Molecular Cell
Resource

K11-Linked Polyubiquitination in Cell Cycle Control Revealed by a K11 Linkage-Specific Antibody

Marissa L. Matsumoto,[1,6] Katherine E. Wickliffe,[5,6] Ken C. Dong,[2] Christine Yu,[2] Ivan Bosanac,[2] Daisy Bustos,[3] Lilian Phu,[3] Donald S. Kirkpatrick,[3] Sarah G. Hymowitz,[2] Michael Rape,[5] Robert F. Kelley,[1] and Vishva M. Dixit[4,*]
[1]Department of Antibody Engineering
[2]Department of Structural Biology
[3]Department of Protein Chemistry
[4]Department of Physiological Chemistry
Genentech, Inc., South San Francisco, CA 94080, USA
[5]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA
[6]These authors contributed equally to this work
*Correspondence: dixit@gene.com
DOI 10.1016/j.molcel.2010.07.001

SUMMARY

Polyubiquitination is a posttranslational modification where ubiquitin chains containing isopeptide bonds linking one of seven ubiquitin lysines with the C terminus of an adjoining ubiquitin are covalently attached to proteins. While functions of K48- and K63-linked polyubiquitin are understood, the role(s) of noncanonical K11-linked chains is less clear. A crystal structure of K11-linked diubiquitin demonstrates a distinct conformation from K48- or K63-linked diubiquitin. We engineered a K11 linkage-specific antibody and use it to demonstrate that K11 chains are highly upregulated in mitotic human cells precisely when substrates of the ubiquitin ligase anaphase-promoting complex (APC/C) are degraded. These chains increased with proteasomal inhibition, suggesting they act as degradation signals in vivo. Inhibition of the APC/C strongly impeded the formation of K11-linked chains, suggesting that a single ubiquitin ligase is the major source of mitotic K11-linked chains. Our results underscore the importance of K11-linked ubiquitin chains as critical regulators of mitotic protein degradation.

INTRODUCTION

Ubiquitin is a 76 amino acid protein that is highly conserved from yeast to humans. Covalent linkage of the C terminus of ubiquitin to the ε-amino group of a lysine on a substrate plays an essential role in many cellular pathways, including the DNA-damage response, protein turnover, receptor downregulation, cell cycle progression, NF-κB signaling, and apoptosis (Hershko and Ciechanover, 1998). Three enzymes mediate ubiquitination—an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase (Deshaies and Joazeiro, 2009; Schulman and Harper, 2009; Ye and Rape, 2009)—whereas deubiquitinating enzymes (DUBs) reverse it (Reyes-Turcu et al., 2009).

Multiple forms of ubiquitination exist (Komander, 2009). Monoubiquitination refers to the attachment of a single ubiquitin to one or more lysines within the substrate, while polyubiquitin chains are formed when additional ubiquitins are linked to lysines or the N terminus of ubiquitin itself. Despite having identical subunits, ubiquitin chains linked through K48 and K63 have distinct structures. K48-linked polyubiquitin forms a compact structure where its hydrophobic patch, centered around I44, is buried (Cook et al., 1992; Eddins et al., 2007; Phillips et al., 2001; Tenno et al., 2004; Varadan et al., 2002). In contrast, K63-linked polyubiquitin forms extended chains lacking intersubunit interfaces (Datta et al., 2009; Komander et al., 2009; Tenno et al., 2004; Varadan et al., 2004). These conformational differences influence the fate of the modified protein by promoting interactions with distinct ubiquitin-binding proteins (Dikic et al., 2009). K48-linked chains target proteins for proteasomal degradation (Hershko and Ciechanover, 1998), whereas K63-linked chains typically facilitate protein-protein interactions required for signaling (Skaug et al., 2009). The structure and function of polyubiquitin chains linked through K6, K11, K27, K29, and K33 are less understood.

Ubiquitin chains linked through K11 were identified as critical regulators of cell division (Jin et al., 2008; Kirkpatrick et al., 2006) and were proposed to signal degradation of ubiquitin ligase anaphase-promoting complex (APC/C) substrates, which is essential for eukaryotic cell division (Jin et al., 2008; Williamson et al., 2009). The APC/C recruits two E2 enzymes, the ubiquitin chain-initiating UbcH10 and the chain-elongating Ube2S, which assemble K11-linked chains with high specificity (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010). Loss of this APC/C-specific E2 module leads to strong defects in mitotic progression (Song and Rape, 2010; Williamson et al., 2009). While these results suggest that K11-linked chains drive protein degradation during mitosis, characterization of ubiquitin chains assembled by the APC/C, UbcH10, and Ube2S has relied largely on in vitro experiments. Direct evidence of K11-linked chains regulating protein degradation in cells is lacking. In addition, it is unclear the extent to which the APC/C assembles K11-linked chains compared to canonical K48-linked chains.

The advent of antibodies capable of recognizing endogenous K48- and K63-linked polyubiquitin chains provided a simple and direct means to interrogate polyubiquitin signaling (Newton et al., 2008). Here, we describe the engineering of a K11-linked polyubiquitin-specific antibody. We illustrate the utility of this antibody by demonstrating upregulation of K11-linked chains during mitosis when APC/C substrates are degraded. Unexpectedly, loss of APC/C activity interfered with the synthesis of most K11-linked chains in cells, suggesting that a single ubiquitin ligase, the APC/C, assembles the bulk of K11-linked chains. These results emphasize a crucial role for K11-linked ubiquitin chains as essential regulators of mitotic protein degradation.

RESULTS

Crystal Structure of K11-Linked Diubiquitin

The generation of K48- and K63-linked polyubiquitin-specific antibodies (Newton et al., 2008) was possible because K48- and K63-linked chains adopt distinct structures, thus presenting different surfaces to binding partners. To determine whether K11-linked chains also adopt a unique conformation, we crystallized enzymatically synthesized K11-linked diubiquitin (Figure S2). The structure, which was solved to 2.2 Å (Table 1), reveals that K11-linked diubiquitin can adopt a compact structure in which I44 of the hydrophobic patch of each ubiquitin is solvent accessible and on the same face of the dimer (Figures 1A and 1B). This structure differs markedly from that of extended K63-linked chains (Datta et al., 2009; Komander et al., 2009; Tenno et al., 2004; Varadan et al., 2004) and compact K48-linked chains, where I44 residues are buried within the interface (Cook et al., 1992; Eddins et al., 2007; Phillips et al., 2001; Tenno et al., 2004; Varadan et al., 2002) (Figure 1B). The interface between the K11-linked ubiquitins is composed primarily of residues from loops L1 and L3 and the C-terminal tail (Figure S1A) and has a hydrophobic core (Figure S1B). Given the distinct conformation adopted by K11-linked diubiquitin, we hypothesized that antibodies specific for K11-linked polyubiquitin could be engineered.

Table 1. X-Ray Data Collection and Refinement Statistics

Data Collection	
Space group	C2
Cell dimensions	
a, b, c (Å)	156, 56, 66
β (°)	95
Resolution (Å)	30–2.2 (2.28–2.20)
R_{sym}	5.7 (46.0)
$\langle I/\sigma I \rangle$	18.7 (3.0)
Completeness (%)	99.9 (99.9)
Redundancy	3.7 (3.6)
Refinement	
Resolution (Å)	2.19
Diubiquitins in asu	4
No. reflections	29,153
R_{work}/R_{free} (%)	24.51/28.12
No. atoms	
Protein	4806
Solvent	200
Rmsds	
Bond lengths (Å)	0.008
Bond angles (°)	0.932
Ramachandran plot	
Preferred regions (%)	96.0
Additionally allowed regions (%)	4.0
Generously allowed regions (%)	0
Disallowed regions (%)	0

Values in parentheses are for highest-resolution shell.

Generation of a K11-Linked Polyubiquitin-Specific Antibody

A synthetic bivalent Fab antibody fragment phage display library (Lee et al., 2004) was sorted using a competitive selection strategy (Figure S3A). A K11-linked diubiquitin-specific clone (G3), when produced as a soluble Fab fragment, had a dissociation constant (K_D) of 105 nM for binding K11-linked diubiquitin and demonstrated no detectable binding to either K48- or K63-linked diubiquitin (Table S1). This clone was used as a template for affinity maturation libraries encoding amino acid diversity within different combinations of complementarity-determining regions (CDRs). The highest-affinity K11-specific clone containing CDR L1 mutations, 2A3, had a K_D of 25 nM for K11-linked diubiquitin, while the highest-affinity K11-specific clone containing CDR H2 mutations, 2E6, had a K_D of 47 nM (Table S1 and Figure S3B). Since the affinity maturation libraries generated were not designed to have both CDR L1 and H2 diversity, the light chain of 2A3 was combined with the heavy chain of 2E6. The resulting hybrid, 2A3/2E6, had a further improvement in affinity for K11-linked diubiquitin over both parental clones (K_D = 12 nM) and showed no detectable binding to K48- or K63-linked diubiquitin (Table S1).

Western Blot and Immunoprecipitation with the K11-Linked Polyubiquitin-Specific Antibody 2A3/2E6

2A3/2E6 detected as little as 16 ng of K11-linked diubiquitin by western blot without showing cross-reactivity to 1 μg of monoubiquitin, linear, K48-, or K63-linked diubiquitin (Figure 2A). It also detected enzymatically synthesized (Figure 2B) and endogenous K11-linked polyubiquitin (Figure 2C). To confirm 2A3/2E6 specificity, we synthesized polyubiquitin chains of multiple linkages in vitro with the E2 UbcH5c and E3 MuRF1. In the absence of a substrate, MuRF1 undergoes autoubiquitination with chains composed predominantly of K11, K48, and K63 linkages (Kim et al., 2007). Reactions were done using WT, K11R, K48R, or K63R ubiquitin (Figures 3A and 3B). Lysine-to-arginine mutations prevent the synthesis of polyubiquitin chains linked through that lysine, as confirmed by mass spectrometry (Figure 3C). Autoubiquitination reactions were immunoprecipitated by 2A3/2E6, and total ubiquitin was detected by western blot (Figure 3B). When 2A3/2E6 immunoprecipitated autoubiquitinated MuRF1 from the WT, K48R, and K63R reactions, the polyubiquitin linkage profiles remained similar to the input material (Figure 3C),

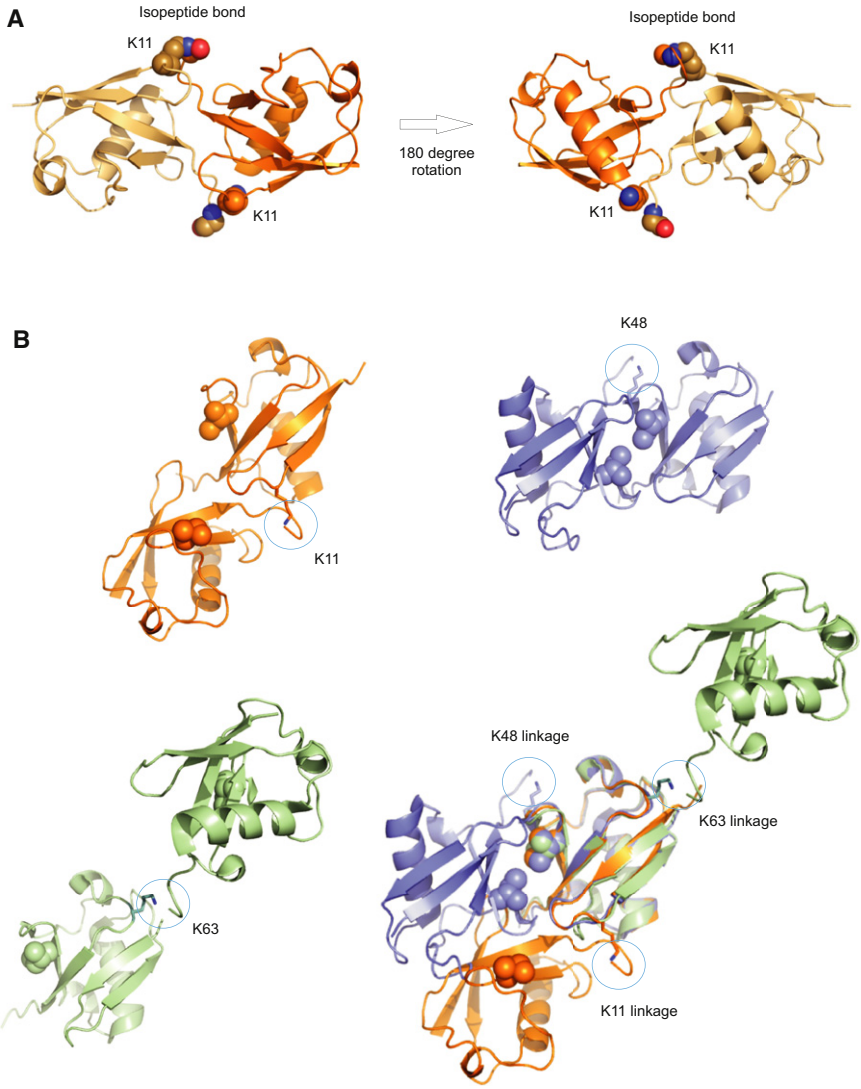

Figure 1. Crystal Structure of K11-Linked Diubiquitin

(A) The structure of K11-linked diubiquitin with the proximal ubiquitin (provides K11 to the isopeptide bond) in light orange and the distal ubiquitin (provides the C terminus) in dark orange. The K11 side chains and the C-terminal glycines are shown as spheres, and the isopeptide bond is labeled. The figure on the right is rotated 180° about a vertical axis relative to the figure on the left. (B) The structures of K11-, K48- (from tetraubiquitin, PDB code 2O6V), and K63-linked diubiquitin (PDB code 2JF5) are shown in orange, blue, and green, respectively. The side chains of I44 are shown as spheres, lysines as sticks, and isopeptide bonds circled. Superposition of the three structures with proximal ubiquitins aligned is shown. See also Figure S1.

suggesting the majority of MuRF1 molecules carry each of the primary linkages. By contrast, 2A3/2E6 failed to immunoprecipitate any polyubiquitin linkages from the K11R reaction, owing to the lack of the target linkage (Figure 3C). Next, we determined whether 2A3/2E6 could immunoprecipitate K11-linked polyubiquitin from HEK293T cells (Figure 3D). Immunoblotting revealed immunoprecipitation of ubiquitinated species, which was increased upon ubiquitin overexpression (Figure 3E). Mass spectrometry confirmed enrichment of K11 linkages in immunoprecipitates relative to the percentage of K11 linkages present in the input lysates (Figure 3F).

Upregulation of K11-Linked Chains Correlates with APC/C Substrate Degradation

Having established that 2A3/2E6 specifically recognizes K11-linked polyubiquitin, we used it to probe the formation of K11-linked chains in vivo. Asynchronous HeLa cells contained relatively little K11-linked polyubiquitin compared to K48-linked polyubiquitin (Figure 4A). By contrast, K11-linked chains were strongly upregulated in HeLa cells released from a nocodazole-induced prometaphase arrest. This increase correlated precisely with the degradation of APC/C substrates securin, cyclin B1, Aurora A, and Plk1 and was enhanced by proteasomal inhibition (Figures 4A and 4B). In contrast, K48- and K63-linked chains did not increase dramatically upon proteasome inhibition in cells exiting from mitosis (Figure 4B). These data suggest that activation of the APC/C results in a sharp increase in the overall concentration of K11-linked chains and that these function as degradation signals in vivo.

The aforementioned experiments relied on drug-induced synchronization. To determine whether the abundance of K11-linked chains is cell-cycle regulated in cells never exposed to spindle toxins, we analyzed the levels of K11-linked chains by immunofluorescence microscopy. Confirming the immunoblotting, K11-linked chains were upregulated in mitosis (Figure 4C). Interestingly, we observed a strong accumulation of K11-linked chains at the spindle midbody from late anaphase on, consistent with a role for the APC/C in targeting Plk1 and passenger complex proteins for degradation (Figure 4C). These experiments further suggest that K11-linked chains act in mitotic control, in a manner consistent with being assembled by the APC/C.

The human APC/C synthesizes K11-linked chains on its substrates to promote their degradation (Jin et al., 2008; Williamson et al., 2009; Wu et al., 2010); however, most experiments to date have been performed in vitro with ubiquitin mutants. To determine whether the APC/C is responsible for formation of K11-linked chains in cells, we knocked down the APC/C-specific E2s, *UbcH10* and *Ube2S*, or the APC/C subunits *Cdc27*, *Apc11*, and *Cdh1*. As reported (Williamson et al., 2009), simultaneous

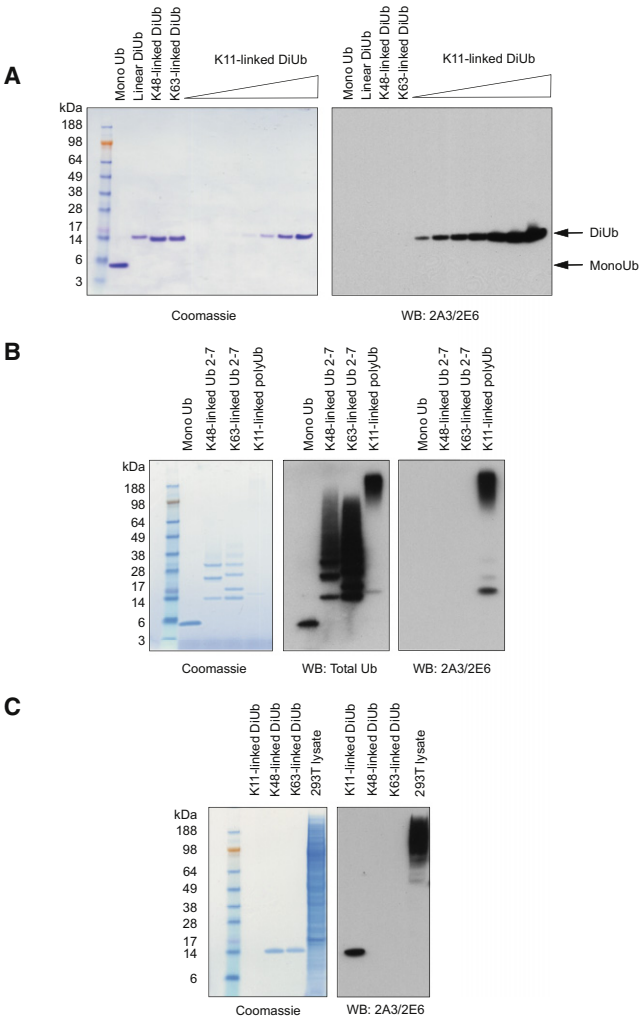

Figure 2. The 2A3/2E6 Antibody Is Specific for K11-Linked Polyubiquitin in Western Blots

(A) Monoubiquitin, linear diubiquitin, K48-linked diubiquitin, and K63-linked diubiquitin (1 μg/lane) and K11-linked diubiquitin (2-fold dilutions, 16–1000 ng/lane gradient) were immunoblotted with 2A3/2E6 (right) or Coomassie stained (left).

(B) Monoubiquitin, K48-linked polyubiquitin 2–7 (2–7 ubiquitins), K63-linked polyubiquitin 2–7, and K11-linked polyubiquitin (1 μg/lane) were immunoblotted with a pan-ubiquitin antibody (middle) or with 2A3/2E6 (right) or were Coomassie stained (left).

(C) K11-linked diubiquitin (50 ng), K48-linked diubiquitin (1 μg), K63-linked diubiquitin (1 μg), and HEK293T cell lysate (100 μg) were immunoblotted with 2A3/2E6 (right) or Coomassie stained (left). See also Figure S2 and Table S1.

depletion of *UbcH10* and *Ube2S* strongly impaired APC/C activity in cells, leading to stabilization of all APC/C substrates tested (Figure 4D). Strikingly, loss of these E2s also blocked upregulation of K11-linked chains in synchronized cells while having no effect on K48-linked chains (Figure 4D). Similar results were obtained upon depletion of APC/C subunits (Figure 4E). To exclude the possibility that the decrease in K11-linked chains resulted from a cell-cycle arrest, rather than from APC/C inhibition, we released the *UbcH10*/*Ube2S*-siRNA-treated cells from mitosis in the presence of a CDK inhibitor, roscovitine, which allows mitotic exit in the absence of APC/C activity (Potapova et al., 2006). Under these conditions, depletion of *UbcH10* and *Ube2S* caused a striking decrease in the abundance of K11-linked chains (Figure 4F). Furthermore, APC/C activation by overexpression of both Cdh1 and Ube2S in asynchronous HeLa cells resulted in upregulation of K11-linked chains (Figure 4G). This demonstrates that the E2s UbcH10 and Ube2S and the E3 APC/C are the major contributors to K11-linked polyubiquitin synthesis in mitosis and underscores the importance of K11-linked chain formation by the APC/C.

Consistent with the critical role of the APC/C in assembling K11-linked ubiquitin chains, we found this chain type to be highly enriched in affinity-purified APC/C (Figure 4H). To directly test whether APC/C assembles K11-linked chains, we investigated the kinetics of K11-linked polyubiquitin synthesis in cell extracts where APC/C activity can be controlled by addition of activator or inhibitor proteins. In synchronized HeLa cell lysates, where the APC/C is relatively inactive due to the spindle assembly checkpoint, long K11-linked chains were formed, and addition of APC/C-inhibitors UbcH10^{C114S} and Emi1 proved that chain formation was APC/C dependent (Figure 4I). When p31 and UbcH10 were added to fully activate the APC/C, cyclin B1 was degraded and there was a dramatic increase in the abundance of K11-linked chains (Figure 4I). The abundance of K48-linked ubiquitin chains was not altered by these treatments. More detailed kinetic analysis revealed that K11-linked chains formed in two distinct phases (Figure 4J), both of which depended on APC/C activity (Figure 4I). Together, our K11 linkage-specific antibody allowed us to demonstrate that APC/C assembles K11-linked chains in extracts as well as in cells. As activation of the APC/C, a single ubiquitin ligase, led to a dramatic increase in the bulk of K11-linked chains, our experiments suggest that the APC/C is a major source for this chain type in human cells.

DISCUSSION

We describe the crystal structure of K11-linked diubiquitin and demonstrate that it can adopt a conformation distinct from diubiquitins of other linkages. While the structure has captured a single conformation, it is likely that there is some flexibility in the diubiquitin such that other conformations could be adopted, as seen with K48-linked chains (Eddins et al., 2007; Varadan et al., 2002). Given its unique structure, it is likely that K11-specific binding proteins and DUBs will be identified.

Analysis of the crystal-packing contacts suggests how K11-linked tetraubiquitin might be formed (Figure S1C). Two diubiquitins in the asymmetric unit are packed in a single plane with their free K11 and C termini pointing toward each other. Tetraubiquitin could be formed by linking the four adjacent ubiquitin subunits into a ring. This arrangement represents a structure distinct from K48-linked tetraubiquitin (PDB 2O6V), where the first two ubiquitins are oriented 90° relative to the last two ubiquitins. Longer K11-linked chains could be formed with multiple rings stacked against each other, forming a coil-like structure. Each turn of the coil would be made up of four ubiquitins, and the connectivity between the turns would occur at the center of the coil.

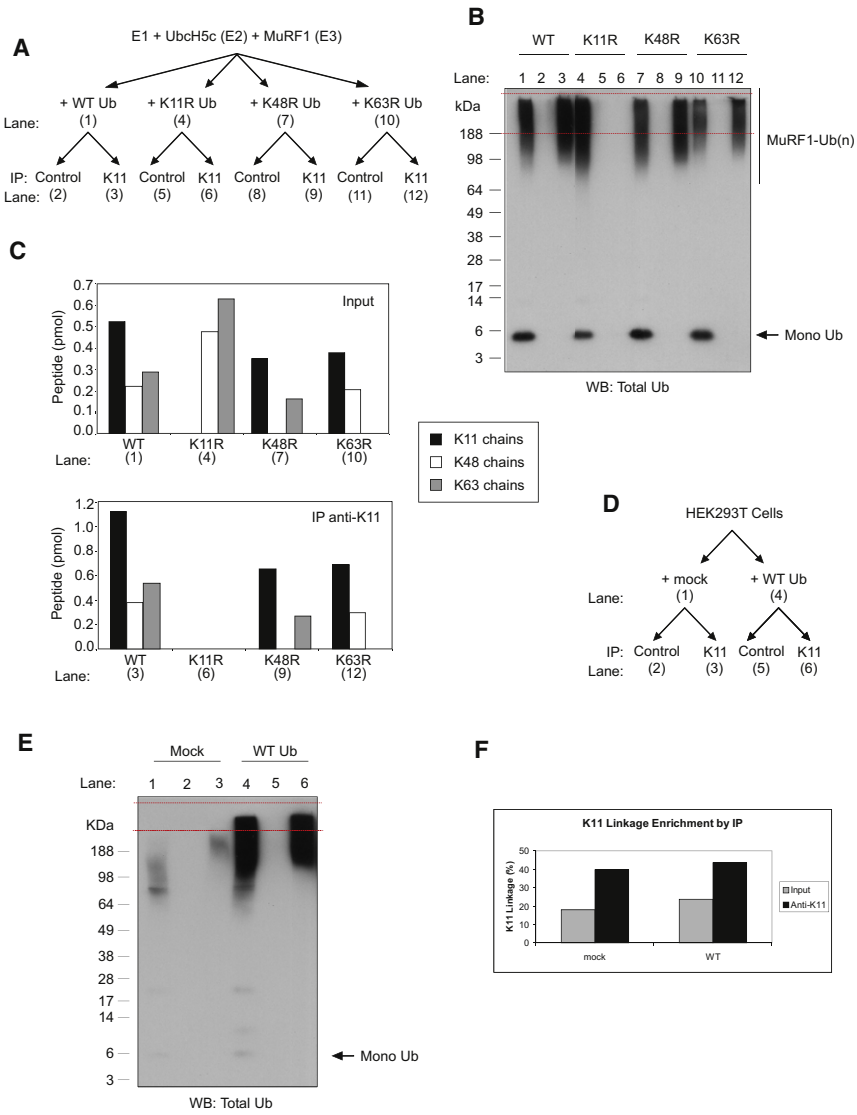

Figure 3. The 2A3/2E6 Antibody Is Specific for K11-Linked Polyubiquitin in Immunoprecipitations

(A–C) MuRF1 autoubiquitination reactions with WT, K11R, K48R, or K63R ubiquitin were immunoprecipitated with 2A3/2E6 (K11) or an isotype control antibody and then analyzed by western blot with a pan-ubiquitin antibody (B) and mass spectrometry (C). Numbers in parentheses in (A) indicate relevant lanes and columns in (B) and (C). Red lines in (B) indicate the region excised for mass spectrometry.

(D–F) Cell lysates from HEK293T cells either mock transfected or transfected with WT human ubiquitin were immunoprecipitated with 2A3/2E6 (K11) or an isotype control antibody and then analyzed by western blot with a pan-ubiquitin antibody (E) and mass spectrometry (F). Numbers in parentheses in (D) indicate the relevant lanes in (E). Red lines in (E) indicate the region excised for mass spectrometry. The percentage of K11 linkages relative to total ubiquitin linkages measured in the cell lysates (input) and immunoprecipitations (anti-K11) are compared (F). See also Figure S3.

The unique conformation adopted by K11-linked diubiquitin prompted efforts to engineer a K11 linkage-specific antibody using a phage display strategy. Specificity was ensured through counter-selection with monoubiquitin and alternative-linkage forms of polyubiquitin. The resulting antibody, 2A3/2E6, should prove critical for studying K11-linked polyubiquitination not only in mitosis but also other cellular pathways.

When we analyzed K11-linked ubiquitin chain abundance in human cells with 2A3/2E6, we recognized a striking cell-cycle-dependent regulation of chain assembly. While K11-linked chains were relatively low in asynchronous cells, they increased significantly as cells exited mitosis. As this is the time of the cell cycle when the majority of substrates of the ubiquitin ligase APC/C are degraded (Peters, 2006), our results suggest an unexpectedly strong link between activation of a single E3, the APC/C, and formation of K11-linked chains.

K11-linked chains were recently proposed to drive the degradation of APC/C substrates during mitosis (Jin et al., 2008; Williamson et al., 2009). Switching APC/C on and off in extracts, we confirmed these earlier findings, which were largely based on the use of ubiquitin mutants. Similar to the experiments in cells, the extract assays suggested that the APC/C is a major E3 for K11-linked chains during mitosis, a finding we confirmed by depleting APC/C subunits from cells. In addition, the E2s UbcH10 and Ube2S have to date only been shown to collaborate with the APC/C, suggesting it is their major, if not only, partner E3. Loss of *UbcH10* and *Ube2S* ablates APC/C activity in cells (Williamson et al., 2009; Song and Rape, 2010) (Figure 4D) and, strikingly, also abrogates the upregulation of mitotic K11-linked chain formation. Interestingly, both APC/C-specific E2s are tightly cell-cycle regulated. Ube2S binds to the APC/C coactivator Cdc20 in early mitosis and to Cdh1 in late mitosis and early G1, and this interaction is required for Ube2S activity (Williamson et al., 2009). In addition, both UbcH10 and Ube2S undergo APC/C-dependent degradation during G1, which occurs subsequent to the degradation of most, if not all, known APC/C substrates (Rape and Kirschner, 2004; Williamson et al., 2009). As seen here, the time window of UbcH10 and Ube2S activity on APC/C coincides with the time in the cell cycle when most K11-linked chains are formed. Together, our observations support the surprising view that a single ubiquitin ligase, the APC/C, is a major source of K11-linked chain formation in cells. The APC/C can intimately regulate K11-linked chain formation by controlling both K11-specific E2s, UbcH10 and Ube2S.

Our results demonstrate that K11- and K48-linked chains differ in many aspects, including their abundance, regulation,

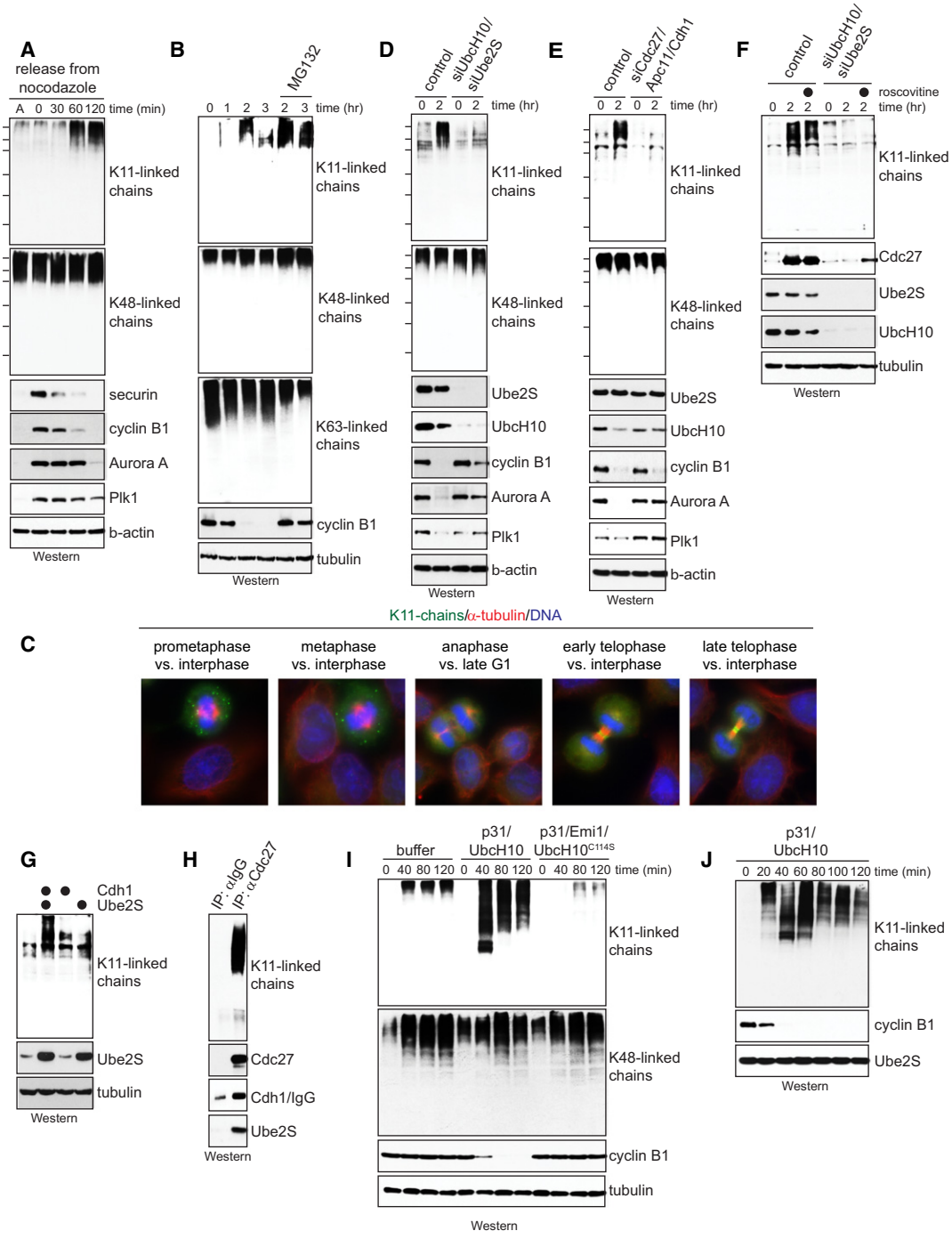

Figure 4. The 2A3/2E6 Antibody Reveals Cell-Cycle Regulation of K11-Linked Polyubiquitination

(A) Western blots of asynchronous (A) or synchronized and released HeLa cells.

(B) Western blots of synchronized and released HeLa cells with or without MG132.

(C) Immunofluorescence microscopy of HeLa cells. K11-linked polyubiquitin staining is shown in green, α-tubulin in red, and Hoechst DNA staining in blue.

(D) Western blots of synchronized and released control or *UbcH10*/*Ube2S* siRNA-transfected HeLa cells.

(E) Western blots of synchronized and released control or *Cdc27*/*Apc11*/*Cdh1* siRNA-transfected HeLa cells.

(F) Western blots of synchronized and released control or *UbcH10*/*Ube2S* siRNA-transfected HeLa cells, with or without roscovitine treatment.

(G) Western blots of asynchronous HeLa cells mock transfected or overexpressing Cdh1 and Ube2S.

(H) Western blots of immunoprecipitations with an anti-Cdc27 or isotype control IgG from synchronized HeLa cells 2 hr after release.

(I and J) Western blots of synchronized HeLa cell extracts incubated with buffer alone, p31 and UbcH10, or p31, Emi1, and $UbcH10^{C114S}$ for the times indicated.

synthesis, and structure. However, chains of both topologies promote degradation by the 26S proteasome, which raises the important question of why the APC/C assembles K11-linked chains, especially to the extent seen here. Human APC/C likely targets more than a hundred substrates for degradation, and we speculate that dedicating a specific ubiquitin chain type for this overburdened E3 might allow this degradation to be completed in the short time span of mitosis and early G1 (Rape et al., 2006). It is also possible that K11-linked chains support functions other than proteasomal degradation, which may be required for APC/C activity at certain locations or cell cycle stages. Our structure implies that specific, yet-to-be identified proteins recognize K11-linked chains, and our K11-specific antibody provides a powerful tool for their identification.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for more detail.

K11-Linked Diubiquitin

K11-linked polyubiquitin was produced using a modification of the procedure described by Pickart and Raasi (2005). Purified diubiquitin was crystallized in 0.2 M ammonium sulfate and 20% polyethylene glycol 3350. The structure was solved by molecular replacement.

Phage Display

Phage display libraries were generated and sorted as described previously (Lee et al., 2004) with later rounds containing soluble monoubiquitin, linear diubiquitin, K48-linked polyubiquitin, and K63-linked polyubiquitin for counter-selection. Surface plasmon resonance (SPR), Fab conversion to IgGs, and purification were done as before (Newton et al., 2008).

Western Blots with the 2A3/2E6 Antibody

Proteins were separated by SDS-PAGE and transferred at 30 V for 2 hr by wet transfer in 10% methanol to nitrocellulose. Nonspecific binding sites were blocked with 5% milk/PBS + 0.05% Tween 20 (PBST) and then incubated with 1 μg/ml 2A3/2E6 IgG in 5% milk/PBST for 1 hr at 25°C. Signal was detected by a goat anti-human peroxidase-conjugated $F(ab')_2$ secondary (Jackson ImmunoResearch).

MuRF1 Autoubiquitination and Immunoprecipitation

MuRF1 autoubiquitination reactions and immunoprecipitations were carried out as previously described (Newton et al., 2008). All immunoprecipitations with 2A3/2E6 were done in the presence of 4 M urea.

Mass Spectrometry

Mass spectrometry was performed as described previously (Blankenship et al., 2009; Kirkpatrick et al., 2006).

Immunofluorescence Microscopy

HeLa cells were methanol fixed, PBST permeabilized, Abdil blocked, and incubated with anti-K11 and anti-α-tubulin antibodies overnight at 4°C. After washing, cells were incubated with fluorescently labeled secondary antibodies and mounted on slides with ProLong Gold Antifade (Invitrogen).

Extract Experiments

Extracts of HeLa S3 cells synchronized in mitosis by thymidine/nocodazole arrest were prepared as described (Jin et al., 2008). The mitotic extracts were activated by addition of UbcH10, $p31^{comet}$, ubiquitin, and energy mix, as indicated. Reactions were incubated for 2 hr at room temperature, stopped by addition of gel loading buffer, and analyzed by western blotting using antibodies against K11-linked chains, cyclin B1, or Ube2S.

ACCESSION NUMBERS

Coordinates have been deposited in the Protein Data Bank (code 3NOB).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, three figures, and one table and can be found with this article online at doi:10.1016/j.molcel.2010.07.001.

ACKNOWLEDGMENTS

We thank G. Fuh for the VH Fab phage display library, W. Wang for X-ray data collection, I. Wertz for helpful discussions, and K. Newton for editorial assistance. M. Matsumoto, K. Dong, C. Yu, I. Bosanac, D. Bustos, L. Phu, D. Kirkpatrick, S. Hymowitz, R. Kelley, and V. Dixit are all employees of Genentech, Inc.

Received: April 2, 2010
Revised: June 2, 2010
Accepted: June 29, 2010
Published online: July 22, 2010

REFERENCES

Blankenship, J.W., Varfolomeev, E., Goncharov, T., Fedorova, A.V., Kirkpatrick, D.S., Izrael-Tomasevic, A., Phu, L., Arnott, D., Aghajan, M., Zobel, K., et al. (2009). Ubiquitin binding modulates IAP antagonist-stimulated proteasomal degradation of c-IAP1 and c-IAP2(1). Biochem. J. *417*, 149–160.

Cook, W.J., Jeffrey, L.C., Carson, M., Chen, Z., and Pickart, C.M. (1992). Structure of a diubiquitin conjugate and a model for interaction with ubiquitin conjugating enzyme (E2). J. Biol. Chem. *267*, 16467–16471.

Datta, A.B., Hura, G.L., and Wolberger, C. (2009). The structure and conformation of Lys63-linked tetraubiquitin. J. Mol. Biol. *392*, 1117–1124.

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

Dikic, I., Wakatsuki, S., and Walters, K.J. (2009). Ubiquitin-binding domains - from structures to functions. Nat. Rev. Mol. Cell Biol. *10*, 659–671.

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.

Garnett, M.J., Mansfeld, J., Godwin, C., Matsusaka, T., Wu, J., Russell, P., Pines, J., and Venkitaraman, A.R. (2009). UBE2S elongates ubiquitin chains on APC/C substrates to promote mitotic exit. Nat. Cell Biol. *11*, 1363–1369.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Jin, L., Williamson, A., Banerjee, S., Philipp, I., and Rape, M. (2008). Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell *133*, 653–665.

Kim, H.T., Kim, K.P., Lledias, F., Kisselev, A.F., Scaglione, K.M., Skowyra, D., Gygi, S.P., and Goldberg, A.L. (2007). Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. J. Biol. Chem. *282*, 17375–17386.

Kirkpatrick, D.S., Hathaway, N.A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R.W., and Gygi, S.P. (2006). Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nat. Cell Biol. *8*, 700–710.

Komander, D. (2009). The emerging complexity of protein ubiquitination. Biochem. Soc. Trans. *37*, 937–953.

Komander, D., Reyes-Turcu, F., Licchesi, J.D., Odenwaelder, P., Wilkinson, K.D., and Barford, D. (2009). Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Rep. *10*, 466–473.

Lee, C.V., Liang, W.C., Dennis, M.S., Eigenbrot, C., Sidhu, S.S., and Fuh, G. (2004). High-affinity human antibodies from phage-displayed synthetic Fab libraries with a single framework scaffold. J. Mol. Biol. *340*, 1073–1093.

Newton, K., Matsumoto, M.L., Wertz, I.E., Kirkpatrick, D.S., Lill, J.R., Tan, J., Dugger, D., Gordon, N., Sidhu, S.S., Fellouse, F.A., et al. (2008). Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. Cell *134*, 668–678.

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

Phillips, C.L., Thrower, J., Pickart, C.M., and Hill, C.P. (2001). Structure of a new crystal form of tetraubiquitin. Acta Crystallogr. D Biol. Crystallogr. *57*, 341–344.

Pickart, C.M., and Raasi, S. (2005). Controlled synthesis of polyubiquitin chains. Methods Enzymol. *399*, 21–36.

Potapova, T.A., Daum, J.R., Pittman, B.D., Hudson, J.R., Jones, T.N., Satinover, D.L., Stukenberg, P.T., and Gorbsky, G.J. (2006). The reversibility of mitotic exit in vertebrate cells. Nature *440*, 954–958.

Rape, M., and Kirschner, M.W. (2004). Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry. Nature *432*, 588–595.

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.

Reyes-Turcu, F.E., Ventii, K.H., and Wilkinson, K.D. (2009). Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu. Rev. Biochem. *78*, 363–397.

Schulman, B.A., and Harper, J.W. (2009). Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. Nat. Rev. Mol. Cell Biol. *10*, 319–331.

Skaug, B., Jiang, X., and Chen, Z.J. (2009). The role of ubiquitin in NF-kappaB regulatory pathways. Annu. Rev. Biochem. *78*, 769–796.

Song, L., and Rape, M. (2010). Regulated degradation of spindle assembly factors by the anaphase-promoting complex. Mol. Cell *38*, 369–382.

Tenno, T., Fujiwara, K., Tochio, H., Iwai, K., Morita, E.H., Hayashi, H., Murata, S., Hiroaki, H., Sato, M., Tanaka, K., and Shirakawa, M. (2004). Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains. Genes Cells *9*, 865–875.

Varadan, R., Walker, O., Pickart, C., and Fushman, D. (2002). Structural properties of polyubiquitin chains in solution. J. Mol. Biol. *324*, 637–647.

Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C., and Fushman, D. (2004). Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. J. Biol. Chem. *279*, 7055–7063.

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.

Wu, T., Merbl, Y., Huo, Y., Gallop, J.L., Tzur, A., and Kirschner, M.W. (2010). UBE2S drives elongation of K11-linked ubiquitin chains by the anaphase-promoting complex. Proc. Natl. Acad. Sci. USA *107*, 1355–1360.

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