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Supplementary Information

Structural basis for the E4 enzyme Ufd2-catalyzed K48/K29 branched ubiquitin chains

Zebin Tong[1,2,3,5], Xiangwei Wu[1,2,5], Hongyi Cai[1,2,5], Shidian Wu[2], Tianyi Zhang[2], Zhiheng Deng[1,2], Ziyu Xu[2], Rujing Yuan[2], Huasong Ai[1*], Lei Liu[2*] &Man Pan[1,4,6*]

[1] Institute of Translational Medicine, School of Pharmaceutical Sciences, School of Chemistry and Chemical Engineering, National Center for Translational Medicine (Shanghai), Shanghai Key Laboratory for Antibody-Drug Conjugates with Innovative Target, Shanghai Jiao Tong University, Shanghai 200240, China.

[2] New Cornerstone Science Laboratory, Tsinghua-Peking Joint Center for Life Sciences, Ministry of Education Key Laboratory of Bioorganic Phosphorus Chemistry and Chemical Biology, Center for Synthetic and Systems Biology, Department of Chemistry, Tsinghua University, Beijing 100084, China

[3] Present address: Department of Molecular Machines and Signaling, Max Planck Institute of Biochemistry, Martinsried, Germany

[4] Center for Future Foods, Muyuan Laboratory, Zhengzhou, Henan Province, China

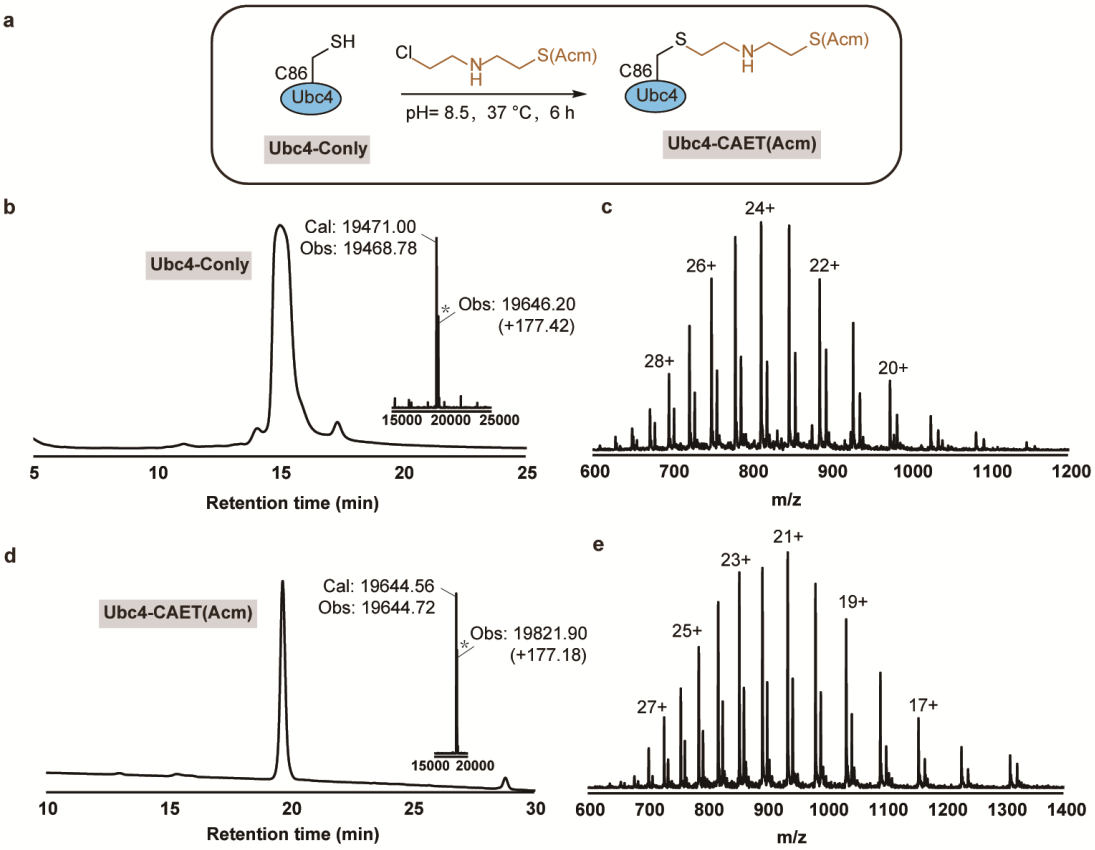
[5] These authors contributed equally to this work: Zebin Tong, Xiangwei Wu, and Hongyi Cai

[6] Lead Contact.

* To whom correspondence should be addressed: Huasong Ai (huasongai@sjtu.edu.cn); Lei Liu (lliu@mail.tsinghua.edu.cn); Man Pan (panman@sjtu.edu.cn)

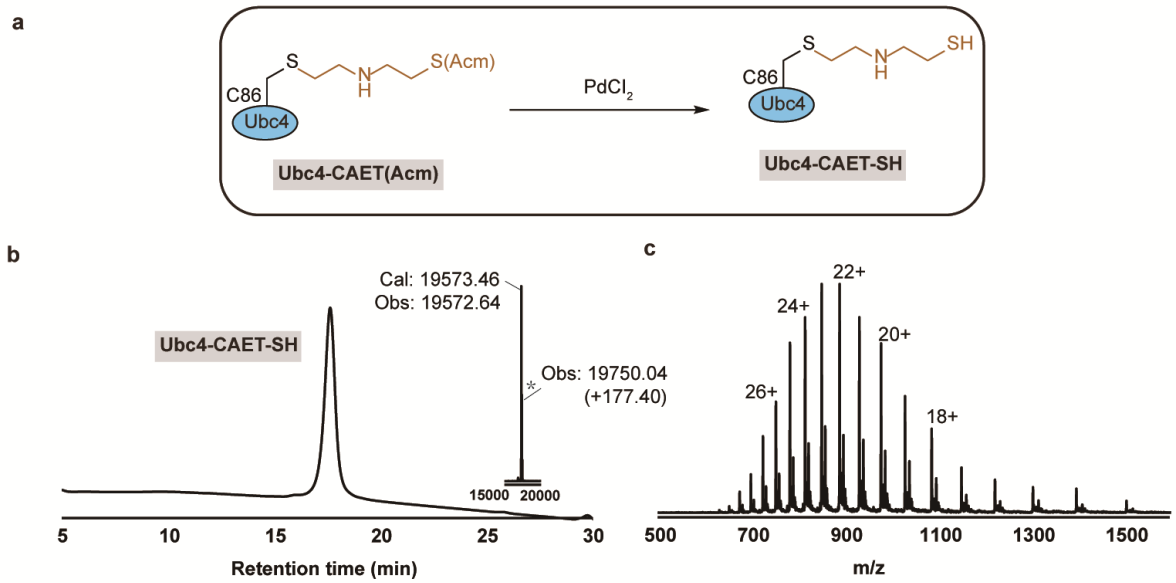
1. Supplementary Figures

1.1 Preparation of Ubc4-CAET-S(Acm)



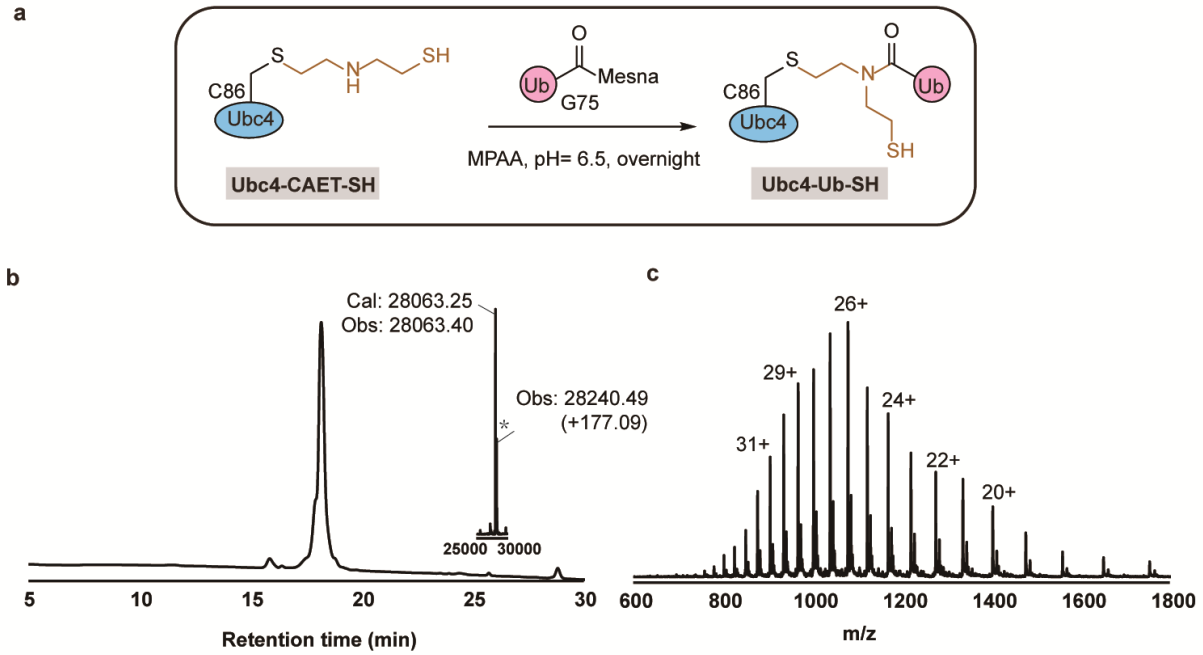
Supplementary Fig. 1 | Preparation of Ubc4-CAET-S(Acm). **a.** Schematic representation of Ubc4-CAET(Acm). **b.** HPLC and deconvoluted MS spectra traces for purified Ubc4-Conly. By-product labelled * results from the spontaneous a-N-6-gluconoylation[1] of His6-tagged Ubc4-Conly during its expression in Escherichia coli. **c.** MS trace for purified Ubc4-Conly. **d.** HPLC and deconvoluted MS spectra traces for purified Ubc4-CAET(Acm). By-product labelled * results from the spontaneous a-N-6-gluconoylation[1] of His6-tagged Ubc4-Conly during its expression in *Escherichia coli*. **e.** MS trace for purified Ubc4-CAET(Acm).

1.2 Preparation of Ubc4-CAET-SH



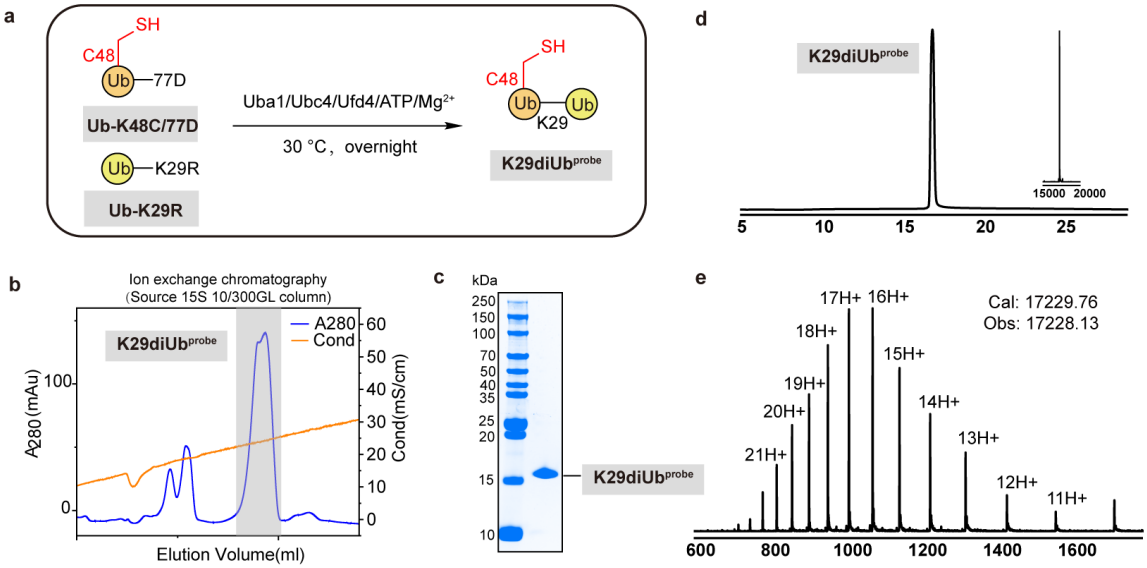
Supplementary Fig. 2 | Preparation of Ubc4-CAET-SH. a. Schematic representation of Ubc4-CAET-SH. **b.** HPLC and deconvoluted MS spectra traces for purified Ubc4-CAET-SH. By-product labelled * results from the spontaneous a-N-6-gluconoylation[1] of His6-tagged Ubc4-Conly during its expression in Escherichia coli. **c.** MS trace for purified Ubc4-CAET-SH.

1.3 Preparation of Ubc4-Ub-SH



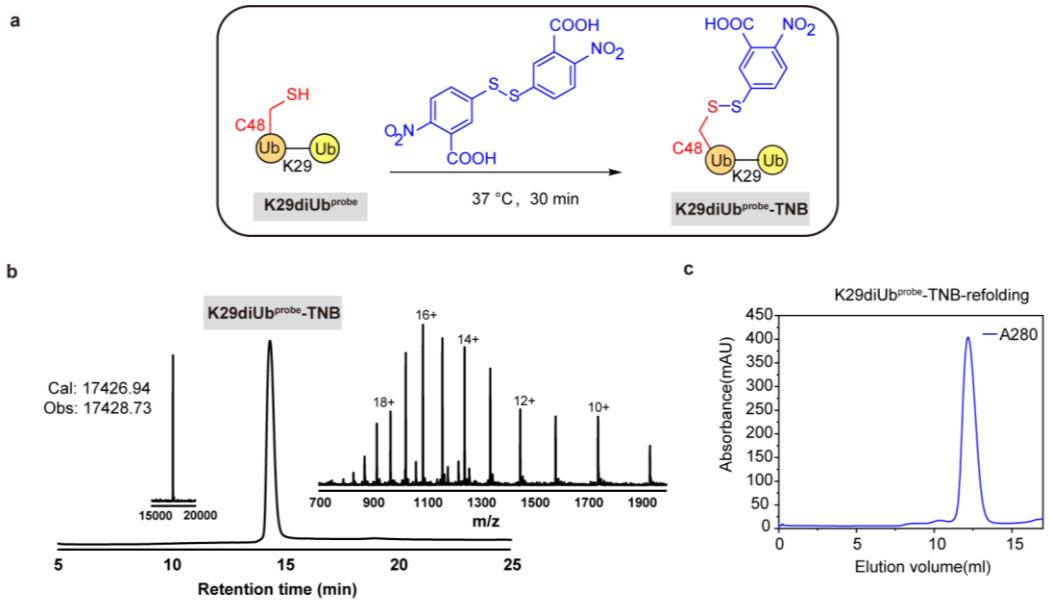
Supplementary Fig. 3 | Preparation of Ubc4-Ub-SH. a. Schematic representation of Ubc4-Ub-SH. **b.** HPLC and deconvoluted MS spectra traces for purified Ubc4-Ub-SH. By-product labelled * results from the spontaneous a-N-6-gluconoylation[1] of His6-tagged Ubc4-Conly during its expression in Escherichia coli. **c.** MS trace for purified Ubc4-Ub-SH.

1.4 Preparation of K29diUb^{probe}



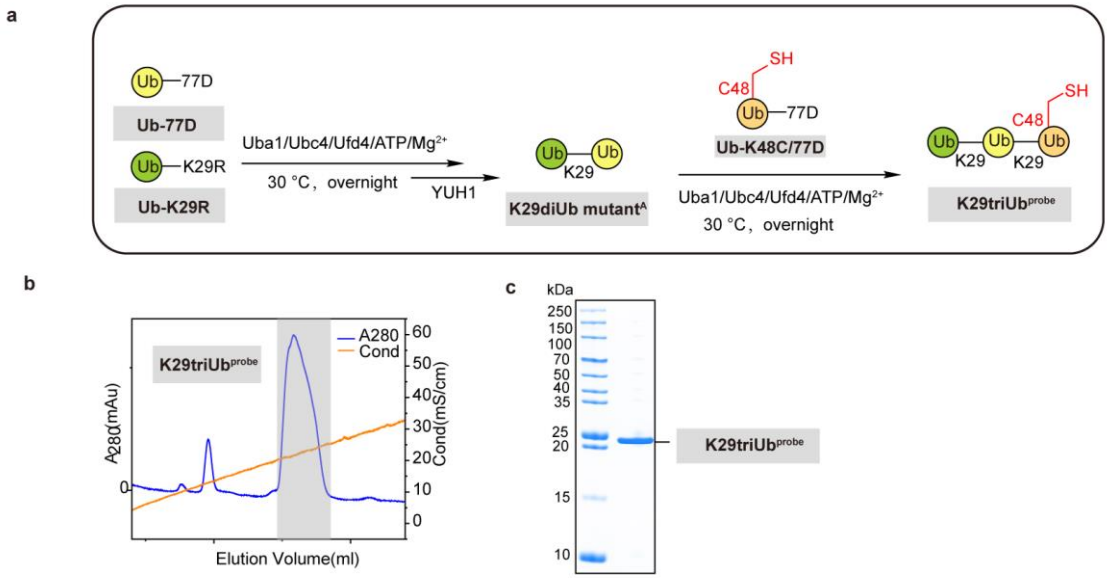
Supplementary Fig. 4 | Preparation of K29diUb^{probe}. a. A Schematic representation of K29 diUb^{probe}. **b.** Ion-exchange chromatography was used for the separation of K29diUb^{probe} with Source 15S 10/300GL column. The peak marked in grey refers to K29diUb^{probe}. **c.** SDS-PAGE analysis of purified K29diUb^{probe}. **d.** HPLC and deconvoluted MS spectra traces for purified K29diUb^{probe}. **e.** MS trace for purified K29diUb^{probe}.

1.5 Preparation of K29diUb^{probe}-TNB



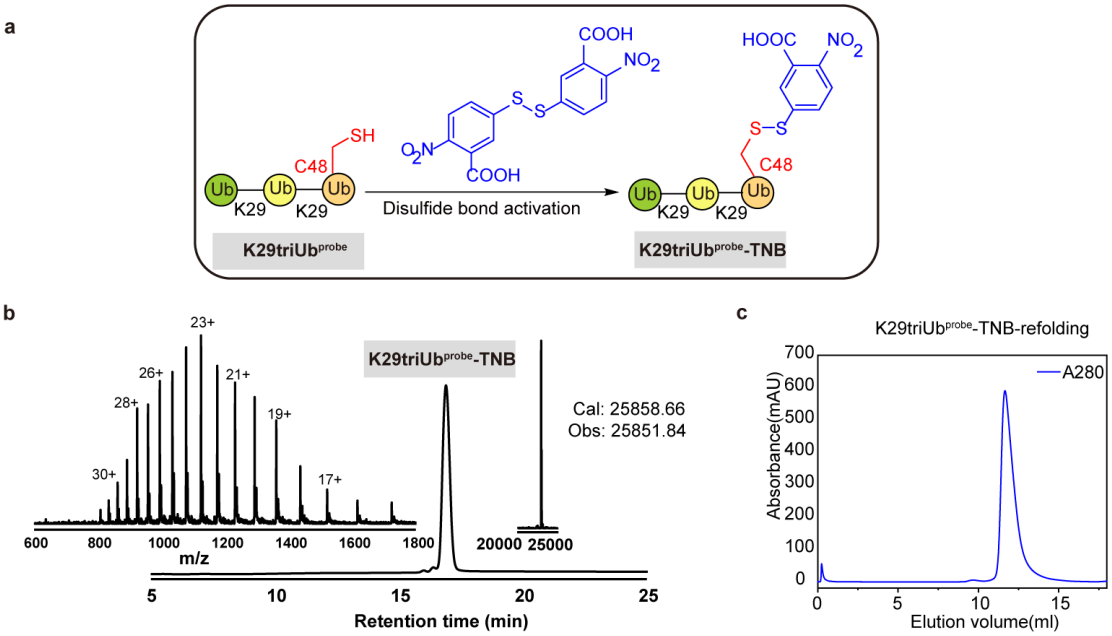
Supplementary Fig. 5 | Preparation of K29diUb^{probe}-TNB. a. A Schematic representation of K29diUb^{probe}-TNB. **b.** HPLC, MS traces and deconvoluted MS spectra for purified K29diUb^{probe}-TNB. **c.** Gel filtration chromatography of the refolded K29diUb^{probe}-TNB.

1.6 Preparation of K29triUb^{probe}



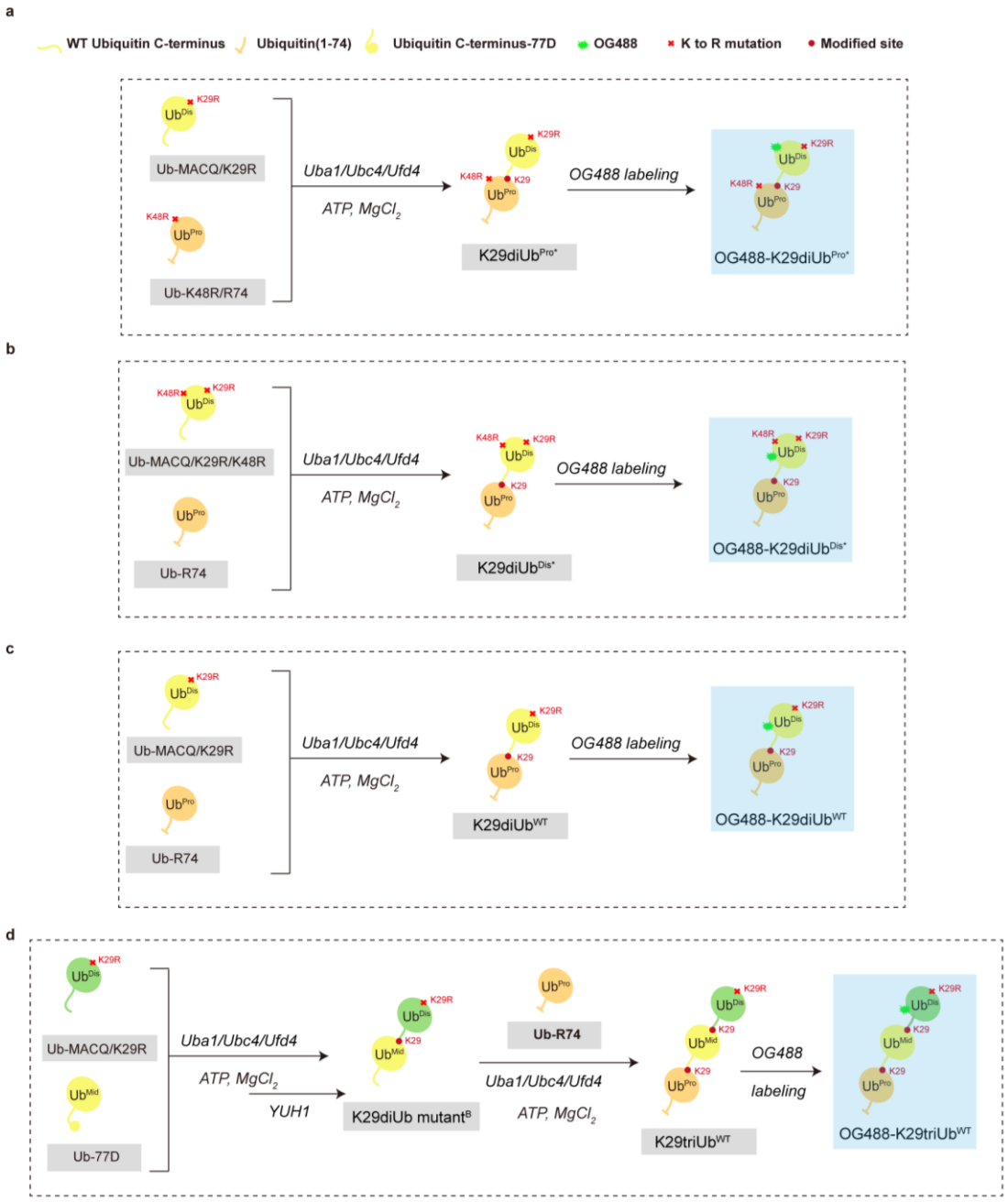
Supplementary Fig. 6 | Preparation of K29 triUb^{probe}. a. A Schematic representation of K29 triUb^{probe}. **b.** Ion-exchange chromatography was used for the separation of K29triUb^{probe} with Source 15S 10/300GL column. The peak marked in grey refers to K29triUb^{probe}. **c.** SDS-PAGE analysis of purified K29triUb^{probe}.

1.7 Preparation of K29triUb^{probe}-TNB



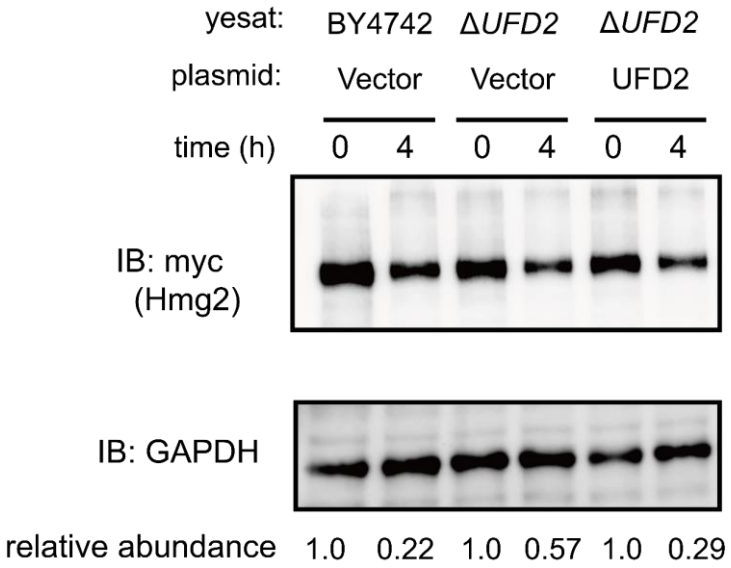
Supplementary Fig. 7 | Preparation of K29triUb^{probe}-TNB. a. A Schematic representation of K29 triUb^{probe}-TNB. **b.** HPLC, MS traces and deconvoluted MS spectra for purified K29triUb^{probe}-TNB. **c.** Gel filtration chromatography of the refolded K29triUb^{probe}-TNB.

1.8 Preparation of fluorescently labelled K29diUb^{Pro*}, K29diUb^{Dis*} and K29diUb^{WT} and K29triUb^{WT}



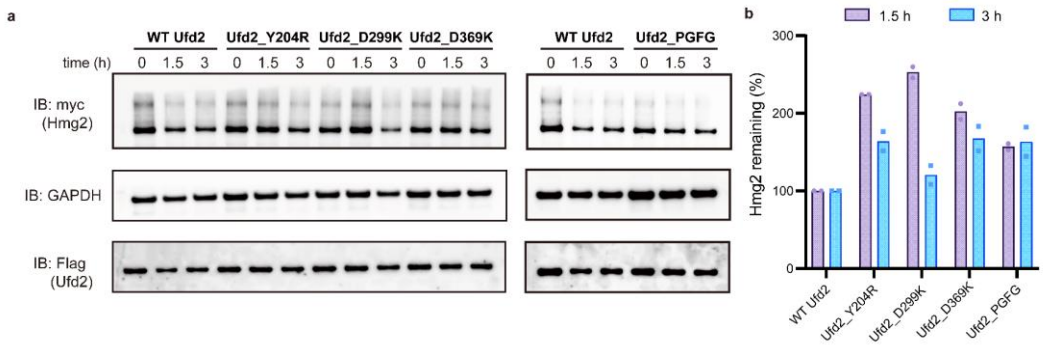
Supplementary Fig. 8 | Synthesis routes of fluorescently labelled K29diUb^{Pro*}, K29diUb^{Dis*}, K29diUb^{WT} and K29triUb^{WT}. **a**. A Schematic representation of fluorescent OG488 labelled K29diUb^{Pro*}. **b**. A Schematic representation of fluorescent OG488 K29diUb^{Dis*}. **c**. A Schematic representation of fluorescent OG488 labelled K29diUb^{WT}. **d**. A Schematic representation of fluorescent OG488 labelled K29triUb^{WT}.

1.9 Ufd2 and mutants regulate the protein homeostasis of Hmg2



Supplementary Fig. 9 | Chase experiments of Hmg2 in wildtype or Ufd2-knockout yeast strains.

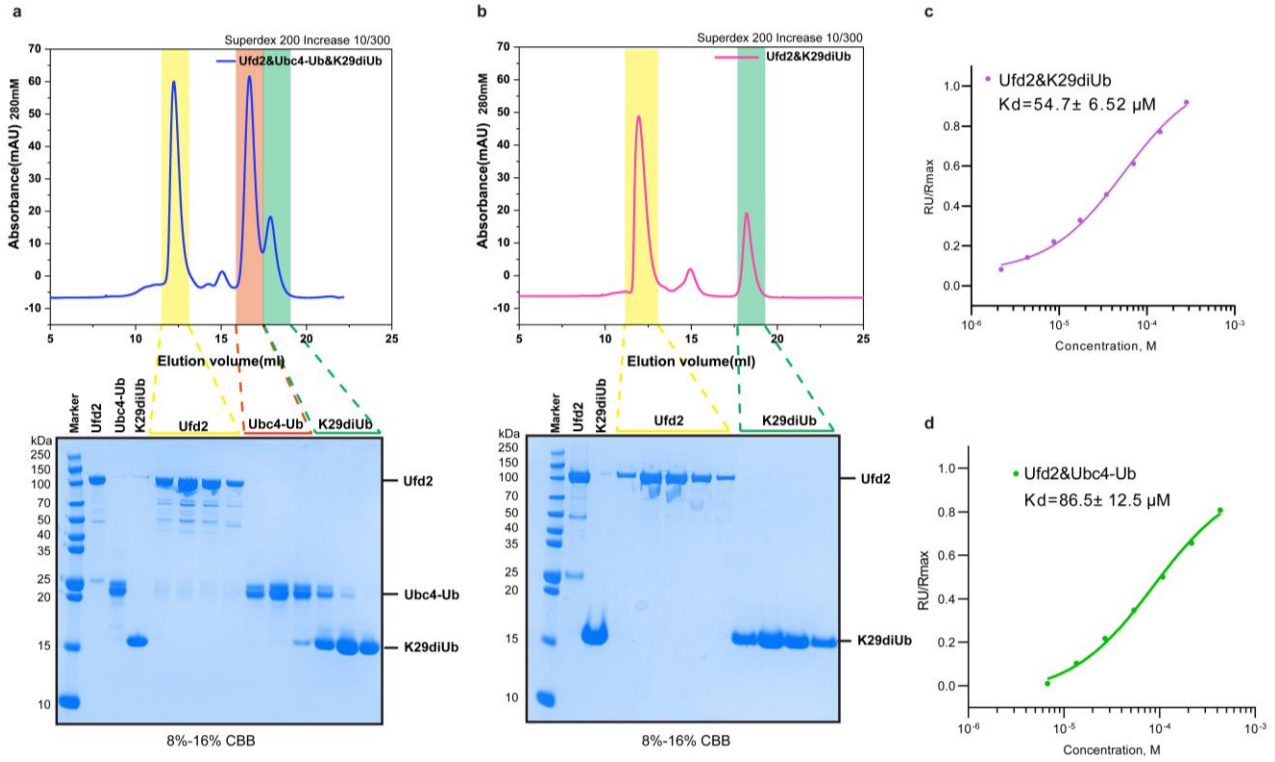
Wild-type yeast strains were complemented with an empty vector, while Ufd2 knockout yeast strains were complemented with either an empty vector or the *UFD2* gene. Hmg2 was detected using an anti-myc tag at 0, and 4 hours of chase, with GAPDH serving as a loading control. The relative amounts of Hmg2 to GAPDH at each time point were calculated, with the relative Hmg2 amount at 0 hours used as the baseline reference for quantifying remaining Hmg2. The gel image is representative of independent biological replicates (n = 2).



Supplementary Fig. 10 |. Validation of branched Ub chain assembly mechanism in yeast.

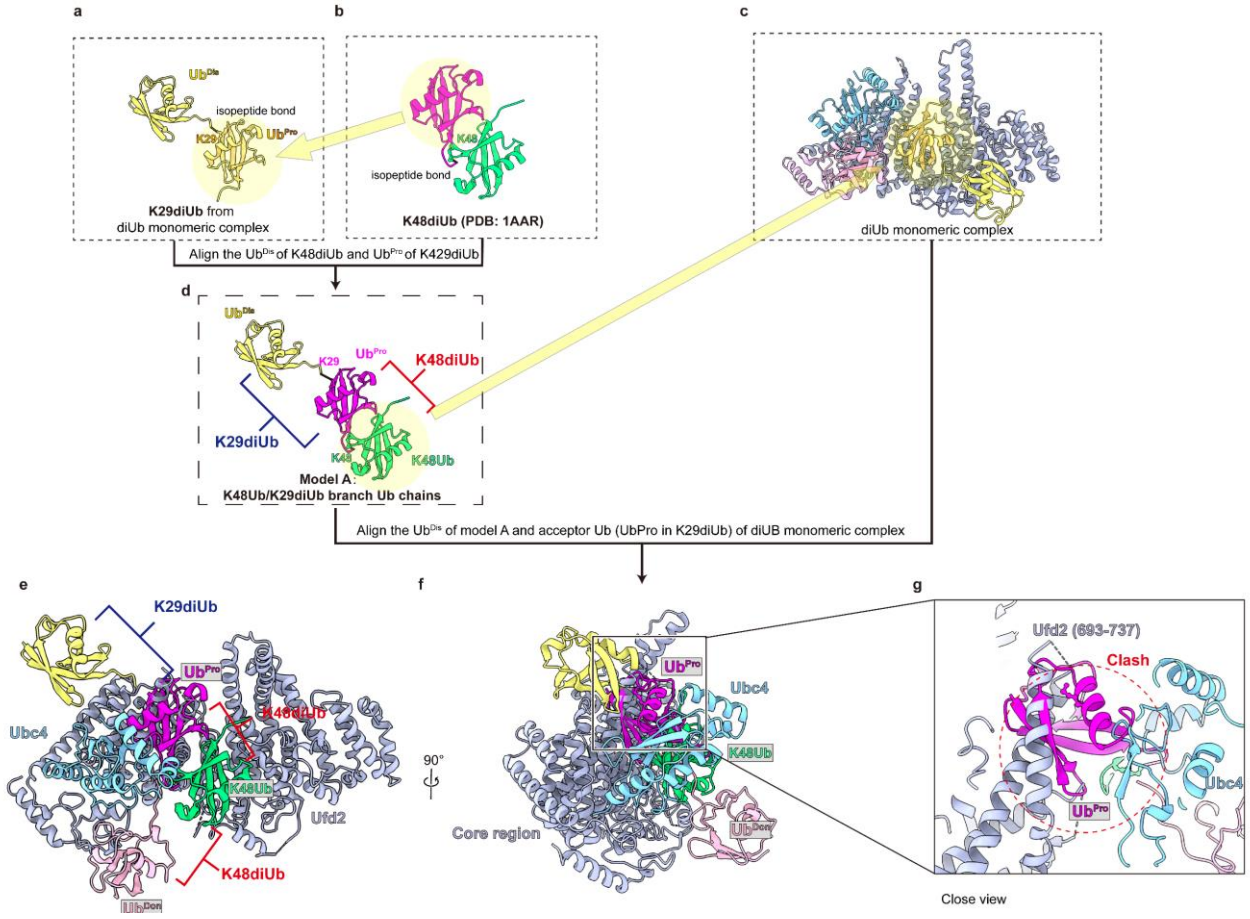
a, Chase experiments of Hmg2 in Ufd2-knockout yeast strains complemented with wild-type Ufd2 or its mutants. Hmg2 was detected using an anti-myc tag at 0, 1.5, and 3 hours of chase, with GAPDH serving as a loading control. Ufd2 and its mutants were detected using an anti-Flag tag. The gel image is representative of independent biological replicates (n = 2). **b**, Quantification of the remaining Hmg2 in 1.5 and 3 hours of chase. The relative amounts of Hmg2 to GAPDH at each time point were calculated, with the relative Hmg2 amount at 0 hours used as the baseline reference for quantifying remaining Hmg2. Each data point is shown as a colored dot.

1.11 Complexes assembly of Ufd2, Ubc4-Ub, and K29diUb by size exclusion chromatography.



Supplementary Fig. 11 | Attempts to assemble complexes of Ufd2, Ubc4-Ub, and K29diUb. a. *Top: s*ize exclusion chromatography (Superdex™ 200 Increase) of Ufd2, Ubc4-Ub, and K29diUb co-injection. From left to right, the peaks correspond to Ufd2 (~12 mL), Ubc4-Ub (~16 ml), and K29diUb (~18 ml). Bottom: SDS-PAGE revealed the components corresponding to different peaks of size exclusion chromatography. **b.** *Top: s*ize exclusion chromatography (Superdex™ 200 Increase) of Ufd2, and K29diUb co-injection. From left to right, the peaks correspond to Ufd2 (~12 mL), and K29diUb (~18 ml). Bottom: SDS-PAGE revealed the components corresponding to different peaks of size exclusion chromatography. c. Surface Plasmon Resonance (SPR) measurement of Ufd2 binding to K29diUb. d. SPR measurement of Ufd2 binding to Ubc4-CAET-Ub.

1.11 A possible model of Ufd2 complex with Ubc4 and K48Ub/K29diUb.



Supplementary Fig. 12 | Structural alignment obtains a possible model in which Ufd2 elongates Ub on K48Ub of the K48Ub/K29diUb branched ubiquitin chain. a. K29diUb module from our diUb monomeric complex (PDB: 9KHS). **b.** The crystal structure of K48diUb (PDB: 1AAP). **c.** The model of diUb monomeric complex (PDB: 9KHS). **d.** The alignment of Ub^{Dis} from K48diUb with Ub^{Pro} from K29diUb results in a model for the K48Ub/K29diUb branched ubiquitin chain. **e, f.** By aligning Ub^{Dis} from K48diUb with Ub^{Pro} from diUb monomeric complex to obtain a possible model of Ufd2 elongates Ub on K48Ub of the K48Ub/K29diUb branched ubiquitin chain. **g.** Close view of the model reveals a significant spatial conflict between the Ub^{Pro} of K29diUb and Ufd2.

2. Supplementary Tables

2.1 Table 1: Cryo-EM data collection, refinement, and validation statistics.

Table 1. Cryo-EM data collection, refinement, and validation statistics				
	diUb monomeric complex (EMD- 62353, PDB 9KHS)	diUb dimeric complex (EMD- 62356)	TriUb dimeric complex (EMDB- 62354, PDB 9KHT)	TriUb monomeric complex (EMD- 62355, PDB: 9M7O)
Data collection and processing				
Magnification	64000	64000	64000	64000
Voltage (kV)	300	300	300	300
Spherical aberration (mm)	0.01	0.01	0.01	0.01
Detector	K3	K3	K3	K3
Electron exposure (e–/Å2)	50.0 e^{-}, 32 frames	50.0 e^{-}, 32 frames	50.0 e^{-}, 32 frames	50.0 e^{-}, 32 frames
Defocus range (μm)	-1.5 to -2.0	-1.5 to -2.0	-1.5 to -2.0	-1.5 to -2.0
Pixel size (Å)	1.097	1.097	1.097	1.097
Symmetry imposed	C1	C2	C2	C1
Box size (pixel)	200	200	256	256
Micrographs (no.)	6640	6640	7112	7112
Initial particle images (no.)	3021092	3021092	5114997	5114997
Final particle images (no.)	191564	48544	125600	347924
Map resolution (Å)	4.31 Å	7.84 Å	4.85 Å	4.14 Å
Map resolution range	3.5-7 Å	4-12 Å	3.5-5.5 Å	3.5-5.5 Å
FSC threshold	0.143	0.143	0.143	0.143
Refinement				
Initial model use (PDB code)	1UBQ, 3M62, 1QCQ		1UBQ,3M62, 1QCQ	1UBQ,3M62, 1QCQ
Model resolution (Å)	4.31		4.85	4.14
FSC threshold	0.5		0.5	0.5
Map sharpening B factor (Å2)	-250		-270	-220
Model composition				
Nonhydrogen atoms	9328		19960	9328
Protein residues	1179		2737	1179
Nucleotides	0		0	0
B factors (Å2)				
Protein	30.00/542.42/213.96		30.00/759.53/384.28	20.73/331.68/116.71
Nucleotide	---		---	---
R.m.s. deviations				
Bond lengths (Å)	0.006		0.004	0.006
Bond angles (٩)	1.477		0.989	1.033
Validation				
MolProbity score	1.60		1.91	1.40
Clashscore	6.24		11.49	7.00
Poor rotamers (%)	0.1		0.00	0.00
Ramachandran plot				
Favored (%)	96.21		95.13	97.93

Allowed (%)	3.70		4.83	2.07
Disallowed (%)	0.09		0.04	0.00

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2.2 Table 2: K_D value and error estimates for all SPR measurements.

K29 diUb-Ufd2 (or mutant)	K_D (μM)	SE (μM)	K_D ± SE (μM)
WT Ufd2	54.7	6.52	54.7 ± 6.52
Y204R Ufd2	222	8.74	222 ± 8.74
H300G Ufd2	228	30.6	228 ± 30.6
D360A Ufd2	179	12.8	179 ± 12.8
D365A Ufd2	/	/	N.D.
Ubc4-Ufd2			
WT-WT	174	9.56	174 ± 9.56
WT-LMY(3G)	/	/	>724
WT-PGFG	/	/	>724
WT-R828G	4432	35.7	432 ± 35.7
SPA mut-WT	/	/	N.D.
Ufd2-Ufd2 (or mutant)			
WT-WT	291	104	291 ± 104
WT-Dimer mutant			>612
Ufd2- Ubc4-Ub			
Ufd2- Ubc4-CAET-Ub	86.5	12.5	86.5 ± 12.5

2.3 Table 3: Sequence of Ub and mutants

Name	Sequence
WT-Ub	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQR LIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub-K29R	MQIFVKTLTGKTITLEVEPSDTIENVKAR(29)IQDKEGIPPDQ QRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub-MACQ/K29R	MACQIFVKTLTGKTITLEVEPSDTIENVKAR(29)IQDKEGIPP DQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub-MACQ/K29R/K48R	MACQIFVKTLTGKTITLEVEPSDTIENVKAR(29)IQDKEGIPP DQQRLIFAGR(48)QLEDGRTLSDYNIQKESTLHLVLRLRGG
Ub-R74	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQR LIFAGKQLEDGRTLSDYNIQKESTLHLVLRLR(74)
Ub-K48R/R74	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQR LIFAGR(48)QLEDGRTLSDYNIQKESTLHLVLRLR(74)
Ub-77D	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQR LIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGD(77)
Ub-K48C/77D	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQR LIFAGC(48)QLEDGRTLSDYNIQKESTLHLVLRLRGGD(77)
Ub-G76C	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQR LIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGC(76)

2.4 Table 4: Sequence of K29-linked Ub chains

Name	Sequence	Use
OG488-K29diUb^{Pro*}	**Distal Ub- MACQ/K29R:** MACQIFVKTLTGKTITLEVEPSDTIENVK AR(29)IQDKEGIPPDQQRLIFAGKQLEDG RTLSDYNIQKESTLHLVLRLRGG **Proximal Ub- K48R/R74:** MQIFVKTLTGKTITLEVEPSDTIENVKAKI QDKEGIPPDQQRLIFAGR(48)QLEDGRTL SDYNIQKESTLHLVLRLR(74)	**Extended Data Fig.2b;**
OG488-K29diUb^{Dis*}	**Distal Ub- MACQ/K29R/K48R:** MACQIFVKTLTGKTITLEVEPSDTIENVK AR(29)IQDKEGIPPDQQRLIFAGR(48)QLE DGRTLSDYNIQKESTLHLVLRLRGG **Proximal Ub- R74:** MQIFVKTLTGKTITLEVEPSDTIENVKAKI QDKEGIPPDQQRLIFAGKQLEDGRTLSDY NIQKESTLHLVLRLR(74)	**Extended Data Fig.2b;**
OG488-K29diUb^{WT}	**Distal Ub- MACQ/K29R:** MACQIFVKTLTGKTITLEVEPSDTIENVK AR(29)IQDKEGIPPDQQRLIFAGKQLEDG RTLSDYNIQKESTLHLVLRLRGG **Proximal Ub- R74:** MQIFVKTLTGKTITLEVEPSDTIENVKAKI QDKEGIPPDQQRLIFAGKQLEDGRTLSDY NIQKESTLHLVLRLR(74)	**Fig.2g;** **Fig.3e, g;** **Fig4i;** **Extended Data Fig.2b;**
OG488-K29triUb^{WT}	**Distal Ub- MACQ/K29R:** MACQIFVKTLTGKTITLEVEPSDTIENVK AR(29)IQDKEGIPPDQQRLIFAGKQLEDG RTLSDYNIQKESTLHLVLRLRGG **Middle Ub- WT:** MQIFVKTLTGKTITLEVEPSDTIENVKAKI QDKEGIPPDQQRLIFAGKQLEDGRTLSDY NIQKESTLHLVLRLRGG **Proximal Ub- R74:** MQIFVKTLTGKTITLEVEPSDTIENVKAKI QDKEGIPPDQQRLIFAGKQLEDGRTLSDY NIQKESTLHLVLRLR(74)	**Fig.4j** **Extended Data Fig.9b, g;**
K29diUb^{probe}	**Distal Ub- K29R:** MQIFVKTLTGKTITLEVEPSDTIENVKAR(29)IQDKEGIPPDQQRLIFAGKQLEDGRTL SDYNIQKESTLHLVLRLRGG **Proximal Ub- K48C/77D:** MQIFVKTLTGKTITLEVEPSDTIENVKAKI QDKEGIPPDQQRLIFAGC(48)QLEDGRTL SDYNIQKESTLHLVLRLRGGD(77)	Intermediate of K29 diUb-Ubc4-Ub probe in **Fig.1b**
K29TriUb^{probe}	**Distal Ub- K29R:**	Intermediate of K29

	MQIFVKTLTGKTITLEVEPSDTIENVKAR(29)IQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG **Middle Ub- WT:** MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG **Proximal Ub- K48C/77D:** MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGC(48)QLEDGRTLSDYNIQKESTLHLVLRLRGGD(77)	triUb-Ubc4-Ub probe used in **Extended Fig.3b**
K29diUb mutant[A]	**Distal Ub- K29R:** MQIFVKTLTGKTITLEVEPSDTIENVKAR(29)IQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG **Proximal Ub- WT:** MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG	Intermediate of K29triUb[probe] used in **Supplementary Fig. 6**
K29diUb mutant[B]	**Distal Ub- MACQ/K29R:** MACQIFVKTLTGKTITLEVEPSDTIENVKAR(29)IQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG **Proximal Ub- WT:** MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG	Intermediate of K29triUb[WT] used in **Supplementary Fig. 8**

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2.5 Table 5: Sequence of Ubc4 and mutants

Name	Sequence
WT-Ubc4	MSSSKRIAKELSDLERDPPTSCSAGPVGDDLYHWQASIMGPADSP YAGGVFFLSIHFPTDYPFKPPKISFTTKIYHPNINANGNICLDILKDQ WSPALTLSKVLLSICSLLTDANPDDPLVPEIAHIYKTDRPKYEATAR EWTKKYAV
Ubc4-Conly	MSSSKRIAKELSDLERDPPTSS(23)SAGPVGDDLYHWQASIMGPAD SPYAGGVFFLSIHFPTDYPFKPPKISFTTKIYHPNINANGNICLDILK DQWSPALTLSKVLLSIS(108)SLLTDANPDDPLVPEIAHIYKTDRPKY EATAREWTKKYAV
Ubc4-F63G	MSSSKRIAKELSDLERDPPTSCSAGPVGDDLYHWQASIMGPADSP YAGGVFFLSIHFPTDYPG(63)KPPKISFTTKIYHPNINANGNICLDIL KDQWSPALTLSKVLLSICSLLTDANPDDPLVPEIAHIYKTDRPKYEA TAREWTKKYAV
Ubc4-SPAtoGGG	MSSSKRIAKELSDLERDPPTSCSAGPVGDDLYHWQASIMGPADSP YAGGVFFLSIHFPTDYPFKPPKISFTTKIYHPNINANGNICLDILKDQ WG(95)G(96)G(97)LTLSKVLLSICSLLTDANPDDPLVPEIAHIYKTDR PKYEATAREWTKKYAV
Ubc4-S23R	MSSSKRIAKELSDLERDPPTSR(23)SAGPVGDDLYHWQASIMGPAD SPYAGGVFFLSIHFPTDYPFKPPKISFTTKIYHPNINANGNICLDILK DQWSPALTLSKVLLSICSLLTDANPDDPLVPEIAHIYKTDRPKYEAT AREWTKKYAV

2.6 Table 6: Sequence or definition of Ufd2 and mutants

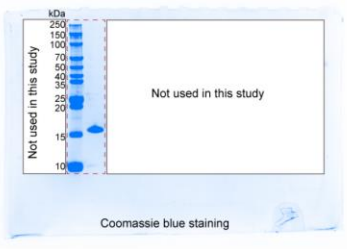
Name	Sequence or Definition
WT-Ufd2	MTAIEDILQITTDPSDTRGYSLLKSEEVPQGSTLGVDFIDTLLLY QLTENEKLDKPFEYLNDCFRRNQQQKRITKNKPNAESLHSTFQE IDRLVIGYGVVALQIENFCMNGAFINYITGIVSNVNSYTDFLSQII QRAILEGTALDLLNAVFPTLLEYCNKHVSHFDLNESVIYNNVLTI FELFVTFKPIAEIFTKIDGFFADYSCKPQDFERKTILGPILSLSPIEA AVAIRNYGDNLLRSKQQTAMIHESLQAEHKVVIDRLFFIVDKLV RGSLNSRTDMISYFAHIANKNHLRRADHPPFKELSSNGFMSNIT LLLVRFSQPFLDISYKKIDKIDANYFNNPSLFIDLSGETRLNSDFK EADAFYDKNRKTADSKPNFISDCFFLTLTYLHYGLGGTLSFEEK MGSEIKALKEEIEKVKKIAANHDVFARFITAQLSKMEKALKTTE SLRFALQGFFAHRSLQLEVFDFICGASTFLIRVVDPEHEFPFKQIK LPLIPDQIGVENVDNADFLRAHAPVPFKYYPEFVVEGPVNYSLY ISKYQTSPIFRNPRLGSFVEFTTMVLRCPELVSNPHLKGKLVQLL SVGAMPLTDNSPGFMMDIFEHDELVNKNLLYALLDFYVIVEKT GSSSQFYDKFNSRYSISIILEELYYKIPSYKNQLIWQSQNNADFFV RFVARMLNDLTFLLDEGLSNLAEVHNIQNELDNRARGAPPTRE EEDKELQTRLASASRQAKSSCGLADKSMKLFEIYSKDIPAAFVT PEIVYRLASMLNYNLESLVGPKCGELKVKDPQSYSFNPKDLLK ALTTVYINLSEQSEFISAVAKDERSFNRNLFVRAVDILGRKTGLA SPEFIEKLLNFANKAEEQRKADEEEDLEYGDVPDEFLDPLMYTI MKDPVILPASKMNIDRSTIKAHLLSDSTDPFNRMPLKLEDVTPN EELRQKILCFKKQKKEEAKHKASE
Ufd2-L347G	Ufd2-L347G mutant was constructed with Leu347 replaced by Gly.
Ufd2-F361G	Ufd2-F361G mutant was constructed with Phe361 replaced by Gly.
Ufd2-Y204R	Ufd2-Y204R mutant was constructed with Tyr204 replaced by Arg.
Ufd2-D365K	Ufd2-D365K mutant was constructed with Asp365 replaced by Lys.
Ufd2-D369K	Ufd2-D369K mutant was constructed with Asp369 replaced by Lys.
Ufd2-D299K	Ufd2-D299K mutant was constructed with Asp299 replaced by Lys.
Ufd2-H300G	Ufd2-H300G mutant was constructed with His300 replaced by Gly.
Ufd2-D299K&H300G	Ufd2-D299K&H300G mutant was constructed with Asp299 replaced by Lys and His300 replaced by Gly.
Ufd2-H576G	Ufd2-H576G mutant was constructed with His576 replaced by Gly.
Ufd2-D360A	Ufd2-D360A mutant was constructed with Asp360 replaced by Ala.
Ufd2-R828G	Ufd2-R828G mutant was constructed with Arg828 replaced by Gly.
Ufd2-R926G	Ufd2-R926G mutant was constructed with Arg926 replaced by Gly.
Ufd2-Y766G	Ufd2-Y766G mutant was constructed with Tyr766 replaced by Gly.
Ufd2-LMYtoGGG	Ufd2-LMYtoGGG mutant was constructed with Leu889 replaced by Gly, Met890 replaced by Gly and Tyr891 replaced by Gly.
Ufd2-PFtoGG	Ufd2-PFtoGG mutant was constructed with Pro923 replaced by Lys and Phe924 replaced by Gly.

2.7 Table 7: Reagents Information

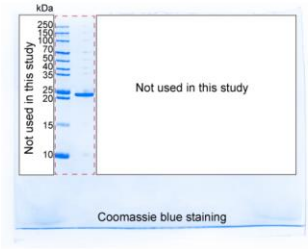
Reagents	Company
Trans-5α Chemically Competent Cell	TransGen Biotech (CD201-01)
BL21 (DE3) cells Chemically Competent Cell	TransGen Biotech (CD601-02)
LB powder	Coolaber (PM0010)
Kanamycin sulfate	LABLEAD (K9316)
Ampicillin sodium salt	LABLEAD (A9314)
Isopropyl-beta-D-thiogalactopyranoside (IPTG)	LABLEAD (0487)
Primer synthesis and gene sequencing	Tsingke
Glutathione beads 4FF	LABLEAD (G10510)
Ni-NTA Agarose resin	DiNing Biotech (SA00501L)
Oregon Green 488 (OG488) maleimide	Thermo Fisher Scientific(O6034)
(2-((2-Chloroethyl) amino) ethane-1-(S-acetaminomethyl) thiol) (CAET-SAcm)	Zheng et al.[2, 3]
Guanidine hydrochloride (Gn·HCl)	Adamas-beta (Shanghai, China)
4-mercaptophenylacetic acid (MPAA)	Sigma Aldrich (653152)
sodium nitrite ($NaNO_2$)	Adamas-beta (Shanghai, China)
5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB)	Sigma Aldrich (D8130)
Tris(2-carboxyethyl) phosphine hydrochloride (TCEP)	Bidepharm (BD155793)
2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic Acid (HEPES)	Shanghai Titan Scientific Co., Ltd
$NaH_2PO_4 \cdot 12H_2O$	J&K Scientific (Beijing, China)
$Na_2HPO_4 \cdot 12H_2O$	J&K Scientific (Beijing, China)
Dithiothreitol (DTT)	J&K Scientific (Beijing, China)
Palladium Chloride ($PdCl_2$)	J&K Scientific (Beijing, China)
Sodium 2-mercaptoethanesulfonate (MesNa)	Energy Chemical(E120446)
Bis(sulfosuccinimidyl) suberate sodium salt (BS^3)	Thermo Fisher Scientific(A39266)
anti-Myc antibody	Cell Signaling Technology (2272)
anti-Flag antibody	Cell Signaling Technology (14793)
anti-GAPDH antibody	Abcam (ab125247)

3. Uncropped scans of blots and gels in supplementary information

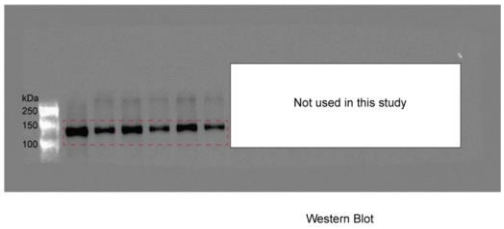
Supplementary Fig. 4c



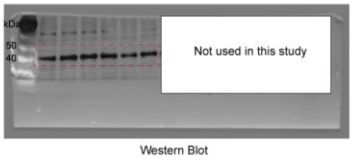
Supplementary Fig. 6c



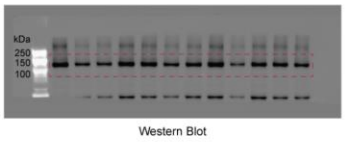
Supplementary Fig. 9 Up



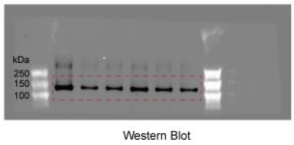
Supplementary Fig. 9 Down



Supplementary Fig. 10 First row, left

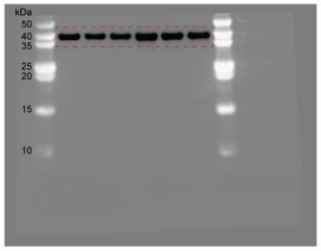


Supplementary Fig. 10 First row, right

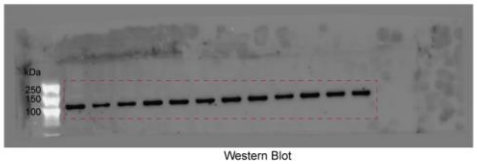


Supplementary Fig. 10 Second row, left

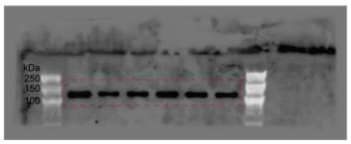
Supplementary Fig. 10 Second row, right



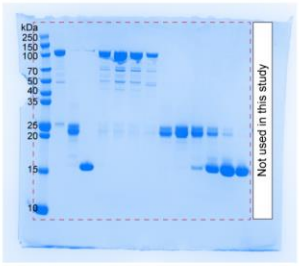
Supplementary Fig. 10 Third row, left



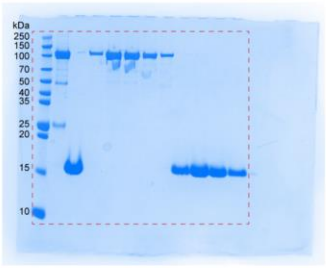
Supplementary Fig. 10 Third row, right



Supplementary Fig. 11a Down



Supplementary Fig. 11b Down



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2. Zheng, Q. *et al.* A bifunctional molecule-assisted synthesis of mimics for use in probing the ubiquitination system. *Nat. Protoc.* **18**, 530-554 (2022).
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