NEDD8 links cullin-RING ubiquitin ligase function to the p97 pathway

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The AAA+ ATPase p97 and its UBA-UBX cofactors are thought to extract ubiquitinated proteins from membranes or protein complexes as a prelude to their degradation. However, for many cofactors ubiquitinated targets have not yet been identified, leaving their biological function unclear. Previous analysis has linked the p97 pathway to cullin-RING ubiquitin ligases (CRLs); here we demonstrate that the human p97 cofactor UBXD7 mediates the p97-CRL interaction through its conserved ubiquitin-interacting motif (UIM). UBXD7 and its yeast ortholog, Ubx5, associate only with the active, NEDD8- or Rub1-modified form of cullins. Disruption of the Ubx5 UIM results in a loss of CRL binding and consequently impedes degradation of a Cul3 substrate. These results uncover an unexpected and conserved role for NEDD8 in linking CRL ubiquitin ligase function to the p97 pathway.

The abundant homohexameric AAA+ ATPase p97/VCP (Cdc48 in yeast) participates in a wide range of cellular processes, including cell cycle regulation, endoplasmic reticulum (ER)-associated degradation, membrane fusion and autophagy1. In many of these processes, p97 is thought to recognize ubiquitinated substrates and separate them from tightly bound partner proteins. Substrate specificity is established through interactions with a plethora of p97 cofactors. In humans, the largest group of cofactors consists of at least 13 proteins that interact with the N-terminal region of p97 through a ubiquitin regulatory X (UBX) domain. Five of these proteins (p47, UBXD7, UBXD8, FAF1 and SAKS1) also have a ubiquitin-binding (UBA) domain, classifying them as UBA-UBX proteins. A recent proteomic analysis revealed that in addition to binding ubiquitin conjugates, UBA-UBX proteins interact with over two dozen ubiquitin ligases2, including several members of the CRL family.

CRLs are multisubunit complexes comprising three core components—a RING-finger protein, a cullin and, except for Cul3-based CRLs, a cullin-specific adaptor protein3. The adaptor protein binds interchangeable substrate specificity factors, which in turn recruit substrates for ubiquitination. For example, the Cul1 adaptor SKP1 recruits over 42 different F-box proteins to Cul1 (refs. 4,5), whereas elongin C recruits ~41 BC-box proteins to Cul2 and Cul5 (ref. 6). CRL activity is stimulated following the covalent attachment of a ubiquitin-like molecule, NEDD8, to a conserved lysine residue (ref. 6). CRL activity is stimulated following the covalent attachment of a ubiquitin-like molecule, NEDD8, to a conserved lysine residue (ref. 6). CRL activity is stimulated following the covalent attachment of a ubiquitin-like molecule, NEDD8, to a conserved lysine residue (ref. 6).

UBXD7 preferably binds Cul2 and Cul4

UBA-UBX adaptor interactions with CRLs may be mediated indirectly via p97. To understand how the p97 network is connected to CRLs, we examined whether CRL binding is specific for a certain UBA-UBX adaptor. We deleted the UBX domain from Flag-tagged versions of the five human UBA-UBX domain proteins (p47, UBXD8, FAF1, UBXD7 and SAKS1) to minimize cross-association with other p97-bound proteins. The expressed proteins were recovered by immunoprecipitation and evaluated by immunoblotting. As expected, only p47 ∆UBX, which has a second p97 contact site11, retained its ability to interact with p97 (Fig. 1a). Unexpectedly, only UBXD7 ∆UBX interacted with endogenous Cul2 and Cul4a.

To assess the cullin-binding preference of UBXD7, V5-tagged cullin constructs (Cul1–5) were coexpressed with Flag-tagged UBXD7. Even though similar levels of p97 were found in association with UBXD7, strikingly different amounts of V5-tagged cullins were recovered (Fig. 1b). UBXD7 displayed the most efficient binding toward Cul2, Cul4a and Cul4b (Fig. 1b and Supplementary Fig. 1), weaker interaction with Cul1 and Cul3, and no interaction with Cul5. UBXD7’s binding preference for Cul2 and Cul4 was confirmed in a reciprocal pull-down assay. Notably, even though cullins

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Received 6 September 2011; accepted 18 February 2012; published online 1 April 2012; doi:10.1038/nsmb.2269
UBXD7 interacts with all cullins except CUL5. (a) Flag-tagged UBA-UBX proteins (p47, UBXD8, FAF1, UBXD7 and SAKS1) lacking the UBX domain (UBX) expressed in 293T cells were immunoprecipitated (IP) with anti-Flag antibodies and probed to detect endogenous binding partners CUL2, CUL4 and p97 as indicated. –, no transfection. (b) Same as in a, except that cells were transfected with (+) or without (−) vectors encoding Flag-UBXD7 and V5 epitope–tagged CUL1–5. Immunoprecipitations were carried out with antibodies against the Flag or V5 epitope.

Figure 1 UBXD7 associates with all cullins except CUL5. (a) Flag-tagged UBA-UBX proteins (p47, UBXD8, FAF1, UBXD7 and SAKS1) lacking the UBX domain (UBX) expressed in 293T cells were immunoprecipitated (IP) with anti-Flag antibodies and probed to detect endogenous binding partners CUL2, CUL4 and p97 as indicated. –, no transfection. (b) Same as in a, except that cells were transfected with (+) or without (−) vectors encoding Flag-UBXD7 and V5 epitope–tagged CUL1–5. Immunoprecipitations were carried out with antibodies against the Flag or V5 epitope.

UBXD7 interacts exclusively with the active form of cullins

Whether UBXD7 associates with active or inactive CRLs is unclear. Coexpression of Flag-UBXD7 with hemagglutinin-tagged CUL2 (HA-CUL2) resulted in a slight increase in neddylated HA-CUL2 (Fig. 2a). This may arise from the ability of UBXD7 to inhibit deneddylation of CUL1 by COP9 signalosome in a purified system (R.J.D. and E. Emberley, unpublished data). However, despite the presence of more unneddylated than neddylated HA-CUL2 in the lysate, UBXD7 exclusively bound the neddylated form. Because of this apparent selectivity for neddylated cullins, we also probed Flag-UBXD7 pull-downs with NEDD8-specific antibodies and detected multiple endogenously neddylated species in the cullin size range but no unconjugated NEDD8 (Fig. 2b).

We next examined whether cullin neddylation mediates the CRL–UBXD7 interaction. Neddylation-deficient CUL1 K720R and CUL2 K689R mutants exhibited minimal UBXD7 binding (Fig. 2c), and inhibition of NEDD8 conjugation via the NEDD8–E1 inhibitor MLN4924 (ref. 10) resulted in accumulation of deneddylated CUL2 and CUL4a proteins that failed to bind UBXD7 (Fig. 2d). Altogether, these results clearly identified UBXD7 as a neddylation-dependent CRL-binding protein.

UBXD7 interacts with neddylated cullins via its UIM

Because UBXD7 has a UBA domain and UIM (Fig. 3a), it could interact with neddylated CRLs via their bound, ubiquitinated substrates. Using pull-down assays with purified K48–linked ubiquitin chains and Flag-tagged UBXD7 mutant proteins, we found that the UBA domain and not the UIM contributed to polyubiquitin binding (Fig. 3b). Next, we looked at binding to purified CUL2 and CUL4a in the absence of polyubiquitin chains. When mixtures with equal amounts of neddylated and unneddylated cullins were incubated with recombinant Flag-UBXD7, only the neddylated forms were recovered after Flag pull-down (Fig. 3c). This interaction largely depended on the UIM; deletion of the ubiquitin-associating (UAS) domain had a modest effect on CUL4a binding but no effect on CUL2 binding. By contrast, removal of the UBA or the UBX domains had no effect. The UIM dependence was also seen with recombinant CUL1–RBX1 (Supplementary Fig. 2a) and CUL3–RBX1 complexes (data not shown). Additional mapping experiments showed that a fragment containing just the UBXD7 UIM plus surrounding sequences bound neddylated CUL2 (Supplementary Fig. 2b).

To evaluate further the role of UBXD7’s four domains, we determined the ability of the constructs employed above to bind endogenous CUL2, p97 and ubiquitin conjugates (Fig. 3d). ΔUBIM mutant UBXD7 exhibited a large reduction in binding to endogenous CUL2, a moderate reduction in binding to ubiquitin conjugates and normal binding to p97, indicating that the UIM mediates assembly of UBXD7 with CUL2. Unexpectedly, both the ΔUAS and ΔUBX mutants showed binding defects that were not seen with purified proteins (the former with CUL2 and the latter with ubiquitin conjugates). We do not understand the
The UIM of UBXD7 associates with conjugated NEDD8

The UIM is a ~20-amino-acid sequence motif\(^{13}\) that forms a single \(\alpha\)-helix, and hydrophobic residues within the helix interact with the Leu8-Ile44-Val70 hydrophobic patch of ubiquitin\(^{14,15}\). Notably, these three residues are conserved in NEDD8 and form a hydrophobic surface identical to the one on ubiquitin\(^{16}\), which could potentially be recognized by a UIM. Sequence alignment confirmed that UBXD7 contained the conserved residues characteristic for a UIM (Supplementary Fig. 3). To examine the possibility of a direct interaction between NEDD8 and the UIM of UBXD7, we used as a template the crystal structure of the UIM of hepatocyte growth factor–regulated tyrosine kinase substrate (HRS) bound to ubiquitin\(^{17}\) and superimposed the structures of ubiquitin with NEDD8 and the HRS UIM with the UIM of UBXD7 (Fig. 4a). The resulting UBXD7 UIM–NEDD8 model was computationally refined using RosettaDock\(^{18}\). The final low-energy model showed that residues in the UIM of HRS and the structurally equivalent residues in UBXD7 made similar contacts with ubiquitin and NEDD8, respectively.

To validate this, we generated single-substitution (A293Q) and triple-substitution (E286R L290E A293Q) mutants in either full-length UBXD7 or UBX7-DUBX (Fig. 4b and Supplementary Fig. 3). When compared to wild-type UBXD7, both mutants exhibited reduced affinity for endogenous neddylated CUL2 in a pull-down assay, and this effect was more striking in the context of UBXD7-DUBX (Fig. 4b). Conversely, purified CUL2–RBX1 neddylated with a NEDD8 protein mutated in the hydrophobic patch (N8–L8) showed a lower binding affinity for purified UBXD7 than CUL2–RBX1 neddylated with wild-type NEDD8 (Fig. 4c). This decrease in association was not due to a change in the NEDD8-induced conformation; a mutant CUL1AWHB–RBX1 complex that spontaneously adopted the active conformation without neddylation\(^{8,19}\) did not bind UBXD7 (Supplementary Fig. 4). Together, these results support the idea that formation of a UBXD7–CRL complex is stabilized by a direct interaction between conjugated NEDD8 and the UIM of UBXD7.

Next we tested whether the UIM of UBXD7 is unique in its ability to recognize NEDD8 by replacing it with UIMs from the ubiquitin-binding protein HRS or the proteasomal subunit S5a. In low-stringency binding conditions (Fig. 4d, CUL2 (lo)) little difference was seen in the amount of recovered CUL2. When the stringency was increased, however (Fig. 4d, CUL2 (me)), both HRS UIM and the first UIM of S5a lost almost all their CUL2-binding ability. In contrast, the second UIM of S5a (S5a-2) was equivalent to UBXD7’s UIM.

The UIM replacement experiment suggested that the UIM of UBXD7 is not NEDD8 specific, but rather the recognition of NEDD8 is context dependent. This predicts that replacing conjugated NEDD8 on CUL2 with ubiquitin would not affect UBXD7 binding. The E2 enzyme UBCH5c can transfer ubiquitin onto the NEDD8 acceptor lysine of CUL1, and this mimics the activating effect of neddylation\(^{7,8}\). Using conditions that favor this monoubiquitination reaction, we generated a mixture that contained both unmodified and monoubiquitinated

Figure 3 UBXD7 directly interacts with neddylated CRLs via its UIM. (a) Domain organization of wild-type UBXD7 protein. UBA, ubiquitin-associated; UAS, ubiquitin-associating; UIM, ubiquitin-interacting motif; UBX, ubiquitin regulatory X. (b) Recombinant wild-type (WT) Flag-UBXD7 or a deletion mutant was incubated with Lys48-linked polyubiquitin chains before immunoprecipitation and western blotting with the indicated antibodies. (c) A mix (input) of unmodified and neddylated recombinant full-length CUL2–RBX1 or CUL4–RBX1 complex was incubated with recombinant Flag-tagged WT UBXD7 or deletion mutants. Following immunoprecipitation with anti-Flag antibodies, recovered proteins were detected by western blotting with the indicated antibodies. Ax Ub refers to ubiquitin chains of increasing length. (d) Full-length Flag-tagged UBXD7 or the indicated UBXD7 deletion mutants were expressed in cells treated with MG132. Lysates were immunoprecipitated with anti-Flag antibodies, and co-precipitated endogenous proteins were detected by western blotting with the indicated antibodies. Ub, ubiquitin. (e) Binding assays were performed as in c, using a mix of unmodified and neddylated recombinant CUL2 CTD–RBX1 or CUL2 CTD–RBX2 complex and recombinant Flag-tagged WT or deletion mutant UBXD7.
The UIM of Ubx5 is required for the degradation of Rpb1

To address whether the association between UBXD7 UIM and NEDD8-conjugated cullins contributes to degradation of CRL substrates, we turned to *Saccharomyces cerevisiae*, in which the first, and so far only, UBX-dependent CRL substrate has been described (other established CRL- and p97-dependent substrates, including CDT1 (data not shown), are not dependent on UBXD7). We recently reported that UV-induced, CUL3-dependent proteolysis of the large subunit of RNA polymerase II (Rpb1) depends on the Cdc48 cofactor Ubx5 (ref. 20). Ubx5, like UBXD7, contains the UBA, UAS, UBX and UIM domains (Supplementary Fig. 5a,b), which is consistent with the suggestion that it is the yeast equivalent of mammalian UBXD7 (ref. 21). Moreover, Ubx5 binds yeast Cul3 (ref. 20), which associates with Elongin C and therefore is functionally most closely related to human CUL2 and CUL5 (ref. 22). We incubated purified Flag-Ubx5 protein with a 1:1 mixture of unmodified SCFCdc4 and SCFCdc4 modified with the yeast NEDD8 ortholog Rub1. SCFCdc4 consists of yeast CUL1 (Cdc53) and Rbx1 (Hrt1), Skp1 and the F-box protein Cdc4. Analogous to UBXD7, Ubx5 only bound to rubylated Cdc53, and this interaction was disrupted by deletion or point mutation of the UIM domain (Fig. 5a).

To assess the role of Ubx5’s UIM, we compared UV-induced degradation rates of Rpb1 in wild type, in ubx5∆ and in a strain, ubx5SIM3, in which the UIM of endogenous UBX5 was eliminated by homologous recombination. Whereas Rpb1 was rapidly degraded in wild-type cells, its degradation was delayed in ubx5SIM3 and further impaired in a ubx5∆ strain (Fig. 5b). Notably, tagging the endogenous loci with a Myc epitope confirmed that both wild-type and Ubx5∆UIM proteins were properly folded and expressed at identical levels (Supplementary Fig. 5c,d).

Figure 5 The UIM in yeast Ubx5 promotes UV-dependent degradation of Rpb1. (a) A mix (input) of unmodified SCF and rubylated recombinant SCF (R-SCF) was incubated with recombinant Flag-tagged wild-type (WT) Ubx5 or the various Ubx5 mutants. Following immunoprecipitation with anti-Flag beads, the recovered proteins were probed with the indicated antibodies. Single and triple mutants are A368Q and E286R L290E A293Q, respectively. (b) Yeast cultures (WT, ubx5∆, ubx5SIM3) were UV irradiated, and total cell lysates were prepared at the indicated times before detection by western blotting using antibodies against Rpb1 and tubulin (tub). (c) Same as in b except that western blots were carried out with infrared dye–linked secondary antibodies and quantified with a LI-COR Odyssey imager following normalization with tubulin. Error bars show s.e.m.; n = 3 for each genotype.
The intermediate effect on Rpb1 degradation in the ubx5uimΔ strain was also observed in a rub1Δ strain23, suggesting that Cul3, Rub1 and the UIM of Ubx5 function in a common pathway. Indeed, a rub1Δ ubx5uimΔ strain behaved identically to the single-mutant rub1Δ strain, indicating an epistatic relationship between these mutations (Fig. 5c). These results are consistent with a functional, rubylation-dependent interaction between Ubx5 and cullins and demonstrate a role for the Ubx5 UIM in promoting degradation of Rpb1 in response to UV radiation.

DISCUSSION

In our efforts to understand how the p97 pathway is linked to CRLs we discovered that the UBA-UBX protein UBXD7 selectively associated with neddyalted cullins. UBXD7 is the only p97 adaptor with a UIM, and this motif enables UBXD7 and its yeast ortholog Ubx5 to bind neddyalted cullins.

Several lines of evidence indicate that the UIM-NEDD8 interaction, though critical, is insufficient by itself to mediate the binding of UBXD7 to neddyalted CRLs. This is not surprising, as UIM-ubiquitin interactions are typically of low affinity (Kₐ > 100 μM)24. We propose that weak interactions between other sequences in UBXD7 and surfaces of the CRL that become exposed upon neddylation place the UIM in proper register to bind NEDD8. In this manner, the UIM-NEDD8 interface stabilizes a multidentate interaction between UBXD7 and active, neddylated CRLs. In support of this hypothesis, UBXD7’s UIM can be swapped for a canonical ubiquitin-binding UIM, or NEDD8 can be replaced by ubiquitin, with little or no effect on UBXD7-CRL association. The exact nature of the rest of the UBXD7 binding surface remains unknown, but we note two things: first, it is likely to reside adjacent to the UIM-NEDD8 interface, because the UIM plus flanking sequences are sufficient to bind neddyalted CUL2 (Supplementary Fig. 2b); and second, UBXD7 is acidic (pI ~ 5), which could facilitate interaction with the basic canyon in cullins25. Mutating the basic canyon impairs UBXD7 binding (Supplementary Fig. 2a) while maintaining ubiquitin ligase activity25. A crystal structure will be needed to fully resolve the details of the UBXD7-CRL interaction.

The NEDD8-dependent recruitment of UBXD7 biases the p97 pathway to engage CRLs that are active and potentially involved in substrate ubiquitination. However, this raises the question of how UBXD7-p97 targets are selected. Our data point to some selectivity with respect to the cullin, with UBXD7 preferentially interacting with CUL2 and CUL4. The reason for this preference is unclear but could be related to differences in sequence or subcellular localization, possibly regulated by post-translational modification. For instance, two proteomics studies identified UBXD7 as a target of the ATM-ATR pathway26,27, which fits well with the known function of mammalian CUL4 in DNA replication and DNA-damage signaling and repair28. However, UBXD7 associated with polyubiquitin conjugates in the absence of radiation, suggesting that not all targets are specific to the DNA-damage pathway (Fig. 3d).

Additional control over recruitment could come from the substrate itself. CRL substrates with tightly folded domains, substrates that are part of multisubunit assemblages or substrates associated with subcellular structures (for example, chromatin) might require p97 unfoldase activity for proficient proteasomal degradation. We posit that when the proteasome encounters a difficult-to-resolve structure, the rate of degradation slows. According to this model, a temporarily stalled neddyalted CRL-polyubiquitinated substrate–proteasome complex might comprise a signal that attracts UBXD7, and the lifetime of such a stalled complex would determine the statistical likelihood that the UBXD7-p97 pathway is engaged. For cullin complexes whose substrates do not require p97 for degradation, the cycle of neddylation, substrate ubiquitination and de neddylation might occur very quickly, providing limited opportunity for UBXD7 to bind.

Our data point to a positive role for the UBXD7 ortholog Ubx5 in the degradation of polyubiquitinated Rbp1 stalled at UV-induced lesions. However, we wish to note that all three determinants of the CRL complex (neddylation, Rbx1 and the basic canyon) that are important for UBXD7-CRL interaction also contribute to recruitment of CDC43, raising the possibility that UBXD7 might antagonize CRL activity. Notably, UBXD7 modestly inhibited SCF–TrCP and CDC43–dependent ubiquitination of a β-catenin peptide in vitro (G.K., unpublished data). If UBXD7 can function as a CRL antagonist in some contexts, it could explain our prior observation that levels of the CRL2VHL substrate HIF1α are lower in UBXD7-depleted cells than in nondepleted cells29. Studies on the CRL regulators COP9 signalosome and CAND1 have revealed that these factors, which inhibit CRL activity in vitro, paradoxically behave as positive regulators in cells29. UBXD7 may possess a similar dual nature that manifests itself depending upon the substrate and the manner in which it is assayed.

Our study revealed an unexpected role for NEDD8. As an activating signal, NEDD8 conjugation causes a conformational change in the cullin, eliminating the CAND1 binding site and locking the enzyme in an active state30. We propose that following this conformational change, NEDD8 and the newly exposed surfaces on cullin and RBX1 recruit UBXD7/Ubx5, which in turn links p97/Cdc48 to the CRL. For substrates whose dislocation from other factors and subsequent proteolysis are strongly dependent on p97/Cdc48 activity, recruitment of UBXD7/Ubx5 via NEDD8 promotes degradation. Selective recruitment of UBXD7 to neddyalted CRLs may also be employed in some circumstances to restrain their activity. Uncovering additional CRL substrates that engage the p97 pathway will be required to gain deeper insight into the full range of biological functions regulated by UBXD7.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

We thank M. Rome for providing Lys48-linked polyubiquitin chains, N. Pierce for purified SCFαTrCP, T. Hagen (National University of Singapore) and E. Emberley for cullin expression constructs, and D. Duda and B. Schulman (St. Jude Children’s Research Hospital) for providing purified recombinant CUL2, CUL3, CUL4a, CUL1ΔWHB and NCE2 proteins. We are grateful to S. Lewis for help with the Rosetta Dock server, Millennium Pharmaceuticals for MLN4924 and NEDD8 antibody, and the members of the Dessen laboratory for helpful discussion during the course of this work. This work was supported by the Howard Hughes Medical Institute (HHMI) and a National Institute of Health Ruth Kirschstein Postdoctoral Fellowship (F32 GM088975; W.d.B.). R.J.D. is an HHMI Investigator.

AUTHOR CONTRIBUTIONS

W.d.B. and R.J.D. conceived and designed the experiments; W.d.B. performed most of the experiments, except that G.K. performed the structural modeling in Figure 3a, and R.V. and R.S.O. made the yeast strains and carried out the Rbp1 turnover studies in Figure 5b and c; W.d.B. and R.J.D. wrote the manuscript with editorial input from the other authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/nsmb/.
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ONLINE METHODS

Cell culture. Human kidney 293T cells were maintained in DMEM (BioWhittaker) supplemented with 10% (v/v) FBS (Atlanta Biologicals), 4 mM l-glutamine and 100 units each of penicillin and streptomycin (Invitrogen) in a 5% CO2–humidified incubator. Where it is indicated, cells were treated with 1 μM MLN4924 (Millennium Pharmaceuticals) for 1 h or with 20 μM MG132 (EMD Biosciences) for 3 h.

Expression constructs. Constructs were generated using standard techniques and verified by DNA sequencing. Cloning details and primer sequences are provided in the Supplementary Methods. Constructs for the expression of V5 tagged CUL1–5 were provided by T. Hagen10 (see Acknowledgments). All constructs used in this study are listed in Supplementary Table 1 with their corresponding Deshaies laboratory database (RDB) number.

Immunoprecipitation and immunoblotting. Cells were lysed in either buffer A (50 mM HEPES, pH 7.5, 5 mM Mg(OAc)2, 70 mM KOAc, 0.2% (v/v) Triton X-100, 10% (v/v) glycerol and 0.2 mM EDTA) or EBC buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1 mM EDTA and 0.5% (v/v) NP-40) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma) and phosphatase inhibitors (10 mM β-glycerophosphate, 1 mM NaF and 0.1 mM NaVO4). For Figure 4d, buffer A and EBC buffer are the low- and medium-stringency buffers, respectively. Cleared lysates were immunoprecipitated with antibodies directed against the following (Immobilon-P, Millipore). Proteins were detected using antibodies against CUL1 (71-8700, Zymed/Invitrogen), CUL2 (51-1800, Zymed/Invitrogen), CUL4a (2527-1, Epitomics), HA (3F10-HRP, Roche), V5 (V5-HRP, Sigma), p97 (H-120, Santa Cruz), β-actin (AB-8243, Sigma), Flag (M2-HRP, Sigma) and ubiquitin (SPA-200, Stressgene) and incubated at 42 °C for 16 h. The mixture was bound and eluted from MonoQ resin using a linear salt gradient with a final concentration of 800 mM NaCl. Peak fractions containing tetraubiquitin chains were pooled and dialyzed into Tris and stored at −80 °C.

For the in vitro binding assay with ubiquitin chains, Flag-UBD7 protein (1 μM) was incubated with 400 nM ubiquitin chains in binding buffer A. Binding reactions were carried out at 4 °C for 1 h followed by 1 h in the presence of anti-Flag beads (M2-agarose, Sigma). Beads were collected by centrifugation at 2,000g for 1 min, washed three times with binding buffer and boiled in 2× Laemmli sample buffer. Samples were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon-P, Millipore). Proteins were detected using antibodies against ubiquitin (SPA-200, Stressgene) and Flag (M2-HRP, Sigma).

Synthesis of K48-linked ubiquitin chains and in vitro binding assay. K48-linked ubiquitin chains were synthesized as previously described34 using 0.8 μM yeast ubiquitin E1 (Ubα1), 10 μM yeast Cdc34 and 0.7 mM ubiquitin (Sigma) in Tris buffer (30 mM Tris, 5 mM MgCl2, 200 mM NaCl, 2 mM DTT and 40 μM ATP) and incubated at 42 °C for 16 h. The mixture was bound and eluted from MonoQ resin using a linear salt gradient with a final concentration of 800 mM NaCl. Peak fractions containing tetraubiquitin chains were pooled and dialyzed into Tris and stored at −80 °C.

For the in vitro binding assay with ubiquitin chains, Flag-UBD7 protein (1 μM) was incubated with 400 nM ubiquitin chains in binding buffer A. Binding reactions were carried out at 4 °C for 1 h followed by 1 h in the presence of anti-Flag beads (M2-agarose, Sigma). Beads were collected by centrifugation at 2,000g for 1 min, washed three times with binding buffer and boiled in 2× Laemmli sample buffer. Samples were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon-P, Millipore). Proteins were detected using antibodies against ubiquitin (SPA-200, Stressgene) and Flag (M2-HRP, Sigma).

Protein expression and purification. Recombinant proteins for CUL2–RBX1 complex, Flag-UBD7 and Flag-UBx5 (WT, domain deletion, and single and triple mutants) were expressed in bacteria and purified by standard methods. Detailed protocols are in Supplementary Methods. Procedures for purification of NEDD8 (ref. 31), NEDD8-82 (UBCH12) (ref. 31), NEDD8-E1 (APPBP1/UBA3) (ref. 31), Split-n-Coexpress CUL1 (ref. 32), UBCH5 (ref. 7), human ubiquitin E1 (ref. 7), UBA1 (yeast ub-E1) (ref. 33), yeast Cdc34 (ref. 25) and SCFCdc4 (refs 26, 27) were described previously. The purified proteins CUL2CTD–RBX1, CUL2CTD–RBX2, Split-n-Coexpress CUL1a, CUL1AWH and NCE2 were generously provided by D. Duda and B. Schulin (see Acknowledgments).

Neddylation conditions and in vitro binding assay. Cullins were neddylated by incubating cullin–RBX1 complexes (300 nM) with NEDD8 (250 nM, 400 μM ATP, 2 mM DTT and 5 mM MgCl2). For neddylation CUL2–RBX2 complexes, UBCH12 was substituted by NCE2/UB2E2. These neddylation reactions were complete in 5 min at 25 °C and resulted in a 1:1 mixture of unneddylated:neddylated cullin–RBX complex. Monoubiquitination of the NEDD8 acceptor lysine in CUL2 was carried out by mixing Ubc-E1 (250 nM), UBCH1c (1 μM), CUL2–RBX1 (300 nM) and ubiquitin (20 μM) in 30 mM Tris-Cl, pH 8.0, containing 2 mM ATP, 2 mM DTT and 5 mM MgCl2. Reactions were incubated at 25 °C for 1 h and stopped by the addition of 100-fold excess of binding buffer. These reaction conditions resulted in a 1:1 mix of unmodified:mono-Ub CUL2.

Recombinant Flag-UBD7 protein (1 μM) was mixed with 10 nM cullin–RBX complex (directly taken from neddylation reaction) in binding buffer A (50 mM HEPES, pH 7.5, 5 mM Mg(OAc)2, 70 mM KOAc, 0.2% (v/v) Triton X-100, 10% (v/v) glycerol and 0.2 mM EDTA). Binding reactions were carried out at 4 °C for 1 h followed by 1 h in the presence of anti-Flag beads (M2-agarose, Sigma). Beads were collected by centrifugation at 2,000g for 1 min, washed three times with binding buffer and boiled in 2× Laemmli sample buffer. Samples were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon-P, Millipore). Proteins were detected using antibodies against CUL1 (71-8700, Zymed/Invitrogen), CUL2 (51-1800, Zymed/Invitrogen), CUL4a (2527-1, Epitomics) and Flag (M2-HRP, Sigma).

Yeast transformation and genomic integration. The yeast integration vector pRS306-UBX5SUMA (RDB2610) was linearized with EcoRI and transformed into the W303 yeast strain. Transformants that grew on SD plates lacking uracil were analyzed by PCR to confirm correct integration at the UBX5 locus using a forward primer (5′-tcacctgtaaagccttcctctc-3′) and reverse primer (5′-tcctgttataagtgcttacatac-3′). Yeast strains with the correct integration were counter-selected on 5-fluoroorotic acid, surviving clones were screened for uracil auxotrophy and genomic DNA was analyzed by PCR at UIM deletion using the following primers: forward, 5′-tcacctcgtgtaaagccttcctc-3′ and reverse, 5′-gtgacttcagctgcttcagcagac-3′.

The chromosomal UBX5 open reading frame was tagged with Myc epitopes according to the procedure of ref. 36, using the following primers: forward, 5′-gtcctatgatgttctactaggatatgcagaggctctgctgctgatggttaaag-3′ and reverse, 5′-atctactaactaatatatatatatattatttctactagcactacatgttgctgcttgatggttaaag-3′.

Rbp1 degradation assay. Rbp1 degradation assays were performed as described in ref. 30.

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