Control of mitochondrial morphology by a human mitofusin

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SUMMARY

Although changes in mitochondrial size and arrangement accompany both cellular differentiation and human disease, the mechanisms that mediate mitochondrial fusion, fission and morphogenesis in mammalian cells are not understood. We have identified two human genes encoding potential mediators of mitochondrial fusion. The mitofusins (Mfn1 and Mfn2) are homologs of the *Drosophila* protein *fuzzy onion* (Fzo) that associate with mitochondria and alter mitochondrial morphology when expressed by transient transfection in tissue culture cells. An internal region including a predicted bipartite transmembrane domain (TM) is sufficient to target Mfn2 to mitochondria and requires hydrophobic residues within the TM. Co-expression of Mfn2 with a dominant interfering mutant dynamin-related protein (Drp1^{K38A}) proposed to block mitochondrial fission resulted in long mitochondrial filaments and networks. Formation of mitochondrial filaments and networks required a wild-type Mfn2 GTPase domain, suggesting that the Mfn2 GTPase regulates or mediates mitochondrial fusion and that mitofusins and dynamin related GTPases play opposing roles in mitochondrial fusion and fission in mammals, as in yeast.

Key words: Mitochondrial fusion, Fzo, Human, GTPase, Dynamin related protein, Mitofusin

INTRODUCTION

Mitochondria differ in size and arrangement with cell type, physiological condition and pathological state (Arbustini et al., 1998; Inagaki et al., 1992). Developmentally regulated changes in mitochondrial morphology accompany cellular differentiation of diaphragm and skeletal muscle (Bakeeva et al., 1981; Kirkwood et al., 1986), retinal cones (Knabe and Kuhn, 1996), and male gametes (Olson and Winfrey, 1992; Tokuyasu, 1975). Mitochondria can rapidly change size and morphology in yeast, apparently regulated by a dynamic balance between fusion and fission events (Hermann and Shaw, 1998; Nunnari et al., 1997; Yaffe, 1999).

The first mediator of mitochondrial fusion identified was the *Drosophila* Fuzzy onions protein (Fzo), a large predicted transmembrane GTPase required for formation of the giant mitochondrial derivative during spermatogenesis (Hales and Fuller, 1997). The *S. cerevisiae* homolog of *fzo* mediates mitochondrial fusion events during mitotic growth and mating and is required for long-term maintenance of mitochondrial DNA (Hermann et al., 1998; Rapaport et al., 1998). Yeast Fzo1p behaves as an integral protein of the outer mitochondrial membrane, with its N-terminal GTPase domain facing the cytosol (Hermann et al., 1998). For both the *Drosophila* and yeast proteins, missense mutations in key residues of the GTPase motif indicated that mitochondrial fusion is dependent on GTPase activity (Hales and Fuller, 1997; Hermann et al., 1998).

In vegetatively growing yeast, mitochondrial fusion mediated by the Fzo1p GTPase is balanced by mitochondrial fission involving action of Dnm1p, a dynamin-related GTPase that assembles at fission sites on the cytoplasmic face of the outer mitochondrial membrane (Bleazard et al., 1999; Otsuga et al., 1998; Sesaki and Jensen, 1999). *dnm1* mutations abolish fission activity, and characteristic networks of interconnected mitochondrial tubules form. In contrast, loss of fusion activity in a temperature sensitive *fzo1-1* mutant strain caused rapid fragmentation of mitochondria (Hermann et al., 1998). This fragmentation is due in part to ongoing Dnm1p-fission activity, as it is suppressed in *fzo1, dnm1* double mutant strains (Bleazard et al., 1999; Sesaki and Jensen, 1999). Dynamin-related proteins from human cells (Drp1, Dlp1) and *C. elegans* (Drp-1) have also been implicated in controlling mitochondrial morphology. Transient expression of a dominant interfering form of human Drp1 carrying a mutation in the GTPase domain induces perinuclear clustering of mitochondria in tissue culture cells (Pitts et al., 1999; Smirnova et al., 1998). The *C. elegans* Drp-1 protein appears to mediate fission of the mitochondrial outer membrane (Labrousse et al., 1999).

Recombination between mitochondrial genomes after fusion of human cells suggests that mitochondrial fusion also takes place in mammals (Tang et al., 2000). However, no mammalian mediators of mitochondrial fusion have been identified. We show that a human homolog of Fzo has GTPase-dependent effects on mitochondrial morphology when expressed in tissue culture cells, consistent with a role in mitochondrial fusion. Co-transfection with human mitofusin and dominant interfering GTPase mutant human Drp1 suggest that mitofusins and dynamin-related GTPases play a reciprocal role in shaping mitochondrial morphology in mammals, as in yeast.

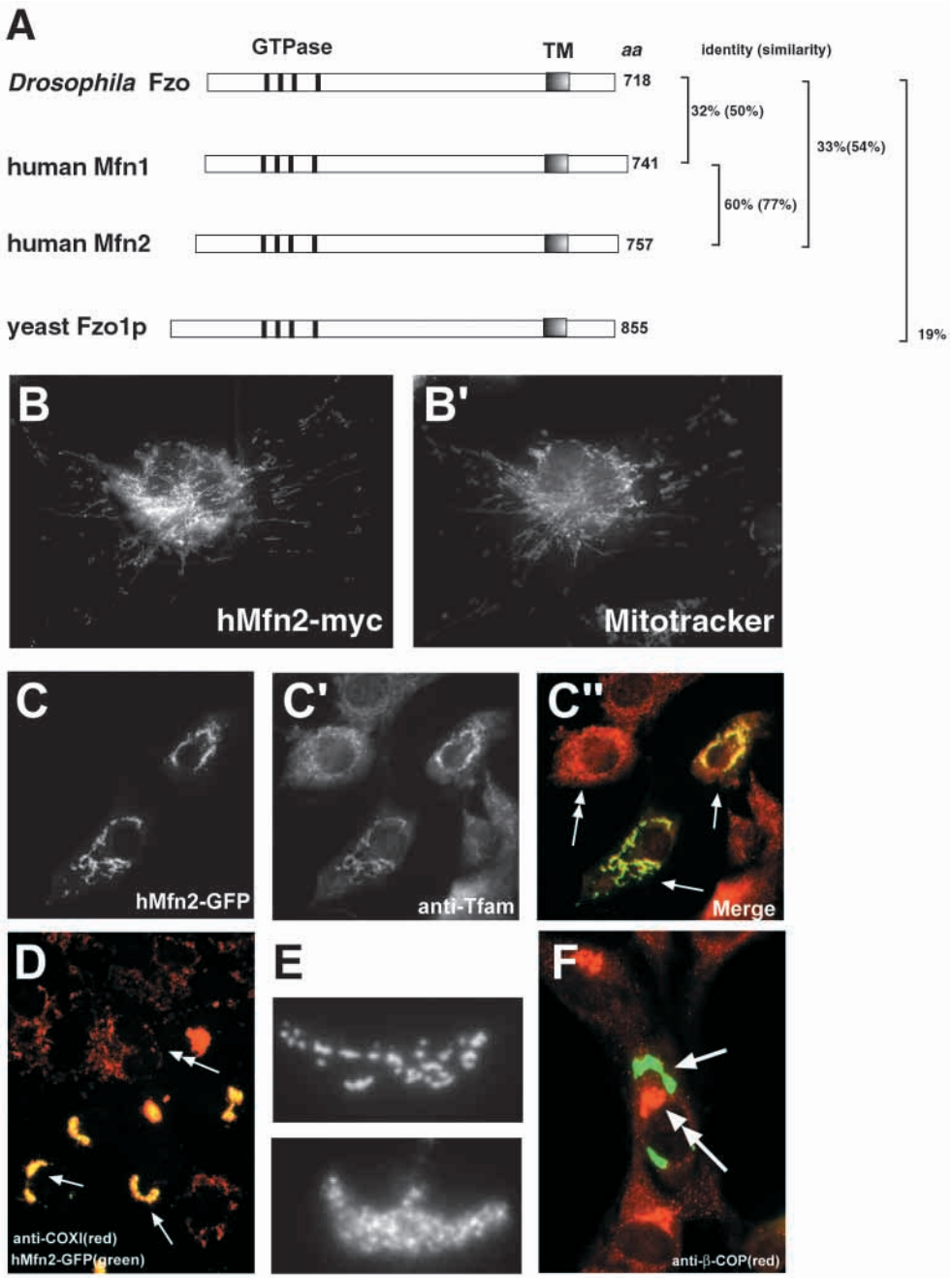

Fig. 1. Human Mfn proteins associate with mitochondria. (A) Homology and domain structure of human Mfn1 and Mfn2 compared with *Drosophila* Fzo and *S. cerevisiae* Fzo1p. Black bars, the four signature GTPase domain motifs; TM, predicted transmembrane domain; aa, predicted protein size in amino acids; brackets, homology among family members. (B) Stably transfected COS-7 cell expressing Mfn2-Myc stained with anti-Myc (B) and Mitotracker Red (B′). (C-F) Perinuclear aggregation of mitochondria after expression of full length Mfn2 in transiently transfected cells. (C,C′,C″) Mouse 10T1/2 fibroblasts transiently transfected with Mfn2 fused to GFP at the C terminus (Mfn2-GFP). (C) Subcellular localization of Mfn2-GFP fusion protein (GFP fluorescence). (C′) Distribution of mitochondria (anti-Tfam). (C″) Merge: GFP (green), T-fam (red). (D) COS-7 cells transiently transfected with Mfn2-GFP (green). Mitochondria counterstained with anti-COXI (red). (C″,D) Compare mitochondrial morphology between transfected (single arrows) and non transfected (double arrows) cells. (E) High magnification view of Mfn2-GFP-positive mitochondrial clusters from transfected HeLa cells. (F) Mouse 10T1/2 cells transfected with Mfn2-GFP (green, arrow) and counterstained for Golgi with anti-β-COP (red, double arrow).

MATERIALS AND METHODS

Human mitofusin genes

Mfn1 sequences were identified in the human genome and EST databases by TBLASTN (Altschul et al., 1990). The predicted Mfn1 open reading frame (ORF) (GenBank Accession Number: AF329637) was amplified by OneStep RT-PCR (Qiagen) from 50 ng of human heart poly-A^+-RNA (Clontech) using appropriate primers. The resulting 2.2 kb cDNA fragment was subcloned into pCR2.1TOPO (Invitrogen) and sequenced. Human Mfn2 was identified as the product of the KIAA0214 cDNA (Nagase et al., 1996) by searching the GenBank database with the *Drosophila* Fzo protein sequence using TBLASTN. The 4.55Kb Mfn2/KIAA0214 cDNA clone (kindly provided by T. Nagase, GenBank Accession Number D86987) covers the entire 2274 bp ORF, as well as additional sequences of the 5′- and 3′-UTR.

Expression constructs

The human Mfn1-coding region was excised with *Eco*RI from pCR2.1TOPO::Mfn1 and cloned into pEGFP-C2 for GFP fusion constructs or inserted into pIRES-GFP (Clontech) to express untagged Mfn1. To generate GFP- or Myc-tagged forms of Mfn2, the Mfn2 ORF was amplified using appropriate primers (PAN facility, Stanford University) and Turbo-*Pfu*-polymerase (Stratagene) and subcloned into pcDNA3.1/myc-HIS A (Invitrogen) or appropriate pEGFP expression vectors (Clontech) for N- or C-terminal GFP-fusion constructs. Deletion constructs were derived by dropping out fragments or using PCR to amplify defined regions of the Mfn2 cDNA. Missense mutations were introduced in Mfn2 by Quickchange Mutagenesis (Stratagene). The ANT-GFP expression construct (clone VLP32) was from T. Rapoport (Rolls et al., 1999). Expression plasmids for HA-tagged human Drp1 and mutant $Drp1^{K38A}$ were from A. van der Bliek (Smirnova et al., 1998).

Cell culture

COS-7 or HeLa cells and mouse 10T1/2 and LA9 fibroblasts were grown in DMEM with 10% fetal bovine serum, 100U/ml streptomycin, 100 U/ml penicillin. Cells were seeded on LabTekII glass slides and transfected using SuperFect (Qiagen), as per manufacturer's instructions. Pools of stably transfected cell populations expressing Mfn2-Myc were prepared by incubating transfected COS-7 cells in DMEM +10% FBS, 0.8 mg/ml Geniticin, maintained in selection medium for several passages over a period of 10 weeks, then processed for immunofluorescence.

Immunofluorescence

Primary antibodies were mouse monoclonal anti-cytochrome C-oxidase I subunit 1:60 (anti-COXI, Molecular Probes); rabbit polyclonal anti-mouse Tfam 1:1000 (Larsson et al., 1998); mouse monoclonal anti-myc-9E10 1:40; mouse monoclonal anti-HA 1:500 (Covance); mouse monoclonal anti-protein disulfide isomerase 1:50 (anti-PDI; Sigma); rabbit polyclonal anti-β-COP 1:100 (Sigma); and mouse monoclonal anti-α-tubulin 4AI 1:100 (Piperno and Fuller, 1985). Mitotracker Red CXRos (Molecular Probes) was used to label mitochondria, as per manufacturer's protocol. 19-24 hours after transfection, cells were rinsed twice with Dulbecco's PBS (phosphate buffered saline), fixed with 4% formaldehyde/PBS 15 minutes at room temperature, rinsed three times and permeabilized for 15 minutes in PBTD (PBS with 0.1% Triton X-100, 0.05% sodium deoxycholate), then blocked for 30 minutes with PBTB (PBS with 0.1% Trition X-100, 3% bovine serum albumin). Slides were incubated with primary antibody 2 hours at 37°C, rinsed four times with PBTB, incubated 2 hours at 37°C in FITC-/TRITC/Rhodamine-conjugated anti-rabbit or anti-mouse IgG (Jackson Immunochemicals; 1:200 in PBTB), washed extensively in PBS, and examined by epifluorescence on a Zeiss Axiophot microscope. Images recorded by CCD camera (Princeton Instruments; IPLab Sofware, Spectrum Software Signal Analytics) and processed with Adobe Photoshop.

RESULTS

Two human genes encode Fzo homologs

Two human genes designated mitofusin 1 and 2 (Mfn1 and Mfn2) encode homologs of *Drosophila fzo*. The human Mfn1 and Mfn2 predicted proteins have highest homology to *Drosophila fzo* protein in the predicted GTPase-domain (Fig. 1A). Overall, Mfn1 (741 amino acids) was 32% identical and 50% similar to *Drosophila fzo* protein, while Mfn2 (757 amino acids) was 33% identical and 54% similar to *Drosophila fzo* protein. The predicted Mfn1 and Mfn2 proteins were 60% identical and 77% similar to each other, with the most extensive homology in the predicted GTPase domain and the least conserved regions in the N- and C-terminal ends. Expressed sequence tags (ESTs) homologous to Mfn1 and Mfn2 were also found in mouse, cow, *Xenopus* and zebrafish (not shown).

Mammalian mitofusins associate with mitochondria

Human Mfn proteins co-localized with mitochondrial markers when expressed in stably transfected COS-7 cells (Myc-tagged Mfn2, Fig. 1B,B′) or after transient transfection with constructs encoding full-length Mfn2 protein fused to GFP (Fig. 1C) in

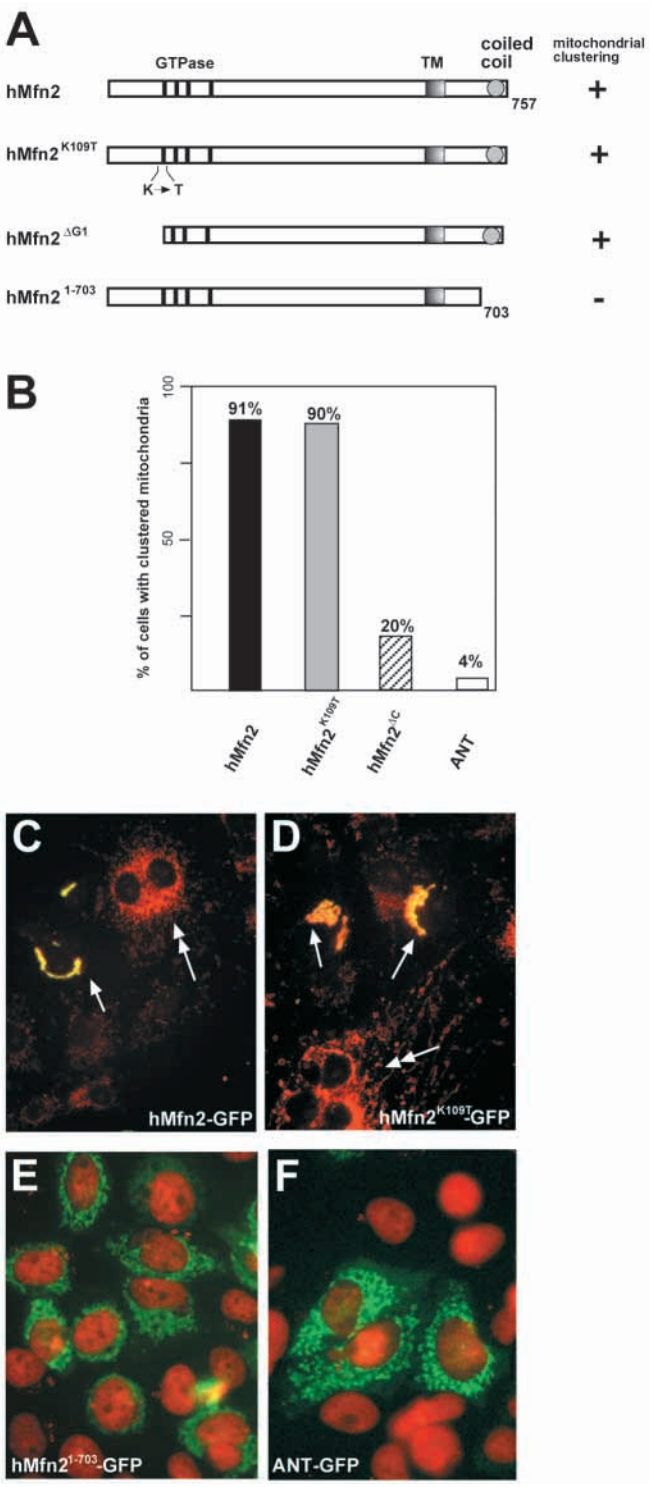

Fig. 2. Mitochondrial clustering independent of Mfn GTPase. (A) Effects of Mfn2-GFP variants on mitochondrial clustering. Blue circle, predicted C-terminal coiled coil region; black bars, the four signature GTPase domain motifs; TM, predicted transmembrane domain. Numbers indicate amino acid length. (B) Percent transfected COS-7 cells with clustered mitochondria in over 200 cells counted per construct. ANT, ATP-translocase-GFP control. (C,D) Representative COS-7 cells expressing either wild-type Mfn2-GFP (C, arrow) or mutant Mfn2^{K109T}-GFP (D, arrow). GFP (green); anti-COXI (red; double-arrows, non-transfected cells). (E,F) HeLa cells transfected with Mfn2-GFP variant lacking the last 54 C-terminal amino acids (E) or ATP-translocase-GFP fusion construct (F). GFP (green); nuclei counterstained with Hoechst (red).

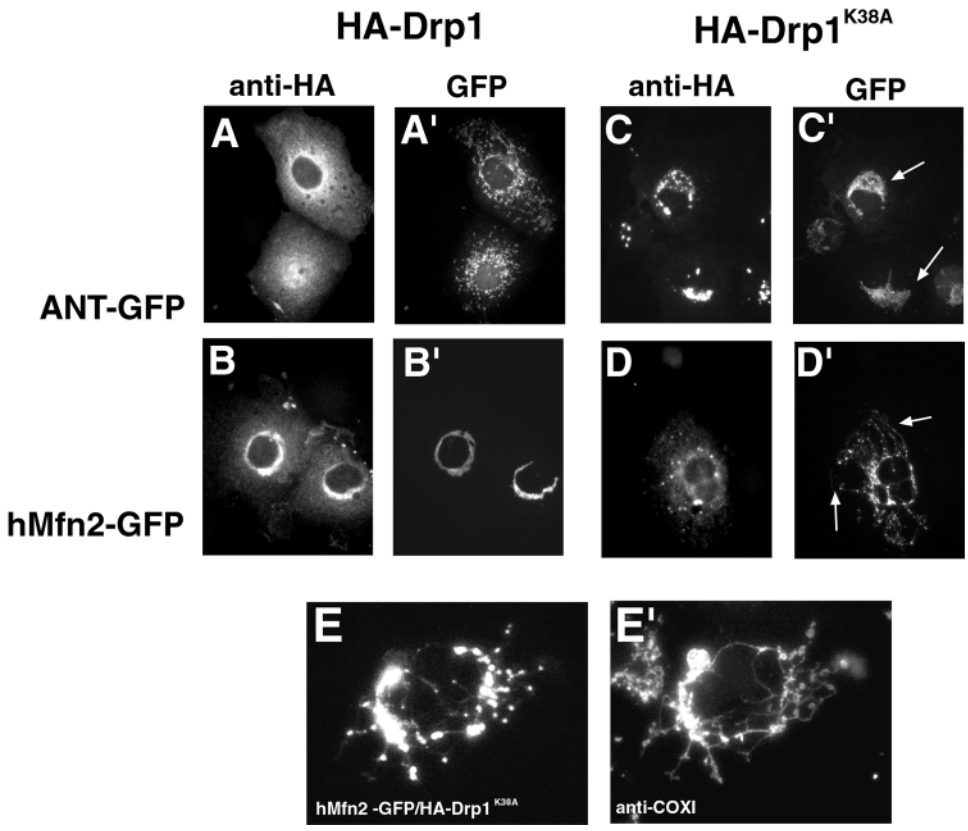

Fig. 3. Mitochondrial threads and networks caused by to co-expression of Mfn2-GFP with dominant interfering mutant dynamin-related protein Drp1. COS-7 cells co-transfected with ATP-nucleotide-translocase-GFP (ANT-GFP, A,A′,C,C′) or Mfn2-GFP (B,B′,D,D′) and HA-tagged wild-type (A,A′,B,B′) human Drp1 or HA-Drp1^{K38A} (C,C′,D,D′). (A,B,C,D) anti-HA counterstain to reveal sub-cellular distribution of Drp1 or Drp1^{K38A}. (A′,C′) GFP-fluorescence marks mitochondria (ANT-GFP). (B′,D′,E) GFP marks Mfn2-GFP fusion protein. (E,E′) Doubly transfected COS-7 cell expressing Mfn2-GFP and HA-Drp1^{K38A} stained with anti-COXI antibodies to show association of Mfn2-GFP (E) with mitochondrial threads and networks (E′).

all cell types tested (HeLa, LA9, 10T1/2, COS-7). Mfn2 epitope tagged with Myc and Mfn1, untagged or tagged with N-terminal GFP (GFP-Mfn1), also associated with mitochondria after transient transfection (not shown).

Expression of human Mfn proteins alters mitochondrial morphology

Mitochondrial morphology was dramatically altered in transiently transfected cells expressing Mfn2 (Fig. 1C), ranging from conversion of the normal dispersed distribution of punctate mitochondia to more reticular structures (Fig. 1C), to extensive perinuclear clustering (Fig. 1D-F). Perinuclear clustering of mitochondria was observed in over 90% of transiently transfected cells expressing either Mfn2-Myc or Mfn2-GFP. Perinuclear clusters usually appeared to contain most of the mitochondria in the cell, based on counterstaining with an independent mitochondrial marker (Fig. 1D; arrow). The perinuclear clusters varied in size and shape from cell to cell, but were usually crescent-shaped aggregates or perinuclear rings of distinct fluorescent particles (Fig. 1D,E).

Expression of C-terminal Myc-Mfn2, N-terminal GFP-Mfn2 and untagged or N-terminal GFP-Mfn1 protein after transient transfection also caused perinuclear mitochondrial clustering (not shown). Mitochondrial morphology appeared unaffected in parallel transient transfections with an ATP-translocase-GFP construct, which drives overexpression of a protein of the inner mitochondrial membrane (not shown; see Fig. 2F). Although the shape of the clustered mitochondrial mass in Mfn2-GFP-tranfected cells was reminiscent of the Golgi apparatus, counterstaining with a Golgi marker revealed that the Golgi and the mitochondrial cluster in Mfn2-GFP-expressing cells did not co-localize (Fig. 1F). Transient expression of Mfn2-GFP did not grossly affect the structure and subcellular localization of the Golgi apparatus, microtubule cytoskeleton or endoplasmic reticulum, based on co-staining with anti-β-COP (Fig. 1F), anti-α-tubulin or anti-PDI (not shown).

The Mfn2 GTPase is not required for mitochondrial clustering

Mitochondrial clustering after expression of Mfn2 may not be due to excessive mitochondrial fusion activity in transfected cells. Conserved residues in the predicted GTPase domain of *Drosophila* Fzo protein and yeast Fzo1p are required for mitochondrial fusion (Bourne et al., 1991; Hales and Fuller, 1997; Hermann et al., 1998). A corresponding missense mutation in the predicted GTPase domain of Mfn2 (Mfn2^{K109T}-GFP; Fig. 2A) did not reduce mitochondrial clustering or association of the fusion protein with mitochondria (Fig. 2B,D), suggesting that the predicted GTPase activity is not responsible for mitochondrial clustering. In addition, N-terminal deletion constructs encoding human Mfn2 or mouse Mfn1 lacking parts of the predicted GTPase domain (deletion of Mfn2 through G1 – Fig. 2A; deletion of mouse Mfn1 through G3 (not shown) still showed mitochondrial clustering upon transient expression.

Residues near the Mfn2 C terminus, however, were required for mitochondrial clustering. Both the Mfn1 and Mfn2 predicted proteins have a high probability of forming a coiled-coil structure in the C-terminal 20 to 30 amino acids, based on the algorithms of Lupas and Wolf (Lupas et al., 1991; Wolf et al., 1997), a feature

conserved in *Drosophila* Fzo. Truncated Mfn2, which lacks the last C-terminal 54 residues fused to GFP (Mfn2$^{1-703}$-GFP (Fig. 2A) co-localized with the mitochondrial marker COXI when expressed in COS-7, HeLa or 10T1/2 cells (not shown) after transient transfection. However, mitochondrial clustering was strongly reduced in Mfn2$^{1-703}$-GFP transfected cells compared with cells transfected with full-length Mfn2-GFP (Fig. 2B,E), suggesting a role for the predicted coiled-coil domain in perinuclear mitochondrial clustering.

GTPase dependent Mfn function in mitochondrial morphology revealed by altering activity of the predicted mitochondrial fission protein Drp1

In vegetatively growing yeast cells, mitochondrial fusion is balanced by ongoing mitochondrial fission mediated by action of the dynamin-related protein, Dnm1 (Bleazard et al., 1999; Sesaki and Jensen, 1999). To test if effects of overexpression of Mfn on mitochondrial fusion might be masked by rapid mitochondrial fission, we co-expressed Mfn fusion protein and a mutant form of the mammalian dynamin-related protein Drp1, which is predicted to dominantly interfere with function of endogenous Drp1 activity (Smirnova et al., 1998; Pitts et al., 1999).

Transiently expressed, HA-tagged wild type human Drp1 protein was distributed ubiquitously in the cytoplasm of COS-7 cells transiently transfected with HA-Drp1 and the mitochondrial marker ATP-Translocase-GFP (Fig. 3A). Expression of wild-type HA-Drp1 after transient transfection did not visibly affect mitochondrial morphology compared with transfection with the ANT-GFP mitochondrial marker alone (not shown). Co-expression of wild-type HA-Drp1 with wild-type Mfn2-GFP resulted in mitochondrial clustering resembling that observed after expression of Mfn2-GFP alone. HA-Drp1 partially co-localized with the clustered mitochondria (Fig. 3B).

A point mutation in a key GTPase residue of Drp1 resulted in an altered subcellular distribution of the Drp1 protein. HA-Drp1^{K38A} appeared in dot-like structures after transient transfection, in contrast to the diffuse cytoplasmic distribution observed for the wild type fusion protein (Fig. 3C,D; see also Pitts et al., 1999). In addition, transient transfection with the HA-Drp1^{K38A} construct affected mitochondrial morphology, resulting in formation of perinuclear aggregates (Fig. 3C′).

When Mfn2-GFP was co-expressed with GTPase-mutant HA-Drp1^{K38A}, mitochondria formed thin GFP-positive tubules extending from the perinuclear mitochondrial cluster towards the cell periphery (Figs 3D′,E, 4A). Many cells displayed an interconnected network of tubular GFP-positive structures (Fig. 4B,C). The GFP-positive threads and networks were mitochondria based, as shown by co-staining with anti-COXI (Fig. 3E).

Formation of mitochondrial threads and networks (Fig. 4A-C) depended on a wild-type Mfn2-GTPase domain. Mutation of a key predicted GTPase residue in Mfn2 blocked formation of mitochondrial threads and networks upon co-transfection with Drp1^{K38A} (Fig. 4F), resulting in mitochondrial clusters indistinguishable from those in cells expressing Mfn2-GFP alone (Fig. 4D,E). The HA-tagged Drp1^{K38A} mutant fission protein appeared in dot-like structures in the Mfn2^{K109T}-GFP/HA-Drp1^{K38A} doubly transfected cells (Fig. 4D,E; red) but did not appear to be associated with the perinuclear mitochondria.

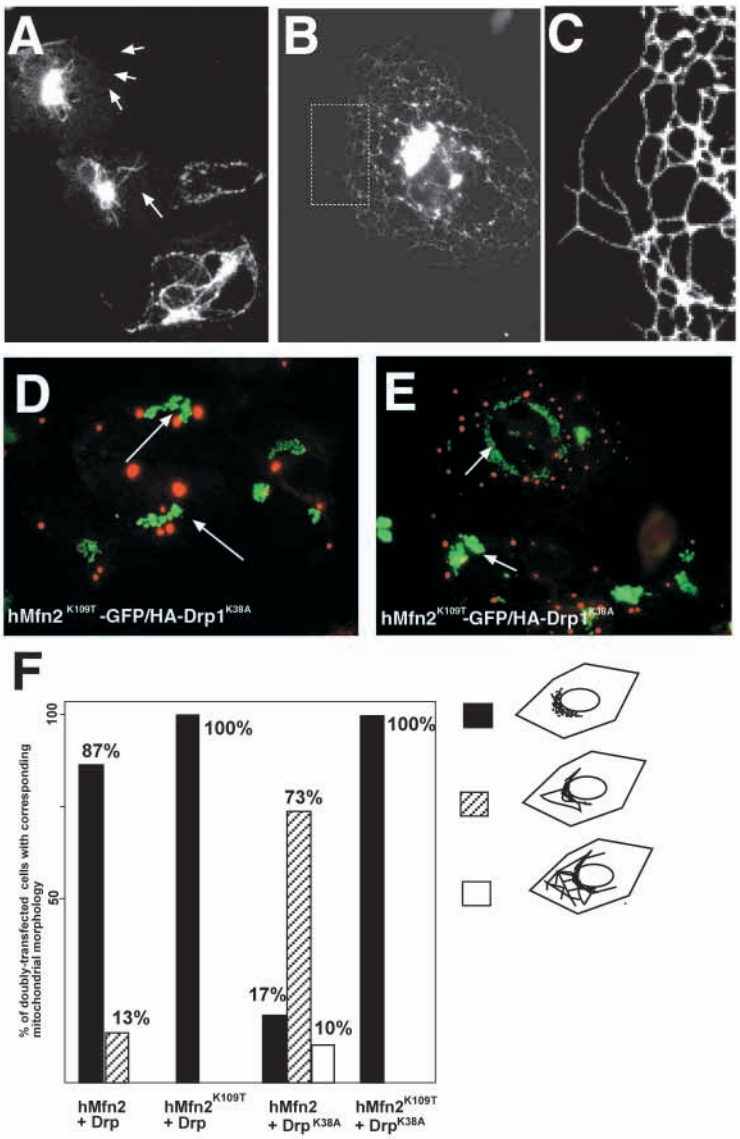

Fig. 4. Formation of mitochondrial threads and networks depends on wild type human Mfn2 GTPase domain. (A,B) GFP-fluorescence in representative COS-7 cells transfected with wild-type Mfn2-GFP and mutant HA-hDrp1^{K38A}. Arrows, mitochondrial threads. (C) Higher magnification view of rectangle in B. (D,E) Representative COS-7 cells expressing GTPase mutant Mfn2^{K109T}-GFP fusion protein (green) and HA-tagged mutant Drp1^{K38A} (red; anti-HA). Arrows, mitochondrial clusters. (F) Percent doubly transfected COS-7 cells with indicated mitochondrial morphology for combinations of wild-type and mutant Mfn2-GFP and HA-Drp1. 19 hours after transfection, cells were fixed and counterstained with anti-HA. Mitochondrial distribution was scored by examination of Mfn-GFP. Phenotypic categories based on GFP distribution on the right (black box, clustered mitochondria; hatched box, clustered mitochondria with string-like extensions; white box, interconnected, web-like mitochondrial structures). 200 doubly transfected cells were counted for each combination. Numbers of cells with corresponding mitochondrial distributions shown as a percentage of doubly transfected cells.

An internal region of Mfn2 is sufficient for mitochondrial targeting

Little is known about the pathways governing targeting of nuclear encoded proteins to the mitochondrial outer membrane. To identify sequences required for association of Mfn proteins with mitochondria, we tested Mfn2 protein fragments expressed after transient transfection (Fig. 5A). Although analysis of Mfn2 by PSORT (Nakai and Horton, 1999) and MITOPROT (Scharfe et al., 1999) indicated a predicted N-terminal mitochondrial targeting signal, the N-terminal 96 amino acids of Mfn2 were neither sufficient nor necessary for association with mitochondria (Fig. 5A). Mfn2$^{97\text{-}757}$-Myc protein co-localized with mitochondrial markers and caused perinuclear clustering of mitochondria when expressed after transient transfection (not shown).

A 93 amino acid internal fragment of Mfn2 containing the predicted transmembrane domain (TM) and C-terminal flanking sequences was sufficient to target a GFP reporter to mitochondria (Fig. 5A,B), although a small amount of GFP fluorescence also appeared throughout the cell (Fig. 5B). Expression of Mfn2$^{610\text{-}703}$-GFP did not cause perinuclear mitochondrial clustering (Fig. 5B). The predicted transmembrane domain contains two stretches of hydrophobic residues separated by one (Fzo) or two (Mfns) charged residues (Fig. 5C). This bipartite hydrophobic domain precedes a predicted alpha-helical region (AH) characterized by a conserved stretch of charged residues. When the bipartite hydrophobic region was deleted from otherwise full-length Mfn2-GFP, the protein no longer localized to mitochondria (Fig. 5D), as expected if the region acted as a transmembrane domain. Although the first hydrophobic region is relatively short, it appeared important, as changing three hydrophobic amino acids into charged residues abolished mitochondrial targeting of the full-length fusion protein (Mfn2^{RRE}-GFP; Fig. 5D,E). Instead, the GFP reporter was distributed diffusely in the cytoplasm and appeared high in the nucleus, either because the cells were thicker over the nuclear region or because the mutant protein was preferentially

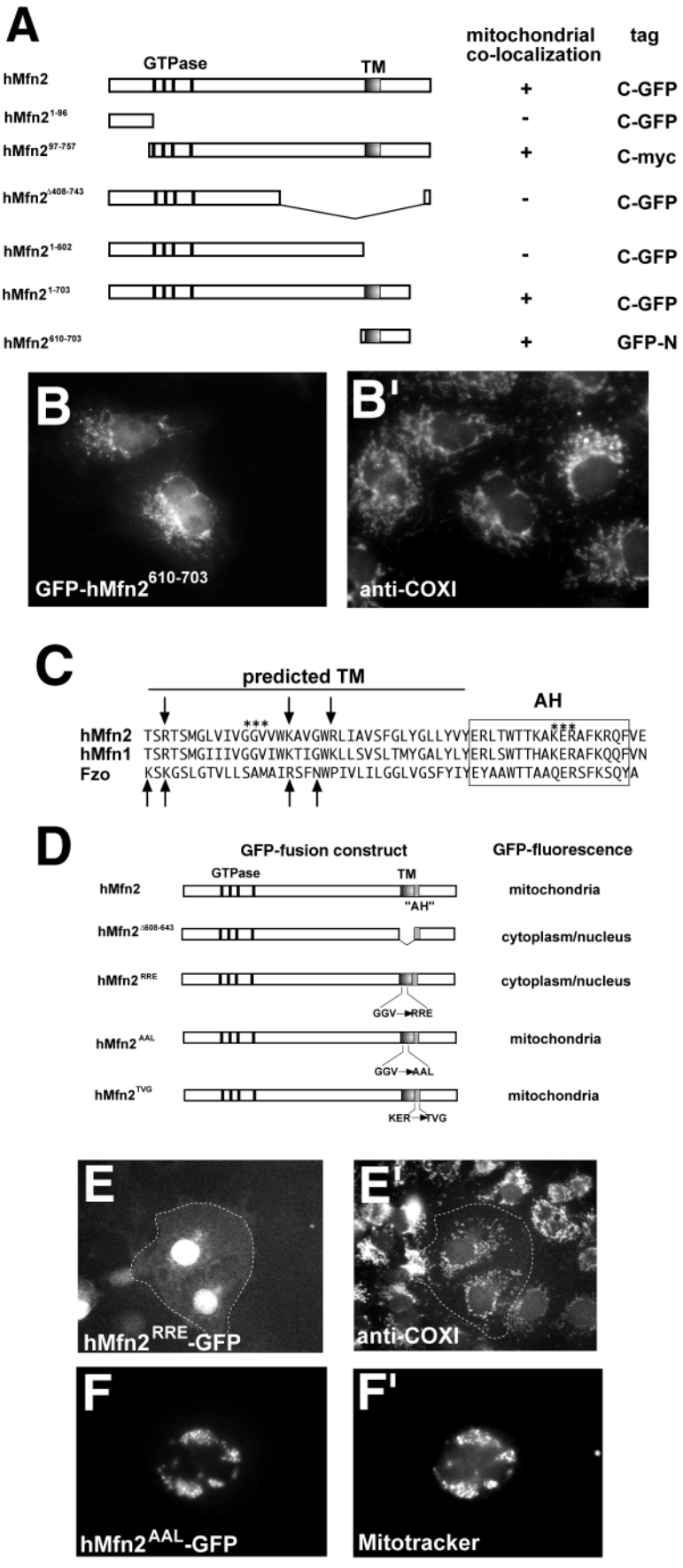

Fig. 5. Association of Mfn2 with mitochondria via an internal targeting domain. (A) Expression constructs tested for association of Mfns with mitochondria after transient transfection in COS-7 cells, position and tag indicated at right. (B,B′) GFP fluorescence in COS-7 cells expressing a 93aa internal region of Mfn2 (amino acids 610-703) fused to GFP. (B) GFP fluorescence. (B′) Same field stained with anti-COXI to reveal mitochondria. (C) Alignment of predicted transmembrane domain (TM) and adjacent conserved region (AH, boxed) in human Mfns and *Drosophila* Fzo. Asterisks mark positions of amino acid substitutions; arrows mark charged residues in predicted TM. (D) Subcellular localization of mutant Mfn2-GFP fusion constructs tested (all C-terminal GFP). (E,E′) COS-7 cells expressing mutant Mfn2^{RRE}-GFP. (E) GFP. (E′) Anti-COXI to reveal location and morphology of mitochondria. (F,F′) COS-7 cell expressing mutant Mfn2^{AAL}-GFP fusion protein. (F) GFP. (F′) Same field with Mitotracker.

localized to the nucleus. In contrast, substituting other hydrophobic residues for these same amino acids (construct Mfn2$^{\Delta AL}$-GFP) did not affect mitochondrial targeting (Fig. 5F). Mutations in the AH-region that changed conserved charged residues to non-charged residues did not abolish either targeting of the Mfn2-GFP fusion protein to mitochondria (Fig. 5D) or formation of perinuclear mitochondrial clusters (not shown). Although many precursor proteins are imported into mitochondria by an N-terminal cleavable pre-sequence, internal targeting sequences have been described for other nuclear-encoded mitochondrial outer membrane proteins, including mammalian OMP25, which also has a C-terminal transmembrane domain-containing region that is capable of correct mitochondrial localization (Nemoto and De Camilli, 1999).

DISCUSSION

The functional similarities between the yeast and *Drosophila* Fzo proteins suggest that mammalian *fzo* homologs might also mediate mitochondrial fusion. Consistent with this hypothesis, we found that expression of the human Mfn2 protein by transient transfection in cells also expressing a dominant interfering form of *Drp1* caused formation of long strings and extensive networks of mitochondria. Formation of the mitochondrial cables and networks was dependent on a wild-type GTPase domain of Mfn2, suggesting a central role for GTPase activity in the mechanism of action of the Mfn proteins, as in their homologs *yfzo1* and *Drosophila fzo*. The mitochondrial networks closely resembled those formed by mitochondrial fusion in *dnm1* mutant yeast cells, owing to unopposed activity of the Mfn homolog yFzo1p (Bleazard et al., 1999). *Dnm1* encodes a yeast homolog of human Drp1 required for mitochondrial fission. We propose that in mammalian cells, as in yeast, the size and morphological arrangement of mitochondria are due to a dynamic balance between Mfn-dependent mitochondrial fusion and Drp1-dependent mitochondrial fission. Expression of Drp1^{K38A} may interfere with mitochondrial fission by blocking function of the endogenous Drp1 protein. Accordingly, we propose that the formation of mitochondrial cables and networks observed in the doubly transfected cells resulted from excessive mitochondrial fusion due to overexpression of Mfn protein, combined with decreased mitochondrial fission due to interference by the mutant Drp1.

In cells transfected with wild-type Mfn alone, or co-transfected with Mfn2 and wild type Drp1, continuous mitochondrial fission may mask effects of overexpression of Mfn by rapidly cleaving any long mitochondria that form. In this case, a second, GTPase-independent role of Mfn proteins in mitochondrial morphogenesis or function may be revealed in the perinucelar aggregation of mitochondria. The predicted C-terminal coiled-coil domain and other parts of the Mfn protein could be involved in mechanisms essential for normal mitochondrial distribution, such as transport or positioning of mitochondria or lipid metabolism of mitochondrial membranes. Disruption of the mouse kinesin motor protein KIF5B also results in perinuclear mitochondrial clustering (Tanaka et al., 1998). Likewise, transient overexpression of the mammalian mitochondrial outer membrane protein OMP25 leads to perinuclear aggregation of mitochondria, possibly due to recruitment of synaptojanin 2A and local modulation of inositol phospholipid metabolism (Nemoto and De Camilli, 1999). Alternatively, overexpression of Mfn proteins could cause perinuclear clustering if high levels of the protein expressed after transient transfection block mitochondrial import channels, causing defects in import or function of other mitochondrial proteins. It is also possible that the perinuclear collapse of mitochondria is due to a negative effect of expressing the protein in a cell type in which it is not normally present. However, northern blot analysis revealed that both Mfn1 and Mfn2 transcripts were expressed in different cultured cell lines (mouse 10T1/2 fibroblasts, HeLa cells), as well as in a variety of adult tissues (A. S. and M. T. F., unpublished observations).

Biochemical analysis of the yeast Fzo1p homolog indicated that the protein is anchored to the mitochondrial outer membrane with both its N-terminal GTPase domain (Hermann et al., 1998) and C-terminal ends exposed to the cytoplasm (J. Thatcher and J. Shaw, personal communication; Rapaport et al., 1998). If Mfns are associated with mitochondria via similar topology, the bipartite hydrophobic region could pass through the mitochondrial outer membrane and back out. The C-terminal predicted coiled-coil region (trimer-type for Mfn1; dimer-type for Mfn2) could form an integral part of the fusion mechanism, reminiscent of membrane fusion by influenza virus hemagglutinin or the SNARE protein complex. If so, we propose that the Mfn GTPase domain displayed on the cytoplasmic face of the mitochondrial outer membrane could interact with the C-terminal coiled-coil region to regulate mitochondrial fusion.

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