

Supplemental Figure 1

Reproducibility of biological replicates.

(A) Pairwise comparisons of three independent *cdc48-3*:WT analyses, demonstrating low coefficients of variation between biological replicates. (B) Same as (A), except two independent analyses for each of the *ubxΔ* mutants are plotted. In these replicates the SILAC labels were switched, hence the negative correlation.

Supplemental Figure 2

Curation of Ub conjugates and overlap with previous screens listed in SCUD.

(A) 2-D plot of protein ratios from the ^{His8}Ub tagged vs untagged control experiment (x-axis) and the WT ^{His8}Ub vs WT ^{His8}Ub control (y-axis). Proteins that were recovered in significantly greater amounts (p-value < 0.05) from the ^{His8}Ub expressing cells in the tagged vs untagged control experiment and whose tagged vs untagged ratios did not differ by significantly greater than 10% (p-value ≥ 0.01) were deemed candidate Ub conjugates. (B) Overlap of Ub conjugates in the SCUD database with those identified in this publication.

Supplemental Figure 3

Changes in the Ub proteome in *cdc48-3* mutants.

(A) Box and whisker plot of annotation terms demonstrating a significant enrichment in the Ub proteome for a majority of their identified members in the *cdc48-3* mutant relative to WT. Significance is defined as the probability that a random subset of *cdc48-3*:WT ratios would have a 25th percentile at least as high

as the 25th percentile of the ratios of proteins annotated with the term. Enriched GO Cellular Component (CC) and GO Biological Process (BP) terms are shown. **(B)** Fold changes in enzymes of the Ubiquitin-Proteasome System. Error bars indicate the standard error of the ratios and asterisks indicate significant deviation from 1:1 (p-value < 0.05 after correction by the Benjamini and Hochberg method). Colors indicate magnitude of change.

Supplemental Figure 4

Changes in the Ub proteome in *ubxΔ* mutants

(A-E) Box and whisker plots of annotation terms demonstrating a significant enrichment in the Ub proteome for a majority of their identified members in the corresponding *ubx* mutant. Significance is defined as the probability that a random subset of *ubxΔ*:WT protein ratios would have a 25th percentile at least as high as the 25th percentile of the ratios of proteins annotated with the term. Enriched GO Cellular Component (CC), GO Biological Process (BP), GO Molecular Function (MF), KEGG Pathway (KFFF), Uniprot Sequence feature (SF), Uniprot Keyword (KW), and Protein Superfamily (PIR) terms are shown.

Supplemental Figure 5

Orthogonal validation of Mga2 ubiquitin conjugate accumulation. WT, *ubx2Δ*, and *ubx4Δ* cells expressing ^{myc}Mga2 from a YEplac181 plasmid were grown in YPD at 30°C and native lysates were fractionated on a TUBE2 resin. The input extract and bound fractions were immunoblotted for the myc epitope, Ub, and Dpm1 (input loading control) as indicated.

Supplemental Table 1 (provided as Excel workbook)

List of all protein groups identified in this study with corresponding ratios in each in mutant.

Supplemental File 1

Protein groups file table legend.

Supplemental File 2

MS/MS spectra of proteins in screen identified by a single peptide sequence.

Supplemental Table 2 (provided as Excel workbook)

List of all di-Gly modified lysine sites identified in this study. Modified sites were compared to previous studies and listed if previously identified.

Supplemental File 3

di-Gly lysine sites table legend.

Supplemental File 4

MS/MS spectra of the 67 ubiquitinated peptides.

Supplemental Table 3

Strains used in this study.

Strain	Genotype	Source
RJD 4614	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, MATa</i>	Open Biosystems
RJD 6137	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0 ubx2::KANMX MATa</i>	Open Biosystems

RJD 6138	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS] MATa</i>	This study
RJD 6139	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx2::KANMX MATa</i>	This study
RJD 6140	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx1::KANMX MATa</i>	This study
RJD 6141	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx3::KANMX MATa</i>	This study
RJD 6142	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx4::KANMX MATa</i>	This study
RJD 6143	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx5::KANMX MATa</i>	This study
RJD 6144	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx6::KANMX MATa</i>	This study
RJD 6145	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx7::KANMX MATa</i>	This study
RJD 6146	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx2::KANMX, ubx4::NATMX, MATa</i>	This study
RJD 6147	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx2::KANMX, ubx4::KANMX MATa</i>	This study
RJD 4781	<i>CAN1, lys2::HISMX, arg4::KANMX, leu2-3,-112 his3-11,-15, trp1-1, ura3-1, ade2-1, [pRS316-His8-Ubiquitin], MATa</i>	D. Chan
RJD 4977	<i>cdc48-3, CAN1, lys2::HISMX, arg4::KANMX, leu2-3,-112 his3-11,-15, trp1-1, ura3-1, ade2-1, [pRS316-His8-Ubiquitin], MATa</i>	This study
RJD 6148	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, arg4::HYG, [pRS316-His8-Ubiquitin], MATa</i>	This study
RJD 6149	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, arg4::HYG, ubx2::KANMX, [pRS316-His8-Ubiquitin], MATa</i>	This study
RJD	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0,</i>	This study

6150	<i>arg4::HYG, ubx3::KANMX, [pRS316-His8-Ubiquitin], MATa</i>	
RJD 6151	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, arg4::HYG, ubx4::KANMX, [pRS316-His8-Ubiquitin], MATa</i>	This study
RJD 6152	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, arg4::HYG, ubx5::KANMX, [pRS316-His8-Ubiquitin], MATa</i>	This study
RJD 6153	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, arg4::HYG, ubx6::KANMX, [pRS316-His8-Ubiquitin], MATa</i>	This study
RJD 6154	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, arg4::HYG, ubx7::KANMX, [pRS316-His8-Ubiquitin], MATa</i>	This study
RJD 6155	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, [pMGA2-MYC-Mga2-LEU], MATa</i>	This study
RJD 6156	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, [pMGA2-MYC-Mga2-LEU], ubx2::KANMX, MATa</i>	This study
RJD 6157	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, [pMGA2-MYC-Mga2-LEU], ubx4::KANMX, MATa</i>	This study
RJD 6158	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx2::KANMX, rsp5-1, MATa</i>	This study
RJD 6159	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx4::KANMX, rsp5-1, MATa</i>	This study
RJD 4472	<i>rsp5-1, his4-914ΔR5, lys2-128Δ, ura3-52, GAL2+, MATalpha</i>	J. Huibregtse
RJD 6160	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, rsp5::KANMX, MATa</i>	N. Shcherbik
RJD 6161	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, [pGAL-FLAG-SPT23-HA-URA3], MATa</i>	This Study
RJD 6162	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, [pGAL-FLAG-SPT23-HA-URA3], ubx2::KANMX, MATa</i>	This Study
RJD 6163	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, [pGAL-FLAG-SPT23-HA-URA3], ubx4::KAN, MATa</i>	This Study
RJD	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0,</i>	This Study

6164	<i>[pGAL-FLAG-MGA2-HA-URA3], MATa</i>	
RJD 6165	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, [pGAL-FLAG-MGA2-HA-URA3], ubx2::KAN, MATa</i>	This study
RJD 6166	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, [pGAL-FLAG-MGA2-HA-URA3], ubx4::KAN, MATa</i>	This study
RJD 6167	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], cdc48-3, MATa</i>	This study
RJD 6168	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], npl4-1, MATa</i>	This study
RJD 6169	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ufd1-2, MATa</i>	This study
RJD 6170	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], cim3-1, MATa</i>	This study
RJD 6171	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], rsp5-1, MATa</i>	This study
RJD 3166	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, sel1::SEL1-TAP-HIS3MX, MATa</i>	Open Biosystems
RJD 6172	<i>his3Δ1, leu2Δ0, met15Δ0, ura3-1, lys2Δ0, ura3::pGAL-MYC-SPT23-HA, MATa</i>	This study
RJD 6173	<i>his3Δ1, leu2Δ0, met15Δ0, ura3-1, lys2Δ0, ura3::pGAL-MYC-SPT23-HA, sel1::SEL1-TAP-HIS3MX, MATa</i>	This study
RJD 6174	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx2::KANMX, leu::pUBX2-UBX2(FL), MATa</i>	C. Wang
RJD 6175	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, SPT23::MYC-SPT23-V5[HIS], ubx2::KANMX, leu::pUBX2-UBX2(FL), MATa</i>	This Study
RJD 6176	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx2::KANMX, leu::pUBX2-UBX2(ΔUBA), MATa</i>	C. Wang
RJD 6177	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx2::KANMX, SPT23::MYC-SPT23-V5[HIS], leu::pUBX2-UBX2(ΔUBA), MATa</i>	This Study
RJD	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0,</i>	C. Wang

6178	<i>ubx2::KANMX, leu::pUBX2-UBX2(ΔUBX), MATa</i>	
RJD 6179	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx2::KANMX, SPT23:MYC-SPT23-V5[HIS], leu::pUBX2-UBX2(ΔUBX), MATa</i>	This Study
RJD 6180	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx2::KANMX, leu::pUBX2-UBX2(ΔUBAΔUBX), MATa</i>	C. Wang
RJD 6181	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx2::KANMX, SPT23:MYC-SPT23-V5[HIS], leu::pUBX2-UBX2(ΔUBAΔUBX), MATa</i>	This Study
RJD 6182	<i>his3Δ1, leu2Δ0, met15Δ0, ura3-1, lys2Δ0, pGAL-MYC-SPT23-HA::URA, ubx2::KANMX, MATa</i>	This Study
RJD 6183	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, arg4::HYG, MATa</i>	This Study
RJD 5246	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx4::KANMX, MATa</i>	Open Biosystems
RJD 5772	<i>his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ubx2::KANMX, ubx4::KANMX, MATa</i>	This Study

Supplemental Table 4

Plasmids used in this study.

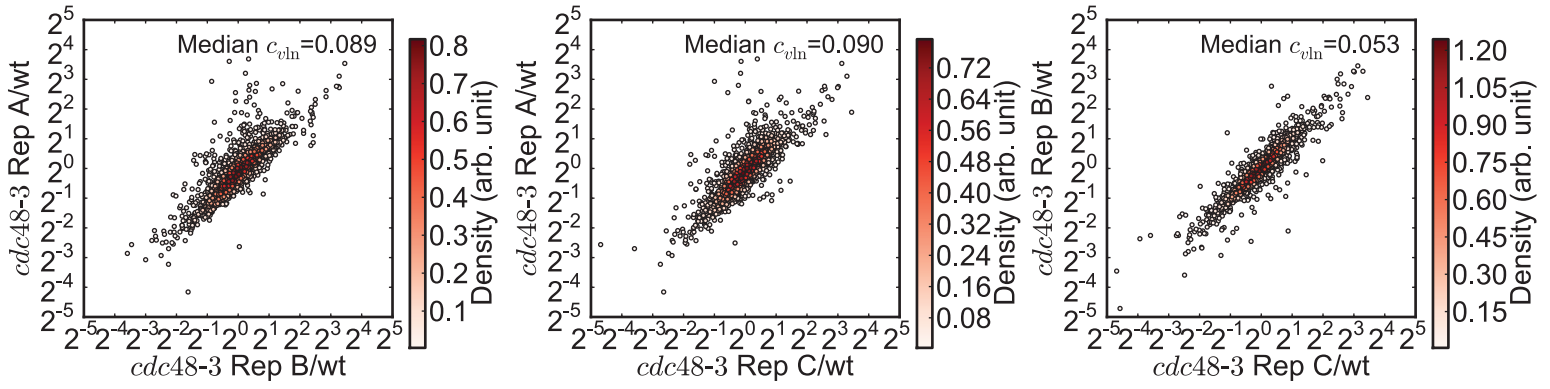
Plasmid	Description	Source
RDB 2818	<i>MYC-MGA2 in YEPlac181</i>	S. Jentsch
RDB 2819	<i>GAL-MYC-SPT23-HA in YIPlac211</i>	S. Jentsch
RDB 1937	<i>GAL-FLAG-SPT23-HA in YEPlac181</i>	D. Haines
RDB 1938	<i>GAL-FLAG-MGA2-HA in YEPlac181</i>	D. Haines
RDB 1851	<i>HIS8-Ubiquitin in pRS316</i>	Our Laboratory

Supplemental Table 5

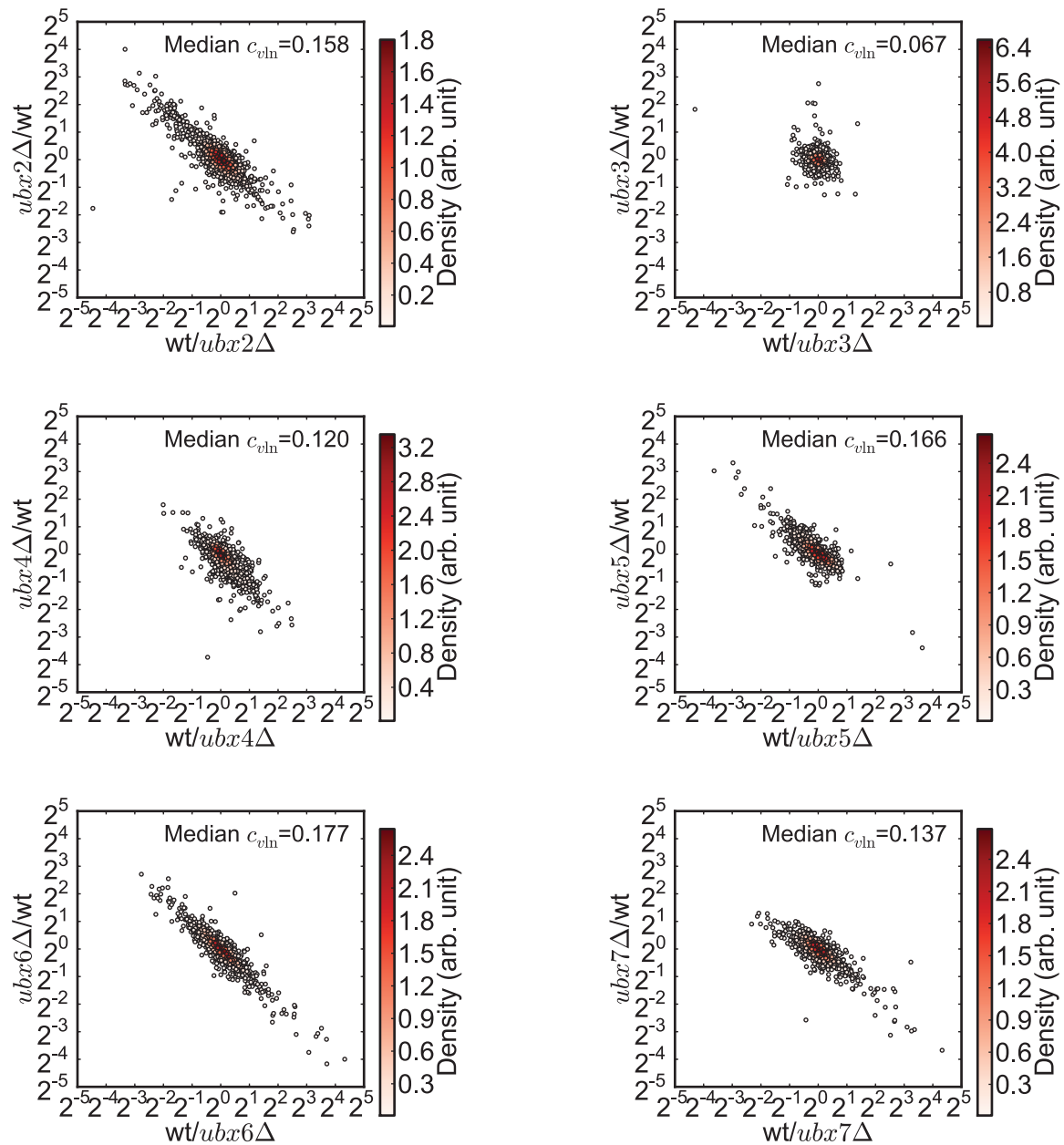
Primers used for quantitative PCR in this study.

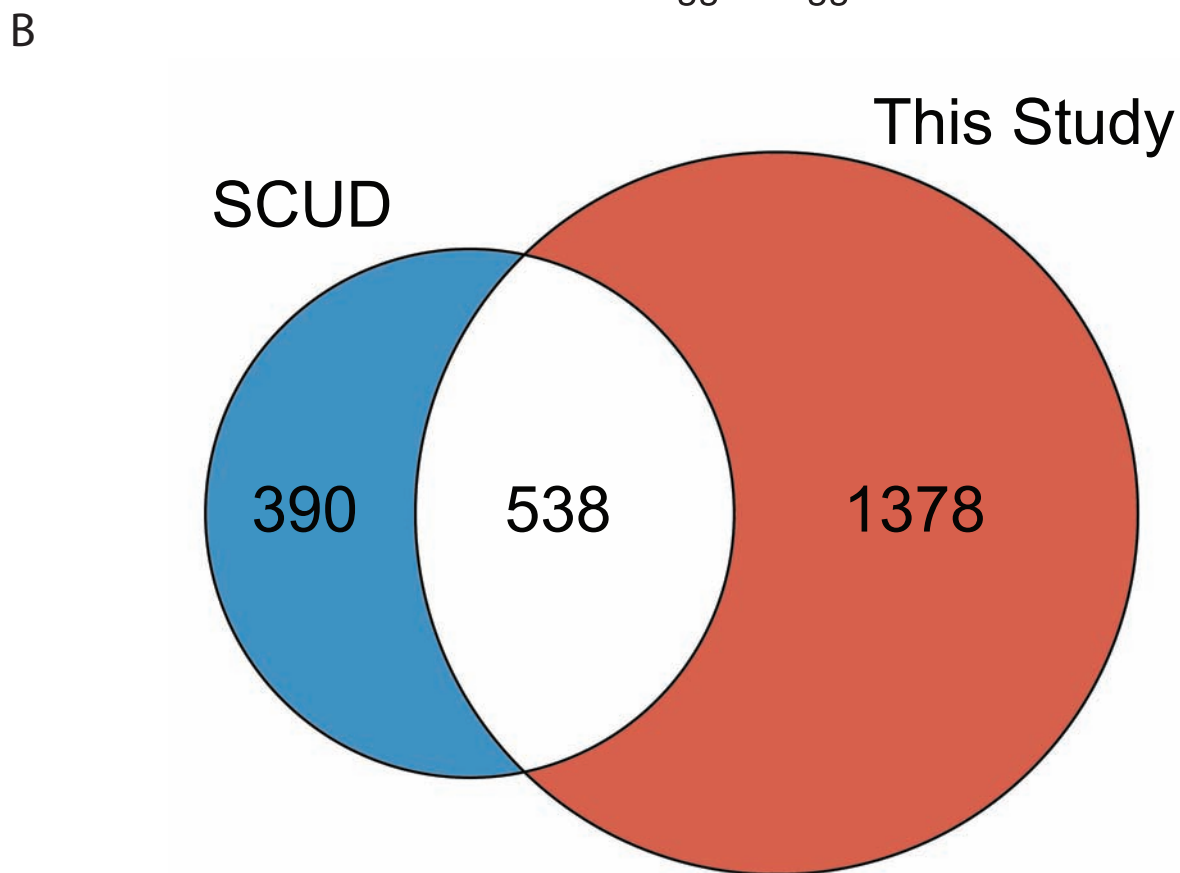
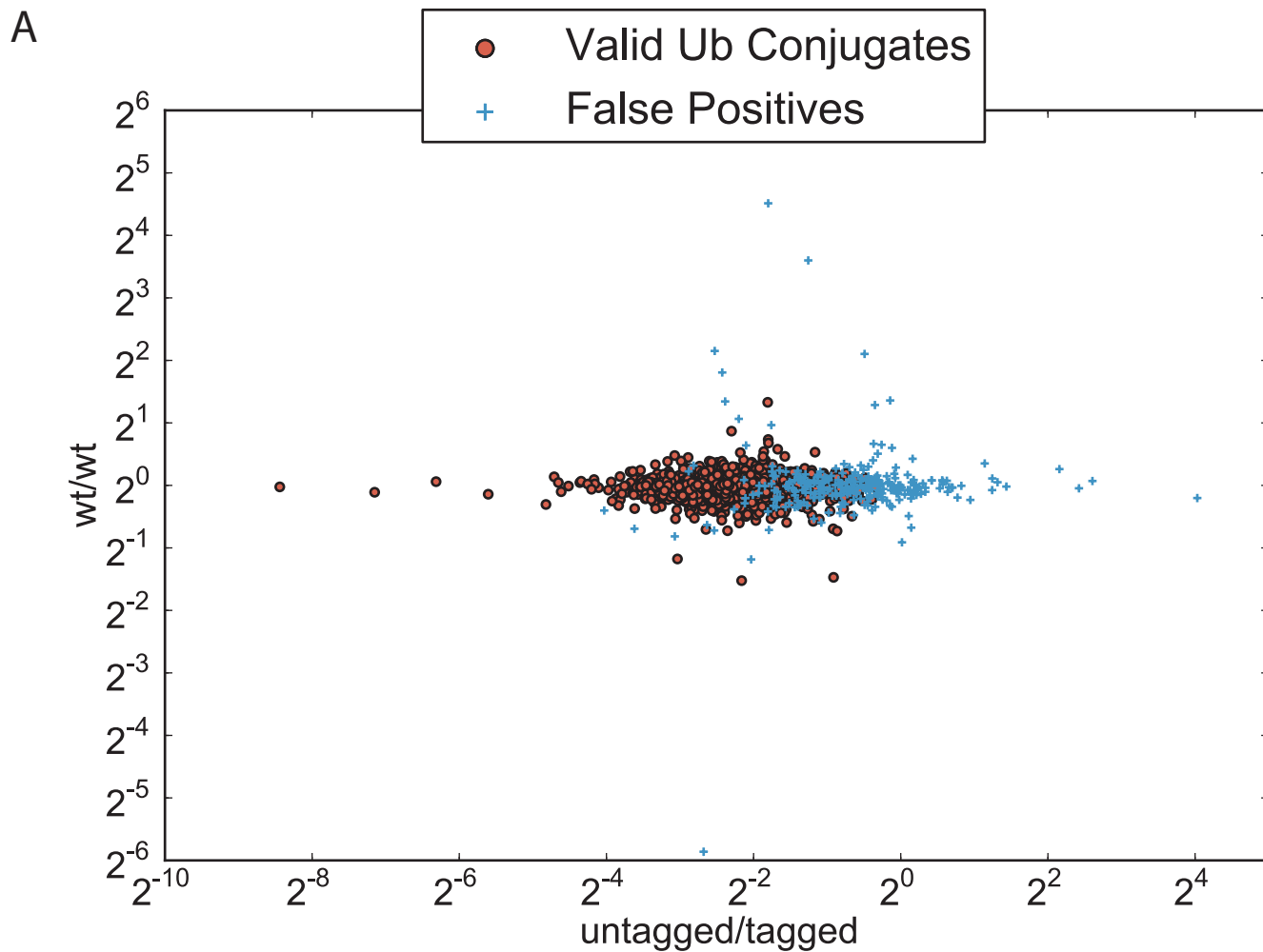
Gene	Forward Primer	Reverse Primer
ACT1	5' -CTGCCGGTATTGACCAAAC- 3'	5'-CGGTGATTCCTTTTGCATT- 3'
OLE1	5'-TAATGGGCTCCAAGGAAATG- 3'	5'- CATGGTTGTTCCGGAGATGTG- 3'

A

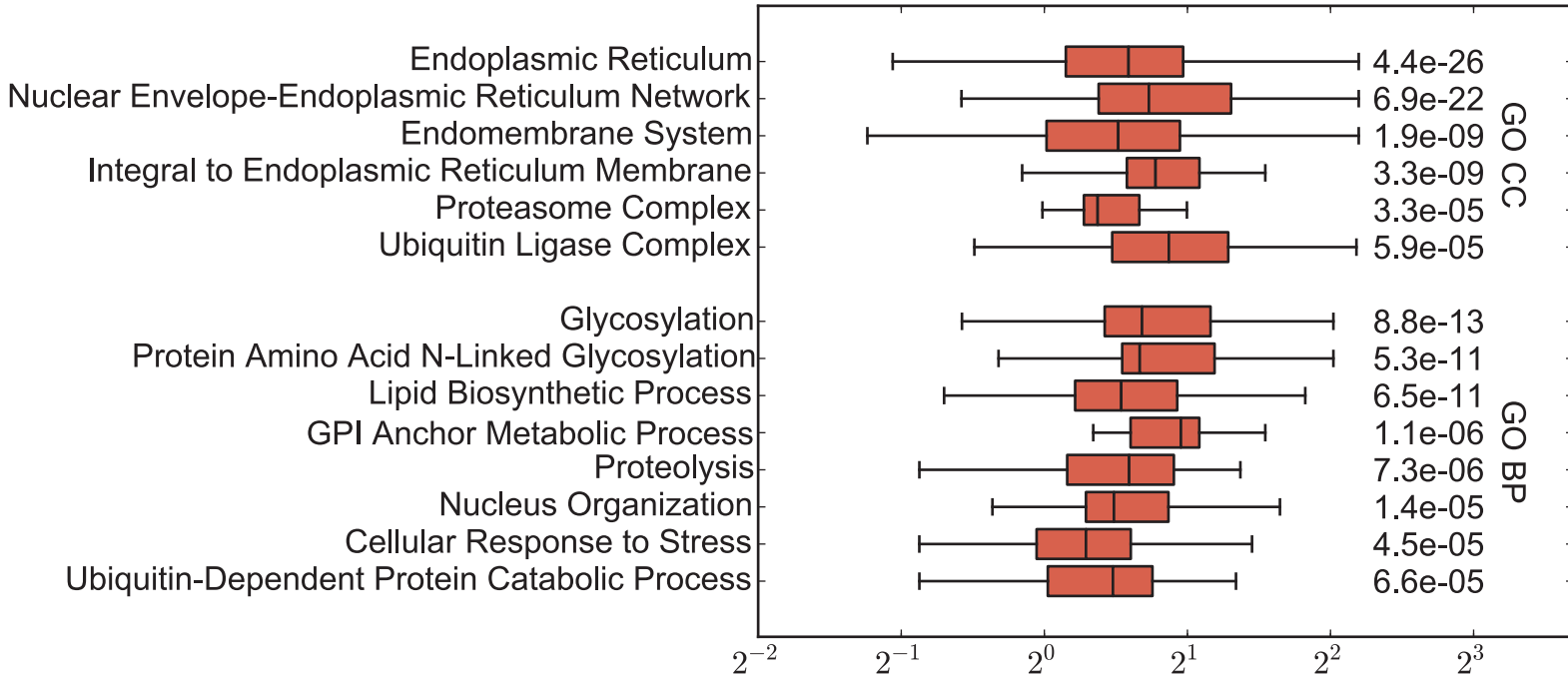


B

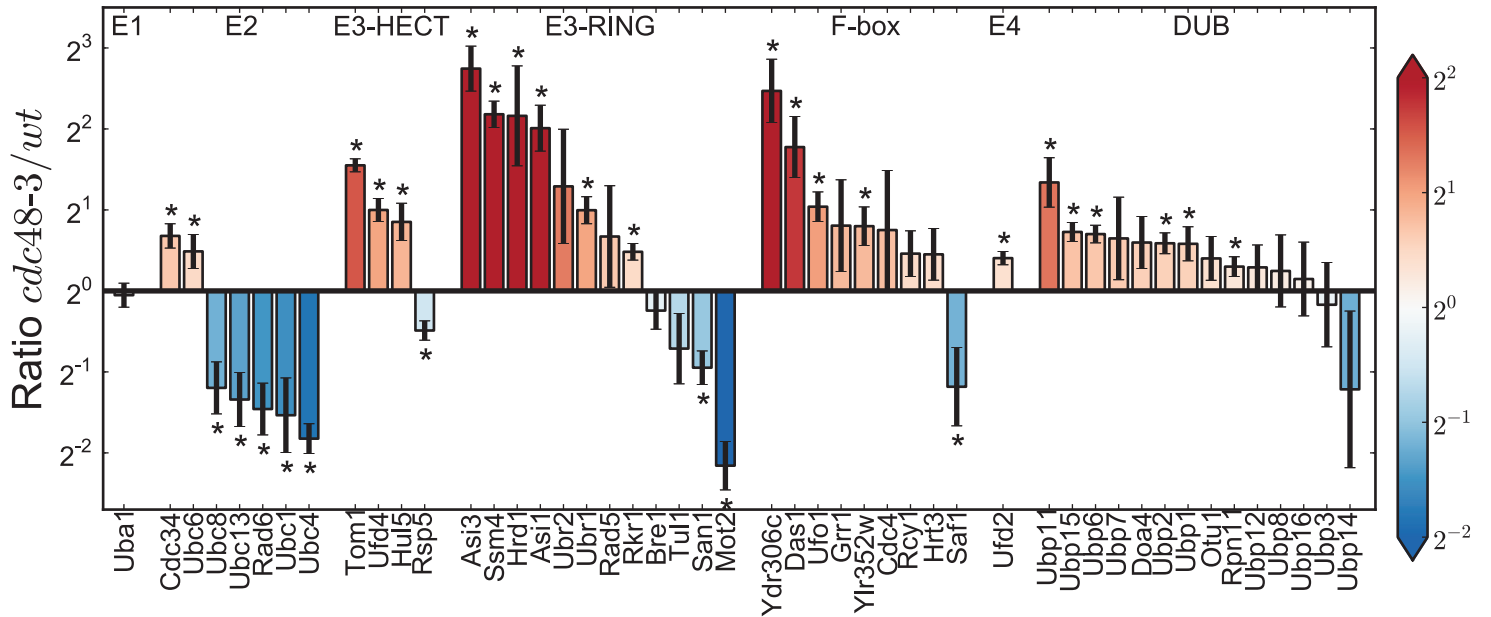




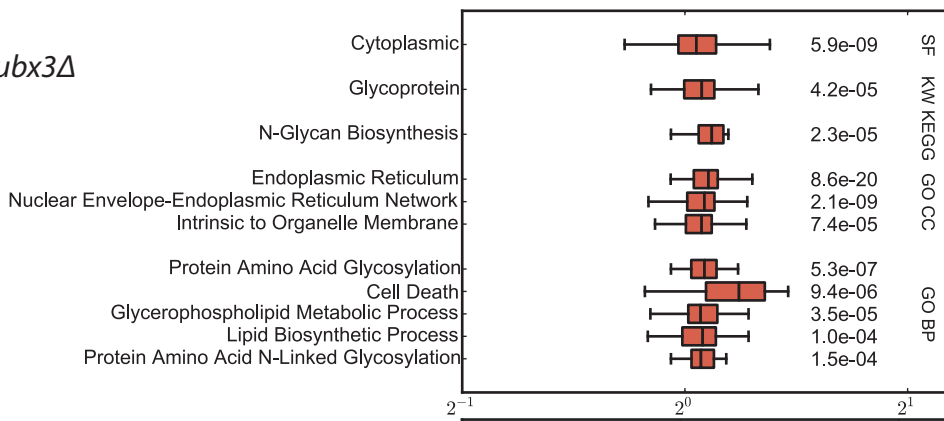
A



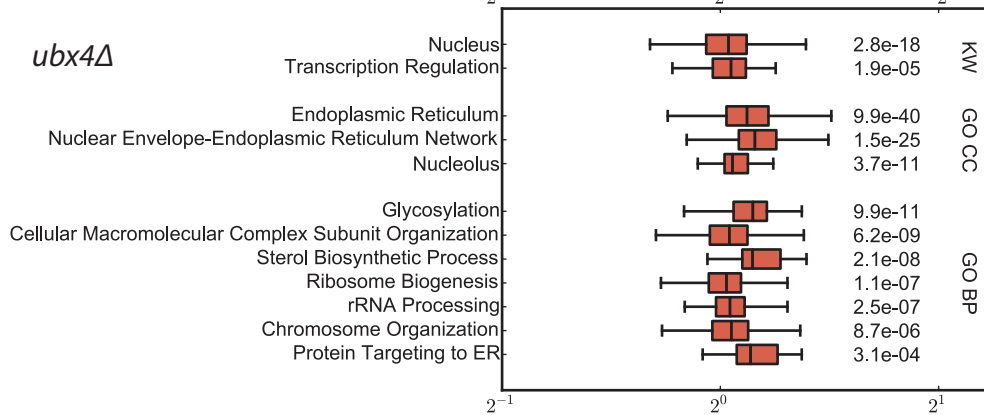
B



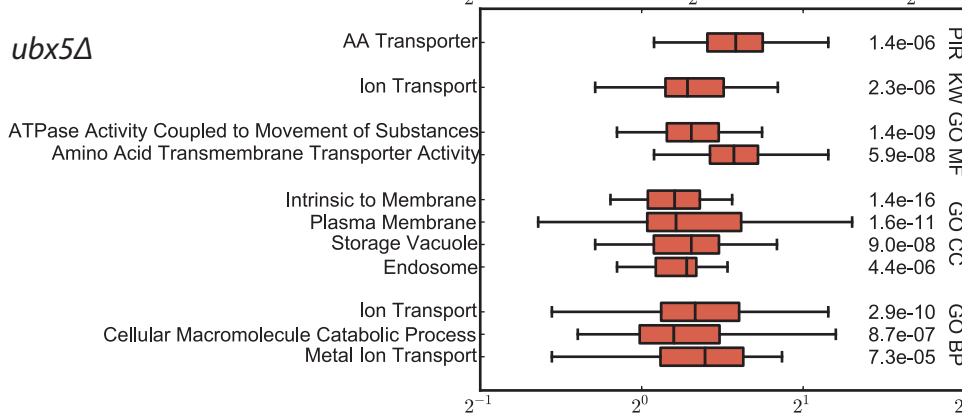
A. *ubx3Δ*



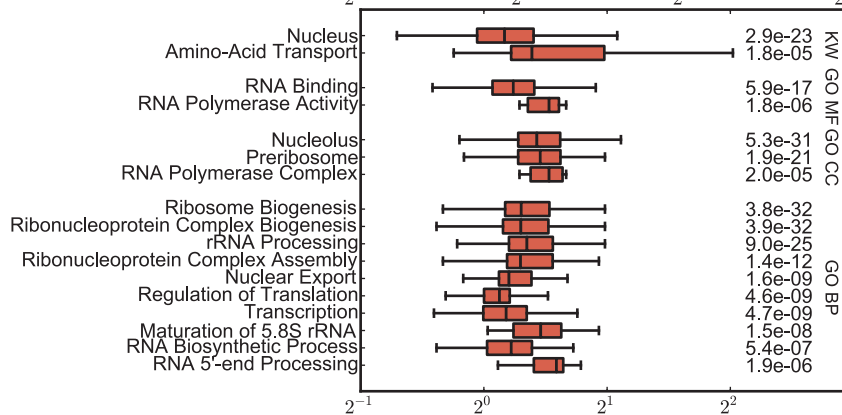
B. *ubx4Δ*



C. *ubx5Δ*



D. *ubx6Δ*



E. *ubx7Δ*

