Two Deubiquitylases Act on Mitofusin and Regulate Mitochondrial Fusion along Independent Pathways

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SUMMARY

Mitofusins, conserved dynamin-related GTPases in the mitochondrial outer membrane, mediate the fusion of mitochondria. Here, we demonstrate that the activity of the mitofusin Fzo1 is regulated by sequential ubiquitylation at conserved lysine residues and by the deubiquitylases Ubp2 and Ubp12. Ubp2 and Ubp12 recognize distinct ubiquitin chains on Fzo1 that have opposing effects on mitochondrial fusion. Ubp2 removes ubiquitin chains that initiate proteolysis of Fzo1 and inhibit fusion. Ubp12 recognizes ubiquitin chains that stabilize Fzo1 and promote mitochondrial fusion. Self-assembly of dynamin-related GTPases is critical for their function. Ubp12 deubiquitylates Fzo1 only after oligomerization. Moreover, ubiquitylation at one monomer activates ubiquitin chain formation on another monomer. Thus, regulation of mitochondrial fusion involves ubiquitylation of mitofusin at distinct lysine residues, intermolecular crosstalk between mitofusin monomers, and two deubiquitylases that act as regulatory and quality control enzymes.

INTRODUCTION

Mitochondria are organelles that constantly undergo fusion and fission events. This dynamic behavior is critical in healthy cells for mitochondrial trafficking, DNA inheritance, and cellular homeostasis (Nunnari and Suomalainen, 2012). In sickness, mitochondrial plasticity orchestrates apoptosis and cellular stress resistance. Fusion and fission of mitochondria rely on dynamin-related GTPases (DRPs) (Escobar-Henriques and Anton, 2012; Hoppins and Nunnari, 2009; Palmer et al., 2011). DRPs are large GTPases whose activities are stimulated by self-oligomerization (Gasper et al., 2009) and which are involved in a variety of membrane scission and fusion events (Praefcke and McMahon, 2004). DRPs responsible for fusion processes are mitofusins (Mfn1/Mfn2/Fzo1) in the mitochondrial outer membrane (OM), OPA1/Mgm1 in the mitochondrial inner membrane, and atlastin/Sey1 in the ER (Hu et al., 2011; Low and Löwe, 2010; Westermann, 2010). Dysfunction of these core components is associated with both common and rare neurological disorders (Chan, 2006; Chen and Chan, 2009; Salinas et al., 2008; Züchner et al., 2004).

Mitochondrial dynamics is regulated by several posttranslational modifications, such as ubiquitylation, sumoylation, and phosphorylation (Elgass et al., 2012; Escobar-Henriques and Anton, 2012). While well studied for mitochondrial fission, their role in mitochondrial fusion is only emerging. Phosphorylation and ubiquitylation of mitofusins triggers their proteolysis in response to several stimuli (Cohen et al., 2008; Gegg et al., 2010; Leboucher et al., 2012; Poole et al., 2010; Tanaka et al., 2010; Ziviani et al., 2010). Ubiquitylation depends on the E3 ligases parkin in mammals and in flies, Huwε1 in mammals, and SCFγ130 in yeast. It is currently unclear whether ubiquitylation only affects the stability of mitofusins or exerts additional regulatory roles during mitochondrial fusion. In fact, the variety of ubiquitin chains that can be formed is immense, and so are their biological consequences (Komander and Rape, 2012). Contributing to this complexity is the fact that formation of ubiquitin chains via K48 linkages typically marks proteins for destruction by the proteasome but can also play nonproteolytic roles (Finley, 2009; Flick et al., 2006). Ubiquitylation is regulated by specific proteases, so-called deubiquitylases (DUBs), which remove ubiquitin chains (Komander et al., 2009). It is currently thought that the specificity of most DUBs is determined in vivo by interactions with the substrate (Marfany and Denuc, 2008; Ventii and Wilkinson, 2008). DUBs render ubiquitylation reversible and offer possibilities for regulation. However, as neither the ubiquitylation sites in mitofusins nor DUBs acting on mitofusins have been identified, the role of ubiquitylation for mitochondrial fusion remained unclear.

Here, we mapped distinct ubiquitylation sites in Fzo1 and identified two DUBs recognizing ubiquitylated mitofusin in vivo. This allowed us to unravel a new regulatory role of ubiquitylation during mitochondrial fusion. Our findings uncover two ubiquitylation pathways, which either promote or inhibit mitochondrial fusion.
RESULTS

Mitochondrial Function Depends on Ubiquitylation of Mitofusin

It was previously shown that Fzo1 is ubiquitylated, as confirmed after coexpression of HA-Fzo1 with Myc-Ubiquitin (Figure 1A; Cohen et al., 2008). To examine if mitochondrial fusion depends on Fzo1 ubiquitylation, we generated an Fzo1 variant deprived of ubiquitin. Ubiquitylation of a substrate can be inhibited by fusing it to ubiquitin-specific proteases (Ernst et al., 2011; Stringer and Piper, 2011). We therefore fused to N-terminally HA-tagged Fzo1 the catalytic domain of the Ub7 protease and, for control, a catalytically inactive variant thereof (Fzo1-Ubp7C618S). The resulting chimeric proteins were coexpressed with Myc-Ubiquitin in Δfzo1 cells and precipitated from mitochondrial extracts using HA-coupled beads. Samples were analyzed by SDS-PAGE and immunoblotting using HA- and Myc-specific antibodies. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated by a black arrowhead or black arrows, respectively. A red arrowhead indicates unmodified HA-Fzo1-Ubp7. The red line indicates ubiquitylated HA-Fzo1-Ubp7C618S.

Yeast Mitofusin Is Ubiquitylated at Conserved Lysine Residues

Mitofusins display an unusual and conserved ubiquitylation pattern consisting of at least three major ubiquitylated forms of Fzo1 (Cohen et al., 2008; Rakovic et al., 2011; Ziviani et al., 2010). Expression of Myc-ubiquitin instead of ubiquitin confirmed that Fzo1 forms slowly migrating during electrophoresis contain ubiquitin (Figure 2A, see Figure S1A online). The lower modified form likely represents monoubiquitylation of Fzo1, while the slower migrating forms contain K48-linked ubiquitin chains, as expected, because they depend on the ubiquitin-conjugating enzyme Cdc34, which builds K48 linkages (Figures S1B and S1C; Cohen et al., 2008). To identify the modified

Figure 1. Ubiquitylation Is Essential for Fzo1 Function

(A) Ubiquitylation of HA-Fzo1-Ubp7. HA-Fzo1, HA-Fzo1-Ubp7, and its corresponding catalytic inactive variant, HA-Fzo1-Ubp7C618S, were coexpressed with Myc-ubiquitin in Δfzo1 cells and precipitated from mitochondrial extracts using HA-coupled beads. Samples were analyzed by SDS-PAGE and immunoblotting using HA- and Myc-specific antibodies. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated by a black arrowhead or black arrows, respectively. A red arrowhead indicates unmodified HA-Fzo1-Ubp7. The red line indicates ubiquitylated HA-Fzo1-Ubp7C618S.

(B) Mitochondrial morphology of Δfzo1 cells expressing HA-Fzo1-Ubp7 fusion proteins. Δfzo1 cells expressing the indicated Fzo1 variants were created by mating and tetrad dissection. For analysis of mitochondrial morphology, a mitochondrial targeted GFP plasmid was coexpressed. Cellular (Nomarski), and mitochondrial (GFP) morphology were visualized by fluorescence microscopy. Lower panel, data are presented as mean ± SE from three different experiments (with more than 200 cells each), as described (Cumming et al., 2007).

(C) Respiratory capacity of Δfzo1 cells expressing HA-Fzo1-Ubp7 fusion proteins. The cells analyzed in (B) were spotted on selective media supplemented with glucose or glycerol.

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Ubp2 and Ubp12 Deubiquitylate Fzo1

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lysine residues in Fzo1, we purified Fzo1 by immunoprecipitation and analyzed the precipitate by mass spectrometry (Xu and Peng, 2006). Two different lysine residues, K398 and K464, were identified as ubiquitylation sites in Fzo1, both positioned downstream of the GTPase domain. K398 and K464 are located in close proximity to each other in a structural model of Fzo1 (Figure 2B), which was generated based on the crystal structure of the bacterial dynamin BDLP (Low and Löwe, 2006; Roy et al., 2010). The c score ranges from −5 to +2 where a more positive score reflects a model of better quality. A truncated variant of Fzo1 (including amino acid residues 106–720 and 751–855, indicated with a gray bar above the linear structure) was modeled on BDLP in its open conformation (PDB ID code 2W6D) using UCSF chimera (estimated rmsd 25.421) (Low et al., 2009; Yang et al., 2012). The arrows indicate the two-hinged domain rearrangement as suggested for BDLP; both rotations are in plane (Low et al., 2009).

The conservation of ubiquitylated lysine residues is indicated by black boxes. Sc, Saccharomyces cerevisiae; Ca, Candida albicans; Dm, Drosophila melanogaster; Ce, Caenorhabditis elegans; Hs, Homo sapiens. See also Figure S1.

In contrast, Fzo1K398R was still ubiquitylated (Figure 3A and Figure S2D) and rescued partially the growth defect of Δfzo1 cells on glycerol-containing medium (Figure 3B and Figure S2C). Consistently, chase experiments revealed accelerated proteolysis of Fzo1K398R (Figure 3C). Ubiquitylation and turnover of Fzo1 depends on the F box protein Mdm30 (Cohen et al., 2008; Escobar-Henriques et al., 2006; Fritz et al., 2003), and we found that Fzo1K398R was degraded in an Mdm30-dependent manner (Figure 3C). As Δfzo1 cells expressing Fzo1K398R are not fully functional, the accelerated proteolysis of Fzo1K398R appears to be detrimental for mitochondrial fusion. This indicates that ubiquitylation of Fzo1 has additional functions beyond its role in determining the stability of Fzo1. These experiments thus identify two ubiquitylated lysine residues in Fzo1 and reveal that Fzo1 activity requires ubiquitylation at these sites.
Ubiquitylation of Mitofusins Occurs Sequentially and Involves Crosstalk between Monomers

As ubiquitylation of Fzo1 occurs after self-assembly of its monomers (Anton et al., 2011), we analyzed how the intermolecular crosstalk within the Fzo1 complex influences its ubiquitylation. To this aim, we compared ubiquitylation of HA-Fzo1K464R, HA-Fzo1K398R, or HA-Fzo1K398R,K464R in Δfzo1 (Figure 3A) and wild-type cells, i.e., in the presence of nontagged endogenous Fzo1 (Figure 3D). Ubiquitylation of Fzo1 was assessed after immunoprecipitation using HA-specific antisera. Although lacking the crucial lysine residue K464, both HA-Fzo1K464R and HA-Fzo1K398R,K464R were ubiquitylated when expressed in wild-type cells (Figure 3D and Figure S2E). This shows, first, that K464 does not need to be present in all subunits of an Fzo1 oligomer; second, that ubiquitin chains of Fzo1 are not linked to K464; and, third, that Fzo1 has additional ubiquitylation sites. Interestingly, HA-Fzo1K398R was ubiquitylated similarly in Δfzo1 (Figure 3A and Figure S2D) or in wild-type cells (Figure 3D and Figure S2E), demonstrating that K398 critically determines the ubiquitylation pattern of Fzo1.

Together, our experiments are consistent with a stepwise, intermolecular ubiquitylation process: Fzo1 is ubiquitylated at K464, which triggers formation of ubiquitin chains at K398 of an associated Fzo1 monomer.

Ubp12 Deubiquitylates Fzo1

To further define the role of Fzo1 ubiquitylation for mitochondrial fusion, we searched for DUBs acting on Fzo1. We expressed HA-Fzo1 or HA-Fzo1K398R in yeast cells lacking DUBs and assessed ubiquitylation of Fzo1 by immunoprecipitation. While deletion of most DUBs had no effect on Fzo1 steady-state levels nor on ubiquitylation, we observed an accumulation of Fzo1 and Fzo1K398R as well as the ubiquitylated forms in cells lacking UBP12 (Figure 4A and Figure S3A). To examine if Ubp12 binds directly to Fzo1, we performed coimmunoprecipitation studies. HA-Fzo1 was coexpressed with Ubp12-Flag or the catalytic inactive variant Ubp12C372S-Flag, which is expected to act as a substrate trap. HA-Fzo1 was coprecipitated with Flag-specific antibodies with Ubp12C372S-Flag, demonstrating physical interaction (Figures 4B and Figure S3B). To exclude posttranslational modification, Fzo1 and each of the two Ubp12 variants were expressed separately, and mitochondrial lysates containing either HA-Fzo1 or a Ubp12-Flag variant were mixed prior to immunoprecipitation. This strongly impaired Fzo1 binding to Ubp12C372S-Flag, substantiating the specificity of the observed interaction between both proteins (Figure S3B). We therefore conclude that Ubp12 directly regulates ubiquitylation of Fzo1.

Ubp2, a Second Deubiquitylase Acting on Fzo1

Screening DUB-deficient yeast cells for Fzo1 ubiquitylation, we noted that cells lacking Ubp2 accumulated less Fzo1 or Fzo1K398R (Figure 4A and Figure S3A). Thus, two DUBs appear to regulate Fzo1 ubiquitylation antagonistically. We reasoned that Ubp2 might recognize different ubiquitin chains than Ubp12. To identify such ubiquitin chains, we expressed a catalytically inactive Ubp2 variant, Ubp2C745S-Flag, in Δubp2 cells. Strikingly, previously undetected Fzo1 ubiquitin chains accumulated upon expression of Ubp2C745S (Figure 4C and Figure S3C). Coimmunoprecipitation experiments after coexpression of HA-Fzo1 and Ubp2C745S-Flag revealed that Ubp2C745S physically interacts with these Fzo1 ubiquitin variants (Figure 4B and Figure S3D). This interaction was specific and did not occur after lysis of mitochondria (Figure S3D). Therefore, different ubiquitylated forms of Fzo1 were precipitated with Ubp2C745S or
Ubp12C372S (Figure 4B). Thus, ubiquitylation of Fzo1 is regulated by two DUBs, Ubp2 and Ubp12, which bind and deubiquitylate different ubiquitin chains.

**Fzo1 Is Ubiquitylated along Two Independent Pathways**

To corroborate the regulation of Fzo1 by two ubiquitylation pathways, we studied the roles of the GTPase domain of Fzo1 and of the SCF<sup>Mdm30</sup> ubiquitin ligase complex for its ubiquitylation. Ubp12 recognizes previously described ubiquitin chains on Fzo1, which are formed after GTP hydrolysis by Fzo1 (Amitot et al., 2009; Anton et al., 2011; Cohen et al., 2011) and depend on the presence of K464 in Fzo1 (Figure 3A). Consistently, mutations in the GTPase domain of Fzo1, affecting GTP binding (Fzo1D195A) or GTP hydrolysis (Fzo1T221A), prevented ubiquitylation in the absence of Ubp12 and thus were epistatic to the deletion of UBP12 (Figure 4D). Strikingly, ubiquitylation of Fzo1 was not abolished in Δfzo1Δubp2 cells expressing Fzo1K464R or Fzo1D195A together with the catalytically inactive mutant Ubp2C745S (Figure 4E and Figure S3E), demonstrating that the GTPase activity or the presence of K464 in Fzo1 is not critical for the formation of ubiquitin chains recognized by Ubp2.

The ubiquitin chains on Fzo1 recognized by Ubp12 are formed by the SCF<sup>Mdm30</sup> ubiquitin ligase complex (Cohen et al., 2008). To examine whether the ubiquitin chains recognized by Ubp2 are generated by the same E3 ubiquitin ligase, we deleted MDM30 in Δubp2 cells expressing the catalytically inactive variant Ubp2C745S. Fzo1 was still ubiquitylated in these cells (Figure 4F and Figure S3F), demonstrating that Ubp2 and Ubp12 act on two independent pathways.

**Ubp2 Protects Fzo1 against Proteasomal Degradation**

To define the function of the different ubiquitylation pathways acting on Fzo1, we analyzed the stability of Fzo1 in cells lacking Ubp2 or Ubp12. Whereas Fzo1 accumulated at moderately increased levels in Δubp2 cells, significantly less Fzo1 accumulated in Δubp12 cells (Figure 5A). Cycloheximide chase experiments revealed an increased turnover rate of Fzo1 in Δubp2 cells (Figure 5B). To assess if the increased degradation of Fzo1 in Δubp2 cells is performed by the proteasome, cells were treated with the proteasome inhibitor MG132, and the turnover of Fzo1 was monitored. Strikingly, Fzo1 was stabilized in Δubp2 cells treated with MG132, whereas treatment of wild-type cells with MG132 had only a mild stabilizing effect on Fzo1 (Figure 5B and Escobar-Henriques et al., 2006). This indicates that Fzo1 is targeted to the proteasome for degradation by ubiquitin chains recognized by Ubp2. Consistently, Δubp2 cells presented a striking increase in formation of ubiquitin chains via K48 linkages, as determined by mass spectrometric analysis of ubiquitin linkages after precipitation of Fzo1 (Figure 5C and Figure S4). We conclude from these experiments that Ubp2 protects Fzo1 from proteasomal degradation, cleaving off ubiquitin chains that are not recognized by Ubp12.

**Ubp2 and Ubp12 Have Opposite Effects on Mitochondrial Fusion**

We examined in further experiments how Ubp2 and Ubp12 modulate the activity of Fzo1 in mitochondrial fusion. To assess the morphology of the mitochondrial network, mitochondrial targeted GFP was expressed in cells lacking Ubp2 or Ubp12 or both. We observed an increased interconnectivity of the mitochondrial network in Δubp12 cells, consistent with an increase in mitochondrial fusion (Figure 6A). This suggests that the ubiquitin chains accumulating in the absence of UBP2 (Figure 4A and Figure 6B) promote mitochondrial fusion. On the other hand, the mitochondria appeared fragmented and aggregated in cells lacking UBP2 (Figure 6A). Δubp2 cells were deficient in respiratory growth and mitochondrial fusion, as monitored upon mating of haploid yeast cells (Figures SSA and SSB). Importantly, the absence of UBP2, of UBP12, or of both did not affect the accumulation of components of the mitochondrial fission machinery (Figure S5C). Thus, ongoing fusion events appear to trigger fragmentation of the mitochondrial network upon inhibition of fusion, suggesting that the limited levels of Fzo1 ubiquitin chains observed in Δubp2 cells do not suffice for mitochondrial fusion. Strikingly, deletion of UBP12 restored the reticulated mitochondrial morphology in Δubp2 cells, as it is observed in wild-type cells (Figure 6A). These findings confirm the opposing role of each DUB in promoting or inhibiting mitochondrial fusion. It should be noted that Δubp2Δubp12 cells resembled Δubp2 with respect to Fzo1 stability (Figure 5A). However, Fzo1 ubiquitylation was similar in Δubp2Δubp12 and in wild-type cells (Figure 6B). As mitochondrial morphology was normal in Δubp2Δubp12 cells, we conclude that Fzo1 ubiquitylation rather than Fzo1 turnover appears important for mitochondrial fusion. This conclusion was substantiated by overexpression of Fzo1, which did not restore a tubular mitochondrial network in Δubp2 cells (Figures SSD and SSE). On the other hand, treatment of Δubp2 cells with MG132 increased Fzo1 ubiquitylation and restored tubular mitochondria (Figures 6C and 6D).

**DISCUSSION**

We identified Ubp2 and Ubp12 and unraveled two independent ubiquitylation pathways that act on Fzo1 and control mitochondrial fusion in vivo (Figure 7A). Ubiquitylation along both pathways involves different E3 ubiquitin ligases and distinct lysine residues of Fzo1. Ubp2 and Ubp12 have different specificities and opposite effects on mitochondrial fusion. Ubp12 recognizes Fzo1 ubiquitylation that is necessary for mitochondrial fusion, depending on the SCF<sup>Mdm30</sup> E3 ligase, and occurs after GTP hydrolysis by Fzo1. Ubiquitin chains are attached to K398 of Fzo1 and may stabilize Fzo1 oligomers (Figure 7B). In contrast, Ubp2 recognizes ubiquitin chains on functional and nonfunctional Fzo1, whose formation does not depend on GTP hydrolysis by Fzo1, nor on the SCF<sup>Mdm30</sup> E3 ligase or K398 of Fzo1.

**A Regulatory Role of Mitofusin Ubiquitylation for Mitochondrial Fusion**

Our experiments revealed that Mdm30-dependent ubiquitylation, but not the steady-state level and turnover rates of Fzo1 variants, correlated with mitochondrial fusion: First, Fzo1 ubiquitylation is not affected in Δubp2Δubp12 cells, which contain a reticulated mitochondrial network despite decreased Fzo1 protein levels. Second, and in contrast, ubiquitylation and the morphology of mitochondria are impaired in Δubp2 cells, but...
Figure 4. Ubp2 and Ubp12 Deubiquitylate Fzo1

(A) Ubiquitylation of HA-Fzo1 and HA-Fzo1\(^{K398R}\) in \(\Delta ubp2\) and \(\Delta ubp12\) cells. HA-Fzo1 variants were expressed in the indicated yeast strains and analyzed by immunoprecipitation, as in Figure 1A. The asterisk indicates an unspecific crossreaction of the HA antibody.

(B) Differential interaction of Fzo1 with Ubp2 and Ubp12. HA-Fzo1 and Ubp2\(^{C745S}\)-Flag or Ubp12\(^{C372S}\)-Flag were coexpressed in \(\Delta ubp2\) or \(\Delta ubp12\) cells, respectively. Crude mitochondrial extracts were lysed, and Flag-tagged Ubp2\(^{C745S}\) or Ubp12\(^{C372S}\) were precipitated using Flag-coupled beads and analyzed by immunoblotting using HA- and Flag-specific antibodies. In, Input; IP, immunoprecipitation; WB, western blot.

(C) Effect of Ubp2\(^{C745S}\) on Fzo1 ubiquitylation. HA-Fzo1 was immunoprecipitated as in (A) from \(\Delta ubp2\Delta fzo1\) cells harboring only the vector control or expressing HA-Fzo1 alone or in combination with Ubp2-Flag or Ubp2\(^{C745S}\)-Flag.

(D) Dependence of Ubp12-specific ubiquitylation on Fzo1 GTPase activity. HA-Fzo1 variants were expressed in \(\Delta fzo1\) or \(\Delta fzo1\Delta ubp12\) cells and analyzed as in (A).

(legend continued on next page)
Fzo1 is present at levels similar to those in \( \text{Dubp2} \) cells. Third, stabilization of ubiquitylated Fzo1 by MG132 restored fusion in \( \text{Dubp2} \) cells. Fourth, although overexpression of Fzo1 impairs fusion (Fritz et al., 2003), an increased turnover of Fzo1 in \( \text{Dubp2} \) cells or in the presence of Fzo1\( ^{K398R} \) was detrimental for mitochondrial morphology. Fifth, deletion of either \( \text{UBP12} \) or \( \text{MDM30} \) stabilizes Fzo1, but while the deletion of \( \text{UBP12} \) promotes ubiquitylation and formation of a reticulated mitochondrial network, ubiquitylation of Fzo1 and mitochondrial fusion are inhibited in the absence of Mdm30 (Escobar-Henriques et al., 2006; Fritz et al., 2003). We therefore conclude that Mdm30-dependent ubiquitylation of Fzo1 has a nonproteolytic function and promotes mitochondrial fusion.

The Mdm30-dependent ubiquitin chains of Fzo1 present an uncommon pattern, which is conserved in flies and mammals (Cohen et al., 2008; Rakovic et al., 2011; Ziviani et al., 2010). Here, we demonstrate that these ubiquitin chains stabilize Fzo1 against proteolysis and are important for Fzo1 function. They are formed in a stepwise process that is intimately connected to the oligomerization of Fzo1 (Figure 7B). One subunit is ubiquitylated at K464 in Fzo1, which is conserved among mitofusins. Modification at K464 activates ubiquitin chain formation on K398 in another Fzo1 subunit. These findings are reminiscent of the transcription factor Met4, whose activity is regulated by ubiquitylation in an unusual way (Kaiser et al., 2000; Rouillon et al., 2000). Ubiquitin chains are formed by the F box protein Met30 on a defined lysine residue of Met4 (Flick et al., 2004; Kuras et al., 2002). Intramolecular association with a ubiquitin binding domain in Met4 prevents elongation of the ubiquitin chains and protects Met4 against proteasomal degradation (Flick et al., 2006; Tyrrell et al., 2010). Similarly, Fzo1

(F) Dependence of Ub2-specific ubiquitylation on Mdm30. HA-Fzo1 was genomically expressed in the indicated strains, and Fzo1 ubiquitylation was analyzed as in (A). The arrowhead and filled arrows are as in Figure 3, and arrows with no fill indicate ubiquitylated Fzo1 species specifically accumulating upon expression of Ub2\( ^{274S} \), WT, wild-type. See also Figure S3.
ubiquitylation is linked to its oligomerization and results in its stabilization (Anton et al., 2011). Therefore, it is possible that steric constraints within the Fzo1 oligomer restrict ubiquitin chain elongation, promote mitochondrial fusion, and limit accessibility of Fzo1 to proteasomal degradation, despite the presence of K48-linked chains.

Formation of ubiquitin chains after mitochondrial docking is consistent with the structural model of Fzo1 that is based on the crystal structure of its bacterial homolog BDLP (Low and Löwe, 2006; Low et al., 2009). K464 is hidden in the closed conformation, which is believed to represent prefusion states, but exposed after the predicted conformational changes occurring at the docking stage. In this way, Mdm30-dependent ubiquitylation of Fzo1 can be restricted to a distinct step in the fusion process, i.e., after GTP hydrolysis and oligomerization of Fzo1. It is an attractive possibility that Fzo1 ubiquitylation contributes to the stabilization of Fzo1 oligomers at the docking stage and thereby provides a time frame for both membranes to fuse. The specific stabilization of a docking intermediate may differentiate DRPs involved in fusion events from those mediating membrane fission.

**The Quality Control Function of Mitofusin Ubiquitylation**

We identified additional ubiquitylated forms of Fzo1 that destabilize Fzo1 and promote its degradation by the proteasome. They are formed independently of the SCF^{Mdm30} complex and are recognized by Ubp2. The identification of Ubp2 thus allows us to resolve seemingly contradicting interpretations on the role of the proteasome for the turnover of Fzo1 (Cohen et al., 2008; Escobar-Henriques et al., 2006). While inhibition of the proteasome has only a moderate effect on the turnover rate of Fzo1 in wild-type cells, the absence of UBP2 and the accumulation of different ubiquitin forms rendered Fzo1 very susceptible to proteasomal inhibition. Similarly, Fzo1 degradation occurred in a proteasome-dependent manner during cell-cycle arrest that has been induced in the presence of α factor.
and impaired mitochondrial fusion (Escobar-Henriques et al., 2006; Neutzner and Youle, 2005). Consistently, we observed that mitochondrial fusion is impaired after mating of Δubp2 cells. Our findings therefore suggest that two different proteolytic systems determine the stability of mitofusins and that proteasomal degradation inhibits mitochondrial fusion.

Nonfunctional variants of Fzo1, which cannot be ubiquitylated by Mdm30 (Fzo1K464R) or cannot oligomerize (Fzo1D195A), are ubiquitylated in an Ubp2-dependent manner. This suggests the existence of checkpoints at different stages of the mitochondrial fusion process. Because the absence of UBP2 impairs mitochondrial fusion, we propose that Ubp2 protects Fzo1 against degradation during the normal fusion cycle.

Ubiquitylation of Mitofusins Emerges as a Regulatory Hub

Ubiquitylation of mitofusins was observed in several organisms, in response to several stress situations, and is of major importance in apoptosis and mitophagy (Youle and Narendra, 2011). Namely, the E3 ligase Parkin ubiquitylates Mfn1 and Mfn2 after mitochondrial depolarization (Gegg et al., 2010; Glauser et al., 2011; Poole et al., 2008, 2010; Rakovic et al., 2011; Ziviani et al., 2010), while Huwe1 ubiquitylates Mfn2 after its phosphorylation by JNK during apoptosis (Leboucher et al., 2012).

Taken together, these findings unravel a central role of mitofusins ubiquitylation as a major determinant in mitochondrial dynamics and cellular adaptation to a multitude of conditions. We show that in order to fuse, yeast mitochondria absolutely require regulatory ubiquitylation of mitofusins. In contrast, quality control ubiquitylation and proteasomal elimination of mitofusins inhibits mitochondrial fusion. Our identification of Ubp2 and Ubp12 as specific factors for these two pathways shows that analysis of the different DUBs will certainly allow us to distinguish a regulatory versus a quality control role for mitofusins ubiquitylation in response to different metabolic inputs.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media

Yeast strains are isogenic to the S288c or W303 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 H2O) or MG132 (Calbiochem) (100 μM from a stock at 10 mM in DMSO) was added when indicated. For the analysis of Fzo1.

Figure 7. Model for the Role of Fzo1 Ubiquitylation in OM Fusion

(A) Ubp2 and Ubp12 regulate two distinct ubiquitylation pathways, which inhibit or promote mitochondrial fusion. Ubp2 acts on a quality control pathway and removes destabilizing ubiquitin moieties from Fzo1. Ubp12 regulates fusion cleaving off ubiquitin chains which stabilize Fzo1 and promote fusion.

(B) Intermolecular ubiquitylation of Fzo1. GTP binding promotes Fzo1 homodimerization and further oligomerization upon tethering of two mitochondria. GTP hydrolysis likely triggers a conformational change in Fzo1, allowing initial ubiquitylation of Fzo1 at K464. This induces ubiquitin chain formation on K398 of a neighboring Fzo1 molecule, which promotes OM fusion and can be reversed by Ubp12. Ub, ubiquitylation.

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ubiquitylation upon exclusive expression of Myc-Ubiquitin, the strain YD466, isogenic to SUB328, was used (Spence et al., 1995).

**Plasmids**

Fzo1 harboring an N-terminal hemagglutinin (HA) tag was expressed under the control of its endogenous promoter using the centromeric plasmid pRS316 (cen; URA3) (Escobar-Henriques et al., 2006). For genomic integration of HA-Fzo1, the nat1T2 resistance cassette (Janké et al., 2004) was cloned downstream of the coding sequence of HA-Fzo1 on pRS316 followed by 500 aa of the FZO1 3’UTR. The resulting vector was linearized with PvuI and integrated into the genome by homologous recombination. HA-Fzo1-Ubp7 was generated by fusing aminoads 561 to 1,071 of Ubp7 to the C-terminus of Fzo1, separated by a GlyArg linker, and cloned in the plasmid pRS316 with the FZO1 promoter. Ubp2 and Ubp12 harboring a C-terminal FLAG tag were expressed from the ADH1 promoter using the plasmid YEpplac181 (2μ; LEU2). All point mutants were generated by site-directed mutagenesis, and verified by DNA sequencing. For analyzing Fzo1 ubiquitylation, an N-terminally Myc-tagged ubiquitin expressed from the CUP1 promoter on Yep112 was used (2μ; TRP1). For visualizing mitochondria, the centromeric plasmid pYX142-mtGFP (cen; LEU2 and TP1 promoter) encoding mitochondrial matrix-targeted GFP was used.

**Spot Tests**

For growth assays with Fzo1-Ubp7, Δfzo1 cells expressing the different Fzo1 plasmids were generated by tetrad dissection. For growth assays with Fzo1-HA200 and Fzo1-K464R, the respective HA-Fzo1 variants were integrated into the genome, replacing the endogenous gene. Five serial dilutions of logarithmically growing cells were spotted on media containing glucose or glycerol and grown for 2 days on glucose and 3 days on glycerol.

**Ubiquitylation Assay**

Immunoprecipitation of ubiquitylated Fzo1 was performed as previously described (Anton et al., 2011). Briefly, crude membrane extracts from 50 OD600 cells, grown in synthetic media to the logarithmic growth phase, from yeast strains transformed with different HA-Fzo1 variants, were solubilized in 1% (w/v) digitonin and cleared by centrifugation. Lysates were immunoprecipitated overnight with 20 μl of the χHA-coupled beads (E6776; Sigma-Aldrich). Bound protein was eluted in 40 μl Laemmli buffer at 4°C for 20 min, subjected to SDS-PAGE, and subsequently immunoblotted using HA-specific antibodies.

**Mass Spectrometry**

HA-Fzo1 was immunoprecipitated essentially as described above with the exception that 10,000 OD600 cells were used, precipitated with 500 μl of the χHA-coupled beads (E6776; Sigma-Aldrich), and eluted with three times 600 μl 200 mM ammonium hydroxide. The eluted protein was dried in a speed vac and resuspended in denaturing buffer (6 M Urea, 2 M thiourea, 20 mM HEPES [pH 8.0]). The proteins were converted to peptides in a two-step protease digestion with Endoproteinase LysC (Wako) and sequencing grade trypsin (Promega) (de Godoy et al., 2008). The resulting peptides were desalted and injected to a C18-reverse phase chromatography (75 μm column, 15 cm length in house packed, 3 μm beads Reprosil, Dr. Maisch). The separated peptides were ionized on a Proxeon ion source and analyzed on a Velos-Orbitrap (Thermo-Scientific) mass spectrometer. The recorded spectra were analyzed using the MaxQuant software package. The coverage of Fzo1 was 88.3%.

**Mass-Spectrometry-Based Ubiquitin Chain Analysis**

Eluted proteins were supplemented with a mixture of reference peptides (JPT, Spike-Tides MQL) (Mirzaei et al., 2010). The isotope-labeled reference peptides were released during the tryptic digest of the samples. Each sample was desalted, and the peptides were separated on a 75 μm inner diameter (C18-reverse phase column, packed in house with 5 μm C18 beads, Dr. Maisch) using a 5%–50% acetonitrile gradient. The peptides were ionized on a nanosource III and sprayed into a Q-TRAP 5500 mass spectrometer (ABSciences). The recorded data were quantified using the MultiQuant software package, and the statistical analysis was performed using the R-Software package (http://www.R-project.org/).

**Coimmunoprecipitation**

Lysates prepared as for the ubiquitylation assay were immunoprecipitated using 20 μl of the χFlag-coupled beads (F2426; Sigma-Aldrich), subjected to SDS-PAGE as described above, and immunoblotted with HA- and Flag-specific antibodies. For the postlysis control, solubilized lysates, from cells expressing only Ubp2-Flag or Ubp12-Flag or HA-Fzo1, were mixed after solubilization and subsequently subjected to immunoprecipitation.

**Protein Steady-State Levels and Synthesis Shutoff**

For analysis of Fzo1 steady-state levels, total proteins from 3 OD600 logarithmic cells grown in synthetic media were extracted at alkaline pH (Escobar-Henriques et al., 2006) and analyzed by SDS-PAGE and immunoblotting. To monitor Fzo1 turnover, cycloheximide was added to logarithmic cells grown on synthetic media. For proteasome inhibition, cells were grown to the logarithmic growth phase in synthetic media containing SDS to allow cell premeabilization to MG132 (Liu et al., 2007). Cells were treated with MG132 1 hr before adding cycloheximide. Samples of 3 OD600 cells were collected at the indicated time points, and total proteins were extracted and analyzed as described above. Western blots were quantified using Quantity One (Bio-Rad). Levels of wild-type Fzo1 at time zero were set to 1. Mean values of at least three different experiments are shown. The error bars reflect the standard error (Cumming et al., 2007).

**Microscopy**

Yeast strains were transformed with mitochondrial-targeted GFP, grown on complete media to the logarithmic phase, and analyzed as described (Escobar-Henriques et al., 2006) by epifluorescence microscopy (Axioplan 2; Carl Zeiss Microlmaging, Inc.) using a 100X oil-immersion objective. Images were acquired with a camera (AxioCam MRm, Carl Zeiss Microlmaging, Inc.) and processed with Axiovision 4.7 (Carl Zeiss Microlmaging, Inc.). For MG132 treatment, cells were grown on synthetic media containing SDS as described above.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found online at http://dx.doi.org/10.1016/j.molcel.2012.12.003.

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Supplemental Information

Two Deubiquitylases Act on Mitofusin and Regulate Mitochondrial Fusion along Independent Pathways

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Inventory of Supplemental Information

Figure S1, related to Figure 2
Figure S2, related to Figure 3
Figure S3, related to Figure 4
Figure S4, related to Figure 5
Figure S5, related to Figure 6

Supplemental Experimental Procedures
**Figure S1 (related to Figure 2).** Fzo1 is ubiquitylated. (A) Short exposure of Figure 2A decorated with HA specific antibodies. (B) Contribution of K48 linked ubiquitin chains to Fzo1 ubiquitylation. Wild type (wt) cells expressing HA-Fzo1 and overexpressing ubiquitin or ubiquitin K48R, respectively, were analyzed as in Figure 1A. (C) Dependence of Fzo1 ubiquitylation on the ubiquitin conjugating enzyme Cdc34. Wild type and cdc34-2 cells expressing HA-Fzo1 were grown at non-permissive temperature and analyzed as in Figure 1A. Unmodified and ubiquitylated forms of HA-Fzo1 are indicated by an arrowhead or an arrow, respectively.
Figure S2 (related to Figure 3). Function of Fzo1 ubiquitylation sites. (A) Short exposure of Figure 3A. (B) Ubiquitylation profile of Fzo1K464R and Fzo1K398R,K464R. The HA-Fzo1 variants were expressed in Δfzo1 cells and analyzed as in Figure 1A. (C) Respiratory capacity of cells expressing HA-Fzo1, HA-Fzo1K398R or HA-Fzo1K464R. Spot test of Figure 3B performed at the indicated temperatures. (D) Ubiquitylation profile of Fzo1K398R. The HA-Fzo1 variants were expressed in Δfzo1 cells and analyzed as in (B). (E) Long (**) and short (*) exposure of Figure 3D. The
arrowhead marks unmodified Fzo1, arrows with black fill or white fill refer to Fzo1 ubiquitylated species detected upon expression of Fzo1 or Fzo1^{K398R}.

Figure S3 (related to Figure 4). Ubp2 and Ubp12 deubiquitylate Fzo1. (A) Long (**) and short (*) exposure of Figure 4A. (B) Interaction of Ubp12 and Fzo1. HA-Fzo1 and Ubp12-Flag or Ubp12^{C372S}-Flag were expressed in Δubp12 cells as
indicated. Crude mitochondrial extracts were lysed and Ubp12 was precipitated using Flag-specific antibodies. Samples were analyzed by SDS-PAGE and immunoblotting using HA- and Flag-specific antibodies. To control post-lysis binding, extracts containing either HA-Fzo1 or Ubp12-Flag were mixed after lysis and then subjected to immunoprecipitation. IP, immunoprecipitation (C) Identification of ubiquitylated forms accumulating in dependence of Ubp12 and Ubp2. The indicated strains expressing HA-Fzo1 and if indicated Ubp2<sup>C745S</sup>-Flag were analyzed as described in Figure 1A and decorated with HA and ubiquitin specific antibodies. (D) Interaction of Ubp2 and Fzo1. HA-Fzo1 and Ubp2-Flag or Ubp2<sup>C745S</sup>-Flag were expressed in Δ<textit{ubp2}</textit> cells as indicated and analyzed as in (B) (E) Long (***) and short (*) exposures of Figure 4E. (F) Short exposure of Figure 4F. The arrowhead marks unmodified Fzo1, arrows with black fill or white fill refer to Fzo1 ubiquitylated species detected upon expression of Fzo1 or Fzo1<sup>K398R</sup>. Arrows with no fill indicate ubiquitylated Fzo1 species specifically accumulating upon expression of Ubp2<sup>C745S</sup>. 
Figure S4 (related to Figure 5). Type of linkages from ubiquitin chains bound to Fzo1. The indicated strains expressing HA-Fzo1 and Ubp2<sup>C745S</sup>-Flag or the corresponding empty vector were analyzed as in Figure 5C, except that absolute values of ubiquitin linkages detected in each sample are shown. Amount of detected peptides is shown in arbitrary units. The average of K48-linked peptides detected in wild type, represented with light grey bars, was set as 1. Data are presented as mean ± SE from two different experiments.
Figure S5 (related to Figure 6). Ubp2 and Ubp12 regulate mitochondrial morphology via Fzo1 on independent and antagonistic pathways. (A) Mitochondrial fusion in Δubp2 cells. Cells of opposing mating type of the indicated strains were transformed with mitochondrial targeted GFP or dsRed respectively and mixing of mitochondrial content was analyzed by fluorescent microscopy, which was performed as in Figure 1B. The left panel shows examples of the three different degrees of
content mixing observed. The right panel shows a quantification of content mixing of approximately 60 zygotes per strain. (B) Respiratory growth of Δubp2 cells. The growth of wild type and Δubp2 cells was analyzed as in Figure 1C. (C) Steady-state levels of fission proteins in cells lacking UBP12 and UBP2. The indicated strains expressing Dnm1-GFP if indicated, were analyzed as in Figure 5A using GFP-, Mdv1-, Fis1- and as a loading control Tom40 specific antibodies. (D) Steady-state levels of Fzo1 upon overexpression or proteasome inhibition. Wild type (wt) or Δubp2 cells expressing the indicated Fzo1 variants, untreated or treated with MG132 were analyzed as in (C) using Fzo1 and as loading control Aco1-specific antibodies. oe, overexpression. (E) Mitochondrial morphology upon overexpression of Fzo1. Wild type and Δubp2 cells expressing mitochondrial GFP and if indicated overexpressing Flag-Fzo1 were analyzed as in Figure 1B. Lower panel, data are presented as mean ± SE from three different experiments (with more than 200 cells each).
Supplemental Experimental Procedures

**Plasmids**

Fzo1 harboring an N-terminal Flag tag was overexpressed under the control of the \( CUP1 \) promoter using the 2\( \mu \) plasmid pJDCEX2 (Escobar-Henriques et al., 2006). Ubiquitin and Ubiquitin\(^ {K48R} \) were overexpressed using the plasmid YEp110 (2\( \mu \), \( TRP1 \))(Ellison and Hochstrasser, 1991). For visualizing mitochondrial fusion after mating, the pvt100-mtGFP (2\( \mu \); \( URA3 \)) or the pvt100-mtRFP (2\( \mu \); \( URA3 \)) plasmids encoding mitochondrial matrix-targeted GFP or RFP, respectively, were used. To monitor the Dnm1-GFP protein, the centromeric plasmid pH520 (cen; \( LEU2 \) and \( DNM1 \) promoter) was used.

**Analysis of mitochondrial fusion in vivo**

Mitochondrial fusion was essentially analyzed as described previously (Fritz et al., 2003). Exponentially growing cells of opposite mating types expressing mtGFP or mtRFP, respectively, were mixed on YPD medium and incubated for 3h at 30\(^ \circ \)C. Mitochondrial fusion was analyzed by fluorescence microscopy.

**Bibliography**