Supplementary information

Lysine 63-linked polyubiquitin chain may serve as a targeting signal for the 26S proteasome

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Supplementary methods

Yeast strains

All *Saccharomyces cerevisiae* strains used in this study were manipulated using standard techniques (Sherman, 1991). The yeast strains used in this study are shown in Table S1. The *UBP2*, *UFD2*, *PDR5*, or *HUL5* genes were disrupted by gene replacement with a PCR product from the *Δubp2::KanMX4*, *Δufd2::KanMX4, Δpdr5::KanMX4, or Δhul5::KanMX4* deletion strain of the BY4741 background (Open Biosystems). The correct disruption was verified by PCR. YYS823 was constructed by transformation of the marker-swap plasmid followed by Ura- Leu+ selection. YYS340 was created as follows: the 3' segment *RPB1* ORF was amplified by PCR from the W303 genome and cloned into p3xT7 IU vector (pOKA401). Then, pOKA401 was linearized at the *Bgl*II site within *RPB1* and integrated into the *RPB2-TAP* strain (Open Biosystems). For disruption of *LYS2* gene, pOKA703 was created by three fragment ligation of the upstream (941 bp, *Bam*HI-*Not*I) and downstream (702 bp, *Sal*I-*Bam*HI) regions of the *LYS2* gene and the *Not*I-*Sal*I digested the YIp vector pRS305 (Sikorski and Hieter, 1989). Then, pOKA703 was linearized at the *Bam*HI site and introduced to the respective strains. YYS1325 was constructed from YYS1246 by replacing the *KanMX* marker to the *HphMX* marker. YYS1265, YYS1327, YYS1328, and YYS1339 were generated by genetic cross among the strains described above.

Construction of plasmids

The plasmids used in this study are listed in Table S2. For expression of yeast E2s in *Escherichia coli*, the ORFs of all E2s for ubiquitin (Ub) were amplified by PCR from the yeast genome using appropriate primers. In the case of *UBC4*, *UBC5*, *UBC8*, *UBC13*, and *MMS2*, primers were designed to exclude their introns. The PCR products were then cloned into the pGEX-6P1 vector (pOKA101-pOKA112). For expression and purification of Ub in *E. coli*, the native Ub expression vector was first constructed by deleting the sequence encoding the GST tag of pGEX-human Ub (Murata et al., 2001). The resultant plasmid expressed the native Ub precisely from the start codon of Ub under the *tac* promoter (named pOKA132). We also constructed a series of Ub mutants by site-directed mutagenesis (Quick Change, Stratagene): Lys to Arg altered Ubs (pOKA133-135), Lys-less (pOKA141), and single- or double-Lys Ubs (pOKA142-149). The PY motif-inserted single Lys Sic1 (pET21a-Sic1K36^{PY}, named pOKA125) was constructed by inserting the 5'CCACCGCCGTAT3' sequence between +18 and +25 bp of *SIC1K36* (Petroski and Deshaies, 2003) as described previously. The PY-motif fused green fluorescence protein (GFP) was constructed by PCR using pGEX6P1-GFP (pOKA126) as the template with the following set of primers; (5'-CCGGAATTCATGAGTAAAGGAGAAGAAC-3' and 5'-ACGCGTCGACTTTGTAGAGCTCATCCATGC-3' for pET21a-GFP, 5'-CCGGAATTCCCACCGCCACCGTATAGCGGTGGTATGAGTAAAGGAGAAGAACTTTTCAC-3' and 5'- ACGCGTCGACTTTGTAGAGCTCATCCATGC-3' for pET21a-^{PY}GFP, 5'-CCGGAATTCATGAGTAAAGGAGAAGAAC-3' and 5'-GTCGACTCCTCCACTATATGGTGGCGGTGGTTTGTAGAGCTCATCCATGC-3' for pET21a-GFP^{PY}). The PCR products were digested with *Eco*RI and *Sal*I, then inserted into the *Eco*RI-*Sal*I gap of the pET21a vector (pOKA127 and 128). The ^{PY}GFP has an additional

amino acid sequence, Pro-Pro-Pro-Tyr-Ser-Gly-Gly, between the Met1 and Ser2 residues of the original GFP, while GFP^{PY} has the extended amino acids sequence, Pro-Pro-Pro-Pro-Tyr-Ser-Gly-Gly, after the Lys238 residue of GFP. The plasmid, GST-rpn10N5 (pOKA202), was generated by replacing the Ub interacting motif sequence encoding LAMAL to NNNNN as described previously (Fu et al., 1998).

For overproduction of Ub in yeast, the yeast Ub gene was cloned into the *Eco*RI-*Xho*I sites of pKT10 vector to be expressed under the *TDH3* promoter (pOKA601). The Lys to Arg altered Ubs were also constructed (pOKA602-604). For expression of Mga2 under the *GAL1* promoter, the *MGA2* ORF was cloned into pYF6 vector, a pYF3 vector-based plasmid with the alternation of multiple cloning sites (Saeki et al., 2002b).

Protein purification

Purification of recombinant ubiquitin

E. coli Rosetta2 (DE3) cells (Novagen) transformed with each plasmid encoding one of Ub and its derivatives were precultured overnight at 37°C in 10 ml of LB medium supplemented with 100 μg/ml ampicillin and 24 μg/ml chloramphenicol, and then transferred to a 200 ml of fresh medium. After incubation for 2 hr at 37°C, IPTG was added at a final concentration of 1 mM, and the cells were further cultured for 6 hr at 37°C. The cells were harvested, suspended in 20 ml of 20 mM Tris-HCl (pH 7.5) and lysed by sonication. After centrifugation at 12,000 x *g* for 20 min, the supernatant was recovered and mixed with 10 ml of DE32 resin (Whatman), pre-equilibrated with the same buffer. After rotation for 1 hr at 4°C, the flow-through fractions were collected and pH was adjusted to ~4.5 by 10% acetic acid, and then centrifuged again. The supernatant was recovered and mixed with 500 μl of CM-Sephadex (Amersham), pre-equilibrated with 50 mM ammonium acetate (pH 5.1). After washing with 100 mM ammonium acetate (pH 5.1), the bound proteins were eluted with 0.4 M ammonium acetate (pH 5.1). The purified Ub and its derivatives were dialyzed against buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10% glycerol) and stored at -80°C.

Purification of RNA polymerase II

The yeast RNA polymerase II was purified from YYS341 cells as follows. The cells were grown to mid-log phase in 1 L of YPD medium, harvested and lysed by glass beads in 10 ml of lysis buffer A containing 0.1% Triton X-100 and the protease inhibitor cocktail (Roche). After removal of the cell debris by centrifugation, the lysate was incubated with 100 μl of IgG Sepharose (Amersham) for 1 hr at 1°C. The resin was washed with the same buffer and packed into a spin column (Amersham). The RNA polymerase II on beads was eluted by incubation with 8 units of TEV protease (Invitrogen) in 120 μl of TEV buffer, buffer A containing 1 mM dithiothreitol (DTT), 0.5 mM EDTA, and 0.02% Triton X-100 for 2 hr at 25°C. The eluate was stored at -80°C.

Purification of Mga2-p120 for in vitro ubiquitination

To purify Mga2-p120, we used *rsp5* mutant cells to prevent contamination of Mga2-p120 Ub conjugates. The *rsp5-101* temperature-sensitive mutant cells (YAT2-1C) carrying P_{GAL1}-$^{FLAG}MGA2^{V5His6}$ plasmid were cultured to an OD_{600} between 0.6-0.8 in 1 l of SRaf-Trp medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 20 mg/ml uracil, 2% raffinose, 400 mg/l adenine, and 10 mM phosphate buffer, pH 7.5). Mga2 was expressed by the addition of galactose (2% final) at 37°C for 3 hr. Cells were harvested and washed once with 10 ml of buffer B (50 mM HEPES-Na, pH 7.5, 100 mM NaCl, 10% glycerol) containing 2 x concentration of the protease complete inhibitor cocktail. The cells (~3 g) were suspended with 10 ml of the same buffer and lysed by glass beads with Multibeads shocker at -5°C (Yasui Kikai Corp., Japan). After removal of the glass beads,

Triton-X100 was added to a final concentration of 1%. After incubation for 30 min at 0°C, the extracts were cleared by centrifugation. Mga2 was first captured on 200 µl of anti-FLAG M2 agarose (Sigma) by rotation for 2 hr at 4°C. Beads were washed twice with 10 ml of buffer B containing 1% Triton-X100 and collected to a Microspin Empty column (Amersham) with buffer B containing 0.2% Triton-X100. Mga2 was eluted with 200 µl of 200 µg/ml Flag peptide in the same buffer. Next, Mga2 was further purified with 50 µl of TALON cobalt resins (Clontech). After washing with buffer B containing 0.2% Triton-X100, Mga2-p120 was eluted by 50 mM HEPES-Na, pH 7.5, 100 mM NaCl, 10% glycerol, and 150 mM Imidazole. The purified Mga2-p120 was directly used for ubiquitination assay.

Purification of 26S proteasomes

The 26S proteasomes were purified from YYS40 or YYS99 cells as described previously (Saeki et al., 2005). Briefly, the FLAG-tagged 26S proteasome was recovered from the cell extract by adsorption to an anti-FLAG M2 agarose (Sigma), followed by elution with 3xFLAG peptide.

Purification of ubiquitinating enzymes and ubiquitin receptors

Yeast Uba1 (E1) was purified from the *UBA1-3xFLAG* strain (YYS41) (Saeki et al., 2004). Recombinant E2 enzymes, Rsp5, and Ub receptor proteins were expressed as GST-fused protein in *E. coli*, then adsorbed with glutathione Sepharose (Amersham), followed by elution with PreScission protease (Amersham) or with glutathione (Saeki et al., 2005). The His_6-tagged proteins were expressed in *E. coli* and purified by using TALON resin (Clontech) as described previously (Saeki et al., 2005).

GST pull-down assay of free ubiquitin chains

Forty pmol each of GST, GST-Rpn10, -rpn10N5, -Rad23, and -UbLRad23 were incubated with 5 µg of either Lys63- or Lys48-linked polyubiquitin chains (BioMol) for 1 hr at 25°C in 100 µl of buffer A plus 0.5% Triton X-100 and 1 mM DTT, then, pulled down with 10 µl of glutathione Sepharose (Amersham), which was preblocked with 5% non-fat milk in the same buffer. After extensive washing, bound proteins were eluted with SDS sample buffer. To avoid the aggregation of polyUb chains, samples were incubated for 1 h at 25°C and subjected to SDS-PAGE (BisTris 4-12% gradient gel, Invitrogen) followed by Western blot with anti-Ub antibody (P4D1, Santa Cruz).

Ubiquitination of $Sic1^{PY}$, Rpb1, Mga2 and Rsp5 *in vitro*

The ubiquitination of $Sic1^{PY}$ was performed as described previously (Saeki et al., 2005). The reaction mixtures contained 1 pmol Uba1 (E1), 60 pmol Ubc4 (E2), 10 pmol Rsp5 or GST-Rsp5 (E3), 10 pmol T7-$Sic1^{PY}$-His_6 (substrate), and 1 nmol Ub in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM DTT, 2 mM ATP, and 5 mM $MgCl_2$ for 3 h at 25°C in 20 µl total volume. The ubiquitination of Rpb1 and Mga2-p120 was carried out under the same condition with 5 pmols of RNAPII and Mga2, respectively. Self-ubiquitination assay for Rsp5 or GST-Rsp5 was conducted under the same condition without a substrate. Bovine Ub and methylated Ub were purchased from Sigma and Calbiochem, respectively.

SDS-PAGE and Western blotting

Multiple SDS-PAGE systems were used for resolving proteins. In Figures 1, 2B-E, 4, 5E-F, 6, S1, S2, S3, S6, S7, S8, S11and S17, hand-made Laemlli gels (10% polyacrylamide) with Tris-Glycine buffer were used. In Figures 2A, a precast gel (12% E-PAGEL, ATTO Corp.) with Tris-Glycine buffer were used. In Figure 3A, a precast gel (4-12% Bis-Tris gel, Invitrogen) with MOPS buffer was used. In Figures 3C-D, 5A-B, S5A, S10, S12, and S15,

precast gels (4-12% Bis-Tris gel, Invitrogen) with MES buffer were used. Western blotting was performed as described previously (Saeki et al., 2005) with the following antibodies; Horseradish peroxidase (HRP)-conjugated anti-T7 antibody (Novagen), HRP-conjugated anti-Flag antibody (M2, Sigma), anti-Ub antibody (P4D1, Santa Cruz), anti-Pgk1 antibody (Molecular Probes), anti-TAP antibody (Open Biosystems), anti-Rpn10 antibody (Kominami et al., 1997), and anti-20S proteasome antibody (Tanaka et al., 1988). Polyclonal antisera against Rpn2, Ubp6, and Rad23 were raised in rabbits against a C-terminal fragment of Rpn2 (104 amino acids), full-length Ubp6, and full-length Rad23, respectively.

Supplementary Tables

Table S1. Yeast strains used in this study

Strain	Genotype	Background	Reference
W303-1A	*MATa ura3-1, trp1-1, leu2-3,112, his3-11,15, ade2-1, can1-100*	W303	Our stock
YYS40	*MATa rpn11::RPN11-3xFLAG-HIS3*	W303	(Saeki et al., 2002b)
YYS99	*MATa Δrpn10::KanMX6 rpn11::RPN11-3xFLAG-HIS3*	W303	(Sone et al., 2004)
YYS449	*MATa Δhul5::KanMX4*	W303	This study
YYS41	*MATa uba1::UBA1-3xFLAG-HIS3*	W303	(Saeki et al., 2004)
Y202	*MATα cdc48-3*	W303	A gift from Dr. Y. Kimura
YAT1892	*MATa rpn2::URA3*	W303	(Yokota et al., 1996)
YYS823	*MATa rpn2::LEU2*	W303	This study
YYS849	*MATa Δufd2::KanMX4*	W303	This study
KA31	*MATa ura3 trp1 leu2 his3*	KA31	(Yashiroda et al., 1996)
YAT2-1C	*MATα rsp5-101*	KA31	(Yashiroda et al., 1996)
YYS263	*MATa Δubp2::KanMX4*	KA31	This study
BY4741	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	BY4741	OpenBiosystems
YYS340	*MATa rpb1::RPB1-3xT7-His$_6$-URA3 RPB2-TAP::HIS3*	BY4741	This study
YYS1119	*MATa Δpdr5::KanMX4*	W303	This study
YYS1246	*MATa Δpdr5::KanMX4 Δlys2::LEU2*	W303	This study
YYS1301	*MATa Δpdr5::KanMX4 Δlys2::LEU2* [pKT10-Ub] [pYF6]	W303	This study
YYS1302	*MATa Δpdr5::KanMX4 Δlys2::LEU2* [pKT10] [pYF6-MGA2]	W303	This study
YYS1303	*MATa Δpdr5::KanMX4 Δlys2::LEU2* [pKT10-Ub] [pYF6-MGA2]	W303	This study
YYS1304	*MATa Δpdr5::KanMX4 Δlys2::LEU2* [pKT10-UbK48R] [pYF6-MGA2]	W303	This study

YYS1305	*MATa Δpdr5::KanMX4 Δlys2::LEU2* [pKT10-UbK63R] [pYF6-MGA2]	W303	This study
YYS1306	*MATa Δpdr5::KanMX4 Δlys2::LEU2* [pKT10-UbK48R K63R] [pYF6-MGA2]	W303	This study
YYS1314	*MATa Δpdr5::KanMX4 Δlys2::LEU2* [pKT10-Ub]	W303	This study
YYS1320	*MATa rpn11::RPN11-3xFLAG-HIS3 Δpdr5::KanMX4 Δlys2::LEU2* [pKT10-Ub] [pYF6-MGA2]	W303	This study
YYS1325	*MATa Δpdr5::HphMX4 Δlys2::LEU2*	W303	This study
YYS1327	*MATa Δubp2::KanMX4 Δpdr5::HphMX Δlys2::LEU2*	W303	This study
YYS1328	*MATa Δufd2::KanMX4 Δpdr5::HphMX Δlys2::LEU2*	W303	This study
YYS1333	*MATa Δubp2::KanMX4 Δpdr5::HphMX Δlys2::LEU* [pKT10-Ub] [pYF6-MGA2]	W303	This study
YYS1334	*MATa Δufd2::KanMX4 Δpdr5::HphMX Δlys2::LEU2* [pKT10-Ub] [pYF6-MGA2]	W303	This study
YYS1265	*MATa rpn11::RPN11-3xFLAG-HIS3 Δpdr5::kanMX Δlys2::LEU2*	W303	This study
YYS1319	*MATa rpn11::RPN11-3xFLAG-HIS3 Δpdr5::kanMX Δlys2::LEU2* [pKT10]	W303	This study
YYS1320	*MATa rpn11::RPN11-3xFLAG-HIS3 Δpdr5::kanMX Δlys2::LEU2* [pKT10-Ub]	W303	This study
YYS1326	*MATa rpn11::RPN11-3xFLAG-HIS3 Δpdr5::hphMX Δlys2::LEU2*	W303	This study
YYS1339	*MATa rpn11::RPN11-3xFLAG-HIS3 Δhul5::kanMX4 Δpdr5::hphMX Δlys2::LEU2*	W303	This study
YYS1389	*MATa rpn11::RPN11-3xFLAG-HIS3 Δhul5::kanMX4 Δpdr5::hphMX Δlys2::LEU2* [pKT10-Ub]	W303	This study

Table S2. Plasmids used in this study.

Plasmid	Details	Reference
pOKA101	pGEX6P1-UBC1	This study
pOKA102	pGEX6P1-UBC2 (RAD6)	This study
pOKA103	pGEX6P1-UBC3 (CDC34)	(Sone et al., 2004)
pOKA104	pGEX6P1-UBC4	(Saeki et al., 2004)
pOKA105	pGEX6P1-UBC5	This study
pOKA106	pGEX6P1-UBC6	This study
pOKA107	pGEX6P1-UBC7	This study
pOKA108	pGEX6P1-UBC8	This study
pOKA109	pGEX6P1-UBC10	This study
pOKA110	pGEX6P1-UBC11	This study
pOKA111	pGEX6P1-UBC13	(Saeki et al., 2004)
pOKA112	pGEX6P1-MMS2	(Saeki et al., 2004)
pOKA121	pGEX6P1-RSP5	(Saeki et al., 2005)
pOKA122	pET28a-SIC1 (T7-Sic1-His_6)	(Saeki et al., 2005)
pOKA123	pET28a-$SIC1^{PY}$ (T7-$Sic1^{PY}$-His_6)	Saeki et al., 2005
pOKA124	pET28a-SIC1 (T7-Sic1K36-His_6)	(Petroski and Deshaies, 2003)
pOKA125	pET28a-$SIC1K36^{PY}$ (T7-$Sic1K36^{PY}$-His_6)	This study
pOKA126	pET28a-GFP (T7-GFP-His_6)	(Saeki et al., 2004)
pOKA127	pET28a-^{PY}GFP (T7-PY motif-GFP-His_6)	This study
pOKA128	pET28a-GFP^{PY} (T7- GFP-PY motif-His_6)	This study
pOKA131	pGEX-human Ub (GST-wild-type human ubiquitin)	(Murata et al., 2001)
pOKA132	P_{tac1}-human Ub (wild-type human ubiquitin)	This study
pOKA133	P_{tac1}-UbK48R (human ubiquitin with a Lys48Arg mutation)	This study
pOKA134	P_{tac1}-UbK63R (human ubiquitin with a Lys63Arg mutation)	This study
pOKA135	P_{tac1}-UbK48R K63R (human ubiquitin with Lys48Arg and Lys63Arg mutations)	This study
pOKA141	P_{tac1}-UbK0 (Lys-less human ubiquitin)	This study
pOKA142	P_{tac1}-UbK6 (Lys6 only human ubiquitin)	This study
pOKA143	P_{tac1}-UbK11 (Lys11 only human ubiquitin)	This study
pOKA144	P_{tac1}-UbK27 (Lys27 only human ubiquitin)	This study
pOKA145	P_{tac1}-UbK29 (Lys29 only human ubiquitin)	This study
pOKA146	P_{tac1}-UbK33 (Lys33 only human ubiquitin)	This study
pOKA147	P_{tac1}-UbK48 (Lys48 only human ubiquitin)	This study
pOKA148	P_{tac1}-UbK63 (Lys63 only human ubiquitin)	This study
pOKA149	P_{tac1}-UbK63 K63 (Lys48 and Lys63 only human ubiquitin)	This study
pOKA201	pGEX6P1-RPN10	(Saeki et al., 2002a)
pOKA202	pGEX6P1-rpn10N5 (Rpn10 with UIM mutation)	This study

pOKA203	pGEX6P1-RAD23	(Saeki et al., 2002a)
pOKA204	pGEX6P1-UbL^{RAD23} (N-terminal 100 amino acids of Rad23)	(Saeki et al., 2002a)
pKT10	P_{TDH3}-multi cloning site-T_{TDH3} 2μm ori URA3	(Tanaka et al., 1990)
pOKA601	pKT10-Ub (wild-type yeast ubiquitin)	This study
pOKA602	pKT10-UbK48R (yeast ubiquitin with a Lys48Arg mutation)	This study
pOKA603	pKT10-UbK63R (yeast ubiquitin with a Lys63Arg mutation)	This study
pOKA604	pKT10-UbK48R K63R (yeast ubiquitin with Lys48Arg and Lys63Arg mutations)	This study
pOKA605	pKT10-^{T7}Ub (T7-tagged yeast ubiquitin)	This study
p3xT7-IU	Multi-cloning site-3xT7-His_6-T_{TDH3} YIplac211	A gift from Dr. T. Araki
pOKA401	p3xT7-IU-5'*Δrpb1* (Rpb1-3xT7-His_6)	This study
pYF6	P_{GAL1}-FLAG-multi cloning site-V5-His_6-T_{CYC1} 2μm ori TRP1	This study
pOKA606	pYF6-*MGA2* (FLAG-Mga2-V5-His_6)	This study
pRS305	LEU2 Amp^r	(Sikorski and Hieter, 1989)
pOKA703	pRS305-lys2Δ (-868~+73 and +4048~+4750)	This study

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Supplementary results

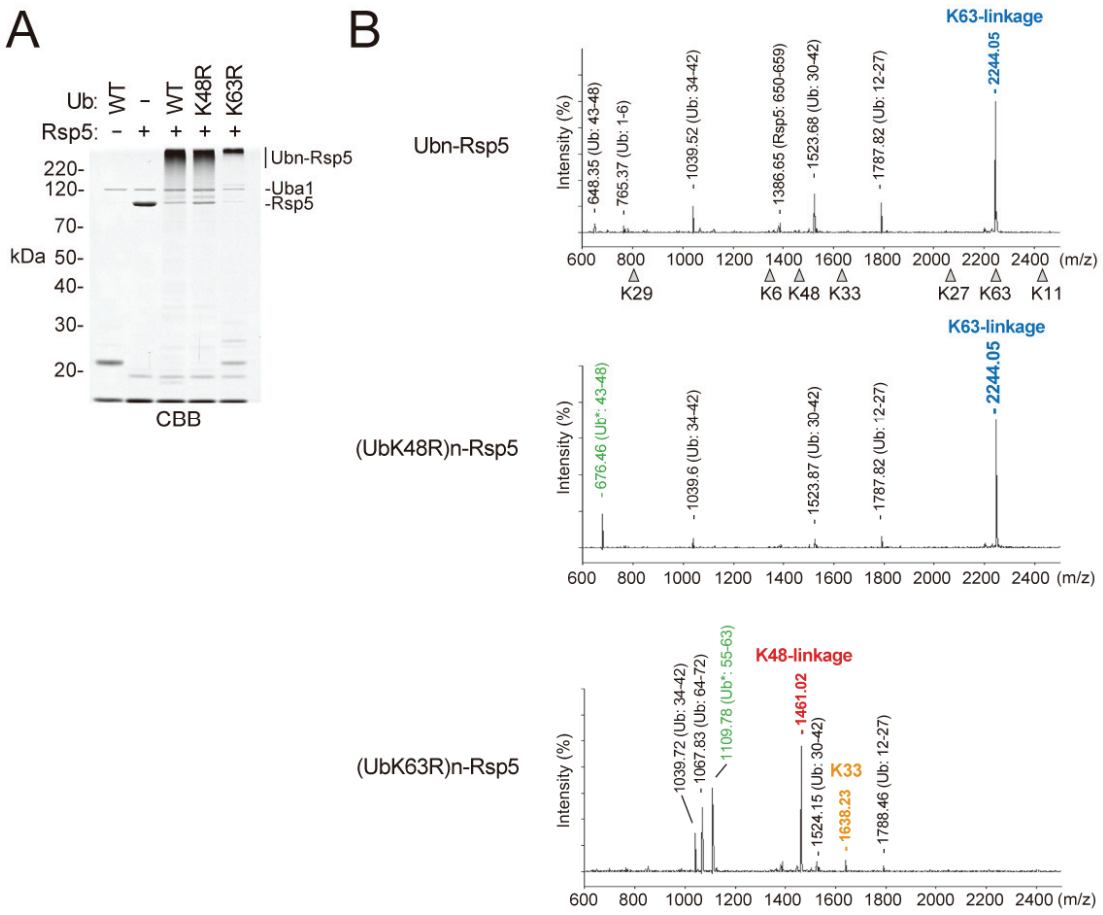

Figure S1. Self-ubiquitinated Rsp5 contains K63-linked Ub chains.
(A) Self-ubiquitination of Rsp5. Recombinant Rsp5 was incubated with E1 (Uba1), E2 (Ubc4), and wild-type (WT) or mutant Ubs (K48R or K63R) in the presence of ATP-Mg^{2+} at 25°C for 3 hr. The reaction mixtures were subjected to SDS-PAGE followed by Coomassie brilliant blue (CBB) staining.
(B) Mass spectrometric analysis of the self-ubiquitinated Rsp5. Gel regions of the ubiquitinated-Rsp5s (Ubn-Rsp5 in A) were excised and subjected to in gel-digestion with trypsin. The resulting peptides were analyzed by MALDI-TOF mass spectrometry (MS). The major peaks are labeled. The peaks corresponding to K63-, K48-, and K33-linkages are indicated in blue, red, and yellow, respectively. The Ub fragments derived from the mutants are indicated by Ub* in green text. The ideal masses (*m/z*) of all seven specific ubiquitin linkages are indicated by the triangles.

Although the self-ubiquitination of Rsp5 occurs using any of wild-type Ub, UbK48R, or K63R Ub (Figure S1A), MS analysis revealed that Rsp5 preferentially assembles the K63-linked Ub chains under normal condition. It is worth nothing that when K63 of Ub is mutated, Rsp5 undergoes K48- and K33-linked chain synthesis.

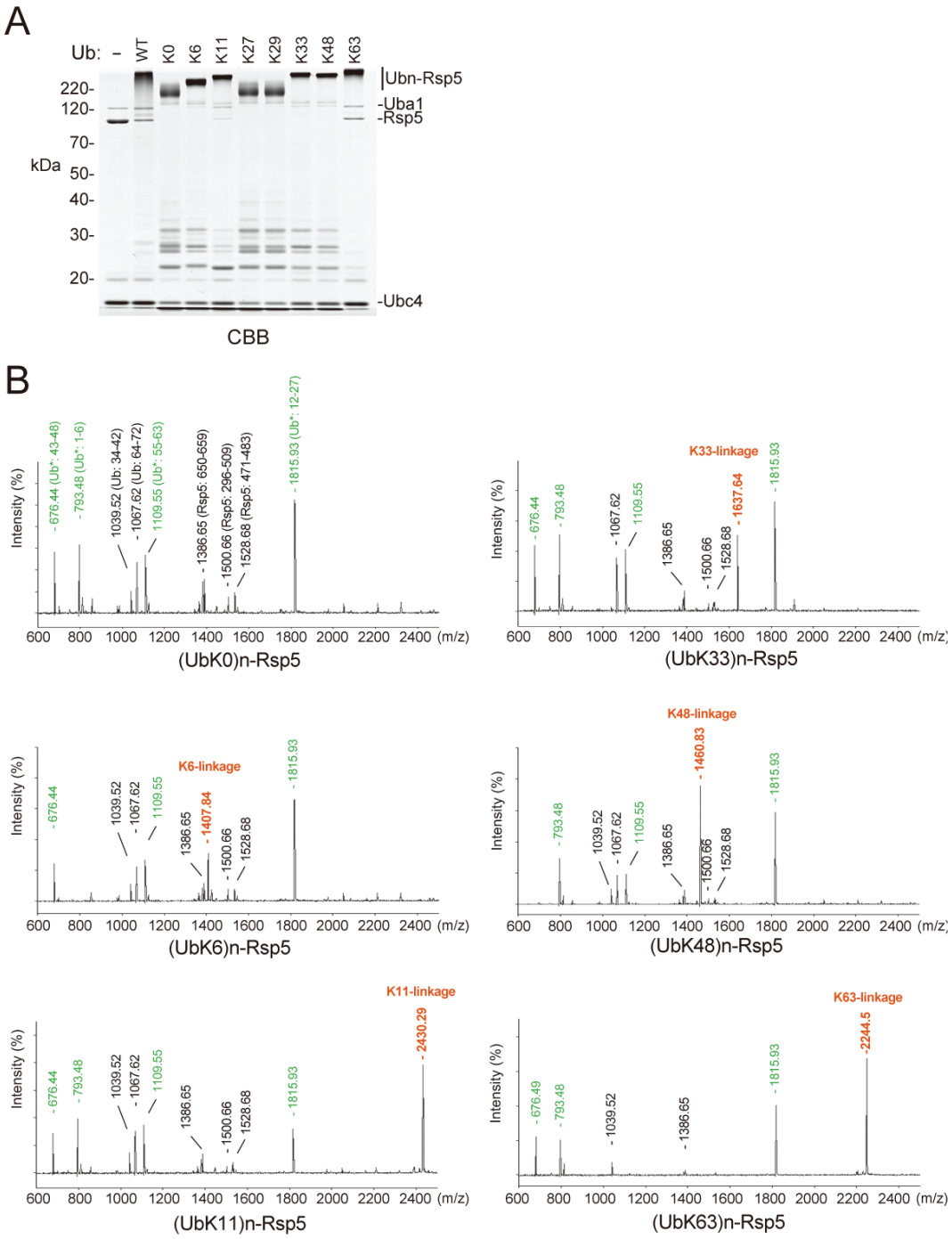

Figure S2. MS analysis of self-ubiquitinated Rsp5 with single-lysine ubiquitin mutants. (A) CBB-stained gel of the self-ubiquitinated Rsp5 with single-lysine Ub mutants. (B) MS analysis of the self-ubiquitinated Rsp5. The ion peaks corresponding to Ub linkages are indicated in an orange. The ion peaks derived from mutant Ub peptides are indicated as "Ub*" in a green.

Specific ion peaks corresponding to K6-, K11-, K33-, K48-, and K63-linkages were easily detected. Thus, the MALDI-MS analysis is a simple and reliable method for determination of Ub-linkages.

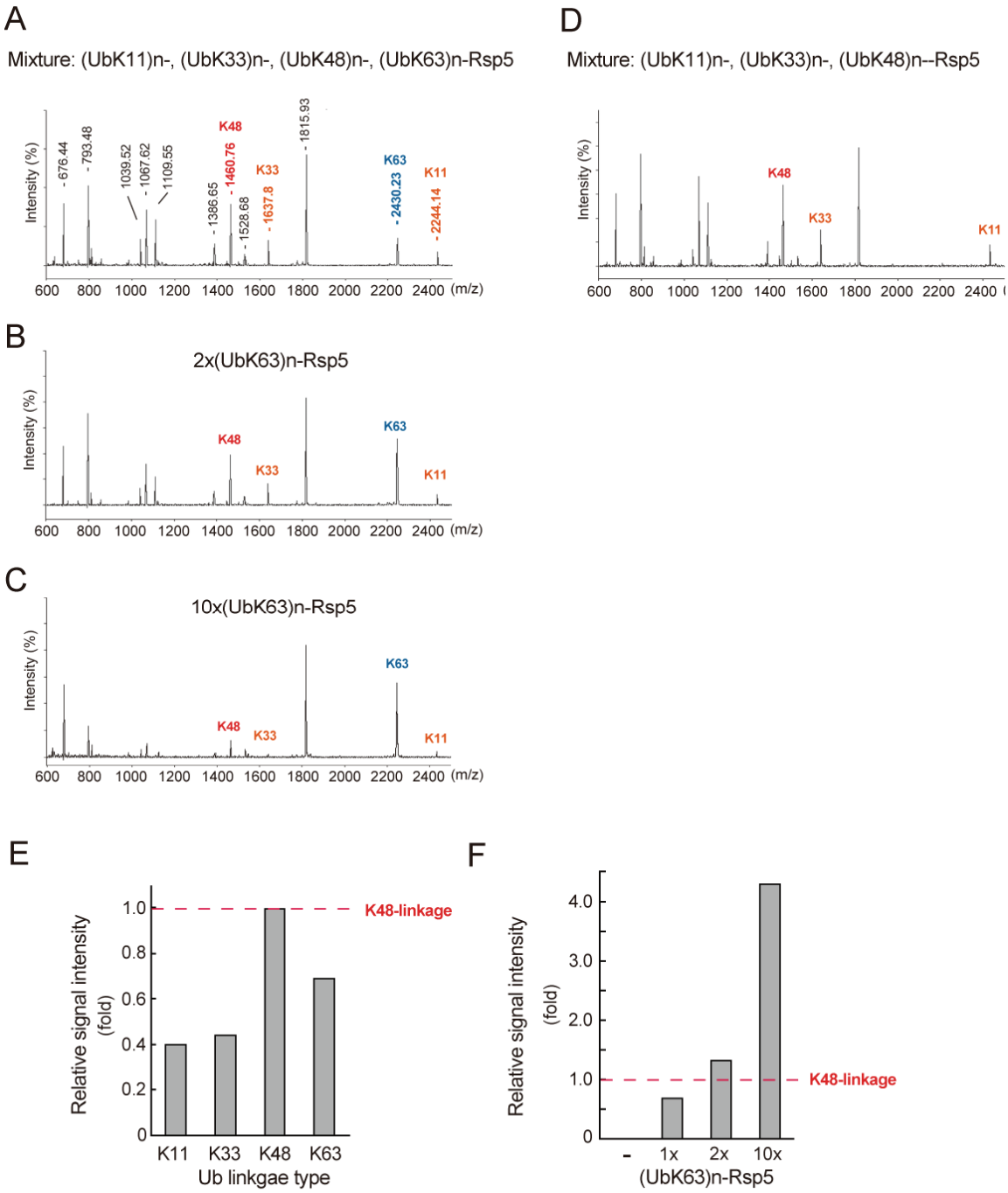

Figure S3. Semiquantitative MS analysis by MALDI-TOF to detect various ubiquitin chains.

(A-D) MS analyses of mixtures of four different Ub-linkages. The tryptic-peptides from the self-ubiquitinated Rsp5 with UbK11, UbK33, UbK48, and UbK63 in Figure S2 were mixed at an equal molar of all four Ub-linkages (A), 2-molar excess of K63-linkage (B), 10-molar excess of K63-linkage (C), and absence of K63-linkage (D). (E) Relative peak intensities of four types of Ub linkages in (A) normalized to the K48-linkage. (F) Relative peak intensities of different amounts of K63-linkage in the mixture, normalized to the K48-linkage shown in (A-D).

The ion intensities of these Ub linkages are correlated with their abundance. Thus, the MALDI-MS is applicable for semiquantitative analysis of Ub-linkages.

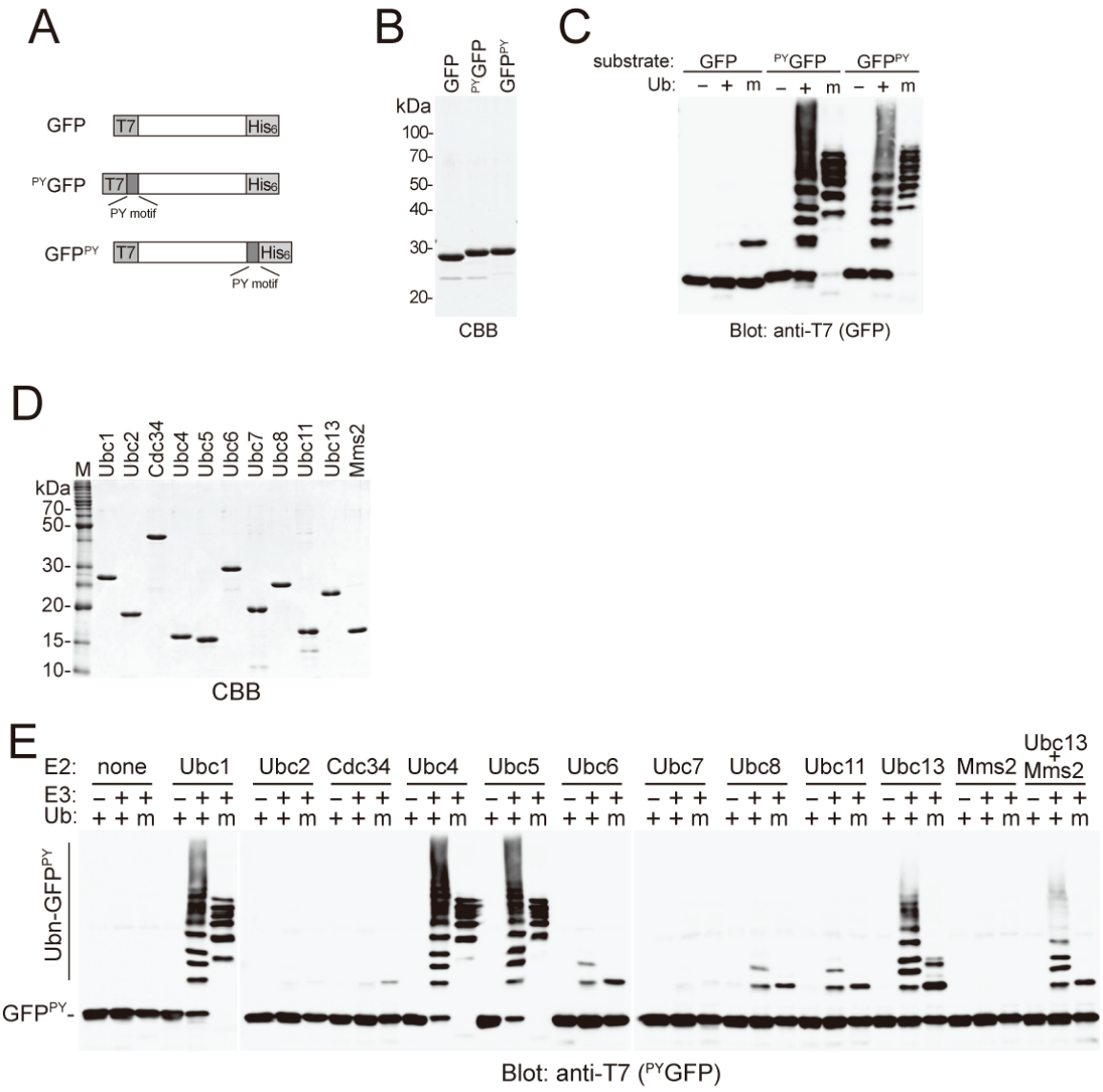

Figure S4. Rsp5 functions with Ubc1, Ubc4, and Ubc5 *in vitro*.
(A) Schematic representation of the PY-motif fused green fluorescence protein (GFP). The PY-motif was fused to the N-terminal or C-terminal region of GFP, designated as ^{PY}GFP and GFP^{PY}, respectively. (B) SDS-PAGE analysis of the purified GFP, ^{PY}GFP, and GFP^{PY}. (C) Ubiquitination of the PY-motif fused GFP. Ubiquitination assay was performed with wild-type Ub (+), methylated Ub (m), or Ub-omitted reaction (-). Ubc4 was used for E2. (D) SDS-PAGE analysis of the purified E2s and Mms2. (E) Ubiquitination assay of GFP^{PY} was performed with different E2s.

We investigated the E2 specificity of Rsp5 for ubiquitination *in vitro* because it has not been defined so far. Three of ten E2s, Ubc1, Ubc4, and Ubc5, stimulated almost equivalently the Rsp5-catalyzed ubiquitination towards ^{PY}GFP (E), as implied by previous genetic studies (Dunn and Hicke, 2001; Horak, 2003; Rotin et al., 2000). These E2 enzymes are known to play an overlapping role (Seufert et al., 1990). Although Ubc13 also stimulates this ubiquitination to a lesser extent, the Ubc13-mediated ubiquitination was considerably suppressed by the addition of Mms2 (E). Because Ubc13 is known to complex with Mms2 tightly (Hofmann and Pickart, 1999), Ubc13 is not likely to function with Rsp5.

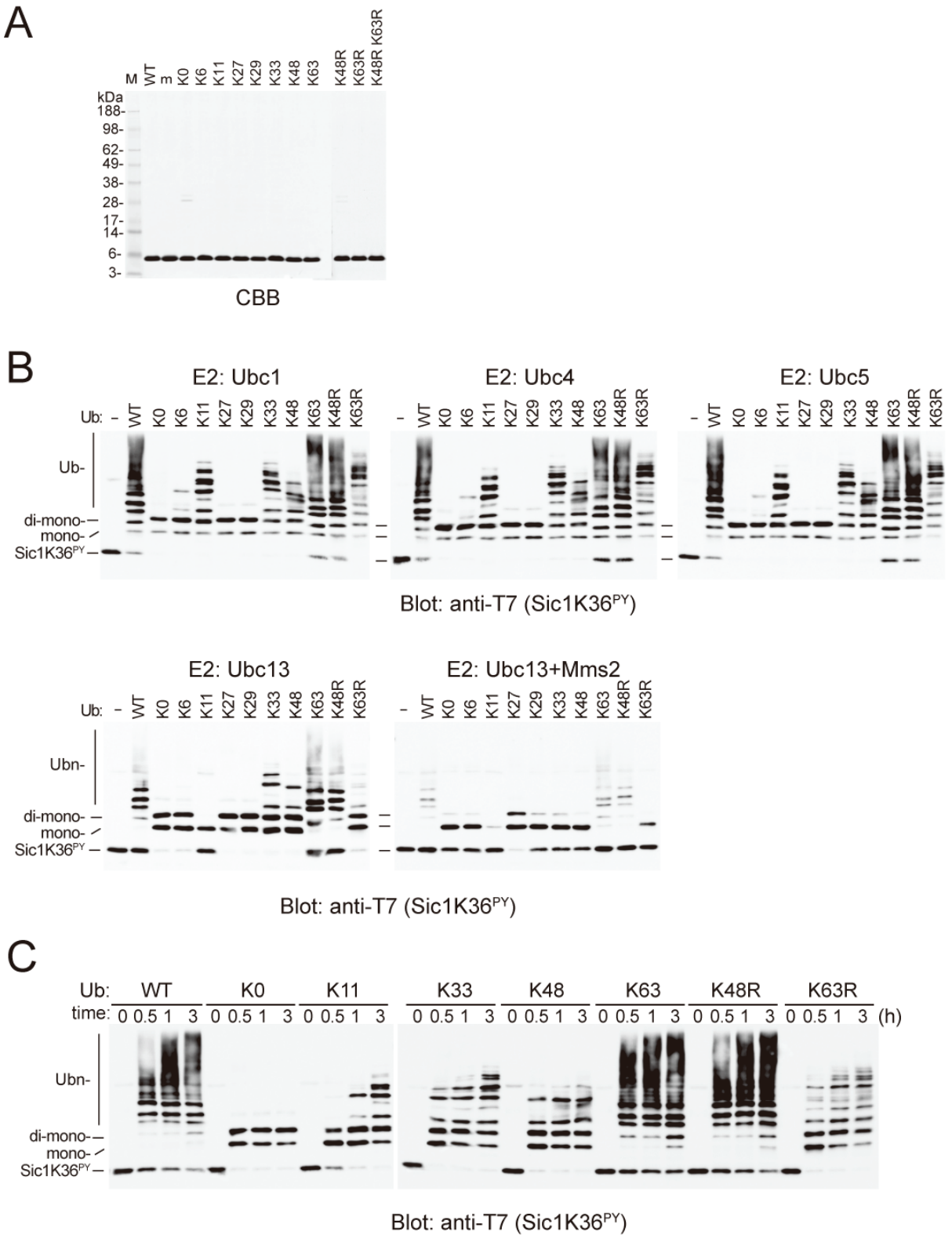

Figure S5. Ubc1, Ubc4, and Ubc5 show the same linkage-specificities *in vitro*.
(A) SDS-PAGE analysis of the purified Ub mutants and a Ub derivative; wild-type Ub (WT), methylated Ub (m), lysine-less Ub (K0), single-lysine Ub (K6, K11, K27, K29, K33, K48, and K63), and Lys to Arg altered Ub mutants (K48R, K63R, and K48R K63R). (B) Ubiquitination assay of Sic1K36^{PY} using various E2s and Ub mutants. (C) Kinetic analysis of Sic1K36^{PY} ubiquitination with Ub mutants. Ubc4 was used as E2.

Recent systematic analysis suggests that some E3s function together with different E2s for substrate ubiquitination and E2 specifies the Ub chain-types *in vitro* (Kim et al., 2007). To test whether E2s affect the chain-type specificity for Rsp5, we performed the ubiquitination assay of Sic1K36^{PY}, K36 only Sic1^{PY}, to monitor the chain length. We found that the types and length of Ub chains did not change irrespective of Ubc1, Ubc4, and Ubc5 with single-lysine Ub mutants (B). We also tested a native Rsp5 substrate Mga2-p120 with two different E2s, Ubc1 and Ubc4 (see Figure S6). Because several Rsp5 substrates are known to stabilize in the *ubc4 ubc5* double mutant (Gwizdek et al., 2005; Horak, 2003; Somesh et al., 2005), and Ubc4 is substantially abundant in growing cells, relative to Ubc5 (Seufert and Jentsch, 1990), we routinely used Ubc4 as E2 for the ubiquitination assays.

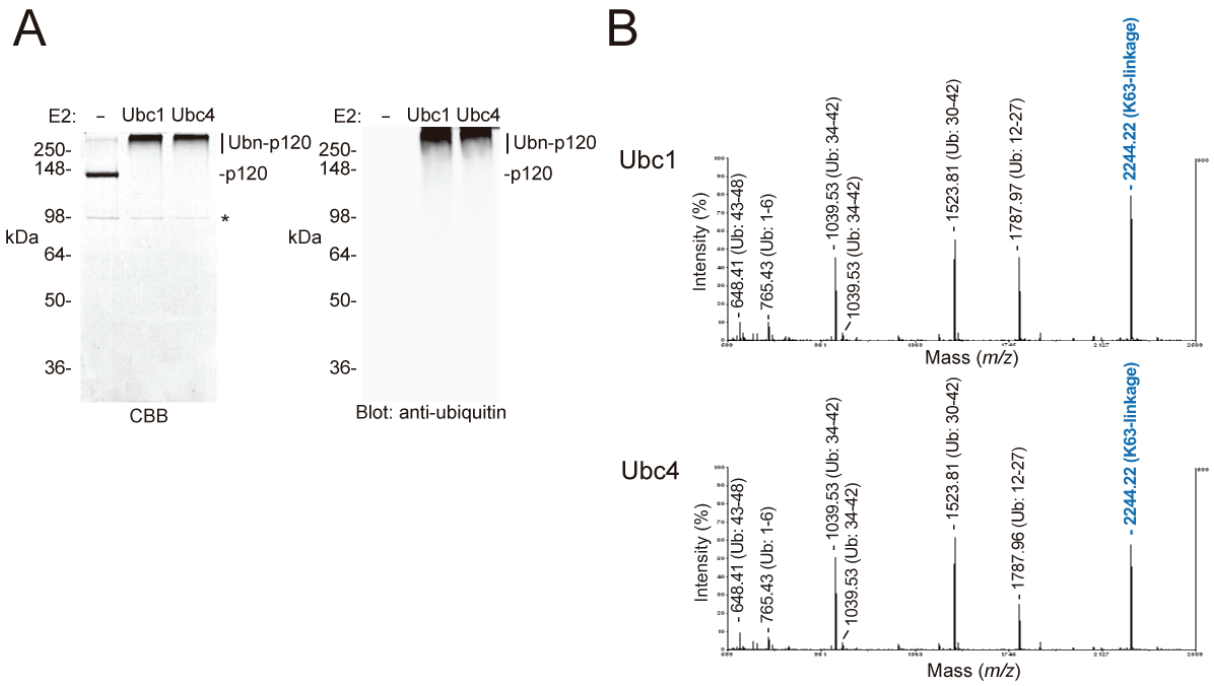

Figure S6. **Ubiquitin chain topologies on Mga2-p120 with Ubc1 as E2 *in vitro*.**
(A) Ubiquitination of ^{Flag}Mga2-p120 *in vitro*. The purified ^{Flag}Mga2-p120 was ubiquitinated by Rsp5 and two different E2s, Ubc1 or Ubc4. E2-omitted reaction was also performed as a control (-). After the ubiquitination, ^{Flag}Mga2-p120 and its Ub conjugates were pulled down with anti-Flag antibody-coupled agarose, then were analyzed by SDS-PAGE (left) and Western blot with anti-Ub antibody (right).Contaminant is marked as *. (B) MS analysis of the ^{Flag}Mga2-p120 Ub conjugates. Gel portion containing the ^{Flag}Mga2-p120 Ub conjugates were analyzed by MS spectrometry as in Figure 1.

Although Ubc1 is reported as a specialized E2 that can assemble the K48-linked chains itself (Hodgins et al., 1996; Rodrigo-Brenni and Morgan, 2007), only K63-linkage was detected in the ubiquitinated Mga2-p120 by Rsp5 even if Ubc1 was used as E2.

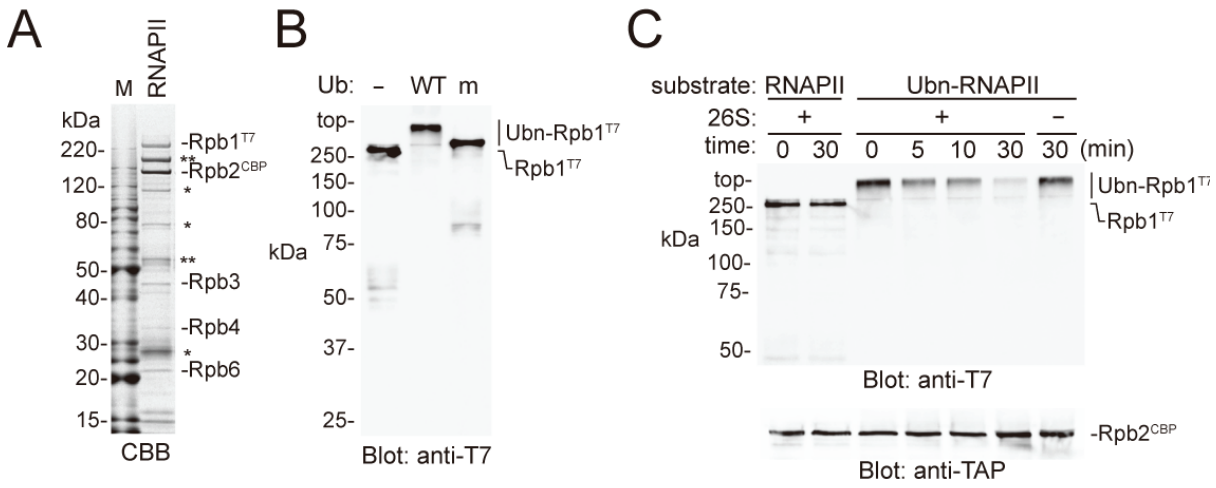

Figure S7. Degradation of the ubiquitinated Rpb1 by the 26S proteasome *in vitro*. (A) SDS-PAGE analysis of the purified yeast RNA polymerase II. RNA polymerase II (RNAPII) was purified from the *RPB1-T7 RPB2-TAP* strain (YYS340). The identified RNAPII subunits are indicated. Contaminants and degradation products of Rpb1 are marked as * and **, respectively. (B) Ubiquitination of Rpb1, the largest subunit of the RNAPII complex, by Rsp5 *in vitro*. The purified RNAPII was subjected to the ubiquitination assay with wild-type Ub (WT), methylated-Ub (m), or the Ub-omitted reaction (-). Ubiquitination of $Rpb1^{T7}$ was monitored by Western blotting with anti-T7 antibody. (C) Degradation assay of polyubiquitinated Rbp1 by the 26S proteasome. After ubiquitination as in (B), the ubiquitinated RNAPII was incubated with the wild-type 26S proteasome (Ubn-RNAPII). The Ub-omitted reaction was also tested as a control (RNAPII). The degradation of the ubiquitinated $Rpb1^{T7}$ was monitored by Western blotting with anti-T7 antibody (top). The second largest subunit, $Rpb2^{CBP}$, was also monitored by Western blotting with anti-TAP antibody (bottom).

The reaction mixture containing the polyubiquitinated Rpb1 was also subjected MS analysis. As expected, only K63-linkage was detected, suggesting that Rpb1 is attached with the K63-linked Ub chains by Rsp5 (data not shown). Notably, $Rpb2^{CBP}$ was not degraded, suggesting that the 26S proteasome specifically segregated and degraded the ubiquitinated Rpb1 (Figure S6C), and that the K63-linked polyUb chain(s) is sufficient to sustain segregation of the complex to be degraded from the stable complex and processive degradation.

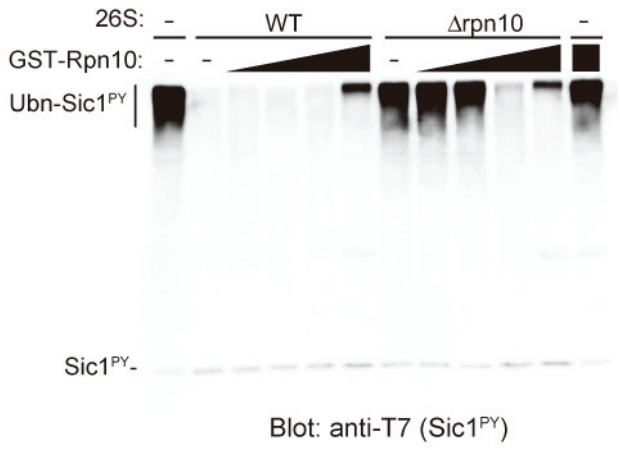

Figure S8. Rpn10 promotes the degradation of K63-linked ubiquitinated Sic1^{PY}
The wild-type and *Δrpn10* 26S proteasomes (50 nM each) were preincubated with various amounts of GST-Rpn10 (10, 50, 250, and 1000 nM) at 4°C for 1 hr. Then, the degradation assay was performed as in Figure 2D.

Rpn10 is an intrinsic Ub receptor subunit of the 26S proteasome and plays an crucial role in the binding and degradation of K48-linked ubiquitinated Sic1 *in vitro* (Verma et al., 2004). Recombinant Rpn10 protein restores the defect of the 26S proteasome lacking Rpn10, suggesting that Rpn10 facilitates the recognition of K48-linked Ub chains and substrate recruitment (Verma et al., 2004). We found that the *Δrpn10* proteasome also failed to degrade K63-linked ubiquitinated Sic1^{PY}. To test whether recombinant Rpn10 promotes the degradation of K63-linked ubiquitinated substrate, we next performed an add-back experiment using recombinant GST-Rpn10. The wild-type and *Δrpn10* proteasomes were preincubated with GST-Rpn10, and then incubated with K63-linked ubiquitinated Sic1^{PY} for 10 min. The *Δrpn10* proteasome failed to degrade K63-linked ubiquitinated Sic1^{PY}, but adding-back GST-Rpn10 restored the activity. The effect of GST-Rpn10 was dose-sensitive as in the case of K48-linked ubiquitinated Sic1 (Verma et al., 2004). At a high concentration of Rpn10 (1000 nM), 20-fold molar excess over the proteasome, the inhibitory effect was observed in both wild-type and mutant proteasomes, and at 5-fold molar excess (250 nM), the restoration was observed in the mutant proteasome. Thus, Rpn10 facilitates the recognition of K63-linked Ub chains and substrate recruitment, as well as K48-linked chains.

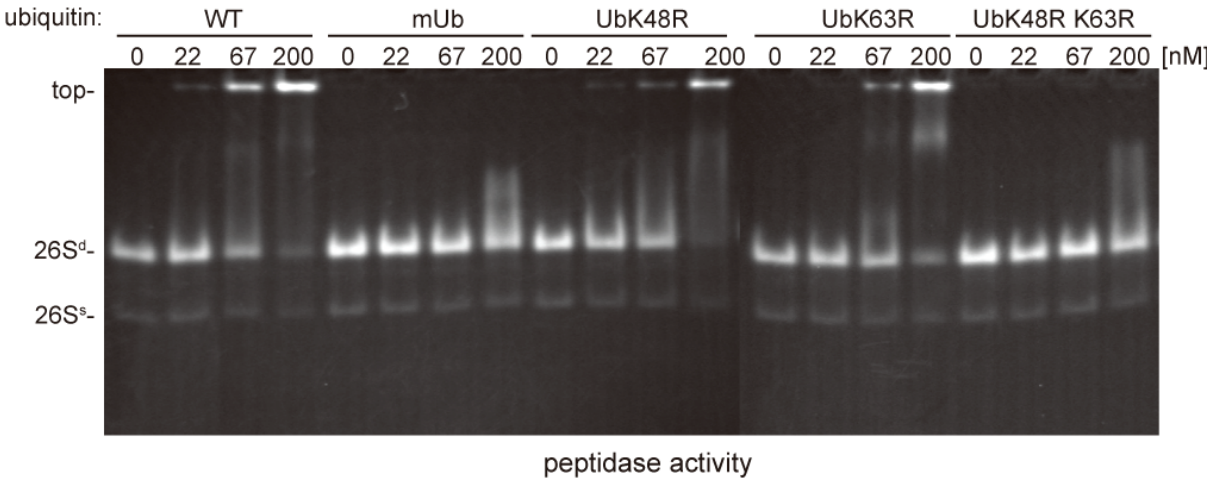

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Figure S9. The 26S proteasome binds with high affinity to both K48- and K63-linked polyubiquitin chains.

Association of the 26S proteasome with polyubiquitin chains on GST-Rsp5 was monitored by a gel-shift assay as in Figure 3B. The 26S proteasome (200 nM) was incubated with
440 various amounts of each GST-Rsp5 Ub conjugates (final concentration; 22 to 200 nM) and then subjected to native-PAGE followed by an in-gel peptidase assay.

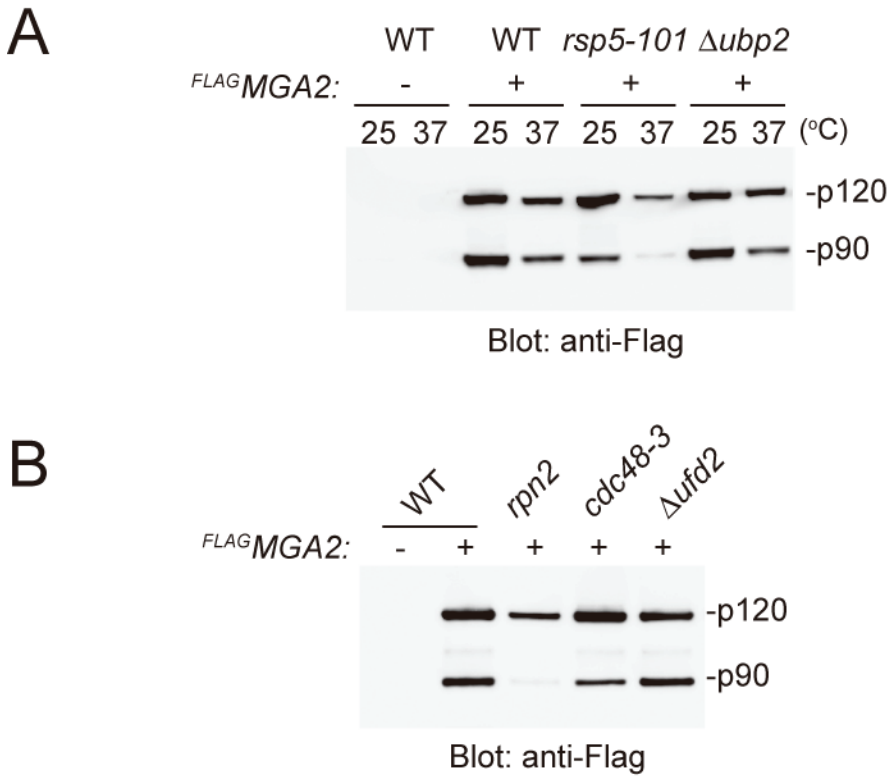

Figure S10. Retardation of Mga2-p120 processing in *rsp5* and proteasome mutant cells. (A and B) The indicated strains carrying either pYF6-MGA2 (+) or pYF6 (-) were cultured to an OD_{600} ~0.8 in SRaf-Trp medium. Then, the expression of Mga2 was induced by the addition of galactose (2% final) for 2 hr. Cells corresponding to 1 OD_{600} were harvested and extracted by the mild-alkali method (Kushnirov, 2000). The extracts were analyzed by Western blotting with anti-Flag antibody. (A) For the *rsp5-101* temperature-sensitive mutant, *Δubp2* mutant, or their parent wild-type strain (KA31), the cells were placed at the indicated temperature for 1 hr prior to the addition of galactose. (B) For the *rpn2* mutant, *cdc48-3* mutant, *Δufd2*, or their parent wild-type strain (W303-1A), the cells were cultured at 25°C.

In *rsp5* and *rpn2* mutant cells, the processing of Mga2-p120 was retarded even at permissive temperatures, suggesting that both Rsp5 and the 26S proteasome regulate the overexpressed ^{FLAG}Mga2-p120 processing as reported previously (Hoppe et al., 2000). The expression level of ^{Flag}Mga2 was significantly low in *rsp5* cells at restrictive temperature, probably due to a defect in mRNA export (Neumann et al., 2003). The level of p90 was slightly low in the *cdc48-3* cells, suggesting that Cdc48-mediated p90 segregation from ER membrane affects p120-procesing as reported previously (Rape et al., 2001; Shcherbik and Haines, 2007). In contrast, the steady-state level of ^{FLAG}Mga2-p120 was slightly decreased in *Δubp2* cells (Kee et al., 2005), see also Figure S11.

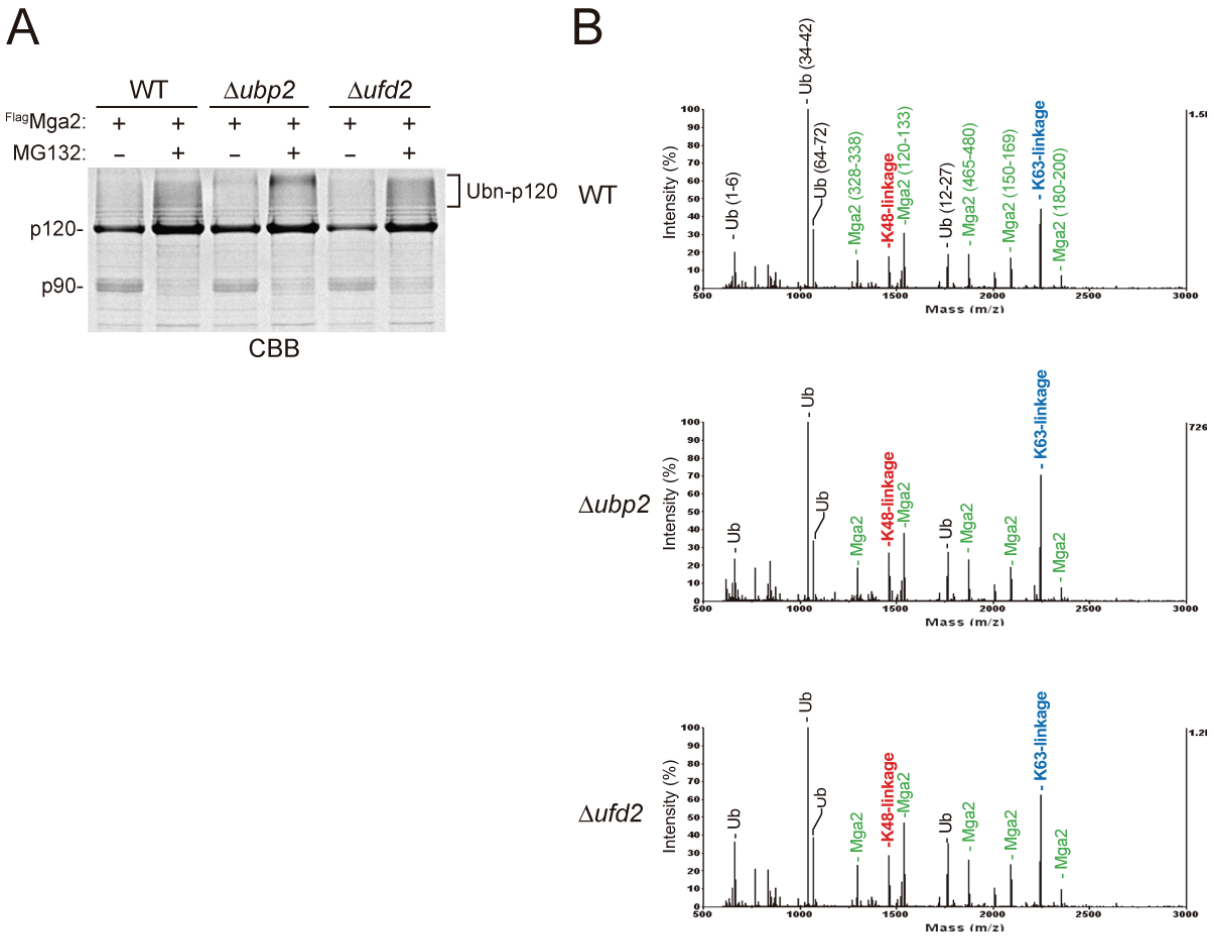

Figure S11. Analysis of the Mga2-p120 Ub conjugates from *Δubp2* and *Δufd2* cells. (A) SDS-PAGE analysis of the purified Mga2 from *Δubp2* and *Δufd2* cells. YYS1303 (wild-type, WT), YYS1327 (*Δubp2*), or *Δufd2* (YYS1334) cells carrying pKT10-Ub and pYF6-MGA2 (^{Flag}Mga2) were analyzed as in Figure 4A. (B) MS spectra of the ubiquitinated-Mga2 from wild-type (WT), *Δubp2*, or *Δufd2* cells. The gel portion of the Mga2-p120 Ub conjugates from MG132-treated cells, indicated by a blanket in (A), was analyzed by MALDI-TOF MS. Ion peaks corresponding to K48-, K63-linkages, and Mga2 peptides are indicated in red, blue, and green, respectively.

Since Ubp2, a deubiquitinating enzyme, is known to interact and regulate Rsp5 (Kee et al., 2006), we investigated whether Ubp2 affects the ubiquitination levels of Mga2-p120. We found that the significant accumulation of polyubiquitinated Mga2, probably K63-linkages, was observed from MG132-treated *Δubp2* cells (B, middle panel). These results suggest that the construct used in this study is a *bona fide* substrate of Rsp5. The p120 processing is normally occurred in *Δubp2* cells (data not shown), suggesting that the Rsp5 activity to Mga2-p120 overcomes the Ubp2 activity in wild-type cells. It is known that Ufd2, an E4 that assembles K48-linked Ub chains, regulates Spt23-p90 degradation (Richly et al., 2005). To exclude the possibility that the existence of K48-linked chains in the Mga2 Ub conjugates is due to Ufd2 activity, the Ub conjugates from *Δufd2* cells were subjected to MS analysis. However, the K48-linkage was still detected in the preparation from *Δufd2* cells. The result suggests that other E3(s) still exits or Rsp5 can assemble the K48-linked chains in a certain situation.

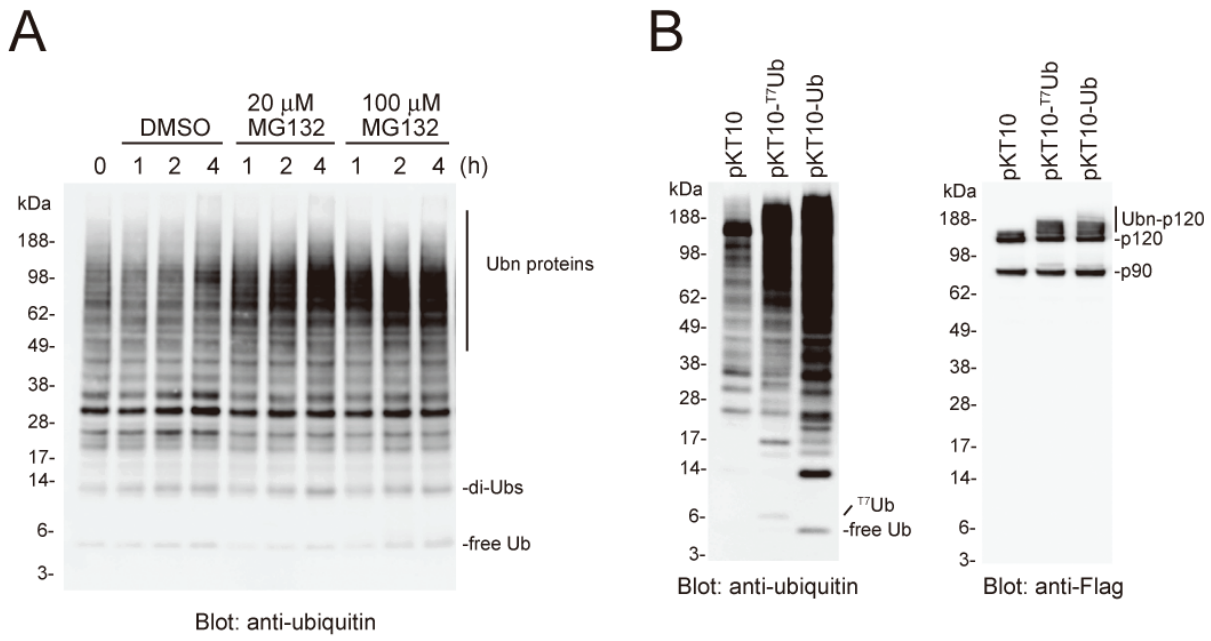

Figure S12. Conditions to stabilize and enhance the protein ubiquitination.
(A) Accumulation of the ubiquitinated proteins in MG132-treated *Δpdr5* cells. To increase sensitivity to the proteasome inhibitor MG132, the *PDR5* gene was disrupted (Fleming et al., 2002). The *Δpdr5* cells were treated with 20 μM or 200 μM MG132 for the indicated period. Then, the extracts were analyzed by Western blotting with anti-Ub antibody.
(B) Ubiquitination of Mga2-p120 was enhanced in the Ub-overproduced cells. Wild-type cells were transformed with one of the plasmids, pOKA601 (pKT-Ub), pOKA605 (pKT-^{T7}Ub), or control plasmid (pKT10), together with pOKA606 (pYF6-MGA2). The transformants were cultured to ~0.8 OD_{600} in SRaf-Ura-Trp medium. Then, the expression of Mga2 was induced by the addition of galactose (2% final) for 2 hr. Extracts were analyzed by Western blotting with anti-Ub or anti-Flag antibody.

We optimized the concentration of MG132 for *Δpdr5* cells. At a concentration as high as 100 μM, the ubiquitin conjugates were reached to maximal accumulations within 2 h (A). We also investigated whether the overproduced Ub enhances the p120 ubiquitination. The ubiquitination levels of p120 were markedly increased in both the T7-tagged Ub (^{T7}Ub) and wild-type Ub expressed cells (B). Epitope-tagged Ubs are useful tool for their purification, but, the following MS analysis revealed that the T7-tagging of Ub prevented the efficient formation of Ub chains *in vivo* (data not shown) as reported previously (Ellison and Hochstrasser, 1991). Therefore, we used untagged wild-type Ub in this study.

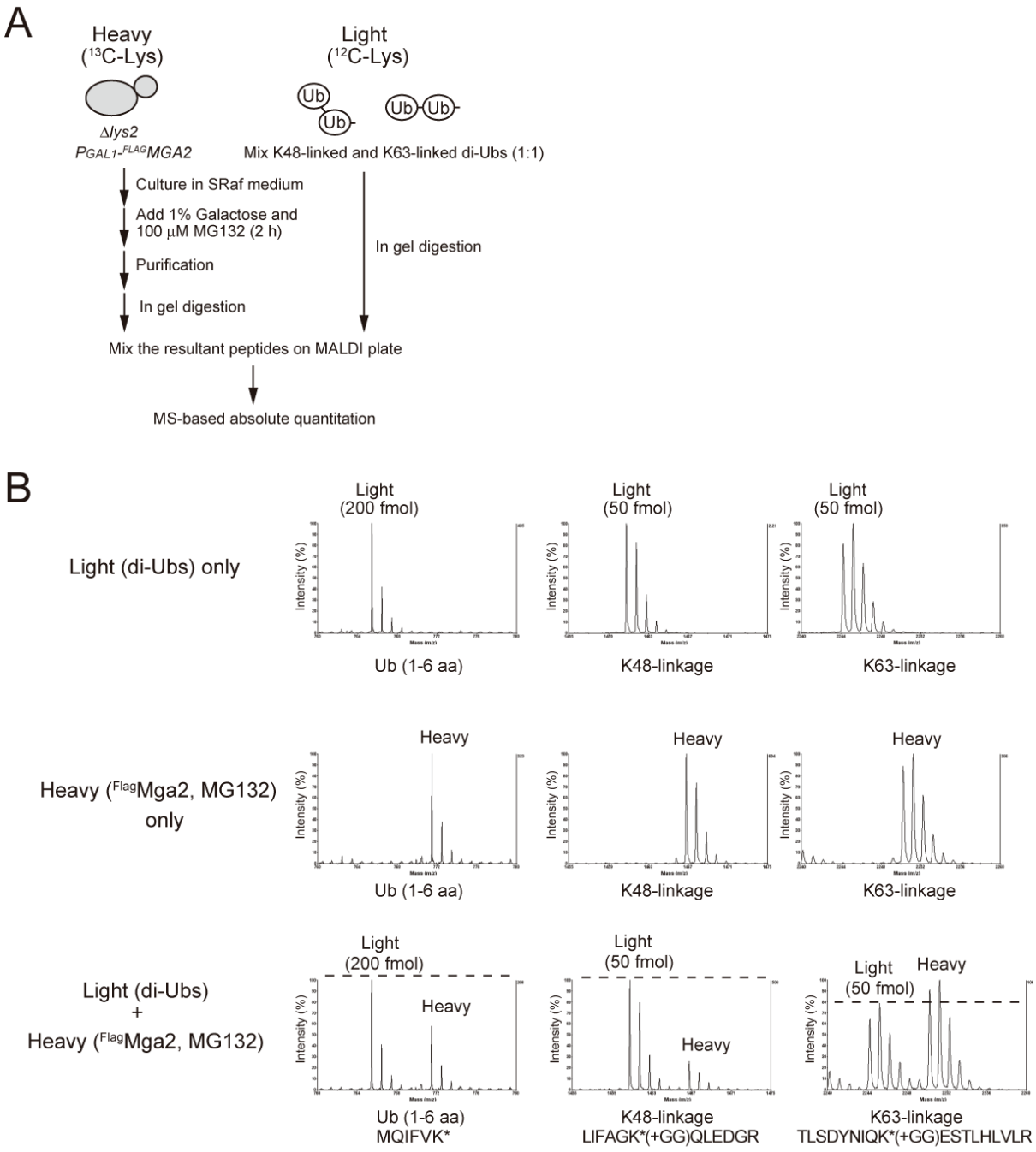

Figure S13. Absolute quantitation of the Ub chains on the Mga2.
(A) Depiction of workflow for absolute quantitaion of the Ub chains. (B) SILAC ion pairs of a linear Ub peptide (1-6 amino acids), K48-, and K63-linkages. Since these peptides contain only a single Lys, incorporation of ^{13}C-Lys generates a mass shift of 6 Da. Peptide sequences were also determined by MS/MS analysis.

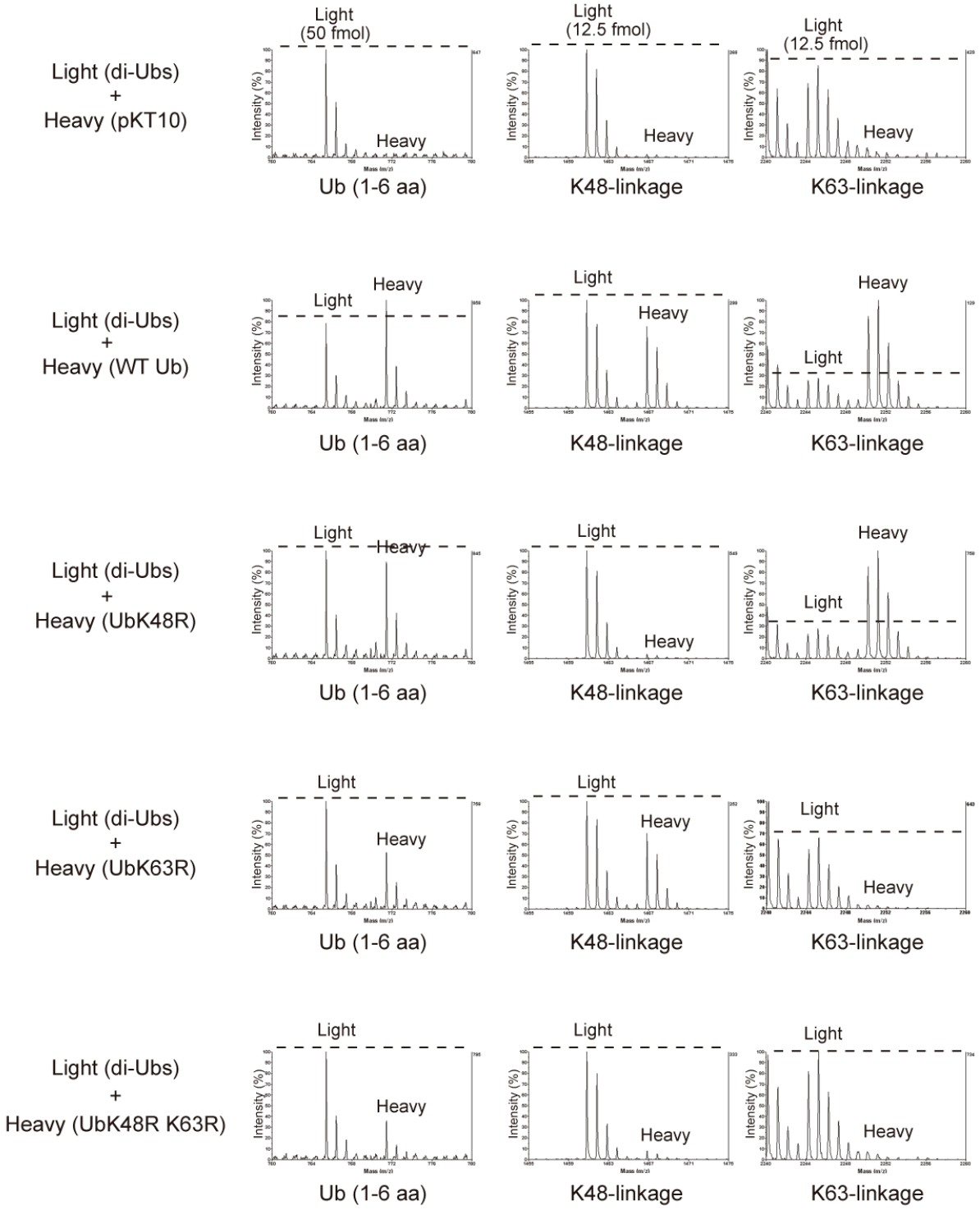

Figure S14. Absolute quantitation of the Ub chains in the Mga2 Ub conjugates from the mutant Ub-expressed cells.

The Mga2 Ub conjugates from the cells expressing the respective mutant Ub were analyzed as in Figure S13. See also, Figure 5E.

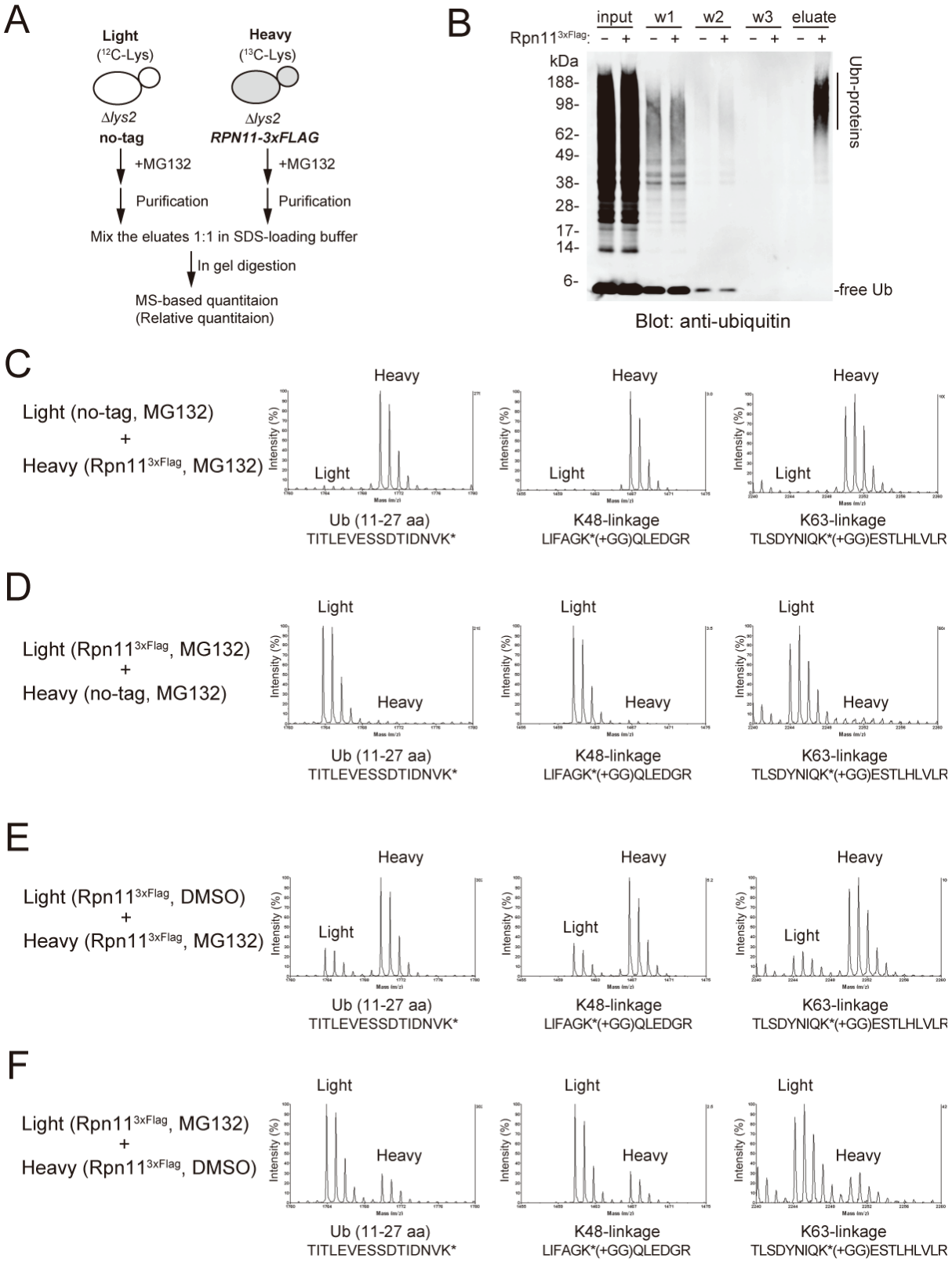

Figure S15. Relative quantitation of the Ub chains in the proteasome-bound ubiquitinated proteins

(A) Depiction of workflow for relative quantitaion of the Ub chains. (B) Western blot analysis of the proteasome-bound ubiquitinated proteins. The *RPN11-3xFLAG* cells (YYS1320) grew in "light" medium and the control cells (YYS1314) grew in "light" medium

were treated with 100 μM MG132 for 3 h. Then, the 26S proteasome was purified under mild conditions. The total extracts (input), the supernatants of each washing step (w1-w3), and the eluates were analyzed by Western blot with anti-ubiquitin antibody. Polyubiquitinated proteins were specifically enriched in the eluates from the *RPN11-3xFLAG* cells. (C-F) SILAC ion pairs of a linear Ub peptide (11-27 amino acids), K48-, and K63-linkages in the proteasome-bound ubiquitinated proteins from the indicated conditions.

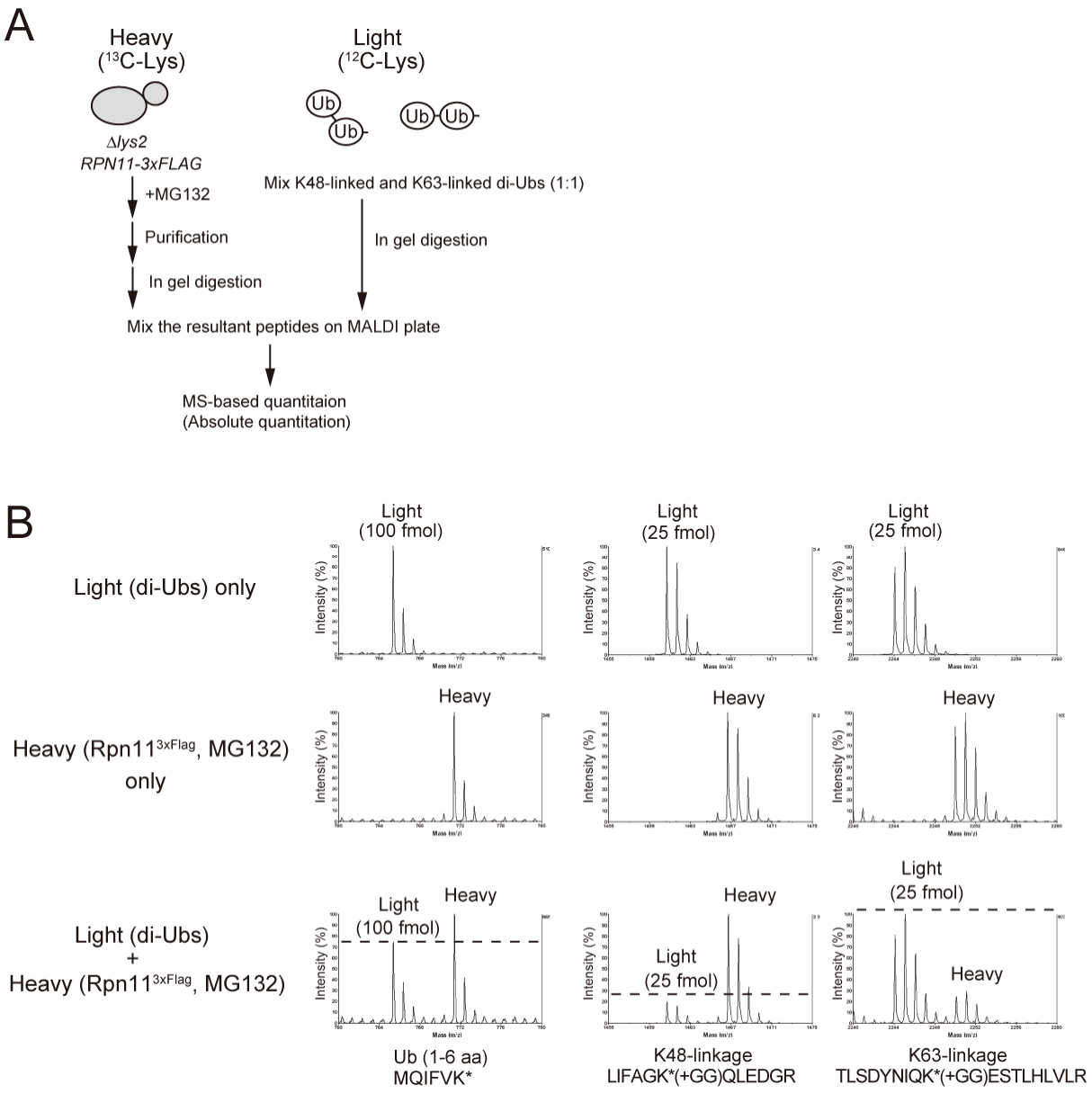

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Figure S16. Absolute quantitation of the Ub chains within the proteasome-bound ubiquitinated proteins

(A) Depiction of workflow for absolute quantitaion of the Ub chains. (B) SILAC ion pairs of a linear Ub peptide (1-6 amino acids), K48-, and K63-linkages.

580

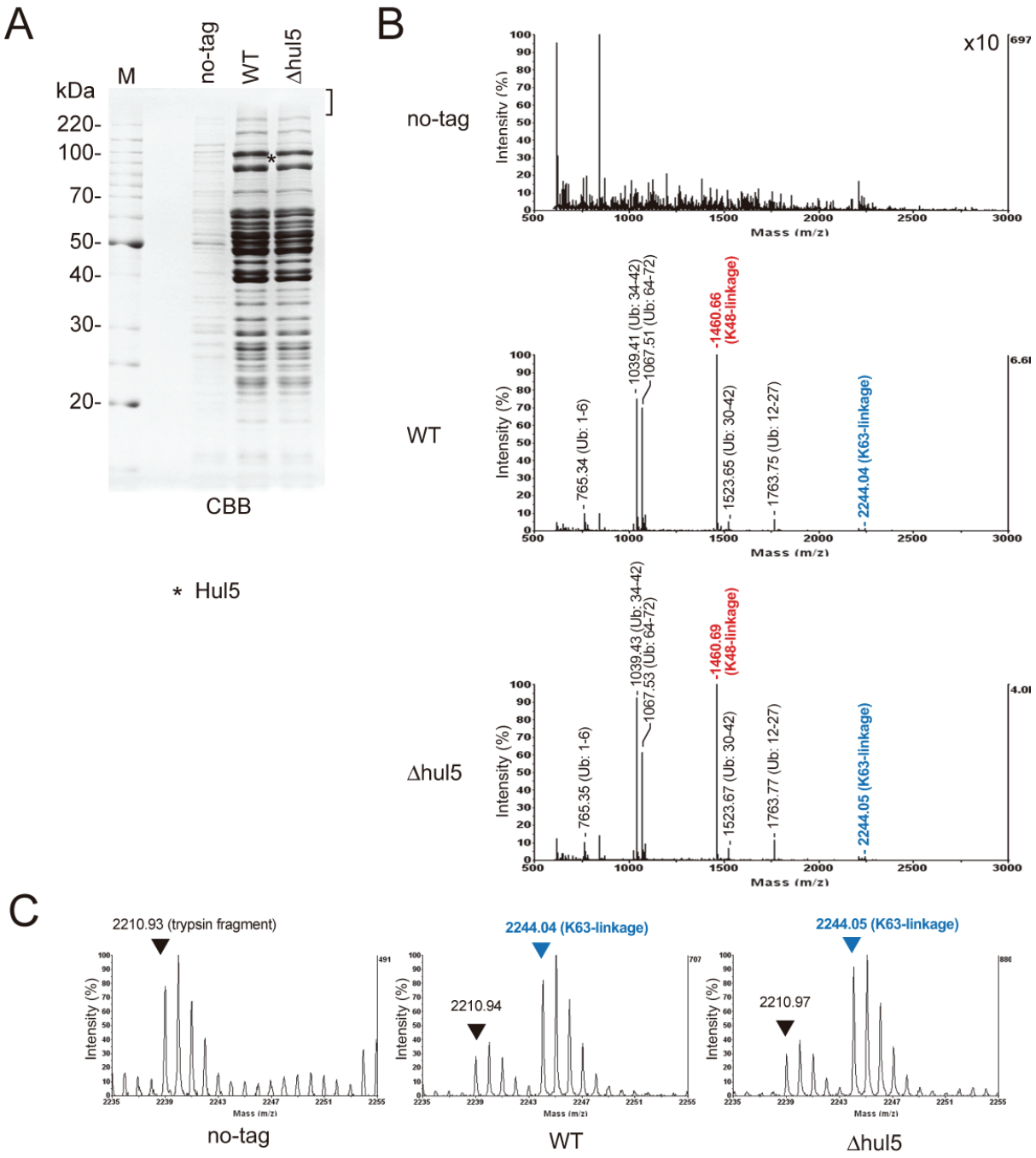

Figure S17. Analysis of the proteasome-bound ubiquitinated proteins from *Δhul5* cells

(A) YYS1320 (WT), YYS1339 (Δhul5), and their control cells YYS1314 (no-tag) were grown to OD_{600} between 0.6-0.8 in SC-Ura medium, then, the cells were treated with 100 μM MG132 for 3 h. The polyubiquitinated proteins bound to 26S proteasomes were analyzed as in Figure S16. (B) MS spectra. The peak corresponding to K48- and K63-linkages are indicated in red and blue, respectively. All MS were confirmed by MS/MS(C) MS regions of the K63-linkage ions.