

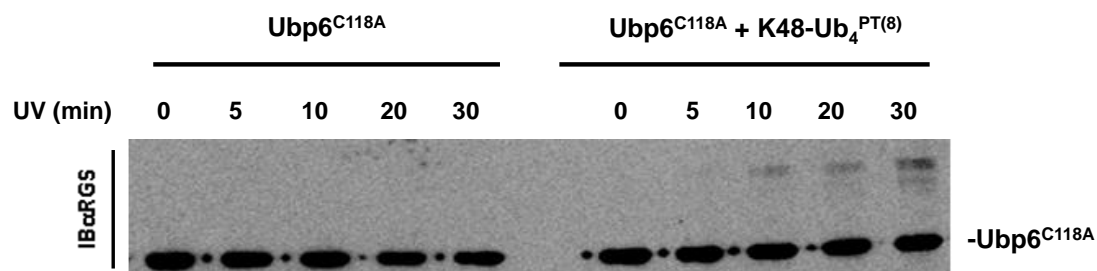
Supplemental Information

Polyubiquitin-Photoactivatable Crosslinking

Reagents for Mapping Ubiquitin Interactome Identify

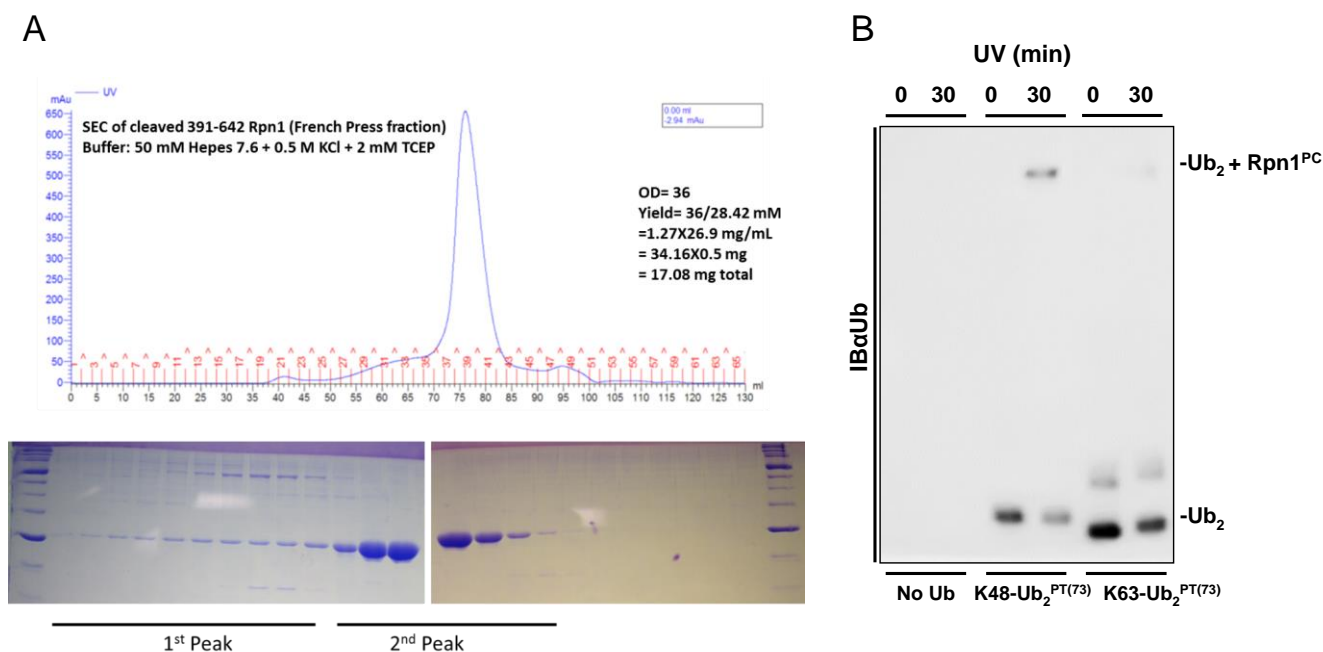
Rpn1 as a Proteasome Ubiquitin-Associating Subunit

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Supplemental Figure S1. Ubp6^{C118A} binding to polyUb^{PT}, related to Figure 3.

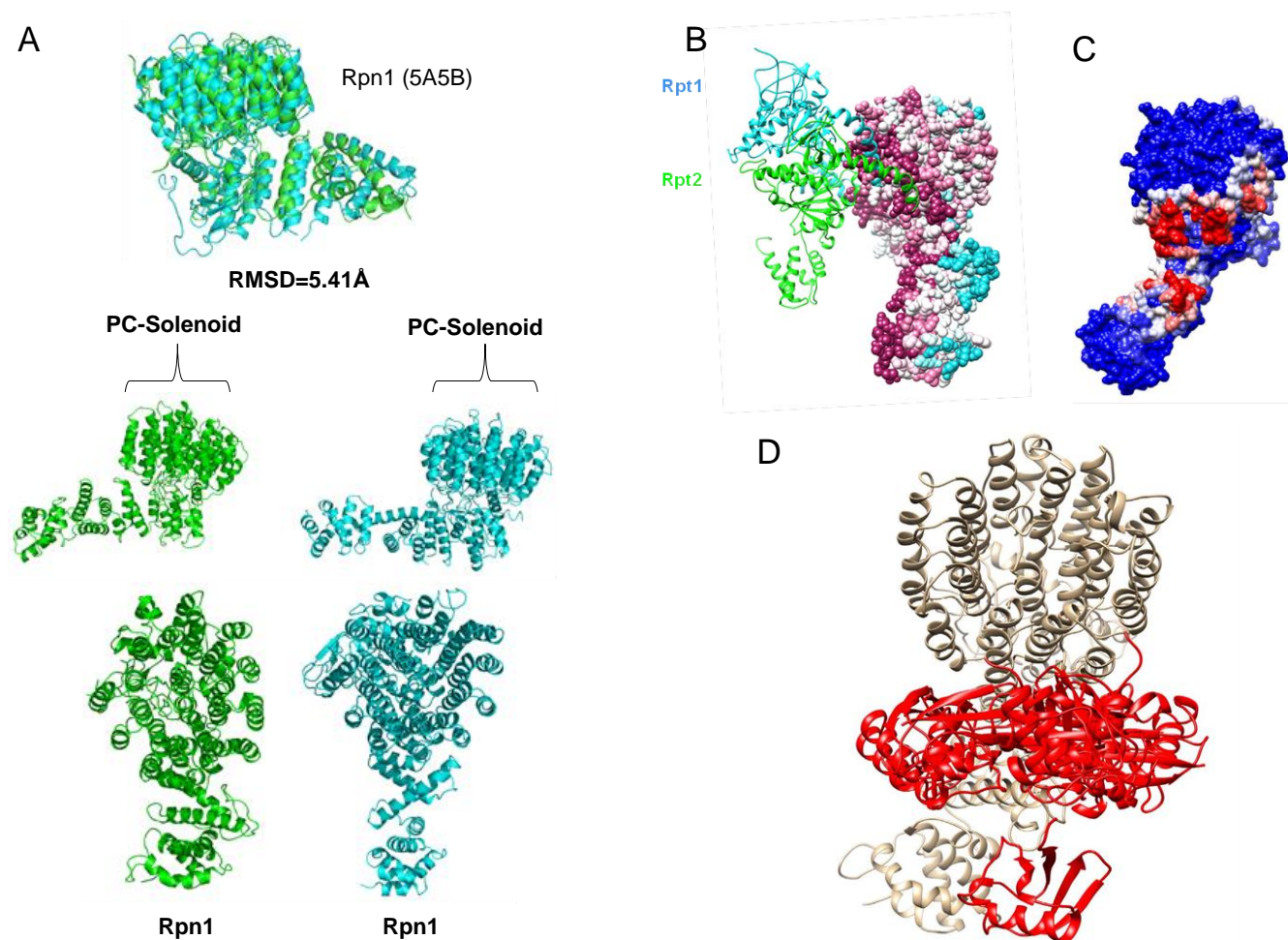
K48-linked polyUb^{PT} crosslinked to purified recombinant Ubp6^{C118A} under similar conditions to those described in Figure 1B.



Supplemental Figure S2. Rpn1 fragments associate with diUb. Related to Figure 4.

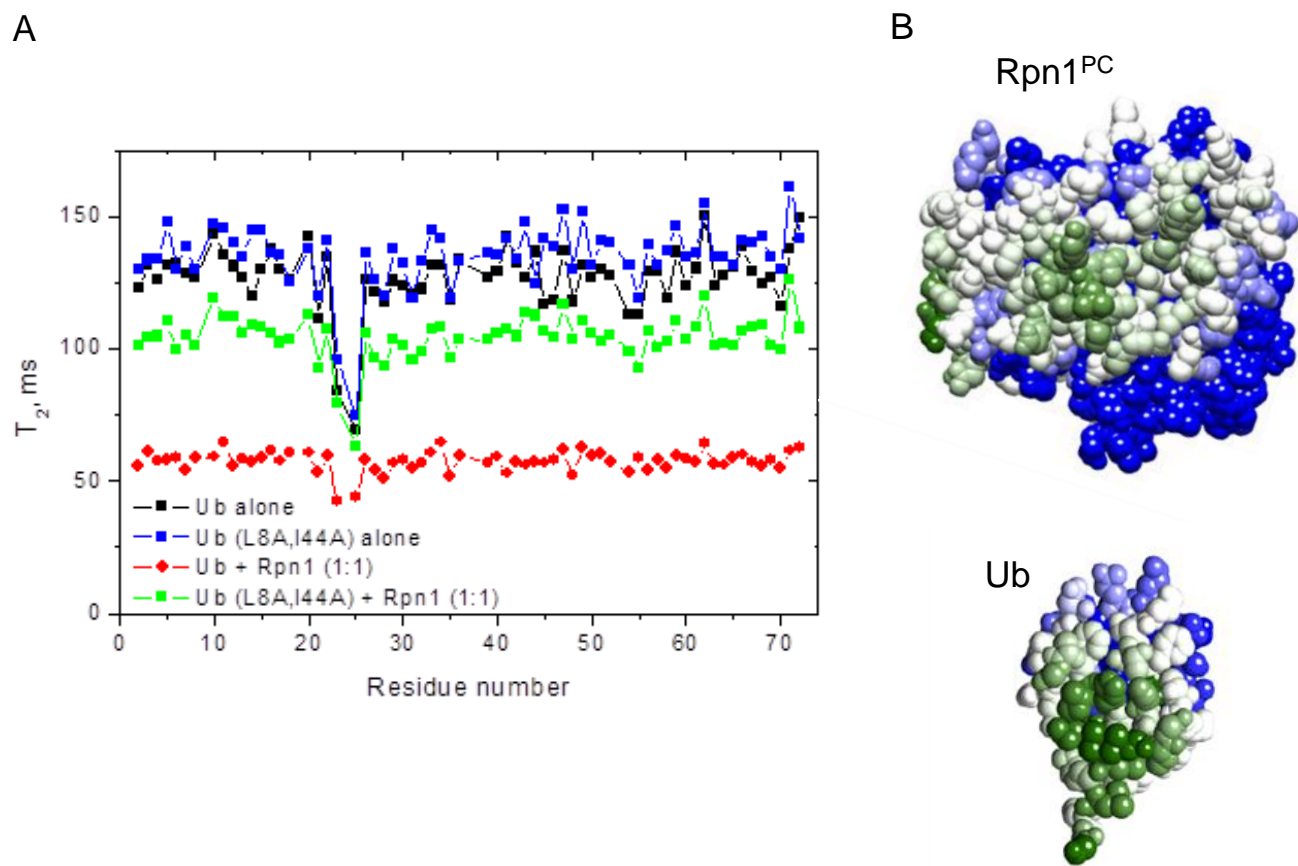
A. Truncated Rpn1 fragments, Rpn1⁴¹⁶⁻⁴⁸⁷, Rpn1⁹⁰⁵⁻⁹³³, Rpn1³⁹¹⁻⁶⁴², and Rpn1³⁵⁶⁻⁹⁰⁵ were created by applying the appropriate primer pair to the full length Rpn1 for ligation into the smt3-pET28b vector transformed and expressed in BL-21 (DE3) Rosetta II cells. 2 L cultures were grown to OD₆₀₀~0.6 at 37°C, induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and expression was carried out for 18 hrs. at 16°C. Cell lysate was fractionated first by His-Trap. Following elution, fractions containing Rpn1 fragment were pooled and separated using a Superdex 200 16/60 in PBS pH 7.4 buffer. Rpn1 eluted as sharp peak consistent with uniform behavior of a soluble monomer (top panel). Purity was confirmed with SDS-PAGE (bottom panel). Moreover, Rpn1³⁹¹⁻⁶⁴² migrates at more or less similar position where diUb dimer runs, supporting the conclusion that Rpn1³⁹¹⁻⁶⁴² is soluble as well as monomeric.

B. Dimeric Ub^{PT} cross-linking with Rpn1³⁵⁶⁻⁹⁰⁵ fragment. K48-linked or K63-linked diUb^{PT} was crosslinked to Rpn1³⁵⁶⁻⁹⁰⁵ fragment according to protocol described in Figure 1B.



Supplemental Figure S3. Potential protein-protein interaction sites on Rpn1 surface. Related to Figure 4.

A. Alignment of Rpn1 and Rpn2. The structural information from Rpn2 (PDB-4ADY) was effective in locating electron density assigned to Rpn1 in cryoEM-derived proteasome models (5A5B). Rpn1 (green) and Rpn2 (cyan, PDB-4ADY), RMSD=8.60Å. **B. Residue conservation on Rpn1 surface based on current 3D structural Model of Rpn1 in 26S proteasomes.** Evolutionary conservation of amino acid positions on Rpn1 surface based on the phylogenetic relations between homologous sequences positioned on putative 3D conformation of Rpn1 from yeast as it appears in EM models of the 26S proteasome (using the ConSurf program and PDB:5A5B). Most highly conserved residues are painted dark red; least conserved positions are in light blue. The position of two nearest neighbors in the 19S, Rpt1 and Rpt2, are shown in light blue and green respectively according to the EM model. Most evolutionary conserved residues on the surface of Rpn1 contacted neighboring subunits Rpt1 and Rpt2. However, patches of conservation were identified on predicted solvent-exposed surfaces of the PC domain (e.g. residues painted dark red facing forward). **C. Protein interaction site on Rpn1 (NIPS).** A docking experiment with PyDock WEB server (Jimenez-Garcia et al., 2013) revealed a surface on Rpn1 with low-energy docking poses for complexes with monoUb. Normalized interface propensity (NIP) values derived from rigid body docking with electrostatics and desolvation scoring for the prediction of interaction hotspots. Rigid-body docking solutions generated by the simulations were subsequently used to project the docking energy landscapes onto full-length Rpn1 without any restraints. Most of the predicted hot-spot residues are above NIP values of 0.3. Even though possible interactions were not restricted to any region of Rpn1, the energetically preferred Ub docking poses mapped to the exposed region of the PC domain and to the hinge between the PC domain and the N-terminal extension of Rpn1 (Red). **D. Molecular docking analysis of ubiquitin docking onto Rpn1 structure.** Best 10 docking pyDockWEB results of Ub (PDB 1UBQ for ubiquitin ribbon, colored red) to Rpn1 (PDB 4CR2 for the Rpn1 structure, colored tan) suggested a specific association site on PC region covering residues ~350-640. Center of mass (spheres) of 100 best Ub-Rpn1 pyDockWEB results. Ten lowest energy Ub results are colored red showing location of center of mass and relative orientation to Rpn1 ribbon in tan. When performing docking of Ub on the PC domain structure, the 100 most preferred docking results distribute along the exposed edge of the PC ring particularly along the segment aa ~350-640 that encompasses the highly conserved hydrophobic residues (Figure 4G). Of these, the orientation of Ub docked at the ten most preferred sites fall into two clusters wedged between the circular PC repeat region and the extended N-terminus, in a solvent-exposed area of Rpn1.

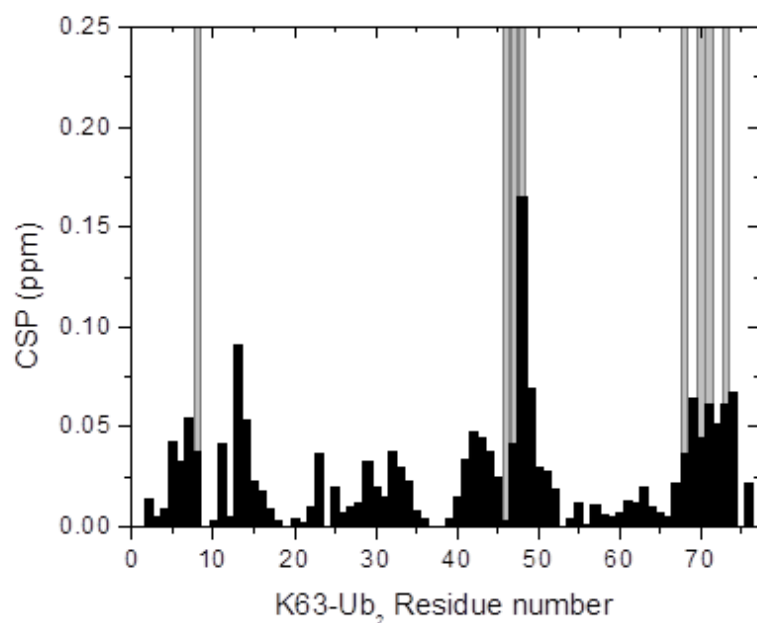


Supplemental Figure S4 - Rpn1 binds Ub via hydrophobic patch of Ub. Related to Figure 5.

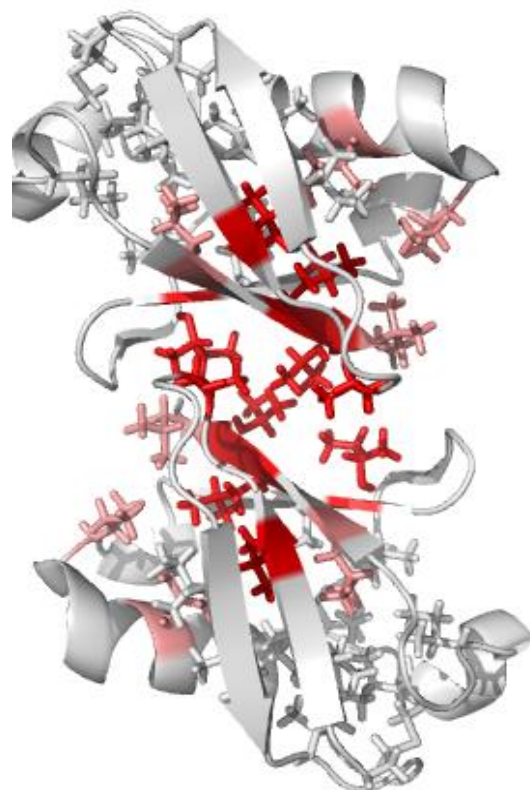
A. Site-directed mutagenesis confirms that recognition by Rpn1 involved hydrophobic patch of Ub. Mutations of the hydrophobic-patch residues L8 and I44 in Ub reduce Rpn1 binding. ^{15}N transverse relaxation time (T_2) of backbone amides in WT Ub (black) and mutant (Ub^{L8A,I44A}) (blue) alone and in the presence of Rpn1³⁹¹⁻⁶⁴² at 1:1 molar ratio (red and green, respectively).

B. Predicted protein/protein interaction site on Ub and Rpn1^{PC}, NIPS. Normalized interface propensity (NIP) values derived from rigid body docking with electrostatics and desolvation scoring for the prediction of interaction hotspots. The ensembles of the rigid-body docking solutions generated by the simulations were subsequently used to project the docking energy landscapes mapping sites on Ub displaying preferable energy of interaction with Rpn1^{PC}. NIP values represent the frequency of a given residue to be located at the interface among the lowest energy solutions of docking points to Helices 586-590, 540-552 on the surface of Rpn1^{PC} upon interaction with free Ub as highly populated (dark green; left). The reciprocal experiment mapped sites on Ub displaying preferable energy of interaction with full-length Rpn1 picks out the hydrophobic patch of Ub and some neighboring residues(right). NIP values greater than 0.3 point to the canonical hydrophobic patch centered on L8, I44, and V70 (dark green) extending to some neighboring residues on one face of free Ub (light green, grey).

A



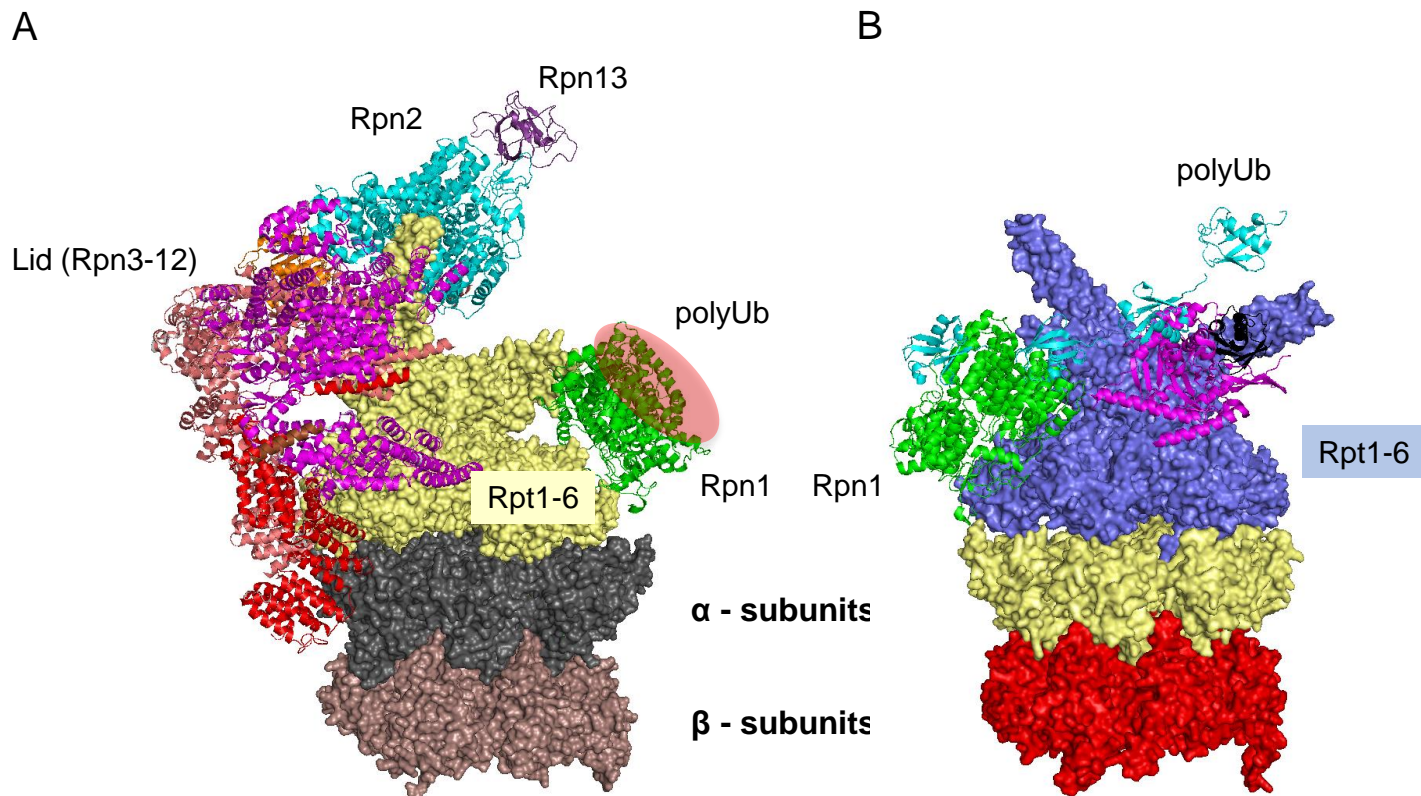
B



Supplemental Figure S5. Nature of association between Rpn1 and diUb. Related to Figure 6.

A. **NMR analysis of the binding interactions between Rpn1³⁹¹⁻⁶⁴² and K63-linked diUb.** Amide CSPs (black bars) in the distal Ub of K63-Ub₂ at the endpoint of titration with Rpn1³⁹¹⁻⁶⁴², as a function of residue number. Residues showing strong signal attenuations during the titration are marked with grey bars. Signal perturbations in K63-Ub₂ upon titration with Rpn1³⁹¹⁻⁶⁴² are similar to those in K48-Ub₂ (Figure 6).

B. **Interaction of K48-linked Ub₂ with Rpn1³⁹¹⁻⁶⁴² by Methyl TROSY.** Residues that shift upon addition of Rpn1 are colored according to the magnitude of CSP (red to white).



Supplemental Figure S6 – Model of how Rpn1 may position polyUb at 19S RP of proteasome for aiding substrate processing. Related to Figure 6.

(A) CryoEM model of the proteasome (PDB 5A5B) highlighting position of Rpn1 (green), and Rpn2-Rpn13 (cyan-purple) on the Six ATPases that make up the base of the 19S RP (Rpt1-6, as yellow surface). When incorporated into the proteasome, a wide swath of Rpn1 (encompassing the majority of its first PC stretch, rich in hydrophobic and conserved residues) does not contact other 19S subunits and is exposed to solvent. This region is positioned perfectly to concentrate proteasome auxiliary factors, proteasome-interaction proteins, and polyUb-conjugates at the entry point of proteasome entry (tan cloud). We suggest that through manifold protein-protein interactions, Rpn1 coordinates substrate recruitment and hand-over to proteasome-associated DUBs for chain removal and the substrate to RPT ATPases for unfolding and translocation. **(B)** Model demonstrating possible orientation of extended tetra-ubiquitin chain spanning Rpn1 (green) and Rpn11 (black) in the 26S proteasome. Proteasome EM model 5A5B was stripped of most lid and base 19S subunits to expose USP domain of Ubp6 (violet) and Rpn1 (green) in relation to RPT ring of ATPases (blue) situated on alpha (yellow) and beta (red) rings of 20S CP. Approximate size and orientation of extend tetra-ubiquitin chain (cyan) is superimposed to demonstrate that chains of three or longer Ub units are sufficient to tether UBA domains of shuttles or the USP domain of Ubp6/USP14 to Rpn1.