Supporting Information

Two Alternative Mechanisms Regulate the Onset of Chaperone-mediated Assembly of the Proteasomal ATPases

Asrafun Nahar[1], Xinyi Fu[1], George Polovin[1], James D. Orth[1] and Soyeon Park[1,*]

Supporting Information contains 11 Figures and 1 Table.

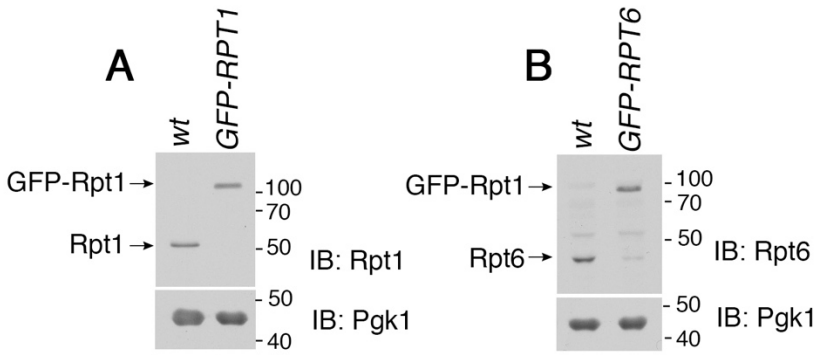

Figure S1. Chromosomally GFP-tagged Rpt subunits are expressed at the same level as untagged Rpt subunits in wild-type cells.
A, B, To confirm that expression levels of chromosomal GFP-Rpt subunits are identical to those of untagged Rpt subunits, whole cell extracts (20 μg) were subjected to 10% Bis-Tris SDS-PAGE and immunoblotting for indicated proteins. Pgk1, loading control.

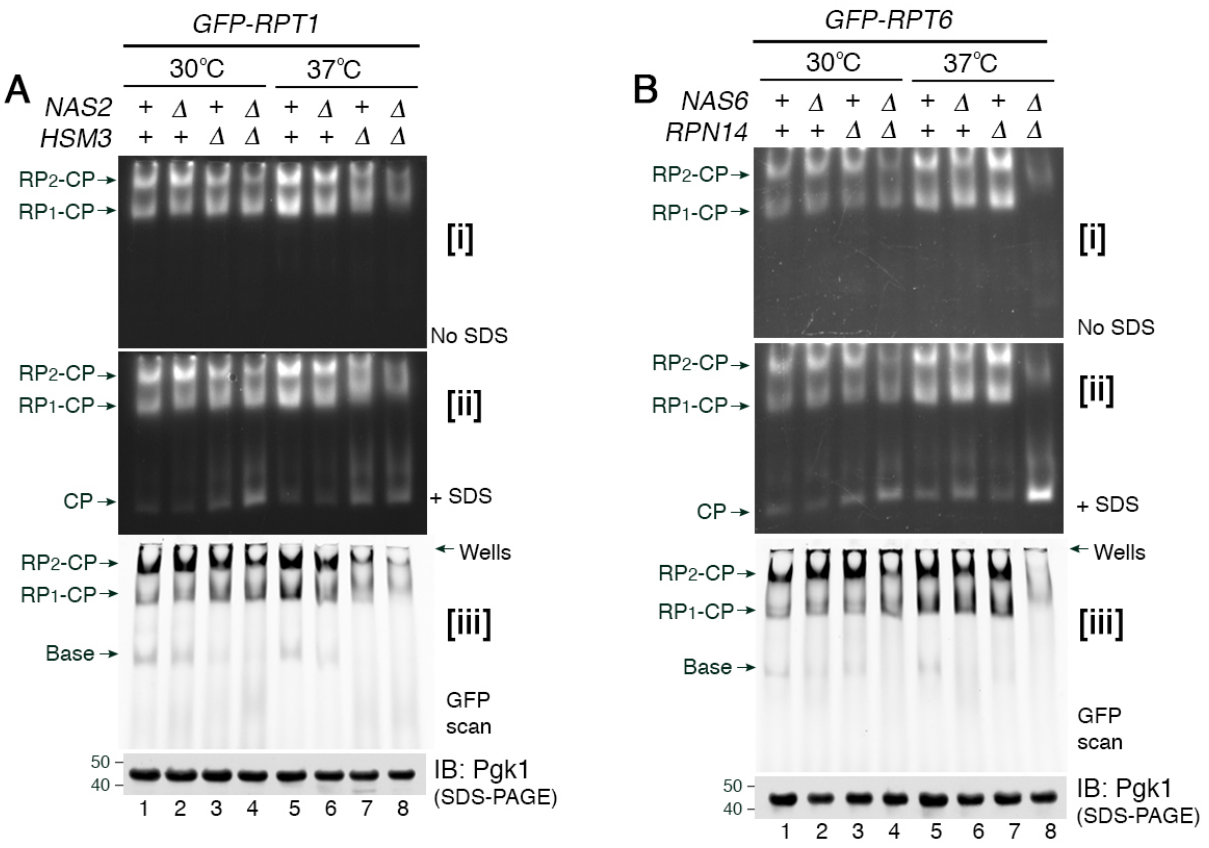

Figure S2. GFP-tagged Rpt subunits incorporate into the proteasome the same as untagged Rpt subunits.

A, B, Experiments were conducted essentially as in Fig. 1A and 1B, except that Rpt1 and Rpt6 harbor a GFP tag in their own chromosomal loci. Yeast strains were grown at 30°C (lane 1-4), and then exposed to heat stress at 37°C for 4 hrs (lane 5-8). Whole cell lysates (60 μg) were subjected to native PAGE and in-gel peptidase assays using LLVY-AMC in the absence and presence of 0.02% SDS ([**i**] and [**ii**], respectively). Native gels were then scanned using a Typhoon scanner to track incorporation of GFP-tagged Rpt subunits into the proteasome holoenzyme [**iii**]. Pgk1, loading control.

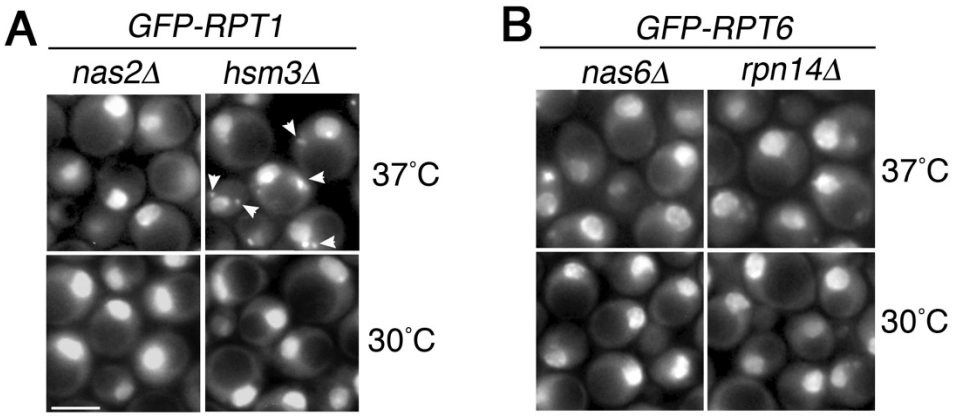

Figure S3. Deletion of *HSM3* leads to Rpt subunit-puncta formation upon heat stress.
Live-cell epi-fluorescence images of GFP-Rpt1 **(A)** and GFP-Rpt6 **(B)** in cells lacking individual chaperones. Cells were grown at 30°C, and then were exposed to heat stress at 37°C for 15 min. Arrowheads indicate the punctate structures of GFP-Rpt1. Scale bar = 5 μm for all panels.

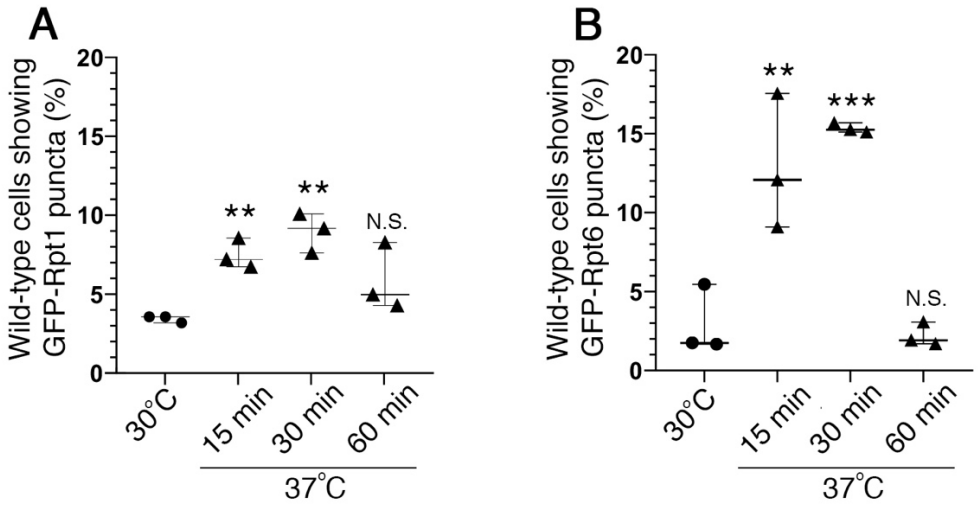

Figure S4. Upon heat stress, Rpt subunits-puncta form transiently in wild-type cells.
A, B, GFP-Rpt1 puncta (**A**) and GFP-Rpt6 puncta (**B**) were individually counted in approximately 150 wild-type cells in each indicated condition. The percent of cells showing puncta are depicted in scatter plots (average ± SD; n=3, biological replicates). Approximately 10-15% of wild-type cells exhibit puncta at 15 and 30 min upon heat stress at 37°C, but not at 60 min. These results suggest that Rpt subunits-puncta may form transiently during proteasome assembly in stressed conditions. Student's t-test was conducted between 30°C and each time point at 37°C (** < p=0.05, ***< p=0.005, N.S.=not significant).

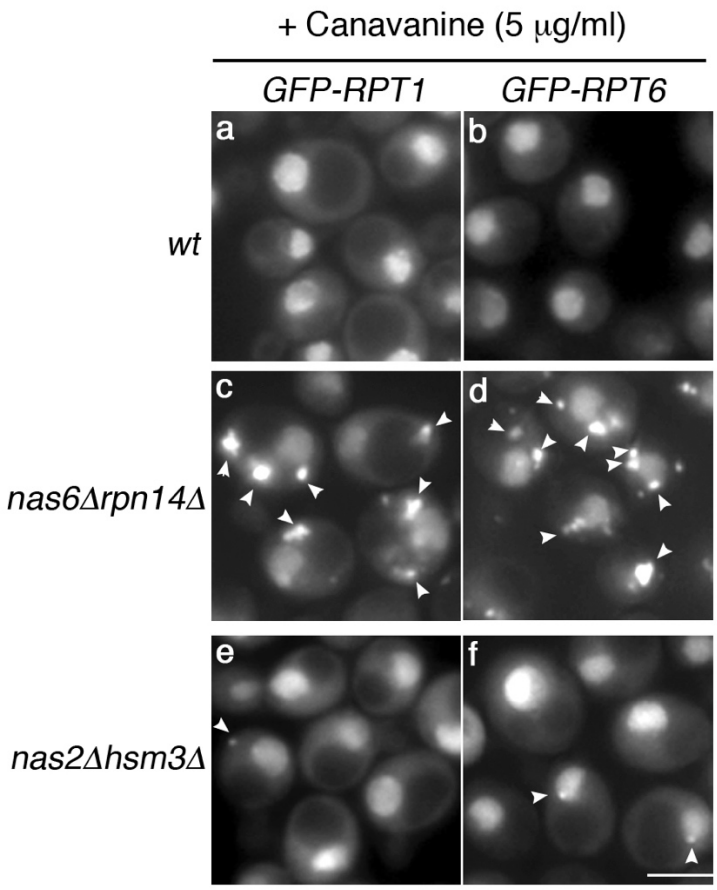

Figure S5. In the presence of canavanine, both GFP-Rpt1 and GFP-Rpt6 form puncta in *nas6Δrpn14Δ*, but minimally in *nas2Δhsm3Δ* cells.
Live-cell epi-fluorescence images of GFP-Rpt1 and GFP-Rpt6 in the indicated chaperone mutants. Canavanine (5 μg/ml) was added to these strains for 2.5 hours at 30°C. ***a, b,*** Puncta are not readily detectable in wild-type strains. ***c-f,*** Arrowheads indicate the punctate structures of GFP-fused Rpt subunits. Scale bar = 5 μm for all panels.

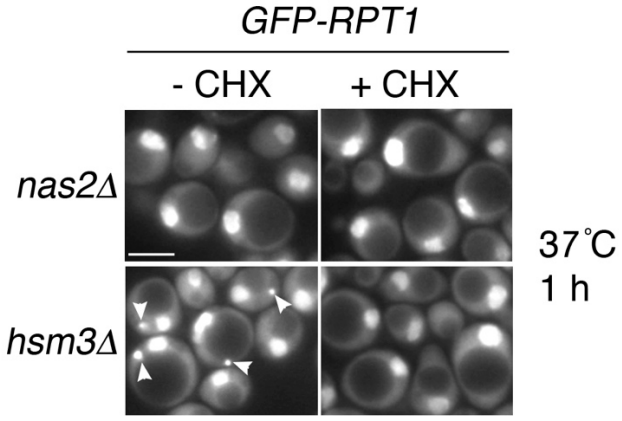

Figure S6. In the *hsm3Δ* mutants, Rpt1 is no longer sequestered upon heat stress when protein translation is inhibited.
Live-cell epi-fluorescence images of GFP-Rpt1 in the indicated chaperone mutants. Cycloheximide (CHX, 150 μg/ml) was added to these strains for 1 hr at 37°C. Arrowheads indicate the punctate structures of GFP-tagged Rpt1. Scale bar = 5 μm for all panels.

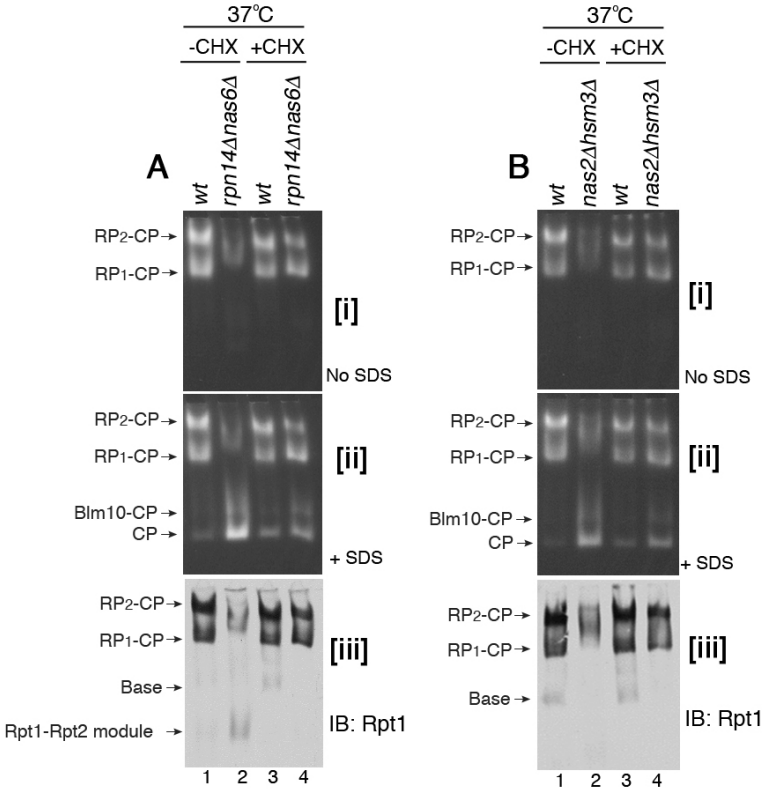

Figure S7. Untagged Rpt subunits assemble into the proteasome holoenzyme upon translation inhibition.

A, B, Experiments were conducted as in Fig. 3C and 3D, except that Rpt subunits are not GFP-tagged in the indicated yeast strains. In both *rpn14Δnas6Δ* and *nas2Δhsm3Δ* cells, RP_2-CP and RP_1-CP are detectable at a similar level as wild-type upon cycloheximide treatment (lanes 3, 4). These results confirm that untagged Rpt subunits also resume their incorporation into the proteasome holoenzyme.

Rpt1-Rpt2 module accumulates in *nas6Δrpn14Δ* cells (**A**, [**iii**], lane 2), reflecting disruption during hexameric Rpt ring assembly upon heat stress. When proteasome assembly resumes, this module also incorporates into the proteasome holoenzyme (**A**, [**iii**], lane 4). The Rpt1-Rpt2 module is undetectable in *nas2Δhsm3Δ* cells (**B**, [**iii**], lanes 2, 4) since the formation of this module requires Rpt1's cognate chaperone Hsm3 (1-3).

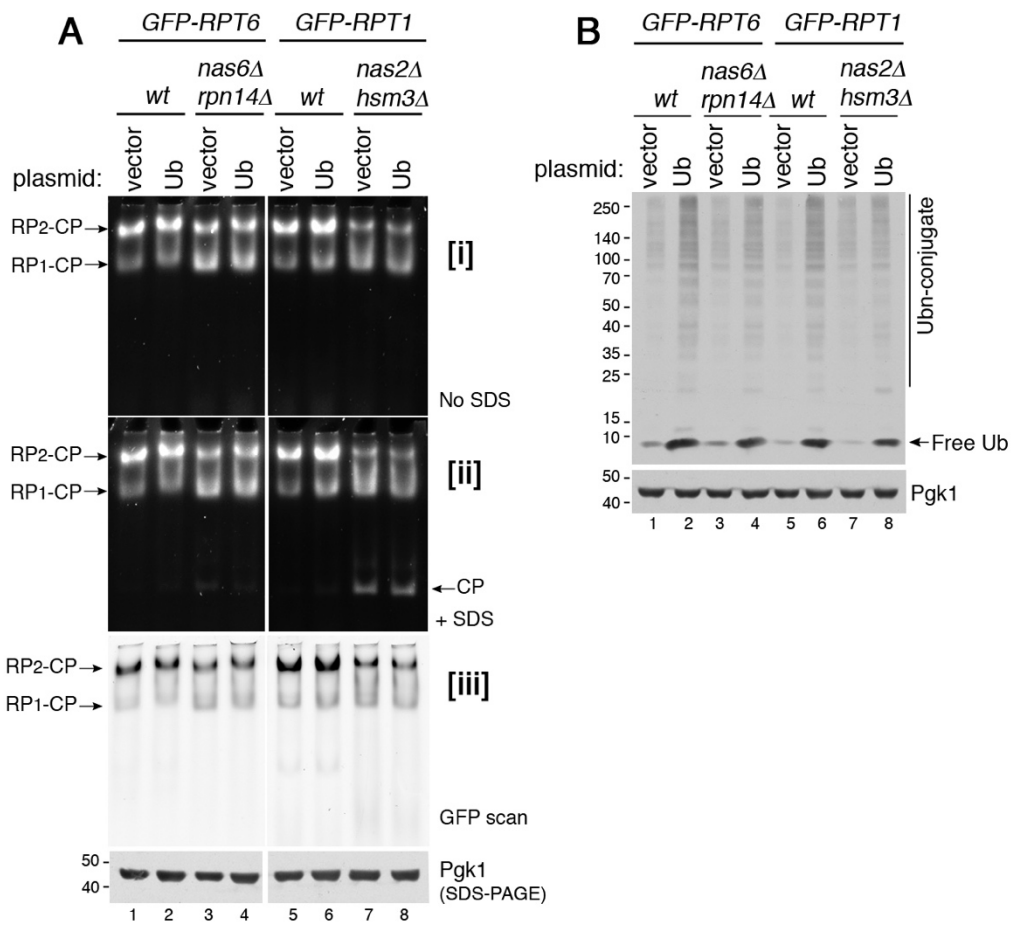

Figure S8. Cellular ubiquitin levels do not influence proteasome assembly and activity in general. ***A,*** Proteasome assembly and activity remain largely unaffected upon ubiquitin induction. The indicated yeast strains were transformed with a high-copy plasmid harboring copper-inducible ubiquitin (YEp96) or vector alone (pES12) as a control (4,5). Ubiquitin expression was induced by adding $CuSO_4$ at 100 μM to yeast cultures for 4 hrs at 30°C. To examine proteasome assembly and activity, whole cell extracts (60 μg) were subjected to 3.5% native PAGE and in-gel peptidase assays in the absence and presence of 0.02% SDS in [**i**] and [**ii**], respectively. Proteasome levels were examined using a GFP scan for GFP-Rpt6 and GFP-Rpt1 in [**iii**]. Pgk1 is a loading control. The Pgk1 blot in lanes 1-4 and 5-8 derives from two different gels, which were processed the same in parallel during immunoblotting and signal detection. ***B,*** Confirmation that samples in (**A)** indeed exhibit ubiquitin induction from plasmid-born ubiquitin. Whole cell extracts (20 μg) were subjected to 10% Bis-Tris SDS-PAGE and immunoblotting for ubiquitin. Pgk1, a loading control.

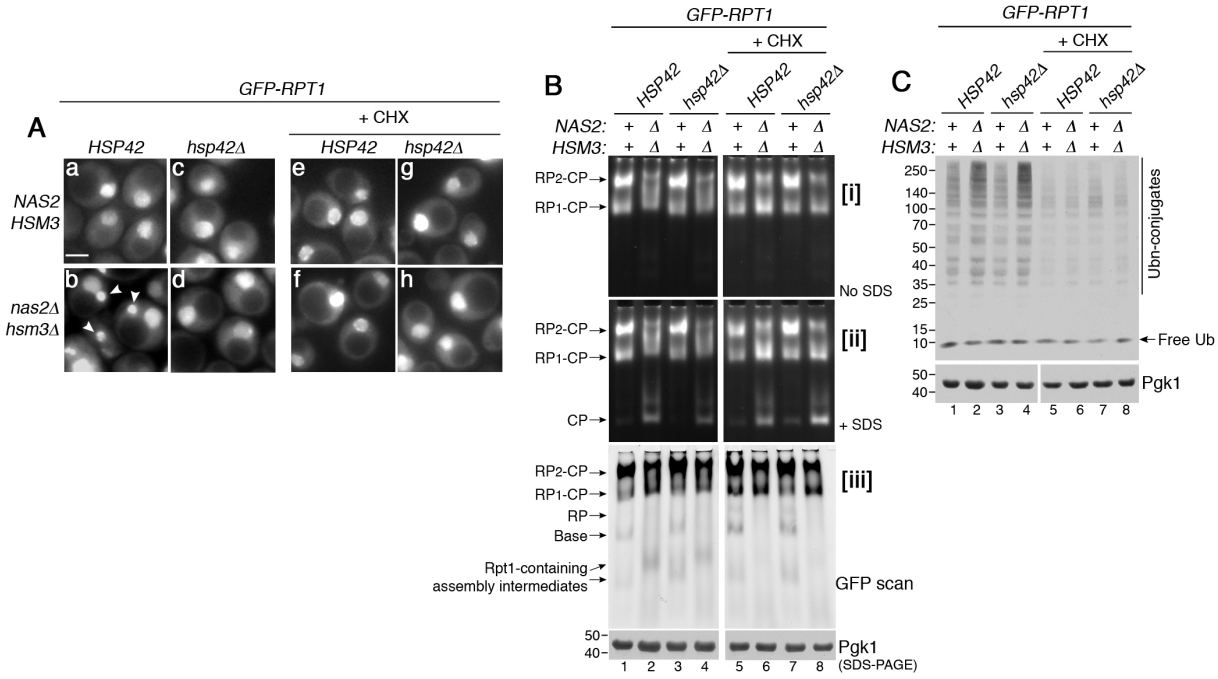

Figure S9. Rpt1 proceeds to proteasome assembly when the pool of free Rpt subunits is reduced. Experiments (**A-C**) were conducted as in Fig. 4, to examine proteasome assembly using GFP-Rpt1 in the *nas2Δhsm3Δ* mutants.

Sequestration of Rpt1 is blocked upon deletion of *HSP42* in the *nas2Δhsm3Δ* mutants (**A,** see b, d). However, proteasome holoenzyme assembly remained deficient in the corresponding samples (**B**, lane 2, 4). Only after addition of cycloheximide, proteasome holoenzyme assembly can be restored to some extent in the *nas2Δhsm3Δ* mutants (**B**, compare lane 2 to 6, and lane 4 to 8). Rpt1-containining assembly intermediates are largely comparable in the *nas2Δhsm3Δ* mutants whether or not Hsp42 is expressed (**B** [**iii**] lanes 2, 4), and incorporate into the holoenzyme complexes during resumption of proteasome assembly (**B** [**iii**] lanes 6, 8). Improved assembly of the proteasome holoenzyme contributes to degradation of cellular ubiquitinated proteins (**C**). In **B** and **C**, their respective Pgk1 blots in lanes 1-4 and 5-8 derive from two different gels, which were processed the same in parallel during immunoblotting and signal detection.

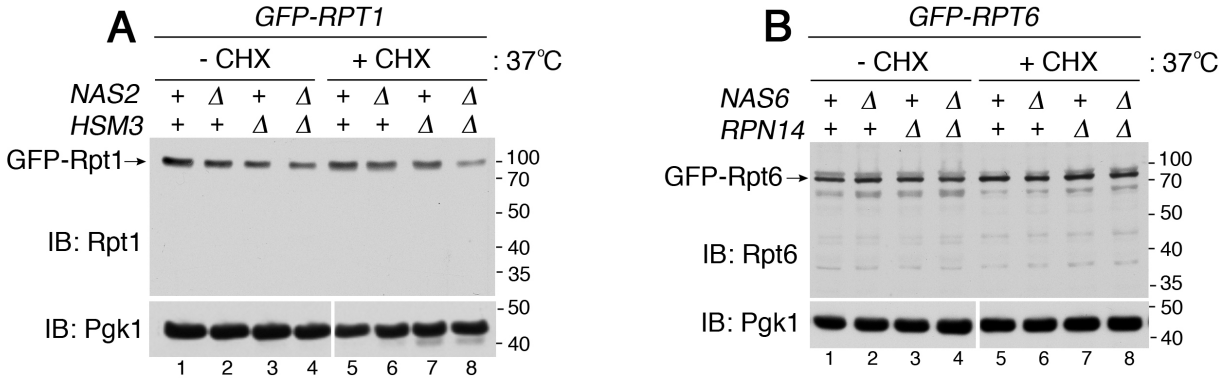

Figure S10. Excess Rpt subunits are degraded processively.

A, B, Experiments were conducted as in Fig. 6A and 6B, except with antibodies specific to Rpt1 and Rpt6 (6). Both anti-Rpt1 and anti-Rpt6 antibodies are rabbit polyclonal. These antibodies were generated using full-length Rpt1 and Rpt6, which were expressed and purified in *E. coli* (6); please note that CIM3 is Rpt6, and that CIM5 is Rpt1 in Ref. (6). These antibodies specifically recognize their targets, as seen in Fig. 3 in the Ref. (6) (also see Supplementary Table 1). Given that anti-Rpt1 and anti-Rpt6 antibodies are polyclonal, they should be able to detect partially cleaved Rpt1 and Rpt6, respectively. However, such Rpt1 or Rpt6 fragments were not readily detected in the chaperone mutants relative to wild-type cells, suggesting that these Rpt subunits are degraded processively. Bands at 70 kDa in (**B**) are likely to be non-specific signal since they are detected even in wild-type cells, and regardless of cycloheximide treatment. In **A** and **B**, their respective Pgk1 blots in lanes 1-4 and 5-8 derive from two different gels, which were processed the same in parallel during immunoblotting and signal detection.

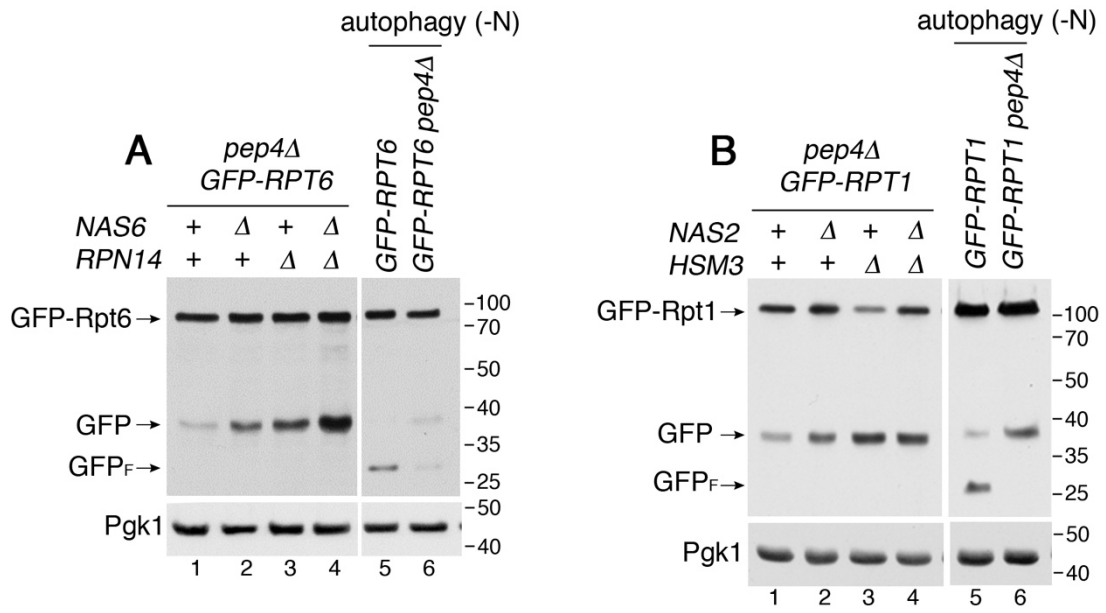

Figure S11. Deletion of *PEP4*, a vacuolar protease, does not block degradation of Rpt subunits. ***A, B,*** Excess Rpt subunits are still degraded upon deletion of *PEP4*, as evident from free GFP release, indicating that they do not undergo vacuole-mediated proteolysis. Yeast strains were grown at 30°C. Whole cell lysates were analyzed by 10% Bis-Tris SDS-PAGE, followed by immunoblotting with anti-GFP antibody. Pgk1 is a loading control. In **A** and **B**, their respective Pgk1 blots in lanes 1-4 and 5-6 derive from two different gels, which were processed the same in parallel during immunoblotting and signal detection.

As a positive control for Pep4, we induced autophagy by growing yeast strains in nitrogen-starvation media (-N, lanes 5, 6) (7,8). As shown previously, free GFP is released at 27 kDa (GFP_F) during autophagy (lane 5), in which the fused Rpt subunit is fully degraded, and can be stabilized upon *PEP4* deletion (lane 6) (7,8). Unlike autophagy, the proteasome leaves approximately 10 kDa remnant of Rpt on GFP, 27 kDa, resulting in the combined 37 kDa fragment (Fig. 6A-D). This 10 kDa fits the distance from the substrate entrance (RP) and the peptidase site (CP) in the proteasome (9), suggesting this remnant releases with GFP whose tight folding is refractory to RP-mediated unfolding.

Supplementary Table 1. Antibody list

	Source (product number)	Reference	Species
Anti-Rpt1	Generated in Carl Mann laboratory	Ref. (6,10,11)	Rabbit polyclonal
Anti-Rpt6	Generated in Carl Mann laboratory	Ref. (6,10-12)	Rabbit polyclonal
Anti-Rpt5	Enzo Lifesciences (BML-PW8245)	Ref. (12-14)	Rabbit polyclonal
Anti-Ubiquitin	Enzo Lifesciences (BML-PW0930)	Ref. (13,15)	Mouse monoclonal
Anti-His	Sigma (H1029)	Ref. (13)	Mouse monoclonal
Anti-GFP	Roche (11814460001)	Ref. (7,8)	Mouse monoclonal
Anti-Pgk1	Life Technologies (459250)	Ref. (8,12,13)	Mouse monoclonal

References

1. Roelofs, J., Park, S., Haas, W., Tian, G., McAllister, F. E., Huo, Y., Lee, B. H., Zhang, F., Shi, Y., Gygi, S. P., and Finley, D. (2009) Chaperone-mediated pathway of proteasome regulatory particle assembly. *Nature* **459**, 861-865
2. Funakoshi, M., Tomko, R. J., Jr., Kobayashi, H., and Hochstrasser, M. (2009) Multiple assembly chaperones govern biogenesis of the proteasome regulatory particle base. *Cell* **137**, 887-899
3. Le Tallec, B., Barrault, M. B., Guerois, R., Carre, T., and Peyroche, A. (2009) Hsm3/S5b participates in the assembly pathway of the 19S regulatory particle of the proteasome. *Mol Cell* **33**, 389-399
4. Ecker, D. J., Khan, M. I., Marsh, J., Butt, T. R., and Crooke, S. T. (1987) Chemical synthesis and expression of a cassette adapted ubiquitin gene. *J Biol Chem* **262**, 3524-3527
5. Hochstrasser, M., Ellison, M. J., Chau, V., and Varshavsky, A. (1991) The short-lived MAT alpha 2 transcriptional regulator is ubiquitinated in vivo. *Proc Natl Acad Sci U S A* **88**, 4606-4610
6. Ghislain, M., Udvardy, A., and Mann, C. (1993) S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. *Nature* **366**, 358-362
7. Marshall, R. S., McLoughlin, F., and Vierstra, R. D. (2016) Autophagic Turnover of Inactive 26S Proteasomes in Yeast Is Directed by the Ubiquitin Receptor Cue5 and the Hsp42 Chaperone. *Cell Rep* **16**, 1717-1732
8. Waite, K. A., De-La Mota-Peynado, A., Vontz, G., and Roelofs, J. (2016) Starvation Induces Proteasome Autophagy with Different Pathways for Core and Regulatory Particles. *J Biol Chem* **291**, 3239-3253
9. Lander, G. C., Estrin, E., Matyskiela, M. E., Bashore, C., Nogales, E., and Martin, A. (2012) Complete subunit architecture of the proteasome regulatory particle. *Nature* **482**, 186-191
10. Glickman, M. H., Rubin, D. M., Fried, V. A., and Finley, D. (1998) The regulatory particle of the Saccharomyces cerevisiae proteasome. *Mol Cell Biol* **18**, 3149-3162
11. Park, S., Li, X., Kim, H. M., Singh, C. R., Tian, G., Hoyt, M. A., Lovell, S., Battaile, K. P., Zolkiewski, M., Coffino, P., Roelofs, J., Cheng, Y., and Finley, D. (2013) Reconfiguration of the proteasome during chaperone-mediated assembly. *Nature* **497**, 512-516
12. Hanssum, A., Zhong, Z., Rousseau, A., Krzyzosiak, A., Sigurdardottir, A., and Bertolotti, A. (2014) An inducible chaperone adapts proteasome assembly to stress. *Mol Cell* **55**, 566-577

13. Fu, X., Sokolova, V., Webb, K. J., Old, W., and Park, S. (2018) Ubiquitin-dependent switch during assembly of the proteasomal ATPases mediated by Not4 ubiquitin ligase. *Proc Natl Acad Sci U S A* **115**, 13246-13251
14. Park, S., Roelofs, J., Kim, W., Robert, J., Schmidt, M., Gygi, S. P., and Finley, D. (2009) Hexameric assembly of the proteasomal ATPases is templated through their C termini. *Nature* **459**, 866-870
15. Li, F., Tian, G., Langager, D., Sokolova, V., Finley, D., and Park, S. (2017) Nucleotide-dependent switch in proteasome assembly mediated by the Nas6 chaperone. *Proc Natl Acad Sci U S A* **114**, 1548-1553