

SUPPORTING ONLINE MATERIAL

Supplementary Material

Materials and Methods

Preparation of Ub-Sic1 and 26S proteasomes. Sic1 was isolated in a complex with GST-Cdc28HA/Clb5 (S-Cdk) from insect cells, phosphorylated by G1-Cdk, and ubiquitinated by SCF^{Cdc4} ubiquitin ligase as described in (1). In some instances the trimeric Sic1 complex was supplemented with CAK, in which case the requirement for the G1 Cdk was bypassed (since Sic1 was isolated in a phospho form), and the complex was ubiquitinated by SCF^{Cdc4} directly. In Fig.1 E, MbpSic1 was isolated from E.coli, and phosphorylated and ubiquitinated as previously described (2). 26S proteasomes were purified from budding yeast strain RJD 1144 (3) containing FlagHis6 epitope tagged *PRE1* by anti-Flag immunoaffinity chromatography.

Yeast strains used in this study. RJD 1144: *MATa his3Δ200 leu2-3,112 lys2-801 trpΔ63 PRE1^{FH}::YIplac211 (URA3)* (3). RJD 1694: *MATalpha his3-Δ200 leu-3,112 ura3-52 lys2-801 trp1-1 gal2 ubp6::HIS3*. RJD 1736: RJD 1694 transformed with BsmI-linearized RDB 930 plasmid which resulted in tagging of the chromosomal *PRE1* locus with the Flag-His6 epitope yielding *PRE1^{FH}::YIplac211 (URA3)* (3). RJD 1877: wild type *RPN11* was subcloned into pRS305 [*LEU2*], which was subsequently linearized with EcoRV to target integration at the *leu2* locus in *mpr1-1* strain RJD 1786 to yield *MAT-alpha, mpr1-1, can1-100, leu2::RPN11 LEU2, his3-11,-15, trp1-1, ade2-1 rho+*. RJD 1878: same as 1877, except *leu2::rpn11AXA LEU2*. RJD 1867 and 1868: RJD 1877 and 1878, respectively, were transformed with linearized RDB 930 yielding *PRE1^{FH}::YIplac211 (URA3)*. RJD 1869: *MAT-alpha can1-100, leu2-3,-112, his3-11,-15, trp1-1 ade2-1 PRE1^{FH}::YIplac211 (URA3) rho+*. RJD 1900: *MAT-alpha can1-100, leu2-3,-112 his3-11,-15 trp1-1 ade2-1 mpr1-1*

PRE1^{FH}::YIplac211 (URA3) rho+; obtained by crossing RJD 1869 X RJD 1877. RJD 1901, 1902, 1903, and 1904: RJD 1785 (*MPR*), 1786 (*mpr1-1*), 1877, and 1878, respectively, transformed with reporter [URA3] plasmid encoding the Ub-V76-Val-eK-B-Gal fusion protein. RJD 1996: *MATa ade2-1 trp1-1 can1-100 leu2-3, 122 his3-11, 15 GAL ura3::URA3 GAL-CLB2-HA3*. RJD 2002: *mpr1-1 leu2::LEU2 RPN11 ura3::URA3 GAL-CLB2-HA3*; derived from cross between RJD 1996 and RJD 1877. RJD 2004 *mpr1-1 leu2::LEU2 rpn11AXA ura3:: URA3 GAL-CLB2-HA3*; derived from cross between RJD 1996 and RJD 1878.

Generation of *RPN11* mutants. A 1.3 kB fragment containing the *RPN11* ORF flanked by 300 bp of upstream and 100 bp of downstream DNA was amplified by PCR (High Fidelity Taq, Roche Molecular Biochemicals) from yeast genomic DNA, restricted with BamHI and HindIII at sites introduced into the primers (Rpn11A and Rpn11B respectively, see supplemental Table 3 for sequences) and sub-cloned into the *URA3* vector pRS316 to yield RDB 1485 and the *LEU2* vector pRS315 to yield RDB 1492. The wild-type ORF was confirmed both by sequencing and by complementation assay (see Fig. 2C). Bridge PCR (4) was carried out to introduce the two mutations resulting in His to Ala substitutions at positions 109 and 111 (oligos Rpn11A, MuR2, Rpn11B) using RDB 1485 as template. Mutant *rpn11AXA* was sub-cloned into pRS315 (*LEU2*) to yield RDB 1493, and the mutations confirmed by sequencing. A heterozygous *RPN11/rpn11::KanMX4* diploid strain (RJD 1791: *MATa/MATa his3D1/his3D1 leu2D0/leu2D0 ura3D0/ura3D0 met15D0/MET15 lys2D0/LYS2 rpn11::KanMX4/RPN11*) was transformed with RDB 1485, sporulated, and tetrads dissected. Haploid strains containing the *rpn11Δ::KanMX4* deletion covered by RDB1485 were selected, and transformed with RDB 1492 and RDB 1493.

Preparation of the Lid subcomplex of the 19S regulatory subparticle. The chromosomal *RPN8* locus was tagged using the *TEV2MYC9* cassette described in (3). Cell lysates were prepared and bound to 9E10 beads. Bound complex was washed in a buffer containing 25 mM Tris, 0.2 % Triton, and 2.0 M NaCl. The high salt resulted in the removal of the 20S core and 19S base subparticle. The remaining bound lid subcomplex was washed twice with 1X tobacco etch virus protease (TEV) buffer, and then eluted by cleaving with recombinant His-tagged TEV. TEV was removed by binding to Ni-NTA beads, and the supernatant containing the lid subunits was stored in a buffer that was supplemented with 15 % glycerol.

Pulse-Chase analysis. Briefly, cultures grown at 25°C were shifted to 36°C for one hour and pulse-labeled for 5 min. Aliquots were withdrawn at the indicated time of chase, and cell lysates were immunoprecipitated with anti -Gal monoclonal antibody. Immunoprecipitated proteins were evaluated by SDS-PAGE followed by autoradiography.

Supplemental Figure 1. Ub-Sic1 is deubiquitinated by epoxomicin-treated 26S proteasomes. Reaction conditions were exactly as described in Fig.1 A except that a 10% polyacrylamide gel was used to better visualize the low molecular weight reaction products and to determine if there were products of higher mobility than unmodified Sic1.

Supplemental Figure 2. Deubiquitination of Ub-Sic1 is independent of Ubp6. Mock- and epoxomicin-treated 26S proteasomes isolated from wild-type (*UBP6*; RJD 1144) and *ubp6Δ* (RJD 1736) strains were assayed for their ability to degrade/deubiquitinate Ub-Sic1 as in Fig. 1A.

Supplemental Figure 3. Alignment of the JAMM domains of the eukaryotic Rpn11 proteins. The multiple alignment, which was constructed using the T-coffee program, includes all available sequences of Rpn11 orthologs. For each protein, the position of the aligned region

in the sequence is shown by numbers. The consensus includes amino acid residues conserved in all Rpn11 sequences; a indicates aromatic residues (F,Y,W; yellow shading), l indicates aliphatic residues (A,I,L,V; yellow shading), h indicates hydrophobic residues (F,Y,W, A,I,L,V,M; yellow shading), p indicates polar residues (D,E,N,Q, K,R,H,S,T; red), + indicates positively charged residues (K,R,H; red), - indicates negatively charged residue (D,E; red), s indicates small residues (G,A,C,S,D,N,V,P; blue), and u indicates “tiny” residues (G,A,S; blue). The predicted metal-chelating and catalytic residues (see text) are shown in yellow against a dark-blue background. Each protein is denoted by the GenBank identifier (GI) followed by an abbreviated species name. Species abbreviations: Hsa, *Homo sapiens*, Mmu, *Mus musculus*, Dme, *Drosophila melanogaster*, Cel, *Caenorhabditis elegans*, Sma, *Schistosoma mansoni* (trematode worm), Ava, *Aphrocallistes vastus* (sponge), Gcy, *Geodia cydonium* (sponge), Ddi, *Dictyostelium discoideum* (slime mold), Sce, *Saccharomyces cerevisiae*, Spo, *Schizosaccharomyces pombe*, Ecu, *Encephalitozoon cuniculi* (Microsporidium), Osa, *Oryza sativa*, Ath, *Arabidopsis thaliana*, Tbr, *Trypanosoma brucei*, Gth, *Guillardia theta* (cryptomonade nucleomorph), Gin, *Giardia intestinalis* (Diplomonade).

Supplemental Table 1: MudPIT analysis of 26S preparations from wild-type and *rpn11* mutant strains. 26S proteasomes were prepared from RJD 1869 (*MPRI*), RJD 1900 (*mpr1-1*), RJD 1867 (*mpr1-1/RPN11*) and RJD 1868 (*mpr1-1/rpn11AXA*) strains and analyzed by MudPIT as previously described (1). Numbers in Table report the percentage of residues in protein sequence that are represented by at least one sequenced peptide. Only proteins that

were present in all four 26S preparations (except the preparation from *mpr1-1*, which did not yield an intact 26S) are reported.

Supplemental Table 2: The lid subunits are either absent or present in very low abundance in 26S preparations from *mpr1-1* containing truncated Rpn11. The % sequence coverage of each subunit was normalized to wild-type using the following calculation: (% seq coverage RpnX in *mpr1-1*) divided by (% sequence coverage in *RPN11*) / (average % coverage of 20S subunits in *mpr1-1*) divided by (avg % seq coverage 20S subunits in *RPN11*).

Supplemental Table 3. Sequences of Oligos used in the study.

References and Notes

1. R. Verma, H. McDonald, J. R. Yates, 3rd, R. J. Deshaies, *Mol Cell* **8**, 439 (2001).
2. J. Seol *et al.*, *Genes Dev* **13**, 1614 (1999).
3. R. Verma *et al.*, *Mol Biol Cell* **11**, 3425 (2000).
4. R. Verma, Y. Chi, R. J. Deshaies, *Methods Enzymol* **283**, 366 (1997).

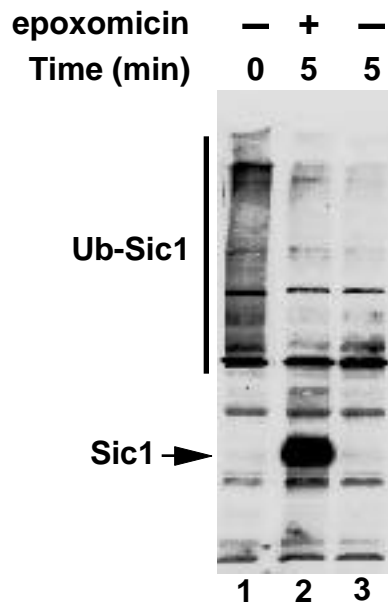


FIG. S 1

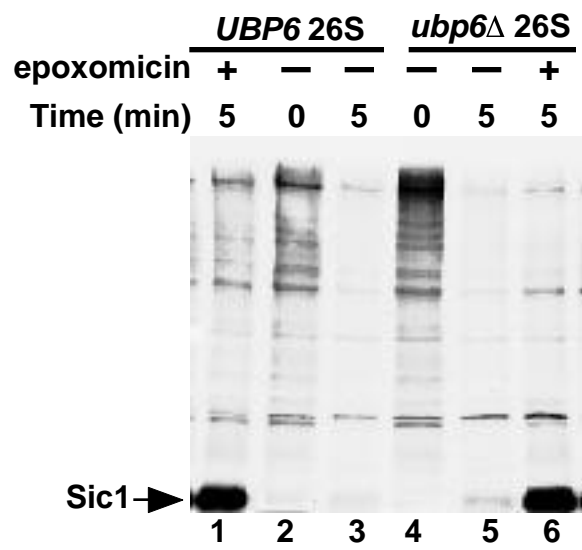


FIG. S 2

TABLE S1
26S PROTEASOME SUBUNITS

Locus	W303	RPN11	rpn11AXA	mpr1-1
YER012W / PRE1	47	47.5	44.4	45.5
YPR103W / PRE2	57.8	57.5	64.1	73.9
YJL001W / PRE3	64.7	75.3	67.9	58.6
YFR050C / PRE4	74.1	69.9	69.2	72.6
YMR314W / PRE5	86.8	87.2	87.2	87.2
YOL038W / PRE6	66.5	72.8	76.4	79.5
YBL041W / PRE7	78	75.9	70.5	74.7
YML092C / PRE8	66.8	59.6	72.4	63.2
YGR135W / PRE9	79.5	86	79.8	83.3
YOR362C / PRE10	64.9	55.2	69.1	66.3
YOR157C / PUP1	47.1	47.1	41.8	52.1
YGR253C / PUP2	88.8	86.2	88.8	86.5
YER094C / PUP3	46.8	47.3	46.8	45.9
YGL011C / SCL1	92.5	77.8	82.5	92.5
YKL145W / RPT1	63.6	50.7	51.6	51.4
YDL007W / RPT2	77.1	65	61.6	66.4
YDR394W / RPT3	71.5	65.2	68.2	71.7
YOR259C / RPT4	60.9	48.7	61.1	59.3
YOR117W / RPT5	68.7	72.4	57.4	70.7
YGL048C / RPT6	62	60.2	61.7	57.3
YHR027C / RPN1	57.5	61.4	56.7	65.5
YIL075C / RPN2	48.7	48	47.2	48.9
YER021W / RPN3	48.8	42.8	21.2	7.8
YDL147W / RPN5	40.9	31	15.7	8.1
YDL097C / RPN6	59	47.2	55.1	23.5
YPR108W / RPN7	33.8	29.8	28.7	
YOR261C / RPN8	69.5	64.2	69.5	8.6
YDR427W / RPN9	43	44.3	40.7	12.2
YHR200W / RPN10	71.6	66.8	46.6	
YFR004W / RPN11	62.1	55.6	48.4	
YFR052W / RPN12	62.4	58	71.2	25.9
YLR421C / RPN13	41.7	64.7	37.8	37.8
NON-PROTEASOMAL PROTEINS				
YBR080C / SEC18	1.6	1.6	1.6	1.6
YFL007W / BLM3	2.6	4.7	2.5	5.3
YFR010W / UBP6	26.1	7.6	48.9	18.8
YGL009C / LEU1	43	22.6	9	43.3
YGL244W / RTF1	11.6	19.9	5.6	15.1
YGR192C / TDH3	39.8	15.7	8.4	39.2
YGR254W / ENO1	34.6	16.7	7.6	20.4
YHR174W / ENO2	38.2	24.7	11.9	46.5
YLL039C / UBI4	12.3	4.2	12.9	3.9
YLR044C / PDC1	30.6	15.3	8.9	28.2
YLR199C / ?	23.2	23.2	28.6	23.2
YLR418C / CDC73	14.2	9.9	7.9	7.9
YOR123C / LEO1	12.7	11.6	8.6	8.2
YPR173C / VPS4	2.7	2.7	2.7	2.7

YAL005C / SSA1	7.8	11.4	5.3	8.4
YOL055C / THI20	40.3	31	41	
YPL258C / THI21	11.1	7.8	12.9	

PROTEIN **Normalized % sequence coverage in 26S prep. from *mpr1-1***

Rpn1	1.14
Rpn2	1
Rpn3	0.15
Rpn5	0.19
Rpn6	0.38
Rpn7	0
Rpn8	0.12
Rpn9	0.27
Rpn10	0
Rpn11	0
Rpn12	0.3
Rpn13	0.9
Rpt1	0.8
Rpt2	0.82
Rpt3	0.96
Rpt4	0.99
Rpt5	0.99
Rpt6	0.89

Table S3

Sequences of Oligos used:

RPN11A 5' CGC GGA TCC TCT GAT CCC ATA GCC ATT TGA 3'

RPN11B 5' CCCAAG CTT TCC ATT AAC TAC TTC ATT AGA 3'

RPN11C 5' ATG GAA CGA CTA CAG AGA TTG 3'

MuR2 5' AGC CAA CAG CCA AAC CCT GGA GCA GAG GCG TAC CAG CCA ACG
AC 3'

MuR3 5' GCC AAA CCC TGG AGC AGA AAC 3'

RPN11E 5' GCC AAA CCC TGG ATG AGA GTG 3'