

Supplementary Information for:

**Cryo-EM of mammalian PA28 $\alpha\beta$ -iCP
immunoproteasome reveals a distinct mechanism of proteasome
activation by PA28 $\alpha\beta$**

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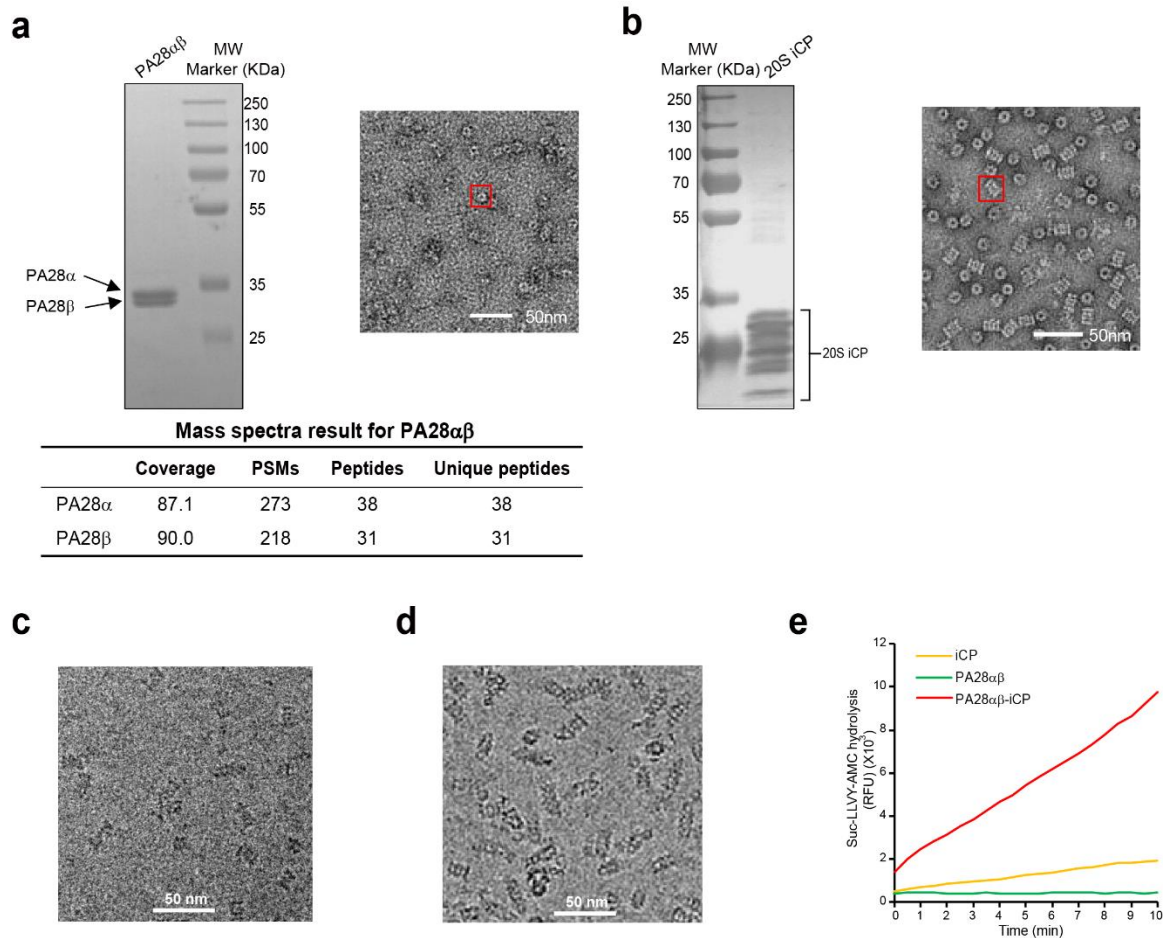
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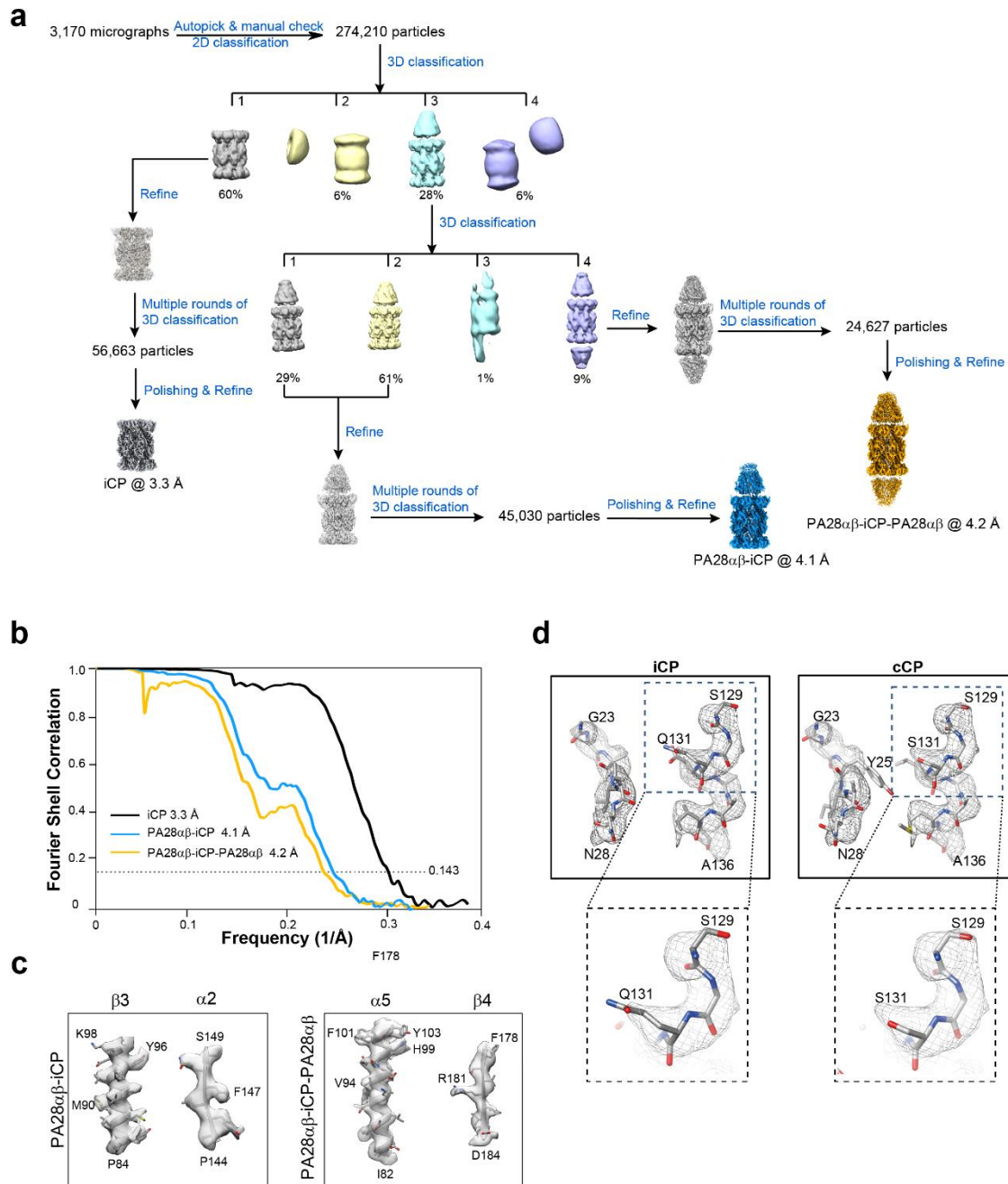
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This Supplementary Information PDF includes:
Supplementary Figure 1 – Supplementary Figure 8
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Supplementary Figure 1. Purification and characterization of human PA28αβ and bovine spleen iCP. (a) Characterizations of human PA28αβ using SDS-PAGE, negative-staining electron microscopy (NS-EM), and mass spectrometry analyses. PSMs stands for peptide spectrum matches, which were used to indicate the relative abundance of a certain protein. Unique peptides were used for protein identification. **(b)** Characterizations of bovine spleen iCP using SDS-PAGE and NS-EM. **(c-d)** A representative cryo-EM image (low pass filtered for better visualization) of the *in vitro* reconstituted PA28αβ-iCP proteasome from purified PA28αβ and iCP in the absence of cross-linker **(c)** and presence of cross-linker **(d)**. **(e)** Proteolytic activity assay of the reconstituted PA28αβ-iCP complex against the fluorogenic peptide Suc-LLVY-AMC. RFU, relative fluorescence units. The experiment was repeated three times with the same outcome.



Supplementary Figure 2. Structural analysis of the PA28 $\alpha\beta$ -iCP proteasome. (a)

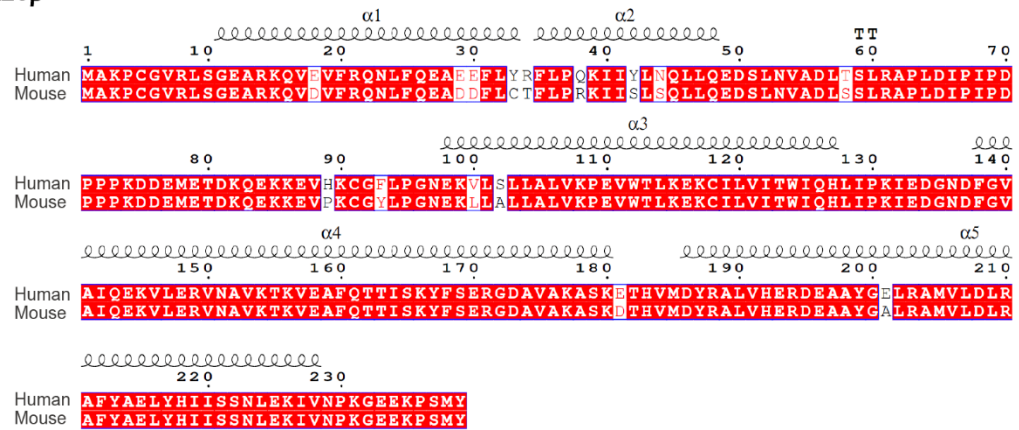
Work-flow of the PA28 $\alpha\beta$ -iCP proteasome data processing. More details are available in **Methods**. **(b)** Resolution assessment of our cryo-EM maps using Fourier shell correlation (FSC) at the 0.143 criterion. **(c)** Model-map fitting for PA28 $\alpha\beta$ -iCP and PA28 $\alpha\beta$ -iCP-PA28 $\alpha\beta$ complexes in the iCP portion. **(d)** The zoomed in views in the second row shows that for the same CP density, when cCP $\beta 2$ S131 is fitted in, there appears to have extra

density (right panel); while this extra density can be partially filled up by iCP Q131 (left panel), implying the partial contribution of the S131 from cCP in this site in addition to that of Q131 from iCP. Still, Q131 of iCP is the better fit to this density possibly due to the dominate population of iCP particles. Also, in the first row, the Y25 in cCP (right panel) is completely out of the density.

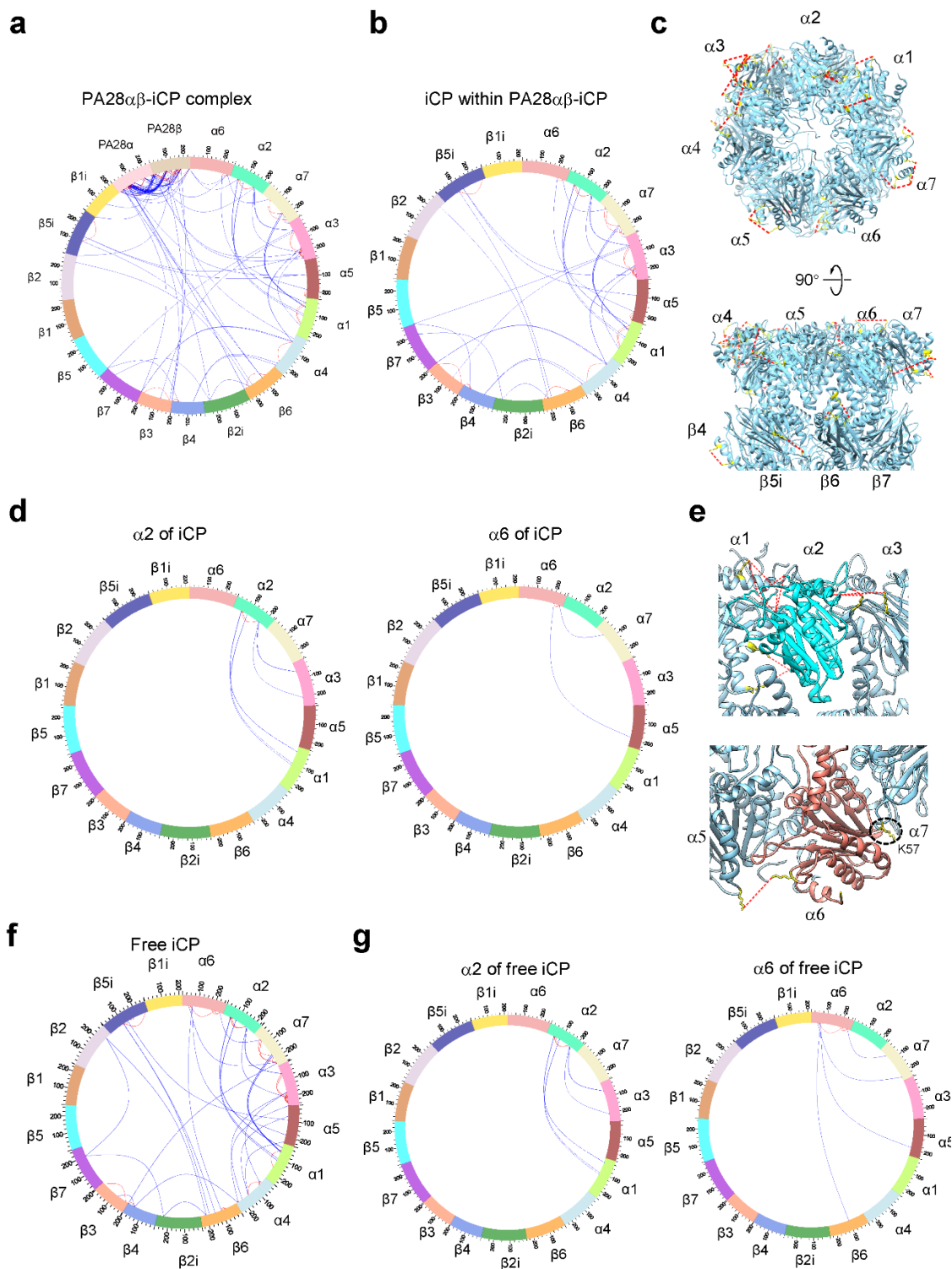
PA28 α



PA28 β

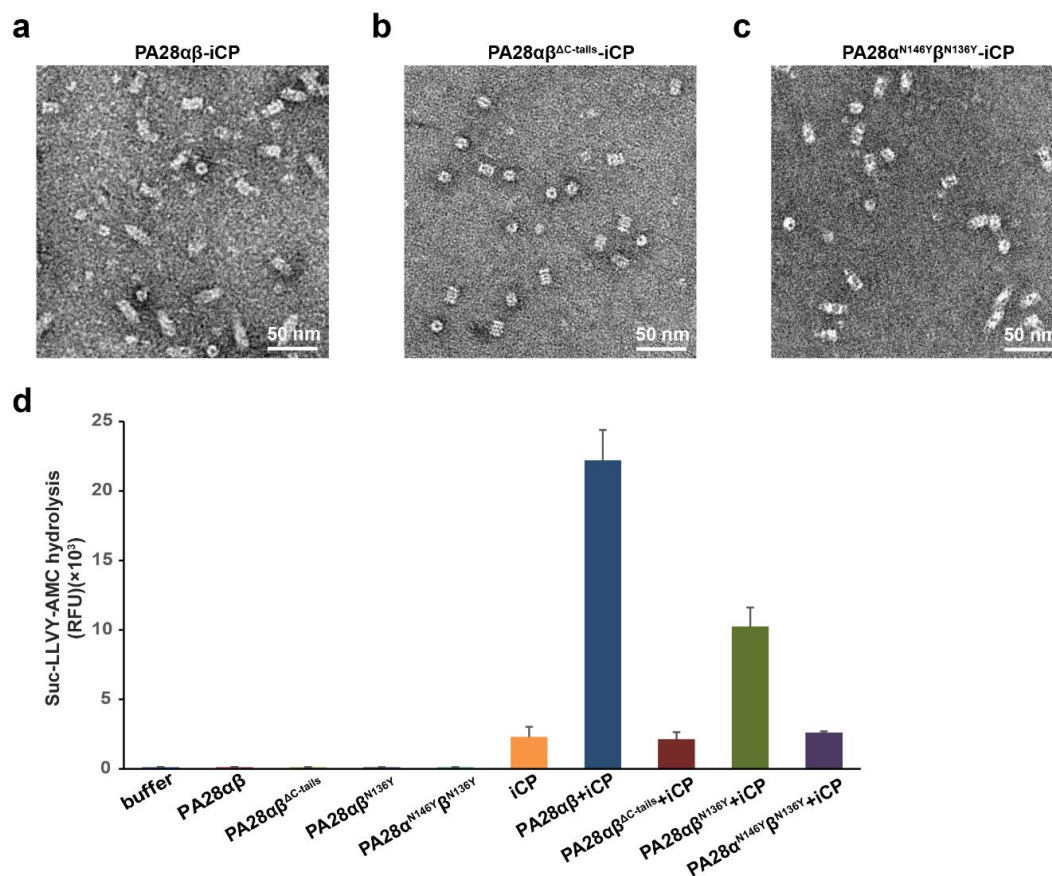


Supplementary Figure 3. High sequence identity between human and mouse PA28 $\alpha\beta$. Sequence alignments of human and mouse PA28 α (top) and of human and mouse PA28 β (bottom), generated by Espright. Showing sequence identities of 94.78% for PA28 α between human and mouse, and 93.72% for PA28 β between human and mouse.

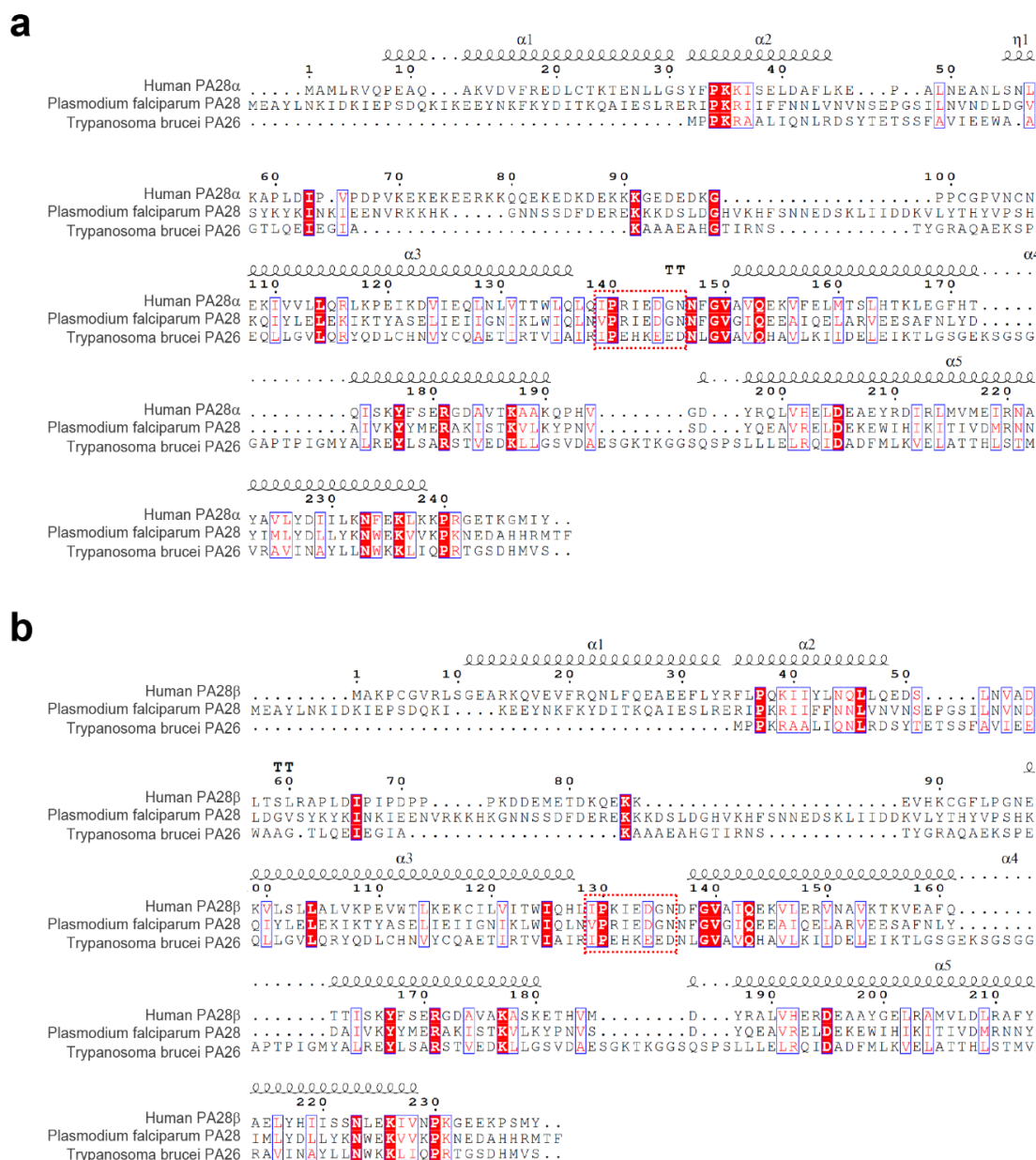


Supplementary Figure 4. Results of XL-MS analysis of PA28 $\alpha\beta$ -iCP proteasome complex shown by circular plots. (a) Circular plot of all cross-links of PA28 $\alpha\beta$ -iCP complex detected by XL-MS analysis, with intra-subunit XLs shown in red, and inter-

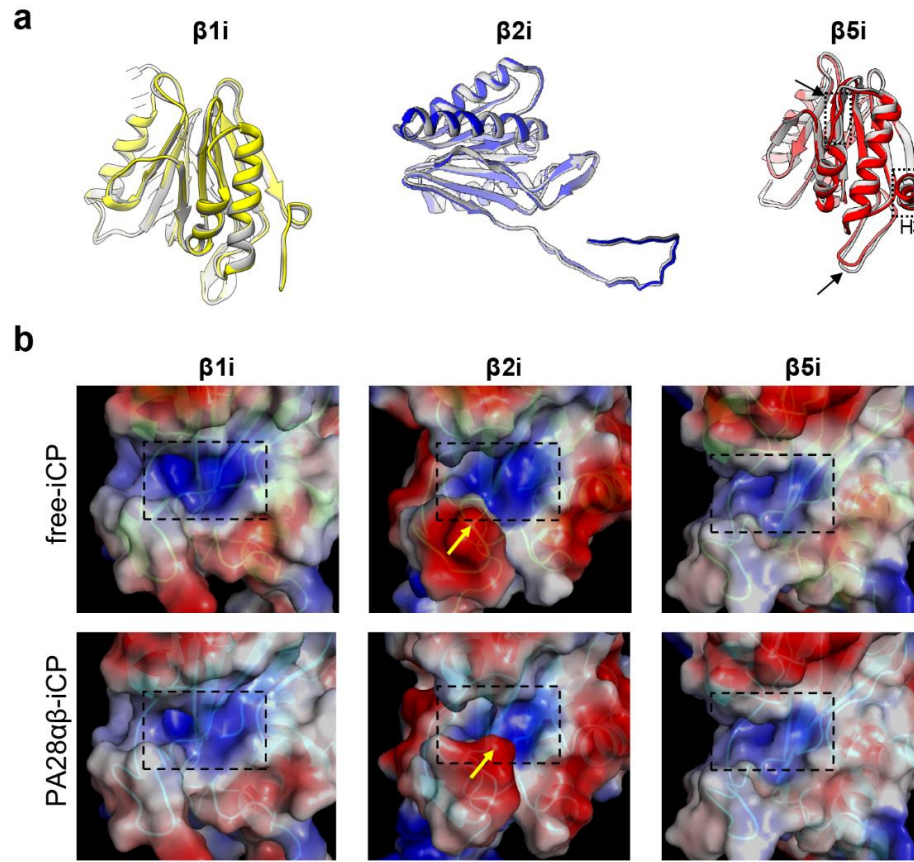
subunit XLs in blue. We only show the spectra data with E-value below 1.00E-02 in the circular plot, which is followed throughout. **(b)** XLs of the iCP portion from the XL-MS results of the PA28 $\alpha\beta$ -iCP complex. **(c)** Plot with detected intra subunit cross-links mapped on the core structure. **(d)** We take $\alpha 2$ and $\alpha 6$ core subunits from PA28 $\alpha\beta$ -iCP complex as examples to illustrate the detected XLs between the subunits of the core proteasome. **(e)** Mapping of those detected intra- and inter-subunit XLs for $\alpha 2$ and $\alpha 6$ subunits on the core particle structure. Due to the dynamic nature of the C-terminus, the $\alpha 6$ C-terminal residue K243 was not resolved in our structure, thus we could not display the XL of $\alpha 6$ (K243)- $\alpha 7$ (K57) in the model; still the $\alpha 7$ (K57) (indicated by dotted black ellipsoid) is fairly close to $\alpha 6$ subunit. **(f)** Circular plot of detected XLs from the free iCP by XL-MS analysis. **(g)** Detected XLs for $\alpha 2$ (left) and $\alpha 6$ (right) from the free iCP XL-MS data, which overall match the XLs detected in the PA28 $\alpha\beta$ -iCP complex with slightly increased number of detected XLs that might be related to the increased crosslinker concentration. This further validate the reliability of our XL-MS results.



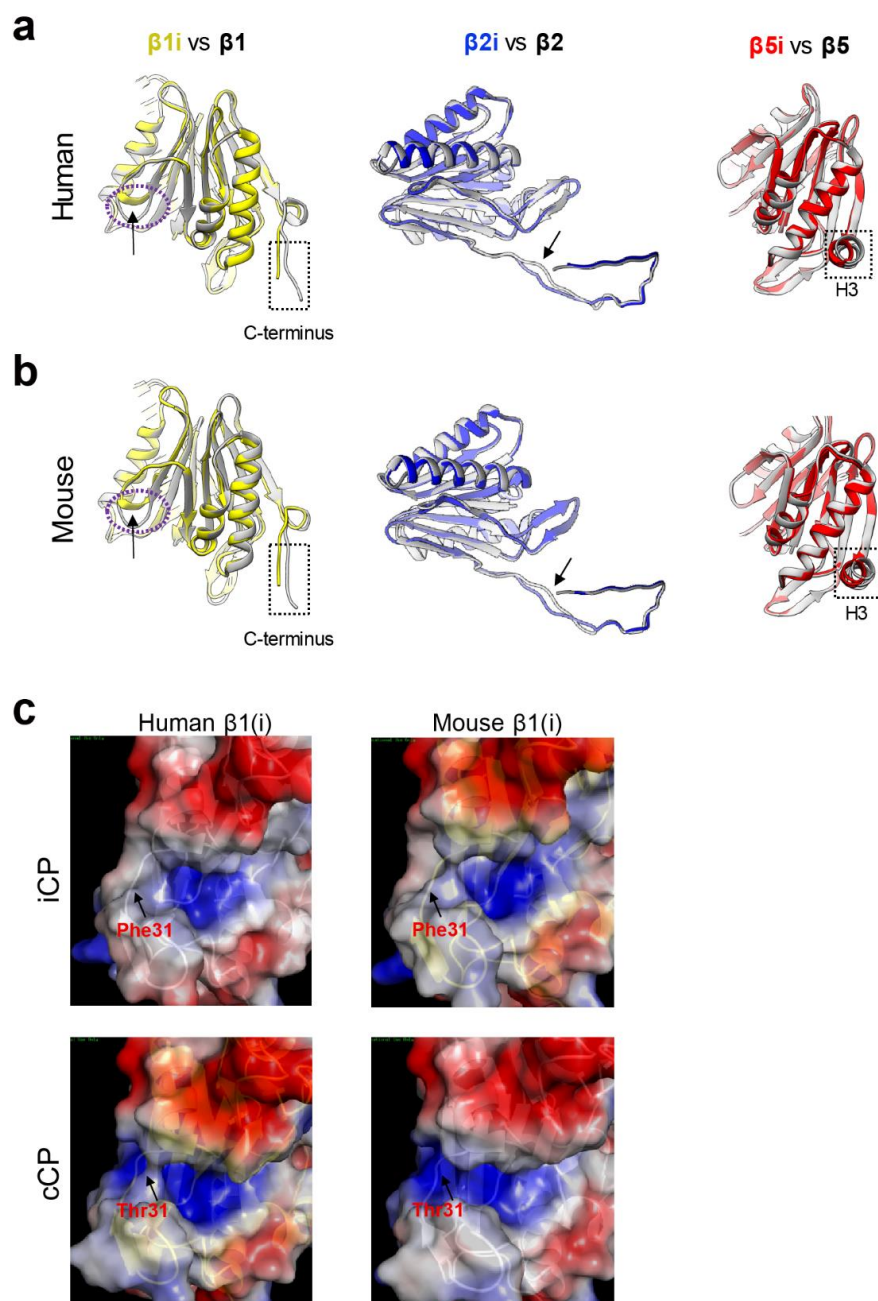
Supplementary Figure 5. Binding assay (through NS-EM analysis) and proteolytic activity assay of wild type PA28αβ or mutated PA28αβ in complex with iCP. (a) Representative NS-EM image of iCP incubated with PA28αβ. **(b)** Similar analyses as in **(a)** but for iCP incubated with the C-terminal truncated PA28αβ^{ΔC-tails} (truncated K245-Y249 for PA28α and E234-Y239 for PA28β). **(c)** Similar analyses as in **(a)** but for iCP incubated with the activation loop mutated PA28α^{N146Y}β^{N136Y}. **(d)** Proteolytic activity assay of the iCP with PA28αβ, PA28αβ^{ΔC-tails}, PA28α^{N146Y}β^{N136Y}, and PA28αβ^{N136Y} against the fluorogenic peptide Suc-LLVY-AMC. RFU, relative fluorescence units. The experiment was repeated three times with the same outcome.



Supplementary Figure 6. Sequence alignments of human, *Plasmodium falciparum* and *Trypanosoma brucei* 11S activator protein chains. (a) Esprict representation of sequence alignment of human PA28α, *Pf*PA28 and *Tb*PA26, showing sequence identities of 26.76% between human PA28α and *Pf*PA28, and 15.64% between human PA28α and *Tb*PA26. Here the position of the activation loop was indicated by dotted red frames. This style was followed. **(b)** Esprict representation of sequence alignment of human PA28β, *Pf*PA28 and *Tb*PA26, showing sequence identities of 24.38% between human PA28β and *Pf*PA28, and 12.46% between human PA28β and *Tb*PA26.



Supplementary Figure 7. Variations of conformation and electrostatic surface property of the three catalytic subunits of bovine iCP induced by PA28 $\alpha\beta$ binding. (a) Structural superpositions of the three catalytic subunits of our PA28 $\alpha\beta$ -bound iCP (with $\beta 1i$, $\beta 2i$, and $\beta 5i$ in color) on the corresponding subunits of our bovine free iCP (in gray). The observable conformational changes are indicated by black arrow and dotted frames. The rendering style is followed throughout. **(b)** Comparison of electrostatic surface property of $\beta 1i$, $\beta 2i$, and $\beta 5i$ between free bovine iCP (top row) and PA28 $\alpha\beta$ -iCP (bottom row). The proteolytic site location is indicated by black dashed line. In $\beta 2i$, the surrounding negatively charged loop (indicated by yellow arrow) shows inward movement toward the proteolytic site after binding the PA28 $\alpha\beta$.



Supplementary Figure 8. Structural comparison and electrostatic surface property comparison of the catalytic subunits between iCP and cCP. (a-b) Superpositions of the structures of **(a)** human iCP (PDB:6AVO) versus human cCP (PDB: 4R3O), and **(b)** mouse iCP (PDB: 3UNH) versus mouse cCP (PDB: 3UNE). **(c)** Electrostatic surface property representations of human $\beta 1i$ versus human $\beta 1$, and mouse $\beta 1i$ versus mouse $\beta 1$. With the most distinct residues between $\beta 1i$ and $\beta 1$ in this region indicated.

Supplementary Table 1. Mass spectrometry analysis of the full composition of the free 20S proteasome from bovine spleen.

Bovine spleen 20S	Coverage	PSMs	Peptides	Unique peptides
PSMB9 / β 1i	76	377	12	12
PSMB10 / β 2i	42	252	8	8
PSMB8 / β 5i	60	215	14	14
PSMB6 / β 1	70	72	11	11
PSMB7 / β 2	64	74	15	15
PSMB5 / β 5	68	74	17	17
PSMB3 / β 3	53	242	14	14
PSMB2 / β 4	94	390	24	23
PSMB1 / β 6	73	304	19	19
PSMB4 / β 7	50	305	9	9
PSMA6 / α 1	74	497	20	20
PSMA2 / α 2	68	318	15	15
PSMA4 / α 3	77	419	18	18
PSMA7 / α 4	70	375	21	21
PSMA5 / α 5	66	355	15	15
PSMA1 / α 6	90	324	25	25
PSMA3 / α 7	44	181	14	14

Supplementary Table 2. Statistics of cryo-EM data collection, processing, and model validation.

	iCP (EMDB-30825) (PDB 7DR7)	PA28 $\alpha\beta$ -iCP (EMDB-30824) (PDB 7DR6)	PA28 $\alpha\beta$ -iCP-PA28 $\alpha\beta$ (EMDB-30828) (PDB 7DRW)
Data collection and processing			
Magnification	18,000x	18,000x	18,000x
Voltage (kV)	300	300	300
Electron exposure (e ⁻ /Å ²)	38	38	38
Defocus range (μm)	-1.5 ~ -2.8	-1.5 ~ -2.8	-1.5 ~ -2.8
Pixel size (Å)	1.32	1.32	1.32
Symmetry imposed	C2	C1	C1
Initial particle images (no.)	274,210	274,210	274,210
Final particle images (no.)	56,663	45,030	24,627
Map resolution (Å)	3.3	4.1	4.2
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	2.9-4.4	3.5-6.5	3.5-6.5
Refinement			
Initial model used (PDB code)	1IRU	1IRU, 5MX5	1IRU, 5MX5
Model resolution (Å)	3.3	4.1	4.3
FSC threshold	0.143	0.143	0.143
Model resolution range (Å)	3.0-3.6	4.0-6.0	4.0-7.4
Map sharpening <i>B</i> factor (Å ²)	-65	-96	-82
Model composition			
Non-hydrogen atoms	48,321	59,979	71,673
Protein residues	6,220	8,169	9,108
Ligands			
<i>B</i> factors (Å ²)			
Protein	48.73	97.65	47.87
Ligand			
R.m.s. deviations			
Bond lengths (Å)	0.0045	0.0038	0.0035
Bond angles (°)	0.95	0.89	0.92
Validation			
MolProbity score	1.78	1.86	1.61
Clashscore	9.68	9.63	7.91
Poor rotamers (%)	0.35	0.05	0.00
Ramachandran plot			
Favored (%)	96.07	94.91	96.92
Allowed (%)	3.78	4.94	2.97
Disallowed (%)	0.15	0.15	0.11

Supplementary Table 3. Results of XL-MS analysis of PA28 $\alpha\beta$ -iCP proteasome complex*

Protein1(site)-Protein2(site) Inter-Proteins	Peptides	E-value	Spec count
α 3(210)-PA28 α (245)	LSAEKVEIATLTR(5)- GETKGMIIY(4)	1.40E-05	13
α 6(243)-PA28 β (235)	KAQPTQPADEPAEK(1)- GEEKPSMYL(4)	2.39E-05	2
α 2(171)-PA28 α (245)	NYVNGKTFLEK(6)-GETKGMIIY(4)	6.95E-05	15
α 2(53)-PA28 β (235)	QKSILYDER(2)-GEEKPSMYL(4)	2.18E-04	2
α 6(243)-PA28 β (15)	KAQPTQPADEPAEK(1)- KQVEVFR(1)	1.93E-08	1
α 7(57)- α 6(243)	LVLSKLYEEGSNKR(5)- KAQPTQPADEPAEK(1)	7.08E-11	5
α 1(30)- α 2(53)	LYQVEYAFKAINQGGLTSVAVR(9)- QKSILYDER(2)	5.49E-17	75
α 2(92)- α 1(104)	KLAQQYYLVYQEPIPTAQLVQR(1) -YKYGYEIPVDMCLK(2)	1.49E-31	42
α 3(176)- α 4(52)	ATCIGNNSAAVSMKQDYK(16)- SVAKLQDER(4)	4.28E-22	21
β 5i(133)- α 5(91)	LLAKECR(4)-TLIDKAR(5)	7.96E-31	45
β 6(204)- β 2i(233)	LVKDVFISAAER(3)- ALSSPTKPIER(7)	2.67E-20	63
β 7(201)- β 2i(233)	EVLEKQPVLSQTEAR(5)- ALSSPTKPIER(7)	5.00E-07	11
β 6(204)- β 2(237)	LVKDVFISAAER(3)- LDFLRPYSVPNKK(12)	8.48E-11	8
PA28 β (180)-PA28 α (190)	ASKETHVMDYR(3)- AAKQPHVGDYR(3)	2.64E-21	168
PA28 β (180)-PA28 α (187)	ASKETHVMDYR(3)- GDAVTKAAK(6)	3.48E-11	109
PA28 α (190)-PA28 β (177)	AAKQPHVGDYR(3)- GDAVAKASK(6)	1.03E-13	75
...	...	...	...

* We used E-value (1.00E-02) as the threshold to remove extra XL-MS data with lower confidence. For the detected cross-links within iCP or PA28 $\alpha\beta$ units, due to limited space of the table, here we listed representative results.

Supplementary Table 4. Occupancy status of the 20S pockets of PA28 $\alpha\beta$, *Pf*PA28 and *Tb*PA26.

20S pockets	PA28 $\alpha\beta$	<i>Pf</i> PA28	<i>Tb</i> PA26
$\alpha 1/ \alpha 2$	++	-	-
$\alpha 2/ \alpha 3$	++	-	++
$\alpha 3/ \alpha 4$	++	-	++
$\alpha 4/ \alpha 5$	++	-	++
$\alpha 5/ \alpha 6$	+	-	++
$\alpha 6/ \alpha 7$	-	-	-
$\alpha 7/ \alpha 1$	-	+	-

++: strong density, +: weak density; -: no density

Supplementary Table 5. Stabilization status of activation loops of PA28 $\alpha\beta$, *Pf*PA28, and *Tb*PA26.

20S subunits	PA28 $\alpha\beta$	<i>Pf</i> PA28	<i>Tb</i> PA26
α 1	++	+	++
α 2	+	-	++
α 3	++	-	++
α 4	++	-	++
α 5	+	+	++
α 6	++	++	++
α 7	+	++	++

++: well resolved, +: less well resolved; -: not resolved.