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APC/C-mediated multiple monoubiquitination provides an alternative degradation signal for cyclin B1

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Abstract

The Anaphase-Promoting Complex/Cyclosome (APC/C) initiates mitotic exit by ubiquitinating cell-cycle regulators such as cyclin B1 and securin. Lys48-linked ubiquitin chains represent the canonical signal targeting proteins for degradation by the proteasome, but they are not required for the degradation of cyclin B1. Lys11-linked ubiquitin chains have been implicated in degradation of APC/C substrates, but the Lys11-chain forming E2 UBE2S is not essential for mitotic exit, raising questions about the nature of the ubiquitin signal that targets APC/C substrates for degradation. Here we demonstrate that multiple monoubiquitination of cyclin B1, catalyzed by UBCH10 or UBC4/5, is sufficient to target cyclin B1 for destruction by the proteasome. When the number of ubiquitinatable lysines in cyclin B1 is restricted, Lys11-linked ubiquitin polymers elaborated by UBE2S become increasingly important. We therefore explain how a substrate that contains multiple ubiquitin acceptor sites confers flexibility in the requirement for particular E2 enzymes in modulating the rate of ubiquitin-dependent proteolysis.

Introduction

A uniform Lys48-linked ubiquitin polymer was the first signal identified to target substrates for destruction by the 26S proteasome[1–3]. Recent work has demonstrated that the repertoire of proteolytic signals encompasses chains of other linkage types, including Lys11-linked ubiquitin chains[4–10] and short chains of mixed linkage types[11]. In contrast, Lys63-linked chains have non-proteolytic roles in DNA repair[12, 13], kinase activation[14], protein trafficking[15, 16] and translation[17]. Similarly, the transfer of a single ubiquitin moiety to one

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Competing Interests Statement

The authors declare that they have no competing financial interests.

Author Contributions

N.D. and R.W.K. designed and interpreted the experiments. N.D. carried out and analyzed all experiments except those outlined below. N.A.H. carried out cyclin B1 ubiquitination for Ub-AQUA analysis and degradation assays with these species in APC/C-depleted extract. D.S.K. performed the Ub-AQUA analysis on cyclin B1 ubiquitinated *in vitro* with the E2 UBC4 and different Ub types in the lab of S.G. B.H.L. provided purified human proteasomes with oversight from D.F. M.B. helped with cloning of different cyclin B1 mutants. The manuscript was written by N.D. and R.W.K with input from all authors.

(monoubiquitination) or to multiple sites (multiple monoubiquitination) in a substrate has been implicated in mostly non-proteolytic processes[18,19], although multiple monoubiquitination can target receptor tyrosine kinases (RTKs) to the lysosome[20–22]. More recently, multiple monoubiquitination has been shown to control proteasomal processing of the p105 NF-kB precursor to the shorter p50 subunit[23]. To date, multiple monoubiquitination has not been coupled with rapid and complete proteolysis of a proteasome substrate.

The E3 ligase activities of the Skp1-Cullin-F-box complex (SCF) family and the Anaphase-Promoting Complex/Cyclosome (APC/C) are essential for cell-cycle progression[24, 25]. While the SCF cooperates with the E2 Cdc34 to assemble uniform Lys48-linked ubiquitin polymers on substrates[26], the APC/C works in conjunction with UBCH10 (also known as UBE2C) and enzymes of the UBC4/5 family to catalyze chain formation through three lysine residues of ubiquitin (Lys11, Lys48 and Lys63)[11]. UBCH10 builds multiple short ubiquitin chains on cyclin B1, which are sufficient to target the protein for degradation by the proteasome[11]. In this context, Lys48-linked ubiquitin polymers are dispensable for binding of modified cyclin B1 to ubiquitin receptors and degradation by the proteasome[11]. More recent work suggests that the assembly of a proteolytic signal on APC/C substrates may occur in two stages. In budding yeast, Ubc4 initiates ubiquitin conjugation, whereas Ubc1 elongates ubiquitin chains[27]. Similarly, in metazoans, UBCH10 has been proposed to initiate monoubiquitination of the substrate, followed by UBE2S-dependent extension of Lys11-linked ubiquitin chains[7, 8, 10]. Consistent with this idea, depletion of UBE2S from *Drosophila* S2 cells prolongs metaphase and stabilizes cyclin B1 at the spindle poles[7]. In contrast, UBE2S is not essential for normal mitosis in human HeLa cells, but rather may be important for proteolysis under conditions where APC/C activity is compromised, such as during recovery from drug-induced spindle-assembly checkpoint (SAC) activation[8]. Using a novel approach in which *Xenopus* extracts are made dependent on exogenous ubiquitin, we sought to understand whether APC/C-catalyzed proteolysis requires Lys11 or other ubiquitin linkages to efficiently degrade cyclin B1.

Results

Inhibiting ubiquitin chain formation has only a modest effect in stabilizing cyclin B1 in *Xenopus* extract

To quantitatively evaluate the role of different ubiquitin (Ub) chain linkages in targeting cyclin B1 for degradation in mitotic *Xenopus* extracts, we measured the degradation of a purified, ^{35}S-labeled N-terminal fragment of human cyclin B1 (cycB1-NT), which was degraded in an APC/C- and proteasome-dependent fashion (Supplementary Fig. S1a–d). Using ubiquitin-AQUA measurements[11, 28], we calculated that free ubiquitin is present at 5–10 μM concentration in *Xenopus* extracts (D. K. and N. H., unpublished observations). When added at 44 μM or 116 μM final concentration, wild-type ubiquitin and different ubiquitin mutants containing a single lysine-to-arginine substitution at position 11, 48, or 63, or at all three positions simultaneously (Ub^{triR}), stimulated cycB1-NT proteolysis, albeit with different kinetics (Supplementary Fig. S1e, f). These results were unexpected, as mass spectrometric analysis indicated that elimination of all three principal sites of Ub-Ub linkage

by the APC/C rendered Ub incapable of forming ubiquitin chains in reconstituted reactions (Supplementary Fig. S2). In contrast, addition of methylated ubiquitin (Ub^{me}) was slightly inhibitory. These findings indicate that ubiquitin levels are limiting for proteolysis, and raise the question of whether ubiquitin chains are essential for cyclin B1 degradation in *Xenopus* extract.

We next sought to make *Xenopus* extracts dependent on exogenous ubiquitin by inhibiting ubiquitin recycling. Pre-treatment of extracts with ubiquitin vinyl sulfone (UbVS), a general inhibitor of ubiquitin isopeptidases[29], inhibited cyclin proteolysis in a dose-dependent fashion, such that 20 μM UbVS inhibited degradation by 90–95% (Supplementary Fig. S3a). Degradation was fully restored by addition of 44 μM wild-type ubiquitin with partial rescue observed at lower concentrations (Fig. 1a). Concentrations of UbVS greater than 20 μM dampened the ability of 44 μM of wild-type ubiquitin to restore degradation (Supplementary Fig. S3b). Mutations that interfere with recognition of ubiquitin by proteasome receptors (L8A, I44A, or V70A)[30–32] hampered its ability to support proteolysis (Fig. 1b). In contrast, mutations that interfere with ubiquitin's non-proteolytic functions (F4A[33, 34] or D58A[35, 36]) had no effect on its ability to stimulate degradation (Fig. 1b). UbVS treatment led to loss of ubiquitinated species in the extract (Fig. 1c) and near complete discharge of E2~Ub thioesters (Fig. 1d, e); both effects were reversed following addition of exogenous ubiquitin.

We next tested the ability of chain-terminating ubiquitin mutants to rescue degradation in UbVS-treated extracts. Addition of wild-type ubiquitin, Ub^{48R} and Ub^{63R} rescued degradation efficiently, showing a half-life of approximately 15 minutes (Fig. 2a). After 20 minutes, the rate of degradation slowed, likely a consequence of ubiquitin depletion in the UbVS-treated extract, as supplementation with additional ubiquitin restored degradation to the initial rate (Fig. 2b). Extracts supplemented with Ub^{11R} or Ub^{triR} degraded substrate only somewhat more slowly. Even addition of methylated ubiquitin (Ub^{me}) (Fig. 2c) or a lysine-less ubiquitin (Ub^{K0})(Supplementary Fig. S3c) supported degradation, with a half-life of approximately 30 minutes. We next assessed how constraining the topology of ubiquitin chains to a single lysine residue affected degradation of cycB1-NT (Fig. 2c). Addition of $Ub^{K11only}$ or $Ub^{K48only}$ to UbVS-treated extract restored cycB1-NT proteolysis, but with slower kinetics compared to wild-type ubiquitin. Because mutation of Lys6 of ubiquitin may have an inhibitory effect on proteasomal degradation[37], we tested the effect of restricting chain formation to one of the three principle sites of ubiquitin-ubiquitin attachment mediated by UBCH10 by mutating the remaining two (Supplementary Fig. S2). $Ubiquitin^{48,63R}$ stimulated degradation efficiently, consistent with the ability of Lys11 linkages to support degradation (Fig. 2d). Ubiquitin forms supporting Lys48 and Lys63 linkages ($Ub^{11,63R}$ and $Ub^{11,48R}$, respectively) and Ub^{triR} supported proteolysis with somewhat slower kinetics. Together these findings indicate that the ability to construct Lys11-linked chains provides a kinetic advantage for degradation, but the advantage is modest. In principle this advantage could arise from the utilization of Lys11 in chain-forming reactions catalyzed by UBCH10, or from a role of UBE2S, which elongates ubiquitin chains exclusively through Lys11 linkages.

Ubiquitin chains are required for cyclin B1 degradation only when the number of available lysine residues in cyclin B1 is restricted

Cyclin B1 contains 18 lysine residues in its unstructured N-terminal region upstream of the cyclin box; 15 of these lysine residues are located within the first 88 amino acids close to the destruction box (Fig. 3a). To rule out the possibility that our results were influenced by use of an N-terminal fragment of cyclin B1, we first examined proteolysis of full-length wild-type cyclin B1 in UbVS-treated extracts (Fig. 3c). Addition of Ub^{11R} or Ub^{triR} stimulated degradation, albeit at slightly reduced rates relative to wild-type ubiquitin. Ub^{me} also supported degradation of cyclin B1, although a small fraction of the protein accumulated in a triply-ubiquitinated species. To examine whether reducing the number of lysines in cyclin B1 renders its proteolysis dependent on ubiquitin-chain formation, we measured degradation of cyclin B1 mutants that contained either one or four lysine residues in the first 115 amino acids at position 64 only ($cyc^{K64only}$) or at positions 59, 63, 64, and 67 ($cyc^{K59,63,64,67only}$). We chose these positions as mass spectrometry studies indicated that these lysine residues become ubiquitinated early in the course of reconstituted ubiquitination reactions (D. K., N. H., unpublished observations). $Cyc^{K64only}$ was degraded rapidly in untreated *Xenopus* extract, and was fully stabilized in UbVS-treated extract (Fig. 3c). However, unlike the case for wild-type cyclin B1, Ub^{11R} did not support efficient degradation of $cyc^{K64only}$. Similar results were obtained with Ub^{triR} and Ub^{me}, with the latter causing quantitative accumulation of cyclin B1 in a monoubiquitinated form. Restoration of three additional lysine residues in cyclin B1 ($cyc^{K59,63,64,67only}$) partially rescued its degradation in UbVS-treated extracts supplemented with Ub^{me} (Fig. 3d). We conclude that when deubiquitinating enzymes are inhibited, the attachment of single ubiquitin molecules to multiple lysine residues in cyclin is sufficient to target the substrate for degradation. Strict dependence on elaboration of ubiquitin chains appears to occur only when the number of available substrate lysines is restricted.

Multiple monoubiquitination can target cyclin B1 for efficient degradation in a reconstituted system and in *Xenopus* extract

We next assessed whether the effects of ubiquitin mutants on proteolysis paralleled effects on ubiquitin conjugation in reconstituted ubiquitination reactions. Elimination of Lys48 or 63 of ubiquitin had no effect on the mass of conjugates generated by UBCH10, whereas elimination of Lys11 reduced the mass of conjugates, consistent with the previously reported preference of UBCH10 for synthesizing Lys11 ubiquitin-ubiquitin linkages[6, 11] (Fig. 4a). In the presence of ubiquitin types that do not support ubiquitin polymer assembly (Ub^{triR} and Ub^{me}), the maximal extent of substrate modification (5–6 ubiquitins per cyclin B1 molecule) was observed at early time-points and remained unchanged in longer reactions (Fig. 4a), implying that only a subset of the 18 lysine residues in the cyclin B1 N-terminal domain become ubiquitinated. A time-course of ubiquitination with either wild-type Ub or Ub^{triR} (Fig. 4b) revealed that the conjugation of ubiquitin monomers to distinct lysines in cyclin B1 occurs with rapid kinetics. Furthermore, conjugates bearing 4 or more ubiquitin moieties were capable of binding proteasome-associated ubiquitin-receptors[38–46] including Rpn10 (Fig. 4c, d) and Rad23 (Fig. 4e, f). For conjugates of a similar molecular mass, substrate ubiquitinated with Ub^{triR} bound to receptors more efficiently than substrate ubiquitinated

with Ub^{me} suggesting that methylation of ubiquitin may compromise its affinity for ubiquitin receptors. We found similar binding patterns with cyclin B1-ubiquitin conjugates generated with UBC4 as the E2 (data not shown). Together these results indicate that multiple monoubiquitination occurs rapidly and can result in a productive signal for binding ubiquitin receptors.

We next sought to determine whether multiple monoubiquitination can target cyclin B1 for degradation in a reconstituted system. Full-length cyclin B1 in complex with CDK1 was ubiquitinated with either UBCH10 or UBC4, in conjunction with wild-type or mutant ubiquitin. The resulting conjugates were incubated with purified human proteasomes that were washed with high salt to eliminate USP14, a deubiquitinating enzyme that can antagonize cyclin B1 degradation *in vitro*[47, 48]. These proteasomes, which retain the deubiquitinating enzymes RPN11 and UCH37 (ref. 47), rapidly degraded polyubiquitinated cyclin B1 generated with UBCH10 (Fig. 5a) or UBC4 (Fig. 5b). Conjugates formed with Ub^{triR} or Ub^{K0} were efficiently degraded, but those generated with methylated ubiquitin were degraded less rapidly, consistent with the defect in the ability of these conjugates to bind ubiquitin receptors (Fig. 4c, e). Similar results were obtained in a quantitative assay using radiolabeled full-length cyclin B1 (Supplementary Fig. S4). Degradation of cyclin B1 in the reconstituted system was confirmed to be both APC/C- and Ub-dependent (Supplementary Fig. S4, S5c). Similar experiments performed with radiolabeled cycB1-NT pre-ubiquitinated by UBCH10 (Fig. 5c) revealed that that degradation of multiply monoubiquitinated cyclin B1 was sensitive to addition of the deubiquitinating enzyme USP14 (Fig. 5d). This effect was reversed by IU1, an inhibitor of the catalytic activity of USP14 (ref. 47) (Supplementary Fig. S5). Together these results indicate that purified proteasomes can efficiently degrade multiply monoubiquitinated cyclin B1 and that USP14 can deubiquitinate this substrate to suppress degradation.

To determine whether multiple monoubiquitination can target cyclin B1 for degradation under physiological conditions in the presence active deubiquitinating enzymes, we added the ubiquitinated species analyzed in Fig. 5c to interphase *Xenopus* extract, a state in which the APC/C is inactive (Fig. 5e). Conjugates generated with wild-type Ub or Ub^{triR} were efficiently degraded, whereas conjugates generated with Ub^{me} were degraded less efficiently. Similar results were obtained when the ubiquitin conjugates were introduced into extracts supplemented with excess nonubiquitinated unlabeled competitor to prevent additional APC/C-mediated ubiquitination (Supplementary Fig. S6b, c) or to extracts that had been immunodepleted of APC/C (Supplementary Fig. S6f). Pre-treatment with UbVS, at a concentration identical to that used to deplete free ubiquitin, did not accelerate degradation of conjugates (Fig. 5e). Similarly, addition of an inhibitor of USP14, IU1, failed to accelerate degradation of cyclin B1 in *Xenopus* extracts (Supplementary Fig. S7). Together these findings indicate that *Xenopus* extracts can rapidly degrade cyclin B1 bearing multiple ubiquitin monomers attached to distinct lysine residues.

Analysis of the role of UBE2S in cyclin B1 degradation

The ability of Ub^{11R} and chain-terminating ubiquitins to support proteolysis raised a question as to whether the E2 enzyme UBE2S is required for cyclin B1 degradation in

Xenopus extract. We therefore immunodepleted the protein and measured how this affected cyclin degradation. Antibodies efficiently depleted the UBE2S protein, as observed by the absence of signal following 25-fold enrichment of E2 enzymes on Ub agarose (Fig. 6a), without affecting levels of the APC/C or the E2 UBCH10 (Fig. 6a). UBE2S depletion caused only a slight increase in the half-life of cycB1-NT as compared to control-depleted extract (Fig. 6b), which was reversed by adding back 10 nM of the recombinant enzyme. We hypothesized that UBE2S is not essential for rapid degradation of cyclin B1 because the substrate contains multiple lysine residues that can serve as sites of attachment of short ubiquitin chains. We therefore examined the effect of UBE2S depletion on the rate of degradation of $cyc^{K64only}$ compared to that of wild-type cyclin B1 (Fig. 6c). Degradation of full-length wild-type cyclin B1 was unaffected by depletion of UBE2S, but proteolysis of $cyc^{K64only}$ was highly sensitive to the depletion of UBE2S, and addition of recombinant UBE2S fully restored degradation (Fig. 6c). Together these findings indicate that UBE2S is present in *Xenopus* extract at sufficient levels to support cyclin proteolysis, but becomes essential only when the number of ubiquitinatable lysine residues in cyclin B1 is restricted. Similarly, we found that immunodepletion of UBCH10 (Supplementary Fig. S8a) had a modest effect on degradation of wild-type cyclin B1, but more significantly delayed turnover of $cyc^{K64only}$ (Supplementary Fig. S8b). The delay in degradation of $cyc^{K64only}$ was rescued by addition of 50 nM of recombinant UBCH10. These findings suggest that other E2 enzymes in the extract may be sufficient to support APC/C-dependent degradation of cyclin B1 in *Xenopus* extracts. Enzymes of the UBC4/5 class are the best candidates for such a role, but we were unable to identify an antibody that could efficiently deplete endogenous UBC4/5 and thus could not evaluate their role in cyclin B1 degradation.

Discussion

Here we have evaluated the role of ubiquitin-chain topology in targeting cyclin B1 for degradation. Our study was motivated by recent findings suggesting that Lys11 linkages, mediated by the chain-forming E2 enzyme UBE2S, may be important for APC/C-dependent proteolysis. However, our earlier work suggested that APC/C, solely in conjunction with the E2 enzyme UBCH10 or the enzyme UBC4/5, can build a ubiquitin signal that is sufficient for degradation by purified proteasomes[11]. Here we provide a resolution to this paradox, demonstrating that conjugation of ubiquitin to multiple lysine residues of cyclin B1 provides an alternative degradation signal for cyclin B1 that does not require chain extension by the Lys11-specific E2 UBE2S. Lysine11-ubiquitin chain formation becomes essential only when the number of available lysine residues in cyclin B1 is restricted.

Dominant-negative effects of different ubiquitin types may be difficult to observe when examined in a background that contains wild-type ubiquitin. By inhibiting ubiquitin recycling, we were able to impose a state of ubiquitin deficiency in extract sufficient to stabilize cyclin B1. The strong dependence of cyclin proteolysis on ubiquitin availability has not been previously appreciated, and suggests the possibility that control of ubiquitin availability could regulate the rate of APC/C substrate degradation. Addition of ubiquitin fully rescues cyclin degradation in UbVS-treated extracts, but the rate of cyclin degradation is no faster in a UbVS-treated extract relative to a non-treated extract. This finding suggests

that for deubiquitinating enzymes to be able to antagonize degradation, it may be crucial that the rate of ubiquitination be constrained by limiting the availability of free ubiquitin.

The UbVS system enabled us to define the role of different chain linkages in targeting substrates for degradation by the proteasome. In agreement with earlier work in a reconstituted system[11], Lys48 ubiquitin linkages were not required for efficient cyclin proteolysis in UbVS-treated extract. Surprisingly, in the light of recent studies[6, 7, 10], ubiquitin incapable of forming Lys11 linkages (Ub^{11R}) also supported efficient degradation of cyclin B1. Importantly, we found that chain-terminating ubiquitins (Ub^{triR} and lysine-less ubiquitin) also supported robust rates of cyclin proteolysis. Methylated ubiquitin was less capable of supporting rapid degradation, which may reflect less efficient recognition by ubiquitin receptors and the proteasome due to modification of Lys6 of ubiquitin[37]. However, upon restriction of ubiquitination to a single lysine residue in cyclin as in $cyc^{K64only}$, chain-terminating ubiquitins were no longer able to support substrate degradation. Together these findings suggest that ubiquitin-chain formation is not essential for cyclin proteolysis unless the number of available lysine residues is restricted.

We propose that attachment of monoubiquitin to multiple lysines in cyclin B1 has the potential for generating a high density of ubiquitin that promotes receptor binding (Fig. 6d). In such an arrangement, the hydrophobic patches on distinct ubiquitin units may be able to engage multiple ubiquitin receptors. Multiply monoubiquitinated cyclin B1 thus resembles a ubiquitin chain, except that the cyclin B1 polypeptide chain is used as a backbone to link one ubiquitin molecule to another. Whether particular spacing of ubiquitinated lysine residues is essential for recognition by ubiquitin receptors remains unknown. In our pull-down experiments, there may be some enhanced avidity resulting from a dimeric GST moiety positioning two ubiquitin-associated domains (UBAs) in close proximity[49]. However, the ability of the multiply monoubiquitinated protein to be degraded by purified proteasomes and in *Xenopus* extracts suggests that this substrate must have sufficient affinity for proteasome-associated ubiquitin receptors to support proteolysis.

The capacity of purified proteasomes to rapidly degrade multiply monoubiquitinated cyclin B1 was significantly suppressed by USP14, suggesting that USP14 can efficiently remove monoubiquitin, as well as trim Ub chains. However, USP14 does not appear to strongly antagonize proteasome function in *Xenopus* extract, as treatment of extract with UbVS or the USP14-specific inhibitor IU1 did not appreciably enhance turnover of pre-ubiquitinated cyclin. Although present in *Xenopus* extracts (N.V.D., R.W.K., unpublished data), levels of USP14 associated with proteasomes in extract may be insufficient to impede proteolysis. Together, these findings suggest the proteasome does not impose a requirement for ubiquitin-chain formation for efficient proteolysis of cyclin B1 in *Xenopus* extract. Our study further strengthens the view that the proteasome has the capacity to recognize and degrade substrates bearing ubiquitin modifications distinct from the canonical Lys48-linked polyubiquitin chains[4, 50–53]

Although UBE2S is sufficient to elongate Lys11-ubiquitin chains to promote the degradation of APC/C substrates[7–10], we found that UBE2S depletion of *Xenopus* egg extract had no impact on degradation of wild-type cyclin B1. Our findings are consistent

with the report that UBE2S is largely dispensable for cyclin B1 degradation in unperturbed mitosis in human cells[8]. The lack of requirement for UBE2S and Lys11-linked ubiquitin chains for robust degradation of cyclin B1 in the *Xenopus* system may be a consequence of higher levels of UBCH10 than seen in other biological contexts[7, 8]. Furthermore, the relative importance of UBCH10 and UBE2S in degradation of different APC/C substrates may vary. Human cyclin B1 is lysine-rich in its N-terminal domain, containing 18 lysine residues, whereas cyclin A2 has 12 lysine residues in the same region, which may make the latter protein more dependent on the chain-elongating enzyme UBE2S for degradation. Similarly, *S. cerevisiae* Clb2 is relatively lysine-poor in its N-terminal domain, containing only 6 lysine residues, potentially explaining the importance of a chain-elongating E2 in this system[27]. A greater dependence on chain-elongating E2s may impact the sensitivity of different substrates to deubiquitination. In this regard, cyclin A2 degradation during interphase is specifically impeded by the deubiquitinating enzyme USP37[54], but this enzyme does not appear to antagonize cyclin B1 degradation. An interesting future question is how the balance between multiple monoubiquitination and ubiquitin-chain formation affects sensitivity of degradation to deubiquitinating enzymes. Finally, our work raises the possibility that the degree of dependence on UBE2S could be regulated by post-translational modification of the substrate. For example, acetylation is known to affect degradation of the spindle-checkpoint protein BubR1[55]. By restricting the number of ubiquitinatable lysine residues, acetylation could increase the dependence of degradation pathways on UBE2S-catalyzed chain formation.

Methods

Antibodies and reagents

Proteins were separated by SDS-PAGE on NuPAGE 4–12 % or 12 % Bis-Tris gels (Invitrogen), followed by wet transfer to PVDF. Sources of antibodies for immunoblotting were as follows: anti-cyclin B1 (Ab-2; RB-008-P, Neomarkers), anti-Cdc27 (610455, BD Transduction Laboratories), anti-UBCH10 (A-650, Boston Biochem; AB3861, Millipore), anti-UBE2S (N-14; sc-131354, Santa Cruz Biotechnology), anti-UBCH5 (A-615; Boston Biochem), anti-ubiquitin (P4D1; sc-8017; Santa Cruz Biotechnology). Secondary antibodies included anti-goat IgG-HRP (sc-2020; Santa Cruz Biotechnology); anti-rabbit IgG-HRP (NA934) and anti-mouse IgG-HRP (NA931) were from GE Healthcare. Antibodies for immunoprecipitation or immunodepletion included: anti-Cdc27 (AF3.1; sc-9972) and anti-UBE2S (N-14; sc-131354) from Santa Cruz Biotechnology; anti-UBCH10 (gift of Hongtao Yu); and for control depletions, normal rabbit (sc-2027) and normal goat (sc-2028) IgG, both from Santa Cruz Biotechnology. UBE2S antibodies were coupled to UltraLink Immobilized Protein A/G beads (53132, Pierce). UBCH10 and CDC27 antibodies were coupled to Affiprep protein A beads (156-0006, Bio-Rad). Ub agarose (U-405), ubiquitin vinyl sulfone (U-202), MG262 (I-120), Ub^{me} and ubiquitin mutants except for $Ub^{11,48R}$, $Ub^{11,63R}$, and Ub^{triR} were purchased from Boston Biochem. TAME (T4626) and ubiquitin (U6253) were purchased from Sigma.

Preparation of recombinant proteins

Ubiquitin mutants $Ub^{11,48R}$, $Ub^{11,63R}$ and Ub^{triR} were generated by introducing arginine codons (AGA, AGG) via PCR-mediated mutagenesis of the human ubiquitin sequence (cloned in pET3a). Plasmids were verified by sequencing and the purified proteins analyzed by mass spectrometry. To ensure efficient arginine incorporation, BL21(DE3) cells were co-transformed with pJY2 (ref.58) [56], which carries T7 lysozyme (LysS) and a gene encoding $tRNA_{UCU}^{Arg}$. Cultures were grown at 37 °C to an OD_{600} of ~0.5, and induced with 100 μM IPTG at an OD_{600} of 0.6 at 25 °C for 5 h. Cells were ruptured by sonication in QA lysis buffer (50 mM HEPES (pH 7.7), 100 mM KCl, protease-inhibitor cocktail, 5 mM 2-mercaptoethanol, 10 μg/ml DNase), lysozyme was added to 1 mg/ml concentration, and incubated with rotation at 4 °C for 15 min. Following sonication, cell lysates were clarified by centrifugation and the resulting supernatants applied to a Q column. The flow-through containing ubiquitin was concentrated and purified by size-exclusion chromatography. Fractions containing ubiquitin were typically > 95% pure.

To generate full-length cyclin B1-CDK1 complex, human cyclin B1 and CDK1 baculoviruses were used as described[11]. To generate mutants of cyclin B1, DNA fragments encoding the N-terminal 124 amino acids of wild-type human cyclin B1 (AACCGGTCCGAAACCGTCGACATGTCGCATCACCATCACCATCACGGCTCGAT
GGCGCTCCGAGTCACGCGTAACTCGAAAATTAATGCTGAAAATAAAGCGAAAA
TCAACATGGCAGGCGCCAAGCGCGTTCCTACGGCACCGGCGGCAACCTCCAAAC
CCGGGCTGAGGCCAAGAACAGCTCTTGGGGACATTGGTAACAAAGTCAGTGAA
CAGCTACAGGCCAAAATGCCTATGAAAAAAGAAGCAAAACCTTCAGCTACCGG
TAAAGTCATTGATAAAAAACTACCAAAACCTCTTGAAAAGGTACCTATGCTGGT
GCCAGTGCCAGTGTCTGAGCCAGTGCCAGAGCCAGAACCTGAGCCAGAACCTG
AGCCTGTTAAAGAAGAAAAACTTTCGCCTGAGCCTATTTTGGTTGATACTGCTA
GCAATA) or the same region of the protein with arginine substitutions at all lysine residues (cyc^{allR};
AACCGGTCCGAAACCGTCGACATGTCGCATCACCATCACCATCACGGCTCGATG
GCGCTCCGAGTCACGCGTAACTCGAGAATTAATGCTGAAAATAGAGCGAGAAT
CAACATGGCAGGCGCCAGGCGCGTTCCTACGGCACCGGCGGCAACCTCCAGAC
CCGGGCTGAGGCCAAGAACAGCTCTTGGGGACATTGGTAACAGAGTCAGTGAA
CAGCTACAGGCCAGAATGCCTATGAGAAGAGAAGCAAGACCTTCAGCTACCGG
TAGAGTCATTGATAGAAGACTACCAAGACCTCTTGAAAGGGTACCTATGCTGGT
GCCAGTGCCAGTGTCTGAGCCAGTGCCAGAGCCAGAACCTGAGCCAGAACCTG
AGCCTGTTAGAGAAGAAAGACTTTCGCCTGAGCCTATTTTGGTTGATA
CTGCTAGCAATA) preceded by 6xHis tag were synthesized (GenScript). Using restriction enzyme digest with NheI and RsrII, fragments were subcloned into pFASTBac containing the C-terminus (125–433 amino acids) of cyclin B1. To generate $cyc^{K64only}$, primers 5′-TCCAGACCCGGGCTGAGGCCAAGAACAGCTCTTGGGGACATTGGTAACAGAGT
CAGTGAACAGCTACAGGCC-3′ and 5′ –
AATGACTCTACCGGTAGCTGAAGGTCTTGCTTCTTTTCTCATAGGCATTCTGGCC
TGTAGCTGTTCACTGAC-3′ were used for an extension reaction and the resulting fragments cloned into Xma I and Age I cleavage sites of pFASTBac carrying full-length cyc^{allR}. Plasmids were verified by restriction enzyme mapping and sequencing.

Baculoviruses were generated according to the Bac-to-Bac manual (Invitrogen). Wild-type cyclin B1 was ^{35}S-labeled in Sf9 cultures by resuspending cells (1.5×10^6 cells/ml) in media containing 10 % SF-900 II SFM and 90 % SF-900 II SFM without methionine or cysteine (both from Invitrogen). Baculovirus was added to cells, along with 50 μCi of ^{35}S methionine and cysteine (NEG772; Perkin Elmer), and cyclin B1 expression was allowed for 2.5 days. CDK1 was expressed separately in Sf9 cells without radiolabeling and then combined with lysate from cells expressing cyclin B1 to allow formation of complex, which was then purified via Ni-NTA affinity and gel filtration chromatography.

N-terminal fragment of human cyclin B1 (cycB1-NT) (1–88) containing an HA-tag at the N-terminus and 6xHis-tag at the C-terminus was generated using PCR amplification with forward primer (5′-CCAGGACCATGGGTTACCCATACGATGTTCCAGATTACGCTGGCTCGATGGCGCTCCGAGTCACG-3′) and reverse primer (5′-GGGAGCCTCGAGCTAGGGAGCGTGATGGTGATGGTGATGCATAGGTACCTTTTCAAGAGG-3′). The resulting PCR product was digested with Nco I and Xho I for subcloning into pET28a. Plasmids were verified by restriction enzyme mapping and sequencing. For ^{35}S labeling in *E. coli*, cultures (50 ml) were grown at 37 °C to OD_{600} = 0.8, then harvested by centrifugation (3,700×*g* for 15 min, 4 °C) and resuspended in modified M9 medium (50 ml final volume). Cells were allowed to grow for 15 min at 37 °C before 5 mCi of Easy Tag™ L-[35S]-Methionine (NEG709A005MC; Perkin Elmer) was added. Expression was induced with 0.5 mM IPTG for 2.5 h at 37 °C. Cells were ruptured in 5 ml/g of pellet guanidine-HCl lysis buffer (pH 8.0) and lysates rotated for at 24 °C until the lysate became slightly translucent; approximately 45 minutes. Lysates were cleared by centrifugation and cycB1-NT was purified using Ni-NTA affinity chromatography (Qiagen). Eluted protein was desalted into XB buffer (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.8 with KOH), supplemented 2% glycerol, protease inhibitors and phenylmethylsulfonyl fluoride, and stored at − 20 °C.

Maltose-binding protein (MBP)-tagged E1 was expressed in *E. coli* inducing cultures at an OD_{600} of 0.6 with 300 μM IPTG for 5 h at room temperature. Purification was carried out using a standard MBP purification protocol. For expression of his-tagged UBCH10 and his-tagged UBC4, bacterial cultures were induced at an OD_{600} of 0.6 at 37 °C with 500 μM IPTG for 4 h. The enzymes were purified via Ni-NTA affinity and gel filtration chromatography. PET28a expressing human wild-type UBE2S was kindly provided by M. Kirschner. Cultures were grown to an OD_{600} of 0.4 and induced with 500 μM IPTG at 37 °C for 4 h. His-UBE2S was purified by Ni-NTA purification. Glutathione-*S*-transferase (GST)-fusion proteins for Rpn10 and Rad23 were purified as reported previously[39, 57].

Preparation of *Xenopus* egg extract

Interphase extract was prepared as described[58] but using 2 μg/ml calcium ionophore (A23187, Calbiochem) for egg activation. Interphase extract was induced to enter mitosis by addition of nondegradable cyclin B (MBP-Δ90)[59] as described previously[60] except that extracts were treated for 45–60 min prior to assay.

Reconstitution of ubiquitination and degradation of cyclin B1

Ubiquitination reactions were performed as described previously[11], but to minimize loss of Cdc20, immunoprecipitated APC/C was washed quickly three times with XB containing 500 mM KCl, two times with XB, and then three times with reaction buffer. Ubiquitination reactions were performed at 24 °C with agitation at 1500 rpm and contained APC/C on 30 μl beads, and 30 μl of a mix containing recombinant MBP-human E1 (1.3 μM), His-tagged UBCH10 or Ubc4 (100 nM – 4 μM), ubiquitin (118–145 μM), and 450–500 nM cyclin B1-CDK1 or cycB1-NT. For ubiquitin-receptor binding and degradation assays, reaction supernatants were combined with the first 20 μl of reaction buffer wash. For analysis of cyclin B1 ubiquitination with different ubiquitins, entire reactions were processed for immunoblotting or autoradiography. Dried gels were analyzed by phosphorimaging (Bio-Rad PMI); quantitation was performed with Quantity One software (Bio-Rad).

For binding experiments with ubiquitin receptors, cyclin B1-CDK1 was pre-ubiquitinated with purified *Xenopus* APC/C, UBCH10 (3 μM), and ubiquitin (118 μM) for 90 min. Binding was performed essentially as described previously[11] in the presence of 0.1 % Tween20. Supernatants were collected and mixed with the first wash to make the flow-through fraction. Beads were washed twice and diluted with SDS sample buffer to analyze the bound fraction.

For degradation assays with purified proteasomes, human proteasomes (10–20 nM), purified as reported previously[47] but non-UbVS treated, were incubated with cyclin B1-Ub_n in buffer (50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 5 mM ATP)[47] and incubated at 24 °C. Recombinant GST-USP14 (ref. 47) was incubated with proteasome for ~5–10 min before initiating degradation. In experiments with USP14-inhibitor IU1 (ref. 47), GST-USP14 was pre-incubated with IU1 or DMSO for 5 min at 24 °C before adding proteasomes. For degradation of cycB1-NT, reactions (20 μl proteasome mix and 4 μl cycB1-NT-Ub_n for each time-point) were quenched with 81 μl of 32 % TCA and 20 μl bovine serum albumin (BSA) (10 mg/ml stock). For degradation of full-length cyclin B1, 90 μl proteasome mix was added to 10 μl cyclin B1-Ub_n for each time-point, and the reaction quenched with 112 μl pre-chilled 2 % perchloric acid (PCA) and 12 μl of BSA (10 mg/ml stock). For 0 min time-point, substrate and proteasome mixtures were individually added to acid. Following acid addition, samples were incubated on ice ≥ 30 min and centrifuged at 14,000 ×*g*, 4 C. A fraction (50–80 %) of supernatants and NaOH to neutralize TCA (or Tris Base to neutralize PCA) were added to Ultima Gold scintillation fluid (6013327, Perkin Elmer). A Packard scintillation counter was used to take measurements. Acid-soluble counts were compared to total radioactive counts and results were plotted as percent released counts. For degradation assays with unlabeled full-length cyclin B1, samples were analyzed by anti-cyclin B1 immunoblot.

Cyclin B1 degradation in *Xenopus* extract

Degradation assays with nonubiquitinated cyclin B1 were performed by adding ~200–250 nM of cyclin B1 in 40 μl reactions, with extract constituting 75–80 % of the total volume. Pre-treatment of extract with TAME or Mg262 was done at 24 °C for 15 min. UbVS treatment was performed for 30 min at 24 °C, 1250 rpm. Extracts contained 100 μg/ml

cycloheximide to prevent re-incorporation of free labeled amino acid. Degradation experiments were performed at 24 °C, 1250 rpm. Samples for proteolysis of unlabeled cyclin B1-CDK1 were processed for anti-cyclin B1 immunoblot. In degradation assays with ^{35}S-labeled cycB1-NT, reactions (3 μl per time-point) were quenched with 97 μl of 20 % TCA, vortexed and incubated on ice before centrifugation at 14,000 ×*g*, 4 °C and the radioactivity in the supernatant measured by scintillation counting.

For degradation of preubiquitinated cycB1-NT, interphase extract was pre-treated with UbVS or buffer for 30 min at 24 °C with agitation and supplemented with 100 μg/ml cycloheximide. In experiments with IU1, IU1 or DMSO was added to extract for 15 min at 24 °C prior to addition substrate. Extract (~ 14 μl) was added to 4 μl of cyclin B1 NT-Ub_n conjugates for each time-point. Reactions were quenched with 107 μl of 20 % TCA, vortexed and incubated on ice before centrifugation at 14,000 ×*g*, 4 °C for 30 min. A fraction of supernatants was combined with NaOH and scintillation fluid.

To deplete APC/C, 100 μl of interphase extract was mixed with 2 μg of anti-Cdc27 antibody coupled to 5 μl of Affiprep Protein A beads and incubated at 4 °C for 3 h. Approximately 10 μl of pre-ubiquitinated radiolabeled cyclin B1 was added to 90 μl of APC/C- depleted extract. Reactions were incubated at 22 °C for the indicated times and stopped by the addition of equal volume of chilled 2 % PCA. Reactions were then incubated on ice for ≥ 30 min and centrifuged at 15,000 rpm for 10 min, 4 °C and radioactivity in the supernatant measured by scintillation counting.

Immunodepletion of E2 enzymes

For UBE2S immunodepletion, 10 μg of anti-UBE2S antibody or control goat IgG antibody was bound to 25 μl UltraLink Immobilized Protein A/G and incubated with 250μl extract at 4 °C for ~ 1 h. For UBCH10 immunodepletion, 100 μl of anti-UbcX antibody or equivalent amount of control rabbit IgG coupled to Affiprep protein A support was used to deplete 170 μl extract. Cyclin B1-CDK1 (~200 nM) was added to E2- or control-depleted extract and time-course of degradation performed at 24 °C (1250 rpm) and the equivalent of 1 μl of extract was analyzed by SDS-PAGE/anti-cyclin B1 immunoblot. Depletion of UBE2S and UBCH10 were confirmed by western blot using anti-UBE2S and anti-UBCH10, respectively. To confirm the efficiency of E2 depletion, extract was incubated with Ub agarose, at a ratio of ~10:1 (extract:resin). Ub agarose was pre-washed 4 times with 1x energy mix (for 20x stock:150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA, 20 mM $MgCl_2$, pH 7.7) in XB buffer (100 mM KCl). Extract and 2x energy mix were added to Ub agarose and incubated at 1250 rpm, 24 C for 45 min. Samples were centrifuged, extract removed and Ub agarose washed once with 10-fold volume of 1x energy mix. Bound proteins were eluted by boiling in SDS sample buffer and analyzed by immunoblot.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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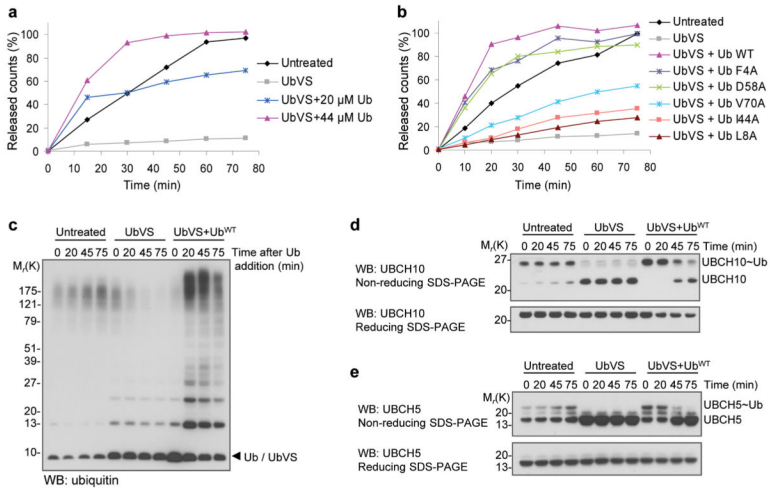

Figure 1.

Ubiquitin vinyl sulfone (UbVS) inhibits cyclin B1 degradation by depleting available ubiquitin. (**a**) ^{35}S-labeled cycB1-NT and 20 or 44 μM of wild-type (WT) Ub were introduced into mitotically-arrested *Xenopus* extract that had been pre-treated with UbVS (20 μM) or buffer (untreated) for 30 min. Proteolysis was measured by release of trichloroacetic acid (TCA) soluble counts, and plotted as percent of input radiolabeled cycB1-NT. Trends are representative of three or more independent experiments. (**b**) Wild-type ubiquitin or forms of ubiquitin (44 μM) bearing single-point mutations in distinct interaction surfaces on ubiquitin, along with radiolabeled substrate, were added to UbVS-treated extract. For panels (**c**–**e**), mitotically-arrested *Xenopus* extract was pre-treated with UbVS (20 μM) or buffer (untreated) for 30 min. Wild-type ubiquitin (44 μM) was added to extract, as indicated. Aliquots were withdrawn at indicated times and analyzed by SDS-PAGE and western blot. (**c**) Ubiquitin status in *Xenopus* extract was examined by anti-ubiquitin western blot. (**d**) Levels of Ub-charged endogenous UBCH10 were examined by anti-UBCH10 western blot. Aliquots were removed at the indicated times and quenched with either nonreducing sample buffer to examine levels of UBCH10~Ub or reducing sample buffer to examine total levels of UBCH10. (**e**) Same as in **d**, but levels of endogenous ubiquitin-charged UBCH5 were examined by western blot. Uncropped images of immunoblots are presented in Supplementary Fig. S9

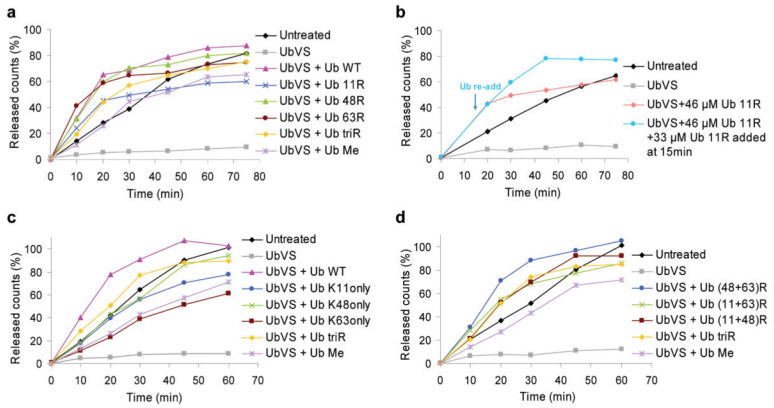

Figure 2.

Ubiquitin chain formation is not essential for cyclin B1 degradation in UbVS-treated *Xenopus* extract. ^{35}S-labeled cycB1-NT and different forms of Ub, where indicated, were introduced concomitantly into mitotically-arrested *Xenopus* extract that had been pre-treated with UbVS (20 μM) or buffer (untreated) for 30 min. Proteolysis was measured by release of trichloroacetic acid (TCA) soluble counts, and plotted as percent of input radiolabeled cycB1-NT. Trends are representative of three or more independent experiments. (**a**) Ubiquitin types (44 μM) with single lysine-to-arginine mutations at indicated positions or at all three positions Lys11, 48 and 63 (Ub^{triR}) were added to UbVS-treated extract. Ub^{WT} refers to wild-type ubiquitin and Ub^{me} refers to methylated ubiquitin. (**b**) Ub^{11R} and substrate were introduced into UbVS-treated extract, and supplemented with Ub^{11R} or buffer control 15 min after initiation of degradation. (**c**) Ubiquitin types (44 μM) used, as indicated. $Ubiquitin^{Konly}$ refers to ubiquitin that has all of its lysines, except for those specified, mutated to arginines. (**d**) Degradation was measured in the presence of different ubiquitin types (44 μM) containing arginine substitutions at two of the three principle sites of ubiquitin-ubiquitin conjugation by the APC/C.

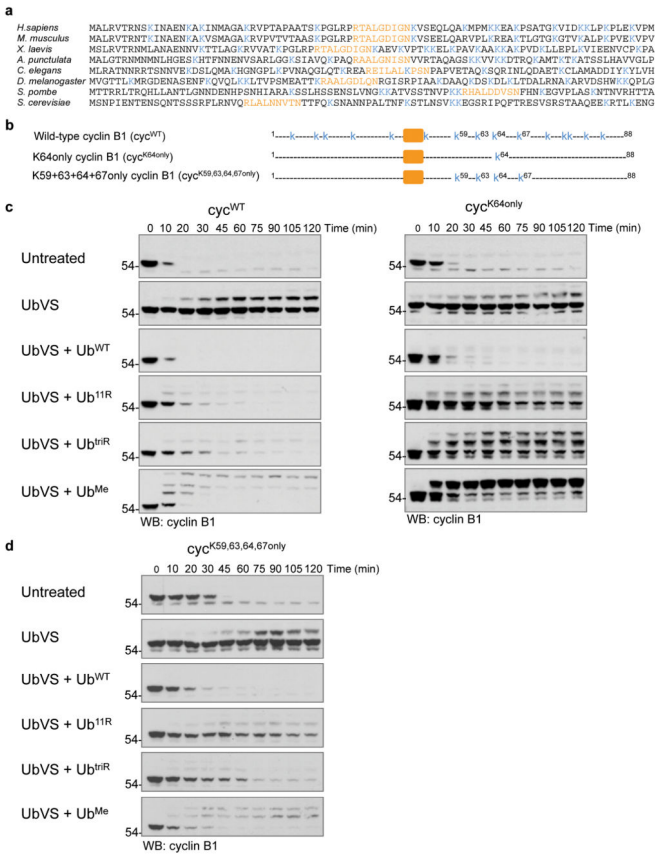

Figure 3.

Cyclin B1 proteolysis depends on Lys11-linked Ub chain formation only when the number of available lysine residues is restricted. (**a**) Sequence comparison of the cyclin B1 N termini from multiple species. Lysine residues are colored in blue; the destruction box (D-box) is colored in orange. (**b**) Schematic representation of the N terminal region (residues 1–88) of human cyclin B1 with lysine residues denoted K in blue and with the D-box motif denoted with an orange rectangle. Cyclin B1 mutants (cyc^{K64only} and cyc$^{K59,\ 63,\ 64,\ 67only}$) were generated by substituting lysine with arginine at all but the specified lysine residues within the first 115 amino acids (residues 89–115 not shown). (**c**) Purified full-length wild-type (cyc^{WT}) or single-lysine (cyc^{K64only}) cyclin B1, in complex with CDK1, and forms of Ub (20 μM) as indicated were added to mitotic *Xenopus* egg extract that had been pre-treated with UbVS (20 μM) for 30 min. Stability of the exogenous substrate over time was assessed by SDS-PAGE and cyclin B1 western analysis. (**d**) Performed as in **c** except the behavior of full-length cyc$^{K59,\ 63,\ 64,\ 67only}$, in complex with CDK1, was analyzed. Uncropped images of immunoblots are shown in Supplementary Fig. S9.

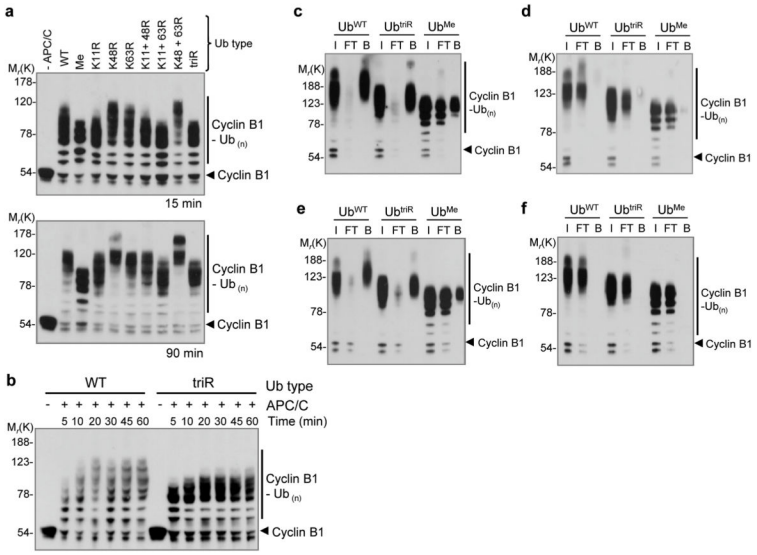

Figure 4.

UBCH10 and APC/C catalyze rapid multiple monoubiquitination of cyclin B1 that is sufficient for binding ubiquitin receptors. (**a**) Western analysis of *in vitro* ubiquitination reaction containing full-length cyclin B1, APC/C immunopurified from mitotically-arrested *Xenopus* extract, recombinant UBCH10 (100 nM), and forms of Ub (118 μM), as indicated. Ubiquitin types with lysine-to-arginine mutations at one, two, or at all three positions Lys11, 48 and 63 (Ub^{triR}), as well as methylated ubiquitin (Ub^{me}) were used. Control "−APC/C" reactions containing all components except for the E3 ligase were performed in parallel. Reactions were allowed to proceed for 15 or 90 min before analysis by SDS-PAGE/western blotting against cyclin B1. (**b**) Time-course of the *in vitro* ubiquitination of full-length wild-type cyclin B1 with Ub^{WT} or Ub^{triR} and remaining components as in **a**. (**c**–**f**) Binding of ubiquitinated cyclin B1 to GST-tagged Ub receptors. Cyclin B1-Ub conjugates were incubated with immobilized receptor proteins for 1 h at 4 °C before reaction products were subjected to SDS-PAGE/western blot analysis against cyclin B1. Equivalent amounts of input (I), flow-through (FT) and bound (B) were loaded in adjacent lanes. Binding experiments with wild-type Rpn10 (**c**) and Rad23 (**e**). Binding with corresponding versions of the receptors lacking the Ub recognition domains, with engineered block substitution of the UIM domain (LAMAL → NNNNN) of Rpn10 (**d**) or deletion of the UBA domains of Rad 23 (**f**).

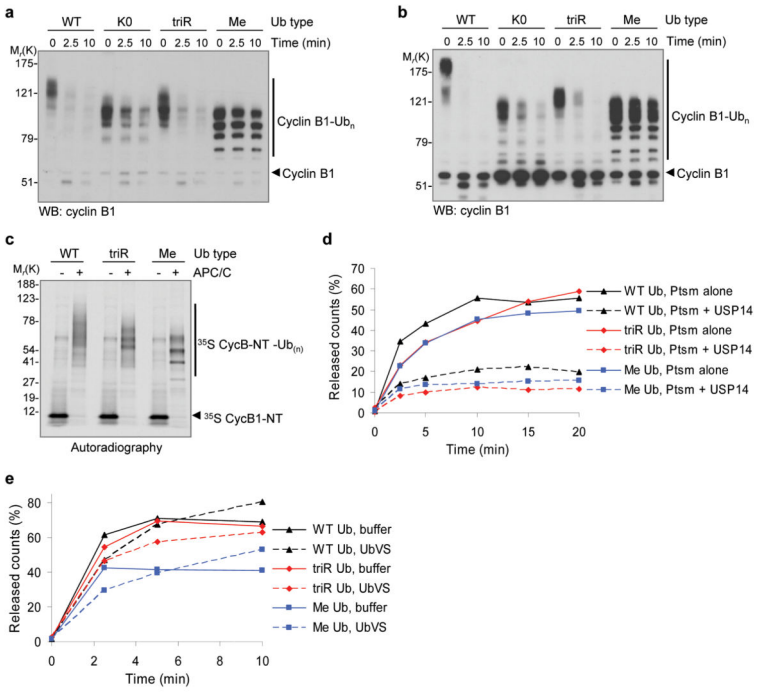

Figure 5.

Multiply monoubiquitinated cyclin B1 is rapidly degraded by purified proteasomes and in *Xenopus* extract. (**a**) *In vitro* degradation assay with cyclin B1-Ub species generated with immunopurified *Xenopus* APC/C, recombinant UBCH10 (250 nM), and forms of Ub (145 μM), as indicated and USP14-deficient human proteasomes (20 nM). Aliquots were removed at the indicated times and reaction products analyzed by SDS-PAGE and anti-cyclin B1 western. (**b**) Same as in **a**, but conjugates were generated with UBC4 as the E2 enzyme. (**c**) Autoradiograph of *in vitro* APC/C-UBCH10 catalyzed ubiquitination of ^{35}S-cycB1-NT (1–88) with immunopurified *Xenopus* APC/C, recombinant UBCH10 (100 nM) and forms of Ub (145 μM) as indicated. Products from a 60-minute ubiquitination assay were separated by SDS-PAGE and analyzed using a phosphorimager. (**d**) CycB1-NT-Ub species from **c** were incubated with purified human proteasomes (20 nM) reconstituted with or without 20-fold molar excess of GST-tagged wild-type USP14. At indicated times, reactions were terminated by addition of trichloroacetic acid (TCA). Proteolysis was measured by release of TCA soluble counts, and plotted as percent of input radiolabeled cyclin B1 protein. See Supplementary Fig. S5c for additional controls. (**e**) CycB1-NT-Ub species from **c** were added to interphase *Xenopus* extract that had been pre-treated with UbVS (15 μM) or buffer control for 30 min. Reactions were terminated by addition of TCA at indicated times. Proteolysis was measured by release of TCA soluble counts, and plotted as percent of input radiolabeled cycB1-NT.

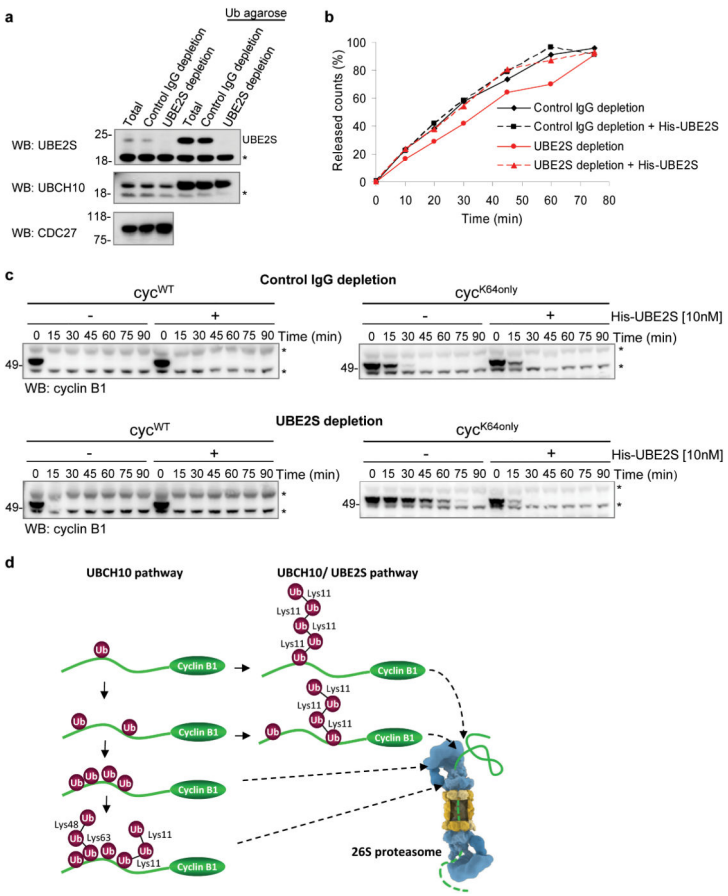

Figure 6.

UBE2S is required for cyclin B1 proteolysis only when ubiquitination is constrained to a single lysine. (**a**) Mitotically-arrested *Xenopus* extract was immunodepleted with UBE2S antibody or control IgG. Samples were further incubated with Ub agarose (10:1 ratio of extract to resin) to enrich for E2 enzymes and bound proteins were analyzed by SDS-PAGE/ immunoblotting. Lanes 4–6 represent 25-fold enrichment of E2 enzymes on Ub agarose. Levels of UBE2S, UBCH10 and APC/C were examined by immunoblotting. Asterisks, nonspecific signal. Uncropped images of immunoblots are shown in Supplementary Fig. S9. (**b**) Rate of degradation of ^{35}S-labeled cycB1-NT in UBE2S- or control-depleted mitotic *Xenopus* extract from **a**. Recombinant His-UBE2S (10 nM), where indicated, was added to reactions concomitantly with substrate. Proteolysis was measured by release of TCA soluble counts, and plotted as percent of input radiolabeled cycB1-NT. (**c**) Time-course of degradation of full-length cyclin B1 (cyc^{WT}) or single-lysine-containing mutant (cyc^{K64only}), each in complex with CDK1, in control- or UBE2S-depleted mitotic *Xenopus* extract. Recombinant His-UBE2S (10 nM), where indicated, was added to reactions concomitantly with substrate. Cyclin B1 proteolysis was analyzed by SDS-PAGE and immunoblotting. Asterisks represent nonspecific signal. Uncropped images of immunoblots are shown in Supplementary Fig. S9. (**d**) Model of cyclin B1 degradation in *Xenopus* cell-cycle extract. The APC/C and the E2 UBCH10 collaborate to transfer ubiquitin monomers to multiple lysine residues on cyclin B1, with subsequent elaboration of short ubiquitin

chains containing K63, K48 and K11 linkages, with K11 linkages predominating[11]. Upon achievement of a threshold of ubiquitin mass, which appears to be 4–5 ubiquitin monomers, multiply monoubiquitinated substrate can associate with proteasome-associated ubiquitin receptors and be degraded efficiently. However, when the number of lysine residues in cyclin B1 is restricted, ubiquitination catalyzed by UBCH10 is insufficient for rapid proteolysis and the activity of UBE2S in extending K11-linked ubiquitin polymers becomes important for efficient degradation.