

# Chaperone-mediated pathway of proteasome regulatory particle assembly

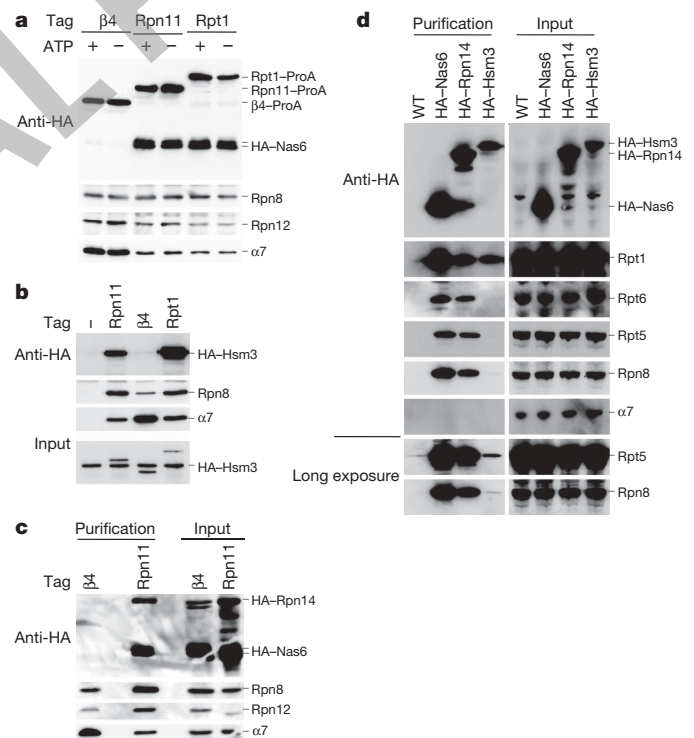
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The proteasome is a protease that controls diverse processes in eukaryotic cells. Its regulatory particle (RP) initiates the degradation of ubiquitin–protein conjugates by unfolding the substrate and translocating it into the proteasome core particle (CP) to be degraded<sup>1</sup>. The RP has 19 subunits, and their pathway of assembly is not understood. Here we show that in the yeast *Saccharomyces cerevisiae* three proteins are found associated with RP but not with the RP–CP holoenzyme: Nas6, Rpn14 and Hsm3. Mutations in these genes confer proteasome loss-of-function phenotypes, despite their virtual absence from the holoenzyme. These effects result from deficient RP assembly. Thus, Nas6, Rpn14 and Hsm3 are RP chaperones. The RP contains six ATPases—the Rpt proteins—and each RP chaperone binds to the carboxy-terminal domain of a specific Rpt. We show in an accompanying study<sup>2</sup> that RP assembly is templated through the Rpt C termini, apparently by their insertion into binding pockets in the CP. Thus, RP chaperones may regulate proteasome assembly by directly restricting the accessibility of Rpt C termini to the CP. In addition, competition between the CP and RP chaperones for Rpt engagement may explain the release of RP chaperones as proteasomes mature.

Affinity purification has allowed identification of proteins that associate with proteasomes but are not true subunits<sup>1,3–5</sup>. Nas6, one such protein<sup>4</sup>, is the apparent homologue of gankyrin (PSMD10, also known as p28), a liver oncoprotein. Gankyrin is thought to interact with retinoblastoma protein (RB), MDM2, CDK4 and the proteasome<sup>6</sup>. Using recombinant glutathione S-transferase (GST)–Nas6 we purified all 19 RP subunits from yeast lysates (Supplementary Table 1). However, to our surprise, no CP subunit was recovered. Similarly, affinity purified samples from RP-tagged strains contained Nas6 whereas those from CP-tagged strains did not (Fig. 1a). Thus, Nas6 may bind a subpopulation of RP that is not associated with CP (hereafter ‘free RP’). The specific association of Nas6 with the RP was validated through additional experiments (Supplementary Fig. 1).

Mass spectrometric analysis of immunoprecipitates of haemagglutinin (HA)-tagged Nas6 revealed two other proteins that bind specifically to free RP, Hsm3 and Rpn14. Hsm3 was recently identified as a proteasome-interacting protein<sup>7</sup>. Hsm3 co-purified with the RP but not CP (Fig. 1b), confirming that it is a second RP-specific component. Although we cannot exclude the possibility that a fraction of these proteins shows bona fide proteasome holoenzyme association, their previous assignment as proteasome subunits may reflect that proteasome subcomplexes are often found in holoenzyme preparations. The mammalian homologue of Rpn14, PAAF1, was recently suggested to bind free RP<sup>8</sup>. Likewise, Rpn14 associated specifically with free RP (Fig. 1c). Hsm3 differed from Rpn14 and Nas6 in associating most strongly with only a subset of RP subunits, potentially representing an RP assembly intermediate (Fig. 1d).

The identification of multiple free RP-binding proteins indicates a common function distinct from those of known proteasome-associated proteins. Free RP might be actively sequestered from CP by an inhibitor of the proteasome, a function proposed for PAAF1 (refs 8 and 9). Alternatively, free RP might provide a non-proteolytic pathway of RP function, suggested for transcriptional control and for PAAF1 in particular<sup>9,10</sup>. Third, free RP-binding proteins might act as RP assembly factors. The mechanisms involved in RP assembly<sup>11–13</sup> are largely unknown. In contrast, CP assembly has been studied intensively and involves specific chaperones<sup>14</sup>.



**Figure 1 | Nas6, Hsm3 and Rpn14 bind to free RP.** **a**, Proteasomes were affinity-purified from strains expressing HA-tagged Nas6. Samples were resolved by SDS–PAGE and immunoblotted for Nas6 (anti-HA), RP (anti-Rpn8 and anti-Rpn12) and CP (anti- $\alpha$ 7). **b**, Proteasomes were affinity-purified from strains expressing HA-tagged Hsm3, eluted and analysed for the presence of Hsm3, RP and CP. **c**, Proteasomes were affinity-purified from strains expressing HA-tagged Nas6 and Rpn14. After elution, samples were analysed as in **a**. **d**, HA-specific immunoprecipitations were tested for RP and CP by immunoblotting. ProA, protein A; WT, wild type.  $\alpha$ 7 and  $\beta$ 4 are CP subunits.

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An initial test for a role of RP-binding proteins in the main proposed non-proteolytic function of the RP, transcriptional elongation, proved negative (Supplementary Fig. 2). We therefore tested whether RP-binding proteins have a more general role in proteasome function. Proteasome inhibitor mutants should behave as proteasome hypermorphs, whereas assembly factor mutants should be hypomorphs. Double mutants of the RP-binding factors showed sensitivity to increased temperature, as typical of proteasome hypomorphs (Fig. 2a). Additional phenotypes are described in Supplementary Fig. 3. To verify that *nas6Δ*, *hsm3Δ* and *rpn14Δ* mutants are hypomorphic for proteasome function, we deleted the gene for Rpn4, which mediates homeostatic proteasome regulation<sup>15</sup>. Mutant phenotypes were exacerbated, suggesting that the severity of *nas6Δ*, *hsm3Δ* and *rpn14Δ* phenotypes is masked through Rpn4-based compensation (Fig. 2a). Accordingly, accumulation of ubiquitin conjugates was observed in total cell lysates from many of the mutants in the *rpn4Δ* background (Supplementary Fig. 4).

The results above indicate that Nas6, Hsm3 and Rpn14 are positive regulators of proteasome function in yeast, despite showing little or no association with the proteasome holoenzyme. The function of one RP-binding protein is not strictly dependent on other proteins in this group, in agreement with the results of Supplementary Fig. 5, which show that they can bind RP or RP subassemblies independently of one another.

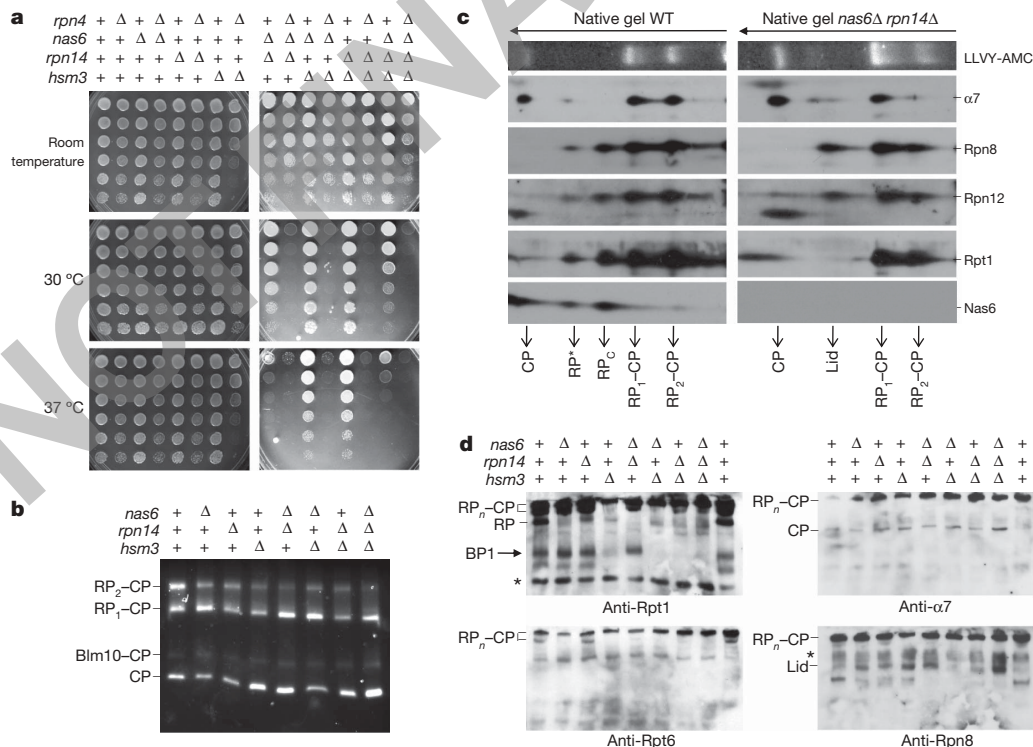
To analyse proteasomes in the mutants, cell lysates were resolved by native polyacrylamide gel electrophoresis (PAGE) and active proteasome species visualized. The mutants showed reduced levels of doubly capped proteasomes, whereas CP levels were increased (Fig. 2b). Thus, RP-binding proteins are important for maintaining normal proteasome levels, indicating a role in assembly.

The activity assay only shows CP-containing proteasome species. To follow RP species, we ran lysates on native gels, followed by second-dimension SDS-PAGE and immunoblotting. In wild-type

lysates we detected RP<sub>0</sub>, which behaves as a true RP species<sup>16</sup>, and RP\*, which appears to contain a subset of RP subunits (Fig. 2c). Importantly, Nas6 co-migrates with these two species and is virtually absent from the holoenzyme. In *nas6Δ rpn14Δ* mutants, both species are undetectable (Fig. 2c), consistent with defective RP assembly. Instead of RP, we observed a species that migrated in the position of the nine-subunit lid subassembly of the RP<sup>16</sup>, and tested positive only for lid subunits. The lid mediates substrate deubiquitination<sup>1</sup>. Size-exclusion chromatography of lysates from *nas6Δ rpn14Δ hsm3Δ* mutants showed results consistent with Fig. 2c (Supplementary Fig. 6). In addition to the lid, the RP contains a ten-subunit assembly known as the base. Free lid complex could indicate a base assembly defect<sup>2,12,13</sup>, assuming that the assembled base consumes free lid as the final step in RP assembly. In summary, these data indicate that Nas6, Rpn14 and Hsm3 function collectively as RP chaperones.

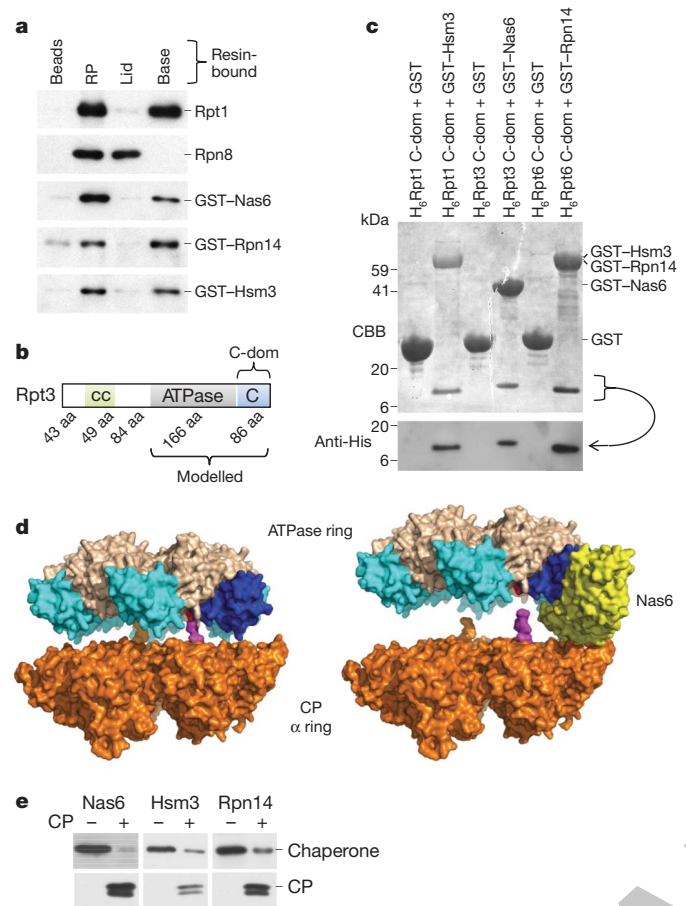
To identify base precursors we used native gels with higher acrylamide content and observed a specific species, the presence of which strongly depended on Hsm3 but was independent of Nas6 and Rpn14 (Fig. 2d). This species, which we termed base precursor 1 (BP1), consists of Rpn1, Rpt1, Rpt2, Hsm3 and Rpt5 (ref. 2).

A role for RP chaperones in base formation was corroborated by the finding that all three RP chaperones bound specifically to the base (Fig. 3a). Six ATPases of the base, the Rpt proteins, are thought to contact the CP through their C termini<sup>17–19</sup> and to form a hetero-hexameric ring. Because Nas6 binds the C-domain (Fig. 3b, ref. 20) of Rpt3 (ref. 21), we tested the binding of the RP chaperones to C-domains of all Rpt subunits by pairwise co-expression in *Escherichia coli*. Although the six Rpt C-domains are closely related<sup>21,22</sup>, we observed strong discrimination among Rpt proteins by Nas6, Hsm3 and Rpn14: Nas6 bound to Rpt3 uniquely, Hsm3 bound Rpt1, whereas Rpn14 bound Rpt6 and, less robustly, Rpt4 (Fig. 3c and Supplementary Fig. 7). These results indicate a related mechanism of action of the three chaperones in base assembly.



**Figure 2 | Phenotypic analysis of *nas6Δ*, *hsm3Δ* and *rpn14Δ* mutants.** **a**, Strains with the indicated genes deleted were spotted on plates in fourfold dilutions and grown at the indicated temperature. **b**, Cell lysates (75 μg) were resolved on native gels. Gels were stained for hydrolytic activity using the fluorogenic substrate LLVY-AMC. RP<sub>2</sub>-CP and RP<sub>1</sub>-CP indicates singly capped and doubly capped proteasomes. Blm10 is an activator of the

proteasome<sup>1</sup>. **c**, Cell lysates of wild-type or *nas6Δ rpn14Δ* strains were resolved by two-dimensional native SDS-PAGE followed by immunoblotting. **d**, Cell lysates were resolved on 5.25% native gels and immunoblotted. BP1 assignment is based on ref. 2. Asterisk, background band. RP<sub>n</sub>-CP indicates singly and double capped proteasomes, which do not separate well on this gel.



**Figure 3 | RP chaperones bind to the C-domains of Rpt proteins in proximity to the CP.** **a**, RP, lid and base bound to IgG beads or untreated IgG beads were incubated with GST–Nas6, GST–Rpn14 or GST–Hsm3 purified from *E. coli*. Beads were analysed for bound proteins. **b**, Domain composition of Rpt3; other Rpt proteins have a similar architecture. aa, amino acids; C, C-domain<sup>20</sup>; cc, predicted coiled-coil region. **c**, His-tagged C-domains were co-expressed with GST or GST-tagged Nas6, Rpn14 or Hsm3 in *E. coli*. Glutathione–Sepharose-purified samples were immunoblotted using His-tag antibody followed by Coomassie blue staining (CBB). **d**, Modelling of the interaction of the Rpt ring with CP in the presence (right) and absence (left) of Nas6: Nas6 binding to the ATPase ring appears to block the C-terminal tail of Rpt3 from docking into the CP. The CP  $\alpha$  ring (beige)<sup>24</sup> was combined with the PAN hexameric ATPase<sup>23</sup> (ATPase domain, grey; C-domains, blue). The Nas6–Rpt3 structure<sup>21</sup> was mapped onto the ATPase ring by aligning the Rpt3 structure with the PAN C-domain (dark blue; C terminus, red), with root mean squared deviation of 1.1 Å over 283 atoms. The last 12 residues of the proteasome activator PA26 C terminus<sup>17</sup> (magenta) are docked into CP<sup>17–19</sup>. The length corresponds to that of Rpt3 C-terminal tail that is absent from the Nas6–Rpt3 structure. **e**, Affinity-purified, resin-bound base was incubated with indicated RP chaperone, washed and then loaded with purified CP. Resin-bound proteins were eluted and analysed by immunoblotting.

The direct interaction of all three RP chaperones with distinct Rpt proteins is consistent with their ability to function in each other's absence (Fig. 2a). These results also explain why Hsm3 but not Nas6 or Rpn14 associates with BP1; their binding partners are absent from BP1 (ref. 2). In support of the functional significance of the Hsm3–Rpt1 interaction and of BP1, we found that overexpressed Rpt1 rescued phenotypes of *rpn4Δ hsm3Δ*, *nas6Δ hsm3Δ* and *rpn14Δ hsm3Δ* mutants but not a *nas6Δ rpn14Δ* mutant (Supplementary Fig. 8). Thus, Rpt1 overexpression relieves the deleterious effects of the absence of Hsm3. Taken together, the data indicate that Hsm3 chaperones Rpt1 or an Rpt1-containing complex.

The Rpt C termini mediate the interaction of RP with the CP<sup>17–19</sup>, and are moreover critical for base assembly<sup>2</sup>, indicating that assembly

is templated on the CP. In contrast to the RP–CP interaction, binding of RP chaperones to C-domains of their cognate ATPases does not involve the extreme C-terminal residues (Supplementary Fig. 7). We propose that, despite non-overlapping binding sites, RP chaperones compete with CP for binding to Rpt proteins, thus controlling the exposure of Rpt C termini to the CP.

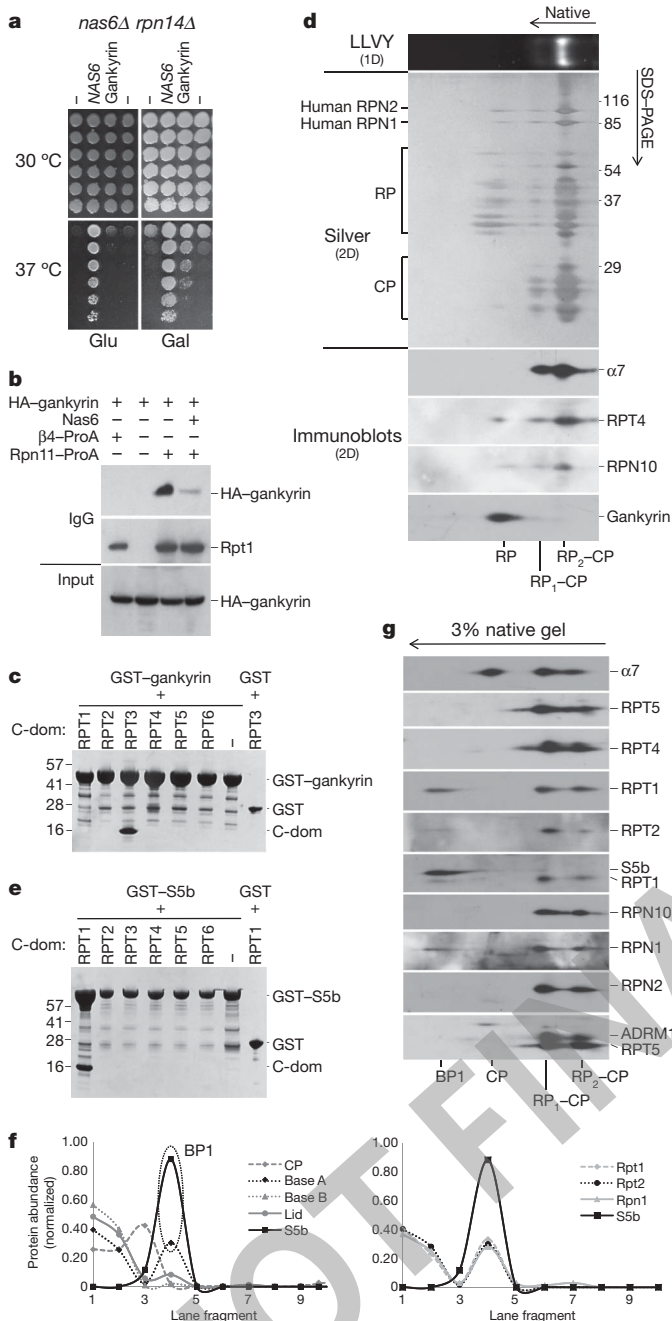
To study the effect of chaperone binding to the Rpt on RP–CP interactions, we modelled the Rpt3–Nas6 structure<sup>21,22</sup> into an ATPase ring based on recent structural studies of the proteasome-activating nucleotidase (PAN), a closely related ATPase from the archaeon *Methanocaldococcus jannaschii*<sup>23</sup>. The ATPase ring was lowered onto the known structure<sup>24</sup> of the CP  $\alpha$  ring. The modelling predicts that Nas6 physically occludes the formation of proper CP–RP contacts (Fig. 3d), because much of Nas6 projects from the RP towards the CP, to prevent close RP–CP apposition.

A prediction of the physical occlusion model is that the CP should be able to expel RP chaperones from the RP. To test this, a base sample loaded with chaperones was immobilized on resin. Application of purified CP to the resin resulted in efficient elution of the RP chaperones (Fig. 3e). This experiment may mimic key steps in proteasome assembly. Moreover, the direct competition between the RP chaperones and the CP shown here appears to explain the absence of RP chaperones from mature proteasomes. Additional tests of the model are described in the accompanying study<sup>2</sup>.

The yeast protein Nas6 and the human oncoprotein gankyrin are closely related by structure, and bind orthologous Rpt proteins<sup>21,22</sup>. In contrast to Nas6, however, gankyrin is thought to be a major proteasome component<sup>6</sup>. To test for functional conservation between Nas6 and gankyrin, we expressed gankyrin in a *nas6Δ rpn14Δ* strain. Gankyrin rescued this strain's temperature sensitivity, showing strong evolutionary conservation (Fig. 4a). Gankyrin expressed in yeast associated specifically with free RP (Fig. 4b). Binding of gankyrin to RP was reduced when Nas6 was overexpressed, indicating that gankyrin and Nas6 bind the same RP surface in yeast (Fig. 4b). Consistently, gankyrin bound the C-domain of human RPT3 (PSMC4) but not those of other Rpt proteins<sup>22</sup> (Fig. 4c). Moreover, preparations of human proteasomes also showed specific association of endogenous gankyrin with free RP and not holoenzyme (Fig. 4d). Considering the low levels of free RP in the preparation, the specificity of gankyrin for free RP is appreciable. Taken together, these data indicate that the functions of gankyrin and Nas6 have indeed been conserved in evolution.

Although BLAST searches did not reveal a human orthologue of Hsm3, the only remaining mammalian proteasome subunit not found in *S. cerevisiae*, S5b (PSMD5), contains ARM repeats, like Hsm3. S5b can apparently bind to a human RPN1–RPT1–RPT2 complex<sup>25</sup>, further indicating that these two proteins are orthologous. When tested against a panel of human Rpt proteins, S5b bound specifically to human RPT1 (PSMC2), as predicted from the specificity of Hsm3 for yeast Rpt1 (Fig. 4e). To test for BP1 in human cells, we analysed HeLa cells lysates on native gels, and subjected gel slices to mass spectrometry. The results showed that S5b co-migrated with human RPN1, RPT1 and RPT2 (PSMD2, PSMC2 and PSMC1, respectively) in a complex that was clearly resolved from proteasomes and RP (Fig. 4f). The existence of BP1 in human cells was confirmed through immunoblotting (Fig. 4g).

Here we identify three factors that assist in assembly of the proteasome base through a common mechanism of action: they associate with C-domains of Rpt proteins, thus negatively regulating interaction of the C-domain with specific pockets within the CP  $\alpha$  ring. Modelling indicates that RP chaperones prevent CP interaction by steric occlusion, and as a result compete with the CP for occupancy of C-domains. This model was tested in three ways: free CP releases RP chaperones from the base complex (Fig. 3e); single amino acid deletions of Rpt C termini fail to expel their cognate RP chaperones<sup>2</sup>, indicating that proper docking of the C terminus is needed to expel the chaperone; and insertions of as little as one residue proximal to



**Figure 4 | Evolutionary conservation of the proteasome assembly.** **a**, *nas6Δ rpn14Δ* strains expressing *NAS6* or gankyrin from a *GAL* promoter were spotted onto plates containing rich medium with glucose or galactose in fourfold dilutions, and incubated at the indicated temperatures.

**b**, Proteasomes were purified from yeast strains expressing HA-tagged gankyrin and analysed by immunoblotting. IgG, immunoglobulin G. **c**, His-tagged C-domains of the indicated human Rpt proteins were co-expressed in *E. coli* with GST-tagged gankyrin. Glutathione-Sephrose-purified samples were separated by SDS-PAGE, and stained with Coomassie blue. Band assignments were confirmed by immunoblotting (data not shown).

**d**, Human proteasomes purified via HTBH-tagged Rpn11 (ref. 5) were resolved by two-dimensional (2D) native SDS-PAGE gel electrophoresis. Proteins were silver-stained or analysed by immunoblotting. **e**, As in **c**, only GST-gankyrin was replaced by GST-S5b. **f**, HeLa cell lysate was resolved on Blue native gels. Fractions were analysed by mass spectrometry, and protein abundance was estimated using spectral counting. On the basis of their abundance profiles, most proteasome subunits can be divided into four groups (left): CP (human (h) $\alpha$ 1-7 and h $\beta$ 1-7), lid (hRPN3, 5, 6, 7, 8, 9, 10, 11), base B (hRPT3, 4, 5, 6 and hRPN2) and base A (hRPT1, 2 and hRPN1). Interestingly, S5b is absent from proteasomes (fraction 1 and 2), but abundant in fraction 4. Right panel, S5b and individual profiles of Base A members. **g**, HeLa cell lysates from **e** were separated by two-dimensional native-SDS-PAGE and analysed by immunoblotting. Official HGNC symbols for the proteins in the figures are: RPT1 (also known as PSMC2), RPT2 (PSMC1), RPT3 (PSMC4), RPT4 (PSMC6), RPT5 (PSMC3), RPT6 (PSMC5), RPN1 (PSMD2), RPN2 (PSMD1),  $\alpha$ 7 (PSMA3) and RPN10 (PSMD4).

unexpectedly implies a more general conservation, that the subunit compositions of yeast and mammalian proteasomes are actually identical. While this paper was under review, the participation of Hsm3 and S5b in RP assembly was reported, consistent with our findings<sup>26</sup>.

Nas6, Rpn14 and Hsm3 fit the definition of a molecular chaperone closely<sup>27</sup>. They assist in assembly of an oligomeric complex, do not act as templates for the complex, and are absent from the mature complex. Chaperones typically function to prevent undesired and non-specific interactions of their target proteins, but RP chaperones represent an interesting case in which the interaction suppressed by the chaperone is a proper interaction in the mature complex, and moreover an interaction that is specifically required for assembly itself. These properties of RP chaperones point to a role in controlling the timing and ordering of maturation events.

How does competition of RP chaperones with the CP for occupancy of the Rpt C-domains promote proper base or Rpt ring assembly? In the simplest version of templated assembly, the ring would assemble in contact with the CP in a growing arc, subunit by subunit, until, with the inclusion of its last subunit, the ring is closed. In this 'one-template model', the Rpt-CP interaction, in combination with Rpt-Rpt interactions, would guide the proper choice of subunit and stabilize the nascent, open ring. Such a model was proposed for assembly of the CP  $\beta$  ring<sup>28</sup>. Interestingly,  $\beta$  ring assembly is, like Rpt ring assembly, guided by the  $\alpha$  ring, albeit the opposite face of the  $\alpha$  ring. In the case of the Rpt ring, the one-template model may not apply, based in part on our identification of an apparent precursor to the base<sup>2</sup>, BP1. This complex contains Rpn1, Rpt1, Rpt2, Rpt5 and Hsm3. Thus, the ring is likely to be assembled in patchwork fashion, partly from preformed ring segments.

The scaffold for BP1 appears to be a non-Rpt subunit, Rpn1 (ref. 2). Rpn2 is related to Rpn1, and may have a complementary scaffolding role for most or all base components that are absent from BP1. In this three-scaffold model, the CP  $\alpha$  ring, Rpn1 and Rpn2 each help to template the ring. Both Rpn1 and Rpn2 are toroidal<sup>29,30</sup>, a property likely to underlie their ability to nucleate ring assembly. A key feature of the three-template model is that the ordering of Rpt addition is achieved by interactions among multisubunit base precursors. An example of how RP chaperones could order the assembly pathway is that Hsm3 might help BP1 to assemble with other base precursors late in the assembly pathway by suppressing premature interaction of Rpt1 with the CP.

Rpt C termini prevent chaperone expulsion, even when the Rpt C terminus is productively docked in the CP pocket, indicating that RP chaperone expulsion is precise and requires a close approach of the CP to the RP<sup>2</sup>. Despite the explanatory potential of this model, we do not exclude the possibilities that the RP chaperones may promote assembly through additional mechanisms, and perhaps regulate proteasomes in additional ways.

The ability of the all chaperones to bind Rpt C termini, compete with CP and promote assembly may reflect convergent evolution, considering that the chaperones are unlikely to have diverged from a common ancestor. For example, Nas6 is composed mainly of ankyrin repeats, whereas Hsm3 is rich in ARM repeats and Rpn14 in WD40 repeats. Key features of base assembly, including the BP1 intermediate, are conserved in mammals. The chaperone functions of yeast proteins Rpn14, Hsm3 and Nas6 are apparently paralleled in mammals by PAAF1, and S5b, and most interestingly the oncoprotein gankyrin. It is likely that gankyrin and S5b are misassigned as proteasome subunits. Thus, conservation of the assembly pathway

## METHODS SUMMARY

**Yeast strains, plasmids and antibodies.** See Supplementary Table 2 for genotypes of strains used. Standard methods were used for strain construction and growth. The reported sequence of Hsm3 in the *Saccharomyces* genome database differs from the sequence in our strain background at the C terminus (see Supplementary Information). Plasmids and antibodies used are described in Supplementary Tables 3 and 4, respectively.

**Immunoprecipitations and protein purifications.** Recombinant proteins were expressed in and purified from Rosetta 2 cells (Novagen) as detailed in Supplementary Methods. For pulldown experiments, yeast cells were lysed by French press or glass beads. Lysate was cleared and incubated with the specific resin. For details, see Supplementary Information.

**Native gels.** For native gels, cell lysates were made in an ATP-containing buffer, cleared by centrifugation, and loaded on a 3.6% or 5.25% polyacrylamide gel. To transfer proteins from native gels to PVDF (polyvinylidene difluoride), the gels were pre-soaked for 10 min in 1× SDS–PAGE running buffer and transferred according to standard protocols. For two-dimensional immunoblotting, a lane was excised and loaded on top of an SDS–PAGE gel. Proteins were transferred from the second-dimension gel to PVDF and probed with the indicated antibodies. Detailed protocols for all experimental procedures can be found in Supplementary Information.

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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