Supporting Information

Ub-ProT reveals global length and composition of protein ubiquitylation in cells.

Tsuchiya et al.

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Supplementary References

Other supplementary items for the manuscript includes the following:

Supplementary Data 1 (Dataset of Supplementary Fig. 2d)

- Supplementary Data 2 (Dataset of Fig. 3b)
- Supplementary Data 3 (Dataset of Fig. 3d)
- Supplementary Data 4 (Dataset of Supplementary Fig. 7a)

Supplementary Data 5 (Dataset of Fig. 4b)

- Supplementary Data 6 (Dataset of Fig. 4c)
- Supplementary Data 7 (Dataset of Fig. 6c)

Supplementary Figures

а TUBE (tandem ubiquitin binding entities)

UBA His6 UBA UBA UB/

TR-TUBE (trypsin resistant-TUBE)



h										
	10	20	30	40	50	60	70	80	90	100
	ATGCGGGGTT	GTCATCATCA	TCATCATCAT	GGTATGGCTA	GCATGACTGG	TGGACAGCAA	ATGGGTGATA	TCGGAGGTGG	AGGATCTGGA	GGTGGAGTAA
	METArgGly <mark>C</mark>	ysHisHisHi	sHisHisHis	GlyMETAlaS	erMETThrGl	yGlyGlnGln	METGlyAspI	leGlyGlyGl	yGlySerGly	GlyGlyValA
	110	120	130	140	150	160	170	180	190	200
	ATCCTCAGCT	ACAGAATCCA	GAAGTCGCGT	TTCAGCAACA	ACTGGAACAA	CTCAGTGCAA	TGGGATTTTT	GAACGCGGAA	GCAAACTTGC	AAGCTCTAAT
	snProGinLe	uGInAsnPro	GIUVAIAIAP	hegingingi	nLeuGluGIn	LeuSerAlaM	ETGLyPheLe	uAsnAlaGlu	AlaAsnLeuG	InAlaLeull
	210	220	230	240	250	260	270	280	290	300
	AGCAACAGGA	GGTGATATTA	ATGCAGCTAT	TGAAGCGTTA	CTGGGCTCCC	AGCCATCAGG	AGGTGGAGGA	TCTGGAGGTG	GAGTAAATCC	TCAGCTACAG
	eAlaThrGly	GIYASPIICA	SNALAALALL	eGIuAlaLeu	LeuGlySerG	InProSerGI	YGIYGIYGIY	SerGIYGIYG	lyvalAsnPr	oGInLeuGIn
	310	320	330	340	350	360	370	380	390	400
	AATCCAGAAG	TCGCGTTTCA	GCAACAACTG	GAACAACTCA	GTGCAATGGG	ATTTTTGAAC	GCGGAAGCAA	ACTTGCAAGC	TCTAATAGCA	ACAGGAGGTG
	AShProgluv	alalaphegi	nginginLeu	GIUGINLEUS	eralametgi	yPheLeuAsh	ALAGIUAIAA	ShLeuginai	aLeuiieAia	THEGIYGLYA
	410	420	430	440	450	460	470	480	490	CREARCHERE
	ATATTAATGC	AGCTATTGAA	GLGTTACTGG	GUTUUUAGUU	ATCAGGAGGT	GGAGGATCTG	GAGGTGGAGT	AAATCUTCAG	Lauchadarc	CAGAAGTUGU
	SpileAshAi	aAlalleGlu	Alabeubeug	TyserGINPr	OSerGIYGIY	GIYGIYSerG	TAGTAGTAA	TASHPTOGIN	LeugInAshP	FOGLUVALAL
	CULCOCOCO	CAACTCCAAC	DOCTO CTCC	D4U	TTCAACCCCC	700	CCDACCTCTA	ATTACCAACAC	CACCECAEAE	TATCCACCT
	aBhaClaCla	CIRLOUCING	Interfordige	AMETCLUDE	InuAnnalac	LUDIODODIO	UCIPALITA	TICALOTT	LuClulanti	CARDIANIS
	arnegingin 610	GINDeuGiug	THLEUSETAT	ane i Gryfile	LeuASIIATAG	TUATAASIILE	401IIAIabeu	LIEATAINIG	LYGIYASPII	EASIIATAATA 700
	ATTGAACCCT	TACTCCCCTC	CCACCCATCA	CCACCTCCAC	CATCTCCACC	TCCACTABAT	COTCAGOTAC	AGAATCCAGA	AGTOGOGTTT	CACCAACAAC
	TleClublaL	euLeuGluSe	rGlnProSer	GlyGlyGlyG	lySerClyCl	vGlvValAen	ProGlaLauG	InAenProGl	uValAlaPho	GinGinGinL
	710	720	730	740	750	760	770	780	790	800
	TGGAACAACT	CAGTGCAATC	GGATTTTGA	ACCCCCAACC	ABACTTCCBA	CCTCTAATAC	CAACAGGAGG	тсататтаат	GCAGCTATTG	AAGCGTTACT
	euGluGlnLe	uSerAlaMET	GlyPheLeuA	snAlaGluAl	aAsnLeuGln	AlaLeuTleA	laThrGlvGl	VASnTleAsn	AlaAlaTleG	luAlaLeuLe
	810	820	830	840	850	860	870	880	890	900
	GGGCTCCCAG	CCATCAGGAG	GTGGAGGATC	TGGAGGTGGA	GTAAATCCTC	AGCTACAGAA	TCCAGAAGTC	GCGTTTCAGC	AACAACTGGA	ACAACTCAGT
	uGlvSerGln	ProSerGlvG	lvGlvGlvSe	rGlvGlvGlv	ValAsnProG	lnLeuGlnAs	nProGluVal	AlaPheGlnG	lnGlnLeuGl	uGlnLeuSer
	910	920	930	940	950	960	970	980	990	1000
	GCAATGGGAT	TTTTGAACGC	GGAAGCAAAC	TTGCAAGCTC	TAATAGCAAC	AGGAGGTGAT	ATTAATGCAG	CTATTGAAGC	GTTACTGGGC	TCCCAGCCAT
	AlaMETGlyP	heLeuAsnAl	aGluAlaAsn	LeuGlnAlaL	euIleAlaTh	rGlyGlyAsp	IleAsnAlaA	laIleGluAl	aLeuLeuGly	SerGlnProS
	1010	1020	1030	1040	1050	1060	1070	1080	1090	1100
	CAGGAGGTGG	AGGATCTGGA	GGTGGAGTAA	ATCCTCAGCT	ACAGAATCCA	GAAGTCGCGT	TTCAGCAACA	ACTGGAACAA	CTCAGTGCAA	TGGGATTTTT
	erGlyGlyGl	yGlySerGly	GlyGlyValA	snProGlnLe	uGlnAsnPro	GluValAlaP	heGlnGlnGl	nLeuGluGln	LeuSerAlaM	ETGlyPheLe
	1110	1120	1130	1140	1150	1160	1170	1180	1190	1200
	GAACGCGGAA	GCAAACTTGC	AAGCTCTAAT	AGCAACAGGA	GGTGATATTA	ATGCAGCTAT	TGAAGCGTTA	CTGGGCTCCC	AGCCATCAGG	AGGTGGAGGA
	uAsnAlaGlu	AlaAsnLeuG	lnAlaLeuIl	eAlaThrGly	GlyAspIleA	snAlaAlaIl	eGluAlaLeu	LeuGlySerG	lnProSerGl	yGlyGlyGly
	1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
	TCCTA A									
	Sortt t									



Supplementary Figure 1. Structure, sequence, and purification of trypsin-resistant (TR)-TUBE.

(a) Illustrations of the original TUBE construct and TR-TUBE. TUBE, which contains four tandem repeats of the UBA domain of human UBQLN1 with flexible linkers (top), was originally developed by Hjerpe et al.¹. TR-TUBE, used in this study, contains a biotin (the yellow box), a hexahistidine tag (pale green), and six tandem repeats of the mutated UBA domain (aqua; bottom).

(b) DNA and amino-acid sequences of TR-TUBE. The Cys residue for biotinylation, hexahistidine tag, and mutated UBA domains are highlighted as in (a).

(c) Expression and purification of 4×TUBE, 6×TUBE, and 6×TR-TUBE. Purified proteins were analyzed by SDS-PAGE.

(d) Trypsin digestion of TUBEs. Purified TUBEs (1 μ g) indicated by red asterisks were incubated overnight at 37°C with trypsin (200 ng). Black asterisks indicate the degradation product of trypsin.



Supplementary Figure 2. Linkage-type selectivity of TR-TUBE and Ub-ProT assay of di-ubiquitins.

(*a*) Binding assay of TR-TUBE and di-ubiquitins. TR-TUBE (2 μ g) was immobilized to streptavidin-conjugated Dynabeads, and then incubated for 30 min with eight different types of di-ubiquitins linked through K6, K11, K27, K29, K33, K48, K63, or M1 (2 μ g). Unbound (U) and bound (B) proteins were analyzed by SDS-PAGE. The protein bands corresponding to TR-TUBE, di-Ubs, and streptavidin are indicated.

(*b*) Optimization of the amount of trypsin for complete cleavage of K48-linked diubiquitin. K48-linked di-ubiquitin (500 ng) was incubated overnight at 37°C with trypsin (25, 50, 100, or 200 ng). Asterisks indicate the degradation product of trypsin.

(c) Ub-ProT assay of di-ubiquitins. Di-ubiquitin (500 ng) was incubated overnight at 37°C with TR-TUBE (5 µg) and trypsin (50 ng). Di-ubiquitins are marked with red asterisks.

(d) Signal intensities of each di-Ubs, trypsinized sample, or TR-TUBE protection sample as in (c) were quantified and relative protection levels of each di-Ub are represented (means \pm SEM of three independent experiments; Supplementary Data 1).



Supplementary Figure 3. Trypsinizaton of polyubiquitin chains.

Unanchored K48 or K63-linked polyubiquitin chains (500 ng) were incubated with trypsin (250 ng) at 37°C for the times indicated. The chains were visualized by Oriole staining (left) or analyzed by immunoblotting with anti-Ub antibody (P4D1; right).



Supplementary Figure 4. Ub-ProT assay using TR-TUBEs with different numbers of UBA domains.

(*a*) Expression and purification of 4×TR-TUBE, 6×TR-TUBE, and 8×TR-TUBE. Purified proteins were analyzed by SDS-PAGE.

(*b*) M1-linked chains were subjected to Ub-ProT assay with 4×TR-TUBE, 6×TR-TUBE, and 8×TR-TUBE, followed by immunoblotting with anti-ubiquitin antibody.



Supplementary Figure 5. Ub-ProT assay of K48/K63 branched chains.

K48/K63 branched chains were subjected to Ub-ProT assay with TR-TUBE, followed by immunoblotting with linkage specific antibodies (K48-linked: EP8589, K63-linked: EPR8590-448).



Supplementary Figure 6. Gel mobilities of K48-, K63-, and M1-linked polyubiquitin

chains.

Different amounts of K48-, K63-, and M1-linked chains were electrophoresed on 4–12% NuPAGE gel with MES running buffer (Life Technologies). The chains were visualized by Oriole or CBB staining (left and left middle). The chains were also analyzed by immunoblotting with anti-Ub antibody (P4D1; right middle). Clearly separated chains were numbered by different colors. We used two different protein standards (SeeBlue Plus 2 and Novex Sharp Unstained; Life Technologies) and the relative positions in the gel are summarized at right.



Supplementary Figure 7. Standard curves of Ub-AQUA/PRM and the effect of iodoacetamide treatment.

(*a*) Determination of the lower limit of quantitation (LLOQ) of Ub peptides. Ub peptides were spiked at concentrations of 50, 75, 100, 125, 150, or 200 amol in 500 ng of yeast matrix. The calculated standard curve regression line concentrations are presented. The acceptable range of variation was set by the FDA guidelines for the LLOQ (\pm 20%). Thus, the LLOQ of all peptides was determined to 50 amol.

(*b*) Effect of iodoacetamide treatment on ubiquitin quantitation. Previous studies have shown that iodoacetamide (IAA) treatment at high concentrations (55 mM) can lead to artifacts that chemically induce pseudo-ubiquitylation²⁻⁴. We used 10 mM IAA for preparation of our yeast lysates. To investigate whether IAA treatment affects ubiquitin quantitation, ubiquitin monomers were treated with 50 mM IAA for 30 min at room temperature and analyzed by ubiquitin PRM. Chromatograms of selected product ions of light (IAA-treated Ub) and heavy (internal AQUA) peptides are represented. Under this condition, pseudo-Ub peptides (light in each panel) were not detected, suggesting that pseudo-ubiquitylation was negligible in this study.



Supplementary Figure 8. Microscopic analysis of EGFR trafficking.

HeLa cells stably expressing EGFR-EGFP-3×FLAG were stimulated with 100 ng ml⁻¹ of Texas red–conjugated EGF for the indicated times. Cells were fixed and stained with antibody against EEA1, an early endosome marker. Insets show higher magnification images of regions indicated by squares. Arrowheads indicate co-localization of EGF, EGFR, and Baf A1. Scale bars = 40 μ m.



(continued)

Supplementary Figure 9. Uncropped blot and gel images in this study.





(continued)



(continued)



Blot: anti-polyUb (K63)

(continued)



Supplementary Figure 6



Oriole-staining

CBB-staining

Blot: anti-Ub (P4D1)

Strain name	Genotype	Reference
W303-1A	MATa ura3-1 trp1-1 leu2-3,112 his3-11,15 ade2-1 can1-100	Our stock
YYS1325	MATa ura3-1 trp1-1 leu2-3,112 his3-11,15 ade2-1 can1-100	5
	pdr5A:: HphMX lys2A::LEU2 (W303 background)	
YHT38	MATa ura3-1 trp1-1 leu2-3,112 his3-11,15 ade2-1 can1-100	This study
	$ubp6\Delta::KanMX pdr5\Delta::HphMX lys2\Delta::LEU2 (W303$	
	background)	
YHT41	MATa ura3-1 trp1-1 leu2-3,112 his3-11,15 ade2-1 can1-100	This study
	dsk2A::TRP1 rad23A::URA3 pdr5A::KanMX	
	lys2A::LEU2 (W303 background)	
Y202	MATα ura3-1 trp1-1 leu2-3,112 his3-11,15 ade2-1, can1-	6
	100 cdc48-3 (W303 background)	
YYS2060	MATα ura3-1 trp1-1 leu2-3,112 his3-11,15 ade2-1, can1-	7
	100 pdr5Δ::KanMX lys2Δ::LEU2 cdc48-3 (W303	
	background)	
YHT235	MATα ura3-1 trp1-1 leu2-3,112 his3-11,15 ade2-1, can1-	This study
	100 npl4::npl4-1-KanMX (W303 background)	
BY4741	MATa his $3\Delta 1$ leu $2\Delta 0$ met $15\Delta 0$ ura $3\Delta 0$	Open
		Biosystems
Y5665	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 otu1Δ::KanMX4	Open
	(BY4741 background)	Biosystems
Y3888	MATa his $3\Delta 1$ leu $2\Delta 0$ met $15\Delta 0$ ura $3\Delta 0$ ufd 2Δ ::KanMX4	Open
	(BY4741 background)	Biosystems
Y5063	MATa his $3\Delta 1$ leu $2\Delta 0$ met $15\Delta 0$ ura $3\Delta 0$ ufd 3Δ ::KanMX4	Open
	(BY4741 background)	Biosystems
Y3084	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 shp1Δ::KanMX4	Open
	(BY4741 background)	Biosystems

Supplementary Table 1. Yeast strains used in this study.

Supplementary References

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