Ugo1 and Mdm30 act sequentially during Fzo1-mediated mitochondrial outer membrane fusion

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Accepted 23 November 2010
Journal of Cell Science 124, 1126-1135

doi:10.1242/jcs.073080

Summary

Dynamin-related GTPase proteins (DRPs) are main players in membrane remodelling. Conserved DRPs called mitofusins (Mfn1/Mfn2/Fzo1) mediate the fusion of mitochondrial outer membranes (OM). OM fusion depends on self-assembly and GTPase activity of mitofusins as well as on two other proteins, Ugo1 and Mdm30. Here, we define distinct steps of the OM fusion cycle using in vitro and in vivo approaches. We demonstrate that yeast Fzo1 assembles into homo-dimers, depending on Ugo1 and on GTP binding to Fzo1. Fzo1 homo-dimers further associate upon formation of mitochondrial contacts, allowing membrane tethering. Subsequent GTP hydrolysis is required for Fzo1 ubiquitylation by the F-box protein Mdm30. Finally, Mdm30-dependent degradation of Fzo1 completes Fzo1 function in OM fusion. Our results thus unravel functions of Ugo1 and Mdm30 at distinct steps during OM fusion and suggest that protein clearance confers a non-cycling mechanism to mitofusins, which is distinct from other cellular membrane fusion events.

Key words: Fusion, Dynamin-related proteins, Mitochondria, Proteolysis, Ubiquitylation

Introduction

A proper control of membrane dynamics and content mixing between organelles is essential for cellular communication (Martens and McMahon, 2008). Endocytosis and mitochondrial fusion and fission are processes promoted by dynamin-related GTPase proteins (DRPs), whose regulation differs significantly from other GTPases (Hinshaw and Schmid, 1995; Hoppins et al., 2007; Lackner and Nunnari, 2008; Okamoto and Shaw, 2005; Praefcke and McMahon, 2004). Whereas conventional GTPases function as molecular switches, cycling between inactive GDP-bound and active GTP-bound forms (Vetter and Wittinghofer, 2001), the activity and downstream signalling of DRPs occur concomitantly with GTP hydrolysis (Praefcke and McMahon, 2004). In addition, the GTPase activities of DRPs are stimulated by homo-oligomerization, and evidence for co-regulators is scarce (Gasper et al., 2009). Recent findings provide profound insight into the role of Dnm1/Drp1 and mitochondrial genome maintenance protein 1 (Mgm1/Opa1), i.e. the DRPs promoting mitochondrial fission or inner membrane (IM) fusion, respectively (DeVay et al., 2009; Ingerman et al., 2005; Lackner et al., 2009; Meglei and McQuibban, 2009; Rujiviphat et al., 2009; Zick et al., 2009). However, the mechanisms underlying the fusion of mitochondrial outer membranes (OM), triggered by DRPs called mitofusins, remain poorly understood.

Mitochondrial fusion occurs as a multistep process starting with organelle tethering followed by OM and IM fusion (Detmer and Chan, 2007). IM fusion relies on long and short isoforms of Mgm1/Opa1, which are anchored to the IM or are soluble in the inner membrane space (IMS), respectively (Herlan et al., 2003; Meeusen et al., 2006; Sesaki et al., 2003; Wong et al., 2003). Mgm1 isoforms oligomerize and associate with phospholipids to form a functional GTPase complex (DeVay et al., 2009; Meglei and McQuibban, 2009; Rujiviphat et al., 2009; Zick et al., 2009). OM fusion depends on the assembly and GTPase activity of mitofusins, termed Mfn1 and Mfn2 in mammals and Fzo1 (fuzzy onions homolog 1) in yeast (Griffin and Chan, 2006; Hales and Fuller, 1997; Hermann et al., 1998; Meeusen et al., 2004; Neuspiel et al., 2005; Rojo et al., 2002). Both in vitro and recent in vivo data demonstrate that GTP hydrolysis is necessary for OM fusion (Amiott et al., 2009; Meeusen et al., 2004). Moreover, in vitro assays have shown that tethering of Fzo1 in trans, i.e. between opposed mitochondria, is also required (Meeusen et al., 2004). Mitofusins are anchored to the OM by two transmembrane domains and are exposed to the cytoplasm, allowing regulation of OM fusion by cytosolic proteins like mitochondrial distribution and morphology protein 30 (Mdm30). Heptad repeats both at the N- and C-terminus of mitofusins mediate self-association and probably the trans tethering of mitochondria (Griffin and Chan, 2006; Koshiba et al., 2004).

In yeast, two additional proteins have been identified that are required for OM fusion, Ugo1 (for fusion in Japanese) and Mdm30 (Dimmer et al., 2002; Fritz et al., 2003; Sesaki and Jensen, 2001). Ugo1 is a modified carrier protein in the OM that appears to operate after OM tethering (Coonrod et al., 2007; Hoppins et al., 2009). Ugo1 possesses three transmembrane domains and exposes its N-terminus to the cytosol (Coonrod et al., 2007; Hoppins et al., 2009). In vitro binding assays with recombinant proteins revealed that the N-terminus of Ugo1 directly interacts with the cytosolic regions adjacent to the transmembrane domains in Fzo1 (Sesaki

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and Jensen, 2004). Interestingly, Ugo1 physically interacts with both Fzo1 and Mgm1, is required for both IM and OM fusion, and might coordinate both events (Hoppins et al., 2009; Sesaki and Jensen, 2004; Wong et al., 2003). Mdm30 is a cytosolic, mitochondria-associated F-box protein that binds to Fzo1 (Escobar-Henriques et al., 2006). The absence of Mdm30 impairs fusion, leading to aggregation of fragmented mitochondria, as revealed by electron microscopy analysis (Durr et al., 2006). Mdm30 regulates mitochondrial fusion by mediating Fzo1 ubiquitylation and degradation in an F-box-dependent manner (Cohen et al., 2008; Escobar-Henriques et al., 2006; Fritz et al., 2003). Moreover, Mdm30 is also responsible for the ubiquitylation of Mdm34, one of the OM components of the ERMES (ER-mitochondria encounter structure) complex, involved in the tethering between mitochondria and the endoplasmic reticulum (Kornmann et al., 2009; Ota et al., 2008). Independent of its mitochondrial roles, Mdm30/Dsg1 promotes ubiquitin-mediated degradation of Gal4 and stimulates nuclear export of specific mRNAs (Li et al., 2010; Muratani et al., 2005; Shukla et al., 2009).

Here, we further define the role of the mitofusin GTPase and its interacting partners Ugo1 and Mdm30 in OM fusion, using a series of Fzo1 mutant forms. Our findings integrate the GTPase cycle of Fzo1 and the function of Ugo1 and Mdm30 with the successive self-assembly and degradation of Fzo1, and provide novel insights into the mechanism of OM fusion in vivo.

Results

Fzo1 dimerizes in the OM of mitochondria

Mitofusins from yeast to mammals assemble in the mitochondrial OM to high molecular weight complexes of unknown composition (Eura et al., 2003; Rapaport et al., 1998). When solubilized yeast mitochondria were subjected to size-exclusion chromatography, the mitofusin Fzo1 eluted in fractions that corresponded to an apparent molecular weight of ~400 kDa (Fig. 1A). This complex probably represents a 'cis' form in one membrane because mitochondria become fragmented during the isolation procedure, disrupting tethered structures from intermitochondrial associations (here called 'trans' associations) (Fuchs et al., 2002; Ishihara et al., 2004; Meeusen et al., 2004). To examine whether mitofusins assemble into homo-oligomeric complexes, as is the case with other dynamins (Praefcke and McMahon, 2004), a FLAG–Fzo1 protein was expressed in yeast and purified to near homogeneity (Fig. 1B). Purified Fzo1 eluted in the same fractions as mitochondrial Fzo1 during size-exclusion chromatography, indicating that the endogenous Fzo1 complex is homo-oligomeric (Fig. 1C, compare with Fig. 1A). We then performed crosslinking experiments to determine the number of Fzo1 subunits in this complex. Glutaraldehyde was chosen as a crosslinking agent in order to detect close contacts and to ensure efficient crosslinking. Purified Fzo1 could be crosslinked to an Fzo1 dimer, as revealed by its electrophoretic mobility in SDS-PAGE (Fig. 1D). We observed an identical apparent molecular mass of the endogenous Fzo1 complex and the crosslinked purified Fzo1 dimer (Fig. 1A,E, compare fractions 11 to 19 from both panels), demonstrating that the Fzo1 complex does not contain proteins other than two Fzo1 subunits under the conditions used. It should be noted that the apparent molecular mass of ~400 kDa for the Fzo1 complex in size-exclusion chromatography exceeds the size of Fzo1 dimers, given the molecular weight of Fzo1 subunits of 98 kDa. The difference in the apparent molecular mass and the calculated mass of the dimer probably reflects detergent micelle artefacts. Similar observations have indeed been made for other membrane proteins (Arlt et al., 1996; DeVay et al., 2009; Leonhard et al., 1996). For instance, two Mgm1 variants differing in the presence of a hydrophobic transmembrane region of 12 kDa, show differential behaviour during size-exclusion chromatography, although both

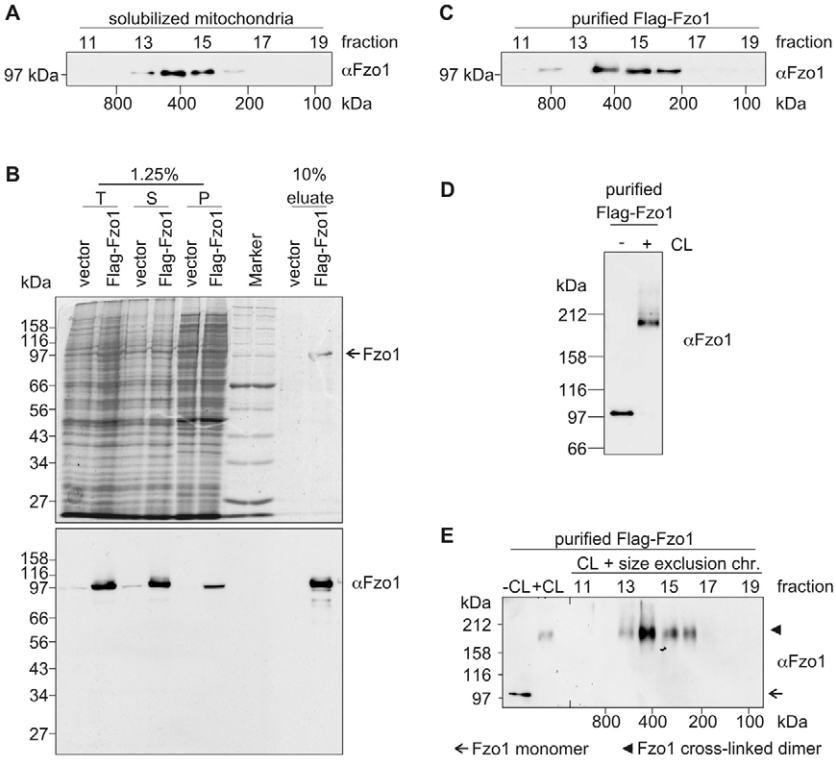

Fig. 1. Fzo1 assembles into a homo-dimer. (**A**) Complex formation of Fzo1. Solubilized mitochondrial extracts were fractionated by size-exclusion chromatography and eluate fractions analysed by SDS-PAGE and immunoblotting using Fzo1-specific antibodies (αFzo1). (**B**) Purification of the Fzo1 protein. Solubilized crude mitochondrial extracts of wild-type yeast cells overexpressing FLAG–Fzo1 were subjected to immunoprecipitation using FLAG-specific antibodies. Immunoprecipitated FLAG–Fzo1 was eluted with a FLAG-peptide and analysed by Coomassie Blue staining (top) or immunoblotting with Fzo1-specific antibodies (bottom). Total extracts (T), supernatant (S) and pellet (P) fractions after solubilization as well as proteins eluted with the triple FLAG-peptide (eluate) are shown. (**C**) Complex formation of purified Fzo1 protein. Purified FLAG–Fzo1 was subjected to size-exclusion chromatography and eluate fractions were analysed by SDS-PAGE and immunoblotting using Fzo1-specific antibodies. (**D**) Crosslinking of purified Fzo1. FLAG–Fzo1 was purified as described for B and eluates treated or not with glutaraldehyde as indicated (+CL or –CL) and analysed by SDS-PAGE followed by immunoblotting using Fzo1-specific antibodies. (**E**) Complex formation of the crosslinked Fzo1 dimer. Purified Fzo1 was crosslinked and analysed by size-exclusion chromatography. The Fzo1 monomer and crosslinked dimer are indicated by arrow and arrowhead, respectively.

forms were demonstrated to be monomers (DeVay et al., 2009). We therefore conclude that Fzo1 assembles into homo-dimeric complexes in the mitochondrial OM.

Fzo1 dimerization depends on GTP binding

To assess the importance of GTP binding and hydrolysis for Fzo1 homo-dimerization, we introduced point mutations in the P-loop (GxxxxGK[S/T]) and switch motifs of the GTPase domain of Fzo1 (Fig. 2A). It has previously been demonstrated that consensus mutations in the P-loop affect GTP binding, whereas in general mutations in the switch 1 region impair GTP hydrolysis (Amiott et al., 2009; Ishihara et al., 2004; Marks et al., 2001; Praefcke et al., 2004). Moreover, we created seven further Fzo1 variants introducing mutations in non-consensus, hydrophilic residues of the P-loop and switch regions of Fzo1 (depicted in Fig. 2A). The Fzo1 mutants were expressed in cells lacking Fzo1 (Δ*fzo1*) and the respiratory cell growth was assessed. The absence of functional Fzo1 protein leads to respiratory deficiency, i.e. impaired growth on non-fermentable carbon sources, as a consequence of mitochondrial DNA loss (Griffin and Chan, 2006; Hermann et al., 1998). This analysis confirmed that three canonical mutations in the P-loop (K200A and S201N) and switch region (T221A) inactivated Fzo1, and identified three additional residues (D195, N197 and D320) to be essential for Fzo1 functionality (Fig. 2A, bold, and supplementary material Fig. S1A).

It is conceivable that mutations inactivating Fzo1 affect its dimerization. We therefore examined complex formation of these Fzo1 mutants by sucrose gradient centrifugation. Fzo1 P-loop mutants were recovered in less-dense fractions than was wild-type Fzo1 (Fig. 2B, left panel, and supplementary material Fig. S1B), indicating impaired assembly. Crosslinking experiments with purified Fzo1 or Fzo1^{D195A} demonstrated an impaired dimerization of the Fzo1 P-loop mutant form (Fig. 2C). These results indicate that Fzo1 dimerization requires GTP binding. By contrast, the assembly of Fzo1 dimers was not affected by mutations in the switch regions (Fig. 2B, right panel, and supplementary material

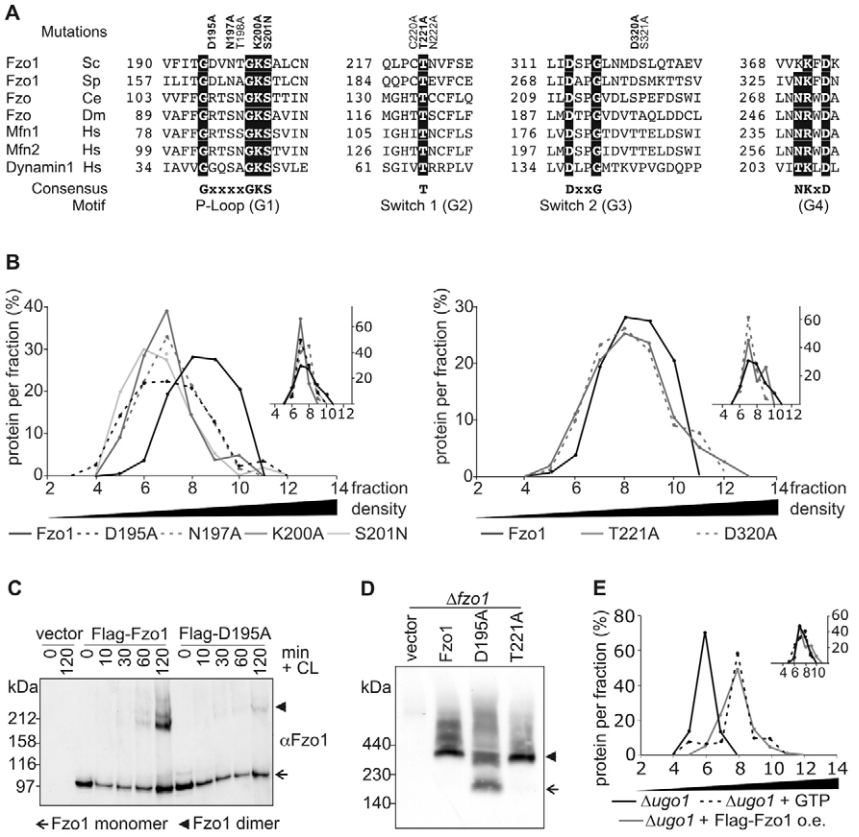

Fig. 2. Integrity of the P-loop of Fzo1 and Ugo1 are necessary for Fzo1 homo-dimerization. (**A**) Multiple protein sequence alignment (score matrix: Blosum62) of the GTPase domains of Fzo1 and Mfn proteins from various eukaryotic species and of human dynamin 1. Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens.* Consensus motifs within the GTPase domain are highlighted in black. Numbers refer to amino acid positions. Mutations that were introduced in *S. cerevisiae* Fzo1 are indicated. Mutations in Fzo1 inhibiting respiration are shown in bold. (**B**) Complex formation of Fzo1 mutants. Crude mitochondrial extracts from Δ*fzo1* cells expressing the indicated Fzo1 forms were solubilized and analysed by sucrose gradient centrifugation. All fractions were recovered and subjected to SDS-PAGE and immunoblotting (supplementary material Fig. S1B). Fractionation of a crossreactive band, used as an internal control, is shown in the inset. Fzo1 P-loop mutants are grouped in the left panel, whereas switch mutants are depicted in the right panel. Total Fzo1 detected was set to 100%. (**C**) Crosslinking of purified Fzo1 or Fzo1^{D195A}. Purified Fzo1 or Fzo1^{D195A} were crosslinked for the indicated time as described in Fig. 1D and analysed by SDS-PAGE and immunoblotting using Fzo1-specific antibodies. (**D**) Complex formation of Fzo1, Fzo1^{D195A} or Fzo1^{T221A} monitored by BN-PAGE. Solubilized mitochondria, which were isolated from Δ*fzo1* cells expressing the indicated Fzo1 forms or containing the expression vector only, were analysed by BN-PAGE and immunoblotted using Fzo1-specific antibodies. (**E**) Complex formation of Fzo1 in Δ*ugo1* cells. Crude mitochondrial extracts from Δ*ugo1* cells overexpressing Fzo1 and supplemented with 1 mM GTP when indicated were analysed by sucrose gradient centrifugation as in B.

Fig. S1B), including the canonical mutant Fzo1^{T221A} that does not hydrolyse GTP (Amiott et al., 2009). The assembly properties of the P-loop Fzo1^{D195A} and the switch Fzo1^{T221A} mutants were corroborated by blue native PAGE (BN-PAGE) analysis (Fig. 2D). Together, these results indicate that Fzo1 dimerization relies on GTP binding but does not require GTP hydrolysis.

Ugo1 promotes Fzo1 dimerization

The mitofusin-interacting proteins Ugo1 and Mdm30 are required for mitochondrial OM fusion (Fritz et al., 2003; Sesaki and Jensen, 2001). Because an essential role of Mdm30 for the assembly of the Fzo1 complex has been excluded previously (Escobar-Henriques et al., 2006), we assessed a possible role of Ugo1 for Fzo1 assembly using sucrose gradient centrifugation. Interestingly, we observed a similar sedimentation profile of the Fzo1 complex in cells lacking Ugo1 (Δ*ugo1*) (Fig. 2E, solid black line) and in cells expressing the Fzo1 P-loop mutants (Fig. 2B), suggesting that Ugo1 is necessary for Fzo1 dimerization. However, Fzo1 levels are decreased in Δ*ugo1* cells (Amiott et al., 2009; Escobar-Henriques et al., 2006) and indirect effects cannot be excluded because DRP self-assembly is concentration-dependent. We therefore examined Fzo1 dimerization in cells lacking Mgm1 (Δ*mgm1*), which show a similar reduction of Fzo1 levels as Δ*ugo1* cells (Amiott et al., 2009; Escobar-Henriques et al., 2006). We observed a normal Fzo1 assembly in the absence of Mgm1 (supplementary material Fig. S1C), demonstrating that Ugo1 participates specifically in Fzo1 assembly. Interestingly, overexpression of Fzo1 (~30-fold) alleviated the requirement of Ugo1 for Fzo1 assembly (Fig. 2E, compare grey and black solid lines). Similarly, the Fzo1 assembly was restored in Δ*ugo1* mitochondrial extracts upon addition of GTP (Fig. 2E, compare solid and dashed black lines). Consistent with Ugo1 interaction with the C-terminal part of Fzo1 (Sesaki and Jensen, 2004), a partial destabilization of complex formation was observed upon truncation of the C-terminal coiled-coil domain of Fzo1 (supplementary material Fig. S1D). Therefore, we conclude that Fzo1 dimerization depends on Ugo1 and relies on the C-terminal coiled-coil of Fzo1.

Fzo1 cis dimers further interact in trans providing mitochondrial OM tethering

In vitro assays have demonstrated that mitochondrial proximity allows the fusion of opposing OM by facilitating the formation of Fzo1 tethering complexes in trans, i.e. between two mitochondria (Meeusen et al., 2004). Moreover, co-immunoprecipitation studies using mammalian mitochondria-derived vesicles expressing HA– or FLAG–Mfn1 revealed the formation of mitofusin tethering

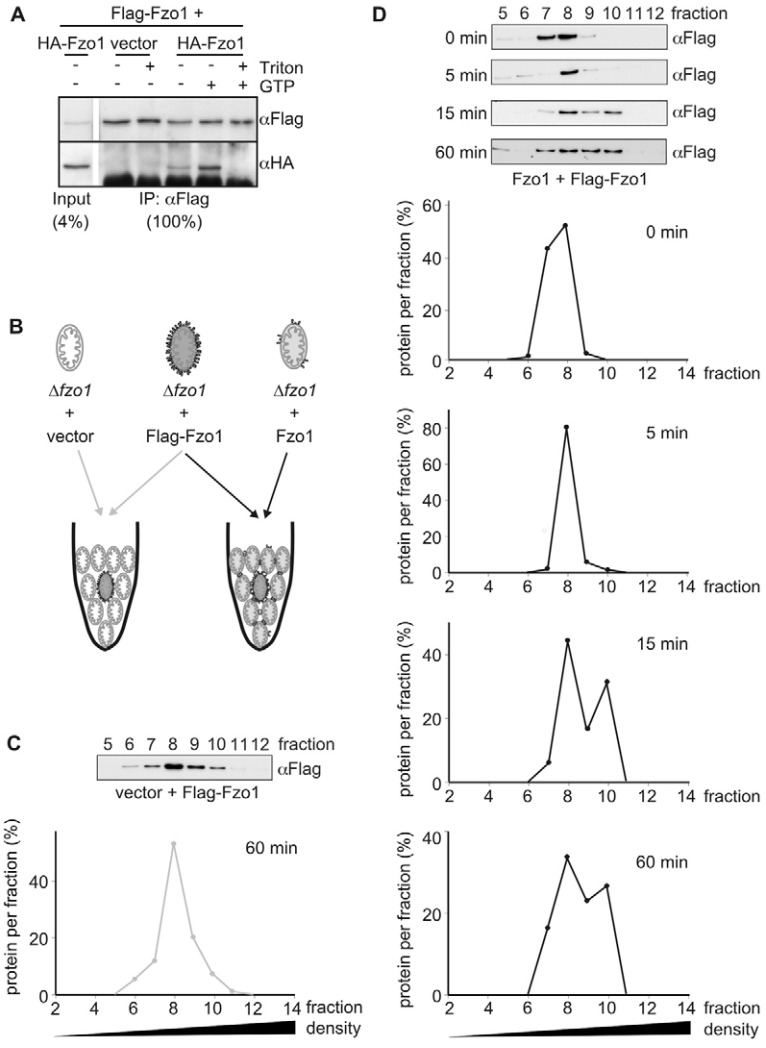

Fig. 3. Fzo1 trans interactions. (**A**) Binding of HA–Fzo1 containing vesicles to vesicles harbouring FLAG–Fzo1. Crude mitochondria from Δ*fzo1* cells expressing FLAG–Fzo1 or HA–Fzo1 were sonicated. Vesicles from FLAG–Fzo1-expressing mitochondria were mixed with vesicles derived from Δ*fzo1* mitochondria expressing HA–Fzo1 or not. The reaction mixtures were treated with 1% Triton X-100 when indicated and subjected to immunoprecipitation using FLAG-specific antibodies. Input and immunoprecipitates were analysed by immunoblotting using HA-specific antibodies. (**B**) Schematic representation of the mitochondrial tethering assay. (**C**,**D**) Formation of trans Fzo1 complexes. Sucrose gradient centrifugation analysis were performed as described in Fig. 2B but using ~10 mg/ml crude mitochondrial extracts, i.e. tenfold more concentrated mitochondria. Mitochondria harbouring FLAG–Fzo1 were mixed for the indicated times in a 1:9 ratio with either Δ*fzo1* mitochondria (in C) or Δ*fzo1* mitochondria expressing Fzo1 (in D). Immunoblots were performed using FLAG-specific antibodies.

complexes in trans (Ishihara et al., 2004). To assess trans tethering of OMs, we performed similar co-immunoprecipitation assays using yeast mitochondria-derived vesicles prepared by sonication of Δ*fzo1* mitochondria harbouring HA– or FLAG–Fzo1. The FLAG–Fzo1-containing vesicles were mixed with either Δ*fzo1*-derived vesicles or with vesicles harbouring HA–Fzo1. The FLAG–Fzo1 vesicles were precipitated using FLAG-specific antibodies. We successfully recovered HA–Fzo1-containing vesicles using FLAG-specific antibodies, indicating trans tethering (Fig. 3A). Addition of GTP increased tethering, whereas no signal could be recovered if Δ*fzo1* vesicles were used as the binding partner (Fig. 3A). Moreover, no HA signal was recovered if the vesicles were detergent-solubilized before immunoprecipitation (Fig. 3A), confirming a specific interaction between Fzo1 vesicles in trans.

We further investigated the nature of the Fzo1 trans complex using sucrose gradient centrifugation assays. We expressed differently tagged Fzo1 proteins in Δ*fzo1* cells, isolated mitochondria, and mixed mitochondria harbouring different Fzo1 variants (Fig. 3B). To promote trans tethering, ~10 mg/ml mitochondrial extracts were used, i.e. a tenfold higher concentration than in the previous sucrose gradient centrifugation assays. Image analysis and co-immunoprecipitation experiments were consistent with the presence of tethered organelles under these conditions (data not shown). To directly assess trans Fzo1 associations, we compared the assembly status of FLAG–Fzo1 when mixed with either Δ*fzo1* mitochondria (only allowing cis interactions) or mitochondria from Δ*fzo1* cells expressing Fzo1 (allowing Fzo1-dependent trans interactions). To monitor the formation of Fzo1 trans complexes over time, the reaction was stopped at different time points by increasing the volume, thus diluting the mitochondrial extracts. Mitochondrial membranes were then solubilized and analysed by sucrose gradient centrifugation. Importantly, if FLAG–Fzo1-containing mitochondria were mixed with Δ*fzo1* mitochondria, FLAG–Fzo1 was exclusively detected in fractions corresponding to the cis dimeric Fzo1 complex (Fig. 3C). FLAG–Fzo1 was ~30-fold overexpressed in these cells (compare the two first lanes in Fig. 1B), demonstrating that high Fzo1 concentrations in cis do not change the Fzo1 assembly behaviour.

By contrast, FLAG–Fzo1 formed larger complexes in a time-dependent manner if FLAG–Fzo1 mitochondria were mixed with Δ*fzo1* mitochondria containing Fzo1 at endogenous levels (Fig. 3D). These results indicate that Fzo1 tethers two mitochondrial membranes in trans.

Fzo1 trans assembly requires GTP binding but not GTP hydrolysis

We next studied the role of guanine nucleotides in the formation of Fzo1 trans complexes in concentrated mitochondria. When wild-type cells were analysed by sucrose gradient centrifugation, Fzo1 was recovered in a significantly broader peak under these conditions (Fig. 4A and supplementary material Fig. S2A), consistent with the presence of both cis dimer and trans complexes. Whereas nucleoside triphosphate (NTP) depletion inhibited the trans complexes (supplementary material Fig. S2B), addition of GTP or the non-hydrolysable GTP analogue GTPγS both resulted in a significant increase of the apparent molecular mass of the Fzo1 complex (Fig. 4A,B and supplementary material Fig. S2A,C). This indicated that trans association does not rely on GTP hydrolysis. By contrast, $Fzo1^{D195A}$ harbouring a mutation in the P-loop did not form Fzo1 trans complexes under these conditions and was detected in fractions corresponding to the monomer (Fig. 4B and supplementary material Fig. S2C). Notably, the equal behaviour of $Fzo1^{D195A}$ in experiments using low or high concentrations of mitochondria demonstrates that the protein:detergent ratio does not influence Fzo1 assembly per se. In conclusion, these experiments suggest that trans association depends on GTP binding to Fzo1, whereas GTP hydrolysis allows membrane fusion after membrane tethering.

Further, the role of the Fzo1-interacting proteins Ugo1 and Mdm30 for trans assembly of Fzo1 complexes was analysed. Fzo1 formed mainly the higher order complexes when concentrated Δ*mdm30* mitochondria were used (Fig. 4C and supplementary material Fig. S2D). Therefore, Mdm30 appears to be required after Fzo1 dimerization and trans tethering. Consistently, electron microscopy analysis revealed aggregation of fragmented mitochondria in Δ*mdm30* cells (Durr et al., 2006). By contrast, when concentrated Δ*ugo1* mitochondria were analysed upon sucrose gradient centrifugation, Fzo1 distribution included

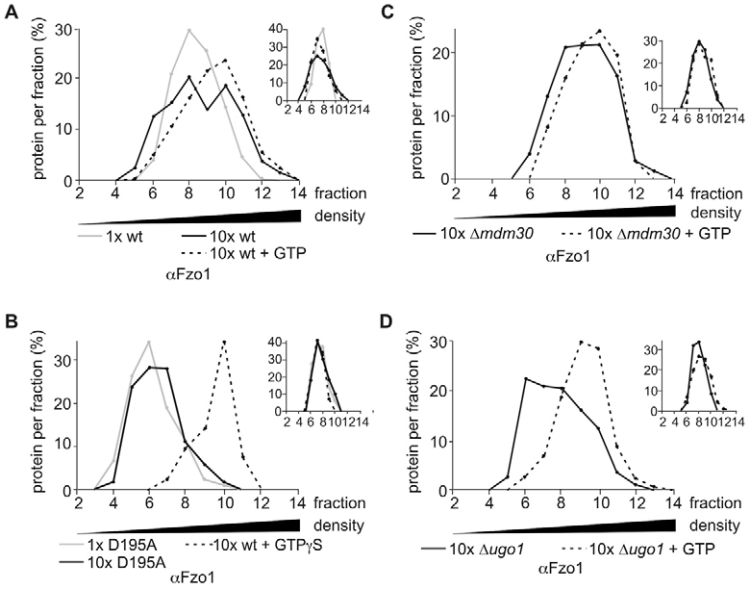

Fig. 4. Endogenous Fzo1 trans interactions. (**A**) Fzo1 complex formation in concentrated wild-type mitochondria. Approximately 1 mg/ml crude mitochondrial extracts from wild-type cells were fractionated by sucrose gradient centrifugation (1× wt). Alternatively, mitochondria were analysed at a tenfold higher concentration (10× wt) and 1 mM GTP was added when indicated. Immunoblots were performed using Fzo1-specific antibodies. (**B**) Effect of GTP binding and hydrolysis on Fzo1 complex formation. Crude mitochondrial extracts from wild-type cells or from Δ*fzo1* cells expressing $Fzo1^{D195A}$ were analysed by sucrose gradient centrifugation as described in A. GTPγS (1 mM) was added when indicated. Immunoblots were performed using Fzo1-specific antibodies. (**C**,**D**) Fzo1 trans association in the absence of Mdm30 or Ugo1. Crude mitochondrial extracts from Δ*mdm30* (C) or Δ*ugo1* (D) cells with or without 1 mM GTP were analysed at high protein concentrations (10×). Immunoblots were performed using Fzo1-specific antibodies. Insets show fractionation of a crossreactive band as internal control.

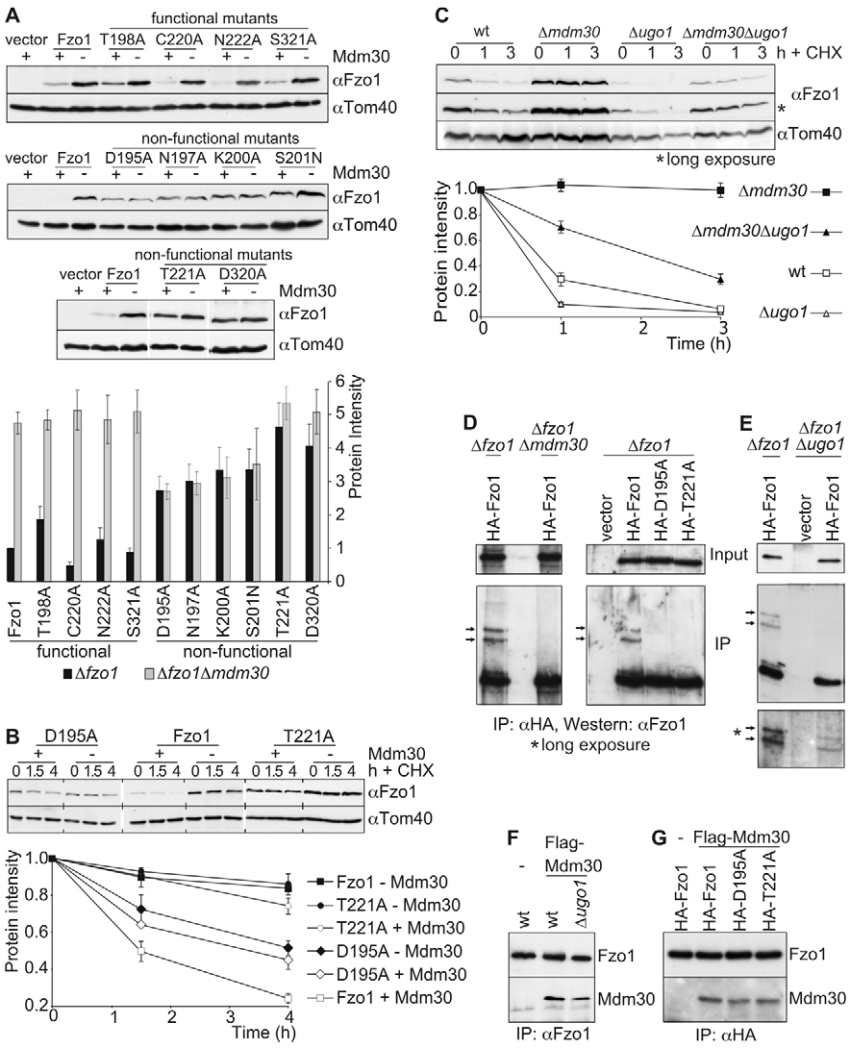

Fig. 5. Mutations in the P-loop or the switch region of Fzo1 abolish the Mdm30-dependent degradation and ubiquitylation of Fzo1. (**A**) Steady-state levels of Fzo1 mutant proteins. Total cellular extracts of Δ*fzo1* (+Mdm30) and Δ*fzo1*Δ*mdm30* (–Mdm30) strains expressing Fzo1 point mutants as indicated were analysed by SDS-PAGE and immunoblotting using Fzo1-specific antibodies. Tom40-specific antibodies were used as loading controls. A quantification including s.e.m. of four to seven independent experiments is shown in the bottom panel. (**B**) Stability of Fzo1^{D195A} and Fzo1^{T221A}. The stability of Fzo1 mutants was assessed after inhibition of cytosolic protein synthesis with cycloheximide (CHX) for the indicated times in exponentially growing Δ*fzo1* (+Mdm30) and Δ*fzo1*Δ*mdm30* (–Mdm30) cells (Escobar-Henriques et al., 2006). A quantification including s.e.m. of three independent experiments is shown in the lower panel. (**C**) Mdm30-dependence for Fzo1 turnover in the absence of Ugo1. Cycloheximide chase experiments in the presence or absence of Mdm30 and Ugo1 were performed and quantified as described in B. Three independent experiments using wild-type, Δ*mdm30* and Δ*ugo1* strains, and seven independent experiments for the Δ*mdm30*Δ*ugo1* strain were quantified. Lower panel shows quantification of results with s.e.m. (**D**) Ubiquitylation of Fzo1^{D195A} and Fzo1^{T221A}. The indicated N-terminally HA-tagged Fzo1 forms, expressed in the indicated strains, were immunoprecipitated using HA-specific antibodies and analysed by SDS-PAGE and immunoblotting using Fzo1-specific antibodies. (**E**) Ubiquitylation of Fzo1 in Δ*ugo1* cells was assayed as described for D. (**F**) Binding of Mdm30 to Fzo1 in the absence of Ugo1. Co-immunoprecipitation experiments were performed as previously described using crude mitochondrial preparations from wild-type (wt) or Δ*ugo1* cells expressing Mdm30 harbouring an N-terminal FLAG-epitope, when indicated. Immunoprecipitation was performed using Fzo1-specific antibodies and analysed by SDS-PAGE and immunoblotting, using both Fzo1- and FLAG-specific antibodies (Escobar-Henriques et al., 2006). (**G**) Binding of Mdm30 to Fzo1 GTPase mutants. Immunoprecipitation of HA–Fzo1 forms was essentially performed as described for D from Δ*fzo1* cells expressing the indicated Fzo1 variants. The immunoblotting was also analysed using FLAG-specific antibodies.

monomeric, dimeric or even larger complexes (Fig. 3D, solid line, and supplementary material Fig. S2E). Moreover, as observed for Fzo1 dimerization (Fig. 2E), addition of GTP completely compensated for the lack of Ugo1, as shown by Fzo1 recovery in trans tethering complexes under these conditions (Fig. 4D and supplementary material Fig. S2E).

We conclude from these experiments that cis dimers of Fzo1 further associate in trans to larger Fzo1 complexes. Trans tethering depends on GTP binding and on Ugo1, whereas Mdm30 and GTP hydrolysis are required only after the tethering step.

Fzo1 ubiquitylation and turnover occurs only after GTP hydrolysis

Mdm30 is responsible for Fzo1 turnover in growing cells, which is required to maintain fusion-competent mitochondria (Cohen et al., 2008; Escobar-Henriques et al., 2006; Fritz et al., 2003).

Moreover, Fzo1 turnover depends on GTP hydrolysis (Amiott et al., 2009). To further examine the link between the degradation of Fzo1 and its GTPase function, we expressed various Fzo1 mutants (Fig. 2A) in Δ*fzo1* and in Δ*fzo1*Δ*mdm30* cells and monitored their steady-state concentration. Fzo1 accumulated in the absence of Mdm30 as compared to wild-type cells (Fig. 5A, compare lanes 2 and 3) (Fritz et al., 2003). Similarly, functionally active Fzo1 mutants were present at increased concentrations in Mdm30-deficient mitochondria (Fig. 5A). By striking contrast, the presence of Mdm30 did not significantly affect the accumulation of non-functional Fzo1 mutants (Fig. 5A). Inhibition of protein synthesis by cycloheximide (CHX) revealed degradation of Fzo1^{D195A}, which occurred in an Mdm30-independent manner and proceeded at slightly lower rates than in wild-type cells (Fig. 5B). The Fzo1^{T221A} and Fzo1^{D320A} forms accumulated to similar levels as Fzo1 in Δ*mdm30* cells (Fig. 5A). Consistently, cycloheximide chase experiments confirmed that the Fzo1^{T221A} represents a stable protein, even in the presence of Mdm30 (Fig. 5B). These findings are in agreement with recently published data (Amiott et al., 2009) and demonstrate that both binding and hydrolysis of GTP by Fzo1 are essential to allow Mdm30-dependent degradation of Fzo1 and to maintain low Fzo1 protein levels.

Next, we examined the importance of Ugo1 for the Mdm30-dependent degradation of Fzo1 because the deletion of *UGO1* impaired Fzo1 dimerization, as did the Fzo1 mutation D195A (Fig. 2). Similarly to Fzo1^{D195A}, wild-type Fzo1 was degraded in an Mdm30-independent manner in the absence of Ugo1 (Fig. 5C). These results corroborate the idea that Fzo1 degradation mediated by Mdm30 depends on the formation of a functional Fzo1 complex. Experiments using truncated variants of Fzo1 further supported the conclusion that Fzo1 turnover requires a functional Fzo1 protein (supplementary material Fig. S3). Non-functional Fzo1 variants lacking N- or C-terminal amino acid residues were degraded regardless of the presence of Mdm30 (supplementary material Fig. S3A,C), although the truncated Fzo1 variants were still able to bind Mdm30 (supplementary material Fig. S3D). A further hint that Mdm30 acts as a part of the fusion events came from the observation that indirect blocking of tethering, i.e. inducing mitochondrial fragmentation by disrupting the actin cables with Latrunculin A (Boldogh et al., 1998), was accompanied by Fzo1 stabilization (supplementary material Fig. S3E,F).

Previous studies have identified distinct modified forms of Fzo1 that were demonstrated to contain ubiquitin (Cohen et al., 2008; Neutzner et al., 2007). Ubiquitylation of Fzo1 depends on Mdm30 (Cohen et al., 2008) (Fig. 5D, left panel) and requires GTP binding and hydrolysis by Fzo1 (Amiott et al., 2009) (Fig. 5D, right panel). Therefore, we examined the role of Ugo1 in Fzo1 ubiquitylation. Consistent with the tethering (Fig. 4D) and the turnover (Fig. 5C) data, immunoprecipitation experiments revealed that ubiquitylated Fzo1 was almost absent in Δ*ugo1* cells (Fig. 5E). However, neither the absence of Ugo1 nor the mutations in the Fzo1 GTPase domain abolished Mdm30 binding to Fzo1 (Fig. 5F,G).

Taking the results together, we conclude that Mdm30-dependent Fzo1 ubiquitylation and degradation requires Ugo1 and occurs only after GTP hydrolysis by Fzo1 at late stages of OM fusion.

Discussion

Our findings identify distinct steps in the mitochondrial OM fusion cycle mediated by mitofusins and refine the roles of two regulatory proteins, Ugo1 and Mdm30, in this process (Fig. 6). We demonstrate that mitofusins form homo-dimers in the mitochondrial OM. Fzo1

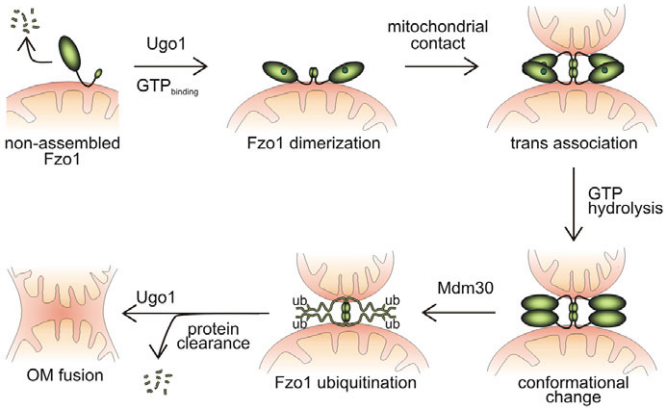

Fig. 6. Model for the role of Ugo1 and Mdm30 during GTP-dependent OM fusion. GTP binding and the Ugo1 protein mediate the cis dimerization of Fzo1, protecting it against degradation. Fzo1 dimers present in two different mitochondria can associate in trans and tether the organelles. The stoichiometry and composition of Fzo1-containing trans complexes is speculative. Subsequently, GTP hydrolysis might induce a conformational change that allows Mdm30 to promote Fzo1 ubiquitylation (ub). Degradation of Fzo1 terminates its role in fusion.

homo-dimerization depends on Ugo1 and binding of GTP to Fzo1, and is essential for OM fusion to proceed. Moreover, dimerization protects Fzo1 against Mdm30-independent turnover, indicating efficient quality control surveillance of non-assembled Fzo1. Mitochondrial close contacts induce mitochondrial tethering, which is triggered by Fzo1 trans complexes, probably composed of four Fzo1 subunits. GTP hydrolysis and Mdm30-dependent degradation complete the fusion of the OM. Thus, Ugo1 and Mdm30 act sequentially during Fzo1-mediated OM fusion.

A dual role of Ugo1 during OM fusion

Our findings demonstrate that Ugo1 ensures efficient homo-dimerization of Fzo1 and therefore assign a function to Ugo1 in the early steps of OM fusion. Consistently, deletion of the C-terminal domain of Fzo1, one of the two regions mapped for the Ugo1–Fzo1 interaction (Sesaki and Jensen, 2004), destabilized dimerization. This suggests that Ugo1 might directly facilitate dimerization of Fzo1, perhaps by promoting GTP binding to Fzo1 or by ‘chaperoning’ newly imported, monomeric Fzo1 molecules. At the same time, in vitro fusion assays using Ugo1 point mutant variants point to a role of Ugo1 for the last, lipid-mixing step in OM fusion (Hoppins et al., 2009). In agreement with a role of Ugo1 at late stages of the fusion cycle, we observed residual levels of ubiquitylated Fzo1 in the absence of Ugo1. In fact, Ugo1 is absolutely essential for OM fusion (Hoppins et al., 2009), which suggests that in Δ*ugo1* cells fusion is also blocked downstream of Fzo1 ubiquitylation. In mammals, the membrane merging step requires the activity of mitochondrial phospholipase D, which facilitates fusion by the hydrolysis of cardiolipin and the generation of phosphatidic acid (Choi et al., 2006). Although changes in cardiolipin levels per se do not interfere with Fzo1 dimerization (data not shown), it is conceivable that Ugo1 regulates the local lipid composition in the OM and thereby affects membrane fusion at early and late stages.

Mdm30 acts after membrane tethering

Several lines of evidence point to a post-tethering role of Mdm30. First, formation of Fzo1 dimers and, most importantly, of Fzo1-

containing tethering complexes do not require the F-box protein Mdm30, which is consistent with the accumulation of aggregated mitochondrial fragments in Δ*mdm30* cells (Durr et al., 2006). Second, these mitochondrial clusters represent stalled fusion-competent intermediates (Fritz et al., 2003) (and data not shown). Third, GTP hydrolysis is only required after trans tethering. Finally, Mdm30 triggers the ubiquitylation and degradation of Fzo1 only after trans tethering and GTP hydrolysis, pointing to an intimate coupling of the GTPase function of Fzo1 and its degradation during OM fusion. This is in agreement with the recently reported effect of a mutation in the GTPase domain of Fzo1 created in analogy to mutations from Charcot-Marie-Tooth hereditary neuropathy type 2A (CMT2A) patients (Amiott et al., 2009). In analogy to other GTPases, we speculate that a modified conformation of Fzo1 after GTP hydrolysis might allow its ubiquitylation and degradation. Interestingly, other F-box proteins have been connected to the regulation of GTPases (Kleijnen et al., 2007; Lafourcade et al., 2003; Mahlert et al., 2009; Yu et al., 2008). For instance, the F-box protein Rcy1 activates the GTPase Ypt6, the yeast homologue of the human Rab6 protein, which is required for fusion of endosome-derived vesicles with the late Golgi (Lafourcade et al., 2003).

Mdm30-dependent and Mdm30-independent Fzo1 turnover

Different proteolytic pathways have been described for the degradation of Fzo1 under various physiological conditions, and are distinct in their dependence on Mdm30. Fzo1 is degraded in an Mdm30-independent manner upon mating factor treatment of yeast cells (Neutzner and Youle, 2005). Under these conditions, degradation of the fusion protein Fzo1 is accompanied by fragmentation of the mitochondrial network due to ongoing fission processes. By contrast, Fzo1 proteolysis in proliferative yeast cells is regulated by Mdm30 and promotes mitochondrial fusion (Cohen et al., 2008; Escobar-Henriques et al., 2006). This observation seems paradoxical but is explained by our findings. During OM fusion, Fzo1 ubiquitylation and degradation take place only after membrane tethering and GTP hydrolysis, i.e. after Fzo1 has accomplished its biological function. Although the ATP-dependent dissociation of SNARE complexes allows a new fusion cycle during SNARE-mediated membrane fusion, Fzo1 tethering complexes are ubiquitylated and degraded. The physical interaction between Mdm30 and Fzo1 suggests that Fzo1 ubiquitiylation occurs at the fusion site. Ubiquitylation of Fzo1 complexes after GTP hydrolysis might be important in overcoming a steric hindrance for membrane fusion imposed by the tethering structures. It is noteworthy that crystal structure studies performed with the C-terminal cytosolic domain of Mfn1 revealed antiparallel helical interactions between their C-terminal coiled-coil domains (Koshiba et al., 2004), therefore oriented perpendicular to the membrane surface. This is in contrast to SNAREs tethering complexes, which are formed by parallel coiled-coil interactions, oriented parallel to the membrane surface and allowing closer membrane contacts (Sutton et al., 1998). Our findings suggest that ubiquitylation and clearance of mitofusins facilitate close membrane approximation and increase the efficiency of OM fusion, therefore pointing to an unusual and irreversible mechanism of a GTPase.

Materials and Methods

Yeast strains and growth media

Yeast strains are isogenic to the S288c (Euroscarf) and were grown according to standard procedures on complete or synthetic media supplemented with 2% (w/v) glucose or with 2% (w/v) glycerol. Cycloheximide (Sigma) (100 μg/ml from a stock at 10 mg/ml in ethanol) or Latrunculin A (Calbiochem) (10 μM from a stock at 2 mM in DMSO) were added when indicated.

Plasmids

Fzo1 or Mdm30 harbouring an N-terminal FLAG-tag were expressed from the multicopy vector pJDCEX2 (2 μ, *LEU2*, *CUP1* promoter) (Escobar-Henriques et al., 2006). The mutant FLAG–Fzo1^{D195A} was generated using this construct by site-directed mutagenesis. Fzo1 or the indicated Fzo1 truncated forms harbouring an N-terminal hemagglutinin (HA) tag were expressed under the control of the endogenous Fzo1 promoter using the centromeric plasmid pRS316. Fzo1 point mutants with or without the HA tag were generated by site-directed mutagenesis using this Fzo1 construct in pRS316. For visualizing mitochondria, the centromeric plasmid pYX142-mtGFP, encoding the mitochondrial matrix-targeted GFP, was used (Westermann and Neupert, 2000). All newly created constructs were verified by DNA sequencing.

Size-exclusion chromatography

Mitochondrial extracts (600 μg) (Tatsuta and Langer, 2007) were solubilized in 1% (v/v) Triton X-100 using buffer A (150 mM potassium acetate, 4 mM magnesium acetate, 30 mM Tris-HCl pH 7.4, 0.5 mM PMSF) and fractionated by size-exclusion chromatography on a Superose 6 column (Amersham Biosciences), as described (Rapaport et al., 1998). Fractions of 0.5 ml were collected, precipitated with trichloroacetic acid, and analysed by SDS-PAGE and immunoblotting. The column was calibrated using the soluble proteins thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa) as standards.

Purification and crosslinking of Fzo1

FLAG–Fzo1 or FLAG–Fzo1^{D195A} were expressed in wild-type or *Δfzo1* yeast cells and 100 OD_{600} of exponentially growing cultures were collected. Crude mitochondria were isolated as described (Tatsuta and Langer, 2007) and lysed for 45 minutes at 4°C in 500 μl solubilization buffer A, containing 1% Triton X-100 (Rapaport et al., 1998). Lysates were cleared by centrifugation at 4°C and 16,000 ***g*** for 10 minutes and incubated for 2 hours at 4°C with EZview Red ANTI-FLAG M2 Affinity Gel (Sigma). The resin was washed three times with 500 μl of the same buffer but containing 0.2% (v/v) Triton X-100. The bound protein was eluted with 5 μl of 3× FLAG peptide (Sigma) (stock 5 mg/ml in TBS) in a total volume of 45 μl buffer A at 4°C for 2 hours. The crosslinking reaction of purified Fzo1 protein was performed at 4°C with 0.02% glutaraldehyde in buffer A, for 2 hours if not indicated otherwise. The reaction was stopped by addition of 4× Laemmli buffer (Laemmli, 1970) if subjected to SDS-PAGE and the sample subsequently immunoblotted. If followed by size-exclusion chromatography, the crosslinking reaction was stopped by adding glycine to 1% (w/v). Aliquots of 20 μl purified Fzo1, crosslinked or not, were then diluted to 100 μl in buffer A and fractionated by size-exclusion chromatography as described above.

Sucrose density gradient centrifugation

Crude mitochondrial preparations (400–600 μg) (Rapaport et al., 1998), obtained from 50–75 OD_{600} exponentially growing cells of the indicated strains, were lysed for 30 minutes at 4°C in 500 μl of solubilization buffer B [1% (w/v) digitonin, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals)]. When indicated, crude mitochondria were first incubated with 1 mM GTP for 1 hour at 30°C in 400 μl buffer B without detergent and then solubilized for 30 minutes at 4°C by further addition of 100 μl 5% (w/v) digitonin in buffer B. The lysates were cleared by centrifugation at 16,000 ***g*** and 4°C for 10 minutes, and the supernatant loaded on top of an 11-ml 5–25% (w/v) sucrose gradient prepared in solubilization buffer B but with 0.1% (w/v) digitonin. The gradients were centrifuged at 174,000 ***g*** and 4°C for 14 hours, and afterwards 28 fractions of 400 μl were collected from the top, precipitated with trichloroacetic acid and analysed by SDS-PAGE and immunoblotting. Blots were quantified using Quantity One (BioRad, Hercules, CA). A crossreactive band detected with the Fzo1 antibody was used as an internal standard for comparison of the fractionation between two or more different gradients.

Blue native gel electrophoresis

The native molecular mass of the Fzo1 complex was analysed as previously described (Schägger, 2001), using modified buffer conditions (Tatsuta et al., 2005). Essentially, 100 μg of mitochondrial extracts were lysed in 25 μl solubilization buffer C [1% (w/v) digitonin, 50 mM NaCl, 5 mM aminohexanoic acid, 50 mM imidazol/HCl pH 7.4, 10% glycerol, 50 mM potassium phosphate buffer pH 7.4] for 20 minutes at 4°C. The lysates were cleared by centrifugation at 16,000 ***g*** and 4°C for 10 minutes. The supernatant was mixed with 1/10 1% (w/v) Coomassie Blue in solubilization buffer C and loaded onto a 3–13% acrylamide (w/v) blue native gel.

Immunoisolation of membrane vesicles containing Fzo1 proteins

Trans Fzo1 physical interactions were analysed essentially as previously described for mitofusins (Ishihara et al., 2004). FLAG–Fzo1 or HA–Fzo1 were expressed in *Δfzo1* yeast cells and 150 OD_{600} of exponentially growing cultures were collected. Crude mitochondrial pellets (~1.2 mg), prepared as described (Rapaport et al., 1998),

were resuspended in 100 µl of buffer B and sonicated with a Branson sonifier (output control 5.5, duty cycle 40%, 75 seconds, output level 20 W, 5-mm diameter microtip, on ice). Unbroken organelles were removed by two centrifugations at 4,000 ***g*** and 4°C for 4 minutes, and the supernatants containing vesiculated membranes were recovered. FLAG–Fzo1 vesicles (10 µl) were mixed with 90 µl of either *Δfzo1* or HA–Fzo1 vesicles and incubated with or without 1 mM GTP at 30°C for 1 hour. The samples were then further incubated for 20 minutes on ice with or without 1% Triton X-100. After incubation, 400 µl buffer B without detergent was added and samples were centrifuged at 1,500 ***g*** and 4°C for 4 minutes to remove membrane aggregates. The supernatant was incubated overnight at 4°C with EZview Red ANTI-FLAG M2 Affinity Gel (Sigma). The resin was washed three times with 500 µl of buffer B without detergent. The bound protein was eluted with 40 µl Laemmli buffer at 65°C for 20 minutes, subjected to SDS-PAGE, and subsequently immunoblotted using HA- and FLAG-specific antibodies. About 30% of the total FLAG–Fzo1-containing vesicles were immunoprecipitated and ~4% of the total HA–Fzo1-containing vesicles could be co-immunoprecipitated.

Analysis of Fzo1 assembly in trans

Crude mitochondria from 50–75 OD_{600} exponentially growing *Δfzo1* yeast cells overexpressing FLAG–Fzo1 or expressing Fzo1 were prepared as described (Rapaport et al., 1998). Each mitochondrial preparation was resuspended in 50 µl of buffer B without detergent, obtaining ~10 mg/ml of protein. FLAG–Fzo1 mitochondria (5 µl) were mixed with 45 µl of either *Δfzo1* or HA–Fzo1 mitochondria and incubated at 30°C for the indicated times. The tethering reaction was stopped by addition of 350 µl buffer B and further solubilized by addition of 100 µl 5% (w/v) digitonin in buffer B for 30 minutes at 4°C. The lysates were cleared and analysed by sucrose density gradient centrifugation as described above.

For analysis of endogenous Fzo1, crude mitochondria from 50–75 OD_{600} exponentially growing cells were prepared. For the experiments labelled 10×, mitochondria were resuspended in 40 µl buffer B without detergent (i.e. to ~10 mg/ml). When indicated, samples were incubated for 1 hour at 30°C with or without 1 mM GTP or GTPγS or alkaline phosphatase (2 µl calf intestinal phosphatase; New England Biolabs, Ipswich, MA). Then, samples were solubilized for 30 minutes at 4°C by further addition of 10 µl 5% (w/v) digitonin in buffer B. The lysates were cleared and analysed by sucrose gradient centrifugation as described above. Experiments labeled 1× refer to the sucrose gradient analysis described above, i.e. in 500 µl of buffer B with 1% (w/v) digitonin.

Protein steady-state levels and synthesis shutoff

For analysis of Fzo1 steady-state levels, cells were grown in SD medium and samples collected during the exponential growth phase. To monitor Fzo1 turnover, cycloheximide was added to logarithmically growing cells. Samples were collected at the indicated time points, and total proteins extracted at alkaline pH (Tatsuta and Langer, 2007) were analysed by SDS-PAGE and immunoblotting. Western blots were quantified using Quantity One (BioRad). Mean values of three to seven different experiments are shown. The error bars reflects the s.e.m. (Cumming et al., 2007). Fzo1 levels in *Δfzo1* cells expressing Fzo1 from a centromeric plasmid and its endogenous promoter were set to 1.

Ubiquitylation assay

Immunoprecipitation of ubiquitylated Fzo1 was performed as previously described (Neuber et al., 2005) except that 25 mM *N*-ethylmaleimide was added before crude membrane extraction. Yeast strains were transformed with HA–Fzo1, HA–$Fzo1^{D195A}$ or HA–$Fzo1^{T221A}$ and grown in synthetic media to the logarithmic growth phase. Crude membrane extracts (Neuber et al., 2005) from 400 OD_{600} cells were solubilized in 1% (w/v) digitonin in buffer A and cleared by centrifugation. Lysates were immunoprecipitated overnight with 5 µl of the HA antibody (H 6908; Sigma-Aldrich). Bound protein was eluted in 40 µl Laemmli buffer at 65°C for 20 minutes, subjected to SDS-PAGE and subsequently immunoblotted using Fzo1-specific antibodies.

We are grateful to Jodi Nunnari (University of Californis, Davis, CA) for the Fzo1 antibody, and to Steffen Augustin, Sascha Dargazanli, Carsten Merkwirth, Oliver Neuber, Christof Osman and Takashi Tatsuta for technical advice. We also thank Bertrand Daignan-Fornier, Sven Saupe and Julia Hunn for critical reading of the manuscript, Bertrand Daignan-Fornier for support to M.E.-H, and to Jürgen Dohmen and Martin Graef for stimulating discussions. This work was supported by grants of the Deutsche Forschungsgemeinschaft (ES 338/1-1 to M.E.-H., SFB635 to G.J.K.P. and T.L.) and the European Research Council to T.L.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/7/1126/DC1

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