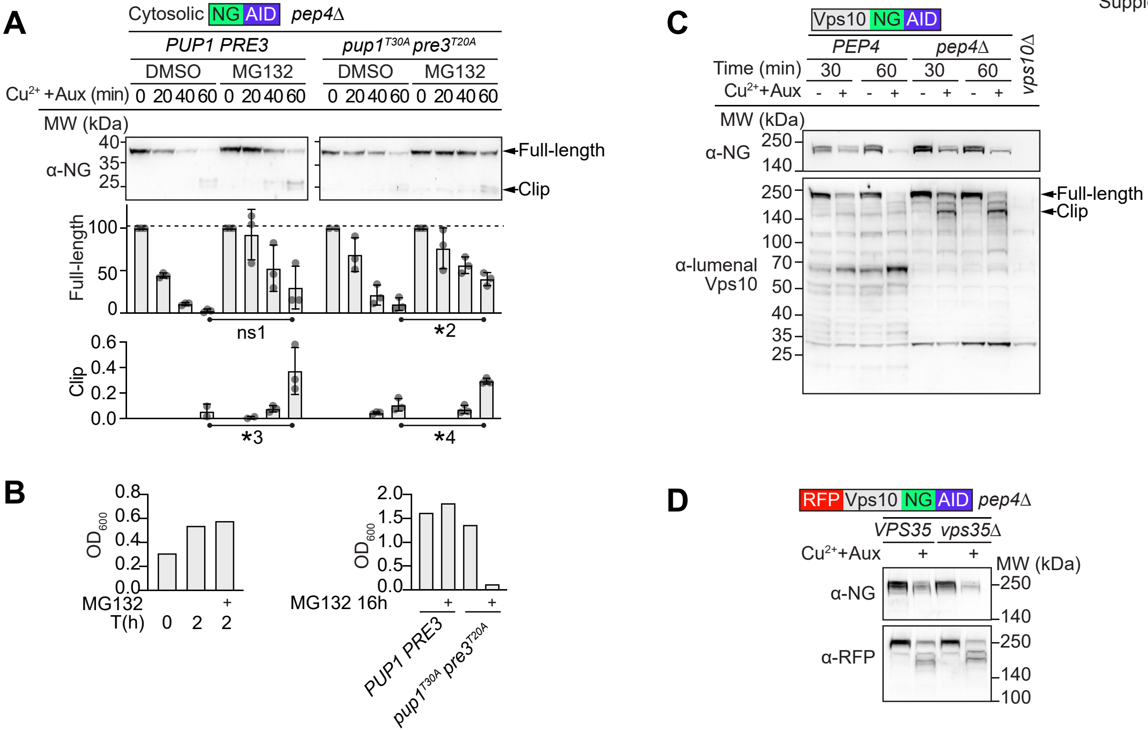
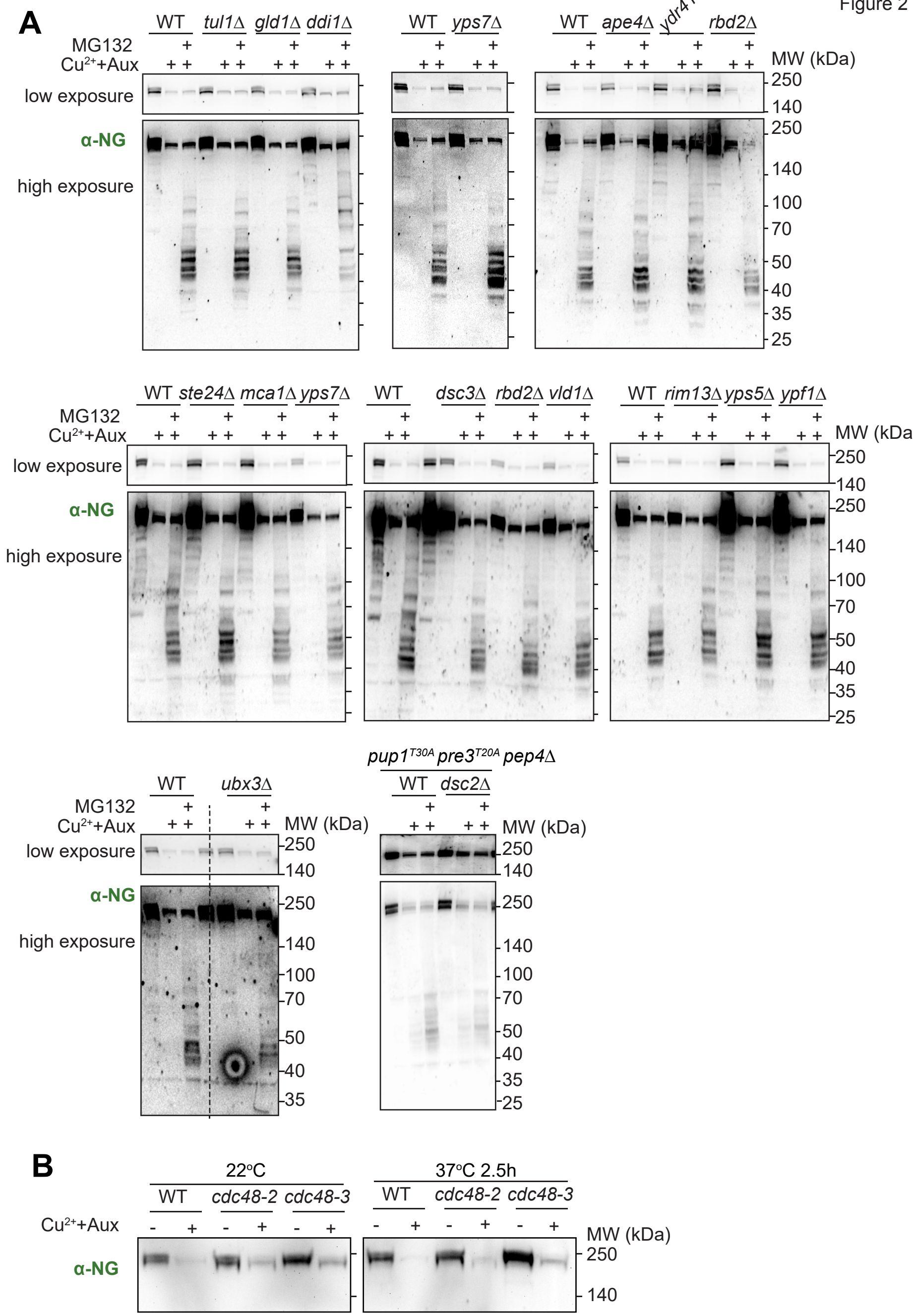
Supplementary Figure 1



Supplementary Figure 1. Proteasome inhibition by MG132 and Vps35 and Pep4-independent degradation of Vps10-NG-AID

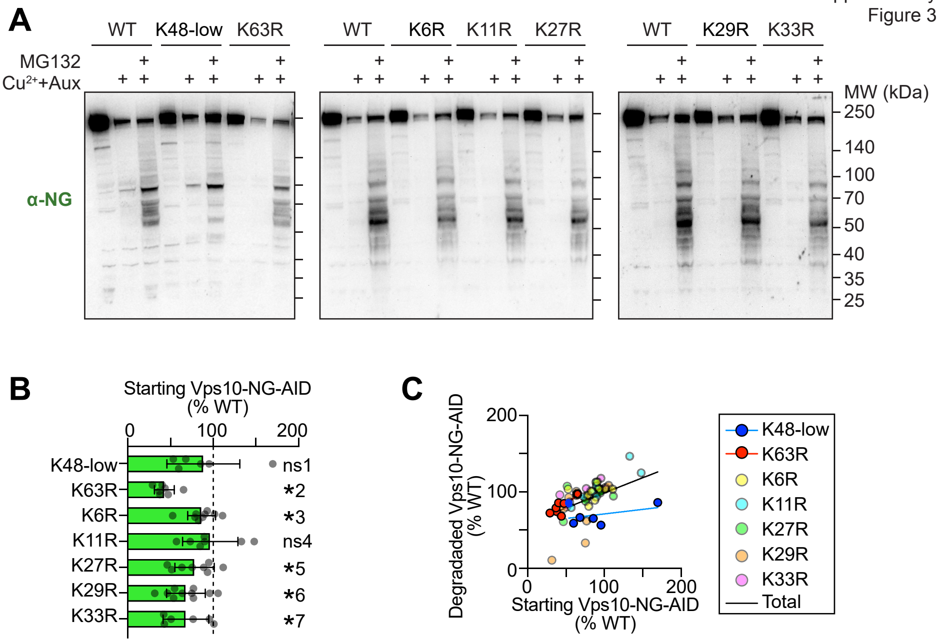
A) SCF^{TIR1} dependent degradation of a cytosolic protein (NG-AID) was monitored in *PUP1 PRE3* or *pup1-T30A pre3-T20A* cells. MG132 was added 30 min prior to induction of SCF^{Tir1} with copper and auxin. Representative immunoblot and graph of full-length and clipped fragments are shown. Bar graphs show mean ± SD, overlaid are grey circles representing values from independent experiments (n) of yeast strains described in Supplementary Tables 1 and 4 (n = 3). Statistical comparisons are indicated by numbered labels, with asterisks or "ns" as prefix to denote significant differences ($p < 0.05$) or not significant, respectively. Pairwise comparisons were performed using two-sided unpaired *t*-tests, with Holm–Šidák correction following a one-way ANOVA. NG FL: $F (15, 32) = 16.84$, $p < 0.0001$; adj. pairwise *p*-values: *1 = 0.0418, *2 = 0.0418. NG Clip: $F (15, 32) = 14.61$, $p < 0.0001$. adj. pairwise *p*-values: *1 = < 0.0001, *2 = < 0.0001. **B)** Effect of MG132 on cell growth. Left, cells (*pup1-T30A pre3-T20A pep4Δ)* at OD_{600} of 0.25 were grown for 0 h or 2 h in the absence and presence of MG132. Growth continued with or without MG132 in this short time window. Right, in contrast MG132 inhibited overnight growth of *pup1-T30A pre3-T20A,* but not *PUP1 PRE3* cells. **C)** Immunoblot of SCF^{Tir1} dependent degradation of Vps10-NG-AID in *PEP4* and *pep4Δ* cells using α-NG and α-Vps10 antibodies. **D)** Immunoblot of RFP-Vps10-NG-AID degradation in *VPS35* and *vps35Δ* cells. Source data are provided as a Source Data files.

Supplementary Figure 2



Supplementary Figure 2. Genetic screen for mediators of Vps10-NG-AID degradation
A) Immunoblot of SCF^{Tir1}-dependent degradation of Vps10-NG-AID degradation in the indicated yeast mutants. MG132 was added 30 min prior to induction of SCF^{Tir1} with copper and auxin. A low and high exposure of the same immunoblot using α-NG antibody is shown. **B)** Temperature-sensitve *cdc48* mutants were grown at 22 °C and then shifted to 37 °C mutants for 2.5 h at 37 °C prior to induction of SCF^{Tir1}-dependent degradation of Vps10-NG-AID. For each strain in A and B, the *CUP1*-Skp1-TIR1 cassette was integrated into the *PDR5* locus, and endogenous Vps10 tagged with NG-AID. Source data are provided as a Source Data files.

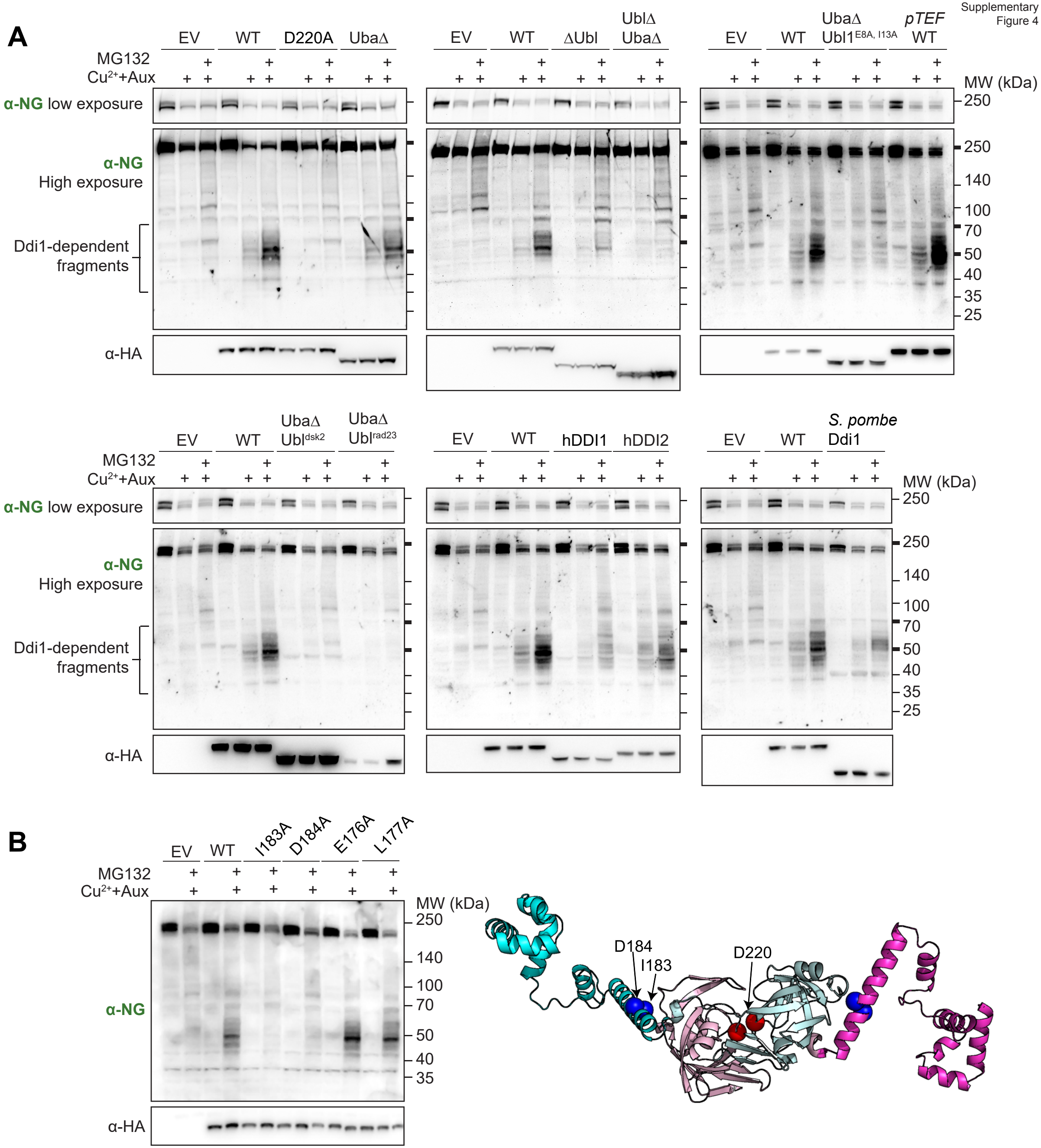
Supplementary Figure 3



Supplementary Figure 3. Vps10-NG-AID degradation in strains defective for specific Ub-linkages.

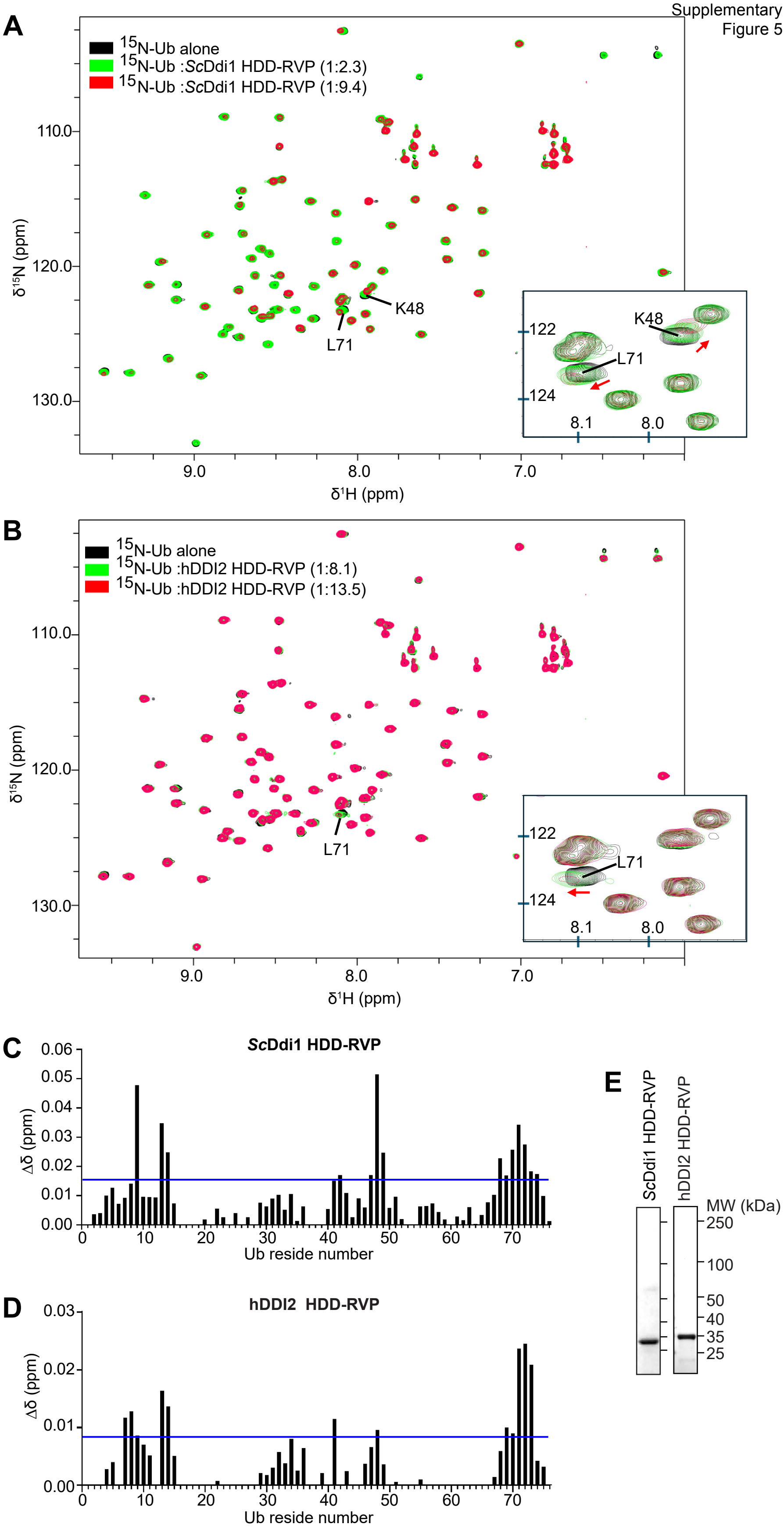
A-C) Supplemental analyses of the experiments presented in Figure 3B. **A)** Representative immunoblot of Vps10-NG-AID degradation in strains carrying the indicated K>R Ub mutants, except for K48R Ub, which also carries low levels of wildtype Ub to sustain viability. **B)** Quantification of initial levels of full-length Vps10-NG-AID in strains carrying the indicated K>R Ub mutants. Data were normalized to WT in each biological experiment. Bar graphs show mean ± SD, overlaid are grey circles representing values from independent experiments (n) of yeast strains described in Supplementary Tables 1 and 4 (n = 3). Statistical comparisons are indicated by numbered labels, with asterisks or "ns" as prefix to denote significant differences ($p < 0.05$) or not significant, respectively. Data were analyzed by a two-sided one sample *t*-test comparing the sample mean with the theoretical value of 100. ns1: $t(5) = 0.6470$, $p = 0.5461$; *2: $t(6) = 13.30$, $p = < 0.0001$; *3: $t(8) = 2.477$, $p = 0.0383$; ns4: $t(6) = 0.2782$, $p = 0.7902$; *5: $t(7) = 2.677$, $p = 0.0317$; *6: $t(9) = 4.470$, $p = 0.0016$, *7: $t(5) = 2.931$, $p = 0.0326$. **C)** Scatter plot of initial levels of full-length Vps10-NG-AID versus the amount degraded. Individual replicates are plotted, with the legend indicating the K>R Ub mutant for each data point. Source data are provided as a Source Data files.

Supplementary Figure 4



Supplementary Figure 4. Ddi1-dependent cleavage of Vps10-NG-AID
A) Representative immunoblots of Vps10-NG-AID cleavage and degradation in *pup1-T30A pre3-T20A ddi1Δ* cells with an empty plasmid vector (EV) or one expressing the indicated Ddi1 variants that were quantified and presented in Figure 3C. **B)** *Left,* immunoblot of Vps10-NG-AID *pup1-T30A pre3-T20A ddi1Δ* cells expressing Ddi1 variants with mutations in HDD domain. *Right,* Alphafold schematic of HDD-RVP domain of Ddi1 as a dimer, with inactivating mutations in HDD highlighted blue and catalytic site highlighted red. Source data are provided as a Source Data files.

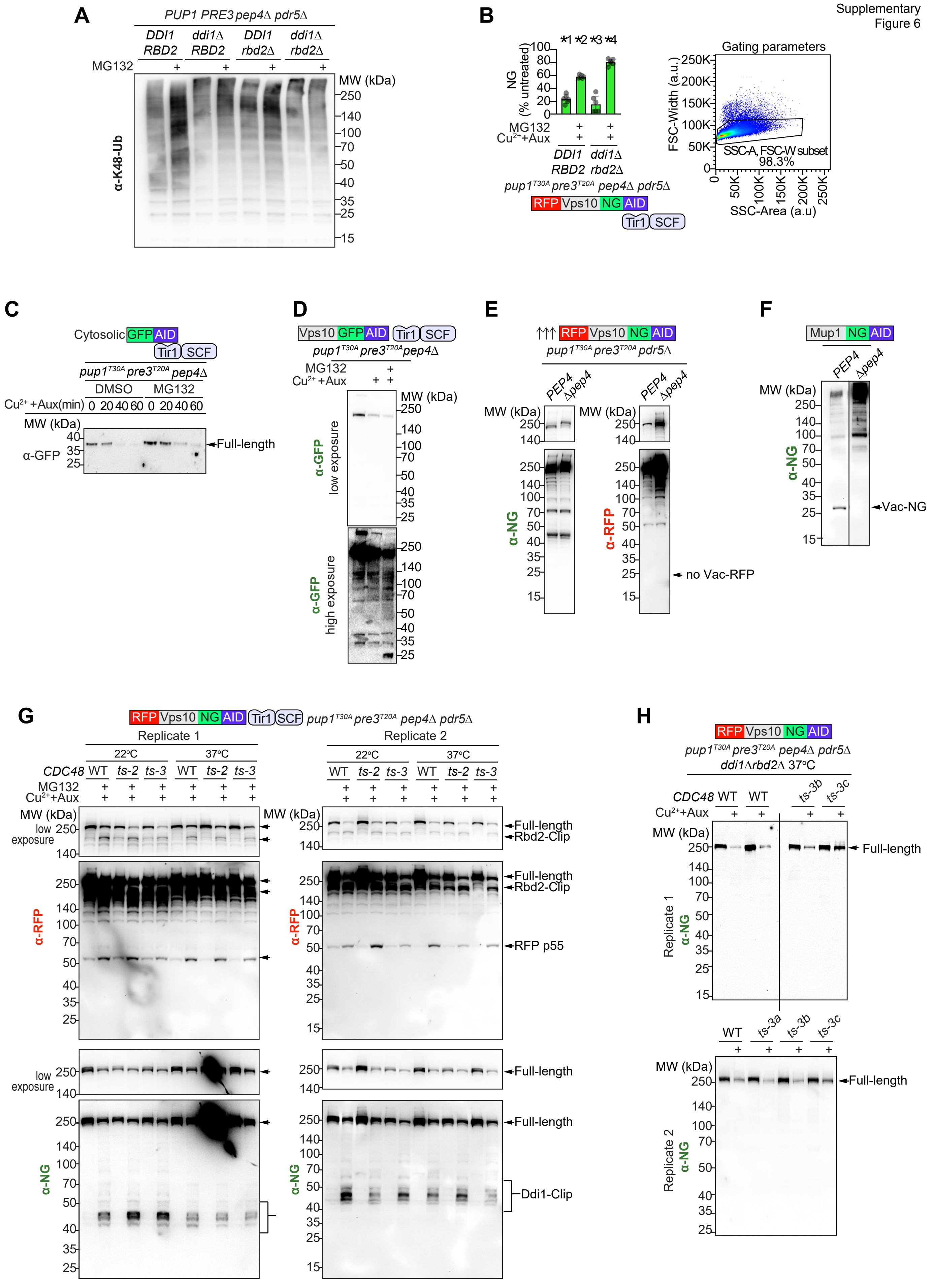
Supplementary Figure 5



Supplementary Figure 5. HSQC analysis of ^{15}N-Ub in the presence of the HDD-RVP domains from *Sc*Ddi1 and human DDI2

A) Overlay of full $^{15}N/^{1}H$ HSQC spectra, showing that *Sc*Ddi1 HDD-RVP binding caused selective chemical shift perturbations in the backbone amides of Ub residues, particularly at K48 and L71. Inset: zoomed-in spectral overlay highlighting peaks shifts at K48 and L71. All spectra are plotted at the same contour level. Notably, peak intensities especially for the residues in the binding interface such as L71, are reduced upon binding, which is expected since *Sc*Ddi1 HDD-RVP is a much larger protein (~29 kDa for monomer and ~58 kDa for dimer) than Ub. **B)** Overlay of full $^{15}N/^{1}H$ HSQC spectra, showing that human DDI2 HDD-RVP binding also caused selective chemical shift perturbations of Ub residues, notably at L71, as highlighted in the inset. **C)** Chemical shift perturbations [$\Delta\delta$(ppm)] of ^{15}N-Ub (30 μM) upon binding *Sc*Ddi1 HDD-RVP at a 1:9.4 (Ub:HDD-RVP) molar ratio. **D)** Chemical shift perturbations [$\Delta\delta$(ppm)] of ^{15}N-Ub (30 μM) upon binding human DDI2 HDD-RVP at a 1:9 (Ub:HDD-RVP) molar ratio. C-D) Blue lines indicate the cutoff values used to define the affected residues, which are mapped in red in Figure 3D and 3E. **E)** Representative Coomassie-stained gel of purified *Sc*Ddi1 HDD-RVP and human DDI2 HDD-RVP used in the NMR binding studies. Source data are provided as a Source Data files.

Supplementary Figure 6



Supplementary Figure 6. K48-polyubiquitinated proteins in Ddi1 mutants, SCF^{Tir1}-mediated degradation of GFP, vacuolar processing of NeonGreen and RFP, and assessment of the role of Cdc48 in the CUT-UP pathway.

A) Immunoblot of whole cell lysates in *PUP1 PRE3 pep4Δ pdr5Δ* cells lacking Ddi1, Rbd2 or both probed with α-K48-polyUb antibody. MG132 was added for 1 h prior to lysis.

B) Quantification (left) of NG fluorescence by flow cytometry 1 h after induction of SCF^{TIR1}-dependent ubiquitination of RFP-Vps10-NG-AID, in the presence or absence of proteasome inhibition with MG132 in the indicated strains. Data were normalized to untreated sample in each biological experiment. Bar graphs show mean ± SD, overlaid are grey circles representing values from independent experiments (n) of yeast strains described in Supplementary Tables 1 and 4 (n = 3). Statistical comparisons are indicated by numbered labels, with asterisks or "ns" as prefix to denote significant differences ($p < 0.05$) or not significant, respectively. Data were analyzed by a two-sided one sample *t*-test comparing the sample mean with the theoretical value of 100. *1: $t(5) = 28.97$, $p = < 0.0001$; *2: $t(5) = 40.91$, $p = < 0.0001$; *3: $t(5) = 16.12$, $p = < 0.0001$; *4: $t(5) = 9.964$, $p = 0.0002$. (Right) The forward and side scatter (arbitrary units) gating strategy used for NG-expressing cells. **(C-D)** Immunoblot using α-GFP antibody of GFP-AID (C) or Vps10-GFP-AID (D) expressed from the *Vps10* promoter in *pup1-T30A, pre3-T20A pep4Δ pdr5Δ* cells. MG132 was added 30 min prior to induction of SCF^{Tir1} with copper and auxin. GFP-AID was only slightly stabilized by MG132 in contrast to the stabilization of NG-AID observed in Supplemental Figure 1A. Stabilization of a C-terminal fragment of Vps10-GFP-AID was also observed upon proteasome inhibition but had low abundance likely due to the vulnerability of GFP to proteasome degradation. **E)** In *pep4Δ* and *PEP4*+ cells, RFP-Vps10-NG-AID expressed from the *MET25* promoter was overproduced by growth in methionine-free medium to deliver it to the vacuolar membrane. Overproduction did not result in a stable *PEP4*-dependent vacuolar RFP fragment. **F)** A stable vacuolar-produced NG fragment (vac-NG) was generated when NG-AID was fused to the methionine transporter Mup1 and induced to undergo MVB sorting by addition of 10 mg/ml methionine for 2 h. **G**) Production of Ddi1-dependent cleavage products and RFPp55 in strains carrying *cdc48-2, cdc48-3,* or wildtype *CDC48* at permissive (25 °C) or restrictive temperature (37°C) 60 min before SCF^{TIR1}-mediated ubiquitination of RFP-Vps10-NG-AID. Immunoblotting for RFP and NG was used to follow the lumenal RFPp55 derived product and the Ddi1-cleaved cytosolic fragments, respectively. **H**) Proteolysis of the NG-tagged cytosolic tail of RFP-Vps10-NG-AID is unaffected by inactivation of Cdc48 in the absence of Ddi1 and Rbd2. Cells (*ddi1Δ rbd2Δ)* carrying the *cdc48-3* allele were grown at 37°C for 1 h prior to SCF^{TIR1}-mediated ubiquitination of RFP-Vps10-NG-AID and analysis by α-NG immunoblotting. Source data are provided as a Source Data files.

Supplementary Table 1: Yeast Strains used in this study

Yeast Strain	Name and Important mutations	Figure used	Genotype	Source
SEY6210	Parental Strain		MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-80 uc2-Δ9	1
BY4742	Parental Strain (PLY5818)	1G, 4E	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	2
DS368	WT Ub SGA strain		MATα his3 ura3 ubi1Δ:: GPDpr-L40A ubi2Δ:: HYGR GPDpr-L40A GPDpr-L40A ubi3Δ::URA3 GPDpr-3xUb-S31-Cyc1tr GPDpr-S31 ubi4Δ:: 2X (Cyc1tr-Ub-GDPpr)-NATr	3
DS369	K6R Ub SGA strain		DS368: ubi3Δ::URA3 GPDpr-3xUb(K6R)-S31-Cyc1tr GPDpr-S31; ubi4Δ:: 2X (Cyc1tr-Ub(K6R)-GDPpr)-NATr	3
DS370	K11R Ub SGA strain		DS368: ubi3Δ::URA3 GPDpr-3xUb(K11R)-S31-Cyc1tr GPDpr-S31; ubi4Δ:: 2X (Cyc1tr-Ub(K11R)-GDPpr)-NATr	3
DS371	K27R Ub SGA strain		DS368: ubi3Δ::URA3 GPDpr-3xUb(K27R)-S31-Cyc1tr GPDpr-S31; ubi4Δ:: 2X (Cyc1tr-Ub(K27R)-GDPpr)-NATr	3
DS372	K29R Ub SGA strain		DS368: ubi3Δ::URA3 GPDpr-3xUb(K29R)-S31-Cyc1tr GPDpr-S31; ubi4Δ:: 2X (Cyc1tr-Ub(K29R)-GDPpr)-NATr	3
DS373	K33R Ub SGA strain		DS368: ubi3Δ::URA3 GPDpr-3xUb(K33R)-S31-Cyc1tr GPDpr-S31; ubi4Δ:: 2X (Cyc1tr-Ub(K33R)-GDPpr)-NATr	3
DS374	K48R Ub (low WT_Ub) SGA strain		DS368: ubi3Δ::URA3 GPDpr-2XUb(K48R)-Ub-S31-Cyc1tr GPDpr-S31; ubi4Δ:: 2X (Cyc1tr-Ub(K48R)-GDPpr)-NATr	3
DS376	K63R Ub SGA strain		DS368: ubi3Δ::URA3 GPDpr-3xUb(K63R)-S31-Cyc1tr GPDpr-S31; ubi4Δ:: 2X (Cyc1tr-Ub(K63R)-GDPpr)-NATr	3
MHY1177	(Widtype) PUP1 PRE3		MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre3-Δ2::HIS3 pup1Δ::leu2::HIS3 [pRS317-PUP1] [Ycplac22-PRE3]	4
MHY1178	pup1-T30A pre3-T20A		MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre3-Δ2::HIS3 pup1Δ::leu2::HIS3 [pRS317-pup1-T30A] [Ycplac22-pre3-T20A]	4
	tul1Δ		BY4742: tul1Δ::KanMX	5
	gld1Δ		BY4742: gld1Δ::KanMX	5
	ddi1Δ		BY4742: ddi1Δ::KanMX	5
	yps7Δ		BY4742: yps7Δ::KanMX	5
	ape4Δ		BY4742: ape4Δ::KanMX	5
	ydr415cΔ		BY4742: ydr415cΔ::KanMX	5
	rbd2Δ		BY4742: rbd2Δ::KanMX	5
	ste24Δ		BY4742: ste24Δ::KanMX	5
	mca1Δ		BY4742: mca1Δ::KanMX	5
	yps7Δ		BY4742: yps7Δ::KanMX	5
	dsc3Δ		BY4742: dsc3Δ::KanMX	5
	vld1Δ		BY4742: vld1Δ::KanMX	5
	rim13Δ		BY4742: rim13Δ::KanMX	5
	yps5Δ		BY4742: yps5Δ::KanMX	5
	ypf1Δ		BY4742: ypf1Δ::KanMX	5
	cdc48-2		BY4742: cdc48-2	6
	cdc48-3		BY4742: cdc48-3	6
RPY34	pep1Δ::URA3 pep4-3		pep1Δ::URA3 pep4-3 leu2-3,112 ura3-52 his4-519 ade6	7
PLY2166	VPS10-GFP	1C,1E	SEY6210: vps10::VPS10-GFP::TRP	8
PLY6264	VPS10-GFP pep4Δ	1E	SEY6210: vps10::VPS10-GFP::TRP pep4Δ	8
PLYL6018	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	1D,1F,S1C	BY4742: vps10::VPS10-NEON-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6022	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ	1F,2A,2B,S1C	BY4742: vps10::VPS10-NEON-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY676	vps10Δ(pep1-Δ3)	2B	SF838 leu2-3,112 his4-519 ade6 ura3-52 pep4-3	
PLY6072	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	2A,3A	MHY1177: vps10::VPS10-NEON-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3 pep4Δ::LEU2	This study
PLY6069	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ::LEU2 pup1-T30A pre3-T20A	2A,3A	MHY1178: vps10::VPS10-NEON-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3 pep4Δ::LEU2	This study
PLY6225	VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ pup1-T30A pre3-T20A	S1A,S1B	MHY1178: vps10::NEON-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ	This study
PLY6225	VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ	3F,S1A,S1B	MHY1178: vps10::NEON-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ	This study
PLY6173	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ	2B,2C,2D,4A,S1D	BY4742: vps10::(Kl)LEU2::MET25pr-mCherry-VPS10-NEON-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3 pep4::KanMX	This study
PLY6312	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ vps35Δ	2C,4A	BY4742: vps10::(Kl)LEU2::MET25pr-mCherry-VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3 pep4::KanMX vps35Δ::loxP-HIS3-loxP	This study
PLY6197	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pdr5Δ	3A	MHY1177: vps10::VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3 pep4Δ ddi1Δ::loxP-LEU2-loxP	This study
PLY6199	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pdr5Δ pup1-T30A pre3-T20A	3A,3C,4F,S4A,S4B	MHY1178: vps10::VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3 pep4Δ ddi1Δ::loxP-LEU2-loxP	This study
PLY6454	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 WT_Ub pdr5Δ	3B,S3A,S3B,S3C	DS368: vps10::VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6456	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K6R_Ub pdr5Δ	3B,S3A,S3B,S3C	DS369: vps10::VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6458	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K11R_Ub pdr5Δ	3B,S3A,S3B,S3C	DS370: vps10::VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study

PLY6460	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K27R_Ub pdr5Δ	3B,S3A,S3B, S3C	DS371: vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6462	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K29R_Ub pdr5Δ	3B,S3A,S3B, S3C	DS372: vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6464	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K33R_Ub pdr5Δ	3B,S3A,S3B, S3C	DS373: vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6468	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K48R_Ub (low WT_Ub) pdr5Δ	3B,S3A,S3B, S3C	DS374: vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6466	Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K63R_Ub pdr5Δ	3B,S3A,S3B, S3C	DS376: vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6653	VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ ddi1Δ	3F	MHY1178: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID::D29 pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ	This study
PLY6249	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ	4A	MHY1177: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID::KanMX pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ	This study
PLY6251	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A	4A,S6E	MHY1178: vps10Δ::neon-aid-kURA pre3-Δ2::HIS3 pup1Δ::leu2::HIS3 [pRS317-pup1-T30A] [YCplac22-pre3-T20A] Δpep4 prd5Δ::cup1-yskp1-tir1-loxp ddi1Δ::loxp	This study
PLY6390	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A	4A,4B,5A,6A, 6B,7A,7B,7E, S6B, S6G	MHY1178: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID::KanMX pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ	This study
PLY6392	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pep4Δ pdr5Δ pup1-T30A pre3-T20A	4A,5A,7B	MHY1178: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID::KanMX pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ ddi1Δ::loxP	This study
PLY6266	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ	4A,4C	BY4742: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID-(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ::KanMX	This study
PLY6369	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 rbd2Δ::natMXloxP pep4Δ pdr5Δ	4A,4C	BY4742: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID-(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ::KanMX rbd2Δ::natMXloxP	This study
PLY6359	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ vps35Δ rbd2Δ	4A	BY4742: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ::KanMX vps35Δ::loxP-HIS3-loxP rbd2Δ::loxP-hphMX-loxP	This study
PLY6394	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ rbd2Δ pup1-T30A pre3-T20A	4B,5A,7B	MHY1178: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID::KanMX pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ rbd2Δ::loxP-hphMX-loxP	This study
PLY6450	mCherry-Vps10 pep4Δ	4E	BY4742: vps10::(KI)LEU2::MET25pr-mCherry-Vps10 pep4Δ::KanMX	This study
PLY6396	mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ rbd2Δ ddi1Δ pup1-T30A pre3-T20A	5A,6A,6B,7B, 7E,S6B, S6H	MHY1178: vps10::(KI)LEU2::MET25pr-mCherry-Vps10-NG-AID::KanMX pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ ddi1Δ::loxP rbd2Δ::natMXloxP	This study
PLY6622	(riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A	5B,5C,7A,7C, 7D	MHY1178: vps10::KanMX::TEF1*pr-(riboswitch)-mCherry-Vps10-sfGFP-DHFR::loxp pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ	This study
PLY6624	(riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A ddi1Δ	5B	MHY1178: vps10::KanMX::TEF1*pr-(riboswitch)-mCherry-Vps10-sfGFP-DHFR::loxp pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ ddi1Δ::loxP	This study
PLY6626	(riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A rbd2Δ	5B,7A,7D	MHY1178: vps10::KanMX::TEF1*pr-(riboswitch)-mCherry-Vps10-sfGFP-DHFR::loxp pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ rbd2Δ::loxP	This study
PLY6628	(riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A rbd2Δ ddi1Δ	5B,5C,7C,7D	MHY1178: vps10::KanMX::TEF1*pr-(riboswitch)-mCherry-Vps10-sfGFP-DHFR::loxp pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ rbd2Δ::loxP ddi1Δ::loxP	This study
PLY6134	tul1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: tul1Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6144	gld1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: gld1Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6154	ddi1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: ddi1Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6077	yps7Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: yps7Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6132	ape4Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: ape4Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6130	ydr415cΔ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: ydr415cΔ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6126	rbd2Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: rbd2Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6148	ste24Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: ste24Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6138	mca1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: mca1Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6128	yps7Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: yps7Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6140	dsc3Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: dsc3Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6150	vld1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: vld1Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6146	rim13Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: rim13Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study
PLY6142	yps5Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ	S2A	BY4742: yps5Δ::KanMX vps10::VPS10-NG-AID::(KI)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3	This study

PLY6136	*ypf1*Δ Vps10-NeonGreen-AID *CUP1pr*-Skp1-TIR1 *pdr5*Δ	S2A	BY4742: *ypf1Δ::KanMX vps10::VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3*	This study
PLY6038	*cdc48-2* Vps10-NeonGreen-AID *CUP1pr*-Skp1-TIR1 *pdr5*Δ	S2A	BY4742: *cdc48-2 vps10::VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3*	This study
PLY6039	*cdc48-3* Vps10-NeonGreen-AID *CUP1pr*-Skp1-TIR1 *pdr5*Δ	S2A	BY4742: *cdc48-3 vps10::VPS10-NG-AID::(Kl)URA3 pdr5Δ::CUP1pr-SKP1-TIR1::HIS3*	This study
PLY6368	*vps10*Δ::VPS10pr-GFP-AID CUP1pr-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A*	S6C	MHY1178: *vps10::VPS10pr-GFP-AID::KanMX pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6448	Vps10-GFP *CUP1pr*-Skp1-TIR1 *pdr5*Δ *pep4*Δ	S6D	MHY1188: *vps10::VPS10-GFP::loxP-KanMX-loxP pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6183	*CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A*	8A	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6195	*CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A ddi1*Δ	8A	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ ddi1Δ::loxP*	This study
PLY6564	*CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A rbd2*Δ	8B	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ rbd2Δ::loxP-NatMX-loxP*	This study
PLY6566	*CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A ddi1*Δ *rbd2*Δ	8B	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ ddi1Δ::loxP rbd2Δ::loxP-NatMX-loxP*	This study
PLY6414	*CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *PUP1 PRE3*	S6A	MHY1177: pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ	This study
PLY6416	*CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *PUP1 PRE3 ddi1*Δ	S6A	MHY1177: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ ddi1Δ::loxP*	This study
PLY6418	*CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *PUP1 PRE3 rbd2*Δ	S6A	MHY1177: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ rbd2Δ::loxP*	This study
PLY6420	*CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *PUP1 PRE3 ddi1*Δ *rbd2*Δ	S6A	MHY1177: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ ddi1Δ::loxP rbd2Δ::loxP*	This study
PLY6474	(riboswitch)-mCherry-Vps10-neon-aid-loxp *CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A rbd2Δ::loxP*	7F	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6572	(riboswitch)-mCherry-Vps10-neon-aid-loxp *CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A*	7F	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6596	(riboswitch)-mCherry-Vps10-neon-aid-loxp CUP1pr-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A dsc2Δ::loxp*	7F	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6598	(riboswitch)-mCherry-Vps10-neon-aid-loxp *CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A rbd2Δ::loxP dsc2Δ::loxp*	7F	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6600	(riboswitch)-mCherry-Vps10-neon-aid-loxp *CUP1pr*-Skp1-TIR1 *pep4*Δ *pdr5*Δ *pup1-T30A pre3-T20A ddi1Δ::loxp rbd2Δ::loxP dsc2Δ::loxp*	7F	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6661	mCherry-Vps10-NeonGreen-AID *CUP1pr*-Skp1-TIR1 *pep4Δ pdr5Δ pup1-T30A pre3-T20A cdc48-2(A547T, T803A)*	S6G	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6665	mCherry-Vps10-NeonGreen-AID *CUP1pr*-Skp1-TIR1 *pep4Δ pdr5Δ pup1-T30A pre3-T20A cdc48-3(P257L, R387K)*	S6G	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6622	pTef*Riboswitch-mCherry-Vps10_sfGFP_DHFR_loxp MHY1178 pep4Δ prd5Δ::cup1-yskp1-tir1-loxp	5B,5C,7A,7C, 7D	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6624	pTef*Riboswitch-mCherry-Vps10_sfGFP_DHFR_loxp MHY1178 pep4Δ prd5Δ::cup1-yskp1-tir1-loxp ddi1Δloxp	5B	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6628	pTef*Riboswitch-mCherry-Vps10_NG_sfGFP_loxp MHY1178 pep4Δ prd5Δ::cup1-yskp1-tir1-loxp rbd2Δ	5B,5C,7C,7D	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6635	pTef*Riboswitch-mCherry-Vps10_NG_sfGFP_loxp MHY1178 pep4Δ prd5Δ::cup1-yskp1-tir1-loxp ddi1Δloxp rbd2Δ	7F	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6670	cdc48-3 MHY1178 pMet25-mCherry-Vps10-neon-aid-loxp-KanMX pep4Δ prd5Δ::cup1-yskp1-tir1-loxp ddi1Δloxp rbd2Δ::natMXloxP	S6H	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6671	cdc48-3 MHY1178 pMet25-mCherry-Vps10-neon-aid-loxp-KanMX pep4Δ prd5Δ::cup1-yskp1-tir1-loxp ddi1Δloxp rbd2Δ::natMXloxP	S6H	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY6672	cdc48-3 MHY1178 pMet25-mCherry-Vps10-neon-aid-loxp-KanMX pep4Δ prd5Δ::cup1-yskp1-tir1-loxp ddi1Δloxp rbd2Δ::natMXloxP	S6H	MHY1178: *pdr5Δ::CUP1pr-SKP1-TIR1::loxP pep4Δ*	This study
PLY5909	MUP1-NEON-AID-kURA integrated at Mup1 locus	S6F	BY4742 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	This study
PLY5910	MUP1-NEON-AID-kURA integrated at Mup1 locus pep4Δ :: KanR	S6F	BY4742 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	This study

Supplementary Table 2. Plasmids used in this study

Plasmid Nam	**Markers**	**Description**	Figures	**Reference/Source**
pRS316	amp *URA3*	pBluescript, CEN6, ARSH4, *URA3*	4B,7A, S6E	9
pRS315	amp *LEU2*	pBluescript, CEN6, ARSH4, *LEU2*	S4A, S4B	9
pRS313	amp *HIS3*	pBluescript, CEN6, ARSH4, *HIS3*		9
pRS303	amp *HIS3*	pBluescript, *HIS3*		9
pPL7364	amp *HIS3*	GFPnb: pRS313 bicistronic plasmid with TET-on 3G transactivator under control of Tef* promoter, and second ORF with 4xHA epitope tagged GFP nanobody under control of minimal PDR3 promoter and 8 tetO sites.	1C	This study
pPL7349	amp *HIS3*	GFPnb-Rsp5 HECT: pPL7364 expressing 4xHA-GFPnb fused to residues 421-809 of *Sc* Rsp5 (NP_011051.3)	1C,1E,1G	This study
pPL7383	amp *HIS3*	GFPnb-1xPib1 RING: pPL7364 expressing 4xHA-GFPnb fused to residues 123-279 of *S.pombe* Pib1 (NP_595987.1)	1C,1E	This study
pPL7366	amp *HIS3*	GFPnb-2xRNF4 RING: pPL7364 expressing 4xHA-GFPnb fused to residues 75-194 of RnRNF4 (NP_062055.1) and residues 128-190 of HsRNF4 (NP_002929.1)	1C,1E,7A,7D	This study
pPL7377	amp *HIS3*	GFPnb-Rsp5/E6AP HECT chimera: pPL7364 expressing 4xHA-GFPnb fused to residues 421-689 of ScRsp5 (NP_011051.3) and residues 325-435 of HsE6AP (NP_001341480.1)	1C,1E	This study
pCHL642	amp *URA3*	pRS416 expressing Mup1-GFP from MUP1 promoter		10
pPL7449	amp *LEU2*	Ddi1-HA: pRS315 with *Sc DDI1* (residues 1-428) carrying single C-terminal HA epitope	3C,4F,S4A,S4B	This study
pPL7457	amp *LEU2*	Ddi1-HA-D220A: pPL7449 carrying a D220A codon change	3C,4F,S4A	This study
pPL7459	amp *LEU2*	Ddi1ΔUBA-HA: pRS315 with *Sc DDI1* (residues 1-391) carrying single C-terminal HA epitope		This study
pPL7460	amp *LEU2*	Ddi1ΔUbl-HA: pRS315 with *Sc DDI1* (residues 80-428) carrying single C-terminal HA epitope	3C,4F,S4A	This study
pPL7504	amp *LEU2*	Ddi1ΔUblΔUBA-HA: pRS315 with *Sc DDI1* (residues 190-390) carrying single C-terminal HA epitope	3C,4F,S4A	This study
pPL7585	amp *LEU2*	Ddi1Ubl*ΔUBA-HA: pPL7459 with E8A and I13A mutations to block Ubl~Ub binding	3C,4F,S4A	This study
pPL7495	amp *LEU2*	*TEF1*-Ddi1-HA: pRS315 with *Sc Ddi1* (residues 1-428) carrying single C-terminal HA epitope under control of the *TEF1* promoter		This study
pPL7588	amp *LEU2*	*TEF1*-HsDDI1-HA: pRS315 with human Ddi1 (residues 3-396: NP_001001711.1) carrying single C-terminal HA epitope under control of th *TEF1* promoter	3C,4F,S4A	This study
pPL7589	amp *LEU2*	*TEF*-HsDDI2-HA: pRS315 with human Ddi2 (residues 3-398: NP_115717.3) carrying single C-terminal HA epitope under control of th *TEF1* promoter	3C,4F,S4A	This study
pPL7591	amp *LEU2*	*TEF1*-Ddi1 (Dsk2Ubl, ΔUBA)-HA: pRS315 with Hs Dsk2 (residues 26-103: NP_038472.2) and Sc Ddi1 (residues 81-370) with single C-terminal HA epitope under control of the *TEF1* promoter	3C,4F,S4A	This study
pPL7592	amp *LEU2*	*TEF1*-Ddi1 (Rad23Ubl, ΔUBA)-HA: pRS315 with *Sc*Rad23 (residues 1-75) and Sc Ddi1 (residues 81-370) with single C-terminal HA epitope under control of the *TEF1* promoter	3C,4F,S4A	This study
pPL7590	amp *LEU2*	*TEF1*-*S. pombe* Ddi1-HA: pRS315 with *S.pombe* Ddi1/Mud1 (residues 1-332, NP_001018195.1) with single C-terminal HA epitope under control of the *TEF1* promoter	3C,4F,S4A	This study
pPL7568	amp *URA3*	RBD2-GFP: pRS316 carrying RBD2 promoter and coding sequence to GFP and the PHO8 3'UT.	4B,4E	This study
pPL7584	amp *URA3*	RBD2(S124A)-GFP: pPL7568 with S124A mutation.	4B	This study
pPL6634	amp *HIS3*	Sec7-mCherry	4E	This study
pPL7710	amp	pET21A expressing 6xHIS ScDdi1 HDD-RVP domain (residues 142-325)	3D,3E,S5A,S5C,S5E	This study
pPL8078	kanr	pET28b expressing 6xHIS HsDdi2 HDD-RVP domain (residues 134-359; NP_115717.3)	3D,3E,S5B,S5D,S5E	This study
pPL8048	amp *LEU2*	prs315 carrying mCherry-ScYLR001cNeonGreenAID	8B	This study
pPL8049	amp *LEU2*	prs315 carrying mCherry-ScYnd1-NeonGreenAID	8B	This study
pPL8042	amp *LEU2*	prs315 carrying mCherry-ScPex3NeonGreenAID	8B	This study
pPL8047	amp *LEU2*	prs315 carrying mCherry-ScFet5NeonGreenAID	8B	This study
pPL6739	amp *HIS3*	prs303 carrying CUP1pr-SKP1-TIR1 integrates into PDR5 locus		This study
pPL7141	amp *KanMX*	pPL6739 *HIS3* marker switched to *loxP-KanMX-loxP*		This study
pPL8237	amp URA3	loops in cdc48-2 mutations with XhoI cut and integration (A547T, T803A). Deletes N-terminal region from endogenous CDC48		This study
pPL8241	amp URA3	loops in cdc48-3 mutations with XhoI cut and integration (P257L, R387K). Deletes N-terminal region from endogenous CDC48		This study
pTS18	amp URA3	PEP4 on URA3 plasmid		This study
pPL7672		Full Length yDdi1-HA/pRS315 I183A	S4B	This study
pPL7673		Full Length yDdi1-HA/pRS315 D184A	S4B	This study
pPL7674		Full Length yDdi1-HA/pRS315 E176A	S4B	This study
pPL7675		Full Length yDdi1-HA/pRS315 L177A	S4B	This study
pPL5293		pET21a for production of recombinant Ubiquitin (amp)	3D,3E,S5A,S5B,S5C,S5D,S5E	11

Supplementary Table 3: Antibodies used in this study

Antibody	Species	Source	Notes
α-NeonGreen	Rabbit polyclonal	Cell Signaling Technology Cat#53061S	This antibody was discontinued by the manufacturer
α-NeonGreen	Rabbit polyclonal	Cell Signaling mNeonGreen Tag (E6M3D) Rabbit mAb #41236	This antibody performs better than CTS#53061S. Used at a diltuion of 1:1000-1:2000
α-GFP D5.1	Rabbit monoclonal	Cell Signaling Technology Cat#2956S	Used at a diltuion of 1:1000-1:2000
α-HA	Mouse monoclonal	BioLegend, San Diego, CA	Used at a diltuion of 1:1000-1:2000
α-PGK	Mouse monoclonal	Molecular Probes Cat# A-6457, RRID:AB_22154	Used at a diltuion of 1:1000-1:2000
α-mCherry	Rabbit polyclonal	Sigma Aldrich #AB356482 Lot 3536102	Rabbit polyclonal α-mCherry was incubated with lysate from *pep1*Δ *pep4-3* (PLY684) to diminish non-specific binding, the pattern of non-specific binding varied between antibody batches. The effective concentration used as ~1:1000.
α-mCherry E5D8f	Rabbit monoclonal	(#43590S, Cell Signaling Technology	Used at a diltuion of 1:1000-1:2000
α-Vps10	Rabbit polyclonal (raised against aa 306-696	ref. 7	Kept as serum and subtracted against yeast extract made from vps10Δ pep4Δ yeast. Effective dilution was 1:1000
α-CPY	Rabbit polyclonal	Tom Stevens, University of Oregon	Used at a diltuion of 1:1000-1:2000
α-ALP	Rabbit polyclonal	Tom Stevens, University of Oregon	Used at a diltuion of 1:1000-1:2000
α-K48Ub	Rabbit monoclonal	Cell Signaling Technology Cat#8081	Used at a diltuion of 1:1000

Supplementary Table 4: Strains and plasmids used in each figure

Figure	Yeast Strains and Plasmids
Figure 1C	PLY2166: VPS10-GFP
	pPL7364 α-GFPnb-HA only (Ø)
	pPL7349 α-GFPnb-HA-Rsp5 HECT
	pPL7383 α-GFPnb-HA-1xPib RING
	pPL7366 α-GFPnb-HA-2xRNF4 RING
	pPL7377 α-GFPnb-HA-Rsp5/E6AP chimeric HECT
Figure 1E	PLY2166: VPS10-GFP
	PLY6264: VPS10-GFP pep4Δ
	pPL7349 α-GFPnb-HA-Rsp5 HECT
	pPL7383 α-GFPnb-HA-1xPib RING
	pPL7366 α-GFPnb-HA-2xRNF4 RING
	pPL7377 α-GFPnb-HA-Rsp5/E6AP chimeric HECT
Figure 1D	PLYL6018: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ
Figure 1F	PLYL6018: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ
	PLY6022: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ
Figure 1G	BY4742: Parental Strain (PLY5818)
	pPL7349 α-GFPnb-HA-Rsp5 HECT
	pCHL642 Mup1-GFP
Figure 2A	PLY6022: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ
	PLY6072: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ
	PLY6069: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ::LEU2 pup1-T30A pre3-T20A
Figure 2B	PLY6173: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ
	PLY6022: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ
	PLY676: vps10Δ
Figure 2C	PLY6312: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ vps35Δ
	PLY6173: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ
Figure 2D	PLY6173: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ
Figure 3A	PLY6072: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ
	PLY6069: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ::LEU2 pup1-T30A pre3-T20A
	PLY6197: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pdr5Δ
	yPL6199: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pdr5Δ pup1-T30A pre3-T20A
Figure 3B	PLY6454: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 WT_Ub pdr5Δ
	PLY6456: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K6R_Ub pdr5Δ
	PLY6458: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K11R_Ub pdr5Δ
	PLY6460: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K27R_Ub pdr5Δ
	PLY6462: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K29R_Ub pdr5Δ
	PLY6464: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K33R_Ub pdr5Δ
	PLY6468: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K48R_Ub (low WT_Ub) pdr5Δ
	PLY6466: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K63R_Ub pdr5Δ
Figure 3C	yPL6199: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pdr5Δ pup1-T30A pre3-T20A
	pPL7449: Ddi1-HA
	pPL7457: Ddi1-HA (D220A)
	pPL7460: Ddi1ΔUbl-HA
	pPL7504: Ddi1ΔUblΔUBA-HA
	pPL7585: Ddi1Ubl*ΔUBA-HA
	pPL7595: TEF1-Ddi1-HA
	pPL7588: TEF1-human DDI1-HA
	pPL7589: TEF1-human DDI2-HA
	pPL7591: TEF1-Ddi1 (Dsk2Ubl, ΔUBA)-HA
	pPL7592: TEF1-Ddi1 (Rad23Ubl, ΔUBA)-HA
	pPL7590: TEF1-S. pombeDdi1-HA
Figure 3D	pPL7710: expresses recombinant ScDdi1 HDD-RVP
	pPL8078: expresses human DDI2 HDD-RVP
	pPL5293: expresses recombinant Ub
Figure 3E	pPL7710: expresses recombinant ScDdi1 HDD-RVP
	pPL8078: expresses human DDI2 HDD-RVP
	pPL5293: expresses recombinant Ub
Figure 3F	PLY6226: VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ
	PLY6653: VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ ddi1Δ
Figure 4F	yPL6199: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pdr5Δ pup1-T30A pre3-T20A
	pPL7449: Ddi1-HA
	pPL7457: Ddi1-HA (D220A)
	pPL7460: Ddi1ΔUbl-HA
	pPL7504: Ddi1ΔUblΔUBA-HA
	pPL7585: Ddi1Ubl*ΔUBA-HA
	pPL7595: TEF1-Ddi1-HA
	pPL7588: TEF1-human DDI1-HA
	pPL7589: TEF1-human DDI2-HA
	pPL7591: TEF1-Ddi1 (Dsk2Ubl, ΔUBA)-HA
	pPL7592: TEF1-Ddi1 (Rad23Ubl, ΔUBA)-HA
	pPL7590: TEF1-S. pombe Ddi1-HA
	pRS315
Figure 4A	PLY6173: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ

PLY6249: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆
PLY6251: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6392: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1∆ pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6266: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆
PLY6369: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 rbd2∆::natMXloxP pep4∆ pdr5∆
PLY6312: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ vps35∆
PLY6359: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ vps35∆ rbd2∆

Figure 4B PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6394: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ rbd2∆ pup1-T30A pre3-T20A
pPL7568: RBD2-GFP
pRS316
pPL7584: RBD2(S124A)-GFP

Figure 4C PLY6266: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆
PLY6369: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 rbd2∆::natMXloxP pep4∆ pdr5∆

Figure 4E BY4742: Parental Strain (PLY5818)
PLY6450: mCherry-Vps10 pep4∆
pPL6634: Sec7-mCherry
pPL7568: RBD2-GFP

Figure 5A PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6392: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1∆ pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6394: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ rbd2∆ pup1-T30A pre3-T20A
PLY6396: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ rbd2∆ ddi1∆ pup1-T30A pre3-T20A

Figure 5B PLY6622: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6624: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A ddi1∆
PLY6626: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆
PLY6628: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆ ddi1∆

Figure 5C PLY6622: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6628: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆ ddi1∆

Figure 6A PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6396: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ rbd2∆ ddi1∆ pup1-T30A pre3-T20A

Figure 6B PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6396: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ rbd2∆ ddi1∆ pup1-T30A pre3-T20A
pTS18: PEP4

Figure 7A PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6622: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6626: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆
pPL7366 α-GFPnb-HA-2xRNF4 RING
pRS316

Figure 7B PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6392: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1∆ pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6394: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ rbd2∆ pup1-T30A pre3-T20A
PLY6396: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ rbd2∆ ddi1∆ pup1-T30A pre3-T20A

Figure 7C PLY6622: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6628: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆ ddi1

Figure 7D PLY6622: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6626: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆
PLY6628: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆ ddi1∆
PLY6628: (riboswitch)mCherry-Vps10-sfGFP-DHFR CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆ ddi1∆
pPL7366 α-GFPnb-HA-2xRNF4 RING

Figure 7E PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6396: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ rbd2∆ ddi1∆ pup1-T30A pre3-T20A

Figure 7F PLY6572: (riboswitch)-mCherry-Vps10-neon-aid-loxp CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6596: (riboswitch)-mCherry-Vps10-neon-aid-loxp CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A dsc2∆::loxp
PLY6474: (riboswitch)-mCherry-Vps10-neon-aid-loxp CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆::loxP
PLY6598: (riboswitch)-mCherry-Vps10-neon-aid-loxp CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆::loxP dsc2∆::loxp
PLY6635: (riboswitch)-mCherry-Vps10-neon-aid-loxp CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A ddi1∆::loxp rbd2∆::loxP
PLY6600: (riboswitch)-mCherry-Vps10-neon-aid-loxp CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A ddi1∆::loxp rbd2∆::loxP dsc2∆::loxp

Figure 8A PLY6183: CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A

Figure 8B PLY6183: CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A
PLY6195: CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A ddi1∆
PLY6564: CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆
PLY6420: CUP1pr-Skp1-TIR1 pep4∆ pdr5∆ pup1-T30A pre3-T20A rbd2∆ ddi1∆
pPL8048: mCherry-ScYLR001cNeonGreenAID
pPL8049: mCherry-ScYnd1-NeonGreenAID
pPL8042: mCherry-ScPex3NeonGreenAID
pPL8047: pRS315 carrying mCherry-ScFet5NeonGreenAID

SFig1A PLY6225: VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5∆ pep4∆ pup1-T30A pre3-T20A
PLY6226: VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5∆ pep4∆ PUP1 PRE3

SFig1B PLY6225: VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5∆ pep4∆ pup1-T30A pre3-T20A
PLY6226: VPS10pr-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5∆ pep4∆ PUP1 PRE3

SFig1C PLYL6018: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5∆

	PLY6022: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ
SFig1D	PLY6173: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ PLY6312: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ vps35Δ
SFig2A	PLY6134: tul1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6144: gld1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6154: ddi1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6132: ape4Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6130: ydr415cΔ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6126: rbd2Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6148: ste24Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6138: mca1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6128: yps7Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6140: dsc3Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6150: vld1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6146: rim13Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6142: yps5Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6136: ypf1Δ Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ
SFig2A	PLY6038: cdc48-2 Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6039: cdc48-3 Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ PLY6018: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pdr5Δ
SFig3A	PLY6454: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 WT_Ub pdr5Δ PLY6456: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K6R_Ub pdr5Δ PLY6458: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K11R_Ub pdr5Δ PLY6460: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K27R_Ub pdr5Δ PLY6462: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K29R_Ub pdr5Δ PLY6464: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K33R_Ub pdr5Δ PLY6468: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K48R_Ub (low WT_Ub) pdr5Δ PLY6466: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K63R_Ub pdr5Δ
SFig3B	PLY6454: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 WT_Ub pdr5Δ PLY6456: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K6R_Ub pdr5Δ PLY6458: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K11R_Ub pdr5Δ PLY6460: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K27R_Ub pdr5Δ PLY6462: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K29R_Ub pdr5Δ PLY6464: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K33R_Ub pdr5Δ PLY6468: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K48R_Ub (low WT_Ub) pdr5Δ PLY6466: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K63R_Ub pdr5Δ
SFig3C	PLY6454: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 WT_Ub pdr5Δ PLY6456: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K6R_Ub pdr5Δ PLY6458: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K11R_Ub pdr5Δ PLY6460: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K27R_Ub pdr5Δ PLY6462: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K29R_Ub pdr5Δ PLY6464: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K33R_Ub pdr5Δ PLY6468: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K48R_Ub (low WT_Ub) pdr5Δ PLY6466: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 K63R_Ub pdr5Δ
SFig4A	PLY6199: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pdr5Δ pup1-T30A pre3-T20A pPL7449: Ddi1-HA pPL7457: Ddi1-HA (D220A) pPL7460: Ddi1ΔUbl-HA pPL7504: Ddi1ΔUblΔUBA-HA pPL7585: Ddi1Ubl*ΔUBA-HA pPL7595: TEF1-Ddi1-HA pPL7588: TEF1-human DDI1-HA pPL7589: TEF1-human DDI2-HA pPL7591: TEF1-Ddi1 (Dsk2Ubl, ΔUBA)-HA pPL7592: TEF1-Ddi1 (Rad23Ubl, ΔUBA)-HA pPL7590: TEF1-S. pombeDdi1-HA pRS315
SFig4B	PLY6199: Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 ddi1Δ pdr5Δ pup1-T30A pre3-T20A pPL7449: Ddi1-HA pRS315 pPL7672: yDdi1-HA I183A pPL7673: yDdi1-HA I183A pPL7674: yDdi1-HA E176A pPL7675: yDdi1-HA E177A
SFig5 A	pPL7710: expresses recombinant ScDdi1 HDD-RVP pPL5293: expresses recombinant Ub
SFig5C	pPL7710: expresses recombinant ScDdi1 HDD-RVP pPL5293: expresses recombinant Ub
SFig5E	pPL7710: expresses recombinant ScDdi1 HDD-RVP pPL5293: expresses recombinant Ub
SFig5B	pPL8078: expresses human DDI2 HDD-RVP pPL5293: expresses recombinant Ub
SFig5D	pPL8078: expresses human DDI2 HDD-RVP pPL5293: expresses recombinant Ub
SFig5E	pPL8078: expresses human DDI2 HDD-RVP pPL5293: expresses recombinant Ub

SFig6A	PLY6414: CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ PUP1 PRE3
	PLY6416: CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ PUP1 PRE3 ddi1Δ
	PLY6418: CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ PUP1 PRE3 rbd2Δ
	PLY6420: CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ PUP1 PRE3 ddi1Δ rbd2Δ
SFig6B	PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A
	PLY6396: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ rbd2Δ ddi1Δ pup1-T30A pre3-T20A
SFig6C	PLY6368: vps10Δ::VPS10pr-GFP-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A
SFig6D	PLY6448: Vps10-GFP CUP1pr-Skp1-TIR1 pdr5Δ pep4Δ
SFig6E	PLY6251: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A
	pTS18
	pRS316
SFig6F	PLY5909: MUP1-NEON-AID-kURA integrated at Mup1 locus
	PLY5910: MUP1-NEON-AID-kURA integrated at Mup1 locus pep4Δ :: KanR
SFig6G	PLY6390: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A
	PLY6661: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A cdc48-2
	PLY6665: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A cdc48-3
SFig6H	PLY6396: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A rbd2Δ ddi1Δ
	PLY6670: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A rbd2Δ ddi1Δ cdc48-3 (a)
	PLY6671: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A rbd2Δ ddi1Δ cdc48-3 (b)
	PLY6672: mCherry-Vps10-NeonGreen-AID CUP1pr-Skp1-TIR1 pep4Δ pdr5Δ pup1-T30A pre3-T20A rbd2Δ ddi1Δ cdc48-3 (c)

Supplementary Note: Statistical outcomes
Description of statistical tests for all main and Supplementary figures, including exact p-values for comparisons labelled in figures. n = number of independent biological replicates of yeast strains indicated in Supplementary Table 1 & 4

Figure 1E. For Fig. 1E (n = 4 for Rsp5-HECT; n = 3 for others), one-way ANOVA with Holm–Šidák–corrected two-sided t-tests showed: Rsp5-HECT, F(3,12) = 23.23, p< 0.0001 (*1 < 0.0001; *2 = 0.0009); Pib1-RING FL, F(3,8) = 7.378, p= 0.0108 (*3 = 0.0253; ns4 = 0.3376); Rsp5-E6AP-HECT FL, F(3,8) = 216.9, p< 0.0001 (*5 < 0.0001; *6 < 0.0001); RNF4-RING FL, F(3,8) = 481.4, p< 0.0001 (*7 < 0.0001; *8 < 0.0001); Rsp5-HECT Vac-GFP, F(3,12) = 13.41, p= 0.0004 (*9 = 0.0007; ns10 > 0.9999); Pib1-RING Vac-GFP, F(3,8) = 8.143, p= 0.0082 (*11 = 0.0104; ns12 > 0.9999); Rsp5-E6AP-HECT Vac-GFP, F(3,8) = 1.721, p= 0.2396 (ns13 = 0.2042; ns14 > 0.9999); RNF4-RING Vac-GFP, F(3,8) = 2.587, p< 0.0001 (ns15 = 0.9789; ns16 > 0.9999).

Figure 1F. For Fig. 1F (n = 5; normalized to untreated controls), two-sided one-sample t-tests gave ns1: t(4) = 2.141, p= 0.0990; *2: t(4) = 8.696, p= 0.0010; ns3: t(4) = 2.151, p= 0.0979; *4: t(4) = 10.55, p= 0.0005.

Figure 2A. For Fig. 2A (n = 3), ANOVA with Holm–Šidák post-tests showed NG FL, F(8,18) = 52.45, p< 0.0001 (*1–*6 all < 0.0001; ns7 = 0.5442; ns8 = 0.2452; ns9 = 0.5442) and NG Clip, F(8,18) = 32.30, p< 0.0001 (ns10 = 0.9667; ns11 = 0.9667; ns12 = 0.1929; *13 = 0.0002; *14 = 0.0002; *15 < 0.0001; ns16 = 0.5340; *17 = 0.0002; ns18 < 0.0001).

Figure 2B. For Fig. 2B (n = 4 for α-Vps10 blots; n = 3 for others), ANOVA with Holm–Šidák correction or Welch's t-tests showed: NG FL, F(3,8) = 163.1, p< 0.0001 (*1 < 0.0001; *2 < 0.0001); Vps10 FL, F(3,10) = 464.3, p< 0.0001 (*3 < 0.0001; *4 < 0.0001); RFP FL, *5: t(3.918) = 25.27, p< 0.0001; NG Clip, F(3,8) = 27.14, p= 0.0002 (*6 = 0.0040; *7 = 0.0004); Vps10 Clip, F(3,10) = 14.99, p= 0.0005 (*8 = 0.0021; *9 = 0.0121); RFP Clip, *10: t(2.832) = 5.160, p= 0.0243.

Figure 3A. For Fig. 3A (n = 3), ANOVA with Holm–Šidák correction showed NG FL, F(11,33) = 75.14, p< 0.0001 (*1–*4 < 0.0001; ns5 = 0.3556; ns6 = 0.3556) and NG Clip, F(11,36) = 10.32, p< 0.0001 (*7 = 0.0001; ns8 = 0.1834; *9 < 0.0001; ns10 = 0.7158; ns11 = 0.0611; *12 < 0.0001).

Figure 3B. For Fig. 3B (n = 6–10 depending on strain), two-sided one-sample t-tests gave: *1: t(5) = 4.111, p= 0.0093; *2: t(6) = 3.650, p= 0.0107; ns3: t(8) = 0.9854, p= 0.3533; ns4: t(6) = 0.7472, p= 0.4832; ns5: t(7) = 1.289, p= 0.2383; ns6: t(9) = 2.211, p= 0.0544; ns7: t(5) = 1.023, p= 0.3534; *8: t(5) = 7.227, p= 0.0008; ns9: t(6) = 0.4721, p= 0.6536; *10: t(8) = 7.607, p< 0.0001; ns11: t(6) = 0.6867, p= 0.5179; ns12: t(7) = 1.172, p= 0.2794; ns13: t(9) = 2.236, p= 0.0522; ns14: t(5) = 1.963, p= 0.1068.

Figure 3C. For Fig. 3C (n = 3–8 depending on construct), two-sided one-sample t-tests yielded: *1: t(2) = 14.04, p= 0.0050; ns2: t(2) = 0.1565, p= 0.8900; *3: t(7) = 6.469, p= 0.3533; *4: t(5) = 14.74, p< 0.0001; *5: t(3) = 3.662, p= 0.0352; ns6: t(4) = 1.570, p= 0.1916; *7: t(2) = 116.8, p< 0.0001; *8: t(2) = 39.78, p= 0.0006; ns9: t(4) = 1.475, p= 0.2143; ns10: t(4) = 0.7938, p= 0.4717; ns11: t(2) = 0.8937, p= 0.4658.

Figure 3F. For Fig. 3F (n = 3–4), ANOVA with Holm–Šidák tests showed for NG FL (left), F(7,16) = 213.3, p< 0.0001 (*1–*4 < 0.0001; *5 = 0.0033); for NG Ddi1-Clip (left), F(7,16) = 7.817, p= 0.0003 (ns6 = 0.7774; *7 = 0.0002; ns8 = 0.9990; ns9 = 0.9990; *10 = 0.0002); for NG FL (right), F(3,12) = 3.102, p= 0.0672 (ns11 = 0.1442; ns12 = 0.1817; ns13 = 0.9345); for NG High MW (right), F(3,12) = 6.983, p= 0.0057 (*14 = 0.0077; ns15 = 0.0538).

Figure 4B. For Fig. 4B (n = 3–4), ANOVA with Holm–Šidák tests showed RFP FL, F(11,33) = 175.9, p< 0.0001 (*1 < 0.0001; ns2 = 0.1193; *3 < 0.0001; *4 = 0.028; *5–*8 < 0.0001; ns9 = 0.2636) and RFP Rbd2-Clip, F(11,33) = 17.72, p< 0.0001 (*10 < 0.0002; ns11–ns13 = 0.8402, 0.9951, 0.9951; *14 = 0.0004; ns15 = 0.9951; *16 = 0.0043; ns17 = 0.9951; *18–*20 < 0.0001; ns21 = 0.9951).

Figure 4C. For Fig. 4C (n = 3), Welch's t-test gave t(3.921) = 3.205, p= 0.0261.

Figure 5A. For Fig. 5A (n = 3–4), ANOVA with Holm–Šidák tests showed: NG High MW, F(11,24) = 7.295, p< 0.0001 (*8 = 0.0034; others ns); NG FL, F(11,24) = 29.99, p< 0.0001 (*9–*16 < 0.0001); NG Ddi1-Clip, F(11,24) = 11.76, p< 0.0001 (*21 < 0.0001; *23 < 0.0001; others ns); RFP High MW, F(11,36) = 19.03, p< 0.0001 (*32 < 0.0001; others ns); RFP FL, F(11,36) = 62.22, p< 0.0001 (*33–*40 < 0.0001); RFP Rbd2-Clip, F(11,36) = 12.23, p< 0.0001 (*41–*42 < 0.0001; *45 < 0.0001; others ns).

Figure 5B. For Fig. 5B ($n = 3$–4), ANOVA with Holm–Šidák tests showed: GFP High MW, $F(11,36) = 16.94$, $p < 0.0001$ (*2 = 0.0156; *6 < 0.0001; *8 < 0.0001; others ns); GFP FL, $F(11,36) = 25.15$, $p < 0.0001$ (*9–*16 < 0.0001); GFP Ddi1-Clip, $F(11,36) = 11.95$, $p < 0.0001$ (*21 < 0.0001; *23 < 0.0001; others ns); RFP High MW, $F(11,24) = 16.70$, $p < 0.0001$ (*28 = 0.0422; *32 < 0.0001; others ns); RFP FL, $F(11,24) = 109.2$, $p < 0.0001$ (*33–*40 < 0.0001); RFP Rbd2-Clip, $F(11,24) = 24.29$, $p < 0.0001$ (*41–*42 < 0.0001; *45 < 0.0001; *46 = 0.0250; others ns).

Figure 5C. For Fig. 5C ($n = 4$–5), ANOVA with Holm–Šidák tests showed: GFP High MW, $F(7,28) = 20.23$, $p < 0.0001$ (*3 = 0.0002; *4 = 0.0056; others ns); GFP FL, $F(7,28) = 15.32$, $p < 0.0001$ (*5–*8 all significant); GFP Ddi1-Clip, $F(7,28) = 5.545$, $p = 0.0004$ (*9 = 0.0056; *10 = 0.0056; others ns); RFP High MW, $F(7,28) = 15.22$, $p < 0.0001$ (*15 < 0.0001; *16 = 0.0012; others ns); RFP FL, $F(7,28) = 17.51$, $p < 0.0001$ (*17–*20 all significant); RFP Rbd2-Clip, $F(7,28) = 7.213$, $p < 0.0001$ (*21–*22 = 0.0018; others ns).

Figure 6A. For Fig. 6A ($n = 3$), one-sample t-tests of total NG or RFP gave NG: ns1: $t(2) = 0.8319$, $p = 0.4930$; ns2: $t(2) = 1.070$, $p = 0.3965$; ns3: $t(2) = 2.233$, $p = 0.1551$; ns4: $t(2) = 1.965$, $p = 0.1884$; RFP: *17: $t(2) = 9.348$, $p = 0.0112$; *18: $t(2) = 8.516$, $p = 0.0135$; *19: $t(2) = 12.77$, $p = 0.0061$; ns20: $t(2) = 2.315$, $p = 0.1466$. ANOVA with Holm–Šidák tests showed: NG High MW, $F(7,16) = 20.34$, $p < 0.0001$ (*7 = 0.0011; *8 < 0.0001; others ns); NG FL, $F(7,16) = 70.69$, $p < 0.0001$ (*9–*12 all significant); NG Ddi1-Clip, $F(7,16) = 39.96$, $p = 0.0001$ (*13–*14 < 0.0001; others ns); RFP High MW, $F(7,16) = 48.61$, $p < 0.0001$ (*23 = 0.0090; *24 < 0.0001; others ns); RFP FL, $F(7,16) = 70.69$, $p < 0.0001$ (*25–*28 all significant); RFP Rbd2-Clip, $F(7,16) = 22.09$, $p = 0.0001$ (*29 = 0.0038; *30 < 0.0001; others ns).

Figure 7A. For Fig. 7A, degradation assays were performed with $n = 3$ for SCF^{TIR1}-induced degradation of RFP-Vps10-NG-AID, $n = 5$ for SCF^{TIR1}-induced degradation of RFP-Vps10-sfGFP-DHFR-AID, and $n = 3$ for RNF4-RING–dependent degradation in RBD2 or Δrbd2 cells.

Figure 7B. For Fig. 7B ($n = 3$), ANOVA with Holm–Šidák tests gave $F(11,24) = 54.68$, $p < 0.0001$, with ns1 = 0.8876, ns2 > 0.9999, ns3 = 0.1794, ns4 > 0.9999, *5 = 0.0435, ns6 > 0.9999, *7 < 0.0001, ns8 > 0.9999, and *9 < 0.0001.

Figure 7C. For Fig. 7C ($n = 3$), ANOVA with Holm–Šidák tests gave $F(11,24) = 47.41$, $p < 0.0001$, with ns1 = 0.1892, ns2 > 0.9999, *3 = 0.0043, *4 < 0.0001, ns5 > 0.9999, *6 = 0.0003, ns7 > 0.9999, *8 < 0.0001, ns9 > 0.9999, and *10 < 0.0001.

Figure 7D. For Fig. 7D ($n = 5$ WT; $n = 4$ *Δddi1Δrbd2*), ANOVA with Holm–Šidák tests gave $F(7,32) = 222.3$, $p < 0.0001$, with *1 < 0.0001, *2 < 0.0001, ns3 > 0.9999, ns4 > 0.9999.

Figure 7E. For Fig. 7E ($n = 3$), ANOVA with Holm–Šidák tests gave $F(7,16) = 9.580$, $p < 0.0001$, with *1 = 0.0144, *2 = 0.0001, ns3 > 0.9999, ns4 > 0.9999.

Supplementary Figure 1A. For Supplementary Fig. 1A ($n = 3$), ANOVA with Holm–Šidák tests gave NG FL, $F(15,32) = 16.84$, $p < 0.0001$ (*1 = 0.0418; *2 = 0.0418) and NG Clip, $F(15,32) = 14.61$, $p < 0.0001$ (*1 < 0.0001; *2 < 0.0001).

Supplementary Figure 3B. For Supplementary Fig. 3B ($n = 6$–10), two-sided one-sample t-tests showed ns1: $t(5) = 0.6470$, $p = 0.5461$; *2: $t(6) = 13.30$, $p < 0.0001$; *3: $t(8) = 2.477$, $p = 0.0383$; ns4: $t(6) = 0.2782$, $p = 0.7902$; *5: $t(7) = 2.677$, $p = 0.0317$; *6: $t(9) = 4.470$, $p = 0.0016$; *7: $t(5) = 2.931$, $p = 0.0326$.

Supplementary Figure 6B. For Supplementary Fig. 6B ($n = 6$), two-sided one-sample t-tests showed *1: $t(5) = 28.97$, $p < 0.0001$; *2: $t(5) = 40.91$, $p < 0.0001$; *3: $t(5) = 16.12$, $p < 0.0001$; *4: $t(5) = 9.964$, $p = 0.0002$.

Supplementary Discussion: References documenting K48-ubiquitination or proteasomal degradation of post-ER proteins.

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