Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supplemental Information

General Methods

4-20% gradient SDS-PAGE gels were run at 180 V for 45 minutes and stained with Coomassie Blue dye unless used for Western blot.

For Western blots, gels were transferred to a nitrocellulose or PVDF membrane with Bjerrum Schafer-Nielsen buffer using Bio-Rad Transblot Turbo transfer system. Membrane was blocked at room temperature in 5% milk in TBST before 4 C overnight incubation with 1:1000 diluted primary antibody in 3% Bovine Serum Albumin (BSA) in TRIS-Buffered Saline with Tween (TBST) (anti-ubiquitin (PD41), Santa Cruz, anti-K48 linkage specific ubiquitin (D9D5), Cell Signaling Technology [CST], and anti-K63 linkage specific ubiquitin (D7A11, CST). Nitrocellulose membrane was washed 3 x 5 minutes at room temperature in TBST before incubating with 1:1000 diluted appropriate secondary antibody (anti-rabbit [#7074] or anti-mouse [#7076], CST) in 5% milk in TBST at room temperature for 1 hour. Blots were washed again 3 x 5 minutes in TBST before visualization using Amersham ECL Western Blotting Detection Kit with Bio-Rad ChemiDoc XRS+ imaging system.

Cloning of Constructs

Cloning strategy was based on Q5 Site-Directed Mutagenesis (Q5 Hot Start High Fidelity Master Mix, New England Biolabs). All primers (Supplemental Table II) were designed based on this strategy and were purchased from Integrated DNA Technologies (IDT).

pET24a-UbK0 cDNA clone was purchased from MRC-PPU at Dundee and was used as the basis for cloning. Q5 Site-Directed Mutagenesis was used to make Sortase compatible mutants UbKX-Srt to mutate LRLRGG to LLPLTGG. UbKXSrt insert was subcloned into pET28 using BamHI and XhoI strategy and ubiquitin single lysine mutants were generated using Q5 Site-Directed Mutagenesis. C-terminal His-tag was added to these constructs using Q5 Site-Directed Mutagenesis.

pet30b-5M SrtA and pet30b-7M SrtA plasmids were a gift from Hidde Ploegh (Addgene plasmids # 51140 and # 51141). pET28a-sfGFP was a gift from Ryan Mehl (Addgene plasmid # 85492) and V1G mutation and N150TAG mutations were added using Q5 Site-Directed Mutagenesis. pDULE-CNF was a gift from Ryan Mehl (Addgene plasmid # 85494). pET28-UBE1 was a gift from Deschaies lab. pDEST17-Cdc34a was a gift from Wade Harper (Addgene plasmid # 18674). pET28aLIC-UEV1 was a gift from Cheryl Arrowsmith (Addgene plasmid # 25619). pETSUMO-hUbc13 was a gift from Cynthia Wolberger (Addgene plasmid # 51131). pET16b-PCNA was a gift from Andrew Deans (Addgene plasmid # 134898). K164TAG mutation and C-terminal His-tag were added via Q5 Site-Directed Mutagenesis.

Intact protein mass spectrometry

Mass spectrometry was run on an Agilent 6545XT LC-QTOF instrument using a reversed-phase PLRPS column (Agilent) for separation of species for intact protein mass spectrometry. Protein samples were buffer exchanged into 150 mM NaCl and 50 mM TRIS pH 8.0 buffer or MilliQ purified water and concentrated to ~1 mg/mL before injection onto LC-QTOF. Samples were eluted using a gradient from 5-95% acetonitrile in water supplemented with 0.1% formic acid over 13 minutes. All mass spectrometry data was processed using Agilent MassHunter BioConfirm 12.0.

Protein construct sequences

Ub WT
MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNI
QKESTLHLVLRLRGG

UbK0Srt
MQIFVRTLTGRTITLEVEPSDTIENVRARIQDREGIPPDQQRLIFAGRQLEDGRTLSDYNIQ
RESTLHLVLLPLTGG

UbK0Srt-His
MQIFVRTLTGRTITLEVEPSDTIENVRARIQDREGIPPDQQRLIFAGRQLEDGRTLSDYNIQ
RESTLHLVLLPLTGGHHHHHHH

UbK48Srt
MQIFVRTLTGRTITLEVEPSDTIENVRARIQDREGIPPDQQRLIFAGKQLEDGRTLSDYNIQ
RESTLHLVLLPLTGG

UbK48Srt-His
MQIFVRTLTGRTITLEVEPSDTIENVRARIQDREGIPPDQQRLIFAGKQLEDGRTLSDYNIQ
RESTLHLVLLPLTGGHHHHHHH

UbK63Srt
MQIFVRTLTGRTITLEVEPSDTIENVRARIQDREGIPPDQQRLIFAGRQLEDGRTLSDYNIQ
KESTLHLVLLPLTGG

UbK63Srt-His
MQIFVRTLTGRTITLEVEPSDTIENVRARIQDREGIPPDQQRLIFAGRQLEDGRTLSDYNIQ
KESTLHLVLLPLTGGHHHHHHH

SrtA 5M
MQAKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATREQLNRGVSFAEENESLDDQNISIAG
HTFIDRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRNVKPTAVEVLDEQKGKD
KQLTLITCDDYNEETGVWETRKIFVATEVKLEHHHHHH

SrtA 7M
MQAKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATREQLNRGVSFAKENQSLDDQNISIA
GHTFIDRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRNVKPTAVEVLDEQKGK
DKQLTLITCDDYNEETGVWETRKIFVATEVKLEHHHHHH

UBE1
MGSSHHHHHHSSGLVPRGSHMSSSPLSKKRRVSGPDPKPGSNCSPAQSALSEVSSVPTN
GMAKNGSEADIDESLYSRQLYVLGHEAMKMLQTSSVLVSGLRGLGVEIAKNIILGGVKA
VTLHDQGTTQWADLSSQFYLREEDIGKNRAEVSQPRLAELNSYVPVTAYTGPLVEDFLS
SFQVVVLTNSPLEAQLRVGEFCHSRGIKLVVADTRGLFGQLFCDFGEEMVLTDSNGEQP

LSAMVSMVTKDNPGVVTCLDEARHGFETGDFVSFSEVQGMIQLNGCQPMEIKVLGPYT
FSICDTSNFSDYIRGGIVSQVKVPKKISFKSLPASLVEPDFVMTDFAKYSRPAQLHIGFQA
LHQFCALHNQPPRPRNEEDATELVGLAQAVNARSPPSVKQNSLDEDLIRKLAYVAAGD
LAPINAFIGGLAAQEVMKACSGKFMPIMQWLYFDALECLPEDKEALTEEKCLPRQNRY
DGQVAVFGSDFQEKLSKQKYFLVGAGAIGCELLKNFAMIGLGCGEGGEVVVTDMDTIE
KSNLNRQFLFRPWDVTKLKSDTAAAAVRQMNPYIQVTSHQNRVGPDTERIYDDDFFQN
LDGVANALDNIDARMYMDRRCVYYRKPLLESGTLGTKGNVQVVIPFLTESYSSSQDPPE
KSIPICTLKNFPNAIEHTLQWARDEFEGLFKQPAENVNQYLTDSKFVERTLRLAGTQPLE
VLEAVQRSLVLQRPQTWGDCVTWACHHWHTQYCNNIRQLLHNFPPDQLTSSGAPFWS
GPKRCPHPLTFDVNNTLHLDYVMAAAANLFAQTYGLTGSQDRAAVASLLQSVQVPEFTP
KSGVKIHVSDQELQSANASVDDSRLEELKATLPSPDKLPGFKMYPIDFEKDDDSNFHMD
FIVAASNLRAENYDISPADRHKSKLIAGKIIPAIATTTAAVVGLVCLELYKVVQGHQQLD
SYKNGFLNLALPFFGFSEPLAAPRHQYYNQEWTLWDRFEVQGLQPNGEEMTLKQFLDY
FKTEHKLEITMLSQGVSMLYSFFMPAAKLKERLDQPMTEIVSRVSKRKLGRHVRALVLE
LCCNDESGEDVEVPYVRYTIR

Cdc34
MSYYHHHHHHLESTSLYKKAGSAAAPFTMARPLVPSSQKALLLELKGLQEEPVEGFRV
TLVDEGDLYNWEVAIFGPPNTYYEGGYFKARLKFPIDYPYSPPAFRFLTKMWHPNIYET
GDVCISILHPPVDDPQSGELPSERWNPTQNVRTILLSVISLLNEPNTFSPANVDASVMYRK
WKESKGKDREYTDIIRKQVLGTKVDAERDGVKVPTTLAEYCVKTKAPAPDEGSDLFYD
DYYEDGEVEEEADSCFGDDEDDSGTEES

His-SUMO-hUbc13/UBE2N
MGHHHHHHPSGVKTENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSM
RQIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGGMAGLPRRIIKETQRLLAEPVPGI
KAEPDESNARYFHVVIAGPQDSPFEGGTFKLELFLPEEYPMAAPKVRFMTKIYHPNVDK
LGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPDDPLANDVAEQWKTNEAQAIETARA
WTRLYAMNNI

UEV1/UBE2V1
MGSSHHHHHHSSGLVPRGSTGVKVPRNFRLLEELEEGQKGVGDGTVSWGLEDDEDMT
LTRWTGMIIGPPRTIYENRIYSLKIECGPKYPEAPPFVRFVTKINMNGVNSSNGVVDPRAI
SVLAKWQNSYSIKVVLQELRRLMMSKENMKLPQPPEGQCYSN

sfGFPV1G
MGSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSV
QLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMD
ELYKGSHHHHHH

sfGFPN150*p*AzF
MVSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNFNSH(***p*AzF**)VYITADKQKNGIKANFKIRHNVE
DGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITH
GMDELYKGSHHHHHH

PCNAK164*p*AzF
MFEARLVQGSILKKVLEALKDLINEACWDISSSGVNLQSMDSSHVSLVQLTLRSEGFDT
YRCDRNLAMGVNLTSMSKILKCAGNEDIITLRAEDNADTLALVFEAPNQEKVSDYEMK
LMDLDVEQLGIPEQEYSCVVKMPSGEFARICRDLSHIGDAVVISCA(***p*AzF**)DGVKFSASG
ELGNGNIKLSQTSNVDKEEEAVTIEMNEPVQLTFALRYLNFFTKATPLSSTVTLSMSADV
PLVVEYKIADMGHLKYYLAPKIEDEESSGSHHHHHH

Expression of protein constructs

All ubiquitin constructs were expressed as described previously [1]. Briefly, Ub constructs were expressed in Rosetta2 *E. coli* cells in Lura-Bertani (LB) Miller media (Sigma) supplemented with appropriate antibiotic (Wild-type Ub plasmid contained an Ampicillin resistance marker while all single-lysine and sortase-compatible Ub variants contained a Kanamycin resistance marker). Cells were grown at 37 °C in a shaker to an OD_{600} of 0.6-0.8 before inducing with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and expressing at 37 °C for 4 hours or 16 °C for 16 hours before collecting cells by centrifugation. Cell pellets were resuspended in 50 mM TRIS pH 8.0, 150 mM NaCl, protease inhibitor cocktail pellet (Thermo), and 0.1% Triton-X 100, and stored at -80 °C until lysis. Cells were lysed by sonication on ice and lysates were cleared by centrifugation at 13,000 x g for 45 minutes. Supernatant was filtered through an Amicon 30 kDa MWCO centrifugal filtration unit (Millipore) before concentration in a 3 kDa MWCO centrifugal filter. Ubiquitin was further purified by size-exclusion chromatography (SEC, Superdex HiLoad 16/600 S75 column, Cytiva) in a final buffer of 10 mM TRIS pH 8.0 + 150 mM NaCl. Purified protein was concentrated to 5 mg/mL-50 mg/mL (construct-dependent) before flash-freezing in liquid nitrogen and storing at -80 °C for future use.

UBE1 was expressed and purified based off of methods described in [2]. Briefly, UBE1 was expressed in Rosetta2 *E. coli* cells in LB Miller media supplemented with Kanamycin. Cells were grown at 37 °C in a shaker to an OD_{600} of 0.6 before cooling the shaker to 16 °C. Cells were induced with 0.5 mM IPTG and expressed protein overnight (~16 hours) before harvesting via centrifugation. Cell pellets were resuspended in UBE1 buffer (50 mM TRIS pH 8.0, 150 mM NaCl, 1 mM DTT) supplemented with a protease inhibitor cocktail pellet (Thermo) and stored at -80 °C until purification. For purification, cells were lysed by sonication on ice and lysates were cleared by centrifugation at 13,000 x g for 45 minutes. Supernatant was incubated with NiNTA-Agarose (Qiagen) at 4 °C for 1 h before purification by immobilized metal affinity chromatography (IMAC). Resin was washed with UBE1 lysis buffer and eluted with lysis buffer supplemented with 100 mM imidazole. Eluted protein was dialyzed into UBE1 lysis buffer overnight before concentration in an Amicon 30 kDa MWCO centrifugal filtration unit (Millipore) and purification by SEC in UBE1 buffer (Superdex HiLoad 16/600 S200 column, Cytiva). Protein was concentrated to ~13 mg/mL before flash-freezing in liquid nitrogen and storing at -80 °C for future use.

Cdc34 was expressed and purified based off of methods described in[3, 4]. Briefly, Cdc34 was expressed in Rosetta2 *E. coli* cells in LB Miller media supplemented with Ampicillin. Cells were grown in a shaker at 37 °C until reaching an OD_{600} of 0.6. The temperature in the shaker was adjusted to 16 °C before inducing the cells with 0.5 mM IPTG. Cells expressed protein overnight (~16 hours) before harvesting by centrifugation and resuspension in Cdc34 lysis buffer (450 mM NaCl, 50 mM TRIS pH 7.5, 1 mM DTT, 0.5% Triton X, Protease inhibitor cocktail pellet (Thermo). Cells were either stored at -80 °C for later use or lysed by sonication. Lysates were cleared by centrifugation at 13,000 xg for 45 minutes. Supernatant was dialyzed overnight at 4 °C into Cdc34 ion exchange buffer A (50 mM TRIS pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA). Cdc34 was purified using a gradient from buffer A to buffer B (50 mM TRIS pH 7.5, 2 M NaCl, 1 mM DTT, 1 mM EDTA). Purification was performed using a Mono Q 5/50 GL anion exchange column with 20 column volume (CV) wash of buffer A, then a gradient of 0-25% B over 25 CV followed by a gradient of 25-100% B over 50 CV. Cdc34 should elute at ~470 mM NaCl. Purified Cdc34 was then concentrated using an Amicon 3 kDa MWCO centrifugal filtration unit (Millipore) before purification by SEC (Superdex HiLoad 16/600 S75 column, Cytiva) in Cdc34 sizing buffer (50 mM TRIS pH 7.5, 1 mM DTT, 450 mM NaCl). Protein was concentrated in Cdc34 sizing buffer supplemented with 5% glycerol before flash-freezing in liquid nitrogen and storage at -80 °C for future use.

UEV1 and His-SUMO-hUbc13 were expressed in Rosetta 2 *E. coli* cells in LB Miller media supplemented with appropriate antibiotics (UEV1 plasmid contains a Kanamycin selection marker while Ubc13 plasmid contains an Ampicillin resistance marker). Cells were grown in a shaker at 37 °C to an OD_{600} of 0.6 before induction with 0.5 mM IPTG. The shaker temperature was adjusted to 16 °C and cells expressed for ~20 hours before harvesting by centrifugation. Cells were resuspended in UEV1/Ubc13 lysis buffer (50 mM TRIS pH 8.0, 300 mM NaCl, 1 mM DTT, protease inhibitor cocktail pellet [Thermo]) and stored at -80 °C for future use. Cells were lysed by sonication, lysates cleared by centrifugation as above, and the supernatant was purified by IMAC (Qiagen). Eluted protein was dialyzed overnight into UEV1/Ubc13 sizing buffer (50 mM TRIS pH 8.0, 150 mM NaCl, 1 mM DTT) and purified by SEC (Superdex HiLoad 16/600 S75 column, Cytiva). Purified protein was concentrated and flash-frozen in liquid nitrogen before storage at -80 °C for future use.

Sortase variants were expressed in Rosetta2 *E.coli* cells in LB Miller media supplemented with Kanamycin and induced at OD_{600} = 0.6 with 0.5 mM IPTG before expression overnight at 16 °C. Cells were harvested by centrifugation and stored at -80 °C until purification. For purification, cells were resuspended in lysis buffer (150 mM NaCl, 50 mM TRIS pH 7.5, protease inhibitor cocktail pellet [Thermo]), lysed by sonication, and lysates cleared as above. Sortase variants were purified by IMAC (Qiagen), dialyzed overnight into their respective reaction buffers (SrtA 7M in 150 mM NaCl, 50 mM TRIS pH 7.5, and SrtA 5M in 150 mM NaCl, 50 mM TRIS pH 7.5, and 5 mM $CaCl_2$), and concentrated before use in assays. Sortase was concentrated to 20 mg/mL, flash frozen in liquid nitrogen, and stored at -80 °C until use in assays.

sfGFPV1G was expressed in Rosetta2 *E.coli* cells, grown at 37 °C to an OD_{600} =0.6, induced with 0.5 mM IPTG, expressed at 37 °C overnight, and pelleted as above. Cells were resuspended in sfGFP lysis buffer (300 mM NaCl, 50 mM TRIS 8.0, and protease inhibitor cocktail pellet [Thermo]) lysed by sonication, lysates cleared by centrifugation, and the sfGFP was purified by nickel IMAC (Qiagen). Eluted protein was dialyzed overnight into sfGFP buffer (300 mM NaCl, 50 mM TRIS pH 8.0) before SEC (Superdex HiLoad 16/600 S75 column, Cytiva), flash freezing in liquid nitrogen, and storage at -80 °C.

Genetic code expansion in protein constructs

sfGFP-N150*p*AzF was expressed in BL21AI *E. coli* cells in 250mL auto-inducing media (AIM, supplemental table I, solutions made according to [5]) supplemented with Kanamycin, Spectinomycin, and 1 mM para-azido-L-phenylalanine (CAS # 33173-53-4, Click Chemistry Tools Cat # 1406). BL21AI cells were co-transformed with pDULE2-CNF machinery plasmid and pET28a-sfGFP-N150TAG plasmid before plating on Kanamycin/Spectinomycin selective media. For expression, cells were shaken at 37 °C in AIM and expressed for 16 hours before collection by centrifugation. Cells were lysed in GFP lysis buffer (50 mM TRIS 8.0, 300 mM NaCl, supplemented with a protease inhibitor cocktail pellet[Thermo]) and lysates cleared by centrifugation. sfGFP was purified using Qiagen Ni-NTA agarose resin for immobilized metal affinity chromatography (IMAC). Briefly, cleared cell lysates were incubated with lysis buffer-equilibrated Ni-NTA agarose resin at 4 C for 1 h before washes with lysis buffer and lysis buffer supplemented with 20 mM imidazole. Protein was eluted with lysis buffer supplemented with 100 mM imidazole and dialyzed into 50 mM TRIS 8.0, 300 mM NaCl at 4 °C overnight. Protein was concentrated and stored at -80 °C or used directly in assays.

PCNA-164*p*AzF-His was expressed in BL21AI cells as mentioned above. Briefly, cells were grown in 250 mL autoinduction media (AIM, supplemental table I) in the presence of 100 ug/mL ampicillin, 100 ug/mL of spectinomycin, and para-azido-L-phenylalanine (CAS # 33173-53-4, Click Chemistry Tools Cat # 1406) to a final concentration of 1 mM. The culture was incubated for an additional 16 hours at 37 °C before cells were harvested by centrifugation. Cells were resuspended in PCNA lysis buffer (300 mM NaCl, 50 mM TRIS pH 8.0, protease inhibitor cocktail pellet [Thermo]) before lysis by sonication as outlined above. Lysates were cleared by centrifugation and supernatant was purified by nickel affinity chromatography (Qiagen). Purified protein was dialyzed overnight at 4 °C into 300 mM NaCl, 50 mM TRIS pH 8.0 before purification by SEC (Superdex HiLoad 16/600 S200 column, Cytiva). Purified proteins were concentrated to 5 mg/mL, flash frozen in liquid nitrogen, and stored at -80 °C until further use.

Generation of wild-type K48-linked polyubiquitin

Polyubiquitin synthesis reactions were conducted using 1 µM UBE1, 5 µM Cdc34, 2 mM ATP, 4 mM $MgCl_2$, and 400 µM total ubiquitin in 50 mM TRIS pH 8.0. Reactions took place at 37 °C for 4 hours before flash-freezing the reaction in liquid nitrogen and storing at -80 °C for purification. For purification, polyubiquitin chains were isolated using an adapted method from [6]. Briefly, the reactions were thawed from -80 ° on ice and concentrated to ~100 µL before diluting to 1 mL in Buffer A (50 mM Sodium Acetate, pH 4.5). Any precipitated protein was pelleted by centrifugation (10 min at 21,000 x g) before injection onto a HiTrap Capto S 1 mL strong cation exchange column (Cytiva) using the AktaPure system. Protein was purified from 0-50% buffer B (1 M NaCl, 50 mM TRIS pH 4.5) over 370 mL. Purified polyubiquitin chains were adjusted to pH 8.0 by addition of 1 M TRIS, pH 10 and were flash-frozen in liquid nitrogen before storing at -80 °C.

Generation of His-tagged K48-linked polyubiquitin

Synthesis of His-tagged polyubiquitin was performed as above, but with the modification that wild-type monoubiquitin was reacted with UbK48Srt-His in a 3:1 ratio. Reactions were conducted at 37 °C for 4 h on a benchtop shaker before flash freezing in liquid nitrogen and storage at -80 °C

before later purification. His-tagged polyubiquitin was purified using Zymo mini His-spin kit for nickel affinity chromatography. The His-tag was subsequently removed using 20 µM SrtA 5M in SrtA 5M reaction buffer (50 mM TRIS pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$) for 4 hours at 37 °C in the presence of 1 mM GG peptide (Sigma). Individual mono-, di-, and tri-ubiquitin species bearing the C-terminal sortase recognition motif were isolated from unreacted species by reverse nickel affinity chromatography (briefly, collecting unbound polyubiquitin species from the Zymo mini His-spin kit as product) and further isolated using cation exchange chromatography (HiTrap Capto S 1 mL column, Cytiva) using the same method as mentioned above.
For triubiquitin and higher molecular weight chains, we applied n-1 length chains of wild-type polyubiquitin into the reaction where n= the final length of the desired chain (for example, $UbK48_2$-UbK48Srt was made from purified K48-linked wild-type diubiquitin and UbK48Srt-His). The His-tagged polyubiquitin was isolated using nickel affinity chromatography, and the His-tag was cleaved via SrtA 5M using the same method as above. For final purification, sortase-compatible triubiquitin was isolated by size-exclusion chromatography (Superdex HiLoad 16/600 S75 column, Cytiva) over 1.1 column volumes in 10 mM TRIS pH 8.0, 150 mM NaCl. Final triubiquitin was flash frozen in liquid nitrogen and stored at -80 °C until used.

Generation of wild-type K63-linked polyubiquitin
K63-linked polyubiquitin was synthesized using 1 µM UBE1, 5 µM SUMO-hUbc13, 5 µM UEV1, 2 mM ATP, 4 mM $MgCl_2$, and 400 µM total ubiquitin in 50 mM TRIS, pH 8.0. Reactions were conducted at 37 °C for 1 hour on a tabletop shaker before flash freezing in liquid nitrogen and storage at -80 °C before later purification. Purification was performed in the same manner as for K48-linked polyubiquitin.

Generation of His-tagged K63-linked polyubiquitin
Polyubiquitin synthesis for His-tagged K63-linked polyubiquitin was conducted similarly to the K48-linked wild-type polyubiquitin, however the wild-type ubiquitin was reacted in a 3:1 ratio with UbK63Srt-His. Purifications were conducted in the same manner as outlined above for K48-linked polyubiquitin.

Sortylation of ubiquitin onto sfGFP-V1G
Sortylation reactions were conducted using 5 µM SrtA 7M, 20 µM sfGFP-V1G, and 60 µM UbK0Srt. Reactions took place in SrtA 7M reaction buffer (50 mM TRIS pH 7.5, 150 mM NaCl) while shaking at 37 °C for 16 h before flash-freezing and storage at -80 °C. Purification of UbK0Srt-sfGFP was performed by SEC (Superdex HiLoad 16/600 S75 column, Cytiva) in 50 mM TRIS pH 8.0, 300 mM NaCl. Purified protein was analyzed by SDS-PAGE, flash frozen in liquid nitrogen, and stored at -80 °C until further use.

Sortase-mediated cleavage or hydrolysis of C-terminal His tag and UbK0Srt-sfGFP
Sortase cleavage experiments were performed using SrtA 5M derivative unless otherwise indicated for the experiment. Reactions were conducted using 5 µM SrtA 5M, 5 µM purified substrate, and 1 mM GG or GGG while shaking at 37 °C for 4 h unless otherwise indicated. Reaction samples were quenched with the addition of 2X Laemmli loading dye before SDS-PAGE analysis.

Strain-promoted Azide-DBCO click chemistry

GGG-DBCO (Click chemistry tools/Vector Laboratories, CCT-1551) and dissolved to a stock concentration of 2.5 mM in DI water. GGG-DBCO and *p*AzF click chemistry was conducted in 50 mM TRIS pH 8.0, 300 mM NaCl. For sfGFP-150*p*AzF, 35 µM sfGFP-150*p*AzF (1 equivalent) was reacted with 175 µM GGG-DBCO (5 equivalents) at room temperature for 16 h before buffer exchange to remove unreacted GGG-DBCO and subsequent analysis by LC-QTOF. For PCNA-164*p*AzF, 35 µM PCNA-164*p*AzF was reacted with 175 µM GGG-DBCO at room temperature for 6 hours. An additional 5 equivalents of GGG-DBCO was added to the reaction before reacting at room temperature for an additional 16 hours. Reaction was buffer exchanged by diluting 4x into 300 mM NaCl, 50 mM TRIS 8.0 and re-concentrating using Amicon Ultracentrifuge filter concentrator tubes (Cat # UFC5003) to remove excess GGG-DBCO before analysis by LC-QTOF.

Conjugation of ubiquitin chains to POI-*p*AzF-DBCO-GGG

For both PCNA-164*p*AzF and sfGFP150*p*AzF, sortase-mediated conjugation of polyubiquitin was performed by SrtA 7M. For the reactions, 12 µM POI-*p*AzF-DBCO-GGG was reacted with 12 µM $UbKX_nSrt$ and 5 µM SrtA 7M in SrtA 7M reaction buffer (50 mM TRIS pH 7.5, 150 mM NaCl). Conjugation took place at 37 °C on a benchtop shaker for 16 hours before analysis by SDS-PAGE and LC-QTOF.

Supplemental Tables and Figures

Supplemental Table I: Auto-inducing media contents
Autoinducing media was generated using the following components referenced from [2]:

Media Components	Volume (mL)
5% (w/v) aspartate pH 7.5	20
10% (v/v) glycerol	20
25x 18 amino-acid mix [a]	16
20% (w/v) arabinose	1
10% (w/v) lactose	8
25x M salts [b]	16
1M $MgSO_4$	0.8
40% (w/v) glucose	0.5
5000x trace metal stock solution [c]	0.08
Sterile water	167.62

[a] 25x 18 amino acid is a 1 L solution mixture consisting of the following:

Amino Acid	MW	Mass (g)
Alanine	89.1	5
Aspartic acid	133.1	5
Glutamic acid, sodium salt	169.1	5
Phenylalanine	165.2	5
Glycine	75.1	5
Histidine	155.2	5
Isoleucine	131.2	5
Lysine-HCl	182.6	5
Leucine	131.2	5
Methionine	149.2	5
Asparagine	132.1	5
Proline	115.1	5
Glutamine	146.1	5
Arginine	174.2	5
Serine	105.1	5
Threonine	119.1	5
Valine	117.1	5
Tryptophan	204.2	5

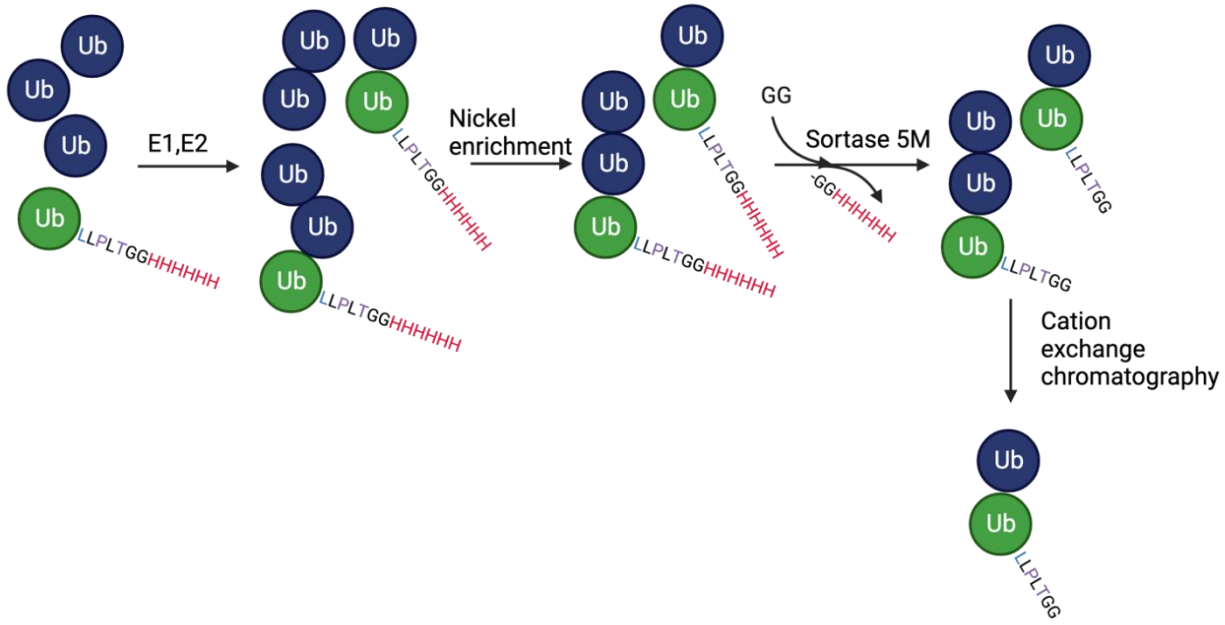
[b] 25x M salts consist of the following: 0.625 M Na_2HPO_4, 0.625 M KH_2PO_4, 1.25 M NH_4Cl, and 0.125 M Na_2SO_4

[c] 5000x trace metals solution consists of the following:

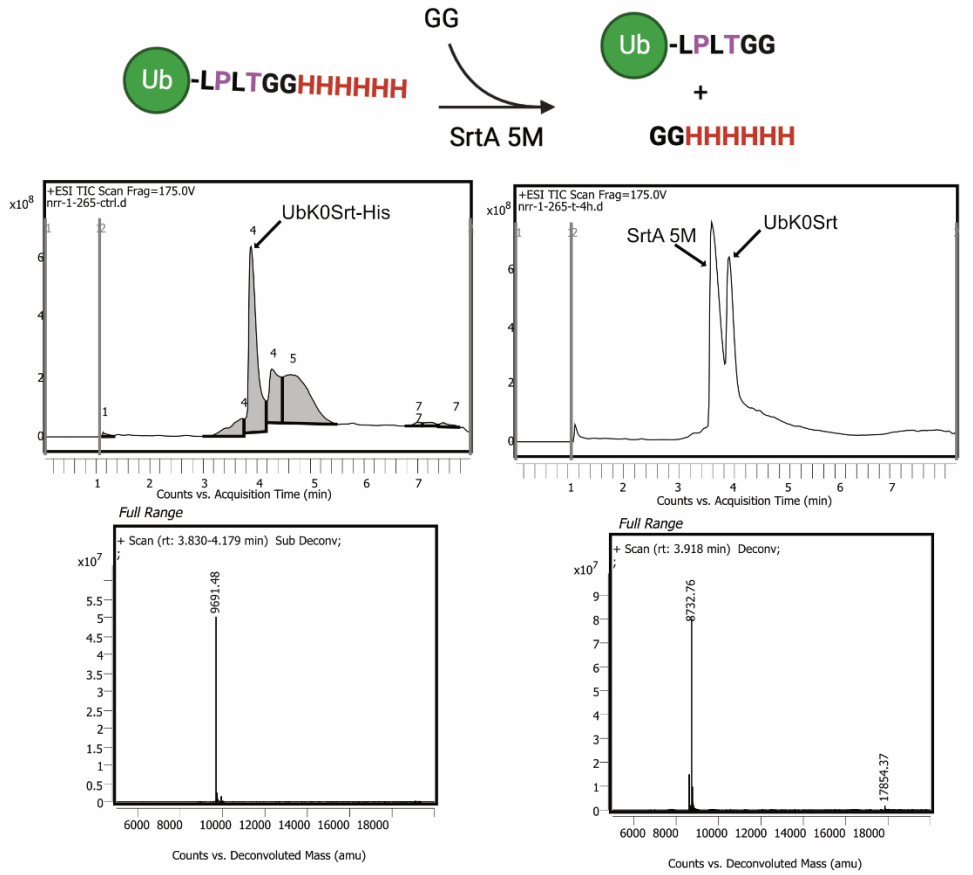
Metal	Concentration (mM)
$FeCl_3$	50
$CaCl_2.2H_2O$	20
$MnCl_2.4H_2O$	10
$ZnSO_4.7H_2O$	10
$CoCl_2.6H_2O$	2
$CuCl_2$	2
$NiCl_2$	2
Na_2SeO_3	2
$Na_2MoO_4.2H_2O$	2
H_3BO_3	2

Supplemental Table II: Primers used for cloning constructs

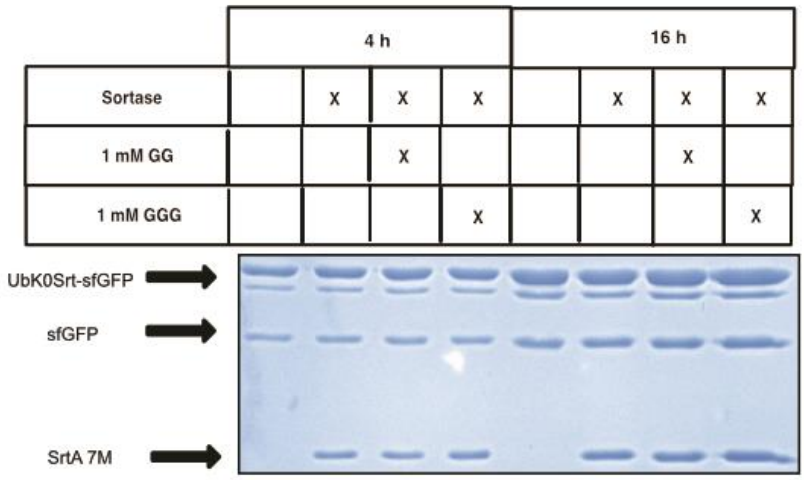
Name of primer	Sequence (5'-3')	Template plasmid
UbK0-LPLTGG-FWD	TCCCGCTCACAGGTGGGTGAT	pET28a-UbK0
UbK0-LPLTGG-REV	GGACCAGGTGCAGGGTGGACTCT	pET28a-UbK0
UbK0-LLPLTGG-FWD	CTGCTCCCGCTCACAGGTG	pET28a-UbK0LPLTGG
UbK0-LLPLTGG-REV	GACCAGGTGCAGGGTGGACTCT	pET28a-UbK0LPLTGG
PCNA-AddHis-FWD	CCACCACCACTAATGACATATGCTCGAGGATCCG	pET16b-PCNA
PCNA-AddHis-REV	TGGTGGTGATGGCTGCCGCTACTTTCTTCATCTTCAATTTTCGG	pET16b-PCNA
PCNA-K164TAG-FWD	TAGCTGTGCATAGGATGGCGTGA	pET16b-PCNA
PCNA-K164TAG-REV	ATAACAACTGCATCACCAATATGGCTCAG	pET16b-PCNA
UbK0Srt-AddHis-FWD	CCACCACCACTGATAAAGATCCGAATTCGAGCTC	pET28a-UbKX-Srt
UbK0Srt-AddHis-REV	TGGTGGTGATGCCCACCTGTGAGCGGGAG	pET28a-UbKX-Srt
sfGFP-V1G-FWD	AAGAACTGTTTACCGGCGTTGTG	pET28a-sfGFP
sfGFP-V1G-REV	CACCTTTGCTGCCCATGGTATAT	pET28a-sfGFP



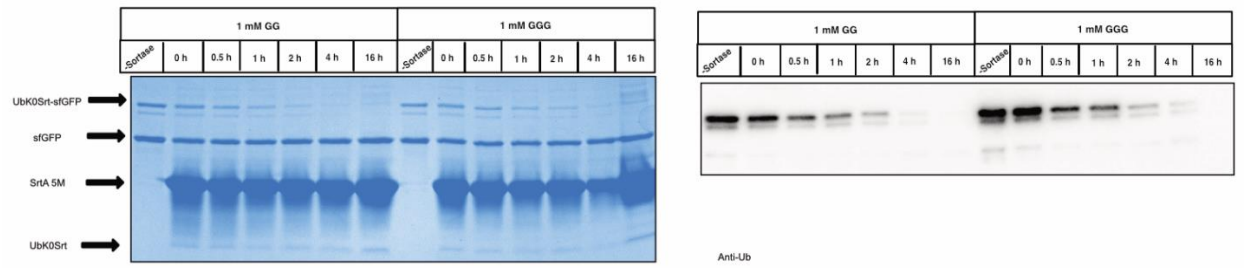
Supplemental Scheme I: Schematic showing overall workflow of the generation of polyubiquitin chains with C-terminal sortase recognition motif.



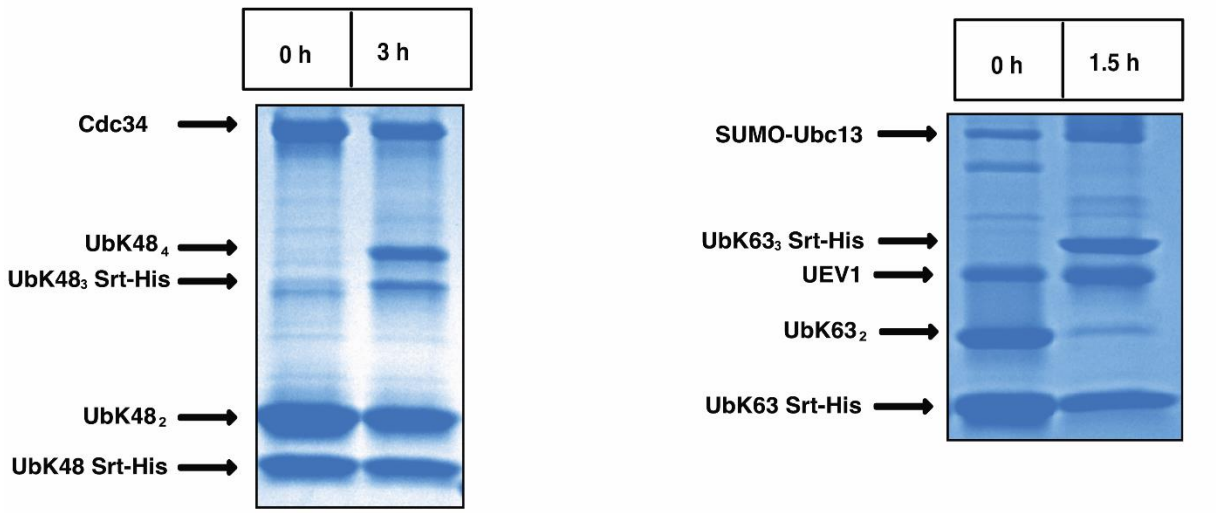
Supplemental Figure I: Mass spectrometry of His-tag cleaved from UbK48SrtHis by GG transamidation. We observed a small species that corresponded to the hydrolyzed UbK48LPLT, but in the presence of 1 mM GG peptide, ~80% of cleaved product contained the UbK48Srt with complete LPLTGG sequence at the C-terminus. The observed mass of 17853 Da corresponds to SrtA 5M in the experiment. TIC peak that was used for mass deconvolution is indicated with an arrow.



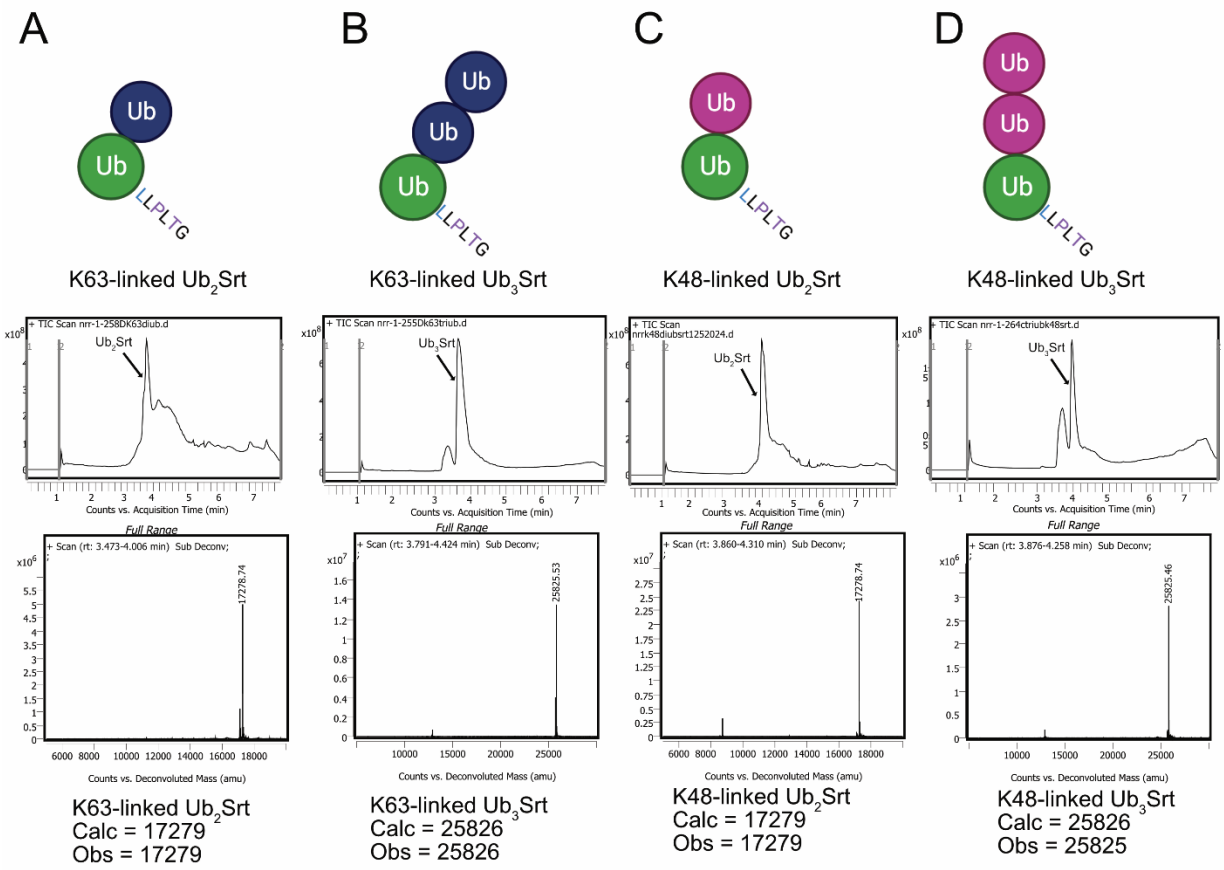
Supplemental Figure II: SrtA 7M does not hydrolyze UbK0Srt-sfGFP. Purified UbK0Srt-sfGFP was subjected to hydrolysis by SrtA 7M at 37 °C for either 4 hours (lanes 2-5) or 16 hours (lanes 6-9). UbK0Srt-sfGFP remained intact when subjected to SrtA 7M in the presence of SrtA 7M reaction buffer, SrtA 7M reaction buffer + 1 mM GG, and SrtA 7M reaction buffer + 1 mM GGG.



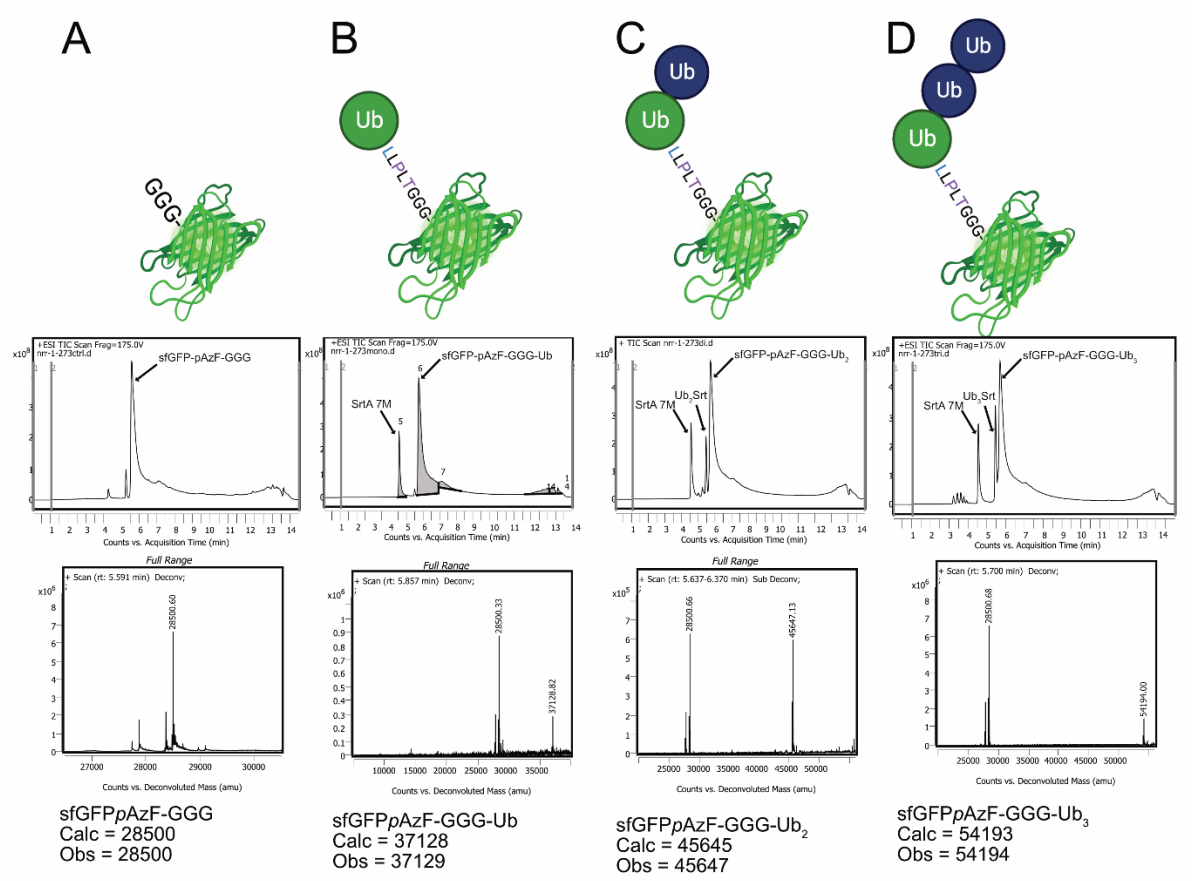
Supplemental Figure III: SrtA 5M hydrolyzes UbK0Srt-sfGFP in the presence of GG and GGG nucleophiles. Purified UbK0Srt-sfGFP was subjected to hydrolysis by SrtA 5M at 37 °C for the specified times in the presence of SrtA 5M buffer as well as 1 mM GG or 1 mM GGG. UbK0Srt-GFP was gradually cleaved, with near-complete cleavage observed after 4 hours and complete cleavage observed after 16 hours in the presence of both GG and GGG. Left: 4-20% SDS-PAGE gel stained with Coomassie blue. Right: Anti-Ubiquitin Western blot.



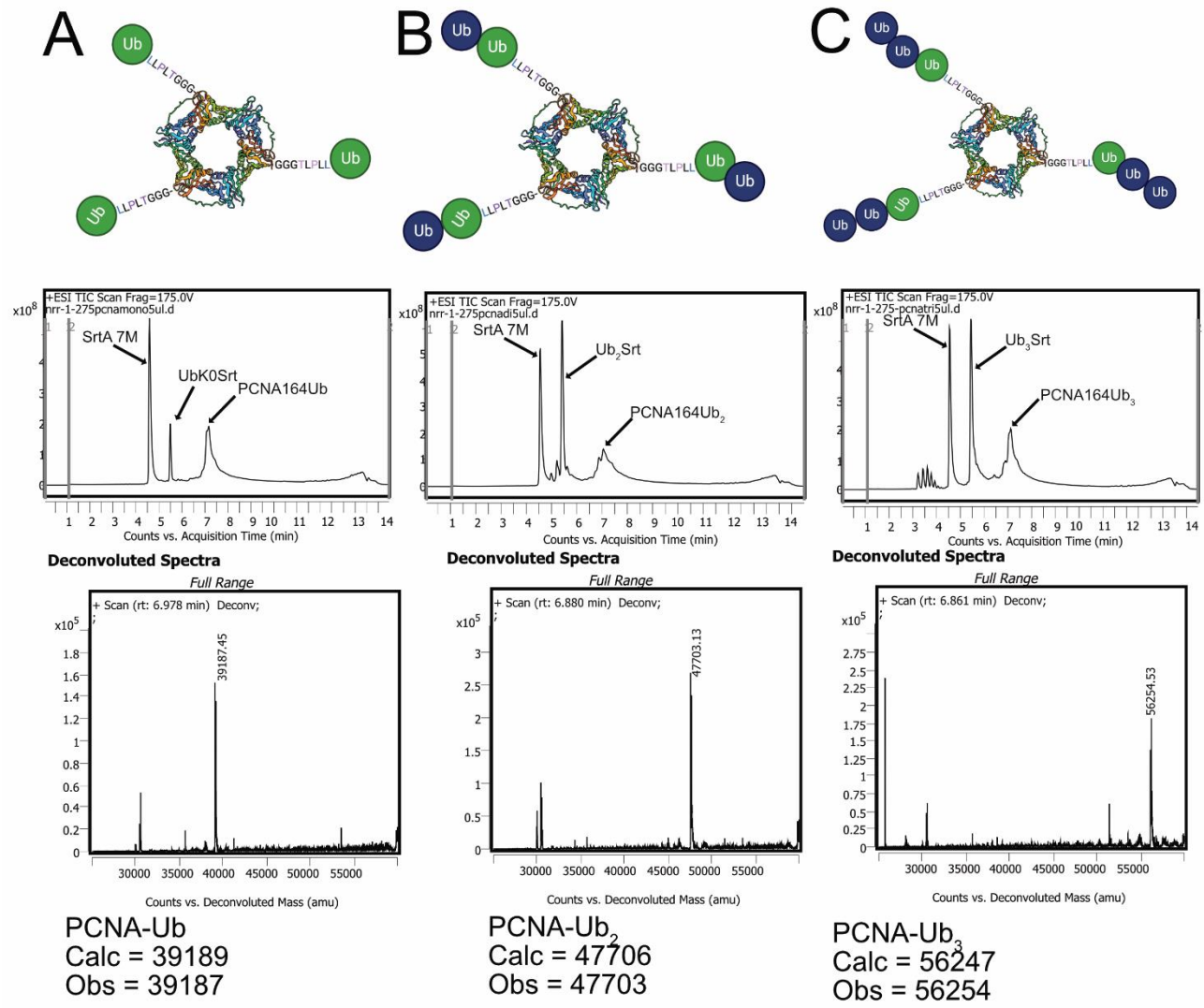
Supplemental Figure IV: Triubiquitin and higher chains are readily achieved for both K48-linked polyubiquitin (left) and K63-linked polyubiquitin (right) by using purified wild type Ub(n-1) in lieu of monoubiquitin.



Supplemental Figure V: Intact protein LC-QTOF of polyubiquitin chains. A) TIC and deconvoluted spectra of K63-linked di-ubiquitin. B) TIC and deconvoluted spectra of K63-linked tri-ubiquitin. C) TIC and deconvoluted spectra of K48-linked di-ubiquitin. D) TIC and deconvoluted spectra of K48-linked tri-ubiquitin.



Supplemental Figure VI: Intact protein LC-QTOF of sortase-mediated ubiquitination of sfGFP150*p*AzF-DBCO-GGG. TIC peak that was used for mass deconvolution is indicated with an arrow. (A) Control reaction with no SrtA present. (B) Monoubiquitination of sfGFP150*p*AzF-DBCO-GGG. (C) Di-ubiquitination of sfGFP-150*p*AzF-DBCO-GGG. (D) Tri-ubiquitination of sfGFP-150*p*AzF-DBCO-GGG.



Supplemental Figure VII: Intact protein LC-QTOF of sortase mediated ubiquitination of PCNA. TIC peak which was used for the deconvoluted spectra is indicated by an arrow. (A) Monoubiquitination of PCNA-164*p*AzF-DBCO-GGG. (B) Di-ubiquitination of PCNA-164*p*AzF-DBCO-GGG . (C) Tri-ubiquitination of PCNA-164*p*AzF-DBCO-GGG .

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