

OTUB1 non-catalytically stabilizes the E2 ubiquitin-conjugating enzyme UBE2E1 by preventing its autoubiquitination

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Nagesh Pasupala^{‡1}, Marie E. Morrow^{‡1}, Lauren T. Que[‡], Barbara A. Malynn[§], Averil Ma[§], and  Cynthia Wolberger^{‡2}

From the [‡]Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185 and the [§]Department of Medicine, University of California San Francisco, San Francisco, California 94117

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OTUB1 is a deubiquitinating enzyme that cleaves Lys-48–linked polyubiquitin chains and also regulates ubiquitin signaling through a unique, noncatalytic mechanism. OTUB1 binds to a subset of E2 ubiquitin-conjugating enzymes and inhibits their activity by trapping the E2~ubiquitin thioester and preventing ubiquitin transfer. The same set of E2s stimulate the deubiquitinating activity of OTUB1 when the E2 is not charged with ubiquitin. Previous studies have shown that, in cells, OTUB1 binds to E2-conjugating enzymes of the UBE2D (UBCH5) and UBE2E families, as well as to UBE2N (UBC13). Cellular roles have been identified for the interaction of OTUB1 with UBE2N and members of the UBE2D family, but not for interactions with UBE2E E2 enzymes. We report here a novel role for OTUB1–E2 interactions in modulating E2 protein ubiquitination. We observe that *Otub1*^{−/−} knockout mice exhibit late-stage embryonic lethality. We find that OTUB1 depletion dramatically destabilizes the E2-conjugating enzyme UBE2E1 (UBCH6) in both mouse and human *OTUB1* knockout cell lines. Of note, this effect is independent of the catalytic activity of OTUB1, but depends on its ability to bind to UBE2E1. We show that OTUB1 suppresses UBE2E1 autoubiquitination *in vitro* and in cells, thereby preventing UBE2E1 from being targeted to the proteasome for degradation. Taken together, we provide evidence that OTUB1 rescues UBE2E1 from degradation *in vivo*.


Ubiquitin signaling plays an essential role in nearly all aspects of eukaryotic biology. Substrate ubiquitination is tightly regulated temporally and in response to different stimuli through the opposing actions of enzymes that attach and remove ubiquitin from substrate proteins. Ubiquitin is conjugated to lysines via E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases, resulting in an isopeptide linkage between the ubiquitin C terminus and a substrate lysine

or, in some cases, with the substrate N terminus (1, 2). Polyubiquitin chains can be homopolymers that are linked through one of ubiquitin's seven lysines or its N-terminal methionine, or heterotypic chains that contain a mixture of linkages (1, 3). The ubiquitin modification is reversed by deubiquitinating enzymes (DUBs),³ which remove ubiquitin from proteins and cleave polyubiquitin chains (4, 5), thus terminating a ubiquitin signaling event and recycling ubiquitin monomers. The ~90 DUBs in human cells fall into six classes based on catalytic domain architecture: ubiquitin C-terminal hydrolases, ubiquitin-specific proteases, ovarian tumor domain containing proteases (OTU), Machado–Joseph domain proteases, JAB1/MPN/Mov34 (JAMM) metalloproteases, and the recently discovered MIU-containing DUB family, MINDY (4, 6).

OTUB1 is an OTU class cysteine protease (7) that specifically cleaves Lys-48–linked polyubiquitin chains (8, 9) and is one of the most abundant human DUBs (10). OTUB1 has been shown to regulate a diverse set of cellular processes through its deubiquitinating activity. Among the proteins that OTUB1 stabilizes are the transcription factors, FOXM1 (11) and ER α (12), and the small GTPase, RhoA (13). OTUB1 also deubiquitinates some E3 ubiquitin ligases, such as c-IAP1, a regulator of NF- κ B and mitogen-activated protein kinase signaling pathways (14), TRAF3 and TRAF6, regulators of virus-triggered interferon induction (15), and GRAIL, which regulates T-cell anergy (16).

In addition to its deubiquitinating activity, OTUB1 has the unique ability to bind to a subset of E2 ubiquitin-conjugating enzymes and inhibit ubiquitin transfer in a manner that does not depend upon the catalytic activity of OTUB1 (17–19). During the double strand break response, OTUB1 binds to UBE2N and suppresses synthesis of Lys-63–linked polyubiquitin at DNA double strand breaks, thereby regulating the DNA damage response (17). OTUB1 inhibits UBE2N by binding to the charged E2~Ub thioester intermediate and preventing ubiquitin transfer (17, 19, 20). OTUB1 has also been shown to non-catalytically inhibit UBCH5 *in vitro* (18) and in cells (21). In other examples of noncatalytic inhibition, OTUB1 stabilizes p53 by inhibiting UBCH5/MDM2-mediated ubiquitination of

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This article contains Tables S1 and S2 and Figs. S1–S5.

¹ These authors contributed equally to the results of this work.

² To whom correspondence should be addressed. E-mail: cwolberg@jhmi.edu.

³ The abbreviations used are: DUB, deubiquitinating enzymes; TBP, TATA box-binding protein; OTU, ovarian tumor domain-containing proteases; MEF, mouse embryonic fibroblasts; HA, hemagglutinin; Ni²⁺-NTA, nickel-nitrilotriacetic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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p53 (22, 23), activates RAS isoforms (24), regulates the transforming growth factor β pathway by stabilizing the signal transducer SMAD2/3 (25), and stabilizes DEPTOR, an mTORC1 inhibitor (26). Mass spectrometry studies have revealed that OTUB1 can form complexes with several other E2s in cells, including UBE2E1 (UBCH6), UBE2E2 (UBCH8), and UBE2E3 (UBCH9), in addition to UBE2N (UBC13) and the UBCH5 (UBE2D1, -2, and -3) isoforms (17, 27). When the E2 partners of OTUB1 are not charged with ubiquitin, E2 binding to OTUB1 stimulates its Lys-48-specific deubiquitinating activity (28), although the physiological role of this stimulation remains to be shown.

UBE2E1 has been identified as a binding partner of OTUB1 by MS analysis of OTUB1 binding partners in cells (17) and because it stimulates the deubiquitinating activity of OTUB1 *in vitro* (28). UBE2E1 is a class III E2 ubiquitin-conjugating enzyme that belongs to the UBE2E family of E2s, comprising UBE2E1/UBCH6, UBE2E2/UBCH8, and UBE2E3/UBCH9 (29). UBE2E family members share a highly conserved UBC domain but are distinguished from one another by their unique N-terminal extensions. These N-terminal extensions are sites for intramolecular autoubiquitination *in vitro*, which has been shown to limit the catalytic activity of UBE2E family E2s (30, 31). When lysine residues within their N termini are mutated to arginine, or their N termini are deleted entirely, UBE2E E2s switch from primarily monoubiquitination of substrates to robust polyubiquitination (30, 31). There are several reports on the cellular functions and substrates of UBE2E1. In cells, UBE2E family E2s are imported into the nucleus when charged with ubiquitin (32). In addition to its ubiquitin-conjugating activity, UBE2E1 can also act as an ISG15-conjugating enzyme *in vitro*, although this activity has not been shown to occur *in vivo* (33). UBE2E1 can be covalently modified with either ISG15 or ubiquitin, both of which interfere with the ubiquitin-conjugating activity of UBE2E1 (30, 33). UBE2E1 has also been reported to monoubiquitinate histone H2A at Lys-119 in concert with the PRC1 E3 ligase complex (34).

We report here a novel role for OTUB1 in maintaining E2 levels in cells. We find that *Otub1*-deficient (*Otub1*^{-/-}) mice exhibit late embryonic lethality. Proteomic analysis of *Otub1*^{-/-} mouse embryonic fibroblasts (MEFs) shows that levels of the E2, UBE2E1, are dramatically lower in the absence of OTUB1. In U2OS cells, knockdown or knockout of *OTUB1* similarly leads to dramatically lower levels of UBE2E1 protein. This regulation of UBE2E1 stability depends on the ability of OTUB1 to bind to UBE2E1 but does not depend upon OTUB1 catalytic activity. We show that UBE2E1 is ubiquitinated *in vivo* and that in the absence of OTUB1, UBE2E1 is targeted to the proteasome for degradation. *In vitro*, UBE2E1 is autoubiquitinated in both the presence and absence of an E3 ligase, but this autoubiquitination activity is suppressed by OTUB1. Taken together, our data suggest that OTUB1 protects UBE2E1 from degradation by inhibiting E2 autoubiquitination activity. These observations reveal a novel role for OTUB1 binding to E2 ubiquitin-conjugating enzymes in regulating E2 stability within the cell.

Table 1

Embryonic lethality of *Otub1*-deficient mice

Numbers of embryos or live born mice of the indicated genotypes obtained from *Otub1*^{+/-} interbreeding. Percentages of each genotype are indicated within parentheses and total numbers of pups indicated at the right.

	<i>Otub1</i> ^{+/+}	<i>Otub1</i> ^{+/-}	<i>Otub1</i> ^{-/-}	Total
E14.5	29 (26%)	51 (46%)	30 (27%)	110 (100%)
Live born	44 (35%)	80 (65%)	0 (0%)	124 (100%)

Results

Deletion of *OTUB1* causes late stage embryonic lethality in mice

OTUB1 is the most abundant DUB in mouse and human cells and is present at concentrations of about 1 μM (10). To gain insight into the function of OTUB1 *in vivo*, we generated *Otub1*^{+/-} mice by gene targeting in C57BL/6 embryonic stem cells (Fig. S1, A and B). Heterozygous *Otub1*^{+/-} mice were then interbred to study the phenotypic effects of *Otub1* deficiency *in vivo*. We screened 124 live born mice and found that 35% were *Otub1*^{+/+}, 65% were heterozygous *Otub1*^{+/-}, and 0% were homozygous *Otub1*^{-/-} mice (Table 1). As we found no live born *Otub1*^{-/-} mice, we sacrificed pregnant females from timed matings of *Otub1*^{+/-} mice at day 14.5 of gestation, and genotyped the E14.5 embryos. These analyses revealed that the mutant *Otub1*⁻ allele segregated in perfect Mendelian ratio (1:2:1) among 110 embryos (Table 1). These results indicate that *Otub1* deficiency causes lethality in the late stages of embryonic development. We then used E14.5 embryos to generate *Otub1*^{-/-} and control MEFs.

Depletion of *OTUB1* destabilizes UBE2E1

We took advantage of the ability to generate *Otub1*^{-/-} null MEFs to search for proteins whose stability depends on the presence of OTUB1. Thus far, there are only a handful of proteins whose stability is known to be regulated by OTUB1, the majority by a noncatalytic mechanism (21, 25, 26). We used tandem mass tag MS (35) to search for proteins whose stability was altered in the absence of OTUB1. Proteins were extracted from both WT and *Otub1*^{-/-} knockout MEF and then modified with tandem mass tags prior to digestion with trypsin and LC-MS analysis. A volcano plot of the relative changes in protein abundance in knockout versus WT cells (Fig. 1A) revealed that levels of several E2 ubiquitin-conjugating enzymes are lower in *Otub1* knockout cells, including UBE2E1/UBCH6, UBE2E2/UBCH8, UBE2C/UBCH10, UBE2S, and UBE2D3/UBCH5C. The most dramatic and statistically significant effect was on UBE2E1, which is known to bind to OTUB1 in cells (17, 27). Levels of UBE2C were also much lower in OTUB1 knockout cells, but this E2 is not known to bind to OTUB1 *in vivo* or *in vitro*. A handful of proteins were enriched upon the loss of OTUB1 (Fig. S2 and Table S1), but none are verified targets of either OTUB1 or UBE2E1 *in vivo*. Further study may clarify whether these enriched proteins are direct OTUB1 or UBE2E1 substrates.

We focused on the effect of *OTUB1* deletion on UBE2E1 levels because of its reported association with OTUB1 in cells (17, 27). To confirm the MS results, we used immunoblotting to compare steady-state levels of UBE2E1 in whole cell lysates of

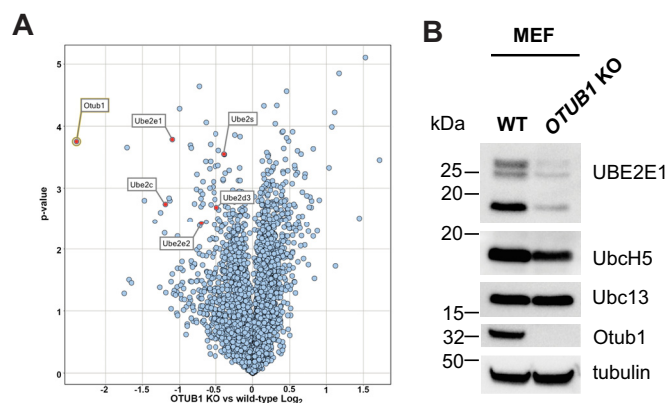


Figure 1. UBE2E1 levels are reduced in *OTUB1* knockout MEFs. A, tandem mass tag MS analysis of MEF WT and *OTUB1*^{-/-} knockout cells. B, Western blotting of the whole cell lysate of MEF WT and *OTUB1*^{-/-} knockout cells with the indicated antibodies.

WT and *Otub1*^{-/-} knockout MEFs (Fig. 1B). Consistent with the MS results (Fig. 1A), we observed markedly lower UBE2E1 protein levels in *Otub1*^{-/-} MEFs as compared with WT cells (Fig. 1B). By contrast, the *Otub1* knockout has a minimal effect on the levels of UBCH5 (UBE2D; all isoforms) and no effect on UBC13 (UBE2N) (Fig. 1B), two known binding partners of OTUB1 in cells (17, 27).

To ascertain whether the effect on UBE2E1 levels of an *Otub1* deletion was specific to MEFs, we used CRISPR-Cas9 (36, 37) to knock out *OTUB1* gene expression in human osteosarcoma (U2OS) cells. As shown in Fig. 2A, UBE2E1 levels were dramatically reduced in the U2OS knockout. To verify that the reduced UBE2E1 levels were not an adaptation to the absence of OTUB1, we used siRNA to transiently knock down OTUB1 expression in U2OS cells. As shown in Fig. 2B, siRNA knockdown of OTUB1 expression similarly reduced levels of UBE2E1 (Fig. 2B). Taken together, these results suggest that OTUB1 regulates UBE2E1 stability and that this regulation is not specific to a particular cell type.

As has been observed in previous studies (34, 38, 39), the anti-UBE2E1 antibody (see “Materials and methods”) reacts with three different molecular weight bands in Western blots (Fig. 1). The middle band corresponds to the canonical UBE2E1, as can be seen by a comparison with recombinant UBE2E1 (Fig. S3A). The remaining cross-reacting bands are also E2 enzymes, as they migrate as higher molecular weight species in whole cell extracts prepared under conditions that preserve the thioester linkages (28, 40) (Fig. S3B), consistent with E2 ubiquitin-conjugating activity. The upper band migrates at the expected molecular weight of UBE2E2 and UBE2E3, as confirmed by immunoblotting using specific antibodies against those isoforms (Fig. S3C). Knockdown of OTUB1 has little to no effect on either of these isoforms (Fig. S3C), suggesting that all three bands may represent splice variants of *UBE2E1*.

Expression of WT or catalytically inactive OTUB1 restores UBE2E1 levels

To confirm that OTUB1 directly regulates the stability of UBE2E1, we generated stable cell lines expressing siRNA-resistant FLAG-OTUB1 and knocked down endogenous

OTUB1 by siRNA. The expression of siRNA-resistant FLAG-OTUB1 restores UBE2E1 protein levels when OTUB1 is knocked down (Fig. 2C). UBE2E1 protein levels could also be restored in *OTUB1* CRISPR knockout cell lines by expressing either FLAG-OTUB1 or untagged OTUB1 (Fig. 2D). To further test whether OTUB1 stabilizes UBE2E1, we overexpressed FLAG-OTUB1 in U2OS cells and probed for UBE2E1. As shown in Fig. 2E, overexpression of OTUB1 significantly enhanced the steady-state levels of UBE2E1 (Fig. 2E). By contrast, levels of UBCH5 were unaffected when OTUB1 was overexpressed (Fig. 2E). Taken together, our results confirm that OTUB1 specifically regulates the stability of the E2 ubiquitin-conjugating enzyme, UBE2E1.

To test whether the catalytic activity of OTUB1 is required for its regulation of UBE2E1 levels, we assayed the ability of OTUB1 mutants defective in noncatalytic inhibition to complement siRNA knockdown of endogenous OTUB1 expression. Substitution of the active site cysteine with serine (C91S) inactivates OTUB1 (9), whereas a T134R substitution abrogates OTUB1 binding to E2 ubiquitin-conjugating enzymes (18, 28), thereby disrupting the ability of OTUB1 to prevent ubiquitin transfer. As shown in Fig. 2F, expression of a catalytic mutant, FLAG-OTUB1^{C91S}, restores levels of UBE2E1, whereas expression of OTUB1^{T134R}, fails to restore WT levels of UBE2E1 (Fig. 2F). We therefore conclude that OTUB1 regulates UBE2E1 levels through the previously reported ability of OTUB1 to bind to E2 enzymes (18–20), and not as a consequence of OTUB1 DUB activity.

OTUB1 rescues UBE2E1 from proteasomal degradation

Because previous studies had identified a role for OTUB1 in stabilizing the transcription factor FOXM1 in cells (11), we considered the possibility that OTUB1 may regulate UBE2E1 protein levels at the level of transcription. We extracted total mRNA from WT U2OS cells, a CRISPR *OTUB1* knockout, and siRNA OTUB1 knockdown cells and compared transcript levels for *OTUB1* and *UBE2E1* to that of TATA box-binding protein (*TBP*). *UBE2E1* transcript levels are not affected by *OTUB1* knockout or knockdown (Fig. S4), indicating that OTUB1 does not regulate UBE2E1 levels at the level of transcription.

To test more directly whether OTUB1 stabilizes the UBE2E1 protein, we treated cells with cycloheximide to inhibit protein synthesis and monitored UBE2E1 stability in siRNA knockdown versus control cells. As shown in Fig. 3A, UBE2E1 is rapidly degraded when endogenous OTUB1 expression is knocked down. Quantification of the canonical UBE2E1 protein band revealed that it has a shorter half-life compared with the control cells (Fig. 3B). However, expression of FLAG-OTUB1 stabilized UBE2E1 in OTUB1-depleted cells. To test whether UBE2E1 is degraded by the proteasome, we treated cells with the proteasome inhibitor, MG132, and compared steady-state levels of UBE2E1 in cells in which OTUB1 expression was knocked down by siRNA as compared with cells transfected with siRNA control. As shown in Fig. 3C, UBE2E1 levels increase in the OTUB1 knockdown cells after 2 h of MG132 treatment as compared with cells treated with DMSO. Taken together, our results suggest that OTUB1 protects UBE2E1 from protea-

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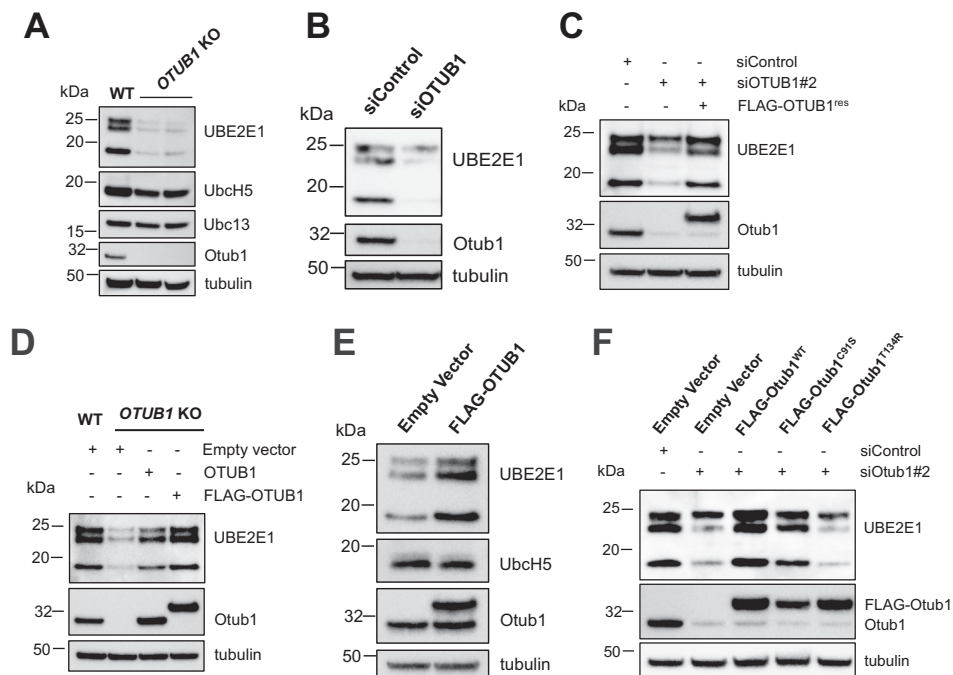


Figure 2. OTUB1 regulates the stability of UBE2E1 protein levels. *A*, Western blot analysis of the whole cell lysate of U2OS wildtype (WT) and CRISPR-Cas9-based *OTUB1* knockout (KO) cells. *B*, U2OS cells were transfected with control or SMART pool siRNA against the *OTUB1* gene. Whole cell lysates were then analyzed for the expression of the indicated proteins by immunoblotting. *C*, expression of siRNA-resistant FLAG-*OTUB1* rescues UBE2E1 protein levels in *OTUB1* knockdown cells. U2OS stable cell lines with control plasmid or FLAG-*OTUB1*^{res} were transfected with nontarget siRNA or individual siRNA against endogenous *OTUB1* gene and assayed for the steady-state levels of the indicated proteins by immunoblotting. *D*, introduction of untagged *OTUB1* or FLAG-*OTUB1* in *OTUB1* knockout U2OS cells rescues UBE2E1 levels. *E*, overexpression of *OTUB1* enhances UBE2E1 protein levels. U2OS WT cells were transiently transfected with either empty vector or FLAG-*OTUB1* plasmid and whole cell lysate was analyzed by Western hybridization. *F*, WT FLAG-*OTUB1*^{WT} and catalytic mutant FLAG-*OTUB1*^{C915} but not E2 interacting mutant FLAG-*OTUB1*^{T134R} rescued the steady-state levels of UBE2E1 in *OTUB1* knockdown U2OS cells.

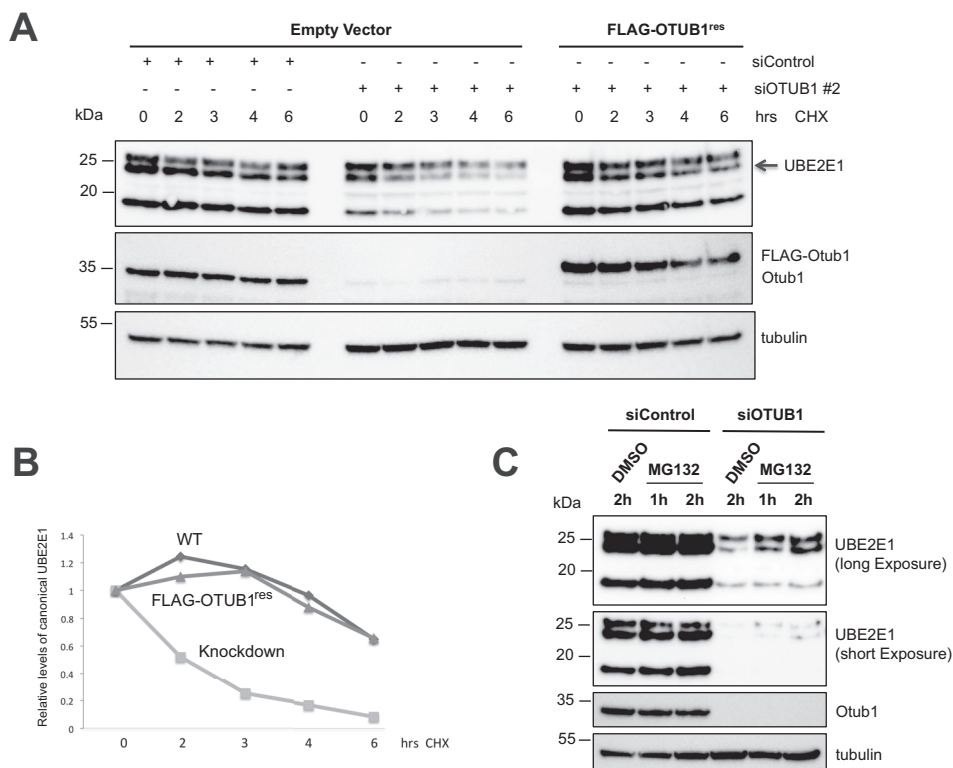


Figure 3. OTUB1 rescues UBE2E1 from proteasomal degradation. *A*, cycloheximide chase to assess the half-life of UBE2E1 in control and *OTUB1* knockdown U2OS cells. Expression of siRNA-resistant *OTUB1* increases the half-life of UBE2E1 in *OTUB1* knockdown cells. UBE2E1 (middle band) levels were normalized against the tubulin loading control and graphed on the bottom left (*B*). *C*, control and *OTUB1* knockdown U2OS cells were treated with MG132 (10 μ M) for 1- and 2-h time points and whole cell lysate was assayed for the steady-state levels of UBE2E1 by immunoblotting.

somal degradation through the ability of OTUB1 to noncatalytically inhibit E2 enzymes (18–20).

OTUB1 inhibits UBE2E1 autoubiquitination

In previous studies in which OTUB1 has been shown to stabilize a substrate through its noncatalytic activity, OTUB1 inhibits the activity of an E2 that conjugates Lys-48–linked polyubiquitin to the substrate (21–26). Because UBE2E1 is known to bind OTUB1 (17), this raises the possibility that OTUB1 may stabilize UBE2E1 directly by forming an OTUB1–UBE2E1 complex. Although it has been demonstrated that UBE2E1 can stimulate OTUB1 DUB activity (28), it has not been shown whether OTUB1 inhibits UBE2E1 in the manner observed for UBE2N/UBC13, UBE2D2, and UBE2D3 (17, 18, 20). Recently, UBE2E1 was shown to autoubiquitinate itself *in vitro* in the presence or absence of the E3 ligase, RNF4 (30, 31), raising the possibility that OTUB1 may stabilize UBE2E1 by preventing autoubiquitination.

To test the hypothesis that OTUB1 may directly inhibit UBE2E1, we assayed the autoubiquitinating activity of UBE2E1 in the presence and absence of OTUB1 *in vitro*. In the absence of an E3 ligase, UBE2E1 primarily monoubiquitinates itself (Fig. 4, top panel). Addition of the E3 ligase RNF4 stimulates synthesis of polyubiquitin chains by UBE2E1 (Fig. 4, top and middle panel). Immunoblotting with an antibody specific for UBE2E1 shows smears characteristic of polyubiquitin chains attached to UBE2E1, whereas immunoblotting with an antibody specific for Lys-48–linked polyubiquitin shows the presence of higher molecular weight Lys-48–linked polyubiquitin (Fig. 4, bottom panel). The Lys-48 chains may either be anchored to UBE2E1 or RNF4, although we cannot rule out the possibility that RNF4 stimulates UBE2E1 to form free Lys-48–linked chains. Catalytically inactive OTUB1^{C91S} completely inhibits UBE2E1 ubiquitination activity in both the presence and absence of E3 (Fig. 4, top panel). These results confirm that OTUB1 can noncatalytically inhibit UBE2E1 as has been observed for UBE2N and UBCH5 isoforms (17–20).

Although it is not known whether RNF4 stimulates UBE2E1 *in vivo*, our *in vitro* results raised the interesting possibility that OTUB1 could inhibit UBE2E1 autoubiquitination in cells, thereby accounting for the ability of OTUB1 to prevent UBE2E1 from being targeted to the proteasome. To test this hypothesis, we co-transfected U2OS cells with plasmids expressing HA-tagged UBE2E1 and His₆-tagged ubiquitin, and then treated the cells with MG132 to enrich for ubiquitinated proteins. His₆-tagged ubiquitinated proteins were pulled down by Ni²⁺-NTA resin under denaturing conditions and the results analyzed by Western blots were probed with an anti-HA antibody. We found that HA-UBE2E1 is primarily monoubiquitinated in cells, with a small amount of higher molecular weight bands also observed (Fig. 5A). To determine whether monoubiquitination of UBE2E1 in cells is due to autoubiquitination activity, we compared autoubiquitination of WT HA-UBE2E1 and a catalytically inactive mutant HA-UBE2E1^{C131A} under the same experimental conditions as described above. As shown in Fig. 5B, WT HA-UBE2E1 is ubiquitinated but not the catalytically inactive mutant HA-UBE2E1^{C131A}, supporting the idea that UBE2E1 is

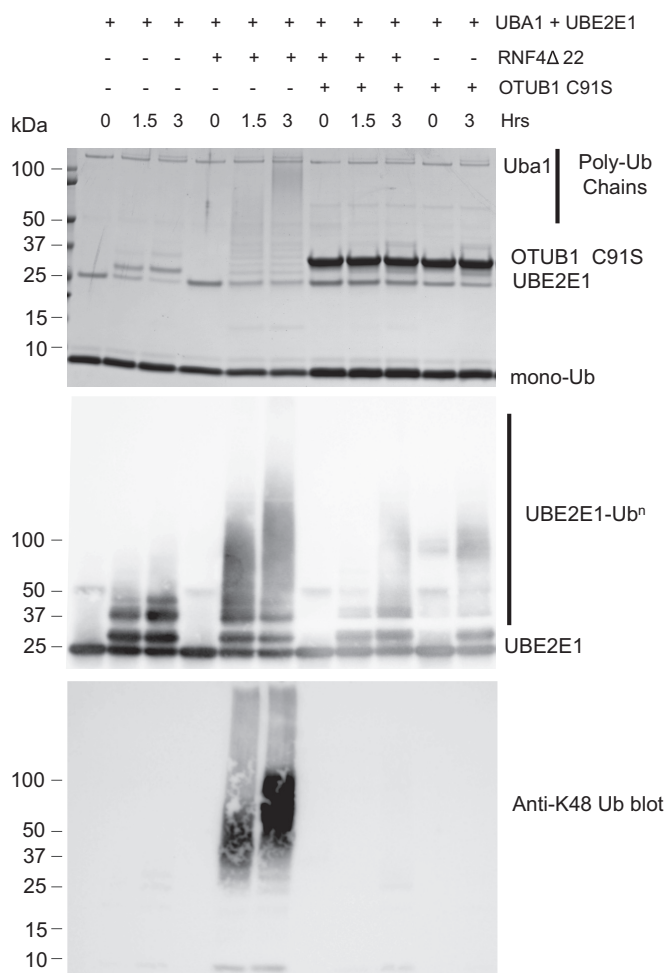


Figure 4. OTUB1 suppresses autoubiquitination of UBE2E1. *In vitro* assay of recombinant proteins showing autoubiquitination of UBE2E1 in the presence and absence of the E3 ligase, RNF4, and OTUB1-C91S. Coomassie-stained gel of ubiquitination reactions containing 0.1 nM UBA1, 5 μM UBE2E1, 1 μM RNF4Δ22, 50 μM ubiquitin, and 10 μM OTUB1 C91S. Western blotting of reactions shown in top panel using antibodies against UBE2E1 (middle) and Lys-48–polyubiquitin (bottom).

autoubiquitinated in cells. We also analyzed the half-life of WT HA-UBE2E1 and a catalytically inactive mutant HA-UBE2E1^{C131A} in the presence of cycloheximide. The result (Fig. 5, C and D) shows that the half-life of WT HA-UBE2E1 is shorter than the catalytically inactive mutant, supporting the idea that autoubiquitination of UBE2E1 is responsible for its degradation through proteasome.

Histone ubiquitination is not affected by OTUB1 or UBE2E1

UBE2E1 has been reported to monoubiquitinate histone H2A at Lys-119 (34), a histone mark that regulates gene silencing (42). In light of our observation that the absence of OTUB1 destabilizes UBE2E1, we tested whether decreased cellular levels of OTUB1 reduce ubiquitination of H2A Lys-119. However, we find no detectable difference in levels of ubiquitinated H2A when OTUB1 is knocked down by siRNA in U2OS cells (Fig. 6A and Fig. S5A), or in a CRISPR-Cas9 OTUB1 knockout cell line (Fig. 6B and Fig. S5B). As a control to rule out possible indirect effects impacting H2A ubiquitination levels, we used siRNA to knock down expression of UBE2E1 directly and examined the

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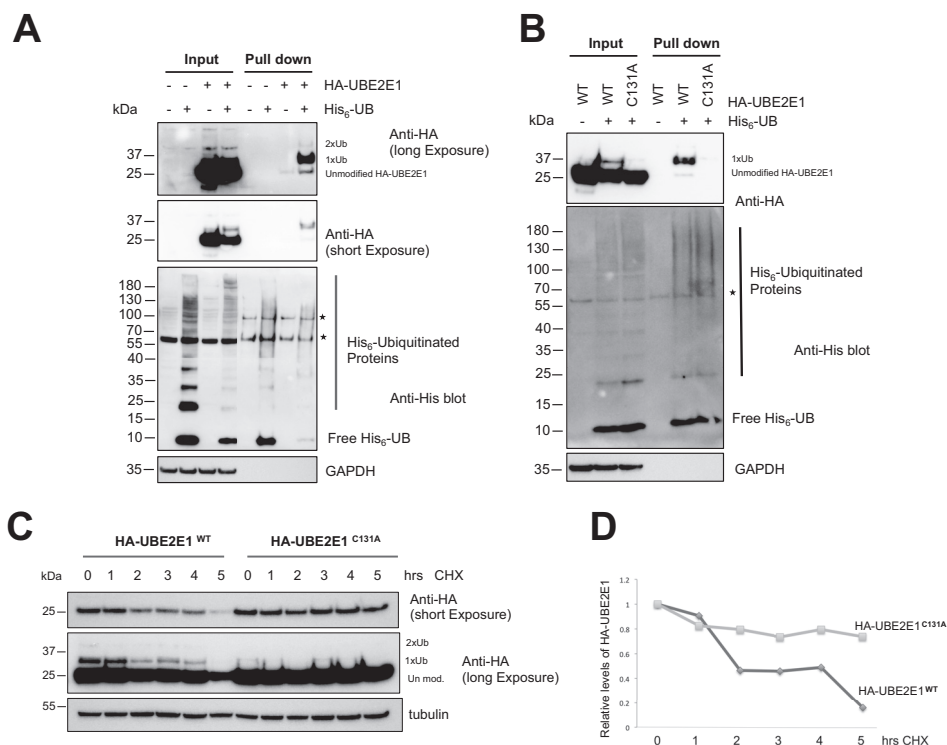


Figure 5. UBE2E1 is autoubiquitinated in U2OS cells. *A*, U2OS cells were co-transfected with plasmids expressing HA-UBE2E1 and His₆-ubiquitin. His₆-tagged ubiquitinated proteins in the whole cell lysate were enriched using Ni²⁺-NTA-agarose beads and analyzed by Western hybridization with anti-HA antibody. *B*, U2OS cells were co-transfected with plasmids expressing either WT HA-UBE2E1 or catalytically inactive HA-UBE2E1 (C131A) and His₆-ubiquitin. His₆-tagged ubiquitinated proteins and whole cell lysate were analyzed as described in *A*. Asterisk denotes cross-reactive bands. *C* and *D*, cycloheximide chase to assess the half-life of WT HA-UBE2E1^{WT} and catalytic mutant HAUBE2E1^{C131A} in U2OS cells.

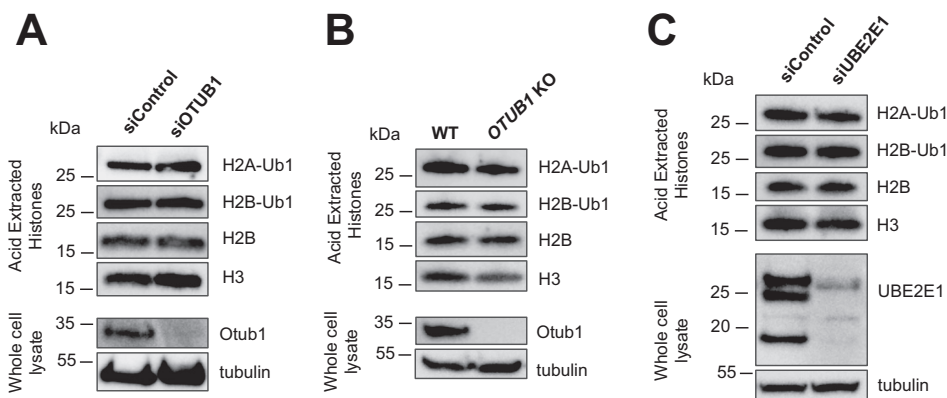


Figure 6. Depletion of OTUB1 or UBE2E1 does not affect global H2A-Ub and H2B-Ub levels. Whole cell lysates and acid-extracted histones were analyzed by immunoblotting with the indicated antibodies. *A*, control and OTUB1 knocked down U2OS cells. *B*, U2OS WT and CRISPR OTUB1 knockout cells. *C*, control and UBE2E1 knockdown U2OS cells.

steady-state levels of ubiquitinated H2A. Contrary to a previous report (34), we did not find that UBE2E1 siRNA knockdown affected ubiquitination of H2A Lys-119 (Fig. 6C and Fig. S5C). We similarly found that a UBE2E1 knockdown did not impact histone H2B monoubiquitination (Fig. 6C), a mark of actively transcribed chromatin (43). Although we cannot rule out additional factors that may account for the lack of an effect of UBE2E1 or OTUB1 knockdown on histone H2A monoubiquitination, we note that the RING1B/BMI1 E3 ligase complex that monoubiquitinates H2A Lys-119 also ubiquitinates H2A together with the E2, UBCH5C (44, 45), which may compensate for the absence of UBE2E1 in our experiments. Our results are

therefore more consistent with a role for UBCH5C, rather than UBE2E1, in monoubiquitinating H2A Lys-119.

Discussion

The ability of OTUB1 to bind to E2 enzymes and inhibit ubiquitin transfer in a manner that does not depend on OTUB1 catalytic activity was first discovered in studies of DNA damage signaling, in which OTUB1 inhibits the E2, UBE2N/UBC13 (17). OTUB1 was also shown to bind to members of the UBE2D and UBE2E E2 families, although the *in vivo* significance of that observation was not known (17, 20, 28). Subsequent studies

have shown that OTUB1 stabilizes proteins such as p53 (21, 22), SMAD2/3 (25), and DEPTOR (26) in a noncatalytic manner by preventing transfer of ubiquitin from an E2 to the substrate. The E2 that ubiquitinates these substrates *in trans* is thought to belong to the UBE2D (UBCH5) family, based on the promiscuous activity of these E2 enzymes with most E3 ligases and the ability of OTUB1 to inhibit members of this family (17, 18). In this study, we show that OTUB1 stabilizes UBE2E1 (UBCH6) in a unique manner by preventing this E2 from ubiquitinating its own lysines, thereby protecting UBE2E1 from proteasomal degradation.

The ability of OTUB1 to suppress UBE2E1 autoubiquitination explains why knockdown or knockout of *OTUB1* dramatically stabilizes UBE2E1 protein levels but has little to no effect on levels of other E2 partners of OTUB1 such as UBE2N and UBCH5 isoforms (17, 18, 20) (Fig. 1). Previous studies have shown that UBE2E1 is autoubiquitinated *in vitro* in the absence or presence of an E3 ligase (30, 31), although the relevance of this activity *in vivo* was not clear. We show here that OTUB1 noncatalytically inhibits UBE2E1 autoubiquitination *in vitro* in either the presence or absence of E3 ligase (Fig. 4). Analysis of whole cell lysates showed that the dramatic reduction in UBE2E1 protein levels in *OTUB1* knockout or knockdown cells could be rescued by expressing either WT OTUB1 or its catalytically inactive mutant, OTUB1^{C91S}, but not the OTUB1^{T134R} mutant that is defective in binding to E2 enzymes (18, 28) (Fig. 2). When OTUB1 is depleted, autoubiquitination of UBE2E1 leads to rapid degradation by the proteasome and shortened half-life in the cell (Fig. 3). In pull-downs of ubiquitinated proteins from cells, we found that WT HA-UBE2E1 was ubiquitinated, but not the catalytically inactive mutant, HA-UBE2E1^{C131A} (Fig. 5), consistent with autoubiquitination in cells. Taken together, these results support a role for OTUB1 in regulating autoubiquitination and proteolytic degradation of UBE2E1.

UBE2E1/UBCH6 belongs to the UBE2E family of E2s, comprised of UBE2E1, UBE2E2, and UBE2E3. Our proteomics data uncovered lower protein levels for UBE2E2 in addition to UBE2E1 (Fig. 1A), indicating that OTUB1 probably also regulates the stability of this E2. Because all members of the UBE2E family can autoubiquitinate themselves *in vitro* (30, 31), the mechanism of stabilization we observed for OTUB1-UBE2E1 likely extends to UBE2E2 and UBE2E3. All three UBE2E family members can interact with a broad set of E3 ubiquitin ligases (46), suggesting that OTUB1 may have further downstream effects on other substrates that are ubiquitinated by UBE2E enzymes.

We have also demonstrated that neither OTUB1 nor UBE2E1 has a detectable effect on ubiquitination of histone H2A, contrary to a recent report (34). Despite assays of multiple biological replicates in *OTUB1* siRNA knockdown and CRISPR-Cas9 knockout U2OS cells, we detected no difference in histone ubiquitination using highly selective antibodies against H2A Lys-119Ub. We speculate that differences in methods for extracting histones may give rise to these discrepancies. In this study, we analyzed histones that were acid extracted and fully solubilized from chromatin fractions, and normalized loading relative to histone H3. Based on our obser-

ations, we hypothesize that UBE2E1 is likely not the primary cognate E2-conjugating enzyme for the PRC1 E3 ligase. The UBCH5 isoforms ubiquitinate histone H2A in concert with PRC1 *in vitro* (44, 45), and TRIM37, an E3 ligase that associates with PRC2, ubiquitinates H2A in human breast cancer cell lines where RING1B of PRC1 is down-regulated (47). Further experiments targeting both UBE2E1 and the UBCH5 isoforms may shed light on which E2 is primarily responsible for H2A Lys-119 ubiquitination in cells.

The results of our OTUB1 knockout in mice indicate that OTUB1 plays a critical role in embryonic development. However, the molecular basis for this late embryonic lethal phenotype is currently unclear. Based on our cell culture experiments, it is tempting to speculate that OTUB1 depletion causes embryonic lethality by destabilizing UBE2E1 and impairing ubiquitination of its targets, one of which might play an essential role in embryogenesis. However, *Ube2e1* knockout mice are viable (IMPC database), making it unlikely that the mechanism we describe here is the only contributing factor to the embryonic lethal phenotype. It is possible that a combination of the catalytic activity of OTUB1 and its ability to inhibit E2-conjugating enzymes contribute to its importance in development. Further studies will be needed to determine definitively the developmental pathways that are disrupted in the absence of OTUB1.

In summary, we have shown that OTUB1 noncatalytically regulates the stability and protein levels of UBE2E1 in the cell. Evidence suggests that ubiquitin-conjugating enzymes, ubiquitin ligases, and deubiquitinating enzymes exist in complex *in vivo* (27, 48). Our results demonstrate that these complexes may not only serve to regulate ubiquitinated substrates, but also affect the levels of ubiquitin machinery in the cell. OTUB1 was previously known to noncatalytically prevent ubiquitination of substrates by binding to E2-conjugating enzymes. Here we have shown a new role for OTUB1 noncatalytic inhibition, namely by preventing E2 autoubiquitination that leads to proteasomal degradation. Our findings add a new layer of complexity to the increasingly complex mechanism of OTUB1-regulated ubiquitin signaling in the cell.

Materials and methods

Generation of *Otub1* knockout mouse and mouse embryonic fibroblasts

The gene targeting construct (Fig. S1A) was generated from a bacterial artificial chromosome from the C57BL/6J strain containing the *Otub1* gene by recombineering, replacing exons 4–7 with a loxP-flanked neomycin-resistance gene (Neo). C57BL/6 embryonic stem cells (PRX-B6T) were transfected with this construct and successfully targeted ES cells were identified by novel XbaI fragments identified by Southern blots (Fig. S1B). Correctly targeted clones were injected into blastocysts by the UCSF Transgenic Core. Chimeras were bred to albino C57BL/6J mice and non-albino progeny were screened for the presence of the targeted allele by Southern blotting (*Otub1*^{+/-}). Heterozygous (*Otub1*^{+/-}) mice were interbred and plugged females were sacrificed at day 14.5 of gestation and generate MEFs. All animal studies were reviewed and approved by the

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University of California, San Francisco Institutional Animal Care and Use Committee.

Cell lines, plasmids, and antibodies

U2OS and MEF cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The U2OS cell line was tested for authenticity by STR profile analysis and found 100% identical to the ATCC STR profile database. The following antibodies were used for immunoblotting: anti-OTUB1 (Abcam, catalog number ab175200), anti-UBE2E1/UBCH6 (Boston Biochem, catalog number A630), anti-UBCH5 (Boston Biochem, catalog number A615), anti-UBE2N (Invitrogen, catalog number 371100), anti-UBE2E2 (Abcam, catalog number 177485), anti-UBE2E3 (Invitrogen, catalog number PA5-51889), ubiquitinyl-histone H2A (Lys-119) (Cell Signaling Technology, catalog number 8240), ubiquitinyl-histone H2B (Lys-120) (Cell Signaling Technology, catalog number 5546), histone H3 (Cell Signaling Technology, 4499), histone H2B (Cell Signaling Technology, 12364), anti-GAPDH (Cell Signaling Technology, catalog number 5174), anti- α -tubulin (Sigma catalog number T6199), anti-HA (Invitrogen catalog number 32-6700), anti-ubiquitin (P4D1), and anti-pentaHis-HRP (Qiagen). The pCI-His₆-ubiquitin plasmid was obtained from Addgene (catalog number 31815). Construction of FLAG-OTUB1 plasmids was previously described (9, 20). HA-UBE2E1 was cloned into pCDNA between HindIII and XhoI restriction sites. Catalytically inactive HA-UBE2E1^{C131A} mutant was constructed by site-directed mutagenesis PCR.

RNAi

All siRNAs employed in this study were purchased from GE Dharmacon. Transfections were performed using Lipofectamine RNAiMAX (Life Technologies) following the manufacturer's protocol and cells were analyzed after 3 days post-transfection. The sequences of SMARTpool siRNA for *OTUB1* (catalog number M-021061-01-0005) were 5'-GACAAC-AUCUAUCAACAGA-3' (siRNA#1), 5'-CCGACUACCU-UGUGGUCUA-3' (siRNA#2), 5'-GACGGCAACUGUUUC-UAUC-3' (siRNA#3), 5'-GACGGACUGUCAAGGAGUU-3' (siRNA#4), the sequence of individual siRNA for *UBE2E1* was 5'-GACCAAGAGAUACGCUACA-3'. Nontarget siRNA (catalog number D-001210-01-05) was used as control. Complementation assays for *OTUB1* were done by introducing siRNA#2 in U2OS stable cell lines expressing siRNA-resistant FLAG-*OTUB1*^{res}. To generate siRNA-resistant FLAG-*OTUB1*, the target sequence in *OTUB1* was re-coded to CCGACTACCTCGTTGTCTA by site-directed mutagenesis.

CRISPR/Cas9-based OTUB1 knockout

Guide RNA targeting the exon II of *OTUB1* gene was designed using online MIT tool (<http://crispr.mit.edu>).⁴ The sequence of guide RNA is TCGGTCTCTGAGCCATGA. Oligos for guide RNA were annealed and ligated into BbsI-digested pSpCas9(BB)-2A-Puro vector (Addgene number 62988) as described (37, 49). Integration of the guide RNA into

the plasmid was confirmed by restriction digest and sequencing. Guide RNA plasmid was transfected into U2OS cells using Lipofectamine 2000 reagent following the manufacturer's protocol and incubated for 3 days. Single cells were isolated and cultured for 3–4 weeks in puromycin (2 μ g/ml) medium to generate isogenic stable cell lines. Genomic DNA was extracted from individual colonies and guide RNA targeted exon II region was PCR amplified (FWD primer: CTAAGCCTGTCTTCCTG-ACCCT and REV primer: AGCTTCCAAAGTAGAGACAGAC) and sequenced to screen for the presence of indel mutations. Furthermore, all possible exonic OFF target sites that are predicted by the MIT tool were PCR amplified and sequenced. Primers sequence for OFF target screening are listed in Table S2.

Mass spectrometry

Three biological replicates of WT and *Otub1*^{-/-} MEF cells were grown to 70% confluence, then harvested and washed with ice-cold PBS. Cells were lysed by incubation in PBS/SDS lysis buffer (1 \times PBS, 2% SDS, 1 \times protease inhibitors (Roche EDTA-free), 25 mM *N*-ethylmaleimide (NEM), 5 mM *o*-phenanthroline, 1 mM EDTA, 1 mM PMSF) at 4 $^{\circ}$ C rocking for 20 min. Lysate was briefly sonicated, then cellular debris was pelleted by centrifugation at 13,000 rpm for 10 min at 4 $^{\circ}$ C. Total protein was quantified by Pierce 660-nm protein assay. 100 μ g of total protein was reduced and alkylated by treatment with tris(2-carboxyethyl)phosphine and methyl methanethiosulfonate, followed by TCA precipitation of proteins at -20 $^{\circ}$ C. Precipitated proteins were pelleted and dried, and then submitted to the JHMI MS core facility for trypsinization, tandem mass tag labeling, and LC-MS analysis.

Quantification of mRNA levels by real-time PCR

Equal numbers of WT or *OTUB1* CRISPR knockout U2OS cells were plated, and the next day were transfected with either control siRNA or *OTUB1* SMARTpool siRNA with Lipofectamine RNAiMAX. Two days after transfection, cells were harvested and total RNA was extracted with a GenElute kit (Sigma), following the manufacturer's protocol. cDNA was synthesized from 200 ng of total RNA using the NEB ProtoScript First Strand cDNA synthesis kit, following the manufacturer's protocol. RT-PCR contained 1 μ l of cDNA, 500 nM forward and reverse primers, and iTaq SYBR Green premix (Bio-Rad). Reactions were run on a QuantStudio RT-PCR machine (Thermo Fisher). Relative mRNA values were quantified from $\Delta\Delta C_t$ values extracted for *UBE2E1* and *OTUB1* genes compared with *TBP* using 3 experimental and 2 technical replicates. Primers used for RT-PCR were: 5'-AGATGTTATCGCCTTT-GGGA-3' and 5'-TCCAAACTCCTCTCCACCAG-3' for *UBE2E1*; 5'-GCCATAAGGCATCATTGGAC-3' and 5'-AAC-AACAGCCTGCCACCTTA-3' for *TBP*; 5'-AACACGTTCA-TGGACCTGATTG-3' and 5'-TGCTCTGGTCATTGAAGG-AGG-3' for *OTUB1* primer set 1; and 5'-CCATCATGGCTC-AGCAGGA-3' and 5'-GAGGTCCTTGATCTTCTGTTGAT-AGATG-3' for *OTUB1* primer set 2.

⁴ Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.

Pulldown of ubiquitinated UBE2E1

U2OS cells were co-transfected with plasmids expressing HA-UBE2E1 (pCDNA) and His₆-ubiquitin for 48 h. Cells were then treated with 25 μ M MG132 (Cayman Chemical) for 3 h before harvesting. Whole cell lysate was made in denaturing conditions as described (21). His₆-ubiquitin-conjugated species were enriched by incubating with Ni²⁺-NTA-agarose beads for 3 h on rotator and analyzed by Western blotting.

Acid extraction of whole cell histones

After 72 h of post-transfection with siRNA, U2OS cells were harvested by trypsinization and re-suspended in culture medium. Cells were pelleted by centrifugation at 1400 rpm for 5 min at room temperature. Cell pellet was washed twice with ice-cold PBS containing 2 mM PMSF. Whole cell lysate was made by adding 100 μ l of RIPA buffer containing 1 \times protease inhibitor (Roche) and 10 mM NEM to one-third of the cell pellet. Lysate was clarified by centrifuging at 13,000 rpm for 10 min, at 4 $^{\circ}$ C and boiled in SDS sample buffer for 5 min. Lysate was analyzed for efficient knockdown of the protein of interest. The remaining two-thirds cell pellet was used for acid extraction of histones (50). Cell pellet was resuspended in 1 ml of ice-cold hypotonic lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 2 mM PMSE, 10 mM NEM, and 1 \times Protease inhibitor mixture) and incubated for 30 min on rotator at 4 $^{\circ}$ C. After hypotonic lysis, intact nuclei were pelleted by centrifuging in a cooled table top microcentrifuge at 10,000 \times *g* for 10 min. Nuclei pellet was resuspended in 400 μ l of ice-cold 0.4 N H₂SO₄ and incubated on rotator for 30 min at 4 $^{\circ}$ C to extract histones. Sample was spun in a cooled microcentrifuge at 16,000 \times *g* for 10 min to remove nuclear debris. TCA was added to the supernatant to a final concentration of 33% and then incubated on ice for 30 min to precipitate histones. Histones were pelleted by centrifuging at 16,000 \times *g* for 10 min and 4 $^{\circ}$ C. Histones were washed twice with 1 ml of ice-cold acetone and air-dried for 20 min at room temperature. Histones were dissolved in 100 μ l of 1 \times SDS sample buffer and analyzed by Western hybridization.

Preparation of whole cell lysate

Cells were scraped on ice in RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40) supplemented with 1 \times complete protease inhibitor (Roche catalog number 11873580001), 10 mM *N*-ethylmaleimide, and 2 mM PMSF. Extracts were clarified by centrifuging in cooled microcentrifuge at 16,000 \times *g* for 10 min and analyzed by Western hybridization.

Western blotting

Whole cell lysate or acid extracted histones were separated on SDS-PAGE (4–12% BisTris, Pre-cast gel, Criterion XT) and then transferred to polyvinylidene difluoride membrane by a Bio-Rad Gel transfer system. Membranes were blocked with 4% Bio-Rad blotting grade blocker in TBST and hybridized with the indicated primary antibodies overnight at 4 $^{\circ}$ C. Membrane was washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies. Super Sig-

nal Pico ECL (Thermo Scientific) was used for chemiluminescent detection of horseradish peroxidase-conjugated antibody. Chemiluminescent signals were detected with the Bio-Rad ChemiDoc Touch imaging system.

Cyclohexamide chase assay

Cycloheximide was added to the cell culture at a final concentration of 50 μ g/ml after 48 h of transfection with either siRNA or plasmid. Cells were harvested at various time points and whole cell lysate was prepared in RIPA lysis buffer supplemented with protease inhibitor. Lysates were subject to Western hybridization to analyze the protein stability. The intensity of the protein bands was quantified using ImageJ software (National Institutes of Health).

Extraction of ubiquitin-charged E2s from cells

Whole cell lysate was prepared in low-pH, nonreducing lysis buffer (50 mM MES, pH 4.5, 150 mM NaCl, 0.2% Nonidet P-40 and protease inhibitor) and immediately loaded onto SDS-PAGE gel with and without adding β -mercaptoethanol. Ubiquitin-charged E2 levels were analyzed by Western blotting.

Protein expression and purification

UBE2E1 was cloned into the pETSUMO vector containing an N-terminal His tag followed by a SENP2 protease cleavage site. The catalytic mutant of OTUB1 and the N-terminal deletion (22 amino acids) of RNF4 were generated by site-directed mutagenesis with the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's protocol. Cloning of human OTUB1 was performed as described previously (9).

All proteins were expressed in *Escherichia coli* Rosetta 2 DE3 cells and grown in M9ZB media. Cultures were grown at 37 $^{\circ}$ C to an A₆₀₀ of 1.5. Addition of 1 mM isopropyl β -D-1-thiogalactopyranoside initiated protein expression and the cells were incubated overnight at 16 $^{\circ}$ C. Cells were harvested by pelleting at 4000 rpm at 4 $^{\circ}$ C. Purification of the catalytic mutant OTUB1^{C91S} (9), RNF4 (51), human E1 enzyme (52), and ubiquitin (41) were performed as previously described. Cell pellets containing His-UBE2E1 were resuspended in lysis buffer (20 mM HEPES, pH 7.3, 300 mM NaCl, 25 mM imidazole, 2 mM β -mercaptoethanol). 0.1 mM Phenylmethylsulfonyl fluoride was added to cells before lysing with a microfluidizer (Microfluidics). After lysis, cellular debris was pelleted by centrifugation and the lysate was then run over a 5-ml HisTrap (GE Biosciences) for affinity purification. His-UBE2E1 was eluted using a linear gradient of 250 mM imidazole over 10 column volumes. The His tag was removed by incubation with 10 mM SENP2 overnight at 4 $^{\circ}$ C during dialysis into lysis buffer. Another round of HisTrap purification was used to remove cleaved protein from SENP2. After the His tag subtraction, flow-through fractions of UBE2E1 were pooled and dialyzed overnight into 25 mM sodium phosphate buffer, pH 7.4, 25 mM NaCl, and 7.5 mM β -mercaptoethanol at 4 $^{\circ}$ C. UBE2E1 was further purified using cation exchange chromatography, eluting off a linear gradient at 100 mM NaCl. Clean fractions were dialyzed overnight at 4 $^{\circ}$ C in 25 mM Tris buffer, pH 8, 150 mM NaCl, and 7.5 mM β -mercaptoethanol, concentrated, and stored at -80° C.

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In vitro ubiquitination assays

Ubiquitination assays were done at 37 °C in a buffer of 50 mM HEPES, pH 7.5, 250 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT, and 0.005% Tween 20. All reactions contained 0.1 nM Uba1, 5 μM UBE2E1, 1 μM RNF4^{Δ22}, and 50 μM ubiquitin. Inhibited reactions contained 10 μM catalytically inactive OTUB1^{C91S}. Assays were initiated by the addition of 5 mM ATP and Uba1. Aliquots were removed at specific time points and quenched with SDS-PAGE loading buffer containing β-mercaptoethanol. Samples were run on 4–12% polyacrylamide BisTris Criterion XT gels (Bio-Rad) and stained with Coomassie Brilliant Blue.

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