The mitochondrial protein MTP18 contributes to mitochondrial fission in mammalian cells

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Summary
Mitochondria are dynamic organelles that change morphology by controlled fission and fusion events. Mitochondrial fission is regulated by a conserved protein complex assembled at the outer membrane. Human MTP18 is a novel nuclear-encoded mitochondrial membrane protein, implicated in controlling mitochondrial fission. Upon overexpression of MTP18, mitochondrial morphology was altered from filamentous to punctate structures suggesting excessive mitochondrial fission. Mitochondrial fragmentation was blocked in cells coexpressing either the mitochondrial fusion protein Mfn1 or Drp1K38A, a dominant negative version of the fission protein Drp1. Also, a loss-of function of endogenous MTP18 by RNA interference (RNAi) resulted in highly fused mitochondria. Moreover, MTP18 appears to be required for mitochondrial fission because it is blocked after overexpression of hFis1 in cells with RNAi-mediated MTP18 knockdown. In conclusion, we propose that MTP18 functions as an essential intramitochondrial component of the mitochondrial division apparatus, contributing to the maintenance of mitochondrial morphology.

Key words: Mitochondrial morphology, Mitofusin, Apoptosis, hFis1, Drp1, shRNA

Introduction
Mitochondria are involved in a variety of cellular processes such as energy supply, β-oxidation, calcium as well as iron homeostasis. Mitochondria play also an important role in controlling apoptosis and cellular aging. It has been suggested that there is a close correlation between morphology and function of mitochondria (Bossy-Wetzel et al., 2003; Chen and Chan, 2004; Karbowski and Youle, 2003). So, the maintenance of mitochondrial morphology is important to ensure mitochondrial homeostasis. Mitochondrial dysfunction owing to inhibition of morphology-controlling proteins is often linked to neurodegenerative diseases and cancer. For example, genetic and molecular studies of the neurodegenerative disease torsional dominant optic atrophy uncovered mutations in the PAI gene, which encodes a large mitochondrial GTPase that participates in the control of mitochondrial fusion (Alexander et al., 2000; Delettre et al., 2000). In addition, mutations in the human gene for the mitochondrial fusion protein Mfn2 have recently been found in patients with syndromes of the Charcot-Marie-Tooth neuropathy type 2A (Zuchner et al., 2004). Evolutionary conserved cellular components of mitochondrial fusion and fission that maintain mitochondrial morphology have been identified and characterised in yeast, fly and mammals (Chen and Chan, 2004; Hales and Fuller, 1997; Karbowski and Youle, 2003; Mozdy and Shaw, 2003; Scott et al., 2003; Shaw and Nunnari, 2002). A class of large GTPases controls the maintenance of mitochondrial morphology through antagonizing fusion and fission events in a GTPase-dependent manner. Mitochondrial mitofusin GTPases regulate uncontrolled mitochondrial fission. By contrast, the cytoplasmic GTPase dynamin 1-like protein (DNM1L, here referred to as Drp1) is recruited to the mitochondrial outer membrane to exert fission of mitochondria. In *Caenorhabditis elegans*, the assembly of Drp1 on mitochondria results in severing the outer membrane (Labrousse et al., 1999). Similarly, genetic and biochemical studies in yeast uncovered a multi-step pathway for mitochondrial fission, whereby preassembled Drp1 structures, in concert with other mitochondrial outer membrane components, are sufficient to cut apart both mitochondrial membranes (Mozdy et al., 2000; Sesaki and Jensen, 1999; Shaw and Nunnari, 2002; Tieu and Nunnari, 2000; Tieu et al., 2002). However, exactly how Drp1 is recruited to mitochondria and how the fission process is then executed is still not fully understood.

Like Drp1, the mitochondrial outer-membrane protein Fis1 appears to be a key player during mitochondrial fission. Human Fis1 [tetratricopeptide repeat protein 11 (TTC11), hereafter referred to as hFis1] is a highly conserved 17-kDa integral mitochondrial protein of the outer membrane. Recently, several reports have provided strong evidence that hFis1, like its yeast homologue Fis1p, is involved in mediating mitochondrial fission, presumably by setting up the fission complex on the cytoplasmic face of the outer membrane through (direct or indirect) recruitment of Drp1 (Frieden et al., 2004; James et al., 2003; Stojanovski et al., 2004; Suzuki et al., 2003; Yoon et al., 2003). In addition, hFis1 has been implicated in the control of Drp1-mediated fission during apoptosis. Mitochondrial fission and mitochondrial-membrane remodeling have been shown to be the initiating step of the apoptotic pathway (Bossy-Wetzel et al., 2003; Fantin et al., 2002; Frank et al., 2001;
arbowksi et al., 2002; Scorrano et al., 2002). Transient pression of hFis1 in mammalian cells leads to excessive itochondrial fission. Consequently, cytochrome c is released on mitochondria and triggers the onset of programmed cell death, highlighting the direct link between mitochondrial action and apoptosis (James et al., 2003). In addition to the oposed interaction of Drp1 with Fis1 during recruitment of Drp1 to the mitochondrial membrane, another report discusses Drp1 as a target for protein modification processes through nteraction with modifying proteins such as Ubc9 (ubiquitin-njugating enzyme 9) and Sumol (small ubiquitin-like editor 1) (Harder et al., 2004). Furthermore, a few viral proteins have been reported to be tively involved in fragmentation of mitochondria after their reess in mammalian cells, however, with different effects on the apoptotic behavior of mitochondria (Karbowksi and eule, 2003). We have recently discovered another novel man mitochondrial protein, MTP18, that is implicated in the ntrol of mitochondrial morphology (Tondera et al., 2004). In this study, we present further experimental evidence for TP18’s function in maintaining the balance of fission and sion in mammalian cells. MTP18-induced mitochondrial gmentation upon its overexpression can be inhibited by reasing counteracting mitochondrial fusion activity. knockdown of MTP18 by RNA interference (RNAi) blocks is-1-induced mitochondrial fission, therefore suggesting that TP18 is required to facilitate the complete fission step.

Materials and Methods

Asmids and short hairpin RNA expression constructs
TP18-myc expression plasmids have been previously described (Tondera et al., 2004). The expression vectors pEGFP-Mfn1, pGFP-Mfn1K88T (Santel et al., 2003) and vectors for hemagglutinin (HA)-Drp1 and HA-Drp1K38A (Smirnova et al., 1998) expression were kindly provided by Minx Fuller (Stanford University, Stanford, CA), pectively. Gene-specific, short hairpin RNA (shRNA)-encoding single-stranded oligonucleotides for shRNA-mediated RNAi have been described in Czauderna et al. (Czauderna et al., 2003). The specific shRNA insert was cloned by using a non-palindromic restriction yme (BsmI) with a 5′ overhang TTTT and a 3′ overhang GGGCA. erts were generated by annealing two synthetic oligonucleotides with CCGT and 3′-AAAA overhangs (Czauderna et al., 2003). Ten referent shRNA-expression constructs for MTP18 were generated and tested for RNAi activity by real-time PCR and western-blotting (data not shown). Four out of ten constructs showed RNAi activity. e following sequences were used for the loss-of-function etotype study (Fig. 5). hFis1: 5′-CCGTCAGTCTGAGAAGGCCA-AGGCAAAAAAAGGCTGCTGCTTCTCAGACTG-3′, AAAACAGTTCAGGAGGGAAGGCCAGAAGCTTTTTTTTTTTGC- GTCGGCTTCTCAGACTG-3′; Drp1: 5′-CCGATATGGGAAAGG- TTATTTCCAGTCAAAAACAAAAAAGTGACTGGAATTCC- CCCAAAT-3′, 5′-AAAAATGGGAAGGGAAGTTTCCGTCATTTT- TTTTTGATGTGAATTCCGGAAAGAAATGGCAATTCCCGGGAAATGCGCCAAATTTCCCTCATACCA-3′; Mfn1: 5′-CCGTCATGGAATGGCCACAGAGGCTAGG-3′, 5′-AAAAATCTCTAGG- CTTGTGCCCACATTTTTTTTTTTTTTTGAAAGGCACAGGGCAG-3′.

The p1100-shRNA sequence has been previously described (Czauderna et al., 2003a). The hFis1 open reading frame was obtained by PCR using human prostate cDNA library using following primers and was subsequently subcloned into pGCM-HA for expression in mammalian cell culture. hFis1 5′ primer: 5′-CTTCTGAGATG-GCTCATATGGGAGGGCTGCTAAGAGGAC-3′, hFis1 3′ primer: 5′-ATGGATCCCTCCAGGCCCCGATTTGGACGTTGGACACA-3′.

Cell culture and transfections

COS-7 and HeLa cells were cultured as described (Tondera et al., 2004). Transfection of MTP18-expression constructs using the Effectene transfection reagent (Qiagen) was carried out according to manufacturer’s instructions. For transient coexpression of specific shRNAs and recombinant proteins, HeLa cells were first transfected with shRNA-expressing plasmids for 24 hours and then again with shRNA-expressing plasmids and overexpression plasmids (ratio 9:1) for another 24 hours.

Subcellular fractionation and proteinase K treatment

Isolation of mitochondria was done as described (Tondera et al., 2004). For membrane protein analyses, mitochondrial pellets were resuspended in 400 μl of 0.1 M Na2CO3 or 0.5 M NaCl, vortexed and incubated on ice for 30 minutes. The insoluble membrane fractions were centrifuged at 100,000 × g for 10 minutes, and the supernatant was precipitated with 10% (v/v) trichloroacetic acid (TCA). For proteinase K digestion, isolated mitochondria were suspended in isotonic mitochondrial buffer (MB; 250 mM sucrose, 10 mM HEPES, 10 mM KCl, 2 mM MgCl2, 1 mM EGTA pH 7.4) and incubated at room temperature with 5 μg/ml proteinase K for the times indicated. Digestion was terminated with 2 mM phenylmethylsulfonyl fluoride (final concentration). Mitochondrial proteins were separated by SDS-PAGE and proteins were detected by western blotting with anti-MTP18, anti-hFis1 and anti-Hsp60 antibodies.

Antibodies, dyes and immunoblotting

Rabbit polyclonal antisera against MTP18 has been previously described (Tondera et al., 2004). Monoclonal anti-cytochrome c, anti-Hsp60 and anti-Drp1/DLPL1 antibodies were purchased from BD Transduction Laboratories. The rabbit polyclonal anti-Mfn1 antibody was kindly provided by Minx Fuller (Santel et al., 2003). Anti-hFis1 was obtained from Alexis Inc. (Portland, OR). Mouse- and rabbit-specific secondary antibodies coupled to Alexa fluor488, Alexa fluor596 or Alexa fluor633, as well as mitochondria-specific dye MitoTracker Red were purchased from Molecular Probes. Preparation of cell extracts and immunoblot analysis was carried out as described (Klippel et al., 1998).

Immunofluorescence microscopy

Before fixation, cells were treated for 1 hour with 5 μM nocodazole (Figs 5, 6, and 7) and for 15 minutes with 1 μM MitoTracker Red at 37°C. For immunofluorescence, cells were fixed for 15 minutes at room temperature in 4% formaldehyde-phosphate-buffered saline. All subsequent steps for indirect immunofluorescence were carried out as previously described (Santel and Fuller, 2001). An LSM 510 META confocal microscopy system (Zeiss) was used for microscopy. Multi-track scanning mode was used to record double-labelled cells.

Results

Drp1-mediated fission occurs on mitochondria with high levels of MTP18

Changes in the expression levels of the mitochondrial protein MTP18 affects mitochondrial morphology and distribution of
Mtp18, a novel mitochondrial fission protein

Tondera et al. (2004) reported that transient expression of MTP18-myc in COS-7 cells resulted in Drp1-mediated mitochondrial fission giving rise to punctated mitochondria. In contrast to long filamentous mitochondria in untransfected cells, the mitochondria in transfected cells expressing MTP18-myc appeared to be bend and circular (Fig. 1A, three small images) and showed distinct Drp1 foci that assembled before fragmentation (Fig. 1A). MTP18 was not particularly localised to Drp1 fission sites. In transfected cells exhibiting fragmented mitochondria, Drp1 was detected at the end of punctated mitochondria (Fig. 1B). A close look at mitochondria with several Drp1 foci revealed ring-like Drp1 structures at constriction sites (Fig. 1B, three small images). Hence, mitochondria with high levels of MTP18-myc underwent Drp1-mediated fission.

MTP18 is an internal mitochondrial membrane protein

Sequence analyses suggest that MTP18 contains three α-helical stretches of high hydrophobicity that are probably transmembrane (TM) domains (Fig. 2A). Interestingly, for the annotated Drosophila melanogaster MTP18-homologue CG7772 only one TM domain (corresponding to the C-terminally located TM3) was predicted (data not shown). Other protein domain motifs, such as coiled-coil domains, were not predicted (not shown). The association of MTP18 with membranes was analysed by treating mitochondrial cell fractions with NaCl or Na2CO3, which caused the release of peripheral membrane associated proteins. MTP18 and the outer-membrane protein hFis1 still precipitated with the membrane fraction after centrifugation, in contrast to Hsp60, a marker protein for the soluble fraction (Fig. 2B). MTP18 becomes degraded like cytochrome c upon osmotic shock. Confocal microscopy of mitochondria from MTP18-myc transfected COS-7 cells stained with anti-MTP18 (green) and anti-Hsp60 (red) did not completely colocalise with the mitochondrial matrix protein Hsp60. Arrows indicate areas of mitochondria where MTP18 does not colocalise with Hsp60, double arrow indicates mitochondria from an untransfected cell.
treated with proteinase K and trypsin to digest the outer-membrane protein Mfn1 (Fig. 2C, lanes 2, 3 compared to untreated control lane 1). MTP18, however, the mitochondrial inter-membrane-space (IMS) protein cytochrome c and the mitochondrial matrix protein Hsp60, were not affected, suggesting that MTP18 is an internal mitochondrial protein. Heating mitochondria with proteinase K after having subjected them to osmotic shock (to selectively break the mitochondrial outer membrane) resulted in the degradation of MTP18 and cytochrome c, while Hsp60 was still protected (lanes 4, 5). This suggests that MTP18 is mainly exposed to the intermembrane space. Furthermore, the submitochondrial localisation of MTP18 was investigated by confocal microscopy to support the biochemical data. Co-localisation experiments were performed in COS-cells transfected with MTP18-myc because polyclonal anti-MTP18 antibody only recognizes recombinant MTP18 but not the lower levels of endogenous MTP18. In transfected cells with very low levels of recombinant MTP18-myc (cells that still exhibit filamentous mitochondria morphology), TP18-myc did not completely colocalise with the matrix protein Hsp60 (Fig. 2D) supporting the above biochemical data that identified the location of MTP18 in the IMS. In addition, in cells that had been co-transfected with GFP-Mfn1 and MTP18-myc, TP18-myc was distinctly localised beyond the outer membrane (Fig. 4D). Taken together, these results indicate that MTP18-myc is not associated with the outer membrane facing the cytoplasm, but exposed to the intermembrane space. We refer to TP18 as an intramitochondrial protein because different models for its topology are conceivable (see discussion).

The C-terminus of MTP18 is crucial for localisation and function of MTP18

The transient expression of a truncated version of TP18 lacking the C-terminus, affects proper mitochondrial localisation of MTP18 and blocks mitochondrial fission. Microscopic studies were carried out with COS-7 cells transfected with MTP18-myc because polyclonal anti-MTP18 antibody only recognizes recombinant MTP18 but not the lower levels of endogenous MTP18. In transfected cells with very high levels of recombinant MTP18-myc, both variants caused fragmentation of mitochondria (Fig. 4D). Taken together, these results indicate that MTP18-myc is not associated with the outer membrane facing the cytoplasm, but exposed to the intermembrane space. We refer to TP18 as an intramitochondrial protein because different models for its topology are conceivable (see discussion).

Fig. 3. Effect of the MTP18 C-terminus on protein localisation and mitochondrial morphology. (A) (left) diagram of myc-tagged (C-terminal red boxes) MTP18 wild-type and mutants that lack the N-terminus (including the predicted TM1) or the C-terminus (including the predicted TM2 and TM3). Transient protein expression was confirmed by western blot (right panel) probed with anti-MTP18 (*, endogenous MTP18; arrow, full-length MTP18–myc; arrowhead, truncated protein variants. (B) Representative confocal immunofluorescence images of transiently transfected COS-7 cells expressing wild-type (upper row), N-terminally truncated (middle row) and C-terminally truncated MTP18 (lower row). Shown is distribution of MTP18 (green, anti-MTP18) and mitochondrial morphology (red, anti-Hsp60). Bars, 20 µm. (C) Immunofluorescence staining of Δ91-166-MTP18-myc in transfected COS-7 cells with anti-MTP18 (green) and anti-Hsp60 (red) to investigate its subcellular localisation. Arrowheads, untransfected cells; arrow, cell with low expression levels of Δ91-166-MTP18-myc; double-arrow, cell with high expression levels of Δ91-166-MTP18-myc.
protein is not required for proper targeting and fragmentation (Fig. 3B). By contrast, when high levels of an MTP18 variant that lacks the C-terminal half of the protein (∆91-166-MTP18-myc) were transiently expressed, it did not colocalise with cytochrome c, but instead showed a conspicuous cytoplasmic pattern. Moreover, the mitochondria did not appear fragmented but clustered (Fig. 3B), even when ∆91-166-MTP18-myc is transiently expressed at very high levels. By contrast, a more detailed analysis of transfected cells revealed that low levels of ∆91-166-MTP18-myc associated with mitochondria (Fig. 3C, arrow), but did not colocalise with sp60 (Fig. 3C, inset). Increasing amounts of this variant localised to the cytoplasm and nucleus in transfected cells as revealed by immunofluorescence staining with anti-MTP18 (Fig. 3C, double-arrow). Therefore, MTP18 might behave as a mitochondrial membrane protein containing more than one particular import signal (see Neupert, 1997). Notably, mitochondria remained intact and did not become fragmented cells with low levels of recombinant ∆91-166-MTP18-myc; mis-localisation of ∆91-166-MTP18-myc presumably due to impaired import might affect the mitochondrial translocation machinery resulting in commonly observed perinuclear clumping of mitochondria upon transient protein expression (Santel and Fuller, 2001). Taken together, the COOH-terminus of MTP18 bearing the predicted TM2 and TM3 regions contributes to the correct mitochondrial protein targeting and mitochondrial fragmentation activity.

Mitochondrial fission caused by transient overexpression of MTP18 is blocked by dominant negative Drp1 (Drp1K38A) overexpression of MTP18 in cell culture induced fragmentation of mitochondria (Tondera et al., 2004), as shown by transient coexpression of MTP18-myc and the GFP-tagged mitochondrial marker protein ANT-GFP (adenosine nucleotide transporter (Fig. 4A) (Santel and Fuller, 2001). Mitochondrial morphology is maintained by controlled fusion and fission events. Both events are controlled by two large GTPases the mitofusins (Mfn1 and Mfn 2) and Drp1. Whereas fission is executed by the Drp1 protein, mitochondrial fusion is mediated by the mitochondrial outer membrane GTPase Mfn1. To study the effect of these proteins on MTP18-induced mitochondrial fragmentation, the following expression constructs were used: myc-tagged MTP18, HA-tagged Drp1, a dominant negative variant of Drp1 (HA-Drp1K38A), GFP-tagged Mfn1 and GFP-tagged variant of the GTPase-defective mutant Mfn1 (GFP-Mfn1K88T) (Fig. 4B).

Transient coexpression of MTP18-myc with Drp1K38A interfered with unopposed mitochondrial fission. Drp1K38A carries a missense mutation in the predicted GTPase domain of Drp1 and acts as dominant negative by blocking mitochondrial fission and promoting fusion (Smirnova et al., 1998; Pitts et al., 1999). By contrast, transiently overexpressed wild-type Drp1 has no effect on altering mitochondrial morphology (data not shown) (Pitts et al., 1999; Smirnova et al., 1998). After transient expression of Drp1K38A in COS-7 cells, 67% of the transfected cells showed highly interconnected mitochondria (Fig. 4C, upper row; Fig. 4E). Consequently, loss of fission activity resulted in uncontrolled mitochondrial fission. When MTP18-myc was transiently coexpressed with Drp1K38A, mitochondria in 30% of doubly transfected cells appeared as a network of long and highly interconnected filaments that partially clustered around the nucleus and scattered throughout the cytoplasm. By contrast, of MTP18-myc transfected cells less than 5% exhibited this mitochondrial morphology (Fig. 4E). Notably, 50% of doubly transfected cells exhibited fragmented mitochondria like seen in transfected cells that overexpressing MTP18-myc alone (>95% showing fragmented mitochondria) (Fig. 4E). The appearance of mitochondrial networks indicates that Drp1K38A overexpression can partly suppress MTP18-mediated fission activity.

MTP18-mediated mitochondrial fragmentation is inhibited by opposed mitochondrial fusion Whereas the transient expression of MTP18-myc alone resulted in mitochondrial fragmentation to be seen in more than 90% of transfected cells, 80% of COS-7 cells that co-expressed MTP18-myc and GFP-Mfn1 showed a perinuclear mitochondrial network (Fig. 4D middle row, Fig. 4E). The observed perinuclear mitochondrial network was reminiscent of Mfn1-induced (fused) ‘grape-like’ mitochondrial clusters (93% in GFP-Mfn1 transfected cells, Fig. 4D upper row) (Santel et al., 2003). To prove that the change from fragmented to highly fused mitochondria in co-transfected cells depended on Mfn1-fusion activity, the mitochondrial morphology in COS-7 cells co-transfected with MTP18-myc and GTPase mutant – and therefore fusion-incompetent Mfn1-variant Mfn1K88T (Santel et al., 2003) – were analysed by immunofluorescence microscopy. The majority (87%) of Mfn1K88T-MTP18-myc expressing cells exhibited clearly fragmented mitochondria reflecting the MTP18-overexpression phenotype (Fig. 4D, lower row; Fig. 4E). The characteristic morphology of grape-like mitochondria as shown for MTP18-Mfn1-expressing cells was not observed, when GTPase-mutant Mfn1 was transiently coexpressed with MTP18.

Knockdown of MTP18 results in mitochondrial fusion The presented overexpression and coexpression studies demonstrated that MTP18 might participate in controlling mitochondrial morphology by modulating the balance of fission and fusion. We have previously shown that overexpression of MTP18 can mediate mitochondrial fragmentation (Tondera et al., 2004). To test whether mitochondrial fission depends on MTP18 action, we analysed the effect of MTP18 loss-of-function in mammalian cells by shRNA-mediated RNA interference (for a review, see Dorsett and Tuschl, 2004; Shi, 2003). For this purpose, we transiently transfected HeLa cells with an expression construct that gives rise to high levels of double-stranded RNA specifically designed to target MTP18 mRNA (shRNA-MTP18). In the same manner, we generated shRNA expression constructs to target Mfn1 (shRNA-Mfn1) and Drp1 (shRNA-Drp1) as controls to compare loss-of-function effects on mitochondrial morphology. An unrelated shRNA (PI 3-kinase subunit p110α) was used as negative control (Czauderna et al., 2003). The 2-day-long transient expression of these shRNA-expressing constructs in HeLa cells resulted in a clear and target-specific...
reduction of target protein levels as assessed by western blot; Fig. 5A shows two representative examples. At the same time, protein levels of a non-target control protein, hFis1, were not affected (Fig. 5A).

For microscopic analysis, HeLa cells were transfected with the above mentioned shRNA expression constructs together with a GFP expression construct (to identify transfected cells) at the ratio 9:1. Transfected cell that showed reduced levels of Mfn1, Drp1 and MTP18 showed dramatic changes in mitochondrial morphology when compared with the non-related shRNA control sample (Fig. 5B). Reduction of the general mediator of mitochondrial fusion Mfn1 resulted in the fragmentation of mitochondria in approximately 80% of transfected – GFP positive – cells, whereas shRNA-Drp1-expressing cells (~78%) displayed long interconnected filamentous mitochondrial network (Fig. 5B). For shRNA-Mfn1, the observed morphology resembled the phenotype described for mitochondria in Mfn1−/− mouse embryonic fibroblast (MEFs) (Chen et al., 2003) and cells transfected with synthetic siRNA molecules for RNAi (Eura et al., 2003), whereas shRNA-Drp1 displayed the anticipated opposite effect of mitochondrial morphology.

Transient expression of shRNA-MTP18 gave rise to long mitochondrial filaments in approximately 90% of transfected cells, similar to the described effect for RNAi against Drp1 (Fig. 5B). The same result was confirmed with another shRNA-MTP18 sequence (data not shown). Therefore, we propose that RNAi-mediated MTP18 protein reduction results in uncontrolled fusion due to suppressed MTP18 fission activity. This observation is in agreement with our initial knockdown analysis of MTP18 in COS-7 cells using antisense molecules (Tondera et al., 2004).

Fig. 4. MTP18-induced mitochondrial fission is inhibited by counteracting fusion activity. (A) Representative picture of a COS-7 cell transiently expressing MTP18-myc (red) and the mitochondrial adenosine nucleotide transporter as a GFP-fusion protein (ANT-GFP, green). The merged picture shows fragmented mitochondria (yellow). (B) Western blot with extracts from COS-7 cells that had been transiently transfected with MTP18, Drp1 or Mfn1 expression constructs. Endogenous and recombinant proteins were detected with appropriate antibodies. Arrow, MTP18-myc; double arrow, GFP-Mfn fusion protein; arrowheads as labelled. (C) COS-7 cell transfected with dominant negative HA-Drp1K38A alone (green, anti-Drp1; red, Mitotracker) or together with MTP18-myc (MTP18-myc, red). (D) Representative confocal microscopy images of COS-7 cells transiently transfected with GFP-Mfn1 alone (mitochondrial morphology shown with Mitotracker dye) or together with MTP18-myc, or with GFP-Mfn1K88T and MTP18-myc. MTP18 was detected with anti-MTP18 (red); GFP-Mfn1 fusion protein is shown in green. (E) Percentage of transfected cells that exhibit indicated mitochondrial morphologies.
Mtp18, a novel mitochondrial fission protein 

al., 2004). These RNAi data along with the overexpression results suggest that MTP18 is involved in mitochondrial fission.

hFis1 controlled mitochondrial fission appears to depend on MTP18

The level of MTP18 is crucial for hFis1-mediated mitochondrial fission. To study the influence of MTP18 on hFis1-mediated mitochondrial fission, we studied the knockdown effects of MTP18/hFis1 in combination with overexpression on mitochondrial morphology in transiently transfected HeLa cells (Fig. 6A). The mitochondrial hFis1 protein controls mitochondrial fission. Consequently, knockdown of hFis1 by shRNA-induced RNAi (Fig. 6A) resulted in the formation of long filamentous fused mitochondria (Fig. 6B upper row). By contrast, overexpression of hFis1 induced fragmented mitochondria in more than 90% of transfected cells (Fig. 6B, lower row). The fragmented mitochondria appeared round and swollen with an average size of 1.4 µm (s.d. 0.4 µm; n=60 mitochondria from two cells). Fragmented mitochondria in hFis1 transfected cells differed in size and morphology from those in MTP18 transfected cells giving rise to mitochondria with an average size of 0.7 µm (s.d. 0.1 µm; n=60). Notably, while overexpression of hFis1 finally led to cytochrome c release and apoptosis (data not shown) (James et al., 2003), overexpression of MTP18 had no effect on viability or apoptosis in transfected cells (Tondera et al., 2004).

Transient overexpression of myc-tagged MTP18 in HeLa cells along with RNAi-mediated knockdown of hFis1 led to fragmentation in many of the transfected cells (~50%). This result suggests that low levels of hFis1 are sufficient to facilitate MTP18-induced mitochondrial fission (Fig. 6C,E, red bars). By contrast, reduction of MTP18 opposed the hFis1-induced mitochondrial fission in cells that had been doubly transfected for the transient coexpression of shRNA-MTP18 and hFis1 protein (Fig. 6D,E, green bars). Compared with control cells and MTP18-shRNA-expressing HeLa cells that express endogenous hFis1 (Fig. 6C, arrows), elevated levels of hFis1 (Fig. 6C, double arrow; and Fig. 6A for western blot) had no effect on the highly filamentous morphology phenotype (~84% of hFis1-overexpressing cells with fused mitochondria and 4% with fragmented mitochondria in shRNA-MTP18/hFis1 doubly transfected cells) because of the suppressed MTP18 expression (Fig. 6E, green bars). Thus, the effect of hFis1 overexpression on mitochondrial morphology was suppressed by knockdown of MTP18. Based on these data, we conclude that reduced MTP18 expression abolished the mitochondrial fragmentation. Therefore, MTP18 appears to be required for the mitochondrial fission process.

MTP18 as a novel intramitochondrial component for mitochondrial fission

Drp1 is responsible for mitochondrial fission. As shown above, dominant negative activity of Drp1K38A can partially block MTP18 induced mitochondrial fission. Overexpression of MTP18 in HeLa cells with reduced Drp1 levels (as assessed by western blot see Fig. 7A) revealed fused mitochondrial networks. Owing to loss of Drp1 function, fusion of mitochondria occurred (Fig. 7B, double arrow) but could not be disrupted by additional levels of MTP18 fission activity.
Fig. 6. For legend see next page.
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Discussion

Mitochondrial fission is a complex process for the coordinated vision of two mitochondrial membranes. So far, only a few conserved protein components of the outer membrane, such as the integral protein hFis1 and the peripheral recruited Drp1 protein, are known to be key mediators of mitochondrial fission.

We propose that MTP18 is a novel mitochondrial protein component involved in the maintenance of mitochondrial morphology by contributing to the control of mitochondrial fission. Changes in the expression levels of MTP18 by either overexpression or RNAi-mediated knockdown in mammalian cells affect mitochondrial morphology. The changes most probably interfere with the balance of mitochondrial fission and fusion. The effects on mitochondrial morphology caused by different levels of MTP18 are reminiscent of mitochondrial fission proteins: loss of MTP18 results in pronounced mitochondrial fusion due to the block of fission and consequently unopposed mitochondrial fusion. By contrast, high levels of MTP18 stimulate frequent mitochondrial fission, which results in a high number of fragmented mitochondria by overriding endogenous fusion processes. Furthermore, an increase of mitochondrial fusion activity, by either overexpressing the main mitochondrial fusion protein Mfn1 or the dominant negative form of the fission protein Drp1, antagonises MTP18-induced mitochondrial fission. These results indicate a functional role of MTP18 in controlling the balance of fission and fusion processes. Our applied transient assays in mammalian cells proves the contribution of MTP18 to the maintenance of mitochondrial morphology and resembles the genetic system in yeast used to study novel regulatory components of the mitochondrial morphology machinery. In addition, by using RNAi (shRNA-MTP18) together with protein overexpression (hFis1), we were able to show that MTP18 might be required for the mitochondrial fission process (Fig. 5). Nevertheless, RNAi only allows to assess the effect of protein reduction (knockdown), in contrast to ‘real’ loss-of-function effects caused by null mutations in the endogenous gene. Therefore, further genetic analysis (e.g. gene knockout) is needed to confirm the hFis1-independent requirement of MTP18 in regulating mitochondrial fission.

Our biochemical and microscopic data imply that MTP18 is an integral protein residing inside mitochondria. MTP18 acts most probably as a component of the inner membrane (IM) facing the inner membrane space (IMS), but it is also conceivable that MTP18 is anchored to the mitochondrial outer membrane (OM) from the inner leaflet and exposed to the IMS. Since the number of predicted TM domains has not been fully determined, the exact localisation and TM topology of MTP18 can be hypothesised.

Since the number of predicted TM domains has not been fully determined, the exact localisation and TM topology of MTP18 remains to be clarified. Thus, it remains unclear, in which mitochondrial compartment the N-terminus and C-terminus reside. Since the antibody used in this study was raised against the entire protein, the exact localisation of the protein cannot be resolved in detail. A putative association of MTP18 as a bipartite TM protein of the outer membrane – with both protein termini facing the intermembrane space – is conceivable, if cytoplasmic portions of MTP18 were protected by peripheral outer membrane proteins from proteinase K digestion. Nevertheless, our results indicate that MTP18 does not act like...
abrogates complete fission and might only result in partial
Conversely, lack of the inner mitochondrial fission machinery
membrane with intramitochondrial division components.
In other words, it is conceivable that MTP18
containing part cannot execute the fission step for both
this scenario, a hypothesised mitochondrial division machinery
the surface of the outer membrane, if MTP18 was lacking. In
function is not sufficient in setting up the division complex on
levels of hFis1 are present. This result suggests that hFis1
is required to allow hFis1-induced fission, even if elevated
overexpressed, MTP18 possibly promotes the assembly of
division of the mitochondrial outer membrane. This idea is in
agreement with the observation, that overexpression of hFis1,
in contrast to MTP18, results in the formation of differently
appearing fragmented mitochondria, which finally release
cytochrome c. For this reason, we speculate that MTP18 action
might be the limiting step during the fission process. When
overexpressed, MTP18 possibly promotes the assembly of
complete fission machineries that are responsible for faithful
mitochondrial division. Consequently, release of cytochrome c
does not occur. Interestingly, overexpression of Drp1 itself
does not increase mitochondrial fission, presumably owing to
the lack of sufficient mitochondrial division components such as hFis1 for the outer membrane and MTP18 for the inner
mitochondrial membrane/intramitochondrial compartment.
This proposed mode of MTP18 action is reminiscent of
MDM33, the yeast mitochondrial inner membrane protein.
MDM33 was proposed to be involved in controlling the fission
of the mitochondrial inner membrane by homotypic
interaction, resulting in the constriction of the inner membrane
(Messerschmitt et al., 2003). However, to date no homologue
or orthologue of MDM33 is known in higher eukaryotes. The
elucidation of the topology of MTP18 inside mitochondria and
the identification of interacting proteins will help to clarify
contribution of MTP18 to the fission process.

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