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## Hexameric assembly of the proteasomal ATPases is templated through their C-termini

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### Abstract

Substrates of the proteasome are recognized and unfolded by the regulatory particle (RP), then translocated into the core particle (CP) to be degraded. A hetero-hexameric ATPase ring, containing subunits Rpt1-Rpt6, is situated within the base subassembly of the RP1. The ATPase ring sits atop the CP, with the Rpt C-termini inserted into pockets in the CP2–6. We have identified a novel function of the Rpt proteins in proteasome biogenesis through deleting the C-terminal residue from each Rpt. Our results indicate that assembly of the hexameric ATPase ring is templated on the CP. We have also identified an apparent intermediate in base assembly, BP1, which contains Rpn1, three Rpts, and Hsm3, a chaperone for base assembly. The Rpt proteins with the strongest assembly phenotypes, Rpt4 and Rpt6, were absent from BP1. We propose that Rpt4 and Rpt6 form a nucleating complex to initiate base assembly, and that this complex is subsequently joined by BP1 to complete the Rpt ring. Our studies show that assembly of the proteasome base is a rapid yet highly orchestrated process.

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The core particle (20S CP) of the 26S proteasome is associated with the regulatory particle (19S RP), which in turn consists of two subassemblies, the base and lid. The CP is composed of  $\alpha$ -type and  $\beta$ -type subunits, with each family of subunits arranged into heteroheptameric rings, which are stacked to form a barrel-like  $\alpha_7$ - $\beta_7$ - $\beta_7$ - $\alpha_7$  complex<sup>1,7</sup>. The proteolytic sites are inside the CP barrel, and substrate access to these sites is achieved by opening a gate in the  $\alpha$  ring. Binding of the RP opens the gate. Rpt1-6, six ATPases of the base, are thought to form a hetero-hexameric ring, and the C-termini of these Rpts insert into pockets in the CP  $\alpha$  ring2–6. The extreme C-termini of four Rpts contain the motif Hb-Y-X

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#### Author contributions

S.P. conducted all experiments. J.R. contributed to designing experiments. W.K. performed mass-spectrometry. S.P. and D.F. wrote the paper. All authors provided comments.

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(hydrophobic residue-Tyr-X), which mediates opening of the CP gate<sup>3,4,6</sup> (Supplementary Table 1).

The C-terminal carboxylate of a given Rpt protein forms a salt bridge to a lysine residue at the bottom of the CP  $\alpha$  ring pocket<sup>2,3</sup>. To eliminate these salt bridges, we deleted the C-terminal residue from each Rpt protein, generating six chromosomal mutations (*rpt1- $\Delta$ I* to *rpt6- $\Delta$ I*). Proteasomes in whole cell extracts from each mutant were analyzed by native PAGE, combined with an in-gel peptidase assay using a fluorogenic substrate, LLVY-AMC (Fig. 1a). Free CP was strongly elevated in *rpt3- $\Delta$ I*, *rpt4- $\Delta$ I*, *rpt5- $\Delta$ I*, and *rpt6- $\Delta$ I* mutants as revealed by the addition of 0.02% SDS (Fig. 1a). As previously shown, SDS opens the CP gate, which is closed in the free CP<sup>1,7</sup>. The CP, and a representative CP subunit,  $\alpha$ 7, were most strongly induced in *rpt4- $\Delta$ I* and *rpt6- $\Delta$ I* mutants (Fig. 1a and 1b). Interestingly, Rpt4 and Rpt6 are the two Rpts that lack the gating-directed HbYX motifs<sup>4</sup> (Supplementary Table 1). These mutants also showed strong reductions in proteasome holoenzyme (Fig. 1a, RP-CP and RP<sub>2</sub>-CP).

CP induction was mediated principally by Rpn4, a transcription factor that upregulates the expression of proteasome subunits when proteasome activity is compromised<sup>8,9</sup>; *RPN4* deletion in *rpt4- $\Delta$ I* or *rpt6- $\Delta$ I* mutants markedly decreased the level of  $\alpha$ 7 and free CP (Fig. 1c and Supplementary Fig. 1). Assembly defects in the *rpt* mutant proteasomes correlated with the accumulation of ubiquitin-conjugates and, in the case of the strongest mutant, *rpt6- $\Delta$ I*, a growth defect at 30°C (Fig. 1b and 1d). Phenotypes of *rpt4- $\Delta$ I* and *rpt6- $\Delta$ I* were exacerbated by *RPN4* deletion, indicating that the proteasome stress response masks the severity of these mutations (Fig. 1e).

Proteolytically inactive subassemblies such as the RP and lid cannot be visualized by activity assay. Therefore, to further address the assembly defects in *rpt4- $\Delta$ I* and *rpt6- $\Delta$ I* mutants, proteasomes were analyzed by immunoblot following native PAGE. Given the apparent role of Rpt C-termini in CP binding<sup>2-6</sup>, the increase in free CP and decrease in RP<sub>2</sub>-CP and RP-CP might predict an increase in free RP in *rpt4- $\Delta$ I* and *rpt6- $\Delta$ I* mutants. On the contrary, free RP was not detected in these mutants; instead free lid was observed (Fig. 1f and Supplementary Fig. 2). The production of free lid was unexpected because the Rpt C-termini, if docking to the CP, cannot be in contact with or proximal to the lid. Base-CP complex, previously observed in a lid mutant<sup>10</sup>, was not apparent in *rpt4- $\Delta$ I* or *rpt6- $\Delta$ I* mutants (Fig. 1f and Supplementary Fig. 2). Thus, productive interaction between the CP and the Rpt C-termini of the base is apparently required for stable incorporation of the lid to complete proteasome assembly.

Because base joins the lid to the CP in proteasome holoenzymes, the accumulation of free lid and free CP in *rpt4- $\Delta$ I* and *rpt6- $\Delta$ I* mutants may reflect underlying defects in base assembly<sup>11,12</sup>. The base can be produced by exposing assembled proteasome to 1M NaCl<sup>13,14</sup>. The resulting particle represents the mature form of the base (Supplementary Fig. 3). If the base exists as a free endogenous complex, its isolation should be possible by affinity purification via Protein A-tagged Rpt1, using a physiological salt concentration. Such samples were analyzed by 2-D native/SDS-PAGE. A base variant, which we refer to as base\*, was detected by this method (Fig. 2a, top). Interestingly, mass spectrometric analysis

revealed that base\* contains, in addition to Ubp6 and subunits of the base15, three proteins that are not proteasome components: Rpn14, Nas6, and Hsm3 (Fig. 2a). An accompanying paper<sup>16</sup> shows that this set of proteins functions as molecular chaperones and more specifically as base assembly factors<sup>16</sup>. We show below that the function of these proteins, termed RP-chaperones, is tightly linked to the Rpt C-termini.

In *rpt4-Δ1* mutants, base\* levels are severely reduced, suggesting a defect in base assembly (Fig. 2a and 2b). Base\* can also be visualized in unfractionated extract from untagged wild-type cells (Fig. 2c and 2d). Base\* was deficient in *rpt6-Δ1* cells (Fig. 2c) and undetectable in the RP-chaperone triple mutant, *hsm3Δ nas6Δ rpn14Δ* (Fig. 2d).

The disappearance of base\* in *rpt4-Δ1* mutants suggested an upstream assembly defect that would be manifest in the accumulation of smaller assembly intermediates of the base. Accordingly, affinity purification from *ProA-TeV-Rpt1 rpt4-Δ1* mutants revealed a novel species that we termed Base Precursor 1, or BP1 (Fig. 2a and 2b). BP1 has two forms; the lower-mobility form contains deubiquitinating enzyme Ubp6, and is referred to as Ubp6 + BP1 (Fig. 2a and 2b). BP1 was also observed in wild-type, though at lower levels (Fig. 2a and 2b). The proportion of BP1 loaded with Ubp6 is higher in the mutant; most likely base assembly, which consumes BP1, is faster than Ubp6 loading in wild-type, while slower than Ubp6 loading in the mutant (see below). By mass spectrometry following 2-D native/SDS-PAGE, BP1 was found to contain Rpn1, Rpt1, Rpt2, Rpt5, and the RP-chaperone Hsm3 (Fig. 2a). The presence of Hsm3 in BP1 suggests that BP1 is an assembly intermediate. BP1 was detected in untagged whole cell extracts, and thus, like base\*, it is not an artifact of tagging or purification (Fig. 2c and 2d). BP1 levels in extracts are elevated in *rpt4-Δ1* and *rpt6-Δ1* mutants (Fig. 2c). In contrast, *HSM3* deletion resulted in an apparently complete elimination of BP1 (Fig. 2e). Thus, Hsm3 is not a bystander in BP1, but strongly regulates its abundance.

The absence of RP-chaperones Rpn14 and Nas6 from BP1 can be explained by their binding specificities: whereas Hsm3 binds to the BP1 component Rpt112,16, the other RP-chaperones bind to Rpt proteins that are absent from BP1: Nas6 to Rpt316,17, and Rpn14 to Rpt4 and Rpt616. RP-chaperones bind to the C-domains of Rpt proteins, but not to their extreme C-termini<sup>16</sup>. In contrast to *HSM3*, deletion of *RPN14* or *NAS6* had little effect on the level of BP1 (Fig. 2e), indicating that their roles in base assembly are distinct from the regulation of BP1.

The absence of Rpt4 and Rpt6 from BP1 is interesting because these are the Rpt proteins whose C-termini are most critical for base assembly. The accumulation of BP1 in *rpt4-Δ1* and *rpt6-Δ1* mutants likely reflects perturbation of an assembly event upstream of BP1 incorporation into nascent base. Also absent from BP1 were CP subunits (Supplementary Fig. 4); the failure to observe a stable BP1-CP complex suggests that such complexes are not involved in assembly. Rather, BP1 may load onto a separate, complementary assembly intermediate that includes Rpt4 and Rpt6 and is capable of direct interaction with CP.

To verify that BP1 is a nascent complex utilized for proteasome assembly, we performed a pulse-chase experiment using *in vivo* S<sup>35</sup> metabolic labeling (Fig. 3). BP1 was visualized by

pulse-labeling, and after a 30-minute chase with excess unlabeled methionine, S<sup>35</sup>-labeled BP1 was significantly decreased (Fig. 3). Thus, BP1 is a transient complex in vivo. Consistent with steady-state experiments, S<sup>35</sup>-labeled BP1 was more prominent in *rpt4-ΔI* than in wild-type, suggesting that its incorporation into nascent base depends on proper interaction between Rpt4 C-termini and CP.

The interaction of CP with Rpt4 and Rpt6 is regulated by Rpn14, which specifically interacts with the C-domains of these two Rpts<sup>16</sup>. Rpn14 associates with RP or base, but little or no Rpn14 is found on CP-containing complexes such as proteasome holoenzyme<sup>16</sup>. Strikingly, Rpn14 was readily detected on Rpt6-ΔI proteasome holoenzyme that had been affinity-purified via a CP tag (Fig. 4a and Supplementary Fig. 5). In contrast, Hsm3 and Nas6 did not associate with Rpt6-ΔI proteasomes (Fig. 4a), showing that the effect of the *rpt6-ΔI* mutation was local, and restricted to Rpt6's binding partner. We conclude that Rpn14 is released as a specific consequence of the engagement of its cognate Rpt's C-terminus with the CP. Proteasome holoenzyme or base-CP assembly *per se* is not sufficient for displacement. The RP-chaperone competes with CP for occupancy of the Rpt C-domain, providing a simple mechanism by which RP-chaperones can regulate base assembly and subsequently exit the complex<sup>16</sup>. Could the failure to release Rpn14 account for the phenotypic defects of *rpt4-ΔI* and *rpt6-ΔI* mutants? This is unlikely, because *rpn14Δ* is not a suppressor of either allele (data not shown).

Analysis of Rpt3-ΔI proteasomes also revealed a defect in chaperone expulsion during assembly. Rather than Rpn14, Rpt3-ΔI proteasomes retained Nas6, the binding partner of Rpt3<sup>16,18</sup> (Fig. 4b). The *rpt3-ΔI* mutation had no effect on the association of Hsm3 or Rpn14 with proteasomes. The association of Rpn14 or Nas6 with the holoenzyme was not due to an increase in their protein expression level in *rpt6-ΔI* or *rpt3-ΔI* mutants, respectively (data not shown).

Our structural modeling suggested that competition between CP and RP-chaperones for Rpt binding reflects the proximity of their binding sites<sup>16</sup>. The model predicts that separating the two binding sites would abrogate competition, and thus allow retention of RP-chaperones on the proteasome. Remarkably, insertion of a single residue into Rpt6 (*rpt6-S<sup>+</sup>*), four residues from the C-terminus, prevented expulsion of Rpn14 (Fig. 4c). Thus, the expulsion mechanism is very precise. Importantly, the failure to expel Rpn14 was not a result of failure to dock the mutant Rpt6 C-terminus at the CP, because *rpt6-S<sup>+</sup>* mutants did not phenocopy *rpt6-ΔI* mutants (Fig. 4d). The release of non-cognate RP-chaperones was unaffected by C-terminal insertions (Fig. 4c).

In bacterial AAA proteins, formation of the ATPase ring is thought to be spontaneous and unassisted<sup>19</sup>. In contrast, the proteasome's ATPase ring is assembled through a programmatic and remarkably intricate pathway. This and the accompanying study<sup>16</sup> suggest that the pathway of base assembly involves four distinct classes of factors, each found in multiple forms: the seven-membered subunit ring of the CP, the six ATPases of the Rpt ring, two scaffolding factors (Rpn1 and Rpn2), and three RP-chaperones.

The importance for assembly of the extreme C-termini of the Rpts indicates a prominent role of CP templating in Rpt ring formation. We propose that one subset of Rpts assembles directly on the CP, including Rpt4 and Rpt6, whose mutants confer the strongest assembly phenotypes. In contrast, another group of Rpts, including Rpt1, Rpt2, and Rpt5, is proposed to initially assemble free of the CP in the BP1 complex (Fig. 4e). Because BP1 disappears rapidly in pulse-chase experiments, it behaves as a true *in vivo* assembly intermediate. The interaction of the Rpt binding proteins with the  $\alpha$  pockets of the CP appears to be negatively regulated by the RP-chaperones<sup>16</sup>. These chaperones may suppress  $\alpha$  pocket-Rpt interactions during certain stages of assembly, and, during other stages, disengage from the Rpt to permit assembly to proceed forward (Fig. 4). In this way the RP-chaperones may temporally sequence the assembly process.

A simple though hypothetical scenario is that the base is formed by addition of BP1 to a preformed complex containing some or all of the base components absent from BP1. This proposed assembly intermediate is termed BP2 in our three-template model (Fig. 4e). Rpt4 and Rpt6 are proposed to mediate BP2 formation by CP templating. Slow maturation of BP2 may explain the accumulation of BP1 observed in *rpt4- $\Delta$ 1* and *rpt6- $\Delta$ 1* mutants, consistent with this model. We also propose that Rpn2 functions as a scaffold to organize BP2, based on evidence found in this and previous studies. For example, Rpt420 and Rpt621 have been shown to specifically interact with Rpn2 (whereas Rpt1 and Rpt2 interact with the Rpn122 scaffold of BP1). Moreover, Rpn2 binds CP directly<sup>23</sup>. Thus, a complex containing Rpn2, Rpt4, and Rpt6 is well suited to nucleate base assembly. Rpn2 and the CP may jointly template early ring assembly as the CP-Rpt binding events are most likely not strong enough to single-handedly specify Rpt ring assembly. A base subassembly containing Rpn1 and Rpn2 was isolated by *in vitro* dissociation of proteasomes<sup>23</sup>, but according to our findings it might not be formed during proteasome biogenesis. However, the Rpn1-Rpn2 interaction could be important for driving association of the BP1 complex with a complementary Rpn2-containing complex, such as the putative BP2.

## Methods summary

### Purification of BP1

Strains carrying ProA-TeV-Rpt1 were grown in 6 L of SD medium to OD<sub>600</sub> 0.8 to 1.2. Cells were harvested, washed once with ice-cold water and drop-frozen in liquid nitrogen. Frozen yeast samples were then ground using an MM301 grinding mill (Restch) under liquid nitrogen following manufacturer's instructions, or using a mortar and a pestle as previously described<sup>24</sup>. Ground powder was hydrated in proteasome buffer (50 mM Tris-HCl [pH7.5], 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 10% glycerol) supplemented with 2 mM ATP, protease inhibitor tablets (Complete, Roche), 2 mM PMSF, 1 mM benzamidine, 10  $\mu$ g/ml pepstatin A, and 1  $\mu$ g/ml antipain. Cell extracts were cleared at 30,000  $\times$  g for 30 min at 4°C, and the supernatants were mixed with rabbit IgG resin (Cappel, MP Biomedicals) for 90 min at 4°C. Resins were collected at 900  $\times$  g for 2 min at 4°C and washed with proteasome buffer containing 50 mM NaCl three times, followed by a final wash with proteasome buffer alone. BP1 was then released from the resin by incubating with AcTEV protease (Invitrogen) at 2.5 unit/L culture in proteasome buffer containing 2 mM ATP and protease inhibitors for 1 hr at

30°C. Eluates were concentrated using Ultrafree-0.5 centrifugal filter device with 30 kDa NMWL (Millipore).

### **Pulse-chase analysis of BP1, native PAGE and immunoblot**

Detailed methods and strain list are described in Supplementary information.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

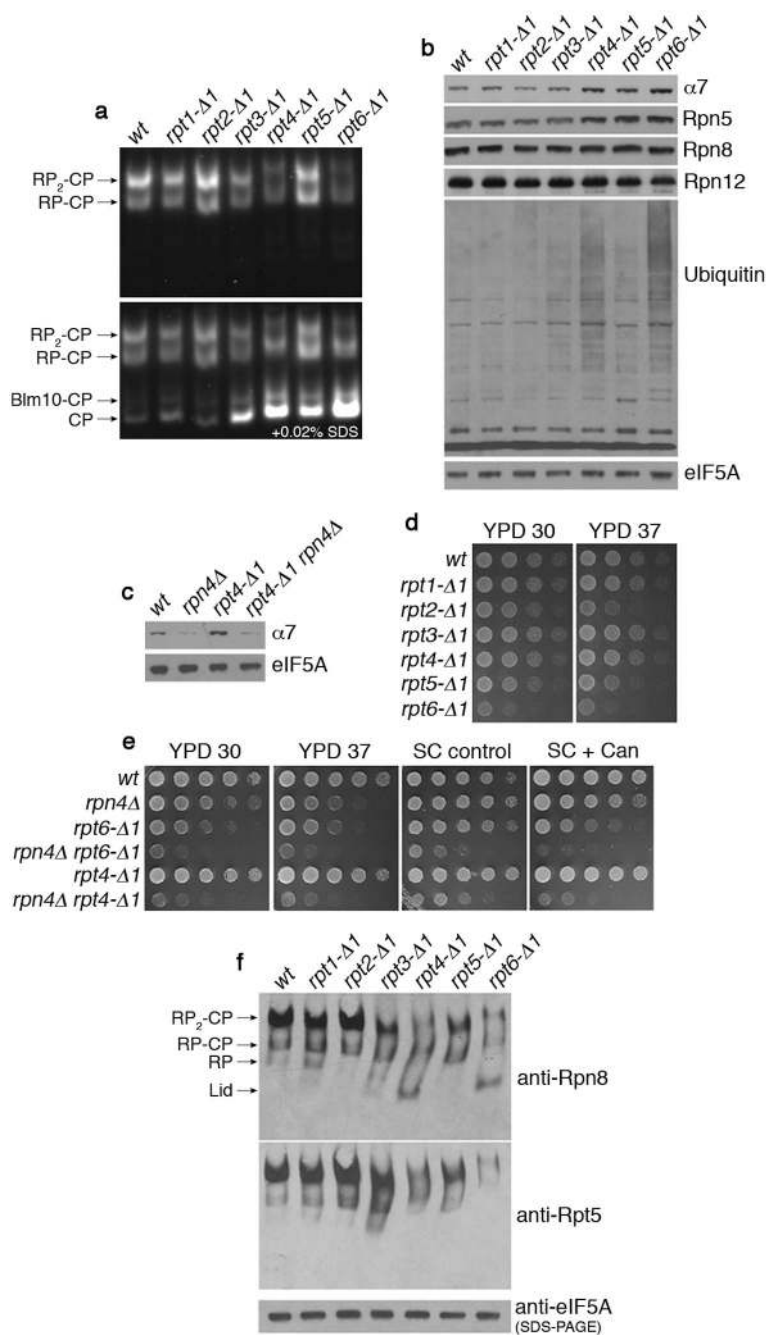
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**Figure 1. *rpt4-Δ1* and *rpt6-Δ1* mutants are proteasome hypomorphs with defective proteasome assembly**

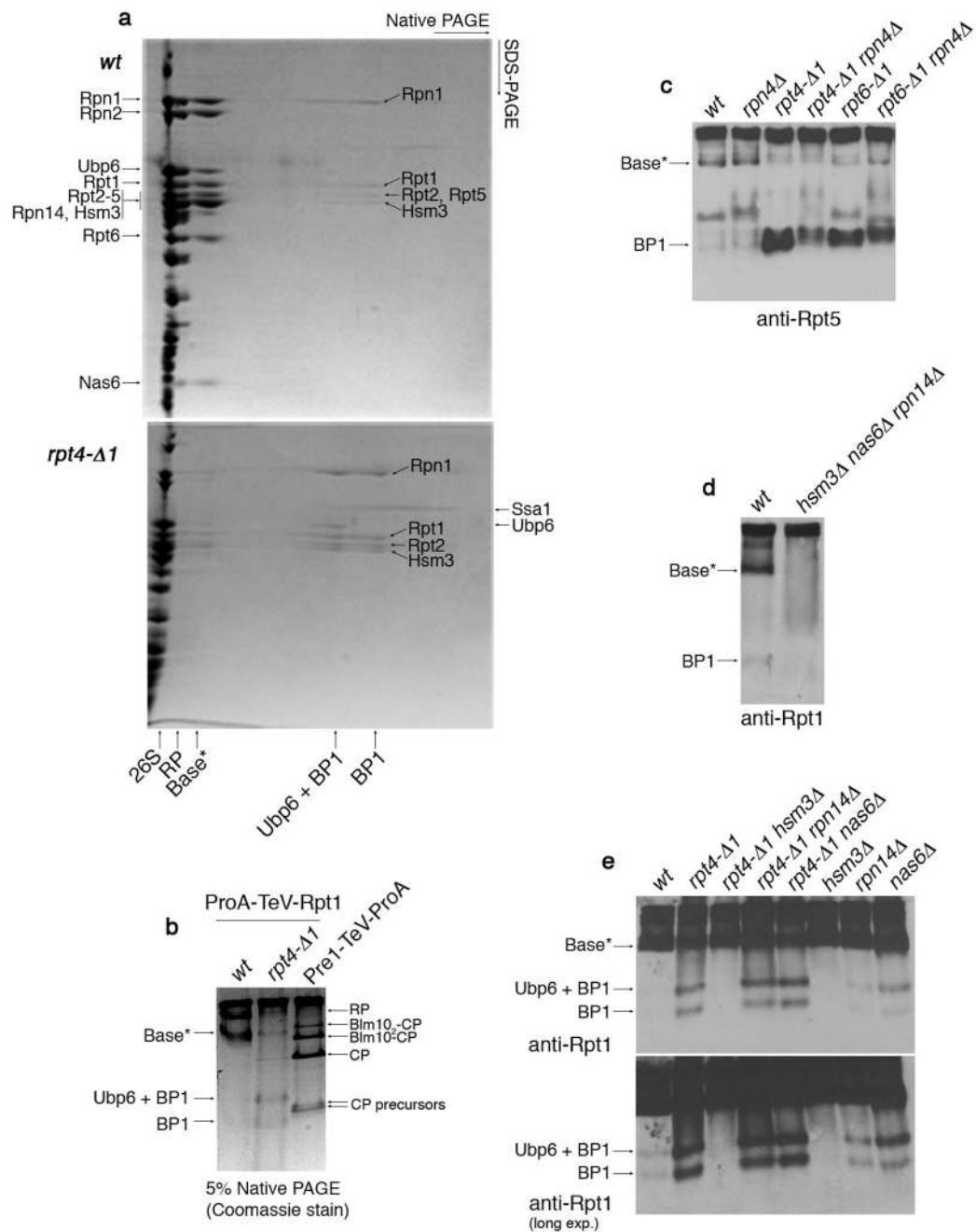
**a**, Native PAGE (3.5%) and two consecutive LLVY-AMC assays with whole cell extracts (85 μg). Immediately after the first LLVY-AMC assay (top panel), the second assay was conducted in the presence of 0.02% SDS (bottom panel).

**b, c**, SDS-PAGE and immunoblotting with whole cell extracts. eIF5A is a loading control.



**d, e,** Growth phenotypes of *rpt* mutants. Cells were spotted in 4-fold dilutions on YPD, synthetic medium (control), or synthetic medium containing canavanine at 0.5 µg/ml. Plates were incubated for 2–4 days at 30°C or at 37°C (YPD 37).

**f,** 3.5% native PAGE and immunoblotting of whole cell extracts (85 µg) against Rpn8 (lid subunit) and Rpt5 (base subunit). Extracts (20 µg) were resolved by SDS-PAGE and immunoblotting for loading control.



### Figure 2. Identification of a base assembly intermediate

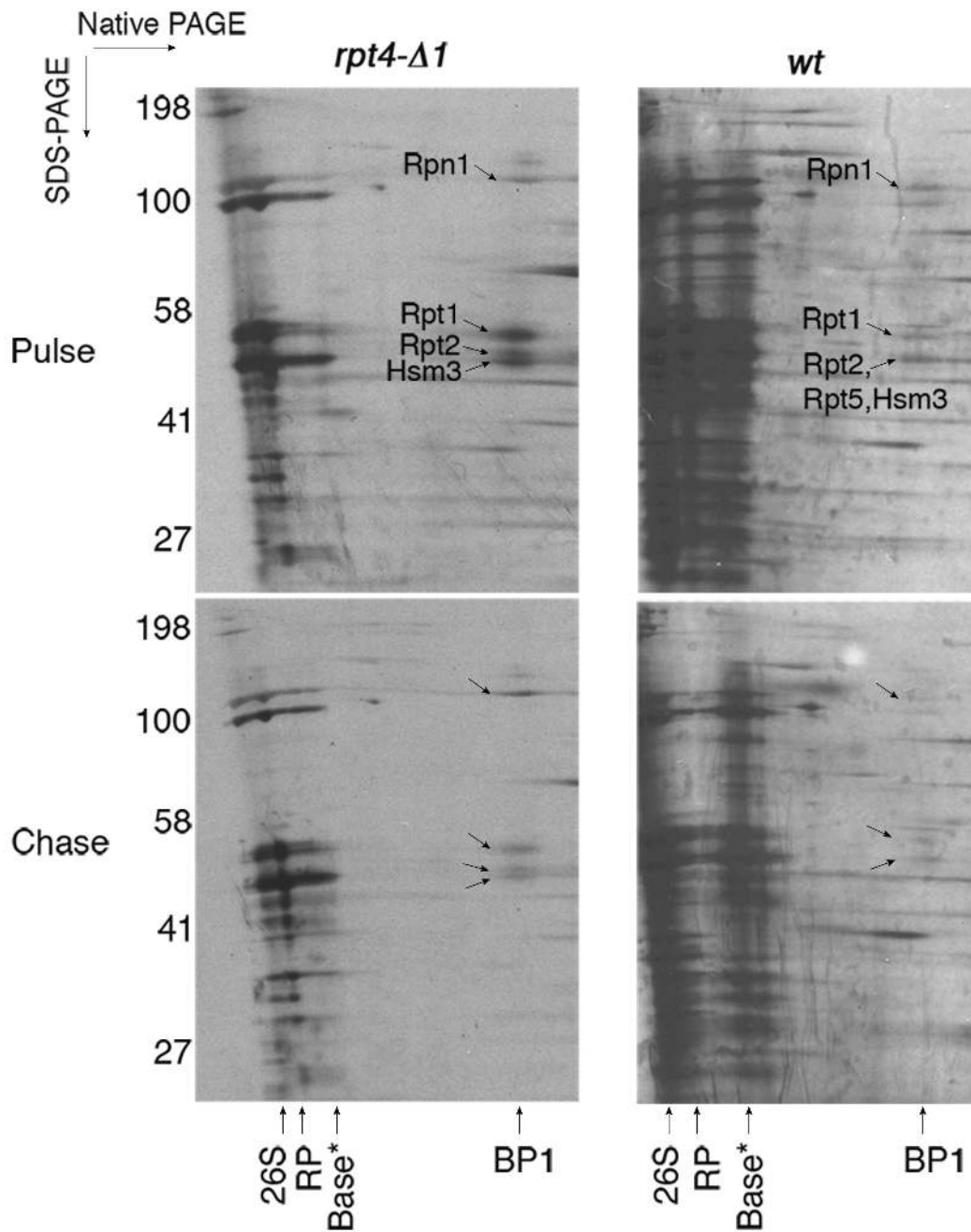
**a**, 2-D native/SDS-PAGE of affinity-purifications with ProA-TeV-Rpt1 (Protein A tag appended to the N-terminus of Rpt1, a base subunit). Following a first-dimension native PAGE (5%), native gel lanes were individually excised and subjected to second-dimension SDS-PAGE (12.5%). Gels were stained with Coomassie blue. Individual spots of the base\*, BP1, and Ubp6 + BP1 complexes were excised for mass spectrometry. Labels on the left indicate spots within base\*. The presence of Rpn10 and Rpn13 in base\* was not determined. Note that Rpt5 is absent from BP1 purified from *rpt4-Δ1* mutants, but present in BP1 in

untagged *rpt4-ΔI* extracts (panel 4c). This appears to reflect a labile association of Rpt5 with BP1 in *rpt4-ΔI* mutants. The presence of Ubp6 in BP1 is explained by its interaction with Rpn125. Note: Base\* and BP1 species appear comparable between *rpt6-ΔI* and *rpt4-ΔI* mutants in their level and composition.

**b**, 5% Native PAGE following affinity purification via a ProA-TeV-Rpt1 as in **(a)** or a Pre1 (CP subunit)-TeV-ProA. Native gels were stained with Coomassie blue.

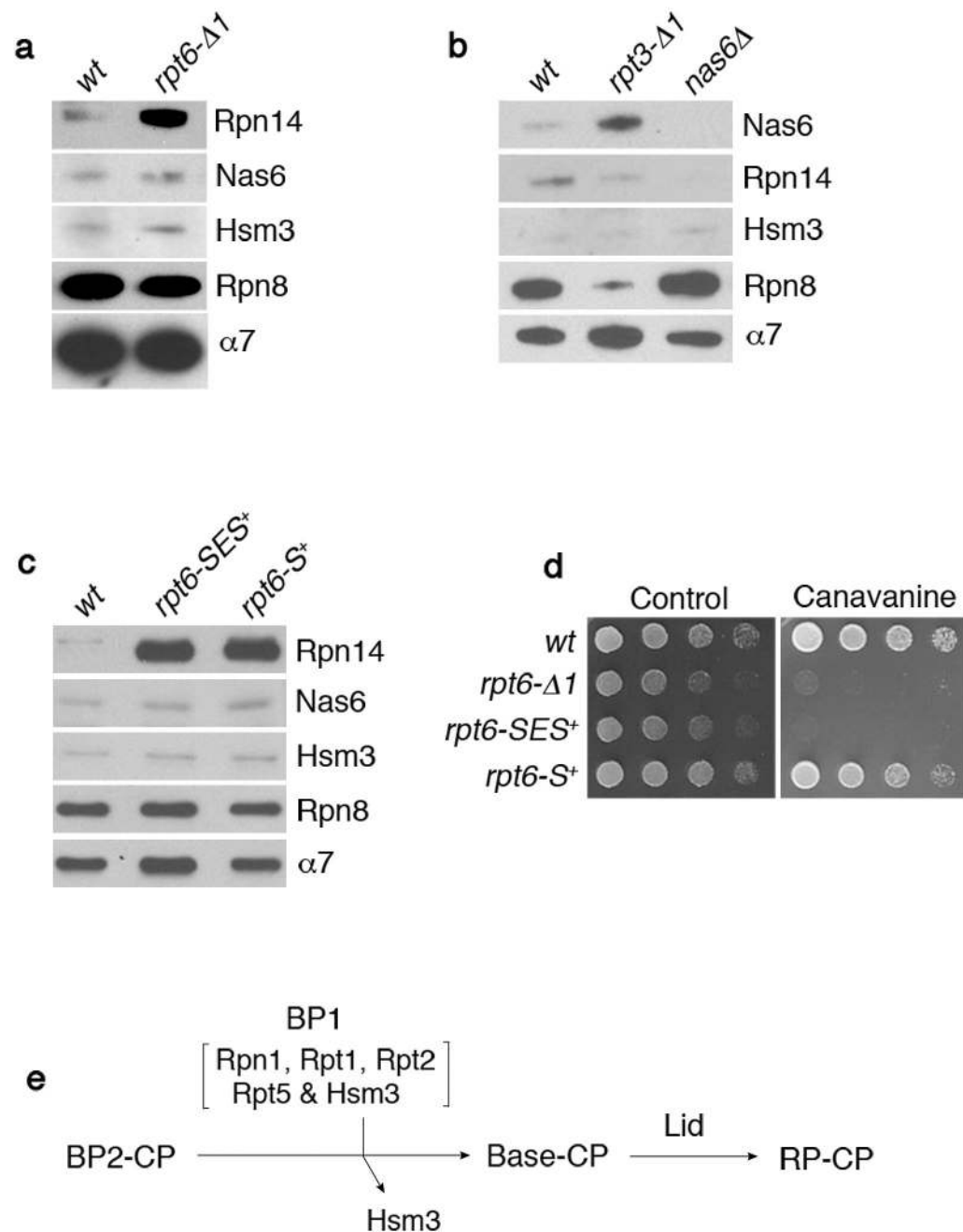
**c, d**, Immunoblotting following 5% native PAGE of whole cell extracts (100 μg).

**e**, Immunoblotting following 5% native PAGE of affinity-purified samples (2 μg) from indicated strains carrying ProA-TeV-Rpt1.



**Figure 3. Pulse-chase analysis of the BP1 base assembly intermediate**

Strains were metabolically labeled with  $S^{35}$ -methionine in vivo for 4–5 min (pulse) and chased with excess methionine for 30 min (chase). Samples were then subjected to affinity-purification with ProA-TeV-Rpt1 and 2-D native/SDS-PAGE (5% and 4–12%, respectively) followed by autoradiography. BP1, but not Ubp6 + BP1, was labeled with  $S^{35}$ -methionine during the pulse in both wild-type and the *rpt4-Δ1* mutant.



**Figure 4. Interactions between Rpt C-termini and the CP control RP-chaperone release**  
**a-c**, SDS-PAGE and immunoblotting followed by affinity purification using a CP tag (Pre1-TeV-ProA). Rpn8 (lid subunit), and  $\alpha 7$  (CP subunit) are loading controls. Nas6 $\Delta$  proteasome (**b**) is a negative control. The *rpt6-SES+* or *rpt6-S+* mutants in (**c**) contain either a three (Ser-Glu-Ser) or one (Ser) residue insertion prior to the fourth residue from the C-terminus.

**d**, Phenotypic analysis of *RPT6* insertion mutants. 4-fold serial dilutions were spotted on synthetic medium (control) or canavanine (1  $\mu\text{g/ml}$ ) plates. Cells were grown for 2–4 days at 30°C.

**e**, Model for late-stage base assembly. It is proposed that the base is formed by the addition of the BP1 complex, containing Rpn1, Rpt1, Rpt2, Rpt5, and Hsm3, to the putative BP2 complex. Initial contact with the CP is provided by BP2, whereas BP1 exists independently of the CP until it joins the BP2-CP complex. BP2 has not been identified and its exact composition is unknown. However, likely components of BP2 are Rpt4, Rpt6, Rpn2, and perhaps Rpt3. Rpn14 and Nas6 (not shown) may function prior to the formation of BP2-CP.