Supplemental Table 1

Supplementary Table 1

Protein Description	gi Number	Accession	P16-Ubn			P16			P16-Ubn + Usp2cc		
			#Scans	Unique Peptides	%Sequence Coverage	#Scans	Unique Peptides	%Sequence Coverage	#Scans	Unique Peptides	%Sequence Coverage
Ubiquitin	51703339	P62988.1	175	21	92.1%				5	4	43.4%
ALIX/Programmed cell death 6-interacting protein (PDCD6-interacti	**31076831**	**Q8WUM4.1**	**59**	**27**	**34.7%**						
Trypsin precursor	136429	P00761	53	8	25.1%	38	7	25.1%	28	7	25.1%
Pyruvate kinase isozymes M1/M2 (Pyruvate kinase muscle isozyme)	20178296	P14618.4	33	15	36.0%	76	27	46.1%	38	17	45.6%
Actin, cytoplasmic 1 (Beta-actin)	46397333	P60709.1	30	13	36.5%	35	13	39.5%	35	16	49.6%
UV excision repair protein RAD23 homolog B (hHR23B) (XP-C repair	**1709985**	**P54727.1**	**25**	**8**	**51.3%**						
Alpha-enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural eno	119339	P06733.2	25	9	30.4%	65	18	51.8%	22	8	26.5%
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	120649	P04406.3	24	8	35.8%	35	11	49.3%	14	7	38.2%
ubiquilin 1 isoform 2 [Homo sapiens]	16753205	NP_444295.1	18	2	6.4%				4	1	3.6%
KH domain-containing, RNA-binding, signal transduction-associated	62511098	Q07666.1	16	8	24.6%				16	8	23.3%
Heterogeneous nuclear ribonucleoprotein R (hnRNP R)	12230547	O43390.1	16	6	11.7%				8	5	13.0%
Polyadenylate-binding protein 1 (Poly(A)-binding protein 1) (PABP 1	3183544	P11940.2	14	7	10.5%				12	8	14.0%
pyruvate kinase, muscle isoform M1 [Homo sapiens]	33286422	NP_872271.1	14	6	12.8%	15	7	13.6%			
Actin, cytoplasmic 2 (Gamma-actin)	54036678	P63261.1	13	6	23.7%	11	5	22.9%			
Vacuolar protein sorting-associated protein 37B (hVps37B)	**74734015**	**Q9H9H4.1**	**13**	**4**	**28.4%**						
ubiquitin and ribosomal protein S27a precursor [Homo sapiens]	4506713	NP_002945.1	12	2	17.3%				8	3	21.8%
Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulate	21264428	P38646.2	9	6	12.7%	11	6	12.1%	1	1	1.8%
ATP synthase subunit beta, mitochondrial precursor	114549	P06576.3	9	6	21.0%	28	14	46.7%	5	3	10.0%
Profilin-1 (Profilin I)	130979	P07737.2	9	3	30.0%	18	6	54.3%	5	3	24.3%
Cell division protein kinase 4 (Cyclin-dependent kinase 4) (PSK-J3)	1168867	P11802.2	9	7	35.6%	14	8	41.6%	7	4	17.2%
Hepatocyte growth factor-regulated tyrosine kinase substrate (Pro	**71152119**	**O14964.1**	**9**	**5**	**7.9%**						
Creatine kinase B-type (Creatine kinase B chain) (B-CK)	125294	P12277.1	8	4	19.2%	24	10	39.9%	3	2	12.9%
PREDICTED: similar to pyruvate kinase, muscle [Homo sapiens]	169218111	XP_0017199	8	5	13.5%				2	1	4.4%
L-lactate dehydrogenase B chain (LDH-B) (LDH heart subunit) (LDH-H	126041	P07195.2	8	5	15.3%	12	8	29.0%	6	4	13.2%
Cyclin-dependent kinase inhibitor 2A, isoforms 1/2/3 (Cyclin-depend	3041660	P42771.2	7	2	18.6%	14	4	34.6%	14	4	34.0%
Tumor susceptibility gene 101 protein	**9789790**	**Q99816.2**	**7**	**5**	**15.1%**						
synaptotagmin binding, cytoplasmic RNA interacting protein [Homo	23397427	NP_006363.1	7	4	8.7%				9	3	5.1%
Elongation factor 1-beta (EF-1-beta)	119163	P24534.3	6	3	20.4%	12	4	37.8%	2	1	6.7%
Ubiquilin-2 (Protein linking IAP with cytoskeleton 2) (PLIC-2) (hPLIC-2	124056593	Q9UHD9.2	6	2	5.8%	23	1	3.2%	3	1	3.2%
Cyclin-dependent kinase 4 inhibitor B (p14-INK4b) (p15-INK4b) (p15	1168869	P42772.1	6	2	18.8%						
Target of Myb protein 1	**25091396**	**O60784.2**	**5**	**3**	**14.2%**						
40S ribosomal protein S10	1173177	P46783.1	5	2	14.5%	3	2	14.5%	2	1	5.5%
Ski-like protein (Ski-related protein) (Ski-related oncogene)	**134594**	**P12757.1**	**4**	**2**	**1.3%**						
Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) (Ubiqui	**136681**	**P09936.2**	**4**	**3**	**15.7%**						
heterogeneous nuclear ribonucleoprotein K isoform a [Homo sapien	14165439	NP_002131.1	4	1	2.6%	12	6	17.7%	4	2	5.8%
Polyadenylate-binding protein 4 (Poly(A)-binding protein 4) (PABP 4	12229875	Q13310.1	4	3	5.0%				5	2	4.8%
Coiled-coil domain-containing protein 50 (Protein Ymer)	**73619722**	**Q8IVM0.1**	**4**	**2**	**7.5%**						

Supplemental Table S2

		Raw Data (height ratio to AQUA std)				pMc	
		K48	**K63**	**Linear**	**ESTL**	K48	K63
WT V	1	0.026	0.897	0.068	0.221	0.25595	8.96619
	2	0.029	0.871	0.049	0.205	0.29414	8.71189
	3	0.021	0.687	0.044	0.159	0.20717	6.8739
6A Mutar	1	0.008	0.061	0.048	0.030	0.08115	0.60817
	2	0.009	0.065	0.075	0.027	0.08798	0.65156
	3	0.011	0.076	0.061	0.034	0.11288	0.75722
TUBE1	1	0.069	0.048	0.012	0.108	0.6929	0.47928
	2	0.092	0.094	0.203	0.143	0.91911	0.93656
	3	0.080	0.057	0.012	0.121	0.79926	0.57476
Bead onl	1	0.001	0.003	0.001	0.003	0.01143	0.02833
	2	0.002	0.005	0.000	0.007	0.0234	0.05023
Input	1	1.512	4.443	2.455	6.089	15.123	44.4283
	2	1.498	4.358	2.610	6.388	14.9752	43.5804
	3	1.526	4.649	2.537	6.313	15.2621	46.4878

ALIX is a Lys63-specific Polyubiquitin Binding Protein That Functions in Retrovirus Budding

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Supplemental Information

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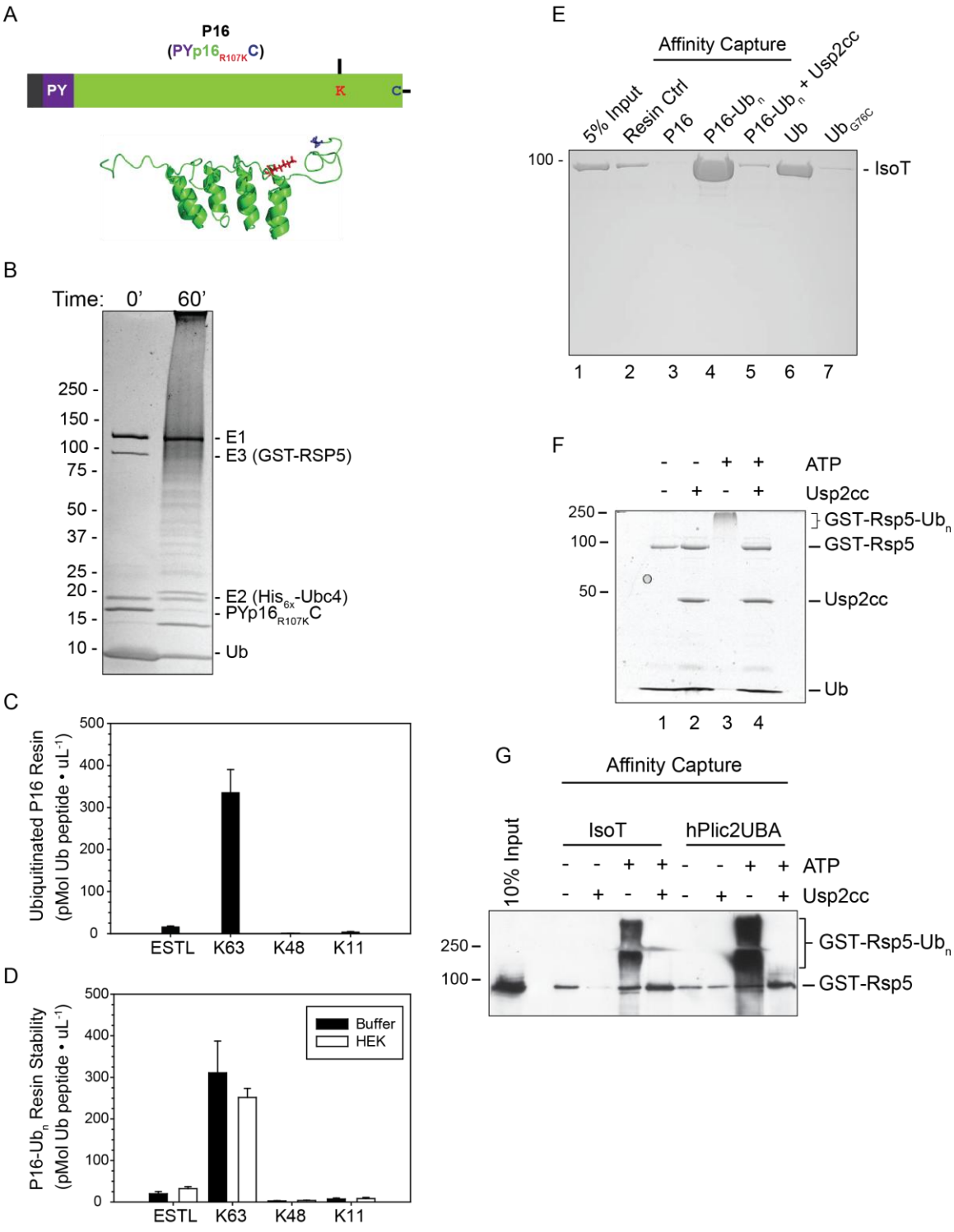
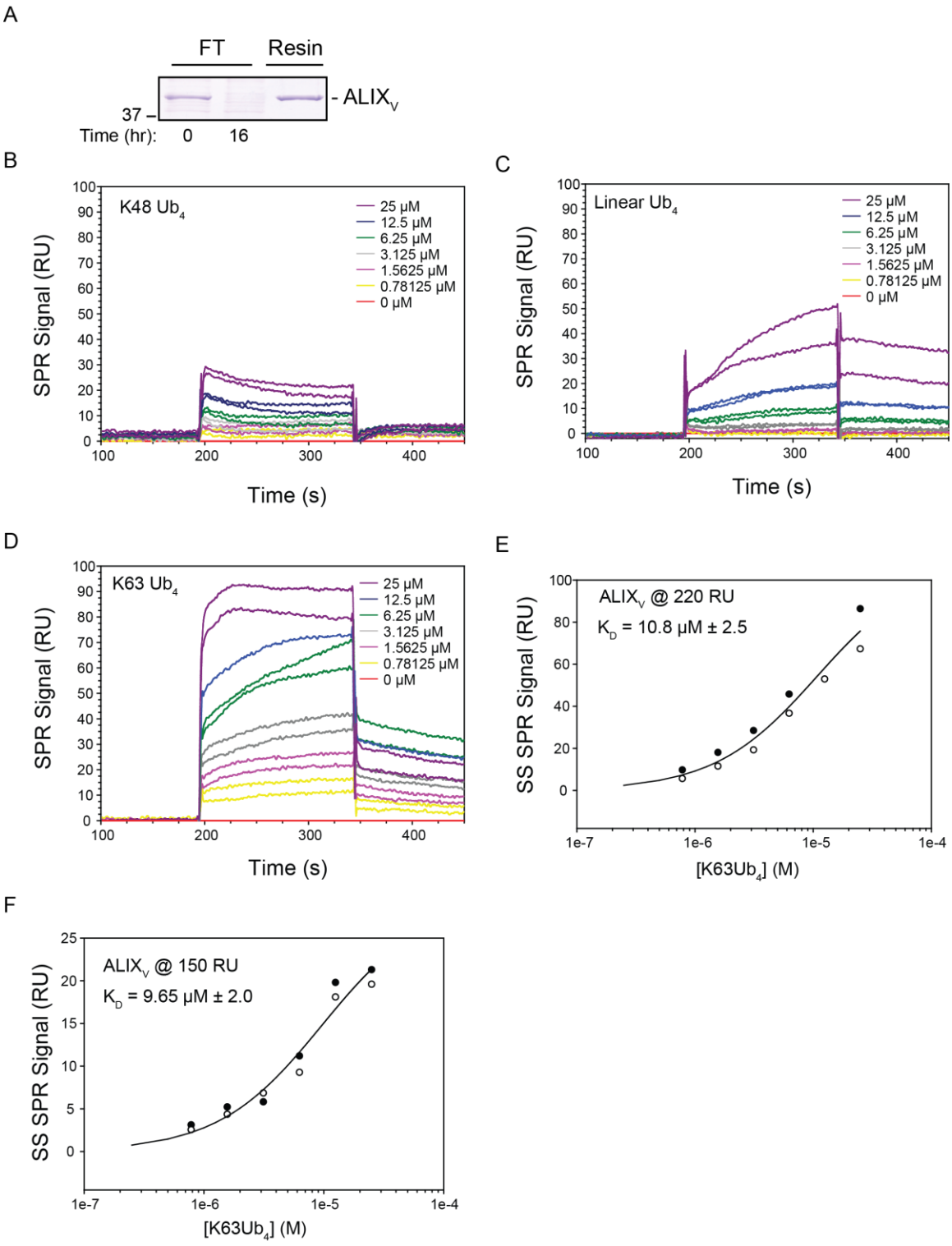


Supplemental Figure 1, related to Figure 1. Generation and Characterization of the P16-Ub$_n$ K63 Polyubiquitin Affinity Capture Reagent

(A) Organization and structure of P16. The lysine-free ankyrin repeat protein p16/INK4a

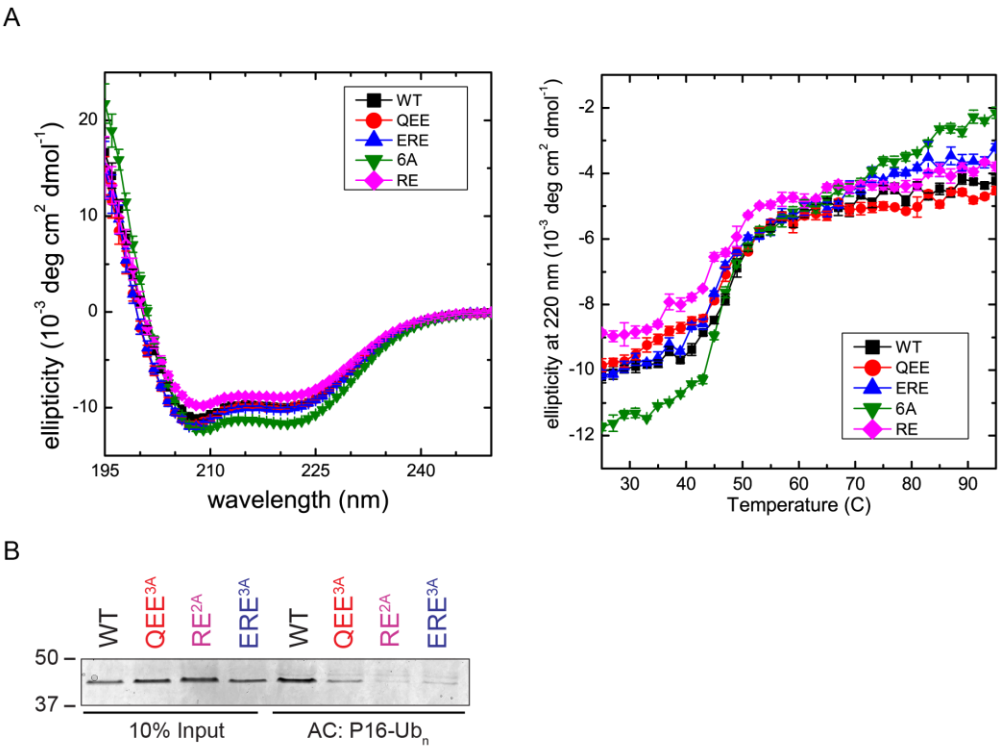
(green; PDB code 2A5E) was engineered to contain a single lysine (R107K, shown in red), a C-terminal cysteine for immobilization (C, shown in dark blue), and an N-terminal PPxY motif (PY, shown in purple). The structure of p16/INK4a was modified in PyMol to show location of the engineered lysine and cysteine. **(B)** Ubiquitylation of P16. P16 ($PYp16_{R107K}C$) protein was incubated at room temperature for 0 or 60 minutes in the presence of E1, His_{6x}-Ubc4, GST-Rsp5, Ub, and ATP, separated by SDS-PAGE and visualized by Coomassie blue staining. **(C)** Immobilized P16 is K63 polyubiquitylated. Ubiquitylated immobilized P16 (used in the experiments shown in Figures 1 and 3) was analyzed by quantitative mass spectrometry using synthetic heavy isoptope labeled AQUA standards. Ub linkages for the P16-Ub_n affinity reagent were determined as previously described (Kirkpatrick et al.), and showed that 97% of Ub was in polymer form, 98% was K63-linked, and the average chain length was 30 Ub molecules. Data here and in Figure S1 are presented as pMoles Ub peptide per µL resin corresponding to K11, K48, K63, and an internal Ub peptide (ESTL). **(D)** Immobilized P16-Ub_n is stable in NEM-treated HEK293 cell lysate. Immobilized P16-Ub_n was incubated overnight at 4°C in either AC buffer (black bars) or NEM-treated HEK293 cell lysate (white bars). Ub peptides were analyzed by Ub AQUA analysis as in panel C. Measurements in panels C and D represent mean ± s.d. (n=3). **(E)** Immobilized P16-Ub_n captures pure recombinant isopeptidase T (IsoT). Pure recombinant IsoT was incubated with the indicated affinity resins and bound proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. (**F**) GST-Rsp5 (used in the experiments shown in Figure 1D) is autoubiquitylated. GST-Rsp5 monomer was converted to high molecular weight Ub conjugates in the presence of ATP (compare lanes 3 and 1), and deubiquitylated

completely by treatment with Usp2cc (compare lane 4 and 3). (**G**) Autoubiquitylated GST-Rsp5 binds to immobilized recombinant IsoT and hPlic2UBA. Immobilized purified recombinant IsoT, or hPlic2UBA were incubated with material from (F), and both bound and eluted proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-GST antibodies.



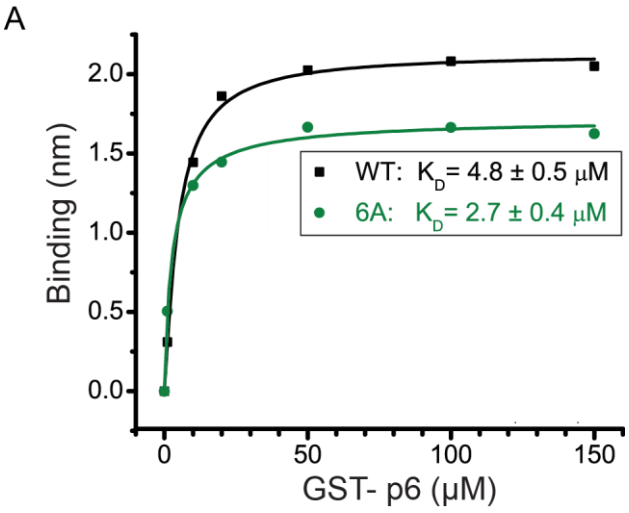
Supplemental Figure 2, related to Figure 2. Supporting Data for Biotin-Tagged ALIX$_V$ Experiments

(**A**) Recombinant $ALIX_V$ (used in experiments shown in Figures 2 and S2) is efficiently biotinylated. Affinity capture of biotinylated $ALIX_V$ with steptavadin-agarose and analyzed by Coomassie stained SDS-PAGE. Shown is flow-through for biotinylated $ALIX_V$ at t=0 and t=16 hours following incubation with strepavadin-agarose resin and protein bound to resin following incubation. (**B-D**) Interaction of immobilized $ALIX_V$ with K48 Ub_4 (**B**), Linear Ub_4 (**C**), K63 Ub_4 (**D**). Sensorgrams are shown for $ALIX_V$ immobilized at density of 220 RU. Ub chains (0.78-25 μM; twofold serial dilutions) were injected in duplicate. (**E-F**) Binding affinity for K63 Ub_4. Equilibrium binding data extracted from panel D (**E**) and Figure 2D (**F**) were fitted to single-site binding models. Errors represent s.d. in the K_D fitting parameter. FT, flow through.



Supplemental Figure 3, related to Figure 3. Analyses of $ALIX_V$ Proteins

(A) The helicity and thermal stability of $ALIX_V$ mutants is similar to wild type ALIX. *Left panel*, CD spectra of WT and mutant $ALIX_V$ proteins. *Right panel*, thermal stability of WT and, mutant $ALIX_V$ proteins. Each wavelength scan represents mean ± s.d. (n=16) collected at 1-nm intervals from 195 to 250 nm. (**B**) $ALIX_V$ triad mutants are impaired in binding to immobilized P16-Ub_n. Bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE and visualized by Coomassie blue staining. $ALIX_V$ RE^{2A} corresponds to residues Arg446 and Glu449 in ALIX substituted to Ala.



Supplemental Figure 4, related to Figure 4. Binding affinity of $ALIX_V$ for HIV $p6^{Gag}$.

(**A**) Mutations in the ALIX Ub binding region do not alter the binding affinity of HIV $p6^{Gag}$ protein for $ALIX_V$. Octet binding data for immobilized, biotin-tagged WT (black) and 6A mutant (green) $ALIX_V$ interacting with pure recombinant GST-p6 fusion protein (GST-p6). Errors represent s.d. in the K_D fitting parameter.

Supplemental Table 1, related to Figure 1. Proteins Identified by LC-MS/MS from Differential Affinity Capture of Proteins from HEK293 Cell Lysates

The set of all proteins captured from HEK293 cell lysates by P16-Ub_n, non-ubiquitylated (P16), and deubiquitylated (P16-Ub_n + Usp2cc) resins and identified by tandem MS analyses was sorted on the basis of total spectral counts (TSC, n>2) for P16-Ub_n. Proteins from were selected from this list if they were (a) affinity-captured by non-ubiquitylated or deubiquitylated P16 or (b) if they were not detected in at least two independent biological replicates. Proteins listed in this representative table that met our selection criteria are shown in bold. Please refer to file **Supplemental Table 1.xls**.

Supplemental Table 2, Related to Figure 2. Processed Data for Ub AQUA Pulldown Reactions

$ALIX_V$ selectively binds to K63 Ub_4 chains. Mass spectral analyses of the Ub_4 species pulled down by $ALIX_V$ (blue), $ALIX_V$ 6A (red), TUBE UBA (orange), resin (background control, green), or input (5%, grey) from a mixture of Ub_4 (K48, K63, and linear). Ub linkages were analyzed using synthetic heavy isoptope labeled (AQUA) standards to determine pMoles of each Ub peptide as previously described (Kirkpatrick et al., 2006). Quantities of peptides derived from each sample were averaged, normalized to levels measured for the input peptides, and corrected for background binding to control resin to produce normalized binding units. The average (avg) and standard deviations (Std Dev) were measured in triplicate except the resin sample, which was measured in duplicate. Please refer to file **Supplemental Table 2.xls**

Supplemental Experimental Procedures

E. coli Expression Plasmids

Plasmids for pET151/D-TOPO-ALIX (360-702; $ALIX_V$) and pGEX-p6 have been described (Fisher et al., 2007; Garrus et al., 2001). DNA encoding a biotin acceptor peptide (BAP) for biotinylation by the BirA enzyme was subcloned into pET151/D-TOPO-ALIX (360-702; $ALIX_V$) to express His6x-$ALIX_V$-BAP. Site-directed mutagenesis was used to introduce alanine mutations into pET151/D-TOPO-$ALIX_V$. pET3a-Ub and pET15b-Ubc4, were kindly provided by the late Cecile Pickart (Johns Hopkins University, Baltimore, MD) and Daniela Rotin (The Hospital for Sick Children, Toronto, Canada), respectively. pHUsp2-cc was a gift from Rohan Baker (The Australia National University, Canberra, Australia). Plasmids for pGEX-4T-ΔC2Rsp5 were previously described (Kaiser et al., 2011). A plasmid for expressing GST-thrombin-INK4a/p16 was kindly provided by Ming-Daw Tsai (Institute of Biological Chemistry, Academia Sinica, Nankang, Taiwan) and DNA encoding GST-thrombin-INK4a/p16 was subcloned into pET28a to express the His_6-GST-thrombin-INK4a/p16 protein. Site-directed mutagenesis was used to introduce a Pro-Pro-Pro-Tyr sequence (PY motif) in front of the p16 sequence, a single Lys (R107K), and a Cys at the C-terminus.

Protein Purification

Recombinant $ALIX_V$(360-702), $ALIX_V$-BAP, and Usp2cc proteins were expressed and purified as previously described (Baker et al., 2005; Fisher et al., 2007). For purification of $ALIX_V$, the fraction of dimer present in the initial purification of the QEE, ERE, and

6A constructs were comparable to that observed for the wildtype construct. Recombinant human E1 enzyme was provided by Boston Biochem. Commercially available recombinant purified tetra-, tri-, and di-ubiquitin chains, and biotin-TUBE2 (UBE-115) were provided by Boston Biochem. $ALIX_V$ was conjugated to Affigel10 resin (BioRad) at 1 mg ml^{-1} following the manufacturer's instructions.

Ubiquitin Preparation

Full-length, wild-type and Ub_{G76C} human ubiquitin was expressed from the pET3a expression vector (Novagen) in *Escherichia coli*. BL21 (DE3) pLysS RIL cells were transformed with expression vector and grown in a 2 l Fernbach flask to OD_{600} 1.0 in LB medium with 0.1 mg ml^{-1} ampicillin at 37 °C. Heterologous protein expression was then initiated with 1 mM IPTG for 4 h. Cells were collected by centrifugation and frozen in liquid nitrogen.

For ubiquitin purification, frozen bacterial cells were resuspended in a lysis buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EDTA and Complete Protease Inhibitor Cocktail Tablets (Roche). Resuspended cells were lysed by sonication followed by two passes through a French press at 10,000 pounds per square inch (p.s.i.). Lysate was clarified by centrifugation at 40,905*g* (18,500 r.p.m. in a Sorvall SS-34 rotor) for 60 min. Glacial acetic acid was added dropwise with mixing until the solution reached pH 4. The supernatant was cleared by centrifugation at 20,199*g* (13,000 r.p.m. for 20 min in a Sorvall SS-34 rotor), dialyzed twice into 25 mM sodium acetate (pH 4.5), loaded onto a HiTrapSP XL 5 ml (GE) column and eluted with a linear gradient from 0 mM to 500 mM NaCl. Ub was purified

by gel filtration chromatography on a Sephacryl S-200 (GE) column equilibrated with 50 mM Tris (pH 7.5), 500 mM NaCl and 1 mM DTT, dialyzed into 25 mM HEPES (pH 7.5), and frozen in liquid nitrogen.

E2–Ubc4 Preparation

BL21 (DE3) cells were transformed with pET15b-Ubc4 and grown to OD_{600} 1.0 in LB medium with 0.1 mg ml^{-1} ampicillin at 37 °C. Heterologous protein expression was then initiated with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Cells were collected by centrifugation and frozen in liquid nitrogen. For purification, cells were resuspended in a lysis buffer consisting of 50 mM Tris (pH 7.6), 300 mM NaCl, 20 mM imidazole, 10% glycerol, 0.2% Triton X-100, 1 mM PMSF and EDTA-free Protease Inhibitor Cocktail Tablets, and lysed by sonication followed by two passes through a French press at 10,000 p.s.i. Lysate was cleared by centrifugation and incubated with nickel-nitrilotriacetic acid resin for 2 h. The resin was washed 5 times with 10 bed volumes of lysis buffer and protein was eluted with lysis buffer supplemented with 250 mM imidazole (pH 8). The eluate was dialyzed into 50 mM Tris (pH 7.6), 150 mM NaCl, 10% glycerol and 1 mM DTT before filtration through a 50,000 molecular weight cutoff (MWCO) ultrafiltration membrane and vacuum concentrated (5,000 MWCO ultrafiltration membrane). The protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce) relative to a BSA standard, and the protein was diluted to 45 μM with dialysis buffer and frozen in liquid nitrogen.

Glutathione-S-transferase (GST)-ΔC2Rsp5(196–809) Preparation

BL21 (DE3) cells were transformed with pGEX-4T-ΔC2Rsp5 and grown to OD_{600} 1.0 in LB medium with 0.1 mg ml^{-1} ampicillin at 37 °C. The temperature was then shifted to 30 °C, and expression was initiated with 1 mM IPTG for 6 h. Cells were collected by centrifugation and frozen in liquid nitrogen. For purification, cells were resuspended in a lysis buffer consisting of 50 mM Tris (pH 8.0), 250 mM NaCl, 5 mM EDTA, 10 mM DTT, 1% Triton X-100 and Protease Inhibitor Cocktail Tablets, and lysed by sonication followed by two passes through a French press at 10,000 p.s.i. Lysate was cleared by centrifugation before incubation with glutathione sepharose resin for 2 h. The resin was washed 3 times with 10 bed volumes of lysis buffer and protein was eluted with lysis buffer supplemented with 20 mM glutathione (pH adjusted to 7.5). After dialysis into 50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM DTT, GST-Rsp5(196–809) was loaded onto a HiTrapQ XL 5 ml column, eluted with a linear gradient from 50 to 500 mM NaCl, and dialyzed into 20 mM Tris (pH 7.5) and 2 mM DTT, and frozen in liquid nitrogen.

P16 Preparation

BL21 (DE3) cells were transformed with His_6-GST-thrombin-INK4a/p16 expression vector and grown to OD_{600} 1.0 in LB medium with 0.1 mg ml^{-1} ampicillin at 37 °C. The temperature was then shifted to 30 °C, and expression was initiated with 1 mM IPTG for 6 h. Cells were collected by centrifugation and frozen in liquid nitrogen. For purification, cells were resuspended in a lysis buffer consisting of 50 mM Tris (pH 8.0), 250 mM NaCl, 5 mM EDTA, 10 mM DTT, 1% Triton X-100 and His_6-GST-thrombin-INK4a/p16, and lysed by sonication followed by two passes through a French press at 10,000 p.s.i. Lysate was cleared by centrifugation and incubated with glutathione sepharose resin for 2 h. The resin was washed 3 times with 10 bed volumes of lysis buffer and protein was

cleaved by addition of thrombin. After dialysis into 50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM DTT, P16 was loaded onto a HiTrapQ XL 5 ml column, eluted with a linear gradient from 50 to 500 mM NaCl, concentrated and purified by gel filtration chromatography (Sephacryl S-200 column, GE Biosciences) equilibrated with 50 mM Tris (pH 7.5), 150 mM NaCl and 1 mM DTT, and then dialyzed into 25 mM HEPES (pH 7.5), supplemented with 0.5 mM TCEP and frozen in liquid nitrogen. P16 was conjugated to UltraLink resin (Pierce) at 1 mg ml^{-1} per the manufacturers instructions.

hPlic2 UBA Domain Preparation

BL21 (DE3) cells were transformed with pet21-hPlic2(575-624) expression vector (gift of E. Bennett, Stanford University) and grown to OD_{600} 1.0 in LB medium with 0.1 mg ml^{-1} ampicillin at 37 °C. The temperature was then shifted to 30 °C, and expression was initiated with 1 mM IPTG for 6 h. Cells were collected by centrifugation and frozen in liquid nitrogen. For purification, cells were resuspended in a lysis buffer consisting of 50 mM Tris (pH 7.5), 650 mM NaCl, 10% glycerol, 1% Triton X-100, 20 mM imidazole and EDTA-free Protease Inhibitor Cocktail Tablets, and lysed by sonication followed by two passes through a French press at 10,000 p.s.i. Lysate was cleared by centrifugation and incubated with nickel-nitrilotriacetic acid resin for 4 h. The resin was washed 3 times with 10 bed volumes of lysis buffer, 3 times with 10 bed volumes of 50 mM Tris (pH 8) and eluted with 50 mM Tris (pH 8) supplemented with 200 mM imidazole (pH 8). The eluted protein was dialyzed into 50 mM HEPES pH 7.0, 100 mM NaCl, 2 mM DTT before conjugation to Affigel10 (Biorad) resin at 1 mg ml^{-1} per the manufacturer's instructions.

Catalytically Inactive IsoT (C335A) Preparation

BL21 (DE3) cells were transformed with the pRS-ISOT (C335A)-His_6 expression vector (gift of K. Wilkinson, Emory University) and grown to OD_{600} 1.0 in LB medium with 0.1 mg ml^{-1} ampicillin and 50 μM $ZnSO_4$ at 37 °C. The temperature was then shifted to 30 °C and expression was initiated with 1 mM IPTG for 6 h. Cells were collected by centrifugation and frozen in liquid nitrogen. For purification of IsoT(163-291)-6His, cells were resuspended in a lysis buffer consisting of 50 mM Tris (pH 7.5), 650 mM NaCl, 10% glycerol, 1% Triton X-100, 20 mM imidazole and EDTA-free Protease Inhibitor Cocktail Tablets, and lysed by sonication followed by two passes through a French press at 10,000 p.s.i. Lysate was cleared by centrifugation before incubation with nickel-nitrilotriacetic acid resin for 4 h. The resin was washed 3 times with 10 bed volumes of lysis buffer and protein was eluted with lysis buffer supplemented with 200 mM imidazole (pH 8). After dialysis into 50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM DTT, IsoT (C335A) was loaded onto a HiTrapQ XL 5 ml column, eluted with a linear gradient from 50 to 500 mM NaCl, concentrated and purified by gel filtration chromatography (Sephacryl S-200 column, GE Biosciences equilibrated with 50 mM Tris (pH 7.5), 500 mM NaCl and 1 mM DTT), dialyzed into 50 mM HEPES (pH 7.2), 100 mM NaCl and 2 mM DTT, and frozen in liquid nitrogen. For pull-down reactions, IsoT was conjugated to Affigel10 beads at 1 mg ml^{-1} following the manufacturer's instructions.

Protein Ubiquitylation

Immobilized P16 (1 mg P16/mL resin) was ubiquitylated at room temperature for 16 hrs in the presence of 50 nM E1, 2.4 μM E2 (His_6-Ubc4), 2 μM E3 (GST-ΔC2Rsp5), 300

μM Ub, and 1X ATP buffer (40 mM Tris-HCl pH 7.2, 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT) and quenched with 5 mM NEM. Autoubiquitylation of GST-ΔC2Rsp5 was performed in the same fashion.

Biotinylation of ALIX V Domain

$ALIX_V$-BAP proteins were incubated with recombinant BirA enzyme (Avidity) following the manufacturer's instructions. Biotinylated $ALIX_V$ proteins were separated from reaction components by size-exclusion chromatography (Sephacryl S-200 column, GE Biosciences). Biotintylation efficiency was determined by depletion of proteins from solution following pull-down with streptavidin-agarose resin and analysis by Coomassie blue stained SDS-PAGE gel.

Biosensor Analyses

Binding measurements were performed using a BIACORE 3000 biosensor system (GE Healthcare). Proteins were diluted in HBS-EP (10mM HEPES at pH 7.4, 150 mM NaCl, 3mM EDTA, and 0.005% v/v Surfactant P20) running buffer. Biotinylated $ALIX_V$ and 6A mutant were immobilized to surface densities of 150 or 220 RU on streptavidin sensor chips following three successive pulses of 1 M NaCl /50 mM NaOH, according to the manufacturer's recommendations. Ub, Ub_2, Ub_3, or Ub_4 analytes were injected into each experimental and control flow cell at a flow rate of μl/min HBS-EP buffer at 25 °C. Dissociation was allowed to occur at the same flow rate by running buffer alone for 7 min until the baseline had stabilized. Biosensor experiments were repeated at least twice. Data were corrected for non-specific binding by subtracting the signal measured in a control

cell lacking immobilized ligand. Background binding for the control cell was 3-35% of the total binding for 0.78 - 25 μM Ub_4 chains to V domain, where higher background binding for the reference cell corresponded to the highest chain concentrations injected. Data processing was performed using BIAevaluation software 4.1 (Biacore), and plotted and fitting using SigmaPlot software. Data shown for K63 Ub_4 binding to $ALIX_V$ were fitted using a one-site binding model as described (Wilkinson, 2004). Non-cooperative binding was confirmed by fitting to the Hill equation, which yielded similar K_D values and Hill coefficients of one.

Octet Biosensor Analyses

Binding of immobilized biotin-tagged wild type and 6A mutant $ALIX_V$ proteins to GST-$p6^{Gag}$ were performed using an Octet QK system (ForteBio) with streptavidin biosensors. Biotin-tagged $ALIX_V$ and 6A mutant were immobilized to streptavidin biosensors following the manufacturer's instructions. Binding measurements were performed in buffer containing 20 mM HEPES 7.4, 150 mM NaCl, 2 mM EDTA, 5 mM β-ME, 0.1 mg/mL BSA, 0.005% v/v Tween-20. Data were plotted and fitted as described for the SPR analysis.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was performed on an Aviv CD spectrometer (Model 202-01) in rectangular cells of 1-mm path length at 25˚C. Protein samples were diluted to 0.2 mg/ml in 4 mM Hepes (pH 7.6), 20 mM NaCl, 0.2 mM EDTA, 0.1 mM β-ME. Mean residue molar ellipticity was calculated using the equation, $\theta = \theta_{obs} \cdot mrw/10\ l$

cw, where θ_{obs} is the observed ellipticity in millidegrees, mrw is the mean residue molecular weight, l is the optical path length of the CD cell (cm), and c is the protein concentration (mg/ml). Each wavelength scan is the average of sixteen spectra collected at 1-nm intervals from 195 to 250 nm. Thermal denaturations were monitored at 220 nm from 25˚C to 90˚C with 2˚C temperature steps and 2 minute equilibration times.

Differential Affinity Capture

293 cells were collected in PBS and lysed in capture buffer containing 150 mM NaCl, 20 mM HEPES, pH 7.4, 2mM EDTA, 40 mM NEM, protease inhibitor cocktail (Roche) and 1% (v/v) Triton X-100 and supplemented with 42 mM DTT to quench NEM shortly after lysis. Lysates were cleared by centrifugation at 20,000*g*. Following centrifugation, proteins (1 mg total) were captured overnight at 4 °C with 80 µL immobilized P16-Ub_n, P16, or deubiquitylated P16-Ub_n. Samples were washed twice in buffer, washed twice in 50 mM ammonium bicarbonante (pH 8.0), supplemented with 50 µL of 0.1% Rapigest (Waters) in 50 mM ammonium bicarbonate pH 8.0 with 1 µg trypsin. After trypsinization overnight at 37°C and hydrolysis of Rapigest following the manufacturer's instructions with HCl, samples were submitted for mass spectrometry analyses.

Ub AQUA Peptides

Synthetic ^{15}N heavy isotope singly labeled peptides corresponding to linkages for linear, K11, K48, K63, and an internal Ub peptide (ESTL) were from Cell Signaling Technologies or Biopeptide LLC and were confirmed by tandem mass spectrometry (MS/MS) and quantified by amino acid analysis.

Mass Spectrometry

For protein identifications, trypsin digested samples were analysed on a LC-MS system that consisted of an ESI-TOF mass spectrometer (MicroTOF System, Bruker Daltonics) coupled to a capillary HPLC (Agilent). The peptide mixtures were separated on a 0.32 mm inner diameter × 150 mm length C-18 reversed-phase column at a flow rate of approximately 10 µl min^{-1} using a linear gradient of acetonitrile (0% to 40%) over 110 min. Eluant from the column was directly electrosprayed into the source of the mass spectrometer at a spray voltage of approximately 4 kV. To identify the peptides, a second portion of each sample was analysed by an undirected LC-MS/MS analysis on a linear ion-trap mass spectrometer (LTQ, ThermoElectron) using the same chromatography conditions as those used in the LC-MS profiling analyses. The acquired MS/MS spectra were searched against a database of protein sequences using the Mascot software package (Matrix Sciences).

Ub AQUA Quantification

Absolute quantification was performed using the ion intensities of the tracked endogenous peptides relative to the spiked labeled synthetic and protein-derived peptides. Peptide identities were confirmed by LC-MS/MS analysis as described previously (Bennett et al., 2007). For AQUA quantification, we measured ions for ESTLHLVLR (ESTL) from unlabeled samples (ESTL *m/z* = 356.55), synthetic AQUA peptide standards with a single ^{15}N-labeled amino acid (ESTL *m/z* = 358.88). We also measured LIFAGK-GG-QLEDGR (ubiquitin Lys48 (UbK48)) isopeptide unlabeled (*m/z* = 487.60) and heavy isotope–labeled AQUA peptide standard (*m/z* = 489.94), TLSDYNIQK-(GG)-ESTLHLVLR (K63) isopeptide unlabeled (*m/z* = 561.81) and heavy-isotope-labeled

AQUA peptide standard (*m/z* = 563.56), TLTGK-(GG)-TITLEVEPSDTIENVK (UbK11) isopeptide unlabeled (*m/z* = 801.43) and heavy-isotope-labeled (*m/z* = 803.43), and GGMQIFVK (UbLinear) peptide unlabeled (*m/z* = 440.24) and heavy-isotope-labeled (*m/z* = 443.23). The peptides measured in this study all eluted together with their corresponding isotope-labeled standards in a single chromatographic peak, and the peak maxima were used to determine the intensities.

Supplemental References

Baker, R.T., Catanzariti, A.M., Karunasekara, Y., Soboleva, T.A., Sharwood, R., Whitney, S., and Board, P.G. (2005). Using Deubiquitylating Enzymes as Research Tools. In Methods in Enzymology, J.D. Raymond, ed. (Academic Press), pp. 540-554.

Bennett, E.J., Shaler, T.A., Woodman, B., Ryu, K.-Y., Zaitseva, T.S., Becker, C.H., Bates, G.P., Schulman, H., and Kopito, R.R. (2007). Global changes to the ubiquitin system in Huntington's disease. Nature *448*, 704-708.

Bohgaki, M., Tsukiyama, T., Nakajima, A., Maruyama, S., Watanabe, M., Koike, T., and Hatakeyama, S. (2008). Involvement of Ymer in suppression of NF-κB activation by regulated interaction with lysine-63-linked polyubiquitin chain. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1783*, 826-837.

Fisher, R.D., Chung, H.-Y., Zhai, Q., Robinson, H., Sundquist, W.I., and Hill, C.P. (2007). Structural and Biochemical Studies of ALIX/AIP1 and Its Role in Retrovirus Budding. Cell *128*, 841-852.

Garrus, J.E., von Schwedler, U.K., Pornillos, O.W., Morham, S.G., Zavitz, K.H., Wang, H.E., Wettstein, D.A., Stray, K.M., CÙtÈ, M., Rich, R.L., *et al.* (2001). Tsg101 and the Vacuolar Protein Sorting Pathway Are Essential for HIV-1 Budding. Cell *107*, 55-65.

Husnjak, K., and Dikic, I. (2012). Ubiquitin-Binding Proteins: Decoders of Ubiquitin-Mediated Cellular Functions. Annual Review of Biochemistry *81*, 291-322.

Kaiser, S.E., Riley, B.E., Shaler, T.A., Trevino, R.S., Becker, C.H., Schulman, H., and Kopito, R.R. (2011). Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools. Nat Meth *8*, 691-696.

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.

Wilkinson, K.D. (2004). Quantitative Analysis of Protein-Protein Interactions Protein-Protein Interactions. H. Fu, ed. (Humana Press), pp. 15-31.