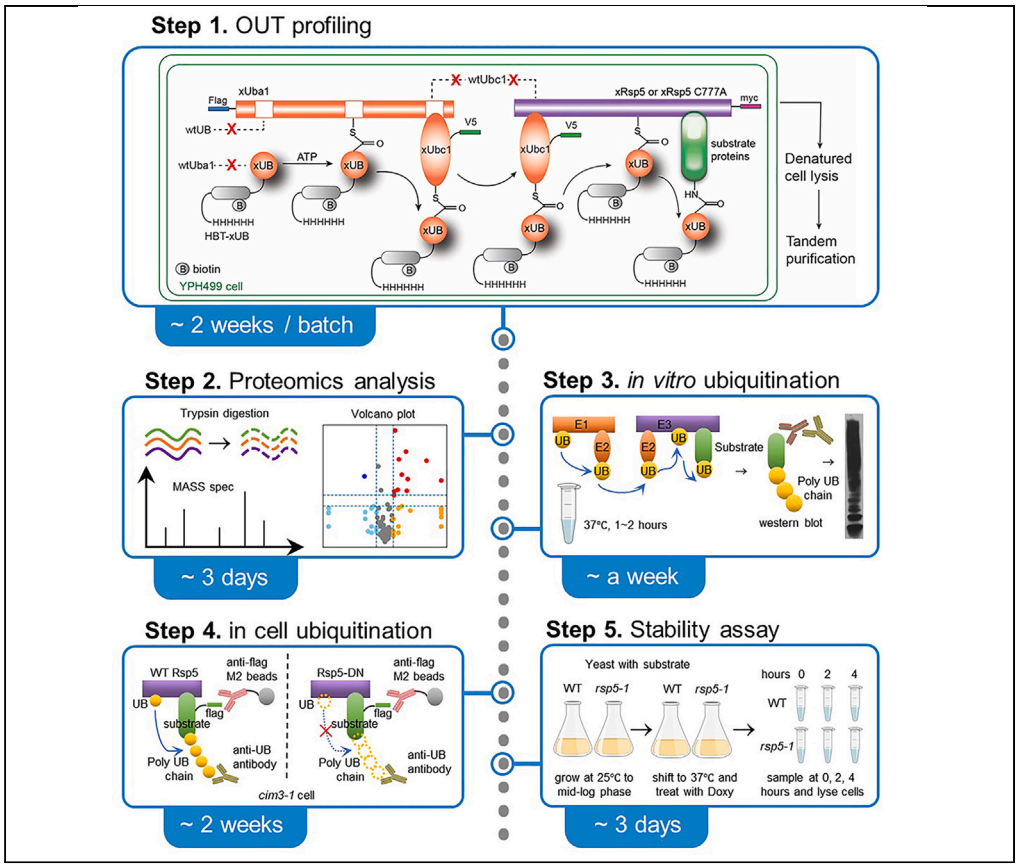
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Profiling and verifying the substrates of E3 ubiquitin ligase Rsp5 in yeast cells



Yeast is an essential model organism for studying protein ubiquitination pathways; however, identifying the direct substrates of E3 in the cell presents a challenge. Here, we present a protocol for using the orthogonal ubiquitin transfer (OUT) cascade to profile the substrate specificity of yeast E3 Rsp5. We describe steps for OUT profiling, proteomics analysis, *in vitro* and in cell ubiquitination, and stability assay. The protocol can be adapted for identifying and verifying the ubiquitination targets of other E3s in yeast.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Shuai Fang, Geng Chen, Yiyang Wang, ..., Natalia Shcherbik, Bo Zhao, Jun Yin

shcherna@rowan.edu (N.S.)
bozhao@sjtu.edu.cn (B.Z.)
junyin@gsu.edu (J.Y.)

Highlights

Identifying E3 substrates by orthogonal ubiquitin transfer (OUT) cascade in yeast

Expressing the OUT cascade of Rsp5 in yeast and preparing samples for proteomics

Verifying the substrates of Rsp5 by ubiquitination assays *in vitro* and in yeast

Measuring the stability of Rsp5 substrates in yeast cells

Fang et al., STAR Protocols 4, 102489
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102489

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Profiling and verifying the substrates of E3 ubiquitin ligase Rsp5 in yeast cells

Shuai Fang,[1,2,11] Geng Chen,[2,3,11] Yiyang Wang,[2,4] Rakhee Ganti,[5] Tatiana A. Chernova,[6] Li Zhou,[2] Savannah E. Jacobs,[2] Duc Duong,[7] Hiroaki Kiyokawa,[8] Yury O. Chernoff,[5] Ming Li,[9] Natalia Shcherbik,[10,*] Bo Zhao,[1,*] and Jun Yin[2,12,13,*]

[1]Engineering Research Center of Cell and Therapeutic Antibody, Ministry of Education, and School of Pharmacy, Shanghai Jiao Tong University, Shanghai, China

[2]Department of Chemistry and Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30303, USA

[3]Kobilka Institute of Innovative Drug Discovery, School of Life and Health Sciences, The Chinese University of Hong Kong, Shenzhen, Guangdong 518172, China

[4]Department of Pathophysiology, School of Medicine, Jinan University, Guangzhou, Guangdong 510632, China

[5]School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA 30332, USA

[6]Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, USA

[7]Integrated Proteomics Core, Emory University, Atlanta, GA 30322, USA

[8]Department of Pharmacology, Northwestern University, Chicago, IL 60611, USA

[9]Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48019, USA

[10]Department of Cell Biology and Neuroscience, Rowan University School of Osteopathic Medicine, Stratford, NJ 08084, USA

[11]These authors contributed equally

[12]Technical contact

[13]Lead contact

*Correspondence: shcherna@rowan.edu (N.S.), bozhao@sjtu.edu.cn (B.Z.), junyin@gsu.edu (J.Y.)
https://doi.org/10.1016/j.xpro.2023.102489

SUMMARY

Yeast is an essential model organism for studying protein ubiquitination pathways; however, identifying the direct substrates of E3 in the cell presents a challenge. Here, we present a protocol for using the orthogonal ubiquitin transfer (OUT) cascade to profile the substrate specificity of yeast E3 Rsp5. We describe steps for OUT profiling, proteomics analysis, *in vitro* and in cell ubiquitination, and stability assay. The protocol can be adapted for identifying and verifying the ubiquitination targets of other E3s in yeast.

For complete details on the use and execution of this protocol, please refer to Wang et al.[1]

BEFORE YOU BEGIN

The yeast genome encodes a single E1, 11 E2, and 54 E3 enzymes for transferring ubiquitin (UB) to cellular proteins. In contrast, two E1s, around 50 E2s, and more than 600 E3s are encoded by the human genome for the UB transfer reaction.[2] The relatively small set of UB-transferring enzymes in yeast makes it a less complex system for deciphering cell regulatory mechanisms mediated by protein ubiquitination. E3 UB ligases decide the timing and targets of the protein ubiquitination reactions and can be categorized into HECT, U-box, RBR, and RING types, based on the domains employed to engage the UB ~ E2 conjugate.[3–6] HECT and RBR E3s rely on a catalytic Cys residue to uptake UB from E2 before transferring UB to substrates, while U-box and RING E3s directly transfer UB from E2 to the substrates. The large number of E3s and the transient nature of their interactions with substrates (K_d ~ 10–100 μM) present a challenge to identifying the direct substrates of each E3 in yeast and other organisms.[7] To profile E3 substrates with high precision, we developed

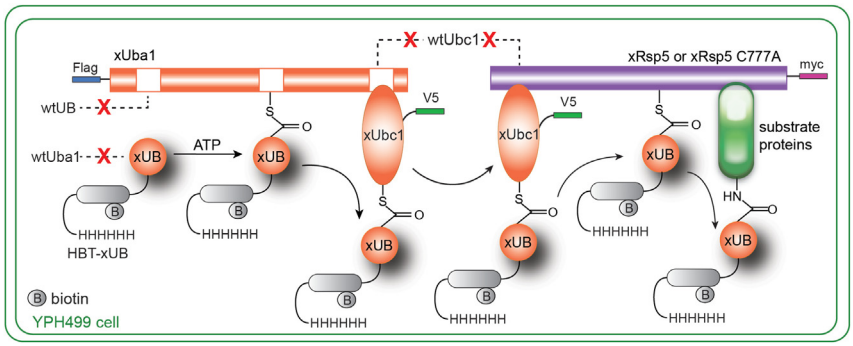


Figure 1. The orthogonal ubiquitin transfer (OUT) pathway of Rsp5 in YPH499 cell

The scheme shows that the HBT-xUB with the 6×His and BCCP tag for biotin labeling is exclusively activated by xUba1 (xE1) and transferred to xUbc1 (xE2) to form thioester conjugates. xUbc1 carries HBT-xUB to engineered xRsp5 (xE3) that mediates the exclusive transfer of HBT-xUB to Rsp5 substrates. By expressing HBT-xUB and the OUT cascade of Rsp5 consisting of xUba1-xUbc1-xRsp5 in the yeast cells, we can achieve the specific labeling of the substrate proteins of Rsp5 by HBT-xUB to enable their purification from the cell lysate for proteomic identification.

orthogonal UB transfer (OUT) cascades for the E3s, in which a UB mutant (xUB) is confined to a single track of xE1, xE2, and xE3 derivatives for its exclusive delivery to the substrates of a specific E3 (Figure 1) ("x" designates engineered UB and enzyme variants orthogonal to their native partners).[8–10] The xUB derivative (HBT-xUB, 25 kDa) is tagged with N-terminal 6×His and biotin carboxyl carrier protein (BCCP) tags, that allow the enrichment of xUB-conjugated proteins by tandem affinity purification (TAP) under denaturing conditions.[11] Subsequently, xUB-conjugated proteins are identified by LC-MS/MS to reveal the substrate profile of the E3 under study. The OUT cascade eliminates the complex cross-reactions among various E2s and E3s and assigns E3 substrates by directly following xUB transfer from a specific E3 to its cellular targets. We engineered an OUT cascade of Rsp5, a canonical HECT E3 in yeast, to identify its substrates associated with endocytosis and prion regulation.[1] This protocol presents procedures for using OUT to profile Rsp5 substrates and verifying E3-regulated substrate ubiquitination and stability in yeast cells.

Prepare the plasmids for the expression of OUT cascade of Rsp5 and its substrates in yeast cells

Timing: 4–5 days for each construct

For expressing the OUT cascade of Rsp5 in yeast cells, we constructed a single pESC vector with a TRP selection marker (pESC-TRP) for expressing HBT-xUB and xUba1 with an N-terminal Flag tag (Flag-xUba1) under the pGAL1 and pGAL10 promoters, respectively (Figure 2A). We have also constructed a single pESC vector with a URA selection marker (pESC-URA) to express xUbc1 with a C-terminal V5 tag (xUbc1-V5) and xRsp5 with an N-terminal myc tag (myc-xRsp5) under the pGAL1 and pGAL10 promoters, respectively (Figure 2B). As a control, we generated a C777A mutant of xRsp5 in the pESC-URA vector with the catalytic Cys of the HECT domain of xRsp5 mutated to Ala. Yeast cells harboring the pESC-TRP-xUB-xUba1 and pESC-URA-xUbc1-xRsp5 vectors (OUT cells) would express a functional OUT cascade for labeling the Rsp5 substrates with HBT-xUB, while yeast cells harboring the pESC-TRP-xUB-xUba1 and pESC-URA-xUbc1-xRsp5-C777A vectors (control cells) would express a nonfunctional OUT cascade due to the expression of the catalytically inactive mutant of xRsp5. We cultured the two cell populations and carried out the tandem purification of xUB-conjugated proteins from the cell lysates in parallel. We then compared the levels of xUB-conjugated proteins from two cell preparations to identify protein targets with a higher level of xUB conjugation in OUT cells relative to control cells and assigned these proteins as the potential substrates of Rsp5.

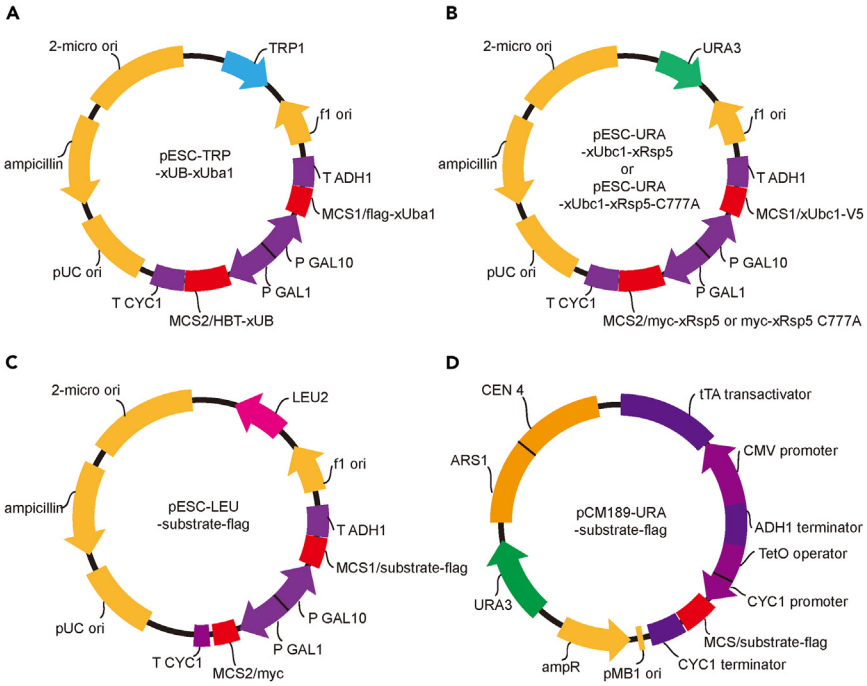


Figure 2. The plasmid maps of the yeast vectors used in this study to express the OUT cascade of Rsp5 and substrate proteins

(A) The plasmid map of the pESC vector for the expression of HBT-xUB and Flag-xUba1.

(B) The plasmid map of pESC vector for the expression of xUbc1-V5 and xRsp5-myc.

(C) The plasmid map of the pESC vector for the expression of substrate proteins to verify their ubiquitination by Rsp5.

(D) The plasmid map of pCM189-URA vector for the expression of substrate proteins to assay their stability in the cell.

The OUT cascade of Rsp5 was expressed with two vectors pESC-TRP-xUB-xUba1 and pESC-URA-xUbc1-xRsp5 and the catalytically inactive OUT cascade of Rsp5 was expressed with the vectors pESC-TRP-xUB-xUba1 and pESC-URA-xUbc1-xRsp5-C777A. The assembly of the vectors is shown in Figures 2A and 2B.

We cloned the gene of potential substrates identified by the OUT screen into the pESC-LEU vector so the substrates could be expressed with a C-terminal Flag tag under the pGAL10 promoter (Figure 2C). For measuring substrate stability in the yeast cells, the genes of the substrates were cloned into the pCM189-URA vector with a pCYC1 promoter (Figure 2D).

The cloned plasmids were sent for sequencing to confirm their sequences as designed. The plasmids were then amplified in XL1-Blue *E. coli* cells, and the plasmid DNA was stored at −20°C before use.

For detailed information on pESC-URA, pESC-TRP, and pESC-LEU vectors, please refer to https://www.agilent.com/cs/library/usermanuals/Public/217451.pdf. For the pCM189-URA vector, please refer to https://www.atcc.org/products/87661.

Prepare the yeast strains

Timing: 1–2 days for each yeast strain

Yeast strains and corresponding plasmids used in this protocol are listed in the key resources table. The YPH499 and SEY6210.1 strains are wildtype strains. The *cim3-1* strain carries a rpt6 thermosensitive proteasome mutant that causes proteasome deficiency at 37°C,[12] and *rsp5-1* (YXY705) is a

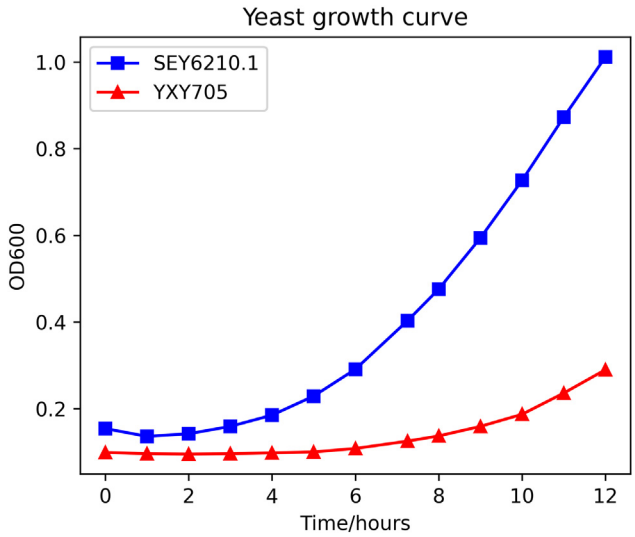


Figure 3. The growth curve of SEY6210.1 and YXY705 under a typical culture condition

strain with a L733S mutation in the HECT domain of Rsp5.[13] These strains should be cultured at 25°C, and they grow much slower than the wild-type strains, especially when *cim3-1* carries Rsp5-DN plasmid. Plotting a growth curve of the yeast strains would be useful to measure their growth rates. Here we provide the growth curve of wild-type strain SEY6210.1 and the *rsp5-1* strain YXY705 under our culture conditions (Figure 3).

Prepare media and reagents

Timing: 1–2 days (as needed)

Prepare the required buffers and medium (see materials and equipment below).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HA-tag antibody (F-7) (1:1,000 dilution)	Santa Cruz	Cat#sc-7392
Anti-Rsp5 antibody (1:1,000 dilution)	Santa Cruz	Cat#sc-26129
Goat anti-mouse IgG-HRP (1:20,000 dilution)	Santa Cruz	Cat#sc-2354
Anti-ubiquitin antibody (P4D1) (1:1,000 dilution)	Santa Cruz	Cat#sc-8017
Goat anti-rabbit IgG-HRP (1:20,000 dilution)	Santa Cruz	Cat#sc-2004
Monoclonal anti-FLAG® M2 antibody (1:5,000 dilution)	Sigma-Aldrich	Cat#F3165
Anti-FLAG M2 Affinity Gel	Sigma-Aldrich	Cat#A2220
F(ab')2-Goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody, HRP conjugate (1:20,000 dilution)	Thermo Fisher Scientific	Cat#31438
Experimental models: Cell lines		
YPH499 (MATa *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*)	Agilent Technologies	Cat#217455
cim3-1 (MATa *cim3-1 ura3-52 trp14Δ63 his3Δ200 leu2Δ1 lys3-801 ade2-10*)	Carl Mann[12]	N/A
SEY6210.1 (MATα *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801; GAL*)	Ming Li	N/A
YXY705 (MATα *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801; GAL, rsp5-1::TRP1*)	Ming Li	N/A
Escherichia coli XL1-Blue	Agilent Technologies	Cat#200228
Chemicals, peptides, and recombinant proteins		
SuperSignal® West Pico Chemiluminescent Substrate	Thermo Scientific	Cat#34079

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plasmid Maxi Kit	Qiagen	Cat#12162
Deoxyribonucleic acid, single-stranded from salmon testes	Sigma	Cat#D9156
Ni Sepharose High Performance	GE Healthcare	Cat#17-5268-01
Glutathione agarose	Thermo Scientific	Cat#16101
High Capacity Streptavidin Agarose Resin	Thermo Scientific	Cat#20359
Pierce® RIPA Buffer	Thermo Scientific	Cat#89900
Pierce™ spin column -Screw Cap	Thermo Scientific	Cat#69705
Pierce™ Lys-C Endoproteinase, MS Grade	Thermo Scientific	Cat#90051
Sequencing Grade Modified Trypsin	Promega	Cat#V511A
0.5 mm acid-washed glass beads	Sigma	Cat#G8772
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	Cat#5000006
Recombinant DNA		
pESC vectors (pESC-LEU2, pESC-URA3 and pESC-TRP1)	Agilent	Cat#217455
pESC-TRP-xUB-xUba1, pESC-URA-xUbc1-xRsp5, pESC-URA-xUbc1-xRsp5-C777A, pESC-LEU-substrate-Flag	This study	N/A
pYES-URA plasmids (pYES-URA, pYES-URA-Rsp5-DN, pYES-URA-wt RSP5)	Natalia Shcherbik	N/A
pCM189-URA vector	ATCC	Cat#87661
pCM189-URA-substrate-Flag	This study	N/A
Other		
Mini-Beadbeater-16	BioSpec Products	Cat#607
Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit	Bio-Rad	Cat#1704272

MATERIALS AND EQUIPMENT

Buffers for purifying 6×His-tagged proteins expressed in *E coli*

Lysis buffer for purifying 6×His-tagged proteins, pH 8.0

Reagent	Final concentration	Amount
Tris Base	50 mM	6.05 g
NaCl	500 mM	29.2 g
Imidazole	5 mM	0.34 g
ddH_2O	N/A	Top to 1 L
Total	**N/A**	**1 L**
The following should be added freshly when use:		
Lysozyme	1 mg/mL	Accordingly
100 mM PMSF	1 mM	Accordingly
protease inhibitor cocktail		Accordingly

Wash buffer for purifying 6×His-tagged proteins, pH 8.0

Reagent	Final concentration	Amount
Tris Base	50 mM	6.05 g
NaCl	500 mM	29.2 g
Imidazole	20 mM	1.36 g
ddH_2O	N/A	Top to 1 L
Total	**N/A**	**1 L**

Elution buffer for purifying 6×His-tagged proteins, pH8.0

Reagent	Final concentration	Amount
Tris Base	50 mM	6.05 g
NaCl	500 mM	29.2 g

(*Continued on next page*)

Continued

Elution buffer for purifying 6×His-tagged proteins, pH8.0

Reagent	Final concentration	Amount
Imidazole	250 mM	17 g
ddH_2O	N/A	Top to 1 L
Total	**N/A**	**1 L**

The wash buffer and elution buffer can be stored at room temperature for six months. The lysis buffer with lysozyme and protease inhibitor added should be prepared in fresh.

Buffers for purifying GST-tagged proteins expressed in *E coli*

Lysis buffer for purifying GST-tagged proteins, pH 8.0

Reagent	Final concentration	Amount
Tris Base	50 mM	6.05 g
NaCl	150 mM	8.8 g
ddH_2O	N/A	Top to 1 L
Total	**N/A**	**1 L**
The following should be added freshly when use:		
Lysozyme	1 mg/mL	Accordingly
1 M $ZnCl_2$	50 μM	Accordingly
100 mM PMSF	1 mM	Accordingly
proteasome inhibitor	1 ×	Accordingly
100 mM DTT	10 mM	Accordingly

Wash buffer for purifying GST-tagged proteins, pH 8.0

Reagent	Final concentration	Amount
Tris Base	50 mM	6.05 g
NaCl	150 mM	8.8 g
ddH_2O	N/A	Top to 1 L
Total	**N/A**	**1 L**

Elution buffer for purifying GST-tagged proteins, pH 8.0

Reagent	Final concentration	Amount
Tris Base	50 mM	6.05 g
NaCl	150 mM	8.8 g
reduced glutathione	10 mM	3.07 g
ddH_2O	N/A	Top to 1 L
Total	**N/A**	**1 L**

The lysis buffers without the addition of protease inhibitors, the wash buffer, and the elution buffer without the addition of glutathione can be stored at room temperature for up to six months. The lysis buffer with protease inhibitors and the elution buffer with glutathione are made fresh just before use.

Dialysis buffer, pH 8.0 (for both purification)

Reagent	Final concentration	Amount
Tris Base	25 mM	3.02 g
NaCl	150 mM	8.8 g
100 mM DTT	0.5 mM	5 mL
ddH_2O	N/A	Top to 1 L
Total	**N/A**	**1 L**

5× reaction buffer, pH 8.0

Reagent	Final concentration	Amount
0.1 M DTT	10 mM	1 μL
0.1 M ATP	25 mM	2.5 μL
1 M $MgCl_2$	25 mM	0.25 μL
PBS	1 ×	6.25 μL
Total	**N/A**	**10 μL**

This buffer is made fresh. 10 μL of 5× Reaction buffer will be needed for each 50 μL of reaction.

Buffers for yeast transformation

10× TE (pH7.5)

Reagent	Final concentration	Amount
Tris Base	100 mM	0.605 g
EDTA	10 mM	0.186 g
ddH_2O	N/A	Top to 50 mL
Total	**N/A**	**50 mL**

10× LiOAc

Reagent	Final concentration	Amount
LiOAc	1 M	3.3 g
ddH_2O	N/A	Top to 50 mL
Total	**N/A**	**50 mL**

TE/LiOAc (1×)

Reagent	Final concentration	Amount
10× TE	1×	50 mL
10× LiOAc	0.1 M	50 mL
ddH_2O	N/A	400 mL
Total	**N/A**	**500 mL**

PEG/TE/LiOAc (1×)

Reagent	Final concentration	Amount
50% PEG 3350 (m/v)	40%	400 mL
10× TE	1×	50 mL
10× LiOAc	0.1 M	50 mL
Total	**N/A**	**500 mL**

These buffers can be stored at 4°C for up to six months.

Buffers for tandem purification

Buffer A, pH 8.0 or pH 6.3

Reagent	Final concentration	Amount
Urea	8 M	240 g
NaCl	300 mM	8.78 g
Tris Base	50 mM	3.02 g

(*Continued on next page*)

Continued

Buffer A, pH 8.0 or pH 6.3

Reagent	Final concentration	Amount
NaH_2PO_4	50 mM	3.9 g
ddH_2O	N/A	Top to 500 mL
Total	**N/A**	**500 mL**
The following should be added freshly when use:		
NP-40	0.5%	Accordingly
Benzonase	125 U/mL	Accordingly
PMSF	1 mM	Accordingly
Imidazole (accordingly)	10 mM	Accordingly

Buffer B, pH 4.3

Reagent	Final concentration	Amount
Urea	8 M	96 g
NaCl	200 mM	2.35 g
Na_2HPO_4	50 mM	3.58 g
SDS	2%	4 g
EDTA	10 mM	0.744 g
Tris Base	100 mM	2.42 g
imidazole	250 mM	3.4 g
ddH_2O	N/A	Top to 200 mL
Total	**N/A**	**200 mL**

Buffer C, pH 8.0

Reagent	Final concentration	Amount
Urea	8 M	48 g
NaCl	200 mM	1.17 g
SDS	2%	2 g
Tris Base	100 mM	1.21 g
ddH_2O	N/A	Top to 100 mL
Total	**N/A**	**100 mL**

Buffer D, pH 8.0

Reagent	Final concentration	Amount
Urea	8 M	48 g
NaCl	1.2 M	7.02 g
SDS	0.2%	0.2 g
Tris Base	100 mM	1.21 g
Ethanol	10%	10 mL
Isopropanol	10%	10 mL
ddH_2O	N/A	Top to 100 mL
Total	**N/A**	**100 mL**

Buffer E, pH 8.0

Reagent	Final concentration	Amount
Urea	8 M	48 g
NH_4HCO_3	100 mM	0.79 g

(*Continued on next page*)

Continued

Buffer E, pH 8.0

Reagent	Final concentration	Amount
ddH_2O	N/A	Top to 100 mL
Total	**N/A**	**100 mL**

The buffers without the protease inhibitors can be stored at room temperature for up to six months. Buffer A with protease inhibitors should be made fresh.

Buffers for on-beads digestion for proteomics, pH7.5

ABC (Ammonium Bicarbonate) buffer

Reagent	Final concentration	Amount
ABC	50 mM	0.4 g
ddH_2O	N/A	Top to 100 mL
Total	**N/A**	**100 mL**

ABC/DTT (Dithiothreitol) buffer

Reagent	Final concentration	Amount
DTT	100 mM	0.154 g
50 mM ABC	50 mM	Top to 10 mL
Total	**N/A**	**10 mL**

ABC/IAA (iodoacetamide) buffer

Reagent	Final concentration	Amount
IAA	500 mM	0.90 g
50 mM ABC	50 mM	Top to 10 mL
Total	**N/A**	**10 mL**

The ABC buffer can be stored at at 4°C for six months. The ABC buffer with DTT or IAA should be freshly prepared.

Buffers for stability assay

2× urea boiling buffer

Reagent	Final concentration	Amount
1 M Tris Base, pH 7.5	50 mM	2.5 mL
0.5 M EDTA (pH 8)	1 mM	100 µL
10% SDS	2%	10 mL
Urea	6 M	18 g
1 M DTT	100 mM	5 mL
ddH_2O	N/A	top to 50 mL
Total	**N/A**	**50 mL**

2× urea sample buffer

Reagent	Final concentration	Amount
0.5 M Tris HCl, pH 6.8	150 mM	12 mL
Urea	6 M	14.4 g
SDS	6%	2.4 g

(*Continued on next page*)

Continued

2× urea sample buffer

Reagent	Final concentration	Amount
50% Glycerol	10%	8 mL
1 M DTT	160 mM	6.4 mL
1% Bromophenol blue solution	0.01%	400 μL
ddH_2O	N/A	top to 40 mL
Total	**N/A**	**40 mL**

These buffers can be stored at 4°C for up to six months.

2×YT media

Reagent	Final concentration	Amount
Trptone	16 g/L	8 g
Yeast extract	10 g/L	5 g
NaCl	5 g/L	2.5 g
Bacto-Agar (optional)	20 g/L	10 g
ddH_2O	N/A	500 mL
Total	**N/A**	**500 mL**

The 2×YT media can be stored at 4°C for a month.

YPD media

Reagent	Final concentration	Amount
Yeast extract	10 g/L	1 g
Dextrose	20 g/L	2 g
Peptone	20 g/L	2 g
Bacto-Agar (optional)	20 g/L	2 g
ddH_2O	N/A	100 mL
Total	**N/A**	**100 mL**

The YPD media can be stored at 4°C for a month.

Drop-out mix (ura- trp- leu- his-)

Reagent	Amount	Reagent	Amount
Adenine	0.5 g	Alanine	2.0 g
Arginine	2.0 g	Asparagine	2.0 g
Aspartic acid	2.0 g	Cysteine	2.0 g
Glutamine	2.0 g	Glutamic acid	2.0 g
Glycine	2.0 g	Inositol	2.0 g
Isoleucine	2.0 g	Lysine	2.0 g
Methionine	2.0 g	Para-aminobenzoic acid	2.0 g
Phenylalanine	2.0 g	Proline	2.0 g
Serine	2.0 g	Threonine	2.0 g
Tyrosine	2.0 g	Valine	2.0 g

The powder was mixed thoroughly and can be kept at room temperature indefinitely.

Complement storages

Reagent	Final concentration	Storage volume
20× Uracil (1.6 g/L)	1×	100 mL
20× Leucine (4 g/L)	1×	100 mL

(*Continued on next page*)

Continued

Complement storages

Reagent	Final concentration	Storage volume
20× Histidine (1.6 g/L)	1×	100 mL
20× Tryptophan (4 g/L)	1×	100 mL

Make four complement storages separately, filter-sterilize, and store at room temperature for up to six months. Keep 20× Histidine out of light.

SC media

Reagent	Final concentration	Amount
Yeast nitrogen base without amino acids	6.7 g/L	1.34 g
Drop-out mix	2 g/L	0.4 g
Bacto-agar (optional)	20 g/L	4 g
Carbohydrate(s) (accordingly):		
Dextrose	20 g/L	4 g
Galactose	20 g/L	4 g
Raffinose	10 g/L	2 g
Complement nutrients (accordingly):		
20× Histidine (1.6 g/L)	0.08 g/L	10 mL
20× Leucine (4 g/L)	0.2 g/L	10 mL
20× Tryptophane (4 g/L)	0.2 g/L	10 mL
20× Uracil (1.6 g/L)	0.08 g/L	10 mL
ddH_2O	N/A	Top to 200 mL
Total	**N/A**	**200 mL**

The media can be stored at 4°C for up to six months.

STEP-BY-STEP METHOD DETAILS

Transformation of the yeast cells for the expression of the OUT cascade

Timing: 3–4 days

1. Revive YPH499 cells from the glycerol stock stored at −80°C.
   a. Thaw the stock and streak the cells on a fresh YPD plate.
   b. Incubate the plate in a 30°C incubator for overnight until colonies appear.
2. Pick a colony from the fresh plate (less than one week old) and use it to inoculate a 5 mL YPD culture in a 15 mL culture tube.
3. Incubate the culture at 30°C with overnight shaking.
4. The next day, measure the optical density of the cell culture at 600 nm (OD_{600}).
5. Yeast cell culture.
   a. Dilute the cell culture to an OD_{600} of 0.2 in 20 mL fresh YPD media with a 50 mL sterilized tube.
   b. Incubate the culture at 30°C with shaking until its OD600 reaches the mid-log phase (OD_{600} = 0.4–0.6).

Note: The growth of the cells may take 4–6 h.

6. Boil 10 mg/mL of ssDNA at 100°C in a water bath to melt the strands and store on ice.
7. Wash the cells.
   a. Spin down yeast cells at 1,800 × *g* for 3 min.
   b. Discard the media and resuspend the cells with 1 mL sterile water.
   c. Transfer 1 mL resuspended cells to a clean, sterile 1.5 mL Eppendorf tube.
   d. Wash cells again with 1 mL TE/LiOAc buffer.

Table1. The plasmids used for cotransformation for OUT expression in YPH499 cell

Transformation	Plasmid #1	Plasmid #2
1 (OUT cells)	pESC-TRP-Flag-xUba1/HBT-xUB	pESC-URA-V5-xUbc1/myc-xRSP5
2 (Control cells)	pESC-TRP-Flag-xUba1/HBT-xUB	pESC-URA-V5-xUbc1/myc-Rsp5-C777A

e. Spin down the cells and remove the remaining liquid.

8. Resuspend the cells with 2-pellet volumes of TE/LiOAc buffer.
9. Aliquot 50 μL of cells to clean and sterile tubes.
10. Add 1 μL (~ 1 μg) of each plasmid and 5 μL of ssDNA to the cells.
11. Pipet the cells to mix them well with the plasmids. Plasmids used for cotransformation are in the note below (Table 1).

Note: The OUT cells are cotransformed with the plasmids encoding the HBT-xUB - xUba1 (xE1) pair and the xUbc1 (xE2) - xRsp5 (xE3) pair. The control cells are cotransformed with the same set of plasmids, except that the xRsp5 gene is replaced with the C777A mutant gene of xRsp5 that is catalytically inactive. A comparison of HBT-xUB conjugated proteins purified from the OUT cells and control cells would enable us to identify proteins with enhanced levels of HBT-xUB conjugation in OUT cells, and these proteins are assigned as the potential substrates of Rsp5.

12. Add 300 μL PEG 4000/LiOAc/TE to the mixture of the cells and the plasmids and vortex the mixture thoroughly.
13. Shake the tubes at 30°C for 30 min and then add 35 μL DMSO to each transformation mixture and vortex for 10–30 s to mix thoroughly.
14. Heat shock the transformation mixture by putting the tubes in a 42°C water bath for 15 min.
15. Spin down the cells at 1,800 × *g* for 3 min and wash the cells with 1 mL of sterile water.
16. Spin down the cells again at 1,800 × *g* for 3 min and resuspend the cell pellet with 100 μL sterile water.
17. Plate resuspensions onto SC Dex ura- trp- plates. Incubate the plates in a 30°C incubator for 1–2 days until colonies appear.

Verification of the expression of the OUT components in the cell

Timing: 5 days

18. Pick colonies from transformation plates and use each colony to inoculate 5 mL SC Dex ura- trp- media.
19. Culture the cells overnight in a 30°C shaker at 220 rpm. The OD_{600} of the overnight culture should reach 1.2 in the next morning.
20. Dilute the culture to OD_{600} 0.3 in 150 mL SC Dex ura- trp- media supplemented with 4 μM biotin in a 500 mL autoclaved flask.
21. Shake the culture at 30°C for 5–6 h until the OD_{600} reaches 1.0.
22. Pellet the cells with an autoclaved centrifuge bottle or three 50 mL Falcon tubes at 1,800 × *g* for 5 min.
23. Wash the cell pellet with 30 mL of deionized water three times.
24. Resuspend cells with a same volume (150 mL in total) of SC ura- trp- media containing 1% raffinose.
25. Incubate the cell culture for 2 h in a 30°C shaker.
26. Induce protein expression by adding 20% galactose to a final concentration of 2% (w/v).
27. Culture the cells in a 30°C shaker overnight.
28. The next day, aliquot the culture to three 50 mL Falcon tubes.
29. Collect the cells by centrifugation at 1,800 × *g* for 5 min and discard the supernatant.
30. Wash the cells.
    a. Resuspend each tube of the cells with 1 mL of DI water.
    b. Transfer the cell suspension to three clean 1.5 mL Eppendorf tubes.

c. Centrifuge each tube to remove DI water.
d. Resuspend the cell pellet with an equal volume of Buffer A (0.2–0.4 mL).

31. Lyse the cells.
    a. Add an equal volume of glass beads.
    b. Lyse the cells with Mini-Beadbeater-16 by beating the cells for 60 s for each cycle and use a total of 4 cycles.
    c. Transfer the cell lysate to a centrifuge tube.

Note: You may add a small amount of defoamer, for example, about 4–5 μL of Antifoam C Emulsion (Sigma, Cat#A8011) to avoid foaming during beads-beating. After each cycle, wait for about 1 min on ice to cool down the samples. Do not put the tubes on ice for long because the high concentration of urea in buffer A may be crystallized at icy temperature.

32. Lysate clear up.
    a. Centrifuge the cell lysate at 20,000 g for 30 min.
    b. Transfer and combine the clear supernatant to a clean tube.
    c. Save 50 μL as "lysate" for western blot analysis.
    d. Measure the protein concentration of the cell lysate by Bradford assay after diluting the lysate 1,000 fold.

Note: The typical protein concentration of the lysate should be around 10 mg/mL. Save 50 μL of the lysate to assay the expression of the components of the OUT cascade by SDS-PAGE gel analysis and western blotting.

⚠ CRITICAL: The urea concentration in the 1,000-fold diluted cell lysate is low and would not affect the Bradford assay. To prepare diluted cell lysate, one may add 0.8 μL cell lysate to 800 μL deionized water, followed by the addition of 200 μL Bio-Rad Protein Assay Dye Reagent Concentrate, and measure the concentration at 595 nm with a spectrophotometer.

Pause point: Now it is a good time to check the expression of xE1, xE2, and xE3 in yeast lysates by SDS-PAGE and western blot probed with anti-Flag (xE1), anti-V5 (xE2) and anti-myc (xE3) antibodies, respectively. Once the expression of the OUT cascade enzymes is confirmed, continue with tandem purification of xUB conjugates from the cell lysate.

Yeast cell lysis and tandem affinity purification of xUB-conjugated proteins

Timing: 3 days

33. Protein binding with Ni-NTA.
    a. Wash Ni-NTA Sepharose resin 3 times with Buffer A (pH 8.0).
    b. Add the resin to the cell lysate at a ratio of 35 μL resin for 1 mg of protein in the lysate.
    c. Incubate the binding mixture with the Ni-NTA resin at room temperature with gentle rocking for 2 h.

Note: At least 10 mg cell lysate proteins are used for binding to the resin.

34. Wash the Ni-NTA resin.
    a. Transfer the resin into a clean CrystalCruz® Chromatography Column (Santa Cruz, sc-205552).
    b. Drain the supernatant into a collection tube.
    c. Save the supernatant as "flowthrough 1" for western blot analysis.
    d. Wash sequentially with 20 bed volumes of Buffer A (pH 8.0), Buffer A (pH 6.3), and Buffer A supplemented with 10 mM imidazole (pH 6.3).

e. Collect all the wash buffer from the column as "wash 1″ for western blot analysis.

Note: Let the first round of wash buffer drain completely through the column before adding the second round of buffer to the column.

35. Elute the protein from the Ni-NTA resin.
    a. Close the top and bottom of the column.
    b. Add 5-bed volume of Buffer B (pH 4.3).
    c. Incubate the mixture on a platform rotator at room temperature for 5–10 min to elute proteins bound to the resin.
    d. After elution, adjust pH of the eluent solution to 8.0 with 0.1 M NaOH.
    e. Save 50 μL as "elution" for western blot analysis.
36. Protein binding with the streptavidin resin.
    a. Add 10 μL streptavidin-agarose beads for each mg of protein in the cell lysate input.
    b. Incubate the mixture with gentle rocking at room temperature.

Note: We usually don't measure the protein concentration again here. We calculate the amount of streptavidin beads to be used according to the input of total protein at the beginning of the purification (step 33). During the subsequent purification steps, the high concentration of SDS in buffer B would interfere with the measurement of protein concentration by Bradford assay.

37. Wash the streptavidin resin.
    a. The next morning, spin down the streptavidin resin at 120 × *g* for 1 min.
    b. Save the supernatant as "flowthrough 2″ for western blot analysis.
    c. Wash the streptavidin resin by resuspending sequentially with 1.5 mL of each of Buffer C, Buffer D, and Buffer E and centrifuge the tubes after each wash to collect the beads.
    d. Save the wash solution as "wash 2″ for western blot analysis.
38. Keep the streptavidin resin bound with HBT-xUB conjugated proteins in 1 mL Buffer E.

Note: This is the sample for trypsin digestion and proteomic analysis in the subsequent steps.

Pause point: You may keep the resin at room temperature temporarily or at −80°C for long-term storage.

39. Save the sample of streptavidin resin for western blot analysis.
    a. Transfer 50 μL of the resin resuspension to a clean tube.
    b. Wash the resin three times with 500 μL of PBS each time.
40. Western blot analysis for tandem purification.
    a. Add 10 μL of 6× SDS loading buffer to 50 μL samples collected from step 32c, 34c, 34e, 35e, 37b, 37d and 39b.
    b. Mix and boil the samples at 100°C for 5 min.
    c. Load 10 μL of each sample to PAGE gel for western blot analysis.
    d. Use an anti-UB antibody to probe for substrate proteins conjugated with HBT-xUB.
    e. Check the western blot.

Note: If there is enough HBT-xUB conjugated protein bound to the resin, as shown by the western blot, the beads can be digested by trypsin to prepare samples for MS analysis.

Sample processing for proteomics analysis

Timing: 2 days

41. Wash beads with ABC buffer.
    a. Add 300 μL streptavidin beads to a Pierce™ spin column with a crew cap.
    b. Wash the beads with 400 μL of 50 mM Ammonium Bicarbonate (ABC) buffer three times.
42. Wash beads with ABC/DTT buffer.
    a. Add 297 μL ABC and 3 μL of 100 mM DTT dissolved in 50 mM ABC buffer to the beads.
    b. Incubate for 1 h with shaking and rotation at 37°C.
43. Wash with ABC/IAA buffer.
    a. Add 3 μL of 500 mM iodoacetamide (IAA) in ABC buffer to the bead mixture to give a final concentration of 5 mM IAA in the solution.
    b. Incubate the mixture in the dark at room temperature with rotation for 40 min.
    c. Remove the solution by centrifugation.
    d. Wash the beads once with 300 μL ABC.
44. Protein digestion with Lys-C.
    a. Dissolve Pierce™ Lys-C Endoproteinase, MS Grade in 50 mM ABC buffer and prepare a 20 μg/mL stock solution.
    b. Add 200 μL ABC and 50 μL stock solution to the beads.
    c. Let the digestion reaction run for 4 h with shaking at 37°C.
45. Protein digestion with trypsin.
    a. Add 2 μg (5 μL) Sequencing Grade Modified Trypsin.
    b. Perform the digestion overnight at 37°C.

Note: The pH of the solution should be in the basic range.

46. Preparation of peptide samples.
    a. Spin down the beads by centrifugation.
    b. Collect the supernatant solution.
    c. Wash the beads twice with 200 μL water each time.
    d. Combine the wash solution with the supernatant solution.
    e. Freeze the solution at −80°C.
    f. Combine the solution and lyophilize.
47. Submit the sample for proteomics analysis.

Analysis of proteomic results and assembly of the substrate profile based on the OUT screen

Timing: 1 day

48. Repeat step 18–47 to generate three datasets of xUB conjugated proteins purified from the OUT cells and control cells.

⚠ CRITICAL: The culture of OUT and control cells for the expression of the OUT cascades is repeated at least three times, followed by the purification of HBT-xUB conjugated proteins by Ni-NTA and streptavidin affinity chromatography.

49. Calculate the ratio of the peptide spectrum matches (PSMs) numbers of the proteins purified from the OUT and control cells.

Note: Proteomic analysis of proteins bound to the streptavidin resin provides the name of the proteins, the sequence of the peptide fragments identified by MS, and the PSMs as an indication of the abundance of the proteins purified from the cell lysates. Software such as Perseus[14] may also be used for analyzing the proteomics data in steps 49–53. Users are suggested to consult resources in the literature to see if they are suitable for their needs.

50. Identify the proteins with a PSM ratio greater than 2 between the two cell populations.

Note: Proteomics analysis of HBT-xUB conjugated proteins purified from the three biological repeats of OUT and control cells is performed to identify proteins with PSM ratio >2 in at least two of three repeated experiments and these proteins are assigned as the potential substrates of Rsp5 from the OUT screen. These proteins show enhanced conjugation to HBT-xUB in OUT cells compared to the control cells and are the likely substrates of Rsp5. Log2 PSM ratio is listed in the table so we prioritize the proteins with Log2 PSM ration > 1 for further analysis

51. Calculate the p-values of PSM by student's t-test for all the proteins identified by proteomics.
52. Convert the p-values to -Log10 p-values.
53. A volcano plot is generated to plot Log2 PSM ratio (x-axis) of each identified protein against the Log10 p-value (y-axis).

Note: A volcano plot is to reveal the statistical significance of the proteins purified from the OUT and control cells to facilitate the analysis of the proteomic results. The volcano plot can be generated with GraphPad, Matplotlib or Microsoft Excel.

Expression of the ubiquitinating enzymes and potential substrates in *E coli*

Timing: ~ 5 days

The UB-transferring enzymes, including Uba1, Ubc1, and potential substrate proteins of Rsp5 to be verified, are expressed in *E. coli* cells with pET vectors. The pET vectors express proteins with with N- or C-terminal 6×His tags for their purification by Ni-NTA beads from the cell lysate. Rsp5 E3 is expressed from a pGEX plasmid and purified from the *E coli* cell lysate using glutathione affinity resin.

54. *E coli* transformation.
    a. Transform the pET expression plasmid of each protein into BL21 electrocompetent *E coli* cells by electroporation, followed by the addition of 1 mL of SOC.
    b. The cells are recovered by shaking in a 37°C incubator for an hour.
    c. Streak 100–200 μL of recovered cells on LB-agar plate supplemented with antibiotics.
    d. The plates are left in a 37°C incubator overnight until colonies appear.

Note: The LB-agar plate and 2XYT used below should be supplemented with Amp or Kan antibiotics matching with the antibiotic resistance gene carried by the pET vector used for transformation.

55. Cell culture and induction of protein expression.
    a. Pick a single colony from the transformation plate and use it to inoculate 5 mL 2XYT in a 15 mL culture tube supplemented with the same antibiotics.
    b. Grow the culture overnight in a 37°C shaker.
    c. The next day, add the overnight culture to 500 mL 2XYT in a 2L sterilized flask with proper antibiotics.
    d. Allow the cell to grow in a 37°C shaker for 4–6 h until OD_{600} reaches 0.6–0.8.
    e. Add 1M IPTG to a final concentration of 1 mM.
    f. Shake the induced culture at 16°C overnight (16–20 h) to induce protein expression.

Note: Reduce IPTG to 500 μM if the yield of protein expression is not good when the cells are induced with 1 mM IPTG.

56. Cell lysing.
    a. The next morning, spin down the cells at 7,000 × *g* for 20 min and discard the supernatant.
    b. Resuspend the cells with 10 mL lysis buffer.
    c. Add 20 mg lysozyme.

Table 2. The ingredients and reaction settings used for *in vitro* ubiquitination assay

Ingredients	Reaction1	Reaction2	Reaction3	Reaction4
20 μM UB	+	+	+	+
1 μM Uba1 (E1)	+	-	+	+
10 μM Ubc1 (E2)	+	+	-	+
2 μM Rsp5 (E3)	+	+	+	-
10 μM Substrate	+	+	+	+
5× reaction buffer	10 μL	10 μL	10 μL	10 μL
PBS	Up to 50 μL			

d. Incubate the cell suspension on ice for 30 min.
e. Sonicate the cells for 30 min (5 s on and 5 s off cycle for 1 h) on ice to avoid overheating.

⚠ CRITICAL: Always put the cells in an ice-water mixture, but not just on ice, as there would be space between ice and the tubes, especially when the ice melts during sonication. This may cause insufficient cooling of the tubes and overheating of the cell lysate.

57. Protein purification.
    a. Transfer the lysate into centrifuge tubes and centrifuge at 30,000 × *g* for 30 min.
    b. Collect the supernatant by transferring it to a new and clean 50 mL tube.
    c. Wash 500 μL of Ni-NTA beads with 1 mL PBS three times.
    d. Add Ni-NTA beads to the cell lysate in step 57b.
    e. Incubate the mixture at 4°C overnight with rotation.
    f. Load the suspension into a clean gravity-flow column.
    g. Wash once with 15 mL lysis buffer and twice with 15 mL wash buffer sequentially.
    h. Elute the protein bound to the Ni-NTA resin with 5 mL of elution buffer.

    Note: For sufficient elution, close the stopper of the column, resuspend beads with 5 mL of elution buffer and wait for 10–15 min so that the proteins can dissociate from the resin. Then open the stopper to collect the eluate.

    i. Put the eluate into a dialysis bag with proper MW cutoff and dialyze in 1 L dialysis buffer at 4°C overnight.
    j. The next day, put the dialysis bag in fresh dialysis buffer and dialyze for 3 h at 4°C.
58. Concentrate the dialyzed protein with a proper size Amicon ultra centrifugal filter unit.
59. Measure the concentration of the protein.
60. Aliquot the purified protein into 1.5 mL tubes and store at −80°C.

Reconstitute substrate ubiquitination catalyzed by Rsp5 *in vitro*

Timing: 2 h

61. Thaw UB, UB-transferring enzymes, and substrate proteins on ice.
62. Follow Table 2 to set up 50 μL *in vitro* ubiquitination reactions for each substrate with the control reactions missing E1, E2, or E3.
63. Incubate the reaction mixture with shaking for 1–2 h at 37°C.
64. Quench the reactions by adding 10 μL 6× SDS loading dye and boil the tubes at 100°C for 5 min.

Western blot of the ubiquitination reactions

Timing: 2 days

Table 3. The plasmids used for cotransformation for the ubiquitination assay in the cell

Transformation #	Plasmid 1	Plasmid 2
1	pYES-URA (empty)	pESC-LEU (empty)
2	pYES-URA (empty)	pESC-LEU-substrate
3	pYES-URA-Rsp5-DN	pESC-LEU-substrate
4	pYES-URA-wt RSP5	pESC-LEU-substrate

65. Load 10 μL of boiled samples to Bio-Rad 4%–20% precast gel for electrophoresis.
66. Transfer the protein bands from the gel to a PVDF membrane with Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit.
67. Block the PVDF membrane with 5% non-fat milk for 1 h.
68. Dilute a primary antibody that binds to the substrate protein or the tag fused to the substrate protein in 5% non-fat milk at a proper dilution ratio.
69. Incubate the PVDF membrane with the antibody solution at 4°C overnight with shaking.
70. The next morning, wash membranes with TBS-T buffer (TBS buffer with 0.1% tween20) three times.
71. Incubate membranes with secondary antibody (1: 20,000, goat anti-mouse or goat anti-rabbit antibody) at room temperature for an hour.
72. Wash the membranes with TBS-T buffer for three times.
73. Prepare 2 mL of fresh developing solution (SuperSignal® West Pico Chemiluminescent Substrate) for each piece of membrane.
74. Apply the developing solution onto the membrane and wait for 2 min. Then remove the excess liquid.
75. Expose the membrane to an autoradiography film in a dark room or use equipment capable of detecting chemiluminescence for imaging the membrane.

cim3-1 cell culturing and transformation

Timing: about 1 week

To verify the ubiquitination of potential substrates by Rsp5, we need to compare the ubiquitination level of the substrates between yeast cells with suppressed and elevated Rsp5 activity. Rsp5 is an essential gene for yeast viability, so the deletion of Rsp5 gene will make the yeast cells nonviable in a standard growth medium without supplementing oleic acid.[15] We thus conditionally express a dominant negative mutant of Rsp5 (Rsp5-DN) to suppress the native Rsp5 activity in the cell. The gene of Rsp5-DN has a premature stop codon at L451 before the HECT domain and would express a truncated Rsp5 without the UB ligase activity. We cloned Rsp5-DN into the pYES vector for expression under the pGAL promoter. The accumulation of ubiquitinated proteins is also important for assaying substrate ubiquitination in yeast. We chose to use yeast strain *cim3-1* for the assay because the strain is deficient in proteasome activity at 37°C.[12] By coexpressing Rsp5 and the substrate proteins in the *cim3-1* cells, we can enhance the level of ubiquitinated substrates of Rsp5 in the cell for their detection by western blots following immunoprecipitation.

76. Use the glycerol stock of the *cim3-1* strain to inoculate 3–5 mL YPD media and culture the cells in a 25°C shaker to prepare the cells for yeast cotransformation.

Note: Please refer to the Preparation Step (steps 3–16) for procedures of yeast cell transformation and refer to Table 3 for the cotransformation of plasmids.

77. Plate transformed yeast cells on the SC Dex ura- leu- plates. Incubate the plates at 25°C for 3–4 days until colonies appear.

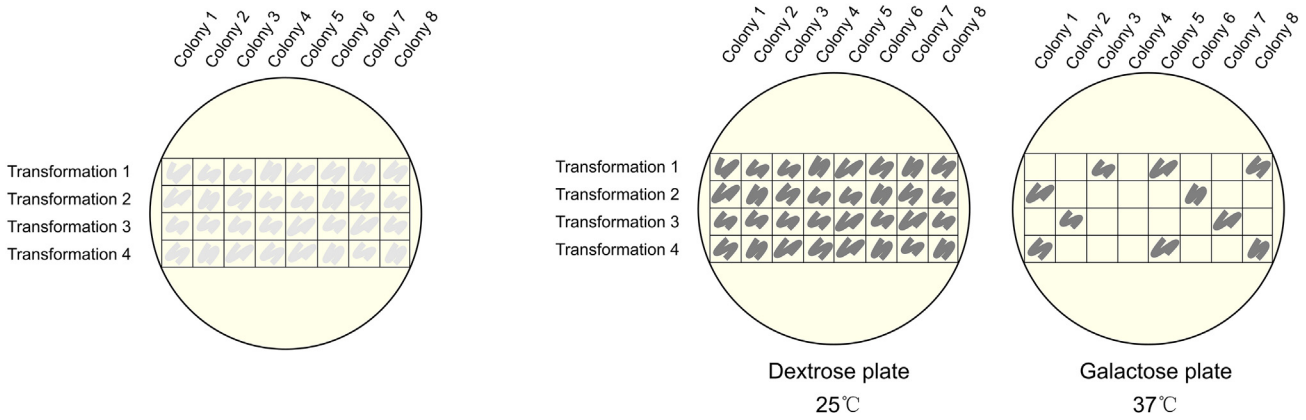


Figure 4. The scheme for phenotype check of *cim3-1* cells co-transformed with the pYES-URA-Rsp5-DN plasmid and the pESC-Leu-substrate plasmid

Transformations 1–4 correspond to the co-transformation reaction of pYES-URA-Rsp5-DN with different substrate expressing plasmids. For each transformation reaction, colonies 1–8 are picked for growing on both the dextrose and galactose plates.

Note: The *cim3-1* cells grow slower than wild-type yeast cells. It will take a longer time for transformed *cim3-1* cells to grow into visible colonies. Since pYES-URA and pESC-LEU vectors are used for cotransformation, Ura- and Leu- plates should be used to select for cells cotransformed with the two plasmids. According to the genotype of *cim3-1*, histidine must be present in the synthetic media, while tryptophane is optional.

Phenotype check

Timing: 2–3 days

Steps 78–82 in the procedures below are to check the phenotypes of *cim3-1* cells transformed with pYES-URA-Rsp5-DN (Figure 4). A phenotype check is performed in order to identify the clones showing growth inhibition due to the expression of Rsp5-DN in the cell. Cells with the pYES-URA-Rsp5-DN plasmid are induced to express Rsp5-DN on the galactose plate but not on the dextrose plate. Because of the lethality associated with Rsp5-DN expression and the deficiency of proteasome activity in the *cim3-1* strain at 37°C, *cim3-1* cells expressing Rsp5-DN would not grow on galactose plates at 37°C but would grow on dextrose plates at 25°C. Colonies cotransformed with the pYES-URA-Rsp5-DN and pESC-Leu-substrate plasmids showing such a phenotype are selected for assaying the ubiquitination of the Rsp5 substrates in the cell. In contrast, cells cotransformed with plasmids expressing wt Rsp5 and the substrate would not show any difference in growth on dextrose and galactose plates at 25°C or 37°C, respectively.

78. Prepare selection plates (SC ura- leu-) with dextrose and galactose, respectively. Draw rows of squares on the back of the plates.
79. For yeast cells co-transformed with the pYES-URA-Rsp5-DN plasmid and pESC-LEU-substrate plasmids (from step 77), pick 6–8 single colonies from each plate.
80. Resuspend each colony in 30 μL of sterile water in separate tubes.
81. Add 1 μL of resuspended cells from each transformation to the center of each square and spread the liquid drop with a sterilized inoculating loop.

Note: Each colony should be assigned the corresponding squares on the dextrose and galactose plates for seeding (Figure 4). Make sure there is no cross-contamination and label the transformations and colonies well.

82. Incubate the dextrose plates at 25°C and galactose plates at 37°C overnight or longer until colonies appear. Colonies showing the Rsp5-DN phenotype should grow on the dextrose plate at 25°C but not on the galactose plate at 37°C (Figure 4).

Substrate pulldown and Western blot for UB

Timing: about 1 week

83. Culture enough yeast cells for the ubiquitination assay.
    a. Pick up colonies from the dextrose plates with cotransformation of plasmids expressing substrate proteins and wt Rsp5 or Rsp5-DN.
    b. Inoculate 5 mL of SC Dex ura- leu- medium with each colony.
    c. Grow the culture overnight at 25°C with shaking.
    d. The next day, use the 5 mL overnight culture to inoculate 35 mL of SC Dex ura- leu- medium for the growth of another overnight culture at 25°C.

⚠ CRITICAL: Only pick the colonies showing the Rsp5-DN phenotype from the cotransformation reaction with the pYES-URA-Rsp5-DN plasmid and the pESC-Leu-substrate plasmid. Use 150 mL Erlenmeyer flasks for the 35 mL culture and shake the culture at 250 rpm for efficient aeration.

Note: The Rsp5-DN cells would grow much slower than the wt Rsp5 cells. If the overnight culture for the wt Rsp5 cells is much higher than that of the Rsp5-DN cells, make a proper dilution of wt Rsp5 cells before inoculating the 35 mL culture so that the starting ODs of the two cell cultures are similar.

84. Change the yeast cells from dextrose medium to galactose medium.
    a. Pellet the cells at 1,800 × *g* for 3 min.
    b. Wash the cells three times with 1 mL DI water, and then remove all the liquid.
    c. Transfer all the cells into 35 mL of SC Gal ura- leu- medium.
    d. Grow the cells overnight in a 25°C shaker.

Note: Again, use 150 mL Erlenmeyer flasks for the 35 mL culture and set the shaker at 160 rpm for good aeration.

Note: We change the cells from dextrose to galactose medium so that the genes under pGAL promoter in the pYES-URA and pESC-LEU plasmids would be induced for expressing Rsp5 and the substrate proteins, respectively.

85. The next morning, shift cells to 37°C and incubate for 7–8 h.

Note: After being induced for protein expression, the *cim3-1* cells are shifted from 25°C to 37°C to induce proteasome-deficiency in the cell so the ubiquitinated proteins in the cell can accumulate. Centrifuge the cells at 1,800 × *g* for 5 min and wash the pellet with sterile water.

Pause point: You can freeze cell pellets at −80°C or lyse them immediately.

86. Yeast cell lysing.
    a. Add 10 pellet-volumes of ice-cold freshly prepared RIPA buffer (containing 1× yeast protease inhibitor cocktail and 1 mM PMSF) to the cell pellet and resuspend the cells in the buffer.
    b. Transfer the cell resuspension to 15 mL tubes for vortexing and keep the tubes on ice.
    c. Add 1 pellet-volume of 0.5 mm acid-washed glass beads to the tubes containing the cell suspension.

d. Vortex the tubes for 30 s and place the tubes on ice for 30 s. Repeat this cycle 12 times.
e. Centrifuge the cell lysate mixture with the glass beads for 5 min at 3,200 × *g*.
f. Collect the supernatant and transfer it to a regular 1.5 mL tube.
g. Precipitate the cell debris in the lysate by centrifugation at 20,000 × *g* for 10–15 min at 4°C.
h. Measure the total protein concentration in the clarified cell lysate by Bradford assay after diluting the lysate by 1,000-fold.

Note: You may need around 1.1 mg of total protein.

87. Expression check.
    a. For each sample, transfer the same amount of protein lysate (around 100 μg) into a fresh tube, add 6xSDS-PAGE loading dye, and boil the tubes at 100°C for 5 min.
    b. Load the cell lysate to a gel and perform SDS PAGE.
    c. Probe the western blots with an anti-Flag or substrate-specific antibody to check the expression of substrates in the co-transformed cells.

Note: The expression of wt Rsp5 can be verified by a western blot probed with an anti-HA antibody that detects the HA tag fused to wt Rsp5. Once the expression of substrate proteins and Rsp5 are confirmed in the cell lysate, co-immunoprecipitation (co-IP) can be carried out with each substrate protein.

88. Substrate ubiquitination check.
    a. Wash M2 agarose anti-Flag beads three times with RIPA buffer and equilibrate the beads with RIPA buffer in a 50% slurry.

Note: For detailed information of M2-agarose Flag beads, please visit: https://www.sigmaaldrich.com/US/en/substance/antiflagm2affinitygel1234598765?gclid=EAIaIQobChMIrJaHo9fh-QIVRdiWCh2Lpw1mEAAYASAAEgK0tfD_BwE.

    b. Prepare another set of cell lysates with approximately 1 mg of lysate proteins in each tube.
    c. Add 25 μL of M2-agarose anti-Flag beads to each tube and fix the tubes on a rotator.
    d. Rotate the sample tubes at 4°C for 2–6 h for thorough mixing of the anti-Flag beads with the cell lysates.

⚠ CRITICAL: Do not incubate the cell lysate with beads overnight to decrease the non-specific binding of proteins in the cell lysate with beads.

    e. Wash the beads 3 times with RIPA buffer.
    f. Boil the beads at 100°C with 25 μL of 1xSDS-PAGE loading dye for 5 min to release the substrate proteins from the beads.
    g. Load 10 μL of the samples onto Bio-Rad precast 8% SDS-PAGE gels for western blot analysis.

Note: Use an anti-UB antibody as the primary antibody to detect ubiquitinated substrate species in the pulldown sample.

Stability assays

Timing: 3 days

Below are the procedures for measuring the stability of Rsp5 substrates in the yeast cells. Rsp5-mainly catalyzes the attachment of K63-linked UB chains on the substrate proteins, which is responsible for non-degradative functions, such as mediating protein-protein interactions in the cells.[16] Still, Rsp5

catalyzed substrate ubiquitination has been associated with protein degradation through proteasome or lysosome pathways.[13,17,18] So we check and compare the stability of Rsp5 substrates in wild-type (wt) yeast strain and *rsp5-1* strain that encodes a L733S mutation in the HECT domain of Rsp5.[19,20] The mutation will render the cell deficient in Rsp5 activity at 37°C. If the Rsp5 substrate is degraded by the proteasome upon their ubiquitination, they would have prolonged stability in *rsp5-1* cells.

89. SEY6210.1 (WT) and YXY705 (*rsp5-1*) cells were cultured in YPD media at 25°C and prepared for the transformation reaction with 1 μL (~ 1 μg) pCM189-URA-substrate-Flag plasmids that express Flag-tagged substrates.

Note: SEY6210.1 is a wt yeast strain with endogenous Rsp5 expression and it is used as a positive control. YXY705 (*rsp5-1*) carries the L733S mutation in the HECT domain of Rsp5 that inactivates its UB ligase activity.[13] The pCM189-URA plasmid has a *tetO* operator and a URA3 marker. Expression of genes in the pCM189-URA plasmid is inhibited when doxycycline is added to the culture media.

90. The transformed yeast cells are plated onto SC Dex ura- plates and incubated at 25°C for 3–4 days until colonies appear.
91. *TET*-off system check.
    a. Pick a single colony from each plate and inoculate 4 mL of SC Dex ura- media.
    b. Aliquot to two separated 15 mL tubes.
    c. Add doxycycline to a final concentration of 2 μg/mL into one of the tubes and mark the tubes well.
    d. Culture the tubes at 25°C overnight with shaking.
    e. Centrifuge at 1,800 × *g* for 3 min to collect the cells.
    f. Resuspend the cells and boil the cells with 30 μL 1× SDS loading dye at 100°C for 10–15 min to break the cell.
    g. Spin down the cell debris at 16,000 × *g* for 1 min.
    h. Load 10 μL supernatant to SDS-PAGE gel for western blot analysis with anti-Flag antibody to check the expression of the substrate under the *TET*-off system.

Note: This step is optional, the aim is to check the substrate expression with or without doxycycline in *TET*-off system and make sure it works before the stability assay.

92. In the morning, pick colonies from plates and use them to inoculate 2 mL of SC Dex ura- media.
93. Incubate the culture tubes at 25°C with shaking.
94. In the evening, dilute the starting culture into 40 mL of media in a 250 mL sterilized flask.
95. Incubate the flask at 25°C overnight with shaking.

Note: A growth curve of each strain should be measured before the stability test in order to estimate the growth time of the yeast cells (Figure 3). Based on the growth curve, the overnight culture should be inoculated with a proper amount of starting culture so it can reach the mid-log phase the next morning.

96. The next morning, measure OD_{600} of the culture, which should be in the mid-log phase (0.4–0.6). If the OD_{600} is too high, dilute the culture to 0.2 and grow it again to mid-log phase.
97. Shift the culture to 37°C for 20 min to prewarm the cells, and prewarm some fresh media to 37°C.

Note: 37°C is the non-permissive temperature for protein degradation in *rsp5-1* cells, while the WT strain is not affected at 37°C.

98. Spin down the cells and resuspend the pellet in 40 mL prewarmed SC Dex ura- media supplemented with 2 μg/mL doxycycline.

99. Measure OD_{600} again and collect 7 OD_{600} of cells. This is marked as time zero.

Note: The volume of the cells collected is calculated by the formula $V = 7/OD_{600}$ of the current culture.

100. Put the culture back in the 37°C shaker.
101. Treat cells with TCA.
    a. Pellet the cells in the collected culture by centrifugation.
    b. Resuspend the cell pellets with 1 mL 10% TCA.
    c. Incubate the cell suspension on ice for 30 min.

Note: TCA is a strong acid, and it is corrosive to the skin. Wear gloves and avoid touching it directly. Cells treated with 10% TCA are dead, and all activities in the cells are stopped.

102. Measure the OD_{600} of the cell culture growing at 37°C every 2 h (0, 2 and 4 h) and collect 7 OD_{600} of cells at each time point.
103. Repeat step 101 with all samples collected.

Pause point: After the last sample was collected, the cells treated with TCA can be stored at 4°C for one week.

104. Cell wash.
    a. Spin down cells in the TCA solution at 16,000 × *g* for 1 min and remove the supernatant.
    b. Wash the cell pellet with 500 μL of 0.1% ice-cold TCA.
    c. Spin down the cells again at 16,000 × *g* for 1 min.
    d. Carefully remove all the supernatant.
105. Cell lysis.
    a. Resuspend the pellet with 70 μL 2× urea boiling buffer and one pellet-volume of glass beads.
    b. Lyse the cells by beads-beating for 5 min using Mini-Beadbeater-16.
    c. Quickly spin down the samples to settle the beads to the bottom.
    d. Heat the tube to 42°C for 5 min to aid the denaturation of the proteins.
    e. Add 70 μL 2× urea sample buffer with DTT.
    f. Repeat steps 105b-d to lyse the cells by bead beating again.
    g. Centrifuge the cell lysates at 16,000 × *g* for 5 min.
    h. Carefully transfer the supernatant to a fresh tube.

Note: This is the sample for analyzing the level of substrate proteins in the cell lysate. Do not boil the samples as the urea in the buffer may be decomposed by high temperature.

106. Analyze the samples by SDS-PAGE and western blot.

Note: Use the anti-Flag antibody to probe for Flag-tagged substrates and use an anti-G6PDH antibody to probe for G6PDH as a loading control. 20 μL of the cell lysate corresponds to 1 OD_{600} of the cells as the source of the lysate.

EXPECTED OUTCOMES

Expression of OUT cascade in YPH499 cells and tandem purification of xUB-conjugated proteins

Following the protocol, we expect the expression of the OUT components, including xUba1 (xE1), xUbc1 (xE2), xRsp5 (xE3) and HBT-xUB, to be strong (Figure 5A). We often see two main bands associated with xUba1 on the Western blot probed with the anti-Flag antibody. The band at 120 kD matches the full-length xUba1 and the band around 80 kD would be a truncated xUba1. The binding

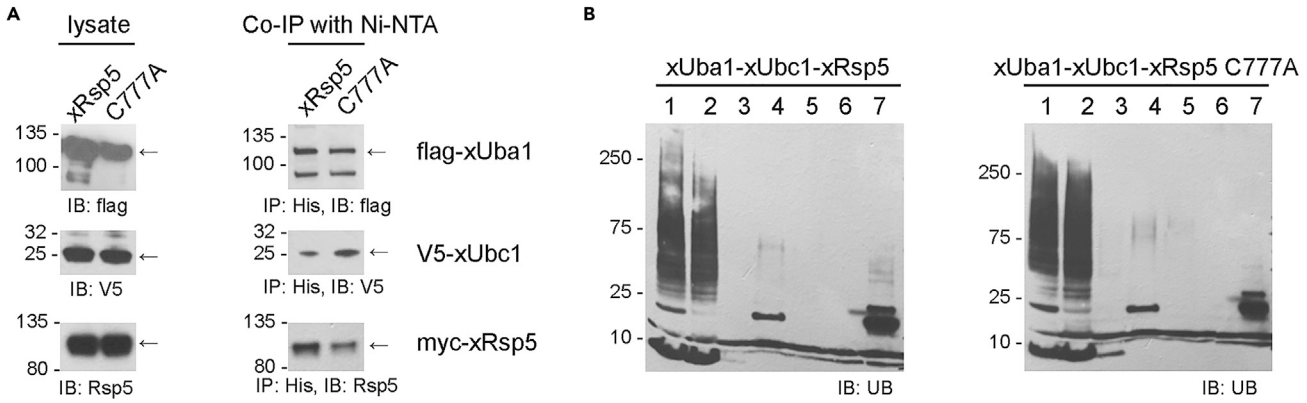


Figure 5. Expression of OUT cascade and tandem purification

(A) The expression of OUT cascade components is confirmed by Western blot with lysates from YPH499 cells. The transfer and conjugation of HBT-xUB to xUba1, xUbc1 and xRsp5 in the cell is confirmed by co-IP with Ni-NTA beads and Western blot analysis.

(B) The xUB-conjugated proteins in each step of tandem purification of lysates of OUT and control cells are analyzed by Western blot probed with an anti-UB antibody. Lane 1: cell lysate from step 32c, Lane2: flowthrough 1 from step 34c, Lane 3: wash 1 from step 34e, Lane 4: elution from step 35e, Lane 5: flowthrough 2 from step 37b, Lane 6: wash 2 from step 37d, Lane 7: sample of streptavidin resin from step 39b. Adapted from the published work (Wang et al., 2021a).

of the cell lysate with Ni-NTA resin would pull down proteins covalently conjugated with HBT-xUB, including xUba1, xUbc1, and xRsp5. Indeed, we expect to observe bands corresponding to these proteins in the pulldown sample when the western blots are probed with specific antibodies binding to the OUT components. The pulldown signal of the C777A mutant of xRsp5 is weaker compared to xRsp5 because the defective UB ligase activity of the C777A mutant would significantly decrease its conjugation to HBT-xUB. The western blots for tandem purification of the cell lysate expressing the OUT cascade of Rsp5 and HBT-xUB are shown in Figure 5B. We would see strong UB signals in the cell lysate and flowthrough fraction of the Ni-NTA column (Lanes 1 and 2) corresponding to proteins modified with wt UB that would not bind to the Ni-NTA resin. There is a weak signal of UB-modified proteins eluted from the Ni-NTA resin corresponding to proteins conjugated with HBT-xUB (Lane 4) and the free HBT-xUB of 25 kD is also visible in the lane. Bands of proteins conjugated with HBT-xUB can also be observed in the fraction bound to the streptavidin resin (Lane 7) and these are the proteins to be subsequently identified by proteomics. It is noted that there is a considerable amount of free HBT-xUB bound to the streptavidin resin as shown by a strong band matching the size of HBT-xUB monomer on the Western blot (Lane 7, Figure 5B). This suggests only a fraction of overexpressed HBT-xUB is conjugated to Rsp5 substrates in the cell. A reason for this could be that HBT-xUB is orthogonal to the wt UB transfer pathways in the cell, so only xRsp5 can use it for substrate conjugation and the amount of HBT-xUB expressed is in much excess for the transfer reaction mediated by the OUT cascade of Rsp5.

Verification of Rsp5 substrates with Pal1 and Pal2 as examples

We verify substrates of Rsp5 from the OUT screen by reconstituting Rsp5-catalyzed ubiquitination *in vitro*. We also assay the change of substrate ubiquitination in *cim3-1* cells with the co-expression of Rsp5 and Rsp5-DN. If there are no adapter proteins needed for bridging the interaction between Rsp5 and substrate, the *in vitro* reaction of the UB transfer cascade, including wt Uba1 (E1), Ubc1 (E2) and Rsp5 (E3) and the substrate proteins, would result in the ubiquitination of the substrates (Figure 6A). The control reactions, eliminating Uba1, Ubc1, or Rsp5, did not show substancial substrate ubiquitination, suggesting the ubiqutination reaction is dependent on all components of the UB-transfer cascade. In the *in vitro* reactions, it is typical to see weak ubiquitination of the substrates when E2 is not present due to the direct transfer of UB from E1 to Rsp5 and then to the substrate. We also carry out ubiquitination assays of Rsp5 substrates in the *cim3-1* cells, taking advantage of their deficiency in proteasome activities at 37°C (Figure 6B). We coexpress substrates with wt Rsp5 or the Rsp5-DN mutant,

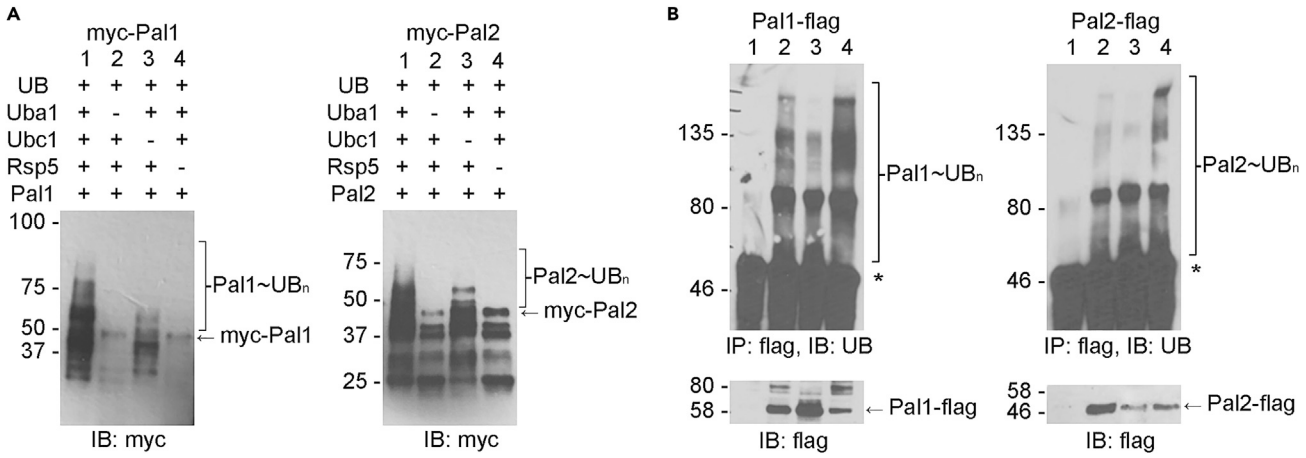


Figure 6. *In vitro* and *in vivo* ubiquitination assays for verifying the substrates of Rsp5

(A) Western blots of the *in vitro* ubiquitination assay. The blots are probed with an anti-myc antibody that recognizes the myc tag on the substrates. Lane 1: *in vitro* ubiquitination assay with all ingredients, Lane 2, 3 and 4: the control reactions eliminating Uba1, Ubc1, or Rsp5, respectively. Lane 1–4 correspond to reactions 1–4 in Table 2 in step 62. Arrows indicate the bands corresponding to unmodified myc-Pal1 and myc-Pal2. Bands below the full-length myc-Pal1 and myc-Pal2 are likely truncated substrate proteins purified from *E coli* cells.

(B) Assay for measuring substrate ubiquitination in cim3-1 cells with the coexpression of the substrates with wt Rsp5 or Rsp5-DN. The Flag-tagged substrates are pulled down by M2 anti-Flag beads and probed with anti-UB antibody on the western blots. Yeast transformation Lane 1: empty + empty, Lane 2: empty + substrate, Lane 3: Rsp5-DN + substrate, Lane 4: wt Rsp5 + substrate. Lane 1–4 correspond to cotransformations in Table 3 and step 77. * designates bands matching the size of the heavy chain IgG of the anti-Flag antibody used for the substrate pulldown. Adapted from the published work with modification (Wang et al.[1]).

immunoprecipitate the substrate with the Flag tag and probe the level of substrate ubiquitination with an anti-UB antibody. We would observe enhanced ubiquitination of the substrate with the coexpression of wt Rsp5 in the cell (comparing Lane 2 and 4) and reduced ubiquitination of the substrate when it is coexpressed with the Rsp5-DN mutant (comparing Lane 2 and 3). These results provide further proof of Rsp5-catalyzed ubiquitination of substrate proteins identified by the OUT screen.

Stability assay of the substrates with Pal1 and Pal2 as examples

We assay the stability of the substrates in wt SEY6210.1 cells and *rsp5-1* cells with defective UB ligase activity in Rsp5. We express substrate proteins in these cells with the pCM189-URA plasmid that regulates the expression of the substrates with a *TET*-off promoter. The addition of doxycycline would inhibit substrate expression and mark the starting point to follow the degradation of the substrates in the cell in the presence of wt Rsp5 or the *rsp5-1* mutant. We first validate the inhibition of substrate expression with the *TET*-off promoter (Figure 7A). We expect to see the expression of the substrate proteins in the media without doxycycline, and no expression of the substrates when doxycycline is added to the media. After establishing the control of substrate expression with doxycycline, we culture the cells to induce substrate expression, add doxycycline to stop the expression, and collect cells at 0, 2 and 4 h after doxycycline addition to follow the change of substrate levels in the cell. If Rsp5- catalyzed ubiquitination signals the degradation of the substrate proteins, we expect to see an accelerated decrease of the substrate protein levels in wt SEY6210.1 cells compared to *rsp5-1* cells. In our assay, we do not see significant differences of Pal1 and Pal2 levels in the two cell types, suggesting that the ubiquitination of these substrates by Rsp5 does not signal their degradation by the proteasome (Figure 7B).

LIMITATIONS

The OUT method takes advantage of the exclusive transfer of HBT-xUB to a designated E3 such as Rsp5 in the yeast cell for the specific labeling of E3 substrates by HBT-xUB. In this way the substrate of the E3 under interrogation could be purified from the cell lysate by tandem Ni-NTA and streptavidin columns and identified by proteomics. We have constructed OUT cascades for human HECT E3 E6AP/UBE3A,

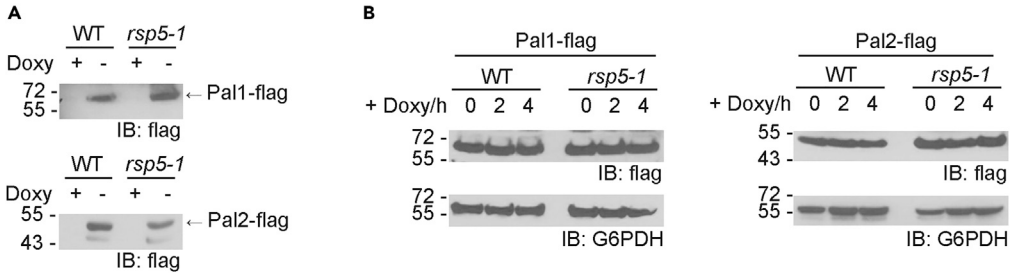


Figure 7. Stability assay of verified substrates of Rsp5

(A) The expression of verified substrates in *TET*-off system with/without doxycycline. 2 μg/mL doxycycline was added to the culture of the WT/*rsp5-1* strains as needed. After overnight culturing with doxycycline, the yeast cells were collected and the expression of Pal1 and Pal2, the substrate proteins of Rsp5, was assayed by Western blot.

(B) The stability of substrates with the coexpression of wt Rsp5 or Rsp5 mutant (L733S) with defective UB ligase activity. The WT and *rsp5-1* cells transformed with plasmids for substrate expressing were cultured to mid-log phase, and doxycycline was added to the 40 mL culture at time 0. Then 7 OD_{600} of cells were collected at 0, 2 and 4 h after doxycycline was added. For each sample, cell lysate of 1 OD_{600} equivalent cells (20 μL) was loaded on the gel for Western blot analysis by an anti-Flag antibody. Adapted from the published work with modification (Wang et al.[1]).

and U-box E3s E4B and CHIP, and profiled their substrates in HEK293 cells.[9,10,21–23] In the method reported here, we express HBT-xUB and the components of the OUT cascade, including xUba1 (xE1), xUbc1 (xE2) and xRsp5 (xE3), in yeast cells under the drive of galactose promoters. The expression of HBT-xUB and the OUT enzymes exceeds the expression of their native counterparts in the yeast cells, and this may lead to the transfer of HBT-xUB to non-physiological substrates in the cell. We reported the identification of 82 potential substrates of Rsp5 with PSM ratios greater than two between OUT cells and control cells expressing a catalytically inactive mutant of xRsp5.[1] Each potential substrate protein need to be further verified with ubiquitination assays *in vitro* and in the cell to authenticate the regulatory relationship between the E3 and the substrates through the UB transfer reaction. This process may take time, and it would not be feasible to verify all the substrates from the OUT screen to generate a list of fully authenticated substrates. Therefore, biologically significant substrates should be curated from the potential substrate list generated by the OUT screen. These substrates can be the focus of the verification process. Regardless, the substrate profile of an E3 generated by OUT provides a shortlist of E3 substrates to guide the investigation of UB transfer pathways started from an E3 hub for piecing together the role of the E3 in cell regulation. In the future, the yeast cell genome may be edited so the expression of engineered xE1, xE2, and xE3 enzymes is under the control of their native promotors. This would enable the OUT components to be expressed at the same level as the native counterparts, and thereby increase the chance for the OUT screen to identify physiologically relevant E3 substrates. If such a system is established, the genome-edited cells expressing the OUT components can also be stimulated with various biological cues to measure how the E3 substrate profile would change in line with the change of the physiological processes in the cell.

TROUBLESHOOTING

Problem 1

Low number of proteins are identified by proteomics after tandem purification of the cell lysates expressing the OUT cascade of Rsp5 (steps 48–53).

Potential solution

The main issue with this problem could be that there is not enough protein bound to the streptavidin beads after the tandem purification. First, make sure there is enough input of the cell lysate protein for tandem purification. Before the purification, the protein concentration of the cell lysate needs to be measured by Bradford assay and the typical concentration would be 10 mg/mL. At least 10 mg of the cell lysate is used as the input for the tandem purification. If the cell lysate concentration is low, one needs to culture the cell and prepare a new batch of cell lysates with high protein concentration.

Furthermore, it is advised to check the expression of each component of OUT cascade to make sure they are properly expressed in the yeast cells and have good activities for mediating the transfer of HBT-xUB (Figure 5A). To ensure the tandem purification of the cell lysate proteins by Ni-NTA and streptavidin resin is properly done, run western blots of samples from each step of purification, as shown in Figure 5B.

There should be strong UB conjugation to the cellular proteins with wt UB and xUB combined, as suggested by the intensive smear of UB-conjugated proteins in the lysate sample (Lane 1 of the Western blot in Figure 5B). The blot of Figure 5B was probed with an anti-UB antibody, and proteins in the cell lysate conjugated with either wt UB or HBT-xUB would be detected by the blot. We found a strong conjugation of cellular proteins with wt UB and xUB combined would be a good indication of healthy cell growth and a weak UB conjugation signal would be an indication of poor cell growth. We suggest the use of freshly transformed cells for the expression of the OUT cascade and growing the cells in correct conditions with proper nutrient selection. One also needs to make sure there is no contamination of the yeast cell culture.

There should be a substantial amount of ubiquitinated protein in the flowthrough fraction of the first step of purification by the Ni-NTA column (Lane 2 of Figure 5B). These proteins are presumably modified by the wt UB so they would not be retained by the Ni-NTA resin.

There should be a detectable amount of wt UB-conjugated protein eluted from the Ni-NTA column on the western bot probed with an anti-UB antibody (Lane 4 of Figure 5B). If no protein bands are detected in the elution fraction of Ni-NTA, it means the first step of tandem purification is not successful. Use freshly prepared cell lysate for Ni-NTA resin binding, and make sure the binding and elution buffer are properly prepared. If necessary, extend the binding time of the Ni-NTA resin with the cell lysate to overnight to enhance the binding of HBT-xUB conjugated proteins with the Ni-NTA resin.

The intensity of the Western blot signals for HBT-xUB conjugated proteins bound to the streptavidin beads should be stronger than the signals from the Ni-NTA elution fraction (compare lanes 4 and 7 in Figure 5B). If the signal is weak for proteins bound to the streptavidin resin, the tandem purification should be repeated with a fresh and larger amount of cell lysate with high protein concentrations (> 10 mg/mL). The concentration of biotin added into the medium can be optimized to promote biotinylation of the HBT-xUB in the yeast cells.

Problem 2

The yields of the substrate proteins expressed from *E coli* cells are low (step 54–60).

Potential solution

We express the substrate proteins from *E coli* cells with N- or C-terminal 6×His tags and other epitope tags such as the myc tag. Sometimes it is useful to switch the tag from one end to the other to improve the yield of protein expression. In addition, some yeast proteins may not express well from *E coli* cells. In this case, order a synthetic gene of the yeast protein with optimized codons for *E coli* expression, or express the protein from a yeast host. Sometimes the substrate proteins or UB-transferring enzymes such as E1, E2, and E3s are available in a purified form from commercial sources.

Problem 3

The *cim3-1* cells grow too slowly (step 76–77 and 83–84).

Potential solution

Make sure to culture the *cim3-1* cells at 25°C instead of 30°C and that the correct medium is used. Use flasks of a large volume to culture the cells and set the shaker to 300 rpm for sufficient aeration. Antibiotics such as ampicillin and kanamycin may be added to the yeast cell culture to eliminate the growth of contaminating bacteria. Plotting a growth curve of the *cim3-1* culture to measure its

growth speed and inoculate the culture with a higher starting OD_{600} is also helpful (Figure 3). To plot the curve, one can culture the cells in a small volume and then use it to inoculate a large volume of culture.

Problem 4

The amount of total protein collected at step 86 is too small for the ubiquitination assay.

Potential solution

Try culturing more cells to prepare for cell lysates of higher protein concentration. When lysing the cells, use a proper cell/bead ratio (about equal volume). Do not add too many acid-washed glass beads to the cell suspension. If the bead volume is too much, a significant amount of the cell lysate maybe trapped by the glass beads and cannot be retrieved by pipetting.

Problem 5

No difference is observed in substrate ubiquitination levels in *cim3-1* cells with the expression of wt Rsp5 and the Rsp5-DN mutant (step 88).

Potential solution

During cell lysis, add protease inhibitor PMSF to the lysis buffer for a final concentration of 1 mM to inhibit the degradation of ubiquitinated proteins. Do not overheat the cell suspension when lysing the cells. In addition, perform a phenotype check of the cell before culturing and lysing the cells. The phenotype check is to identify the clones expressing Rsp5-DN that show growth inhibition on galactose plate at 37°C, and these clones should be used for substrate ubiquitination assays. Also, assay the expression level of wt Rsp5 and Rsp5-DN by western blotting after galactose induction to make sure the E3 enzymes are properly expressed.

Problem 6

For the stability assay with the *TET*-off system, no expression of the substrate proteins is detected in wt SEY6210.1 cells and *rsp5-1* cells (step 106).

Potential solution

Cells may not be growing at the mid-log phase when treated with doxycycline. Once the cells reach the mid-log phase, the cells should be spun down and resuspended in culture media with doxycycline to inhibit the expression of the substrate proteins. If the cell culture has passed the mid-log phase, dilute the culture so it can grow to the mid-log phase again. Samples for stability assay should be collected at the mid-log phase after the addition of doxycycline.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jun Yin (junyin@gsu.edu).

Materials availability

Plasmids for the expression of the OUT components of the Rsp5 cascade will be available upon request from Jun Yin.

Data and code availability

This study did not generate any codes.

The proteomics data with the dataset identifier PXD023688 have been deposited to the ProteomeXchange Consortium via the PRIDE[24] partner repository.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (NIH) of the United States (R01GM104498 to J.Y. and H.K., R01GM114308 to N.S., and R01GM133873 to M.L.) and the National Science Foundation of the United States (NSF) (1817976 to Y.O.C. and 2109051 to J.Y.). B.Z. was supported by grants from the Natural Science Foundation of China (31770921 and 31971187).

AUTHOR CONTRIBUTIONS

S.F., G.C., Y.W., R.G., L.Z., D.D., and N.S. performed experiments. T.A.C., M.L., and H.K. provided technical guidance and/or intellectual input. S.F., G.C., S.E.J., B.Z., N.S., Y.O.C., and J.Y. analyzed data, interpreted results, and drafted and edited the manuscript. Y.O.C., N.S., B.Z., and J.Y. acquired funding. All authors reviewed and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Wang, Y., Fang, S., Chen, G., Ganti, R., Chernova, T.A., Zhou, L., Duong, D., Kiyokawa, H., Li, M., Zhao, B., et al. (2021). Regulation of the endocytosis and prion-chaperoning machineries by yeast E3 ubiquitin ligase Rsp5 as revealed by orthogonal ubiquitin transfer. Cell Chem. Biol. *28*, 1283–1297.e8. https://doi.org/10.1016/j.chembiol.2021.02.005.

2. Lauwers, E., Erpapazoglou, Z., Haguenauer-Tsapis, R., and André, B. (2010). The ubiquitin code of yeast permease trafficking. Trends Cell Biol. *20*, 196–204. https://doi.org/10.1016/j.tcb.2010.01.004.

3. Deshaies, R.J., and Joazeiro, C.A.P. (2009). RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. *78*, 399–434.

4. Hatakeyama, S., and Nakayama, K.i.I. (2003). U-box proteins as a new family of ubiquitin ligases. Biochem. Biophys. Res. Commun. *302*, 635–645.

5. Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat. Rev. Mol. Cell Biol. *10*, 398–409.

6. Spratt, D.E., Walden, H., and Shaw, G.S. (2014). RBR E3 ubiquitin ligases: new structures, new insights, new questions. Biochem. J. *458*, 421–437. https://doi.org/10.1042/BJ20140006.

7. Zhao, B., Tsai, Y.C., Jin, B., Wang, B., Wang, Y., Zhou, H., Carpenter, T., Weissman, A.M., and Yin, J. (2020). Protein Engineering in the Ubiquitin System: Tools for Discovery and Beyond. Pharmacol. Rev. *72*, 380–413. https://doi.org/10.1124/pr.118.015651.

8. Zhao, B., Bhuripanyo, K., Zhang, K., Kiyokawa, H., Schindelin, H., and Yin, J. (2012). Orthogonal Ubiquitin Transfer through Engineered E1-E2 Cascades for Protein Ubiquitination. Chem. Biol. *19*, 1265–1277.

9. Wang, Y., Liu, X., Zhou, L., Duong, D., Bhuripanyo, K., Zhao, B., Zhou, H., Liu, R., Bi, Y., Kiyokawa, H., and Yin, J. (2017). Identifying the ubiquitination targets of E6AP by orthogonal ubiquitin transfer. Nat. Commun. *8*, 2232. https://doi.org/10.1038/s41467-017-01974-7.

10. Bhuripanyo, K., Wang, Y., Liu, X., Zhou, L., Liu, R., Duong, D., Zhao, B., Bi, Y., Zhou, H., Chen, G., et al. (2018). Identifying the substrate proteins of U-box E3s E4B and CHIP by orthogonal ubiquitin transfer. Sci. Adv. *4*, e1701393. https://doi.org/10.1126/sciadv.1701393.

11. Tagwerker, C., Flick, K., Cui, M., Guerrero, C., Dou, Y., Auer, B., Baldi, P., Huang, L., and Kaiser, P. (2006). A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with in vivocross-linking. Mol. Cell. Proteomics *5*, 737–748. https://doi.org/10.1074/mcp.M500368-MCP200.

12. Ghislain, M., Udvardy, A., and Mann, C. (1993). S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. Nature *366*, 358–362. https://doi.org/10.1038/366358a0.

13. Li, M., Rong, Y., Chuang, Y.S., Peng, D., and Emr, S.D. (2015). Ubiquitin-dependent lysosomal membrane protein sorting and degradation. Mol. Cell *57*, 467–478. https://doi.org/10.1016/j.molcel.2014.12.012.

14. Tyanova, S., and Cox, J. (2018). Perseus: A Bioinformatics Platform for Integrative Analysis of Proteomics Data in Cancer Research. Methods Mol. Biol. *1711*, 133–148. https://doi.org/10.1007/978-1-4939-7493-1_7.

15. Shcherbik, N., Zoladek, T., Nickels, J.T., and Haines, D.S. (2003). Rsp5p is required for ER bound Mga2p120 polyubiquitination and release of the processed/tethered transactivator Mga2p90. Curr. Biol. *13*, 1227–1233. https://doi.org/10.1016/s0960-9822(03)00457-3.

16. Kim, H.C., Steffen, A.M., Oldham, M.L., Chen, J., and Huibregtse, J.M. (2011). Structure and function of a HECT domain ubiquitin-binding site. EMBO Rep. *12*, 334–341. https://doi.org/10.1038/embor.2011.23.

17. Liu, J., Sitaram, A., and Burd, C.G. (2007). Regulation of copper-dependent endocytosis and vacuolar degradation of the yeast copper transporter, Ctr1p, by the Rsp5 ubiquitin ligase. Traffic *8*, 1375–1384. https://doi.org/10.1111/j.1600-0854.2007.00616.x.

18. Fang, N.N., Chan, G.T., Zhu, M., Comyn, S.A., Persaud, A., Deshaies, R.J., Rotin, D., Gsponer, J., and Mayor, T. (2014). Rsp5/Nedd4 is the main ubiquitin ligase that targets cytosolic misfolded proteins following heat stress. Nat. Cell Biol. *16*, 1227–1237. https://doi.org/10.1038/ncb3054.

19. Krsmanović, T., and Kölling, R. (2004). The HECT E3 ubiquitin ligase Rsp5 is important for ubiquitin homeostasis in yeast. FEBS Lett. *577*, 215–219. https://doi.org/10.1016/j.febslet.2004.10.006.

20. Wang, G., Yang, J., and Huibregtse, J.M. (1999). Functional domains of the Rsp5 ubiquitin-protein ligase. Mol. Cell Biol. *19*, 342–352.

21. Peng, K., Liu, R., Jia, C., Wang, Y., Jeong, G.H., Zhou, L., Hu, R., Kiyokawa, H., Yin, J., and Zhao, B. (2021). Regulation of O-Linked N-Acetyl Glucosamine Transferase (OGT) through E6 Stimulation of the Ubiquitin Ligase Activity of E6AP. Int. J. Mol. Sci. *22*, 10286. https://doi.org/10.3390/ijms221910286.

22. Wang, Y., Liu, R., Liao, J., Jiang, L., Jeong, G.H., Zhou, L., Polite, M., Duong, D., Seyfried, N.T., Wang, H., et al. (2021). Orthogonal ubiquitin transfer reveals human papillomavirus E6 downregulates nuclear transport to disarm interferon-gamma dependent apoptosis of cervical cancer cells. FASEB J *35*, e21986. https://doi.org/10.1096/fj.202101232RR.

23. Sane, S., Hafner, A., Srinivasan, R., Masood, D., Slunecka, J.L., Noldner, C.J., Hanson, A.D., Kruisselbrink, T., Wang, X., Wang, Y., et al. (2018). UBXN2A enhances CHIP-mediated proteasomal degradation of oncoprotein mortalin-2 in cancer cells. Mol. Oncol. *12*, 1753–1777. https://doi.org/10.1002/1878-0261.12372.

24. Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D.J., Inuganti, A., Griss, J., Mayer, G., Eisenacher, M., et al. (2019). The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res. *47*, D442–D450. https://doi.org/10.1093/nar/gky1106.