Cell Chemical Biology

Supplemental Information

Regulation of the Endocytosis and Prion Chaperoning Machineries by Yeast E3 Ubiquitin Ligase Rsp5 as Revealed by Orthogonal Ubiquitin Transfer

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Supplemental Figures

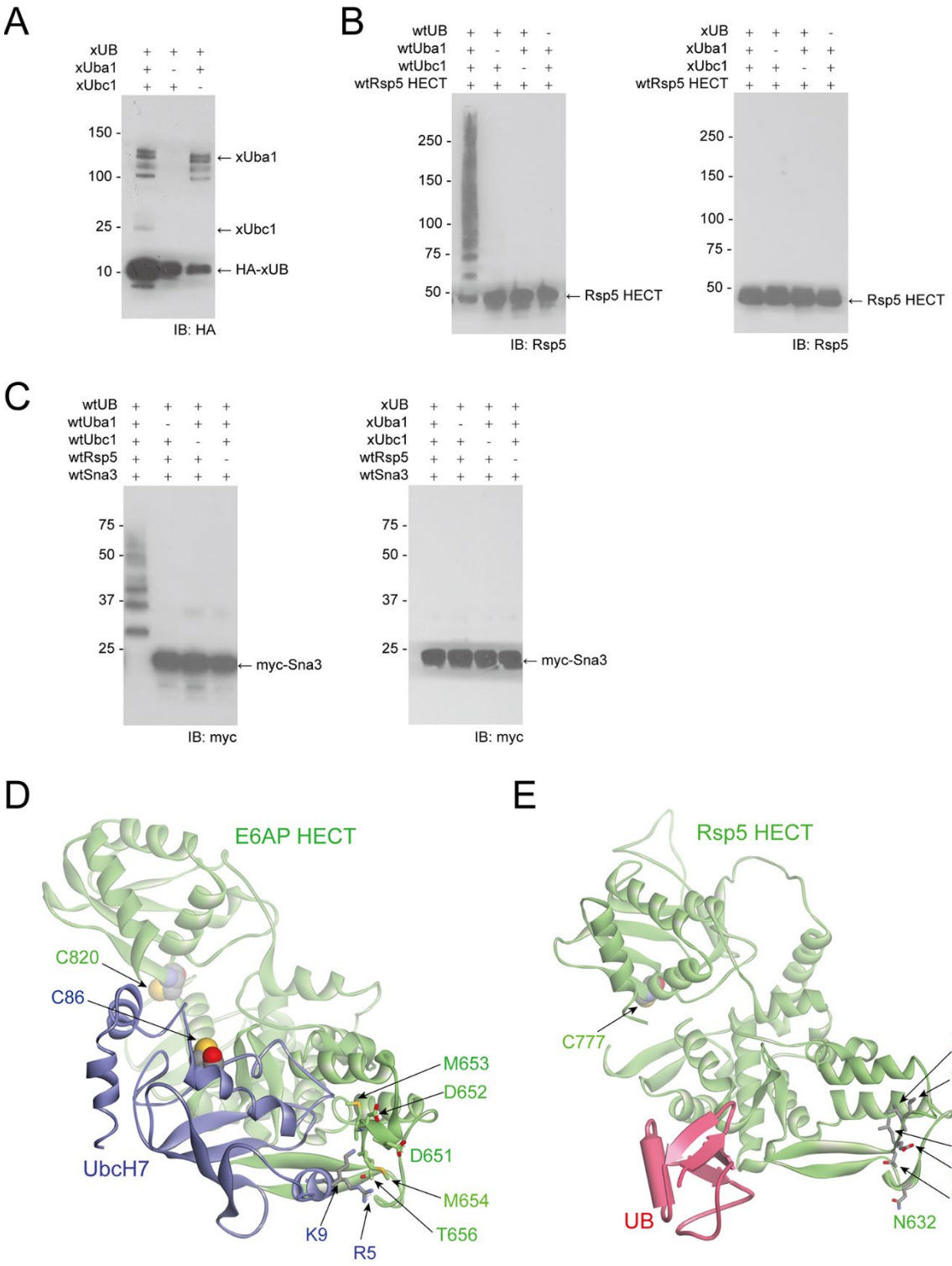


Figure S1. The activity of the xUba1-xUbc1 pair with wt Rsp5 in the transfer of xUB and the design of the Rsp5 HECT domain library for yeast cell surface display, Related to Figure 1. (A) HA-tagged xUB can be loaded onto xUba1 and xUbc1 with the K5E and K9D mutations in the N-terminal helix. (B) The HECT domain of Rsp5 can be actively auto-ubiquitinated with the transfer of HA-wt UB from the wt Uba1-UbcH7 pair while auto-ubiquitination does not happen with the transfer of HA-xUB from the xUba1-xUbc1 pair. (C) Sna3, a known Rsp5 substrate can be actively poly-ubiquitinated with the HA-wtUB transfer from the wt Uba1-Ubc1-Rsp5 cascade while the ubiquitination reaction cannot happen with the crossover cascade of xUba1-xUbc1-wt Rsp5. (D) and (E), Structural analysis of the E2-HECT interface. (D) Crystal structure of the E6AP HECT in complex with UbcH7 (PDB ID 1C4Z) (Huang et al., 1999). Residues D651, D652, M653, M654 and T656 constitute a loop that interacts with R5 and K9 that sit on the N-terminal helix of UbcH7. (E) Crystal structure of the Rsp5 HECT domain in complex with UB (PDB ID 3OLM) (Kim et al., 2011). Residues V606, L607, D608. L609, T610 constitute a loop that would interact with the N-terminal helix of the E2 enzymes. N632 of Rsp5 HECT may also contribute to the interaction with the N-terminal helix of E2.

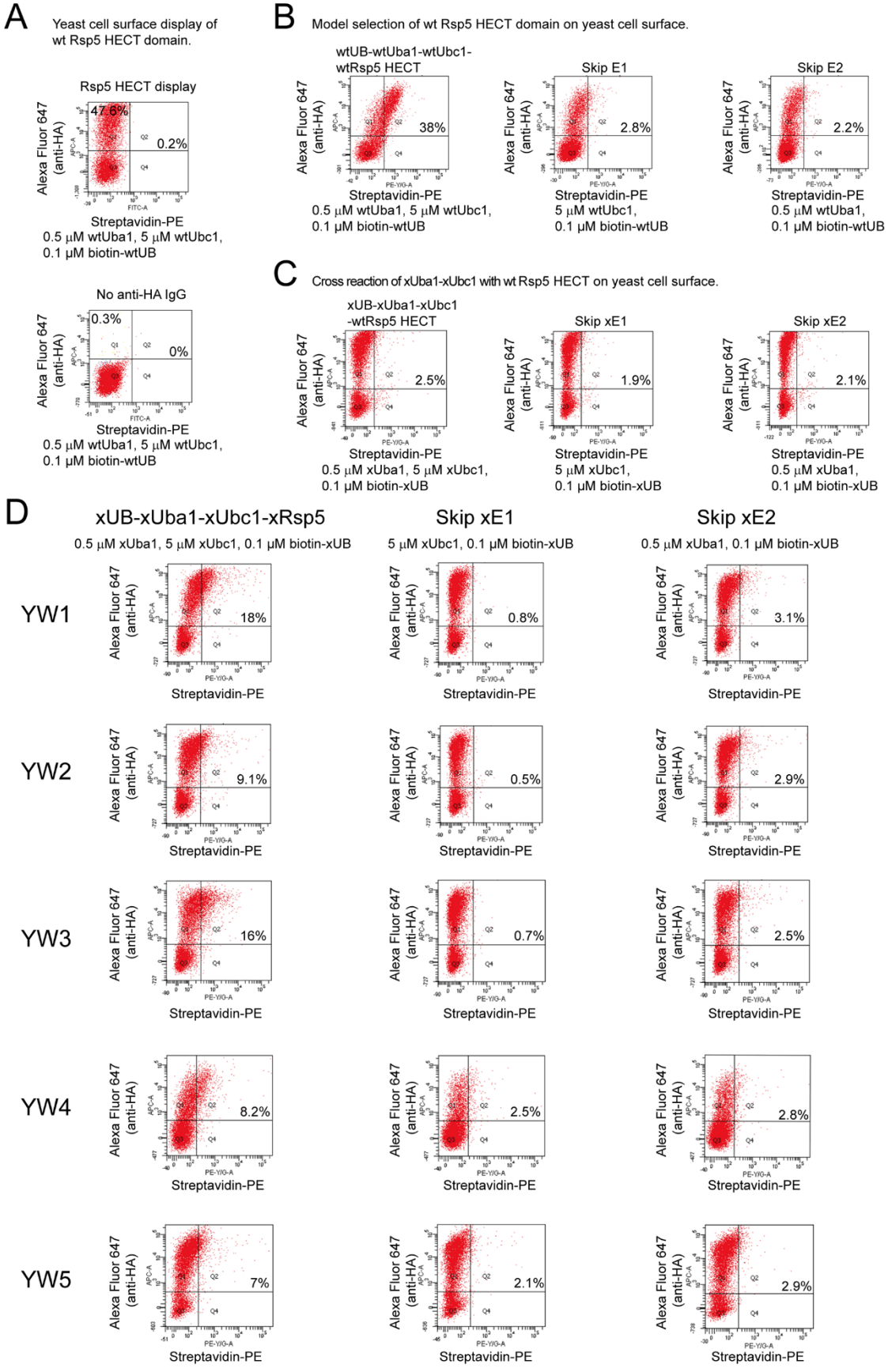

A
Yeast cell surface display of wt Rsp5 HECT domain.
Rsp5 HECT display
47.6%
0.2%
Alexa Fluor 647 (anti-HA)
Streptavidin-PE
0.5 μM wtUba1, 5 μM wtUbc1, 0.1 μM biotin-wtUB
No anti-HA IgG
0.3%
0%
Streptavidin-PE
0.5 μM wtUba1, 5 μM wtUbc1, 0.1 μM biotin-wtUB
B
Model selection of wt Rsp5 HECT domain on yeast cell surface.
wtUB-wtUba1-wtUbc1-wtRsp5 HECT
38%
Streptavidin-PE
0.5 μM wtUba1, 5 μM wtUbc1, 0.1 μM biotin-wtUB
Skip E1
2.8%
Streptavidin-PE
5 μM wtUbc1, 0.1 μM biotin-wtUB
Skip E2
2.2%
Streptavidin-PE
0.5 μM wtUba1, 0.1 μM biotin-wtUB
C
Cross reaction of xUba1-xUbc1 with wt Rsp5 HECT on yeast cell surface.
xUB-xUba1-xUbc1 -wtRsp5 HECT
2.5%
Streptavidin-PE
0.5 μM xUba1, 5 μM xUbc1, 0.1 μM biotin-xUB
Skip xE1
1.9%
Streptavidin-PE
5 μM xUbc1, 0.1 μM biotin-xUB
Skip xE2
2.1%
Streptavidin-PE
0.5 μM xUba1, 0.1 μM biotin-xUB
D
xUB-xUba1-xUbc1-xRsp5
0.5 μM xUba1, 5 μM xUbc1, 0.1 μM biotin-xUB
Skip xE1
5 μM xUbc1, 0.1 μM biotin-xUB
Skip xE2
0.5 μM xUba1, 0.1 μM biotin-xUB
YW1
18%
0.8%
3.1%
YW2
9.1%
0.5%
2.9%
YW3
16%
0.7%
2.5%
YW4
8.2%
2.5%
2.8%
YW5
7%
2.1%
2.9%

Figure S2. Verifying the efficiency of the yeast cell display platform for selecting catalytically active HECT domains and confirming the activity of the selected xHECT clones in xUB transfer, Related to Figure 2. (A) Yeast cells could display the wt Rsp5 HECT domain with high abundance. The cells were labeled with streptavidin-PE and the pair of mouse anti-HA antibody /anti-mouse IgG – Alexa Fluor 647 conjugate and sorted along the PE and Alexa Fluor 647 axis. Percentages in the panels designate the fractions of yeast cells counted in the Q2 region with positive fluorescent signals along both axes. (B) Yeast cells display the wt HECT domain of Rsp5 were reacted with biotin-wt UB, wt Uba1 and wt Ubc1. Cells were then labeled with fluorophores as in (A) and sorted. Strong shift of cell population (38%) was observed on both the PE and Alexa Fluor 647 axis, suggesting the transfer of biotin-UB to wt HECT domain displayed on the yeast cell surface. When wt Uba1 or Ubc1 was skipped in the reaction mixture, cells were only labelled with Alexa Fluor 647 suggesting the UB transfer reaction was dependent on both Uba1 and Ubc1. Such results demonstrated the high efficiency of the yeast sorting method to enrich catalytically active HECT domain clones based on the transfer of biotin-UB from Ubc1 to HECT. (C) Yeast cells displaying wt Rsp5 HECT domain were reacted with biotin-xUB and the xUba1-xUbc1 pair. Cells were labeled with fluorophores and sorted as in (A). No transfer of biotin-xUB to the wt HECT domain was observed suggesting the orthogonality of the wt HECT with the xUba1-xUbc1 pair so xUB would not be transferred from xUbc1 to the wt HECT domain of Rsp5. (D) Verifying the activities of selected xHECT domain clones in xUB transfer. Yeast cells displaying individual mutants of YW1-5 was cultured separately and cells were added to the reaction mixture containing biotin-xUB and xUba1-xUbc1 pair to allow xUB transfer to the HECT domain on cell surface, while either xUba1 or xUbc1 were eliminated in the control reactions. Cells were then washed and dual labeled with streptavidin-PE and the antibody pairs of anti-HA IgG and anti-IgG Alexa Flour 647. Sorting of the cells from the control and UB transfer reactions revealed that all selected clones demonstrate significant shift on the PE axis, suggesting that the Rsp5 HECT domain clones from yeast selection pairs with xUba1-xUbc1 for the transfer of xUB. In contrast, no shift of cell population on the PE axis was observed when either xUba1 or xUbc1 were omitted from the reaction. These results demonstrated that yeast selection based on catalytic turnover of

the UB transfer cascade successfully yielded catalytically active mutants of the Rsp5 HECT domain for pairing with xUbc1 in the OUT cascade.

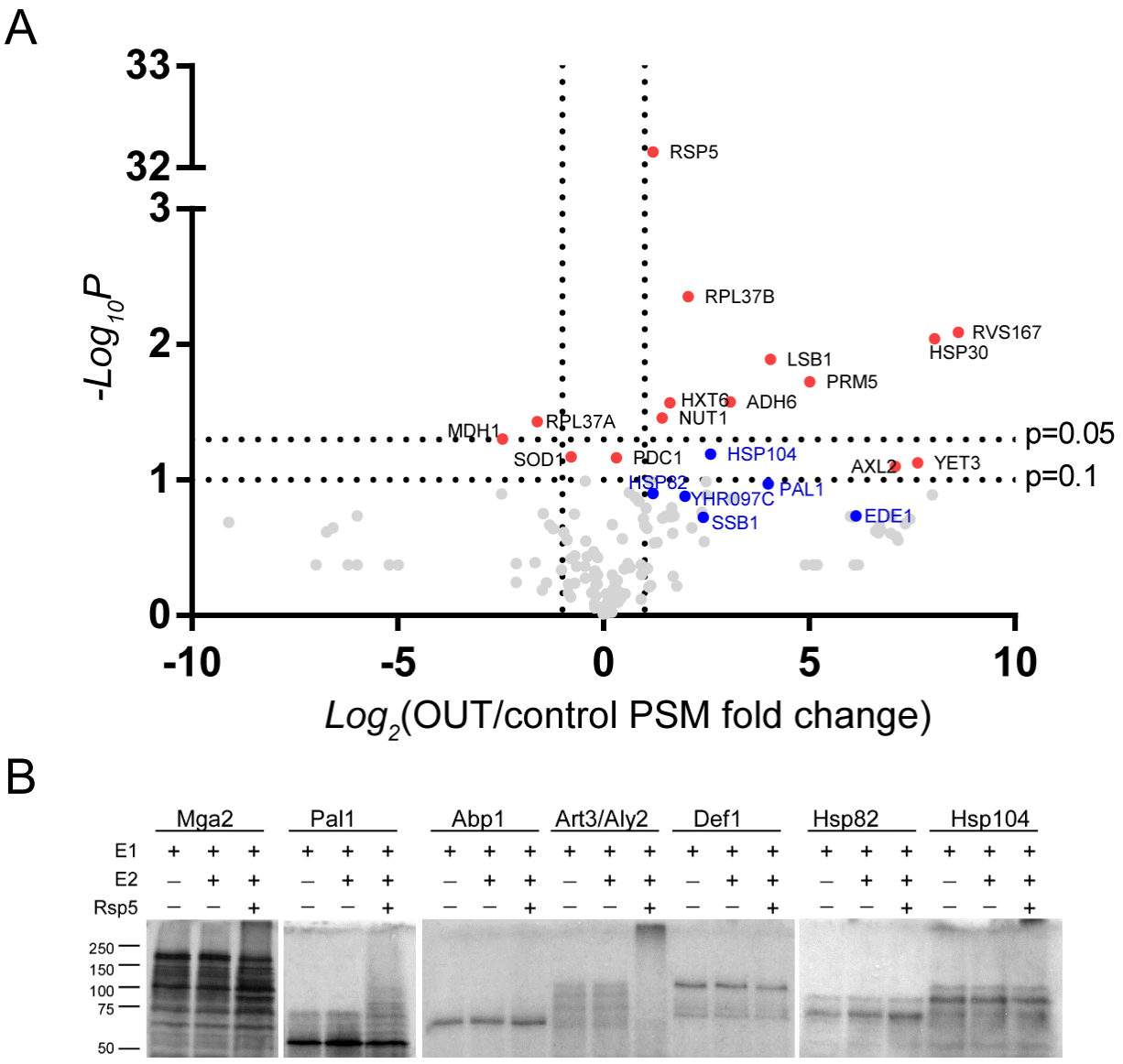
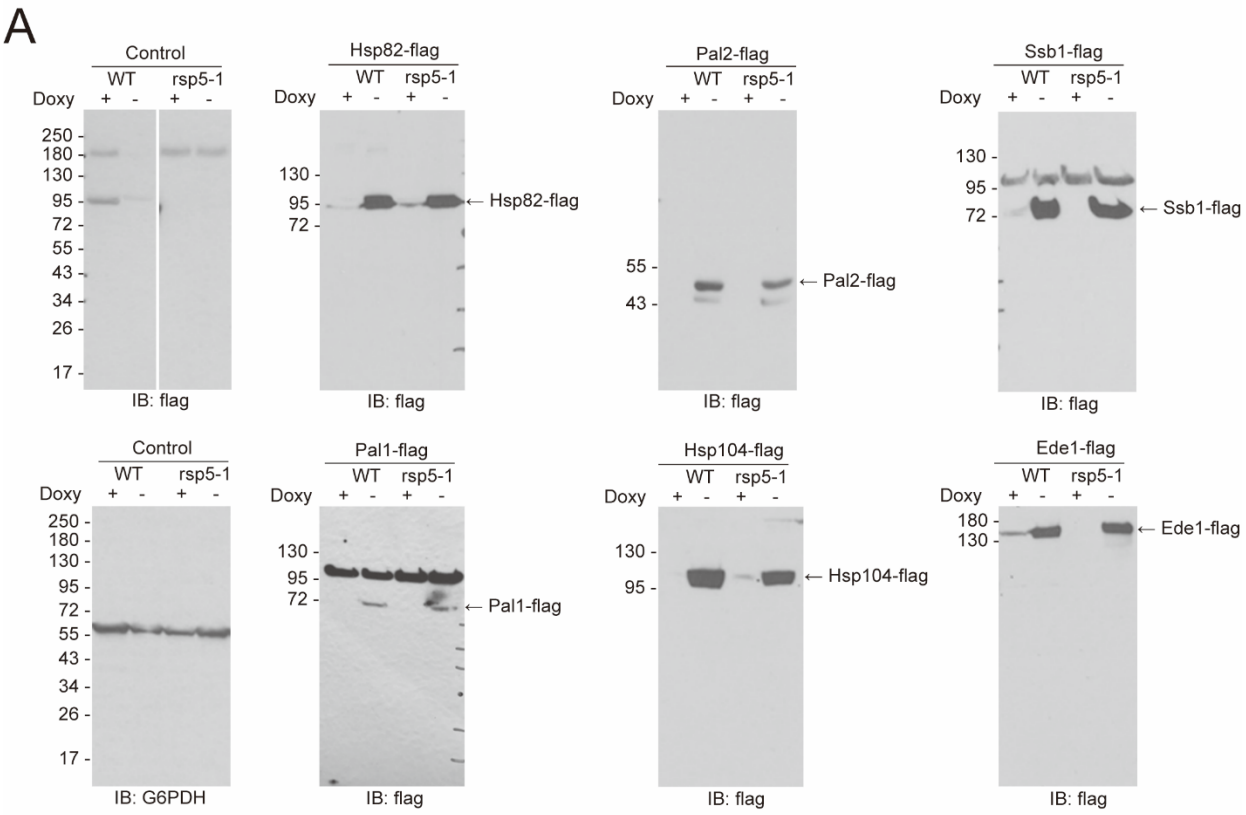


Figure S3. Volcano plot of the proteins identified by the Rsp5 OUT cascade and the *in vitro* ubiquitination assays to verify Rsp5 substrates with ^{35}S-labeled proteins, Related to Figure 3 and 4. (A) The average fold changes of PSM numbers (-log_2[OUT/control PSM fold change]) of proteins purified from three repeats of tandem purifications from OUT and control cells were plotted against the *P* values (-$log_{10}P$) of the corresponding proteins in the Volcano plot. Experimental PSM values were normalized on the average Rsp5 PSM value per sample group (6.67 for control samples and 15.33 for OUT samples). A two tailed paired sample *t*-test was then used to calculate the *P* value. Red dots designate proteins with *P*

< 0.1. Blue dots designate proteins characterized as Rsp5 substrates in this study. (B) Assaying substrate ubiquitination by Rsp5 by *in vitro* ubiquitination assays with ^{35}S-labeled substrates. The substrate proteins were prepared by *in vitro* protein translation in rabbit reticulocytes in the presence of [^{35}S-Met]. wt UB, wt recombinant Uba1, Ubc1 and Rsp5 were added into the reaction mixture, reaction products were resolved by SDS-PAGE and signal was visualized by autoradiography. Known Rsp5 substrate Mga2 was used as a positive control. Pal1 and Art3/Aly3 from the OUT screen were confirmed to be ubiquitinated by Rsp5 while Abp1, Def1, Hsp82 and Hsp104 from the OUT screen were not found to be directly ubiquitinated by Rsp5 *in vitro*.


A
Control
WT rsp5-1
Doxy + - + -
250
180
130
95
72
55
43
34
26
17
IB: flag
Hsp82-flag
WT rsp5-1
Doxy + - + -
130
95
72
← Hsp82-flag
IB: flag
Pal2-flag
WT rsp5-1
Doxy + - + -
55
43
← Pal2-flag
IB: flag
Ssb1-flag
WT rsp5-1
Doxy + - + -
130
95
72
← Ssb1-flag
IB: flag
Control
WT rsp5-1
Doxy + - + -
250
180
130
95
72
55
43
34
26
17
IB: G6PDH
Pal1-flag
WT rsp5-1
Doxy + - + -
130
95
72
← Pal1-flag
IB: flag
Hsp104-flag
WT rsp5-1
Doxy + - + -
130
95
← Hsp104-flag
IB: flag
Ede1-flag
WT rsp5-1
Doxy + - + -
180
130
← Ede1-flag
IB: flag

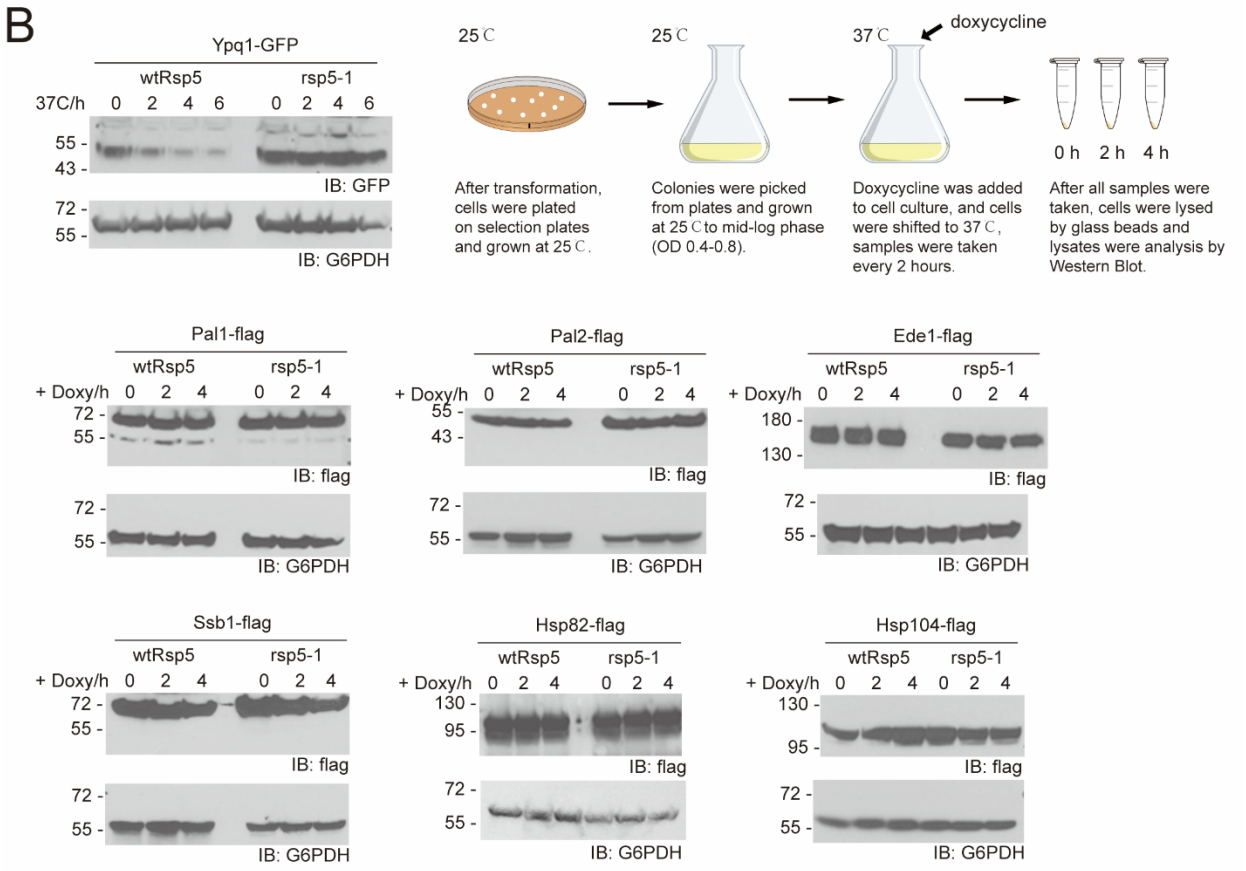

B
Ypq1-GFP
wtRsp5 rsp5-1
37C/h 0 2 4 6 0 2 4 6
55
43
IB: GFP
72
55
IB: G6PDH
25 C
25 C
37 C
doxycycline
0 h 2 h 4 h
After transformation, cells were plated on selection plates and grown at 25 C.
Colonies were picked from plates and grown at 25 C to mid-log phase (OD 0.4-0.8).
Doxycycline was added to cell culture, and cells were shifted to 37 C, samples were taken every 2 hours.
After all samples were taken, cells were lysed by glass beads and lysates were analysis by Western Blot.
Pal1-flag
wtRsp5 rsp5-1
+ Doxy/h 0 2 4 0 2 4
72
55
IB: flag
72
55
IB: G6PDH
Pal2-flag
wtRsp5 rsp5-1
+ Doxy/h 0 2 4 0 2 4
55
43
IB: flag
72
55
IB: G6PDH
Ede1-flag
wtRsp5 rsp5-1
+ Doxy/h 0 2 4 0 2 4
180
130
IB: flag
72
55
IB: G6PDH
Ssb1-flag
wtRsp5 rsp5-1
+ Doxy/h 0 2 4 0 2 4
72
55
IB: flag
72
55
IB: G6PDH
Hsp82-flag
wtRsp5 rsp5-1
+ Doxy/h 0 2 4 0 2 4
130
95
IB: flag
72
55
IB: G6PDH
Hsp104-flag
wtRsp5 rsp5-1
+ Doxy/h 0 2 4 0 2 4
130
95
IB: flag
72
55
IB: G6PDH

Figure S4. Rsp5 expression does not induce the degradation of the substrate proteins identified by OUT, Related to Figure 5. (A) Controlling the expression of substrate proteins in the yeast cells with a Tet-Off system. Genes of the substrates to be verified were cloned after the Tet-Off promoter in the pMC189 plasmid. The plasmids were then transformed into the SEY6210.1 cells expressing wt Rsp5 and YXY705 cells expressing Rsp5-1, a temperature sensitive mutant of Rsp5 with an L733S mutation in the HECT domain. Colonies growing up on selective plates were incubated in 5 ml media with or without 2 μg/ml doxycycline at 25°C for overnight. Cells were collected and lysed, and the same amount of total protein of each sample was analyzed by Western blot. Empty pMC189 plasmid was used as control. (B) Substrates stability assay. Cells were grown in selective media at 25°C to mid-log phase (OD_{600} 0.4-0.6) and prewarmed to 37°C before the addition of doxycycline to a final concentration of 2 μg/ml to shut down the expression of the substrate proteins under the regulation of the Tet-Off promoter. Cells in the amount corresponding to seven OD units were collected every 2 hours from time 0. Cells were lysed and analyzed by Western blot. G6PDH was used as loading control. Positive control Ypq1-GFP was expressed under endogenous promoter, and its degradation was observed when the cell was shifted to 37°C as previously reported (Li et al., 2015). The addition of doxycycline and the temperature switch did not affect yeast cell growth as previously reported (Wishart et al., 2005).

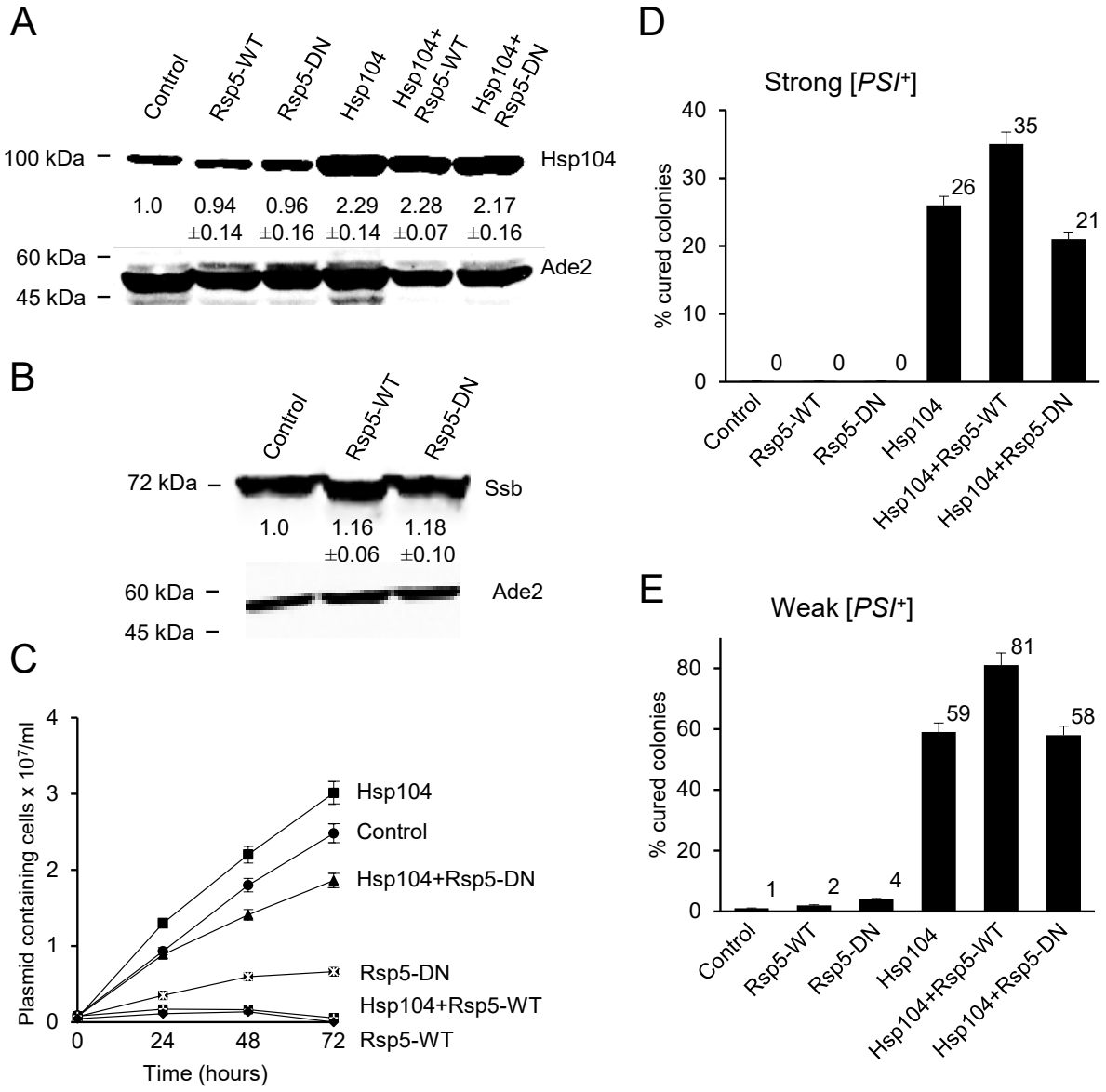


Figure S5. Hsp levels, growth kinetics and [*PSI*$^+$] loss in yeast strains in various yeast cultures with overexpression or inactivation of Rsp5, Related to Figure 6. (A) and (B). Hsp levels. Levels of Hsp104 (A) or Ssb (B) were detected in yeast cultures of the strain OT56 producing Rsp5-WT or Rsp5-DN, in comparison to control, after growing for 48 hours in galactose medium selective for the plasmids where Rsp5 constructs (and in panel A, Hsp104 where indicated) are induced. Representative SDS-PAGE gels reacted to either anti-Hsp104 (A) or anti-Ssb (B) antibodies, or to anti-Ade2 antibodies (used as a loading control) are shown. Numbers under the Hsp104 (A) or Ssb (B) bands indicate intensities of bands relative

to control, as determined by densitometry and normalized by the Ade2 levels. Numbers on the left indicate the positions of molecular weight markers. (C) Quantitation of plasmid retention in the weak [*PSI*$^{+}$] strain (OT55) during the growth in the liquid Gal-Ura-Leu medium. Procedure and designations are the same as for the strong [*PSI*$^{+}$] strain on Figure 6B (main text). Average concentrations of viable plasmid-containing cells in each culture are shown. Error bars indicate SDs. Numbers are shown in Table S3. (D) and (E) [*PSI*$^{+}$] loss after growth on solid medium. Cells from the cultures containing either strong (strain OT56, D) or weak (strain OT 55, E) variant of the [*PSI*$^{+}$] prion and bearing the Rsp5-WT, Rsp5-DN, and/or Hsp104 constructs expressed from the galactose-inducible promoter, as indicated, and/or respective control plasmids, were grown on solid Gal-Ura-Leu medium (selective for the plasmids) for three days, and streaked out for single colonies on the glucose -Ura-Leu medium, followed by velveteen replica plating onto the YPD and -Ade media for the detection of [*PSI*$^{+}$] prion. At least three independent cultures were analyzed per each strain/plasmid combination. Average percentages of colonies originated from cells that have lost [*PSI*$^{+}$] are indicated. Error bars depict SDs. Numbers are shown in Table S4.

Supplemental Tables

Table S1. Mutations in xUB, xUba1, xUbc1, and xRsp5 employed for the assembly of the OUT cascade of Rsp5, Related to Figure 1.

xUB (yeast)	R42E, R72E
xE1	
xUba1 (yeast)	Q576R, S589R, D591R, E1004K, D1014K, E1016K
xE2	
xUbc1 (yeast)	K5D, K9E
xE3	
xRsp5 (YW3)	V606I, D608N, T610K, N632V

Table S2. Plasmid loss after growth on solid galactose medium, Related to Figure 6.

Strain	Construct	Number of colonies*		Plasmid loss (%) ± SD
		Ura$^-$ Leu$^-$	Total	
OT56 (strong [*PSI*$^+$]) **	Control	48	432	10.4 ± 5.1
	Rsp5-WT	156	198	78.0 ± 4.0
	Rsp5-DN	177	249	71.1 ± 2.7
	Hsp104	78	402	19.3 ± 2.1
	Hsp104 + Rsp5-WT	162	222	72.8 ± 4.3
	Hsp104 + Rsp5-DN	153	339	45.0 ± 4.0
OT55 (weak [*PSI*$^+$])	Control	57	351	16.2 ± 1.9
	Rsp5-WT	150	168	90.0 ± 3.6
	Rsp5-DN	177	234	76.2 ± 3.8
	Hsp104	69	420	16.8 ± 5.2
	Hsp104 + Rsp5-WT	216	315	68.3 ± 4.7
	Hsp104 + Rsp5-DN	189	384	49.2 ± 4.2

* Three cultures were analyzed per each strain/construct combination.

** Data for Figure 6A.

Table S3. Concentrations of plasmid-containing cells in cultures growing in liquid galactose medium, Related to Figures 6 and S5.

Strain	Construct	Concentration of plasmid-containing cells per ml (± SD)*			
		0 hrs	24 hrs	48 hrs	72 hrs
OT56 (strong [*PSI*$^+$]) **	Control	9.91(±1.20)x10^5	3.98(±0.37)x10^6	1.37(±0.10)x10^7	2.24(±0.14)x10^7
	Rsp5-WT	9.06(±0.83)x10^5	5.72(±0.85)x10^5	3.44(±0.68)x10^5	< 10
	Rsp5-DN	6.84(±0.83)x10^5	1.09(±0.14)x10^6	2.49(±0.25)x10^6	2.50(±0.43)x10^6
	Hsp104	5.97(±0.50)x10^5	8.66(±0.74)x10^6	1.87(±0.08)x10^7	3.01(±0.35)x10^7
	Hsp104 + Rsp5-WT	5.69(±0.34)x10^5	6.64(±0.88)x10^5	1.22(±0.22)x10^6	1.73(±0.33)x10^6
	Hsp104 + Rsp5-DN	6.13(±0.61)x10^5	1.52(±0.20)x10^6	1.07(±0.04)x10^7	1.98(±0.20)x10^7
OT55 (weak [*PSI*$^+$]) ***	Control	5.66(±0.84)x10^5	9.31(±0.43)x10^6	1.80(±0.12)x10^7	2.48(±0.11)x10^7
	Rsp5-WT	4.66(±1.00)x10^5	1.10(±0.10)x10^6	1.36(±0.06)x10^6	1.13(±0.28)x10^3
	Rsp5-DN	7.47(±0.72)x10^5	3.52(±0.31)x10^6	5.97(±0.28)x10^6	6.63(±0.45)x10^6
	Hsp104	8.78(±0.37)x10^5	1.30(±0.07)x10^7	2.22(±0.05)x10^7	3.01(±0.14)x10^7
	Hsp104 + Rsp5-WT	7.88(±0.69)x10^5	1.71(±0.25)x10^6	1.64(±0.51)x10^6	5.63(±4.10)x10^5
	Hsp104 + Rsp5-DN	7.06(±0.84)x10^5	8.85(±0.68)x10^6	1.41(±0.08)x10^7	1.86(±0.04)x10^7

* Three cultures were analyzed per each time point for each strain/construct combination.

** Data for Figure 6B.

*** Data for Figure S5C.

Table S4. [*PSI*$^+$] loss after growth on solid galactose medium, Related to Figures 6 and S5.*

Strain	Induced construct	Number of colonies		Average [*PSI*$^+$] loss (%) ± SD
		[*psi*$^-$]	Total	
OT56 (strong [*PSI*$^+$]) **	Control	0	228	0
	Rsp5-WT	0	68	0
	Rsp5-DN	0	163	0
	Hsp104	81	307	26.3 ± 4.5
	Hsp104 + Rsp5-WT	28	81	34.5 ± 3.3
	Hsp104 + Rsp5-DN	35	170	20.5 ± 4.9
OT55 (weak [*PSI*$^+$]) ***	Control	3	345	0.8 ± 0.7
	Rsp5-WT	4	213	1.8 ± 1.6
	Rsp5-DN	10	248	4.0 ± 1.0
	Hsp104	237	399	59.3 ± 5.5
	Hsp104 + Rsp5-WT	231	285	81.0 ± 4.2
	Hsp104 + Rsp5-DN	189	326	57.9 ± 3.5

* Three cultures were analyzed for each strain/construct combination.

** Data for Figure S5D.

***Data for Figure S5E.

Table S5. [*PSI*$^+$] loss after growth in liquid galactose medium in the strong [*PSI*$^+$] strain OT56, Related to Figure 6.*

Time (hrs)	Protein	Number of colonies		Average [*PSI*$^+$] loss (%) ± SD
		[*psi*$^-$]	Total	
0	Control	0	951	0
	Rsp5-WT	0	868	0
	Rsp5-DN	0	661	0
	Hsp104	0	575	0
	Hsp104 + Rsp5-WT	0	547	0
	Hsp104 + Rsp5-DN	0	588	0
24	Control	0	451	0
	Rsp5-WT	0	182	0
	Rsp5-DN	0	417	0
	Hsp104	277	831	33.2 ± 3.7
	Hsp104 + Rsp5-WT	105	256	40.9 ± 3.7
	Hsp104 + Rsp5-DN	61	292	20.6 ± 5.3
48	Control	0	820	0
	Rsp5-WT	0	66	0
	Rsp5-DN	0	685	0
	Hsp104	337	897	37.5 ± 2.6
	Hsp104 + Rsp5-WT	115	235	48.9 ± 4.2
	Hsp104 + Rsp5-DN	226	1,023	22.0 ± 2.5
72	Control	0	858	0
	Rsp5-WT	0	0	0
	Rsp5-DN	0	119	0
	Hsp104	383	959	39.8 ± 5.4
	Hsp104 + Rsp5-WT	75	110	67.4 ± 6.6
	Hsp104 + Rsp5-DN	207	758	27.6 ± 5.7

* Data for Figure 6C, with the addition of data for Rsp5-WT and Rsp5-DN without Hsp104; three cultures were analyzed for each strain/protein combination.

Table S6. [*PSI*$^+$] induction after galactose induction on solid galactose medium in the [*psi*$^-$ *PIN*$^+$] strain, Related to Figure 6.*

Induced construct	Number of colonies		Average [*PSI*$^+$] induction (%) ± SD
	[*PSI*$^+$]	Total	
Control	4	585	0.7 ± 0.3
Sup35N	244	1037	23.8 ± 2.8
Sup35N + Rsp5-WT	5	648	0.8 ± 1.1**
Sup35N + Rsp5-DN	37	336	11.4 ± 3.4***

* Data for Figure 6F; results are obtained in the [*psi*$^-$ *PIN*$^+$] strain, originated from GT81-1C; three cultures were analyzed for each strain/construct combination.

** Differences from Sup35N are statistically significant ($P_{Ho} < 0.0005$).

*** Differences from Sup35N are statistically significant ($0.005 < P_{Ho} < 0.01$).