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RESEARCH ARTICLE

The deubiquitylating enzyme Ubp12 regulates Rad23-dependent proteasomal degradation

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ABSTRACT

The consecutive actions of the ubiquitin-selective segregase Cdc48 and the ubiquitin shuttle factor Rad23 result in the delivery of ubiquitylated proteins at the proteasome. Here, we show that the deubiquitylating enzyme Ubp12 interacts with Cdc48 and regulates proteasomal degradation of Rad23-dependent substrates in *Saccharomyces cerevisiae*. Overexpression of Ubp12 results in stabilization of Rad23-dependent substrates. We show that Ubp12 removes short ubiquitin chains from the N-terminal ubiquitin-like domain (UbL) of Rad23. Preventing ubiquitylation of Rad23 by mutation of lysine residues within the UbL domain, $Rad23^{UbLK0}$, does not affect the non-proteolytic role of Rad23 in DNA repair but causes an increase in ubiquitylated cargo bound to the UBA2 domain of Rad23, recapitulating the stabilization of Rad23-dependent substrates observed upon overexpression of Ubp12. Expression of $Rad23^{UbLK0}$ or overexpression of Ubp12 impairs the ability of yeast to cope with proteotoxic stress, consistent with inefficient clearance of misfolded proteins by the ubiquitin–proteasome system. Our data suggest that ubiquitylation of Rad23 plays a stimulatory role in the degradation of ubiquitylated substrates by the proteasome.

KEY WORDS: Rad23, Degradation, Proteasome, Ubiquitin, Ubiquitin shuttle factor, Deubiquitylating enzyme, Yeast

INTRODUCTION

Post-translational modifications with the protein modifier ubiquitin are best known for their critical role in targeting proteins for proteasomal degradation (Hershko and Ciechanover, 1998). More recent work has revealed the existence of an elaborated ubiquitin code that orchestrates not only proteasomal degradation but also a broad array of other cellular processes by regulating protein–protein interactions through ubiquitylation (Komander and Rape, 2012). Because of the wealth of knowledge on the ubiquitin–proteasome system (UPS) and its many players, this essential proteolytic system remains an important paradigm for understanding the spatial and temporal control of interactions between ubiquitylated proteins and ubiquitin-binding proteins. Although ubiquitin modifications have been originally considered as a point of no return, leading inevitably to the destruction of the tagged protein, it has become apparent that a number of proteins not only handle ubiquitylated proteins but can also change their fate (Crosas et al., 2006; Hanna et al., 2006; Richly et al., 2005; Verma et al., 2004). These proteins that share the ability to interact with ubiquitylated proteins – either through the presence of ubiquitin-binding domains or by interacting with proteins that contain these motifs – can, depending on their mode of action, either promote or prevent the degradation of ubiquitylated proteins.

In yeast, the ubiquitin-selective segregase Cdc48 [also known as valosin-containing protein (VCP) or p97 in mammals] is an interaction hub for proteins that modulate ubiquitylated proteins (Jentsch and Rumpf, 2007). While the intrinsic segregase activity of this AAA-ATPase appears to prepare proteins for destruction by extracting them from macromolecular complexes (Dai et al., 1998; Elkabetz et al., 2004) and/or generating unfolded structures that allow efficient proteasomal degradation (Beskow et al., 2009), it also associates with a number of proteins, including ubiquitin ligases, ubiquitin elongation factors and deubiquitylating enzymes (DUBs), that modify the ubiquitin signal on the substrates (Richly et al., 2005). Moreover, Cdc48 binds the ubiquitin shuttle factor Rad23 (Baek et al., 2011), which sequesters ubiquitylated proteins and delivers them to the proteasome (Schauber et al., 1998). The ubiquitin-like (UbL) domain of Rad23, which resembles ubiquitin but has distinct properties, is critical for the interaction of Rad23 with Cdc48 and the proteasome (Lambertson et al., 2003). It has been proposed that Cdc48 and Rad23 facilitate the destruction of proteasome substrates by ensuring that the substrates reach the proteasome in a state allowing efficient degradation (Richly et al., 2005). Consistent with this model, insertion of a short peptide extension that is sufficiently long to function as an unstructured initiation site for proteasomal degradation abrogates the need for both Cdc48 and Rad23 (Gödderz et al., 2015).

Rad23 interacts with the proteasome by means of its N-terminal UbL domain, while the ubiquitin-associated (UBA)-2 domain located at its most extreme C-terminus functions as an intrinsic stabilization signal (Heessen et al., 2005) that hinders initial unfolding events at the entrance of the proteasome, allowing Rad23 to resist proteasomal degradation (Fishbain et al., 2011; Heinen et al., 2011). This endows Rad23 to function as a reusable shuttle that delivers its ubiquitylated cargo, which is bound to the ubiquitin-binding UBA1 and UBA2 domains, to the proteasome where the cargo will selectively bind to the ubiquitin receptors that reside in the regulatory particle of the proteasome (Finley, 2009). Rad23 shares its molecular structure and function with Dsk2 and Ddi1, two other ubiquitin shuttle factors with distinct but overlapping substrate specificities (Crosas et al., 2006). These three ubiquitin shuttle factors potentially provide an additional layer of specificity in substrate targeting that may enable the cell to prioritize a specific set of substrates.

Although it is presently unclear whether the functionality of ubiquitin shuttle factors is differentially regulated, there are at least two molecular mechanisms that may control the activity of these

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scaffold proteins. First, specific binding proteins may interact with the functional domains in the ubiquitin shuttle proteins and prevent them from collecting and/or delivering their ubiquitylated cargo. In this respect, it is noteworthy that the peptidyl-tRNA hydrolase Pth2 has been reported to interact with the UbL domain of Rad23 and Dsk2 restricting their access to the proteasome (Ishii et al., 2006). Moreover, the extraproteasomal population of the ubiquitin-binding receptor Rpn10 selectively binds Dsk2 and prevents it from docking to the proteasome (Matiuhin et al., 2008). Second, post-translational modifications may change the behavior of the ubiquitin shuttle factors and may provide a means to control their activity. Notably, phosphorylation of the UbL domain of Rad23 regulates its interaction with the proteasome (Liang et al., 2014), while modification of Dsk2 with proteolytic ubiquitin chains results in a reduced capacity to bind polyubiquitylated proteins (Sekiguchi et al., 2011). Moreover, recruitment of the ubiquitin-binding receptor Rpn10 is regulated by monoubiquitylation (Isasa et al., 2010). Thus, the functional status of ubiquitin shuttle factors can be modulated by protein–protein interactions and post-translational modifications.

In the present study, we show that Rad23 is modified in its UbL domain with short polyubiquitin chains and identified Ubp12 as a Cdc48-interacting DUB that selectively reverses this modification. Interestingly, abrogating Rad23 ubiquitylation causes an increase in the amount of ubiquitylated cargo associated with Rad23, which, despite being proficient for binding to the proteasome, fails to facilitate proteasomal degradation of the substrates. Our data show that ubiquitylation of Rad23 is important for proper proteasomal degradation and suggest that this Ubp12-controlled mechanism may control the release of ubiquitylated proteins at the proteasome.

RESULTS

The DUB Ubp12 interacts with Cdc48 and stabilizes proteasome substrates

Yeast Cdc48 and the mammalian orthologue VCP/p97 are known to interact with a number of proteins involved in ubiquitylation, including the deubiquitylating enzymes Otu1 (Rumpf and Jentsch, 2006) and ataxin-3 (Wang et al., 2006), respectively. We found that the poorly characterized ubiquitin-specific protease Ubp12 also interacts with Cdc48 in the budding yeast *Saccharomyces cerevisiae*. We observed that immunoprecipitation of endogenous Cdc48 resulted in co-purification of HA-tagged Ubp12 ($Ubp12^{6HA}$) expressed from the endogenous promoter (Fig. 1A). This interaction was further validated by the reciprocal immunoprecipitation, in which endogenous Cdc48 was pulled down with FLAG-tagged Ubp12 ($Ubp12^{FLAG}$) (Fig. 1B).

Ubp12 is a member of the ubiquitin-specific protease (Ubp) family that is comprised of 16 members in yeast, none of which are essential for viability (Reyes-Turcu et al., 2009). These proteins share a conserved catalytic core domain and possess terminal extensions and insertions. Ubp12 is a large protein containing a DUSP domain in its N-terminal part, which has been suggested to be important for protein–protein interactions (Song et al., 2010), and the USP catalytic domain, which compromises the central part of the protein (Fig. 1C). To further characterize the interaction of Ubp12 with Cdc48, two constructs expressing FLAG-tagged fragments of Ubp12 either containing the N-terminal DUSP domain ($Ubp12\text{-}N^{FLAG}$) or the C-terminal USP domain ($Ubp12\text{-}C^{FLAG}$) were generated. Interestingly, Cdc48 primarily interacted with the C-terminal fragment that included the catalytic USP domain (Fig. 1D).

To understand the functional importance of the Ubp12–Cdc48 interaction, we analyzed the degradation of Cdc48-dependent proteasomal substrates. Cdc48 has originally been implicated in the UPS by its identification as a critical factor for proteasomal degradation of ubiquitin fusion degradation (UFD) substrates (Johnson et al., 1995) and N-end rule substrates (Ghislain et al., 1996). In line with a role of Ubp12 in degradation of Cdc48-dependent substrates, turnover analysis revealed that the UFD substrate Ub^{G76V}–GFP was stabilized upon overexpression of Ubp12 (Fig. 1E), while expression of the catalytically inactive $Ubp12^{C372S}$ mutant did not delay degradation, indicating that its deubiquitylating activity is required for the stabilizing effect (Fig. S1A). In addition, degradation of the N-end rule substrate Ub-R–GFP, which is targeted by its N-terminal arginine residue upon cleavage of the ubiquitin moiety by ubiquitin C-terminal hydrolyses (Varshavsky, 1996), was impaired upon overexpression of Ubp12 (Fig. 1F). Surprisingly, the Cdc48-dependent model substrate DEG1–GFP, which is based on the natural degradation signal of the yeast mating factor Matα2 (Chen et al., 1993), was not affected by Ubp12 overexpression (Fig. 1G). Taken together, these data show that Ubp12 is a Cdc48-interacting DUB that stabilizes some, but not all, Cdc48-dependent substrates.

Ubp12 modulates Rad23 ubiquitylation

We noticed that a difference between the Cdc48-dependent substrates assessed in the above experiments is their reliance on the ubiquitin shuttle factor Rad23 for proteasomal degradation. Thus, whereas Ub^{G76V}–GFP (Fig. S1B) and Ub-R–GFP (Fig. S1C), which are stabilized by Ubp12 overexpression, require Rad23 for degradation (Gödderz et al., 2015), DEG1 is degraded in a Rad23-independent fashion (Medicherla et al., 2004). Since Rad23 operates downstream of Cdc48 by delivering ubiquitylated substrates to the proteasome (Richly et al., 2005), an effect of Ubp12 on Rad23-dependent degradation would be consistent with a stabilizing role of Ubp12 in a specific branch of the Cdc48 pathway. To test a possible function of Ubp12 in Rad23-dependent degradation, we determined whether Ubp12 physically interacts with Rad23. Indeed, we found that immunoprecipitation of $Ubp12^{FLAG}$ resulted in the co-precipitation of Rad23 (Fig. 2A). This interaction was further corroborated by the reciprocal immunoprecipitation, in which $Ubp12^{6HA}$ was pulled down with endogenous Rad23 (Fig. 2B). The interaction between Ubp12 and Rad23 appeared to be more robust than the Ubp12–Cdc48 interaction raising the question of whether Ubp12 binds Cdc48 indirectly through its interaction with Rad23. However, this possibility was excluded since we could co-immunoprecipitate Cdc48 and Ubp12 in a strain that lacked Rad23 (Fig. 1B). Interestingly, the interaction with Ubp12 was strictly dependent on the N-terminal UbL domain of Rad23 (Fig. 2C).

Rad23 has been found in a large-scale mass spectrometric analysis to be ubiquitylated at multiple lysine residues in its UbL domain but the functional significance of these modifications has remained elusive (Swaney et al., 2013). Immunoprecipitation of endogenous Rad23 indeed revealed the presence of a band that corresponded in size to ubiquitylated Rad23 and that was shifted to a slightly higher molecular mass in yeast overexpressing Myc-tagged ubiquitin (^{Myc}Ub), consistent with these bands representing Rad23 modified with wild-type or epitope-tagged ubiquitin, respectively (Fig. 2D). To validate the presence of ubiquitylated Rad23 in yeast lysates, we precipitated His-tagged ubiquitin (6×His–Ub) under denaturing conditions using NiNTA beads, in order to avoid co-immunoprecipitation of non-covalently bound ubiquitin conjugates, and probed the precipitated ubiquitin conjugates for endogenous Rad23. This sensitive method revealed

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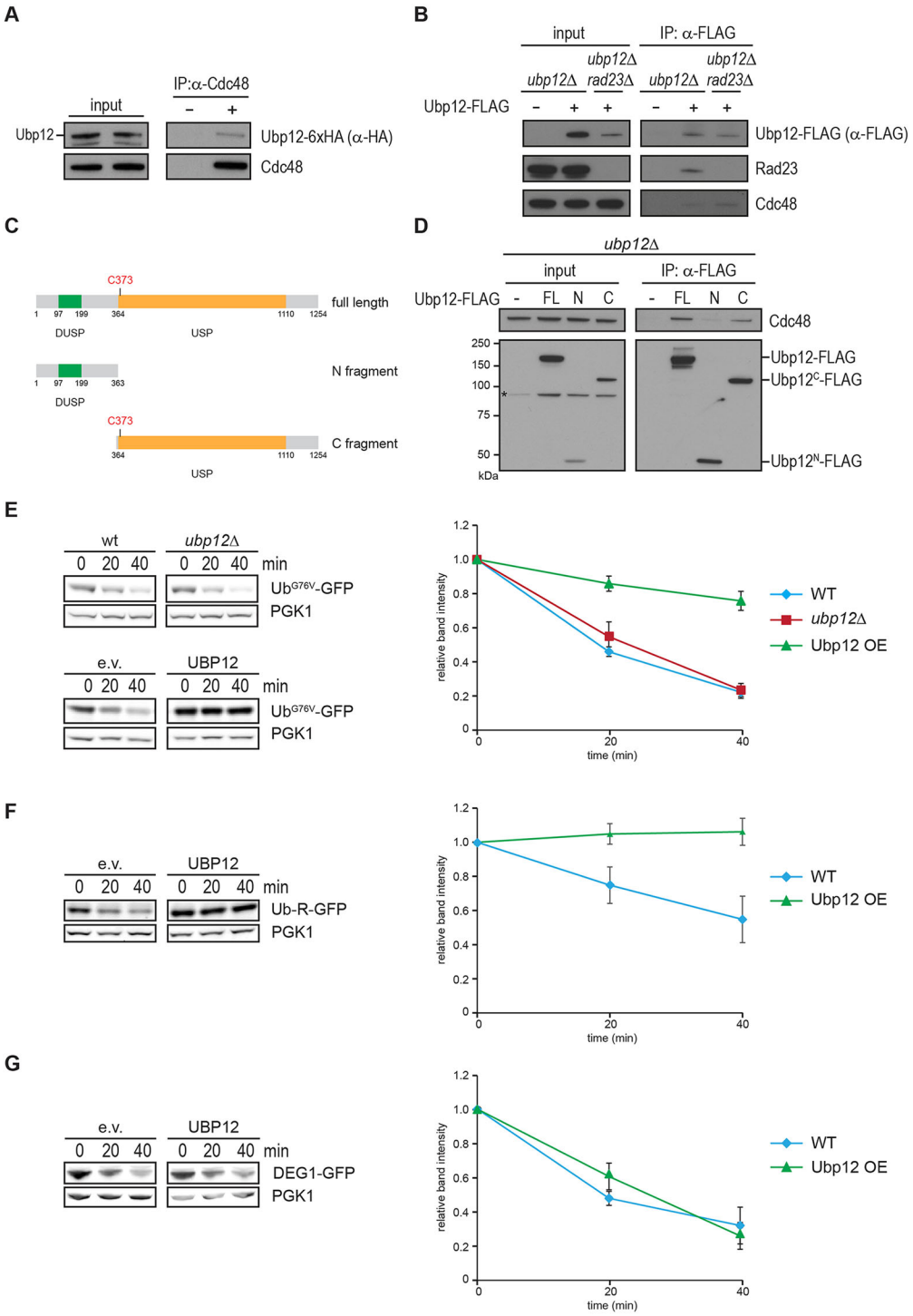

Fig. 1. The DUB Ubp12 interacts with Cdc48 and stabilizes Rad23-dependent substrates. (A) Endogenous Cdc48 was immunoprecipitated (IP) with a Cdc48-specific antibody from wild-type yeast expressing endogenously 6×HA-tagged Ubp12. Non-specific IgG was used as control. Immunoprecipitated proteins were analyzed by immunoblotting using antibodies indicated on the right. As a reference 2% of total input lysate was analyzed in parallel. (B) Anti-FLAG immunoprecipitation was performed from *ubp12*Δ or *ubp12*Δ*rad23*Δ yeast transformed with either empty vector (–) or Ubp12-FLAG-expressing vector (+). Input controls correspond to 2% of total lysate. Immunoprecipitated proteins were analyzed by immunoblotting using antibodies indicated on the right. (C) Schematic representation of the domain structure of the Ubp12 protein. Two fragments were generated for characterization of Ubp12–Cdc48 interaction. (D) Anti-FLAG immunoprecipitation was performed as in B from *ubp12*Δ yeast overexpressing either FLAG-tagged full-length Ubp12 (FL), the N- or the C-terminal fragment as depicted in C. Input controls correspond to 2% of total lysate. Immunoprecipitated proteins were analyzed by immunoblotting using antibodies indicated on the right. The position of molecular mass markers (kDa) is depicted on the left. A cross-reactive band is marked with an asterisk. (E) Turnover of the UFD model substrate Ub^{G76V}-GFP was determined by cycloheximide chase assay in a wild-type strain (wt), in wild-type yeast containing either empty vector (e.v.) or overexpressing Ubp12-FLAG from a high-copy plasmid (UBP12) or *ubp12*Δ yeast. Proteins were analyzed by immunoblotting with GFP- and PGK1-specific antibodies. A quantification (mean±s.e.m.) of GFP levels normalized to PGK1 for three independent experiments is shown on the right. (F) As in E, turnover of N-end rule model substrate Ub-R–GFP was analyzed in wild-type yeast containing either empty vector (e.v.) or overexpressing Ubp12–FLAG (UBP12). (G) As in E, turnover of DEG1–GFP was analyzed in wild-type yeast that did or did not overexpress Ubp12–FLAG.

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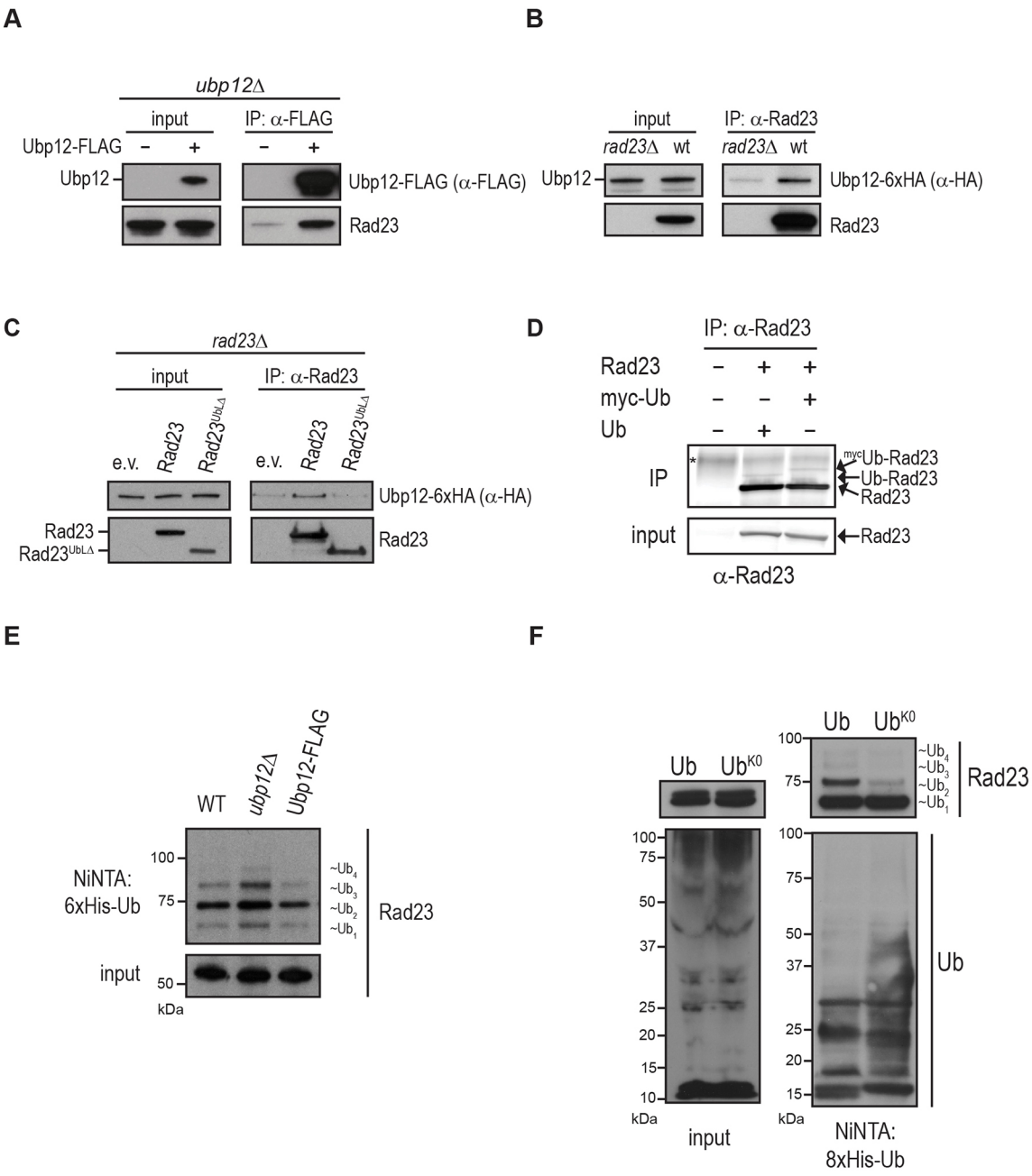

Fig. 2. Ubp12 modulates Rad23 ubiquitylation. (A) An anti-FLAG immunoprecipitation (IP) was performed from *ubp12Δ* yeast containing either empty vector (–) or overexpressing Ubp12–FLAG (+). Input controls correspond to 2% of total lysate. Immunoprecipitated proteins were analyzed by immunoblotting using antibodies indicated on the right. (B) Immunoprecipitation with a Rad23-specific antibody was performed from wild-type (wt) or *rad23Δ* yeast expressing endogenously 6×HA-tagged Ubp12. Immunoprecipitated proteins were analyzed by immunoblotting using antibodies indicated on the right. Input controls correspond to 2% of total lysate. (C) Anti-Rad23 immunoprecipitation was performed as in B from *rad23Δ* yeast with endogenously expressed 6×HA-tagged Ubp12 and containing low-copy plasmids expressing either full-length Rad23 or Rad23 lacking the UbL domain ($Rad23^{UbL\Delta}$). Input controls correspond to 2% of total lysate. Immunoprecipitated proteins were analyzed by immunoblotting using antibodies indicated on the right. (D) Anti-Rad23 immunoprecipitation was performed from either wild-type yeast or yeast expressing Myc-tagged ubiquitin (myc-Ub) as the sole source for ubiquitin. Immunoprecipitated proteins were analyzed by immunoblotting using a Rad23-specific antibody. Detected proteins are indicated on the right. A cross-reactive band is marked with an asterisk. (E) Denaturing Ni-NTA pulldown from wild-type, *ubp12Δ* or Ubp12–FLAG-overexpressing yeast co-expressing 6×His-tagged ubiquitin (6xHis-Ub) was performed. Immunoprecipitated proteins were analyzed by immunoblotting using a Rad23-specific antibody. Input controls correspond to 2% of total lysate. (F) Denaturing Ni-NTA pulldown from wild-type yeast expressing either wild-type (Ub) or lysine-less ubiquitin (Ub^{K0}) tagged with 8×His was performed. Precipitated proteins were analyzed by immunoblotting using antibodies indicated on the right. The position of molecular mass markers (kDa) is depicted on the left for E and F.

Rad23 species that were modified with up to four ubiquitin molecules, confirming that Rad23 is covalently modified with multiple ubiquitin moieties (Fig. 2E). We wondered whether Ubp12 expression levels had an effect on the ubiquitylation status of Rad23. Indeed, we found that the steady-state levels of these ubiquitin-modified Rad23 species were increased in a strain lacking Ubp12 (*ubp12Δ*) and decreased upon overexpression of Ubp12 in line with Ubp12 being a DUB that targets Rad23 ubiquitylation (Fig. 2E). In order to investigate whether this activity is unique to Ubp12, we analyzed the ubiquitylation status of Rad23 using

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deletion strains for each of the non-essential DUBs belonging to the Ubp family, except for Ubp9 and Ubp15, as well as the non-essential DUB Otu1 and the ubiquitin C-terminal hydrolase Yuh1 (Fig. S2A). We found that only deletion of Ubp3 also resulted in an increase of ubiquitylated Rad23 (Fig. S2B). However, unlike the situation in the *ubp12Δ* strain, deletion of Ubp3 was accompanied by a general accumulation of ubiquitin conjugates (Fig. S2C). Moreover, simultaneous deletion of Ubp3 and Ubp12 resulted in a further increase, suggesting that Ubp3 causes an increase in ubiquitylated Rad23 by a different mechanism than Ubp12, consistent with the general effect on ubiquitin conjugates (Fig. S2B). Even though this does not exclude the possibility that Ubp3 plays a role in regulating the ubiquitylation status of Rad23, we decided to focus on Ubp12 as we anticipated that the more general effect of Ubp3 deletion on protein ubiquitylation would complicate deciphering the contribution of Rad23 ubiquitylation to this phenotype.

Instead of a smear of high molecular mass ubiquitylated species, which is typically observed for polyubiquitylated substrates, we predominantly detected a limited number of distinct bands that corresponded in size to Rad23 being modified with one to four ubiquitin moieties. To discriminate whether these ubiquitin-modified species represented Rad23 modified with a short ubiquitin chain or Rad23 that was monoubiquitylated at multiple lysine residues, we analyzed whether overexpression of a His-tagged lysine-lacking ubiquitin (8×His–Ub^{K0}) in yeast expressing endogenous wild-type ubiquitin affected the abundance of these modified species. If Rad23 is subject to multiple monoubiquitin modifications, the expression of 8×His–Ub^{K0} should not affect their relative abundance. However, in the case of short ubiquitin chains, the band corresponding to a single ubiquitin conjugated to Rad23 should not be affected, while the larger species would be expected to be reduced since 8×His–Ub^{K0} will function as a chain terminator. In line with the latter possibility, the bands corresponding to Rad23 with two, three and four ubiquitin moieties were reduced in the presence of 8×His–Ub^{K0} while the monoubiquitylated Rad23 remained at the same level (Fig. 2F). Taken together, our data indicate that Rad23 is modified with short ubiquitin chains that are negatively regulated by Ubp12.

The UbL domain of Rad23 is ubiquitylated

We aimed to identify the lysine residue within Rad23 that is modified with the ubiquitin chain. Rad23 contains 15 lysine residues of which all, except for one, are located in the N-terminal UbL domain (Fig. 3A). Supporting the idea that the UbL domain is the target for this ubiquitylation event, we observed that deletion of this domain abrogated ubiquitylation of Rad23 (Fig. 3B). In order to identify the lysine residue(s) that serves as ubiquitin acceptor site(s) in Rad23, we generated a collection of Rad23 mutants in which one or several lysine residues were simultaneously replaced by arginine residues (Fig. S3). Analysis of the ubiquitylation status of these Rad23 variants showed that all mutants containing lysine residues in the UbL domain were subject to ubiquitin conjugation while ubiquitylation did not occur when all 14 lysine residues in the UbL domain had been replaced by arginine residues ($Rad23^{UbLK0}$) (Fig. 3C). This suggested that the short ubiquitin chain that modifies Rad23 could be conjugated in a promiscuous fashion to multiple lysine residues within the UbL domain suggesting a relaxed stringency of the ubiquitin ligase(s) involved. To empirically address this, we compared the ubiquitylation pattern of three Rad23 mutants that each contained a single lysine residue at different positions in the UbL domain. Each of these Rad23 mutants was ubiquitylated, confirming that a single lysine residue in various positions can restore ubiquitylation of Rad23 (Fig. 3D). We noted differences in the ubiquitylation efficiencies and ubiquitylation pattern depending on the position of the lysine residue, suggesting that the ubiquitin modification may quantitatively and qualitatively differ depending on the lysine residue that is targeted. The exact nature of these differences, as well as their functional significance, awaits further clarification. Importantly, our data show that all lysine residues in the UbL domain have to be removed in order to abrogate Rad23 ubiquitylation.

The UbL domain of Rad23 functions in an analogous manner to a degradation signal without resulting in actual degradation of Rad23 because of its C-terminal UBA2 domain, which acts as an intrinsic stabilization domain that prevents degradation (Heessen et al., 2005), by hindering the initiation of unfolding events required for proteasomal degradation (Fishbain et al., 2011; Heinen et al., 2011). We wondered whether these ubiquitin chains could be responsible for the ability of the UbL domains to target Rad23 for proteasomal degradation upon inactivation of the protective UBA2 domain. We have shown previously that mutation of the leucine residue at position 392 to an alanine residue abrogates the protective effect of the UBA2 domain and converts Rad23 into an efficient substrate for proteasomal degradation (Heessen et al., 2005). However, we found that a Rad23 mutant in which the L392A mutation was combined with replacement of all the lysine residues in the UbL domain ($Rad23^{UbLK0,L392A}$) was still unstable, suggesting that the UbL domain does not require ubiquitylation to target this mutant for degradation (Fig. 3E). This also shows that the mutant UbL domain lacking all lysine residues is proficient in targeting Rad23 for degradation and therefore still capable of interacting with the proteasome.

Preventing Rad23 ubiquitylation stabilizes substrates and enhances levels of ubiquitylated cargo

To investigate the role of ubiquitylation in proteasomal degradation, we compared the functionality of Rad23 and $Rad23^{UbLK0}$ in the turnover of the Rad23-dependent substrate Ub^{G76V}–GFP and Rad23-independent substrate DEG1–GFP. This revealed that wild-type Rad23 fully restored degradation of Ub^{G76V}–GFP in a *rad23Δ* strain, while $Rad23^{UbLK0}$ only had a partial rescuing effect (Fig. 4A) that was comparable in magnitude to what was observed upon Ubp12 overexpression (the effect of Ubp12 overexpression on Ub^{G76V}–GFP can be seen in Fig. 1E). As anticipated, deletion of Rad23 did not affect the degradation of DEG1–GFP but, notably, degradation was neither distorted by overexpression of $Rad23^{UbLK0}$, again suggesting that this mutant does not cause a general impairment of ubiquitin-dependent degradation (Fig. 4B). Thus, replacement of lysine residues in the UbL domain impairs the ability of Rad23 to facilitate degradation of specific substrates, without causing a general impairment of proteasomal degradation, in line with a function of Rad23 ubiquitylation in proteasomal degradation.

Since Rad23 functions as a scaffold protein that mediates degradation by simultaneously binding the proteasome and ubiquitylated substrates, we argued that the stabilizing effect of Rad23 ubiquitylation was likely due to either an impaired binding to the proteasome or an altered interaction with ubiquitylated substrates. Notably, it has been shown previously that replacement of the lysine residue at position 7 in the UbL domain with an alanine residue strongly reduced binding of Rad23 to the proteasome (Kim et al., 2004), raising the question that the interaction between mutant

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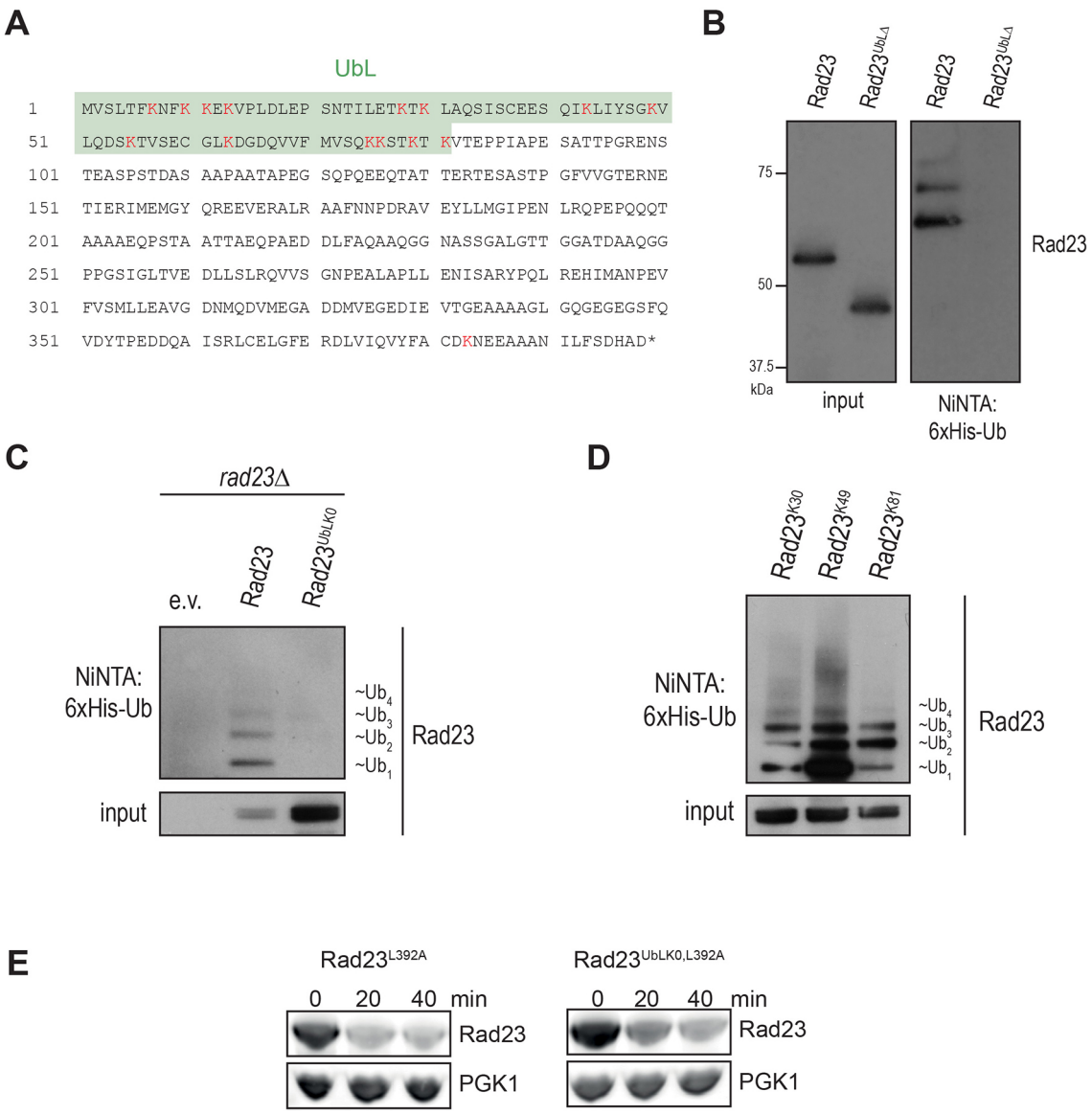

Fig. 3. The UbL domain of Rad23 is ubiquitylated. (A) The amino acid sequence of Rad23 is depicted, with the UbL domain marked by green shading and lysine residues highlighted in red. (B) Denaturing Ni-NTA pulldown from *rad23*Δ yeast co-expressing 6×His–Ub and either full-length Rad23 or Rad23$^{UbL\Delta}$ was performed. Precipitated proteins were analyzed by immunoblotting using Rad23-specific antibody. The position of molecular mass markers (kDa) is depicted on the left. (C) Denaturing Ni-NTA pulldown from *rad23*Δ yeast cells co-expressing 6×His–Ub and either wild-type Rad23 or a variant with all lysine residues in the UbL domain mutated to arginine (Rad23^{UbLK0}) was performed. Bound proteins were analyzed by immunoblotting using Rad23-specific antibody. (D) Same as in B, where denaturing Ni-NTA pulldown was performed, but from yeast expressed Rad23 variants, in which the indicated single lysine residues had been reintroduced. (E) Turnover of Rad23^{L392A} and Rad^{UbLK0,L392A} was determined by a cycloheximide chase assay in wild-type yeast. The membranes were probed with Rad23- and PGK1-specific antibodies.

Rad23^{UbLK0} and the proteasome may be impaired. On the other hand, as mentioned above (see Fig. 3E), we noted that Rad23^{UbLK0,L392A} was degraded by the proteasome, suggesting that the UbLK0 domain is still able to interact with the proteasome. To probe further into this issue, we compared the binding of the proteasome to Rad23 and Rad23^{UbLK0} by immunoprecipitation and observed that indeed both proteins interacted with the proteasome (Fig. 4C). We also analyzed the binding of Cdc48 (Fig. S4A) and Ubp12 (Fig. S4B) to Rad23^{UbLK0} and found that both still interacted with mutant Rad23. This suggests that ubiquitylation of the UbL domain is not required for binding of the UbL domain to the proteasome, Cdc48 or Ubp12 and at the same time confirms that the overall structural integrity of the UbLK0 domain is not affected by the lysine mutations.

We investigated whether binding of ubiquitylated cargo to Rad23 was altered in the absence of lysine residues in the UbL domain. Interestingly, we observed a strong increase in ubiquitylated cargo bound to Rad23^{UbLK0} (Fig. 4D). Introduction of a mutation in the UBA1 domain of Rad23^{UbLK0} caused a substantial reduction in co-immunoprecipitation of ubiquitin conjugates (Fig. 4E). Mutation of the UBA2 domain gave a more subtle reduction in co-immunoprecipitated ubiquitin conjugates, and combining the mutations in the UBA1 and the UBA2 had an additive effect (Fig. 4E). This implies that the UbLK0 domain increases the load of ubiquitylated proteins bound to Rad23, while the ubiquitin conjugates are still bound to the same two UBA domains that facilitate their recruitment to wild-type Rad23 (Bertolaet et al., 2001; Raasi et al., 2005). A possible explanation could be that

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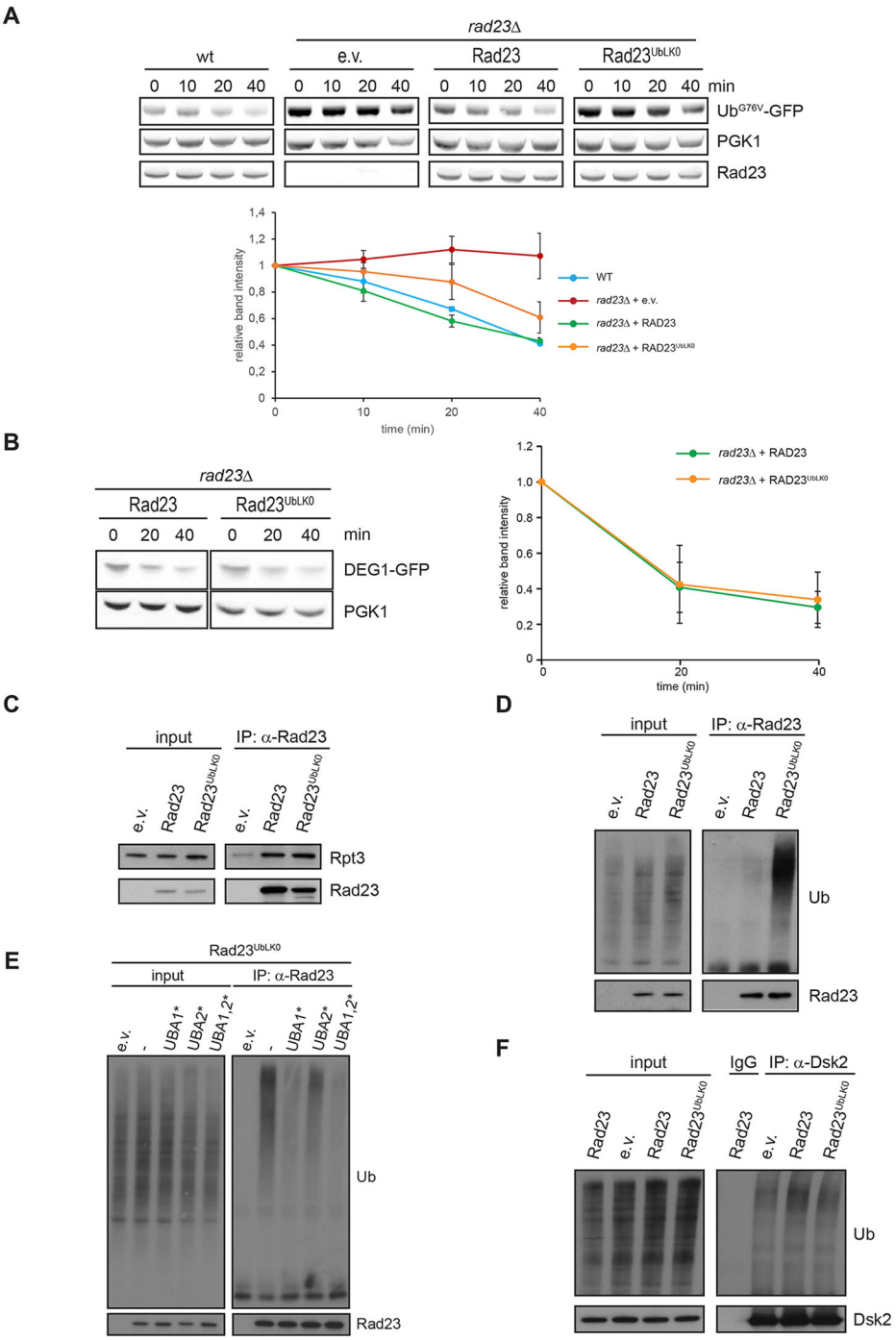

Fig. 4. Preventing Rad23 ubiquitylation stabilizes substrates and enhances levels of ubiquitylated cargo. (A) Turnover of Ub^{G76V}–GFP was determined by a cycloheximide chase assay in wild-type (wt) or *rad23Δ* yeast cells containing either empty vector (e.v.) or a low-copy plasmid expressing Rad23 or $Rad23^{UbLK0}$. Proteins were analyzed by immunoblotting. A quantification (mean±s.e.m.) of GFP levels normalized to PGK1 for three independent experiments is shown. (B) Turnover of DEG1–GFP was monitored by a cycloheximide chase assay in *rad23Δ* yeast expressing either wild-type Rad23 or $Rad23^{UbLK0}$. A quantification (mean±s.e.m.) of GFP levels normalized to PGK1 for three independent experiments is shown. (C) Rad23-specific immunoprecipitation (IP) was performed from *rad23Δ* yeast expressing either wild-type Rad23 or $Rad23^{UbLK0}$. The interaction with proteasome was analyzed by means of an antibody against the specific proteasome subunit Rpt3. The input control corresponds to 2% of total lysate. e.v., empty vector. (D) To assess the binding of ubiquitylated cargo to Rad23, Rad23 was immunoprecipitated from *rad23Δ* yeast containing either empty vector or a low-copy plasmid expressing Rad23 or $Rad23^{UbLK0}$. Immunoprecipitated proteins were analyzed by immunoblotting using antibodies against Rad23 and ubiquitin. The input control corresponds to 2% of total lysate. (E) As in D, Rad23-specific immunoprecipitation was performed from *rad23Δ* yeast expressing either empty vector or a low-copy plasmid expressing Rad23 variants in which the UBA1, UBA2 or UBA1 and UBA2 had been mutated to abrogate their ubiquitin-binding properties. Expression was induced by using 100 µM $CuSO_4$, and proteasomal activity was blocked by addition of 40 µM MG132 in all cultures. (F) Dsk2 was immunoprecipitated from *rad23Δ* yeast containing low-copy plasmids expressing the indicated Rad23 variants. IgG was used as negative immunoprecipitation control. Bound proteins were analyzed by immunoblotting using antibodies against Dsk2 and ubiquitin. The input control corresponds to 2% of total lysate.

$Rad23^{UbLK0}$ impairs the degradation of ubiquitylated proteins resulting in a global increase in ubiquitin conjugates, which may increase the amount of substrates that are captured by Rad23. However, we did not observe a general increase in ubiquitin conjugates in yeast expressing $Rad23^{UbLK0}$, arguing against this scenario (Fig. 4E). Moreover, if this were the case, other ubiquitin shuttle factors would also be expected to display an increase in the load of polyubiquitylated substrates. To probe into this, we immunoprecipitated the related ubiquitin shuttle factor Dsk2 in yeast expressing either wild-type Rad23 or $Rad23^{UbLK0}$. Unlike the situation for Rad23, we found that the levels of ubiquitylated proteins that were bound to Dsk2 were comparable under both conditions (Fig. 4F). We therefore conclude that preventing ubiquitylation of the UbL domain of Rad23 does not interfere with the UbL-dependent binding of Rad23 to the proteasome but selectively increases the pool of ubiquitylated substrates that is associated with the UBA domains of Rad23.

$Rad23^{UbLK0}$ is proficient in DNA repair but defective in proteasomal degradation

Rad23 does not only play important roles in proteasomal degradation but is also involved in nucleotide excision repair, which is the primary pathway for removing helix-distorting lesions from DNA (Dantuma et al., 2009). Although the latter is dependent on the UbL domain of Rad23 and involves also the proteasome, it is believed to be a largely non-proteolytic process as it does not require the degradation capacity

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of the proteasome (Russell et al., 1999). We observed that the ultraviolet (UV) light sensitivity of the *rad23Δ* strain could be rescued by expression of wild-type Rad23 but not by Rad23 lacking the UbL domain (Rad23$^{UbL\Delta}$), consistent with a critical role of the UbL domain in nucleotide excision repair (Fig. 5A). However, expression of Rad23^{UbLK0} gave a comparable rescue of the viability of yeast upon UV exposure to wild-type Rad23 demonstrating that the UbLK0 domain is functional in DNA repair and arguing against a role for Rad23 ubiquitylation in nucleotide excision repair.

Owing to redundancy of the ubiquitin shuttle factors and the intrinsic ubiquitin receptors in the proteasome in substrate delivery, deficiencies in any of these factors give relatively mild phenotypes unless multiple delivery proteins are simultaneously compromised. To unmask the effects of Rad23^{UbLK0} on proteasomal degradation, we therefore studied the effect of this Rad23 variant in yeast that lacked the ubiquitin receptor Rpn10 proteasome subunit. Deletion of both Rpn10 and Rad23 results in a mild growth impairment that is aggravated when yeast was grown at a low temperature, a condition that enhances proteotoxic stress. We expressed Rad23, Rad23 lacking the UbL domain or Rad23^{UbLK0} in an *rpn10Δrad23Δ* strain. Whereas expression of wild-type Rad23 strongly enhanced growth at 18°C as well as 30°C, expression of either ΔUbL or UbLK0 did not rescue growth suggesting that the lysine residues in the UBL domain are required for the function of Rad23 in proteasomal degradation (Fig. 5B). The importance of the lysine residues within the UbL domain for the ability of yeast to deal with proteotoxic stress was confirmed by the observation that expression of wild-type Rad23, but not Rad23^{UbLK0}, restored growth of the *rad23Δ* strain in the presence of canavanine, an arginine analogue that causes severe proteotoxic stress (Fig. 5C). In addition, this phenotype was aggravated when canavanine sensitivity was analyzed in the *rad23Δrpn10-UIM* strain, which in addition to the Rad23 deletion also expressed an Rpn10 subunit that lacked the ubiquitin interaction motif (UIM). Thus, whereas wild-type Rad23 improved the growth of the *rad23Δrpn10-UIM* strain, the Rad23^{UbLK0} variant was unable to restore growth (Fig. 5D). This phenotype is similar to the one observed in wild-type

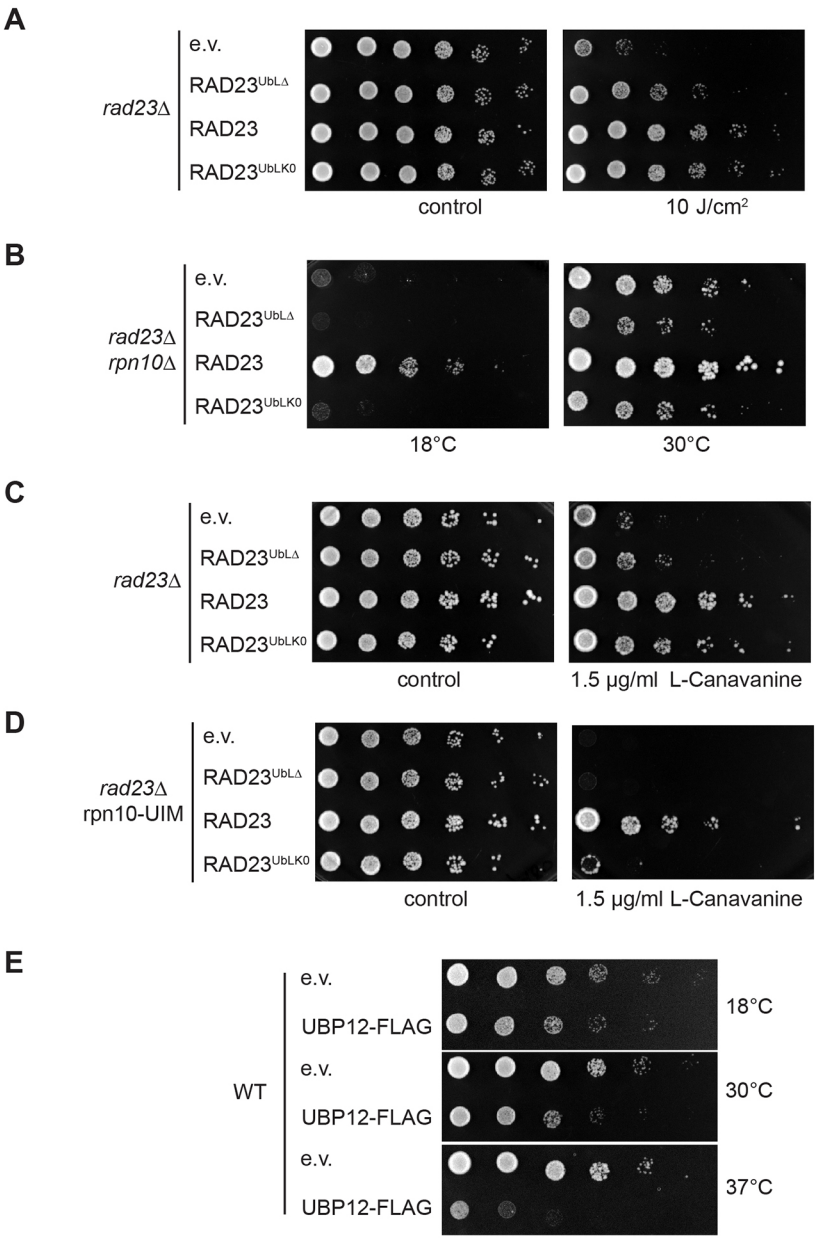

Fig. 5. Rad23^{UbLK0} is proficient in DNA repair but defective in proteasomal degradation. (A) The *rad23Δ* yeast were transformed with empty vector (e.v.) or plasmids expressing either Rad23$^{UbL\Delta}$, Rad23 or Rad23^{UbLK0}. Yeast cultures were grown to exponential phase, and five-fold dilutions were spotted on synthetic medium and exposed to UV light of the indicated intensity prior to incubation at 30°C for 3 days. (B) The *rad23Δ*/*rpn10Δ* strain was transformed with empty vector or plasmids expressing either Rad23$^{UbL\Delta}$, Rad23 or Rad23^{UbLK0}. Yeast cultures were grown to exponential phase, and five-fold dilutions were spotted on synthetic medium and grown at 30°C or 18°C. Cell growth was examined after 3 to 5 days of incubation, respectively, depending on the temperature. (C) As in A, but yeast were spotted on synthetic medium or synthetic medium containing 1.5 µg/ml L-canavanine and incubated at indicated temperatures for 3 days. (D) As in C, but *rad23Δ*/rpn10-UIM yeast were used. (E) Wild-type (WT) yeast were transformed with empty vector or an expression vector for Ubp12–FLAG. Cell growth was examined at 18°C, 30°C or 37°C after 3 to 5 days, respectively.

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yeast overexpressing $Ubp12^{FLAG}$, which displayed reduced growth when yeast was cultured at an elevated (37°C) temperature, while a very mild effect was observed at 18°C or 30°C, suggesting that proteotoxic stress enhances the growth suppressive effect of Ubp12 overexpression (Fig. 5E). We wondered whether the growth defect caused by Ubp12 overexpression could be rescued by simultaneous overexpression of wild-type Rad23 with the rational that this may restore the levels of ubiquitylated Rad23 although at the expense of having very high levels of unmodified Rad23. Unfortunately, overexpression of Rad23 and Ubp12 caused a synthetic growth phenotype (Fig. S5), which may be attributed to the ability of Rad23 to cause a general inhibition of polyubiquitylation (Chen et al., 2001; Ortolan et al., 2000). Consistent with the fact that this function of Rad23 does not require its UbL domain (Ortolan et al., 2000), we found very similar effects when wild-type Rad23 or $Rad23^{UbLK0}$ were overexpressed in combination with Ubp12. Because of this practical limitation, we cannot decipher the contribution of Ubp12-mediated deubiquitylation to the growth defect observed upon overexpression of Ubp12. Taken together, our data support the model that the ubiquitylation status of the UbL domain of Rad23 is important for the function of Rad23 in proteasomal degradation, whereas it is not required for the non-proteolytic role of Rad23 in DNA repair. Thus, ubiquitylation of Rad23, which is regulated by Ubp12, stimulates proteasomal degradation of Rad23-dependent substrates, and interference with this process results in stabilization of Rad23-dependent substrates, increased load of ubiquitylated cargo on Rad23 and an impaired ability to deal with proteotoxic stress.

DISCUSSION

In the present study, we show that Rad23 is ubiquitylated in its N-terminal UbL domain and that this modification is reversed by Ubp12, which we identified as a Cdc48- and Rad23-interacting DUB. We show that preventing ubiquitylation of Rad23 by either replacing acceptor lysine residues in the UbL domain or by overexpressing Ubp12 impairs the degradation of Rad23-dependent substrates. Interestingly, stabilization of Rad23-dependent substrates is accompanied by an increased load of ubiquitylated proteins that are bound to the UBA domains of Rad23. Our data suggest that this defect in degradation of Rad23-dependent substrates is not caused by a reduced interaction with the proteasome or Cdc48, although we can presently not exclude the possibility that impaired interactions with another UbL-interacting protein contribute to substrate stabilization. Despite the fact that Ubp12 interacts with $Rad23^{UbLK0}$, it remains possible that this interaction is qualitatively different, causing the observed effects. If Ubp12 were to act on Rad23-associated substrates, this could also give rise to an increase in ubiquitylated cargo. However, we do not favor this model since it has been shown that ubiquitylated proteins are protected from the action of DUBs through their interaction with the UBA domains of Rad23 (Bertolaet et al., 2001; Ortolan et al., 2000). Furthermore, based on the stimulatory role of ubiquitin chains in the Cdc48–Rad23 delivery pathway (Richly et al., 2005), we would expect that longer polyubiquitin chains on Rad23-associated substrates in the absence of Ubp12 will accelerate their degradation, if the delivery function of Rad23 was not to be affected, whereas we observed the opposite. Therefore, we feel that our data are most consistent with a model where the presence of increased levels of ubiquitylated cargo in cells with a Rad23 mutant that is proficient for binding to the proteasome is due to impaired delivery of ubiquitylated proteins at the proteasome.

It has been proposed that the presence of several ubiquitin shuttle factors with different, though partially overlapping, specificities might function as a regulatory layer that determines the targeting rate of distinct pools of substrate (Verma et al., 2004). Although there are some examples of post-translational modifications and binding proteins that regulate the interaction of ubiquitin shuttle factors with the proteasome (Isasa et al., 2010; Liang et al., 2014; Sekiguchi et al., 2011), it is presently unclear if and how these processes are regulated by internal and/or external cues. It is feasible that, under certain conditions, Rad23 deubiquitylation behaves as a regulatory switch that stalls degradation of Rad23-dependent substrates, thereby giving higher priority to substrates that reach the proteasome by different means, such as alternative shuttle factors. However, we consider such a role for ubiquitylation of Rad23 less likely as we would expect such a regulatory switch to operate before the interaction with the proteasome since Rad23 would otherwise still compete with other ubiquitin shuttle factors for binding to the proteasome and thereby also negatively affect degradation of their cargo. Our data, however, show that $Rad23^{UbLK0}$ still interacts with the proteasome despite its impaired ability to facilitate degradation of substrates. Moreover, at any given time, only a small fraction of Rad23 is modified with ubiquitin conjugates whereas interference with Rad23 ubiquitylation has a strong effect on degradation, which would be more consistent with a transient modification of Rad23 being required for degradation than a conditional regulatory switch.

Based on the present data, we propose an alternative, although not mutually exclusive, model in which deubiquitylation of Rad23 changes its properties in a dynamic fashion that contributes to the directionality in substrate delivery. Interestingly, ubiquitin is not only a signal for delivery of proteins to the proteasome but also targets substrates to Cdc48 and other ubiquitin-binding proteins that act exclusively upstream of the proteasome (Schrader et al., 2009). Earlier work has shown that elongation of the ubiquitin chain ensures that substrates interact with Cdc48, Rad23 and the proteasome in a successive manner since the preferred ubiquitin chain length increases progressively along this route (Richly et al., 2005). However, while ubiquitin chain elongation may be important for the events that occur before the delivery at the proteasome, the proteasome itself has a broad specificity for ubiquitin modifications and hence additional cues may be required to facilitate the final delivery of the substrate (Grice and Nathan, 2016). Our data would be consistent with a model in which Rad23 deubiquitylation switches Rad23 into a mode that allows delivery of its cargo to proteasomes. Although we have been unable to identify a specific ubiquitin ligase responsible for ubiquitylation of Rad23, it is feasible that Rad23 ubiquitylation is generally linked to association with the proteasome since a broad variety of ubiquitin ligases have been shown to interact with the proteasome (Xie and Varshavsky, 2000). It is also noteworthy that Rad23 can adopt open and closed conformations due to intramolecular interactions between its UbL and UBA domains and this process can be regulated by the ubiquitin receptor Rpn10 (Walters et al., 2003). It is tempting to speculate that the ubiquitylation status of the UbL domain modulates this process. Thus, our observation of the ubiquitylation status of Rad23 being governed by Ubp12 and its implications for degradation of Rad23-dependent substrates gives new insights in the molecular mechanism underlying the targeting of substrates for proteasomal degradation.

MATERIALS AND METHODS

Yeast strains and plasmids

Yeast strains were grown according to standard procedures on complete or synthetic media supplemented with 2% (w/v) glucose. Cycloheximide

(Sigma) (100 µg/ml from a stock at 10 mg/ml in ethanol) or MG132 (Enzo) (50 µM from a stock at 10 mM in DMSO) was added when indicated. For the analysis of Rad23 ubiquitylation upon exclusive expression of Myc-tagged ubiquitin, the strain YD466, which is isogenic to SUB328, was used (Spence et al., 1995). Yeast strain SY980 (RPN10-UIM) was a gift from Suzanne Elsasser (Cell Biology, Harvard Medical School, USA) (Elsasser et al., 2004). A complete list of the yeast strains used in this study can be found in Table S1. Ubp12 harboring a C-terminal FLAG tag was expressed from the ADH1 promoter using the plasmid YEplac181 (2µm; LEU2) as reported before (Anton et al., 2013) (kindly provided by Mafalda Escobar-Henriques; (Institute for Genetics, University of Cologne, Germany)). The expression plasmid for Myc-tagged ubiquitin, the YEp112 plasmid (2 µ, URA3, pCUP1-Ub) and plasmids for wild-type and lysine-less ubiquitin (Ub and UbK0; 2 µ, LEU2) were provided by Jürgen Dohmen (Institute for Genetics, University of Cologne, Germany), Daniel Finley (Cell Biology, Harvard Medical School, USA) and Michael Glickman (Department of Biology, Technion-Israel Institute of Technology, Israel), respectively. A list of the plasmids used in this study can be found in Table S2.

Immunoprecipitations

The indicated strains were grown in 25 ml of the appropriate medium, and cells were harvested at an optical density at 600 nm (OD_{600}) of around 1, snap frozen and stored at −80°C, if needed. Cells were harvested, resuspended in 500 µl of ice-cold lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 20 mM NEM, 10 µM MG-132; for Ubp12 interaction, 0.01% digitonin in 1× TBS, 20 mM NEM, 10 µM MG-132) containing a protease inhibitor mix (complete, EDTA free, Roche) and lysed with an equal volume of acid-washed glass beads (0.4–0.6 mm diameter) by vortexing five times for 1 min with 1 min intervals on ice. Lysates were clarified by centrifugation at 16,000 ***g*** for 10 min at 4°C. Protein concentrations were quantified by the Bradford method, and lysates normalized to 200–500 µg total protein. A total of 10 µl lysate was collected as the input sample. For immunoprecipitation, the following antibodies were used: Rad23 (goat polyclonal antibody, 1.5 mg/µl, R&D systems, A302-306), Dsk2 (rabbit polyclonal antibody, 1:500, Abcam, ab4119-100), Cdc48 (rabbit polyclonal antibody, 1:500, Abcam, ab138298), and FLAG C-terminal (M2 mouse monoclonal antibody, 1:500, Sigma, F3165). Protein lysates were incubated with the indicated antibodies for 1 h at 4°C on a rotating wheel. Simultaneously, 40 µl of Protein G Dynabeads (Invitrogen) were blocked with 1% bovine serum albumin and also incubated under rotation for 1 h at 4°C. After blocking, magnetic beads were washed three times with wash buffer (lysis buffer without NEM and MG132) and added to the lysates, which were incubating for 1 h at 4°C on a rotating wheel. Before elution, beads were washed five times with wash buffer. For anti-FLAG immunoprecipitation, elution was performed with 1 µg/ml FLAG peptide in wash buffer for 2 h at 4°C. Otherwise, beads were incubated in 20 µl of SDS buffer for 5 min at 95°C. Input and eluate samples were analyzed by SDS-PAGE and western blotting.

Western blot analysis

The membranes were probed with antibodies directed against ubiquitin (rabbit polyclonal antibody, dilution 1:500, DAKO, Z0458), HA (mouse monoclonal antibody, dilution 1:5000, Biosite, 16B12), GFP (rabbit polyclonal antibody, dilution 1:5000, Molecular Probes, A6455), PGK1 (mouse monoclonal antibody, 1:5000, Life Technologies, H3420), FLAG N-terminal (mouse monoclonal antibody, M5, dilution 1:2000, Sigma, F4042), the FLAG C-terminal (M2 mouse monoclonal antibody, 1:1000, Sigma, F3165), Cdc48 (rabbit polyclonal antibody, dilution 1:2000-1:5000, a gift from Thomas Sommer; Intracellular Proteolysis, Max-Delbruck-Center for Molecular Medicine, Germany), Rad23 (rabbit polyclonal antibody, dilution 1:5000, a gift from Kiran Madura; Department of Pharmacology, Robert Wood Johnson Medical School-Rutgers University, USA), Rpt3 (rabbit polyclonal antibody, dilution 1:5000, Biomol, PW8110). Proteins were detected using either secondary anti-mouse-, anti-goat- or anti-rabbit-IgG coupled to peroxidase (GE), chemiluminescent substrate (GE), and X-ray films or anti-mouse-, anti-goat- or anti-rabbit-IgG coupled to near-infrared fluorophores (LiCOR) and the Odyssey Infrared Imaging System. The latter system was also used for signal quantification.

Cycloheximide chase assay

For cycloheximide chase analysis, exponentially growing cultures were adjusted to 100 µg/ml cycloheximide, and samples were taken at different time points. Protein extracts were prepared by lysis and precipitation in 27.5% trichloroacetic acid and analyzed by SDS-PAGE and western blotting.

Ubiquitylation assay

Yeast were transformed with an expression plasmid for His-tagged wild-type and lysine-less ubiquitin [gifts from Michael Glickman (Department of Biology, Technion-Israel Institute of Technology, Israel) and Daniel Finley (Cell Biology, Harvard Medical School, USA)]. For precipitation of 6×His–Ub modified proteins under denaturing conditions, a yeast culture of 200 OD_{600} units was harvested and lysed on ice in 6 ml lysis buffer [1.91 M NaOH, 7.5% (v/v) β-mercaptoethanol]. After lysis, 6 ml 55% trichloroacetic acid was added and the samples were vortexed and incubated on ice for 15 min. The samples were centrifuged at 16,000 ***g*** for 15 min at 4°C. The pellets were washed two times with 10 ml ice-cold acetone, after which the pellets were resuspended in 12 ml buffer A (6 M guanidiumchloride, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 8.0, 0.05% Tween 20). Imidazole was added to a final concentration of 20 mM together with 100 µl Ni-NTA magnetic agarose beads (Qiagen). The samples were incubated on a rotating wheel for 16 h at 4°C. The beads were washed two times while placed in a magnetic rack with buffer A and two times with buffer B (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 6.3, 0.05% Tween 20). The proteins were eluted by incubating the beads in 30 µl 1% SDS for 10 min at 65°C. The eluates were dried in a speed-vac for 45 min, dissolved in 20 µl loading buffer, incubated for 10 min at 95°C, separated on SDS-PAGE gels and analyzed by western blotting.

Growth assays

For viability assays, 5- or 10-fold serial dilutions of logarithmically growing cells were spotted on appropriate medium containing 1.5 µg/ml canavanine, where indicated, and grown for 3 days at the indicated temperature.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.G., N.P.D.; Methodology: D.G., T.A.G., J.L., N.P.D.; Formal analysis: D.G., T.A.G., J.L., V.M.B., N.P.D.; Investigation: D.G., T.A.G., J.L.; Writing - original draft: D.G., N.P.D.; Writing - review & editing: D.G., T.A.G., J.L., V.M.B.; Supervision: V.M.B., N.P.D.; Project administration: N.P.D.; Funding acquisition: D. G., V.M., N.P.D.

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Supplementary information

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References

Anton, F., Dittmar, G., Langer, T. and Escobar-Henriques, M. (2013). Two deubiquitylases act on mitofusin and regulate mitochondrial fusion along independent pathways. *Mol. Cell* **49**, 487-498.

Baek, G. H., Kim, I. and Rao, H. (2011). The Cdc48 ATPase modulates the interaction between two proteolytic factors Ufd2 and Rad23. *Proc. Natl. Acad. Sci. USA* **108**, 13558-13563.

Bertolaet, B. L., Clarke, D. J., Wolff, M., Watson, M. H., Henze, M., Divita, G. and Reed, S. I. (2001). UBA domains of DNA damage-inducible proteins interact with ubiquitin. *Nat. Struct. Biol.* **8**, 417-422.

Beskow, A., Grimberg, K. B., Bott, L. C., Salomons, F. A., Dantuma, N. P. and Young, P. (2009). A conserved unfoldase activity for the p97 AAA-ATPase in proteasomal degradation. *J. Mol. Biol.* **394**, 732-746.
Chen, P., Johnson, P., Sommer, T., Jentsch, S. and Hochstrasser, M. (1993). Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MAT alpha 2 repressor. *Cell* **74**, 357-369.
Chen, L., Shinde, U., Ortolan, T. G. and Madura, K. (2001). Ubiquitin-associated (UBA) domains in Rad23 bind ubiquitin and promote inhibition of multi-ubiquitin chain assembly. *EMBO Rep.* **2**, 933-938.
Crosas, B., Hanna, J., Kirkpatrick, D. S., Zhang, D. P., Tone, Y., Hathaway, N. A., Buecker, C., Leggett, D. S., Schmidt, M., King, R. W. et al. (2006). Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities. *Cell* **127**, 1401-1413.
Dai, R.-M., Chen, E., Longo, D. L., Gorbea, C. M. and Li, C.-C. H. (1998). Involvement of valosin-containing protein, an ATPase Co-purified with IκBα and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IκBα. *J. Biol. Chem.* **273**, 3562-3573.
Dantuma, N. P., Heinen, C. and Hoogstraten, D. (2009). The ubiquitin receptor Rad23: at the crossroads of nucleotide excision repair and proteasomal degradation. *DNA Repair (Amst)* **8**, 449-460.
Elkabetz, Y., Shapira, I., Rabinovich, E. and Bar-Nun, S. (2004). Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplamic reticulum-bound p97/Cdc48p and proteasome. *J. Biol. Chem.* **279**, 3980-3989.
Elsasser, S., Chandler-Militello, D., Müller, B., Hanna, J. and Finley, D. (2004). Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. *J. Biol. Chem.* **279**, 26817-26822.
Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu. Rev. Biochem.* **78**, 477-513.
Fishbain, S., Prakash, S., Herrig, A., Elsasser, S. and Matouschek, A. (2011). Rad23 escapes degradation because it lacks a proteasome initiation region. *Nat. Commun.* **2**, 192.
Ghislain, M., Dohmen, R. J., Levy, F. and Varshavsky, A. (1996). Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in Saccharomyces cerevisiae. *EMBO J.* **15**, 4884-4899.
Gödderz, D., Heinen, C., Marchese, F. P., Kurz, T., Acs, K. and Dantuma, N. P. (2015). Cdc48-independent proteasomal degradation coincides with a reduced need for ubiquitylation. *Sci. Rep.* **5**, 7615.
Grice, G. L. and Nathan, J. A. (2016). The recognition of ubiquitinated proteins by the proteasome. *Cell. Mol. Life Sci.* **73**, 3497-3506.
Hanna, J., Hathaway, N. A., Tone, Y., Crosas, B., Elsasser, S., Kirkpatrick, D. S., Leggett, D. S., Gygi, S. P., King, R. W. and Finley, D. (2006). Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. *Cell* **127**, 99-111.
Heessen, S., Masucci, M. G. and Dantuma, N. P. (2005). The UBA2 domain functions as an intrinsic stabilization signal that protects Rad23 from proteasomal degradation. *Mol. Cell* **18**, 225-235.
Heinen, C., Ács, K., Hoogstraten, D. and Dantuma, N. P. (2011). C-terminal UBA domains protect ubiquitin receptors by preventing initiation of protein degradation. *Nat. Commun.* **2**, 191.
Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-479.
Isasa, M., Katz, E. J., Kim, W., Yugo, V., González, S., Kirkpatrick, D. S., Thomson, T. M., Finley, D., Gygi, S. P. and Crosas, B. (2010). Monoubiquitination of RPN10 regulates substrate recruitment to the proteasome. *Mol. Cell* **38**, 733-745.
Ishii, T., Funakoshi, M. and Kobayashi, H. (2006). Yeast Pth2 is a UBL domain-binding protein that participates in the ubiquitin-proteasome pathway. *EMBO J.* **25**, 5492-5503.
Jentsch, S. and Rumpf, S. (2007). Cdc48 (p97): a "molecular gearbox" in the ubiquitin pathway? *Trends Biochem. Sci.* **32**, 6-11.
Johnson, E. S., Ma, P. C., Ota, I. M. and Varshavsky, A. (1995). A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.* **270**, 17442-17456.
Kim, I., Mi, K. and Rao, H. (2004). Multiple interactions of Rad23 suggest a mechanism for ubiquitylated substrate delivery important in proteolysis. *Mol. Biol. Cell* **15**, 3357-3365.
Komander, D. and Rape, M. (2012). The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203-229.
Lambertson, D., Chen, L. and Madura, K. (2003). Investigating the importance of proteasome-interaction for Rad23 function. *Curr. Genet.* **42**, 199-208.
Liang, R.-Y., Chen, L., Ko, B.-T., Shen, Y.-H., Li, Y.-T., Chen, B.-R., Lin, K.-T., Madura, K. and Chuang, S.-M. (2014). Rad23 interaction with the proteasome is regulated by phosphorylation of its ubiquitin-like (UbL) domain. *J. Mol. Biol.* **426**, 4049-4060.
Matiuhin, Y., Kirkpatrick, D. S., Ziv, I., Kim, W., Dakshinamurthy, A., Kleifeld, O., Gygi, S. P., Reis, N. and Glickman, M. H. (2008). Extraproteasomal Rpn10 restricts access of the polyubiquitin-binding protein Dsk2 to proteasome. *Mol. Cell* **32**, 415-425.
Medicherla, B., Kostova, Z., Schaefer, A. and Wolf, D. H. (2004). A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep.* **5**, 692-697.
Ortolan, T. G., Tongaonkar, P., Lambertson, D., Chen, L., Schauber, C. and Madura, K. (2000). The DNA repair protein Rad23 is a negative regulator of multi-ubiquitin chain assembly. *Nat. Cell Biol.* **2**, 601-608.
Raasi, S., Varadan, R., Fushman, D. and Pickart, C. M. (2005). Diverse polyubiquitin interaction properties of ubiquitin-associated domains. *Nat. Struct. Mol. Biol.* **12**, 708-714.
Reyes-Turcu, F. E., Ventii, K. H. and Wilkinson, K. D. (2009). Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu. Rev. Biochem.* **78**, 363-397.
Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C. and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell* **120**, 73-84.
Rumpf, S. and Jentsch, S. (2006). Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. *Mol. Cell* **21**, 261-269.
Russell, S. J., Reed, S. H., Huang, W., Friedberg, E. C. and Johnston, S. A. (1999). The 19S regulatory complex of the proteasome functions independently of proteolysis in nucleotide excision repair. *Mol. Cell* **3**, 687-695.
Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W. and Madura, K. (1998). Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature* **391**, 715-718.
Schrader, E. K., Harstad, K. G. and Matouschek, A. (2009). Targeting proteins for degradation. *Nat. Chem. Biol.* **5**, 815-822.
Sekiguchi, T., Sasaki, T., Funakoshi, M., Ishii, T., Saitoh, Y. H., Kaneko, S. and Kobayashi, H. (2011). Ubiquitin chains in the Dsk2 UBL domain mediate Dsk2 stability and protein degradation in yeast. *Biochem. Biophys. Res. Commun.* **411**, 555-561.
Song, E. J., Werner, S. L., Neubauer, J., Stegmeier, F., Aspden, J., Rio, D., Harper, J. W., Elledge, S. J., Kirschner, M. W. and Rape, M. (2010). The Prp19 complex and the $Usp4^{Sart3}$ deubiquitinating enzyme control reversible ubiquitination at the spliceosome. *Genes Dev.* **24**, 1434-1447.
Spence, J., Sadis, S., Haas, A. L. and Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell. Biol.* **15**, 1265-1273.
Swaney, D. L., Beltrao, P., Starita, L., Guo, A., Rush, J., Fields, S., Krogan, N. J. and Villén, J. (2013). Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. *Nat. Methods* **10**, 676-682.
Varshavsky, A. (1996). The N-end rule: functions, mysteries, uses. *Proc. Natl. Acad. Sci. USA* **93**, 12142-12149.
Verma, R., Oania, R., Graumann, J. and Deshaies, R. J. (2004). Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. *Cell* **118**, 99-110.
Walters, K. J., Lech, P. J., Goh, A. M., Wang, Q. and Howley, P. M. (2003). DNA-repair protein hHR23a alters its protein structure upon binding proteasomal subunit S5a. *Proc. Natl. Acad. Sci. USA* **100**, 12694-12699.
Wang, Q., Li, L. and Ye, Y. (2006). Regulation of retrotranslocation by p97-associated deubiquitinating enzyme ataxin-3. *J. Cell Biol.* **174**, 963-971.
Xie, Y. and Varshavsky, A. (2000). Physical association of ubiquitin ligases and the 26S proteasome. *Proc. Natl. Acad. Sci. USA* **97**, 2497-2502.