Transfer of Ho Endonuclease and Ufo1 to the Proteasome by the UbL-UbA Shuttle Protein, Ddi1, Analysed by Complex Formation In Vitro

Olga Voloshin, Anya Bakhrat, Sharon Herrmann, Dina Raveh*

Department of Life Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel

Abstract

The F-box protein, Ufo1, recruits Ho endonuclease to the SCF\(^{-Ufo1}\) complex for ubiquitylation. Both ubiquitylated Ho and Ufo1 are transferred by the Ubl-UbA protein, Ddi1, to the 19S Regulatory Particle (RP) of the proteasome for degradation. The Ddi1-Ubl domain binds Rpn1 of the 19S RP and Ddi1-UbA domain binds ubiquitin chains on the degradation substrate. Here we used complex reconstitution in vitro to identify stages in the transfer of Ho and Ufo1 from the SCF\(^{-Ufo1}\) complex to the proteasome. We report SCF\(^{-Ufo1}\) complex at the proteasome formed in the presence of Ho. Subsequently Ddi1 is recruited to this complex by interaction between the Ddi1-Ubl domain and Ufo1. The core of Ddi1 binds both Ufo1 and Rpn1; this interaction confers specificity of SCF\(^{-Ufo1}\) for Ddi1. The substrate-shield model predicts that Ho would protect Ufo1 from degradation and we find that Ddi1 binds Ho, Ufo1, and Rpn1 simultaneously forming a complex for transfer of Ho to the 19S RP. In contrast, in the absence of Ho, Rpn1 displaces Ufo1 from Ddi1 indicating a higher affinity of the Ddi1-Ubl for the 19S RP. However, at high Rpn1 levels there is synergistic binding of Ufo1 to Ddi1 that is dependent on the Ddi1-UbA domain. Our interpretation is that in the absence of substrate, the Ddi1-Ubl binds Rpn1 while the Ddi1-UbA binds ubiquitin chains on Ufo1. This would promote degradation of Ufo1 and disassembly of SCF\(^{-Ufo1}\) complexes.

Introduction

The Ubiquitin-proteasome system has a major role in regulation of cellular processes, in particular the cell cycle and many signaling pathways [1,2]. Proteins targeted for degradation are conjugated to ubiquitin (Ub) by a cascade of enzymes, an E1 Ub activating- and E2 Ub conjugating enzyme, and an E3 Ub ligase responsible for substrate identification [3]. In some instances an E4 Ub chain elongating activity is also involved [4]. Ub chains comprising at least four K48-linked Ub molecules are recognized by the 19S Regulatory particle (RP) of the proteasome, either by an endogenous 19S RP subunit [5–7], or by a member of the Ub-L-UbA protein family. Ub-L-UbA proteins bind specific 19S RP subunits through their Ub-like (Ubl) domain and K48-Ub chains on the substrate through their Ub-associated (UbA) domain. The yeast family of Ub-L-UbA proteins comprises Rad23, Dsk2, and Ddi1, and each family member participates in the degradation of a range of substrates either by itself, or as a Rad23-Dsk2 pair (reviewed in [8]).

Ub-L-UbA proteins are often referred to as shuttle proteins based on their recruitment of the ubiquitylated substrate from the E2-E3 complex and transfer to the 19S RP. This is supported particularly by the interaction between Rad23 and Dsk2 with the chain elongating E2, Ufd2, that occurs in the framework of a complex between Ufd2 and the AAA-ATPase ring hexamer, Cdc48 [9]. However, many E3s bind the 19S RP directly; these include Ubr1 and Ufd4 [10], Hul5 [11], Ufo1 [12], SCF (Skp1-Cullin1-F-box protein) and APC (Anaphase Promoting complex) [13,14]. In the case of Ufd4, direct interaction between the E3 and the proteasome is essential for substrate degradation [15]. In some instances the Ub-L-UbA protein may be an essential stoichiometric subunit of the E3 complex, as reported for KPC2 (Kip1 ubiquitylation-promoting complex 2) that regulates degradation of the p27 cell cycle inhibitor [16]. These reports raise the question whether other Ub-L-UbA proteins may also occur as intrinsic components of an E3-19S RP complex and if so whether it is possible to detect additional interactions between the core domain of the Ub-L-UbA protein and subunits of this complex. In the event of such interactions they are a prerequisite for interaction of the E3 complex with the 19S RP.

The SCF complex comprises a rigid cullin scaffold, in S. cerevisiae Cdc53, with the RING protein, Rbx1, attached to a C-terminal domain [17]. The RING domain serves as a landing pad for the Ub-charged E2, Cdc34 [18]. Substrate recruitment is executed by a series of different F-box proteins (FBPs), each of which binds a subset of targets many of which are recognized by phosphorylation [19–21]. FBPs have a F-box domain and a WD40- or LRR substrate-binding domain. The F-box domain binds the Skp1 adaptor that interacts with the N-terminal domain of Cdc33 [17,22–25]. Exchange of FBPs within the SCF complex is achieved by auto-ubiquitylation of the FBP followed by degradation in the proteasome [26,27]. A number of FBPs of SCF complexes and the related BTB/3-box domain receptor proteins...
Transfer of Ho and Ufo1 to the Proteasome by Ddi1

We observed a robust interaction of GFPFL-Ufo1 with GSTFL-Ufo1 and with GSTUfo1-UIMs whereas the interaction between GFPFL-Ufo1 and GSTUfo1-UIM1-WD40 domain was extremely weak. GFPFL-Ufo1, Ufo1-WD40, and GSTUfo1-UIMs did not interact with GSTFL-Ufo1 or with GSTUfo1-UIM1. However, in contrast to GFPFL-Ufo1, truncated GSTUfo1-UIMs interacted robustly with GSTUfo1-WD40 (Figure 1A).

These results suggest both a positive and a negative role for the Ufo1-UIMs in Ufo1 dimerization. The positive role is indicated by the ability of FL-Ufo1 to dimerize with both FL-Ufo1 and with the isolated Ufo1-UIM fragment, whereas the negative role is indicated by the absence of dimerization between FL-Ufo1 and the Ufo1-WD40 domain fragment. This may indicate that the Ufo1-UIMs regulate access to the WD40 domain. To test directly whether the Ufo1-UIMs dimerize we incubated yeast extract with GFPFL-Ufo1-UIMs with recombinant GSTUfo1-UIMs on beads. We observed a robust interaction that was not found with the control GST beads indicating that isolated Ufo1-UIMs fragments dimerize (Figure 1B).

The interaction between GFPUfo1-UIMs and GSTUfo1-WD40 (Figure 1A) suggests that the Ufo1-WD40 domain by itself can dimerize. Indeed when we expressed the Ufo1-WD40 domain in bacteria with two different epitope tags we observed that GSTUfo1-WD40 bound to HBsUfo1-WD40 (Figure 1C). Thus Ufo1 resembles other FBPs in forming dimers and both the unique Ufo1-UIMs and the Ufo1-WD40 domain participate in dimerization. Dimerization via the Ufo1-WD40 domains is supported by our previous finding of turnover of Ho in ufo1A mutants that produce plasmid-encoded Ufo1-UIMs [35].

SCF[Ufo1] Complexes Interact with the 19S RP in vitro Only in the Presence of Substrate

Despite its nuclear role Ho must exit the nucleus to be degraded [46] and in ufo1A mutants stabilized Ho accumulates in the cytoplasm as an ubiquitylated conjugate [34]. SCF[Ufo1] complexes that have bound Ho may associate with the 19S RP as reported for SCF[Cdc4]-Sic1 complexes [14], or alternatively Ddi1 could shuttle ubiquitylated Ho from a SCF[Ufo1]-Ho complex to the proteasome. We therefore reconstituted SCF[Ufo1] complexes in vitro in the presence or absence of Ho. Recombinant GSTFL-Ufo1 and the GSTUfo1-WD40 domain proteins on GSH beads were incubated with yeast extract from cells that produced mycCdc53 and with the 19S RP complex tagged with Rpn11GFP. The experiment was performed both in the presence and the absence of GFPHo endonuclease. Experimental conditions are such that the 19S RP complex with a single tagged subunit remains intact in the yeast extract [5,13,14,36,51]. Both FL-Ufo1 and the Ufo1-WD40 domain on beads supported the formation of SCF[Ufo1]-Ho-19S RP complexes and interacted with yeast mycCdc53, GFPHo, and with the tagged 19S RP complex. In addition endogenous Ddi1 was present as a major component of the GSTFL-Ufo1 and the GSTUfo1-WD40 domain bead fractions of complexes formed in the presence of Ho. In the absence of Ho, we found an interaction of GSTUfo1 with mycCdc53, but there was no interaction with Rpn11GFP. Ddi1 could still be detected in the GSTFL-Ufo1 and the GSTUfo1-WD40 domain bead fractions, although in a considerably diminished amount (Figure 2A). A similar result was observed using tagged Rpn11GFP (Figure S2). Rpn12 was present in the bead fraction indicating that 19S RP complexes and not just the tagged subunit were interacting with SCF[Ufo1] (Figure S3).

Ddi1 is involved in the final stages of transfer of Ho and of Ufo1 to the 19S RP and could be recruited to the SCF[Ufo1]-Ho-19S RP complex after its assembly. We therefore repeated the above experiment using extracts of transformed ddi1A mutants. As in w.t.
cells, Ho was crucial for formation of complex between SCF^{Ufo1} and the 19S RP, however, there was no requirement for Ddi1 for formation of the SCF^{Ufo1-Ho-19S RP} complex (Figure 2B). These results taken together and supported by our \textit{in vivo} data that show that both Ho and Ufo1 accumulate as ubiquitylated conjugates in \textit{ddi1} mutants suggest that \textit{in vivo} Ddi1 is recruited to the SCF^{Ufo1-Ho-19S RP} complex after its assembly.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Ufo1 forms a homodimer via its UIMs. A. GST-FL-Ufo1, GST-Ufo1-WD40 domain, GST-Ufo1-UIMs or GST beads were incubated with yeast extract from cells expressing full-length pGAL-GFP-UFO1 or pGAL-GFP-UFO1.uims. The bead fraction was analysed by Western blotting with anti-GFP and anti-GST antibodies. T is 10\% of yeast extract with which the beads were incubated. \textasteriskcentered denotes contaminant band. B. Recombinant GST-Ufo1-UIMs or control GST beads were incubated with yeast extract with GFP-Ufo1-UIMs and analysed as above. T is 10\% of yeast extract as above. C. Recombinant GST-Ufo1 WD40 domain protein or control GST on GSH beads were incubated with bacterial lysate from cells that expressed HIS-Ufo1-WD40 and the bead fraction was analysed by Western blotting initially with anti-HIS and then with anti-GST antibodies. T is 10\% of yeast extract as above. The brackets around HIS-Ufo1-WD40 in the anti-GST Western blot indicate that these bands were observed after incubation with anti-HIS antibodies as shown in the upper part of the blot.}
doi:10.1371/journal.pone.0039210.g001
\end{figure}

\textbf{SCF^{Ufo1-Ho-Ddi1-19S RP} Complexes can be Reconstituted \textit{in vitro} with Immobilized GST-Ddi1 or GST-Rpn1}

Reconstitution of SCF^{Ufo1-Ho-19S RP} complexes \textit{in vitro} in the above experiments was achieved with GST-FL-Ufo1 or the GST-Ufo1-WD40 domain on beads. To determine whether complex reconstitution is also possible with immobilized GST-Ddi1 or GST-Rpn1, the 19S RP subunit bound by Ddi1 [36,52], we incubated GST-Ddi1 or control GST on GSH beads with yeast
extract from cells that produced \textit{myc}Cdc53 and \textit{GFP}Ufo1. We observed a robust interaction of both proteins with GSTD\textit{di1} that was not observed with the GST control beads (Figure 3A). Similarly, \textit{GST}\textit{Ufo1} on beads could reconstitute SCF \textit{Ufo1-GFP-Ho-GSTD}n1 complexes that included endogenous \textit{Ddi1} present in the yeast extract (Figure 3B). No complexes were formed with the control GST beads. Thus it is possible to reconstitute complexes \textit{in vitro} irrespective of which component is immobilized.

The Core of \textit{Ddi1} Binds Cdc53, the \textit{Ufo1-WD40} Domain, and \textit{Rpn1}

The \textit{Ufo1-UIMs} fragment in isolation interacts with all three UbL-UbA proteins, Rad23, Dsk2, and \textit{Ddi1}, however, FL-\textit{Ufo1} discrimimates between them [35]. This suggests that the initial interaction between \textit{Ufo1} and Ddi1 occurs via interaction of its UIMs with the Ddi1-UbL domain and that specificity of UbL-UbA protein may be conferred by further interactions between \textit{Ufo1} and the core of \textit{Ddi1}. We subcloned \textit{HIS}\textit{DD}\textit{Ddi1} without the UbL and UbA domains comprising residues 180–325. Indeed both the \textit{GST}\textit{Cdc53} and \textit{GST}\textit{Ufo1-WD40} domain bound core \textit{HIS}\textit{DD}\textit{Ddi1} (Figure 3C). \textit{Ddi1} binds the LRR domain of the \textit{Rpn1} subunit of the 19S RP [36,52] via its UbL domain and here we found that the core \textit{HIS}\textit{DD}\textit{Ddi1} fragment bound \textit{GST}\textit{Rpn1} robustly but showed only extremely weak binding to control \textit{GST}\textit{Rpn10} (Figure 3D). Thus after the initial interaction between the \textit{Ufo1-UIMs} and the \textit{Ddi1-UbL} these additional interactions with the \textit{Ddi1} core could secure \textit{Ddi1} within the SCF \textit{Ufo1-GFP-Ho-Ddi1-19S RP} complex. They could also allow flexibility to the \textit{Ddi1-UbL} allowing it to switch to binding \textit{Rpn1} for substrate or FBP transfer.

\textbf{Figure 2. SCF} \textit{Ufo1-Ho-19S RP} complex formation \textit{in vitro}. A. \textit{GST}\textit{Ufo1}, \textit{GST}\textit{Ufo1-WD40} domain, or control GST beads were incubated with yeast extract from cells with tagged genomic \textit{RPN11-GFP} that were transformed with \textit{pGAL-MYC-CDC53} either with \textit{pGAL-GFP-HO} or alone. The bead fraction was analysed by Western blotting with anti-GFP antibodies to detect \textit{Rpn11GFP} and \textit{GFP}Ho, with anti-myc antibodies to detect \textit{myc}Cdc53, and with anti-Ddi1 and anti-GST antibodies. T is 10% of total yeast extract with which the beads were incubated (Lanes 1 and 2). Lane 3: \textit{GST}\textit{Ufo1} beads incubated with \textit{myc}Cdc53, \textit{Rpn11GFP} and \textit{GFP}Ho; Lane 4: \textit{GST}\textit{Ufo1} WD40 domain incubated with \textit{myc}Cdc53, \textit{Rpn11GFP} and \textit{GFP}Ho; Lane 5: control GST beads incubated with these yeast extracts; Lane 6: \textit{GST}\textit{Ufo1} beads incubated with \textit{myc}Cdc53 and \textit{Rpn11GFP}; Lane 7: \textit{GST}\textit{Ufo1} WD40 domain incubated with \textit{myc}Cdc53 and \textit{Rpn11GFP}; Lane 8: control GST beads incubated with these yeast extracts. B. \textit{GST}\textit{Ufo1}, \textit{GST}\textit{Ufo1-WD40}, or control GST beads were incubated with yeast extract from \textit{ddi1} mutant cells that expressed \textit{pGAL-MYC-CDC53}, \textit{pGFP-RPN11}, with or without \textit{pGAL-GFP-HO}. The bead fractions were analysed by Western blotting with anti-myc, anti-GFP, anti-\textit{Ddi1}, and anti-GST antibodies as in A. T is 10% of total yeast extract with which the beads were incubated (Lanes 1-3). Lane 4: \textit{GST}\textit{Ufo1} beads incubated with \textit{myc}Cdc53, \textit{Rpn11GFP} and \textit{GFP}Ho; Lane 5: \textit{GST}\textit{Ufo1} WD40 domain incubated with \textit{myc}Cdc53, \textit{Rpn11GFP} and \textit{GFP}Ho; Lane 6: control GST beads incubated with these yeast extracts; Lane 7: \textit{GST}\textit{Ufo1} beads incubated with \textit{myc}Cdc53 and \textit{Rpn11GFP}; Lane 8: \textit{GST}\textit{Ufo1} WD40 domain incubated with \textit{myc}Cdc53 and \textit{Rpn11GFP}; Lane 9: control GST beads incubated with these yeast extracts.

doi:10.1371/journal.pone.0039210.g002
Figure 3. Immobilized Ddi1 and Rpn1 reconstitute SCF^{Ufo1} complexes *in vitro*. A. GSTDdi1 or control GST on GSH beads were incubated with yeast extract from cells that produced mycCdc53 and GFPUfo1. Analysis was by Western blotting with anti-myc, anti-GFP, and anti-GST antibodies. T represents 10% of the yeast extract with which the beads were incubated. B. GSTRpn1, GSTRpn10, or GST beads were incubated with yeast extract from cells that produced mycCdc53 and GFP^{Ufo1} mixed with bacterial lysate with recombinant HIS^{Ufo1}. The bead fraction was analysed by Western blotting with anti-myc, anti-HIS, anti-GFP, anti-Ddi1, and anti-GST antibodies. T represents 10% of the yeast extract incubated with the beads. C. GSTUfo1 WD40 domain, GSTCdc53, or control GST on GSH beads were incubated with bacterial lysate from cells that produced recombinant HIS^{Ufo1}ΔDdi1. The bead fraction was analysed by Western blotting with anti-HIS and anti-GST antibodies as indicated. T is 10% of the HIS^{Ufo1}ΔDdi1 bacterial lysate incubated with the beads. D. The HIS^{Ufo1}ΔDdi1 bacterial lysate was incubated with GSTRpn1, GSTRpn10, or GST beads and analysed as above.
doi:10.1371/journal.pone.0039210.g003
SCF\textsuperscript{Ufo1-Ddi1}-19S RP Complex Subunits Immunoprecipitate Together in the Presence of Ho

The above experiments demonstrate that in the presence of Ho a SCF\textsuperscript{Ufo1-Ho-Ddi1-19S RP} complex is formed in vitro. To verify that this is indeed a complex we prepared a reaction mix comprising yeast extract with mycCdc53, with or without GFPHo, and bacterial lysate with GSTUfo1 and HISRpn1, and immunoprecipitated each tagged protein separately. In the presence of Ho, immunoprecipitation of mycCdc53, of GFPHo, of GSTUfo1 or of HISRpn1 led to reciprocal coimmunoprecipitation of the other three proteins and of Ddi1 present in the yeast extract. In the absence of Ho, immunoprecipitation of mycCdc53, GSTUfo1 or HISRpn1 led to coimmunoprecipitation of endogenous Ddi1 from the yeast extract, but not of any of the other proteins of the complex formed in the presence of substrate. This result indicates that in the presence of Ho a bona fide complex is formed between SCF\textsuperscript{Ufo1-Ho-Ddi1-Rpn1} and Rpn1. This complex does not form in the absence of Ho (Figure 4).

Ufo1 and Rpn1 Bind Ddi1 in Both a Competitive and a Synergistic Manner

(a) Competitive interaction: GSTRpn1 abrogates binding of GPFUfo1 to HISDdi1. The Ddi1-UbL domain binds both the Ufo1-UMs and Rpn1 [35,52], however, interaction between Ddi1 and Rpn1 is essential for turnover of Ufo1 [36]. Both Ufo1 and Rpn1 bind the core of Ddi1 (Figure 3C and 3D) and this interaction may facilitate the switch of the Ddi1-UbL domain from the Ufo1-UMs to Rpn1 for transfer of Ho or Ufo1 to the 19S RP. We therefore examined whether there is competition between Ufo1 and Rpn1 for interaction with Ddi1. Each protein incubated separately with Ddi1 beads was present in the HISDdi1 bead fraction (Figure 5A, Lanes 4-6). However, Rpn1 displaced Ufo1 from Ddi1 when both GSTUfo1 and GSTRpn1 were incubated together with the HISDdi1 beads (Lane 7). In contrast addition of yeast extract with ubiquitylated GFPHo to the reaction mix with either GSTUfo1 or GSTRpn1 did not affect the binding of either protein to HISDdi1 (Lanes 8 and 9). Furthermore, Ho in the reaction mix comprising Ufo1, Rpn1, and Ddi1, abrogated the competition between Ufo1 and Rpn1 and all three proteins bound the HISDdi1 beads (Lane 10) and Figure 2. Thus Ho protects Ufo1 from displacement from Ddi1 by Rpn1. In this complex the Ddi1-UbL would bind Rpn1, Ufo1 would be bound via its WD40 domain to Ho and to the Ddi1 core, and further interactions would occur between the Ddi1-UbA and the Ub chains on Ho. This is the complex we predict to underlie transfer of ubiquitylated Ho to the 19S RP (Figure 6).

(b) Synergistic interaction: GSTRpn1 and GFPUfo1 bind HISDdi1 in a tertiary complex that requires the Ddi1 UbA domain and does not involve the Ddi1 UbL domain. The competitive interaction between Ufo1 and Rpn1 may occur during handover of the FBP to the 19S RP after degradation of Ho. To explore this hypothesis we examined whether exclusion of GSTUfo1 from binding to HISDdi1 by GSTRpn1 is concentration dependent. We calibrated the system by determining an amount for each lysate/extract that would give detectable binding of protein to the Ddi1 beads (x1, Figure 5B, Lanes 3 and 4). Then keeping the amount of GPFUfo1 extract constant in a fixed reaction volume we increased the amount of GSTRpn1 lysate two- and threefold. In this experiment we used ubiquitylated GPFUfo1 produced in yeast [35]. GSTRpn1 at x1 and x2 in the reaction mix gave a similar amount bound to the Ddi1 beads. Both these GSTRpn1 concentrations abrogated binding of GPFUfo1 to Ddi1 (Figure 5B, Lanes 5 and 6 and as observed in Figure 5A, Lane 7). However, x3 the amount of GSTRpn1 lysate induced synergistic binding of GSTRpn1 and GPFUfo1 to the HISDdi1 beads. A similar although considerably weaker signal was obtained when core HISADdi1 beads were used. In contrast binding of GSTRpn1 to the HISDdi1 beads was not affected by GSTUfo1 nor was any synergistic effect observed between them in binding to Ddi1 (Figure 5C). In contrast to Ddi1 [36] there is no direct binding between Ufo1 and Rpn1 (Figure 5D).

The competition between Ufo1 and Rpn1 for binding Ddi1 may involve the Ddi1-UbL which binds both proteins (above). To address this question we repeated the synergistic binding experiment described in Figure 5B but this time in addition to GSTFL-Ddi1 beads we used Ddi1 that lacked either the UbL or UbA domain: GSTDdi1AUBL, and GSTDdi1AUBA, respectively (Figure 5E, Lanes 1–3). Ddi1AUBL exhibited severely reduced binding to Rpn1 and did not bind Ufo1 when each protein was incubated separately with the beads. In contrast, Ddi1AUBA bound both Rpn1 and Ufo1 synergistically when both were present in the reaction mix. This suggests a role for the Ddi1-UbA in the synergistic binding of Rpn1 and Ufo1 to Ddi1. Surprisingly although Rpn1 binds the Ddi1-UbL, when we incubated HISRpn1 with GSTDdi1AUBA beads it interacted less strongly than with GSTFL-Ddi1 beads (Figure 5E, compare Lane 1 with Lane 4). GPFUfo1 bound GSTDdi1AUBA beads and there was an extremely
Figure 5. Rpn1 and Ufo1 exhibit synergistic binding to Ddi1. A. Bacterial lysate with \( \text{GST} \text{Rpn1} \) or \( \text{GST} \text{Ufo1} \) and yeast extract with \( \text{GFP} \text{Ho} \) were incubated with \( \text{HIS} \text{Ddi1 beads} \) alone (Lanes 4–6), in pairs (Lanes 7–9), or all three together (Lane 10). The bead fractions were analysed by Western transfer of Ho and Ufo1 to the Proteasome by Ddi1.
Figure 6. Model for sequential interactions of Ho, Ufo1, and Rpn1 with Ddi1. Panel 1. Active SCF\textsubscript{Ufo1}-Ho complexed with the 19S RP recruits Ddi1 by interaction of the Ufo1-UIMs with the Ddi1-UbL domain ([35] and Figures 2, 4 and 5F). Subsequently the core of Ddi1 binds the Ufo1-WD40 domain and Rpn1 (Figure 3C and D). Both Ufo1 (Figure 1) and Ddi1 [38] form dimers but are drawn here as monomers for clarity. Panel 2. The Ddi1-UbA domain interacts with ubiquitin chains on Ho and the Ddi1-UbL binds Rpn1 for transfer of ubiquitylated Ho to the 19S RP [34]. At this stage Ho, Rpn1, and Ddi1 bind Ddi1 simultaneously (Figure 5A). Panel 3. After degradation of Ho, Ufo1 can no longer bind Ddi1 in the presence of Rpn1 (competitive interaction, Figure 5B and C). However, at high levels of Rpn1 there is synergistic binding that is supported to a small extent by the Ddi1 core (Figure 5B) and is totally dependent on the Ddi1-UbA domain (Figure E). Based on the higher affinity of the Ddi1-UbL for Rpn1 seen in the competitive interaction we propose that at this stage the Ddi1-UbL binds Rpn1 and the Ddi1-UbA binds ubiquitin chains on Ufo1. This would lead to competitive binding of Rpn1 to GSTDdi1 and GSTUfo1 in vitro experiments indicated that Ufo1 and Ddi1 interact via the Ufo1-UIMs and the Ddi1-UbL [35]. We therefore substituted GFPUfo1\textsubscript{UIMs} for GFP\textsubscript{FL-Ufo1}. Indeed GFPUfo1\textsubscript{UIMs} did not interact with GST\textsubscript{FL-Ddi1}, GSTDdi1\textsubscript{UbL} or GSTDdi1\textsubscript{UbA} beads both in the presence or the absence of Rpn1 (Figure 5F).

Discussion

Complex reconstitution in vitro indicated that SCF\textsubscript{Ufo1} complexes that contain their substrate, Ho, are associated with the 19S RP. These complexes can assemble in the absence of Ddi1, however, in experiments with extracts from w.t. cells Ddi1 is found in association with the SCF\textsubscript{Ufo1}-Ho-19S RP complex. Our interpretation is that Ddi1 is recruited to preformed SCF\textsubscript{Ufo1}, Ho-19S RP complexes. Based on our previous experiments in vivo we propose that Ddi1 enters the SCF\textsubscript{Ufo1}-Ho-19S RP complex via initial interaction between the Ufo1-UIMs and the Ddi1-UbL ([35] and Figure 5F). Subsequent interaction between the Ufo1-WD40 and the core of Ddi1 detected here could explain the specificity of the interaction of SCF\textsubscript{Ufo1} for Ddi1 [35]. The recruitment of Ddi1 after formation of the SCF\textsubscript{Ufo1}-Ho-19S RP complex supports our in vivo results that suggested Ddi1 is required for disassembly of SCF\textsubscript{Ufo1} complexes after substrate degradation. This hypothesis is based on accumulation of ubiquitylated Ho in the cytoplasm of ddi1A mutants [34], stabilization of full-length Ufo1 in ddi1A mutants, cell cycle arrest at the G1-S interphase by overexpression of Ufo1\textsubscript{Aums} in wild type cells or of full-length Ufo1 in ddi1A mutants, and by the accumulation of Cln2, a substrate of the FBP, Grr1 [21], in cells with a high level of Ufo1\textsubscript{Aums} [35]. The Ufo1-UIMs promote dimerization of Ufo1 and are crucial for all interactions of Ufo1 with Ddi1. They may fulfill two roles in dimerization: one is physical interaction between the UIMs of two Ufo1 molecules to initiate dimerization. The other is regulation of access to the Ufo1-WD40 domain as full-length Ufo1 did not dimerize with an Ufo1-WD40 domain fragment. Thus dimerization may start at the C-terminal UIMs and proceed to include the Ufo1-WD40 domains. We previously reported that SCF complexes from cells that produced Ufo1\textsubscript{Aums} are capable of degrading Ho [35]. Given that dimerization of FBPs has been shown to be a prerequisite for substrate ubiquitylation in some instances [28,32,33], our current results support an interpretation that in the absence of its UIMs the Ufo1-WD40 domains of each monomer are able to interact with one another in vivo. The Ufo1-WD40 domain alone is sufficient for formation of complexes that include GFPHo, the 19S RP, and Ddi1 and indeed in our yeast two-hybrid experiments we reported an interaction between Cdc53 and the Ufo1-WD40 domain [35]. This is unusual as the solved SCF structures do not display interaction between the cullin and the WD40 domain of the FBP [23,25] or with the related BTB/3-box domain receptor protein [28]. The Ufo1 WD40 sequence has a rather degenerate b-propeller sequence and a full analysis of this unusual interaction awaits solution of the 3D structure of Ufo1. A dimerization sequence has been identified in weak synergistic binding of Rpn1 and Ufo1 to GSTDdi1\textsubscript{UbA} beads when both were present in the reaction mix. Our previous in vivo experiments indicated that Ufo1 and Ddi1 interact via the Ufo1-UIMs and the Ddi1-UbL [35]. We therefore substituted GFPUfo1\textsubscript{UIMs} for GFP\textsubscript{FL-Ufo1}. Indeed GFPUfo1\textsubscript{UIMs} did not interact with GST\textsubscript{FL-Ddi1}, GSTDdi1\textsubscript{UbL} or GSTDdi1\textsubscript{UbA} beads both in the presence or the absence of Rpn1 (Figure 5F).
the N-terminal region of certain FBPs [26,27] and it is conceivable that there is one in Ufo1 too that could serve for dimerization in the absence of the Ufo1 UIMs.

The Ddi1-UbL – Ufo1-UIMs interaction is essential for recruitment of Ddi1 to the SCF<sup>Ufo1</sup>-Ho-19S RP complex ([35] and Figure 5F). However, degradation of the ubiquitylated substrate requires transfer of the Ddi1-UbL from its interaction with the Ufo1-UIMs to Rpn1 [36]. In the presence of Ho a complex is formed that includes Ufo1, Rpn1, and Ddi1. Binding of Ho to Ddi1 is mediated by interaction of its ubiquitin chains with the Ddi1-UbA domain [34]. We propose that interaction of the Ddi1-UbA with a critical amount of Ub chains on Ho could lead to switching of the Ddi1-UbL domain from the Ufo1-UIMs to Rpn1 for transfer of Ho to the 19S RP. Transfer of the Ddi1-UbL without disruption of the complex between these proteins would be supported further by concurrent binding of the Ddi1 core to both Ufo1 and Rpn1 and by interactions of Ho with both the Ufo1-WD40 domain [40] and with the Ddi1-UbA domain via its Ub chains ([34] and Figure 6).

The “substrate shield” model proposes that the substrate protects the FBP from degradation [47]. In the reaction lacking Ho (comparable to an in vivo situation after substrate degradation but prior to SCF<sup>Ufo1</sup> complex disassembly) we observed two different modes of interaction of Ufo1 and Rpn1 with Ddi1: (a) competitive - Rpn1 excludes Ufo1 from binding to Ddi1; (b) synergistic - high levels of Rpn1 form a tertiary complex between Ufo1, Ddi1, and Rpn1. The competitive interaction indicates that the Ddi1-UbL has higher affinity for Rpn1 than for the Ufo1-UIMs. The dependence of synergistic binding of Ufo1 and Rpn1 on the Ddi1-UbA domain suggests that Ub chains on Ufo1 are involved. The higher ratio of Rpn1 to Ufo1 in the in vitro Ddi1 synergistic binding experiment could parallel molecular crowding within the SCF<sup>Ufo1</sup>-19S RP complex. Thus in the absence of Ho our data support a complex in which the Ddi1-UbL is bound to Rpn1 while the Ddi1-UbA domain binds Ub chains on Ufo1. This model for sequential transfer of Ho and of Ufo1 to the 19S RP is presented in Figure 6.

**Materials and Methods**

### Yeast Strains

Wild type BY4741 (MATa; his3A1; leu2A0; metl5 Δ0; ura3A0, and ddi1A (MATa, leu3 Δ1, leu2 Δ0, lys2 Δ0, ura3 Δ0, TRY1(3xkanMX4)) were purchased from Euroscarf. Strains with genomic RPNI-GFP and RNPI1-GFP are from the library of [48].

### Bacterial Strains

Rosetta bacteria (Novagen) (F<sup>−</sup>, amp<sup>T</sup>, hisS<sub>Ry</sub><sup>Ry</sup>, mp<sub>Ry</sub>, dcm, gal, lacY<sup>T</sup>, pRARE (argU, argW, ileX, glyT, leuW, proL<sup>C</sup>)) were used for expression of GST-His<sub>Ry</sub> proteins. His-tagged recombinant proteins were expressed in BL21 (Promega) (F<sup>−</sup>, amp<sup>T</sup>, hisS<sub>Ry</sub><sup>Ry</sup>, mp<sub>Ry</sub>, dcm, gal, lacY<sup>T</sup>, pRARE (argU, argW, ileX, glyT, leuW, proL<sup>C</sup>)) were used for most recombinant protein expression. His-tagged recombinant proteins were expressed in BL21 (Promega) (F<sup>−</sup>, amp<sup>T</sup>, hisS<sub>Ry</sub><sup>Ry</sup>, mp<sub>Ry</sub>, dcm, gal, lacY<sup>T</sup>, pRARE (argU, argW, ileX, glyT, leuW, proL<sup>C</sup>)).

### Yeast Plasmids

YPGKAL-GFP (GFP-UFO1, GFP-AUMs, GFP-HO) are described in [35]; pMT2999 for expression of MHC-CD3<sub>C</sub> from the GAL promoter was obtained from M. Tyers [21]; pYE-RPN11-GFP in which expression of RNPI1-GFP is from the ADH1 promoter was obtained from M. Glickman [49].

**Growth media and yeast transformation** by LiOAc are as in [50].

**Bacterial Plasmids**

* pHB2-GST-CD3<sub>C</sub>, pGST-DDI1, pGST-DDI1-AUBL, pGST-DDI1-AUBL, pHIS-DDI1, pHIS-AADDI1, pGST-RPN1, and pGST-RPN10 (gift of Dorota Skowyra); pGEX-3X-1 (Amersham Biosciences) was used to construct pGST-UFO1 by ampling the UFO1 gene from genomic DNA using primer pair Ufo1F (GAACTTATGAGCGGCGCTGGTGTGATT) and Ufo1R (CTCGAGTCAATTGATTTCACTAATGCAACG).

**Immunoprecipitation and immunoblotting** were performed as described in [3,40]. Briefly, proteins were induced from the GAL promoter by overnight growth in minimal medium with 2% galactose. Next morning the culture was diluted 1:3 and grown for a further 1.5 hours. 50 ml of logarithmic culture served as the source of a 300 μl extract with 80 μg/μl protein. 200 μl were taken for immunoprecipitation (IP) with the appropriate antibody and the immunoprecipitate was run in a single lane for Western blotting (WB).

**Antibodies**

Mouse anti-GFP (Roche Applied Science), mouse 9E10 antmyc (Enzo), and mouse anti-HIS (Sigma) antibodies were used at a dilution of 1:250 for IP and at 1:1,000 for WB; mouse anti-GST (Santa Cruz Biotechnology) antibodies were diluted 1:1,000 for IP and 1:2,000 for WB, rabbit anti-DDI1 (gift from Jeffrey Gerst) was used at 1:5,000 for WB. Goat anti-mouse and anti-rabbit antisera, used at 1:1,000 were from Santa Cruz Biotechnology. Protein A- sepharose was purchased from Amersham and used at 50%; 30 μl were added to each sample.

**TCA precipitation** proteins were precipitated from 300 μl cell extract by adding TCA to 10% with 10 minutes incubation on ice. The pellet was centrifuged at 12,000 g for 10 minutes and five volumes of cold acetone were added. The protein pellets were harvested and dried. For WB analysis the pellets were dissolved in 30 μl of sample buffer and 5 μl of each fraction was separated by SDS-PAGE.

**Expression of GST and HIS Fusion Proteins in Rosetta**

**Bacteria**

Bacteria were transformed by electroporation and the colonies selected on LB-agar plates with ampicillin and kanamycin, each at 100 μg/ml and chloramphenicol at 34 μg/ml. A single colony was grown in 1 liter of LB (with ampicillin and chloramphenicol) to an OD<sub>600</sub> of 0.6–0.8 (3–5 hours) with vigorous agitation at 37°C. IPTG was added to 0.4 mM to induce expression and the culture was incubated overnight at 20°C. The cells were harvested by centrifugation at 4°C for 10 min at 6,000 rpm. The cell pellet was washed with 20 ml of ice-cold PBS and resuspended in 3 ml yeast extract buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub> and 2 mM dithiothreitol).
5 mM EDTA, 0.1% NP40, 1:25 Protease Inhibitor cocktail (Roche). The cell suspension was disrupted by an ultra-sonic sonicator on ice using 6 cycles each of 10 seconds and clarified by centrifugation for 10 min at 4,000 rpm at 4°C. The supernatants were incubated with Glutathione-Sepharose 4B (GSH) beads (Amersham Biosciences) prewashed in yeast extract buffer with 1% Triton-X100; HIS-fusion proteins were incubated with washed Ni-sepharose (Clontech) for 1 hr at 4°C. The bead fractions were washed 5 times in extract buffer with 2.5% Triton-X100. The GST- and HIS-fusion proteins on beads were stored at -20°C after addition of glycerol to 5%.

**GST in vitro Binding Assay**

Yeast cells were grown overnight to late log phase (OD600 = 0.8) in 2% galactose medium for the G63-regulated constructs, or in YePD. The cells were harvested by centrifugation at room temperature for 5 minutes at 4,000 rpm, washed in 50 ml TE and resuspended in 600 μl extract buffer, 0.5-0.6 mg of glass beads were added and the cells were broken by vigorous vortexing for 25 minutes at 4°C. The extract was clarified by centrifugation at 12,000 g for 20 minutes at 4°C and protein concentration was measured with the Bio-Rad protein reagent. 5–10 mg of protein extract were taken for each GST pull-down in a total volume of 350–400 μl extract buffer. 30–50 μl of 50% Glutathione Sepharose 4B beads coupled to GST fusion protein were added to each sample and incubated at 4°C for 1–2 hours with very mild shaking. The samples were washed 6 times with extract buffer with 2.5% Triton X100. The pellet was resuspended in 30–50 μl sample buffer x2, boiled for 5 minutes and centrifuged for 3 minutes at high speed to remove insoluble material. The supernatant was separated on a 12% polyacrylamide SDS gel with protein size standards followed by WB analysis.

**References**

1. Kornitzer D, Ciechanover A (2000) Modes of regulation of ubiquitin-mediated protein degradation. J Cell Physiol 182: 1–11.
2. Kirklin V, Diik I (2007) Role of ubiquitin- and Ubl-binding proteins in cell signaling. Curr Opin Cell Biol 19: 199–205.
3. Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem 67: 425–479.
4. Koegl M, Hoppe T, Schlenker S, Ulrich H, Mayer T, Jentsch S (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell 96: 635–644.
5. Voloshin O, Gocheva Y, Gutnick M, Movshovich N, Bakhrat A, et al. (2010) Tubulin chaperone E binds microtubules and proteasomes and protects against misfolded protein stress Cell and Mol Life Sci 67: 2025–2038.
6. Hussein K, Elasser S, Zhang N, Chen X, Randles L, et al. (2008) Proteasome subunit Rpn13 is a novel ubiquitin receptor. Nature 453: 481–480.
7. Isala M, Kaur EJ, Kim W, Yago V, Gonzalez S, et al. Monoubiquitination of RPN10 regulates substrate recruitment to the proteasome. Mol Cell 38: 733–745.
8. Finley D (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu Rev Biochem 78: 477–513.
9. Hanzelmann P, Stingel J, Hofmann K, Schindelin H, Raasi S (2010) The yeast E4 ubiquitin ligase Ufd2 interacts with the ubiquitin-like domains of Rad23 and Dsk2 via a novel and distinct ubiquitin-like binding domain. J Biol Chem 285: 29390–29398.
10. Xie Y, Varsavsky A (2000) Physical association of ubiquitin ligases and the 26S proteasome. Proc Natl Acad Sci U S A 97: 2497–2502.
11. Leggett DS, Hanna J, Borodovsky A, Crosas B, Schmidt M, et al. (2002) Multiple associated proteins regulate proteasome structure and function. Mol Cell 10: 495–507.
12. Baranes-Bachar K, Khaliha I, Ivantsh Y, Lavut A, Voloshin O, et al. (2008) New interacting partners of the F-box protein Ufo1 of yeast. Yeast 25: 733–743.
13. Verma R, Chen S, Feldman R, Schielze D, Yates J, et al. (2000) Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol Biol Cell 11: 3425–3439.
14. Wold B, Gass A, Delfraux NA, Cheng YH, Bally E, et al. (2005) ATP hydrolysis-dependent disassembly of the 26S proteasome is part of the catalytic cycle. Cell 121: 533–563.
15. Xie Y, Varsavsky A (2002) UFD4 lacking the protease-binding region catalyses ubiquitination but is impaired in proteolysis. Nat Cell Biol 4: 1003–1007.
16. Harms T, Kamura T, Kato T, Saka Y, Fujikawa K, et al. (2005) Role of the UBL-UBA Protein RP2 in Degradation of p27 at G1 Phase of the Cell Cycle. Mol Cell Biol 25: 9292–9303.
17. Zheng N, Schulman BA, Song L, Miller JJ, Jeffery PD, et al. (2002) Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature 416: 703–709.
18. Kamura T, Koepp DM, Conrad MN, Skowyra D, Moreland RJ, et al. (1999) Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science 284: 657–661.
19. Bai C, Sen P, Hofmann K, Ma L, Goehl M, et al. (1996) SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell 86: 263–274.
20. Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell 91: 209–219.
21. Patton E, Willems A, Sa D, Kuras L, Thomas D, et al. (1998) Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell cycle. Mol Cell 20: 9–19.
22. Paton E, Willems A, Sa D, Kuras L, Thomas D, et al. (1998) Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methane biosynthesis in yeast. Genes Dev 12: 692–705.
23. Hao B, Zheng N, Schulman BA, Wu G, Miller JJ, et al. (2003) Structural basis of the Cdc53-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase. Mol Cell 20: 9–19.
24. Orlicky S, Tang X, Willems A, Tyers M, Sibiri S (2003) Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. Cell 112: 243–256.

**Supporting Information**

**Figure S1 Domains of Ufo1 and Ddi1 used in experiments.** The protein fragments used in the experiments depicted in the Figures are shown.

**Figure S2 Formation of SCF(Ufo1)-Ho-19S RP complex with yeast extract from RPN1-GFP cells.** GST-Ufo1 or control GST beads were incubated with yeast extract from cells with tagged genomic RPN1-GFP that were cotransformed with pGFP-Ho and with pMYC-CDC53. The bead fraction was analysed by Western blotting with anti-GFP antibodies to detect Rpn1 and Ho, with anti-myc antibodies to detect Cdc53, and with anti-Ddi1 antibodies.

**Figure S3 Rpn12 is present in the GST-Ufo1 bead fraction.** A further experiment in which GST-Ufo1 and GST beads were incubated with yeast extract in the presence of GFP-Ho as in Figures 2 and S2. Here the Western blot employed antibodies made to GST-Rpn12. The presence of Rpn12 is an indication that the 19S RP is intact.

**Author Contributions**

Conceived and designed the experiments: OV SH AB DR. Performed the experiments: OV SH AB DR. Analyzed the data: OV SH AB. Contributed reagents/materials/analysis tools: OV SH. Wrote the paper: SH DR.
24. Schultman BA, Carrano AC, Jeffrey PD, Bowen Z, Kinnamon ER, et al. (2000) Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. Nature 408: 381–386.

25. Wu G, Xu G, Schultman BA, Jeffrey PD, Harper JW, et al. (2003) Structure of a beta-TrCP-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP) ubiquitin ligase. Mol Cell 11: 1445–1456.

26. Mathias N, Johnson S, Byers B, Goebel M (1999) The abundance of cell cycle regulatory protein Cdc1p is controlled by interactions between its F box and Skp1p. Mol Cell Biol 19: 1759–1767.

27. Galan JM, Peter M (1999) Ubiquitin-dependent degradation of multiple F-box proteins by an autocatalytic mechanism. Proc Natl Acad Sci U S A 96: 9124–9129.

28. Zhuang M, Calabrese MF, Liu J, Waddell MB, Nourse A, et al. (2009) Structures of SPOP-substrate complexes: insights into molecular architectures of BTB-Cul3 ubiquitin ligases. Mol Cell 36: 39–50.

29. Hao B, Oehlmann S, Sowa ME, Harper JW, Pavletich NP (2007) Structure of a F-box/Skp1-cyclin E complex: multisite-phosphorylated substrate recognition by SCF ubiquitin ligases. Mol Cell 26: 131–145.

30. Wolf DA, McKeon F, Jackson PK (1999) F-box/WD-repeat proteins Pop1p and Sud1p/Pop2p form complexes that bind and direct the proteolysis of cdc18p. Curr Biol 9: 373–376.

31. Seibert V, Prohl C, Schoutz I, Rhee E, Lopez R, et al. (2002) Combinatorial diversity of fission yeast SCF ubiquitin ligases by homo- and heterooligomeric assemblies of the F-box proteins Pop1p and Pop2p. BMC Biochem 3: 22.

32. Wang M, Calabrese MF, Liu J, Waddell MB, Nourse A, et al. (2005) Suprafacial orientation of the SCF Cdc4 dimer accommodates multiple geometries for substrate ubiquitination. Cell 129: 1163–1176.

33. Wolkow M, Charman BE. (2007) Pbc7/BCDC4 dimerization regulates its substrate interactions. Cell Div 2: 7.

34. Kaplun L, Tzirkin R, Bakhrat A, Shahak N, Ivantsiv Y, et al. (2005) The DNA damage-inducible Ubl-Uba protein Ddi1 participates in Mec1-mediated degradation of Ho endonuclease. Mol Cell Biol 25: 5355–5362.

35. Ivantsiv Y, Kaplun L, Tzirkin-Goldin R, Shahak N, Raveh D (2006) Turnover of SCF complexes requires the Ubl-Uba motif protein, Ddi1. Mol Cell Biol 26: 1579–1588.

36. Gomez T, Kolawa N, Gee M, Svedrasoki M, Deshaies R (2011) Identification of a functional docking site in the Rpn11 LRR domain for the UBA-UBL domain protein Ddi1. BMC Biol 9: 33.

37. Krylov DM, Koonin EV (2001) A novel family of predicted retroviral-like aspartyl proteases with a possible key role in eukaryotic cell cycle control. Curr Biol 11: R584–587.

38. Elsasser S, Gali RR, Schwickart M, Larsen CN, Leggett DS, et al. (2002) Participation of the proteasomal lid subunit Rpn11 in mitochondrial morphology and function is mapped to a distinct C-terminal domain. Biochem J 361: 275–283.

39. Hirano S, Kawasaki M, Ura H, Kato R, Raiborg C, et al. (2006) Double-sided ubiquitin binding of Hs-UIM in endosomal protein sorting. Nat Struct Mol Biol 13: 272–277.

40. Oldham CE, Moloney RP, Miller SL, Hanes RN, O’Bryan JP (2002) The ubiquitin-interacting motifs target the endocytic adaptor protein epsin for ubiquitination. Curr Biol 12: 1112–1116.

41. Kozak M, Shiehman K, Tal-Or P, Yakir L, Mosesson Y, et al. (2002) Ligand-independent degradation of epidermal growth factor receptor involves receptor ubiquitylation and Hgs, an adaptor whose ubiquitin-interacting motif targets ubiquitylation by Nedd4. Traffic 3: 740–751.

42. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, et al. (2003) Global analysis of protein localization in budding yeast. Nature 425: 686–691.

43. Oldham CE, Mohney RP, Miller SL, Hanes RN, O’Bryan JP (2002) The ubiquitin-interacting motifs target the endocytic adaptor protein epsin for ubiquitination. Curr Biol 12: 1112–1116.

44. Regan-Klapisz E, Soreskina I, Voortman J, de Keizer P, Roovers RC, et al. (2005) Ubiquitin recruits Eps15 into ubiquitin-rich cytoplasmic aggregates via a UIM-UBL interaction. J Cell Sci 118: 4437–4450.

45. Kaplun L, Ivantsiv Y, Kornitzer D, Raveh D (2006) Functions of the DNA damage response pathway target Ho endonuclease of yeast for degradation via the ubiquitin-26S proteasome system. Proc Natl Acad Sci U S A 97: 10077–10082.

46. Deshaies R (1999) SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Biochem 68: 435–467.

47. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, et al. (2003) Global analysis of protein localization in budding yeast. Nature 425: 686–691.

48. Oldham CE, Mohney RP, Miller SL, Hanes RN, O’Bryan JP (2002) The ubiquitin-interacting motifs target the endocytic adaptor protein epsin for ubiquitination. Curr Biol 12: 1112–1116.

49. Rinaldi T, Pick E, Gambadoro A, Zilli S, Maytal-Kivity V, et al. (2004) Ubiquitination of Ho endonuclease. Mol Cell Biol 25: 5355–5362.

50. Adams A, Gottschling DE, Kaiser CA, Stearns T (1997) Methods in Yeast Genetics. CSHL Press, NY.

51. Verma R, Oania R, Graumann J, Deshaies RJ (2004) Multiubiquitin chain independent monoubiquitination of ubiquitin-binding proteins. Mol Cell 26: 891–896.

52. Elsasser S, Gali RR, Schwickart M, Larsen CN, Leggett DS, et al. (2002) Participation of the proteasomal lid subunit Rpn11 in mitochondrial morphology and function is mapped to a distinct C-terminal domain. Biochem J 361: 275–283.

53. Adams A, Gottschling DE, Kaiser CA, Stearns T (1997) Methods in Yeast Genetics. CSHL Press, NY.

54. Verma R, Oania R, Graumann J, Deshaies RJ (2004) Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. Cell 118: 99–110.

55. Elsasser S, Gali RR, Schwickart M, Larsen CN, Leggett DS, et al. (2002) Proteasome subunit Rpn11 binds ubiquitin-like protein domains. Nat Cell Biol 4: 725–730.