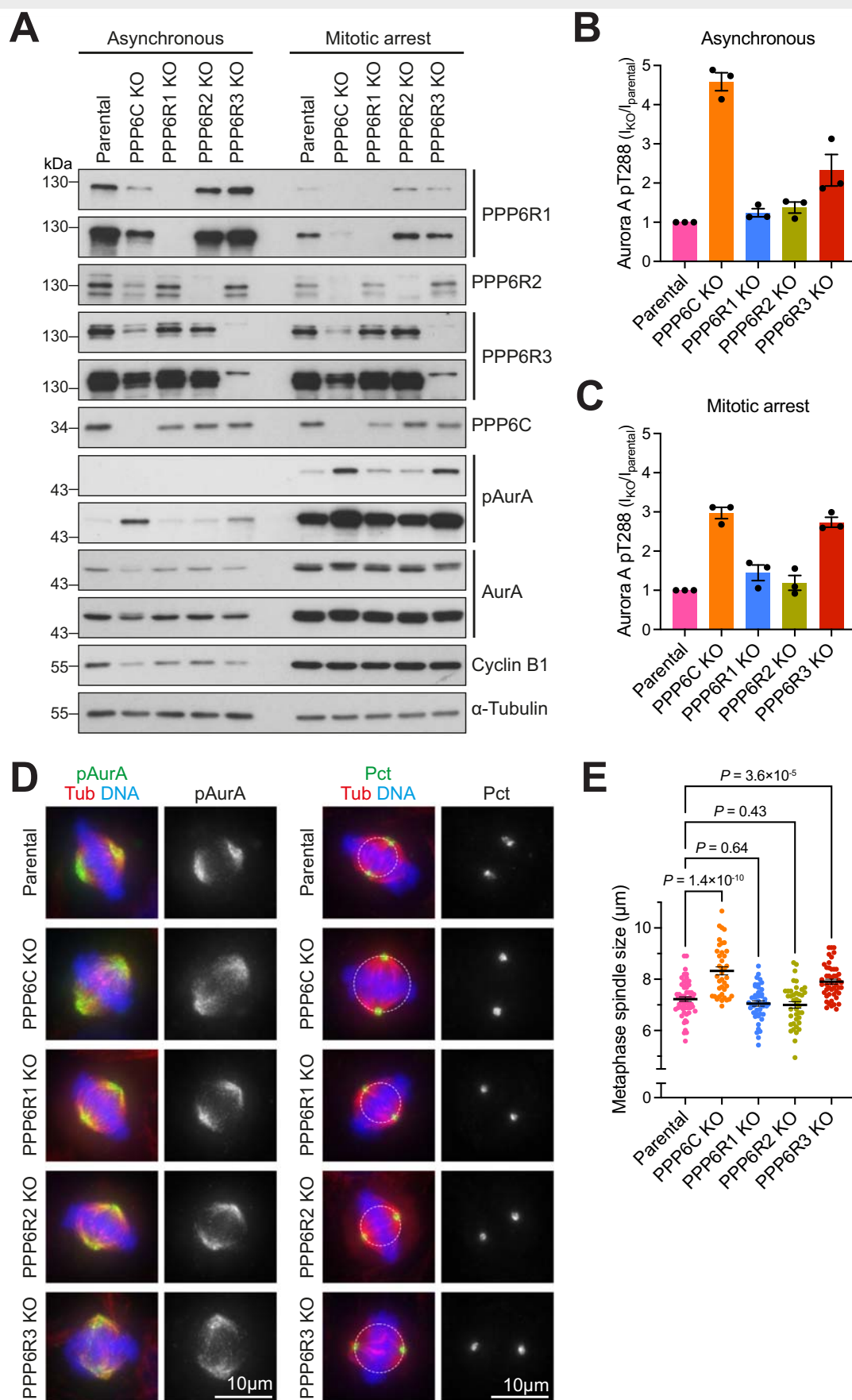


Expanded View Figures

Figure EV1. Increased Aurora A activity and enlarged mitotic spindles in PPP6C and PPP6R3 knock-out cell lines.

(A) HeLa cells with CRISPR/Cas9 knock-outs (KO) for the PP6 catalytic (PPP6C) or regulatory subunits (PPP6R1-3) in asynchronous culture or mitosis were blotted for the indicated proteins. Two different exposures for total Aurora A and pT288 (pAurA) are shown. (B) The fold-increase of Aurora A pT288 in the asynchronous KO cells over the parental cells was plotted (mean $\pm$ SEM; $n = 3$ independent experiments). (C) The fold-increase of pT288 in the mitotic KO cells over the parental cells was plotted (mean $\pm$ SEM; $n = 3$ independent experiments). (D) The cells from (A) were fixed and stained with the indicated antibodies. Aurora A pT288 (pAurA) staining is dispersed along the spindle only in cells lacking PPP6C or PPP6R3. The scale bar is 10 μ m. (E) Metaphase spindle size in cells in (D) as indicated by dashed circles in (D) was quantitated and plotted (mean $\pm$ SEM; $n \geq 39$ cells, $n = 3$ independent experiments). Significance was determined by a one-way ANOVA test followed by Dunnett's multiple comparison test against parental cells.



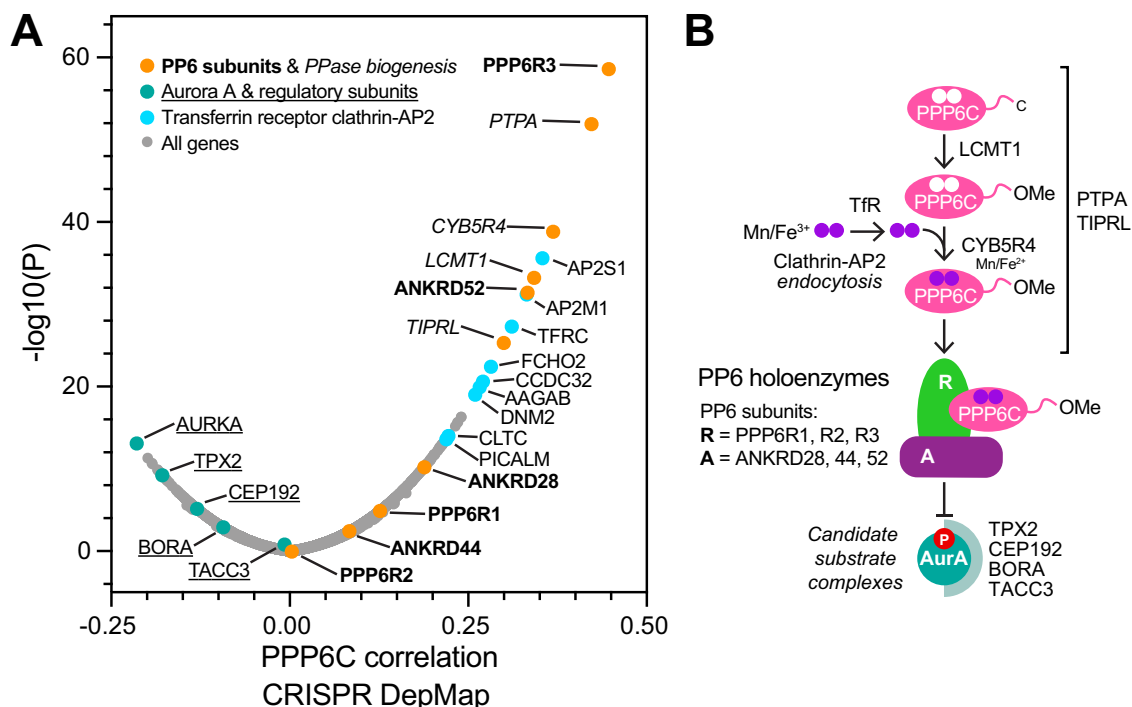
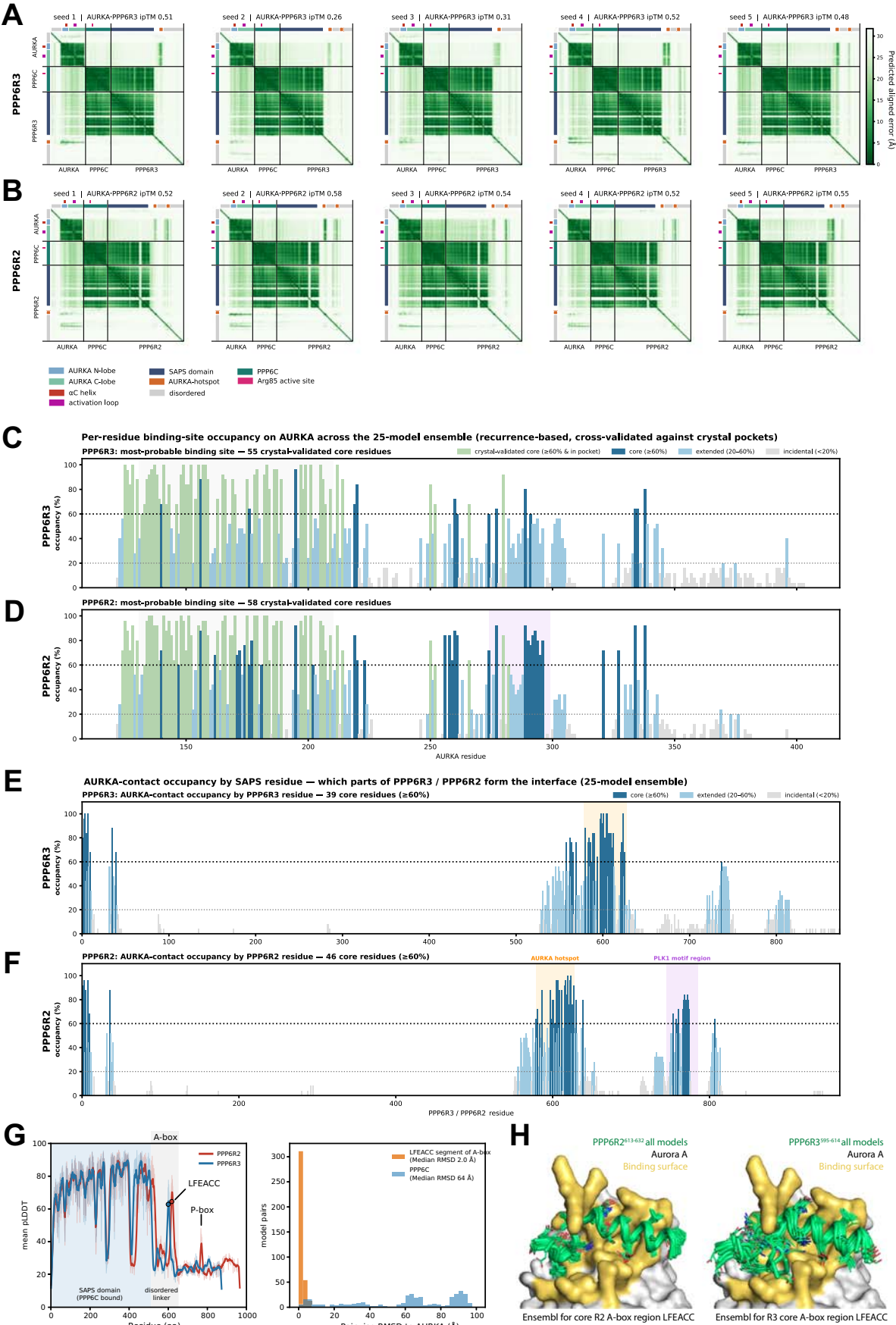


Figure EV2. DepMap analysis of PPP6C reveals components required for PP6 biogenesis and function of the Aurora A pathway.

(A) DepMap CRISPR correlations with PPP6C KO were calculated using the tools at <https://depmap.org/portal> and DepMap release 25Q3 and then plotted as Volcano plot. Positive associations suggest related biological function whereas negative associations suggest opposing biological function. PPP6R3 was identified as the strongest positive correlation with PPP6C, followed by several proteins involved in the PP6 biogenesis pathway outlined in (B). The strongest negative association is the known PP6 target Aurora A (AURKA), followed by known Aurora A activators and binding partners TPX2, CEP192, BORA and TACC3. PPP6R1 and PPP6R2 showed only weak correlation with PPP6C under the conditions tested. (B) Schematic representation of the PP6 biogenesis pathway. Like the PP2A catalytic subunit, the PP6 catalytic subunit is C-terminally methylated, and Mn^{2+} and Fe^{3+} ions, supporting catalysis, are incorporated into the catalytic subunit before assembly of the functional PP6 holoenzyme complex. Holoenzyme complex assembly is supported by PP2A-family phosphatase regulatory proteins PTPA and TIPRL.



◀ **Figure EV3. Modelling of PP6 holoenzyme Aurora A substrate binding.**

(A, B) Extended view data for a PP6 core holoenzyme complex with PPP6C, PPP6R3 or PPP6R2 and the substrate Aurora A (AURKA). AlphaFold models were created using 5 random seeds with 5 models per seed. PAE plots are shown for the top-ranked model for each seed for PPP6R3 (A) and PPP6R2 (B) complexes. Interfaces for PPP6C-PPP6R3 and -PPP6R2 SAPS domains have an ipTM ~ 0.90 in every model and are essentially identical across all seeds, consistent with the idea that this is the rigid core of the holoenzyme. The PPP6R3-AURKA interface has a moderate ipTM of ~ 0.51 – 0.55 . Long disordered C-terminal regions of PPP6R3 and PPP6R2 reduce the overall ipTM for the models. (C, D) Graph of PPP6R3 and PPP6R2 occupancy along Aurora A for the 25 models defines common interactions with the kinase N-lobe. (E, F) Graph of Aurora A contact occupancy along PPP6R3 (E) or PPP6R2 (F) for the 25 models defines a common Aurora A binding hotspot (the A box region; orange) and a more proximal binding region containing a PLK1 docking motif in PPP6R2 (P-box; purple). (G) Mean pLDDT $\pm$ SD for all models was plotted as a measure of per residue confidence for PPP6R3 and R2 (left). This highlights the confident prediction for the structured PPP6C-binding SAPS domain and divides the A-box into a lower confidence region interpreted as a disordered linker followed by a more ordered LFEACC sequence within the A-box region. Relative positions of the LFEACC A-box compared to Aurora A and PPP6C are plotted in the bar graph as pairwise RMSD. (H) Ensemble structural models showing the PPP6R2 and PPP6R3 LFEACC A-box core regions (green) and common binding surface on Aurora A (yellow). Models were aligned to Aurora A as a reference.

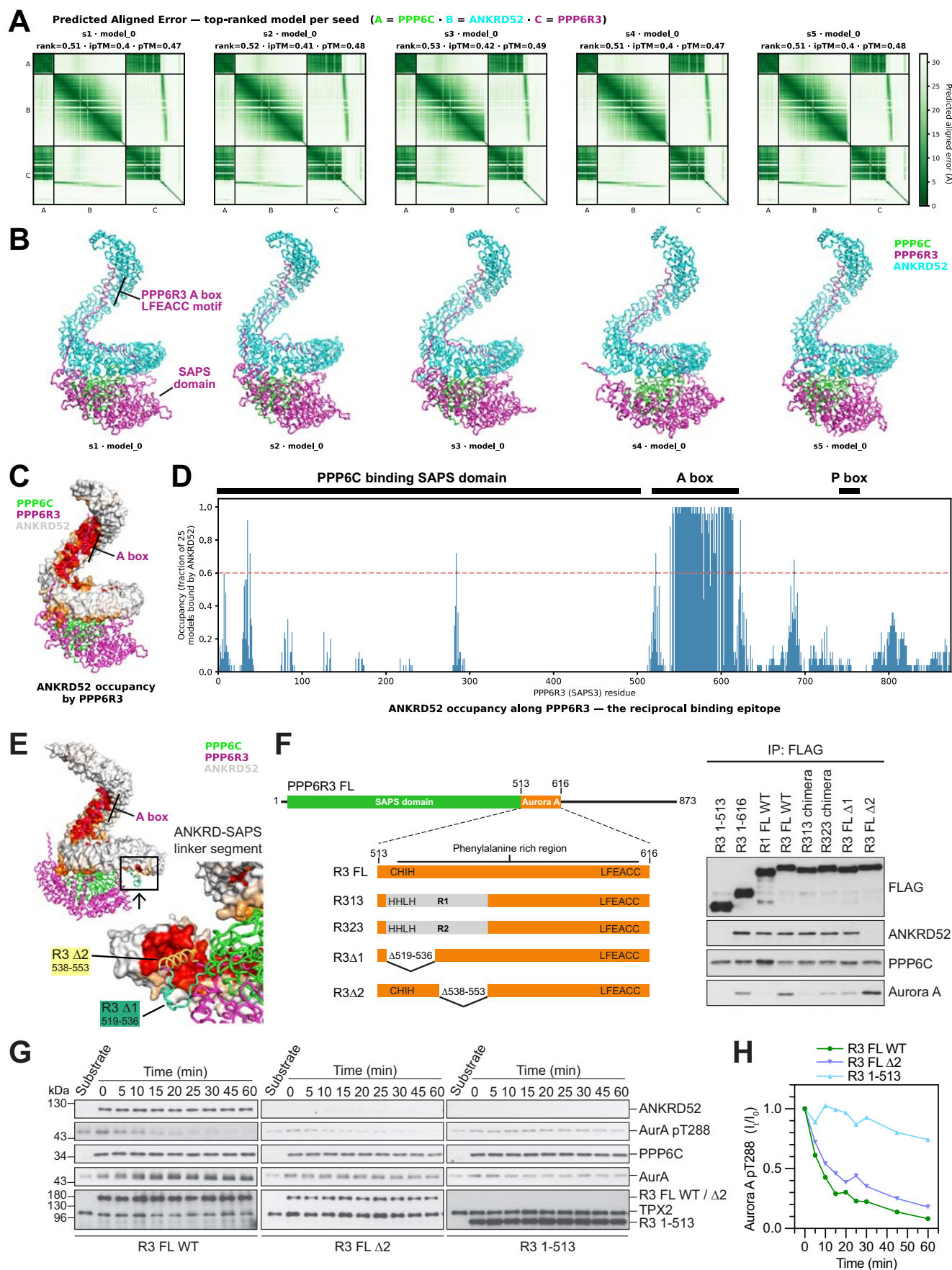


Figure EV4. ANKRD subunits scaffold the C-terminus of PPP6R3 in PP6 holoenzymes but are not essential for substrate binding.

(A) Extended view data for a PPP6R3 holoenzyme complex with PPP6C, PPP6R3 and the ANKRD52 subunit. AlphaFold 3 models were created using 5 random seeds with 5 models per seed. PAE plots are shown for the top-ranked model for each seed. The overall ranking is from 0.51 to 0.53 for 24 of 25 models. The best model of the 25 is seed 3, model_0 with a ranking score of 0.530, ipTM 0.42, pTM 0.49. Interface A-C (PPP6C-PPP6R3) has an ipTM -0.90 in every model and is essentially identical across all seeds, consistent with the idea that this is the rigid core of the holoenzyme. Interface B-C (ANKRD52-PPP6R3) has a moderate ipTM of -0.42 and is consistent across the seeds. The A-B interface (PPP6C-ANKRD52): ≈ 0.20 -0.30 is the weakest pairing, with ANKRD52 placement against the catalytic subunit being the main source of uncertainty. This reduces the overall ipTM for the models. AlphaFold 3 therefore predicts a confident PPP6C-PPP6R3 sub-complex with ANKRD52 attached at a reproducible interface on PPP6R3 at aa 542-623 within the A box region, but a less certain global orientation. (B) The five top models for each seed were aligned on ANKRD52 to show how the PPP6C-PPP6R3 SAPS domain core position varies. Whereas the ANKRD52 ankyrin domain agrees to 1.6-2.8 Å across all seeds, the PPP6C-PPP6R3 core albeit rigid swings by 7.6-14.3 Å relative to the seed 3, model_0 reference position. (C) Common contacts between PPP6R3 and ANKRD52 were mapped and the surface of ANKRD52 colour coded grey (no contacts) to red (occupancy 1.0). (D) Graph of ANKRD52 occupancy along PPP6R3 for the 25 models defines a common binding epitope from aa 542-623 within the A box region. (E) The region at the start of the A-box segment can be divided into two segments, R3Δ1 519-536 which forms a flexible linker to the structured SAPS domain and R3Δ2 538-553 which forms a short helix making contact to ANKRD52 and may anchor that interaction. (F) Left: Schematic on PPP6R3 and expanded region showing the A-box with R3Δ1 519-536 flexible linker and suggested R3Δ2 538-553 ANKRD52 anchor helix. In R313 and R323 chimeric constructs this region was replaced with the equivalent sequences in R1 and R2. Right: FLAG-tagged PPP6R3 constructs as indicated were transiently transfected into HeLa cells. Complexes were isolated by FLAG IP and analysed by western blotting. (G) In vitro phosphatase assays with PPP6R3 FL WT, Δ2 and 1-513. Phosphorylated Aurora A-TPX2 substrate complexes were incubated with PP6-R3 holoenzymes as indicated. Samples were western blotted for Aurora A, Aurora A pT288 and PP6 subunits. (H) Aurora A pT288 signal was quantitated over the time course shown in (G) and plotted relative to $t = 0$. The western blots and graph in (G, H) are representative examples of 2 independent experiments with different protein preparations.

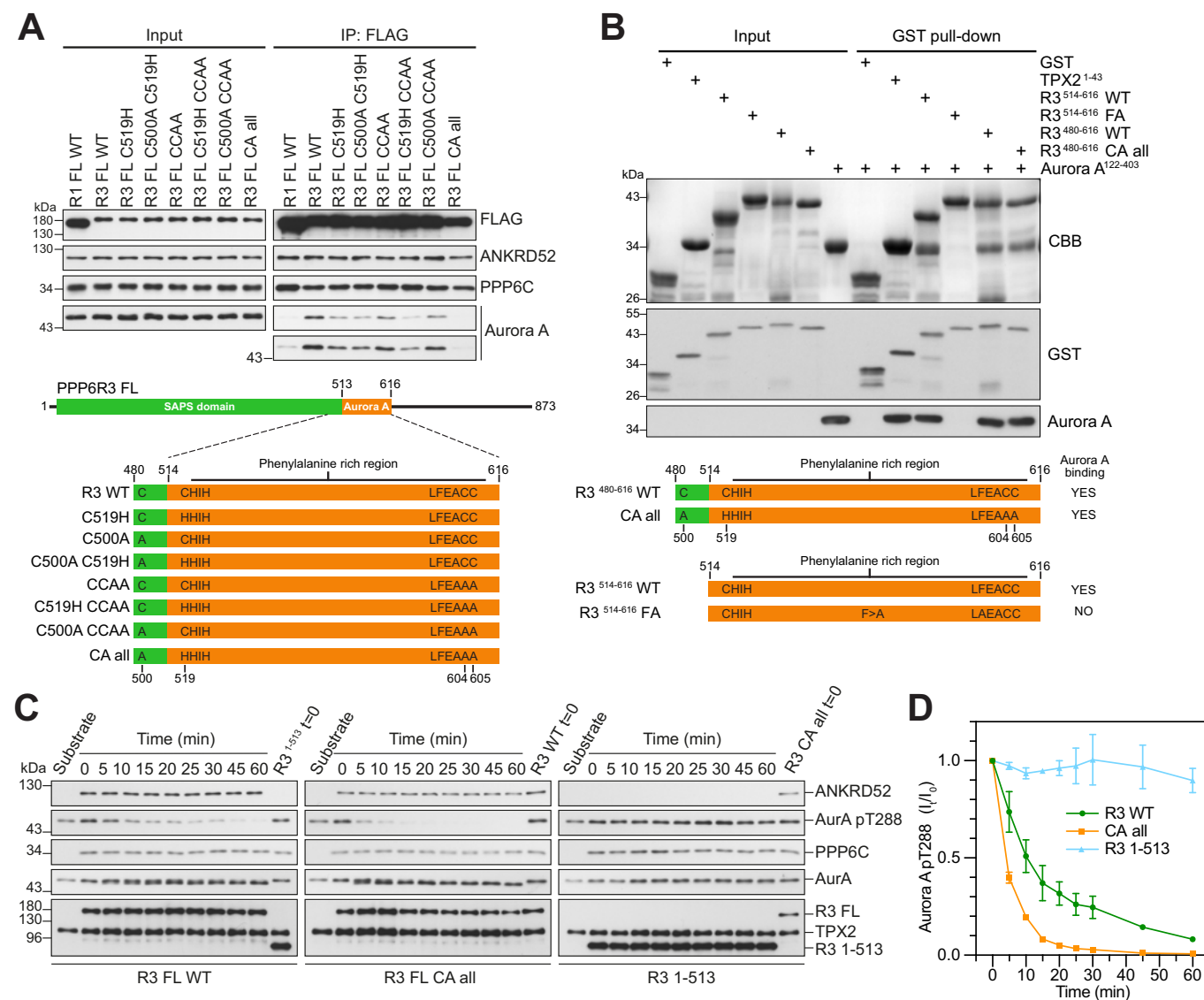


Figure EV5. Mutagenesis of PPP6R3 cysteine patches enhances phosphatase activity.

(A) Full-length FLAG-tagged PPP6R1- and PPP6R3 WT and point mutants as depicted schematically were precipitated from transiently transfected HeLa cells and western blotted. Two different exposure times are shown for Aurora A. (B) In vitro binding assay with recombinant GST-tagged TPX2, GST alone, and R3 wild-type (WT) or mutant (FA and 'CA all') fragments were incubated with recombinant His-tagged Aurora A. Note that R3 aa 480-616 'CA all' carries C500A, C519H, C604A, C605A mutations. Protein complexes were isolated on glutathione-Sepharose 4B beads and analysed by western blotting or CBB staining. (C) In vitro phosphatase assays with PPP6R3 FL WT, 'CA all' mutant and 1-513 fragment. Phosphorylated Aurora A-TPX2 substrate complexes were incubated with PP6-R3 holoenzymes as indicated. Samples were western blotted for Aurora A, Aurora A pT288 and PP6 subunits. (D) Aurora A pT288 signal was quantitated over the time courses shown in (C) and plotted relative to $t = 0$ (mean $\pm$ SEM; $n = 3$ independent experiments).

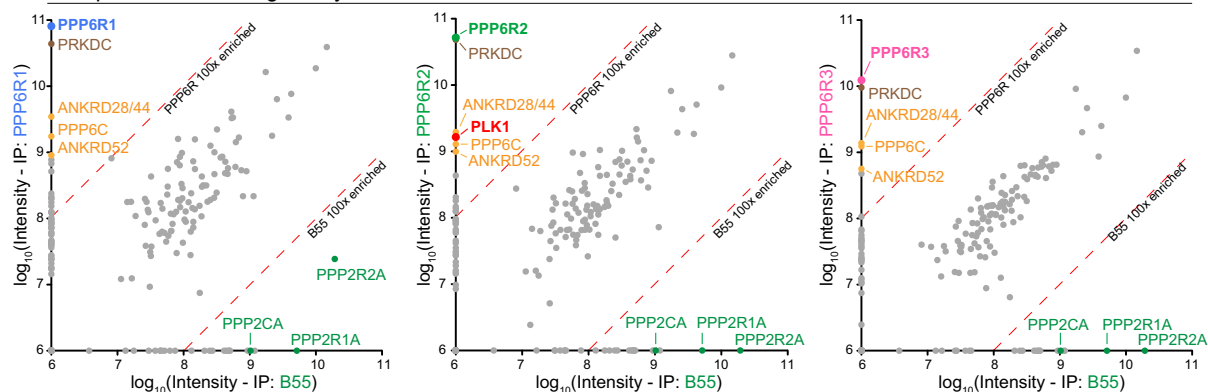
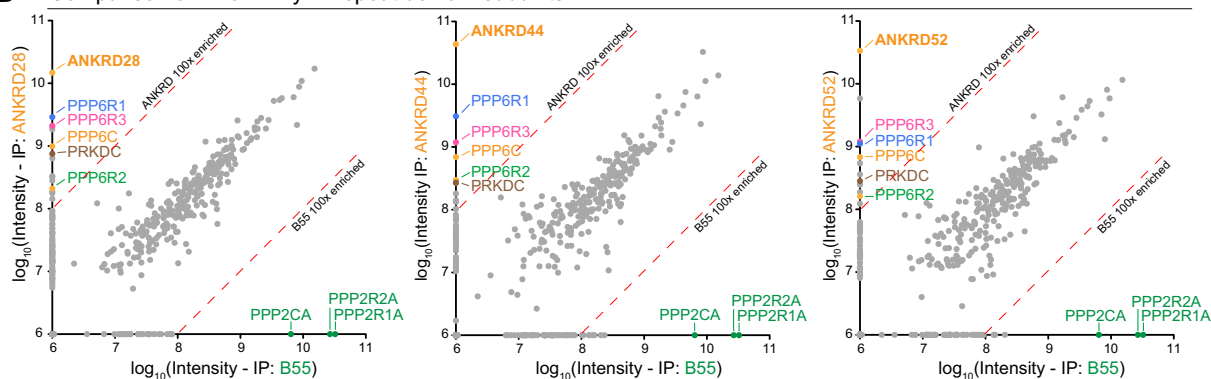
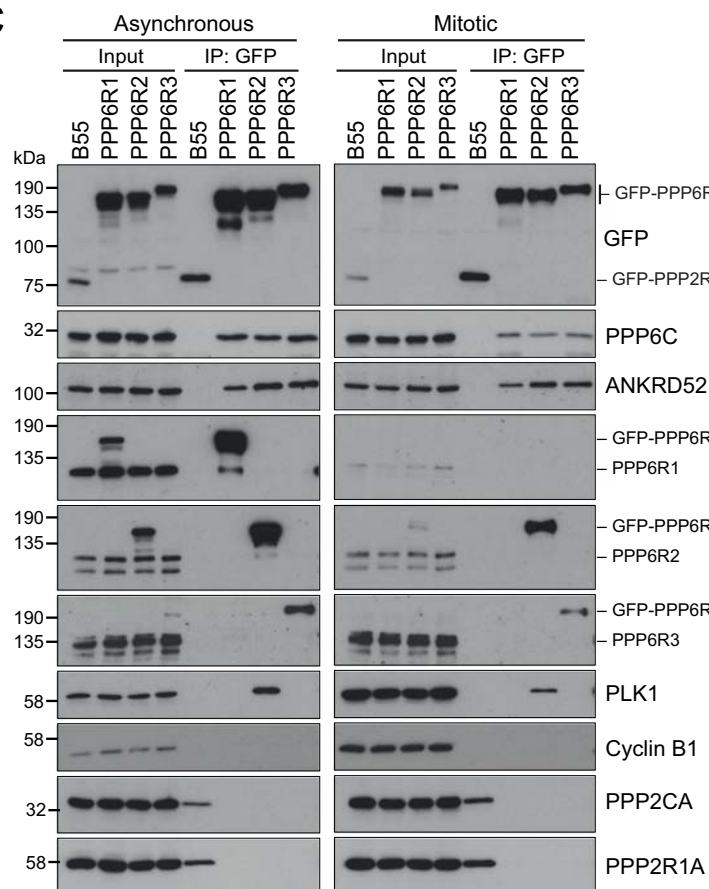
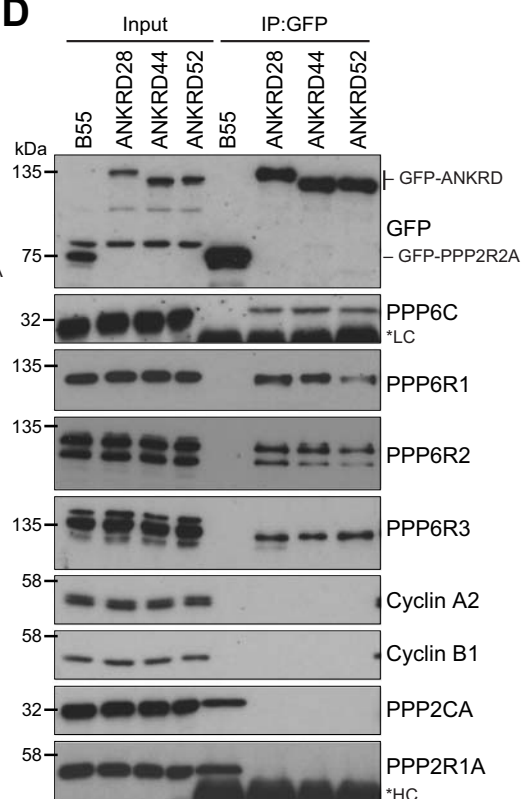
A Comparison of PP6 regulatory subunits**B** Comparison of PP6 Ankyrin-repeat domain subunits**C****D**

Figure EV6. Proteomic analysis of the different PP6 holoenzymes.

(A) HeLa cells were transfected with GFP-tagged PP6 or PP2A-B55 regulatory subunits as indicated for 48 h for asynchronous populations or 36 h before a nocodazole arrest for mitotic populations, followed by lysis, anti-GFP immunoprecipitation and preparation for mass spectrometry. Output data were computationally annotated and protein group intensities found in asynchronous PPP6R1/2/3 were compared to the PP2A-B55 (B55) control IP. Note that missing intensity values for assigned protein groups were replaced with a minimal background value of 6. Candidates were defined as specific if enriched more than 100-fold (2 logarithmic units) over the control PP2A-B55 sample. (B) As in (A), but cells were transfected with FLAG-tagged PP6 ankyrin-repeat domain (ANKRD) subunits and immunoprecipitated by a FLAG antibody. (C) Western blot analysis of the samples as in (A). (D) Western blot analysis of the GFP IP samples from HeLa cells transiently transfected with GFP-tagged ANKRD proteins. LC and HC denote antibody light chain and heavy chain, respectively.

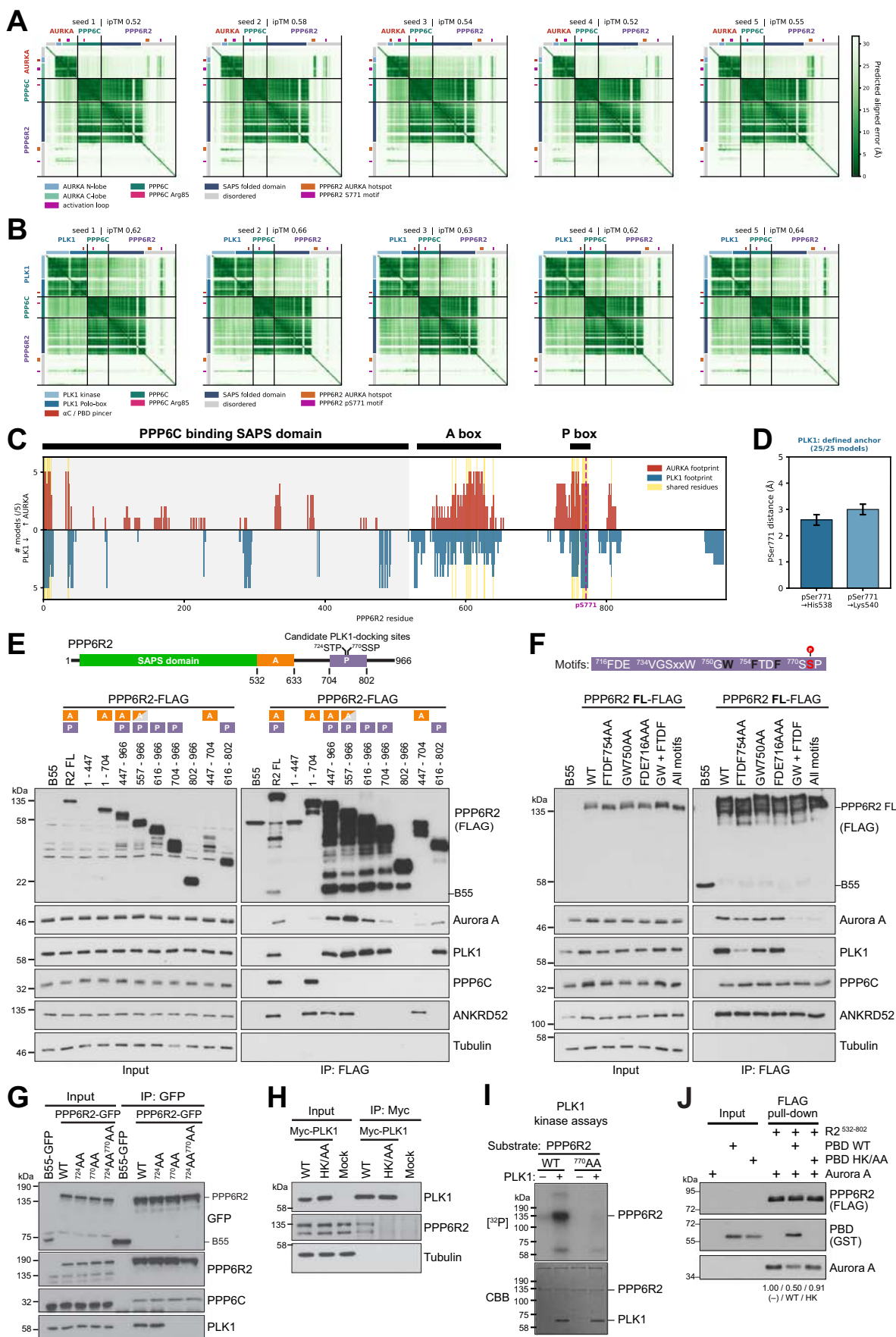



Figure EV7. Mapping the PLK1 and Aurora A binding motifs in PPP6R2.

(A) Extended view data comparing the PPP6R2 interfaces with Aurora A and PLK1. AlphaFold 3 models for PPP6C with PPP6R2 and Aurora A (AURKA) or (B) PLK1 were created using 5 random seeds with 5 models per seed. PAE plots are shown for the top-ranked model for each seed. (C) Graph of Aurora A or PLK1 occupancy along PPP6R2 for the top 5 models for each seed defines common binding epitopes within the A box and P box regions. The PLK1 docking site centered at S771 is marked. (D) PPP6R2 pS771 is captured by the PLK1 polo box domain H538/K540 pincer in all models. (E) A schematic of PPP6R2 showing the P-box and candidate PLK1-docking sites. FLAG-tagged PPP6R2 full-length (R2 FL), R2 fragments indicated by amino acid, or FLAG-B55 as a negative control were transiently transfected into HeLa cells. Complexes were isolated by FLAG IP and analysed by western blotting. (F) Schematic showing conserved residues in the R2 P-box region. FLAG-tagged PPP6R2 full-length (R2 FL) WT and point mutants in conserved residues or FLAG-B55 as a negative control were transiently transfected into HeLa cells. Complexes were isolated by FLAG IP and analysed by western blotting. (G) GFP-tagged PPP6R2 full-length (R2 FL), candidate PLK1 docking-site mutants, or GFP-tagged B55 as a negative control were transiently transfected into HeLa cells. Complexes were isolated by IP with an anti-GFP antibody and analysed by western blotting. (H) Myc-tagged PLK1 wild-type (WT) or “pincer”-mutant (H538A/K540A, HK/AA) were precipitated by an anti-Myc antibody from transiently transfected HeLa cells and probed for PLK1, PPP6R2 and tubulin by western blotting. Anti-Myc immunoprecipitations from mock transfected (empty plasmid) cells were used as controls. (I) PLK1 kinase assays with PPP6R2 WT and ⁷⁷⁰AA. Purified recombinant PPP6R2 proteins were incubated with [³²P]-ATP in the presence or absence of PLK1. (J) Phosphorylated recombinant GFP- and FLAG-tagged PPP6R2⁵³²⁻⁸⁰² was isolated from transiently transfected HEK293T cells using FLAG affinity resin. This purified PPP6R2⁵³²⁻⁸⁰² was prebound with either the wild-type (WT) or H538A/K540A (HK) mutant GST-tagged polo-box domain (PBD) on anti-FLAG antibody beads, and the beads were then incubated with His₆-Aurora A¹²²⁻⁴⁰³. The bead-bound samples were western blotted. The PBD proteins were used at twofold molar excess to R2. Numbers indicate the relative level of Aurora recovered under the different conditions.