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Supplemental Information

The Cdc48 Complex Alleviates the Cytotoxicity of Misfolded Proteins by Regulating Ubiquitin Homeostasis

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SUPPLEMENTAL INFORMATION

Figure S1 (related to Figure 1). The proline-rich region in Huntingtin affects its aggregation and degradation. **A.** Cellular localization of Htt103QP-GFP and Htt103QΔP-GFP in yeast cells. Yeast cells with *P_{GAL}FLAG-Htt103QP-GFP* (3419-1-1) and *P_{GAL}FLAG-Htt103QΔP-GFP* (YYW313-1) were grown in raffinose medium to mid-log phase at 30°C. Galactose (final concentration 2%) was added and cell images were taken after a 6-hour incubation in galactose medium. The GFP signal is shown. Scale bar, 5μm. **B.** The degradation kinetics of Htt103QP and Htt103QΔP. The above yeast strains were grown in raffinose medium to mid-log phase. Galactose (final concentration 2%) was added for 1 hour to induce Htt103QP and Htt103QΔP expression, followed by addition of glucose to shut off the induction. Protein samples were prepared over time; Htt103QP and Htt103QΔP protein levels were monitored over time with anti-FLAG antibody. Pgk1 was used as a loading control. The experiment was repeated three times and the relative abundance of Huntingtin over Pgk1 is shown in **C** as mean ± SD. The Wilcoxon rank sum test was used to calculate *p*-values, and the difference was considered significant (*) when $p < 0.05$.

Figure S2 (related to Figure 3). Increased Hsp104-positive aggregates in *cdc48-3*, *npl4-1*, and *ufd1-2* mutants. **A.** WT (YYW315-2) and *cdc48-3* (3506-1-1) mutants containing *HSP104-GFP* were grown in YPD (glucose medium) to mid-log phase at 25°C. Cells were then shifted to 34°C for 3 hours. Hsp104-GFP foci in WT and mutant cells were imaged (left) and quantified (right). The result is the average from three independent experiments (mean ± SD). Two-way ANOVA analysis with Tukey's multiple comparison test was performed (N = 3). *, $p < 0.05$; **, $p < 0.01$. Scale bar = 5μm. **B.** The Hsp104-GFP signal in WT (YYW316-1), *npl4-1* (3641-2-2), and *ufd1-2* (3642-1-1) mutants was compared using the same protocol as in (A).

Figure S3 (related to Figure 4). The accumulation of Htt103QP on the proteasome increases in *cdc48-3* mutants. WT (3589-1-4), *RPN11-3×FLAG P_{GAL}-Htt103QP-GFP* (3592-3-3), and *cdc48-3 RPN11-3×FLAG P_{GAL}-Htt103QP-GFP* (3592-3-1) cells (80 mL) in *pdr5*Δ background were grown in YPR (raffinose medium) to mid-log phase at 25°C. Cells were shifted to 34°C and galactose was added for 3 hours. The proteasome inhibitor, MG132, was then added for 1 hour. Cells were harvested and lysed, and Rpn11-3×FLAG protein was immunoprecipitated using M2 anti-Flag beads. Htt103QP-GFP was detected using anti-GFP antibody. A 4% SDS-PAGE was

used to visualize Htt103QP-GFP species associated with proteasome protein Rpn11. In the strains used in this experiment, the Htt103QP construct lacked the FLAG tag that is present in most other experiments.

Figure S4 (related to Figure 4). Deletion of *SAN1* and *UBR1* deletion partially suppresses the accumulation of ubiquitinated proteins on proteasomes. Strains with the indicated genotypes (in *pdr5*Δ background) were grown in YPD (glucose medium) to mid-log phase and then shifted to 34°C for 3 hours. MG-132 (50 μM) was then added for 1 hour. The cells were lysed and Rpn11-3×FLAG protein was immunoprecipitated using M2 anti-FLAG beads and ubiquitinated proteins were detected using anti-Ub antibody. We used 4% SDS-PAGE to visualize ubiquitinated high-molecular-weight protein species. A *pdr5*Δ strain (3589-1-4) was used as a negative control. *cdc48-3* (3592-3-1), *san1*Δ *ubr1*Δ (3625-1-2), and two *cdc48-3 san1*Δ *ubr1*Δ strains (3622-1-3 and 3624-1-1) were used to examine the level of high-molecular-weight species that are associated with proteasome protein Rpn11. These strains contain *RPN11-3×FLAG* and P_{GAL}*Htt103QP-GFP* that lacks the N-terminal FLAG tag. In this experiment, yeast cells were grown in glucose medium and no Htt103QP expression is induced.

Figure S5 (related to Figure 4). The increased accumulation of ubiquitinated proteins on proteasomes in *cdc48-3* mutant cells depends on Dsk2 and Rad23. Yeast strains WT (3589-1-4), *RPN11-3×FLAG* (3592-4-4), *cdc48-3 RPN11-3×FLAG* (3592-5-2), and *cdc48-3 rad23*Δ *dsk2*Δ *RPN11-3×FLAG* (3967-2-4) in *pdr5*Δ background were grown in YPD (glucose medium) to mid-log phase and then shifted to 34°C for 3 hours. Proteasome inhibitor MG-132 was then added at 50 μM for 1 hour. The cell lysates were immunoprecipitated using M2 anti-FLAG beads for Rpn11-3×FLAG protein, and ubiquitinated protein species were detected using anti-Ub antibody. We used 4% SDS-PAGE to visualize ubiquitinated high-molecular-weight protein species. The protein levels of Rpn11-3×FLAG and Pgk1 are shown.

Figure S6 (related to Figure 5). **A.** Western blotting results show HA-Ub induction after switch from raffinose (Raff) to galactose (Gal) using anti-HA antibody. * indicates the HA-fusion peptide generated from the control vector. **B.** High-level ubiquitin expression in *cdc48-3* mutants. A empty vector (pRS416) or a P_{ADH1}*RPS31(UBI3)* ubiquitin plasmid was introduced into WT (Y300) and *cdc48-3* (MHY3512) cells, and the transformants were selected on URA dropout plates. Saturated

cultures of the transformants were serially 10-folded diluted and spotted onto URA dropout plates. Images were acquired after incubation at 25°C, 30°C, 34°C and 37°C for 3 days.

Figure S1. Higgins et al.

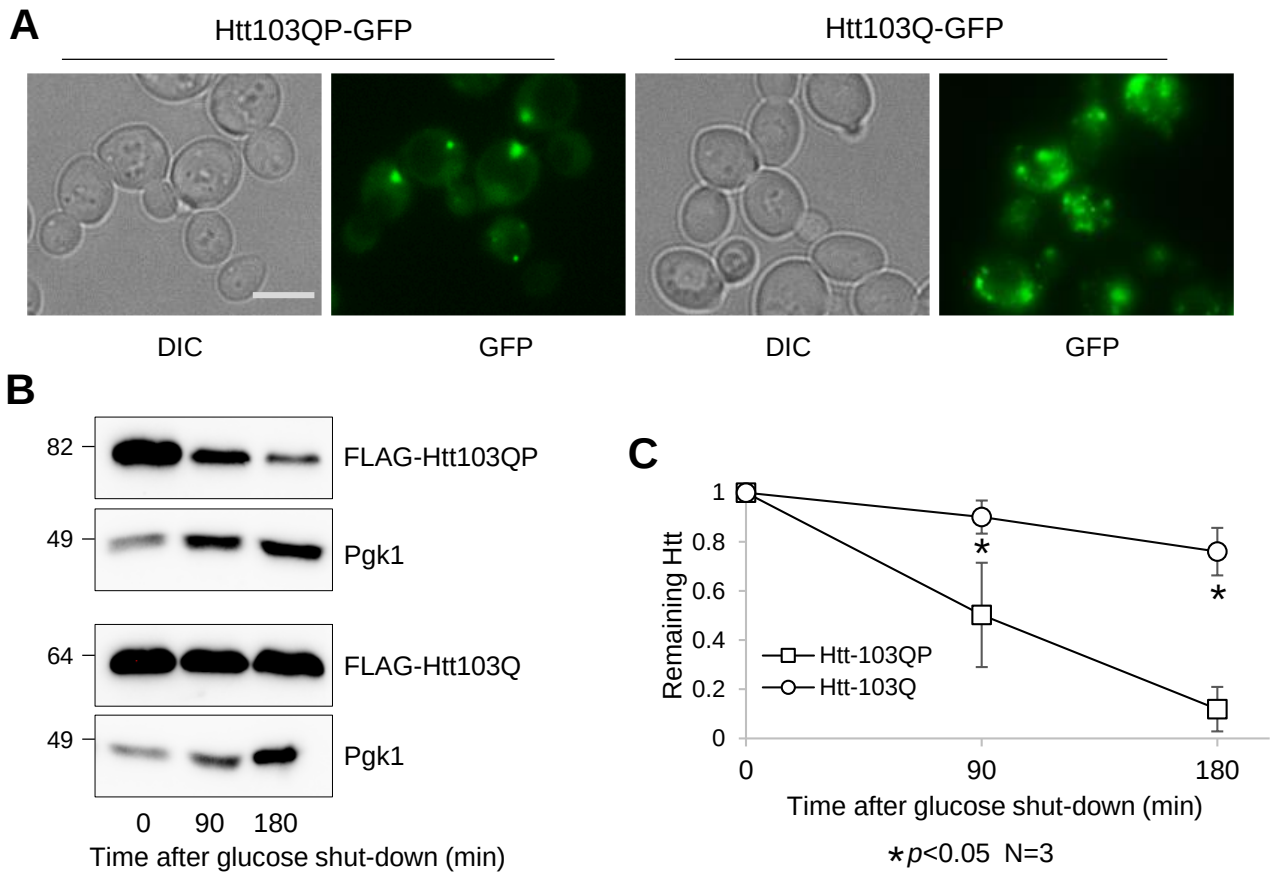


Fig. S2 Higgins et al.

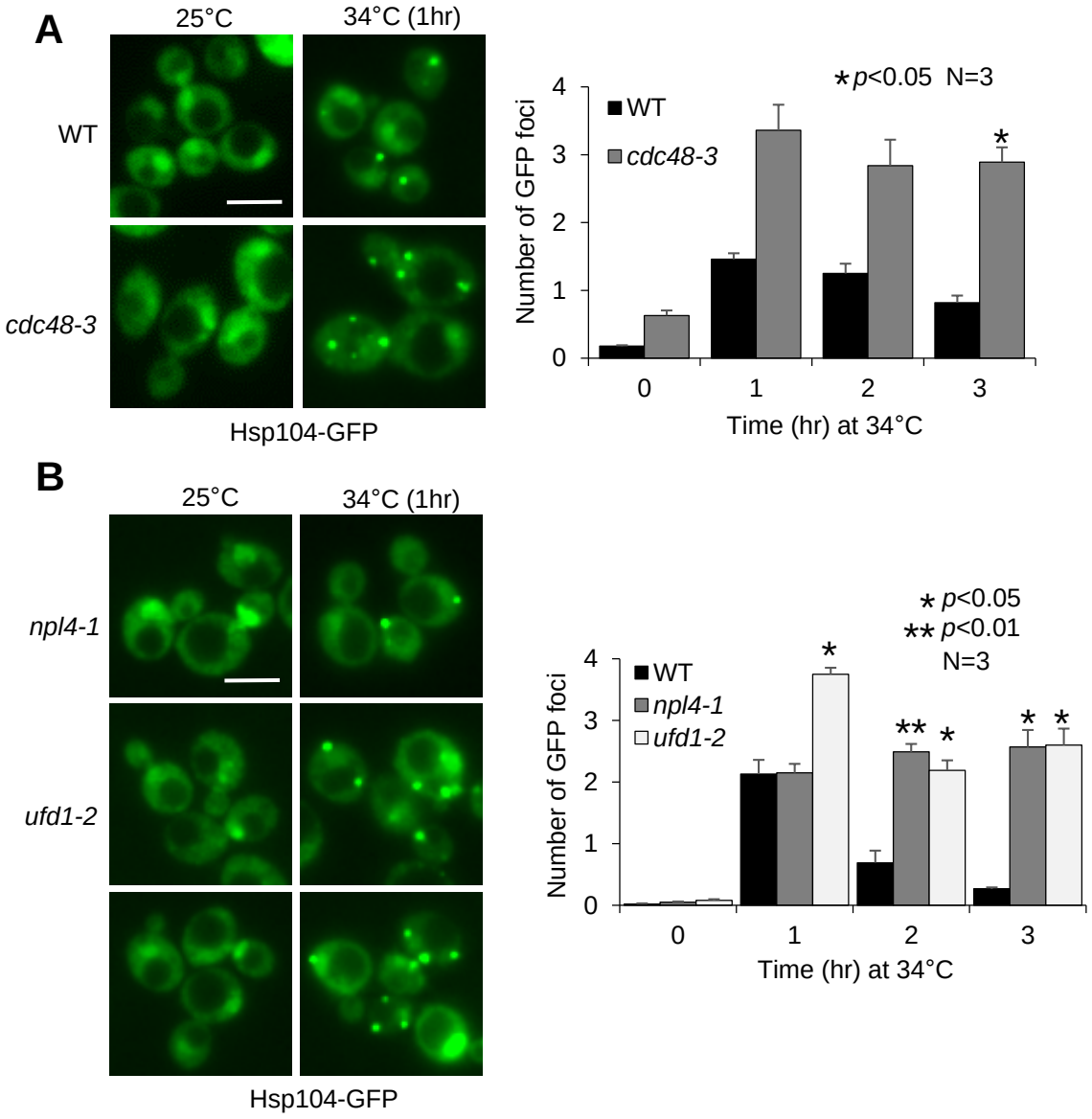
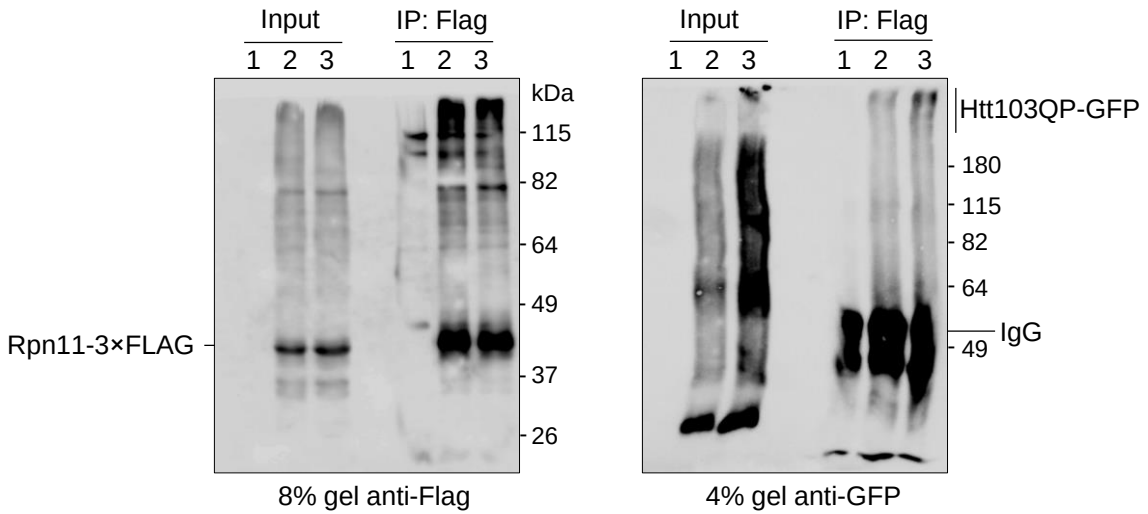
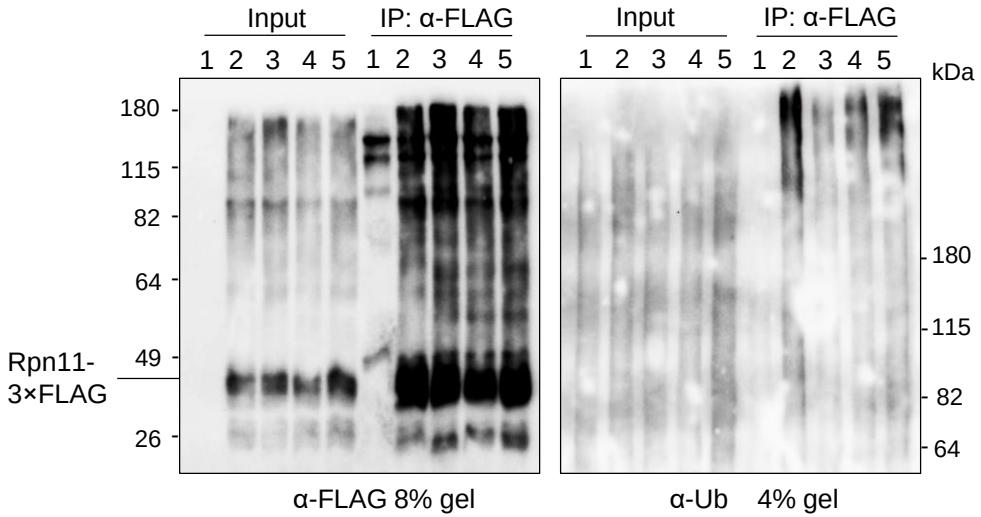


Figure S3. Higgins et al.



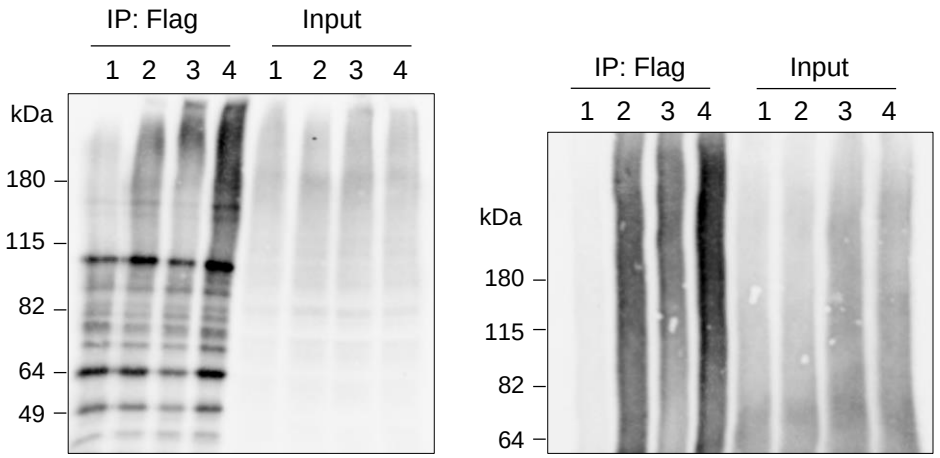
1. WT
2. *Rpn11-3×Flag Htt103QP-GFP*
3. *cdc48-3 Rpn11-3×Flag Htt103QP-GFP*

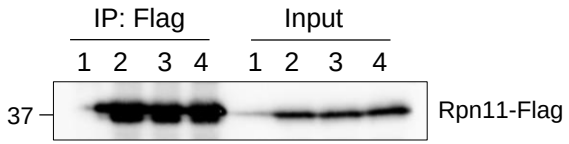
Figure S4. Higgins et al.

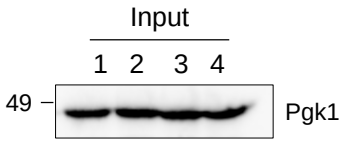


1. WT; **2.** *cdc48-3 RPN11-3×FLAG;* **3.** *san1Δ ubr1Δ RPN11-3×FLAG;* **4.** *san1Δ ubr1Δ cdc48-3 RPN11-3×FLAG;* **5.** *san1Δ ubr1Δ cdc48-3 RPN11-3×FLAG*

Figure S5. Higgins et al.







1: WT
2: *RPN11-3×FLAG*
3: *cdc48-3 dsk2Δ rad23Δ RPN11-3×FLAG*
4: *cdc48-3 RPN11-3×FLAG*

Figure S6. Higgins et al.

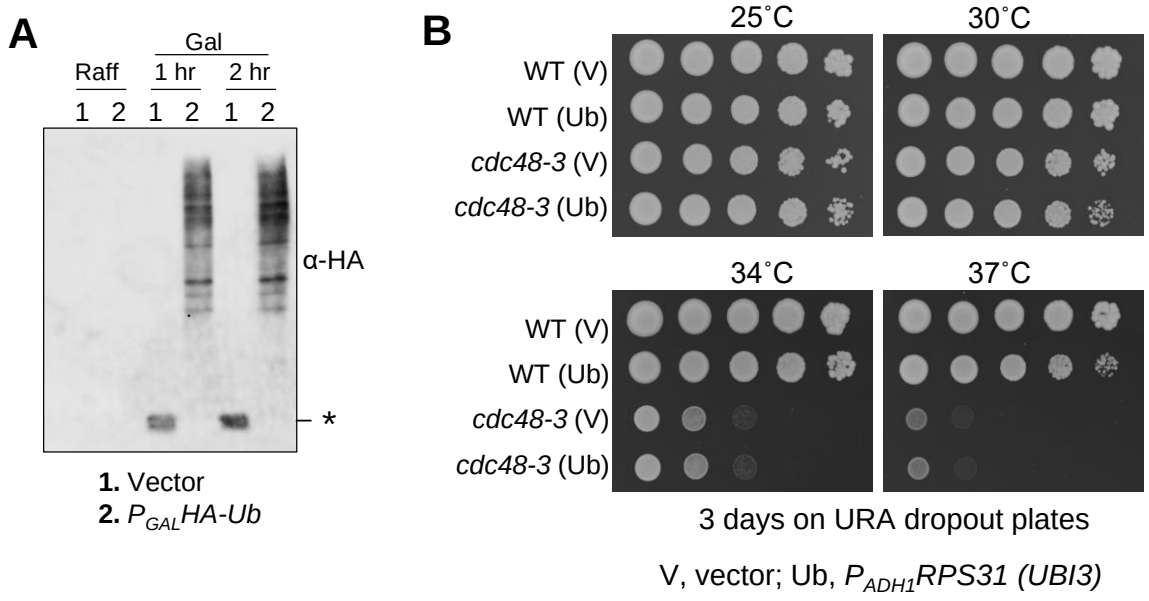


Table S1. Yeast deletion mutants used to screen the E3 ligase for Htt103QP (related to Figure 2A)

ORF	NAME	Motif
YAL002W	VPS8	RING finger
YBR062C	YBR062C	RING finger
YBR114W	RAD16	RING finger
YBR158W	AMN1	F-box
YBR203W	COS111	F-box
YBR280C	SAF1	F-box
YCR066W	RAD18	RING finger
YDL013W	SLX5	RING finger
YDL190C	UFD2	U-box
YDR049W	VMS1	Zinc finger, C2H2
YDR103W	STE5	RING finger
YDR131C	YDR131C	F-box
YDR132C	MRX16	BTB
YDR143C	SAN1	RNF-ring finger
YDR219C	MFB1	F BOX
YDR255C	RMD5	RING finger
YDR265W	PEX10	RING finger
YDR266C	HEL2	RING finger
YDR306C	PFU1	F-box
YDR313C	PIB1	RING finger
YDR457W	TOM1	HECT
YER116C	SLX8	RING finger
YGL003C	CDH1	WD40 repeat, APC/C complex component
YGL131C	SNT2	RING finger
YGL141W	HUL5	HECT
YGR003W	CUL3	CULLIN REPEAT
YGR184C	UBR1	RING finger
YHL010C	ETP1	RING finger
YHR115C	DMA1	RING finger
YIL001W	YIL001W	BTB
YIL030C	SSM4 (DOA10)	RING finger
YJL047C	RTT101	CULLIN
YJL149W	DAS1	F BOX
YJL157C	FAR1	RING finger
YJL204C	RCY1	F BOX
YJL210W	PEX2	RING finger
YJR036C	HUL4	HECT
YJR052W	RAD7	F-box
YJR090C	GRR1	F-box
YKL010C	UFD4	HECT
YKL034W	TUL1	RING finger
YKR017C	HEL1	RING finger
YLR024C	UBR2	RING finger
YLR032W	RAD5	RING finger

YLR097C	HRT3	F-box
YLR108C	YLR108C	BTB
YLR224W	UCC1	F-box
YLR247C	IRC20	RING finger
YLR352W	LUG1	F-box
YLR368W	MDM30	F-box
YLR427W	MAG2	RING finger
YML068W	ITT1	RING finger
YML088W	UFO1	F-box
YMR026C	PEX12	RING finger
YMR119W	ASI1	RING finger
YMR247C	RKR1	RING Zinc finger
YMR258C	ROY1	F-box
YNL008C	ASI3	RING finger
YNL023C	FAP1	RING finger
YNL116W	DMA2	RING finger
YNL230C	ELA1	F-box
YNL311C	SKP2	F-box
YOL013C	HRD1	RING finger
YOL054W	PSH1	RING finger
YOL138C	RTC1	RING finger
YOR080W	DIA2	F-box
YOR191W	ULS1	RING finger
YPL046C	ELC1	ELONGIN C,BTB,SKP1 COMPPNENT
YPR093C	ASR1	RING finger
YMR247C	RKR1	RING finger
YMR080C	NAM7	CH-rich domain (RING-related domain)

Table S2. Yeast strains used in this study (related to Star Methods)

Strains	Genotype	Reference
Y300 (WT)	Mata *ura3-1, his3-11,15 leu2-3,112 trp1-1, ade2-1, can1-100*	Lab Stock
MHY3512	Mata *cdc48-3 ura3-52 leu2-3, 122 ade2-1 trp1-1 his3*	Hochstasser
1126	Mata *npl4-1*	R.H. Chen
1122	Mata *ufd1-2*	R.H. Chen
3419-1-1	Mata P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3598-2-3	Mata *cdc48-3* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3387-3-4	Mata *npl4-1* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3385-4-4	Mata *ufd1-2* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
RH142	Mata *san1::Sphis5*$^+$ P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3301-2-2	Mata *san1::KanMX* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3522-4-4	Mata *ubr1::KanMX* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3287-1-1	Mata *ltn1::KanMX* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3288-1-3	Mata *ufd2::KanMX* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
2925-3-2	Mata *HTA1-mApple-HIS3* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3222-1-1	Mata *dsk2::TRP1* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
3514-1-2	Mata *dsk2::TRP1 san1::KanMX* P_{GAL}*FLAG-Htt103QP-GFP::URA3*	This study
FY-13-1	Mata Y300 (P_{GAL}*HA-CLB5::URA3)*	This study
3504-3-2	Mata *cdc48-3* (P_{GAL}*HA-CLB5::URA3*)	This study
3580-1-3	Mata *cdc48-3 san1::TRP1 ubr1::Sphis5*$^+$ (P_{GAL}*HA-CLB5::URA3*)	This study
3660-1-4	Mata *cdc48-3 ubr2::KanMX* (P_{GAL}*HA-CLB5::URA3*)	This study
229-3-2	Mata *CLB5-HA*	Lab stock
3968-4-3	Mata *cdc48-3 san1::TRP1 ubr1::Sphis5*$^+$ *CLB5-HA*	This study
3968-5-1	Mata *cdc48-3 CLB5-HA*	This study
3969-4-4	Mata *ubr2::KanMX*	This study
3655-2-4	Mata *cdc48-3 ubr2::KanMX*	This study
3658-1-4	Mata *npl4-1 ubr2::KanMX*	This study
3659-1-2	Mata *ufd1-2 ubr2::KanMX*	This study
3550-5-3	Mata *cdc48-3 san1::TRP1*	This study
3550-6-3	Mata *cdc48-3 ubr1::Sphis5*$^+$	This study
3550-2-1	Mata *cdc48-3 san1::TRP1 ubr1::Sphis5*$^+$	This study
3555-5-1	Matα *npl4-1 san1:TRP1 ubr1::Sphis5*$^+$	This study
3556-3-3	Mata *ufd1-2 san1:TRP1 ubr1::Sphis5*$^+$	This study
3556-1-1	Mata *ufd1-2 san1::TRP1*	This study
3556-2-3	Mata *ufd1-2 ubr1::Sphis5*$^+$	This study
YYW14	Mata *dsk2::TRP1*	This study
3553-2-4	Mata *rad23::Sphis5*$^+$	This study
3553-5-3	Mata *dsk2:TRP1 rad23::Sphis5*$^+$	This study
3553-10-3	Mata *cdc48-3 rad23::Sphis5*$^+$	This study
3553-7-2	Mata *cdc48-3 dsk2::TRP1*	This study
3553-3-2	Mata *cdc48-3 rad23::Sphis5*$^+$ *dsk2::TRP1*	This study
RH156	Mata Y300 (*p1217*, empty vector *TRP1*)	This study
RH157	Mata Y300 (P_{GAL}*HA-Ub-TRP1)*	This study
RH158	Mata *cdc48-3* (*p1217*, empty vector *TRP1*)	This study
RH159	Mata *cdc48-3* (P_{GAL}*HA-Ub-TRP1)*	This study
RH160	Mata *npl4-1* (*p1217*, empty vector *TRP1*)	This study
RH161	Mata *npl4-1* (P_{GAL}*HA-Ub-TRP1)*	This study
RH162	Mata *ufd1-2* (*p1217*, empty vector *TRP1*)	This study
RH163	Mata *ufd1-2* (P_{GAL}*HA-Ub-TRP1)*	This study
3589-1-4	Mata *pdr5::KanMX*	This study
3592-4-4	Mata *pdr5::KanMX RPN11-3×FLAG::HIS3*	This study
3592-5-2	Mata *pdr5::KanMX cdc48-3 RPN11-3×FLAG::HIS3*	This study

3592-3-1	Mata *pdr5::KanMX cdc48-3 RPN11-3×FLAG::HIS3* P_{GAL}*Htt103QP-GFP::URA3*	This study
3592-3-3	Mata *pdr5::KanMX RPN11-3×FLAG::HIS3* P_{GAL}*Htt103QP-GFP::URA3*	This study
3625-1-2	Matα *pdr5::KanMX san1::TRP1 ubr1:: Sphis5*$^{+}$ *RPN11-3×FLAG::HIS3* P_{GAL}*Htt103QP-GFP::URA3*	This study
3622-1-3	Mata *pdr5::KanMX cdc48-3 san1::TRP1 ubr1:: Sphis5*$^{+}$ *RPN11-3×FLAG::HIS3* P_{GAL}*Htt103QP-GFP::URA3*	This study
3624-1-1	Mata *pdr5:Kan cdc48-3 san1::TRP1 ubr1::Sphis5*$^{+}$*RPN11-3×FLAG::HIS3* P_{GAL}*Htt103QP-GFP::URA3*	This study
3967-2-4	Mata *pdr5:Kan cdc48-3 dsk2::TRP1 rad23::Sphis5*$^{+}$*RPN11-3×FLAG::HIS3*	This study
YYW315-2	Mata *HSP104-GFP::TRP1*	This study
3506-1-1	Mata *cdc48-3 HSP104-GFP::TRP1*	This study
YYW316-1	Mata *HSP104-GFP::Sphis5*$^{+}$	This study
3641-2-2	Mata *npl4-1 HSP104-GFP::Sphis5*$^{+}$	This study
3642-1-1	Matα *ufd1-2 HSP104-GFP::Sphis5*$^{+}$	This study
YYW313-1	Matα P_{GAL}*Htt103Q-GFP::URA3*	This study
PHY648	Mata *ppz1::KANMX ppz2::NATMX*	MacGurn lab
4023-1-1	Mata *cdc48-3 ppz1::KANMX*	This study
4023-2-4	Mata *cdc48-3 ppz1::KANMX ppz2::NATMX*	This study
4023-8-4	Matα *cdc48-3 ppz1::KANMX ppz2::NATMX*	This study