Ddi1 is a ubiquitin-dependent protease

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The *Saccharomyces cerevisiae* protein Ddi1 and its homologs in higher eukaryotes have been proposed to serve as shuttling factors that deliver ubiquitinated substrates to the proteasome. Although Ddi1 contains both ubiquitin-interacting UBA and proteasome-interacting UBL domains, the UBL domain is atypical, as it binds ubiquitin. Furthermore, unlike other shuttling factors, Ddi1 and its homologs contain a conserved helical domain (helical domain of Ddi1, HDD) and a retroviral-like protease (RVP) domain. The RVP domain is probably responsible for cleavage of the precursor of the transcription factor Nrf1 in higher eukaryotes, which results in the up-regulation of proteasomal subunit genes. However, enzymatic activity of the RVP domain has not yet been demonstrated, and the function of Ddi1 remains poorly understood. Here, we show that Ddi1 is a ubiquitin-dependent protease, which cleaves substrate proteins only when they are tagged with long ubiquitin chains (longer than about eight ubiquitins). The RVP domain is inactive in isolation, in contrast to its retroviral counterpart. Proteolytic activity of Ddi1 requires the HDD domain and is stimulated by the UBL domain, which mediates high-affinity interaction with the polyubiquitin chain. Compromising the activity of Ddi1 in yeast cells results in the accumulation of polyubiquitinated proteins. Aside from the proteasome, Ddi1 is the only known endoprotease that acts on polyubiquitinated substrates. Ddi1 and its homologs likely cleave polyubiquitinated substrates under conditions where proteasome function is compromised.

Ddi1 | ubiquitin | proteasome | protease | Nrf1

Many proteins in eukaryotic cells are degraded by the ubiquitin–proteasome system: A polyubiquitin chain is first attached to a substrate and then serves as a recognition signal for the 26S proteasome. Some polyubiquitinated substrates bind directly to ubiquitin receptors that are integral parts of the 26S proteasome, but others are delivered to the proteasome through shuttling factors (1–4). Three conserved proteins have been proposed to be shuttling factors, called in *Saccharomyces cerevisiae* Rad23, Dsk2, and Ddi1 (5). These proteins contain both UBA (ubiquitin-associated) and UBL (ubiquitin-like) domains. In the case of Rad23 and Dsk2, the UBA and UBL domains bind ubiquitin and the proteasome, respectively, thus allowing these proteins to serve as bridges between substrate and the proteasome (6). The third protein, Ddi1, remains much less studied and understood.

The UBL domain of Ddi1 is unique in that it can bind not only to the proteasome but also to ubiquitin (7, 8). The UBA domains in Ddi1 and its homologs also associate with ubiquitin (7, 8). In addition, Ddi1 family members differ from Rad23 and Dsk2 by the presence of a "helical domain of Ddi1" (HDD) and a predicted "retroviral protease-like" (RVP) domain that is related in its amino acid sequence to retroviral aspartyl proteases, including that of HIV (9, 10). The crystal structure of the RVP domain is indeed similar to that of retroviral proteases (11). In both retroviruses and Ddi1, the RVP domain forms dimers with the catalytic residues at the interface (10, 12). The HIV protease is relatively promiscuous, cleaving viral precursor polypeptides at multiple sites between an aromatic residue and a proline or between two hydrophobic residues (13). By contrast, the RVP domain of Ddi1 is proteolytically inactive when tested with a

broad range of peptides (11, 12). Nevertheless, given the sequence similarity to retroviral proteases, it is possible that Ddi1 is not only a proteasome shuttling factor but also a protease that requires an unknown activation mechanism (14–16).

The best-studied function for Ddi1 family members is in the Nrf1 pathway in metazoans (14–20). Nrf1 is a transcription factor that regulates the expression of proteasomal subunits and other components of the proteasome system, such as the p97/VCP ATPase. Nrf1 is made as an integral endoplasmic reticulum (ER) membrane protein precursor with a large luminal domain. The protein is continuously degraded by ER-associated protein degradation. Specifically, the luminal domain is retrotranslocated into the cytosol, polyubiquitinated, extracted from the membrane by the p97/VCP ATPase, and, finally, degraded by the proteasome. When the proteasome is inhibited, the luminal domain is still retrotranslocated but can no longer be degraded by the proteasome. Instead, it is cleaved in a reaction that is dependent on polyubiquitination and the presence of the Ddi1 homolog DDI2 in mammalian cells or DDI1 in *Caenorhabditis elegans*. Cleavage of the previously intraluminal C-terminal domain releases a fragment that then moves into the nucleus and acts as a transcription factor to up-regulate proteasomal subunits (14–20). Thus, this pathway serves as a compensatory mechanism to rectify the effects of proteasome inhibition. The Nrf1 pathway does not exist in lower organisms, such as yeast, so Ddi1 must have other substrates. Ddi1 is not required for normal growth of *S. cerevisiae* cells, but it plays a role in the control of the cell cycle, mating type switching, and protein secretion (7, 21, 22). For example, Ddi1 has been reported to inhibit secretion, probably by inhibiting the assembly of SNAREs required for

Significance

Many proteins in the cell are tagged with a polyubiquitin chain, which serves as a recognition signal for degradation by the proteasome. Some tagged substrates bind directly to the proteasome, but others are delivered through shuttling factors. Yeast Ddi1 and its homologs in other eukaryotic cells have protein domains typical of shuttling factors, but they also contain a predicted protease domain that is related to those in retroviral proteases. This paper shows that Ddi1 is a ubiquitin-dependent protease, which cleaves substrate proteins only when they are tagged with long ubiquitin chains. Ddi1 is the only known endoprotease besides the proteasome that cleaves polyubiquitinated substrates. Ddi1 might prevent the excessive accumulation of polyubiquitinated proteins in cells.

vesicle fusion with the plasma membrane (22–25). Recent reports show that, along with the metalloprotease Wss1, Ddi1 is involved in the disassembly of DNA–protein cross-links (26, 27). Ddi1's putative target in this pathway is the core component of stalled RNA polymerase II (27). As in other reports, Ddi1 was assumed to be a protease because mutation of the putative active site of the RVP domain compromises its in vivo activities (18, 22). However, proteolytic activity of Ddi1 has never been observed in vitro except for the homolog from *Leishmania major* at nonphysiologically low pH (pH 4) (11, 28).

Here, we demonstrate that *S. cerevisiae* Ddi1 is a ubiquitin-dependent protease, which requires exceptionally long ubiquitin chains on its substrates. The RVP domain is inactive in isolation. Proteolytic activity of Ddi1 requires the HDD domain and is stimulated by the UBL domain, which mediates high-affinity interaction with the polyubiquitin chain. Compromising the activity of Ddi1 in yeast results in the accumulation of polyubiquitinated proteins. Ddi1 and its homologs may thus be involved in the degradation of long-chain polyubiquitinated substrates.

Results

To purify full-length *S. cerevisiae* Ddi1, a construct containing His14 and SUMO tags at the N terminus was overexpressed in *E. coli*, and the protein was purified on a nickel–nitrilotriacetic acid (Ni-NTA) column followed by anion exchange chromatography and gel filtration (*SI Appendix*, Fig. S1). To test the proteolytic activity of Ddi1, we generated a polyubiquitinated substrate, reasoning that previous failures to detect in vitro activity of Ddi1 might have been due to the use of unmodified peptides and proteins. The substrate used for our studies contains a His14–SUMO tag, a 43-residue degron sequence derived from the *E. coli* lac repressor, and superfolder GFP (sfGFP) (Fig. 1*A* and *SI Appendix*, Fig. S2) (29). The protein was expressed in *E. coli* and purified on a Ni–NTA column, and the SUMO tag was cleaved off to expose an N-terminal arginine, rendering the protein a substrate for Ubr1, the ubiquitin ligase in the N-end rule pathway (30). To facilitate detection of the substrate, a fluorescent dye (DyLight800) was attached to a unique cysteine close to the N terminus. The labeled protein was then incubated with a mixture of purified E1 (Uba1), E2 (Ubc2), and E3 (Ubr1) enzymes, as well as ubiquitin and ATP. The ubiquitin chains synthesized by this method are nearly exclusively composed of K48 linkages (29). The polyubiquitinated substrate was purified on streptavidin beads via a C-terminal streptavidin-binding peptide (SBP) tag and eluted from the resin by cleavage with 3C protease (Fig. 1*A* and *SI Appendix*, Fig. S2). Where indicated, the sample was further subjected to gel filtration to isolate fractions with ubiquitin chains of various lengths.

We first incubated purified Ddi1 with DyLight800-labeled, polyubiquitinated substrate. Samples of the reaction were taken at various time points and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence scanning (Fig. 1*B*). The high-molecular-weight fluorescent species disappeared over time, and a low-molecular-weight (~1 kDa) fluorescent peptide accumulated (see arrowheads), indicating that Ddi1 cleaves the substrate. Because the fluorescent dye is attached to Cys6 of the substrate and the ubiquitin chain is attached at Lys19, it is likely that cleavage occurs between these two residues. Inspection of the sequence between Cys6 and Lys19 indeed showed that it contains an AWL motif, similar to that in the cleavage site of Nrf1, where proteolysis occurs between Trp103 and Leu104 (Fig. 1*C*) (17). We therefore mutated both Trp9 and Leu10 in our substrate to Gly residues. Indeed, these mutations abolished the appearance of the low-molecular-weight fluorescent peptide upon incubation with Ddi1 (Fig. 1*D*), confirming that the Trp–Leu sequence is important for this cleavage event. A decrease in the molecular weight of the substrate
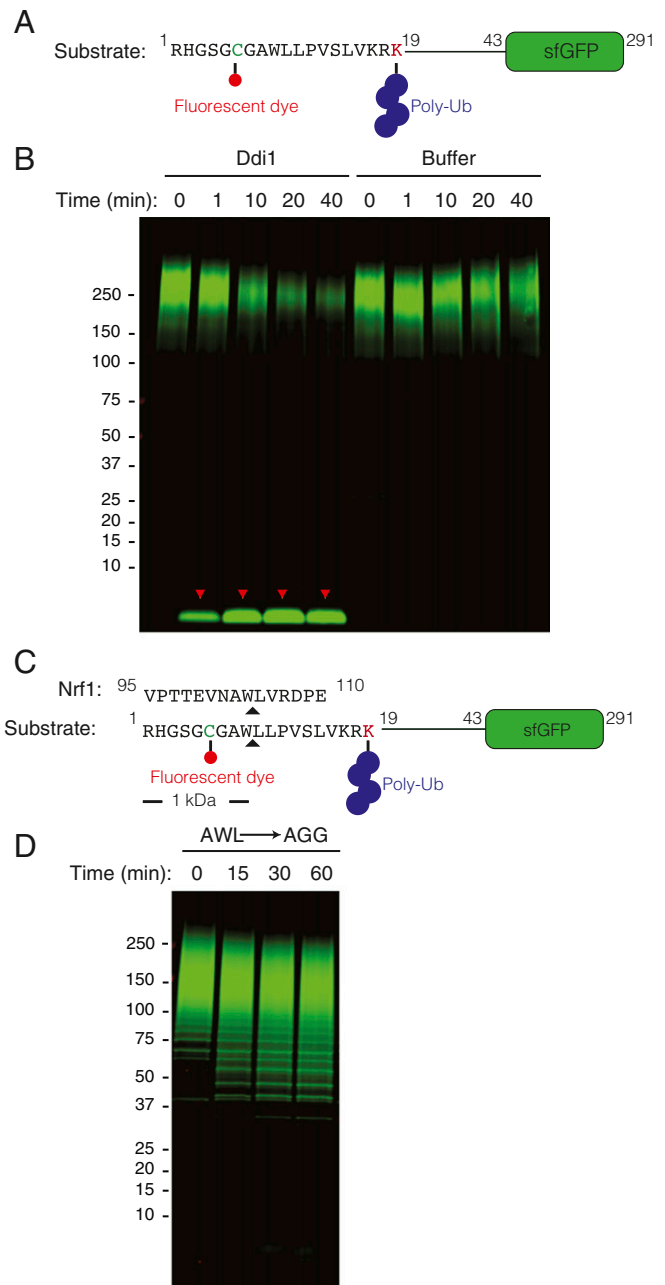
Fig. 1. Ddi1 cleaves a polyubiquitinated model substrate. (*A*) Scheme of the model substrate, carrying a fluorescent dye and a polyubiquitin chain at the indicated positions (see *SI Appendix*, Fig. S2 for how the substrate was synthesized). (*B*) Yeast Ddi1 was purified as a His14–SUMO fusion protein. The purified protein (1 μM dimer) was incubated with 1 μM polyubiquitinated substrate labeled with a fluorescent dye (DyLight800). As a control, substrate was incubated with buffer. Samples were taken at the indicated time points and subjected to SDS-PAGE and fluorescence scanning for DyLight800 fluorescence. The arrowheads indicate a proteolytic fragment generated by Ddi1 activity. (*C*) Comparison of selected sequences from the model substrate and human Nrf1. The arrows indicate the putative substrate cleavage site in the model substrate and the confirmed Nrf1 cleavage site. (*D*) Ddi1 was incubated with substrate in which the AWL motif was mutated to AGG. The samples were analyzed as in *A*.

was still observed with the diglycine cleavage site mutant, suggesting that residual proteolysis might occur at another site in the substrate, probably within ubiquitin molecules. This cleavage reaction was slower than the one in the substrate. In addition,

Yip et al.
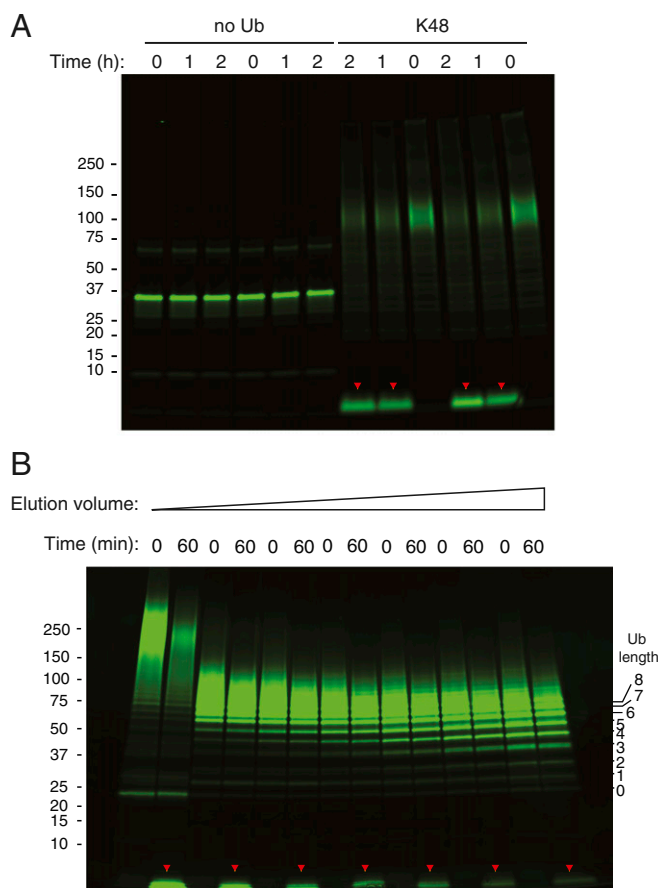
Fig. 2. Substrate cleavage by Ddi1 depends on the ubiquitin chain length. (*A*) Ddi1 was purified as a His14–SUMO fusion protein. It was then incubated either with nonubiquitinated DyLight800-labeled substrate (*Left*) or with polyubiquitinated DyLight800-labeled substrate purified by gel filtration (*Right*). Samples were taken at the indicated time points and subjected to SDS-PAGE and fluorescence scanning. The arrowheads indicate a proteolytic fragment generated by Ddi1 activity. (*B*) As in *A*, but with various fractions of polyubiquitinated substrate separated by gel filtration. Note that cleavage is reduced with decreasing ubiquitin chain length. The number of ubiquitin molecules in the chain is given at the right.

when free ubiquitin chains were used as substrate, only minor degradation products appeared over time, regardless of the length of the ubiquitin chains (*SI Appendix,* Fig. S3). Thus, cleavage within ubiquitin is probably a side reaction and was not studied further.

Generation of the small peptide by Ddi1 was dependent on the presence of the polyubiquitin chain on the substrate, as unmodified substrate was not proteolyzed (Fig. 2*A*). To further explore this dependence, we used substrates with ubiquitin chains of varying lengths, separated by size exclusion chromatography. Ddi1 efficiently cleaved substrates bearing very long ubiquitin chains (15 to 25 moieties) but showed little activity against substrates with chains of fewer than eight ubiquitin moieties (Fig. 2*B*). This length requirement contrasts with that of the proteasome, for which the canonical minimal degradation signal is only four ubiquitin moieties (31). Complete cleavage of the polyubiquitinated substrate required almost stoichiometric amounts of Ddi1, but some cleavage was observed at a 1:100 enzyme-to-substrate ratio (*SI Appendix,* Fig. S4).

We also tested the cleavage of substrate containing K63-linked polyubiquitin chains. To this end, we employed a PPSY sequence present in our model substrate and used the ubiquitin ligase Rsp5 to attach K63-linked polyubiquitin chains (32, 33).

Incubation of this substrate with Ddi1 also generated the small peptide, but less efficiently than with K48-linked chains (*SI Appendix,* Fig. S5). The binding of K63-linked ubiquitin chains to Ddi1 is consistent with a structure of its UBL domain and bound ubiquitin, in which both K48 and K63 linkages are accessible (8). The UBA domain of Ddi1 is also not thought to bind ubiquitin in a linkage-specific manner (34, 35). It remains unclear why Ddi1 cleaves substrates with K63-linked chains less efficiently. Rsp5 likely generates multiple K63-linked chains, some of which might be on the GFP surface, rather than only attached to Lys19, as is the case for the single K48-linked chain. Thus, despite the migration of the polyubiquitinated substrate in SDS gels, the individual K63 chains could be shorter and not optimal for Ddi1 cleavage. Alternatively, the chains might have an unfavorable distance between the ubiquitination and cleavage sites.

Next, we determined the contributions of the individual domains of Ddi1 to the cleavage of polyubiquitinated substrates. Ddi1 has an N-terminal UBL domain, a helical domain (HDD), a predicted protease domain (RVP), and a C-terminal UBA domain (Fig. 3*A*). Ddi1 lacking the UBL domain was less active than the wild-type protein (Fig. 3*B*), although the residual activity was somewhat variable in different experiments. The deletion of the UBA domain had no effect (Fig. 3*B*). These results are consistent with observations made in vivo (23) and support the notion that the UBL domain of Ddi1 binds ubiquitin (8). A construct lacking both the UBL and UBA domains (HDD–RVP) retained some low activity, visible at high protein concentration (*SI Appendix,* Fig. S6*A*), whereas the RVP alone was completely inactive, even at a 10:1 enzyme:substrate ratio (*SI Appendix,* Fig. S6*B*). Together with the observation that a construct lacking only the HDD was also inactive (Fig. 3*B*), these results indicate that the HDD is required for catalytic activity. Quantification of the intensity of the cleaved peptide confirmed that UBA deletion had no effect, UBL deletion had a moderate effect, HDD–RVP had low activity, and HDD deletion completely abolished cleavage (*SI Appendix,* Fig. S7). Interestingly, all proteolytically competent constructs retained the requirement for long ubiquitin chains (*SI Appendix,* Fig. S8). This suggests that the HDD domain licenses the RVP domain for cleavage of polyubiquitinated substrate. As expected, no proteolytic activity was observed for any construct if the active site of the RVP domain was mutated (D220N) (Fig. 3*C*).

Next, we tested the affinity of the different Ddi1 constructs for polyubiquitinated substrate, using microscale thermophoresis (MST). To avoid substrate cleavage, these binding experiments were performed with the enzymatically inactive D220N mutants. Full-length Ddi1 had a very high affinity for substrate bearing long ubiquitin chains (Fig. 4*A*; orange and blue curves, obtained at different substrate concentrations). The apparent binding constant was in the range of 50 pM, although the exact number is uncertain as the stoichiometry of the complex is unknown. Substrate with short ubiquitin chains bound much more weakly (Fig. 4*A*; yellow curve; dissociation constant [K_d] ~ 50 nM), and substrate lacking ubiquitin showed only marginal binding (Fig. 4*A*; green curve, K_d ~ 5 μM).

The deletion of the UBA domain had a negligible effect on substrate binding when tested with substrate bearing long ubiquitin chains (Fig. 4*B*; red curve [K_d ~ 0.3 nM] versus blue curve), whereas the deletion of the UBL domain had a more pronounced effect on substrate affinity (Fig. 4*B*; green curve; K_d ~ 300 nM). These results explain the differences between these constructs in their enzymatic properties (Fig. 3*B*) and indicate again that the UBL domain contributes to the interaction with ubiquitin. The construct lacking both UBL and UBA domains (HDD–RVP) had a very low affinity for substrate (Fig. 4*B*; dark blue curve; K_d ~ 100 μM), although it must have been sufficient to generate the low remaining enzymatic activity (Fig. 3*B* and *SI Appendix,* Fig. S7). The RVP alone had an unmeasurably low
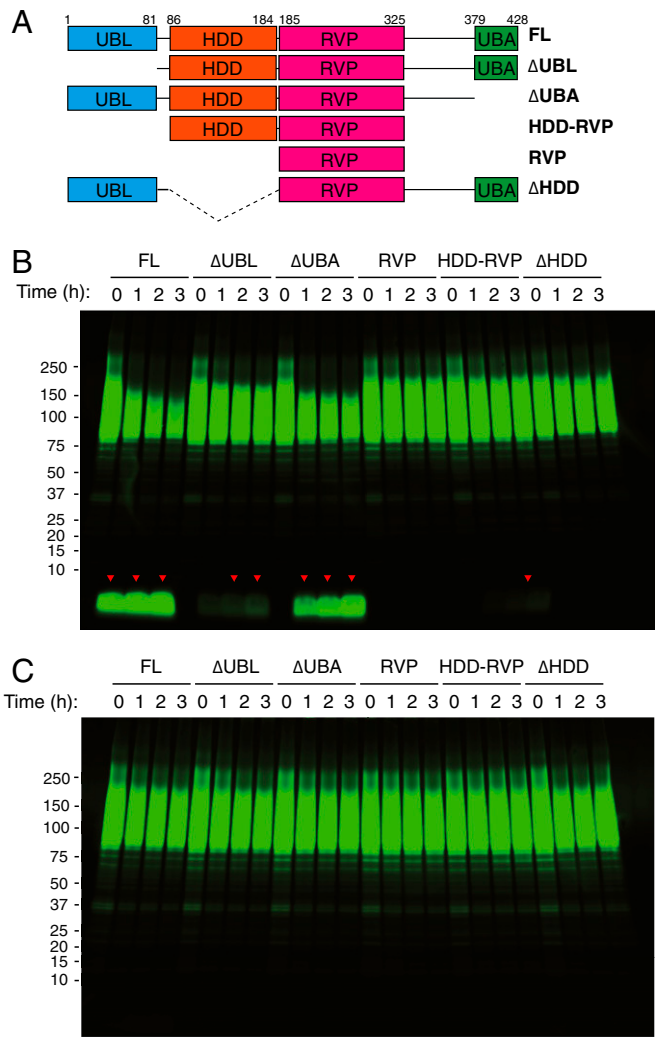
Fig. 3. Analysis of Ddi1 domains required for proteolytic activity. (*A*) Scheme of Ddi1 domain organization. The domains are shown in different colors with the amino acid residue number at the beginning and end of a domain indicated above. (*B*) Wild-type or mutant Ddi1 was purified as a His14–SUMO fusion. The N-terminal SUMO tag was cleaved off with SUMO protease, and the proteins were further purified by gel filtration. They were then incubated with DyLight800-labeled substrate bearing long polyubiquitin chains purified by gel filtration. Samples were taken at the indicated time points and subjected to SDS-PAGE and fluorescence scanning. FL refers to the full-length construct. ΔUBL, ΔUBA, and ΔHDD are mutants lacking the indicated domains. RVP and HDD–RVP are constructs containing only the indicated domains. The arrowheads indicate a generated proteolytic fragment. (*C*) As in *B*, but with constructs containing the D220N mutation in the active site of the RVP domain.

substrate affinity, while deletion of the HDD domain had a moderate effect on the affinity (Fig. 4*B*; yellow curve; $K_d \sim 3.6$ nM), supporting the conclusion that the HDD makes only a small contribution to the overall affinity for polyubiquitin. Taken together, there is a good correlation between substrate affinity and enzymatic activity, indicating that ubiquitin binding is the major determinant for proteolysis by Ddi1.

Our observation that Ddi1 cleaves polyubiquitinated substrates suggests that its function may overlap with that of the proteasome. To test this idea, we used *S. cerevisiae* cells lacking Ddi1. The cells also lack the multidrug transporter Pdr5, so that the added proteasome inhibitor bortezomib is not rapidly pumped out of the cells (36). As expected, the addition of bortezomib

to wild-type cells resulted in the accumulation of polyubiquitinated proteins (Fig. 5*A*, lane 3 versus 1). In the absence of Ddi1, the abundance and molecular weight of the polyubiquitinated species increased, consistent with Ddi1 normally cleaving these proteins (lanes 2 and 4). The accumulation of polyubiquitinated proteins was particularly pronounced in the presence of bortezomib and absence of Ddi1 (lane 4), indicating that Ddi1 and the proteasome indeed act synergistically. Next, we transformed Δpdr5 cells containing endogenous Ddi1 with low-copy plasmids expressing FLAG-tagged versions of wild-type Ddi1 or enzymatically inactive Ddi1 (D220N). The cells were incubated in the absence or presence of bortezomib (Fig. 5*B*). The addition of proteasome inhibitor resulted in the accumulation of polyubiquitinated proteins (lanes 4 to 6 versus 1 to 3), but this effect was enhanced when Ddi1 (D220N) was expressed (lane 6). Again, the length of the ubiquitin chains was increased, consistent with the notion that enzymatically active Ddi1 cleaves substrates bearing long chains. Taken together, these results show that Ddi1 is a protease that cleaves polyubiquitinated proteins.
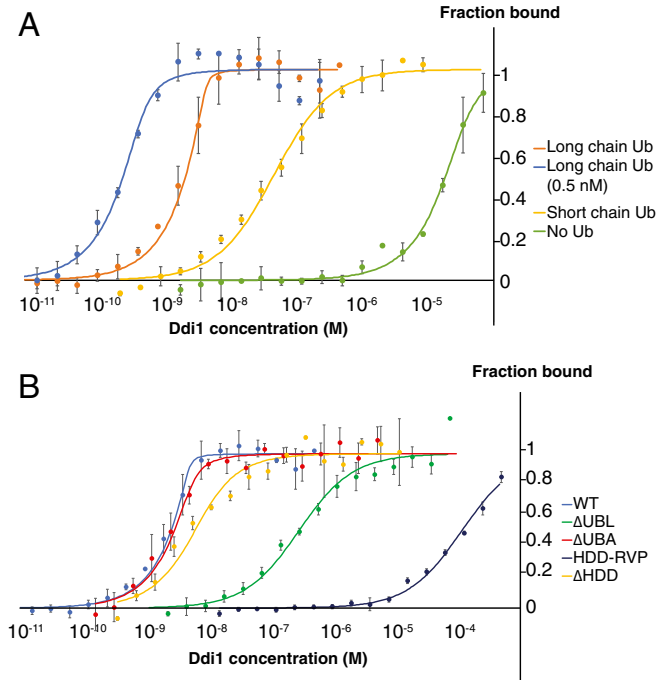
Fig. 4. Interaction of wild-type (WT) and mutant Ddi1 with polyubiquitinated substrate. (*A*) Enzymatically inactive Ddi1 (D220N) was purified as a His14–SUMO fusion protein. The N-terminal SUMO tag was cleaved off with SUMO protease, and the protein was further purified by gel filtration. Different concentrations were then incubated with 5 nM Cy5-labeled substrate bearing long (orange curve), short (yellow curve), or no (green curve) ubiquitin chains. The samples were then subjected to MST, using the fluorescence of the substrate to monitor its movement in a temperature gradient. Each point represents the mean and SD of the mean of three measurements. Apparent binding constants were estimated by fitting these curves with the assumption of a 1:1 complex of the binding partners. Binding with long ubiquitin chains was also performed at 0.5 nm substrate (blue curve), as the orange curve reached an abrupt saturation level, typical of an excessively high substrate concentration in binding experiments. (*B*) Enzymatically inactive full-length Ddi1 (D220N) or inactive Ddi1 mutant constructs, all lacking the His14–SUMO tag, were incubated at different concentrations with 5 nM substrate bearing long ubiquitin chains. The samples were subjected to MST, as in *A*. The blue curve corresponds to the wild-type protein; the green, red, and yellow curves correspond to constructs lacking the UBL, UBA, and HDD domains, respectively, and the dark blue curve corresponds to a construct containing the HDD and RVP domains.

Yip et al.

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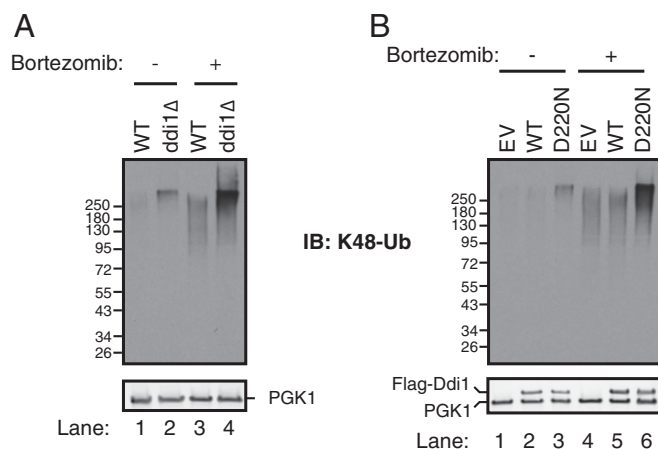
Fig. 5. Ddi1 degrades polyubiquitinated proteins in vivo. (*A*) *S. cerevisiae* cells lacking either only the multidrug resistance transporter Pdr5 (wild-type, WT) or additionally Ddi1 (ddi1Δ) were treated with the proteasome inhibitor bortezomib, as indicated. Equal numbers of cells were analyzed by SDS-PAGE followed by immunoblotting with anti-ubiquitin antibodies specific to K48 linkages. Immunoblotting with antibodies to phosphoglycerate kinase (PGK1) served as a loading control. (*B*) Cells lacking only Pdr5 were transformed either with an empty vector (EV), with a plasmid expressing FLAG-tagged wild-type Ddi1 (WT), or with a plasmid expressing a FLAG-tagged version of an enzymatically inactive Ddi1 mutant (D220N). The cells were incubated with bortezomib, as indicated. The samples were analyzed as in *A* but also immunoblotted with FLAG antibodies.

Discussion

Our results show that Ddi1 is an active protease that cleaves substrates bearing long ubiquitin chains. Besides the proteasome, Ddi1 is the only known endoprotease that acts on polyubiquitinated substrates. Based on our model substrate, it seems that Ddi1 is strictly ubiquitin dependent. In contrast, the proteasome is not an obligatory ubiquitin-dependent protease, as it can degrade unmodified proteins (37–40). Deubiquitinating enzymes (DUBs) also recognize polyubiquitin chains in their substrates, but they cleave isopeptide bonds.

Although proteolytic activity was suggested by the homology of Ddi1's RVP domain with retroviral proteases and by the effect of mutations in the predicted active site on phenotypes observed in vivo, enzymatic activity could not previously be detected because protease activity is dependent on polyubiquitination and only unmodified substrates were tested (11, 12). Although we have not determined the exact cleavage site in our substrate, our mutagenesis data suggest that Ddi1 cleaves a polyubiquitinated substrate at a sequence similar to that in Nrf1. This provides evidence for the previous proposal that Nrf1 is cleaved directly by Ddi1 homologs (16, 18, 19) and not by an unidentified intermediary protease. However, the stringency of the motif recognized by Ddi1 remains to be determined, and it could be rather degenerate, as with retroviral proteases. In our model substrate, the recognition motif is close to the lysine residue at which the polyubiquitin chain is attached, and such proximity might be a general principle of substrate selection. In addition, the cleavage site may have to be present in an unstructured polypeptide region, as is the case in our model substrate. Our data confirm that the RVP domain in isolation is enzymatically inactive (11, 28). In contrast, the RVP domain of the HIV protease can cleave peptides and proteins, perhaps because it has a more closed conformation of its active site that binds peptides more tightly (11). The differences between retroviral proteases and Ddi1 are also supported by experiments with nelfinavir, a competitive inhibitor of HIV protease (*SI Appendix*, Fig. S9): an inhibition of Ddi1 by nelfinavir was only seen at very high

concentrations, which are at least four orders of magnitude higher than required to inhibit the HIV protease in vitro (41, 42).

Ddi1 requires very long ubiquitin chains on the substrate, much longer than those needed by the proteasome. In fact, we are not aware of any other protein that requires ubiquitin chains of eight or more moieties for its function. The mechanism by which Ddi1 interacts with multiple ubiquitins is not entirely clear. Our data indicate that the minimal construct recognizing long ubiquitin chains consists of the HDD and RVP domains, although the enzymatic activity of this construct was very low. The RVP is known to form dimers, so two HDD domains seem to form the minimal ubiquitin-binding unit. The presence of either the UBL or UBA domain drastically increased substrate affinity and proteolytic activity, likely by providing two additional ubiquitin-binding sites per Ddi1 dimer. The UBL domain has a more important role than the UBA domain because deletion of the UBA domain reduced neither ubiquitin binding nor proteolytic activity. Consistent with this notion, the UBA domain is lacking in Ddi1 homologs of several species (8). Perhaps, Ddi1 has two distinct functions, one as a ubiquitin-dependent protease and another as a proteasomal shuttling protein, with the UBA domain only required for the latter.

The apparent binding affinity of wild-type Ddi1 for a polyubiquitinated substrate is extremely high, as determined by microscale thermophoresis. Because the dissociation rate must be very low, it is unclear how the ubiquitinated part of the cleavage product is released from Ddi1. In fact, complete cleavage of polyubiquitinated substrate required almost stoichiometric concentrations of Ddi1, raising the possibility that product release does not occur spontaneously. A possible release mechanism may involve DUBs that trim the ubiquitin chain to a length that no longer interacts with Ddi1. Alternatively, product release might require the proteasome. In this scenario, an interaction of Ddi1's UBL domain with the proteasome could facilitate the transfer of the Ddi1 cleavage product to the proteasome. The microscale thermophoresis technique used by us may generally be useful to measure the affinity of ubiquitin-binding proteins for polyubiquitin chains. Previous studies were performed by pulldown experiments, which are not very quantitative, or they employed monoubiquitin or oligoubiquitin chains and resulted in much lower affinities (in the micromolar range or lower). It remains to be tested whether other ubiquitin-binding proteins have an affinity as high as Ddi1 when tested with polyubiquitin chains.

Our results indicate that Ddi1 proteolytically cleaves polyubiquitinated proteins in yeast cells and that the proteasome and Ddi1 act synergistically. Under normal conditions, polyubiquitinated proteins seem to be mostly processed by the proteasome, explaining why Ddi1 is not essential for cell viability. The expression of an enzymatically inactive Ddi1 mutant leads to the excessive accumulation of polyubiquitinated proteins, indicating that the mutant acts in a dominant-negative manner on wild-type Ddi1. These results also show that Ddi1 substrates exist even in untreated wild-type cells. However, both in yeast and mammals the full spectrum of these substrates remains unclear. Perhaps, Ddi1 and its homologs degrade a large variety of substrates that carry long ubiquitin chains. We favor the possibility that Ddi1 cleaves the substrate to which these long chains are attached, but it is possible that that some cleavage occurs in the ubiquitin chain itself, as we observed slow degradation of such chains in our in vitro system. Because of its preference for very long chains, Ddi1 could serve as a safeguard under conditions where proteasome function is compromised and undegraded polyubiquitinated proteins accumulate. These substrates would be cleaved by Ddi1 into smaller peptides, which might then be further degraded by the remaining proteasome activity or by other proteases.

Yip et al.

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Experimental Procedures

Purification of Wild-Type and Mutant Ddi1. All proteins were expressed in *E. coli* BL21 (DE3) RIPL cells with N-terminal His14–SUMO fusion tags (43) and purified, as described previously for other proteins (29) and further in the *SI Appendix*.

Preparation of Polyubiquitinated, Fluorescent Substrates. The synthesis, labeling, and purification of polyubiquitinated substrates was done as previously described and discussed further in the *SI Appendix*. Free ubiquitin chains were synthesized as described (29).

Ddi1 Cleavage Assays. Reactions were carried out in 50 mM Hepes (pH 7.4), 150 mM NaCl, and 0.5 mM tris(2-carboxyethyl)phosphine at 30 °C. The reactions were quenched at various time points by the addition of SDS sample buffer and analyzed by SDS-PAGE, followed by fluorescence scanning for DyLight800 on an Odyssey CLx imager (Li-COR). The retroviral protease inhibitor nelfinavir was dissolved in methanol at 20 mM or 2 mM and diluted into the assay. The control contained the equivalent methanol concentration (0.5%).

Binding Experiments Using MST. MST experiments were performed with a Monolith NT.115pico (NanoTemper Technologies) instrument with Ddi1 concentrations of 10 pM to 100 μM and final substrate concentrations of either 5 or 0.5 nM. Experimental parameters are described in the *SI Appendix*. The fluorescence change during the temperature jump was plotted against the Ddi1 concentration, and curves were fitted with a K_d model in the MO.Affinity Analysis Software (NanoTemper Technologies).

In Vivo Experiments. Strains used in the study were derived from BY4741. The ddi1Δ strain was obtained from a yeast knockout collection (Clone ID 6141, Dharmacon). The Pdr5 deletion was obtained using PCR-mediated gene replacement. For the overexpression experiments, an N-terminal FLAG epitope was added to either wild-type Ddi1 or the D220N mutant. These genes were cloned into the p426GAL1 vector and transformed into the pdr5Δ strain.

Growth conditions and cell lysis protocols are further described in the *SI Appendix*.

Data Availability. All data used in the study are included in the paper and *SI Appendix*. All protocols are described in the *Experimental Procedures* and *SI Appendix, Supplementary Materials and Methods* or in the references therein. Plasmids and yeast strains used in the study are freely available upon request to the corresponding author.

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