Appendix

E2/E3-independent ubiquitin-like protein conjugation by Urm1 is directly coupled to cysteine persulfidation

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Running title: Structural and functional insights into urmylation

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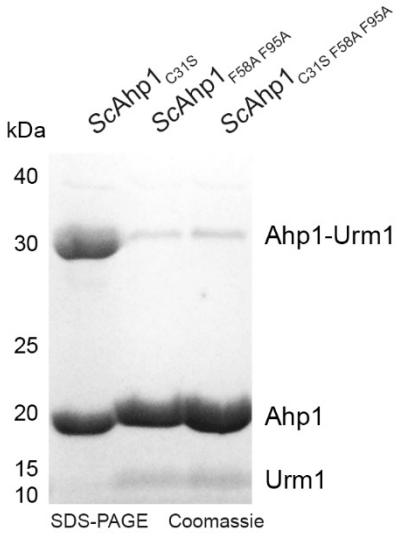
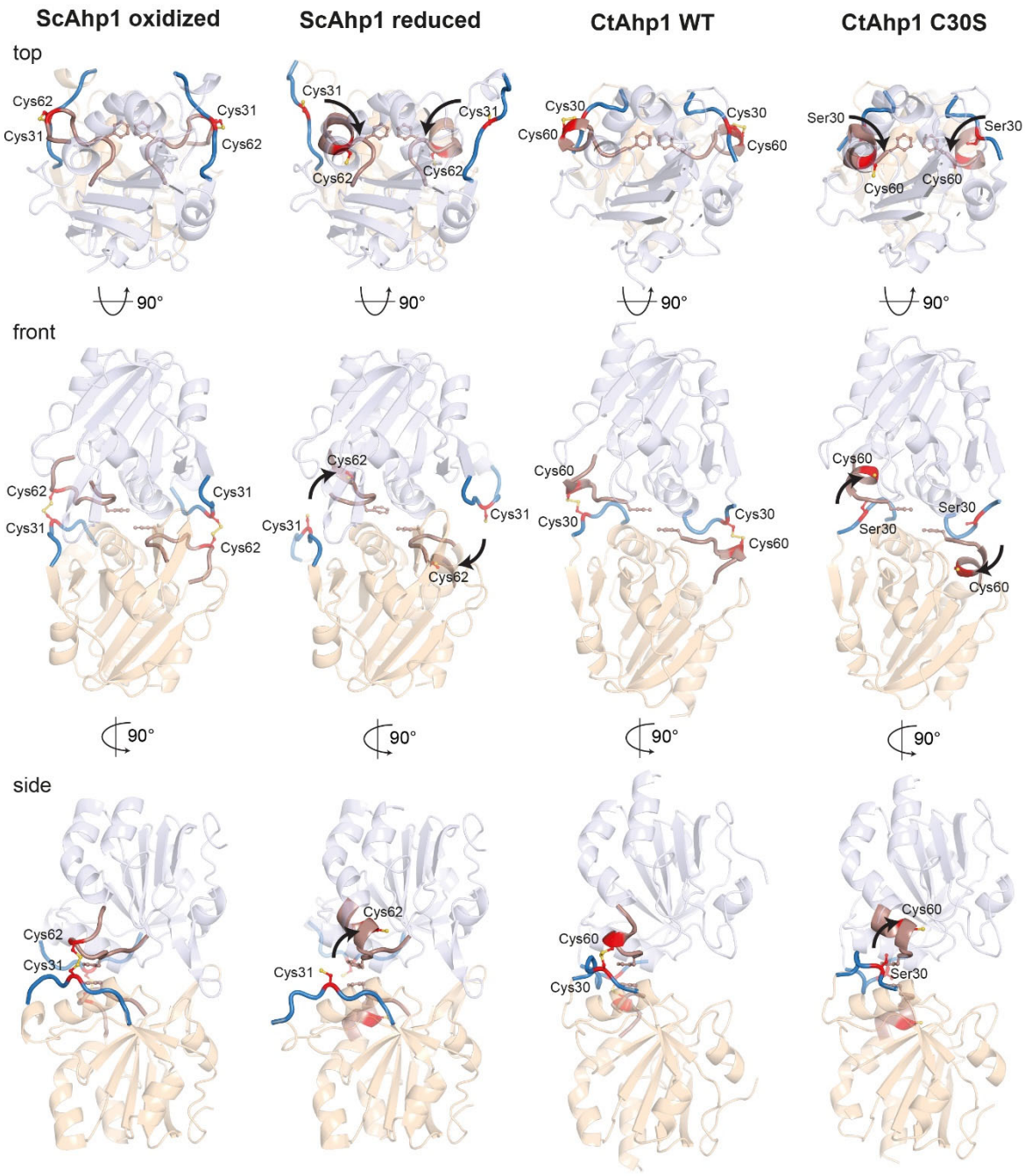


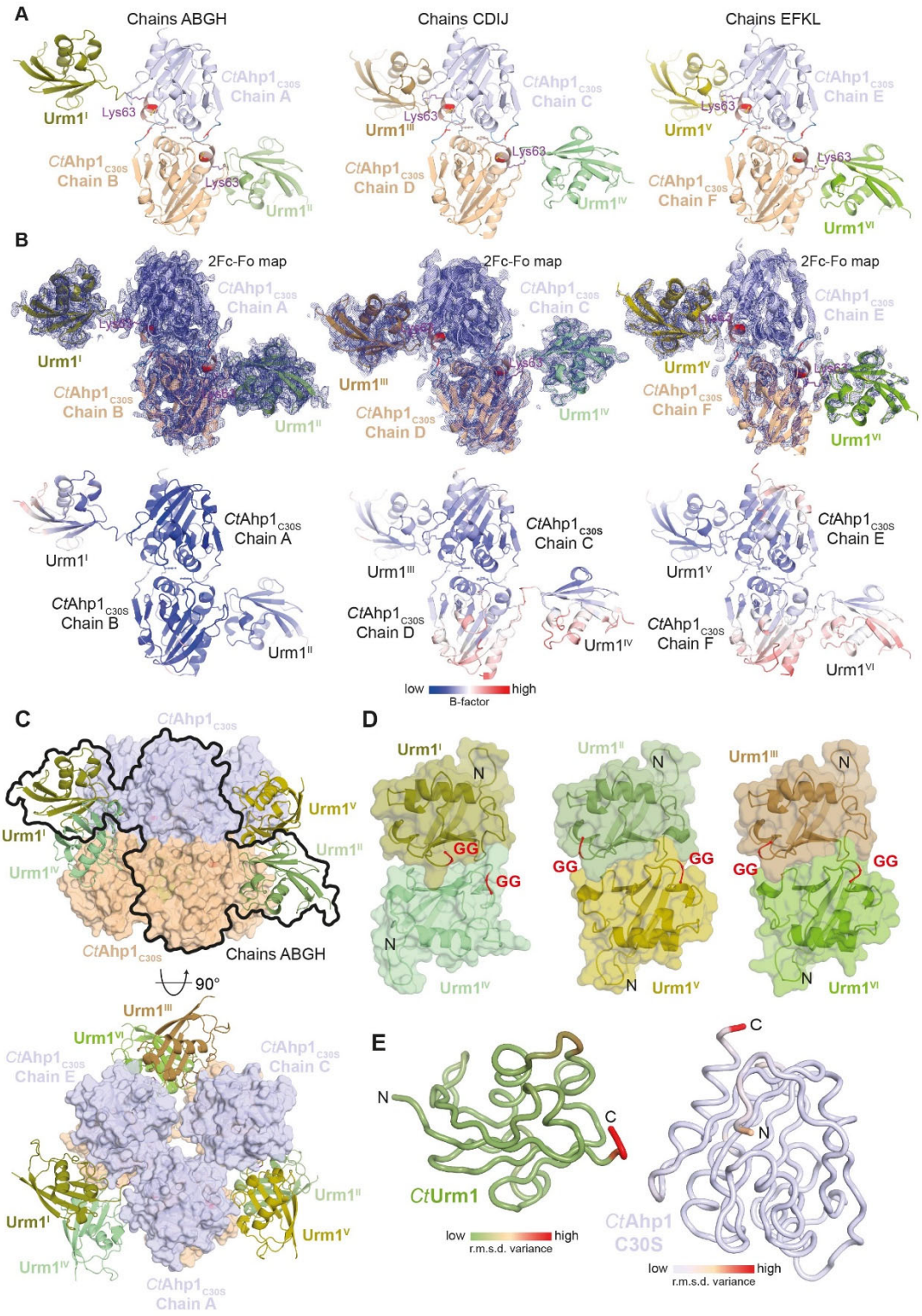
Appendix Figure S1: *In vitro* conjugation activity of monomeric Ahp1 variants

Analysis of conjugation between monomeric *Sc*Ahp1 variants (*Sc*Ahp1$_{F58A\ F95A}$ and *Sc*Ahp1$_{C31S\ F58A\ F95A}$) in combination with thiocarboxylated *Sc*Urm1 in the presence of TBH. Unconjugated Ahp1 and Urm1 as well as the formed conjugates (Ahp1-Urm1) are indicated on the right.



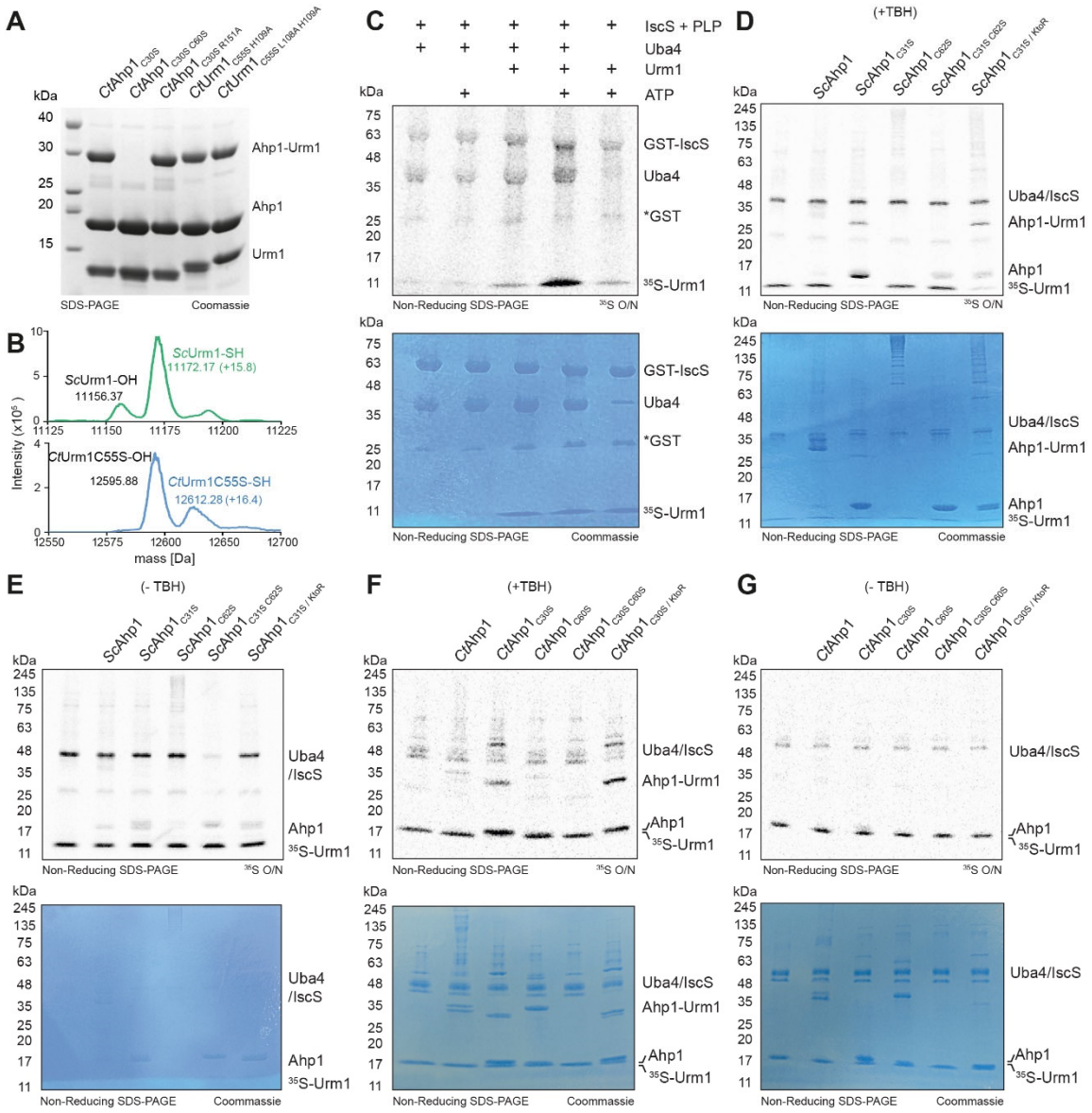
Appendix Figure S2: Comparison between active sites of *Sc*Ahp1 and *Ct*Ahp1 from the crystal structures.

Comparison of the crystal structures of *Sc*Ahp1 in oxidised (PDB ID: 4DSQ) and reduced form (PDB ID: 4DSR) alongside the *Ct*Ahp1 WT and C30S mutant. Cartoon representation of four crystal structures is shown and the catalytic and resolving cysteines are highlighted with ribbon. Catalytic cysteine-containing loops for *Sc*Ahp1 (Cys62) and *Ct*Ahp1 (Cys60) are highlighted in dark brown with red color sticks. The resolving cysteine-containing loop for *Sc*Ahp1 (Cys31) and *Ct*Ahp1 (Cys30) is highlighted in blue with cysteine marked in red sticks.



Appendix Figure S3: Structural variation in Ahp1-Urm1 complexes

A. Comparison of the three individual Ahp1-Urm1 entities in the asymmetric unit.
B. Refined density and B-factor distribution.
C. Hexameric assembly of Ahp1-Urm1 complex.
D. Comparison of Urm1 dimers in the hexameric Ahp1-Urm1 complex.
E. Average structures of all six copies of Urm1 and Ahp1 with the RMSD variance plotted.



Appendix Figure S4: Radioisotope labelling for the *in vivo* urmylation targets

A. Analysis of *in vitro* conjugation of active site mutants. *Ct*Ahp1 variants *Ct*Ahp1$_{C30S}$, *Ct*Ahp1$_{C30S\ C60S}$, *Ct*Ahp1$_{C30S\ R151A}$ and thiocarboxylated Urm1 variants *Ct*Urm1$_{C55S\ H109A}$, *Ct*Urm1$_{C55S\ L108A\ H109A}$ were tested for complex formation in the presence of TBH. The samples were separated by SDS-PAGE and visualized by Coomassie stain. Unconjugated target proteins as well as the formed conjugates are indicated on the right.

B. Deconvoluted mass spectra of (Top) *Sc*Urm1-SH and (Bottom) *Ct*Urm1 $_{C55S}$ -SH produced via IscS and *Sc*Uba4/*Ct*Uba4 using L-cysteine as a sulfur source. The expected and determined masses of the samples are indicated.

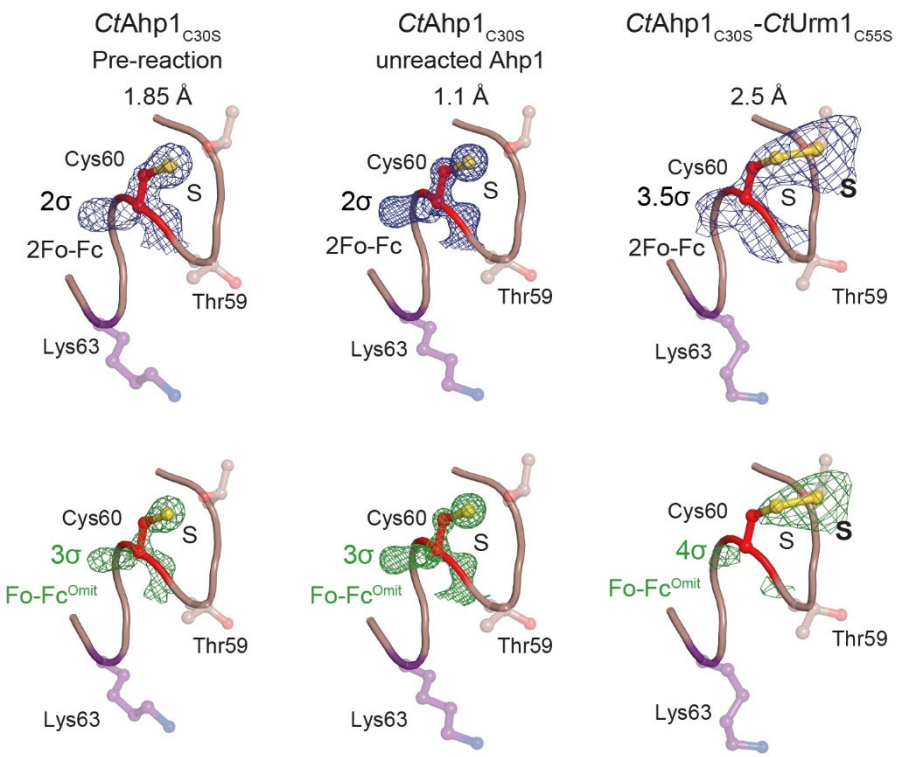
C. Radioisotope labelling. Production of ^{35}S-labelled Urm1 by desulfurase IscS and Uba4 using ^{35}S L-cysteine. Pyridoxal-5'-phosphate (PLP) and ATP were added as mentioned.

D. Radioisotope assay for ^{35}S-labelled Urm1. Analysis of conjugation and sulfur transfer from *Sc*^{35}S-Urm1 onto *Sc*Ahp1, *Sc*Ahp1$_{C31S}$, *Sc*Ahp1$_{C62S}$, *Sc*Ahp1$_{C31S\ C62S}$ and *Sc*Ahp1$_{C31S\ /\ KtoR}$ in presence of TBH. The samples were separated by non-reducing SDS-PAGE and visualized by Coomassie staining. The dried gels were exposed overnight to storage phosphor screens.

E. Radioisotope assay for ^{35}S-labelled Urm1. Analysis of conjugation and sulfur transfer from *Sc*^{35}S-Urm1 onto *Sc*Ahp1, *Sc*Ahp1$_{C31S,}$ *Sc*Ahp1$_{C62S,}$ *Sc*Ahp1$_{C31S\ C62S}$ and *Sc*Ahp1$_{C31S\text{-}KtoR}$ in absence of TBH. The samples were separated by non-reducing SDS-PAGE and visualized by Coomassie staining. Subsequently, the dried gels were exposed overnight to storage phosphor screens.

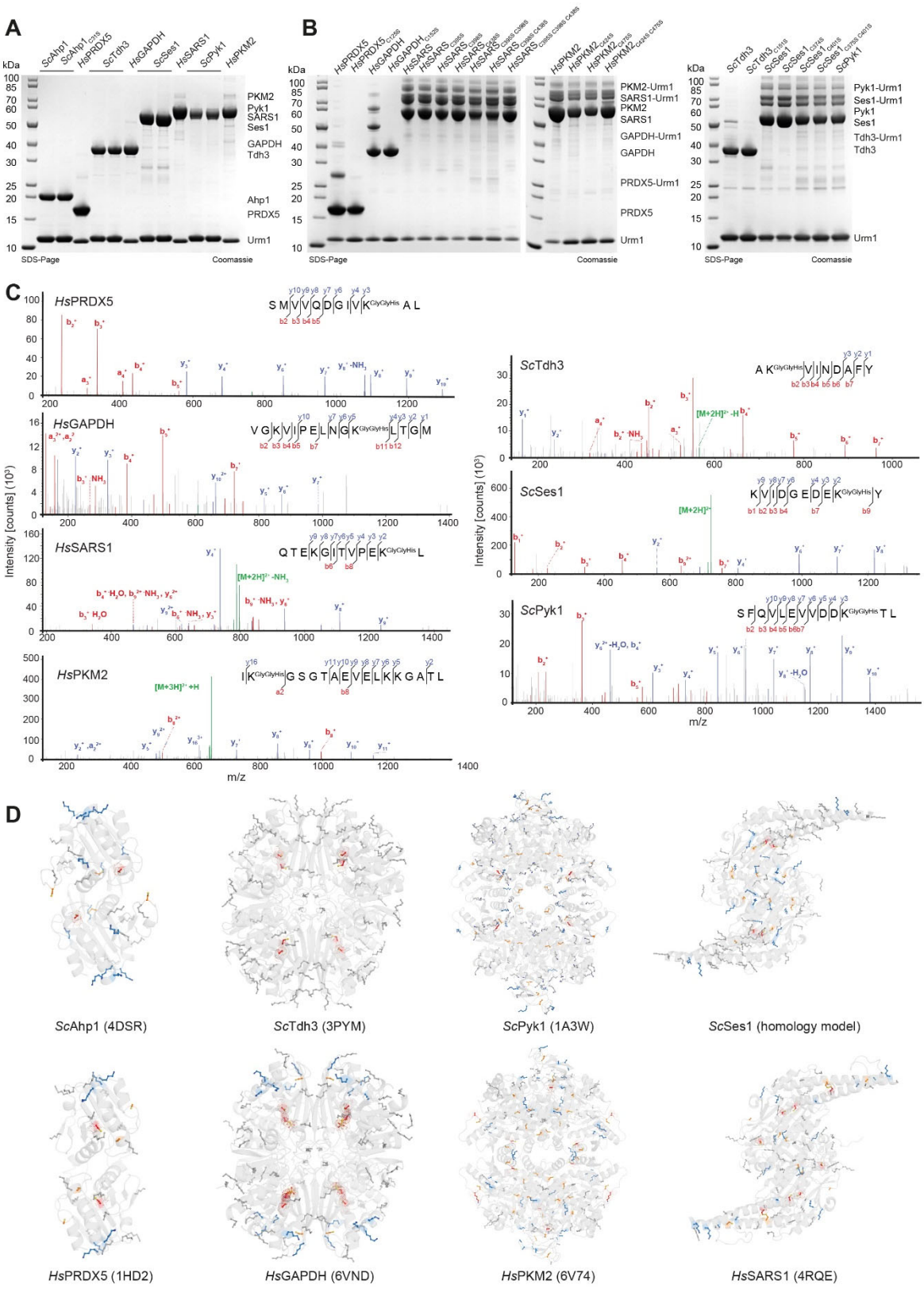
F. Radioisotope assay for ^{35}S-labelled Urm1. Analysis of conjugation and sulfur transfer from *Ct*^{35}S-Urm1$_{C55S}$ onto *Ct*Ahp1, *Ct*Ahp1$_{C30S,}$ *Ct*Ahp1$_{C60S,}$ *Ct*Ahp1$_{C30S\ C60S}$ and *Ct*Ahp1$_{C30S\text{-}KtoR}$ in presence of TBH. The samples were separated by non- reducing SDS-PAGE and visualized by Coomassie staining and then the dried gels were exposed overnight to storage phosphor screens.

G. Radioisotope assay for ^{35}S-labelled Urm1. Analysis of conjugation and sulfur transfer from *Ct*^{35}S-Urm1$_{C55S}$ onto *Ct*Ahp1, *Ct*Ahp1$_{C30S,}$ *Ct*Ahp1$_{C60S,}$ *Ct*Ahp1$_{C30S\ C60S}$ and *Ct*Ahp1$_{C30S\text{-}KtoR}$ in absence of TBH. The samples were separated by non- reducing SDS-PAGE and visualized by Coomassie staining. The dried gels were exposed overnight to storage phosphor screens.



Appendix Figure S5: Crystallographic analyses of Cys60 in different structures

Structural close-up of the peroxidatic cysteine (Cys60) and Lys63 (violet) in *Ct*Ahp1$_{C30S}$ pre-conjugation (left), unreacted Ahp1 from the conjugation reaction (middle) and the *Ct*Ahp1$_{C30S}$-*Ct*Urm1$_{C55S}$ complex (right). Refined 2Fo-Fc density maps (top, blue) and the Fo-Fc omit refinement maps after removal of the active site cysteine (bottom, green) are shown at the indicated σ-levels.



Appendix Figure S6: Specificity of Urm1 conjugation in various *in vivo* targets

A. Analysis of conjugate formation by carboxylated *Sc*Urm1 or *Hs*Urm1 on various urmylation targets (*Sc*Ahp1, *Sc*Ahp1$_{C31S}$, *Hs*PRDX5, *Sc*Tdh3, *Hs*GAPDH, *Sc*Ses1, *Hs*SARS1, *Sc*Pyk1, *Hs*PKM2) in the presence of diamide.

B. Analysis of conjugate formation by thiocarboxylated *Sc*Urm1 or *Hs*Urm1 on various urmylation targets (*Hs*PRDX5, *Sc*Tdh3, *Hs*GAPDH, *Sc*Ses1, *Hs*SARS1, *Sc*Pyk1, *Hs*PKM2) and cysteine mutants thereof in the presence of diamide.

C. MS/MS spectrum of the *Sc*Urm1 or *Hs*Urm1 conjugated peptide K-ε-GGH on *Hs*PRDX5, *Sc*Tdh3, *Hs*GAPDH, *Sc*Ses1, *Hs*SARS1, *Sc*Pyk1, *Hs*PKM2. A representative annotated fragmentation spectrum is shown with the b- and y-ions marked in red and blue, respectively. The peptide sequence is shown at the top with the collision-induced fragmentation pattern. The Urm1 conjugation site was identified by detection of the HGG motif generated by chymotrypsin digestion. m/z: mass to charge ratio. The urmylation was identified as HGG tag on lysines, while persulfidation was searched as a mass shift: +32 Da (persulfidation) or +89 Da (persulfidation + carbamidomethylation) on cysteines.

D. Comparison of the crystal structures of Urm1 targets. *Sc*Ahp1 reduced (PDB ID: 4DSR) alongside *Hs*PRDX5 (PDB ID: 1HD2). *Sc*Tdh3 (PDB ID: 3PYM) alongside *Hs*GAPDH (PDB ID: 6VND). *Sc*Pyk1 (PDB ID: 1A3W) alongside *Hs*PKM2 (PDB ID: 6V74). *Sc*Ses1 (homology model built using 4RQE) alongside *Hs*SARS1 (PDB ID: 4RQE). Cartoon representation of crystal structures. The lysines are shown as grey color sticks and the Urm1 conjugated lysines are highlighted by blue color sticks. The cysteines are shown as orange color sticks and the persulfidated cysteines are highlighted as red color sticks.

Reagents and Tools

Appendix Table S1 Plasmid Constructs

Organism	Protein	Uniprot ID	Source
Homo sapiens	URM1	Q9BTM9	Genscript
Homo sapiens	PRDX5	P30044	Genscript
Homo sapiens	GAPDH	P04406	Genscript
Homo sapiens	SARS1	P49591	Genscript
Homo sapiens	PKM2	P14618	Genscript
Chaetomium thermophilum	Uba4	G0SC54	Pabis et al. (2020)
Chaetomium thermophilum	Urm1	G0SE11	Pabis et al. (2020)
Chaetomium thermophilum	Ahp1	G0S1P8	Genscript
Chaetomium thermophilum	Ahp1-KtoR	G0S1P8	Genscript
Saccharomyces cerevisiae	Urm1	P40554	Termathe and Leidel (2018)
Saccharomyces cerevisiae	Uba4	P38820	Termathe and Leidel (2018)
Saccharomyces cerevisiae	Ahp1	P38013	Brachmann et al. (2020)
Saccharomyces cerevisiae	Ahp1-KtoR	P38013	Genscript
Saccharomyces cerevisiae	Ubiquitin	P0CH09	Genscript
Saccharomyces cerevisiae	Pyk1	P00549	Genscript
Saccharomyces cerevisiae	Ses1	P07284	Genscript
Saccharomyces cerevisiae	Tdh3	P00359	Genscript
Saccharomyces cerevisiae	Smt3 (SUMO)	Q12306	Thermofisher
Escherichia coli	IscS	P0A6B7	Genscript
Aequorea victoria	GFP		Pédelacq et al. (2006)

The above-mentioned proteins of *Homo sapiens, Chaetomium thermophilum, and Saccharomyces cerevisiae* were codon-optimized for expression in *E. coli*, obtained from Genscript and cloned into a series of modified pET-24d (+) vectors (Novagen/Merck) with various tags using the NcoI and KpnI/ XhoI restriction sites (https://www.embl.de/pepcore/pepcore_services/strains_vectors/vectors/bacterial_expression_vectors/popup_bacterial_expression_vectors/). In case of *Sc*Ahp1, the gene from yeast was cloned into pETM-30 vector with no codon-optimization for expression in *E. coli*. We used pETM30 (N-terminal His_6 and GST tag) and pETM11 (N-terminal His_6 tag) vectors (Novagen).

The tags are cleavable with tobacco etch virus protease (TEV). To produce thiocarboxylated proteins we used pTYB1 (IMPACT™ system, NEB) using NdeI and SapI restriction sites, and subsequently subcloned using NdeI and KpnI/ XhoI in pETM30 (N-terminal His_6 and GST tag) and pETM11 (N-terminal His_6 tag) vectors (Novagen). Cloning was conducted by site-specific restriction and/or restriction-free cloning method (Van Den Ent & Löwe, 2006) and mutations in all the genes were introduced using site-directed mutagenesis. All mutations were verified by Sanger-based DNA sequencing at Eurofins.

Appendix Table S2 Constructs of variants

Organism	Protein	Truncations/ Mutants	Vector
Homo sapiens	URM1	wt	pETM-30
Homo sapiens	URM1	wt	pTYB1
Homo sapiens	PRDX5	wt	pETM-30
Homo sapiens	PRDX5	(Δ1-52)	pETM-30
Homo sapiens	PRDX5	(Δ1-52)-C125S	pETM-30
Homo sapiens	GAPDH	wt	pETM-30
Homo sapiens	GAPDH	C152S	pETM-30
Homo sapiens	SARS1	C395S	pETM-11
Homo sapiens	SARS1	C395S_C398S	pETM-11
Homo sapiens	SARS1	C438S	pETM-11
Homo sapiens	SARS1	C398S_C438S	pETM-11
Homo sapiens	SARS1	C395S_C398S_C438S	pETM-11
Homo sapiens	SARS1	C395S_C398S_C438S	pETM-11
Homo sapiens	PKM2	wt	pETM-11
Homo sapiens	PKM2	C424S	pETM-11
Homo sapiens	PKM2	C475S	pETM-11
Homo sapiens	PKM2	C424S_C475S	pETM-11
Chaetomium thermophilum	Uba4	wt	pETM-30
Chaetomium thermophilum	Uba4	wt	pETM-11
Chaetomium thermophilum	Urm1	wt	pTYB1
Chaetomium thermophilum	Urm1	C55S	pTYB1
Chaetomium thermophilum	Urm1	C55S L108A	pTYB1
Chaetomium thermophilum	Urm1	C55S H109A	pTYB1

Chaetomium thermophilum	Urm1	C55S L108A H109A	pTYB1
Chaetomium thermophilum	Urm1	wt	pETM-30
Chaetomium thermophilum	Urm1	C55S	pETM-30
Chaetomium thermophilum	Urm1	C55S G111C	pETM-30
Chaetomium thermophilum	Ahp1	wt	pETM-30
Chaetomium thermophilum	Ahp1	C30S	pETM-30
Chaetomium thermophilum	Ahp1	C60S	pETM-30
Chaetomium thermophilum	Ahp1	C30S C60S	pETM-30
Chaetomium thermophilum	Ahp1	C30S K44R	pETM-30
Chaetomium thermophilum	Ahp1	C30S K63R	pETM-30
Chaetomium thermophilum	Ahp1	C30S K99R	pETM-30
Chaetomium thermophilum	Ahp1	C30S K44/ 63R	pETM-30
Chaetomium thermophilum	Ahp1	C30S K44/ 99R	pETM-30
Chaetomium thermophilum	Ahp1	C30S K63/ 99R	pETM-30
Chaetomium thermophilum	Ahp1	C30S K44/ 63/ 99R	pETM-30
Chaetomium thermophilum	Ahp1	C30S T57A	pETM-30
Chaetomium thermophilum	Ahp1	C30S T59A	pETM-30
Chaetomium thermophilum	Ahp1	C30S T57/ 59A	pETM-30
Chaetomium thermophilum	Ahp1	C30S R151A	pETM-30
Chaetomium thermophilum	Ahp1-KtoR	C30S T57A	pETM-30
Chaetomium thermophilum	Ahp1-KtoR	C30S T59A	pETM-30
Chaetomium thermophilum	Ahp1-KtoR	C30S T57/ 59A	pETM-30
Saccharomyces cerevisiae	Uba4	wt	pETM-30
Saccharomyces cerevisiae	Uba4	wt	pETM-11
Saccharomyces cerevisiae	Uba4	Uba4 (Δ1-38)	pETM-30
Saccharomyces cerevisiae	Uba4	Uba4 (Δ1-38)	pETM-11
Saccharomyces cerevisiae	Ahp1	wt	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S	pETM-30
Saccharomyces cerevisiae	Ahp1	C31R	pETM-30
Saccharomyces cerevisiae	Ahp1	C62S	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S C62S	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K32R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K47/ 48R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K124R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K156R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K124/ 156R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K32/ 124/ 156R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K47/ 48/ 124R	pETM-30

Saccharomyces cerevisiae	Ahp1	C31S K47/ 48/ 156R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K32/ 47/ 48/ 124R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S K32/ 47/ 48/ 124/ 156R	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S S59A	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S T61A	pETM-30
Saccharomyces cerevisiae	Ahp1	C31S S59A T61A	pETM-30
Saccharomyces cerevisiae	Ahp1-KtoR	C31S	pETM-11
Saccharomyces cerevisiae	Ahp1-KtoR	C31S S59A	pETM-11
Saccharomyces cerevisiae	Ahp1-KtoR	C31S T61A	pETM-11
Saccharomyces cerevisiae	Ahp1-KtoR	C31S S59A T61A	pETM-11
Saccharomyces cerevisiae	Urm1	wt	pET30a
Saccharomyces cerevisiae	Urm1	Δ99G	pET30a
Saccharomyces cerevisiae	Urm1	Δ98/ 99G	pET30a
Saccharomyces cerevisiae	Urm1	wt	pTYB1
Saccharomyces cerevisiae	Urm1	Δ98/ 99G	pTYB1
Saccharomyces cerevisiae	Urm1	Δ99G	pTYB1
Saccharomyces cerevisiae	Urm1	G99C	pTYB1
Saccharomyces cerevisiae	Urm1	G99A	pTYB1
Saccharomyces cerevisiae	Urm1	G99V	pTYB1
Saccharomyces cerevisiae	Urm1	G99L	pTYB1
Saccharomyces cerevisiae	Urm1	G99I	pTYB1
Saccharomyces cerevisiae	Urm1	G99M	pTYB1
Saccharomyces cerevisiae	Urm1	G99F	pTYB1
Saccharomyces cerevisiae	Urm1	G99W	pTYB1
Saccharomyces cerevisiae	Urm1	G99P	pTYB1
Saccharomyces cerevisiae	Urm1	G99S	pTYB1
Saccharomyces cerevisiae	Urm1	G99T	pTYB1
Saccharomyces cerevisiae	Urm1	G99Y	pTYB1
Saccharomyces cerevisiae	Urm1	G99N	pTYB1
Saccharomyces cerevisiae	Urm1	G99Q	pTYB1
Saccharomyces cerevisiae	Urm1	G99D	pTYB1
Saccharomyces cerevisiae	Urm1	G99E	pTYB1
Saccharomyces cerevisiae	Urm1	G99K	pTYB1
Saccharomyces cerevisiae	Urm1	G99R	pTYB1
Saccharomyces cerevisiae	Urm1	G99H	pTYB1
Saccharomyces cerevisiae	Urm1	G98/ 99A	pTYB1
Saccharomyces cerevisiae	Urm1	G98/ 99S	pTYB1
Saccharomyces cerevisiae	Urm1	H97A ΔGG	pTYB1

Saccharomyces cerevisiae	Urm1	H97A	pTYB1
Saccharomyces cerevisiae	Urm1	H97A	pTYB1
Saccharomyces cerevisiae	Tdh3	wt	pETM-30
Saccharomyces cerevisiae	Tdh3	C151S	pETM-30
Saccharomyces cerevisiae	Pyk1	wt	pETM-30
Saccharomyces cerevisiae	Pyk1	C419S	pETM-30
Saccharomyces cerevisiae	Ses1	wt	pETM-11
Saccharomyces cerevisiae	Ses1	C374S	pETM-11
Saccharomyces cerevisiae	Ses1	C401S	pETM-11
Saccharomyces cerevisiae	Ses1	C374S C401S	pETM-11
Saccharomyces cerevisiae	Ubiquitin	wt	pETM-30
Saccharomyces cerevisiae	Ubiquitin	wt	pTYB1
Saccharomyces cerevisiae	Smt3 (SUMO)	wt	pETM-30
Saccharomyces cerevisiae	Smt3 (SUMO)	wt	pTYB1
Escherichia coli	IscS	wt	pETM-30
Aequorea victoria	GFP	wt	pTYB1
Aequorea victoria	GFP	wt	pETM-30
GFP fused with *Sc*Urm1	GFP-U6	GFP-Urm1 (94-99)	pTYB1
GFP fused with *Sc*Urm1	GFP-U6	GFP-Urm1 (94-99)	pETM-30
GFP fused with *Sc*Urm1	GFP	GFP-Urm1	pTYB1
GFP fused with *Sc*Urm1	GFP	GFP-Urm1	pETM-30

Appendix Table S3 Chemicals

Chemicals	**Source**	**Identifier**
ATP	Jena Bioscience	NU-1010
Sodium thiosulfate	Sigma-Aldrich	1615107
L-Cysteine	Sigma-Aldrich	168149
tert-Butyl hydroperoxide (TBH)	Sigma-Aldrich	458139
Hydrogen peroxide solution (HP)	Sigma-Aldrich	**H1009**
Diamide	Sigma-Aldrich	**D3648**
Peroxynitrite	Sigma-Aldrich	20-107
Methyl glyoxal	Sigma-Aldrich	M0252
Di-tert-butyl disulfide (DTB-disulfide)	Sigma-Aldrich	247545
Hydroxylamine solution 50 wt. % in H_2O	Sigma-Aldrich	**467804**
N-Ethylmaleimide	Sigma-Aldrich	E3876
Isopropyl β-D-thiogalactoside/Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	I6758
Ni-NTA Resin	Sigma-Aldrich	70666-5

BiGGY Agar	Sigma-Aldrich	73608
Tris (2-Carboxyethyl) phosphine Hydrochloride (TCEP)	GoldBio	51805-45-9
1,4-Dithiothreitol (DTT)	Lab Empire	**DTT001**
Iodoacetamide	Lab Empire	IOD500
^{35}S L-cysteine	Perkin Elmer	NEG022T001MC
Imidazole	Carl Roth Gmbh	3899.4
L-Cysteine hydrochloride	Carl Roth Gmbh	52-89-1
NucleoZOL	Macherey-Nagel	108-95-2
Chitin Resin	New England Biolabs	S6651L
Chymotrypsin	Promega	V1061
Trypsin	Promega	VA9000
([*N*-acryloylamino]phenyl)mercuric chloride (APM)	Synthesized as per literature	Igloi (1988)
PD SpinTrap G-25	Cytiva	28918004

Appendix Table S4 Software and Algorithms

Software	**Source**	**Identifier**
Pymol	Pymol	https://pymol.org/2/
Phenix	Phenix	https://phenix-online.org/download
CCP4	CCP4	https://www.ccp4.ac.uk/
Molprobity	Duke Biochemistry	http://molprobity.biochem.duke.edu/
GraphPad Prism 8.0	GraphPad	https://www.graphpad.com/
Image J	NIH	https://imagej.nih.gov/ij/download.html
ImageStudio	LI-COR	https://www.licor.com/bio/image-studio-lite/
Unicorn 7.0	Cytiva	https://www.cytivalifesciences.com/
Adobe Illustrator	Adobe	https://www.adobe.com/products/illustrator.html
Biorender	Biorender	https://app.biorender.com/
Data Analysis 4.1	Bruker Daltonics	https://www.bruker.com/en/products-and-solutions/mass-spectrometry/ms-software.html
Proteome Discoverer 1.4	Thermofisher	https://www.thermofisher.com/
MASCOT server 2.5.1	Matrix Science	https://www.matrixscience.com/

Appendix Table S5 Yeast strains used in this study

Strain	Genotype	Background	Source
Y10000	BY4742, *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	S288C	Euroscarf
LK36	BY4742, *ahp1Δ::KlLEU2*	S288C	This study
Y11400	BY4742, *urm1Δ::kanMX4*	S288C	Euroscarf
Y12507	BY4742, *tum1Δ::kanMX4*	S288C	Euroscarf
LK37	BY4742, *ncs6Δ::KlLEU2*	S288C	This study
Y16865	BY4742, *cys3Δ::kanMX4*	S288C	Euroscarf
LK29	BY4742, *cys4Δ::KlLEU2*	S288C	This study
LK38	BY4742, *urm1Δ::kanMX4 ahp1Δ::KlLEU2*	S288C	This study
LK30	BY4742, *tum1Δ::kanMX4 urm1Δ::KlLEU2*	S288C	This study
LK39	BY4742, *urm1Δ::kanMX4 ncs6Δ::KlLEU2*	S288C	This study
LK31	BY4742, *cys3Δ::kanMX4 urm1Δ::hphMX*	S288C	This study
LK32	BY4742, *urm1Δ::kanMX4 cys4Δ::KlLEU2*	S288C	This study
FEY16	BY4741, *ahp1Δ::kanMX4 urm1Δ::ScHIS3*	S288C	Jüdes et al. (2015)
FEY18	BY4741, *yap1Δ::kanMX4 ahp1Δ::SpHIS5*	S288C	Brachmann et al. (2020)

Appendix Table S6 Oligonucleotides used in this study for *in vivo* assays

Oligonucleotide	Sequence (5'-3')	Application
FC_Ahp1-YCplac111_Fw	GAAAGTGTCTTGGCTCATTTGTAGATGCTATGTAATAG ACAATAAAACCAT	*AHP1* FC
FC_Ahp1-YCplac111_Rv	ATGGTTTTATTGTCTATTACATAGCATCTACAAATGAG CCAAGACACTTTC	*AHP1* FC
AHP1_FW	CCTCTCCCTTGCCAATTGTG	Sequencing
KO_URM1_FW	CAATACTGATTTCTGATACTAAAACGAGATAGGTTAAT AGCAAAATCGGGCAGCTGAAGCTTCGTACGC	*URM1* ko
KO_URM1_RV	CTTTATATATATATATGTAGCTGCTTCTTAAAAATTATT TGCTGCTATTTGCATAGGCCACTAGTGGATCTG	*URM1* ko
PUrm1VerFw	GCATCGCATCGACCTAATGAAA	*URM1* ve
PUrm1VerRv	CCTTCGAGAAACTGGAATGGTA	*URM1* ve
KO_NCS6_FW	AAAATTTTGGCGATGAGACGATATGGTAAGAGTAAAG CAAAGGAACCGTCCAGCTGAAGCTTCGTACGC	*NCS6* ko

KO_NCS6_RV	TATATTATATTATGTTACGCTGCATTCTTCTACTGCGA GCTATATATATGGCATAGGCCACTAGTGGATCTG	*NCS6* ko
N_NCS6_FW	CGTCGATAACACTGGTAGG	*NCS6* ve
N_NCS6_RV	CAATATGCTTGGTTCGCTC	*NCS6* ve
AHP1KOF	ATTTCAACAAACCAGAACAACACAAGTACTACCAATA ACCACAACAAAACCAGCTGAAGCTTCGTACGC	*AHP1* ko
AHP1KOR	TTTTGAATTTTTTTTATATAAACATGGTTTTATTGTCTA TTACATAGCATGCATAGGCCACTAGTGGATCTG	*AHP1* ko
AHP1_FW_HindIII	GGGAAGCTTCCTTGGCCTCGATCTATTGC	*AHP1* ve
AHP1_RV_EcoRI	GGGGAATTCCTGCTCCAACTCACTCTGTC	*AHP1* ve
KO_CYS4_FW	TAGGCCACTTGCTCAAAGGACATCTAGATAAATACGA CGTAAGAATAAAACAGCTGAAGCTTCGTACGC	*CYS4* ko
KO_CYS4_RV	TGACGGATTTTGCTTCTATGTTTGCTTTTATTTGAAGCG TGGGTTCTTATGCATAGGCCACTAGTGGATCTG	*CYS4* ko
N_CYS4_FW	ATTGCCATCTTCCTCCACAG	*CYS4* ve
N_CYS4_RV	CTTCAGAGTCGGGTAAGAGC	*CYS4* ve
8xHis-3xHA-URM1_FW	GCAAAATCGGGATGAGATCTCATCATCACCATCACCA TCACCATATCTTTTACCCATACGATGTTC	*URM1* SDM
8xHis-3xHA-URM1_RV	GAACATCGTATGGGTAAAAGATATGGTGATGGTGATGGTGATGATG AGATCTCATCCCGATTTTGC	*URM1* SDM
SeqHisUrm1	TTACATTGTCGCCCACTTC	Sequencing

Abbreviations: Fast Cloning (FC), knock-out (ko), ko verification (ve), site-directed mutagenesis (SDM)

Appendix Table S7 Plasmids used in this study for *in vivo* assays

Plasmid	**Description**	**Source**
YCplac111	*ARS1-CEN4, ScLEU2*	Gietz and Akio 1988
pRS426	2µ ori, *ScURA3*	Christianson et al. (1992)
pHA-URM1	pRS426, 3XHA-*URM1*	Furukawa et al. (2000)
pLK20	pRS426, 8XHis-3XHA-*URM1* by SDM-PCR	This study

pAJ31	YCplac111, *ScAHP1*	Brachmann et al. (2020)
pAJ33	YCplac111, *ScAHP1-C31S* by SDM-PCR	Brachmann et al. (2020)
pAJ37	YCplac111, *ScAHP1-C31S/K32R* by SDM-PCR	Brachmann et al. (2020)
pAJ39	YCplac111, *ScAHP1-C62S* by SDM-PCR	Brachmann et al. (2020)
pAJ67	YCplac111, *ScAHP1-C31,62S* by SDM-PCR	Brachmann et al. (2020)
pLK124	YCplac111, *ScAHP1-C31S/K47,48R* by FC	This study
pLK121	YCplac111, *ScAHP1-C31S/K124R* by FC	This study
pLK122	YCplac111, *ScAHP1-C31S/K156R* by FC	This study
pLK123	YCplac111, *ScAHP1-C31S/K124,156R* by FC	This study
pLK129	YCplac111, *ScAHP1-C31S/K32,124,156R* by FC	This study
pLK125	YCplac111, *ScAHP1-C31S/K47,48,124R* by FC	This study
pLK126	YCplac111, *ScAHP1-C31S/K47,48,156R* by FC	This study
pLK127	YCplac111, *ScAHP1-C31S/K47,48,124,156R* by FC	This study
pLK133	YCplac111, *ScAHP1-C31S/K32,47,48,124,156R* by FC	This study

1 Abbreviations: Fast Cloning (FC), site-directed mutagenesis (SDM)