Doa1 targets ubiquitinated substrates for mitochondria-associated degradation

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Mitochondria-associated degradation (MAD) mediated by the Cdc48 complex and proteasome degrades ubiquitinated mitochondrial outer-membrane proteins. MAD is critical for mitochondrial proteostasis, but it remains poorly characterized. We identified several mitochondrial Cdc48 substrates and developed a genetic screen assay to uncover regulators of the Cdc48-dependent MAD pathway. Surprisingly, we identified Doa1, a substrate-processing factor of Cdc48 that inhibits the degradation of some Cdc48 substrates, as a critical mediator of the turnover of mitochondrial Cdc48 substrates. Deletion of DOA1 causes the accumulation and mislocalization of substrates on mitochondria. Profiling of Cdc48 cofactors shows that Doa1 and Cdc48-Ufd1-Npl4 form a functional complex mediating MAD. Biochemically, Doa1 interacts with ubiquitinated substrates and facilitates substrate recruitment to the Cdc48-Ufd1-Npl4 complex. Functionally, Doa1 is critical for cell survival under mitochondrial oxidative stress, but not ER stress, conditions. Collectively, our results demonstrate the essential role of the Doa1–Cdc48-Ufd1-Npl4 complex in mitochondrial proteostasis and suggest that Doa1 plays dual roles on the Cdc48 complex.

Introduction

Owing to their essential metabolic and signaling functions, mitochondria are under close surveillance by complex quality-control mechanisms, including reactive oxygen species removal by anti-oxidation enzymes (Collins et al., 2012), intra-mitochondrial protein turnover by mitochondrial proteases (Quirós et al., 2015), mitophagy (Youle and Narendra, 2011), and the mitochondria-associated degradation (MAD) pathway degrading mitochondrial outer-membrane (MOM) proteins (Karbowsky and Youle, 2011; Taylor and Rutter, 2011).

MAD is analogous to the well-studied ER associated degradation (ERAD) pathway (Hirsch et al., 2009) in that they both require the highly conserved AAA-ATPase Cdc48 (TER94 in Drosophila melanogaster and VCP or p97 in mammals) to dislodge ubiquitinated proteins from organelle membranes and escort their degradation by the proteasome (Karbowsky and Youle, 2011; Taylor and Rutter, 2011; Xu et al., 2011). Cdc48 forms a hexameric ring-like structure to unfold or remodel substrates and is extensively involved in cytoplasmic, nuclear, and organelar protein degradation, autophagy, and intracellular trafficking processes (Meyer et al., 2012). The cellular functions of Cdc48 are regulated by two types of cofactors: the substrate-recruiting factors that contain ubiquitin-binding domains to recognize and recruit ubiquitinated substrates to Cdc48, and the substrate-processing factors that often possess enzymatic activities to modulate the turnover rate of the Cdc48-bound substrates (Stolz et al., 2011; Meyer et al., 2012; Baek et al., 2013; Buchberger, 2013).

Accumulating evidence suggests that Cdc48 has an essential and evolutionarily conserved role in mitochondrial quality control. Cdc48/TER94/VCP mutations in yeast (Braun et al., 2006), Drosophila (Chang et al., 2011; Kim et al., 2013), mouse (Custer et al., 2010; Nalbandian et al., 2012; Yin et al., 2012), and human (Bartolome et al., 2013) all cause severe abnormalities in mitochondria, including mitochondrial swelling and aggregation, production of reactive oxygen species, and reduced ATP production. Pathogenic mutations of human VCP cause several muscular and neural degenerative diseases (Watts et al., 2004; Johnson et al., 2010).

Although the importance of the Cdc48-dependent MAD pathway is being increasingly appreciated, its molecular mechanism remains obscure. First, only a very limited number of mitochondrial Cdc48 substrates have been reported; these include Fzo1 (Cohen et al., 2008; Heo et al., 2010) in yeast and mitofusins (Tanaka et al., 2010; Xu et al., 2011) and Mcl-1 (Xu et al., 2011) in mammals. Second, the regulatory components of MAD remain largely unknown. It has been proposed that in yeast, MAD is mediated by the Vms1–Npl4–Cdc48 complex, in which Npl4 may interact with ubiquitinated substrates (Heo et al., 2010). However, it is noteworthy that the ubiquitin-binding...
NZF domain present in mammalian Npl4 is not conserved in yeast Npl4 (Meyer et al., 2002; Ye et al., 2003), and yeast Npl4 was not classified as a ubiquitin-binding protein (Stolz et al., 2011; Buchberger, 2013). The involvement of Vms1 in MAD has been supported by its requirement for Fzo1 degradation (Heo et al., 2010), but the role of Vms1 in Fzo1 turnover has been debated (Esaki and Ogura, 2012).

To investigate the molecular mechanisms and biological functions of the Cdc48-dependent MAD pathway, we analyzed the turnover of transmembrane MOM proteins and identified mitochondrial Cdc48 substrates in *Saccharomyces cerevisiae*. The newly identified substrates allowed us to establish a genetic screen to identify MAD regulators and characterize their roles in mitochondrial proteostasis and mitochondrial quality control.

**Results**

**Identification of mitochondrial Cdc48 substrates**

To identify mitochondrial Cdc48 substrates, we tagged transmembrane MOM proteins by chromosomal integration with a HA tag (6xHA at the C terminus or 3xHA at the N terminus) and analyzed their turnover. To minimize potential artifacts, we focused on the experimentally verified transmembrane MOM proteins whose mitochondrial localization and functions are not affected by epitope tags (Table S1 and supplemental references therein). We tested 18 such proteins and successfully generated 14 strains that had normal growth and detectable HA signals on Western blot (Table S1). We monitored the degradation of these HA-tagged proteins after stopping new protein synthesis with cycloheximide (CHX) treatment, and we identified nine slow-turnover proteins (Fig. S1 A) and five fast-turnover proteins (Figs. 1 A and S1 B).

We inactivated the Cdc48-proteasome pathway by using the widely used temperature-sensitive (ts) mutants of Cdc48 (cdc48-3) and proteasome (pre1ts pre2ts). Among the five fast-turnover proteins, the degradation of Fzo1-HA, Mdm34-HA, and Msp1-HA was inhibited by ts inactivation of Cdc48 and proteasome and by the proteasome inhibitor MG132 (Fig. 1, B–D). The degradation of the other two proteins (Tom22-HA and Om45-HA) was not inhibited in the cdc48-3 or the pre1ts pre2ts mutants (Fig. S1 C). The degradation of all five proteins was insensitive to the deletion of the autophagy regulator ATG5 (Fig. S1 D) or the vacuolar proteases PEP4 and PBP1 (Fig. S1 E). Therefore, we confirmed Fzo1-HA, Mdm34-HA, and Msp1-HA as Cdc48 substrates. We further did imaging and growth tests to confirm that C-terminal tagging of the three Cdc48 substrates does not affect their localization (Fig. S1 F) or cell growth (Fig. S1 G).

The aforementioned characterization of MOM proteins was conducted in the BY4741 strain. The MOM protein Tom70 was long-lived (Fig. S1 A) in the BY4741 strain but had a fast turnover rate (Fig. 1 E) in the prototrophic CEN.PK strain (van Dijken et al., 2000). The degradation of Tom70-HA was inhibited in the ts mutants of proteasome (pre1ts pre2ts) and Cdc48 (cdc48ts). Therefore, Tom70-HA is another Cdc48 substrate in the CEN.PK strain.

**A genetic screen identifies MAD regulators**

To identify the regulatory components of the Cdc48-dependent MAD pathway, we need a simple and efficient way to monitor the turnover of substrates on a large scale. We thus developed a colony assay for such a purpose (Fig. S2 A) and tested the four substrates (Fzo1-HA, Mdm34-HA, Msp1-HA, and Tom70-HA) with this assay. Among the four substrates, we observed an almost complete loss of Tom70-HA signal after CHX treatment, and the degradation of Tom70-HA was blocked by knocking down CIM3, a proteasome subunit (Fig. S2 B). We therefore performed a transposon-based insertion screen using Tom70-HA as the reporter substrate (Fig. 2 A).

We screened ~5,000 colonies, covering ~15% of the genome, and identified insertional mutations in 16 genes (Table S2). Among these putative hits, we found four genes related to the Cdc48-proteasome pathway. These genes are *DOA1* (a Cdc48 cofactor, Johnson et al., 1995; Ghislain et al., 1996), *PRB1*, *MAD3*, *PRB1*, and *PRB1*.
Figure 2. Doa1 mediates the turnover of mitochondrial Cdc48 substrates, and deletion of DOA1 causes substrate accumulation and mislocalization on mitochondria. (A) Schematic illustration of the colony screen assay (see Materials and methods for details). Blue indicates Tom70-HA signal. In the representative Western blot image, white arrowheads point to colonies with normal degradation of Tom70-HA, and the red arrow points to a colony defective in Tom70-HA degradation. (B) The WT and doa1Δ strains transformed with a high-copy plasmid expressing ubiquitin (UB) or the empty vector (V) were grown to log phase before CHX treatment. (C) The FZO1-HA, MDM34-HA, and MSP1-HA strains in WT or doa1Δ background were transformed with V or UB plasmids and grown to log phase before CHX treatment. (D) Subcellular fractionation of the FZO1-HA and FZO1-HA doa1Δ strains. Lysates from the whole-cell extracts (WCEs), mitochondria-enriched fraction, and postmitochondria supernatant (PMS) were analyzed by Western blot. G6PDH and Por1 are the markers for cytoplasm and mitochondria, respectively. (E) Subcellular fractionation of the MDM34-HA, MDM34-HA doa1Δ, and mdm34ΔPY-HA strains were analyzed as in D. (F) The indicated WT or mutant strains chromosomally expressing Mdm34-GFP and mtDsRed (labeling mitochondria) were grown in glucose media to log phase before imaging. Z projections and differential interference contrast images are shown. Bar, 1 µm. White arrows point to diffusive mitochondrial signals of Mdm34-GFP. (G) Quantitative analysis of the foci intensity of Mdm34-GFP. Data values represent means (green line) ± SEM (red line). n = 100 for each strain. Similar results were obtained in two additional, independent experiments. Data were analyzed by one-way analysis of variance followed by Tukey post tests. (H) Quantitative analysis of the percentage of cells with diffusive mitochondrial signals of Mdm34-GFP. Data values represent means ± SEM from three independent experiments, with at least 200 cells counted in each experiment. Data were analyzed by one-way analysis of variance followed by Tukey post tests. Note that ubiquitin overexpression did not rescue the increased foci intensity or the mislocalization phenotype of Mdm34-GFP in doa1Δ cells.
UBP6 (a proteasome-associated ubiquitin protease; Verma et al., 2000), BRO1 (associated with deubiquitinase Doa4; Luhtala and Odorizzi, 2004), and RSP5 (an E3 ubiquitin ligase; Huijberts et al., 1995). Deletions of BRO1, UBP6, and its inactivation of Rsp5 inhibited the degradation of Tom70-HA, Mdm34-HA, and Msp1-HA, but not that of Fzo1-HA (Fig. S2, C and D). In contrast, deletion of DAO1 inhibited the degradation of all the four substrates (Fig. S2, C and D).

Mdm34 has a highly conserved PY motif, PYYY, at its C terminus (Fig. S2 H), which has been shown to mediate the interaction between Rsp5 and its substrates (Rotin and Kumar, 2009). Consistently, the PYYY to AAAAA (mdm34ΔPY) mutation strongly blocked Mdm34-HA degradation and elevated its steady level (Fig. S2 J), demonstrating that Mdm34 is a bona fide Rsp5 substrate.

Doa1 (also known as Ufd3) is a Cdc48 cofactor known for two decades (Johnson et al., 1995; Ghislain et al., 1996) and was reported to function as a substrate-processing factor (Rumpf and Jentsch, 2006). Another substrate-processing factor, Ufd2, catalyzes the ubiquitination of some Cdc48-bound substrates and promotes their degradation (Koegl et al., 1999; Rumpf and Jentsch, 2006). Doa1 competes with Ufd2 for the same binding site on Cdc48 and thus inhibits the degradation of the Ufd2-dependent substrates (Rumpf and Jentsch, 2006). Apparently, our results cannot be explained by the substrate-processing function of Doa1.

It is also known that deletion of DOA1 reduces free ubiquitin level and thus indirectly inhibits the degradation of some proteins, such as Ubi-Pro-β galactosidase; ubiquitin overexpression rescues the protein degradation defects of doa1Δ cells (Johnson et al., 1995). As reported, the doa1Δ cells had reduced ubiquitin level, which was rescued by ubiquitin overexpression (Johnson et al., 1995; Fig. 2 B) or by the deletion of UFD2 (Rumpf and Jentsch, 2006; Fig. S2 J). Surprisingly, the impaired degradation of mitochondrial Cdc48 substrates (Fzo1-HA, Mdm34-HA, and Msp1-HA) was not rescued by restoring the ubiquitin level (Figs. 2 C and S2 J). In contrast, the degradation of Om45-HA and Tom22-HA and the steady level of the stable MOM protein Por1 were not affected by deletion of DOA1 (Fig. S2 K). Therefore, Doa1 itself is specifically required for the turnover of mitochondrial Cdc48 substrates.

Deletion of DOA1 causes substrate accumulation and mislocalization on mitochondria

The steady levels of Fzo1-HA and Mdm34-HA were significantly elevated in doa1Δ cells (Fig. 2 C). We asked where these proteins accumulate in cells. Sub-cellular fractionation experiment showed that Fzo1-HA accumulated in the mitochondria-enriched fraction, but not in the postmitochondria supernatant (PMS), in doa1Δ cells (Fig. 2 D). Moreover, when cultured in lactate media (YPL), doa1Δ cells had an increased ability to maintain tubular mitochondria in comparison to wild type (WT) cells (Fig. S3 A), indicating that the accumulated Fzo1 is, at least partially, functional.

Mdm34 is an essential component of the ER-mitochondria encounter structure (ERMES) complex that forms punctate foci connecting mitochondria and ER (Kornmann et al., 2009). Similar as Fzo1-HA, Mdm34-HA also accumulated in the mitochondria-enriched fraction in doa1Δ and mdm34ΔPY cells (Fig. 2 E). We directly monitored the localization of Mdm34 by chromosomal integration of a GFP tag to its C terminus. The fluorescence intensity of the Mdm34-GFP foci was greatly increased in doa1Δ and mdm34ΔPY cells (Fig. 2, F and G), whereas the number of the Mdm34-GFP foci did not change in the mutant cells (Fig. S3 B). Moreover, we observed diffuse non-punctate signals of Mdm34-GFP on mitochondria in doa1Δ and mdm34ΔPY cells, but not in WT cells (Fig. 2, F and H), indicating the mislocalization of Mdm34 in the mutant cells. The accumulation of Mdm34 also caused adaptive changes: another two proteins of the ERMES complex, Mmm1 and Mdm12, exhibited increased foci intensities in doa1Δ and mdm34ΔPY cells (Fig. S3, C–E). These fractionation and imaging results show the accumulation of substrates on mitochondria in doa1Δ cells and thus indicate dysfunctions of the Cdc48 complex in extracting and degrading mitochondrial substrates.

The Doa1–Cdc48–Ufd1–Npl4 complex mediates the degradation of mitochondrial Cdc48 substrates

The function of the Cdc48 complex is assisted and diversified by cofactors, including the substrate-recruiting factors (Ufd1-Npl4 heterodimer, Shp1, and Ubx2-7) and substrate-processing factors (Ufd2, Otu1, and Doa1; Stolz et al., 2011; Meyer et al., 2012; Buchberger, 2013). We did a comprehensive analysis of the Cdc48 cofactors and found that only Doa1 and the Ufd1-Npl4 heterodimer are required for the turnover of mitochondrial Cdc48 substrates (Fig. 3, A–C). The Cdc48 cofactor Vms1 was reported to be required for Fzo1 turnover (Heo et al., 2010). However, deletion of VMS1 did not block Fzo1 degradation, a result consistent with what reported by Esaki and Ogura (Esaki and Ogura, 2012).

Doa1 has a Cdc48-binding PUL domain and two ubiquitin-binding domains (UBDs), WD40 and PFU (Fig. 3 D). The structures of these domains have been resolved individually (Mullally et al., 2006; Fu et al., 2009; Zhao et al., 2009; Nishimasu et al., 2010; Pashkova et al., 2010; Qiu et al., 2010). The recombinant UBDs bind monoubiquitin in vitro (Mullally et al., 2006; Fu et al., 2009; Pashkova et al., 2010), but whether they are required for protein turnover in vivo is unknown. We generated Doa1 mutants by mutating the critical residues in each domain according to previous studies (Fig. 3 D) and replaced the endogenous DOA1 with the mutants. The expression levels of the Doa1 mutants were comparable to that of WT Doa1 (Fig. 3 E). Mutating the WD40 (WD40Δub) and PUL (PULΔCdc48) domains inhibited the degradation of all the three substrates (Fzo1-HA, Mdm34-HA, and Msp1-HA); mutating the PUL domain (PULΔub) specifically inhibited the degradation of Msp1-HA, but not the other two substrates (Fig. 3 F). Our results suggest the WD40 UBD is more important than the PUL UBD in substrate turnover, which is consistent with studies showing that the affinity for monoubiquitin of the WD40 domain (Kd ≈ 40 µM) (Pashkova et al., 2010) is substantially higher than that of the PUL domain (Kd ≈ 1 mM; Fu et al., 2009).

The Ufd1–Npl4 heterodimer binds to the N terminus of Cdc48; Doa1 binds to the C terminus of Cdc48 (Stolz et al., 2011; Buchberger, 2013). Immunoprecipitation (IP) of Doa1-FLAG pulled down Cdc48 and Ufd1-FLAG, confirming the formation of the Doa1–Cdc48–Ufd1–Npl4 complex. As expected, this complex was disrupted by the PULΔCdc48 mutation, but not by the WD40Δub mutation (Fig. 3 G). Collectively, our results suggest that the Doa1–Cdc48–Ufd1–Npl4 complex mediates the turnover of mitochondrial Cdc48 substrates and that the UBDs of Doa1, especially the WD40 domain, are indispensable for substrate degradation (Fig. 3 H).
Figure 3. The Doa1–Cdc48–Ufd1–Npl4 complex mediates the degradation of mitochondrial Cdc48 substrates. [A and B] The FZO1-HA, MDM34-HA, and MSP1-HA strains in WT, ufd1-1, or npl4-2 background were grown to log phase at 25°C and then treated with CHX at 28°C. [C] The FZO1-HA, MDM34-HA, and MSP1-HA strains in WT or the indicated deletion mutant background were grown to log phase and then treated with CHX. [D] Domain architecture of Doa1 and the mutations that disrupt the function of the indicated domains. [E] The coding sequence of DOA1 was deleted and then replaced with WT or mutated DOA1 sequences with a C-terminal FLAG tag. Their expression levels were analyzed by anti-FLAG Western blot. [F] The FZO1-HA, MDM34-HA, and MSP1-HA strains in WT or the indicated mutation background were grown to log phase and then treated with CHX. The WD40 and PUL domains of Doa1 were required for the degradation of all the three substrates, whereas the PFU domain was only required for Msp1-HA degradation. [G] The UFD1-HA strains in WT, DOA1-FLAG, doa1WD40ΔUb-FLAG, or doa1PFUΔCdc48-FLAG background were subject to anti-FLAG IP. WCE and immunoprecipitates were analyzed by Western blot. [H] Illustration of the Doa1–Cdc48–Ufd1–Npl4 complex. Doa1 and the Ufd1-Npl4 heterodimer bind to the C and N terminus of the Cdc48 hexamer, respectively.
Ubiquitinylated Fzo1-HA and Mdm34-HA accumulate in the mitochondria-enriched fraction in doa1Δ cells

The aforementioned results suggest that Doa1 is a key Cdc48 cofactor mediating the turnover of mitochondrial substrates. The Cdc48 complex interacts with ubiquitinated substrates and extracts them from organelle membranes. Ubiquitinated substrates accumulate in cells upon the inactivation of Cdc48 (Stolz et al., 2011; Meyer et al., 2012; Buchberger, 2013). If Doa1 is required for the removal of ubiquitinated mitochondrial substrates, accumulation of ubiquitinated substrates in doa1Δ cells would be expected.

We could not detect substrate ubiquitinlation by Western blot analysis of whole-cell extracts (WCEs; unpublished data), indicating that the ubiquitinated species are present in low amounts. We therefore performed IP of substrates. In WT cells, we observed up-shifted bands of Fzo1-HA, which were greatly increased in cdc48-3 cells (Fig. 4 A, lane 1 vs. 2). Deletion of MDM30, the ubiquitin E3 ligase of Fzo1 (Cohen et al., 2008), abolished the formation of Fzo1-HA upshifts in WT and cdc48-3 cells (Fig. 4 B, lane 2 vs. 3 and lane 4 vs. 5), indicating that the upshifts are very likely to be the ubiquitinated forms of Fzo1-HA (Ub-Fzo1-HA). Accumulation of such upshifts was also seen in doa1Δ cells (Fig. 4 A, lane 3). By overexpressing and probing 3×FLAG-tagged ubiquitin, we confirmed that the upshifts of Fzo1-HA in doa1Δ cells are indeed Ub-Fzo1-HA (Fig. 4 C). We rescued ubiquitin level in doa1Δ cells, which actually further enhanced the accumulation of Ub-Fzo1-HA (Fig. 4 A, lane 5 vs. 6), indicating that the accumulation of Ub-Fzo1-HA in doa1Δ cells is not caused by ubiquitin depletion. We then performed IP of Fzo1-HA from different subcellular fractions and found that Ub-Fzo1-HA was only present in the mitochondria-enriched, but not the PMS, fraction of cdc48-3 and doa1Δ cells (Fig. 4 D).

Upshifts of HA signals indicate Ub-Fzo1-HA (Fig. 4, A–C), but we could not see similar upshifts of Mdm34-HA, indicating Ub-Mdm34-HA is present in very low amounts. Therefore, we overexpressed FLAG-ubiquitin using a 2μ plasmid, and after IP of Mdm34-HA, we probed the immunoprecipitates with an anti-FLAG antibody to detect Ub-Mdm34-HA. In this way, we observed for other Cdc48 substrates (Shcherbik and Haines, 2007).

Consistent with the Fzo1-HA IP results (Fig. 5 A), deletion of DOA1 significantly reduced the amount of the Ufd1- and Cdc48-associated nonubiquitinated Fzo1-HA and Ub-Fzo1-HA in cdc48-3 cells (Fig. 5, E [lane 4 vs. 5] and F [lane 4 vs. 5]). In Fig. 5 F, ubiquitin level was restored by ubiquitin overexpression to avoid possible side effects caused by ubiquitin depletion in doa1Δ cells. Collectively, these results strongly suggest that Doa1 is required for the efficient recruitment of mitochondrial substrates to the Cdc48-Ufd1-Npl4 complex.

The Ufd1–Npl4 heterodimer serves as a substrate-recruiting complex in other Cdc48-mediated degradation pathways (Stolz et al., 2011; Buchberger, 2013). To examine its function in MAD, we introduced npl4Δ-2, a conditional mutant allele of Npl4, to cdc48-3 cells. Inactivation of the Ufd1-Npl4 heterodimer enhanced the accumulation of Ub-Fzo1-HA (Fig. 5 G, lane 2 vs. 3) but greatly reduced the interaction between Cdc48 and Ub-Fzo1-HA (Fig. 5 H, lane 2 vs. 3). Thus, the Ufd1-Npl4 heterodimer is also required for the recruitment of MAD substrates to Cdc48. Given that Doa1 is required for the recruitment of Ub-Fzo1-HA to Ufd1 (Fig. 5 E), the Ufd1–Npl4 complex may function downstream of Doa1 in the substrate-recruitment process.
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Doa1 mediates mitochondria-associated degradation (Wu et al., 2005; Schuberth and Buchberger, 2005) and nuclear (Verma et al., 2011) substrates, respectively. If Doa1 plays a similar role in MAD as the UBX proteins, Doa1 may interact with ubiquitinated MAD substrates through its UBDs, which are essential for substrate degradation (Fig. 3 F).

IP of Doa1-FLAG showed that Doa1-FLAG interacted weakly with nonubiquitinated Fzo1-HA and Ub-Fzo1-HA in WT cells. The interaction, especially with Ub-Fzo1-HA, was greatly enhanced in cdc48-3 cells (Fig. 6 A, lane 2 vs lane 3). The Doa1-associated Ub-Fzo1-HA resembles the major forms of Ub-Fzo1-HA accumulated in cdc48-3 cells (the three Ub-Fzo1-HA bands in Fig. 4 A, lane 2, white arrows), and has a distinct pattern from the Ufd1- and Cdc48-associated Ub-Fzo1-HA (compare Fig. 6 A, lane 3, with Fig. 5, C [lane 3] and D [lane 3]). Therefore, it is unlikely that Doa1 indirectly interacts with substrates through its association with the Cdc48-Ufd1-Npl4 complex.

Deletion of MDM30, the E3 ligase of Fzo1 (Cohen et al., 2008), abolished the interaction of Doa1-FLAG with nonubiquitinated Fzo1-HA and Ub-Fzo1-HA in WT (Fig. 6 B, lane 2 vs. 3) and cdc48-3 (Fig. 6 B, lane 4 vs. 5) cells. These results suggest the Doa1-associated nonubiquitinated Fzo1-HA is indirectly pulled down because of the oligomeric nature of Fzo1.

The WD40 UBD of Doa1 is required for Fzo1 turnover (Fig. 3 F). Consistently, mutating the WD40 domain greatly inhibited the interaction between Doa1-FLAG and Fzo1-HA (Fig. 6 C, lane 2 vs. 3). However, the truncated Doa1 containing
the two UBDs (WD40 + PFU) was not able to pull down Fzo1-HA (Fig. 6 D, lane 2 vs. 5), indicating UBDs are not sufficient for substrate-binding in vivo. Similarly, the WD40 domain alone could not interact with Fzo1-HA (Fig. 6 D, lane 2 vs. 4). We further tested the PULΔCdc48 mutant, which disrupts the interaction with Cdc48 but largely preserves the PUL domain. In comparison with the Doa1 fragment containing the UBDs, the PULΔCdc48 mutant had a slightly stronger interaction with Fzo1-HA (Fig. 6 D, lane 3 vs. 5). But the interaction was still much weaker than the binding between WT Doa1 and Fzo1-HA (Fig. 6, C [lane 2 vs. 4] and D [lane 2 vs. 3]), indicating that interaction with Cdc48 is required for Doa1 to bind its physiological ubiquitinated substrates.

**Doa1 accumulates on mitochondria in cdc48-3 cells**

The aforementioned characterizations showed that the interaction between mitochondrial substrates and the Doa1–Cdc48–Ufd1-Npl4 complex is stabilized in cdc48-3 cells. The interaction may be stabilized on mitochondria, because inactivation of Cdc48 prevents the retrotranslocation of ubiquitinated substrates from membranes (Verma et al., 2011; Nakatsukasa et al., 2013). We did subcellular fractionation experiments and detected a significantly increased amount of ubiquitinated proteins and Doa1-FLAG in the mitochondrial-enriched fraction in cdc48-3 cells as compared with WT cells (Fig. 6 E, lane 3 vs. 4). We further showed that Doa1 peripherally associates with MOM because it was completely degraded by Proteinase K added to intact mitochondria (Fig. S4 A) and was extracted from mitochondria by sodium carbonate (Fig. S4 B).

The amount of Ufd1-FLAG in the mitochondrial-enriched fraction was also increased in cdc48-3 cells (Fig. S4 C, lane 3 vs. lane 4), but to a much lesser extent in comparison with Doa1-FLAG. The underlying reason for such a difference could be that a Cdc48 hexamer can only bind one pair of Ufd1-Npl4 heterodimer (Hetzer et al., 2001; Pye et al., 2007), whereas the docking of Doa1 to a Cdc48 hexamer may not have such constraints.

We further analyzed the localization of Doa1 in cdc48-3 cells by imaging. We fused a GFP tag to the C terminus of Doa1, which did not interfere with Doa1 function (Fig. S4 D).
Doa1-GFP was diffusively distributed in the nucleus and the cytoplasm in WT cells, but was enriched on mitochondria in cdc48-3 cells (Fig. 6 F). Mutating the WD40 and PUL domains, which were required for substrate interaction (Fig. 6, C and D), inhibited the mitochondrial enrichment of Doa1-GFP (Fig. 6 F).

Doa1 is not essential for surviving ER stress

Previous studies demonstrate that the Ubx2–Cdc48–Ufd1–Npl4 complex mediates ERAD (Ye et al., 2001; Jarosch et al., 2002; Neuber et al., 2005; Schuberth and Buchberger, 2005). Our study suggests that MAD and ERAD share the core Cdc48–Ufd1–Npl4 complex but have different additional Cdc48 cofactors: MAD requires Doa1, and ERAD requires the ER-resident Ubx2 (Fig. 7 A). To determine whether Doa1 is an ERAD component, we performed the following growth tests.

The lack of ERAD components strongly compromises the viability of cells that cannot elicit unfolded protein response (UPR) at 37°C (Friedlander et al., 2000; Ng et al., 2000). Accordingly, cells lacking UBX2 or IRE1, a key UPR component (Cox et al., 1993), grew normally, but cells lacking both genes showed severe growth defects at 37°C (Fig. 7 B). Similarly, double knockout of IRE1 and HRD1, another key ERAD component (Hampton et al., 1996), caused strong synthetic growth defects at 37°C as shown previously (Friedlander et al., 2000; Fig. 7 C). In contrast, deletion of DOA1 in ire1Δ cells did not affect cell growth (Fig. 7, B and C). Therefore, Doa1 is unlikely an essential ERAD component. Our result is consistent with the previous study that doa1Δ cells were insensitive to the treatment of the ERAD inducer tunicamycin (Mullally et al., 2006).

We further examined the role of Doa1 in the degradation of an ERAD-lumen substrate, CPY* (Hiller et al., 1996), and an ERAD-cytoplasm substrate, Ubc6 (Swanson et al., 2001; Walter et al., 2001). CPY*-FLAG degradation was inhibited in ubx2Δ and doa1Δ cells, but the degradation defect in doa1Δ cells was rescued by ubiquitin overexpression (Fig. S5 A). The

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degradation of Ubc6-HA was inhibited by inactivation of Ufd1 but was normal in doa1Δ cells (Fig. S5 B). Deletion of DOA1 did not affect the degradation of a cytoplasmic Cdc48 substrate (prematurely terminated Gnd1 PTC) either (Verma et al., 2013; Fig. S5 C). These results suggest that Doa1 does not directly mediate the turnover of these nonmitochondrial Cdc48 substrates. Although deletion of DOA1 may indirectly affect the turnover of some ERAD substrates because of ubiquitin depletion, it does not cause global impairment of ERAD as suggested by the growth tests.

Doa1 is required for surviving mitochondrial oxidative stress

The doa1Δ cells had no overt growth defect under normal conditions. We therefore proceeded to investigate whether deletion of DOA1 affects cell survival under mitochondrial stress conditions. We analyzed the synthetic growth phenotype between doa1Δ and the deletion of other mitochondrial quality-control genes, including SOD2, a matrix superoxide scavenger (van Loon et al., 1986); mitochondrial AAA proteases, including YME1, YTA12, AFG3, and PIM1 (Quirós et al., 2015); and ATG32, a mitophagy receptor (Kanki et al., 2009; Okamoto et al., 2009). Deletion of DOA1 did not affect the growth of atg32Δ (Fig. S5 D) or yme1Δ cells (Fig. S5 E), but it mildly inhibited the growth of yta12Δ and afg3Δ cells (Fig. S5 F) and strongly inhibited the growth of pim1Δ cells (Fig. S5 G). Besides the degradation of damaged mitochondrial proteins, Yta12 and Afg3 are required for the assembly of inner membrane complexes (Arlt et al., 1996), and Pim1 is required for the maintenance of mtDNA (Suzuki et al., 1994; Van Dyck et al., 1994). Therefore, the synthetic growth defects of doa1Δ and the deletion of mitochondrial proteases may be caused by compound effects.

In comparison to WT cells, deletion of SOD2, which elevates mitochondrial oxidative stress, compromised cell growth in ethanol and glycerol (YPEG) media (Fig. 7 D). YPEG media are nonfermentable and thus require mitochondrial respiration for cell growth. This growth defect was further exacerbated upon the deletion of DOA1 and could not be rescued by ubiquitin overexpression (Fig. 7 D). The synthetic growth defect of doa1Δ sod2Δ cells was rescued by doa1PFUΔUb, but not by doa1WD40ΔUb, doa1WD40ΔCdc48, or doa1PULΔCdc48 mutants (Fig. 7 E), further supporting that the WD40 and PUL domains are essential for Doa1 function. These results suggest Doa1 is critical for surviving mitochondrial oxidative stress.

Discussion

MAD is an integral part of the mitochondrial quality control system. We analyzed the turnover of a subset of MOM proteins and identified Cdc48 substrates (Fig. 1) and potential Cdc48-independent substrates (Fig. S1). This observation, as well as a recent discovery that another AAA ATPase Msp1 mediates the degradation of mistargeted proteins on MOM (Chen et al., 2014; Okreglak and Walter, 2014), suggests MAD is more complex than expected.

Using the newly identified mitochondrial Cdc48 substrate Tom70, we established a genetic screen assay to unbiasedly characterize the genetic basis of the Cdc48-dependent MAD pathway. Our pilot screen identified several regulators, including the Cdc48 cofactor Doa1 and the ubiquitin E3 ligase Rsp5 (Fig. S2). Although the characterization of the Cdc48-dependent MAD pathway is at its beginning, our study has made several interesting observations.
We provide genetic and biochemical evidence that the Doa1–Cdc48-Ufd1-Npl4 complex mediates the turnover of mitochondrial Cdc48 substrates (Fig. 8). The degradation of Cdc48 substrates is mostly mediated by the core Cdc48-Ufd1-Npl4 complex and additional substrate-recruiting factors, including Ubx2-7 (Stolz et al., 2011; Meyer et al., 2012; Baek et al., 2013; Buchberger, 2013). In the absence of these additional substrate-recruiting factors, substrate recruitment to the Cdc48-Ufd1-Npl4 complex and the subsequent substrate degradation are partially or completely inhibited (Schuberth and Jentsch, 2006). In contrast to these substrates, the degradation of Cdc48 substrates is Ufd2 independent but Doa1 dependent (Fig. 3). Thus, our results and previous characterizations highlight Doa1 as a unique factor playing dual roles in the degradation of Cdc48 substrates: for Ufd2-dependent substrates, it is a substrate-processing factor inhibiting degradation; for Ufd2-independent mitochondrial Cdc48 substrates, it is a substrate-recruiting factor promoting degradation.

Doa1 has an interesting property that only the Cdc48-bound Doa1 can interact with substrates, whereas the Cdc48-unbound Doa1 or its UBDs alone cannot (Fig. 6, C and D), which brings a potential benefit that the Cdc48-unbound Doa1 does not bind substrates to interfere with substrate degradation. The underlying molecular mechanism is not clear, but it is conceivable that conformational changes may occur when Doa1 interacts with Cdc48, which has been suggested by structural studies of the PUL domain (Zhao et al., 2009; Nishimasu et al., 2010) and has been observed for other Cdc48 cofactors (Beuron et al., 2006; Bebeacua et al., 2012).

An intriguing observation is that in cdc48-3 cells, in which Cdc48 substrates accumulate at many subcellular locations, Doa1 selectively enriches on mitochondria (Fig. 6 F). This observation indicates that a significant portion of, if not all, Doa1 substrates is mitochondrial. Considering that our analysis of MOM proteins (14 experimentally verified transmembrane proteins) only covers a small percentage of the MOM proteome (>110 proteins predicted by mass spectrometry analysis; Zahedi et al., 2006), we speculate that Doa1 may have more mitochondrial substrates not identified yet. It is unclear whether the recognition of mitochondrial Cdc48 substrates by Doa1 involves specific molecular mechanisms. A genome-wide genetic screen and further biochemical characterizations are thus necessary for a better understanding of the Cdc48-dependent MAD pathway.

Doa1 is highly conserved during evolution. Structural studies and sequence analyses show that the domain organization and the critical residues are highly conserved between Doa1 and its mammalian orthologue PLAA (Mullally et al., 2006; Fu et al., 2009; Pashkova et al., 2010; Qiu et al., 2010). It will be interesting to examine if PLAA is also involved in the mammalian MAD pathway in the future.

Materials and methods

Yeast strains and media

The yeast strains used in this study are listed in Table S3. Strain transformation was performed using standard protocols (Longtine et al., 1998; Voth et al., 2001; Janke et al., 2004). Yeast strains were grown at 30°C if not otherwise indicated. BY4741, W303-1A, and CEN.PK strains were gifts from B. Tu (University of Texas Southwestern, Dallas, TX).

Media used in this study (Sherman, 2002) included YPD (1% yeast extract, 2% peptone, and 2% glucose), YPL (1% yeast extract, 2% peptone, and 2% lactate), YPEG (1% yeast extract, 2% peptone, 3% ethanol, and 3% glycerol), YPGAL (1% yeast extract, 2% peptone, and 2% galactose), SCD (0.67% yeast nitrogen base without amino acids, 0.079% complete supplement mixture, and 2% glucose), SCEG (0.67% yeast nitrogen base without amino acids, 0.079% complete supplement mixture, 3% ethanol, and 3% glycerol), SCD-LEU (0.67% yeast extract, 2% leucine, and 2% lactate), YPAGAL (1% yeast extract, 2% peptone, and 2% galactose), SMM (1.5% yeast extract, 3% peptone, and 2% galactose), and SC-R (0.67% yeast nitrogen base without amino acids, 0.079% complete supplement mixture, and 2% galactose)

Figure 8. Cartoon illustration of the MAD pathway mediated by the Doa1–Cdc48-Ufd1-Npl4 complex. MOM proteins with different topologies are ubiquitinated by ubiquitin E3 ligases, such as Mdm30 and Rsp5. Doa1 in the Doa1–Cdc48-Ufd1-Npl4 complex is the primary factor to recognize and recruit ubiquitinated MOM proteins to the Ufd1–Npl4 heterodimer and then to Cdc48. By hydrolyzing ATP, Cdc48 dialogues ubiquitinated MOM proteins from membrane and presents them to proteasome for degradation. The dashed lines indicate potential additional mitochondrial substrates of Doa1.
nitrogen base without amino acids, 0.069% -Leu DO supplement, and 2% glucose), SCD-URA (0.67% yeast nitrogen base without amino acids, 0.077% -Ura DO supplement, and 2% glucose), and SCGAL-URA (0.67% yeast nitrogen base without amino acids, 0.077% -Ura DO supplement, and 2% galactose). ClonNAT (100 µg/µl WERNER BioAgents) was added to media for plasmid maintenance as needed.

**Antibodies and chemicals**

The following antibodies were from Sigma-Aldrich: glucose-6-phosphate dehydrogenase (G6PDH), HA-peroxidase, and FLAG M2. Antibodies for ubiquitin (P4D1) and VCP/Cdc48 were from Cell Signaling Technology. The antibody for Por1 was from Thermo Fisher Scientific. The GFP antibody was obtained from CWbio.

Yeast extract, peptone, and yeast nitrogen base without amino acids were purchased from BD. Yeast complete supplement mixture was purchased from MP Biomedicals. Yeast amino acid dropout supplements were obtained from Takara Bio Inc. CHX was obtained from Amresco. MG132 was from EMD Millipore. Other chemicals or reagents were obtained from Sigma-Aldrich if not otherwise indicated.

**Yeast plasmids**

Plasmids used in this study are listed in Table S4.

The ubiquitin expression plasmids pRS42N-UB, pRS42N-3xFLAG-UB, pRS425-UB, and pRS425-1xFLAG-UB were generated as follows: the ubiquitin coding sequence (with or without a 1xFLAG right after the start codon) was first inserted into p417-TEF1 (Wu and Tu, 2011) at the BamHI–EcoRI sites; a 3xFLAG tag was inserted into the resulting plasmid at SpeI–BamHI sites; the ubiquitin coding sequence with or without the 1x or 3xFLAG tag, together with the promoter and terminator sequences, were then excised at the Apal–NotI sites and inserted into pRS42N or pRS425 (Christianson et al., 1992; Taxis and Knop, 2006).

The plasmids expressing full-length or truncated forms of DOA1 were generated as follows: the TEF1 promoter in p417-TEF1-natNT2 (KanMX4 in p417-TEF1 was replaced by natNT2) was replaced with the GAL1 promoter at SacI–XbaI sites; a 3xFLAG tag was inserted into the resulting plasmid at EcoRI–SalI sites; the full-length coding sequence of DOA1, the WD40 domain (1–900 bp of DOA1), and the WD40+pPFU domain (1–1350 bp of DOA1) were then inserted into the plasmid (natNT2-GAL1-3xFLAG-C) from the last step at XbaI–SpeI sites to make plasmids natNT2-GAL1-DOA1-FLAG, natNT2-GAL1-DOA1-WD40-FLAG, and natNT2-GAL1-DOA1+pPFU-FLAG. Point mutations R541A and R669A in the PUL domain were created by QuikChange site-directed mutagenesis (Agilent Technologies).

The pYES2-CPY<sub>G255R</sub> (CPY<sup>+</sup>)-FLAG plasmid was created as follows: the coding sequence for 3xFLAG was inserted into pYES2 (Thermo Fisher Scientific) at the BamHI–EcoRI sites, the CPY coding sequence was then inserted into the resulting plasmid at the HindIII–KpnI sites, and the G255R mutation was introduced by QuikChange site-directed mutagenesis.

The hphNT1-TEF1-UBC6-HA plasmid was generated as follows: a 1xHA tag was inserted in between Leu<sup>50</sup> and Asp<sup>302</sup> of Ubc6 by isothermal assembly (Gibson et al., 2009), and then the full-length coding sequence of Ubc6 with the internal 1xHA tag was inserted to p417-TEF1-hphNT1 (KanMX4 in p417-TEF1 was replaced by hphNT1) at BamHI–SalI sites.

Plasmids pRS42N, pRS425, p417-TEF1, and pYES2 were gifts from B. Tu.

**Colonel screen assay**

To randomly generate insertion mutants by homologous recombination (Burns et al., 1994), the founder strain TOM70-<i>HA</i>, <i>lea2Δ</i> was transformed with the yeast genomic mini-Tnt3::lacZ::LEU2 transposon insertion library (provided by B. Tu). Approximately 5,000 mutants were generated and tested by colony assay. On day 0, the transformed yeast cells were grown on selective glucose (SCD-LEU) plates. On day 3, we replica-plated the cells from the SCD-LEU plates to lactate (YPL) plates. On day 4, these cells were replica-plated onto nitrocellulose membranes that were plated on YPL plates. On day 5, the nitrocellulose membranes were placed on new YPL plates supplemented with 50 µg/ml CHX. On day 6, we lysed the yeast cells by placing the membranes on filter paper soaked with colony lysis buffer (0.1% SDS, 0.2 M NaOH, and 0.5% β-mercaptoethanol; Knop et al., 1996). After incubation for 1 h at room temperature, we cleaned the membranes by rinsing off the cell debris with water. The membranes were then subject to HA immunoblotting. The colonies with relatively stronger HA signals were collected onto a new plate and subsequently reexamined for protein degradation by colony assay and Western blot. Finally, the insertion sites were mapped using the Vectorette PCR method (Riley et al., 1990).

**Yeast WCE preparation**

Cell pellets were resuspended in 300 µl yeast lysis buffer (50 mM NaCl, 50 mM NaF, 100 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 14 mM 2-mercaptoethanol, 2 mM PMSF, 5 µM pepstatin A, and 10 µM leupeptin). After adding ~80 µl of glass beads, cells were lysed via three rounds of bead-beating (40 s of beating followed by 1 min of cooling on ice). For the degradation assay, cycloheximide was used at 50 µg/ml and MG132 was used at 20 µM.

**Yeast IP**

Approximately 100–600 ml of cells grown in SCD media to late log phase (OD<sub>600</sub> ~4) was harvested, flash frozen with liquid nitrogen, and stored at −80°C until cell lysis. Cell pellets were then thawed on ice and resuspended with 2–5 ml yeast lysis buffer. Cells were lysed by nine rounds of bead-beating (20 s beating followed by 1 min of cooling on ice). The crude cell extracts were subject to centrifugation (17,000 g) for 10 min at 4°C. The supernatant was then diluted with 3–4 volumes of yeast lysis buffer, mixed with 8–20 µl anti-FLAG or anti-HA agarose beads (Sigma-Aldrich), and incubated at 4°C for 5–6 h (anti-FLAG) or 8–18 h (anti-HA). The agarose beads were then washed four times with yeast lysis buffer and eluted overnight with FLAG or HA peptide (ChinaPeptides Co. Ltd.) at 4°C.

**Isolation of the mitochondria-enriched fraction and analysis of sub-mitochondrial localization**

Mitochondria were isolated using a previously described method (Dickert et al., 2001), with some modifications. In brief, ~200 ml cells grown in SCD media to late log phase (OD<sub>600</sub> ~3) was collected. Cells were washed once with water and incubated in TD buffer (10 mM DTT and 100 mM Tris·SO<sub>4</sub>, pH 9.4) for 15 min at 30°C. Cells were then washed once with SP buffer (1.2 M sorbitol and 20 mM potassium phosphate, pH 7.4) and treated with Zymolyase 20T/100T (MP Biomedicals) for 40 min at 30°C to generate spheroplasts. After two washes with SP buffer, the spheroplasts were resuspended in SHE buffer (0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 1 mM EGTA, pH 8, and 2 mM MgCl<sub>2</sub>) supplemented with protease inhibitors and homogenized in a glass homogenizer (15 strokes). A small aliquot of spheroplasts was saved for making WCEs. The mitochondria-enriched fraction and PMS were obtained by differential centrifugation. Equal amount of proteins from different fractions was analyzed by Western blot.

To determine the subunit mitochondrial localization of proteins, mitochondria were washed twice with SHE buffer and resuspended in isotonic SHE buffer or hypotonic buffer (20 mM Hepes-KOH, pH 7.4)
to ~1.5–2 mg/ml. Proteasine K (Sigma-Aldrich) was then added to 25 
µg/ml and incubated for 20 min on ice. The digestion was stopped by 
adding TCA to 12% (wt/vol). For alkaline extraction, mitochondria were 
resuspended in control SHE buffer or sodium carbonate (0.1 M) to 
~1 mg/ml, incubated for 30 min on ice, and then subject to ultracent-
trifugation (100,000 g for 20 min) to obtain the supernatant (soluble 
proteins) and pellet fractions. Proteins from both fractions were precipitated with 12% TCA. The precipitated proteins were 
were washed once with 5% TCA and once with 100% ethanol and then 
solubilized in 120 µl of solubilization buffer (40 µl 1 M Tris-HCl, pH 
8.0, and 80 µl 2x sample buffer). An equal amount of samples from 
different treatments was analyzed by Western blot.

Microscopy
Yeast cells were grown in appropriate media (SCD media for Figs. 2, 6 
F, S1 F, and S3 C; YPT media for Fig. S3 A) to log phase, concentrated, 
and immobilized on microscope slides. Fluorescent images were 
captured at 25°C using a 100x objective (CFI Plan Apochromat Lambda, NA 
1.45; Nikon) with immersion oil (type NF) on an inverted fluorescence 
microscope (Eclipse Ti-E; Nikon) with a spinning-disk confocal scanner 
unit (UltraView; PerkinElmer) with 488 (emission filter 525 [W50]) and 
561 (dual-band emission filter 445 [W60] and 615 [W70]) lasers. Z-stack 
images with 0.5-µm increments were acquired with Velocity software 
(PerkinElmer) and processed with Velocity and ImageJ software.

Statistical analysis
Data were processed in Excel and analyzed in GraphPad Prism software 
using the statistical methods as indicated in the figure legends.

Online supplemental material
Fig. S1 provides further characterization of MOM protein degradation 
and shows the effect of C-terminal tagging on protein localization and 
cell growth. Fig. S2 shows the development of the genetic screen assay 
and shows the effect of C-terminal tagging on protein localization and 
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