Fis1 acts as a mitochondrial recruitment factor for TBC1D15 that is involved in regulation of mitochondrial morphology

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Accepted 27 September 2012
Journal of Cell Science 126, 176–185

doi: 10.1242/jcs.111211

Summary

In yeast, C-tail-anchored mitochondrial outer membrane protein Fis1 recruits the mitochondrial-fission-regulating GTPase Dnm1 to mitochondrial fission sites. However, the function of its mammalian homologue remains enigmatic because it has been reported to be dispensable for the mitochondrial recruitment of Drp1, a mammalian homologue of Dnm1. We identified TBC1D15 as a Fis1-binding protein in HeLa cell extracts. Immunoprecipitation revealed that Fis1 efficiently interacts with TBC1D15 but not with Drp1. Bacterially expressed Fis1 and TBC1D15 formed a direct and stable complex. Exogenously expressed TBC1D15 localized mainly in cytoplasm in HeLa cells, but when coexpressed with Fis1 it localized to mitochondria. Knockdown of TBC1D15 induced highly developed mitochondrial network structures similar to the effect of Fis1 knockdown, suggesting that the TBC1D15 and Fis1 are associated with the regulation of mitochondrial morphology independently of Drp1. These data suggest that Fis1 acts as a mitochondrial receptor in the recruitment of mitochondrial morphology protein in mammalian cells.

Key words: Mitochondria, Membrane fission, Fis1, Dynamin-related protein, Drp1

Introduction

Mitochondria play central roles in a variety of cellular processes such as lipid metabolism, apoptosis, Ca^{2+} signaling, and energy production by oxidative phosphorylation (McBride et al., 2006). They dynamically change their morphology by frequent fusion and fission in response to cellular differentiation or pathologic conditions, and these reactions are regulated by evolutionally conserved GTPase proteins (Hoppins et al., 2007; Ishihara et al., 2012; Liesa et al., 2009; Okamoto and Shaw, 2005; Westermann, 2010).

In mammals, mitochondrial fusion is regulated by two outer membrane mitofusin (Mfn) proteins, Mfn1 and Mfn2, which are homologues of yeast and *Drosophila* Fzo proteins (Chen et al., 2003; Eura et al., 2003; Santel and Fuller, 2001), while the inner-membrane-bound GTPase optic atrophy (OPA)1, a homologue of yeast Mgm1, regulates the fusion and cristae formation of the inner membrane (Olichon et al., 2003). Mfn2 and OPA1 have also been identified as causal gene products in the neurodegenerative disorders Charcot-Marie-Tooth neuropathy type 2a and autosomal dominant optic atrophy type-I, respectively (Alexander et al., 2000; Delettre et al., 2000; Züchner et al., 2004). Mice lacking these mitochondrial fusion factors show defects in embryonic development and tissue differentiation (Chen et al., 2003; Chen et al., 2007; Davies et al., 2007; Zhang et al., 2011). Dynamin-related GTPase protein (Drp1, also known as Dlp1), is the homologue of yeast Dnm1 and plays a key role in mitochondrial and peroxisome fission (Koch et al., 2003; Smirnova et al., 1998). Moreover, Drp1-dependent mitochondrial fission is essential for embryonic development and normal neuronal differentiation (Ishihara et al., 2009; Wakabayashi et al., 2009).

Drp1 accumulates at mitochondrial fission sites as punctate structures, then stimulates mitochondrial fission (Smirnova et al., 1998; Westermann, 2010). Recruitment of Drp1 from the cytoplasm to the mitochondria is a key step in fission regulation. In yeast, the mitochondrial outer membrane protein Fis1 plays an essential role in the recruitment of Dnm1 to the mitochondria (Hoppins et al., 2007; Mozdy et al., 2000; Okamoto and Shaw, 2005; Westermann, 2010). Fis1 has tetratricopeptide-repeat (TPR)-like domains at the N-terminus exposed to the cytoplasm, and a transmembrane (TM) domain at the C-terminus to anchor at the mitochondrial outer membrane (James et al., 2003; Jofuku et al., 2005; Suzuki et al., 2003; Yoon et al., 2003). Cytoplasmic Dnm1 assembles with mitochondrial Fis1 via interaction with the WD motif containing proteins Mdv1 or Caf4, and severs the membrane by GTP hydrolysis (Hoppins et al., 2007; Tieu et al., 2002).

Although Drp1 and Fis1 are evolutionally conserved, the mechanism of Drp1 mitochondrial recruitment differs between species (Okamoto and Shaw, 2005; Otera and Mihara, 2011; Westermann, 2010). Drp1 recruitment in higher plants involves the soluble protein Elm1, and no role has as yet been attributed to the recently identified Fis1 homologues (Arimura et al., 2008). Mammalian Drp1 recruitment requires the outer membrane proteins mitochondrial fission factor (Mff), mitochondrial dynamics (MiD)51/mitochondrial elongation factor (MIEF)1 and MiD49 (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010; Palmer et al., 2011; Zhao et al., 2011). Fis1 appears to be dispensable in this role (Otera et al., 2010), although several reports suggest that Fis1 exogenous expression induces mitochondrial fragmentation and that Fis1 knockdown affects mitochondrial morphology (James et al., 2003; Jofuku et al., 2005; Yoon et al., 2003). Thus the molecular function of Fis1 in mammalian mitochondrial morphology regulation still remains to be clarified.

To elucidate the roles of Fis1 and determine its molecular function in mammalian mitochondrial morphology regulation, we searched mammalian cells for Fis1-binding proteins. We identified TBC1D15, which localizes both to the cytoplasm and to the mitochondria via direct interaction with Fis1. TBC1D15 knockdown extended mitochondrial networks without affecting mitochondrial Drp1 foci. Thus we speculate that Fis1 might be

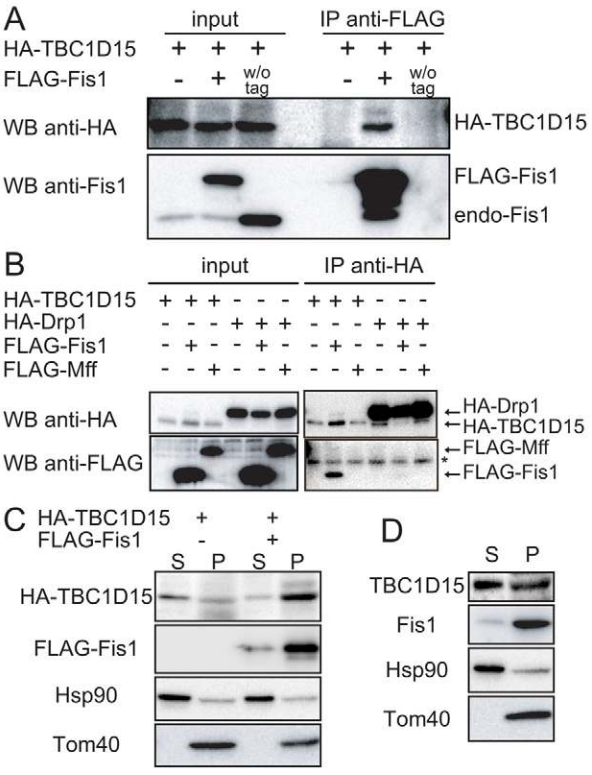

Fig. 1. TBC1D15 interacts with Fis1. (**A**) HeLa cells expressing HA–TBC1D15 and FLAG–Fis1 were subjected to immunoprecipitation using anti-FLAG antibody, then analyzed by SDS-PAGE and detected by immunoblotting using anti-HA and anti-Fis1 antibodies. A non-tagged version of Fis1 (w/o tag) was used as a negative control. (**B**) HeLa cells expressing the indicated constructs were subjected to immunoprecipitation using anti-HA antibody, then analyzed by immunoblotting using anti-HA and anti-FLAG antibodies. The asterisk indicates a nonspecific band. (**C**) HeLa cells were transfected with the indicated expression vectors. After 24 h in culture, cells were recovered, treated with 25 µg/ml digitonin on ice for 15 min, and fractionated by centrifugation. The supernatant fraction (S) and fivefold of the pellet fraction (P) were analyzed by immunoblotting using the indicated antibodies. (**D**) Endogenously expressed TBC1D15 in HeLa cells was analyzed as in C.

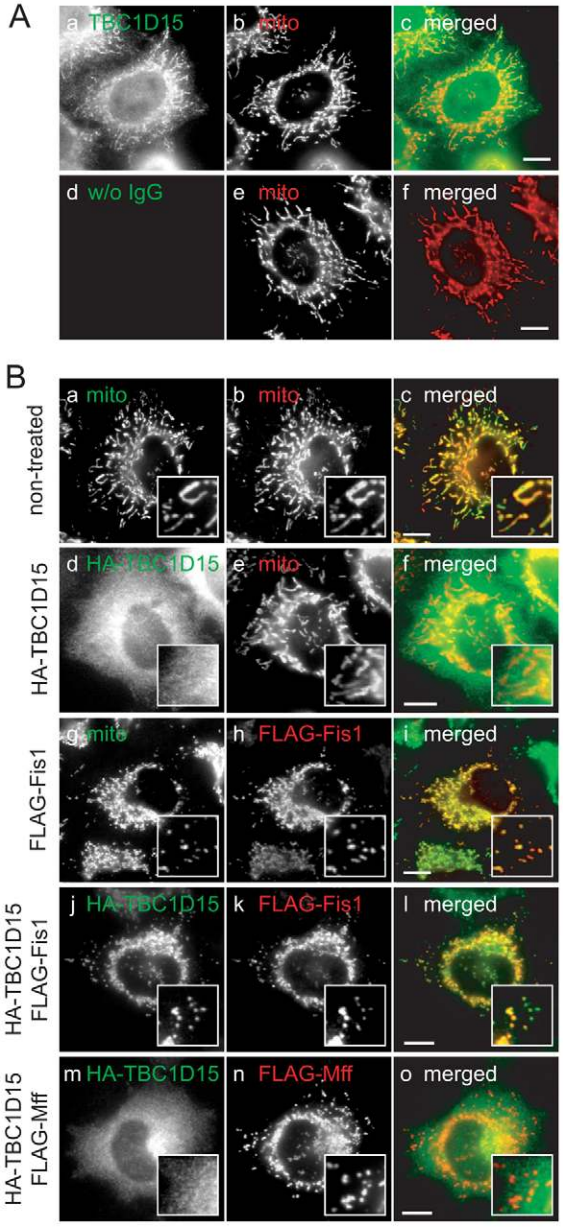

Fig. 2. TBC1D15 localizes to mitochondria dependent on Fis1. (**A**) HeLa cells expressing mitochondrial RFP (mitRFP) were immunostained with anti-TBC1D15 antibodies (a–c) or left unstained (d–f). Merged images of TBC1D15 (green) and mitRFP (red) are shown on the right. (**B**) HeLa cells were transfected with the indicated expression vectors. After 24 h culture, cells were fixed and immunostained using mouse monoclonal anti-HA antibody and rabbit polyclonal anti-FLAG antibody. Mouse monoclonal anti-mtHSP70 antibody and rabbit polyclonal anti-mitofilin antibody were used as mitochondrial markers (mito). Merged images of HA–TBC1D15 or mtHSP70 (green) and mitofilin, FLAG–Fis1 or FLAG–Mff (red) are shown. Scale Bars: 10 µm. Inset, magnified images of a 10×10 µm area.

involved in mitochondrial morphology regulation by recruiting TBC1D15 in a Drp1-independent manner.

Results

Identification of TBC1D15 as a Fis1-binding protein

To analyze the molecular functions of Fis1, we used co-immunoprecipitation with stably expressed FLAG-tagged rat Fis1 (FLAG–Fis1) to search HeLa cells for Fis1-interacting proteins. Immunoprecipitation using a FLAG antibody from digitonin-solubilized lysates revealed an ~75 kDa major specific interacting protein (supplementary material Fig. S1A). Mass spectrometry of the band showed that the majority of fragments were from TBC1D15. TBC1D15 is a member of the TBC (Tre2/Bub2/Cdc16)-domain-containing protein family, which is the domain conserved in the GTPase-activating protein (GAP) for small GTPase Rab family proteins (Barr and Lambright, 2010; Fukuda, 2011) (supplementary material Fig. S1B, shown in blue). No other known homology motifs or domains such as the WD motif were detectable in the molecule. Its calculated molecular size was 77.8 kDa and hydropathy profiling revealed no obvious transmembrane domains (supplementary material Fig. S1C).

The involvement of the GAP activity of TBC1D15 or identification of the partner Rab GTPase proteins in the mitochondrial morphology regulation remains an important issue to be investigated in the future (see Discussion).

TBC1D15 interacts with Fis1

To confirm the interaction of TBC1D15 with Fis1, HA-tagged TBC1D15 (HA–TBC1D15) was coexpressed with FLAG–Fis1 in HeLa cells and subjected to immunoprecipitation after digitonin solubilization. HA–TBC1D15 was recovered with FLAG–Fis1, but not with Fis1 without the epitope tag (Fig. 1A) or with cotransfected FLAG–Drp1 (data not shown).

Immunoprecipitation using anti-HA antibodies co-precipitated FLAG–Fis1 with HA–TBC1D15 (Fig. 1B). TBC1D15 was expressed at higher levels when coexpressed with Fis1, suggesting that it was stabilized by the interaction (Fig. 1B, also see Fig. 4D,G). The Fis1 expression also stabilized the endogenous TBC1D15 (supplementary material Fig. S1E). Consistent with previous report (Otera et al., 2010), HA–Drp1 was not isolated with FLAG–Fis1 (Fig. 1B) in the absence of cross-linker treatment. Moreover, HA–TBC1D15 was not co-precipitated with FLAG–Mff, the outer membrane protein reported to be involved in Drp1 mitochondrial recruitment (Fig. 1B). We also found that the Drp1–Fis1 interaction was not stimulated by TBC1D15 expression (data not shown), suggesting that TBC1D15 is not a functional counterpart of yeast Mdv1 and Caf4 (Hoppins et al., 2007; Okamoto and Shaw, 2005).

Fis1 stimulates mitochondrial localization of TBC1D15

Immunofluorescence microscopy using specific antibodies against a recombinant TBC1D15 fragment (amino acid residues 1–333) revealed staining of the cytoplasm, as reported previously (Zhang et al., 2005), as well as mitochondrial network structures (Fig. 2A), demonstrating that endogenously expressed TBC1D15 localizes to both the mitochondria and cytoplasm in HeLa cells. The vast majority of exogenously expressed HA–TBC1D15 localized to the cytoplasm of HeLa cells, and was also detected at low levels on mitochondria (Fig. 2Bd–f). Mitochondrial morphology was normal in cells expressing the HA–TBC1D15 alone (Fig. 2Bd–f), suggesting no dominant effect following exogenous expression.

We next analyzed the effect of Fis1 on HA–TBC1D15 localization. Exogenous expression of FLAG–Fis1 induced

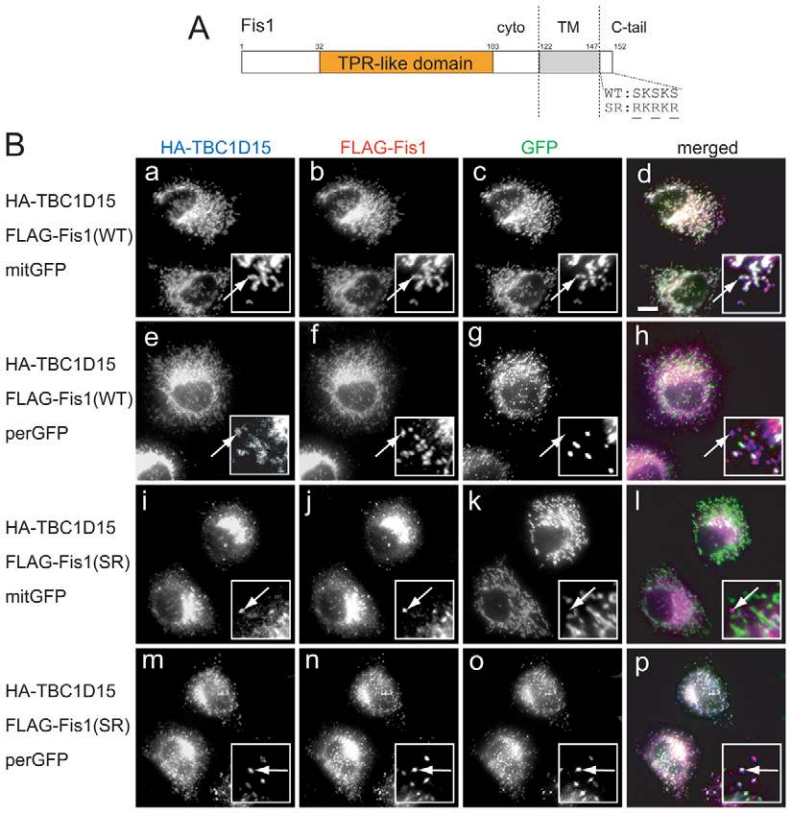

Fig. 3. TBC1D15 localization is dependent on Fis1 localization. (**A**) Schematic representation of Fis1 structure. Orange, tetratricopeptide-repeat (TPR)-like domain; gray, transmembrane (TM) domain. SR, FLAG–Fis1 construct mutated at the C-tail segment. (**B**) HeLa cells were transfected with the indicated expression vectors, and analyzed as in Fig. 2. Mitochondrial–GFP (mitGFP) and peroxisomal GFP (perGFP) were used as organelle markers. Merged images showTBC1D15 in blue; Fis1 in red; GFP in green. Arrows indicate TBC1D15-containing structures. Scale bar: 10 μm. Inset, magnified images of a 10×10 μm area.

mitochondrial fragmentation or aggregation, as reported previously (Jofuku et al., 2005; Yoon et al., 2003) (Fig. 2Bg–i). HA–TBC1D15 localization was shown to change from the cytoplasm to the mitochondria with FLAG–Fis1 expression (Fig. 2Bj–l), but no clear effect was observed on HA–Drp1 distribution (supplementary material Fig. S2). Expression of FLAG–Mff stimulated both mitochondrial fragmentation and Drp1 mitochondrial recruitment, in agreement with a previous study (Otera et al., 2010) (supplementary material Fig. S2), but there was no obvious effect on HA–TBC1D15 distribution (Fig. 2Bm–o). These data indicate that Fis1 stimulates the mitochondrial localization of TBC1D15. We confirm the distribution change of TBC1D15 by subcellular fractionation (Fig. 1C). Expression of FLAG–Fis1 resulted in HA–TBC1D15 localization changing from the soluble supernatant fraction (S) to the mitochondria-enriched pellet fraction (P) along with Fis1, and this fraction of HA–TBC1D15 was released from the membrane by alkali extraction (pH 11.5), indicating that it was peripherally associated with the membrane (supplementary material Fig. S1D). We also confirmed that a significant amount of endogenously expressed TBC1D15 was also cofractionated with Fis1 to the pellet fraction (Fig. 1D). However, overexpression of Fis1 did not enhance mitochondrial localization of endogenous TBC1D15 for unknown reasons (data not shown).

To further analyze the function of Fis1 in TBC1D15 localization, we generated Fis1 mutant that was directed to peroxisomes (Fig. 3). When coexpressed with wild-type (WT) Fis1, both Fis1 and TBC1D15 colocalized with mitGFP (Fig. 3Ba–d), but not with peroxisome GFP (perGFP) (Fig. 3Be–h). In contrast, Fis1-SR, in which three serine residues at the C-terminus were substituted with arginine residues (Fig. 3A), colocalized mainly with perGFP (Fig. 3B,m–p), but not with mitGFP (Fig. 3Bi–l). Fis1-SR had no effect on mitochondrial fragmentation (Fig. 3Bi–l), but induced the perinuclear aggregation of peroxisomes (Fig. 3Bm–p, compare with Fig. 3e–h). Under this condition, most TBC1D15 colocalized with Fis1-SR

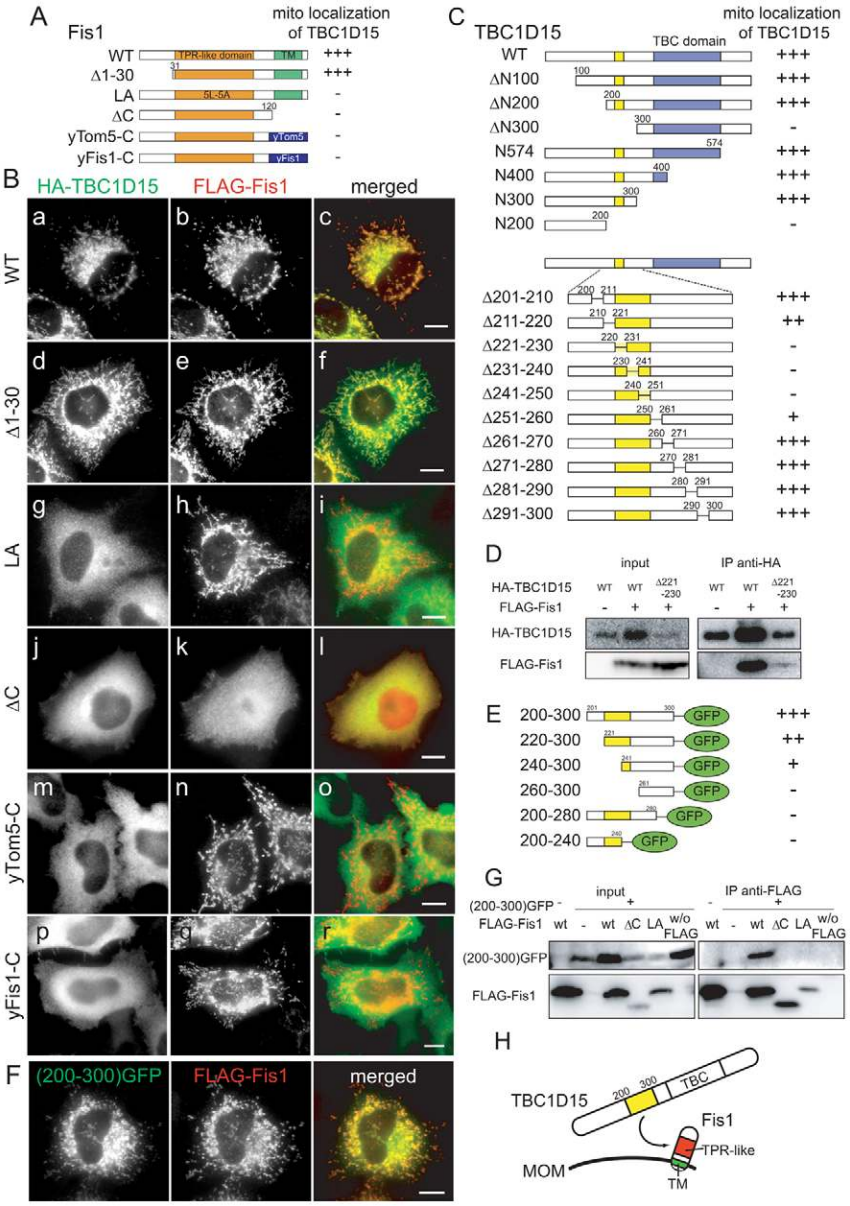

Fig. 4. Structural requirement for interaction between TBC1D15 and Fis1. (**A**) Summary of mutated Fis1 constructs and their potential for TBC1D15 recruitment to mitochondria. (**B**) HeLa cells were co-transfected with HA–TBC1D15 and the indicated FLAG–Fis1 mutants and analyzed by immunofluorescence microscopy. Merged images show HA–TBC1D15 (green) and FLAG–Fis1 mutants (red). Scale bars: 10 µm. (**C**) Summary of mutated TBC1D15 constructs and their mitochondrial localization. Blue, TBC domain; yellow, essential domain for Fis1-binding (amino acids 220–250). The indicated constructs were coexpressed with Fis1 and analyzed by immunofluorescence microscopy. (**D**) HeLa cells were co-transfected with WT or the Δ221–230 mutant of HA–TBC1D15 constructs and FLAG–Fis1. Cell lysates were subjected to immunoprecipitation using an anti-HA antibody, then analyzed by immunoblotting using anti-HA and anti-FLAG antibodies. (**E**) Summary of constructs and the mitochondrial localization of mutated 200–300GFP (amino acids 200–300 of TBC1D15 fused with GFP). The indicated constructs were coexpressed with Fis1 and analyzed by fluorescence microscopy. (**F**) Fluorescence microscopy of 200–300GFP coexpressed with Fis1. Merged images show 200–300GFP (green) and FLAG–Fis1 (red). Scale bar: 10 µm. (**G**) HeLa cells were co-transfected with 200–300GFP and various mutated FLAG–Fis1 constructs. Cell lysates were subjected to immunoprecipitation using an anti-FLAG antibody, then analyzed by immunoblotting using anti-GFP and anti-FLAG antibodies. (**H**) Schematic drawing of interaction between Fis1 and TBC1D15 on mitochondria.

on peroxisomes, but not particularly on mitochondria. These data indicate that the subcellular localization of TBC1D15 is directed by Fis1.

The TPR-like domain and transmembrane domain of Fis1 are required for TBC1D15 recruitment to mitochondria

Fis1 is anchored to the mitochondrial outer membrane through its C-terminus, extruding the bulk of the N-terminal portion containing the tetratricopeptide-repeat (TPR)-like domain into the cytoplasm. To define the structural requirement of Fis1 responsible for TBC1D15 mitochondrial recruitment, we analyzed the effect of Fis1 mutants used in our previous study (Jofuku et al., 2005) (Fig. 4A).

The N-terminus-truncated mutant (Δ1–30) succeeded in the mitochondrial recruitment of TBC1D15 even though mitochondrial fission-stimulating activity was abolished, as reported previously (Jofuku et al., 2005) (Fig. 4B,d–f). The TPR-like domain mutant, 5 leucine substituted by alanine (LA), localized to mitochondria but lost the ability of mitochondrial fission (Jofuku et al., 2005) as well as TBC1D15 recruitment (Fig. 4Bg–i). The C-terminus truncated mutant (ΔC), lacking the transmembrane (TM) and C-tail domains, also lost the ability of mitochondrial fission (Jofuku et al., 2005) and was dispersed in the cytoplasm with coexpressed TBC1D15 (Fig. 4Bj–l). Immunoprecipitation showed that the ΔC lost TBC1D15 affinity (Fig. 4G, see below). We previously showed that Fis1-yTom5-C and Fis1-yFis1-C, the constructs in which the TM and C-tail domains were all replaced with the corresponding regions of mitochondrial C-tail-anchored proteins, yeast Tom5 or yeast Fis1, respectively, lost the ability for mitochondrial fragmentation and homo-oligomer formation (Jofuku et al., 2005). These C-terminus replacement mutants also lost the affinity to TBC1D15 (Fig. 4Bm–r), even the C-tail domain mutant Fis1-SR was still able to recruit TBC1D15 (Fig. 3). Since TBC1D15 has no TM segment (supplementary material Fig. S1B–D), Fis1-TM should not be directly interacted with TBC1D15, but possibly required for formation of active oligomer/conformation of Fis1. Thus, the TM hydrophobic segment of Fis1 has multiple functions, in membrane anchorage, oligomerization, and TBC1D15 interaction. These data also support our conclusion that Fis1 homologs have diverse functions in evolution from yeast to mammals. Together, these data indicate that the Fis1 TPR-like and C-terminal transmembrane domains are essential for TBC1D15 recruitment to the mitochondria (model in Fig. 4H).

Amino acid residues 200–300 of TBC1D15 directly interact with Fis1

To define the TBC1D15 regions responsible for Fis1 interaction, we generated various deletion mutants of TBC1D15 and coexpressed them with Fis1 (summarized in Fig. 4C). Similar to full-length TBC1D15, two of the N-terminal truncated mutants (ΔN100 and ΔN200) localized to the mitochondria when coexpressed with Fis1 (Fig. 4C; supplementary material Fig. S3). However, mitochondrial recruitment was barely detectable for ΔN300 and N200 (supplementary material Fig. S3A). Three of the C-terminal truncated mutants (N574, N400, N300) also localized to the mitochondria. These data clearly show that the TBC domain is not essential for Fis1 interaction and mitochondrial recruitment. A series of deletion mutants of 10 amino-acid residues revealed that the region between residues 220 and 250 contains essential information for TBC1D15 mitochondrial localization (Fig. 4C; supplementary material Fig. S3B). We also confirmed that residues 221–230 were required for the stabilization by, and the physical interaction with, coexpressed Fis1 (Fig. 4D).

Next, we constructed a GFP fusion protein with the 200–300th residue region of TBC1D15 (200–300GFP) and coexpressed this with Fis1 (Fig. 4E,F). As expected, 200–300GFP colocalized with Fis1 (Fig. 4F). Further deletion analysis indicated that the minimal region required for Fis1-dependent mitochondrial

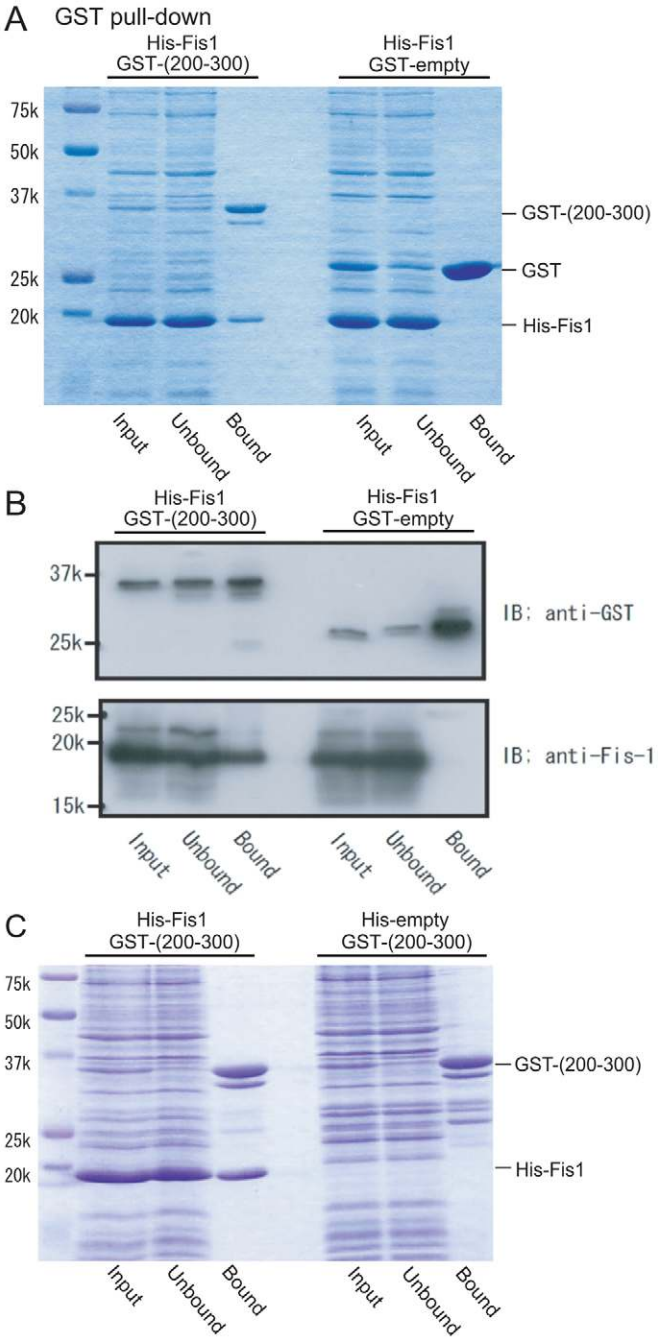

Fig. 5. Physical interaction between TBC1D15 and Fis1. Bacterial coexpression of His-tagged Fis1 and GST-tagged TBC(200–300) region, as indicated, pull-downed with glutathione–Sepharose 4B beads. Unbound and bound fractions were analyzed by CBB staining (**A**,**C**) or immunoblotting with the indicated antibodies (**B**). GST-empty (A,B) and His-empty (C) constructs were used as negative controls.

recruitment was residues 240–300 (Fig. 4E; supplementary material Fig. S3C). We also used immunoprecipitation to confirm the interaction between 200–300GFP and FLAG–Fis1 constructs (Fig. 4G). Thus, TPR-like and transmembrane domains of Fis1, and residues 200–300 of TBC1D15 are necessary for the interaction (model in Fig. 4H).

We further analyzed the physical interaction between TBC1D15 and Fis1 using purified recombinant proteins. N-terminal His-tagged full-length Fis1 (His-Fis1) and/or the N-terminal GST-tagged 200–300 residue segment of TBC1D15 (GST200–300) were coexpressed in bacteria. Bacterial cells were lysed by buffer containing Triton X-100, and the GST fusion protein was isolated by glutathione–Sepharose. Coomassie Brilliant Blue (CBB) staining (Fig. 5A,C) and immunoblot experiments (Fig. 5B) revealed that His–Fis1 was co-purified with GST200–300, but not with GST. The CBB stained gel showed that His–Fis1 and GST200–300 were highly purified as major proteins, strongly indicating that TBC1D15 directly and stably interacts with Fis1.

TBC1D15 is recruited to mitochondria dependent on Fis1 and independent of Drp1

We next analyzed the effect of Drp1 on the TBC1D15 localization using Drp1-knockout (KO) mouse embryonic fibroblasts (MEF). Exogenously expressed HA–TBC1D15 in control MEF was localized in cytoplasm (Fig. 6A,b), but the localization changed to the fragmented mitochondria by coexpression of FLAG–Fis1 (Fig. 6Ad–f), as seen in HeLa cells (Fig. 2). The mitochondrial localization of HA–TBC1D15 was also observed in Drp1-KO MEF when coexpressed with FLAG–Fis1 (Fig. 6Bd–i), clearly showing that Drp1 is dispensable for the mitochondrial localization of TBC1D15. Mitochondria in the Drp1-KO MEF were slightly but clearly shortened by expression of Fis1, or by coexpression of Fis1 and TBC1D15 (Fig. 6Bc,d–i,C,D), suggesting that Fis1 and TBC1D15 have Drp1-independent function in mitochondrial morphology regulation. Mitochondrial morphology was normal in cells expressing the HA–TBC1D15 alone (Fig. 6C,D).

We further examined the effect of Fis1 knockdown on the intracellular localization of TBC1D15. When control HeLa cells were immunostained with anti-TBC1D15 antibodies, the signals partially but clearly overlapped with mitRFP (Fig. 7Aa–c), as shown in Fig. 2A. In contrast, mitochondrial TBC1D15 was clearly released to the cytoplasm in Fis1-RNAi cells (Fig. 7Ad–f). Knockdown of Drp1 and Mff did not affect localization of endogenous TBC1D15 (supplementary material Fig. S4). In support of previous findings (Otera et al., 2010), Drp1 foci on mitochondria were still present in Fis1-RNAi cells (supplementary material Fig. S5Bd–f) but were lost in Mff-RNAi cells (supplementary material Fig. S5Bj–l). As in Fis1-RNAi cells, Drp1 foci on mitochondria were still present in TBC1D15-RNAi cells (supplementary material Fig. S5Bg–i). These data further confirm the above conclusion that Fis1 is not involved in the mitochondrial recruitment of Drp1, but rather functions as a TBC1D15 receptor in the Drp1-independent regulation of mitochondrial morphology.

TBC1D15 knockdown induces changes in mitochondrial network structures

The role of TBC1D15 in mitochondrial morphology was examined by TBC1D15-RNAi cells. Immunoblot analysis revealed that endogenously expressed TBC1D15 was almost completely

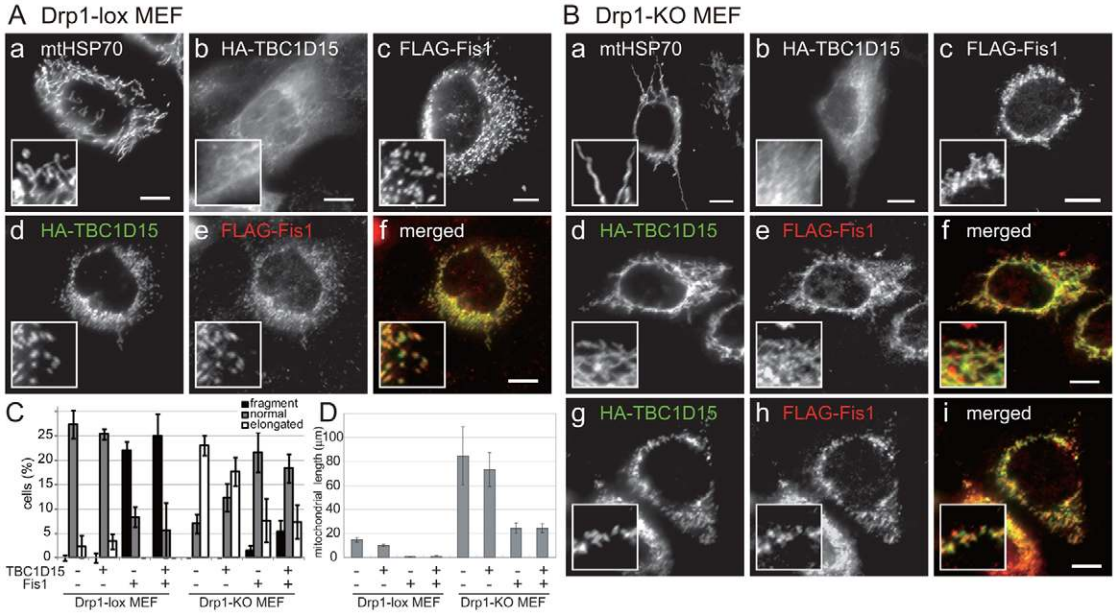

Fig. 6. TBC1D15 localizes to mitochondria independent of Drp1. (**A**,**B**) Control MEFs (A) or Drp1-KO MEFs (B) were transfected without (a) or with HA-TBC1D15 alone (b), FLAG-Fis1 alone (c), or both HA-TBC1D15 and FLAG-Fis1 (d–i), then fixed and immunostained using anti-HA antibody, anti-FLAG antibody or anti-mtHSP70 antibody. Merged images of HA–TBC1D15 (green) and FLAG–Fis1 (red) are shown. The KO cells with normal tubular mitochondria (Bd–f) and with fragmented mitochondria (Bg–i) are shown. Scale bars: 10 μm. Inset, magnified images of a 10 μm x 10 μm area. (**C**) Cell counts. Cells were treated as above. Cells with fragmented mitochondria (fragment), normal tubular mitochondria (normal) or highly elongated mitochondria (elongated) were counted. Over 100 cells were analyzed three times. (**D**) Average lengths of mitochondria in three cells were measured by MetaMorph software.

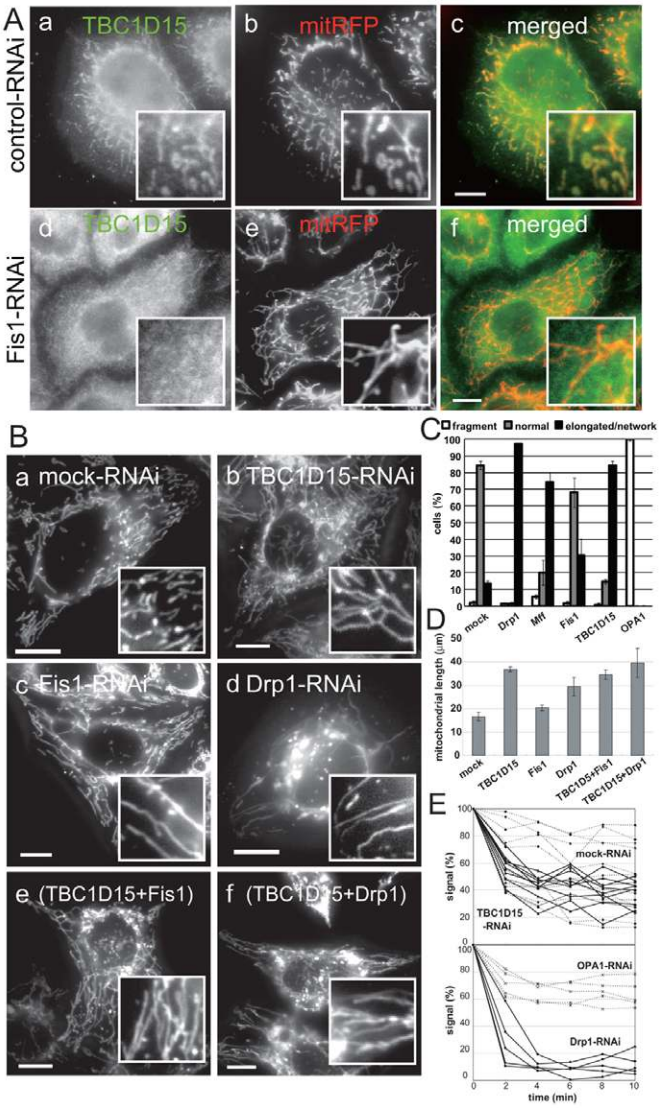

Fig. 7. TBC1D15 regulates mitochondrial morphology with Fis1. (**A**) Localization of endogenously expressed TBC1D15. HeLa cells stably expressing mitRFP were transfected with the indicated siRNA twice over 4 days. Mitochondria were identified using mitRFP, and endogenously expressed TBC1D15 was analyzed by immunofluorescence microscopy using anti-TBC1D15 antibodies. Merged images show TBC1D15 (green) and mitRFP (red). Scale bars: 10 μm. Inset, magnified images of a 10×10 μm area. (**B**) Mitochondrial morphology. HeLa cells stably expressing mitRFP were transfected with the indicated siRNAs twice over 4 days, then mitochondrial morphology was observed in living cells. Scale bars: 10 μm. Inset, magnified images of a 10×10 μm area. (**C**) Cell counting. Cells with fragmented mitochondria (fragment), normal tubular mitochondria (normal), or highly elongated mitochondria or highly connected mitochondrial networks (elongated/network) were counted. Over 100 cells were analyzed three times. (**D**) Average lengths of mitochondria. Mitochondria in three cells were measured. (**E**) Measurement of mitochondrial connectivity using photoactivatable GFP in the mitochondrial matrix. Top panel: control RNAi (dotted lines), TBC1D15-RNAi (solid lines). Bottom panel: OPA1-RNAi (dotted lines), Drp1-RNAi (solid lines).

depleted (supplementary material Fig. S5A). More than 80% of TBC1D15-RNAi cells showed highly connected mitochondrial network structures with many branches (Fig. 7Bb,C). Mitochondrial morphology in TBC1D15-RNAi cells was clearly different from that observed in Drp1-RNAi cells. In Drp1-RNAi cells, less branched and highly elongated mitochondria with bulb-like structures were accumulated in perinuclear region (Fig. 7Bd). The mitochondrial morphology in Fis1-RNAi cells was similar, but milder compared with that in TBC1D15-RNAi cells (Fig. 7Bc). Quantitative analysis of mitochondrial length also showed that TBC1D15 RNAi resulted in severe mitochondrial elongation than in Fis1 RNAi cells (Fig. 7D). Further quantitative assessment of mitochondrial connectivity using mitochondrial photoactivatable GFP (PAmitoGFP) (Karbowski et al., 2004) showed that Drp1-RNAi cells had higher dilution rates of PAmitoGFP fluorescence than control cells, indicating that the mitochondria are highly connected in these RNAi cells (Fig. 7E). TBC1D15-RNAi cells also had higher dilution rates than control cells (Fig. 7E), suggesting that TBC1D15 modulates the mitochondrial fusion–fission balance to higher fission/lower fusion. Neither TBC1D15-RNAi nor Fis1-RNAi cells (Otera et al., 2010) had a clear effect on peroxisome morphology, although Drp1 RNAi induced peroxisome elongation as reported previously (supplementary material Fig. S6) (Koch et al., 2003). In TBC1D15-RNAi cells, Fis1 overexpression moderately induced mitochondrial fragmentation (supplementary material Fig. S7A), to similar mitochondrial length seen in normal cells, (supplementary material Fig. S7B), suggesting that TBC1D15 should play important roles in mitochondrial morphology regulation with Fis1.

Double RNAi using a mixture of TBC1D15 and Fis1 siRNAs resulted in similar mitochondrial morphology with TBC1D15 RNAi cells (Fig. 7Be,D). However, double RNAi using a mixture of TBC1D15 and Drp1 siRNAs resulted in the mixed structures of mitochondria; in these cells, elongated mitochondria form the network and accumulated in perinuclear region with bulb-like structures (Fig. 7Bf). These data suggested that TBC1D15 and Drp1 should independently function in the mitochondrial morphology regulation. Next, TBC1D15, Fis1 or Drp1 was co-repressed with mitochondrial fusion factor OPA1. Co-repression of OPA1 resulted in mitochondrial fragmentation both in TBC1D15 and Fis1 repressed cells, but not in Drp1 repressed cells (Fig. 8A). We further analyzed the roles of TBC1D15 in the regulation of mitochondrial morphology. We previously showed that CCCP induced mitochondrial fragmentation by changing mitochondrial fusion/fission balance (Ishihara et al., 2003) (Fig. 8B, mock RNAi). Both TBC1D15-RNAi cells and Fis1-RNAi cells were sensitive to the CCCP induced mitochondrial fragmentation. However, elongated mitochondria in Drp1-deficient cells were maintained after a 30-minute treatment with CCCP (Fig. 8B,C), suggesting mitochondrial fission was not completely blocked in TBC1D15 or Fis1-RNAi cells.

Together, these data clearly indicate that TBC1D15 and Fis1 work together in the regulation of mitochondrial morphology independently of Drp1.

Discussion

The mitochondrial outer membrane protein Fis1 is widely conserved and plays a role in the formation of the mitochondrial fission complex containing the dynamin-related GTPase Dnm1 and WD motif-containing proteins Mdv1 or Caf4 in yeast (Hoppins et al., 2007; Okamoto and Shaw, 2005). However, the molecular function of mammalian Fis1 is poorly understood. Here, we found that mammalian Fis1 is involved in the mitochondrial recruitment of TBC1D15 in a process associated with mitochondrial morphology changes (summarized in supplementary material Fig. S8).

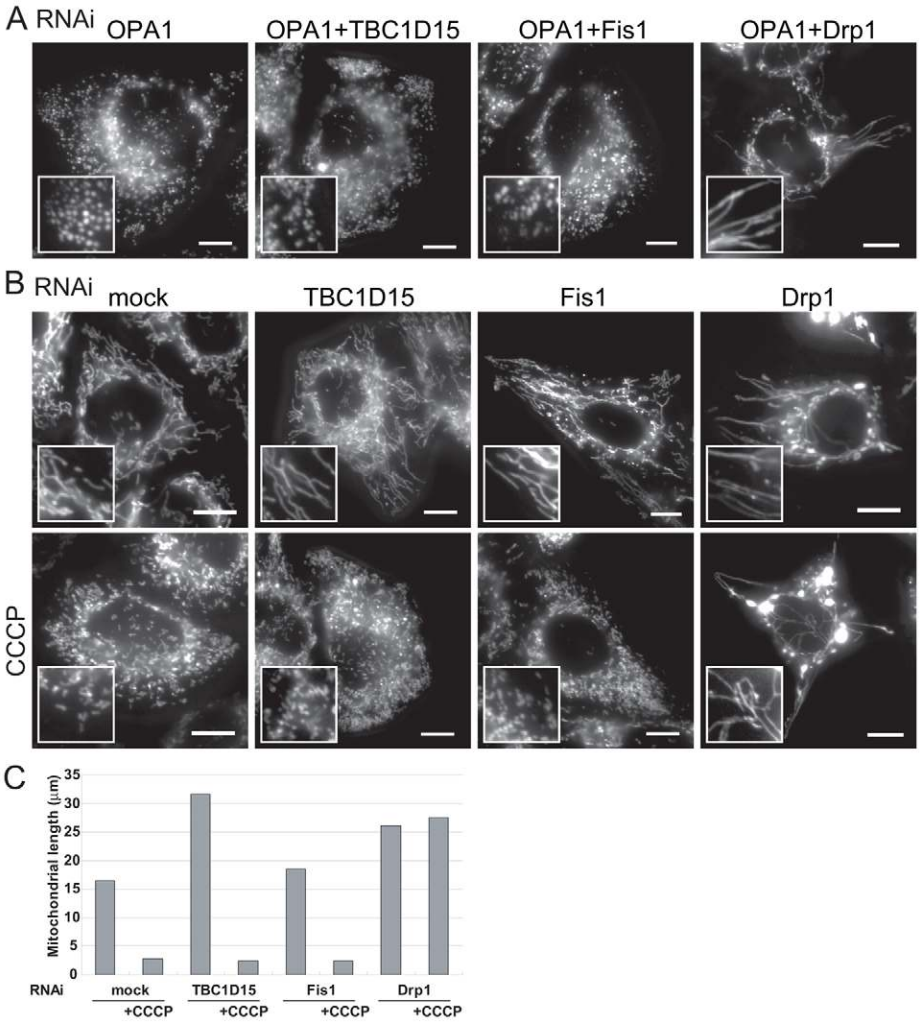

Fig. 8. Mitochondrial morphology in TBC1D15-RNAi cells. (**A**,**B**) HeLa cells stably expressing mitRFP were transfected with the indicated siRNA twice over 4 days. (A) Mitochondrial morphology in double RNAi-treated cells, as indicated, was analyzed by fluorescence microscopy. (B) Cells were treated with the uncoupler CCCP for 30 minutes, then mitochondrial morphology was observed in living cells. Scale bars: 10 μm. Inset, magnified images of a 10×10 μm area. (**C**) The average lengths of mitochondria in cells in B.

TBC domain-containing proteins and mitochondrial morphology

More than 40 TBC domain-containing proteins are expressed in humans (Barr and Lambright, 2010; Fukuda, 2010; Fukuda, 2011). Genome-wide RNAi screening of cultured *Drosophila melanogaster* cells showed that TBC family member protein TBC1D24 affected mitochondrial morphology (Gandre-Babbe and van der Bliek, 2008), although molecular details were unknown. The present study identified TBC1D15 as an Fis1-binding protein, the first TBC-domain-containing protein to be shown to function in mammalian mitochondria. Although here we failed to show the direct interaction of endogenous Fis1 and TBC1D15, possibly by technical problems, or by their weak or transient interaction (data not shown), however, their interactions were supported by many experiments presented here. TBC1D15 homologues have higher sequence similarity among vertebrates (72% homology between human and *Xenopus laevis*), than with the fruit fly, nematodes, and yeast (*D. melanogaster,* 46%; *Caenorhabditis elegans*, 49%; and *Saccharomyces cerevisiae,* 40%). Human TBC family member TBC1D17 shares a high amino acid similarity with TBC1D15 (50% overall identity; (Zhang et al., 2005), although its RNAi in HeLa cells had no obvious effect on mitochondrial morphology (data not shown). The outcome of double TBC1D15 and TBC1D17 RNAi was indistinguishable from that of TBC1D15 alone (data not shown), suggesting that TBC1D15 plays a major role in mitochondrial morphology in HeLa cells.

In the present study, we found that TBC1D15 localizes to mitochondria (Figs 2–4, 7) where it functions in mitochondrial morphogenesis with Fis1 (Fig. 7). TBC1D15 was reported to function in lysosome/endosome fusion as a putative GTPase regulator for Rab7 and Rab11 in mammalian cells (Peralta et al., 2010; Zhang et al., 2005). However, the specificity of TBC proteins for Rab proteins is often promiscuous and they have been demonstrated to have multiple functions (Fukuda, 2011). Rab32 was previously reported to localize to mitochondria where it recruits A-kinase as an A-kinase anchoring protein (AKAP), and participates in mitochondrial dynamics regulation, most likely mitochondrial fission (Alto et al., 2002; Bui et al., 2010). Protein kinase A phosphorylates Ser637 of Drp1 in its GTPase effector domain, stimulates Drp1 GTPase, and releases Drp1 from mitochondria to promote mitochondrial network extension (Chang and Blackstone, 2007; Cribbs and Strack, 2007).

However, expression of TBC domain mutants (D397A and R400K) of TBC1D15 had no effect on mitochondrial morphology as WT TBC1D15 does (K.O., T.I., R.B.I., unpublished data), suggesting that the GTPase regulator activity of TBC1D15 should not be required for these processes. This was previously observed

in the role of another TBC-domain-containing protein, EPI64/TBC1D10A, in microvillar formation independent of their GTPase regulator domain (Hanono et al., 2006). Furthermore, TBC1D2B and GAPCenA/TBC1D11 could interact with Rab22 and Rab36, respectively, while functioning independently of GTPase regulator activity (Kanno et al., 2010). Although it is possible that TBC1D15 functions independently of the TBC domain, further analysis is required to determine the molecular function of TBC1D15 in mitochondrial morphogenesis.

Function of Fis1

It was recently reported that mammalian Drp1 is recruited to the mitochondria by Mff and MiD/MIEF proteins but not by Fis1 (Otera and Mihara, 2011; Otera et al., 2010; Palmer et al., 2011; Zhao et al., 2011). The function of mammalian Fis1, therefore, has become an open question. Here we clearly showed that mammalian Fis1 is the mitochondrial receptor that recruits the GTPase regulator domain-containing protein TBC1D15 and functions in mitochondrial morphology regulation independently of Drp1. As reported previously, Fis1 RNAi had less effect on mitochondrial morphology than Drp1 or Mff (Otera et al., 2010). However, knockdown of the Fis1-binding partner TBC1D15 induced more obvious mitochondrial morphological changes compared with Fis1 knockdown. It is conceivable that part of TBC1D15 is localized on the mitochondria independently of Fis1, and this is supported by the finding that double RNAi of TBC1D15 and Fis1 resulted in similar mitochondrial morphology to that of TBC1-RNAi. We speculate that TBC1D15 is a direct player in the regulation of mitochondrial morphology in cooperation with Fis1.

Fis1 was recently reported to interact with the endoplasmic reticulum (ER) membrane protein Bap31, leading to the induction of apoptosis (Iwasawa et al., 2011). We speculate that Fis1 has several interaction partners in its role as a mitochondrial recruiter of cytoplasmic proteins, that is, in the TBC1D15 complex in mammals and the Mdv1/Caf4/Dnm1 complex in yeast, or for bridge mitochondria with other organelles such as the ER via Bap31.

In conclusion, we showed that mammalian Fis1 acts to recruit the GTPase regulator protein TBC1D15, similar to yeast Fis1 which recruits GTPase regulator proteins Mdv1 and Caf4 to mitochondria for dynamin-related GTPase Dnm1 (supplementary material Fig. S8). Although the function of TBC1D15 in the regulation of mitochondrial morphology, and in the requirement of its GTPase regulator activity remains to be fully elucidated, our findings suggest that the Rab family proteins should play roles in mitochondrial morphology regulation in cooperation with the mitochondrial surface receptor Fis1.

Materials and Methods

Constructs

For mammalian expression, cDNA fragments of human TBC1D15 isoform 3 (NCBI database accession number NP_001139685) encoding a 674-amino-acid protein were isolated from HeLa cells and subcloned into the pHM6 vector at the *Hin*dIII and *Eco*RI sites for N-terminal HA-tagged TBC1D15 (primers: 5′-ATAACGCTAAGCTTAGCGGCGGCGGGTGTTGTGA-3′ and 5′-TGTAGAG-AATTCTCATGCAGGTGTTAATCTGCAGACATCT-3′).

Mammalian expression plasmids of FLAG-Fis1, HA-Drp1 (Jofuku et al., 2005), FLAG-Mff (Otera et al., 2010), mitochondrial GFP (mitGFP: su9-GFP) (Ishihara et al., 2004), and peroxisome GFP (perGFP) (Invitrogen, Carlsbad, CA) have previously been described. Mutated or deleted Fis1 has also been described (Jofuku et al., 2005). Deleted TBC1D15 constructs were also constructed by PCR.

For the bacterial expression of GST-TBC(200–300), cDNA fragments of amino acid residues 200–300 were subcloned into pGEX-KG. (primers: 5′-CCCGGATCCAAGAGTCTTTCACAGTCTTTT-3′ and 5′-GTAGAATTCCTC-ATACCGGTTCTCTCCTTTG-3′). The bacterial expression vector of His-Fis1 has previously been described (Jofuku et al., 2005).

Antibodies and reagents

To generate antibodies against human TBC1D15 proteins, cDNA fragments of amino acid residues 1–333 were subcloned into pGEX-KG (primers: 5′-AAAGGATCCATGGCGGCGGCGGGTGTT-3′ and 5′-GTAGAATTCCTCAT-GCATGACTAAGTCCCCC-3′). Bacterially expressed protein was recovered to insoluble inclusions. These were applied and extracted from an SDS-PAGE gel, then used for immunizing rabbits as described (Eura et al., 2003). Glutathione–Sepharose 4B was purchased from GE Healthcare. All other reagents were of biochemical research grade.

Rabbit polyclonal antibodies for HA (PRB-101C; COVANCE, Princeton, NJ), FLAG, (F-7425; Sigma, St. Louis, MO), Fis1 (ALX-210-907-R100; ALEXIS Biochemicals, Enzo Life Sciences, Exeter, UK), and mouse monoclonal antibodies for FLAG (M2; Sigma), HA (16B12; Babco, Tucson, AZ), Drp1 (D80320; BD Biosciences, Franklin Lakes, NJ), and anti-mtHSP70 (30A5, Abcam) were used.

Cell culture, transfection and RNA interference

HeLa cells and HeLa cells expressing mitRFP (Taguchi et al., 2007) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 incubator. FuGene 6 reagent (Roche Diagnostics, Tokyo, Japan) or Lipofectamine 2000 (Invitrogen) was used for plasmid transfection. For RNAi, siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's protocols. After 2 days, cells were transfected again and cultured for an additional 2 days. The target sequences of RNAi oligonucleotides (stealth RNA, Invitrogen) were as follows:

TBC1D15 5′-AUCCUGUGGAGAUUCACACAAUACC-3′ and 5′-GGUAU-UGUGUGAAUCUCCACAGGAU-3′, Mff: 5′-CCUUGUUCCAGGUCAGCG-UUUGGCG-3′ and 5′-CGCCAAACGCUGACCUGGAACAAGG-3′.

The siRNAs for Fis1 (Jofuku et al., 2005), Drp1 (Taguchi et al., 2007) and OPA1 (Ishihara et al., 2006) have previously been described.

Microscopy

Immunofluorescence microscopy was performed as described (Taguchi et al., 2007). In brief, HeLa cells grown on coverslips were fixed by 4% paraformaldehyde, permeabilized by 0.05% Triton X-100, stained with specific antibodies and visualized by secondary antibody conjugated to Alexa Fluor 488, 568 or 660 (Invitrogen). Note that cells were directly fixed in paraformaldehyde without washing with PBS, to avoid morphological changes to the mitochondria during fixation.

To analyze mitochondrial morphology in RNAi cells, HeLa cells expressing mitRFP cultured on glass-bottomed dishes were analyzed. These cells were observed with a fluorescence microscope (IX81; Olympus, Tokyo, Japan) equipped with a charged-coupled device camera (ORCA R2 Hamamatsu Photonics, Shizuoka, Japan). A 60× PlanApoN oil immersion lens (1.42 NA; Olympus, Tokyo, Japan) was used. Images were acquired using MetaMorph (Molecular Devices, Sunnyvale, CA) image analysis software. To measure mitochondrial length, images of mitochondria were manually traced using 'Traced Line' of the Region Tools in Metamorph software. To measure mitochondrial connectivity, HeLa cells transiently expressing PAmitoGFP were used (Karbowski et al., 2004). A part of mitochondria were stimulated by irradiation at 405 nm for 2 seconds, then fluorescence was measured at 2-minute intervals using a FV1000D confocal laser scanning microscope system (Olympus).

Immunoprecipitation

HeLa cells transiently expressing candidate proteins were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, containing 150 mM NaCl, protease inhibitor cocktails, 1% digitonin). The lysates were cleared by centrifugation and supernatants fractions were subjected to immunoprecipitation using monoclonal FLAG or polyclonal HA antibodies with protein-G– or protein-A–Sepharose, respectively. The precipitants were analyzed by SDS-PAGE and subsequent immunoblotting (Ishihara et al., 2004). For identification of Fis1-binding proteins, HeLa cells stably expressing FLAG–Fis1 were lysed as above and immunoprecipitated using M2-agarose beads (Sigma). A 75-kDa band was eluted from SDS-PAGE gel and subjected to mass spectrometry after in-gel digestion.

Pull-down assay

A physical interaction between TBC1D15 and Fis1 was investigated by a pull-down assay using glutathione–Sepharose 4B affinity beads. *E. coli* cell lysate coexpressed with His-tagged Fis1 and GST-tagged TBC (200–300) was mixed with glutathione–Sepharose 4B beads in 50 mM Tris-HCl buffer (pH 7.2) containing 150 mM NaCl, 5% (w/v) glycerol, 1% (w/v) Triton X-100, and 1 mM DTT for 1 h. The resulting samples were centrifuged and divided into bound and unbound fractions. The bound fraction was washed once with the same buffer, and the samples were subjected to 15% SDS-PAGE.

Acknowledgements

We thank Dr Richard Youle (NIH) for a generous gift of the PAmitGFP plasmid.

Funding

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas [grant numbers 22020010 to N.I. and 22020026 to K.M.]; Grant-in-aid for Special Promoted Research [grant number JPS14002007 to K.M.] from the Ministry of Education, Culture, Sports, Science and Technology, Japan; the Funding Program for Next Generation World-Leading Researchers; the Ono Medical Research Foundation; the Kato Memorial Bioscience Foundation and the Takeda Science Foundation.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.111211/-/DC1

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