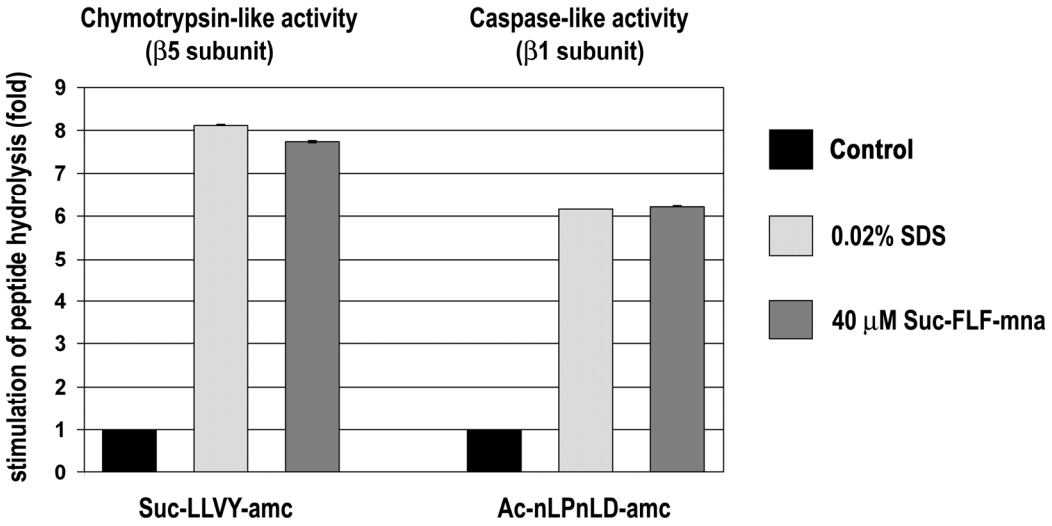
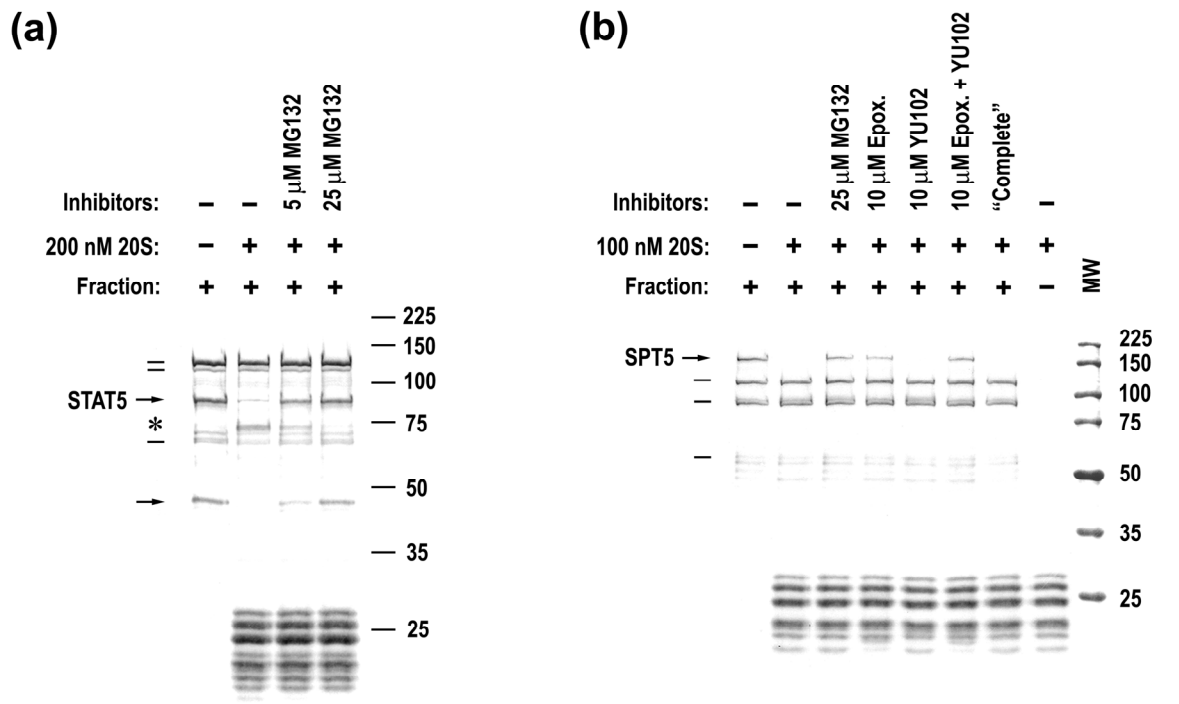
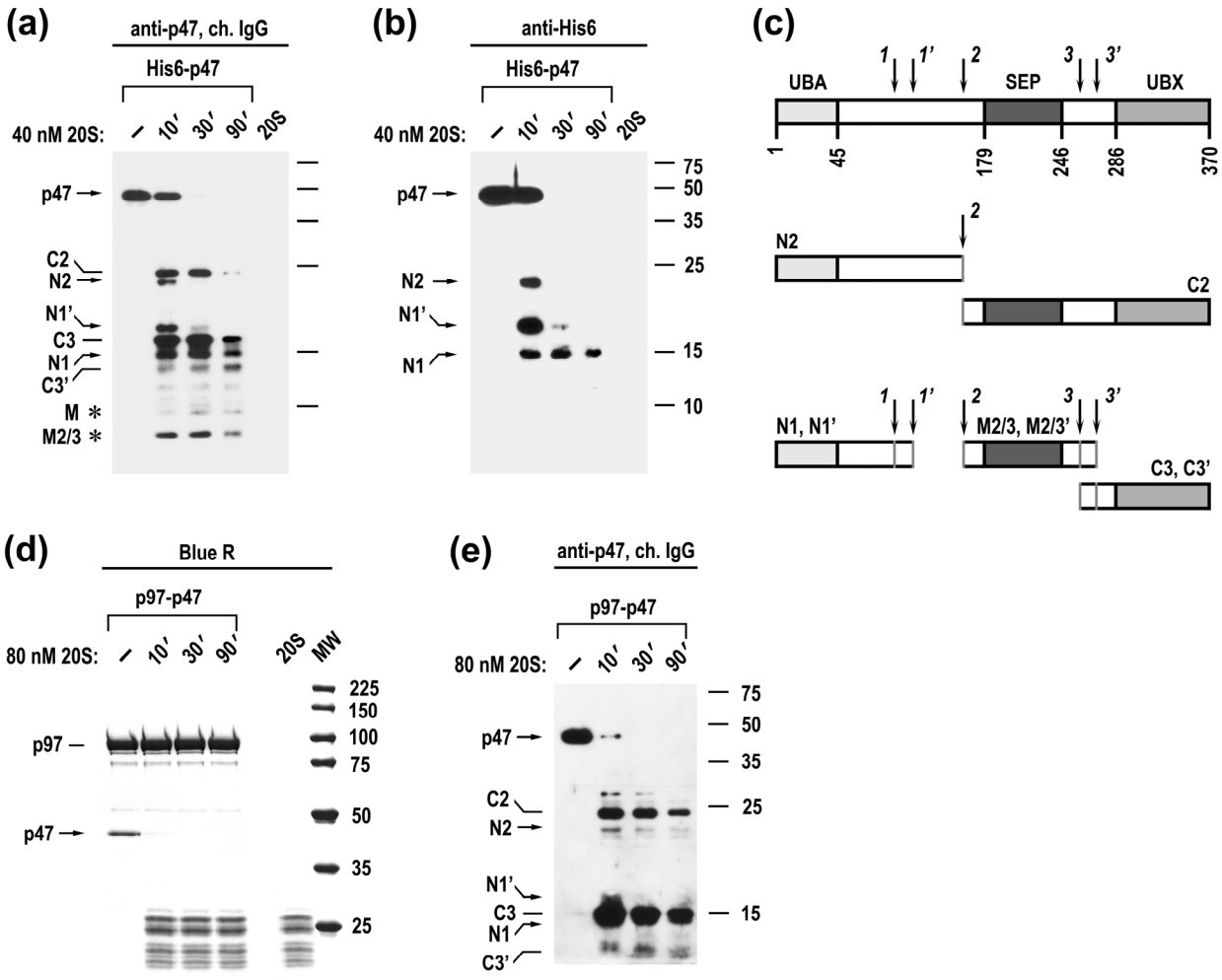



**Supplementary Fig. 1.** The effect of chemical treatments on the activity of 20S proteasomes in hydrolysis of model peptide substrates. Chymotrypsin-like and caspase-like activities were assessed by following degradation of the fluorogenic substrates, Suc-LLVY-amc (5μM) and Ac-nLPnLD-amc (50μM), respectively. Substrates were incubated with 10 nM 20S proteasomes for 1 hour at 37ºC in the absence (control) or presence of 0.02% SDS or 40μM Suc-FLF-mna. Stimulation of peptide hydrolysis is represented as means of fluorescence values collected during 1 minute course of measurements normalized to that of the control. Similar results were obtained in three independent experiments.



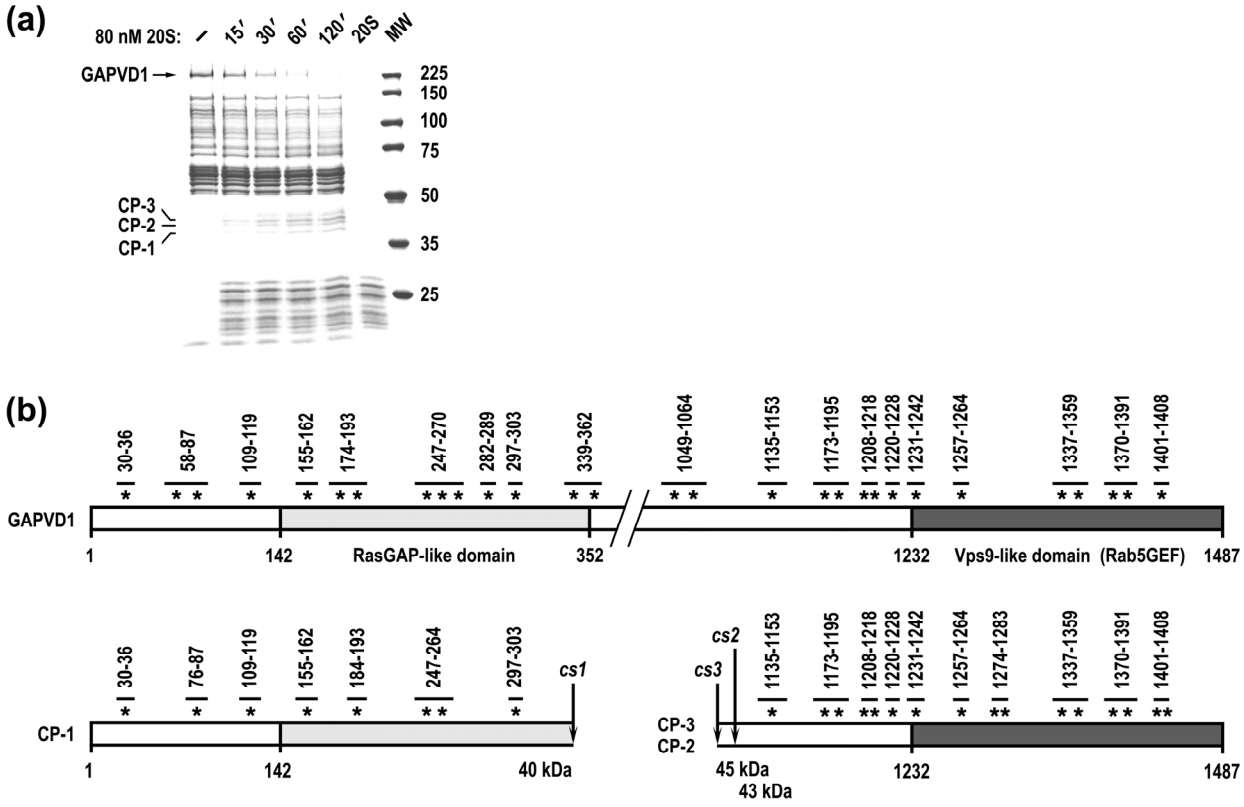

**Supplementary Fig. 2.** IPS cleavage is suppressed by specific proteasome inhibitors. (a) Dose-dependent inhibition of IPS cleavage by specific proteasome inhibitor MG132. STAT5-containing gel filtration elution fraction #26 was incubated for 3 hours with or without 20S proteasomes and stained with Blue R, following SDS-PAGE. Reactions were supplemented or not with the indicated concentration of MG132. Protein species that were cleaved or remained intact are marked with arrows or lines to the left of the reactions. The position of new protein species that appeared after incubation with 20S proteasomes is marked with an asterisk. The positions of MW markers are shown to the right. (b) The effect of specific proteasome inhibitors MG132, epoxomicin, and YU102 along with the Protease Inhibitor Cocktail "Complete" on the cleavage of SPT5 by 20S proteasomes. SPT5-containing fraction was incubated for 90 minutes with or without 20S proteasomes and stained with Blue R, following SDS-PAGE. Reactions were

supplemented with the indicated concentration of proteasome inhibitors. The protease Inhibitor Cocktail “Complete” (Roche) was added at the fivefold excess over the concentration recommended by the manufacturer.



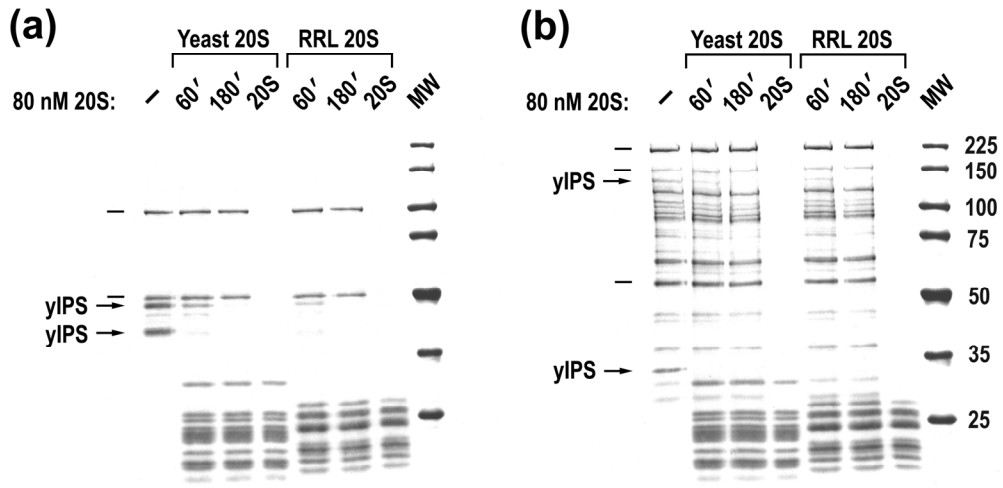

**Supplementary Fig. 3.** 20S proteasome cleaves the p47 cofactor of p97 at internal disordered regions while sparing structured domains. (a, b) Immunoblot analysis of the time course of cleavage of recombinant His6-human p47 protein by 20S proteasomes with chicken anti-serum raised against the recombinant protein and an antibody specific to the N-terminal His6-tag. Reactions were assembled as indicated. The positions of the N-terminal (Nx), middle (M), and C-terminal (Cx) cleavage products are marked with arrows, asterisks, and lines, respectively, to the left of the panels. The positions of the MW markers are shown to the right. (c) The model of p47 protein cleavage. The upper

lane is a schematic representation of the structure of p47, with the empty boxes representing flexible regions and the filled boxes corresponding to the UBA, SEP [domain present in *S. cerevisiae* Shp1, Drosophila melanogaster eyes closed gene *eyc*, and vertebrate p47], and ubiquitin regulatory X (UBX) domains (Yuan, X., Simpson, P., McKeown, C., Kondo, H., Uchiyama, K., Wallis, R., Dreveny, I., Keetch, C., Zhang, X., Robinson, C., Freemont, P. & Matthews, S. (2004). Structure, dynamics and interactions of p47, a major adaptor of the AAA ATPase, p97. *EMBO J.* **23,** 1463–1473). The positions of cleavage sites (arrows with numbers) are tentative, based upon the mobility of cleavage products, time-course of their generation, and mapping of the cleavage products with specific antibodies. (d, e) Blue R stained SDS-PAGE and immunoblot analysis of the time course of cleavage of the native rabbit p47 protein associated with p97/Cdc48 complex. The assignment of cleavage products is based upon their mobility, time course of their generation, and the proposed cleavage model.

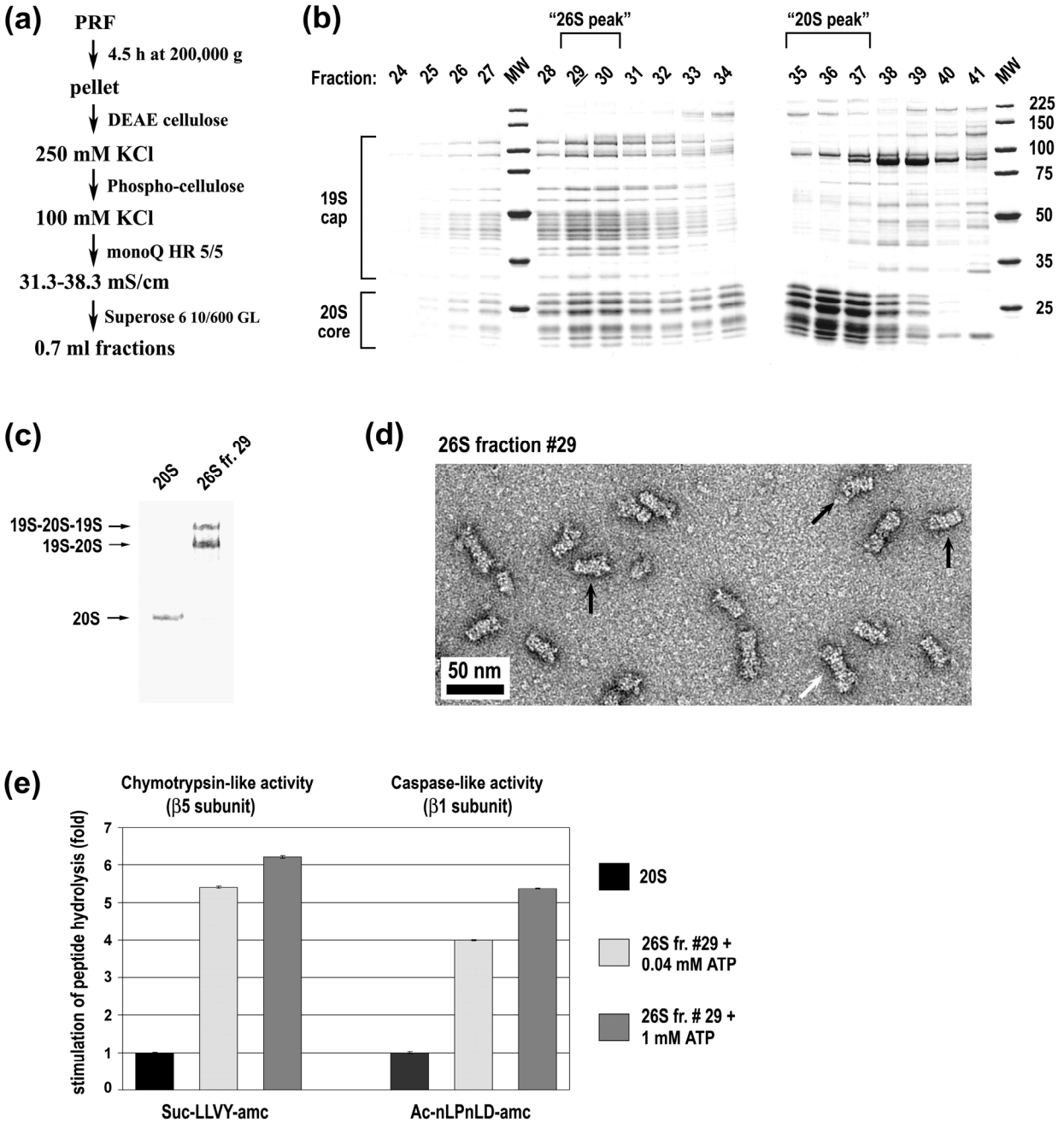



**Supplementary Fig. 4.** 20S proteasome cleaves GAPVD1 protein at internal sites producing cleavage species encompassing structured functional domains. (a) Blue R stained SDS-PAGE of the time course of cleavage of partly purified GAPVD1 by 20S proteasomes. The position of the extracted bands and the identification of the proteins comprised therein are shown to the left (CP, cleavage products of GAPVD1). (b) The model of GAPVD1 protein cleavage. The upper lane schematically represents the structure of the longest splicing form of GAPVD1 (GI:51093832) and the filled boxes correspond to the functional domains (Hunker, C. M., Galvis, A., Kruk, I., Giambini, H., Veisaga, M. L. & Barbieri, M. A. (2006). Rab5-activating protein 6, a novel endosomal protein with a role in endocytosis. *Biochem. Biophys. Res. Commun.* **340,** 967-975).

Mass spectrometry analysis of the trypsin-digested intact protein revealed forty seven distinct peptides. Asterisks denote positions of peptides derived from the N- and C-termini and numbers indicate amino acid sequences covered by the peptides. Mass spectrometry analysis of CP-1 yielded eight distinct peptides that matched peptides characteristic of the very N-terminus of intact GAPVD1 (bottom left lane). Mass spectrometry analysis of CP-2 and CP-3 yielded an identical set of sixteen distinct peptides, which matched all peptides characteristic of the very C-terminal portion of intact GAPVD1 (bottom right lane). Cleavage products were mapped and the positions of cleavage sites (*cs*) predicted based upon correlation of the GAPVD1 amino acid sequence with peptides either observed or missing in its cleavage products, as well as upon the mobility of cleavage products. Of note, GAPVD1 cleavage products encompass Ras GAP or Rab5 GEF domains, indicating that the 20S proteasome splits the intact protein to uncouple two distinct functional activities that have opposite regulatory roles in fluid-phase and receptor-mediated endocytosis.

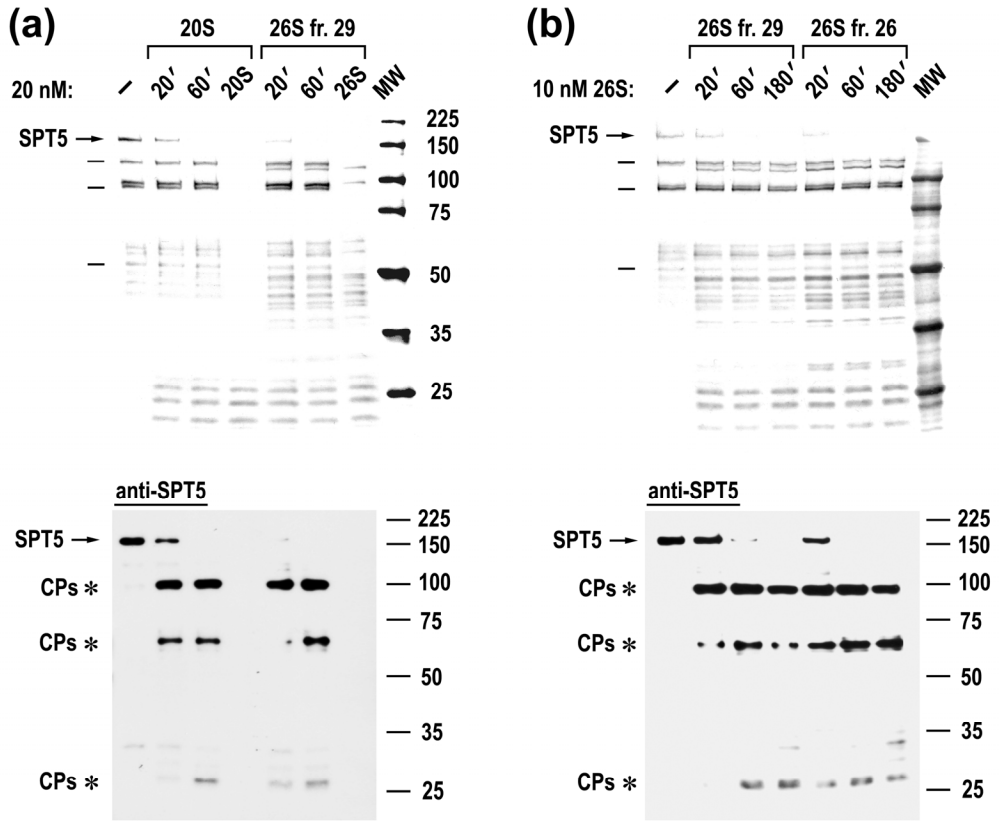



**Supplementary Fig. 5.** Yeast and mammalian 20S proteasomes specifically recognize and cleave the same yeast IPSes (yIPS). Time courses of cleavage of partly purified yIPSes by either yeast or rabbit 20S proteasomes. Reactions were assembled as indicated above the panels, with the positions of some yIPSes (arrows) and stable cellular proteins (lines) marked to the left.

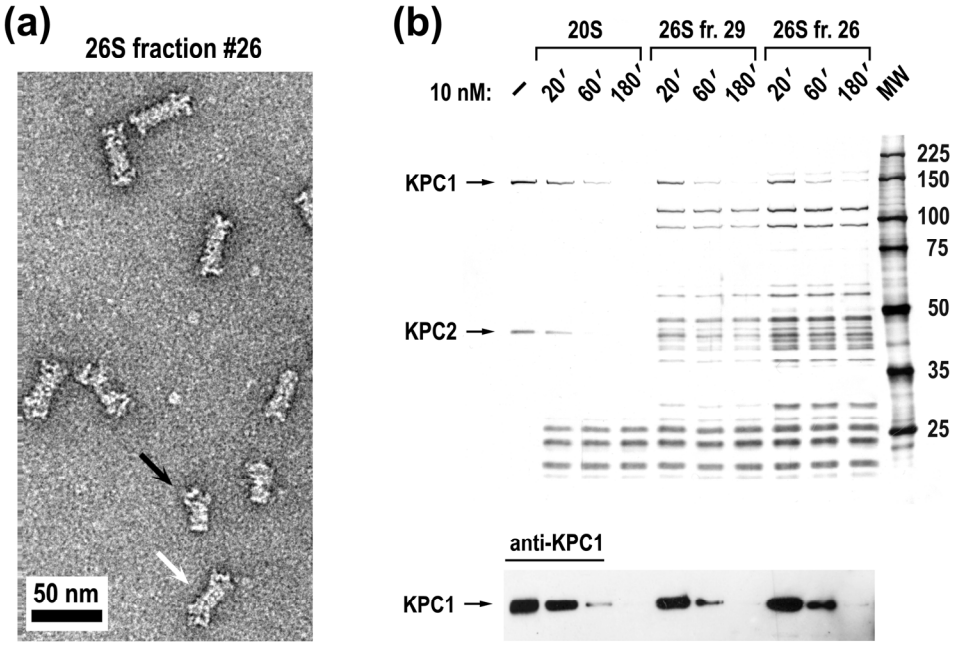



**Supplementary Fig. 6.** Purification and characterization of 26S proteasomes. (a) Purification protocol of 26S proteasomes. (b) Blue R stained SDS-PAGE of fractions eluted from Superose 6 column. 8 μl aliquot was loaded from each fraction. Peak elution

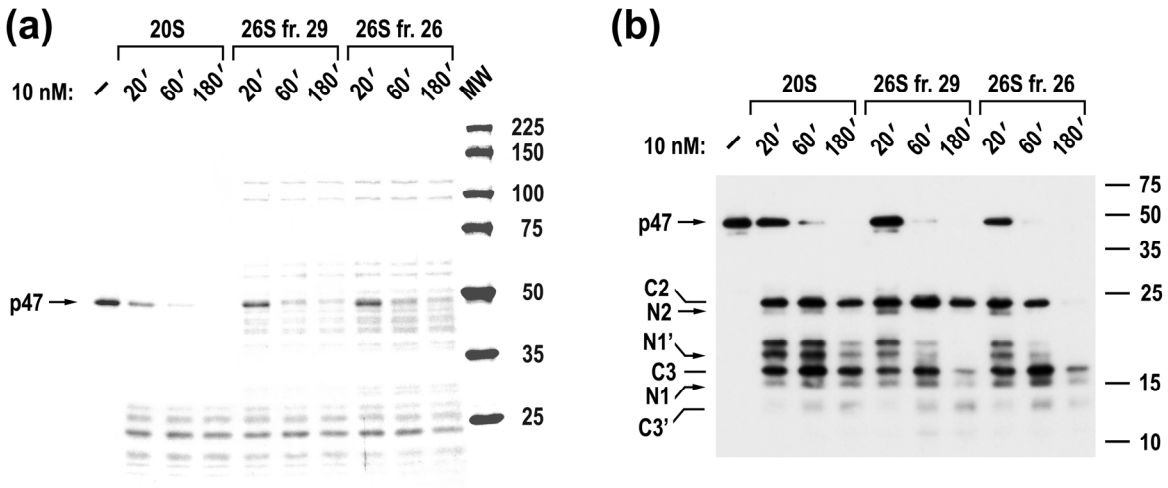
fractions of 26S proteasomes and free 20S particles are indicated on the top. Bands corresponding to subunits of the 19S cap and the 20S proteasome are marked with brackets to the left. (c) Silver stained native PAGE of purified 20S and 26S proteasomes (fraction #29), used in this study. 0.1 pmole of proteasomes was loaded. Bands corresponding to free catalytic core as well as singly and doubly capped 26S proteasomes are marked to the left. Note that fraction #29 contains more singly capped than doubly capped proteasome species. (d) Electron micrograph of 26S proteasomes (fraction #29) stained with 1% uranyl acetate. Some singly and doubly capped proteasomes are marked with black and white arrows, respectively. 207 singly capped and 77 doubly capped particles were counted on two representative micrographs, affording an assessment of their relative abundance at a ratio of 8:3. (e) The activity of 20S and 26S proteasomes in hydrolysis of model peptide fluorogenic substrates. Reactions contained 10 nM proteasomes and were assembled as indicated (see legend to Supplementary Fig. 1 for more detail). Similar results were obtained in two independent experiments.



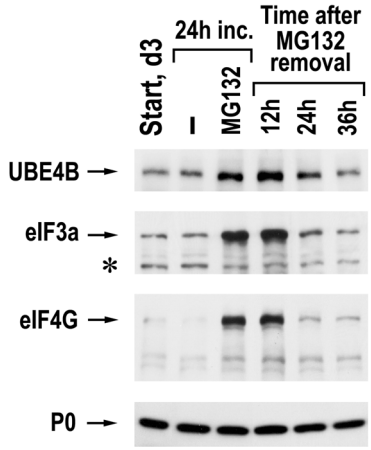

**Supplementary Fig. 7.** SPT5 cleavage by different proteasome species. (a) Time courses of cleavage of partly purified SPT5 with 20S and 26S proteasomes (fraction #29). Upper and bottom panels show Blue R stained SDS-PAGE and immunoblot analysis of these reactions, respectively. The positions of intact SPT5 (arrow), its cleavage products (asterisks), and some of the stable cellular proteins (lines) are marked to the left. (b) Time courses of cleavage of partly purified SPT5 with 26S proteasomes enriched in singly capped species (fraction #29) and 26S proteasomes enriched in doubly capped species (fraction #26; see Supplementary Fig. 8a for more information). Upper and bottom panels show silver stained SDS-PAGE and immunoblot analysis of these reactions, respectively.




**Supplementary Fig. 8.** KPC1 cleavage by different proteasome species. (a) Electron micrograph of 26S proteasomes (fraction #26) stained with 1% uranyl acetate. Representative singly and doubly capped proteasomes are marked with black and white arrows, respectively. 51 singly capped and 126 doubly capped particles were counted on representative micrographs, affording an assessment of their relative abundance at a ratio of 2:5. (b) Time courses of cleavage of highly purified KPC1-KPC2 complex with 20S proteasomes, 26S proteasomes enriched in singly capped species (fraction #29), and 26S proteasomes enriched in doubly capped species (fraction #26). Upper and bottom panels show silver stained SDS-PAGE and immunoblot analysis of these reactions, respectively. Note that KPC2 protein is also cleaved by proteasomes.




**Supplementary Fig. 9.** Time courses of cleavage of recombinant His6-p47 protein with 20S proteasomes, 26S proteasomes enriched in singly capped species (fraction #29), and 26S proteasomes enriched in doubly capped species (fraction #26). (a) Blue R staining of SDS-PAGE and (b) immunoblot analysis of these reactions with chicken anti-serum raised against the recombinant protein. The positions of the N-terminal (Nx) and C-terminal (Cx) cleavage products are marked with arrows and lines, respectively.




**Supplementary Fig. 10.** The accumulation of IPSes is reversible after the relief from proteasome inhibition. Three day old LoVo cells were harvested immediately or after 24 hours of incubation in the absence or presence of 3 μM of reversible proteasome inhibitor MG132. Three flasks with MG132 treated cells were incubated further for 12, 24, or 36 hours after the media was replaced with fresh media containing no MG132. Cells were harvested and lysates analyzed by western blotting, using antibodies specific to the indicated IPSes as well as to ribosomal protein P0 (loading control). The position of intact proteins and cleavage products (asterisk) are indicated to the left of the panel.

**Supplementary Table 1.** Purification, identification, and analysis of Intrinsic Proteasome Substrates (IPSes)

| IPS # Mobility | monoQ mS/cm | Gel filtr. elution, ml | Blue mS/cm | Phenyl mS/cm | IPS ID Protein Name(s) | Accession # MW[g] | IPS complex[h]: IPS examination[i] | CP[k] |
|---|---|---|---|---|---|---|---|---|
| 1[a] 40 kDa | 23.2-26.7 | 14.5-16.5[e] | 60-185 | - | Ribosomal protein SA, RPSA; laminin receptor 1, LR1 | GI:9845502 32.9 kDa | RPSA-S21[j]: 20S & 26S tc, Blue R, IB, MS | + |
| 2[a] 55 kDa | 29.1-32.0 | 12.5-14[e] | 30-55 | 140 (FT) | Actin binding protein 1, ABP-1; SH3 domain-containing protein 7, SH3P7 | GI:51316115 48.2 kDa | homodimer[j]: 20S & 26S tc, Blue R, IB | + |
| 3[a] 47 kDa | 27.2-31.4 | 12.5-14[e] | 6.3 (FT) | 1.9-1.2 | Heterogeneous nuclear ribonucleoprotein F, hnRNP F | GI:1710628 45.7 kDa | homodimer: 20S & 26S tc, Blue R, IB | + |
| 4[a] 52 kDa | 26.1-27.4 | 13.5-15[e] | 6.3 (FT) | 35-5.2 | UV excision repair protein RAD23 homolog A, HR23A | GI:4826964 39.6 kDa | monomer[j]: 20S & 26S tc, Blue R, silver, IB | + |
| 6[d] 120 kDa | 20.7-24.5 | - | - | - | ATP-binding cassette 50, ABC50 | GI:69354671 95.8 kDa | eIF2-ABC50[j]: 20S tc, Blue R | + |
| 7[d] 170 kDa | 45.5-47.8 | - | - | - | Eukaryotic translation initiation factor 5B, eIF5B | GI:84043963 138.7 kDa | monomer[j]: 20S tc, Blue R | + |
| 8[b] 230 kDa | 23.8-27.6 | 7.5-8.5[f] | - | - | Myosin heavy chain 9, MyH9 | GI:6166599 226.4 kDa | homodimer[j]: 20S tc, Blue R, IB | + |
| 9[b] 230 kDa | 23.8-27.6 | 10.5-11.5[f] | - | - | Golgi-specific brefeldin A resistance factor 1, GBF1 | GI:13124260 206.3 kDa | unknown: 20S tc, Blue R, IB | + |
| 10[a] 44 kDa | 40.5-45.5 | 8-10.5[e] | 11.3 (FT) | - | p47 protein, cofactor for AAA ATPase p97/VCP/Cdc48 | GI:62898243 40.6 kDa | p97-p47[j]: 20S & 26S tc, Blue R, IB, EM | + |
| 11[a] 44 kDa | 40.5-45.5 | 8-10.5[e] | 31-65 | - | | | unknown: 20S & 26S tc, Blue R, IB, EM | + |
| 13[a] 160 kDa | 43-44.7 | 11-12.5[e] | - | - | Transcription elongation factor SPT5 (suppressor of Ty 5 homolog) | GI:74735318 121 kDa | unknown: 20S & 26S tc, Blue R, IB | + |
| 14[a] 65 kDa | 40.5-45.5 | 13-14[e] | - | - | Ran GTPase activating protein 1, RanGAP1 | GI:4506411 63.4 kDa | homodimer: 20S tc, Blue R | |
| 16[a] 65 kDa | 40.5-45.5 | 8-11[e] | 31-65 | - | Anaphase promoting complex subunit 8 (APC8); cell division cycle 23 (Cdc23) | GI:118402596 68.7 kDa | APC: 20S & 26S tc, Blue R, IB, EM | + |
| 17[a] 72 kDa | 40.5-45.5 | 8-11[e] | 31-65 | - | Anaphase promoting complex subunit 6, APC6; cell division cycle 16, Cdc16 | GI:37537763 71.7 kDa | APC: 20S & 26S tc, Blue R, IB, EM | + |
| 18[a] 47 kDa | 26.7-30.1 | 15-16[e] | - | - | Cysteine protease Atg4B (Autophagy-related protein 4 homolog B) | GI:61212253 44.2 kDa | monomer: 20S tc, Blue R | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 20[b] 145 kDa | 39.6-41.8 | 9.5-11.5[f] | - | - | Valyl-tRNA synthetase, VRS | GI:12644177 140.3 kDa | VRS-eEF1[j]: 20S tc, Blue R, MS, EM | + |
| 21[b] 33 kDa | 39.6-41.8 | 9.5-11.5[f] | - | - | Eukaryotic translation elongation factor 1B-beta, eEF1Bβ | GI:25453472 31 kDa | tRS-eEF1[j]: 20S & 26S tc, Blue R, IB, MS, EM | + |
| 24[b] 95 kDa | 39.6-41.8 | 9.5-11.5[f] | - | - | Cysteinyl-tRNA synthetase, CRS | GI:62240992 94.5 kDa | CRS/eEF1[j]: 20S tc, Blue R, MS, EM | |
| 25[a] 145 kDa | 21.6-23.6 | 11-12[e] | - | - | Ubiquitin conjugation factor E4B, UBE4B; Ubiquitin fusion degradation protein 2, Ufd2 | GI:24638295 146.2 kDa | unknown: 20S & 26S tc, Blue R, IB | + |
| 26[a] 51 kDa | 21.6-23.6 | 11-12[e] | - | - | NudC domain containing 3, NudCd3 | GI:122939165 40.7 kDa | unknown: 20S tc, Blue R | |
| 27[a] 95 kDa | 21.6-23.6 | 10-11.5[e] | - | - | Adaptor protein complex AP-1 gamma-1 subunit, AP1γ1 | GI:71773010 91.6 kDa | AP-1 complex: 20S tc, Blue R | |
| 28[a] 90 kDa | 21.6-23.6 | 11.5-13.5[e] | - | - | Signal transducer and activator of transcription 5A, STAT5A | GI:21618342 90.5 kDa | dimer: 20S & 26S tc, Blue R, IB | + |
| | | | | | Signal transducer and activator of transcription 5B, STAT5B | GI:21618344 89.7 kDa | dimer: 20S & 26S tc, Blue R, IB | + |
| 29[a] 85 kDa | 23.6-25.8 | 12.5-13[e] | - | - | Eukaryotic peptide chain release factor 3a, eRF3a; G1 to S phase transition 1, GSPT1 | GI:33874734 68.4 kDa | unknown: 20S & 26S tc, Blue R, IB | + |
| | | | | | Eukaryotic peptide chain release factor 3b, eRF3b; G1 to S phase transition 2, GSPT2 | GI:46094014 68.8 kDa | unknown: 20S & 26S tc, Blue R, silver, IB | + |
| 38[a] 225 kDa | 26.7-30.1 | 10-11.5[e] | - | - | GTPase activating protein and VPS9 domains 1, GAPVD1; Rab5-activating protein 6, RAP6 | GI:51093832 166 kDa | unknown: - | |
| 47[b] 225 kDa | 23.8-27.6 | 11.5-13.5[f] | - | - | | | unknown: 20S tc, Blue R, CP ID | + |
| 39[a] 57 kDa | 21.6-23.6 | 11.5-12.5[e] | - | - | Target of Myb-like protein 1, TOM1L1 | GI:25091352 52.9 kDa | unknown: 20S tc, Blue R | |
| 40[a] 155 kDa | 25.5-28.2 | 11.5-12.5[e] | 62-101 | 40.5-17.1 | Kip1 ubiquitylation-promoting complex subunit 1, KPC1; RING finger 123, RNF123 | GI:37588869 148.4 kDa | KPC1-KPC2[j]: 20S & 26S tc, Blue R, silver, IB | |
| 48[a] 48 kDa | 26.8-29.2 | 11.5-12.5[e] | 6.3 (FT) | - | Hsp70-interacting protein, Hip | GI:19923193 41.2 kDa | homotetramer[j]: 20S tc, Blue R | + |
| 49[a] 80 kDa | 27.7-30.9 | 11.5-12.5[e] | 101-152 | 17.1-1.9 | Heme-regulated inhibitor of translation, HRI; eukaryotic translation initiation factor 2-alpha kinase 1, eIF2αK1 | GI:51094455 71 kDa | homodimer[j]: 20S tc, Blue R, silver, IB | + |
| 50[c] 46 kDa | 31.1-35.0 | 10.5-12[e] | 32.5-70 | - | Cell division cycle 37 protein,Cdc37 | GI:5901922 44.3 kDa | Hsp90-cdc37[j]: 20S tc, Blue R, MS | |

[a] IPSes were purified from post-ribosomal fraction (PRF), 0-30% ammonium sulfate (A.S.) precipitation cut-off, 100-250 mM KCl elution fraction from DEAE cellulose, 100 mM KCl flowthrough (FT) fraction from P11 phosphocellulose.

[b] IPSes were purified from PRF, 0-30% A.S. precipitation cut-off, 100-250 mM KCl elution fraction from DEAE cellulose, 100-250 mM KCl elution fraction from P11 phosphocellulose.

[c] IPSes were purified from PRF, 30-45% A.S. precipitation cut-off, 100-250 mM KCl elution fraction from DEAE cellulose, 100 mM KCl FT fraction from P11 phosphocellulose.

[d] IPSes were purified from ribosomal salt wash (RSW), 40-50% A.S. precipitation cut-off, 100-250 mM KCl elution fraction from DEAE cellulose, 400-800 mM KCl elution fraction from P11 phosphocellulose.

[e] Proteins were separated on Superdex 200 10/30 GL column.

[f] Proteins were separated on Superose 6 10/30 GL column.

[g] Accession numbers and MW given are for human IPS homologues.

[h] Composition of complexes is based upon gel-filtration elution profiles, co-purification of its components on all columns, detection of protein species with mobility corresponding to components of well-characterized complexes, EM analysis of known complexes, direct identification of complex components by LC-MS/MS and/or immunoblot analysis using respective antibodies.

[i] tc, the time-course of IPS cleavage by 20S or 26S proteasomes; Blue R (silver), staining of probes with Blue R dye (silver); IB, immunoblot analysis using antibodies specific to IPS and components of IPS-containing complexes; EM, electron microscopy of protein complexes; MS, LC-MS/MS identification of component(s) of IPS-containing complex; CP ID, LC-MS/MS identification of IPS's cleavage product(s).

[j] IPS or IPS-containing complex was highly purified.

[k] Distinct IPS cleavage products were observed (+) by staining of tc probes with dye and/or immunoblot analysis using specific antibodies.