Supporting Information

Pathare et al. 10.1073/pnas.1117648108

SI Materials and Methods

Cloning and Purification of Rpn6. Rpn6 from *Drosophila melanogaster* was cloned as N-terminal 6xHis-tag fusion protein into a modified pTipRC1 plasmid with a tobacco etch virus (TEV) protease cleavage site in between 6xHis-tag and the *rpn6* gene. Protein expression was carried out in *Rhodococcus erythropolis (L-88)* (1), where the resulting strain was grown at 30 °C in 5 L of LB medium. Protein production was induced with 0.5 μg mL^{-1} thiostrepton for another 24 to 48 h. Cells were sedimented at 4,000 g and washed with sterile water. The pellet (20 g) was resuspended in 50 mL lysis buffer (50 mM sodium phosphate pH 8.0, 10 mM imidazole, 300 mM NaCl). Complete protease inhibitor cocktail (Roche Biotech) and 1 mg mL^{-1} lysozyme were added, and the mixture incubated for 2 h on ice. Subsequently, 100 ppm Benzonase was added, followed by ultrasonication on ice. Cell debris was removed by ultracentrifugation at 28,000 × *g*. His-tagged Rpn6 was purified by affinity chromatography using Ni-NTA beads (GE Healthcare) according to the supplier recommendations. Fractions containing Rpn6 were pooled, augmented with TEV protease for removal of the 6xHis-tag, and incubated for 12 h at 4 °C in a dialysis chamber equilibrating against 25 mM Tris HCl pH 7.5. TEV protease was removed by MonoQ anion exchange chromatography, using a linear salt gradient to 1 M NaCl in 25 mM Tris HCl pH 7.5. Size-exclusion chromatography (SEC), using Superose-12 (GE Healthcare) equilibrated with 20 mM Hepes NaOH pH 7.5, 300 mM NaCl, and 1 mM DTT was used as final purification step.

Limited proteolysis. Full-length Rpn6 at 0.5 mg mL^{-1} was subjected to limited proteolysis, using increasing concentrations of Proteinase-K (0.025–0.1 mg mL^{-1}). After 30 min incubation at 20 °C, samples were analyzed by SDS-PAGE followed by in-gel digestion and peptide mass spectrometry. Samples with prominent digestion products were subjected to liquid chromatography–mass spectrometry analysis to measure apparent molecular masses to be able to determine actual proteolytic sites.

Crystallization. Crystals of Rpn6(30–422) were grown by the sitting drop vapor diffusion method at 4 °C and 18 °C, mixing equal volumes of Rpn6(30–422) (25 mg mL^{-1} in 20 mM Hepes NaOH pH 7.5, 300 mM NaCl, and 1 mM DTT) with a precipitant containing 100 mM Tris HCl pH 7.5 200 mM Li_2SO_4, 12% PEG-3350. For cryoprotection, crystals were transferred stepwise into 100 mM Tris HCl pH 7.5, 300 mM Li_2SO_4, 15% PEG-3350 and 20% glycerol before being flash-frozen in liquid nitrogen.

Structure Determination. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The data were processed with XDS (2) and transferred into the CCP4 format using Pointless (3), Scala (4), and Truncate (5). The structure was solved using crystals soaked with 0.5 mM $GdCl_3$. Four Gd sites were found in a MAD dataset using SHELXD (6) as implemented in HKL2MAP (7). This solution was further refined with Sharp (8). Density modification was subsequently carried out using Resolve (9). A preliminary model was manually built in the resulting map using Coot (10). For final model building and refinement, nearly isomorphous native data were used. Iterative cycles of manual model building and refinement with Refmac (11), as implemented in the CCP4 interface (12), were carried out. The final model contains Rpn6 residues 38 to 390, two sulphate, two glycerol, and 49 water molecules. Nonglycine residues facing solvent channels without discernable side-chain density were modeled as alanines.

Site-Directed Mutagenesis. Site-directed mutations in Rpn6 were introduced with the QuikChange site-directed mutagenesis kit (Stratagene) using pTipRC-Rpn6 as the template.

Coprecipitation Assay. Different subunits from the *D. melanogaster* regulatory particle were used for the coprecipitation assay. Binary interactions of these subunits were studied by mixing Rpn6 with other subunits having a 6xHis-tag. Proteins were mixed at 1:1 molar ratio and incubated with Ni-NTA Superflow beads at 20 °C for 45 min. The mixtures were applied to spin column bodies (Qiagen), washed with the washing buffer, and the retained proteins were eluted with the elution buffer. The fractions were analyzed by SDS-PAGE.

EM Density Fitting. The exact position and orientation of Rpn6 in the electron density map of the 26S proteasome was determined by an exhaustive six-dimensional search procedure. The atomic coordinates of Rpn6 were converted into a gray-scale volume by assigning the sums of atomic numbers for all atoms contained in the corresponding voxels. This Rpn6 density volume was low-pass filtered to a resolution of 6.4 Å and used as a template for a cross-correlation based search by screening three translational and three rotational parameters. The rotational search was performed with an angular increment of 2° using MOLMATCH (13). The position of the maximal normalized cross-correlation correlation coefficient ($CCC_{max} = 0.48$) and the corresponding rotational parameters were determined, and the original atomic coordinates of Rpn6 were transformed accordingly (see also Fig. S5). In addition, we computed a Z-score for the orientation-specificity for each of the determined positions as described previously (14).

Bioinformatics Methods. A Dali search (15) of the Protein Data Bank (PDB) using the solenoid part of Rpn6 revealed several prokaryotic proteins with structurally similar repeats but disparate biological functions: DrR162B (PDB ID code 3GW4), PlcR [PDB ID code 2QFC (16)], and MalT [PDB ID code 1HZ4 (17)].

HHpred (18) was used for identification of structural templates and their alignment to the target sequence of *Schizosaccharomyces pombe* Rpn6. Our *D.melanogaster* Rpn6 crystal structure covered residues 38 to 387, and TOM70 (PDB ID code 2GW1) was used as an additional template for residues 4 to 100. The C-terminal residues 388–421 were not modeled. Comparative models were built using MODELLER (19) and further refined in the context of the EM map using MDFF (20).

Structural figures were prepared using PyMOL (http://www.pymol.org) and Chimera (http://www.cgl.ucsf.edu/chimera/). Alignment figures were created with ESPript (21).

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Fig. S1. Comparison of the (proteasome, COP9/signalosome, and eIF3 (PCI) modules of Rpn6, Csn7, and eIF3κ. (*A*) Superposition of Rpn6 and Csn7 [Protein Data Bank (PDB) ID code 3CHM (1)]. The superposed PCI modules of Rpn6 and Csn7 are shown as green and yellow ribbons, respectively. N and C termini are indicated. (*B*) Superposition of Rpn6 and eIF3κ [PDB ID code 1RZ4 (2)]. The superposed PCI modules of Rpn6 and eIF3κ are shown as green and blue ribbons, respectively.

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                                  1        10         20         30        40
D.melanogaster   ..........MAGATLFERAQALSSVNREEQDSSLLNKLVRDQEG..........AENDE
H.sapiens        ........MAAAAVVEFQRAQSLLSTDREASIDILHSIVKRDIQ............ENDE
O.dioica         ..........MAGAACVAQPT.......NNDLQQLYHDLNSAKE............RSDQ
C.albicans       SIHHLQPLQNMSGQQLLEEARKAVTNKQYDEAEIKYKQIINPSESSTSTTTTTTTTTTTA
S.cerevisiae     ........MSLPGS.KLEEARRLVNEKQYNEAEQVYLSLLD...KDSSQSSAAAGASVDD
Y.lipolytica     ..........MTLEETLASAKTAGDKGNLVEAEKLYREILD............QKAGTNE
C.posadasii      .....MAPNNSETAALLKEATALS.KTDPSKAETIFQKVLSLG............TGSTE
P.brasiliensis   .....MAAG.PDDASLLAEAKNLV.KTDPAKAESLYQKILSKG............VGSTE
T.stipitatus     .....MAST..SASQRIEEAKSLA.SKDPAKSEQIYRDVLSSG............VGKTE
N.crassa         .....MAQG..EASERVREAQKVV.STDPRQAEQIYKDIISKPP...........SVTSD
T.melanosporum   .............MGRLEEAQEIQ.KRDPARAEAMYKEMISKP............PGMND
S.pombe          ..........MSSKSSLELANNAVKSNDIEKAIDLYKEVLNKG............VSKDE
D.discoideum     ..........MNWKEQLEEIGNCQ...DSNKAIQDYNKILTIQ................E
A.thaliana       ........MVSYRAT..TETISLALEANSSEAITILYQVLE.............DPSSSP
P.patens         ..................................MLREVLY.............HPASTA
C.rheinhardtii   ........MSAELETRLKSATELGATDVPGAAAQLKGLVLE.............ESSNDA
N.gruberi        MTDNTTSDQQTFYTTLFEKATKEEEKQNTAEAIKLFTQVINGE...........MSKKNE
T.brucei         ........MEKDLDSLWDTAEDFIADGRRTEARGVLEDIVSTD...........VTADDA
C.elegans        ........................MSATPVTLKAVQSEVSAQT............AKSSE
P.tetraurelia    .....MATLIEKFQALLQEVDNARLDTNNLEAALLKLLTFDSK...............SE
T.vaginalis      .MDNEIPFVERLGNIVDPKDPNVPLTQRAALLEDLIDEVIQDG.............AHIE
consensus>50     ......................................v....................e

                        α1                  α2        η1        α3
                                 50         60         70         80         90        100
D.melanogaster   ERIRIKEQGILQQGELYKQEGKAKELADLIKVTRPFLSSISKAKAAKLVRSLVDMFLDMD
H.sapiens        EAVQVKEQSILELGSLLAKTGQAAELGGLLKYVRPFLNSISKAKAARLVRSLLDLFLDME
O.dioica         ETIRKNEAKIIELGEWLQKQGNAKELGELVKNVRPYLGSLSKAKAARLVRTLVDLFLDMD
C.albicans       KQLQQQESAILELGKIYETLNEPTKLSDLIADSRSILGNFAKSKTAKIVKTLIEDFDKLS
S.cerevisiae     KRRNEQETSILELGQLYVTMGAKDKLREFIPHSTEYMMQFAKSKTVKVLKTLIEKFEQVP
Y.lipolytica     KSIQIQESALINLGTLYASNNKPQDLADLIHTSLTVMSGFAKSKTAKIIRNLIDLFAKVP
C.posadasii      AASRDYETALVGLGELYRDQKRPKELAELLRTSRSSFSSFAKAKSAKLVRQLLDFFAAIP
P.brasiliensis   AASRDYEAALVGLGELYRDERKPTELAQLLKTSTSAFSSFAKAKSAKLVRQLLDFFSAIP
T.stipitatus     SASRDYEAALVGLGELYRDNKKAHELADLIKSSRDAFSSFAKAKTAKLVRQLLDLLSEIP
N.crassa         AAIREYETALIHLGELYRDEKNSQGLVDLVTQSRTVLSSFAKAKTAKLVRQLLDLFEAIP
T.melanosporum   AELREYELVLVALGGIYRDARRTGELAELIKASRSIMSSFAKAKTAKIVRQLIDLFTTIP
S.pombe          KVANEQEQALTNLSDLYVRENRHNDLAQLVQQSRPLMANFSKAKSAKIVRTLIDKFSGEK
D.discoideum     SPDDIKEEAILRLAKLFVKIGKGDQLPTLLRSVRPFFDKISKPKTDKIVRNFIDIFSTVP
A.thaliana       EAIRIKEQAITNLCDRLTEEKRGEDLRKLLTKLRPFFSLIPKAKTAKIVRGIIDAVAKIP
P.patens         EVLRVKEQAITALSDLLSQEKKAEELRGLLTDLRPYFNLIPKAKTAKIVRGIIDAVAKIP
C.rheinhardtii   EAVRIKEQAISQLCELYIKQANAQALADLLTSLRGFFNAIPKAKTAKLVRSIIDSIAKVP
N.gruberi        VIIKKKEEAIYSLGKIYATKGDAQAIMELNKSIRPFFQDLPKAKTAKIVRTLIELVGTIK
T.brucei         IGLRAKERAIYRLAEILSVEKQTDMLVQLLSAIRSFFALLPKAKATRMVRKMFDLILNSG
C.elegans        AEVKRCEDLILSYSRQLAKEKDITGIRTLVESIRSFYDLVGKARASKLIRDIVEHALTID
P.tetraurelia    EHLKHKESAYNKLSSLYCKQNKPQLVFQIMKTHD..FSGFNQTRAAKIMRQMIDQVSQLE
T.vaginalis      ERVGDFEQVLNHLLETYVQLANSEKFATLLDHLAEYFTKIPKARTAKLVRIIIQALRKVP
consensus>50     ......E.....l...y........l.dl....r.......kak.ak.vr.li#......

                                 α4                  α5               α6
                          110        120        130        140        150
D.melanogaster   A........GTGIEVQLCKDCIEWAKQEKRTFLRQSLEARLIALYFDTALYTEALALGAQ
H.sapiens        A........ATGQEVELCLECIEWAKSEKRTFLRQALEARLVSLYFDTKRYQEALHLGSQ
O.dioica         S........AIGVEVDLCKECIQWAMEEKRTYLRQALEARLMALYFDTNNYKECLTLGQR
C.albicans       AKD....EKSLDIQIDVTKSSMQWAIESKLSFLRQSLQLKLSDLLYQKKHHHEAIKIINE
S.cerevisiae     D........SLDDQIFVCEKSIEFAKREKRVFLKHSLSIKLATLHYQKKQYKDSLALIND
Y.lipolytica     S.......DCIDLQIATTQKCIEWAVSEKRNFLRQSLQTRLVSLFLEKKTYYDALNLINT
C.posadasii      N........TLDIQISVIKSCIDWAVSERRSFLRQNLETRLVTIYMQKQTYYDALTLINS
P.brasiliensis   N........TLDIQIQVIKSCIEWAVSERRSFLRQNLETRLVTIYMQKQSYYDALTLINS
T.stipitatus     N........TIDIQVAVIKSSIEWAVSERRSFLRQSLETRLVAIYMQKQSYYDALTLINS
N.crassa         D........SLDIQISVTKSCIEWATSERRSFLRQNLETRLVALYMAKQSYYDALTLING
T.melanosporum   D........TLDIQISVTKSCIEWAIQERRSFLRQNLETRLVGLYLQKHSYTEALSLINS
S.pombe          K........SLPLQIEVANDCIKWAIKEKRTFLRQALETKLISLYYDNSSYTDAINLINT
D.discoideum     D........NLTTLIEFVKENIQWCKDTNRIYLRQRLETKLFTLMFEAKDYANALSGLTT
A.thaliana       G........TTDLQITLCKEMVEWTRAEKRTFLRQRVEARLAALLMENKEYVEALALLST
P.patens         G........TSDLQISLCREMVEWTRAEKRMFLRQRVEARLAALLMENKEYSEALSLLTN
C.rheinhardtii   G........STQLQVDVCKGQVEWAKTEKRTFLRQRIELRLASLYMQTRDYPAALALIST
N.gruberi        G........SDAIQIEICRESIEWATTEKRSFLRQRIESRLANLLFIKKEFTESLEIITR
T.brucei         A........SLDRQMEVCRDMIAWARQEKRTFLRQRLQHRLAEVQFARNERQEALTTLQA
C.elegans        QGVGPALDHGKKEKIDLLTNCIGWATSNKREFLRRSLQARLIRLYNDIRDFTNAQKLAQD
P.tetraurelia    G........TVELQVEMCQFLIDQCARDKKNYLKHKMQIRLATLYNEQEKFSQGIEIIDK
T.vaginalis      G........TLEIQADLCQKWITWAKDQERTLLRQRLETELSEILLEQHKYNEAIEILQR
consensus>50     ............qi.......iewa..e.r.fLrq.le.rL..l..q...y.eal.l...
                                                ★
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                 α7                    α8                  α9
                160       170       180       190       200       210
D.melanogaster   LLRELKKLDDKNLLVEVQLLESKTYHALSNLPKARAALTSARTTANAIYCPPKVQGALDL
H.sapiens        LLRELKKMDDKALLVEVQLLESKTYHALSNLPKARAALTSARTTANAIYCPPKLQATLDM
O.dioica         LYSELKKLDDKALLVEIQLTESKAYHSIGNLQNSRAALTSARTTANAIYCPPRTQANLDM
C.albicans       LLREYKKLDDKSSLVEVQLLESKIYHALRNIPKAKAALTSARTSANSIYCPTILQAELDC
S.cerevisiae     LLREFKKLDDKPSLVDVHLLESKVYHKLRNLAKSKASLTAARTAANSIYCPTQTVAELDL
Y.lipolytica     LLKELKKLDDKMVLVEVQLLEAKAYHALRNIPKSRASLTSARTSANAVYCPPLTQAALDT
C.posadasii      LLRELKRLDDKLVLVEVQLLESRVYHALGNQPKARAALTSARTSAASVYTPPLLQAGLDM
P.brasiliensis   LLRELKRLDDKLVLVEVQLLESRVYHALGNQPKARAALTSARTSAASVYTPPLLQAGLDM
T.stipitatus     LLRELKRLDDKVVLVEIQLLESRVYHALGNQPKARAALTSARTSASAVYTPPTLQAGLDM
N.crassa         LLRELKRMDDKLVLVEVQLLESRVYHALGNIAKARAALTSARTSAASVYTPPLLQANLDM
T.melanosporum   LLKELKRLDDKMVLVEVQLLESRAYHALRNIPKSRASLTSARTSANAVYCPPLMQAGLDM
S.pombe          LLSELKRMDDKMLLTEVHLLESKVYHAIRNIPKARASLTAAKTSANAVYCPPMLQGNLDL
D.discoideum     LLTEIKRLDDKPLLVEIQLVESRIQHALKNIPKARAALTSARTNANTIYCPPKLQAEIDM
A.thaliana       LVKEVRRLDDKLLLVDIDLLESKLHFSLRNLPKAKAALTAARTAANAIYVPPAQQGTIDL
P.patens         LVREVRRLDDKLLLVDIDLLECKLYFSLRNLAKAKAALTAARTAANAIYVPPAQQGTIDL
C.rheinhardtii   LLSEVKKLDDKLLLVDIYLLESKVNHALRNVPKARASLTAARTAANAIYVPLQLQAEIDC
N.gruberi        LLREVRKLDDKALLVEIHLLESKVYHSLRNLSKSRAALTSARTDANAIYCPPLLQAEIDM
T.brucei         LLREVRRLDDRALLLDIHLLESHIYYSIRNISRARAALVAARTNANSIYCPPLAQAEIDL
C.elegans        LSKELKKLEDRELLIEVSVEESKSSFNLNNLAKAKTALLTAKTNTNSAFASPQLQASVDM
P.tetraurelia    IVVEVRKADDKHLLLEIYLIESKLHFGQTNLAKAKASLTAARACSNTIYCPPHVQAEIDM
T.vaginalis      LTAELRKVDHKSQLIEVHLIESRTFRGLGDFSRAKASLTAARTNAAAVYTAPLLQGQLDL
consensus>50     ll.E.k.l#dk..Lv#!.llEs..yh.l.#..ka.a.Lt.Art.an.i%.pp..qa..Dm

                       α10                  α11          η2
                220       230       240       250       260
D.melanogaster   QSGILHAADERDFKTAFSYFYEAFEGFDSVD.S...VKALTSLKYMLLCKIMLGQSDDVN
H.sapiens        QSGIIHAAEEKDWKTAYSYFYEAFEGYDSID.S...PKAITSLKYMLLCKIMLNTPEDVQ
O.dioica         QAGILHAAENKDWKTAFSYFYEAFEGFDSCDLK...KKAVQNLRYMIMCKIMNNKEEEVS
C.albicans       QSGILNSED.KDYKTAFSYFYESFEGFNSQDDP....RSIIVLKYMLLTKIMLNLIDDVN
S.cerevisiae     MSGILHCED.KDYKTAFSYFFESFESYHNLTTHNSYEKACQVLKYMLLSKIMLNLIDDVK
Y.lipolytica     MSGILQADD.KDYKTAFSYFYEAFEGYVGQENP....KAGTVLKYMLLCKIMLNLADDVE
C.posadasii      QSGMLHAED.KDFNTAYSYFIEALEGYHSLEEE...RKATSALQYMLLCKIMVNSVNDVN
P.brasiliensis   QSGMLHAED.KDFTTAFSYFIEALEGYHSQEET...SKATSALQYMLLCKIMLNAVNDVN
T.stipitatus     QSGMLHAED.KDFNTAYSYFIEALEGYSSVDDT...EKATAALQYMLLCKIMLNLVDDVT
N.crassa         QSGMLHAED.KDFNTAFSYFIEALDGYHTQEEP...QRAQAALQYMLLCKIMLNLADDVN
T.melanosporum   QSGILHAED.KDYNTAFSYFIEALDGYSAQDET...KKAIAALKYMLLSKIMLNLTDDVH
S.pombe          QSGILHADD.MDFKTAYSYFYEAYEGFTSLDDD...KKALSSLKYMLLSQIMLNSVSEVK
D.discoideum     QSGILHSEE.KDYKTAFSYFFESYETYDSLEDP....FAMKALKYMLLCKIMTNQTDDVH
A.thaliana       QSGILHAEE.KDYKTGYSYFFEAFESFNALGDP....RAVFSLKYMLLCKIMVSQADDVA
P.patens         QSGILHAEE.KDYKTGFSYFFEAFEAFNALDDP....RAVFSLKYMLLCKIMLNQADDVG
C.rheinhardtii   QSGILCAEE.KDYKTAYSYFFESFEQLASMDDP....RAAQVLKYMLLAKVMLDMADDVP
N.gruberi        QSGVLHAEE.KDYKTAFSYFFEAFEGYSNFDDY....TAVMCLKYMLLCKVCTNNPDDVQ
T.brucei         QSGVLHAEE.HDAKTAFSYLYEAFEGFHQLGDQAR..QARKALHYMILAKIATNSPDELS
C.elegans        QSGVLYSAEERDYKTSFSYFYEAFEGFASIGDK...INATSALKYMILCKIMLNETEQLA
P.tetraurelia    MAGVLYAEE.RDYRTSYSYFYEAFEAFNNLEDK....RALGTLKYMLLCKIMIGATDEVK
T.vaginalis      ESGILFNDD.GDYRTASSYFAEAFDAFANAGDP....LALDALKYRLLCRILDGKASECP
consensus>50     qsGilhae#.kDy.TafSYfyEaf#.f...dd......a...LkYml$ck!m.n..d#v.
                                   ▲▲   ▲

                 α12  α13               α14             α15
                270       280       290       300       310
D.melanogaster   QLVSGKLAIT........YSGRDIDAMKSVAEASHKRSLADFQAALKEYK.KELAEDVIV
H.sapiens        ALVSGKLALR........YAGRQTEALKCVAQASKNRSLADFEKALTDYR.AELRDDPII
O.dioica         NLLTAKLAFK........YKGPEIDAMKEICQASADRDVHKLEAAFVKYQ.PQLSDDTVV
C.albicans       NILNNKNVIK........YKSKDIDAMKSIAMAYSNRSLKEFENSLLTYA.NELKSDAII
S.cerevisiae     NILNAKYTKET.......YQSRGIDAMKAVAEAYNNRSLLDFNTALKQYE.KELMGDELT
Y.lipolytica     TILNNKSAQA........YQGRDVDVMKAVAVAYANRSLKEFEHVLELYK.EEVQADPVI
C.posadasii      SLLASKQAIR........YAGTSLEAMKAVAKAHSNRSLEEYERALSDYR.YELGSDVFI
P.brasiliensis   SLLASKQALR........YAGTSLEAMKAVARAHSNRSLEEYEKALNDYR.YELGSDVFI
T.stipitatus     NLLGSKQALK........YASTRLEAMKAVARAHANRSLEEYEKALSDYR.YELGGDVFI
N.crassa         QLMTSKQAVK........YAGKSLEAMKAIARAHSNRSLEEYERALAAYR.YELGSDTFI
T.melanosporum   QIMTGKLAIK........YAGRDIEAMKAVARAHSNRSLAEFEKALETFK.EELRSDPFI
S.pombe          SLLTGKHAIR........YAGRDIEAMRAIAQAHENRSLADFEKALQDYK.PELASDPII
D.discoideum     ALVNGKIGLKY.......HNNRSIEAITQISKSHAKRSLHMFQEVTKEFS.DQLSDDPII
A.thaliana       GIISSKAGLQ........YVGPDLDAMKAVADAHSKRSLKLFENALRDYK.AQLEDDPIV
P.patens         SLISSKAGLK........YTGVELDAMKAVADAYGKRSLKNFEEALNVYR.EQLEEDPIV
C.rheinhardtii   GIISSKAGLK........YTGPEVEAMRAVAAAYHDRSLQAFQDTLSAHK.SQLVDDLVV
N.gruberi        TLLSTKTALK........YTGREVDSMKAVSVAYQERSLHSFEKALSEFE.GELKNDPII
T.brucei         ALLSSKNVLE........YKGADMVALHGVADAYNSQDTHLFNRIMQENKNAPFLEDEVL
C.elegans        GLLAAKEIVAY.......QKSPRIIAIRSMADAFRKRSLKDFVKALAEHK.IELVEDKVV
P.tetraurelia    QILTGKHGLK........YAGRHLEAMKAISNSNQKKSLIEFTKVLEEYR.EEIEGDKVM
T.vaginalis      AIEAAAAIFLQNSKNAIVQKNTQIETMLEIARASEAKSLHDLLDVIKKRK.DDLNADPVV
consensus>50     .....k............y.g...eamk.va.a...rsl.efe..l..y...el..D.ii
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                  α16                  βA   α17        α18           βB
           320         330         340         350         360         370
D.melanogaster   QAHLGTLYDTMLEQNLCRIIEPYSRVQVAHVAESIQLPMPQVEKKLSQMILDKKFSGILD
H.sapiens        STHLAKLYDNLLEQNLIRVIEPFSRVQIEHISSLIKLSKADVERKLSQMILDKKFHGILD
O.dioica         QEHLVSLKSNLLEKNLMRLIEPYSRVEISKIAKLIDLDELEIELKLSQMILDKKLIGILS
C.albicans       KNHFNALYDNLLEQNLLKIIESYSCVELSHISKVIGLNLQQVEGKLSQMILDKVFYGVLD
S.cerevisiae     RSHFNALYDTLLESNLCKIIEPFECVEISHISKIIGLDTQQVEGKLSQMILDKIFYGVLD
Y.lipolytica     RVHFNDLYDSLLEQNLLKVIEPFSCVEINHIAEIIGLDTRQVEGKLSQMILDKGIKGVLD
C.posadasii      RNHLRRLYDAMLEQNLIKVIEPFSRVEIDHIAKMVGLDTQQVERKLSQMILDKVIIGVLD
P.brasiliensis   RNHLRRLYDAMLEQNLIKVIEPFSRVEIDHIAKMVGLDTQQVEKKLSQMILDKVIIGVLD
T.stipitatus     RNHLRRLYDAMLEQNLIKVIEPFSRVEIDHIAKMVGLDTQQVERKLSQMILDKVIVGVLD
N.crassa         RNHLRRLYDAMLEQNLIKVIEPFSRVEIDHIAKMVGLDTQQVERKLSQMILDKVIIGVLD
T.melanosporum   RSHFTALYDTLLEQNLVRVIEPFSRVEIEHVAKLVGLGTPHVEAKLSQMILDKVFSGVLD
S.pombe          RSHLSSLYDNLLEQNLLRVVEPFSRVEVSHIAELIGLSTVQVEGKLSQMILDKIFYGILD
D.discoideum     HSHLTELYSNLLEQNLCRIIEPFSRVEISHIAKLIDLPVDVVERKLSLMILDKKYNGILD
A.thaliana       HRHLSSLYDTLLEQNLCRLIEPFSRVEIAHIAELIGLPLDHVEKKLSQMILDKKFAGTLD
P.patens         HRHLSSLYDTLLEQNLCRLIEPFSRVEISHISELIGLPSENVEKKLSQMILDKKFAGTLD
C.rheinhardtii   GAHLAELYDTLMQQNLVRLIEPFSRVEIAHVASLIGLPRDIVETKLSQMILDGKLAGTLD
N.gruberi        SSHLSELYDNLLEQHLLRIIEPFSRVQITHVSSLIDLPRTRVERKLSQMILDNKLNGILD
T.brucei         QRQLSEMYKSLLEGHLLKLLEPYSRVQISYLAELLKLDVETVESQVSQLILDKKLAGIVD
C.elegans        AVHSQNLERNMLEKEISRVIEPYSEIELSYIARVIGMTVPPVERAIARMILDKKLMGSID
P.tetraurelia    RLHIKQLYEVLLEMNLFQVIQPYSKVQIDYITQRMQIDVEIIQRKLSELILDKKIDGTLD
T.vaginalis      ASNVKNLINSLEEQHLLRIVKPYSAVQLSRIAELIHLDVEQVEAKLVQMILDQKLKASIN
consensus>50     ..h...$yd.$l#qnl..viep%sr!#i.hia.li.l....!#.klsq$ILDk...gvld
                                      ▲  ▲▲                     ★▲  ▲▲

             βC
           380         390
D.melanogaster   QGEGVLIVFEE.........................................TPVDKTYE
H.sapiens        QGEGVLIIFDE.........................................PPVDKTYE
O.dioica         QEDRALQIFEP.........................................SESDKVYE
C.albicans       QGNGWLIIYDE.........................................PRKDAAYE
S.cerevisiae     QGNGWLYVYET.........................................PNQDATYD
Y.lipolytica     QGNGWLFIYDE.........................................PQTDKTYD
C.posadasii      QGAGCLIIFDE.........................................TERDQGYD
P.brasiliensis   QGAGCLIVFDE.........................................VERDQGYD
T.stipitatus     QGAGCLIVFEE.........................................TERDKGYD
N.crassa         QGAGCLIIYDE.........................................THRDEAYD
T.melanosporum   QGSGCLIVFDE.........................................VGRDKSYD
S.pombe          QGSGCLIVYDE.........................................PQQDKTYE
D.discoideum     QGTGTLIVFEE.........................................QKEDKLFN
A.thaliana       QGAGCLIIFED.........................................PKADAIYS
P.patens         QGAGCLIIFDD.........................................VKPDGIYP
C.rheinhardtii   AGAGCLEVFTP.........................................APADAVYP
N.gruberi        QGNDCIIVYEE.........................................TKKDECYP
T.brucei         QQHQCVVVFDEQDAKREKAKQKGDGTSDGSHGVSHSSAYHSGGTGTSGSGDVTSATTLYQ
C.elegans        QHGDTVVVYPK.........................................ADAANQFT
P.tetraurelia    QGNDCLILFDT.........................................VKHDNLYQ
T.vaginalis      QADGILNIFED.........................................EEANEILT
consensus>50     qg.g.liv%de............................................d..yd

            400         410         420
D.melanogaster   RVLETIQSMGKVVDTLYQKAKKLS.
H.sapiens        AALETIQNMSKVVDSLYNKAKKLT.
O.dioica         DSLEMIGALEDVVASLYKKAEKLK.
C.albicans       ASLELVKNLSNVVDLLYEKASSLN.
S.cerevisiae     SALELVGQLNKVVDQLFEKASVLY.
Y.lipolytica     SALETVKHMSTVVDLLYEKASSLN.
C.posadasii      AALETIDKLSSVVDVLYTNQASLLE
P.brasiliensis   AALETLEKLGNVVDVLYTNQASMLE
T.stipitatus     AALQTIEKLNNVVEVLYANQASLLE
N.crassa         AALKTIEKLSNVVDVLYTNQASQLE
T.melanosporum   AALETIKKLSDVVEVLYTNQASMLD
S.pombe          AALEVIKNMGTVVDLLIENKASALL
D.discoideum     CSLETIGALSRVVDLLYEKTNKLS.
A.thaliana       ATLETIANMGKVVDSLYVRSAKIMS
P.patens         STLETLSNISKVVDSLYLKSAKIMA
C.rheinhardtii   AALDVLESLGRVVDTLFARSQRVVA
N.gruberi        AALETIENMDSVMDSLFERAKALTK
T.brucei         DALDALEKYDKLVTALFDKANGKFD
C.elegans        RSLKTIRELTKTVDVSYSRTKHFK.
P.tetraurelia    HSLSLINNLNGVVDKLFDRVKVK..
T.vaginalis      ESIDLIENMDGVVDALYNRCKLMN.
consensus>50     ..le.i..l..vvd.ly........
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Fig. S2. Alignment of representative Rpn6 sequences. Amino acid sequences of selected Rpn6 homologs were aligned using Clustal-X. Secondary structure elements for the *D. melanogaster* Rpn6 are indicated above the sequences. The Rpn6 domain structure is indicated by purple, blue, and green coloring of secondary structure elements in the capping helix, α-solenoid, and PCI module, respectively. Similar residues are shown in red and identical residues in white on red background. Blue frames indicate homologous regions. The consensus sequence is shown at the bottom. The mutation sites, F132L and L377P, in *Saccharomyces cerevisiae* strain *rpn6-2* are indicated by asterisks. The mutations sites for *D. melanogaster* mutants M1, M2, and M3 are indicated by triangles. The position of the predicted C-terminal helix is indicated by a yellow bar below the alignment. The Uniprot accession codes for the sequences are: Q7KLV9, *Drosophila melanogaster*; O00231, *Homo sapiens*; E4XC34, *Oikopleura dioica*; Q59TN7, *Candida albicans*; Q12377, *Sacharomyces cerevisiae*; Q6C9R4, *Yarrowia lipolytica*; C5P9Z7, *Coccidioides posadasii* (strain C735); C1GHW5, *Paracoccidioides brasiliensis* (strain Pb18); B8M6N4, *Talaromyces stipitatus* (strain ATCC 10500/CBS 375.48/QM 6759/NRRL 1006); Q96U28, *Neurospora crassa*; D5GI46, *Tuber melanosporum* (strain Mel28); Q9P7S2, *Schizosaccharomyces pombe*; Q54UB5, *Dictyostelium discoideum*; Q9LP45, *Arabidobsis thaliana*; A9RB85, *Physcomitrella patens* subsp. patens; A8I274, *Chlamydomonas rheinhardtii*; D2UZW5, *Naegleria gruberi*; Q586L6, *Trypanosoma brucei*; P34481, *Caenorhabditis elegans*; A0BT65, *Paramecium tetraurelia*; A2DYJ9, *Trichomonas vaginalis*.

A

area II → ← area III

180º

area I

90º

90º

B

area II

area III

180º

hydrophobic
acidic
basic

Fig. S3. Surface properties of Rpn6. (*A* and *B*) Physicochemical properties of the Rpn6 surface. Rpn6 is shown in surface representation. The same orientations as in Fig. 2 of the main text are shown. Hydrophobic side chains are indicated in yellow. Positively and negatively charged functional groups are colored blue and red, respectively. The rest of the surface is shown in white.

Fig. S4. Rpn6 crystal contacts suggest a linear arrangement of PCI subunits in the proteasome. (*A*) Crystal contacts of Rpn6. Five Rpn6 chains are shown in rainbow colors, as found in the crystal lattice. The sixfold screw axis is oriented perpendicular to the paper plane at the center. The small β-sheets at the tip of the PCI module form a continuous β-ribbon around the screw axis. Slight rearrangements would lead to an open structure. (*B*) Schematic model for the arrangement of the PCI subunits in the proteasome. The proposed PCI subunit order was deduced from (*i*) the lid assembly pathway (1), (*ii*) the PCI-domain-dependent yeast-two hybrid interaction between Rpn5 and Rpn6 (2), and the binary Rpn6–Rpn7 interaction described in this study.

1 Fukunaga K, Kudo T, Toh-e A, Tanaka K, Saeki Y (2010) Dissection of the assembly pathway of the proteasome lid in Saccharomyces cerevisiae. *Biochem Biophys Res Commun* 396:1048–1053.

2 Isono E, Saito N, Kamata N, Saeki Y, Toh EA (2005) Functional analysis of Rpn6p, a lid component of the 26 S proteasome, using temperature-sensitive rpn6 mutants of the yeast Saccharomyces cerevisiae. *J Biol Chem* 280:6537–6547.

Peak #	CCC	z-score
1	0.4813	6.0942
2	0.4308	4.9813
3	0.4097	4.5057
4	0.3923	4.3202
5	0.3898	4.5434

Fig. S5. Docking of Rpn6 into the 26S proteasome EM density map. (*A*) Isosurface representation of the EM density of the 26S proteasome (grayscale) with overlaying cross-correlation function (CCF) (blue). The position of the maximum of the CCF (red) indicates the location of Rpn6. (*B*) Analysis of the highest five peaks of the CCF. (*Left*)The cross-correlation coefficients (CCCs); (*Right*) the corresponding Z-scores (14). (*C*) Table of the five highest CCC peaks and corresponding Z-score.

Fig. S6. Colocalization of secondary structure elements in Rpn6 atomic models and cryo-EM density. (*A*) dmRpn6 crystal structure (green) fitted into the EM density (gold). For better visibility of secondary structure elements, we hi-pass filtered the EM map (>1/17 nm). (*B*) Comparative model of spRpn6 (residues 1–387) after flexible fitting.

Table S1. Data collection and refinement statistics

Dataset	MAD			Native
Beamline	ESRF, ID23-1			ESRF, ID29
	peak	*inflection*	*remote*	
Wavelength, Å	1.71024	1.71072	1.03320	1.00686
Space group	$P6_1$			$P6_1$
Cell dimensions, *a*, *b*, *c*; Å	161.25, 161.25, 42.08;			161.30, 161.30, 42.10;
α, β, γ, °	90, 90, 120			90, 90, 120
Resolution limits, Å*	40.42–3.4 (3.58–3.4)	40.47–3.4 (3.58–3.4)	38.89–3.0 (3.16–3.0)	52.78–2.5(2.65–2.5)
R_{merge}*	0.063 (0.335)	0.054 (0.352)	0.048 (0.489)	0.046 (0.303)
I/sigma*	20.1 (5.2)	13.5 (2.9)	14.5 (1.9)	14.4 (2.6)
Multiplicity[†]	7.3 (7.6)	3.6 (3.7)	3.6 (3.7)	3.3 (3.3)
Completeness, %*	97.9 (98.5)	97.4 (97.7)	97.9 (96.6)	99.3 (99.0)
Phasing				
Sites	4 Gd			—
Phasing power ano	2.172	1.405	0.447	
Phasing power iso	1.158	1.113	—	
Mean FoM			0.277	—
Refinement				
Resolution range	—	—	20–3.0	20–2.5
Reflections (test set)	—	—	11819 (638)	20825 (1146)
R_{work}	—	—	0.205	0.216
R_{free}	—	—	0.251	0.265
No. of atoms	—	—	2731	2788
rmsd bonds, Å	—	—	0.012	0.012
rmsd angles, °	—	—	1.384	1.294
Ramachandran plot[†]				
% most favored region	—	—	88.7	91.8
% additionally allowed	—	—	11.0	8.2

*Values in parenthesis for outer shell.

[†]As defined in Procheck (1).

1 Laskowski RA, MacArthur MW, Moss DS, Thornton JM (1993) PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr* 26:283–291.