Doa1 is a MAD adaptor for Cdc48

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Dislocation of polypeptides from the mitochondrial outer membrane by the p97/Cdc48–Ufd1–Npl4 adenosine triphosphatase complex is essential for mitochondria-associated degradation and Parkin-mediated mitophagy. In this issue, Wu et al. (2016. *J. Cell Biol.* http://dx.doi .org/10.1083/jcb.201510098) identify Doa1 as a pivotal adaptor that recruits substrates to Cdc48 for processing.

Eukaryotic cells use the ubiquitin proteasome system to eliminate misfolded proteins from diverse subcellular compartments to maintain protein homeostasis. Once polyubiquitinated, soluble proteins are readily targeted to the proteasome. However, the degradation of proteins in lipid bilayer or membrane-encircled organelles requires additional steps because the membranes render these substrates, at least in part, inaccessible to the ubiquitin proteasome system. Taking ER-associated degradation (ERAD) as an example, misfolded ER luminal proteins can only become ubiquitinated after they emerge from the ER lumen via a retrotranslocation process; the degradation of ubiquitinated substrates embedded in the membrane then requires their dislocation into the cytosol, a reaction mediated by a conserved ATPase named p97 in mammals or Cdc48 in *Saccharomyces cerevisiae* (Christianson and Ye, 2014). During this process, the hexameric barrel-like ATPase p97/Cdc48, assisted by an array of cofactors, uses the energy from ATP hydrolysis to extract polypeptides from the membranes for targeting to the proteasome. Besides ERAD, p97/Cdc48 is also involved in dislocating polypeptides from the mitochondrial outer membranes (MOMs) to facilitate mitochondria-associated degradation (MAD; Heo et al., 2010; Xu et al., 2011; Hemion et al., 2014). The MAD process can be used to eliminate aberrant proteins for regulation of mitochondria protein homeostasis or to degrade factors (e.g., mitofusin) controlling the turnover of damaged mitochondria by mitophagy (Tanaka et al., 2010). Intriguingly, p97 and the heterodimeric cofactor Ufd1-Npl4 accumulate on the surface of damaged mitochondria, and deficiency in each of these factors causes a defect in Parkin-mediated mitophagy (Kimura et al., 2013). These findings suggest a critical role of p97/Cdc48 in mitochondria homeostasis regulation, but how substrates are recruited to p97/Cdc48 in MAD is unclear. In this issue, Wu et al. identify Doa1 as a critical regulator of Cdc48-dependent MAD in *Saccharomyces cerevisiae* (Wu et al., 2016).

The MAD pathway has been poorly characterized in budding yeast because of a lack of model substrates, so Wu et al. (2016) first measured the half-life of endogenously tagged MOM proteins to identify substrates suitable for mechanistic characterization of this process. The study revealed four short-lived proteins (Fzo1, Mdm34, Msp1, and Tom70) whose rapid turnover depends not only on the proteasome but also on Cdc48. Among these substrates, Tom70, a mitochondrial import receptor, was used to set up a transposon-based genetic screen. The screen, notwithstanding its limited genome coverage (~15%), efficiently uncovered four insertion mutants with elevated Tom70 expression that were likely associated with defects in MAD because the affected genes encode a proteasome-associated deubiquitinase (Ubp6), a deubiquitinase-binding protein (Bro1), an E3 ubiquitin ligase (Rsp5), and the Cdc48 adaptor Doa1. Because Doa1 was the only factor required for efficient degradation of all four MAD substrates, the authors further characterized its function.

DOA1 (also named *UFD3*) was initially reported in a genetic screen that searched for genes required for efficient degradation of a β-galactosidase fusion protein containing a ubiquitin moiety at the N terminus. The screen identified five mutants, namely, *ufd1–5* (ubiquitin fusion degradation; Johnson et al., 1995). Subsequent studies established several of these UFD proteins as key Cdc48-binding proteins required for Cdc48-dependent degradation. These include a substrate-recruiting adaptor (Ufd1) and a substrate-processing cofactor (Ufd2; Koegl et al., 1999; Meyer et al., 2000; Böhm et al., 2011). The WD domain–containing Doa1 was also shown to bind Cdc48, but it competes with Ufd2 in binding to the C terminus of Cdc48, and therefore was proposed to antagonize Ufd2 functionally in Cdc48-mediated degradation (Rumpf and Jentsch, 2006). However, because *DOA1* deficiency also causes a reduction in the level of endogenous ubiquitin, which could indirectly stabilize proteasome substrates (Johnson et al., 1995), whether Doa1 negatively regulates Cdc48-mediated degradation has been unclear. Strikingly, Wu et al. (2016) showed that in the case of MAD, reexpression of ubiquitin in *DOA1* null cells did not restore degradation. Thus, Wu et al. (2016) for the first time reveal a role of Doa1 in Cdc48-dependent degradation that is unrelated to its function in ubiquitin homeostasis regulation. Doa1 contains an N-terminal WD domain that has a strong ubiquitin-binding activity (Pashkova et al., 2010), a weak ubiquitin-binding PFU domain (Fu et al., 2009), and a C-terminal Cdc48-binding PUL domain (Ghislain et al., 1996; Mullally et al., 2006; Zhao et al., 2009; Qiu et al., 2010). Wu et al. (2016) performed complementation experiments with a series of Doa1 truncation mutants and showed that the WD domain and the PUL domain of Doa1 are indispensable for MAD, whereas the PFU domain is only required for degradation of a subset of MAD substrates.

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In ERAD, Cdc48/p97 is known to interact with ubiquitinated substrates and extract them from the ER membranes (Ye et al., 2001). Cdc48–Doa1 may act similarly in MAD because an interaction between Cdc48 and MAD substrates was observed by coimmunoprecipitation and because deletion of *DOA1* caused MAD substrates to accumulate on mitochondrial membranes. Furthermore, biochemical fractionation showed that in cells bearing a temperature-sensitive *cdc48* allele or lacking *DOA1*, MAD substrates enriched in the mitochondrial fraction are highly ubiquitinated.

Comprehensive analyses of other known Cdc48 cofactors showed that in addition to Doa1, the Ufd1–Npl4 complex is also required for degradation of Cdc48 substrates at the mitochondria. As Ufd1–Npl4 binds to Cdc48 via its N-terminal domain, whereas Doa1 interacts with the C-terminal tail of Cdc48, a multiprotein complex consisting of Cdc48, Ufd1, Npl4, and Doa1 could be detected by coimmunoprecipitation. Genetic studies showed that both Doa1 and Npl4 are required for substrate interaction with Cdc48 in MAD, suggesting that these factors may function as a substrate-recruiting cofactor. Interestingly, the interaction of Doa1 with ubiquitinated MAD substrates, while being mediated by its WD40 domain, is also dependent on Cdc48: in *cdc48-3* mutant cells, Doa1 accumulates on the mitochondrial membranes and binds MAD substrates more efficiently, yet deletion of the Cdc48-interacting domain reduced the interaction of Doa1 with MAD substrates. These results suggest that Doa1 may facilitate substrate recruitment to Cdc48 only when it is bound to Cdc48; but upon Cdc48-mediated extraction, substrates are released from this complex (Fig. 1).

The function of Doa1 in targeting proteins for degradation by the proteasome appears specific to MAD as deletion of *DOA1* either had no effect on degradation of nonmitochondrial substrates or, in the case of the ERAD substrate CPY*, the stabilizing effect of *DOA1* deletion could be attributed to the deficiency in ubiquitin. Moreover, unlike Ufd2, *DOA1* deficiency did not sensitize cells to ER stress triggered by deletion of the IRE1 component of the unfolded protein response. In contrast, the growth of cells under increased mitochondrial oxidative stress conditions, such as superoxide dismutase deficiency, was compromised by deletion of the *DOA1* gene, and this phenotype could be rescued by wild-type Doa1, but not by Doa1 mutants lacking either the WD or the Cdc48-binding PUL domain.

Overall, Wu et al. (2016) convincingly establish Doa1 as a key regulator of MAD in *S. cerevisiae*, but whether Doa1's mammalian homologue phospholipase A2 activating protein is similarly involved in MAD as well as in Parkin-mediated mitophagy remains to be tested. Because Doa1 binds to Cdc48 at a site close to the hexameric ring formed by the second ATPase domain (D2; Mullally et al., 2006; Rumpf and Jentsch, 2006; Zhao et al., 2009; Qiu et al., 2010), these findings further suggest that Cdc48 and perhaps its mammalian homologue p97 might first engage substrate using the D2 domain, as proposed previously (DeLaBarre et al., 2006). Intriguingly, the N domain–binding cofactor Ufd1–Npl4 is also required for substrate interaction with Cdc48/p97 in MAD. It is unclear how substrate recruitment to Cdc48/p97 could simultaneously involve two spatially separated adaptors. One possibility is that Ufd1–Npl4 may indirectly promote substrate binding by allosterically activating Cdc48/p97 or Doa1. Alternatively, these adaptors may relay substrate for targeting to Cdc48. The key to distinguish between these models lies in better assays that
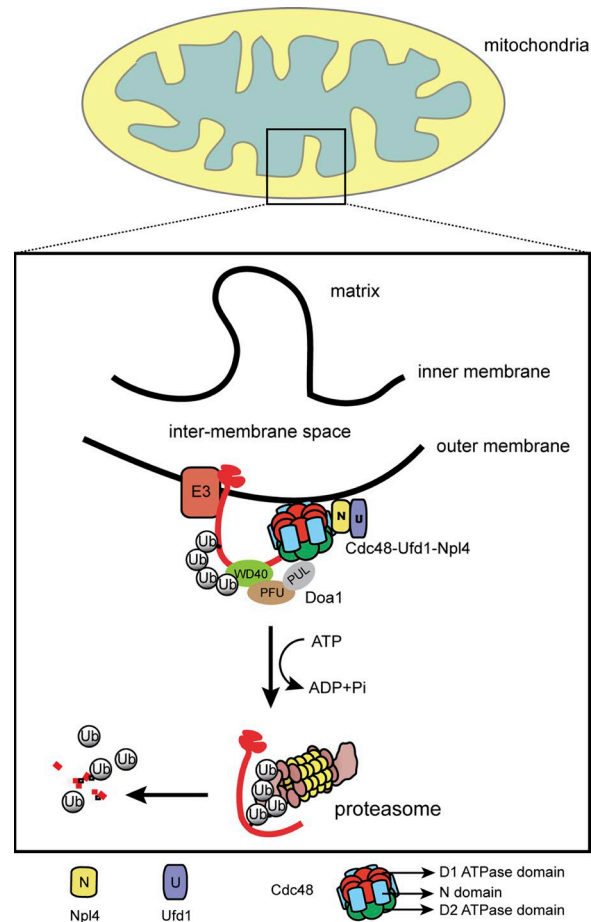

Figure 1. **Cdc48–Doa1–dependent degradation of MOM proteins.** A MOM protein (red) is ubiquitinated by an E3 ubiquitin ligase. The ubiquitin chain is then recognized by the ubiquitin-binding WD domain of Doa1 (light green), which recruits the substrate to the Cdc48–Ufd1–Npl4 complex through an interaction between its PUL domain (gray) and the Cdc48 C terminus. Upon ATP hydrolysis, Cdc48–Ufd1–Npl4 extracts ubiquitinated substrate from the MOM and brings it to the proteasome for degradation.

would allow the mapping of direct interactions between Cdc48 and MAD substrates in the presence or absence of these adaptors. The precise signal for substrate recognition in Cdc48p–Doa1–mediated MAD also remains to be elucidated. Despite these unresolved issues, the newly identified substrates and the demonstration that Doa1 is the MAD adaptor for Cdc48 by Wu et al. (2016) should provide a new handle to advance our understanding of this important yet poorly studied pathway.

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