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Supplemental Information

Dual RING E3 Architectures Regulate Multiubiquitination and Ubiquitin Chain Elongation by APC/C

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Supplemental Experimental Procedures

Protein purification

For ubiquitination assays, human APC/C, CDH1, UBA1, wild type and mutant versions of APC2–APC11, APC11 RING domain (residues 17-84), UBE2C, UBE2S, $CycB^N$ (residues 1-95), UB-$CycB^N$ (UB-fused to N-terminus of $CycB^N$), UB-Securin, and Hsl1 (residues 768-842) were expressed largely as described (Brown et al., 2015; Brown et al., 2014; Jarvis et al., 2016), with some minor differences. APC/C was expressed from a baculovirus expression construct system allowing relatively facile incorporation of mutants (Weissmann et al., 2016). The proteins used in the $SCF^{FBW7\Delta D}$ - and WWP1-dependent ubiquitination assays were expressed and purified as previously described (Scott et al., 2014; Zhang et al., 2016).

For all experiments other than EM, APC/C, mutants and the APC1–APC2–APC4–APC5–APC11 subcomplex were purified with a 3-step scheme: 1) affinity purification with Strep-Tactin Sepharose (IBA Life Sciences) and elution with desthiobiotin (Sigma-Aldrich), 2) anion exchange with gradient NaCl elution, and 3) size exclusion chromatography (SEC) in a final buffer of 20 mM HEPES pH 8.0, 200 mM NaCl, 1 mM DTT. Because some APC11 mutants are difficult to observe by SDS-PAGE during protein purification owing to small size (e.g. APC11 ΔRING has only 17 residues), all experiments examining APC11 mutants used MBP fusions, including the wild type counterpart. This allowed confirming stoichiometric inclusion of MBP-APC11 using Coomassie-stained SDS-PAGE gels. Based on a previous alanine scanning study of APC11 RING domain in the context of APC/C^{CDH1} (Brown et al., 2014), the RING exo mutant harbors M57A and F82A mutations and the RING cE2 mutant harbors an R27A mutation. APC2–APC11 subcomplexes #s 2, #3, and #4 in **Fig. 5H** were obtained by coexpressing APC11 and full-length APC2, APC2 residues 446-822 (C/R + WHB domains but referred to in main text as C/R owing to WHB being dispensable for UB chain elongation by UBE2S (Brown et al., 2014)), or APC2 residues 549-822 (C/R + WHB domains with the SiA-helix deleted) as previously described (Brown et al., 2014; Jarvis et al., 2016).

CDH1 was expressed with a HRV13 3C protease cleavable N-terminal 3x MYC-tag using a baculovirus expression system, and purified by nickel affinity chromatography (Sigma-Aldrich). After HRV13 3C-mediated proteolytic cleavage, the 3X MYC-His_6 tag was removed by purifying CDH1 by cation exchange chromatography and SEC in a final buffer of 20 mM HEPES pH 7.0, 300 mM Ammonium Sulfate, 1 mM DTT, 2.5% Glycerol.

Wild type and mutant versions of UBE2C were expressed in BL21(DE3) Codon Plus (RIL) cells. For assaying ubiquitination and hydrolysis of oxyester-linked UBE2C~UB, wild type and C114S (active site mutant) versions were purified by nickel affinity chromatography based on a flush C-terminal His_6 tag. Wild type UBE2C was purified by SEC in 20 mM HEPES pH 8.0, 200 mM NaCl, 1 mM DTT. The UBE2C~UB oxyester-linked complex used to assay hydrolysis was generated as previously described (Brown et al., 2015). In short, we mixed UBE2C C114S, UBA1, $MgCl_2$, ATP, and UB at concentrations of 560 μM, 2 μM, 5 mM, 5 mM, and 3 mM, respectively, at 30°C overnight. The oxyester-linked UBE2C~UB complex was separated from other reaction components by SEC. For cross-linking, UBE2C harboring a single Cys only at the active site (C102A) was expressed and purified via either a C-terminal flush Strep tag or a C-terminal HRV13 3C protease-cleavable Flag-Strep tag. After the initial purification using Strep-Tactin Sepharose, the protein was then used in the cross-linking reactions described below. The UBE2C-UBE2S CTP chimera had residues 157-222 from UBE2S fused at the C-terminus of UBE2C, as described (Chang et al., 2015), allowing purification by cation exchange chromatography from a cell lysate and subsequent polishing by SEC.

Unless otherwise specified, WT and mutant versions of UBE2S were expressed with an N-terminal multipurpose composite tag in BL21(DE3) Codon Plus (RIL) cells from a modified pRSF duet vector. The tag consisted of an N-terminal His_6 tag, followed by a TEV protease cleavage site, a FLAG tag, and an HRV13 3C protease site. UBE2S and variants were purified by nickel affinity chromatography, subsequently treated with HRV13 3C protease, and purified by cation exchange. For broad mutagenesis screens of UBE2S, such as suite of substitutions for C118 used to identify suitable replacements in a single catalytic Cys only version of UBE2S **(Fig. S2H)**, or the linker deletion series **(Fig. 5I)**, wild type and mutant versions of UBE2S were purified by cation exchange prior to assaying APC/C-dependent ubiquitination activity. The series of UBE2S constructs removing subsets of the 56 linker residues between the UBC domain (residues 1-156) and the CTP sequence (residues 213-222) named Linker 51, Linker 46, Linker 36, Linker 26, Linker 15, Linker 6, and Linker -6 (this deletion includes part of the CTP) correspond

to deletion of residues 182-186, 178-187, 168-187, 161-190, 161-201, 157-206, 157-218, respectively **(Fig. 5I)**. The UBE2S Linker -6 variant could not be purified by cation exchange due to the deletion of 13 strongly basic residues and was instead purified nickel affinity chromatography followed by SEC. Wild type and mutant variants of UBE2S used for kinetic assays, and those selected from broad mutagenesis screening for follow-up studies, were subjected to a third purification step, SEC in a final buffer of 20 mM HEPES pH 8.0, 200 mM NaCl, 1 mM DTT. Two different versions of UBE2S were used for EM studies. For our initial attempts to trap a complex representing UBE2S in action, UBE2S catalytic Cys only (C118A) was expressed with a His_6-MBP-TEV protease site-fused to the N-terminus, and purified by nickel affinity chromatography, treated with TEV protease, further purified by cation exchange chromatography, used in cross-linking reactions described below, an analyzed by negative stain EM. The version of UBE2S used in the cryo EM reconstruction contained only a single Cys at the active site, with C118F based on optimization shown in **Fig. S2H**.

For assays comparing WT UBv, UBv mutant (I42D F44D L68D), and UB, these were expressed in BL21(DE3) Codon Plus (RIL) cells from a modified pRSF duet vector with a TEV protease-cleavable N-terminal His_6-tag. The proteins were purified by nickel affinity chromatography, subjected to TEV-mediated proteolytic cleavage to liberate the tag and purified by SEC in a final buffer of 20 mM HEPES pH 8.0, 200 mM NaCl.

UB was expressed in BL21(DE3) Codon Plus (RIL) cells as previously described (Brown et al., 2014). UB used as a donor in substrate ubiquitination assays and as an acceptor in **Figures 5F and S5G** was purified as described previously (Pickart and Raasi, 2005). Methylated UB was used as a donor in **Figures 1C, 2E, 6F-G,S4B, S4E, S4G, S4I-J, and S6F-G** was reductively methylated as previously described (White and Rayment, 1993). The fluorescent donor UB (*UB) for experiments in **Figures 5F and S5G** and the fluorescent acceptor UB (UB*) for experiments in **Figures 5H, 6F-G, S2D, S2L, S5I-K and S6F-G** were expressed as TEV-cleavable N-terminal GST fusions, purified by glutathione-affinity chromatography, treated with TEV to liberate the GST tag, and polished by SEC. The fluorescent donor and acceptor UBs contain a single cysteine at either position -1 or at 77 (G75S G76S C77), respectively, for fluorescein-5-maleimide or Cyanine5 maleimide labeling as previously described (Brown et al., 2014).

The substrates in ubiquitination assays - $CycB^N$, UB-$CycB^N$, and UB-Securin - were purified as previously described (Jarvis et al., 2016; Yamaguchi et al., 2015). Briefly, the substrates were expressed as N-terminal GST-TEV and C-terminal Cys-His_6 fusions in BL21(DE3) Codon Plus (RIL) cells. The substrates were then purified by glutathione-affinity chromatography, treated with TEV protease to liberate GST, and further purified by nickel-affinity chromatography. The substrates were then labeled with fluorescein-5-maleimide (denoted by an asterisk) as previously described (Brown et al., 2014). Free label was removed by desalting and SEC. For CycB mutants with fewer ubiquitination sites, Lys-to-Arg substitutions were made **(Figs. 1C, 6G, and S4D-J)**. These substrates were expressed with an additional modified N-terminal Strep-tag sequence, GGGSAWSHPQFEGGS, following the TEV proteolysis site (ENLYFQG) and contain only residues 1-88 of CycB. To remove degradation products that are more prevalent when multiple arginines replace existing lysines, an additional purification with Strep-Tactin Sepharose was used instead of SEC after the labeling reaction. For the single ubiquitination site mutant **(Figs. 1C and S4I-J),** the N-terminus of $CycB^N$ was blocked using a sortase-mediated reaction as follows. After TEV-mediate cleavage, 10 μM $CycB^N$ was subjected to 150 nM Sortase and 40 μM Fluorescent-LPETGG peptide in a buffer of 20 mM HEPES pH 8, 50 mM NaCl, and 10 mM $CaCl_2$. After a 2 hour incubation at 4°C, the reaction was quenched with 20 mM EGTA and then purified by Strep-Tactin Sepharose and SEC.

APC11 RING (residues 17-84) and RBX1 RING (residues 36-108) domains were expressed with a TEV-cleavable, N-terminal GST-tag in BL21(DE3) Codon Plus (RIL) cells from a modified pGEX4T1 vector. GST-tagged RING domains purified by glutathione affinity chromatography and SEC and were used for phage display selection and Bio-Layer Interferometry (BLI) experiments.

The APC11-UBv complex for crystallization was purified after coexpression of TEV-cleavable GST-tagged APC11 RING (residues 17-84) and TEV-cleavable His_6-tagged UBv in BL21(DE3) Codon Plus (RIL) cells from modified pGEX4T1 and pRSF duet vectors, respectively. The complex was purified by glutathione affinity chromatography, followed TEV-mediated proteolysis. Subsequent purification steps included dialysis, removal of GST with Glutathione Sepharose, and SEC. The final SEC buffer was 50 mM TRIS pH 7.6, 200 mM NaCl, 1 mM DTT.

For cryo EM, the "substrate" used in the Multiubiquitination trap, $Hsl1^P$(K788C)-UBv was expressed in BL21(DE3) Codon Plus (RIL) cells from a modified pGEX4T1 vector containing a TEV

protease site. After purification by glutathione affinity chromatography, the GST-fusion was treated with TEV protease during overnight dialysis, and free GST was then removed by glutathione affinity chromatography and SEC. The "substrate" used in the UB chain Elongation trap, a UBv (K11C)-Hsl1 D-box fusion, was expressed in BL21(DE3) Codon Plus (RIL) cells from a modified pRSF duet vector with an N-terminal TEV-cleavable His_6-tag. In an initial attempt to visualize UB chain elongation, using negative stain EM and APC/C purified from HeLa cells as described below, the "substrate" His_6-UB(K6R, K11C, K27R, K29R, K33R, K48R, K63R)-Hsl1 (residues 768-842) was expressed in BL21(DE3) Codon Plus (RIL) cells and purified by nickel affinity chromatography prior to crosslinking.

Proxies for "Donor UB" were Flag-UB (1-74, G75C), except the initial attempt to visualize UB chain elongation without a UBv acceptor used Flag-HRV13 3C protease site-UB (1-74, G75C),and were expressed as N-terminal GST fusions. Following glutathione affinity purification and TEV-treatment during overnight dialysis to remove glutathione, the GST-tag was removed by Glutathione Sepharose (GE Life Sciences) chromatography. These proteins were then used in the cross-linking reactions described below. Flag-UB (1-75, G76C) used in disulfide-linked proxies for E2~UB intermediates was purified similarly.

UBv selection

The phage-displayed UBv library used in this study was re-amplified from Library 2 as previously described (Ernst et al., 2013). Protein immobilization and subsequent phage selections were done according to established protocols (Tonikian et al., 2007). Briefly, purified GST-APC11 RING fusion was coated on 96-well MaxiSorp plates (Thermo Scientific 12565135) by adding 100 µL of 1 µM proteins and incubating overnight at 4ºC. Afterwards, five rounds of selections using the phage displayed UBv library were performed against immobilized proteins. A total of 96 phage clones obtained from the fourth and the fifth round of binding selections (48 from each round) were subjected to clonal ELISA to identify individual phages with improved binding properties against 53BP1. Afterwards, UBv sequences were derived through phagemid DNA sequencing (Tonikian et al., 2007). For phage ELISA, proteins in study were immobilized on 384-well MaxiSorp plates (Thermo Scientific 12665347) by adding 30 µL of 1 µM proteins for overnight incubation at 4ºC before adding overnight amplified phages (1:3 dilution in PBS+1%BSA+0.05%Tween). Binding of phage was detected using anti-M13-HRP antibody (GE Healthcare 27942101). The ELISA and sequencing identified a single APC11 RING-binding UBv, with sequence differences from UB shown in **Fig. 2A**.

X-ray Crystallography

The APC11 RING–UBv complex was mixed with reservoir solution at a 1:1 volume:volume ratio for crystallization by the hanging-drop vapor diffusion method. The reservoir solution contained 0.2 M Ammonium acetate, 0.1 M Sodium acetate pH 4.6, 33% PEG4000. For cryoprotection during the flash-freezing in liquid nitrogen, the crystals were harvested in the reservoir solution supplemented with 24% glycerol. Diffraction data were processed with HKL2000 (Otwinowski and Minor, 1997). The structure was determined by molecular replacement using Phaser with APC11 [Protein Data Bank (PDB) ID 4R2Y] and UB (1-70) (PDB ID 1UBQ) as search models (Brown et al., 2014; Vijay-Kumar et al., 1987). Model construction and refinement was performed using COOT, Refmac5, and Phenix (Adams et al., 2010; Emsley et al., 2010; Murshudov et al., 1997). Diffraction data and refinement statistics are provided in **Supplemental Table 1**.

Generation of 3-way crosslinked complexes to trap APC/C^{CDH1}–E2–UB-linked substrate assemblies representing multiubiquitination and UB chain elongation

Ever since our initial efforts to visualize UBE2C and UBE2S bound to APC/C indicated tremendous conformational variability, we and others recognized that incorporating additional stabilizing interactions would be required (Brown et al., 2015; Chang et al., 2015). We turned toward 3-way crosslinking, with the goal of avidly capturing multisite interactions for the catalytic core, which is highly dynamic in CDH1-activated APC/C (Chang et al., 2014). Our initial attempt to visualize placement of UBE2S utilized UB with K11C and other lysines mutated to arginines. Extremely high concentrations of this version of UB can slightly inhibit UB chain elongation. Thus, the initial attempt at an "Elongation trap" contained Strep-UBE2S (C118A) as E2, HRV13 3C protease-cleavable Flag-HRV13 3C protease UB (1-74,G75C) to represent the donor UB, and His_6-UB (K6R, K11C, K27R, K29R, K33R, K48R, K63R)-Hsl1 (residues 768-842) to represent substrate (sequence:

MGSSHHHHHHSQDPGGGSMQIFVRTLTGCTITLEVEPSDTIENVRARIQDREGIPPDQQRLIFAGRQLEDGRTLSDYNIQRESTLHLVLRLRISGVSTNKENEGPEYPTKIEKNQFNMSYKPSENMSGLSSFPIFEKENTLSSSYLEEQKPKRAALSDITNSFNKMN). After 3-way crosslinking, this was added to an IP of APC/C from HeLa cell lysate and recombinant CDH1, and the complex was purified as described previously for APC/C^{CDH1} complexes with EMI1 (Frye et al., 2013). The reconstruction determined by negative-stain EM for the first generation complex is shown in **Movie S2**. While this initial studies enabled localizing UBE2S's UBC domain as binding the APC2–APC11 C/R domain even in the absence of a UBv, obtaining complex suitable for cryo EM involved numerous improvements, including anchoring the mobile RING domain with the UBv.

For the samples used for structure determination by cryo EM, the three-way cross-linked complexes contain E2, a "donor UB", and a surrogate for a polyubiquitination substrate, to "trap" APC/C^{CDH1} architectures for multiubiquitination and UB chain elongation. To visualize Multiubiquitination, the trap contained UBE2C (C102A)-Strep as E2 with only a Cys at the active site, Flag-UB (1-74, G75C) to represent donor UB, and Hsl1^{P}(K788C)-UBv to represent substrate (sequence: GSRENELSAGLSKRKHRGSGSGSGSISGVSTNKENEGPEYPTKIECYLEEQKPKRAALSDITNSFNKMNSGSSGSGSSGMQILVKTPRGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQILFFAVKRLEDGRTLSDYNIQKKSSLLLAMRVPGKMK).

To visualize UB chain elongation, the trap contained UBE2S Linker15 (C118F) as E2 with only a Cys at the active site, Flag-UB (1-74, G75C) to represent the donor UB, and UBv-Hsl1 D-box (K11C) to represent substrate (sequence: GSGGSMQILVKTPRGCTITLEVEPSDTIENVKAKIQDKEGIPPDQQILFFAVKRLEDGRTLSDYNIQKKSSLLLAMRVPGKMKSSYLEEQKPKRAALSDITNSFNKMN).

The Multiubiquitination and UB chain Elongation "traps" were three-way crosslinked using methods similar to those previously described (Kamadurai et al., 2013) except a trifunctional crosslinker containing three sulfhydryl-reactive maleimide groups, TMEA (tris(2-maleimidoethyl)amine, 33043, ThermoFisher Scientific), was used. The proteins were treated with 10 mM DTT for 30 min before they were desalted into 50 mM HEPES pH 7.0, 150 mM NaCl. First, a 5:1 TMEA:UB mixture reacted for 20 min on ice before desalting to remove unreacted TMEA. The final three way product was prepared by reacting the E2 for each trap with this UB at a 1:1 ratio for 20 min on ice and then subsequently adding the third protein that mimics the acceptor lysine at a 2-3 fold molar excess at room temperature (RT). Following a 2-hour incubation of all three proteins, the reaction was quenched with β-mercaptoethanol.

The Multiubiquitination trap was then purified using Strep-Tactin Sepharose and SEC. For use in biochemical assays, a related version of this complex was used with the Strep-tag cleaved from UBE2C by HRV13 3C protease **(Fig. S2A-E)**.

The UB chain elongation trap was purified by cation exchange chromatography and nickel affinity chromatography. The His_6-tag was removed from the complex by TEV-mediated proteolytic cleavage and subsequent SEC.

The initial attempt at a cross-linked complex to represent UB chain elongation, which used a UB rather than UBv mutant, was purified by nickel affinity chromatography and subsequent SEC.

A control complex without the UBv representing Priming, used in **Fig. S2B-D**, was described previously (Brown et al., 2015), except the C-terminal Strep-tag was liberated from UBE2C by HRV13 3C protease.

All cross-linked complexes were ultimately purified by SEC in 20 mM HEPES and 200 mM NaCl. After purification, intact mass spectrometry confirmed correct identity of the three-way cross-linked complex (Hartwell Center for Biotechnology and Bioinformatics, St. Jude Children's Research Hospital, Memphis, TN).

Purification of APC/C^{CDH1} with Multiubiquitination or Elongation traps for cryo EM

Recombinant APC/C^{CDH1} for use in the complex representing multiubiquitination was purified as described previously (Brown et al., 2015). For the complex representing UB chain elongation, APC/C was expressed with HRV13 3C protease-cleavable tags, a Twin-Strep tag at the N-terminus of APC2 and a GST-tag at the N-terminus of APC16, and the complex with CDH1 was purified as previously described (Brown et al., 2015). APC/C^{CDH1} was incubated with the Multiubiquitination or UB chain elongation trap, Anti-FLAG affinity gel (Genscript), and HRV13 3C protease for 1 hour. The resin was thoroughly washed in microspin columns, and complexes eluted by the addition of FLAG peptide.

Cryo Electron Microscopy

For cryo electron microscopic studies of APC/C complexes representing Multiubiquitination or UB chain elongation, 100 µg of purified APC/C was loaded onto a 10%–40% glycerol gradient containing 50 mM HEPES pH 8.0, 200 mM NaCl, 2 mM $MgCl_2$. For particle fixation by GraFix (Kastner et al., 2008), the gradient also contained 0.025% and 0.1% glutaraldehyde in the lighter and denser glycerol solution, respectively, creating an additional glutaraldehyde gradient from top to bottom (0.025–0.1%). Centrifugation was performed at 34,000 rpm in a SW55TI rotor (Beckman) for 15 hr at 4°C. For cryo EM the fractions containing APC/C were subjected to a buffer exchange procedure using Zeba spin columns (Pierce) to remove the sugar prior to EM grid preparation. APC/C particles were allowed to adsorb on a thin film of carbon for 5 min, transferred onto a cryo EM grid (Quantifoil 3.5/1 1 µm, Jena) and then plunged into liquid ethane under controlled environmental conditions of 4 °C and 100% humidity in a vitrification device (Vitrobot Mark IV, FEI Company, Eindhoven). Images were recorded on a Falcon II direct detector under liquid-nitrogen conditions with a Titan Krios electron microscope (FEI, Eindhoven) equipped with a XFEG electron source and a C_s corrector (CEOS, Heidelberg) using 300 kV acceleration voltage. An electron dose of ~40 electrons per $Å^2$, −0.7 to −3.5 µm defocus and a nominal magnification of 94,000× were used, resulting in a final pixel size of ~1.57 Å. The extracted particle images were corrected locally for the contrast-transfer function by classification and averaging of power spectra (Sander et al., 2003). Initial 2D sorting of images was performed based on CTF parameters. Only images showing isotropic Thon rings better than 6 Angstrom were used for further processing. Additional image sorting was performed applying several rounds of multivariate statistics, first without alignment and subsequently after image alignment to remove ice contaminations and bad particle images. The remaining good particle images were used for further processing. 3D classification in RELION 1.2 was used to obtain the particles revealing the highest UBE2S/UBE2C factor occupancy (Scheres, 2012). The best class was then used for the final refinement using the 'gold-standard procedure' in RELION 1.2. The final resolution (6.4 and 6 Å) was calculated applying a mask for the entire APC/C using the FSC 0.143 criterion.

Structure	Picked particles	Remaining particles after 2D sorting	Number of particles in best class after 3D classification in Relion	Resolution without masking (Angstrom)	Resolution with soft mask for the entire APC/C (Angstrom)
Multiubiquitination	784,672	399,597	135,578	7.4	6.4
Elongation	873,870	392,468	125,390	7.2	6.0

For the heat maps, **Fig. S3C**, a sphere of 14 voxel in diameter was run over the entire 3D volume moving it by one voxel in all directions. At each position the calculation of the local FSC values was performed. This then generates the FSC values to generate the heat map and to visualize the local resolution. Local resolution measurements are dependent on the size of the small volume used to calculate the local FSC. The size of the sphere was adjusted to 14 voxel because the mean value of all local correlations agrees with the calculated global resolution.

Docking crystal structures into cryo EM maps representing multiubiquitination and UB chain elongation

Coordinates for APC/C with the mobile catalytic core deleted (from 4UI9.PDB lacking the APC2–APC11 C/R, WHB, and RING domains, (Chang et al., 2015)) were placed as a single unit into the cryo EM maps using Chimera (Pettersen et al., 2004). For the map representing Multiubiquitination, the additional density was fit with structures of three subcomplexes, the APC2–APC11 C/R domain (4UI9.PDB, (Chang et al., 2015)), UBE2C bound to APC2's WHB domain (4YII.PDB (Brown et al., 2015)), and of the APC11 RING-UBv complex **(Fig. 2B)**. For the model representing UB chain elongation, additional density from maps from classes initially calculated to overall 9 Å resolution were segmented into three separate maps, with the density from the best map for each segment fit individually with the corresponding coordinates for the APC2–APC11 C/R domain, the crystal structure of the APC11 RING-UBv complex, and the crystal

structure of UBE2S (Brown et al., 2015; Chang et al., 2015; Lorenz et al., 2016; Pettersen et al., 2004). In the higher resolution map, density corresponding to the N-terminal portion of UBE2S distal from APC2 was lacking, and a truncated version of UBE2S corresponding to residues 46-156 was used for further calculations. The models were rigid body fitted in UCSF Chimera (Pettersen et al., 2004) and then subjected to molecular dynamics flexible fitting (MDFF). The MDFF simulation was run using the Charmm force field applying implicit solvent conditions and using secondary structure, chirality and cis-peptide restraints. First an energy minimization was calculated for 2ps. The simulation was performed for 150 ps using a ξ value of 0.3. Afterwards energy minimization of the resulting model was performed for 2 ps with a ξ of 10 (Trabuco et al., 2008). The resultant models are shown in **Movies S1 and S2**, except that the structural representation for UB chain elongation is shown with the full-length UBE2S in place of the truncated version. To visualize an acceptor Lys relative to UBE2S, the APC11–UBv portion of the map was substituted by isolated APC11 RING domain (and the closest homology model of UB (PDB ID 4RF0)) (Bailey-Elkin et al., 2014; Brown et al., 2014). There is no density in any of the maps corresponding to a donor UB, which is shown to be clash-free in **Fig. S3** for Multiubiquitination based on a prior structure of a RING–E2~UB complex (Dou et al., 2012; Plechanovova et al., 2012), and for UB chain elongation using a model of UBE2S~UB generated based on crystallographic symmetry (Lorenz et al., 2016). The structural data guided biochemical experiments in **Figs. 4-6** and **Figs. S4-S6**, which validate the models. For example, the model for UB chain elongation predicted a charge-swap rescue experiment that confirmed placement of UBE2S, and maintained geometry for UBv's residue 11 and UBE2S's active site imposed by the crosslink despite this not a restraint during molecular dynamics.

Enzyme Assays

"Single encounter" experiments **(Figs. 1C and S4D-J)** were used to examine ubiquitination occurring during a substrate's single binding event with APC/C^{CDH1}. This is monitored using a fluorescently labeled substrate and excess unlabeled Hsl1, such that APC/C^{CDH1} dissociated from the labeled substrate is rapidly sequestered. Two different sets of reaction components were independently made and then mixed to begin the reaction. The first, APC-sub, was made by mixing components such that the final concentrations in the actual reaction were 100 nM APC/C, 400 nM CDH1, and 80 nM fluorescent CycB^N versions for 30 min. The second mix, E2-UB, consisted of components for charging E2 with UB, which in the ultimate reaction mix were at final concentrations of 50 nM E1, 5 μM E2, 5 mM $MgCl_2$, 5 mM ATP, and 80 μM (1000-fold excess) unlabeled Hsl1. UB (or meUB) was added to the E2-UB mix last such that the final concentration was 65 μM. Both sets of components were immediately allowed to warm to room temperature for 3 min and co-mixed to begin the reaction. The ubiquitination reactions were then quenched at 2 min. The "No-encounter" control assay in **Fig. S4D** swaps the labeled CycB^N* and unlabeled Hsl1 but is otherwise performed identically, and shows that unlabeled Hsl1 effectively blocks ubiquitination of labeled CycB^N*.

Qualitative assays probing the function of APC/C and E2 variants were performed with 30 nM APC/C, 1 μM CDH1, 0.2 μM UBE2C or UBE2S (unless otherwise indicated), 0.1 μM E1, 250 μM UB, 0.2 μM CycB^N* or UB-CycB^N* **(Figs. 2D, 2E, 5G, 5I, 6D, S1G, S2B, S2C, S2K, S5C, S5F)**. The assay in **Fig. 2E** was performed similarly except using meUB. **Figs. 2D and 2E** also included UBv titrations from 0.9-25 μM, over 3-fold dilution series. In **Fig. 6B**, the E2 concentrations ranged from 0.26 - 7 μM, over 3-fold dilution series. In **Fig. S2I**, the concentrations of WT and C118F mutant versions of UBE2S ranged from 20 nM to 1.2 μM over 2-fold dilution series. These reactions were quenched at 12 min. Qualitative assays in **Figs. S2H** were performed similarly, except the APC/C concentration is 14 nM and the reaction was quenched at 8 min. The assays monitoring UB transfer to an acceptor UB* **(Figs. 5H, 6F-G, S2D, S2L, S5I-K, and S6F-G)** were performed in similar conditions as assays for UB-CycB^N* ubiquitination assays, except the acceptor substrate was UB-fluorescein (G75S:G76S:C77) and the reaction was quenched at 20 min. Fluorescent labeling of the C-terminal Cys prevents the UB* from conjugating to either the E1 or E2.

Ubiquitination assays monitoring substrate depletion as a measure of Processive Affinity Amplification (PAA) **(Figs. 4E-G)** were performed as described above except concentrations of 40 nM APC/C, 5 μM UBE2C, 0.5 μM E1, 250 μM UB or meUB, and either 6 μM CycB^N* or UB-CycB^N* were used. The reaction mixtures were quenched at the time points indicated. Substrate bands were quantified and normalized to the reaction using the negative control APC/C$^{\Delta RING}$ mutant. Product bands were quantified and APC/C$^{\Delta RING}$ products were subtracted as background. Reactions that were subjected to mass spectrometry **(Fig. 4D and S4B)** analysis were performed similarly except 80 nM APC, 100 nM E1, and 20

μM CycB^{N}* were used and quenched at 30 min. unless otherwise indicated.

In kinetic experiments, apparent K_m (K_m^{app}) and apparent V_{max} (V_{max}^{app}) values were determined by fitting the initial velocities to the hyperbolic Michaelis-Menten, $v = V_{max}^{app}[X]/(K_m^{app}+[X])$, equation, where X is either the UBE2C, UBE2S, or ^{Acceptor}UB concentration, using GraphPad Prism 6 software. Single time points were taken under conditions that satisfy initial velocity regimes. In summary, a time course was monitored at both the minimum and maximum point of each titration to ensure a single time point could be taken where the substrate or UBE2S~UB depletion are minimal and product formation remained linear. The activity for each quantitative assay was normalized to the V_{max}^{app} of wild type UBE2C or UBE2S with APC/C^{CDH1}.

The kinetic parameters, K_m^{app} and V_{max}^{app} values, were determined for UBE2C **(Figs. S1E-F)** and UBE2S **(Figs. 5C, 5E and S5D-E)** in assays monitoring substrate polyubiquitination with APC^{CDH1} to evaluate E2–APC/C binding and the maximum rate of ubiquitination. Concentrations of 10 nM APC/C variant, 50 nM or 1 μM E1, 65 μM or 250 μM UB, and 200 nM UB-CycB^{N}* or 1 μM UB-Securin* were used and the reactions were quenched after 6 min. or 10 min. for UBE2C and UBE2S, respectively. In **Fig. S1E and S1F**, the UBv concentration was 50 μM. These data were normalized to the V_{max}^{app} of wild type UBE2C–APC/C^{CDH1} or UBE2S–APC/C^{CDH1}, respectively.

The K_m^{app} and V_{max}^{app} values for the acceptor UB were determined to evaluate the effects of APC2 mutants on APC/C^{CDH1}-dependent stimulation of UBE2S-mediated di-ubiquitin synthesis, **(Figs. 5F and S5G)**. The N-terminally labeled wild type UB (20 μM *UB) was first loaded onto 10 μM E1 in the presence of 5 mM $MgCl_2$ and 5 mM ATP for 10 min at RT. Formation of the E1~*UB intermediate was quenched with 25 mM EDTA and two passes over microspin desalting columns (Zeba spin column, Pierce) to remove MgATP and chelate any residual Mg^{2+} to prevent reloading of the E1. The E1~*UB was then diluted at a final concentration of 2 μM into a second independent mixture that contained unlabeled UB, BSA, 0.2 μM UBE2S and 0.1 μM APC/C^{CDH1}, and ubiquitination reactions were then carried out for 4 min at RT. The data were normalized to the V_{max}^{app} of wild type UBE2S–APC/C^{CDH1}.

A dual-color assay was used to monitor the activities of UBE2C and UBE2S simultaneously **(Figs. 6F-G and S6F-G)**. Methylated UB was used as the donor UB to prevent UBE2S targeting of CycB^{N}*(fluorescein-labeled)-linked UB. Instead, UBE2S could only form diubiquitin on a Cyanine5-labeled acceptor UB (1-74, G75S G76S C77). For these experiments, 0.25 μM UBA1, 50 nM of APC/C, 1 μM of UBE2S, 10 μM CycB^{N}* or *CycB^{N}-(1K), and 10 μM UB* were mixed on ice, equilibrated to room temperature and initiated by the addition of 250 μM methylated UB. After 30 min, the reaction was quenched and the products were resolved by SDS-PAGE and fluorescent scanning. Inhibition of APC/C^{CDH1}-UBE2S-mediated di-UB synthesis was tested upon addition of varying concentrations of UBE2C.

To demonstrate that the UBv is specific for APC/C, 30 μM UBv was tested for effects on two other E3s, SCF$^{FBW7\Delta D}$, which harbors APC11's closest relative (RBX1) as the catalytic subunit **(Fig. S1H)** and WWP1, which has its own distinct UB-binding exosite **(Fig. S1I)**. SCF$^{FBW7\Delta D}$ -dependent ubiquitination of a phosphopeptide derived from CycE was assayed with 75 nM NEDD8~CUL1-RBX1, 75 nM FBW7ΔD, CycE-Biotin, 1 μM UBCH5B, 500 nM CDC34, 150 nM UBA1, and 1 mg/mL BSA first mixed on ice, then equilibrated to room temperature prior to initiating the reaction by the addition of 140 μM UB. Substrate ubiquitination was monitored over the indicated time course through western blotting for biotinylated CycE with anti-Biotin (Rockland Inc. 600-401-098) and HRP-linked rabbit IgG whole antibody (GE Healthcare NA934). For the HECT-type E3 fluorescent WBP2 was the substrate. 750 nM WWP1, 250 nM *WBP2, 500 nM UBCH7, and 50 nM UBA1 were mixed on ice and equilibrated to room temperature. The reaction was initiated by the addition of 70 μM UB. As a positive control for activation by the WWP1-specific UBv that is distinct from the APC11-specific UBv, 7.5 μM UBv P2.3 (Zhang et al., 2016) was added. SDS-loading buffer was added at 30 min to quench the reaction.

Substrate-independent assay for APC/C^{CDH1} activation of UBE2C, monitoring hydrolysis of oxyester-linked analog of UBE2C~UB

To test effects of shackling the RING away from the position in the architecture for multiubiquitination, it was necessary to assay APC/C^{CDH1} activation of UBE2C without a substrate, because substrate binding is blocked by the Elongation trap. Thus, we assayed the ability of APC/C complexes to activate hydrolysis of oxyster-linked UBE2C~UB (Brown et al., 2015). It was necessary to use the isosteric oxyester mimic rather than the native UBE2C~UB intermediate due to the thioesester rapidly discharging via automodification of UBE2C in the absence of substrate. For the oxyester-linked version of

the UBE2C~UB complex, UB's C terminus is enzymatically linked to a serine substituted for the catalytic Cys114 of UBE2C. The oxyester-linked UBE2C (C114S)~UB was mixed with either wild type or variant versions of APC/C in the absence or presence of CDH1. Experiments were performed at 30°C and monitored the persistence of E2~UB and generation of the hydrolytic products UBE2C and UB over time **(Figs. 6C, S1D, and S2E)**. Reaction mixtures contained 5 μM UBE2C~UB and 1 μM wild type or variant versions of APC/C. Reaction products were separated by SDS-PAGE and visualized by staining with Coomassie blue.

Bio-Layer Interferometry (BLI)

Concentrated analyte and ligand proteins were diluted into BLI reaction buffer (20 mM HEPES pH 8.0, 200 mM NaCl, 0.1 mg/ml BSA, 0.01% Tween20). BLI experiments were performed on an Octet RED96 system (ForteBio) using anti-GST antibody biosensors for GST-tagged ligands (APC11 or RBX1 RING domains) UB, UBv, or UBv mutant analytes were titrated at 25°C. Nine dilution points of analytes covering 0.16 – 7.5 μM concentration range were applied. Sensorgram raw data was processed and extracted by Octet Analysis 9.0 software. Dissociation constants (K_D) were obtained by fitting the response wavelength shifts in the steady-state regions using single-site binding system (Eq. 1) shown below.

$$R_{eq} = R_{max} \frac{[C]}{K_D + [C]} \quad (1)$$

where R_{eq} is value of steady-state response shift in each sensorgram curve, *[C]* is the titrant concentration, R_{max} is the maximal response in the steady-state region, K_D is the binding constant for single-site binding system. R_{max} and K_D values are unknown and Levenberg–Marquardt algorithm was used to perform iterative non-linear least squares curve fitting in Profit 6.2 (QuantumSoft) to obtain the fitted R_{max} and K_D.

Assaying APC/C substrate degradation in *Xenopus* egg extracts

Interphase *Xenopus* egg extract was prepared as described (Rudner and Murray, 2000; Yamaguchi et al., 2015). CycB Δ90 was added at 300 nM and incubated for 120 min. at RT. Buffered (20 mM HEPES pH 8.0, 200 mM NaCl) wild type UB or UBv were added at 10 μM and incubated for 10 min. 110 nM full-length CycB/CDK1 was then added. To monitor substrate degradation, samples were diluted in SDS-PAGE sample buffer at 0 - 60 min. post substrate addition and processed for SDS-PAGE and immunoblotting using antibodies raised against CycB (Thermo Scientific RB-008-P), APC3 (BD Biosciences C40920) and Smc3 (A846, (Sumara et al., 2000)).

Single-molecule assays monitoring APC/C^{CDH1} binding during substrate multiubiquitination

Monitoring the kinetics of the APC/C^{CDH1}-Securin interaction during ubiquitination was performed as previously described (Lu et al., 2015). In brief, biotinylated Securin was immobilized on a passivated slide with 0.2 mg/ml streptavidin. *In vitro* ubiquitination reactions were performed using 20 nM recombinant APC/C^{CDH1} mixed with 100 nM E1, 200 nM UBE2C, 5 mg/ml BSA, and 1.5 μM alexa647-UB in UBAB buffer (25 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) with an ATP regenerating system. Recombinant APC/C was monitored during the reaction using an anti-APC4 antibody (ab97658, Abcam) labeled with dylight 550-NHS (62263, Pierce). Experiments were performed at RT. A Nikon Ti TIRF microscope equipped with three laser lines of 491nM(50 mW), 561 nM(50 mW), 640 nm(125 mW), and a Hamamatsu C9100 EMCCD camera (the non-EM mode was used for a better signal-to-noise). Time-series were acquired at 5 sec/frame for 15 minutes. Exposure time: 3 seconds for the ubiquitin channel; 750 ms for the APC/C channel; 500 ms for the substrate channel.

Generation of disulfide-linked E2~UB for NMR titrations with ^{15}N-labeled APC11 RING domain

Disulfide-linked UBE2C~Flag-UB and UBE2S~Flag-UB complexes were generated using 2,2'-Dipyridyl disulfide (Sigma-Aldrich). Flag-UB (1-75, G76C) and versions of both E2s harboring only a single Cys at the active site (UBE2C-His_6 C102A, and UBE2S C118F) were treated with 10 mM DTT for at least 30 min before they were desalted into 50 mM HEPES pH 7.0, 150 mM NaCl. 2,2'-Dipyridyl disulfide was, first, added to Flag-UB (1-75, G76C) in 5-molar excess. After a 20 minute incubation on ice, the disulfide-linked UBE2C~UB and UBE2S~UB complexes were then purified by nickel affinity chromatography or cation exchange chromatography, respectively, and ultimately sized in 20 mM HEPES pH 7.0 with 100 mM NaCl.

NMR experiments

NMR experiments were measured on a Bruker 600 or 800 MHz spectrometer equipped with a ^{1}H and ^{13}C detect, TCI triple resonance cryogenic probe using standard Bruker pulse programs. ^{1}H, ^{13}C, and ^{15}N backbone resonances were assigned using standard triple resonance experiments, such as HNCA, HNCACB, CBCA(CO)NH, HNCO and HN(CA)CO. All of the 3D experiments were collected with 8 or 16 transients at 298 K. All the ^{1}H chemical shifts were referenced with respect to DSS measured in the same buffer, while the ^{13}C and ^{15}N chemical shifts were referenced indirectly with respect to the DSS shift. All of the spectra were processed using topspin software and analyzed using the computer-aided resonance software, CARA (Keller, 2004).

Assignments of ubiquitin resonances were obtained using BMRB database 17437 and APC11 assignments were done by our group earlier (Brown et al., 2014). Assignment of UBv was carried out at 298 K using uniformly labeled ^{13}C,^{15}N-labeled sample in the apo state and in complex with APC11 (1:2) at 500 μM concentration, independently, since the resonances of the complexes exhibited slow exchange. The NMR experiments were performed in a 20 mM sodium phosphate buffer (pH 7.0) with 100 mM NaCl, 10 mM DTT and 0.1% sodium azide in 90% H_2O and 10% D_2O. Titration experiments were carried out with either ^{15}N-labeled or ^{13}C, ^{15}N-labeled APC11 or UBv at 100 μM concentration in the same buffer. Titration experiments testing different APC/C subcomplexes were carried out with ^{15}N-labeled UBE2S at 50 μM. Resonance assignments for UBv and UBE2S will be published elsewhere. The chemical shift perturbations (CSPs) were calculated using CSP (ppm) = $\sqrt{\Delta\delta_{HN}^2 + 0.2\Delta\delta_N^2}$, which included the difference in the proton and nitrogen chemical shifts between the free and bound resonance.

UB-AQUA/PRM Proteomics

For UB-AQUA/PRM, *in vitro* UB reaction samples were quenched with 20 mM EDTA at the indicated time and flash frozen. Aliquots of samples were subject to TCA precipitation. Samples were digested first with Lys-C [in 100 mM tetraethylammonium bromide, 0.1% Rapigest (Waters Corporation), 10% (vol/vol) acetonitrile (ACN)] for 3 hours at 37°C, followed by the addition of trypsin and further digested overnight. Digests were acidified with an equal volume of 5% (vol/vol) formic acid (FA) to a pH of ~2 for 30 min, dried down, and resuspended in 1% (vol/vol) FA.

UB-AQUA/PRM was performed largely as described previously but with several modifications (Ordureau et al., 2015; Ordureau et al., 2014; Phu et al., 2011). A collection of 16 heavy-labeled reference peptides (Ordureau et al., 2014), each containing a single $^{13}C/^{15}N$-labeled amino acid, was produced at Cell Signaling Technologies and quantified by amino acid analysis. UB-AQUA peptides from working stocks [in 5% (vol/vol) FA] were diluted into the digested sample [in 1% (vol/vol) FA] to be analyzed to an optimal final concentration predetermined for individual peptide. Samples and AQUA peptides were oxidized with 0.05% hydrogen peroxide for 30 min, subjected to C18 StageTip and resuspended in 1% (vol/vol) FA. Experiments were performed in triplicate and MS data collected sequentially by LC/MS on a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled with a Famos Autosampler (LC Packings) and an Accela600 LC pump (Thermo Fisher Scientific). Peptides were separated on a 100-μm i.d. microcapillary column packed with ~0.5 cm of Magic C4 resin (5 μm, 100 Å; Michrom Bioresources) followed by ~20 cm of Accucore C18 resin (2.6 μm, 150 Å; Thermo Fisher Scientific). Peptides were separated using a 45-min gradient of 3–25% ACN in 0.125% FA with a flow rate of ~300 $nL \cdot min^{-1}$. The scan sequence began with an Orbitrap full MS^1 spectrum with the following parameters: resolution of 70,000, scan range of 200–1,000 Thomson (Th), AGC target of 1×10^6, maximum injection time of 250 ms, and profile spectrum data type. This scan was followed by 12 targeted MS^2 scans selected from a scheduled inclusion list with a 5-min retention time window. Each targeted MS^2 scan consisted of high-energy collision dissociation (HCD) with the following parameters: resolution of 35,000, AGC of 1×10^6, maximum injection time of 200 ms, isolation window of 1 Th, normalized collision energy (NCE) of 25, and profile spectrum data type. Raw files were searched, and precursor and fragment ions were quantified using Skyline version 3.1 (MacLean et al., 2010). The UB-AQUA peptides used for quantitation were previously listed by Ordureau et al.(Ordureau et al., 2014). Data generated from Skyline were exported into an Excel spreadsheet and Prism for further analysis as previously described (Ordureau et al., 2014). Total UB was determined as the average of the total UB calculated for each individual locus, unless specified otherwise.

Tandem Mass Tag (TMT) Proteomic analysis

For TMT analysis of *in vitro* ubiquitylation reactions, samples were quenched with 20 mM EDTA

at the indicated time and flash frozen. Aliquots of samples were reduced (10 mM TCEP) and alkylated (20 mM chloroacetamide) prior to TCA precipitation. Samples were digested first with Lys-C [in 200 mM EPPS (pH 8.0), 0.1% Rapigest (Waters Corporation), 10% (vol/vol) ACN] for 3-4 hours at 37°C, followed by the addition of trypsin and further digested overnight. Digests were acidified with an equal volume of 5% (vol/vol) FA to a pH of ~2 for 30 min, dried down, resuspended in 1% (vol/vol) FA, and subjected to C18 StageTip (packed with Empore C18; 3M Corporation) desalting. Eluted peptides were resuspended in 100 µl [200 mM EPPS pH 8.0] and labeled using 10-plex tandem mass tag (TMT) reagents (Thermo Fisher Scientific, Rockford, IL). TMT reagents (0.8 mg) were dissolved in 42 µl dry ACN and 5 µl was added to samples. After 1hr (RT), the reaction was quenched by adding 6 µl of 5% hydroxylamine. Labeled peptides were combined, acidified with final 2% FA (pH ~2), dried down, resuspended in 1% (vol/vol) FA 2.5% (vol/vol) ACN, and subjected to C18 StageTip (packed with Empore C18; 3M Corporation) desalting. Mass spectrometry data were collected using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a Proxeon EASY-nLC II liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were fractionated on a 100 µm inner diameter microcapillary column packed with ~0.5 cm of Magic C4 resin (5 µm, 100 Å, Michrom Bioresources) followed by ~35 cm of GP- 18 resin (1.8 µm, 200 Å, Sepax, Newark, DE). Peptides were separated using a 100 min gradient of 4 to 25% ACN in 0.125% FA at a flow rate of ~425 nL/min. The scan sequence began with an MS^1 spectrum (Orbitrap analysis; resolution 120,000; mass range 400−1400 m/z; AGC target 2×10^5; maximum injection time 100 ms, monoisotopic peak). In addition, unassigned, singly and doubly charged species were excluded from MS^2 analysis and dynamic exclusion (1 min) was set to 5 after 30 sec. A list of targeted mass (m/z (7 ppm mass tolerance), z, 6 min schedule window) for all possible single di-GLY sites in CycB sequence as well as previously observed multiply di-GLY modified CycB peptides found in a test-run were defined and data dependent analysis was performed if no target species were found. By default precursors for MS^2 analysis were selected using the Top10 most abundant peptides. MS2 analysis consisted of high energy collision-induced dissociation (quadrupole ion trap analysis; AGC 4×10^4; CID normalized collision energy (NCE) 35; maximum injection time 50 ms; isolation window 0.7; with injection of all available parallelizable time). We performed multinotch MS3 (McAlister et al., 2012; Ting et al., 2011) with synchronous-precursor-selection (SPS) where precursor ions (n=10) were collected (Orbitrap analysis; resolution 60,000; mass range 100−1000 m/z; HCD normalized collision energy 55; AGC target 5×10^4; maximum injection time 250 ms; with injection of all available parallelizable time).

Sequest-based identification using first a Human UNIPROT database (containing common contaminants) followed by a target decoy-based linear discriminant analysis (FDR 1%) was used for peptide and protein identification as described (Huttlin et al., 2010). A custom database that included all the identified proteins (excluding CycB) during the first search, plus the CycB[1-95]-His_6 sequence was then generated and used for a novel search. Parameters used for database searching include: 50 p.p.m. Precursor mass tolerance; 0.9 product ion mass tolerance; tryptic digestion with up to three missed cleavages; Carboxyamidomethylation of Cys was set as a fixed modification, while oxidation of Met and di-Gly modification of Lys were set as variable modifications. Localization of di-Gly sites used a modified version of the A-score algorithm (Beausoleil et al., 2006) as described (Kim et al., 2011b). A-scores of 13 were considered localized. For quantification, a 0.003 m/z window centered on the theoretical m/z value of each ten reporter ions and the closest signal intensity from the theoretical m/z value was recorded. Reporter ion intensities were adjusted based on the overlap of isotopic envelopes of all reporter ions (manufacturer specifications). Total signal to noise values for all peptides were summed for each TMT channel (with the exception of CycB, UB and common contaminant proteins present in samples), and all values were adjusted to account for variance in sample handling. For each peptide use for protein quantification, a total minimum signal to noise value of 150 and isolation specificity of 0.7 was required (McAlister et al., 2012; Ting et al., 2011). For diGly peptides use for quantification, a total minimum signal to noise value of 100 and isolation specificity of 0.5 was required.

Supplemental Table

Table S1, related to Figure 2. Crystallographic data and refinement statistics for APC11 RING–UBv complex.

Data Collection	
Beamline	NECAT 24-ID-E
Wavelength (Å)	0.97918
Space Group	C121
Cell Dimensions	
a, b, c (Å)	131.1, 35.2, 72.8
α, β, γ (˚)	90, 120, 90
Resolution (Å) (highest shell)	36.3−2.0 (2.07−2.00)
Number of measured reflections	64,390
Number of unique refections	19,650
Overall R_{sym} (%)	10.4 (57.2)
Overall I/σI	10.2 (2.0)
Completeness (%)	99.7 (99.7)
Multiplicity	3.3 (3.3)
Wilson B-factor	25.49
Refinement	
Resolution range (Å)	36.3−2.0
No. of reflections (σ≥0)	19,400
R_{work}/ R_{free} (%)	18.52/21.95
Number of non-solvent atoms	2,163
Number of metal ligands	6
Number of solvent	116
RMSD bond lengths (Å)	0.007
RMSD bond angles (°)	0.947
Isotropic B-factor Min/Maxi	12.2/67.8
Isotropic B-factor Mean	28.1
Molprobity statistics	
All-atom Clashscore	2.37
Ramachandran Plot	
Residues in preferred regions (%)	98.53
Residues in allowed regions (%)	1.47
Residues in disallowed regions (%)	0.0
Rotamer outliers (%)	0.0
C-beta outliers	0

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