

Multiple Assembly Chaperones Govern Biogenesis of the Proteasome Regulatory Particle Base

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SUMMARY

The central protease of eukaryotes, the 26S proteasome, has a 20S proteolytic core particle (CP) and an attached 19S regulatory particle (RP). The RP is further subdivided into lid and base subcomplexes. Little is known about RP assembly. Here, we show that four conserved assembly factors govern biogenesis of the yeast RP base. Nas2 forms a complex with the Rpt4 and Rpt5 ATPases and enhances 26S proteasome formation *in vivo* and *in vitro*. Other RP subcomplexes contain Hsm3, which is related to mammalian proteasome subunit S5b. Hsm3 also contributes to base assembly. Larger Hsm3-containing complexes include two additional proteins, Nas6 and Rpn14, which function as assembly chaperones as well. Specific deletion combinations affecting these four factors cause severe perturbations to RP assembly. Our results demonstrate that proteasomal RP biogenesis requires multiple, functionally overlapping chaperones and suggest a model in which subunits form specific subcomplexes that then assemble into the base.

INTRODUCTION

In eukaryotes, short-lived proteins are degraded primarily by the ubiquitin-proteasome system (Hochstrasser, 1996; Heinemeyer et al., 2004). Defects in the system are linked to a variety of human diseases, and proteasomal inhibitors are used to treat several cancers (Goldberg, 2007; Schwartz and Ciechanover, 2009). Most proteasome substrates are first modified by polyubiquitin chains, allowing recognition by the proteasome and degradation of the substrate. The 26S proteasome consists of a proteolytically active 20S proteasome core particle (CP) bound at one or both ends by a 19S regulatory particle (RP) (Schmidt et al., 2005). In addition to substrate binding, the RP is responsible for substrate unfolding, substrate translocation into the proteolytic chamber of the CP, and release of ubiquitin from the substrate.

High-resolution structural information is available for the CP, and it has been subject to extensive biochemical and genetic

analysis (reviewed in Marques et al., 2009). The CP forms a cylinder of four stacked heptameric rings. Two structurally related classes of subunits make up the rings. The outer rings have α -type subunits, and the inner rings β -type subunits, with the protease sites formed by specific β subunits.

The structure and activities of the RP are less well understood. The lid and base each contain at least nine different subunits, with additional polypeptides, such as Rpn10, associated more loosely or only under specific conditions (Figure 1A) (Schmidt et al., 2005). The base includes six different AAA+ ATPase subunits, Rpt1–Rpt6, as well as the two largest subunits, Rpn1 and Rpn2. Recent structural analysis suggests that Rpn1 and Rpn2 form toroids, with Rpn1 stacked on Rpn2 and Rpn2 in direct contact with the CP α ring (Rosenzweig et al., 2008). The ATPases are thought to form a hexameric ring that encircles the Rpn1–Rpn2 stack and also contacts the α ring (Ferrell et al., 2000; Hartmann-Petersen et al., 2001).

Rpn10 and the base subunit Rpn13 are proteasomal polyubiquitin receptors (Husnjak et al., 2008). Additional proteins function as mobile receptors that bring polyubiquitin-modified substrates to the proteasome. In yeast, the related Rad23, Dsk2, and Ddi1 proteins use their ubiquitin-like domains to dock onto Rpn1 and their UBA domains to bind substrate polyubiquitin chains (reviewed in Hurley et al., 2006). There is considerable redundancy for substrate binding among these receptors, and genetic data indicate additional receptors must exist (Diaz-Martinez et al., 2006; Husnjak et al., 2008).

Although much is known about how the proteasome recognizes and degrades its substrates, investigations have just begun into how this highly abundant complex of ~2500 kD and at least 33 different subunits is assembled in the first place. Understanding proteasome assembly should provide general insight into strategies of multisubunit protein assembly *in vivo*. It will also be crucial for finding ways to inhibit assembly as part of emerging therapies that target the proteasome (Goldberg, 2007). Analysis of CP assembly is far more advanced than for the RP (Marques et al., 2009; Murata et al., 2009). Eukaryotic CP assembly initiates with formation of an α ring followed by ordered addition of β subunits to the α ring heteroheptamer (Hirano et al., 2005; Li et al., 2007). Joining of two half-proteasomes triggers autocatalytic processing of active-subunit propeptides and CP maturation (Chen and Hochstrasser, 1996). Moreover, a least three phylogenetically conserved CP-specific assembly chaperones facilitate proteasome biogenesis, and one of these

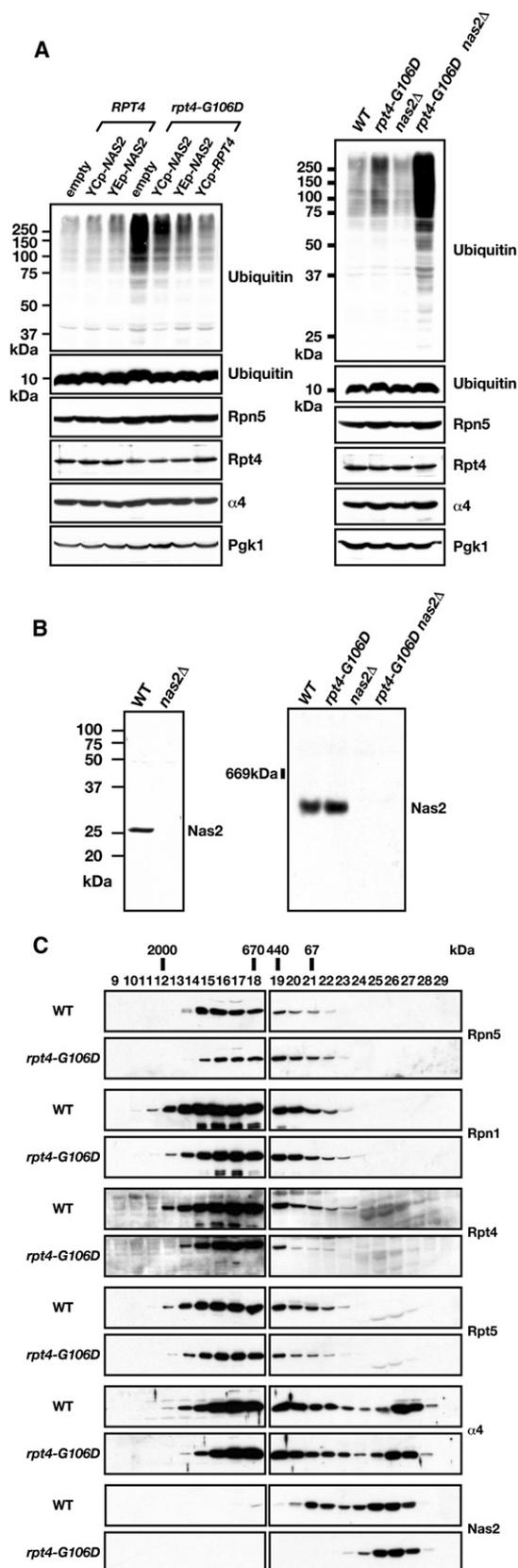


Figure 2. Nas2 Affects Proteasome Function but Is Not Part of the 26S Proteasome

(A) Anti-ubiquitin immunoblots showing polyubiquitin conjugate accumulation in *rpt4-G106D* cells. Left, effect of low-copy (YCp) and high-copy (YEp) expression of *NAS2* on *rpt4-G106D*. Right, effect of *nas2Δ* on ubiquitin-conjugate profiles. Levels of proteasome components in the strains are shown below. Pgk1 serves as a loading control.

(B) Anti-Nas2 immunoblot analysis of yeast proteins separated by SDS-PAGE (left) or by nondenaturing PAGE (right).

(C) Gel filtration analysis of Nas2 and proteasomal proteins from WT and *rpt4-G106D* cells. Equivalent amounts of protein were loaded from each extract onto a Superose-6 column, and fractions were subjected to immunoblotting with antibodies to the indicated proteins.

We first attempted to link Nas2 to the *in vivo* function of the proteasome. Proteasomal defects lead to accumulation of polyubiquitinated proteins that can be detected by anti-ubiquitin immunoblotting. The *rpt4-G106D* mutant showed a pronounced build-up of such species, and these were suppressed by *NAS2* when introduced into the mutant on a low-copy or, to a greater extent, high-copy plasmid (Figure 2A, left). Conversely, *nas2Δ*, which by itself caused no increase in bulk polyubiquitin conjugates, exacerbated the defect due to *rpt4-G106D* (Figure 2A, right). Growth assays yielded parallel results, showing that *nas2Δ* did not cause any detectable growth defects, but it caused an enhanced growth defect when combined with *rpt4-G106D* (Figure S1A). Similarly, when we assayed levels of specific model substrates of the proteasome, *nas2Δ* had no effect, but *rpt4-G106D* mutants had increased amounts of these substrates, consistent with a proteolytic defect (Figure S1B). Levels were further increased in the *rpt4-G106D nas2Δ* double mutant. Based on growth assays, high-copy *NAS2* could also weakly suppress another mutant *rpt4* allele, called *cr113* or *sug2-13*, but could not suppress an *rpt4* allele (*sug2-1*) that is specifically defective in the transcriptional activation function of the RP but not in general proteolysis (Figure S2A and data not shown) (Russell et al., 1996). Loss of Nas2 did not worsen the defects due to mutations in two other RP ATPases, Rpt1 (*cim5-1*) or Rpt6 (*cim3-1*), nor did high-copy *NAS2* suppress these defects (Figures S2B and S2C).

Together, these results implicate Nas2 in the *in vivo* function of the proteasome, which previously had only been suggested from biochemical studies with human p27. Moreover, the genetic data indicate that Nas2 activity is linked to the Rpt4 subunit of the RP.

RP Assembly Defects in *rpt4-G106D* Are Enhanced by Loss of Nas2

An earlier study did not find Nas2 in 26S proteasomes (Russell et al., 1999), but because the protein was epitope tagged, it might have been defective for proteasome association. Affinity-purified antibodies to Nas2 reacted specifically with a single species of the expected size in SDS gel separations (Figure 2B, left). By nondenaturing PAGE, we observed a single band, which migrated more quickly than assembled proteasomes (Figure 2B, right; see Figure 3D for size comparison). Similarly, using Superose-6 gel filtration, we found Nas2 in two peaks in wild-type (WT) cells: one peak that is probably monomeric Nas2, and a larger species that appeared to be less than 400 kDa (Figure 2C). No reactivity was seen in earlier fractions

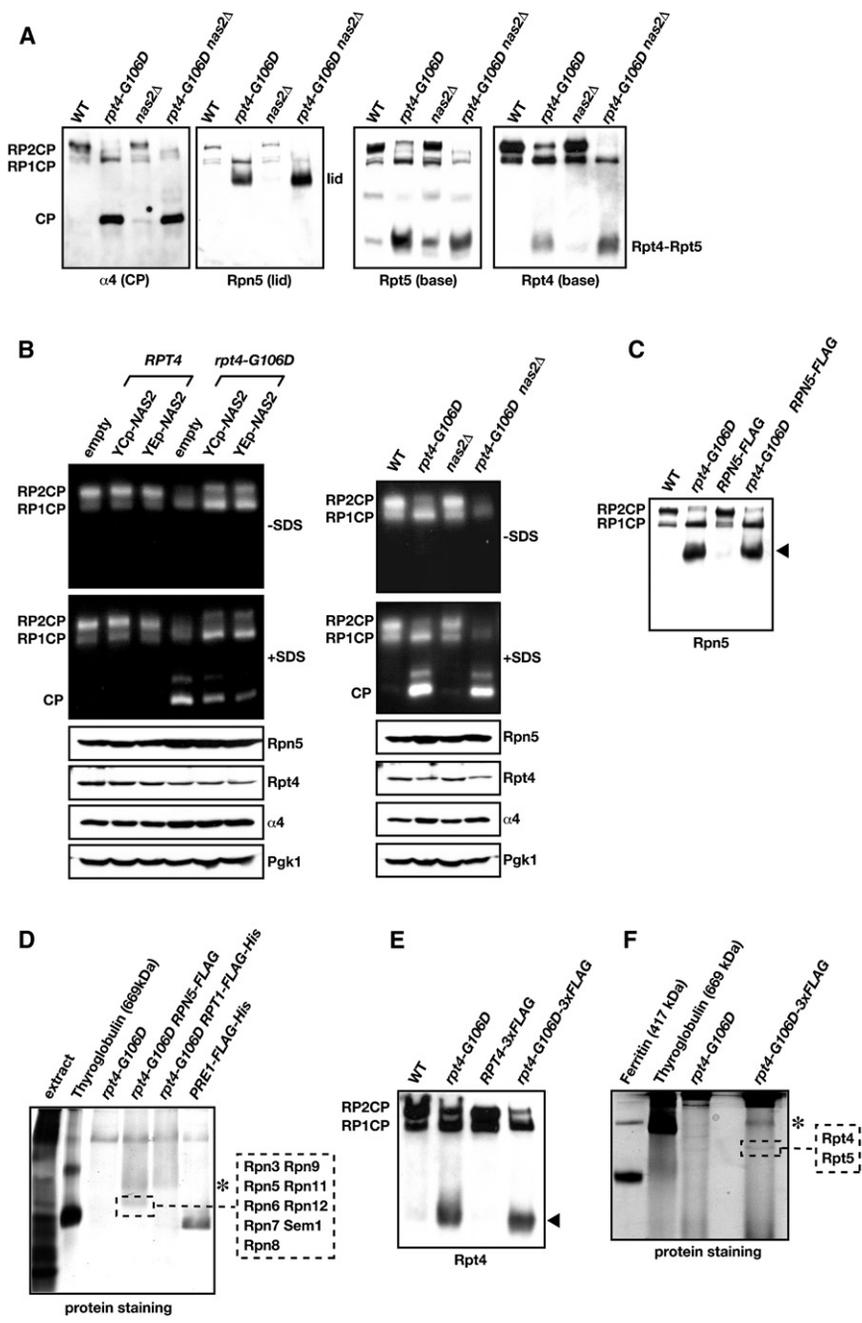


Figure 3. The *rpt4-G106D* and *nas2Δ* Mutants Have Proteasome Assembly Defects

(A) Immunoblot analysis of nondenaturing PAGE-separated proteins from yeast whole-cell extracts. The faint central band in the anti-Rpt5 blot is of unknown composition.

(B) Nondenaturing gel separation of proteins from yeast extracts followed by gel overlay with the fluorogenic substrate Suc-LLVY-AMC to assay proteasomal activity. Left panel, effect of low-copy (YcP) and high-copy (YEp) expression of *NAS2* on functional proteasome formation. Right panel, effect of *nas2Δ* on assembly. Addition of SDS to the reaction buffer activates the otherwise latent CP and B1m10-CP (unlabeled band above free CP).

(C) A C-terminal Flag tag on Rpn5 does not disrupt the Rpn5-containing RP subcomplex (arrowhead) in *rpt4-G106D* cells.

(D) Nondenaturing gel separation of Flag affinity-purified proteins from *rpt4-G106D RPN5-FLAG* cells. MS/MS analysis of the indicated band from the GelCode Blue-stained gel demonstrated that it was the nine-subunit RP lid. Asterisk, a band that is probably the free RP but was not analyzed. Unlike in (C), gel separation included a stacking gel and was run longer.

(E) A triplicated Flag tag on Rpt4 does not disrupt the Rpt4-containing RP subcomplex (arrowhead) in *rpt4-G106D* cells.

(F) Nondenaturing gel separation of Flag affinity-purified proteins from *rpt4-G106D RPT4-3xFLAG* cells. MS/MS analysis of the indicated band from the GelCode Blue-stained gel yielded Rpt4 and Rpt5 from the RP base. Asterisk, as in (D). PAGE was done with a 5.5% PA separating gel instead of the 4% used in other panels, and a stacking gel was used.

from the column where mature proteasomal species migrate. We conclude that yeast *Nas2* is not stably associated with the mature proteasome or RP. Notably, the larger *Nas2* peak disappeared in extracts from *rpt4-G106D* cells, indicating that this species is dependent on functional Rpt4.

Since *Nas2* is not a stable component of the proteasome, we hypothesized that it might function in proteasome assembly. Anti- $\alpha 4$ (CP) immunoblotting of nondenaturing gel-separated whole-cell yeast lysates revealed a very small but reproducible increase in free CP in the *nas2Δ* mutant relative to WT (Figure 3A), consistent with a deficiency in RP relative to CP levels. A much more pronounced increase in free CP, with a concomitant

reduction of doubly capped 26S proteasomes (RP2CP), was seen in an *rpt4-G106D* strain by blotting with antibodies against the CP ($\alpha 4$), RP base (Rpt4, Rpt5), and RP lid (Rpn5) (Figure 3A). These changes were also observed by a fluorogenic peptide substrate overlay assay (Figure 3B). The deficiency in 26S proteasome complexes could be partially suppressed by introduction of extra copies

of the *NAS2* gene (Figure 3B, left) and was exacerbated in the *rpt4-G106D nas2Δ* double mutant (right). These changes in the relative levels of free CP compared to full RP2CP and RPCP particles indicate that *Nas2* contributes to RP (or RP-CP) assembly or stability.

The anti-Rpn5 (lid) and anti-Rpt4/Rpt5 (base) immunoblots in Figure 3A also revealed protein complexes from the *rpt4-G106D* strains that were either absent or barely detectable in WT cells. To purify and determine the molecular composition of these complexes, we modified chromosomal *RPN5* or *rpt4-G106D* with sequences for the Flag epitope. These tags did not interfere with accumulation of the novel RP

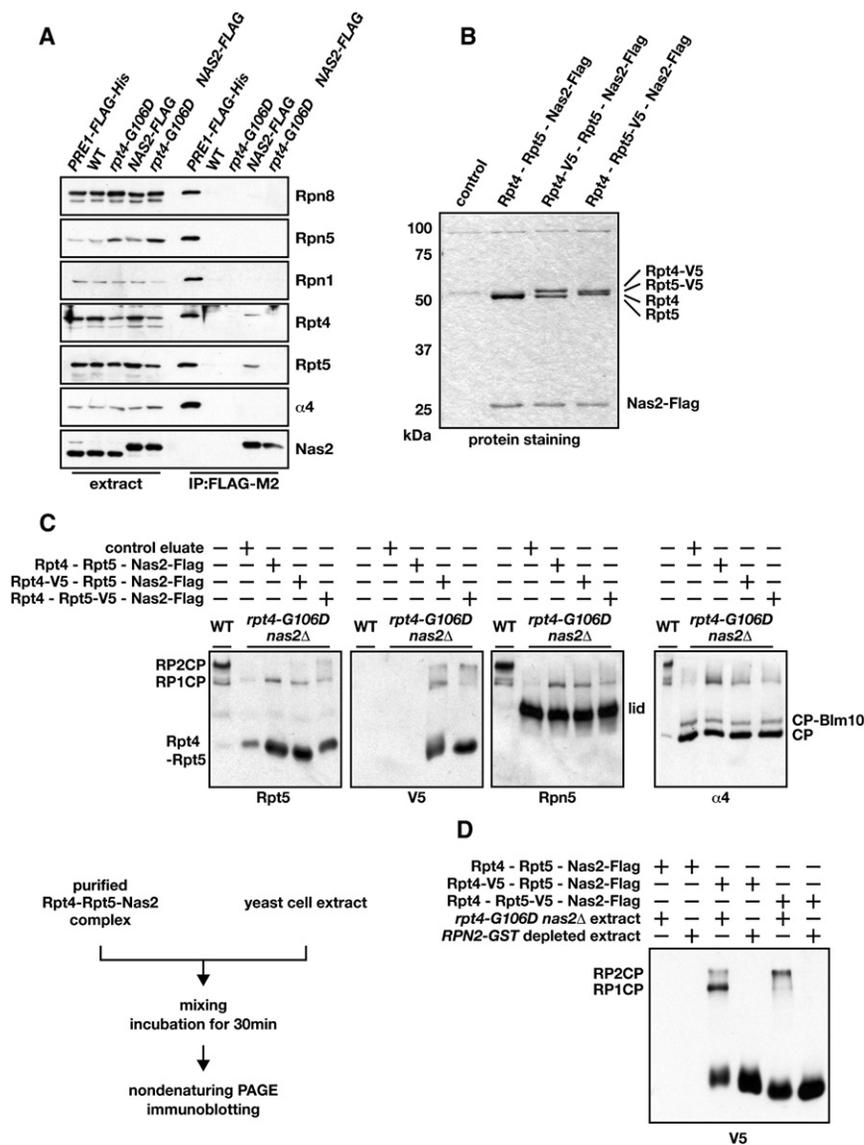


Figure 4. Nas2 Forms a Complex with Rpt4 and Rpt5, which Stimulates 26S Proteasome Formation In Vitro

(A) Coprecipitation of endogenous Rpt4 and Rpt5 with chromosomally expressed Nas2-Flag. Eluted proteins were tested for the indicated proteasomal subunits by immunoblotting. Pre1-Flag (CP β 4 subunit) served as a positive purification control. (B) Affinity-purified Nas2-Flag-Rpt4-Rpt5 complexes resolved on an SDS gel and stained with Coomassie blue. Control, purification from a strain without Flag-tagged Nas2. (C) Nas2-Rpt4-Rpt5 complexes stimulate 26S proteasome formation in vitro. Control eluate is derived from cells lacking the Flag tag on Nas2. Assembly reactions were done at 24°C. A diagram of the experimental scheme is shown. (D) Stimulation of assembly of 26S proteasomes by purified Nas2-Rpt4-Rpt5 requires proteasome components from the target yeast extract. 26S proteasomes and RP-derived components were precleared from RPN2-GST cell extracts by binding to glutathione-Sepharose.

but not to base or CP subunits (Figure 3A). It was further supported by comparing the migration on native gels of Rpn5-containing complexes from *rpt4-G106D* cells to those from *rpn2-ΔN*, a base mutant known to accumulate free lid (Isono et al., 2007) (Figure S3A; see also below other subunits). The identity of the Rpt4-Rpt5 complex was supported by immunoblotting experiments showing that the complex purified via the Flag-tagged *rpt4-G106D* protein reacted with antibodies to Rpt5 but not to Rpt1 or Rpn1 (Figure S3B). Therefore, the *rpt4-G106D* RP base mutation causes excess accumulation of free, fully assembled RP lid plus at least one sub-complex of the base (Rpt4-Rpt5), consistent with a defect in RP base assembly.

Since the mammalian modulator complex consists of the orthologs of Rpt4, Rpt5, and Nas2, we next asked whether the yeast Rpt4-Rpt5 complex, which is of very low abundance but detectable in WT cells, associates in vivo with Nas2. A fully functional Flag-tagged Nas2 expressed from its normal chromosomal locus was able to coprecipitate both Rpt4 and Rpt5 in WT cells but not the CP (α 4), the lid (Rpn5, Rpn8), or another subunit of the base (Rpn1) (Figure 4A). The three proteins, when overexpressed, can be purified as a stoichiometric complex from yeast as well (Figure 4B). These results suggest that yeast contain a complex similar to the mammalian modulator and that Nas2 dissociates from Rpt4-Rpt5 once these proteins are incorporated into the RP. Interestingly, even though the Rpt4-Rpt5 complex was more abundant in *rpt4-G106D* cells, we failed to detect either subunit in the Nas2-Flag coprecipitations (Figure 4A), which is consistent with the loss in this mutant

subcomplexes (Figures 3C and 3E, arrowheads). Extracts from large yeast cultures were then bound to an anti-Flag affinity column, and proteins were eluted with excess Flag peptide and resolved on nondenaturing gels that were stained for protein (Figures 3D and 3F). The indicated complexes (and the same gel regions from purifications from untagged strains) were excised, cleaved with trypsin, and subjected to mass spectrometric (LC-MS/MS) sequencing. On the basis of these analyses, the Rpn5-containing complex was identified as the complete 9-subunit RP lid (Figure 3D; Table S1), and the Rpt4-containing complex had Rpt5 as its only other detectable constituent (Figure 3F; Table S2). (We note that our conditions for nondenaturing PAGE appear to break apart Nas2 and Rpt4-Rpt5, so what we refer to as the “Rpt4-Rpt5 complex” when analyzed by nondenaturing PAGE may have originally also contained Nas2 [see below]).

The assignment of lid identity was consistent with the reactivity of this species on native gels with antibodies to a lid subunit

of the early Nas2 peak seen by gel filtration (Figure 2C). This suggests that the *rpt4-G106D* mutation, while not affecting Rpt4 binding to Rpt5, impairs Rpt4-Rpt5 binding to Nas2.

A Nas2-Rpt4-Rpt5 Complex Facilitates Proteasome Assembly In Vitro

If a Nas2-Rpt4-Rpt5 complex acts as a functional intermediate in RP base assembly, it should be possible to transfer Rpt4-Rpt5 from such purified complexes to proteasomal precursors in vitro, leading to stimulation of 26S proteasome formation. We purified the Nas2-Flag-Rpt4-Rpt5 complexes from yeast strains that overexpressed these three polypeptides and also had chromosomal *RPN2* tagged with a GST coding sequence. We first depleted yeast lysates of assembled proteasomes and RPs by passing them over a glutathione-Sepharose resin; Nas2-Flag-containing complexes in the depleted lysates were then bound to an anti-Flag column and eluted with Flag peptide. This yielded Nas2-Rpt4-Rpt5 complexes of high purity (Figure 4B).

We then made whole-cell lysates in low-salt conditions from *rpt4-G106D nas2Δ* cells, which accumulate excess free CP and RP lid and various RP base intermediates (Figure 3 and below). Purified Nas2-Rpt4-Rpt5 complexes were added to the lysates along with ATP and incubated for 30 min. Reaction products were resolved on nonreducing gels and analyzed by immunoblotting (Figure 4C). Three different preparations of Nas2-Rpt4-Rpt5 complexes all stimulated formation of 26S proteasomes (RPCP and RP2CP). Using V5-epitope tags on either Rpt4 or Rpt5, we could detect incorporation of these ATPase subunits from the purified Nas2-Rpt4-Rpt5 complexes into 26S particles. Importantly, *RPN2-GST* cell extracts depleted of 26S proteasome components failed to assemble 26S complexes in the reconstitution assay, indicating that the 26S particles seen in Figure 4C were not derived from contaminants of the purified Nas2-Rpt4-Rpt5 complexes (Figure 4D). These results suggest that Nas2-Rpt4-Rpt5 complexes can indeed serve as intermediates in the assembly of proteasomes.

Additional RP Subparticles Identified in RP Assembly Mutants

When we compared proteasome profiles from WT and *rpt4-G106D* cells by native gel immunoblotting with an anti-Rpt1 antibody, two species appeared in the mutant whole-cell extracts that were much less abundant in WT extracts (Figure 5A, complexes I and II). These species were still detectable when Rpt1 was fused to a Flag-His6 tag (data not shown); therefore, this tag was used for affinity purification of the two complexes and MS/MS identification of their components. Three base subunits, Rpn1, Rpt1, and Rpt2, comprised the less abundant complex I, whereas these same subunits, together with the protein Hsm3, comprised complex II (Figure 5B; Table S4).

Hsm3 was previously linked to DNA mismatch repair, but its molecular function had not been explored (Fedorova et al., 2000). A proteomic survey of proteins that could be crosslinked in vivo to the yeast 26S proteasome identified 471 proteins, one of which was Hsm3 (Guerrero et al., 2008), and a very recent report also links Hsm3 to RP assembly (Le Tallec et al., 2009). Hsm3 has a large central region with similarity to armadillo

(ARM) or HEAT repeats (Figure 5C). These related motifs form α -solenoid modules that contribute to curved or helical structures (Rosenzweig et al., 2008). Notably, our sequence analysis of Hsm3 indicated weak but significant similarity to the human S5b proteasome subunit (Gorbea et al., 2000) (Figure S4). Although regarded as an RP subunit, S5b is not detected in most MS/MS analyses of proteasome-associated proteins. S5b might associate only with RP-related complexes that are not part of full 26S proteasomes. It had been thought that S5b was specific to mammals. However, the idea that Hsm3 is in fact the yeast ortholog of S5b is strongly supported by the finding that human S5b forms part of a heterotetramer with human Rpt1, Rpt2, and Rpn1 when these proteins are coexpressed in vitro (Gorbea et al., 2000). These correspond exactly to the three base subunits that we isolated in a complex with Hsm3 in yeast (Figure 5B).

Hsm3 Is Also an RP Base Assembly Factor

Cells deleted for the *HSM3* gene had no apparent growth deficit when grown on rich medium at several temperatures, but a growth defect was observed when *hsm3Δ* cells were grown on medium containing the amino acid analog canavanine, especially at temperatures above 30°C (Figure S5A). When *hsm3Δ* was combined with other mutations in the proteasome, more severe growth defects were detected; enhanced growth defects were also evident for the *nas2Δ hsm3Δ* double mutant (Figure S5B, and see Figure 6A). Analysis of proteasomes by native PAGE/immunoblotting revealed a modest deficiency in RP2CP complexes in the *hsm3Δ* mutant and small increases in both free lid and free CP (Figure 5D). These data are consistent with a defect in RP base assembly in *hsm3Δ* cells. A mutation in the Rpt1 ATPase, *cim5-1*, also caused an apparent defect in RP assembly, with two prominent particles migrating below the RPCP complex (arrowheads in Figure 5D, anti-Rpt5). The pattern of particle accumulation in *cim5-1* cells was altered when Hsm3 was also deleted. Interestingly, the growth defect of *cim5-1* cells (and that of the *rpn1-821* mutant) was suppressed by high-copy *HSM3* expression but not by extra copies of *NAS2* (Figures S5C and S5D).

In light of the possible link between Hsm3 and RP base assembly, we used an affinity-purified antibody to Hsm3 to test potential Hsm3 binding to the proteasome or proteasomal intermediates. In WT cells, most Hsm3 is associated with a fast-migrating species of undetermined composition (Figure 5E). A small fraction is present in two slower-migrating bands (marked as I and II). Staining of these latter bands was much more intense in the *cim5-1* mutant, and they migrated similarly to the two Rpt5-containing species highlighted in Figure 5D. By tagging the chromosomal copy of *HSM3* with a Flag epitope sequence, we could affinity purify species I and II and determine their composition by MS/MS (Figure 5F). The sequenced peptides (Tables S5 and S6) indicated that band I was the full RP plus Hsm3 while band II was a complex of the full base, Hsm3, and two additional proteins, Nas6 and Rpn14.

Nas6 and Rpn14 Have Overlapping Roles in RP Assembly

Since neither Nas6 nor Rpn14 has been found in purified 26S proteasomes and both appear to be present at

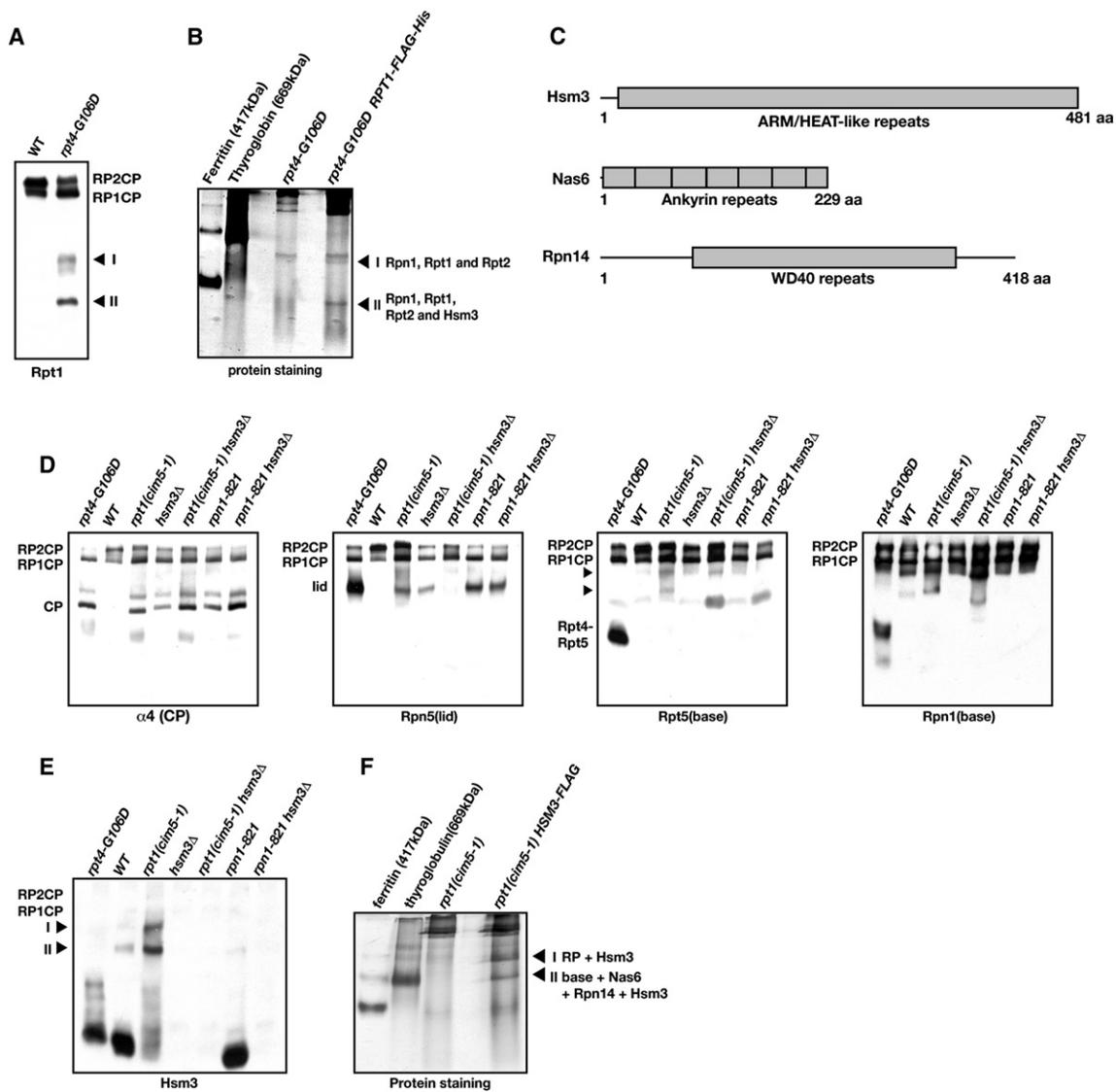


Figure 5. Hsm3 Enhances RP Assembly In Vivo

- (A) Two complexes that include the Rpt1 ATPase accumulate in *rpt4-G106D* cells (labeled I and II in the immunoblot).
- (B) Flag affinity-purified complexes from *rpt4-G106D RPT1-FLAG-His6* separated by native gel PAGE, stained with GelCode Blue, excised, and analyzed by MS/MS. The proteins identified in each band are listed.
- (C) Domain organization of Hsm3, Nas6, and Rpn14 proteins.
- (D) RP assembly defects in *hsm3Δ* and base subunit mutants. Analysis was done as in Figure 3A. Arrowheads, two Rpt5-containing complexes that accumulate in *rpt1ts (cim5-1)*.
- (E) Two slow-migrating complexes from *cim5-1* (and at a lower level, WT) cells contain Hsm3 (arrowheads labeled I and II).
- (F) Purification of complexes I and II from *cim5-1 HSM3-FLAG* cells with analysis as in (B). The protein complexes identified by MS/MS from each band are listed.

substoichiometric levels in RP-like particles, we considered the possibility that they also are RP assembly chaperones. Deletion of either factor alone had no obvious effects on growth or viability (Figure S5A and data not shown). However, combining the two deletions caused a striking temperature-sensitive growth phenotype. At 30°C, the double mutant grew comparably to WT, but at 33°C, minimal growth was observed (Figure 6A). Combining *nas6Δ*, *rpn14Δ*, *nas2Δ*, and *hsm3Δ* in all possible double-mutant combinations revealed varying degrees of temperature sensi-

tivity, with *nas6Δ rpn14Δ* > *nas2Δ hsm3Δ* > *nas2Δ nas6Δ* \cong *hsm3Δ nas6Δ* > *hsm3Δ rpn14Δ* > *rpn14Δ nas2Δ* \cong WT (Figure 6A). The same trends were seen in strains with triple and quadruple deletions of these genes, with the 4xΔ (quadruple) mutant being the most sensitive of all (Figure S6A). These data suggest unique but partially overlapping functions for these four proteins, and they imply particularly close links between Nas6 and Rpn14 on the one hand and Nas2 and Hsm3 on the other.

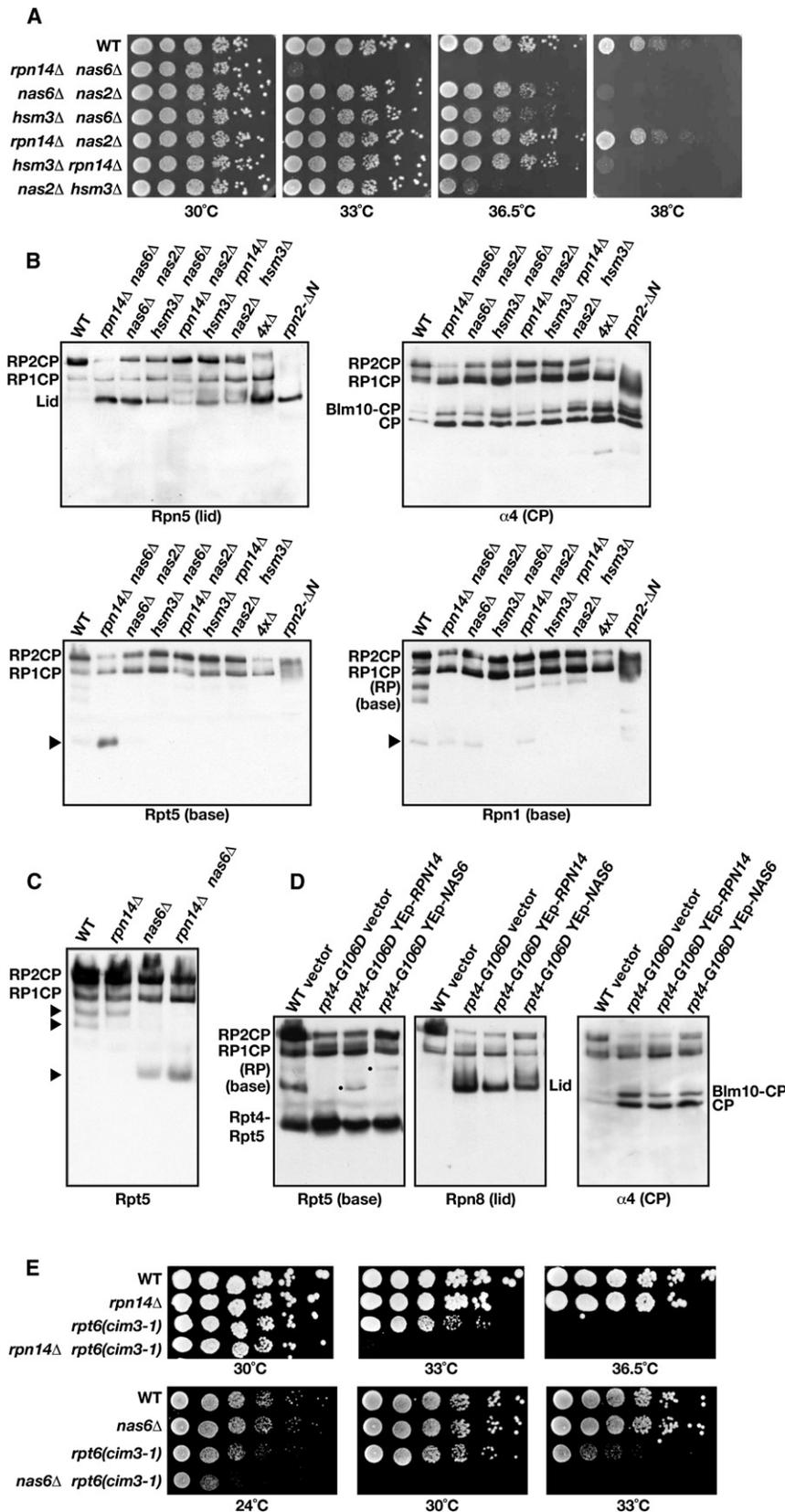


Figure 6. Nas6 and Rpn14 Are Also Important for RP Base Assembly

(A) Serial dilutions of the indicated double mutants were spotted onto YPD plates and incubated at the indicated temperatures for 2 days.

(B) Immunoblot analysis of nondenaturing gel-separated proteins from the indicated mutants. The *4xΔ* strain is a *nas2Δ nas6Δ hsm3Δ rpn14Δ* quadruple mutant.

(C) Comparison of strains by nondenaturing gel/anti-Rpt5 immunoblotting. The free RP and free base are labeled by the upper two arrowheads on the basis of the MS/MS analysis done in Figure 5F and immunoblot analyses. Lower arrowhead, probably the Rpt4-Rpt5 complex (based on comigration on native gels with species observed in *rpt4-G106D*).

(D) High-copy *RPN14* stimulates RP base formation, whereas *NAS6* stimulates full RP accumulation in *rpt4-G106D* cells. Native gel immunoblotting was done as in (B).

(E) Growth of the strains combining mutations in Nas6 or Rpn14 and base subunit Rpt6 (*cim3-1*) was evaluated as in (A).

The synthetic growth interactions between deletions of the four putative RP-base assembly factors were paralleled by their effects on proteasome assembly as assayed by native gel immunoblotting (Figure 6B and Figure S6B). Among the double mutants, anti-Rpn5 immunoblotting revealed the greatest accumulation of free lid, and lowest level of RP2CP, in the *nas6Δ rpn14Δ* mutant, congruent with its strong temperature-sensitive growth defect. The *nas6Δ rpn14Δ* strain also showed the greatest reduction in RP2CP particles, and a strong build-up of free CP (and Blm10-CP), based on anti- α 4 (and anti-Blm10) blotting (Figure 6B and data not shown). All of these results are consistent with impairment of RP base assembly.

Blotting with antibodies to base subunits (Rpn1, Rpt5) revealed that distinct proteasomal subcomplexes accumulated in the various double mutants (Figure 6B). The *nas6Δ rpn14Δ* mutant accumulated high levels of an Rpt5-containing species (Figure 6B, arrowhead) that based on mobility could be equivalent to the Rpt4-Rpt5 complex seen in *rpt4-G106D* cells (Figures 3 and 5D; also seen in *nas6Δ*, Figure 6C). Consistent with this, loss of either Nas6 or Rpn14 exacerbated the *rpt4-G106D* growth defect (Figure S6D), and overexpression of either protein partially suppressed *rpt4-G106D* (data not shown). The *nas6Δ rpn14Δ* strain, and to a slightly lesser extent the *nas6Δ*, *nas6Δ hsm3Δ*, and *nas6Δ nas2Δ* mutants, also accumulated less free RP and free base than WT (Figures 6B and 6C). The loss of free RP and concomitant increase in free lid whenever Nas6 is lost is striking and suggests that Nas6 has a role in lid-base joining or in preventing RP-CP association (or both). In support of these ideas, high-copy *NAS6* stimulated formation of an RP-like species in *rpt4-G106D* cells, whereas high-copy *RPN14* enhanced levels of free base but not the RP (Figure 6D). Native gel immunoblot analysis indicated that Nas6 associates with both free base and RP-like complexes, whereas Rpn14 associated with free base but was not detected in the RP (Figures S7A and S7B). However, tagged Rpn14 could weakly coprecipitate lid subunits (data not shown), suggesting at least some association with the full RP.

We also detected a faster migrating Rpn1-containing band (arrowhead in Figure 6B) that is visible in WT cell extracts and a number of the chaperone mutants and may represent a species similar or identical to the Rpt1-Rpt2-Rpn1-Hsm3 complex that accumulates in *rpt4-G106D* cells (Figure 5). This species is eliminated or altered in mobility when Hsm3 is deleted (Figure 6B). The implication of these results is that blocking full RP base formation leads to a build-up of various base subcomplexes.

PAAF1, the mammalian ortholog of Rpn14, binds most tightly to the Rpt6 ATPase (Park et al., 2005). Consistent with this, we found strong enhancement of the growth defect of an *rpt6ts* (*cim3-1*) mutant when *RPN14* was deleted (Figure 6E). This was first evident at 33°C. Conversely, overexpression of *RPN14* suppressed the *cim3-1* growth defect (Figure S6C). Because *nas6Δ* interacts strongly genetically with *rpn14Δ*, we checked for *nas6Δ cim3-1* interactions and found an even greater growth defect, which is evident already at 24°C (Figure 6E). High-copy *NAS6* also suppressed *cim3-1* (Figure S6C). Collectively, these data strongly suggest that Nas6 and Rpn14 are key factors in RP assembly that have linked but distinct functions.

DISCUSSION

The present results demonstrate that cells have at least four specific factors that are necessary for efficient RP base assembly in vivo. Their functions partially overlap, so the single-gene knockouts have only mild defects. But when they are deleted in particular combinations, striking RP assembly abnormalities result. These factors are all characterized by well-known protein-protein interaction modules, such as coiled-coil and PDZ domains (Nas2), ARM/HEAT repeats (Hsm3), WD40 repeats (Rpn14), and ankyrin repeats (Nas6). These domains are expected to function in RP subunit binding; this is known to be true for Nas6 (Nakamura et al., 2007). Our data show that efficient in vivo proteasomal regulatory particle biogenesis depends on an unexpectedly large array of extrinsic factors and that these evolutionarily conserved proteins all function, at least in part, as RP base assembly chaperones.

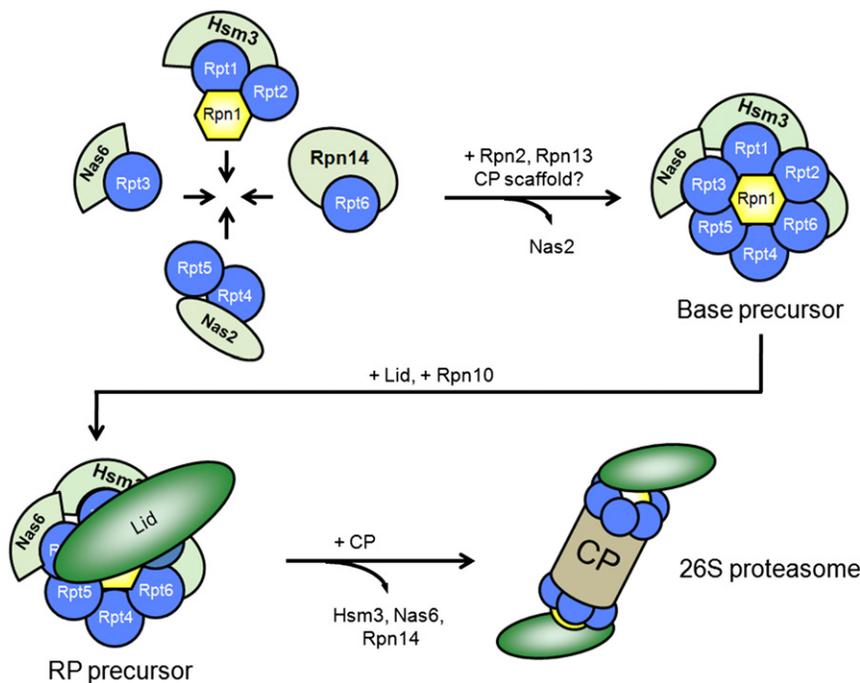
Nas2, Hsm3, Nas6, and Rpn14 Are RP Assembly Factors

Analysis of the mammalian Nas2 ortholog, p27, showed that it could be purified as a complex with an apparent size of ~300 kDa that included equal amounts of p27, Rpt4, and Rpt5 (DeMartino et al., 1996). Addition of this “modulator” to purified RP (called PA700) and CP complexes enhanced capping of the CP with RP complexes. Our in vitro assembly data are broadly comparable with these results. We suggest that in both yeast and mammalian cells, Nas2/p27 and the modulator stimulate RP base assembly from incomplete RP base precursors.

Yeast Nas2 and mammalian p27 have never been detected in fully assembled base, RP, or 26S proteasome complexes, so it is possible that Nas2 binding is mutually exclusive with another component of the RP base. The *rpt4-G106D* CC-disrupting mutation does not interfere with Rpt4-Rpt5 interaction but impairs binding to Nas2. However, *rpt4-G106D* cells have a much stronger assembly defect than do *nas2Δ* cells. The simplest hypothesis to explain these data is that the Rpt4 CC associates directly with the CC in Nas2 as well as another RP subunit(s), although other interactions between Rpt4-Rpt5 and Nas2 are also possible.

Yeast Hsm3, which is not part of mature 26S proteasomes, is related by its sequence and biochemical interactions to mammalian S5b, a protein previously thought to be part of 26S proteasomes (Gorbea et al., 2000). However, the isolation of S5b and biochemical analysis of its protein-protein interactions were done with purified RP complexes, so either the purified mammalian RPs included RP precursors that exclusively contained the S5b protein or S5b can also associate with mature RP when the RP is not bound to the CP. Consistent with these ideas, our MS/MS and anti-Hsm3 native gel immunoblot analyses showed Hsm3 in a complex that appeared to include the complete RP, while no Hsm3 was detected in the RPCP or RP2CP species.

Congruent with what had been shown with in vitro-synthesized human RP proteins, yeast Hsm3 forms a stable complex in vivo with the Rpt1, Rpt2, and Rpn1 subunits (Figure 5B). This heteromer was detectable in WT cells but was much more abundant in the *rpt4-G106D* strain. Potentially, the *rpt4* mutation interferes with the binding of Rpt4-Rpt5(-Nas2) to the



Rpt1-Rpt2-Rpn1-Hsm3 complex, limiting formation of a larger RP-base precursor that has both subcomplexes. Rpt4 and Rpt5 are not believed to be direct ring neighbors with either Rpt1 or Rpt2 (Hartmann-Petersen et al., 2001), but a recent study suggests that Rpn1 fills the center of the ATPase ring (Rosenzweig et al., 2008), so Rpn1 might make contacts with multiple ATPases (see Figure 7). Nas2 may facilitate this initial interaction but could get displaced from Rpt4-Rpt5 by other base subunits. Alternative routes of base assembly are also possible (see next section).

Just prior to the submission of our work, an independent study was published on the potential role of yeast Hsm3 in RP assembly (Le Tallec et al., 2009). The overlapping data on Hsm3 from the two studies are largely in agreement. Le Tallec et al. did not analyze any of the other RP assembly factors identified here, nor did they determine the exact complexes in which Hsm3 resides. Our analysis of assembly defects were all done under conditions where cells were fully viable, whereas the other study required extended cell growth under conditions that are lethal to *hsm3Δ*.

Nas6 and Rpn14 had previously been associated with proteasome function, primarily in mammals (Whitby and Hill, 2007). Yeast Nas6 and its mammalian ortholog, gankyrin, bind directly to the Rpt3 ATPase; gankyrin does not bind detectably to the full 26S proteasome (Dawson et al., 2002). Interestingly, gankyrin also appears to function as an assembly platform for specific kinases and ubiquitin ligases, which is important for the regulation of the p53 and Rb tumor suppressors. From the crystal structure of the Nas6-Rpt3 complex, the ankyrin repeats that comprise most of Nas6 form a curved structure whose concave surface forms an extensive interface with Rpt3 (Nakamura et al., 2007). Modeling Rpt3 as part of a full ATPase ring suggests that Nas6 binds to the outer surface of the ring and would be compat-

Figure 7. Model for the Assembly of the Proteasome Regulatory Particle and the Role of RP Base Assembly Chaperones

The inferred point of each chaperone's release is shown at the step after the latest complex in which it was detected. See the main text for details.

ible with binding to a full ring (Whitby and Hill, 2007). This is consistent with our MS/MS analysis, which identified Nas6 in a base precursor containing all six ATPases (and all three base Rpn subunits). Moreover, anti-Nas6 immunoblotting showed that Nas6 also associates with a full RP-like complex.

Previously, only minimal information was available for Rpn14 or its human ortholog, PAAF1. Deletion of yeast *RPN14* causes an extremely mild proteolytic defect against certain substrates (Seong et al., 2007). In contrast, human PAAF1 has been proposed to act as an inhibitor of RP-CP interaction (Park et al., 2005).

This inhibition contributes to the recruitment of the RP, without the CP, to HIV-1 promoters by the viral Tat protein, which stimulates transcriptional elongation (Lassot et al., 2007). PAAF1 binds most strongly to Rpt6 but shows some association with the remaining five proteasomal ATPases as well (Park et al., 2005). If Rpn14/PAAF1, which has a WD40/ β -propeller domain, makes contacts with multiple ATPases, it is possible that it binds the surface of the ATPase ring that would contact the CP α ring. This could account for the inhibition of RP-CP association when PAAF1 is overexpressed in tissue culture cells (Park et al., 2005).

The other three assembly factors described in our study are also composed substantially or almost entirely of structurally well-characterized protein interaction motifs. In Nas6 binding to the C-terminal domain of Rpt3, over 2250 \AA^2 of surface area are buried between Nas6 and Rpt3, which led to the suggestion that Nas6 is an intrinsic subunit of the proteasome (Nakamura et al., 2007). The fact that it is not, despite this very large interface with complementary charge patches, suggests that Nas6 release might require additional factors, such as molecular chaperones or regulated conformation changes in the ATPase ring. An earlier study indicated that Nas6 is only associated with the RP in the absence of ATP (Verma et al., 2000), and Hsp70 and Hsp90 family members contribute to proteasome assembly, although their exact functions remain uncertain (Murata et al., 2009).

The central region of Hsm3 is composed largely of ARM or HEAT repeats; based on known structures of proteins made of such repeats, the Hsm3 protein is likely to form a curved structure analogous to that formed by the ankyrin repeats of Nas6. We therefore suggest that Hsm3 will also bind to the outer surface of the ATPase ring. The specific genetic interaction between an *rpt1ts* mutation (*cim5-1*) and *hsm3Δ*, as well as the ability of high-copy *HSM3* (but not *NAS2*) to suppress the

cim5-1 growth defect, suggests that Hsm3 might bind the Rpt1 C-terminal domain, similar to the Nas6-Rpt3 complex.

Nas2 does not bear any obvious repeat structure, but residues 96–191 of the 220 residue protein are predicted to comprise a PDZ domain. Most PDZ domains bind C-terminal peptide motifs in their target proteins (Nourry et al., 2003). It is possible that Nas2 binds a C-terminal peptide of one of the base subunits. Rpt5 terminates with the sequence FYA-COOH, which would fit the consensus for a class II PDZ target (X-Hb-X-Hb-COOH, where Hb is a hydrophobic residue; Nourry et al. [2003]). Interestingly, this C-terminal segment of Rpt5 also conforms to the “HbYX motif” recently shown to insert into a pocket between subunits in the CP α ring (Rabl et al., 2008; Gillette et al., 2008). Nas2 might prevent premature association of the Rpt4-Rpt5 complex with the CP in addition to facilitating its incorporation into RP base precursors.

Model for RP Assembly

We propose a general model for RP assembly, as outlined in Figure 7. Although there may be a precise order of addition of subunits or subunit complexes into the RP base, we cannot deduce this from the data in hand. The general roles we envision for the assembly chaperones are (1) stabilizing specific RP base subcomplexes, (2) facilitating incorporation of these subcomplexes into higher-order RP precursors, and/or (3) preventing subunit associations that slow assembly or lead to off-pathway proteasome intermediates. For the subcomplexes shown at the beginning of the pathway in Figure 7, we have provided direct evidence for in vivo formation of Rpt4-Rpt5-Nas2 and Rpt1-Rpt2-Rpn1-Hsm3. These are both detected in WT cells but accumulate to higher levels in specific mutants. An Rpt3-Nas6 complex has been documented in biochemical and structural studies (Nakamura et al., 2007). The simplest RP subcomplex that we had seen containing the Rpt3, Rpt6, Rpn2, and Rpn13 base subunits was a full base precursor, but it is possible that these subunits also participate in other, more transient precursors.

In the preceding section, we suggested that the Rpt4-Rpt5-Nas2 and Rpt1-Rpt2-Rpn1-Hsm3 complexes might assemble cooperatively into an RP base precursor, consistent with the partially overlapping functions of Nas2 and Hsm3 inferred from analysis of multiple mutant combinations. Simultaneous loss of the other two chaperones, Nas6 and Rpn14, has even more striking effects on RP biogenesis. Moreover, high-copy *RPN14* and *NAS6* suppress *rpt6* (*cim3-1*) growth defects, and overexpression of both genes in the same cells further enhances this suppression (Figure S6C and data not shown). This argues for significant functional overlap between these two chaperones. Nevertheless, the individual mutations do have distinct effects. The *rpn14 Δ* strain shows a selective loss of free base, whereas *nas6 Δ* cells lose a large fraction of both the free base and free RP (Figure 6C). These data suggest that while both deletions lead to an RP base assembly defect, *nas6 Δ* also may cause a defect in base-lid joining or may allow unregulated RP-CP binding. Consistent with this, high-copy *NAS6*, but not *RPN14*, can enhance formation of the full RP in certain *rpt* mutants (Figure 6D and data not shown).

It is interesting that Rpt3 and Rpt6, the two ATPases bound by Nas6 and Rpn14, respectively, are not direct neighbors in the

ATPase ring (Figure 7). However, in vitro binding and yeast two-hybrid analyses indicate that these two subunits can associate (Russell et al., 1996; Richmond et al., 1997). Therefore, it is possible that an important function for the Nas6 and Rpn14 chaperones is to limit this potential off-pathway interaction. This could operate indirectly. For example, Rpn14 binding to Rpt6 might facilitate Rpt6 association with Rpt1-Rpt2-Rpn1-Hsm3, and Nas6 binding to Rpt3 might enhance interaction with Rpt4-Rpt5(-Nas2), with either interaction thereby limiting Rpt3-Rpt6 binding. Thus, only after loss of both Nas6 and Rpn14 will a strong assembly defect be detected. Experiments to test these mechanistic hypotheses are underway.

Conservation of RP Assembly Mechanisms

All of the assembly factors described here are conserved across a broad range of eukaryotic species. This is also true for CP assembly chaperones. Therefore, it is highly likely that the assembly mechanisms of the RP and 26S proteasome are very similar in most or all eukaryotes. At present we know of three dedicated CP assembly factors (Murata et al., 2009) and now four RP base assembly factors. Nothing is known yet about whether additional dedicated assembly chaperones will be needed for efficient RP lid biogenesis or lid-base joining in vivo, although this seems probable.

Proteasomal active site inhibitors are in clinical use for treating several cancers and are in trials for managing additional disorders (Goldberg, 2007). Impairing proteasome assembly represents a potential new strategy for inhibiting proteasome function for clinical purposes. This might prove particularly useful when patients develop resistance to active-site inhibitors. Research on the mechanisms of proteasome biogenesis and the roles of the emerging array of assembly chaperones will be essential for the development of effective assembly inhibitors.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

All yeast manipulations were carried out according to standard protocols (Guthrie and Fink, 2002). Strains used in this study are listed in the Supplemental Data. For plating serial dilutions of cells, yeast cultures were grown overnight in rich medium (YPD) or minimal medium (SD) and diluted to an OD₆₀₀ of 0.2 in water. Six-fold dilutions were prepared in water and spotted onto various media. Plasmids (Supplemental Data) were made by standard methods (Guthrie and Fink, 2002).

Immunoblot Analysis

SDS-PAGE and immunoblot analysis of proteins were carried out according to standard procedures (Li et al., 2007). For immunoblotting, gel-separated protein samples were transferred to PVDF membranes (Millipore). Membranes were incubated with antibodies to ubiquitin (M.H. lab), Rpn5 or Rpn8 (D. Finley), Rpt4 (T. Kodadek [rabbit] or W. Tansey [mouse]), $\alpha 4$ (D.H. Wolf), Pgk1 or V5 (Invitrogen), Rpt5 (BIOMOL), Rpn1 or Rpt1 (W. Tansey), or Blm10 (C. Enenkel), or affinity-purified antibodies to Nas2, Nas6, or Hsm3. Proteins were visualized by ECL or ECL-Advance (for mouse anti-Rpt4 antibody; GE Healthcare).

Nondenaturing Gel Analyses of Yeast Extracts

Yeast cell extracts were prepared essentially as described (Kusmierczyk et al., 2008). In brief, mid-to-late log phase cells (OD₆₀₀ 1.0–2.0) were washed with ice-cold water and frozen in liquid nitrogen. The frozen cells were ground with mortar and pestle, and the resultant cell powder was thawed in 26S buffer (50 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 10% glycerol, 1 mM ATP). Extracts

were centrifuged for 10 min at 15,000 × g to remove cell debris. After concentrations were determined with the Bio-Rad Protein Assay, 24–25 μg of protein per sample were used for native PAGE, followed by substrate overlay or immunoblotting; 3–6 μg of total protein were used for immunoblotting after SDS-PAGE. Nondenaturing PAGE was performed as described (Kusmierczyk et al., 2008).

MS/MS Analysis of Protein Complexes

Purified proteasomes and proteasome subparticles were separated by 4%–6% native PAGE and visualized with GelCode Blue as described (Li et al., 2007). Protein bands were excised and sent to Midwest Bio Services LLC for compositional analysis by Nano-LC-MS/MS. Samples were digested in-gel with trypsin, and the resulting peptide mixture was analyzed by LC-MS/MS.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, six tables, and seven figures and can be found with this article online at [http://www.cell.com/supplemental/S0092-8674\(09\)00526-1](http://www.cell.com/supplemental/S0092-8674(09)00526-1).

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