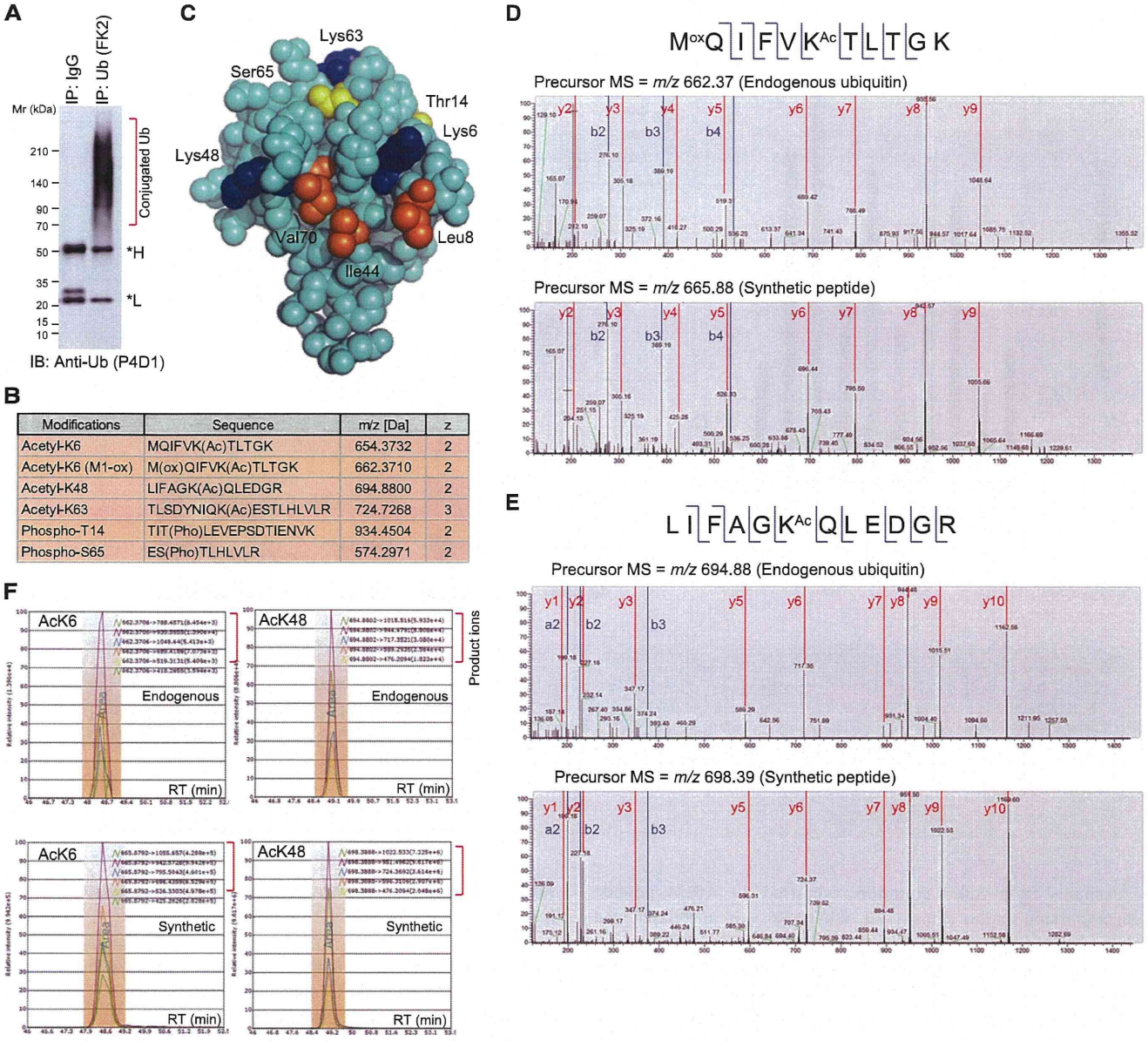

Figure 1. Ubiquitin is acetylated at K6 and K48.

A Preparation of conjugated ubiquitin from cell lines. Cell lysates were subjected to immunoprecipitation using an anti-Ub (FK2) antibody. Asterisks indicate the antibody heavy and light chains.

B Summary of the identified PTMs modifying ubiquitin. More details are provided in Supplementary Fig S2.

C Structural view of the ubiquitin modification sites. The images were drawn from PDB 1F9J. Acetylation sites, blue. Phosphorylation sites, yellow. Hydrophobic patch, orange.

D, E MS/MS spectra identifying acetylation on endogenous ubiquitin at K6 (D) and K48 (E). For each panel, the upper spectra are obtained from cell-derived ubiquitin prepared in (A), and the lower spectra are from synthetic, isotopically labeled AQUA peptides. Identified *b* and *y* fragment ions are shown.

F Sample-derived peptides containing acetyllysine at either K6 (left) or K48 (right), and the synthetic, isotopically labeled counterparts co-eluted at the same retention times. The detected fragment ions are listed.

Source data are available online for this figure.

yielded consistent results (Supplementary Fig S4B and C). A titration analysis further demonstrated the inhibitory effect of acetyl-ubiquitin in chain elongation (Fig 4B). To mimic nonacetylated lysine or acetyllysine, respectively [20], the ubiquitin K6 residue was replaced with arginine (Ub-K6R) or glutamine (Ub-K6Q). Ub-K6Q similarly inhibited polyubiquitylation (Supplementary Fig S4D).

The linkage-specific regulation of polyubiquitylation was monitored using PRM-based quantification (Fig 4C). To quantify

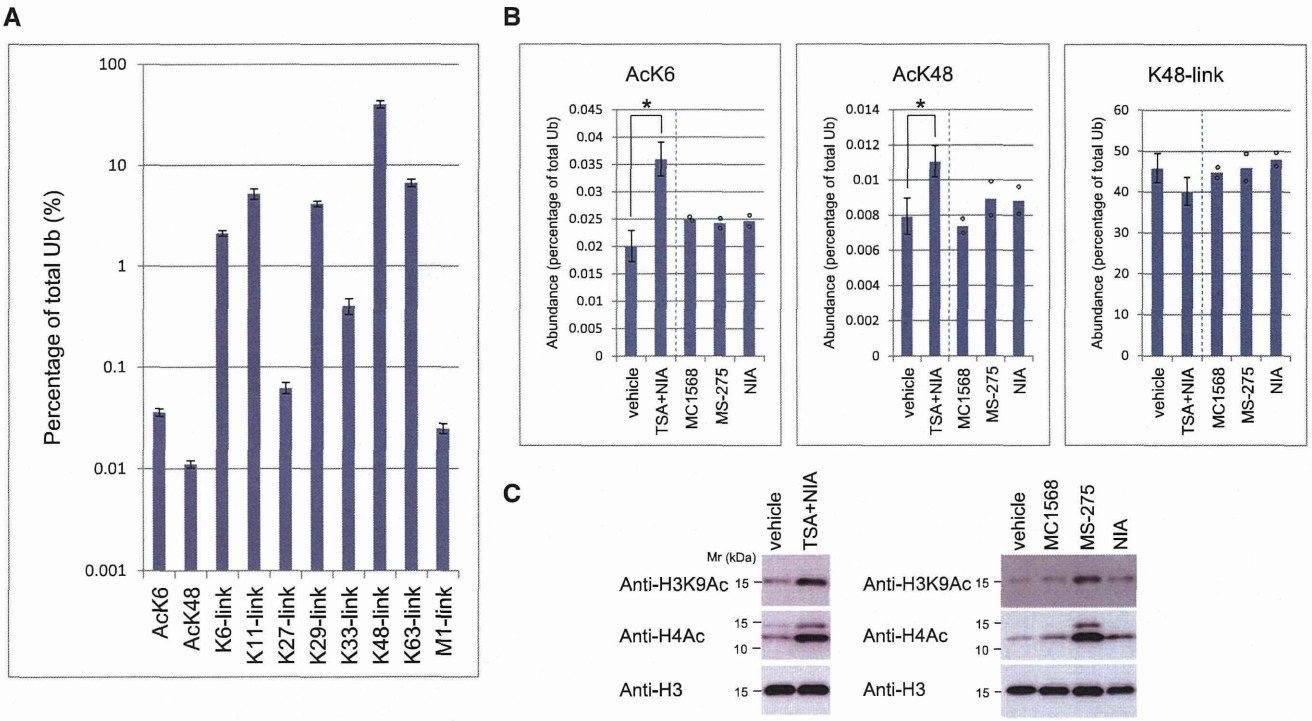

Figure 2. Quantification of ubiquitin acetylation levels in cells.

A Cellular abundance of acetylated ubiquitin and polyubiquitin. Ubiquitin conjugates (> 70 kD) immunoprecipitated from 293F cells treated with TSA/NIA and MG132/PR619 were quantified by parallel reaction monitoring (PRM). The K6-locus was used for normalization. Mean ± SD of seven biological replicates is shown.

B Cellular acetylated-ubiquitin levels are upregulated by HDAC inhibitors. Cells treated with the indicated inhibitors were lysed and subjected to anti-Ub immunoprecipitation and subsequent quantification by PRM. Peptide abundance was calculated as a percentage of total ubiquitin. Data represent mean ± SD or individual data points (n = 6, 7, 2, 2, and 2, respectively). *P < 0.01, Student's t-test. TSA/NIA samples are the same as those used in (A).

C Histone acetylation levels. Total lysates were subjected to immunoblotting as indicated.

Source data are available online for this figure.

K11-chains from AcK6 ubiquitin, we used an AQUA peptide encompassing amino acids 1–26, where K6 was acetylated and K11 was GlyGly-modified (Supplementary Fig S5). Intriguingly, ubiquitin acetylation at K6 or K48 significantly decreased specific chain elongation by CDC34, UBE2K, UBE2S, and UBC13-UEV1a. RAD6 and UBCH5 mainly elongated K11, K48, and K63 chains and, to a lesser extent, K6 chains. These chain elongation activities were also repressed in the presence of either AcK6 or AcK48, although the extent of repression varied depending on the combination of acetylation and ubiquitylation sites.

Based on the published structural analyses, the acetylation sites (K6 and K48) appear to be close to the E2 interaction surface. K6 and K48 of the acceptor ubiquitin are close to the interaction surface of UEV1a (Mms2; Fig 4D) [17,21]. Similarly, structural (UBCH5 and UBE2K) or modeling (CDC34 and UBE2S) analyses indicate that the K6 residue of noncovalently bound ubiquitin is located at the E2 interaction surface (Supplementary Fig S6A) [5,7,22,23]. Our surface plasmon resonance (SPR) analysis revealed that the affinity of noncovalent interactions between UEV1a and ubiquitin is lower with AcK6 or AcK48 (Fig 4D and Supplementary Fig S6B). These insights support our findings that acetylation at K6 or K48 represses E2-mediated polyubiquitin chain assembly.

AcK6 controls mono- or polyubiquitylation of UFD substrates and histone H2B

Next, we asked whether ubiquitin acetylation regulates mono- and polyubiquitylation in cells using the ubiquitin fusion degradation (UFD) pathway as a model system [24]. Turnover of Ub-G76V-GFP, a model UFD substrate [25], was accelerated by the expression of nonacetylatable Ub-K6R but delayed by that of the acetyl-mimetic Ub-K6Q (Fig 5A and Supplementary Fig S7A). Ub-G76V-GFP monoubiquitylated by Ub-K6Q was stabilized, while the levels of total ubiquitin conjugates were not significantly affected by the expression of ubiquitin mutants (Supplementary Fig S7B and C). Consistently, treatment with TSA stabilized Ub-G76V-GFP (Supplementary Fig S7D).

Monoubiquitylated Ub-G76V-GFP was significantly accumulated in cells expressing Ub-K6Q, but decreased in cells expressing Ub-K6R (Fig 5B and Supplementary Fig S8A and B). PRM quantification revealed an increase in K48-chains with expression of Ub-K6R (Fig 5C). Interaction of the proteasome component C2 (PSMA1) correlated with the levels of polyubiquitylated Ub-G76V-GFP (Supplementary Fig S8C). We also analyzed the ubiquitylation of UBB(+1), a naturally occurring human UFD substrate [22]. The ubiquitylation levels of UBB(+1) containing K6Q were decreased as

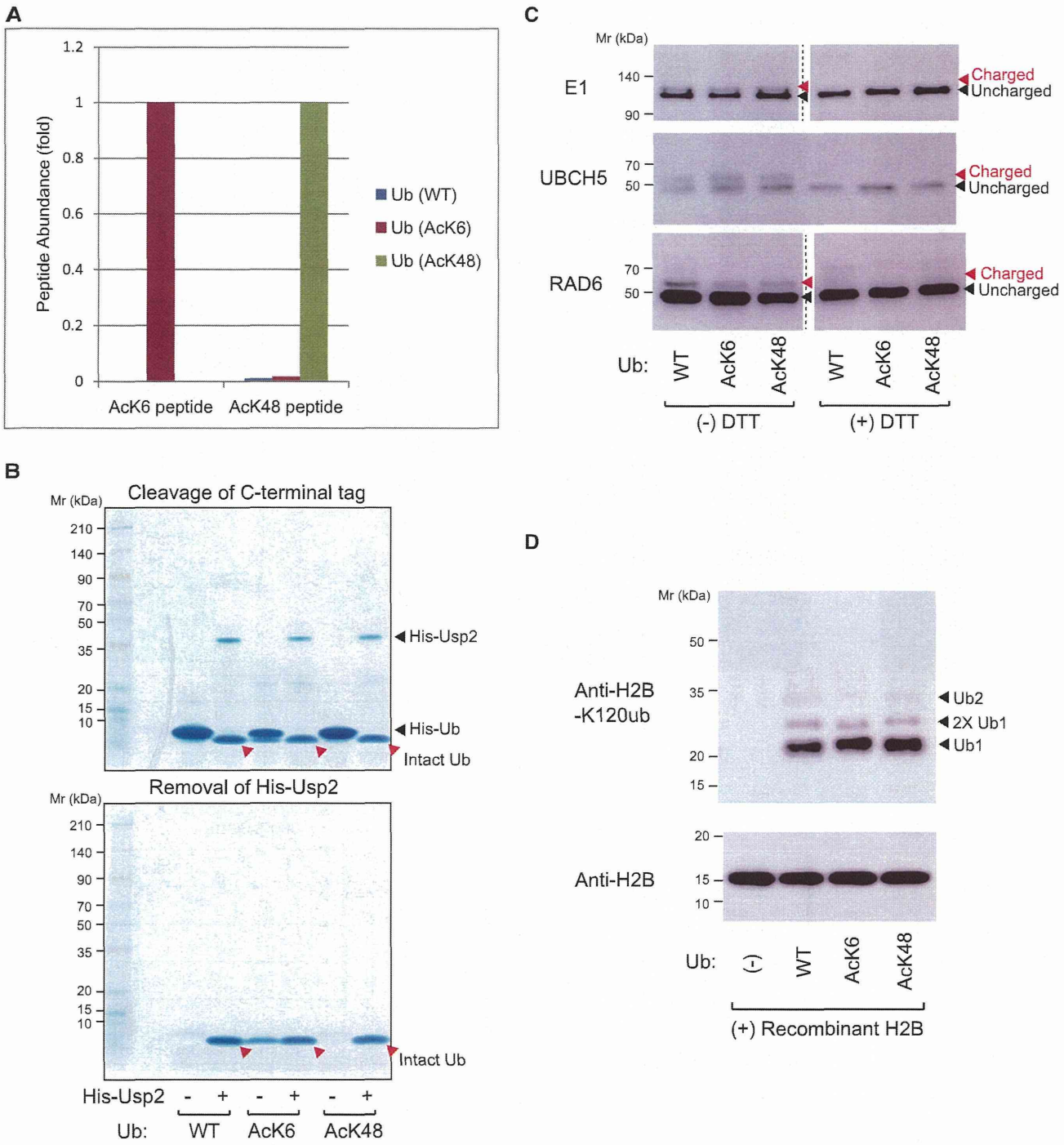

Figure 3. Generation of recombinant acetylated ubiquitins.

A Quantification of the site-specific acetylation of ubiquitin. Acetyllysine is incorporated at the desired residues.

B Purification of intact acetylated ubiquitins. After production of C-terminally His-tagged acetyl-ubiquitin, the tag was cleaved using His-USP2 (upper panel, lanes 2, 4, and 6). Subsequently, His-USP2 and uncleaved His-ubiquitin were absorbed onto Ni^{+} resin to obtain pure ubiquitin proteins (lower panel, lanes 2, 4, and 6).

C Acetylated ubiquitin retains the ability to be charged to E1/E2. Recombinant ubiquitin is charged to E1 or E2s as indicated. The charging of ubiquitin was analyzed by immunoblotting.

D Acetylated ubiquitin can be used to monoubiquitylate histones. Recombinant histone H2B was ubiquitylated by E1, E2 RAD6, and wild-type or acetylated ubiquitin. H2B ubiquitylation was detected by using an anti-H2B-K120ub antibody.

Source data are available online for this figure.

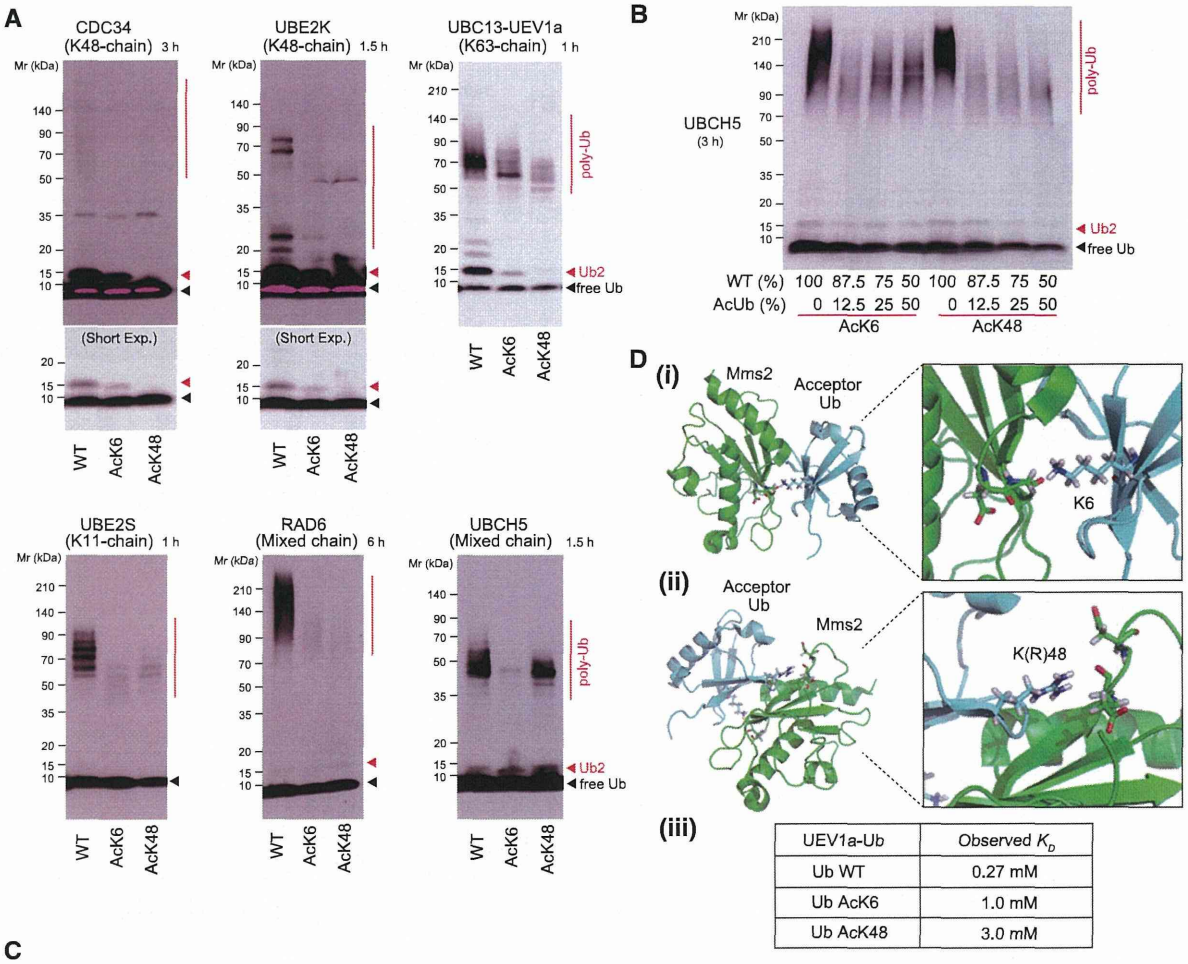
A
CDC34 (K48-chain) 3 h
UBE2K (K48-chain) 1.5 h
UBC13-UEV1a (K63-chain) 1 h
UBE2S (K11-chain) 1 h
RAD6 (Mixed chain) 6 h
UBCH5 (Mixed chain) 1.5 h
Mr (kDa)
(Short Exp.)
poly-Ub
Ub2
free Ub
WT
AcK6
AcK48
B
UBCH5 (3 h)
WT (%) 100 87.5 75 50 100 87.5 75 50
AcUb (%) 0 12.5 25 50 0 12.5 25 50
AcK6
AcK48
D
(i)
Mms2
Acceptor Ub
K6
(ii)
Acceptor Ub
Mms2
K(R)48
(iii)
UEV1a-Ub | Observed K_D
Ub WT | 0.27 mM
Ub AcK6 | 1.0 mM
Ub AcK48 | 3.0 mM
C
CDC34
UBE2K
UBC13-UEV1a
UBE2S
RAD6
UBCH5
Abundance of chains (normalized fmol)
K6 K11 K27 K29 K33 K48 K63

Figure 4.

Figure 4. Ubiquitin K6 or K48 acetylation represses polyubiquitin chain elongation *in vitro*.

A Ubiquitin acetylation represses polyubiquitin chain elongation. E1 and the indicated E2 were incubated with wild-type or acetylated ubiquitin. Polyubiquitin chains were analyzed using an anti-Ub antibody (Dako). Short exposure panels allow the visualization of di-ubiquitin (Ub2).

B Inhibitory effect of acetylated ubiquitin in a titration analysis. A total of 2.0 μg recombinant ubiquitin, comprising a mixture of the indicated percentages of wild-type and acetylated ubiquitin, was subjected to an *in vitro* ubiquitylation assay with an incubation time of 3 h.

C PRM quantification of polyubiquitin linkages. The assembled polyubiquitin chains in (A) were quantified using AQUA peptides. Mean and individual data points of two independent experiments.

D The ubiquitin K6 (i) or K48 (ii) residue is close to the Mms2 interaction surface. Images were drawn from the PDB 1ZGU. (iii) Observed K_D of ubiquitin with UEV1a in SPR analysis.

Source data are available online for this figure.

compared to those of UBB(+1) containing K6R or wild-type lysine (Fig 5D). These results suggest that AcK6 ubiquitin represses ubiquitin chain elongation in cells.

To identify components of the cellular ubiquitin acetylation-related pathway and its substrates, proteins preferentially interacting with Ub-K6Q were screened using SILAC (stable isotope labeling with amino acids in cell culture; Fig 5E). We identified ~50 proteins that preferentially interacted with K6Q over K6R. Gene ontology analysis of the K6Q-interacting proteins revealed the enrichment of chromosome- or chromatin-related factors, including histone H2B and histone-interacting/-modifying proteins (Supplementary Fig S9). To address whether histones could serve as endogenous substrates for acetyl-ubiquitylation, purified histone fractions were analyzed. We observed a more than tenfold enrichment of AcK6 and AcK48 abundance in the histone fraction versus total ubiquitin conjugates (Fig 5F). This indicated that histone H2B, and possibly also H2A, serves as an endogenous substrate of acetyl-ubiquitylation.

Histone H2B is monoubiquitylated at K120 (K120ub), a multifunctional histone PTM regulating histone H3 K4/K79 methylation, transcriptional elongation, and DNA repair response [13,16]. H2B is also polyubiquitylated either at K120 or other residues [26]. We found that expression of nonacetylatable Ub-K6R decreased H2B K120ub levels, while that of Ub-K6Q increased them (Fig 5G). Because inhibition of the proteasome decreases histone monoubiquitylation to compensate the free ubiquitin pool [27], we could not determine whether the observed stabilization of monoubiquityl-H2B involved the repression of steady-state polyubiquitylation or other mechanisms, such as deubiquitylation. Nonetheless, these results suggested that acetyl-ubiquitin can stabilize the monoubiquitylation state of H2B at certain step(s) in its dynamic regulation.

Discussion

This study revealed that ubiquitin is itself a substrate of yet another PTM. Acetylation at K6/K48/K63 and phosphorylation at T14/S65 were unambiguously identified from endogenous ubiquitin. The identified PTMs are in part consistent with results of other proteomic studies [28]. Importantly, these ubiquitin PTMs were identified from ubiquitin fractions conjugated to substrate proteins. This indicates that substrates are modified by acetyl-ubiquitylation or phospho-ubiquitylation. The numerous cellular functions of ubiquitin are achieved by the diversity of ubiquitylation, which includes differences in chain length and linkages [2,3]. Our present findings add PTMs occurring on ubiquitin itself as new factors regulating the diversity of the ubiquitin system.

We found that acetylated ubiquitin was intact in monoubiquitylation but repressed polyubiquitin chain elongation *in vitro* and stabilized the monoubiquitylation state in intact cells. Our data suggest that acetylation, which neutralizes the positive charges of lysine residues, significantly affects the noncovalent interaction of ubiquitin with E2 enzymes. Moreover, a fraction of ubiquitin may also be acetylated after conjugation to substrates. In such cases, AcK6 and AcK48 may affect ubiquitin interactions with other partners, for example, HECT- and RBR-type E3s in specific chain assembly, various DUBs in chain/substrate-specific processivity [8], and at least 16 classes of ubiquitin-binding domains (UBDs) [9]. The protein PTM code, best characterized in histones, dynamically modulates the fate and function of modified proteins [13]. Considering that polyubiquitin chain formation represents the ubiquitylation of ubiquitin itself, we propose that ubiquitin is modified by different PTMs that cross talk with each other—acetylation represses ubiquitylation—to constitute a ubiquitin PTM code (Fig 5H).

Figure 5. Acetylation of ubiquitin K6 regulates mono-/polyubiquitylation of substrates in cells.

A Turnover of UbG76V-GFP was delayed by incorporation of the acetylation-mimic K6Q ubiquitin. 293F cells were transfected with UbG76V-GFP together with either wild-type, K6R-, or K6Q-containing FLAG-ubiquitin as indicated, and a cycloheximide chase analysis was performed. Data represent the mean ± SE of quantified UbG76V-GFP band intensities; $n = 3$. The asterisk indicates a processed form of UbG76V-GFP.

B Incorporation of the acetylation-mimic ubiquitin leads to the accumulation of monoubiquitylated UbG76V-GFP. 293F cells were cotransfected with UbG76V-GFP and the mutant ubiquitins as indicated. Then, the cells were treated with MG132 for 6 h, followed by immunoprecipitation with an anti-GFP antibody. The ubiquitylation of UbG76V-GFP was analyzed using an anti-FLAG antibody. The asterisk indicates a processed form of UbG76V-GFP.

C PRM analysis of UbG76V-GFP prepared as in (B). Data represent mean ± SE; $n = 3$. *$P < 0.05$, Student's *t*-test.

D The acetylation-mimic UBB(+1) represses polyubiquitylation anchored onto UBB(+1). In the lower panel, the band densities were quantified and shown as normalized to the UBB(+1) bands.

E SILAC analysis. FLAG-Ub-K6R or FLAG-Ub-K6Q was expressed in 293F cells cultured in either heavy isotope- or light isotope-containing medium, and cell lysates were immunoprecipitated with an anti-FLAG antibody. The ratio of abundance of each Ub-K6Q/K6R-associated protein is shown from two independent experiments.

F Histones are substrates for acetyl-ubiquitylation. Gel regions corresponding to monoubiquityl-histones were analyzed by PRM. Mean and individual data points; $n = 2$ for histones, $n = 3$ for FK2.

G MCF7 cells were transfected with the indicated ubiquitin mutants, and acid-extracted histones were analyzed.

H A proposed model. Acetylation of ubiquitin at K6 and K48 represses polyubiquitylation at K11/K48/K63, constituting the ubiquitin PTM code.

Source data are available online for this figure.

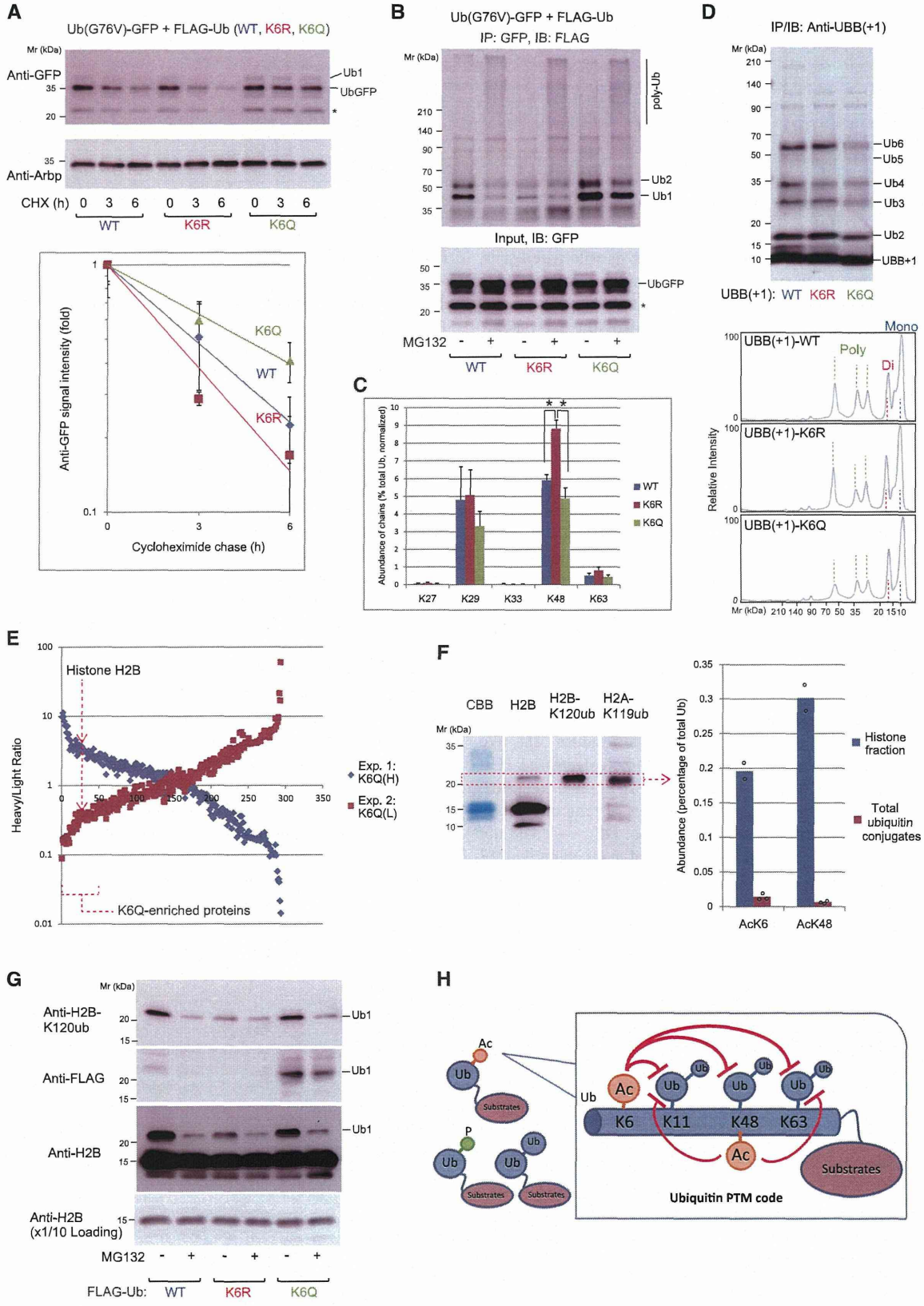

Figure 5.

Histone H2B was identified as a substrate for ubiquitin acetylation. Because the global abundance of acetyl-ubiquitin was very low, we speculate that acetyl-ubiquitylation of histones might be locally regulated (e.g. in responsive chromatin regions) to play a role in histone cross talk. Although we could not exclude the possibility of nonenzymatic acetylation of ubiquitin [29], the significant incorporation of acetyl-ubiquitin in monoubiquityl-histone suggests that ubiquitin acetylation is a regulated process.

Independent studies recently reported that ubiquitin S65 phosphorylation activates the E3 activity of Parkin [30–32]. While these reports support our notion that ubiquitin itself is a substrate for other PTMs, our present study revealed that ubiquitin acetylation controls polyubiquitin chain elongation, demonstrating the cross talk of two PTMs (acetylation and ubiquitylation) on ubiquitin. Taken together, the discovery and characterization of ubiquitin PTMs add a new layer to the molecular complexity of the ubiquitin system.

Materials and Methods

Mass spectrometric analyses

Liquid chromatography–mass spectrometry (LC-MS/MS) analyses and quantification of peptides by parallel reaction monitoring (PRM) were performed essentially as previously described [10]. The Q Exactive was operated using Xcalibur software (Thermo Fisher Scientific), and fragmentation was performed by HCD (higher energy collisional dissociation). To acquire MS/MS data on peptides of interest, inclusion lists were added in the targeted MS2 mode. Standard peptides: AcK6, MQIFVK[AcK]TL[HeavyL]TGK; AcK48, LIFAGK[AcK]QL[HeavyL]EDGR; and AcK6-ubK11, MQIFVK[AcK]TLTGK[di-GlyGly]TITLEVEPSDTIENV[HeavyV]K, were synthesized by Sigma or Operon. Other unmodified or GlyGly-modified standard peptides were previously described [10]. The mass spectrometry datasets are available in PeptideAtlas with identifier PASS00617.

Production of recombinant acetyl-ubiquitin proteins

Ubiquitin variants with acetyllysine were synthesized in *E. coli* cells expressing a UAG-reading tRNA and an enzyme able to attach the unnatural amino acid to the tRNA [33]. The cells were transformed with the variant gene containing UAG in place of a lysine codon at position 6 or 48 and then grown in media supplemented with acetyllysine and nicotinamide at final concentrations of 50 and 5 mM, respectively.

Immunoprecipitation of ubiquitin

Cells were treated with a urea-containing lysis buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 6 M urea) and sonicated (Handy Sonic, Tomy Seiko). The cell lysates were diluted 10-fold in lysis buffer and then immunoprecipitated with an anti-ubiquitin (FK2) antibody [12].

In vitro ubiquitylation and E1/E2 charging assays

To generate active form of recombinant ubiquitin, 20 μg His-ubiquitin was incubated with 2 μg USP2cc [15] in a deubiquitination buffer (50 mM Tris (pH 7.5), 1 mM DTT) at 37°C overnight. Uncleaved His-ubiquitin and His-USP2 cc were removed from the buffer with Ni-NTA resin (Promega) to yield pure wild-type or acetylated ubiquitin proteins. For the *in vitro* ubiquitylation assay, 2 μg ubiquitin, 50 ng E1, and the indicated E2 enzymes (400 ng Ubc13, Uev1a, or UBE2S; 800 ng CDC34; 400 ng Rad6; 200 ng UBCH5c; or 400 ng UBE2K) were incubated in a reaction buffer (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT) at 37°C for the times indicated. For the E1/E2 charging assay, 2 μg ubiquitin, 50 ng E1, and 200 ng of the indicated E2 enzymes were incubated in a reaction buffer at 25°C for 20 or 30 min. Then the samples were divided and boiled in SDS sample buffer either in the presence or absence of 200 mM DTT.

Surface plasmon resonance (SPR) analysis

Analyses were performed using a BIAcore 3000 instrument (GE Healthcare). His-UEV1a protein was immobilized on the surface of a CM5 sensor chip using an amine coupling kit (GE Healthcare). The analyte (wild-type, AcK6, or AcK48 ubiquitin) was injected at different concentrations (5, 10, 20, 40, or 80 μM) at a flow rate of 5 μl per minute [34].

More detailed methods are provided in the Supplementary Methods.

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

FO designed the project, performed most of the experiments, and wrote the paper. YS contributed to the mass spectrometric analysis and provided reagents and experimental advice. HN and TO performed SPR analysis. HT produced proteins. KS and KO optimized the incorporation of acetyllysine and purified acetyl-ubiquitin. KT and JK provided reagents and suggestions. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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