**Supplementary materials for**

# A COMMON SUBSTRATE RECOGNITION MODE CONSERVED BETWEEN KATANIN P60 AND VPS4 GOVERNS MICROTUBULE SEVERING AND MEMBRANE SKELETON REORGANIZATION *

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Running title: Structure of the N-terminal domain of katanin p60

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## SUPPLEMENTARY EXPERIMENTAL PROCEDURES

*Production of Full-length kp60* - Expression vector for the recombinant GST-tagged full-length kp60 of mouse was constructed by standard protocol using PCR, and ligated into *BamH*I-*SalI* sites of pGEX-6P3 (GE Healthcare Bioscience). Ala-substituted mutants were engineered with QuikChange site-directed mutagenesis kit (Stratagene). The fusion proteins were produced in *E. coli* JM109. Expression was induced with 0.1 mM IPTG, and LB cultures were grown overnight at 20 °C. For pull-down assays, GST-tagged proteins were bound to glutathione-Sepharose 4B (GE Healthcare Bioscience) and washed with the storage buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, and 0.1 mM ATP) supplemented with EDTA-free protease inhibitor cocktail (Nacali tesque Inc, Kyoto, Japan) on column. GST-kp60s bound to glutathione-Sepharose were eluted with the elution buffer (50 mM Tris-HCl, 100 mM NaCl, 40 mM reduced glutathione, pH 8.0, and 5% glycerol). The eluents were further used for ATPase assays.

*ATPase assays* - ATPase activity was measured using an ATP regenerating system (1). The reaction mixture containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 1 mM ATP, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase, and 0.2 mM NADH was used. The reactions were initiated by the addition of GST-kp60s (0.5 μM), and the activities were measured by monitoring the decrease of NADH absorption at 340 nm at room temperature using UV-Vis spectrophotometer, UV mini-1240 (Shimadzu, Tokyo, Japan). The data were normalized for further analysis.

*Tubulin Binding Assays* - 5 μg of GST-proteins bound to glutathione-Sepharose 4B (20 μl) were incubated with 10 μg of tubulin in the binding buffer (80 mM PIPES, pH 7.0, 1 mM $MgCl_2$ and 1 mM EGTA) for 30 min at 4°C. The beads were washed four times in the wash buffer (4.3 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.3, and 5% glycerol). The associated proteins were eluted in the elution buffer (50 mM Tris-HCl, 100 mM NaCl, 50 mM reduced glutathione, pH 8.0, and 5% glycerol). The eluted proteins were analyzed by SDS-PAGE and Western blotting.

*Western Blotting* - Proteins were resolved in SDS-PAGE and blotted onto a PVDF membrane. We detected tubulin using 1/2000 diluted anti-α-tubulin antibody (Siguma-Aldrich) followed by HRP-conjugated anti-mouse IgG secondary antibody (Promega). The proteins were visualized using an ECL-Plus kit (GE Healthcare Bioscience) and detected using LAS-1000 detector (Fuji Film, Tokyo, Japan).

*Model building* – A molecular model of the complex of kp60-NTD with a tubulin tetramer was constructed based on the complex between spastin-MIT and CHMP1b (PDB: 3eab). The kp60-NTD structure and the tubulin tetramer (3du7) were superimposed onto the corresponding position of spastin-MIT and the C-terminal helix of CHMP1b (174-193), respectively. The best model fully overrided on helices with binding sites was selected considering steric crash and complementary charge interactions between structures. A hexameric ring model of AAA ATPase domains of kp60 was generated by superimposing the Cα atoms of kp60 onto those of the hexameric ring structure of p97 D1 (PDB: 1s3s) using MODELLER (version 9v6) (http://salilab.org/modeller/). Finally, the complex model structure of hexameric full-length kp60 with tubulin oligomer was constructed using MOLMOL (2) by joining the components manually.

## SUPPLEMENTARY FIGURE LEGENDS

**Supplementary Fig. 1.**
Phylogenetic tree of the AAA protein superfamily. Red circle, kp60 subfamily; Blue circle, Vps4 subfamily. The tree data were calculated by ClustalX (3) and the tree was drawn with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

**Supplementary Fig. 2.**
Ramachandran plot for the phi-psi values of the final 20 structures of kp60-NTD. This figure was produced using PROCHECK-NMR (4).

**Supplementary Fig. 3.**
Interactions between kp60-NTD and MTs/tubulin dimer. *A*, schematic diagram of pull-down assay using Microtubule binding protein spin down assay kit, BK029 (Cytoskeleton) to assess interactions between GST-tagged kp60-NTD and MTs/tubulin *in vitro*. GST-tagged kp60-NTD was mixed with a reaction solution after (upper left) and before (upper right) tubulin polymerization reaction, then ultracentrifuged. Tubulin was separated by molecular weight; polymerized MTs were sedimented at the bottom of tubes, non-polymerized tubulin migrated to the top of the solution (lower panel). Reaction solutions were divided into four fractions (from top to bottom) and each fraction was analyzed by SDS-PAGE. GST-tagged kp60-NTD co-sedimented with non-polymerized tubulin. *B*, the reaction solution after polymerization of only tubulin was ultracentrifuged and analyzed as a control. Lanes 1–4 correspond to fractions from top to bottom, indicated in the lower panels of *A*. *C*, pull-down assay for kp60-NTD mixed after tubulin polymerization reaction. kp60-NTD may possibly bind with a tubulin dimer rather than MTs. *D*, pull-down assays of the GST-tagged kp60-NTD mixed before tubulin polymerization reaction. SDS-PAGEs are Coomassie-stained.

**Supplementary Fig. 4.**
ATPase activity of full-length kp60 and interactions of kp60 with tubulin. *A*, ATPase activities of kp60s (0.5 μM) at 340 nm. Filled diamond (continuous line): wild type, filled box (dotted line): R49A, filled triangle (broken line): K67A. *B*, pull-down assays of tubulin with wild type (WT) of GST-kp60 and Ala mutants *in vitro*. Molecular size is shown in the left. Tubulin was used as the input. Only the buffer and the GST-tag mixed with tubulin as negative controls are shown in lanes 2 and 3. Recombinant proteins used for pull-down are indicated at the top of the gel. Filled and open arrowheads show tubulin and full-length kp60s, respectively. SDS-PAGE was Coomassie-stained (upper panel). Western blotting analysis of tubulin bound to full-length kp60s was visualized by ECL (lower panel).

**Supplementary Fig. 5.**
Comparison of structures and tubulin binding interfaces with other tubulin binding domains. Tubulin binding interfaces are indicated by black and arrows. *A*, stathmin-like domain bound to the tubulin (white) (PDB: 1sa1); *B*, EB1 CH domain (2qjz); *C*, Msps TOG2 domain (2qk2); *D*, CAP-Gly domain bound to the tubulin peptide (white) (2e4h), and *E*, tubulin-specific chaperone cofactor A (1h7c).

**Supplementary Fig. 6.**
Model for α–tubulin helix 12 binding with kp60-NTD. An electrostatic surface potential diagram (top), a ribbon diagram (middle), and a sequence conservation diagram (bottom) for kp60-NTD were shown. α–Tubulin helix 12 is shown as a transparent cylinder (yellow).

**Supplementary Fig. 7.**

Comparison between model for tubulin binding interfaces of kp60-NTD. *A*, model of kp60-NTD bound to α–tubulin at the helix 1/3 interface (see text). Ribbon diagram of the model complex between kp60-NTD and a tubulin tetramer (grey) was constructed based on the complex between spastin-MIT and CHMP1b (PDB: 3eab). α–tubulin helix 12, a putative interface to kp60-NTD, is colored yellow. *B* and *C*, side (top) and top (bottom) views of the ribbon diagram of the complex between kp60-NTD and α–tubulin using the helix 1/3 and helix 2/3 interfaces, respectively. Side chains of key residues for binding tubulin are shown (red). *D*, top view of the ribbon diagram of the complex between spastin-MIT and CHMP1b (yellow) (3eab). Side chains of the residues interacting between spastin and CHMP1b are indicated.

**Supplementary Fig. 8.**
Proposed model for tubulin binding with full-length kp60. Model complex between tubulin origomer (grey) and hexameric full-length kp60, composed of kp60-NTD and AAA ATPase domain (violet) is shown. AAA ATPase domains form hexameric ring. Five of the six kp60-NTDs on the hexameric AAA ATPase domains were not drawn for clarity. One of the tubulin C-terminal tail is shown in yellow. The tail on the surface of MT may bind to the pore of the hexameric AAA ATPase domain of kp60.

**Supplementary Table 1.**

**Oligonucleotides used as primers for Ala substitution.**

| Primer | Sequence |
|---|---|
| Q35A_F | CAGGGAGTTCTTGAC**GCC**ATGAACAAGTACCTGTACTCAGTC |
| Q35A_R | GACTGAGTACAGGTACTTGTTCAT**GGC**GTCAAGAACTCCCTG |
| N37A_F | CAGGGAGTTCTTGACCAAATG**GCC**AAGTACCTGTACTCAGTC |
| N37A_R | GACTGAGTACAGGTACTT**GGC**CATTTGGTCAAGAACTCCCTG |
| D45A_F | CTGTACTCAGTCAAA**GCC**ACACACCTCCGTCAGAAATGG |
| D45A_R | CCATTTCTGACGGAGGTGTGT**GGC**TTTGACTGAGTACAG |
| R49A_F | GTCAAAGATACACACCTC**GCC**CAGAAATGGCAACAG |
| R49A_R | CTGTTGCCATTTCTG**GGC**GAGGTGTGTATCTTTGAC |
| Q53A_F | CTCCGTCAGAAATGG**GCC**CAGGTTTGGCAGGAAATAAATGTG |
| Q53A_R | CACATTTATTTCCTGCCAAACCTG**GGC**CCATTTCTGACGGAG |
| V55A_F | CTCCGTCAGAAATGGCAACAG**GCC**TGGCAGGAAATAAATGTG |
| V55A_R | CACATTTATTTCCTGCCA**GGC**CTGTTGCCATTTCTGACGGAG |
| E58A_F | CAGAAATGGCAACAGGTTTGGCAG**GCC**ATAAATGTGGAAGCTAAG |
| E58A_R | CTTAGCTTCCACATTTAT**GGC**CTGCCAAACCTGTTGCCATTTCTG |
| K64A_F | GTTTGGCAGGAAATAAATGTGGAAGCT**GCC**CAAGTTAAGGATATCATG |
| K64A_R | CATGATATCCTTAACTTG**GGC**AGCTTCCACATTTATTTCCTGCCAAAC |
| K67A_F | GTGGAAGCTAAGCAAGTT**GCC**GATATCATGAAAACATAATAGAGC |
| K67A_R | GCTCTATTATGTTTTCATGATATC**GGC**AACTTGCTTAGCTTCCAC |
| D68A_F | GTGGAAGCTAAGCAAGTTAAG**GCC**ATCATGAAAACATAATAGAGC |
| D68A_R | GCTCTATTATGTTTTCATGAT**GGC**CTTAACTTGCTTAGCTTCCAC |

## SUPPLEMENTARY REFERENCES


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3. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res* **25**, 4876-4882
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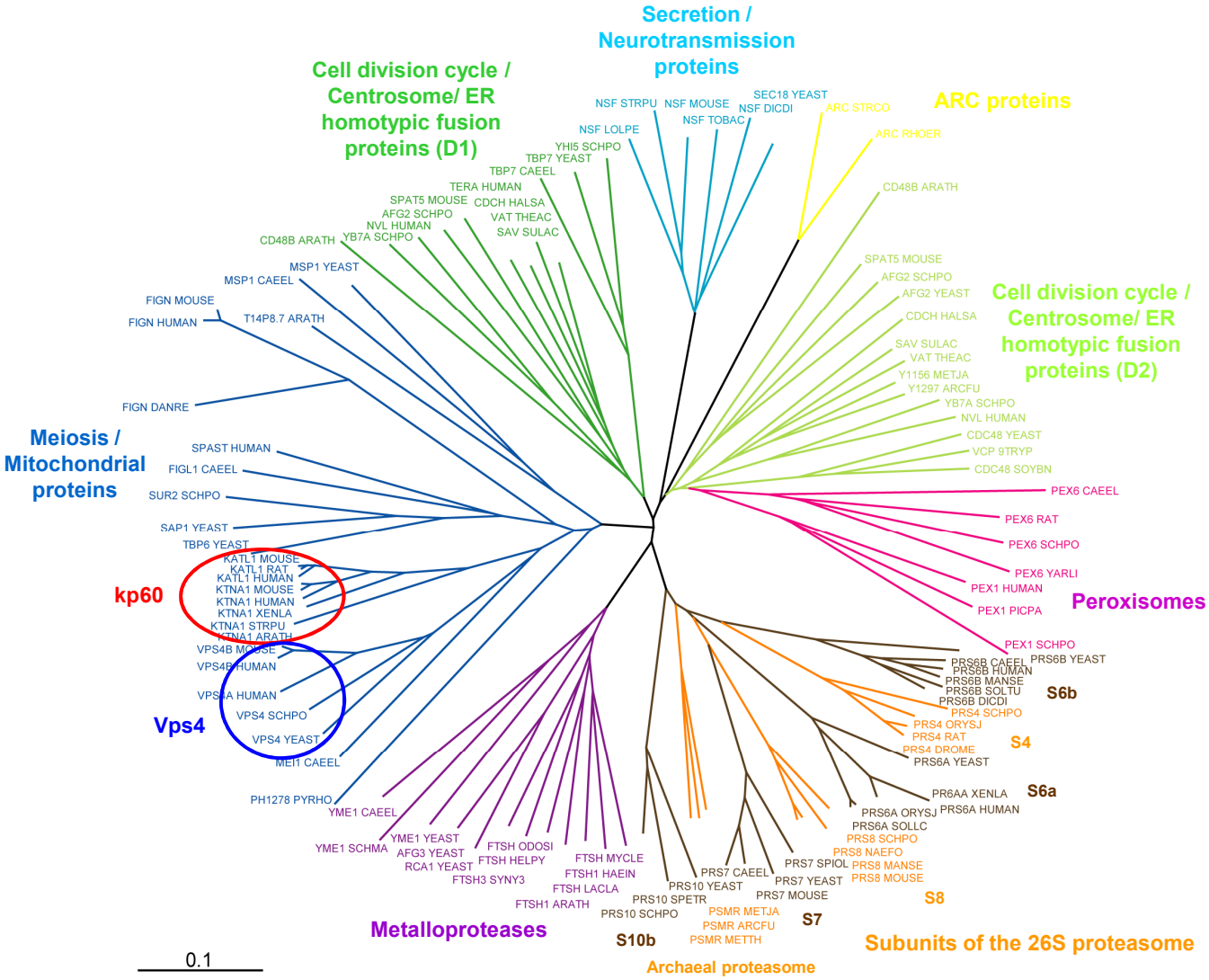
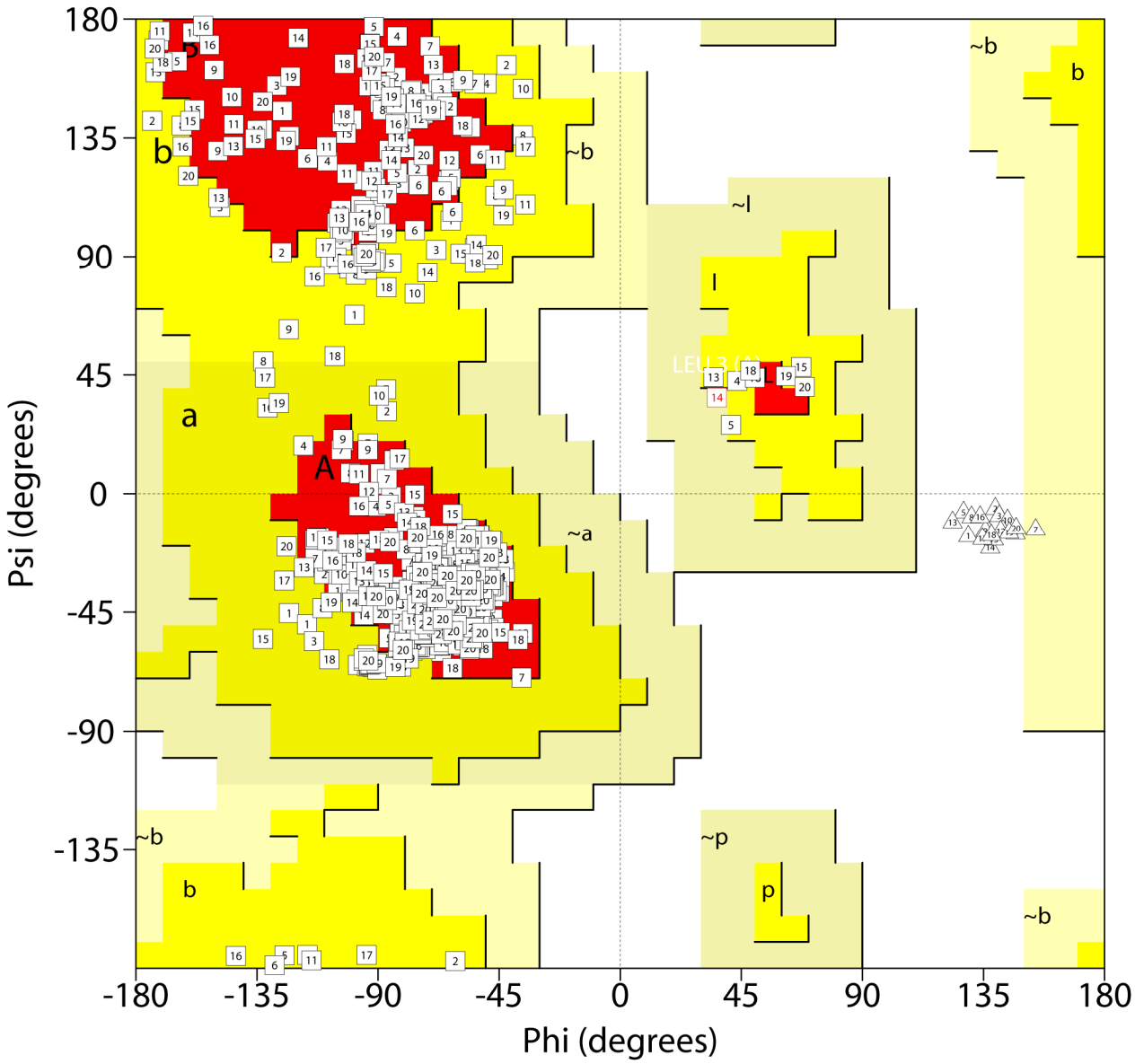
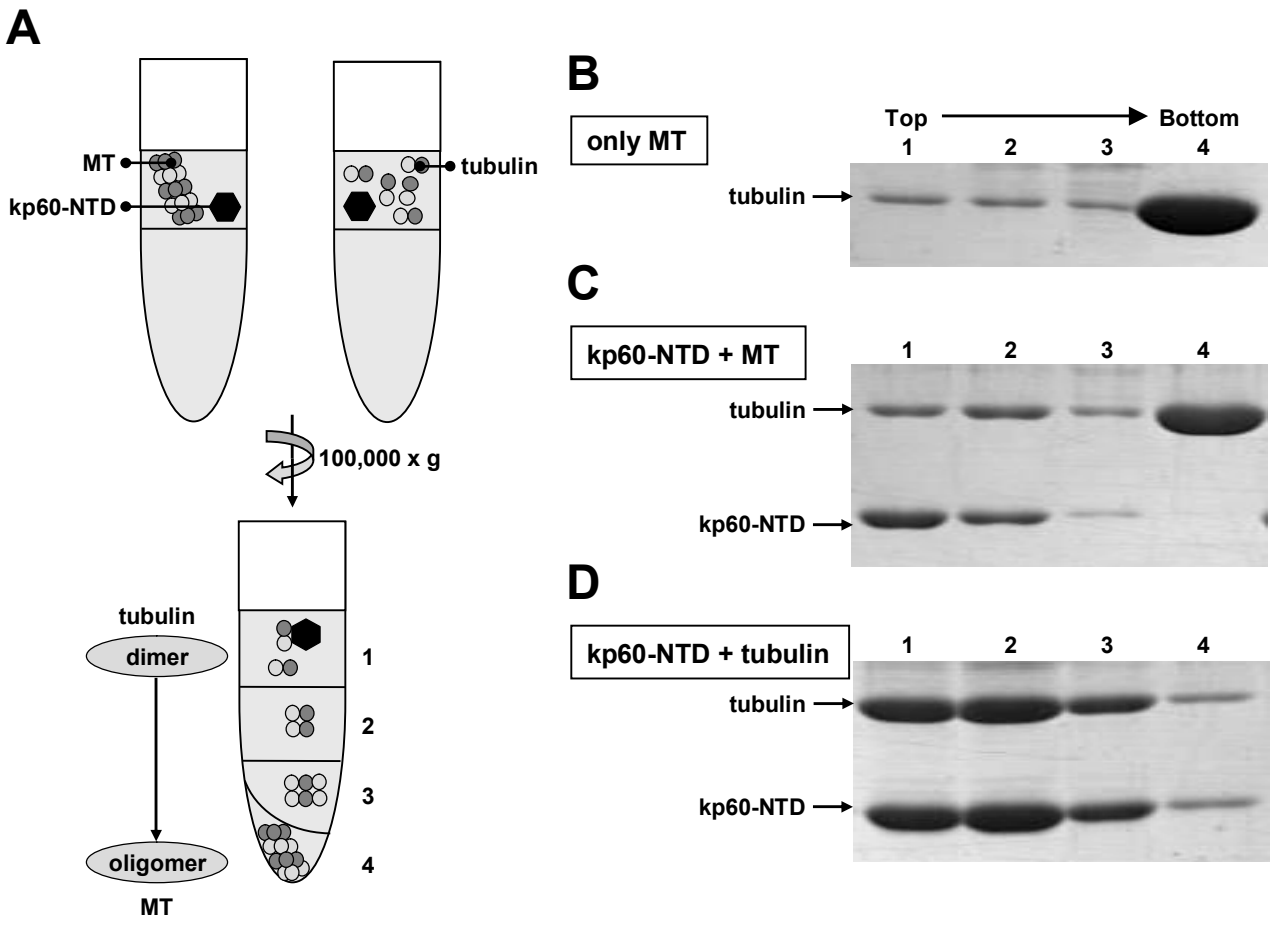



# Supplementary Figure 1




Supplementary Figure 2

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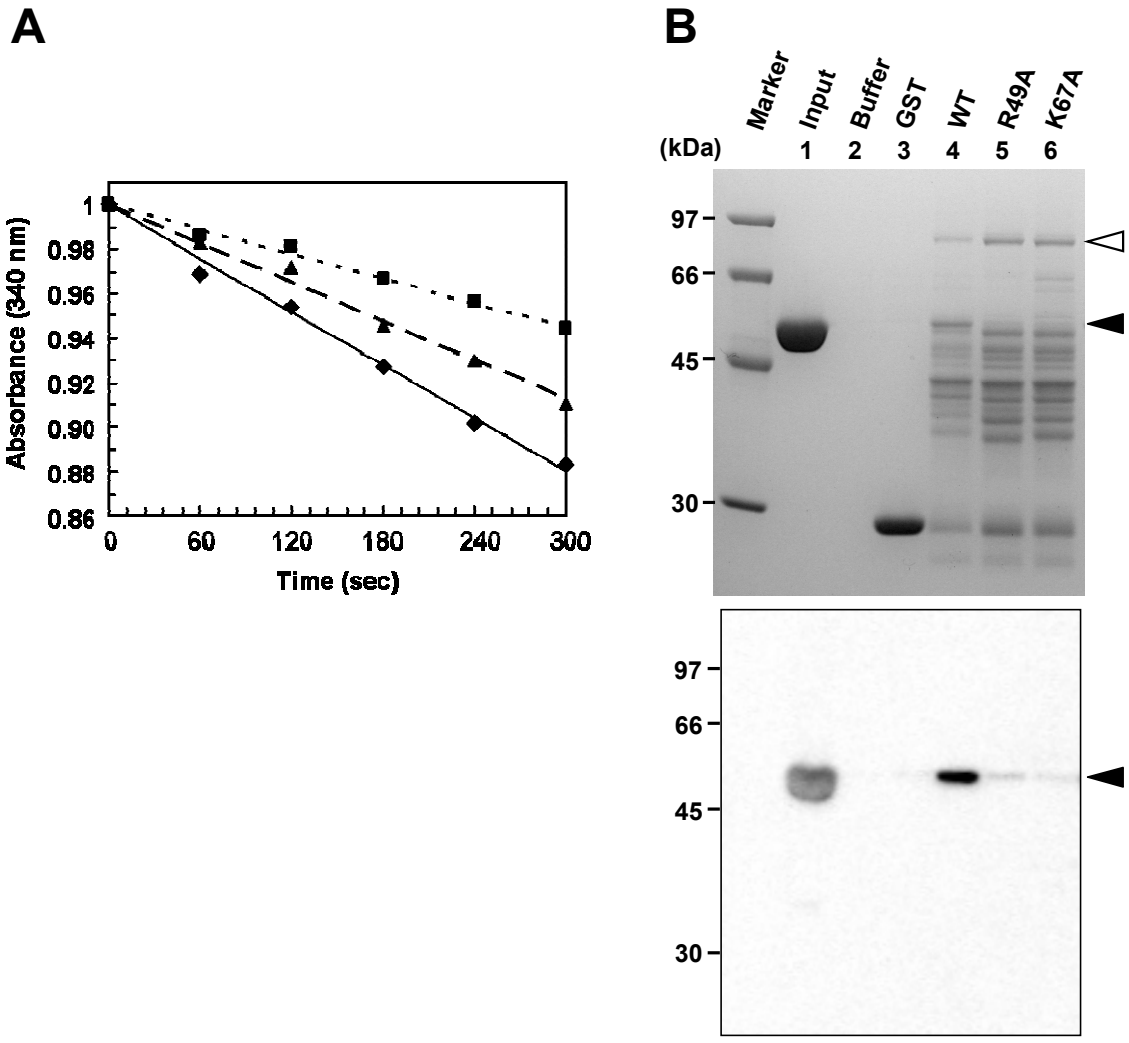


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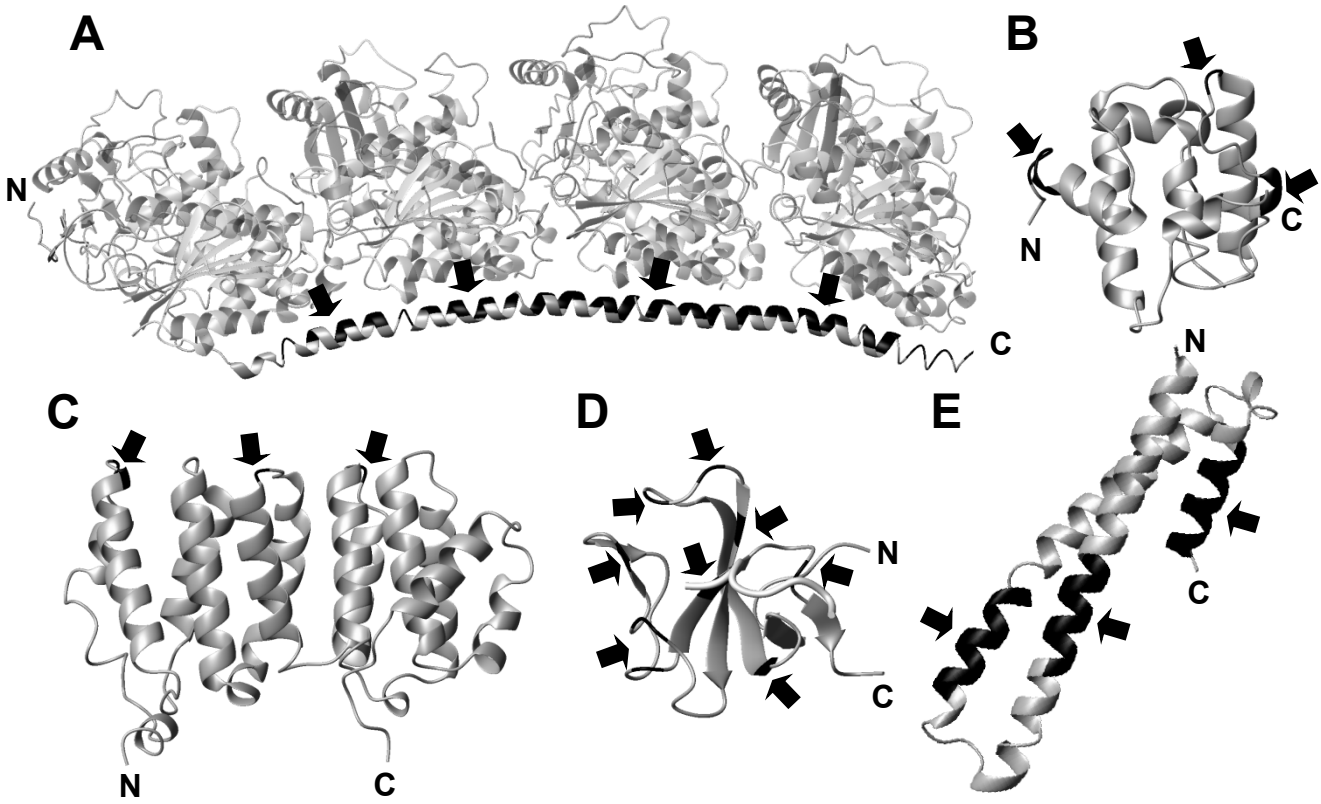


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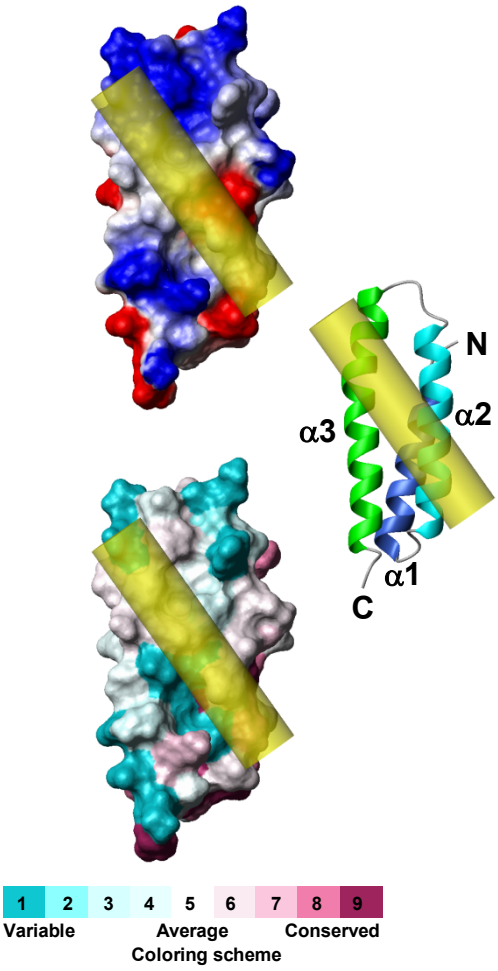


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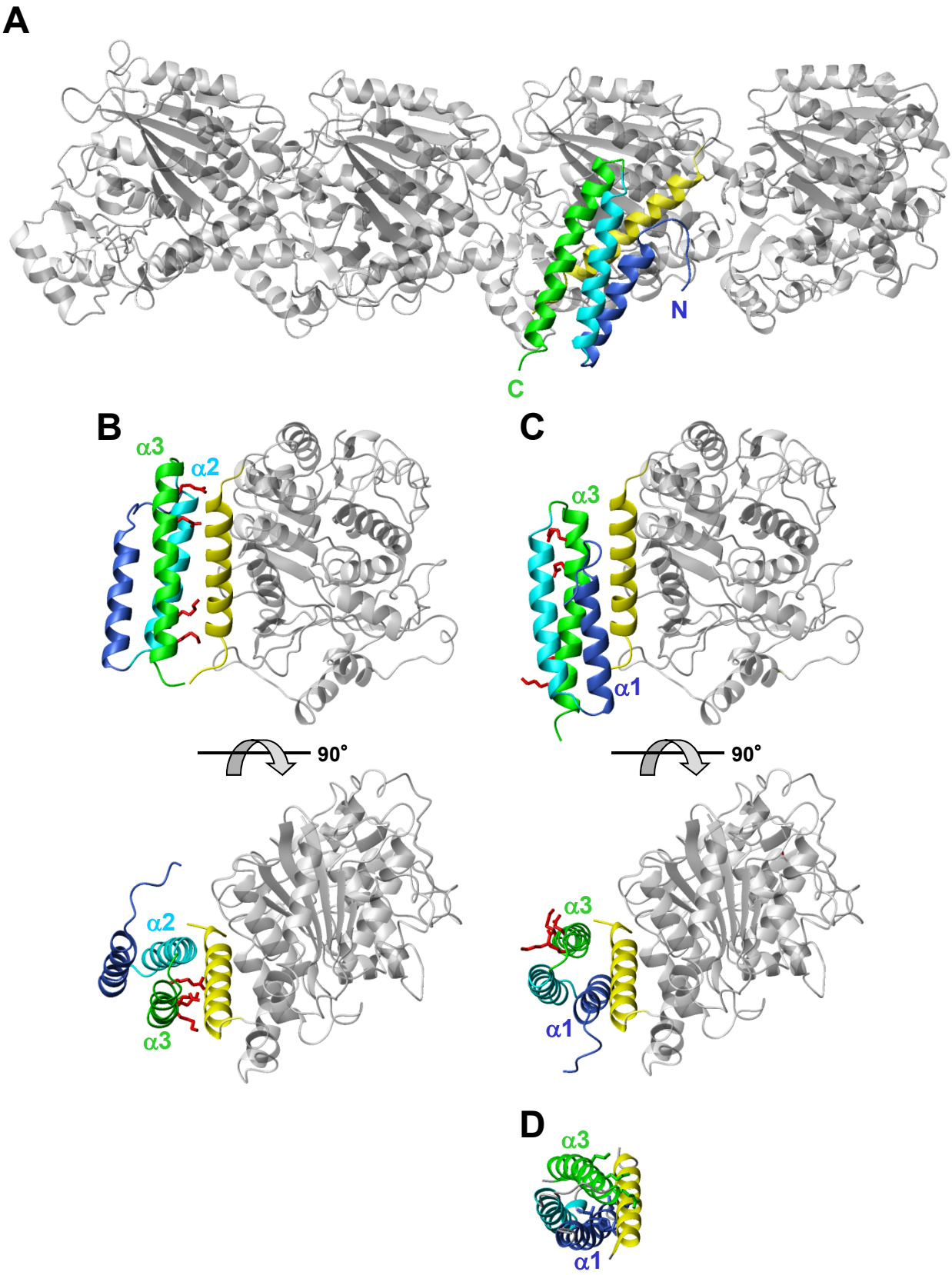
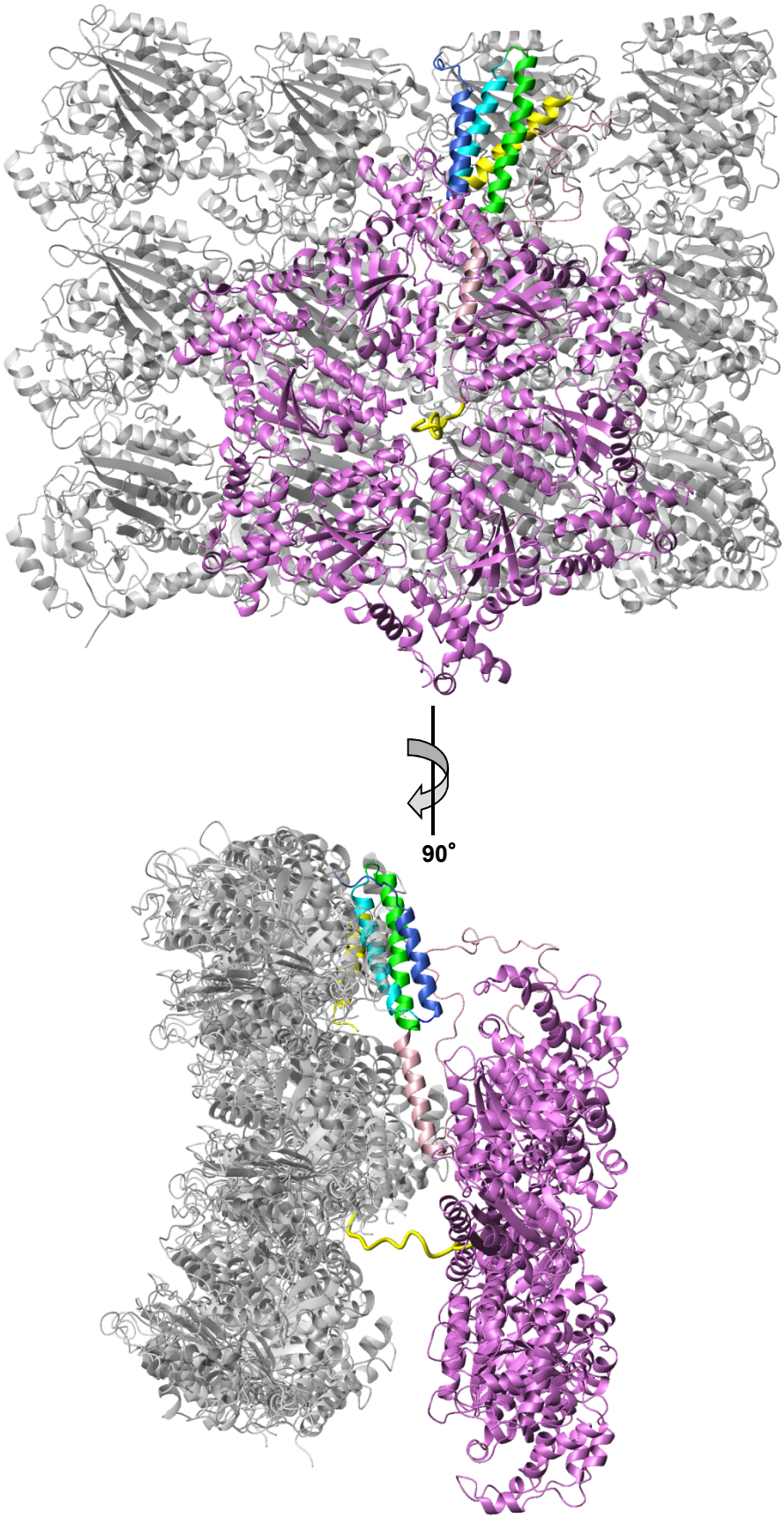



Supplementary Figure 7




Supplementary Figure 8