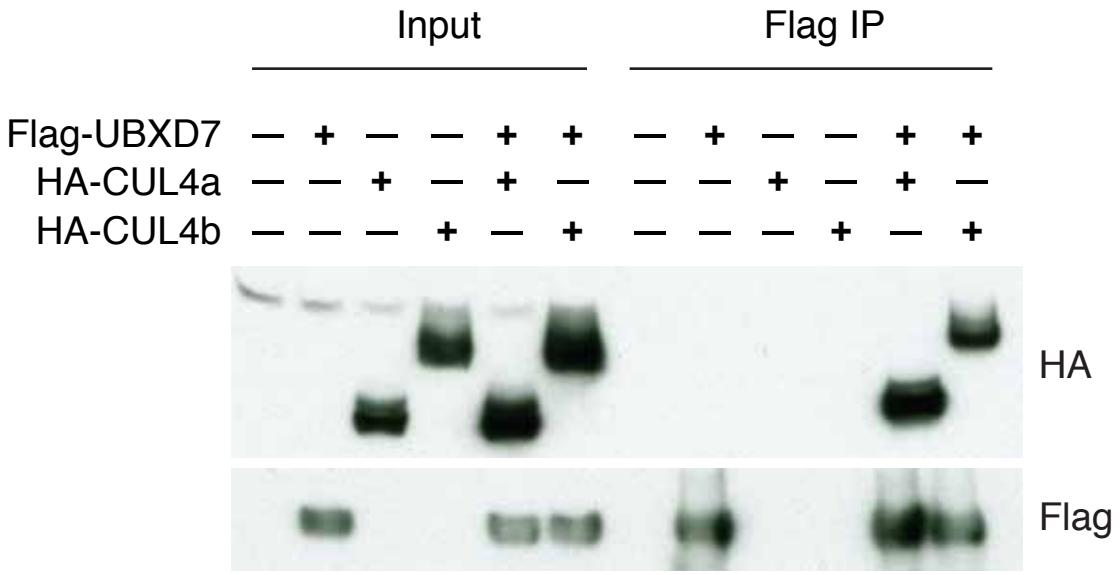
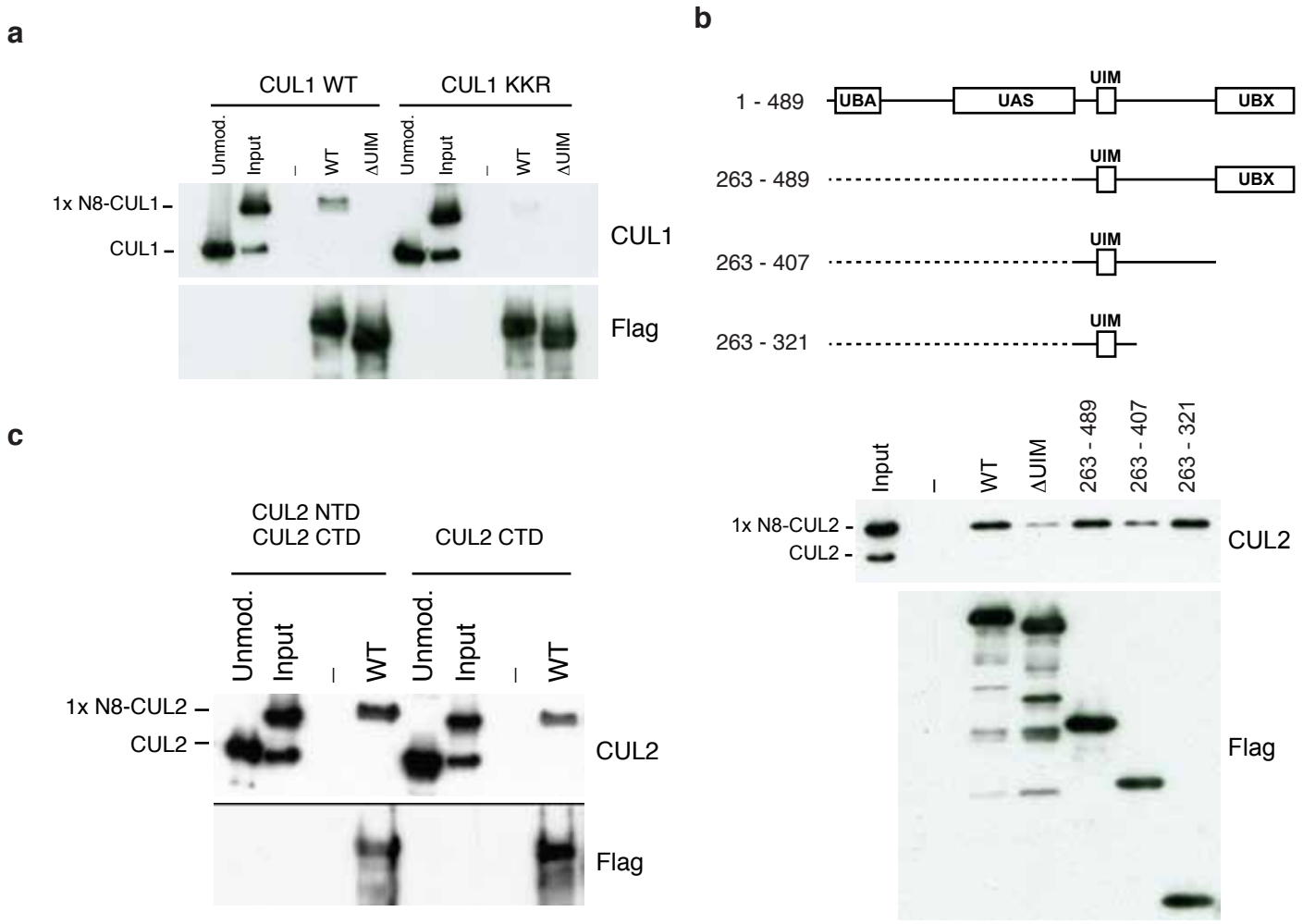


Supplementary Figure 1. UBXD7 associates with similar efficiency to CUL4a and CUL4b. Human 293T cells were transiently transfected with (+) or without (–) the indicated expression constructs. Cell extracts were prepared 48 hours later and immunoprecipitated (IP) with anti-Flag antibodies. Recovered proteins were eluted by boiling in sample buffer, separated by SDS-PAGE, and transferred to PVDF. Membranes were probed with antibodies against HA and Flag.

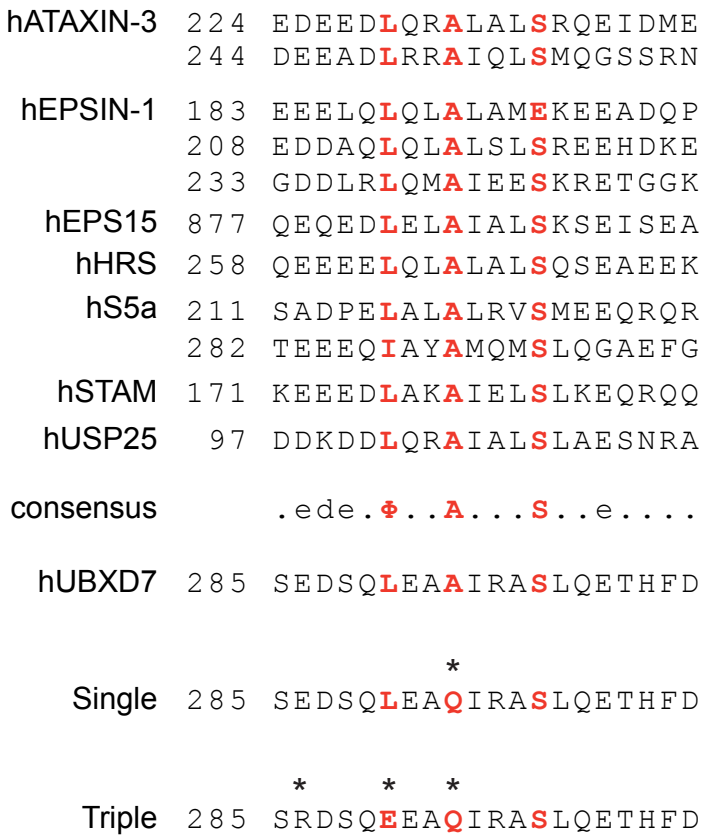


Supplementary Figure 2. Additional feature contribute to the UBXD7 UIM - neddylated CRL interaction.

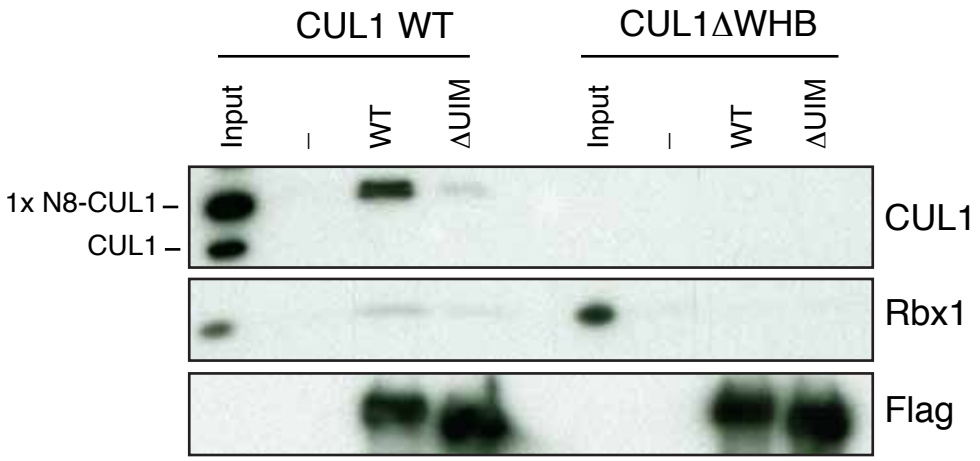
(a) A mix (shown as input) of unneddylated and neddylated recombinant full-length CUL1–RBX1 WT or CUL1-RBX1 KKR complex (10 nM) was incubated with 1 μM of recombinant wild type Flag-tagged UBXD7 or the various UBXD7 deletion mutants for 1 hour. Following immunoprecipitation (IP) with anti-Flag antibodies, the recovered proteins were separated by SDS-PAGE, transferred to membrane, and detected by western blotting with indicated antibodies. Recombinant CUL1 KKR protein contains K678E, K679E, and R681E substitutions.

(b) (Top) Schematic of the different UBXD7 fragments.
(Bottom) A mix (shown as input) of unneddylated and neddylated recombinant full-length CUL2 was incubated with 1 μM of the recombinant UBXD7 fragments for 1 hour. Following immunoprecipitation (IP), the recovered proteins were separated by SDS-PAGE, transferred to membrane, and detected by western blotting with indicated antibodies.

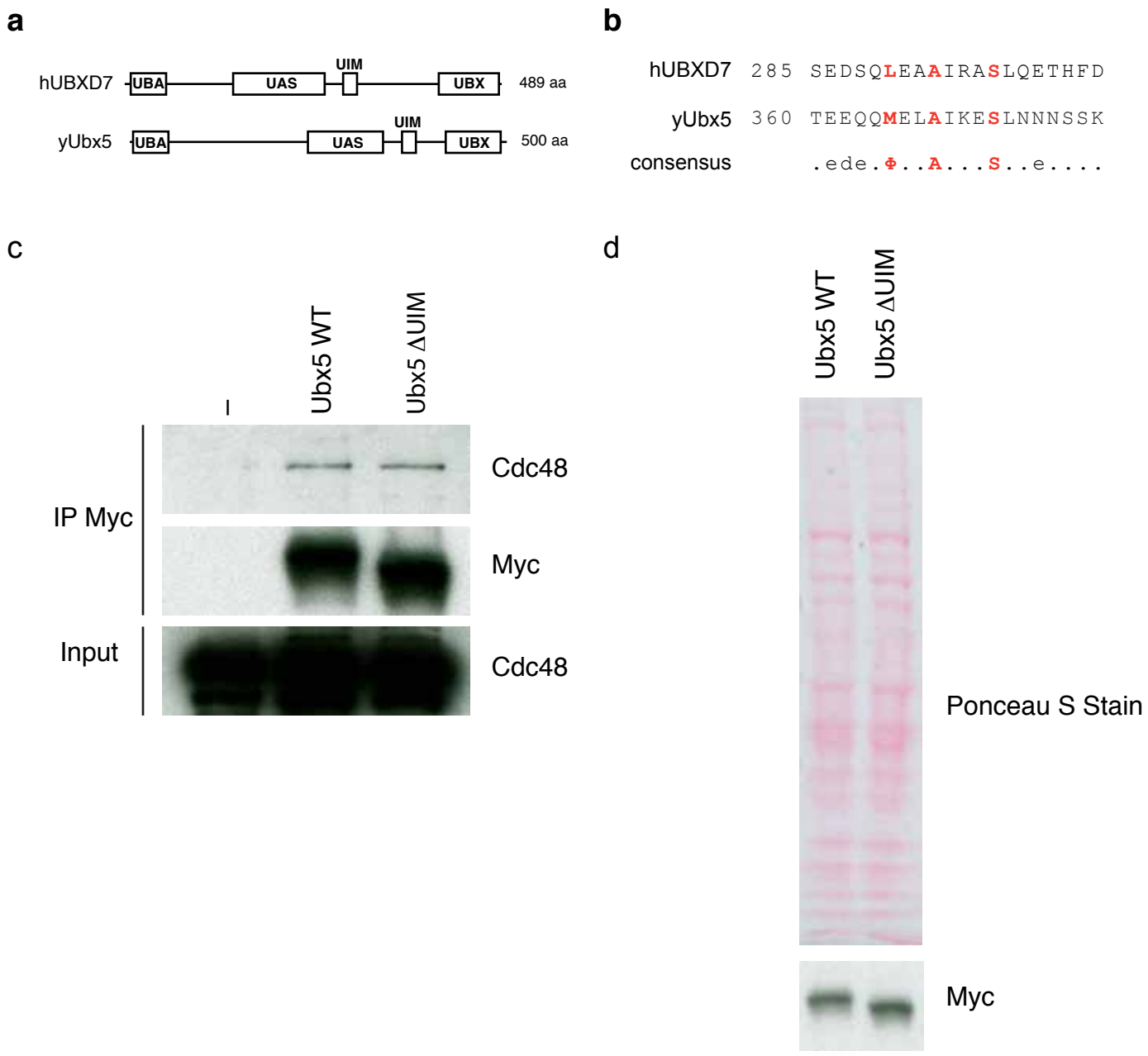
(c) A mix (shown as input) of unneddylated and neddylated recombinant full-length CUL2–RBX1 (NTD + CTD) or CUL2-CTD-RBX1 complex (10 nM) was incubated with 1 μM of recombinant wild type Flag-tagged UBXD7 or the various UBXD7 deletion mutants for 1 hour. Following immunoprecipitation (IP) with anti-Flag antibodies, the recovered proteins were separated by SDS-PAGE, transferred to membrane, and detected by western blotting with indicated antibodies.



Supplementary Figure 3. Alignment of UIMs found in several human proteins. Conserved residues of the core UIM region are colored red; Φ denotes a large hydrophobic residue and (*) indicates the amino acids substituted in single (A293Q) and triple (E286R, L290E, A293Q) point mutants.



Supplementary Figure 4. Conformational change of Cul1 in the absence of neddylation is insufficient to support UBXD7 binding.
A mix (shown as input) of unneddylated and neddylated recombinant full-length CUL1 WT or CUL1ΔWHB complex (10 nM) was incubated with 1 μM of recombinant wild type Flag-tagged UBXD7 or Flag-tagged UBXD7ΔUIM for 1 hour. Following immunoprecipitation with anti-FLAG antibodies, the recovered proteins were separated by SDS-PAGE, transferred to membrane, and detected by western blotting with indicated antibodies. Note: Because CUL1ΔWHB lacks the C-terminal epitope recognized by the CUL1 antibody, binding to UBXD7 was monitored by blotting for Rbx1.



Supplementary Figure 5. Both wild type Ubx5 and Ubx5ΔUIM proteins were properly folded and expressed at identical levels.
(a) Schematic representation of the domains in human UBXD7 and yeast Ubx5.
(b) Alignment between the UIM domain of human UBXD7 and yeast Ubx5. Conserved residues of the core UIM region are colored red; Φ denotes a hydrophobic residue.
(c) Yeast extracts prepared from Ubx5-myc WT or Ubx5-myc ΔUIM strains were immunoprecipitated with Myc antibody. The recovered proteins were separated by SDS-PAGE, transferred to membrane, and endogenous Cdc48, Myc tagged Ubx5 WT, and Myc tagged Ubx5 ΔUIM were detected by western blot with the indicated antibodies.
(d) Yeast extracts prepared from Ubx5-Myc WT or Ubx5-Myc ΔUIM strains were separated by SDS-PAGE and proteins were detected by Ponceau S Stain (loading control) or with antibodies against the Myc epitope.

SUPPLEMENTARY METHODS

Protein expression and purification

Plasmids for Split-n-Coexpress CUL2 (pACYC184 CUL2 NTD and pGEX-RBX1rbs-CUL2 CTD) (RDB2611&2612) were co-transformed into BL21 DE3 (Invitrogen) and selected on LB plates containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml). An overnight starter culture grown at 37°C was diluted 200 fold and incubated at 37°C until an OD_{600} 1.0 was reached. Cells were shifted to 16°C and protein expression induced by the addition of 400 μM IPTG for 16 hours. Bacteria were harvested, resuspended in lysis buffer (50 mM Tris-Cl pH 8.0, 200 mM NaCl, 10% (v/v) glycerol, protease inhibitors (Roche)) and lysed by 4 rounds of sonication. Cleared lysates were incubated with Glutathione Sepharose 4B resin (GE Healthcare) for 1-3 hours after which the beads were washed with lysis buffer and incubated with elution buffer (20 mM glutathione, 50 mM Tris pH 8.0, 200 mM NaCl, 10% (v/v) glycerol). The GST tag on RBX1 was removed by incubating the eluted recombinant protein with Thrombin (Sigma) for 16 hours at 4°C. CUL2-RBX1 complexes were further purified by ion exchange (Resource Q) followed by sizing column (S200) and stored at -80°C in storage buffer (30 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1 mM DTT and 10% (v/v) glycerol).

pGEX4T-2 TEV vectors encoding Flag-UBXD7 and Flag-Ubx5 (wt, domain deletion, single and triple mutants) were transformed into BL21 Rosetta cells and selected on LB plates containing ampicillin (100 μg/ml) and chloroamphenicol (25 μg/ml). An overnight starter culture grown at 37°C was diluted 200 fold and incubated at 37°C until an OD_{600} 0.8 was reached. Protein expression was induced by the addition of 400 μM IPTG for 3 hours. Bacteria were harvested, resuspended in lysis buffer (50 mM Tris-Cl pH 8.0, 200 mM NaCl, 10% (v/v) glycerol, protease inhibitors (Roche)) and lysed by 4 rounds of sonication. Cleared lysates were incubated with gluthatione sepharose (GE Healthcare) for 3 hours after which the beads were washed with lysis buffer and resuspended in TEV-cleavage buffer (50 mM Tris-Cl pH 8.0, 0.5 mM EDTA and 1 mM DTT) to make a 20% slurry. TEV protease (AcTEV, Invitrogen) was added to a final concentration of 75 U/ml and the beads were incubated for 16 hours at 4 °C with gentle agitation. Supernatant containing Flag-tagged UBXD7 or Ubx5 was snap frozen after the addition of glycerol to final concentration of 5% (v/v) and stored at -80°C.

Expression plasmids

<u>Mammalian expression constructs for Flag-UBXD7</u>

Mammalian expression construct, pCMV5-Flag-UBXD7 [1], was used as a template for site directed mutagenesis to generate the following alterations: ΔUBA (deleted for amino acids 9-54), ΔUAS (deleted for amino acids 141-259), ΔUIM (deleted for amino acids 284-301), ΔUBX (deleted for amino acids 408-

489), A293Q, and the triple mutant E286R, L290E, A293Q. For the single ΔUBX and the double (ΔUBAΔUBX, ΔUASΔUBX, ΔUIMΔUBX) domain deletion constructs, a stop codon was introduced upstream of the UBX domain in UBXD7.

Primers used for site directed mutagenesis:
(Nucleotides at the deletion boundary and nucleotides that were substituted are highlighted in red)

UBXD7 ΔUBA
5′-GCCCACGGGGGCTCCGCGATCGCTGAAGAGCCCAGTAC
5′-GTACTGGGCTCTTCAGCGATCGCGGAGCCCCCGTGGGC
UBXD7 ΔUAS
5′-CGATAAGAAATTAACTACCCTTGCAGATGAACATGGACAACTGGATGGACTTTCTAGC
5′-GCTAGAAAGTCCATCCAGTTGTCCATGTTCATCTGCAAGGGTAGTTAATTTCTTATCG
UBXD7 ΔUIM
5′-GTGCCCGTTCAGAGAGCCTTATAGATCATTTTGATTCAACACAGACAAAACAGG
5′-CCTGTTTTGTCTGTGTTGAATCAAAATGATCTATAAGGCTCTCTGAACGGGCAC
UBXD7 ΔUBX
5′-GATGGAGTAGTGGAGGGGTAAGATGTAAATGGACCAAAAGCAC
5′-GTGCTTTTGGTCCATTTACATCTTACCCCTCCACTACTCCATC
UBXD7 A293Q
5′-CAGCCAGCTAGAAGCTCAGATCAGAGCCTCCTTAC
5′-GTAAGGAGGCTCTGATCTGAGCTTCTAGCTGGCTG
UBXD7 E286R, L290E, A293Q
5′-CTTATAGATGCAAGTAGAGACAGCCAGGAAGAAGCTCAGATCAGAGCCTCCTTAC
5′-GTAAGGAGGCTCTGATCTGAGCTTCTTCCTGGCTGTCTCTACTTGCATCTATAAG

MSCV-Flag UBA-UBX ΔUBX expression constructs

Full-length cDNAs for all five UBA-UBX genes (p47, UBXD8, FAF1, UBXD7, and SAKS1) [1] were amplified by PCR using primers that contained attB sites at the 5'-termini. After gel purification, PCR products were cloned into the pDONR223 vector [2] using BP clonase (Invitrogen) according to manufacturers instructions. Correct clones were used for site-directed mutagenesis to introduce a stop codon upstream of the UBX domain in each of the UBA-UBX genes. Lastly, each UBA-UBX cDNA was transferred to an MSCV-Flag-Gateway-Ires-GFP vector with LR clonase (Invitrogen).

Primers used for PCR amplification and cloning into pDONR223 with BP clonase:
(Nucleotide homologs with the cDNA's are highlighted in red)

P47
5′-GGGGACAACTTTGTACAAAAAAGTTGGC**ATGGCGGCGGAGCGACAGGAGG**
5′-GGGGACAACTTTGTACAAGAAAGTTGGG**TTATGTTAACCGCTGCACGATG**
UBXD8
5′-GGGGACAACTTTGTACAAAAAAGTTGGC**ATGGCGGCGCCTGAGGAG**
5′-GGGGACAACTTTGTACAAGAAAGTTGGG**TCATTCGTCAGTTAGGTCCTGAAC**
FAF1
5′-GGGGACAACTTTGTACAAAAAAGTTGGC**ATGGCGTCCAACATGGACC**
5′-GGGGACAACTTTGTACAAGAAAGTTGGG**TTACTCTTTTGCTTCAAGGAAAAGG**
UBXD7
5′-GGGGACAACTTTGTACAAAAAAGTTGGC**ATGGCTGCCCACGGGGGCTCC**
5′-GGGGACAACTTTGTACAAGAAAGTTGGG**TTAATTTCTTTCCTGTACAAAGACAGTCT**

SAKS1
5′-GGGGACAACTTTGTACAAAAAAGTTGGC**ATGGCGGAGCTGACGGCTCTTG**
5′-GGGGACAACTTTGTACAAGAAAGTTGGG**TCACAAAGCCCCTTCTCTTTGCATC**

Primers used for the introduction of a stop codon upstream of the UBX domain:
P47
5′-CTCGTCCATCTTAATCAATTAGGCAGAACCTACCACGAAC
5′-GTTCGTGGTAGGTTCTGCCTAATTGATTAAGATGGACGAG
UBXD8
5′-CCTGATGACCCTGAATGAGTCAAGATCATCTTCAAATTAC
5′-GTAATTTGAAGATGATCTTGACTCATTCAGGGTCATCAGG
FAF1
5′-CCTGAGCCAAAGGAAGAATAAGCTGAGCCTGTGAGCAAAC
5′-GTTTGCTCACAGGCTCAGCTTATTCTTCCTTTGGCTCAGG
UBXD7
5′-GATGGAGTAGTGGAGGGGTAAGATGTAAATGGACCAAAAGCAC
5′-GTGCTTTTGGTCCATTTACATCTTACCCCTCCACTACTCCATC
SAKS1
5′-GAGCCTCCCACCAAGCGGTAGTATGACCAGTGTCGCATAC
5′-GTATGCGACACTGGTCATACTACCGCTTGGTGGGAGGCTC

The gateway destination vector, MSCV-Flag-GW-Ires-GFP, was made by ligating an annealed oligo containing a Flag tag into the BamHI-XhoI sites of MSCV-Ires-GFP. Next the RFA gateway cassette (Invitrogen) was introduced into the SnaBI site.

Flag tag oligo:
5′-GATCCAGATCTGCCACCATGGACTACAAGGACGACGATGACAAGTACGTAG
5′-TCGACTACGTACTTGTCATCGTCGTCCTTGTAGTCCATGGTGGCAGATCTG

Bacterial expression constructs for Flag-UBXD7

Flag-tagged UBXD7, as an NcoI fragment isolated from pCMV5-Flag-UBXD7, was first cloned into pBluescript-SK II-NcoI, then isolated as an EcoRI-XhoI fragment and cloned into pGEX-4T-2 (GE Healthcare). Because thrombin cleaved recombinant Flag-UBXD7 protein, we introduced a TEV cleavage site into the BamHI-EcoRI sites of pGEX-4T-2 Flag-UBXD7. All DNA fragments containing Flag-UBXD7 domain deletions or amino acid substitutions were isolated from the pCMV5 vector as NcoI fragments and used to replace Flag-UBXD7 in the pGEX-4T-2 TEV vector. pBluescript SK II-NcoI was made by introducing an NcoI linker into the EcoRV site of pBluescript SK II (Stratagene).

UBXD7 UIM replacement constructs

Mammalian expression construct, pCMV5-Flag-UBXD7 [1], was used as a template for site directed mutagenesis to introduce recognition sequences for the restriction endonucleases RsrII (at bp 829-835) and NruI (at bp 934-939) located upstream and downstream of the UIM, respectively. Next UBXD7 was digested with RsrII and NruI and annealed oligo's, containing the UIM domain of S5a (S5a-1 residues 211-231; S5a-2 residues 282-302) or HRS (residues 258-278), were introduced.

Primers used for site directed mutagenesis:
(Nucleotides that were substituted are highlighted in red and restriction sites are underlined)

RsrII:
5′-cccaaaaaatgtgcccggtccgagagccttatagatgc
5′-gcatctataaggctctcggaccgggcacatttttggg

NruI:
5′-cacagacaaaacaggattcgcgatcagatgaagaatctgaatc
5′-gattcagattcttcatctgatcgcgaatcctgttttgtctgtg

Sequence of oligo's used to insert the UIM domain of:

S5a UIM-1
5′-gtccgagagccttatagatgcaagtgctgatcctgagctggccttggcccttcgtgtatctatggaagagcagcgg
cagcggtcaacacagacaaaacaggattcg
5′-cgaatcctgttttgtctgtgttgaccgctgccgctgctcttccatagatacacgaagggccaaggccagctcagga
tcagcacttgcatctataaggctctcg

S5a UIM-2
5′-gtccgagagccttatagatgcaactgaggaagagcagattgcttatgccatgcagatgtccctgcagggagcagag
tttggctcaacacagacaaaacaggattcg

5′-cgaatcctgttttgtctgtgttgagccaaactctgctccctgcagggacatctgcatggcataagcaatctgctct
tcctcagttgcatctataaggctctcg

HRS UIM
5′-gtccgagagccttatagatgcacaggaggaggaggagctgcagctggccctggcgctgtcacagtcagaggcggag
gagaagtcaacacagacaaaacaggattcg

5′-cgaatcctgttttgtctgtgttgacttctcctccgcctctgactgtgacagcgccagggccagctgcagctcctcc
tcctcctgtgcatctataaggctctcg

UBX5 constructs for bacterial expression and yeast genomic integration

Full-length yeast *UBX5* was amplified by PCR with primers that contained SalI and NotI sites and the PCR product was cloned into the pGEM-T easy vector (Promega). The pGEM-T easy *UBX5* construct was used for site directed mutagenesis to generate ΔUIM (deleted for amino acids 359-376), as well as single (A368Q) and triple (E361R, M365E, A368Q) point mutant constructs. Wild type and mutant forms of *UBX5* as SalI-NotI fragment were subsequently used to replace UBXD7 in pGEX-4T-2 TEV Flag-UBXD7. The same SalI-NotI fragment of *UBX5ΔUIM* was inserted into pRS306 [3].

Primers used for site directed mutagenesis of yeast *UBX5*:
(Nucleotide at the deletion boundary and nucleotides that were substituted are highlighted in red)

Ubx5 ΔUIM
5′-CCTCTTCCTAAAGTGGATCCAACAACTTCGAGCAAATCAAACCAAGAAGAAGTG

5′-CACTTCTTCTTGGTTTGATTTGCTCGAAGTTGTTGGATCCACTTTAGGAAGAGG

Ubx5 A368Q

5′-CCAACAACTTTGACCGAAGAGCAACAAATGGAATTACAGATTAAAGAGTCATTA

5′-TAATGACTCTTTAATCTGTAATTCCATTTGTTGCTCTTCGGTCAAAGTTGTTGG

Ubx5 E361R, M365E, A368Q

5′-CCAACAACTTTGACCAGAGAGCAACAAGAAGAATTACAGATTAAAGAGTCATTA

5′-TAATGACTCTTTAATCTGTAATTCTTCTTGTTGCTCTCTGGTCAAAGTTGTTGG

Generation of a Split-n-Coexpress CUL2–RBX1 bacterial expression construct

The N-terminal part of human CUL2 (amino acids 6-379) was amplified by PCR using primers that contained AseI and NotI restriction sites. The bacterial expression vector, pACYC184 containing CUL1 NTD [4], was digested with NdeI and NotI to release CUL1 NTD. In its place, the CUL2 NTD PCR product, digested with AseI and NotI, was introduced.

The bacterial expression construct, pGEX-RBX1-rbs-CUL1 CTD [4], was digested with NcoI and NotI and the released CUL1 CTD fragment was cloned into pBluescript-SK II-NcoI. The C-terminal part of human CUL2 (amino acids 380-745) was amplified by PCR using primers that contained AseI and NotI restriction sites. CUL1 CTD was removed from pBluescript-SK II-NcoI with NdeI and NotI and CUL2 CTD PCR product was inserted. Using NotI and NcoI, the CUL2 CTD fragment from pBluescript-SK II-NcoI CUL2 CTD was used to replace CUL1 CTD in the pGEX-RBX1-rbs-CUL1CTD vector.

Table 1. List of plasmids used in this study with their corresponding Deshaies laboratory database (RDB) number.

RDB number	Plasmid name
2155	pCMV5B Flag-UBXD7 wt
2571	pCMV5B Flag-UBXD7 ΔUBA
2572	pCMV5B Flag-UBXD7 ΔUAS
2573	pCMV5B Flag-UBXD7 ΔUIM
2574	pCMV5B Flag-UBXD7 ΔUBX
2575	pCMV5B Flag-UBXD7 ΔUBA UBX
2576	pCMV5B Flag-UBXD7 ΔUAS UBX
2577	pCMV5B Flag-UBXD7 ΔUIM UBX
2578	pCMV5B Flag-UBXD7 A293Q
2579	pCMV5B Flag-UBXD7 E286R, L290E, A293Q
2580	pCMV5B Flag-UBXD7 ΔUBX A293Q
2581	pCMV5B Flag-UBXD7 ΔUBX E286R, L290E, A293Q
2582	pDONR223 p47
2583	pDONR223 UBXD8
2584	pDONR223 FAF1
2585	pDONR223 UBXD7
2586	pDONR223 SAKS1
2587	pDONR223 p47 ΔUBX
2588	pDONR223 UBXD8 ΔUBX
2589	pDONR223 FAF1 ΔUBX
2590	pDONR223 UBXD7 ΔUBX
2591	pDONR223 SAKS1 ΔUBX
2592	MSCV-Flag-Gateway-Ires-GFP
2593	MSCV-Flag p47 ΔUBX-Ires-GFP
2594	MSCV-Flag UBXD8 ΔUBX-Ires-GFP
2595	MSCV-Flag FAF1 ΔUBX-Ires-GFP
2596	MSCV-Flag UBXD7 ΔUBX-Ires-GFP
2597	MSCV-Flag SAKS1 ΔUBX-Ires-GFP
2598	pGEX-4T-2 TEV Flag-UBXD7 wt
2599	pGEX-4T-2 TEV Flag-UBXD7 ΔUBA
2600	pGEX-4T-2 TEV Flag-UBXD7 ΔUAS
2601	pGEX-4T-2 TEV Flag-UBXD7 ΔUIM
2602	pGEX-4T-2 TEV Flag-UBXD7 ΔUBX
2603	pGEX-4T-2 TEV Flag-UBXD7 ΔUBA UBX
2604	pGEX-4T-2 TEV Flag-UBXD7 ΔUAS UBX
2605	pGEX-4T-2 TEV Flag-UBXD7 ΔUIM UBX
2606	pGEX-4T-2 TEV Flag-UBX5 wt
2607	pGEX-4T-2 TEV Flag-UBX5 ΔUIM
2608	pGEX-4T-2 TEV Flag-UBX5 A368Q

2609	pGEX-4T-2 TEV Flag-UBX5 E361R, M365E, A368Q
2610	pRS306 UBX5 ΔUIM
2611	pGEX-RBX1-rbs-CUL2 CTD
2612	pACYC184 CUL2 NTD
2613	pcDNA3.1 CUL 1-V5 [5]
2614	pcDNA3.1 CUL 2-V5 [5]
2615	pcDNA3.1 CUL 3-V5 [5]
2616	pcDNA3.1 CUL 4a-V5 [5]
2617	pcDNA3.1 CUL 5-V5 [5]
2618	pCS2 HA-Cul1
942	pCS2 HA-Cul1 K720R
2619	pcDNA3.1 HA-Cul2
2620	pcDNA3.1 HA-Cul2 K689R
2705	pCMV5B Flag-UBXD7 wt UIM (RsrII and NruI)
2706	pCMV5B Flag-UBXD7 S5a-1 (RsrII and NruI)
2707	pCMV5B Flag-UBXD7 S5a-2 (RsrII and NruI)
2708	pCMV5B Flag-UBXD7 HRS (RsrII and NruI)
2736	pGEX2TK-NEDD8 L8A
2737	MSCV-HA-CUL4A-Ires-GFP
2738	MSCV-HA-CUL4B-Ires-GFP
2739	pGEX4T2-Flag UBXD7 263-489
2740	pGEX4T2-Flag UBXD7 263-407
2741	pGEX4T2-Flag UBXD7 263-321

Yeast strains used in this study with their corresponding Deshaies laboratory database (RJD) number.

RJD 5416: ubx5ΔUIM
RJD 5428: ubx5ΔUIM rub1Δ
RJD 4214: UBX5 wt - myc
RJD 4622: ubx5ΔUIM - myc

References

1 Alexandru, G. *et al.* UBXD7 binds multiple ubiquitin ligases and implicates p97 in HIF1alpha turnover. *Cell* **134**, 804-816 (2008).
2 Rual, J. F. *et al.* Human ORFeome version 1.1: a platform for reverse proteomics. *Genome Res* **14**, 2128-2135 (2004).
3 Sikorski, R. S. & Hieter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**, 19-27 (1989).
4 Zheng, N. *et al.* Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416**, 703-709 (2002).
5 Chew, E. H. & Hagen, T. Substrate-mediated regulation of cullin neddylation. *J Biol Chem* **282**, 17032-17040 (2007).