CellPress

Molecular Cell
Article

Ubiquitin Chain Elongation Requires E3-Dependent Tracking of the Emerging Conjugate

Aileen Kelly,[2,3] Katherine E. Wickliffe,[2,3,4] Ling Song,[2,3] Indro Fedrigo,[2] and Michael Rape[1,2,*]
[1]Howard Hughes Medical Institute
[2]Department of Molecular and Cell Biology
University of California at Berkeley, Berkeley, CA 94720, USA
[3]Co-first author
[4]Present address: Genentech, South San Francisco, CA 94080, USA
*Correspondence: mrape@berkeley.edu
http://dx.doi.org/10.1016/j.molcel.2014.09.010

SUMMARY

Protein modification with ubiquitin chains is an essential signaling event catalyzed by E3 ubiquitin ligases. Most human E3s contain a signature RING domain that recruits a ubiquitin-charged E2 and a separate domain for substrate recognition. How RING-E3s can build polymeric ubiquitin chains while binding substrates and E2s at defined interfaces remains poorly understood. Here, we show that the RING-E3 APC/C catalyzes chain elongation by strongly increasing the affinity of its E2 for the distal acceptor ubiquitin in a growing conjugate. This function of the APC/C requires its coactivator as well as conserved residues of the E2 and ubiquitin. APC/C's ability to track the tip of an emerging conjugate is required for APC/C-substrate degradation and accurate cell division. Our results suggest that RING-E3s tether the distal ubiquitin of a growing chain in proximity to the active site of their E2s, allowing them to assemble polymeric conjugates without altering their binding to substrate or E2.

INTRODUCTION

The attachment of a ubiquitin chain is an essential signaling event that can profoundly affect the fate of a eukaryotic protein. When connected through Lys11 or Lys48, ubiquitin chains direct substrates for degradation by the 26S proteasome, the major cytoplasmic protease (Chau et al., 1989; Meyer and Rape, 2014). In contrast, when linked through Met1 or Lys63, these conjugates promote the reversible assembly of oligomeric complexes, which is at the heart of NF-κB transcription factor activation, DNA repair, or spliceosome maturation (Komander and Rape, 2012). Elucidating mechanisms of ubiquitin chain formation is therefore critical to our understanding of basic principles of cellular regulation.

Mammalian proteins are decorated with ubiquitin chains by one of ~600 E3 ubiquitin ligases. The majority of these E3s contain a RING domain for catalysis and a separate domain or subunit for the recognition of substrate degron motifs (Deshaies and Joazeiro, 2009). The RING domain recruits an E2 conjugating enzyme charged with a thioester-linked ubiquitin and then promotes the transfer of this ubiquitin to a substrate lysine by aligning the catalytic players for nucleophilic attack (Dou et al., 2012; Plechanovová et al., 2012; Pruneda et al., 2012; Reverter and Lima, 2005; Scott et al., 2014). Whether RING domains play additional roles in transferring ubiquitin molecules onto a growing chain is not known.

As RING-E3s do not form a thioester intermediate, the linkage specificity of ubiquitin chain formation is likely conferred by the E2 (Ye and Rape, 2009), and indeed, the topology of ubiquitin chains assembled by a RING-E3 can change with the nature of its collaborating E2s (Christensen et al., 2007; Meyer and Rape, 2014). Illustrating how E2s determine linkage specificity, the heterodimeric Ube2N/Uev1A engages the acceptor ubiquitin through a noncovalent binding site in Uev1A, which orients Lys63 toward the active site of Ube2N (Eddins et al., 2006). Ube2R1 and Ube2G2 utilize an acidic loop in their UBC domains to position Lys48 of a substrate-bound acceptor ubiquitin toward their active site (Liu et al., 2014; Petroski and Deshaies, 2005). Conversely, the cell cycle E2 Ube2S acts through a mechanism of substrate-assisted catalysis, during which an acidic residue of the acceptor ubiquitin activates the target Lys11 (Wickliffe et al., 2011). Thus, all of these E2s depend on specific interactions with the acceptor ubiquitin to determine linkage specificity. However, they bind the acceptor with such low affinity, often in the millimolar range (Pierce et al., 2009; Wickliffe et al., 2011), that it has remained unclear how these enzymes are able to produce long ubiquitin conjugates.

During chain formation, E3 enzymes also face an intriguing steric problem: as the conjugate becomes longer, the distance between the E3-bound substrate and the distal acceptor lysine increases. In contrast to DNA polymerases, which have solved a similar issue by sliding their active site on the substrate, RING-E3s remain associated with both target protein and E2 at defined surfaces. To overcome this topological constraint, ubiquitin chains could be assembled on the active site of an E2 before being transferred en bloc to the substrate (Li et al., 2007; Ravid and Hochstrasser, 2007). However, kinetic analyses suggested that essential ubiquitylation enzymes, including the E3 anaphase-promoting complex (APC/C) and its E2 Ube2S, synthesize ubiquitin chains directly on their target (Pierce et al.,

2009; Rape et al., 2006; Wickliffe et al., 2011). Because the APC/C and Ube2S also produce ubiquitin chains that are connected through a single linkage (Wickliffe et al., 2011), they must repeatedly modify a specific lysine residue in the distal ubiquitin of a growing chain. It is unclear how the APC/C, or any ubiquitin ligase, is able to recognize the tip of the emerging conjugate, and mechanisms of ubiquitin chain elongation remain poorly understood.

Here, we show that the RING-E3 APC/C promotes ubiquitin chain elongation by strongly increasing the affinity of its E2 Ube2S for the distal acceptor molecule in a growing conjugate. The ability of mitotic APC/C to hold on to an emerging chain requires its coactivator Cdc20, a conserved E2-surface, and specific residues of the acceptor ubiquitin. Interfering with this function of the APC/C disrupts chain formation in cells, stabilizes APC/C substrates, and interferes with mitotic progression. Our work therefore suggests that RING-E3s tether the distal ubiquitin of a growing chain in proximity to the E2, a catalytic feature that allows them to build polymeric conjugates without altering the positions of the substrate or the E2.

RESULTS

The Active Site of Ube2S Is in Proximity to Apc2, Apc11, and Cdc20

As Ube2S only modifies ubiquitin, but not substrate lysine residues (Wickliffe et al., 2011), it can serve as a powerful model to reveal catalytic features that are specific to ubiquitin chain elongation. Ube2S binds to its E3, the APC/C, through a C-terminal peptide (CTP), which is connected to its catalytic UBC domain by a long Gly-rich linker (Meyer and Rape, 2014). Due to the linker's structural flexibility, it has remained unclear where the active site of Ube2S is located in relation to the APC/C. To address this issue, we developed a crosslinking strategy using a variant of Ube2S (Ube2S^{C118A}) that contains a single cysteine at its active site and binds the APC/C with affinity similar to that of the wild-type E2 (Wickliffe et al., 2011). We labeled Cys95 in the active site of Ube2S^{C118A} with 1,4-bismaleimidobutane (BMB), a divalent cysteine-reactive crosslinker, and incubated it with APC/C that was affinity purified from mitotic cells synchronized by three independent methods. These experiments revealed specific crosslinks between the catalytic center of Ube2S and the cullin scaffold Apc2, the RING-domain subunit Apc11, and APC/C's mitotic coactivator Cdc20, all of which were dependent on the presence of BMB and the active site Cys residue in Ube2S (Figure 1A; Figure S1A, available online). We did not detect Ube2S-dependent crosslinks for other APC/C subunits.

Several observations indicate that these experiments reported on a specific environment of the active site of Ube2S on the APC/C. The addition of Emi1 or deletion of Ube2S's C-terminal residues, both procedures that are known to impede binding of Ube2S to the APC/C (Frye et al., 2013; Wang and Kirschner, 2013; Williamson et al., 2009), also obliterated all crosslinks between Ube2S and Cdc20, Apc2, or Apc11 (Figures 1B and 1C). The C terminus of Ube2S is enriched in positive charges, and it is thought that Ube2S engages the APC/C through electrostatic interactions (Wang and Kirschner, 2013). Consistent with this notion, we found that the crosslinks were sensitive to increased salt concentrations in the buffer (Figure S1B). In contrast to these inhibitory treatments, the addition of a ubiquitylation substrate did not impair the crosslinking between Ube2S and APC/C subunits (Figure S1C).

To further evaluate the specificity of our observations, we purified mitotic APC/C from cells that were depleted of Cdc20, Apc2, or Apc11 by verified siRNAs. Importantly, the siRNA treatment removed the unmodified APC/C subunit as well as the Ube2S-dependent crosslinked species (Figures 1D and 1E; Figure S1D). Previous analyses had found that the RING subunit Apc11 is recruited to the APC/C via Apc2 (Thornton et al., 2006), and indeed, depletion of Apc2 also eliminated crosslinks between Apc11 and Ube2S (Figure 1E). By contrast, the loss of Apc2 did not affect crosslinks between Ube2S and Cdc20, and a corresponding reduction in Cdc20 levels had no consequence for the crosslinking efficiency between Ube2S and Apc2 or Apc11 (Figures 1D and 1E; Figure S1D). Together, these experiments therefore show that the CTP-dependent interaction between Ube2S and the APC/C places the active site of Ube2S in proximity to the APC/C subunits Apc2, Apc11, and Cdc20 (Figure 1F).

Cdc20 Stabilizes Ube2S Binding to the APC/C

Cdc20 and its homolog Cdh1 are mostly known for their ability to recruit substrates to the APC/C (Peters, 2006). However, they were initially described as coactivators that might regulate the catalytic activity of the APC/C (Schwab et al., 1997; Visintin et al., 1997), a hypothesis that is consistent with our crosslinking data: the proximity between Cdc20 and the active site of Ube2S implied that coactivators could impact ubiquitin chain elongation by the APC/C.

We therefore decided to investigate the interplay between Ube2S and Cdc20 in more detail and tested whether both proteins could directly bind to each other. Indicative of such an association, Ube2S effectively retained purified Cdc20 in pull-down experiments (Figure 2A). In cells, an interaction between Cdc20 and Ube2S could be bridged by the APC/C (Meyer and Rape, 2014). Thus, to determine whether Ube2S and Cdc20 engage each other in vivo, we screened for Cdc20 variants that were defective in APC/C binding and asked whether they retained their capacity to interact with Ube2S (Table S1). We found that mutation of Asp464, a residue that is in proximity to the D-box binding site (Figure S2A), strongly reduced the association of Cdc20 to the APC/C, as seen in CompPASS mass spectrometry or western blot analysis of affinity-purified Cdc20 (Figures 2B and 2C), or by testing the stability of Cdc20 toward APC/C-dependent degradation (Figures S2B and S2C). Despite its failure to bind mitotic APC/C, Cdc20^{D464R} associated with Ube2S with the same efficiency as wild-type Cdc20 in vitro (Figure 2A), and in cells, Cdc20^{D464R} interacted with Ube2S even more strongly than WT-Cdc20 (Figure 2C). Similar results were obtained for the isolated WD40 domain of Cdc20 that was unable to bind the APC/C yet efficiently associated with Ube2S in cells (Figure 2C). These findings indicate that Cdc20 and Ube2S directly bind to each other. Moreover, our results suggest that the APC/C turns over the interaction between Cdc20 and Ube2S, consistent with a role of Cdc20 in delivering Ube2S to a binding site on the APC/C.

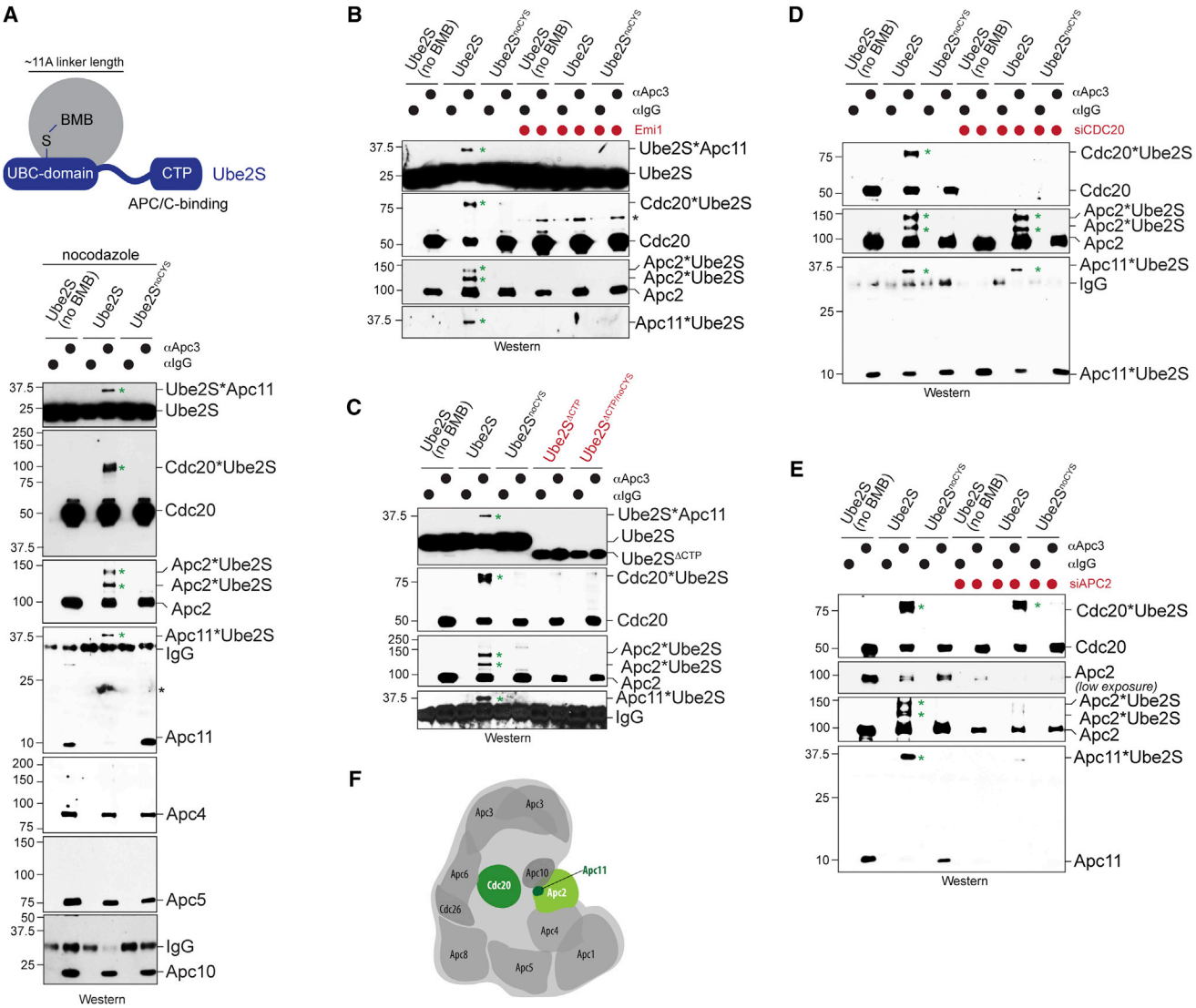

Figure 1. The Active Site of Ube2S Is in Proximity to Apc2, Apc11, and Cdc20

(A) Ube2S forms 1,4-bismaleimidobutane (BMB)-dependent crosslinks with Cdc20, Apc2, and Apc11, as detected by western. Green asterisks indicate Ube2S-specific crosslinked species; the black asterisk denotes a gel impurity in the Apc11 western.

(B) Emi1 blocks Ube2S-specific crosslinks with mitotic APC/C, as determined by western. Green asterisks indicate Ube2S-specific crosslinks; the black asterisk shows a crosslink between Emi1$^{299-447}$ and Cdc20.

(C) The C terminus of Ube2S is required for its crosslinks with APC/C subunits. Ube2S or Ube2S$^{\Delta CTP}$ was used for crosslinking experiments; reactions were analyzed as described.

(D) Depletion of Cdc20 from prometaphase HeLa cells prior to APC/C purification removes Cdc20-specific crosslinks of Ube2S but has no consequences on crosslinks with Apc2 or Apc11. Green asterisks indicate Ube2S-specific crosslinks.

(E) Apc2 is required for Ube2S-specific crosslinks with Apc2 and Apc11, but not for Ube2S-Cdc20 crosslinks. Apc2 was depleted from HeLa cells by siRNA prior to APC/C purification. Green asterisks indicate Ube2S-specific crosslinks.

(F) Schematic overview of the APC/C, depicting in green the APC/C subunits that engaged in BMB-dependent crosslinks with Ube2S. See also Figure S1.

Based on these observations, we asked whether Cdc20 could target endogenous Ube2S to the APC/C. Similar to the depletion of the known Ube2S interactor Apc2 (Wang and Kirschner, 2013), a reduction in Cdc20 levels strongly diminished the steady-state association of Ube2S with the APC/C without impacting the composition of core APC/C (Figure 2D). This was a specific consequence of Cdc20 depletion, as the interaction between Ube2S and the APC/C was rescued by lentiviral expression of siRNA-resistant wild-type Cdc20, but not mutant Cdc20^{D464R} (Figure 2E). To support these observations, we immunodepleted ~60% of Cdc20, which did not affect the concentration, composition, or cell-cycle stage of mitotic APC/C, yet also resulted in Ube2S dissociation from the APC/C (Figure 2F). Finally, we built on the observation that Ube2S does not efficiently interact with

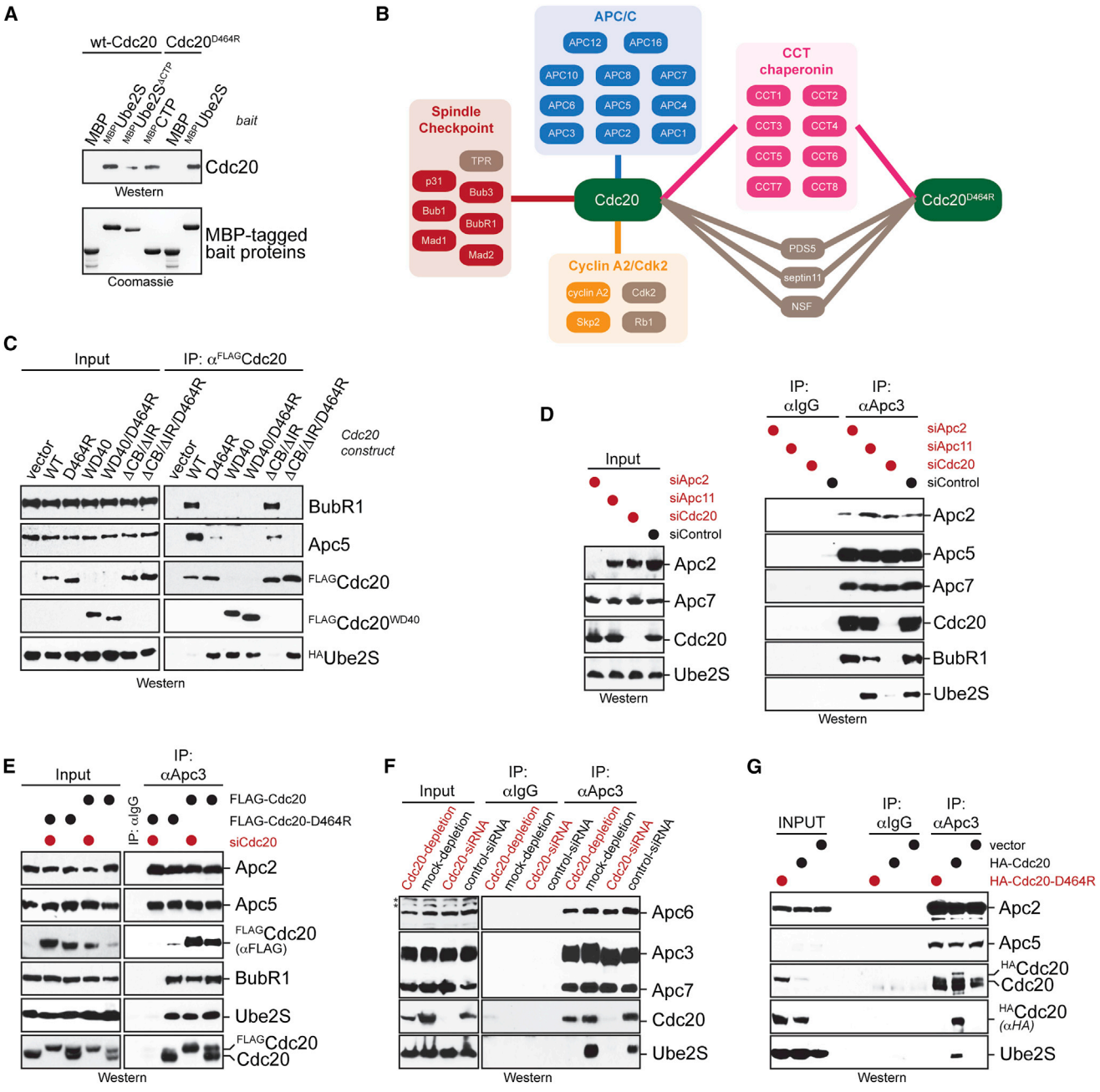

Figure 2. Cdc20 Stabilizes Ube2S Binding to the APC/C

(A) ^{Flag}Cdc20 bound to immobilized ^{MBP}Ube2S in vitro, as detected by western.

(B) Interaction networks of ^{Flag}Cdc20 or ^{Flag}Cdc20^{D464R}, as determined by CompPASS mass spectrometry.

(C) Cdc20 directly binds Ube2S in cells. ^{FLAG}Cdc20 mutants were purified from prometaphase HeLa cells, and interactors were detected by western. ΔCB: deletion of C-box; ΔIR: loss of C-terminal IR residues of Cdc20.

(D) Cdc20 and Apc2 are required for the association of Ube2S with prometaphase APC/C, as detected by immunoprecipitation and western.

(E) APC/C was purified from prometaphase HeLa cells stably expressing siRNA-resistant ^{Flag}Cdc20 or ^{Flag}Cdc20^{D464R} and depleted of endogenous Cdc20, and bound proteins were detected by western.

(F) Cdc20 was immunodepleted from prometaphase HeLa extracts or siRNA-depleted from prometaphase HeLa cells. APC/C was immunopurified, and bound proteins were detected by western.

(G) Cdc20 targets Ube2S to the APC/C. APC/C was purified from 293T cells expressing ^{HA}Cdc20 or ^{HA}Cdc20^{D464R}, and bound proteins were determined by western. See also Figure S2 and Table S1.

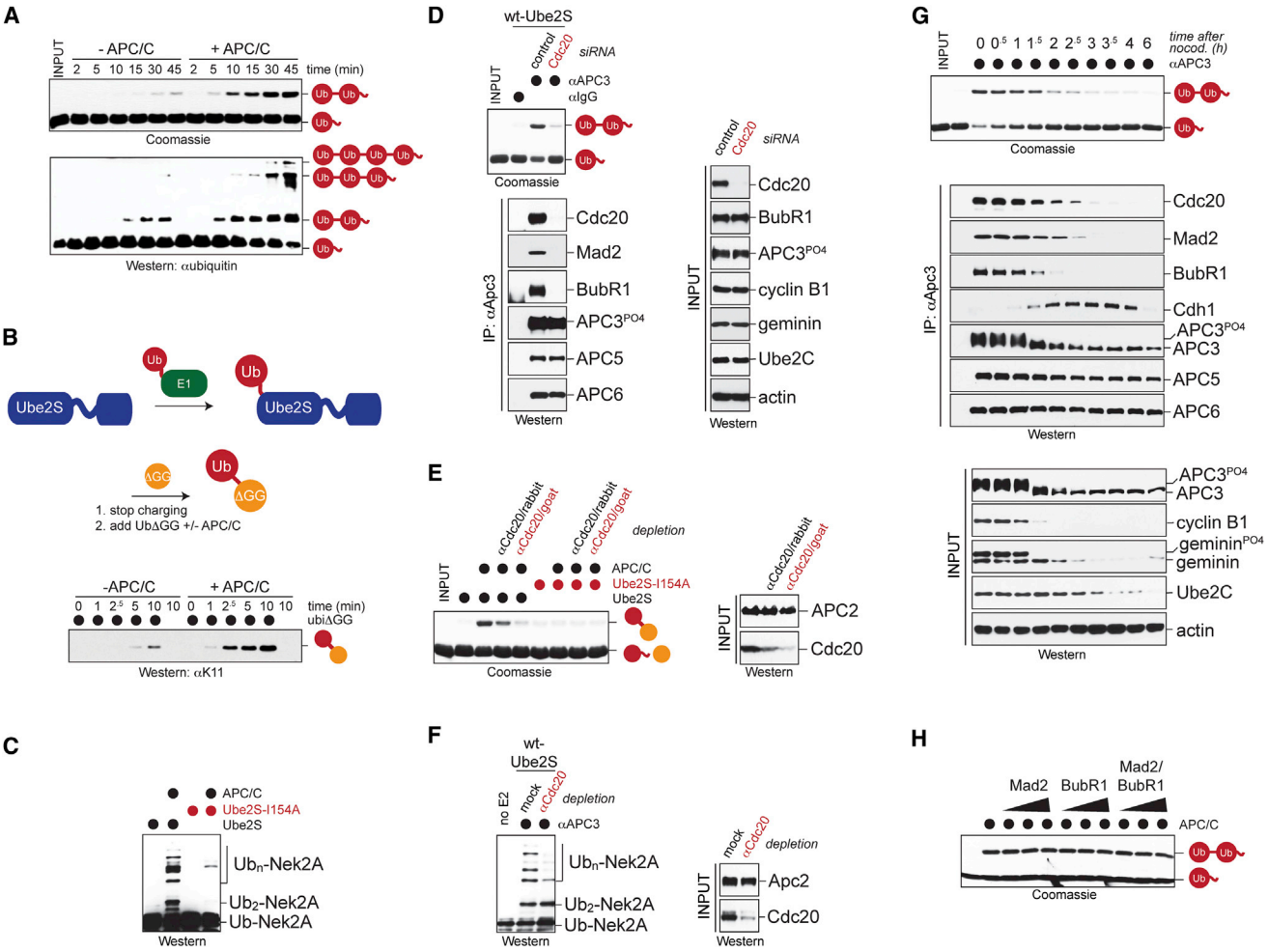

Figure 3. Cdc20 Is Required for the Activation of Ube2S by the APC/C

(A) Diubiquitin formation by Ube2S is increased by mitotic APC/C. Reaction products were visualized by Coomassie or western.

(B) APC/C promotes discharge of ubiquitin from Ube2S, as seen in single turnover assays. Discharge of ubi^{K11R} (red) from Ube2S was initiated with ubi$^{\Delta GG}$ (orange) in the presence or absence of APC/C. K11-linked diubiquitin was visualized by western.

(C) Ub-Nek2A was incubated with Ube2S and prometaphase APC/C, and substrate ubiquitylation was visualized by αNek2 western.

(D) APC/C was purified from prometaphase HeLa cells depleted of Cdc20, and diubiquitin formation was visualized by Coomassie.

(E) APC/C was purified from mitotic HeLa S3 extract immunodepleted of Cdc20, and diubiquitin formation was visualized by Coomassie.

(F) Ubiquitylation of Ub-Nek2A was performed using APC/C purified from control or Cdc20-depleted mitotic extracts.

(G) APC/C was purified from HeLa cells at the indicated times after release from a prometaphase arrest, and its effects on diubiquitin formation by Ube2S were monitored by Coomassie.

(H) Mitotic APC/C activates Ube2S in the presence of the mitotic checkpoint complex. Ube2S was incubated with APC/C and increasing concentrations of Mad2, BubR1, or Mad2/BubR1 complexes. Ubiquitin dimers were visualized by Coomassie. See also Figure S3.

APC/C in asynchronous cell populations (Williamson et al., 2009) and tested whether an increase in Cdc20 levels could overcome this limitation. Indeed, expression of Cdc20, but not mutant Cdc20^{D464R}, was sufficient to induce the association of endogenous Ube2S with the APC/C in asynchronous cells (Figure 2G). Thus, Cdc20 is close to the active site of Ube2S, binds directly to the E2, and stabilizes its association with the mitotic APC/C.

Cdc20 Is Required for Activation of Ube2S by the APC/C

As these observations suggested that Cdc20 could affect ubiquitin chain elongation by Ube2S, we monitored the formation of K11-linked diubiquitin in either the presence or absence of the APC/C. We found that Cdc20-bound APC/C strongly improved the ability of Ube2S to produce diubiquitin conjugates as well as short ubiquitin chains (Figure 3A). A similar APC/C^{Cdc20}-dependent activation was observed in single turnover assays in which we charged Ube2S with ubiquitin-K11R (ubi^{K11R}), stopped charging by addition of EDTA and apyrase, and then added the acceptor ubiquitin-ΔGG75/76 (ubi$^{\Delta GG}$) (Figure 3B). Attesting to the specificity of these reactions, the APC/C-dependent activation of Ube2S was abolished upon deletion of the CTP of Ube2S or addition of Emi1 (Figures S3A–S3C). As an

independent approach, we tested whether the APC/C improved the modification of the model substrate Ub-Nek2A, which requires Cdc20 for catalysis but not for APC/C binding (Hayes et al., 2006). Consistent with our diubiquitin assays, APC/C^{Cdc20} strongly enhanced the synthesis of ubiquitin chains on Ub-Nek2A by Ube2S (Figure 3C).

Notably, if APC/C was purified from mitotic cells that were depleted of Cdc20, it was unable to increase the efficiency of diubiquitin formation by Ube2S (Figure 3D). Similar observations were made after immunodepletion of Cdc20 from extracts of mitotic cells: a reduction in Cdc20 levels quantitatively correlated with a strongly diminished ability of the APC/C to promote Ube2S-dependent ubiquitin dimer formation or ubiquitin chain formation on Ub-Nek2A (Figures 3E and 3F).

To further evaluate the importance of Cdc20 for Ube2S activation, we analyzed the cell-cycle regulation of diubiquitin formation. We synchronized HeLa cells in prometaphase, released them into a new cell cycle, and purified the APC/C at short intervals until cells had reached late G1. Strikingly, Ube2S was most potently activated by prometaphase APC/C, i.e., the form of the APC/C that was most stably charged with Cdc20 (Figure 3G). Degradation of Cdc20 at later stages of mitosis correlated with a reduced propensity of the APC/C to promote diubiquitin formation by Ube2S. The ability of prometaphase APC/C to activate Ube2S was not affected by effector proteins of the spindle checkpoint, as even a large excess of active Mad2 and BubR1 did not inhibit the APC/C^{Cdc20}-dependent increase in diubiquitin formation by Ube2S (Figure 3H; Figure S3D). Thus, our depletion and cell-cycle studies suggest that Cdc20 is required for the APC/C-dependent activation of diubiquitin formation by Ube2S.

APC/C^{Cdc20} Promotes Acceptor Ubiquitin Recognition by Ube2S

As a step toward understanding the molecular basis for Ube2S activation, we measured the kinetics of diubiquitin formation in the presence or absence of APC/C^{Cdc20}. For each concentration of acceptor ubiquitin (ubi$^{\Delta GG}$), we monitored the initial rate of diubiquitin formation with a constant concentration of donor ubiquitin (ubi^{K11R}). Our results showed that mitotic APC/C strongly improved the affinity of Ube2S to the acceptor ubiquitin, as indicated by a 60- to 125-fold decrease in the apparent K_M (Figure 4A). Consistent with substrate-assisted catalysis, the increased affinity for its substrate coincided with a higher rate of diubiquitin formation by Ube2S, yet effects on v_{max} were less pronounced than those on K_M. Together, the mitotic APC/C improved the catalytic efficiency of Ube2S, as expressed in the ratio of v_{max}/K_M, by up to 360-fold. It should be noted that in the absence of the APC/C, the low activity of Ube2S toward ubi$^{\Delta GG}$ complicated the determination of an apparent K_M, and the kinetic analysis likely underestimated the extent of APC/C-dependent Ube2S activation. When these experiments were performed with mitotic extracts that were depleted of ~50% of Cdc20, the ability of the APC/C to improve acceptor recognition and catalysis was strongly reduced (Figure 4A). In a similar manner, degradation of Cdc20 during late mitosis reduced the efficiency of the APC/C to promote acceptor ubiquitin recognition by Ube2S, with Cdc20 depletion and degradation having almost identical effects (Figure 4B). These findings underscore the notion that Cdc20 is required for the APC/C-dependent activation of Ube2S. They further suggest that APC/C^{Cdc20} promotes chain elongation by increasing the affinity of its E2 Ube2S for an acceptor ubiquitin.

To ascertain APC/C's role in promoting ubiquitin recognition by an E2, a novel function for a RING-E3, we searched for residues of the acceptor ubiquitin that might be specifically required for E3-dependent chain elongation (Figure 4C). We mutated ~80% of all surface-exposed residues of ubiquitin (Table S1) and subjected the recombinant proteins to an assay that monitored the Ube2S-dependent formation of diubiquitin in the absence of the APC/C. Ubiquitin variants that allowed diubiquitin formation, and hence did not impair the core catalytic function of Ube2S, were then tested for their ability to support ubiquitin chain elongation in the presence of the APC/C. Mutants that were inactive in the second assay should be specifically impaired in APC/C-dependent chain elongation.

This biochemical screen revealed that mutation of Ala46 of ubiquitin to Glu, Lys, or Ile (ubi^{A46E}; ubi^{A46K}; ubi^{A46I}) allowed diubiquitin formation by Ube2S but interfered with the APC/C-dependent production of ubiquitin chains (Figures 4D and 4E; Figures S4A and S4B). In agreement with these results, ubi^{A46E} did not show any increase in Ube2S-dependent diubiquitin formation that was induced by the APC/C (Figure 4F), an observation that was also made in single-discharge experiments (Figure 4G). In cells, mutation of Ala46 strongly interfered with APC/C-substrate modification during mitosis (Figure 4H), demonstrating that this residue is important in physiological settings. By contrast, Ala46 was not required for Ube2S charging (Figure S4C), nor did it play a crucial role in the formation of ubiquitin chains by the initiating E2, Ube2C (Figure S4D).

As seen in diubiquitin assays containing the APC/C, Ala46 was only required on the acceptor, but not the donor ubiquitin (Figure 4I; Figure S4E). In agreement with these observations, mutation of Ala46 disrupted the stimulatory effects of APC/C on acceptor ubiquitin recognition in kinetic analyses that monitored the rate of diubiquitin formation as a function of increasing acceptor concentrations (Figure S4F). By introducing mutations into defined acceptor ubiquitin molecules, we found that Ile44, Lys48, and Arg54, all of which are utilized for donor ubiquitin recognition, were also required on the acceptor ubiquitin for the APC/C-dependent stimulation of Ube2S activity (Figure S4G). Our kinetic and mutational analyses thus indicate that Cdc20-bound APC/C promotes chain elongation by increasing the affinity of its E2 Ube2S for a specific surface on the acceptor ubiquitin.

APC/C^{Cdc20} Tracks the Distal Ubiquitin of a Growing Chain

Is the APC/C^{Cdc20}-dependent increase in acceptor ubiquitin recognition important if a substrate is already bound through its degron motifs? To address this issue, we introduced the A46E mutation into the ubiquitin moiety of a covalent fusion between ubiquitin and cyclin A (Ub^{A46E}-cyclin A), a protein that stably associates with the APC/C through a D-box as well as an accessory factor, Cks1. We incubated Ub-cyclin A or Ub^{A46E}-cyclin A with APC/C, Ube2S, and the donor ubi^{K11R}, which supports transfer of a single ubiquitin and allowed us to specifically

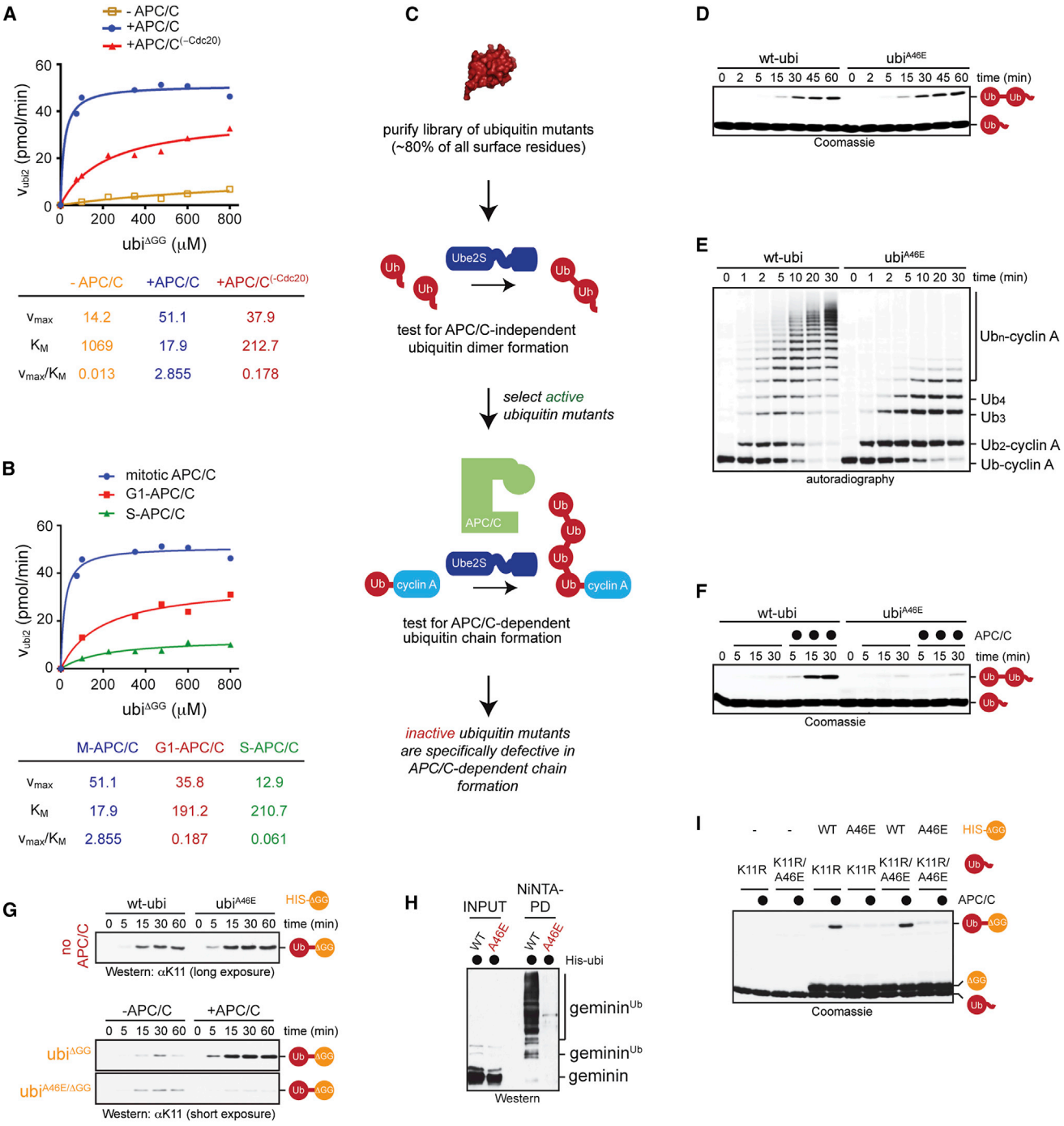

Figure 4. Cdc20-Bound APC/C Promotes Acceptor Ubiquitin Recognition by Ube2S

(A) Rates of diubiquitin formation by Ube2S at different concentrations of ubi$^{\Delta GG}$ were determined in the presence or absence of prometaphase APC/C; where indicated, Cdc20 had been immunodepleted prior to APC/C purification. Apparent kinetic constants were obtained by fitting initial rate constants to a Michaelis-Menten equation.

(B) Diubiquitin formation at different concentrations of ubi$^{\Delta GG}$ was monitored with APC/C purified from extracts of HeLa S3 at different cell-cycle stages (prometaphase, late mitosis/early G1, and S).

(C) Outline of a biochemical screen to identify ubiquitin residues specifically required for APC/C-dependent chain elongation by Ube2S.

(D) Ala46 of ubiquitin is not required for diubiquitin formation by Ube2S in the absence of the APC/C. Reaction products were visualized with Coomassie.

(E) Ala46 of ubiquitin is required for ubiquitin chain elongation on ^{35}S-labeled Ub-cyclin A by Ube2S and the APC/C, as detected by autoradiography.

(F) Ala46 of ubiquitin is required for increased diubiquitin formation by Ube2S in the presence of the APC/C, as visualized by Coomassie.

(legend continued on next page)

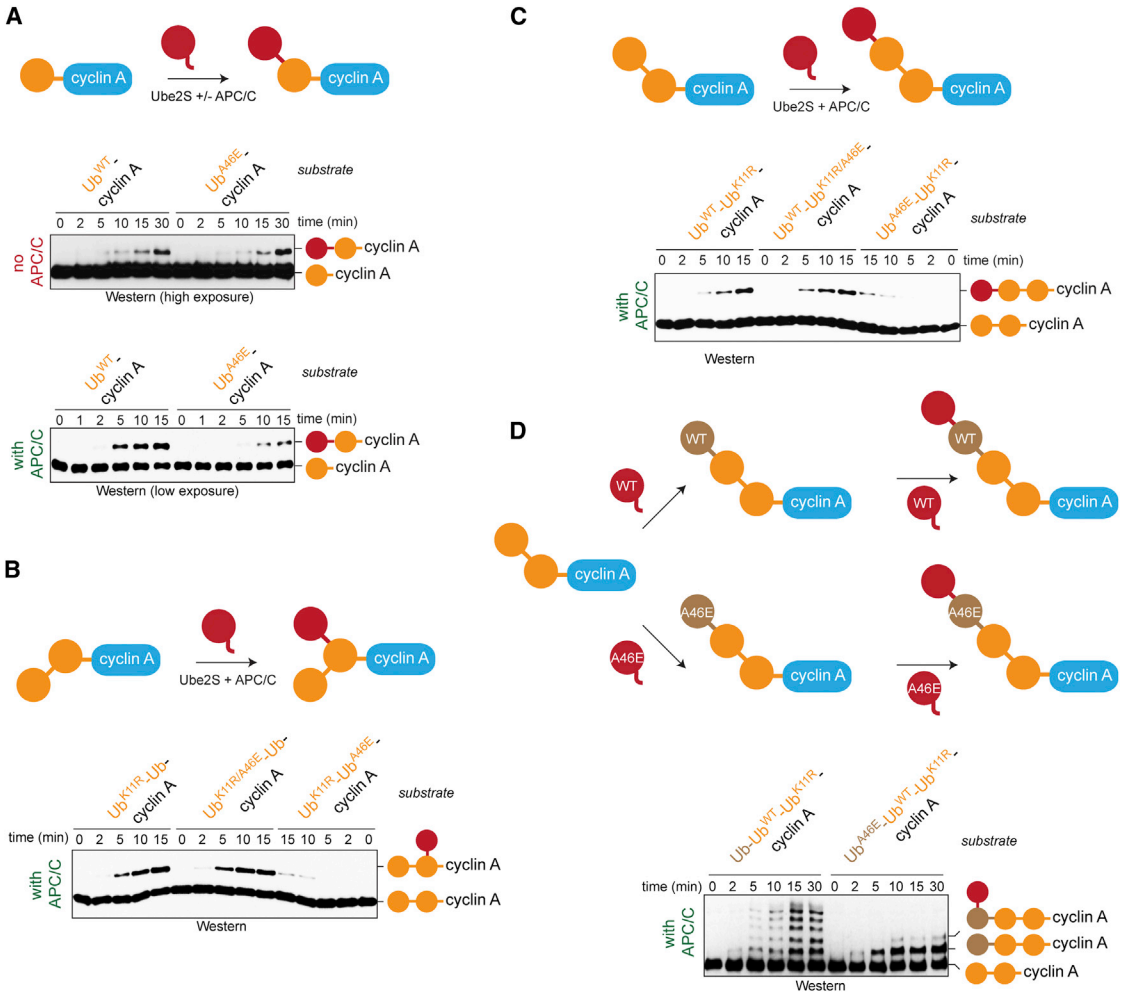

Figure 5. APC/C^{Cdc20} Tracks the Distal Ubiquitin of a Growing Chain

(A) Modification of Ub-cyclin A (WT-ubiquitin or ubi^{A46E} fused to cyclin A^{HA-His}) with ubi^{K11R} by Ube2S and APC/C was detected by western.

(B) Modification of Ub^{K11R}-Ub-cyclin A^{HA} with ubi^{K11R} by Ube2S and APC/C was detected by western.

(C) Ub-Ub^{K11R}-cyclin A^{HA} was incubated with Ube2S, E1, ubiquitin^{K11R}, and ATP in the presence of APC/C. Reaction products were analyzed by western.

(D) Ub-Ub^{K11R}-cyclin A^{HA} was incubated with APC/C, Ube2S, E1, ATP, and either WT-ubiquitin or ubi^{A46E} to first generate Ub-Ub-Ub^{K11R}-cyclin A or Ub^{A46E}-Ub-Ub^{K11R}-cyclin A. Mutation of Ala46 strongly impedes the next step, i.e., formation of the fourth linkage (or any linkage beyond). Reaction products were detected by western. See also Figure S5.

monitor the formation of the second linkage in a growing chain. Notably, whereas Ub-cyclin A was efficiently modified by Ube2S and the APC/C, addition of another ubiquitin molecule to Ub^{A46E}-cyclin A was strongly impaired (Figure 5A, lower panel). Similar observations were made if the formation of a second linkage was detected in the context of a branched chain (Figure 5B). As expected from our earlier observations, the effects of mutating Ala46 were specific for the APC/C-dependent recognition of an acceptor ubiquitin: without the APC/C, Ube2S modified Ub-cyclin A and Ub^{A46E}-cyclin A with equal efficiency (Figure 5A; upper panel), and mutating Ala46 in the donor ubiquitin did not impair APC/C-substrate modification (Figure S5).

As more ubiquitin molecules are added to a growing conjugate, each chain entity could provide a surface for ubiquitin recognition by the APC/C. To determine whether Ala46 was required on a particular molecule, we introduced the A46E mutation into each of the ubiquitin entities of Ub-Ub^{K11R}-cyclin A, a substrate that allowed us to monitor formation of the third linkage

(G) Top: APC/C-independent formation of K11-linked diubiquitin by Ube2S in a single-discharge experiment. Bottom: formation of diubiquitin by the APC/C and Ube2S in a single-discharge experiment, as detected by western using a K11-specific antibody.

(H) Ala46 of ubiquitin is required for APC/C-substrate ubiquitylation in mitotic cells, as seen by denaturing purification of ubiquitin conjugates from prometaphase HeLa cells. The loss of monoubiquitylated geminin in cells expressing ubi^{A46E} is due to high activity of deubiquitylases (Meyer and Rape, 2014).

(I) Ala46 is required on acceptor (ubi$^{\Delta GG}$, orange), but not donor ubiquitin (ubi^{K11R}, red), as visualized by Coomassie. See also Figure S4 and Table S1.

in a growing conjugate. We found that Ala46 was required on the distal, but not the proximal, ubiquitin molecule (Figure 5C). We made similar observations when we analyzed the requirements for longer ubiquitin chains: in these experiments, we utilized Ub-Ub^{K11R}-cyclin A and incubated it with the APC/C, Ube2S, and WT-ubiquitin or ubi^{A46E} to generate either Ub-Ub-Ub^{K11R}-cyclin A or Ub^{A46E}-Ub-Ub^{K11R}-cyclin A. At the next step, i.e., the attachment of the fourth ubiquitin, Ube2S will be presented with a substrate that has either WT-ubiquitin or ubi^{A46E} as its distal chain entity. Also in this case, mutation of Ala46 in the distal acceptor molecule strongly impaired further chain elongation, even though Ala46 was available in the two proximal ubiquitin molecules of Ub^{A46E}-Ub-Ub^{K11R}-cyclin A (Figure 5D). Thus, as a chain is being assembled, Ala46 is recognized on the distal ubiquitin of the emerging conjugate, and residues on a neighboring ubiquitin are unable to compensate for the loss of Ala46 on the actual acceptor molecule. Together with our kinetic studies, these results suggest that the APC/C tethers the distal molecule of an emerging conjugate close to the active site of Ube2S.

Helix D of Ube2S Is Required for Activation by APC/C^{Cdc20}

We next asked whether the APC/C promotes acceptor ubiquitin recognition in cells. Although depletion of Cdc20 would interfere with this reaction, it would also impair substrate delivery to the APC/C, and we were concerned that mutations could disrupt the folding of Cdc20's WD40 domain. In a similar manner, expression of mutant ubiquitin would affect numerous processes, complicating the interpretation of experiments based on Ala46 mutants. To provide an alternative strategy, we hypothesized that APC/C^{Cdc20} might require specific residues in Ube2S to promote chain elongation, and mutation of such E2 residues could block APC/C-dependent chain formation without altering ubiquitin, impairing Cdc20 folding, affecting APC/C composition, or interfering with the intrinsic catalytic activity of Ube2S.

We therefore adopted our ubiquitin mutant screen to Ube2S and interrogated a library of recombinant Ube2S variants for E2s that catalyzed ubiquitin dimer formation in the absence of the APC/C but were unable to produce long chains in its presence (Figure 6A; Table S1). These experiments revealed a cluster of residues close to the C terminus of the catalytic domain of Ube2S that were specifically required for APC/C-dependent chain formation (Figure 6B; Figure S6A). Among these residues, mutation of Ile154 in helix D of the UBC domain of Ube2S had a particularly strong effect on APC/C-dependent chain formation, comparable to characterized mutants that block the core catalytic function of Ube2S (Figure 6C). As expected from these endpoint assays, mutation of Ile154 did not affect the time course of diubiquitin formation by Ube2S alone but dramatically delayed the APC/C^{Cdc20}-dependent formation of diubiquitin or substrate-attached ubiquitin chains (Figures 6D and 6E). A similar lack of APC/C activation was observed in single-turnover assays that compared the activity of WT-Ube2S and Ube2S^{I154A} (Figure 6F). Ile154 of Ube2S was also important in the context of longer substrates (Figure S6B) and for a complete ubiquitylation reaction that was carried out in the presence of the initiating E2 Ube2C (Figure S6C). By contrast, mutation of Ile154 or other residues of helix D had no effects on charging of Ube2S (Figure S6D) or on the CTP-dependent interaction of Ube2S with the APC/C in vitro, as seen in pull-down or crosslink experiments (Figures S6E and S6F).

Further observations supported the notion that mutation of Ile154 disrupted the effects of the APC/C^{Cdc20} on Ube2S activation. Similar to depleting Apc2 or Cdc20, mutation of Ile154 reduced the steady-state association of Ube2S with APC/C in vivo, as seen in prometaphase cells that expressed mutant Ube2S^{I154A} as the sole source of this E2 (Figure 6G). Accordingly, kinetic analyses revealed that mutating Ile154 of Ube2S abrogated the APC/C-dependent improvement of acceptor ubiquitin recognition by Ube2S (Figure 6H), an effect that was highly similar to the absence of the APC/C, depletion of Cdc20, or mutation of Ala46 in ubiquitin. Moreover, we found that Ube2S^{I154A} was unable to promote ubiquitin chain elongation on Ub-Nek2A, a substrate that selectively requires the coactivator, but not the substrate recruitment, function of Cdc20 (Figure 3D). Together, these findings indicate that APC/C^{Cdc20} promotes ubiquitin chain elongation via Ile154 of helix D of Ube2S. Consistent with this important function, Ile154 is conserved among all species known to contain Ube2S (Figure S6G).

Improved Acceptor Ubiquitin Recognition Is a Critical Function of the APC/C in Cells

The discovery of Ube2S^{I154A} allowed us to interfere with APC/C-dependent chain elongation without alterations to Cdc20, the core APC/C, or ubiquitin. Following an established approach (Wickliffe et al., 2011), we depleted endogenous Ube2S using siRNAs against the 3′ UTR of its mRNA and stably expressed siRNA-resistant WT-Ube2S or Ube2S^{I154A}. This strategy resulted in cells that selectively expressed either WT-Ube2S or mutant Ube2S^{I154A}. We synchronized these cell lines in prometaphase and then released them into a new cell cycle, a procedure that leads to full activation of the APC/C and a concomitant massive increase in the formation of K11-linked chains (Matsumoto et al., 2010; Wickliffe et al., 2011). The depletion of Ube2S abrogated the production of K11-linked chains upon APC/C activation, and this phenotype was rescued upon expression of siRNA-resistant WT-Ube2S (Figure 7A). By contrast, the expression of Ube2S^{I154A} did not support K11-linked chain formation after endogenous Ube2S had been depleted, showing that the APC/C-dependent increase in acceptor ubiquitin recognition is essential for substrate modification in vivo.

To determine whether APC/C's effects on chain elongation are required for substrate degradation, we analyzed the stability of Nek2A during prometaphase, the time when Cdc20 binds to the APC/C most stably and Ube2S is activated most efficiently. Previous work had shown that the degradation of Nek2A requires the activator function of Cdc20 (Kimata et al., 2008) as well as branched ubiquitin chains assembled by Ube2S (Meyer and Rape, 2014). Moreover, as depletion of Ube2S affects neither mRNA levels of Nek2A nor synchronization in prometaphase (Meyer and Rape, 2014), a potential stabilization of Nek2A would not be caused by differences in transcription or cell-cycle stage. As seen in cycloheximide chase experiments, we found that Ube2S^{I154A} was strongly impaired in driving the degradation of Nek2A (Figure 7B).

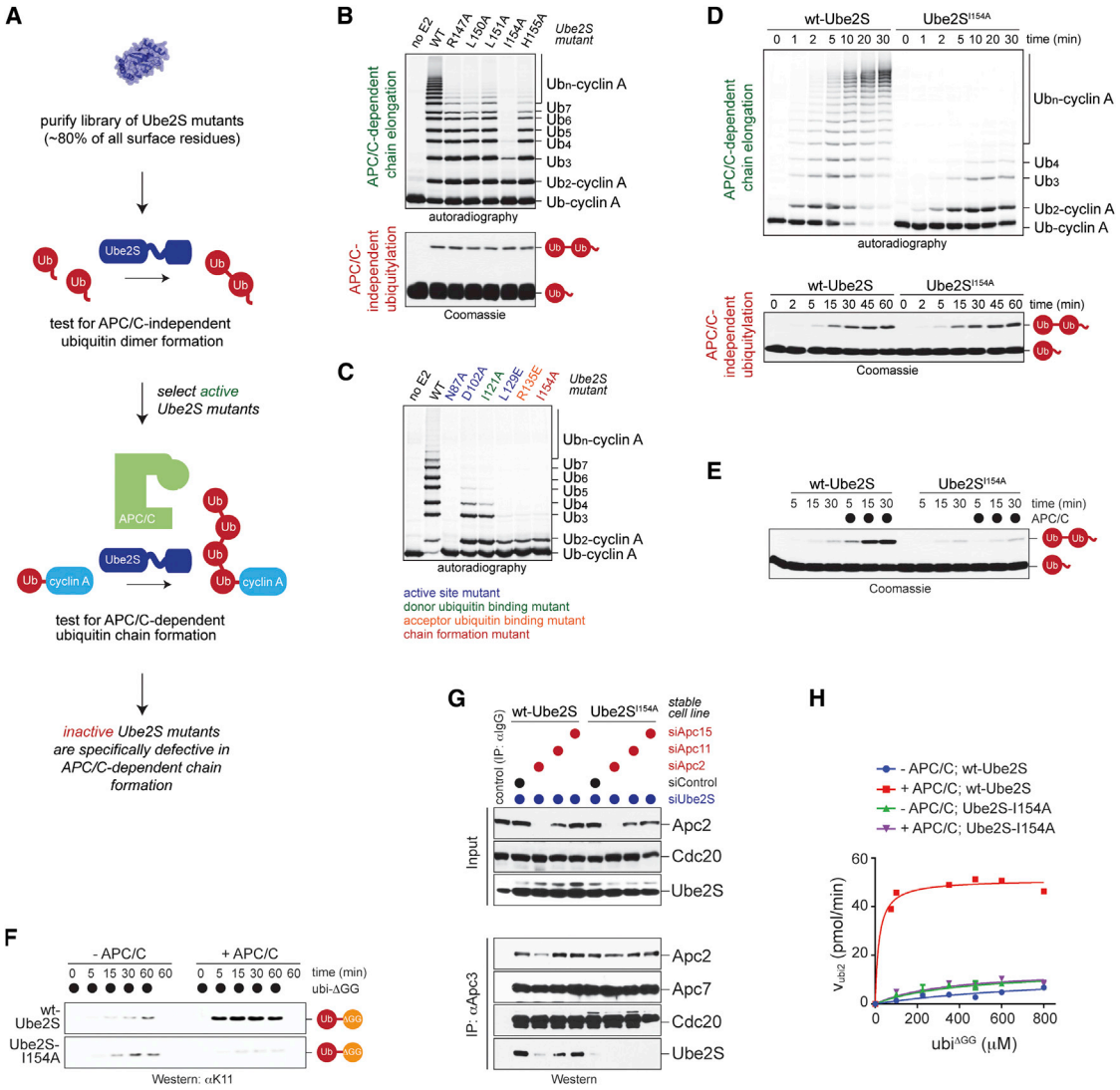

Figure 6. Helix D of Ube2S Is Required for Activation by Cdc20-Bound APC/C

(A) Outline of a biochemical screen to identify Ube2S residues required for APC/C-dependent chain elongation, but not for the core catalytic activity of Ube2S.

(B) Top: ^{35}S-labeled Ub-cyclin A was incubated with Ube2S mutants, ubiquitin, E1, and APC/C. Reaction products were analyzed by autoradiography. Bottom: the same Ube2S mutants were tested for APC/C-independent formation of K11-linked diubiquitin, as detected by Coomassie.

(C) ^{35}S-Ub-cyclin A was incubated with Ube2S mutants, ubiquitin, E1, and the APC/C. Reaction products were analyzed by autoradiography.

(D) Top: ^{35}S-labeled Ub-cyclin A was incubated with Ube2S (WT or I154A), ubiquitin, E1, and the APC/C. Reactions were analyzed by autoradiography. Bottom: Ube2S was incubated with ubiquitin, E1, and ATP. Diubiquitin formation was analyzed by Coomassie.

(E) Diubiquitin formation by Ube2S (WT or I154A) was monitored in the presence or absence of the APC/C by Coomassie.

(F) Helix D of Ube2S is required for APC/C-dependent activation, as seen in single-discharge experiments. Formation of ubiquitin dimers was monitored by western using a K11-linkage-specific antibody.

(G) HeLa cells stably expressing either WT-Ube2S or Ube2S^{I154A} were transfected with siRNAs against endogenous Ube2S, as well as the indicated siRNAs against APC/C subunits. Prometaphase APC/C was immunoprecipitated, and copurifying proteins were detected by western.

(H) Initial rates of ubiquitin dimer formation at different concentrations of acceptor ubi$^{\Delta GG}$ were determined for WT-Ube2S or Ube2S^{I154A} with or without mitotic APC/C and fitted to a Michaelis-Menten equation. See also Figure S6 and Table S1.

To test whether APC/C's role in improving acceptor ubiquitin recognition is important for cell division, we asked whether Ube2S^{I154A} could support spindle checkpoint disassembly, an APC/C-dependent reaction that contributes to the proper onset of sister chromatid separation. Cells expressing only Ube2S^{I154A} were strongly impaired in their capacity to induce the dissociation of spindle checkpoint effectors from the APC/C, as documented by the increased binding of BubR1 to the APC/C during synchronization and the persistent interaction of Mad2 with the APC/C in cells released from the checkpoint arrest (Figure 7C). As a consequence, cells expressing Ube2S^{I154A} were delayed in anaphase initiation following a spindle checkpoint-dependent

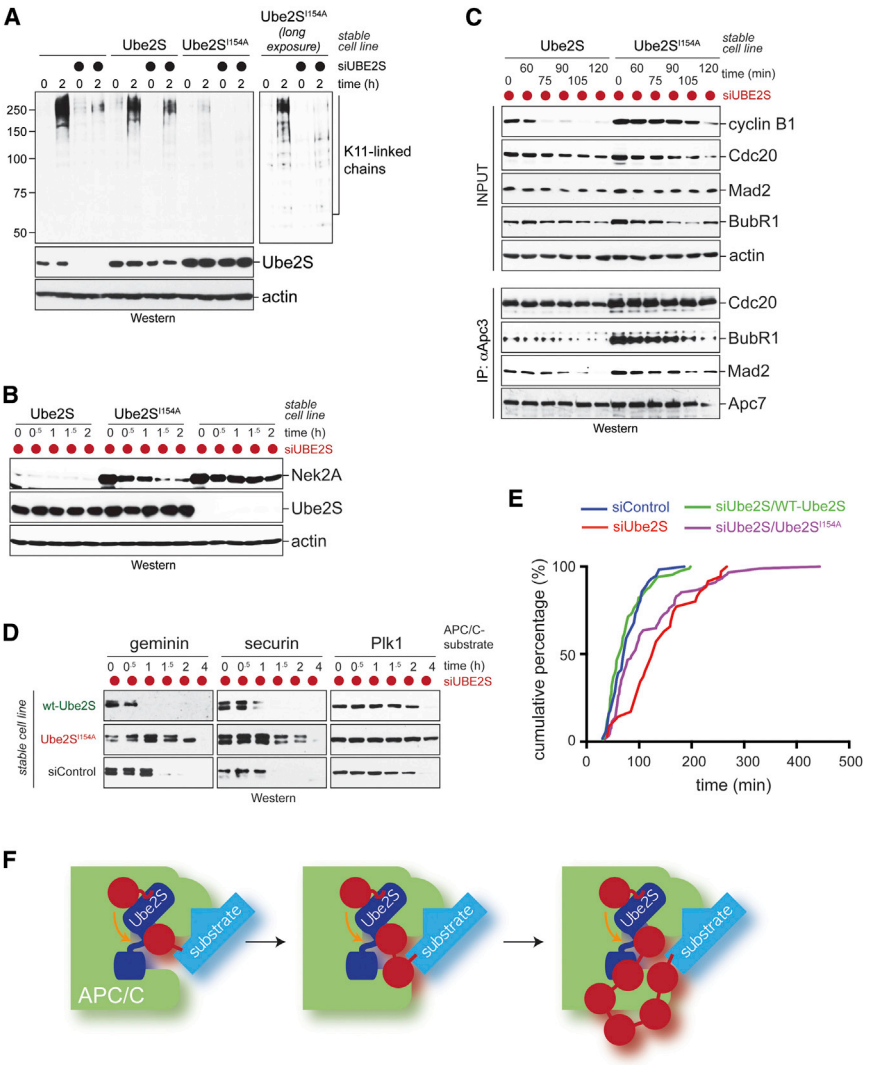

Figure 7. Improved Acceptor Ubiquitin Recognition Is a Critical Function of the APC/C

(A) HeLa cell lines stably expressing either WT-Ube2S or Ube2S^{I154A} were treated with control siRNA or siRNA targeting the 3′ UTR of Ube2S. Cells were released from prometaphase arrest, and formation of K11-linked ubiquitin chains was detected by western.

(B) Parental HeLa cells or stable cell lines expressing WT-Ube2S or Ube2S^{I154A} were transfected with Ube2S siRNA, synchronized in prometaphase, and treated with cycloheximide. Protein abundance was analyzed by western.

(C) WT-Ube2S or Ube2S^{I154A} stable cell lines were transfected with Ube2S siRNA and released from a prometaphase arrest. APC/C was immunoprecipitated from cells at the indicated time points after the release, and APC/C-bound mitotic checkpoint components (Cdc20, BubR1, Mad2) were analyzed by western.

(D) HeLa stable cell lines expressing either WT-Ube2S or Ube2S^{I154A} were treated with siRNA against the 3′ UTR of the Ube2S mRNA to deplete endogenous Ube2S. Prometaphase cells were released into drug-free media, and lysates were analyzed by western.

(E) Hygromycin-resistant HeLa cells and WT-Ube2S or Ube2S^{I154A} stable cell lines were infected with lentiviruses that express H2B-mCherry, transfected with siRNAs, and monitored by live-cell imaging. The time from nuclear envelope breakdown to initiation of sister chromatid separation was determined for ~50–100 cells per condition. The cumulative percentage of cells that have reached anaphase is shown.

(F) Model of APC/C-dependent ubiquitin chain elongation. Growing ubiquitin chains on substrates loop out to position the distal acceptor ubiquitin near the catalytic site of Ube2S. The RING domain of Apc11 is a candidate core subunit that forms part of the acceptor ubiquitin binding site.

mitotic arrest (Figure 7D). Even cells that had never been exposed to microtubule toxins and had not experienced chronic activation of the spindle checkpoint showed a significant delay in the initiation of sister chromatid separation, if the elongation-deficient Ube2S^{I154A} was the only source of Ube2S (Figure 7E). We conclude that the capacity of the APC/C to improve acceptor ubiquitin recognition by Ube2S is required for the degradation of APC/C substrates, and consequently, for the accurate execution of the cell division program.

DISCUSSION

How E3 ligases assemble polymeric ubiquitin chains despite binding their substrates and E2s at invariant surfaces had been poorly understood. Here, we show that the essential E3 APC/C promotes chain elongation by increasing the affinity of its E2 Ube2S to the distal ubiquitin of a growing conjugate. The capacity of the APC/C to hold on to an emerging chain requires its coactivator, a conserved surface of Ube2S, and specific residues of the acceptor ubiquitin. Impeding this function of the APC/C ablates chain formation in cells and interferes with substrate degradation and mitotic regulation. Our findings therefore demonstrate that the APC/C recognizes its substrates at the degron interface as well as at the distal ubiquitin of the growing chain, a capacity that allows this E3 to build polymeric conjugates without altering its binding to the substrate or E2 (Figure 7F).

How Does the APC/C Promote Ubiquitin Chain Elongation?

In line with the original description of Cdc20 and Cdh1 as coactivators (Schwab et al., 1997; Visintin et al., 1997), we found that the APC/C requires Cdc20 for a specific step in ubiquitin chain formation: Cdc20-bound APC/C, but not free APC/C, increased the catalytic activity of Ube2S by more than 200-fold. Our in vitro systems did not allow us to investigate a role of Cdh1: in contrast to Cdc20, whose binding to the APC/C is stabilized by the spindle checkpoint, Cdh1 dissociates from the APC/C more rapidly and is lost to a significant extent during the affinity purification

of the APC/C. However, as Cdh1 binds to Ube2S (Williamson et al., 2009), we anticipate that it serves a role similar to that of Cdc20 in activating Ube2S. Cdc20 and Cdh1 are also required for the recognition of substrate degron motifs known as D- or KEN-boxes (Peters, 2006), suggesting that the APC/C coordinates substrate engagement with ubiquitin chain elongation.

Cdc20 and Cdh1 have been reported to interact with ubiquitin (Pashkova et al., 2010), so they could be involved in stabilizing an acceptor ubiquitin on the APC/C. However, the proposed site for ubiquitin binding on Cdc20 overlaps with residues involved in the recruitment of substrates or spindle checkpoint effectors (Chao et al., 2012; Tian et al., 2012), and our experiments showed that neither APC/C substrates nor checkpoint effectors impeded the ability of APC/C^{Cdc20} to activate Ube2S. Previous studies had suggested that coactivators alter the conformation of the APC/C, which promotes APC/C's association with certain E2 enzymes (Chang et al., 2014; da Fonseca et al., 2011; Van Voorhis and Morgan, 2014). Yet, the APC/C stimulates chain elongation whether or not it is bound to checkpoint proteins, even though checkpoint-bound and free APC/C adopt strikingly different conformations (Herzog et al., 2009).

We therefore suggest that Cdc20 initially engages the CTP of Ube2S and delivers the E2 to the APC/C. After the CTP has been handed over to a core APC/C subunit (see Brown et al., 2014), Cdc20 might continue to stabilize the interaction between the UBC domain of Ube2S and the APC/C. This hypothesis is based on the observation that immunodepletion of Cdc20 dissociated Ube2S from the APC/C and that depletion of Apc2 and Cdc20 or mutation of helix D in the UBC domain destabilized the binding of Ube2S to APC/C independently of the CTP. We anticipate that delivery by Cdc20 positions the catalytic domain of Ube2S in proximity to a ubiquitin-binding site on the core APC/C, which, based on our crosslinking studies and experiments by Brown et al. (2014), likely includes the RING domain of Apc11. In our model, the APC/C therefore has two sites for recognizing its substrates (Figure 7F): the first site is formed by Cdc20/Cdh1 and Apc10 and engages the substrates' degron motifs. The second site, described here, associates with the distal acceptor ubiquitin and allows the APC/C to track the tip of the emerging chain. By recognizing both substrate and growing ubiquitin chain, the APC/C solves the topological problem of assembling conjugates of increasing length, despite binding substrate and E2 at invariant positions.

Consequences of Tracking the Growing Ubiquitin Chain

Tethering the distal ubiquitin in proximity to the active site of Ube2S requires that the conjugate is able to fold back onto the APC/C (Figure 7F). Previous work had suggested that the conformational flexibility of the C-terminal glycine residues of ubiquitin allows such dramatic conformational changes to occur (Ye et al., 2012). However, as the chain length grows, the sample volume for the distal ubiquitin will also increase, and further elongation events will become less likely. Tracking the end of a growing chain is thus predicted to limit the length of a ubiquitin chain, and indeed, the APC/C shows a strong propensity to assemble conjugates containing ~6 ubiquitin molecules in a single substrate-binding event (Meyer and Rape, 2014; Wickliffe et al., 2011).

Intriguingly, the ubiquitin surface recognized by the APC/C overlaps with the hydrophobic patch, which includes Ile44 and mediates donor ubiquitin binding by Ube2S and many other E2s (Dou et al., 2012; Plechanovová et al., 2012; Saha et al., 2011; Wickliffe et al., 2011). Hence, a single ubiquitin molecule can be either an acceptor or donor for APC/C-dependent chain formation, but modification of a thioester-linked ubiquitin to build chains on the active site of Ube2S is impossible. Our observations therefore lend support to the hypothesis that the APC/C synthesizes ubiquitin chains by a sequential mechanism, as previously suggested for the APC/C and other E3s on the basis of kinetic arguments (Pierce et al., 2009; Rape et al., 2006).

In recent years, several studies have noted yet undetermined roles for ubiquitin recognition by chain-elongating enzymes. The U-box containing Ufd2, for example, was purified based on its capacity to adhere to a ubiquitin matrix (Koegl et al., 1999). Adaptors of cullin-RING ligases have been found to bind ubiquitin, which might help these enzymes to drive chain elongation (Pashkova et al., 2010). Finally, the elongating E2s Ube2K or Ube2N/Uev1A can engage ubiquitin using surfaces that are different from their donor ubiquitin-binding motif (Eddins et al., 2007; Merkley and Shaw, 2004). Although further studies are required, these observations suggest that ubiquitylation enzymes might frequently promote ubiquitin chain formation by improving the affinity of their catalytic centers to the distal ubiquitin of a growing conjugate.

Implications for Cell-Cycle Control

In addition to providing insight into the mechanism of ubiquitin chain formation, our work has implications for cell-cycle control. During prometaphase, the spindle checkpoint inhibits substrate recognition by the APC/C, thereby preventing premature entry into anaphase. While the inhibitory function of the checkpoint toward the APC/C has been established, recent studies suggested that the APC/C can inactivate the checkpoint by promoting the disassembly of checkpoint complexes in a ubiquitylation-dependent manner (Reddy et al., 2007; Uzunova et al., 2012; Varetti et al., 2011). However, alternative roles for the APC/C in either protecting the checkpoint or promoting its inactivation independently of ubiquitylation had also been put forward (Mansfeld et al., 2011; Nilsson et al., 2008).

The identification of a Ube2S mutant that disabled a specific catalytic function of the APC/C, i.e., improved recognition of the acceptor ubiquitin, allowed us to distinguish between the aforementioned models without mutating Cdc20, ubiquitin, or the core APC/C. We found that cells expressing Ube2S^{I154A} as the sole source of this E2 were impaired in their capacity to support prometaphase APC/C activity and to disassemble spindle checkpoint complexes. Consequently, these cells were delayed in their entry into anaphase. Our results therefore indicate that ubiquitylation-dependent spindle checkpoint disassembly is an important function of the APC/C in cells.

Intriguingly, it is the checkpoint-inhibited APC/C that is most potent in activating Ube2S, most likely because prometaphase APC/C is most stably bound to Cdc20. Thus, the spindle checkpoint could play a dual role in regulating the APC/C: by altering the position of Cdc20 on the APC/C and by blocking the KEN-box binding motif on Cdc20, it interferes with the recognition of canonical APC/C substrates (Chao et al., 2012; Herzog et al.,

2009; Tian et al., 2012). At the same time, by stabilizing the binding of Cdc20 to the APC/C, it increases the capacity of the APC/C to produce ubiquitin chains on Cdc20, the very signal that triggers the disassembly of spindle checkpoint complexes (Reddy et al., 2007). The regulation of the APC/C by the spindle checkpoint is, therefore, reminiscent of incoherent feedforward regulation, a network motif frequently used by cellular checkpoints to monitor the persistence of the initial insult that triggered the checkpoint activation.

Given that the turnover of checkpoint proteins on the APC/C should accelerate once all kinetochores have accomplished bipolar spindle attachment, it is likely that APC/C-dependent chain formation is regulated beyond what has been discovered in this work. Indeed, whereas APC/C purified from mitotic cells required Cdc20 for stable Ube2S binding, recombinant APC/C, i.e., a form that lacks physiological modifications or binding partners, could associate with Ube2S in the absence of coactivators (Brown et al., 2014). Both dedicated checkpoint-silencing factors, such as $p31^{comet}$, or posttranslational modifications of the APC/C, such as phosphorylation, might play roles in this regulation. As activation of the spindle checkpoint has been an established chemotherapeutic strategy, it will be an exciting avenue for future research to test whether ubiquitin chain elongation, and consequently the dynamic interplay between the APC/C and the spindle assembly checkpoint, could be modulated by small molecules for the more efficient treatment of diseases caused by aberrantly dividing cells.

EXPERIMENTAL PROCEDURES

Supplemental Experimental Procedures can be found in the Supplemental Information.

APC/C Purification and Crosslinking

Human APC/C was purified from extracts of prometaphase HeLa S3 cells (Rape et al., 2006). APC/C was incubated with 2 μM $Ube2S^{C118A}$ or $Ube2S^{C95A/C118A}$ and 10 μM BMB crosslinker (1,4-bismaleimidobutane) for 30 min at 22°C. Ub-$CycA^{HA\text{-}His}$ (4 μM) or $^{His}Emi1^{299–447}$ (20 μM) was added when indicated, or specific APC/C subunits were depleted prior to APC/C purification by 10–25 nM siRNA transfected with Lipofectamine RNAiMAX.

Kinetic Analyses

Time courses of diubiquitin formation were performed with 200 nM E1, 5 μM Ube2S, 75 μM ubi^{K11R}, and various concentrations of $ubi^{\Delta GG}$. APC/C was immunoprecipitated either from extracts of synchronized HeLa S3 cells or, alternatively, from mitotic extract depleted of Cdc20 (Song et al., 2014). Ubi_2 formation was detected by colloidal Coomassie, quantified by ImageJ, and compared to a standard curve of purified Ubi_2. Initial velocity and kinetic constants were calculated with GraphPad Prism and Michaelis-Menten equations.

Single-Discharge Assays

Ube2S (6 μM) was incubated with 50 μM ubi^{K11R} in the presence of 200 nM E1 and 3 mM ATP. Reactions were stopped by 35 U/ml apyrase followed by 65 mM EDTA. Discharge of Ube2S∼ubi was initiated by addition of 120 μM $ubi^{\Delta GG}$, and reactions were monitored by western using a K1-linkage-specific antibody.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.09.010.

AUTHOR CONTRIBUTIONS

A.K., K.E.W., and L.S. designed, executed, and interpreted experiments and helped write the paper. I.F. wrote the scripts for CompPASS mass spectrometry analysis, and M.R. helped to design and interpret experiments and wrote the paper.

ACKNOWLEDGMENTS

We thank Nick Brown and Brenda Schulman for sharing results prior to publication; Julia Schaletzky, Jacob Corn, Roberto Zoncu, Achim Werner, Andrew Manford, Eugene Oh, and Allison Craney for comments on the manuscript; and all members of the Rape lab for their discussions and ideas. This work was funded by grants from the National Institutes of Health and the March of Dimes Foundation. M.R. is an Investigator of the Howard Hughes Medical Institute and is cofounder and consultant to Nurix.

Received: July 19, 2014
Revised: August 27, 2014
Accepted: September 3, 2014
Published: October 9, 2014

REFERENCES

Brown, N.G., Watson, E.R., Weissman, F., Jarvis, M.A., Vanderlinden, R., Grace, C.R.R., Frye, J.J., Qiao, R., Dube, P., Petzold, G., et al. (2014). Mechanism of polyubiquitination by human anaphase-promoting complex: RING repurposing for ubiquitin chain assembly. Mol Cell *56*, this issue, 246–260.

Chang, L., Zhang, Z., Yang, J., McLaughlin, S.H., and Barford, D. (2014). Molecular architecture and mechanism of the anaphase-promoting complex. Nature *513*, 388–393.

Chao, W.C., Kulkarni, K., Zhang, Z., Kong, E.H., and Barford, D. (2012). Structure of the mitotic checkpoint complex. Nature *484*, 208–213.

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576–1583.

Christensen, D.E., Brzovic, P.S., and Klevit, R.E. (2007). E2-BRCA1 RING interactions dictate synthesis of mono- or specific polyubiquitin chain linkages. Nat. Struct. Mol. Biol. *14*, 941–948.

da Fonseca, P.C., Kong, E.H., Zhang, Z., Schreiber, A., Williams, M.A., Morris, E.P., and Barford, D. (2011). Structures of APC/C(Cdh1) with substrates identify Cdh1 and Apc10 as the D-box co-receptor. Nature *470*, 274–278.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. *78*, 399–434.

Dou, H., Buetow, L., Sibbet, G.J., Cameron, K., and Huang, D.T. (2012). BIRC7-E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer. Nat. Struct. Mol. Biol. *19*, 876–883.

Eddins, M.J., Carlile, C.M., Gomez, K.M., Pickart, C.M., and Wolberger, C. (2006). Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. Nat. Struct. Mol. Biol. *13*, 915–920.

Eddins, M.J., Varadan, R., Fushman, D., Pickart, C.M., and Wolberger, C. (2007). Crystal structure and solution NMR studies of Lys48-linked tetraubiquitin at neutral pH. J. Mol. Biol. *367*, 204–211.

Frye, J.J., Brown, N.G., Petzold, G., Watson, E.R., Grace, C.R., Nourse, A., Jarvis, M.A., Kriwacki, R.W., Peters, J.M., Stark, H., and Schulman, B.A. (2013). Electron microscopy structure of human APC/C(CDH1)-EMI1 reveals multimodal mechanism of E3 ligase shutdown. Nat. Struct. Mol. Biol. *20*, 827–835.

Hayes, M.J., Kimata, Y., Wattam, S.L., Lindon, C., Mao, G., Yamano, H., and Fry, A.M. (2006). Early mitotic degradation of Nek2A depends on Cdc20-independent interaction with the APC/C. Nat. Cell Biol. *8*, 607–614.

Herzog, F., Primorac, I., Dube, P., Lenart, P., Sander, B., Mechtler, K., Stark, H., and Peters, J.M. (2009). Structure of the anaphase-promoting

complex/cyclosome interacting with a mitotic checkpoint complex. Science *323*, 1477–1481.

Kimata, Y., Baxter, J.E., Fry, A.M., and Yamano, H. (2008). A role for the Fizzy/Cdc20 family of proteins in activation of the APC/C distinct from substrate recruitment. Mol. Cell *32*, 576–583.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635–644.

Komander, D., and Rape, M. (2012). The ubiquitin code. Annu. Rev. Biochem. *81*, 203–229.

Li, W., Tu, D., Brunger, A.T., and Ye, Y. (2007). A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate. Nature *446*, 333–337.

Liu, W., Shang, Y., Zeng, Y., Liu, C., Li, Y., Zhai, L., Wang, P., Lou, J., Xu, P., Ye, Y., and Li, W. (2014). Dimeric Ube2g2 simultaneously engages donor and acceptor ubiquitins to form Lys48-linked ubiquitin chains. EMBO J. *33*, 46–61.

Mansfeld, J., Collin, P., Collins, M.O., Choudhary, J.S., and Pines, J. (2011). APC15 drives the turnover of MCC-CDC20 to make the spindle assembly checkpoint responsive to kinetochore attachment. Nat. Cell Biol. *13*, 1234–1243.

Matsumoto, M.L., Wickliffe, K.E., Dong, K.C., Yu, C., Bosanac, I., Bustos, D., Phu, L., Kirkpatrick, D.S., Hymowitz, S.G., Rape, M., et al. (2010). K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. Mol. Cell *39*, 477–484.

Merkley, N., and Shaw, G.S. (2004). Solution structure of the flexible class II ubiquitin-conjugating enzyme Ubc1 provides insights for polyubiquitin chain assembly. J. Biol. Chem. *279*, 47139–47147.

Meyer, H.J., and Rape, M. (2014). Enhanced protein degradation by branched ubiquitin chains. Cell *157*, 910–921.

Nilsson, J., Yekezare, M., Minshull, J., and Pines, J. (2008). The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. Nat. Cell Biol. *10*, 1411–1420.

Pashkova, N., Gakhar, L., Winistorfer, S.C., Yu, L., Ramaswamy, S., and Piper, R.C. (2010). WD40 repeat propellers define a ubiquitin-binding domain that regulates turnover of F box proteins. Mol. Cell *40*, 433–443.

Peters, J.M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat. Rev. Mol. Cell Biol. *7*, 644–656.

Petroski, M.D., and Deshaies, R.J. (2005). Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. Cell *123*, 1107–1120.

Pierce, N.W., Kleiger, G., Shan, S.O., and Deshaies, R.J. (2009). Detection of sequential polyubiquitylation on a millisecond timescale. Nature *462*, 615–619.

Plechanovová, A., Jaffray, E.G., Tatham, M.H., Naismith, J.H., and Hay, R.T. (2012). Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. Nature *489*, 115–120.

Pruneda, J.N., Littlefield, P.J., Soss, S.E., Nordquist, K.A., Chazin, W.J., Brzovic, P.S., and Klevit, R.E. (2012). Structure of an E3:E2~Ub complex reveals an allosteric mechanism shared among RING/U-box ligases. Mol. Cell *47*, 933–942.

Rape, M., Reddy, S.K., and Kirschner, M.W. (2006). The processivity of multiubiquitination by the APC determines the order of substrate degradation. Cell *124*, 89–103.

Ravid, T., and Hochstrasser, M. (2007). Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue. Nat. Cell Biol. *9*, 422–427.

Reddy, S.K., Rape, M., Margansky, W.A., and Kirschner, M.W. (2007). Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. Nature *446*, 921–925.

Reverter, D., and Lima, C.D. (2005). Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. Nature *435*, 687–692.

Saha, A., Lewis, S., Kleiger, G., Kuhlman, B., and Deshaies, R.J. (2011). Essential role for ubiquitin-ubiquitin-conjugating enzyme interaction in ubiquitin discharge from Cdc34 to substrate. Mol. Cell *42*, 75–83.

Schwab, M., Lutum, A.S., and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. Cell *90*, 683–693.

Scott, D.C., Sviderskiy, V.O., Monda, J.K., Lydeard, J.R., Cho, S.E., Harper, J.W., and Schulman, B.A. (2014). Structure of a RING E3 trapped in action reveals ligation mechanism for the ubiquitin-like protein NEDD8. Cell *157*, 1671–1684.

Song, L., Craney, A., and Rape, M. (2014). Microtubule-dependent regulation of mitotic protein degradation. Mol. Cell *53*, 179–192.

Thornton, B.R., Ng, T.M., Matyskiela, M.E., Carroll, C.W., Morgan, D.O., and Toczyski, D.P. (2006). An architectural map of the anaphase-promoting complex. Genes Dev. *20*, 449–460.

Tian, W., Li, B., Warrington, R., Tomchick, D.R., Yu, H., and Luo, X. (2012). Structural analysis of human Cdc20 supports multisite degron recognition by APC/C. Proc. Natl. Acad. Sci. USA *109*, 18419–18424.

Uzunova, K., Dye, B.T., Schutz, H., Ladurner, R., Petzold, G., Toyoda, Y., Jarvis, M.A., Brown, N.G., Poser, I., Novatchkova, M., et al. (2012). APC15 mediates CDC20 autoubiquitylation by APC/C(MCC) and disassembly of the mitotic checkpoint complex. Nat. Struct. Mol. Biol. *19*, 1116–1123.

Van Voorhis, V.A., and Morgan, D.O. (2014). Activation of the APC/C ubiquitin ligase by enhanced E2 efficiency. Curr. Biol. *24*, 1556–1562.

Varetti, G., Guida, C., Santaguida, S., Chiroli, E., and Musacchio, A. (2011). Homeostatic control of mitotic arrest. Mol. Cell *44*, 710–720.

Visintin, R., Prinz, S., and Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. Science *278*, 460–463.

Wang, W., and Kirschner, M.W. (2013). Emi1 preferentially inhibits ubiquitin chain elongation by the anaphase-promoting complex. Nat. Cell Biol. *15*, 797–806.

Wickliffe, K.E., Lorenz, S., Wemmer, D.E., Kuriyan, J., and Rape, M. (2011). The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. Cell *144*, 769–781.

Williamson, A., Wickliffe, K.E., Mellone, B.G., Song, L., Karpen, G.H., and Rape, M. (2009). Identification of a physiological E2 module for the human anaphase-promoting complex. Proc. Natl. Acad. Sci. USA *106*, 18213–18218.

Ye, Y., and Rape, M. (2009). Building ubiquitin chains: E2 enzymes at work. Nat. Rev. Mol. Cell Biol. *10*, 755–764.

Ye, Y., Blaser, G., Horrocks, M.H., Ruedas-Rama, M.J., Ibrahim, S., Zhukov, A.A., Orte, A., Klenerman, D., Jackson, S.E., and Komander, D. (2012). Ubiquitin chain conformation regulates recognition and activity of interacting proteins. Nature *492*, 266–270.