BJ Structure

Biochem. J. (2012) **448**, 55–65 (Printed in Great Britain) doi:10.1042/BJ20120542

online data

SUPPLEMENTARY ONLINE DATA

Structural and functional characterization of Rpn12 identifies residues required for Rpn10 proteasome incorporation

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EXPERIMENTAL

Expression and purification of Rpn12

The vector pGEX-6P-1 containing full-length *S. pombe* Rpn12 was transformed into BL21(DE3) cells and plated on to LB (Luria–Bertani) agar containing 100 μg/ml carbenicillin. A single colony was grown overnight at 37 °C in 5 ml of LB supplemented with 100 μg/ml carbenicillin and then used to inoculate 1 litre of autoinduction medium [1] also supplemented with 100 μg/ml carbenicillin. Cultures were grown at 37 °C with vigorous shaking until the $D_{600\,nm}$ reached 0.6. The temperature was then reduced to 20 °C and the incubation continued for a further 20 h. Cells were harvested by centrifugation (3500 ***g*** for 25 min) and the supernatant was discarded. The pellet was re-suspended in 25 ml of buffer (20 mM Hepes, 150 mM NaCl, 0.01 % monothioglycerol and 0.02 % sodium azide, pH 7.5) supplemented with an EDTA-free protease-inhibitor cocktail tablet (Roche) and then flash-frozen in liquid nitrogen and stored at −80 °C. Proteins were purified by affinity chromatography. Cell pellets were thawed under running cold water and then lysed by four cycles of homogenization at 12 000–20 000 psi (1 psi = 6.9 kPa). Cell debris was removed by centrifugation (40 000 ***g*** for 45 mins), the supernatant was loaded on to a glutathione–Sepharose column (GE Healthcare) pre-equilibrated with HBS buffer and then the column was washed to baseline with several column volumes of HBS. GST (glutathione transferase)–Rpn12 was eluted using 20 mM reduced glutathione (pH 8.0) in HBS. The eluate was then concentrated to 10 ml, and the fusion protein cleaved with 3C (molar ratio of 1:500) on a rotating wheel overnight at 4 °C. Rpn12 was subsequently purified by size-exclusion chromatography (Superdex75 HiLoad 26/60) equilibrated in HBS. Rpn12-containing fractions were pooled and re-applied to a fresh glutathione–Sepharose column equilibrated in HBS to remove any residual GST. Rpn12 proteins prepared for NMR spectroscopy were similarly prepared except that PBS buffer (50 mM NaCl, 25 mM sodium phosphate, 0.01 % monothioglycerol and 0.02 % sodium azide, pH 6.5) replaced HBS in the final size-exclusion and affinity-chromatography steps. Full-length *S. pombe* Rpn12 repeatedly purified from recombinant *E. coli* cells as three species as judged by SDS/PAGE suggesting that it is prone to degradation. Using limited proteolysis with subtilisin A followed by N-terminal sequencing and MS we identified three stable fragments truncated at residues 224, 228 and 250. Fragments 1–228 and 1–250 were subcloned into pGEX6P-1 and purified as for the full-length protein.

Affinity purification of the 26S proteasome

26S proteasomes were isolated using a modified protocol as described previously [2]. Both Rpn12 WT and mutant *S. pombe* tagged strains were grown in PMG medium to a D_{595nm} of 0.6–0.8 at 25 °C. Adenine and nourseothicin antibiotic were also added to a final concentration of 20 μg/ml and 100 μg/ml respectively. Cells were harvested and washed twice with distilled water and then frozen at −80 °C until further use. Cells, typically obtained from 250 ml cultures, were thawed and then dissolved in 250 μl of buffer A (50 mM Tris, pH 8.0, 50 mM NaCl, 25 mM $MgCl_2$, 10 % glycerol, 0.1 % Triton X-100 and 10 mM ATP) supplemented with a protease inhibitor cocktail tablet (Roche) and 1 mM PMSF and lysed using glass beads using a FastPrep-24 cell disruptor (MP Biomedicals). Cell extracts were centrifuged at 22 000 ***g*** for 45 min at 4 °C and 30 μl of Sepharose A beads (GE Healthcare) were added to the supernatant. Following 30 min incubation at 4 °C the mixtures were centrifuged at 3 000 ***g*** for 5 min and the supernatants were recovered and incubated (equal amounts of protein were added) with 40 μl of anti-FLAG M2-agarose beads (Sigma) for 2 h at 4 °C. The mixtures were constantly rotated during both incubations. The beads were washed six times with buffer B (as buffer A but with 150 mM NaCl) and the bound 26S proteasome complex was released by boiling the beads in 4× SDS-containing electrophoresis buffer at 95 °C for 5 min. 26S proteasome complex composition was confirmed by SDS/PAGE using a 4–12 % gradient gel (Invitrogen). Following SDS/PAGE, eluted proteins were transferred on to a PVDF membrane for Western blot analysis.

Characterization of Rpn12 by CD

The integrity of the Rpn12 fold was assessed by CD (Figure S1). Full-length WT and K29A/Q76A Rpn12 were expressed and purified as described above except that the proteins were exchanged into 20 mM sodium phosphate buffer, pH 6.0, 100 mM NaCl, 5 mM DTT (dithiothreitol) during the size-exclusion chromatography step and the subsequent glutathione–Sepharose 4B column was also equilibrated in this buffer. All CD measurements were taken on a Jasco J-810 spectropolarimeter at 20 °C. A buffer baseline was subtracted from the protein spectrum for each experiment, the analysis buffer consisting of 10 mM sodium phosphate buffer, pH 6.0, 50 mM NaCl and 2.5 mM DTT. The far-UV CD spectra (190–250 nm), was measured using a 0.2 mm Hellma quartz cuvette, a 2 nm bandwidth and a scan speed of 20 nm/min with a response time of 4 s. The spectra were recorded as three averaged accumulations, and displayed as $\Delta\varepsilon$ ($M^{-1}\cdot cm^{-1}$) calculated using the mean residue concentration.

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The structure of Rpn12 has been deposited with the PDB and assigned the code 4B0Z and the structure factors have been assigned the code r4B0Zsf.

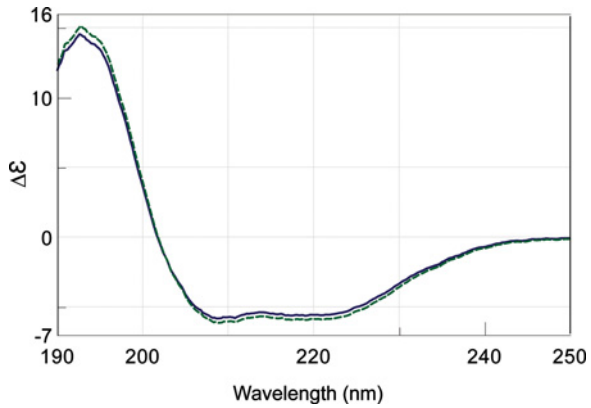

Figure S1 Comparison of the authentic and K29A/Q76A Rpn12 proteins by CD

Overlay of the CD spectra for the authentic and Rpn12 K29A/Q76A mutant protein folds (green dotted, WT; blue solid, mutant). The measurements were taken at 20°C. Each spectrum is an average of five scans and has been corrected for the buffer baseline, a 0.2 mm light path and the protein concentration on a mean residue basis.

Table S1 Strains used in the present study

Strain	Source
mts3-1leu1.32h^{-}	[6]
ade6M210/ade6M216leu1.32/leu1.32rpn12^{+} */rpn12::ura4*$^{+}$ *ura4-D18/ura4-D18h*$^{+}$ */h*$^{-}$	[6]
ade6M210leu1.32plNTrpn12^{+} *rpn12::ura4*$^{+}$ *ura4-D18h*$^{-}$	The present study
ade6M210leu1.32plNTrpn12^{mut}*rpn12::ura4*$^{+}$ *ura4-D18h*$^{-}$	The present study
ade6M210leu1.32plNTrpn12^{+} *rpn1::FLAGrpn12::ura4*$^{+}$ *ura4-D18h*$^{-}$	The present study
ade6M210leu1.32plNTrpn12^{mut}*rpn1::FLAGrpn12::ura4*$^{+}$ *ura4-D18h*$^{-}$	The present study

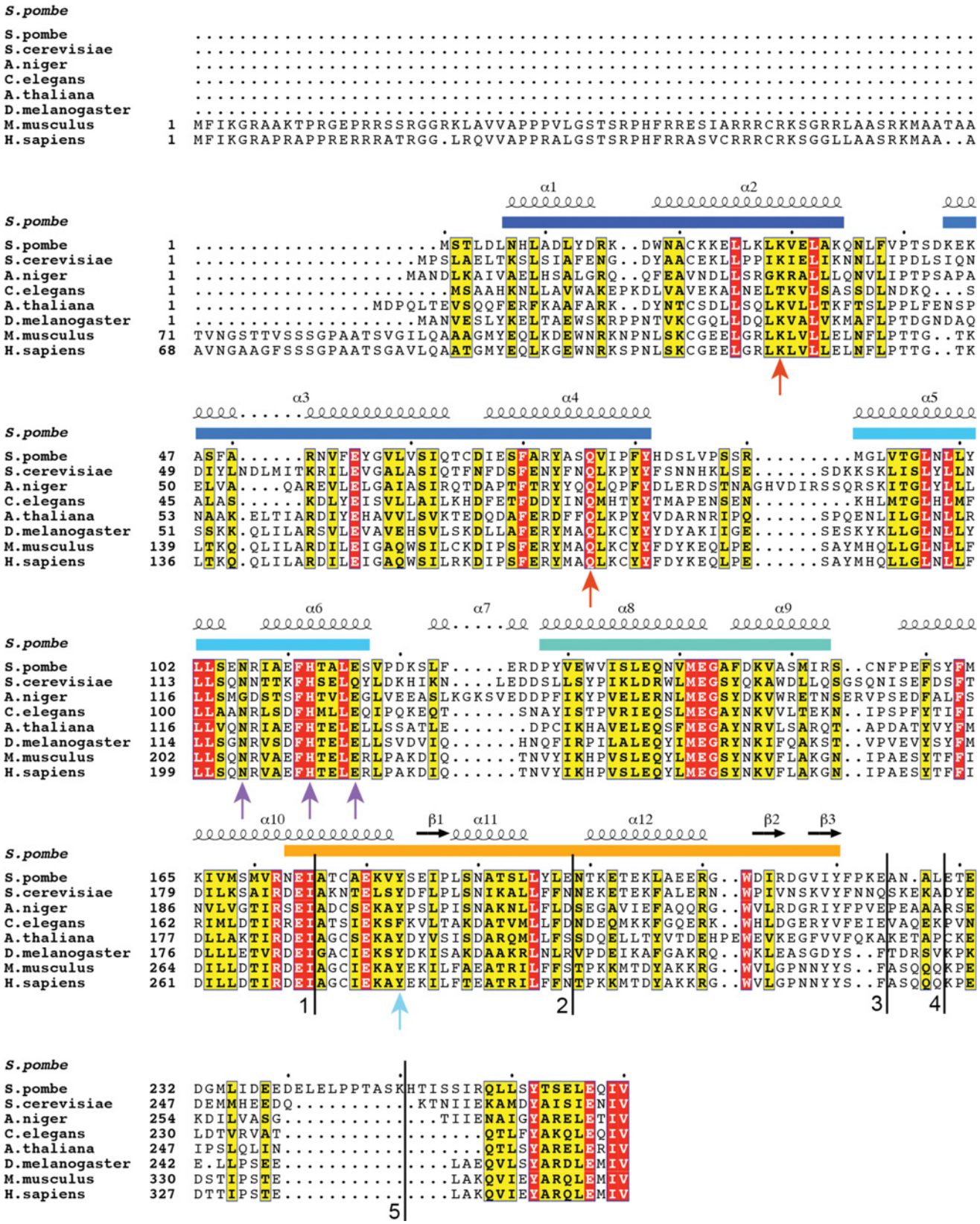

Figure S2 Sequence alignment of Rpn12 orthologues

The sequences of Rpn12 orthologues were aligned with ClustalW2 alignment software [3]. Similarity scores were calculated with the Risler matrix [4] and coloured accordingly. The WH domain is indicated by an orange bar and the repeats of the TPR-like domain are highlighted in different tones of blue. Coloured arrows indicate sets of mutations on the basis of surface conservation analysis. The vertical black bars labelled 3, 4 and 5 mark the sites of subtilisin A cleavage. Bars 1, 4 and 5 also represent the ends of the constructs used for NMR assignment (residue 175), structure determination (residue 228) and biophysical characterization (residue 250) respectively. Bar 2 indicates the end of the Rpn12 sequence present in the *S. pombe Mts3-1* mutant [5].

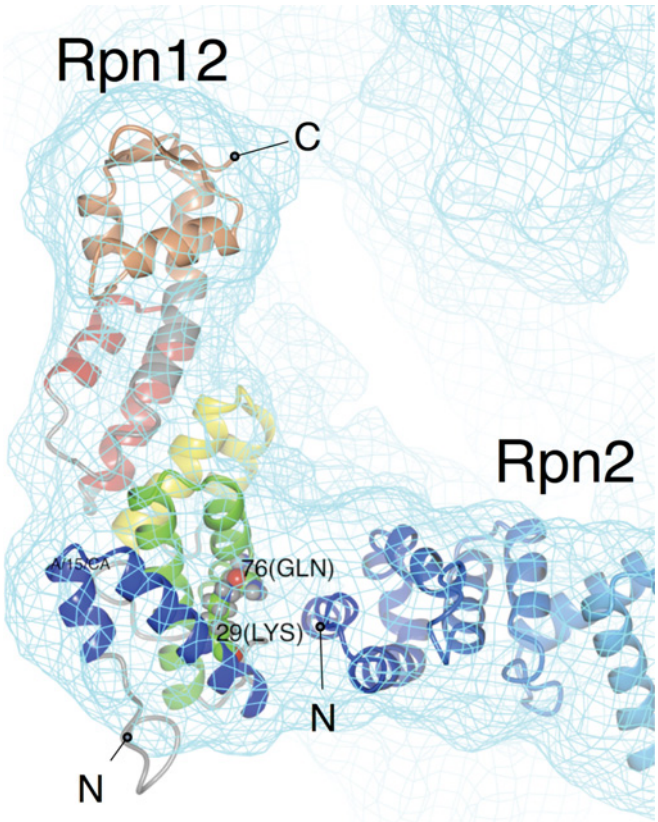

Figure S3 Fit of Rpn12 and Rpn2 in a cryo-EM map of the 19S regulatory particle

Rpn12, coloured according to the scheme defined in Figure 1 of the main text, is shown in ribbon representation, with the locations of the Site 2 residues that we have targeted by mutagenesis (Lys^{29} and Gln^{76}) highlighted in CPK representation. Rpn2 is shown in ribbon representation, colour-ramped from the N- to the C-terminus. The cryo-EM map, contoured at 1 S.D., as well as the docked Rpn subunits were kindly provided as a personal communication (F. Foerster).

Received 29 March 2012/6 August 2012; accepted 20 August 2012
Published as BJ Immediate Publication 20 August 2012, doi:10.1042/BJ20120542

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