial fission, RNA interference was performed. p*Silencer* vectors containing a hairpin-forming sequence specific for hFis1 or a non-specific scrambled sequence (as control) were transfected into COS-7 cells. 72 hours after transfection, cells were harvested and subjected to western blot analysis using antibodies against hFis1 and hTom40 (a control protein of the mitochondrial outer membrane). As can be seen, the levels of hFis1 were reduced to ~40% in cells transfected with p*Silencer*-hFis1 compared with cells transfected with the vector control (Fig. 7A). The reduction in hFis1 levels is anticipated to be higher because not all cells (~20% in our hands) are transfected with the siRNA vector. Nevertheless, the morphology of mitochondrial networks within hFis1-depleted cells was found to differ substantially from those mitochondrial networks from cells transfected with the vector control (Fig. 7B). In particular, we noticed that a large proportion of cells depleted of hFis1 contained long mitochondrial extensions projecting out from the nucleus. These were classified into two separate types: interconnected mitochondria and extended mitochondria. The interconnected mitochondria were reminiscent of the ‘interconnected nets’ phenotype previously

ascribed to mitochondria from cells containing mutant Drp1 (Smirnova et al., 2001). To determine their significance, in excess of 500 cells were scored from both sets of transfected cells. As can be seen (Table 1), 65% of cells depleted of hFis1 displayed these aberrant mitochondria, whereas neither morphological type was observed in control cells. We conclude from these studies that hFis1 is involved in mitochondrial fission and its level of expression on the mitochondrial surface directly influences this process.

Discussion

Although it has been noted that mitochondria undergo cycles of division, several factors involved in this process have only been recently identified in yeast and higher eukaryotes (Sesaki and Jensen, 1999; Fekkes et al., 2000; Griparic and van der Bliek, 2001; Yoon and McNiven, 2001; Shaw and Nunnari, 2002; Jakobs et al., 2003). Here, we report the characterization of hFis1, an integral mitochondrial outer membrane protein involved in the organellar fission. hFis1 inserts into the mitochondrial outer membrane via a C-terminal transmembrane domain, thereby leaving a short, basic C-terminal segment facing the intermembrane space. The two C-terminal basic residues are essential for conferring mitochondrial-targeting information because their substitution leads to mis-targeting to the ER. Such mitochondrial targeting information in hFis1 is similar to that of other C-terminally anchored mitochondrial proteins although the actual process of targeting and membrane insertion of this subset of proteins is not well understood (Horie et al., 2002; Kaufmann et al., 2003). It is interesting, however, that the mitochondrial targeting information of hFis1 is not sufficient for its targeting to mitochondria in yeast. One possibility for this is that the targeting signal within hFis1 is not recognized by the yeast mitochondrial surface receptors such as Tom70 and/or Tom20 (Pfanner and Geissler, 2001) and hence hFis1 is targeted to the ER by default mechanisms (Beilharz et al., 2003). In contrast to hFis1, its yeast counterpart contains four basic residues within its C-terminal segment, and this increase in charge might be what confers specificity to mitochondria in this organism.

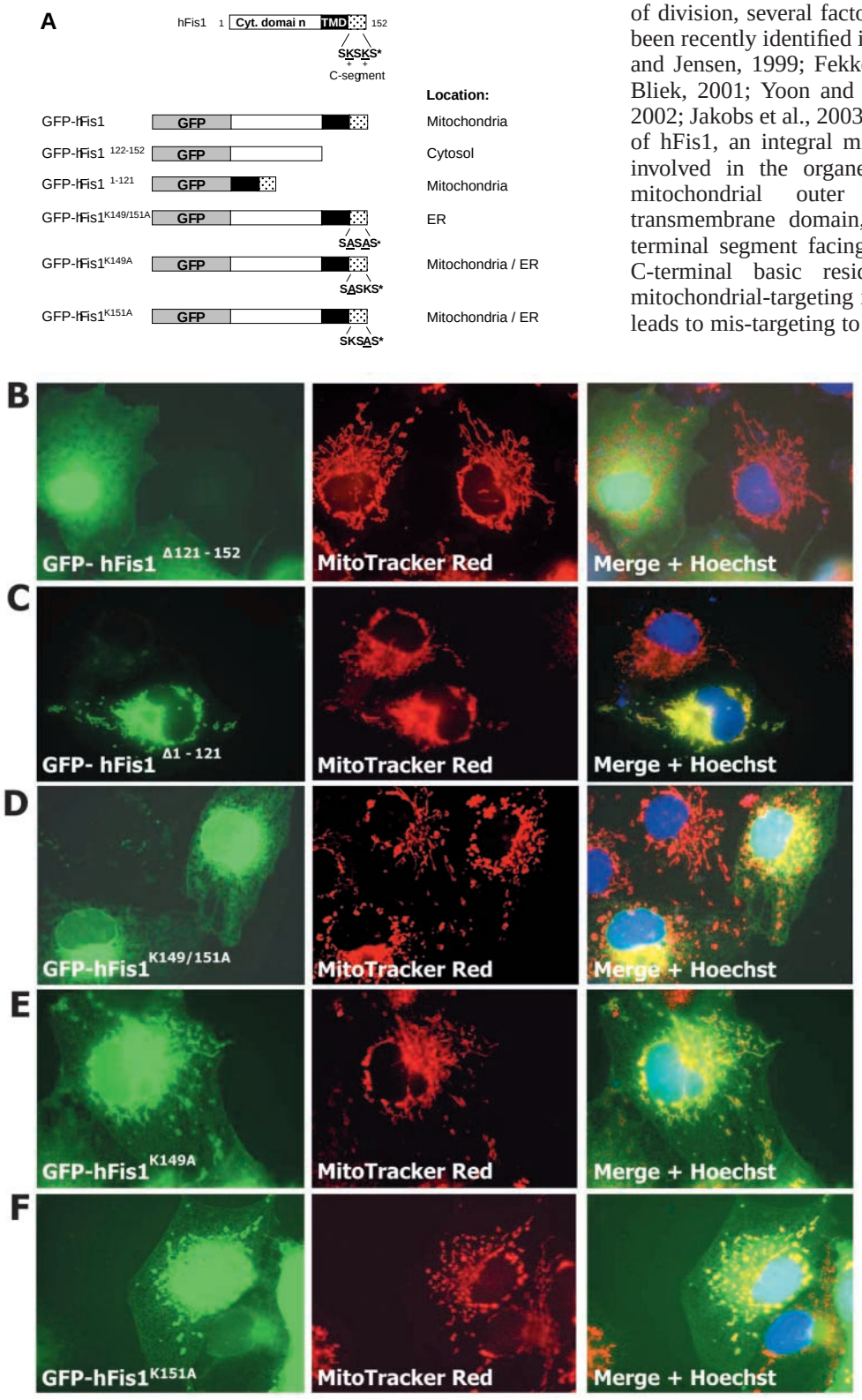

Fig. 4. hFis1 is targeted to the mitochondria via basic residues within its C-segment and its transmembrane domain. (A) Schematic representation of GFP-hFis1 fusion proteins and their subcellular location. (B-F) COS-7 cells expressing the GFP-hFis1 fusion proteins depicted in (A) were incubated with MitoTracker Red and the nuclear stain Hoechst 33258 before analysis by fluorescence microscopy.

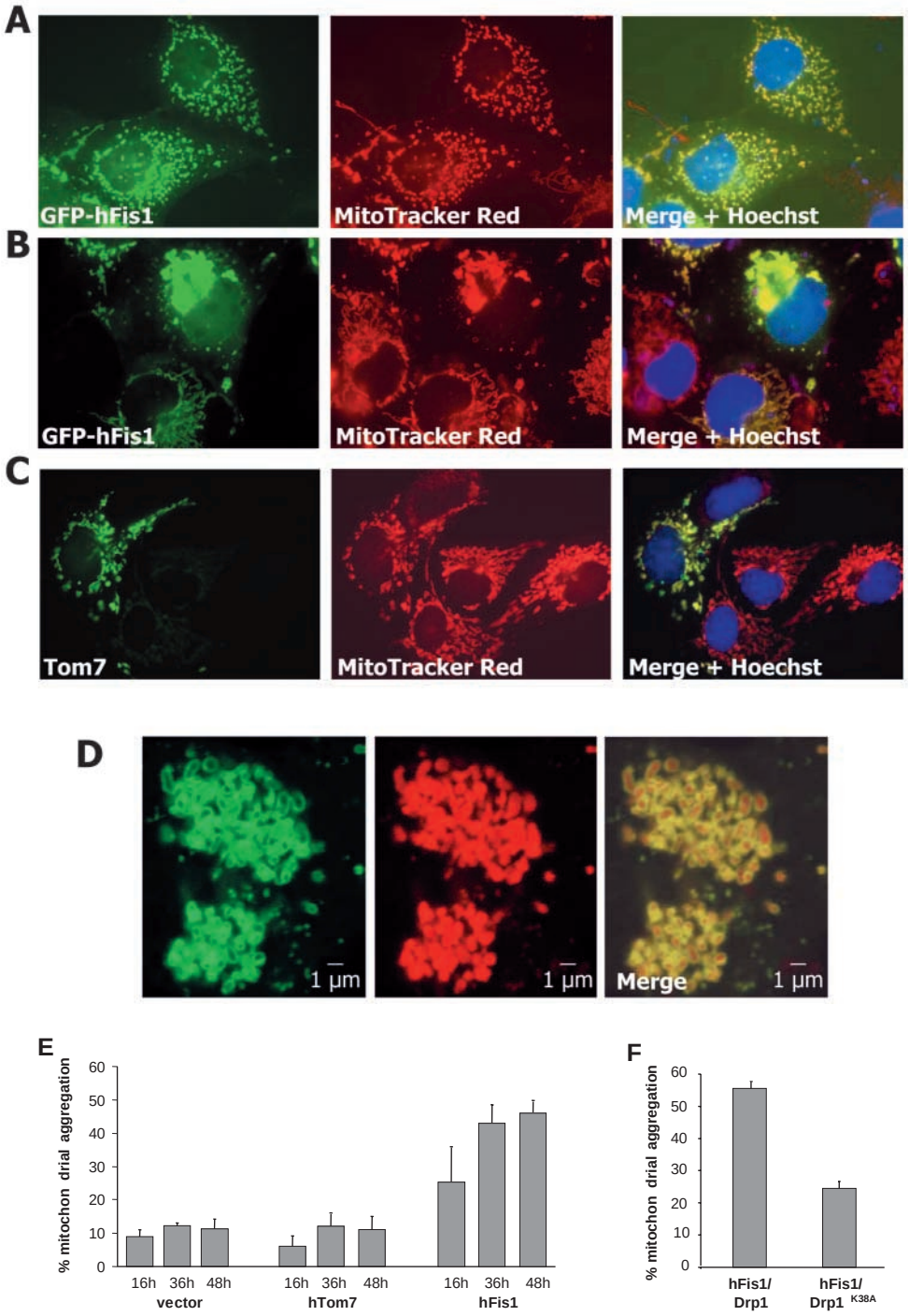

Fig. 5. Overexpression of hFis1 compromises steady state mitochondrial morphology. COS-7 cells overexpressing the GFP-hFis1 chimera were counterstained with MitoTracker Red and Hoechst 33258 and analysed by fluorescence microscopy. Cells expressing hFis1 mainly displayed one of two phenotypes: (A) fragmented mitochondria; or (B) mitochondrial aggregates in the vicinity of the nucleus. (C) Phenotype of cells expressing the control outer membrane protein Tom7. (D) High-resolution confocal microscopy of mitochondrial aggregates similar to those seen in (B) reveals that they are composed of small fragmented mitochondria. (E) Quantitation of mitochondrial aggregates in COS-7 cells overexpressing vector alone, Tom7 or hFis1 was monitored and quantified at 16 hours, 36 hours and 48 hours after transfection (n=3). (F) Quantitation of mitochondrial aggregates in >100 COS-7 cells co-expressing hFis1/Drp1 or hFis1/Drp1 (K38A) (n=3).

Overexpression of hFis1 with or without an N-terminal GFP fusion caused significant mitochondrial morphology defects. In many cells, fragmented mitochondria or aggregates consisting of mitochondrial fragments were observed. These results led us to conclude that overexpression of hFis1 results in an increase in mitochondrial fission. This conclusion is supported by the fact that overexpression of a Drp1 dominant negative mutant causes decreases in the amount of these mitochondrial aggregates and indicates that these two proteins function on the same fission process. During the preparation of this manuscript, two papers were published that reported the involvement of hFis1 in mitochondrial fission (James et al., 2003; Yoon et al., 2003). James et al. (James et al., 2003) noted that overexpression of hFis1 results in organelle fragmentation followed by the formation of punctiform, collapsed mitochondria surrounding the nucleus. Yoon et al. (Yoon et al., 2003) also observed fragmentation as well as the formation of mitochondrial clumps upon overexpression of GFP-Fis1, but they suggested that it was due to an artefact caused by GFP dimer formation on the mitochondrial surface. Our data, however, show that aggregation was still observed upon overexpression of hFis1 alone, whereas overexpression of a control outer membrane

Table 1. Summary of mitochondrial morphologies observed in COS-7 cells transfected with hFis1 siRNA expression vector or vector control (scrambled sequence)

		Phenotype			
	n*	Normal	Fragmented	Interconnected tubules	Extended tubules
Control	537	417 (78%)	120 (22%)	0	0
siRNA	520	92 (18%)	87 (17%)	152 (29%)	189 (36%)

*The number of cells counted.
Relative phenotypes are expressed as percentages in brackets.

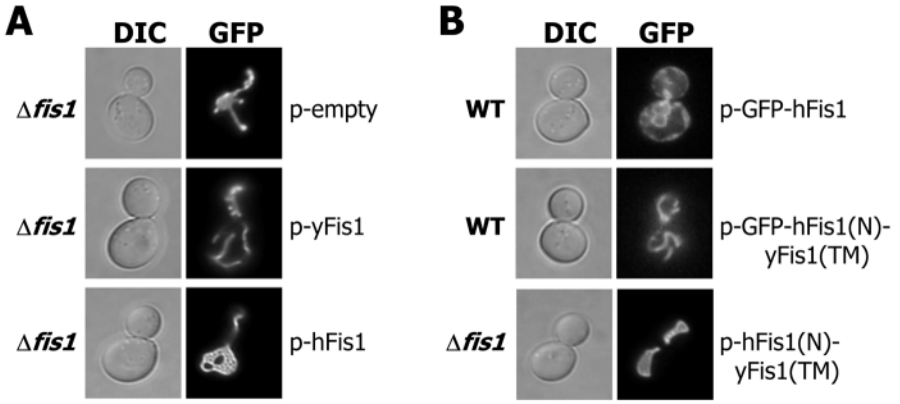

Fig. 6. Complementation studies in *S. cerevisiae*. (A) Yeast *Δfis1* cells expressing a mitochondrial targeted from of GFP as well as either vector alone (p-empty), vector expressing yeast Fis1p or hFis1 were subjected to DIC and fluorescence microscopy. (B) GFP-hFis1 is mis-targeted to the ER in yeast cells. By contrast, GFP-hFis1(N)-yFis1p(TM), a chimeric fusion protein containing the N-terminal cytosolic domain of hFis1 and the transmembrane domain and C-segment of yeast Fis1p, is targeted to the mitochondria but fails to complement in *Δfis1* cells.

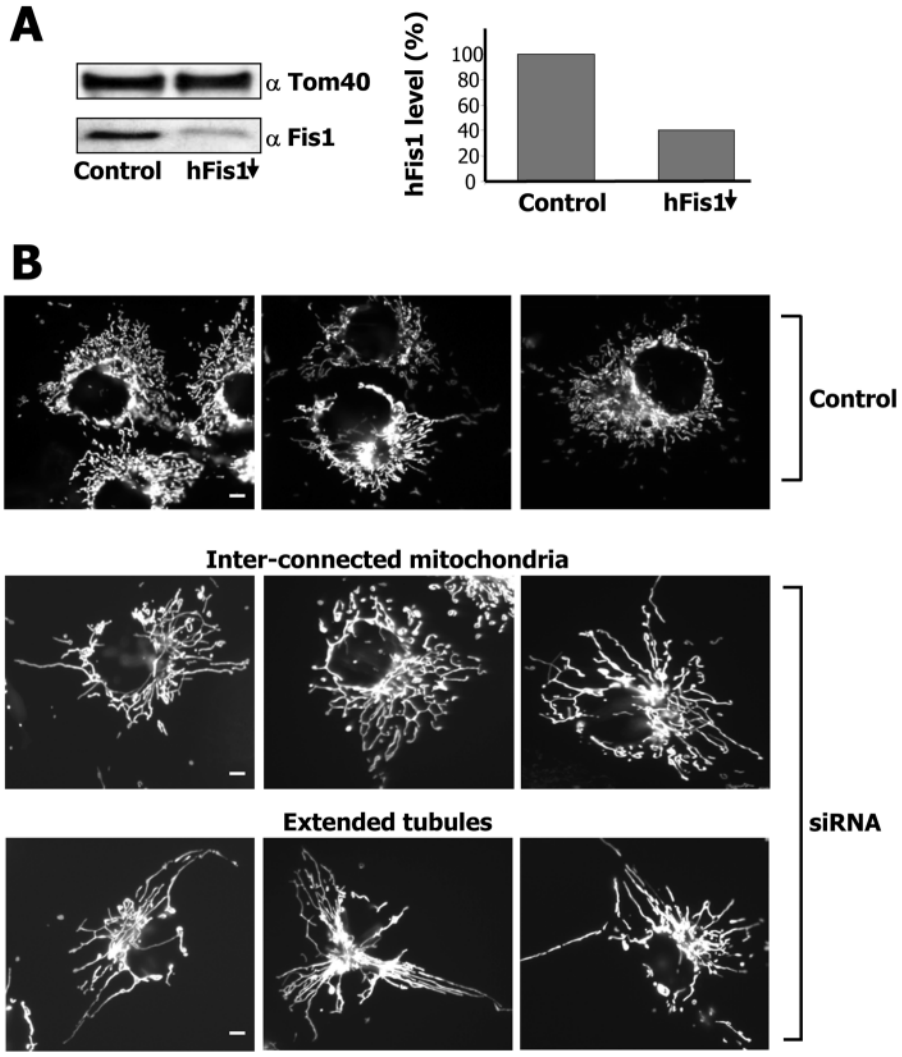

Fig. 7. Knockdown of hFis1 in COS-7 cells leads to the formation of mitochondrial tubule extensions. COS-7 cells were transfected with vector alone (scrambled sequence; control) or vector encoding small interfering RNAs specific for hFis1 knockdown. (A) At 72 hours after transfection, mitochondria were isolated and subjected to SDS-PAGE and western blot analysis using antibodies specific for hFis1 and Tom40 (as control; left). The amount of hFis1 knockdown was quantified (right). (B) Representative mitochondrial morphologies observed in cells transfected with control and hFis1 small interfering RNAs as described in (A). Scale bar, 5 μm.

protein (Tom7), alone or fused to GFP, did not induce organelle aggregation. Furthermore, this aberrant morphology was inhibited by overexpression of $Drp1^{K38A}$ – a dominant negative mutant of Drp1 that is known to block mitochondrial fission (Smirnova et al., 1998). The process of mitochondrial fission has been recently linked to apoptosis with the pro-apoptotic factor Bax, localizing to Drp1-containing puncta (Frank et al., 2001; Karbowski et al., 2002). Interestingly, Bax overexpression causes mitochondrial fragmentation followed by aggregation as part of the cellular apoptotic process (Desagher and Martinou, 2000). Our results are consistent with this model because we observed nuclear blebbing in many cells containing aggregated mitochondrial fragments, suggesting that they were also undergoing apoptosis (data not shown). These conclusions are also consistent with the recent finding that hFis1 overexpression induces apoptosis (James et al., 2003).

It is interesting that the cytosolic domain of hFis1 cannot complement the phenotype of cells lacking yeast Fis1. This indicates that hFis1 is not functionally interchangeable with that of yeast, suggesting that significant evolutionary divergence has occurred. In yeast, Mdv1p is involved in the fission process and also interacts with Fis1p (Tieu et al., 2002). Mammalian cells possess no obvious Mdv1 homologue and hence an Mdv1-like interaction domain might not be present in hFis1, thereby preventing complementation in yeast cells.

Although overexpression of hFis1 leads to mitochondrial fragmentation, we found that its depletion through

siRNA led to increases in mitochondrial extensions and interconnections. The mitochondrial morphology seen in this study is similar to the impaired morphology observed using dominant-negative mutants of Drp1 or when mitochondrial fusion factors are overexpressed in cells (Smirnova et al., 1998; Santel and Fuller, 2001). In both cases, the balance between mitochondrial fission and fusion events were shifted towards fusion, leading to highly interconnected mitochondrial networks within cells. In addition, Yoon et al. (Yoon et al., 2003) have recently shown similar alterations in mitochondrial morphology following the inhibition of endogenous levels of hFis1. Our findings therefore indicate that the steady-state levels of hFis1 on the mitochondrial surface influence the rate of mitochondrial fission. Models of dynamin-mediated membrane fission suggest that pools of dynamin molecules are required for polymerization and subsequent membrane scission to occur (Hinshaw, 2000). Indeed, large concentrations of Drp1 can constrict and tubulate model membranes in vitro in the absence of Fis1 (Yoon et al., 2001). However, in the confines of the cell, specific targeting of Drp1 to the mitochondrial surface is required and yeast studies indicate that this is mediated by Fis1 (Yoon and McNiven, 2001). In support of this, an interaction between hFis1 and Drp1 has also been reported (Yoon et al., 2003).

It has been calculated that only ~3% of Drp1 is found at the mitochondrial surface, with the remainder diffusely distributed throughout the cytosol (Smirnova et al., 2001). Increased levels of hFis1 might subsequently direct more of the cytosolic pool of Drp1 to mitochondria, thereby promoting fission; conversely, decreases in Fis1 might hinder its binding, inhibiting fission. How might Fis1 function in the recruitment of a member of the dynamin family to a membrane surface? Although this is not yet known, the answer might lie in the fact that, unlike other dynamin members, Drp1 lacks a pleckstrin homology domain. This domain has been found to be responsible for promoting association of dynamin with membranes via interactions with phosphatidylinositol-4,5-bisphosphate (Salim et al., 1996; Hinshaw, 2000). Thus, in the absence of this domain, hFis1 might specifically recruit Drp1 to the mitochondrial membrane surface, enabling the process of mitochondrial fission to occur. Interestingly, we have failed to detect a stable interaction using purified Drp1 and hFis1 (M. Lazarou and M.T.R., unpublished), indicating that they might interact transiently or, alternatively, additional proteins or membrane systems are also required. Development of an in vitro fission assay will aid in addressing these aspects.

We thank C. Li and N. Klonis for assistance with confocal microscopy, J. Shaw for yeast strains, B. Westermann for the pVT100U-mtGFP clone, M. Hagiwara for the pME-HA-Dymple (Drp1) clone, A. van der Bliek for the $Drp1^{K38A}$ clone, and P. Nagley for the cytochrome *c* antibody. This work was supported by grants from the Australian Research Council and the Wellcome Foundation. DS acknowledges receipt of an Australian Postgraduate Research Award. K.O. was supported by a grant from the National Institute of Health (GM53466) awarded to J. M. Shaw. The Utah Health Sciences Sequencing Facility is supported by a National Cancer Institute grant (5-P30CA42).

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