Supplementary Fig. 1, related to Fig. 1. Alphafold2 prediction of IER5 structure and PP2A/B55α-IER5 purification for cryo-EM structure determination. A and B, Alphafold2[31] prediction of the IER5 structure, shown in cartoon representation. A, pLDDT coloring of IER5 using alphafold palette: <50, red, very low confidence; 50-70, yellow, low confidence; 70-90, light blue, confident; 90-100, dark blue, very high confidence. B, Cartoon representation of IER5 with the IER-N50 domain colored cyan. The 1-50 region is also highlighted in cyan on the domain representation beneath the cartoon. C, Size exclusion chromatogram of the purified PP2A/B55α-IER5 complex on a Superdex 200 column. An SDS-PAGE gel of the

purified complex is shown to the right of the chromatogram. Both peaks are PP2A/B55α-IER5 complexes; peak 2 was used in all biochemical and structural studies.

Supplementary Fig. 2, related to Fig. 1. Cryo-EM image processing workflow. Cryo-EM processing scheme for PP2A/B55α-IER5 reconstruction. Milestone maps are shown to display progression of processing (the discarded, "junk" class is shown as pink, the monomer as orange, the dimer as blue, and the combined map as purple). The masks used for local refinement are light blue.

Supplementary Fig. 3, related to Fig. 1. Cryo-EM data quality. A, Representative micrograph of PP2A-IER5 in vitreous ice visualized by cryo-EM on a Titan Krios microscope equipped with a Gatan K3 detector. Scale bar indicates 500 Å. B, GS-FSC curves with default CryoSPARC masks. C, Orientation distribution of particles (from CryoSPARC) used in preparing the final map of PP2A/B55α-IER5 for model building. D, 2D class averages of PP2A/B55α-IER5 reconstruction prior to particle subtraction. E, Local resolution map of the final map generated by CryoSPARC (FSC threshold = 0.143). F, map-model FSC curve (line at FSC = 0.5) generated using Phenix[55]. G, Maps around IER5 helix 1 (left), loop (loop) and helix 2 (right). Sigma was set to 3. All maps shown were processed using DeepEMhancer[46].

Supplementary Fig. 4, related to Figs. 1 and 2. Comparison of the PP2A/B55α - IER5 complex with PP2A/B55α structures bound to other partners. A, superposition of PP2A/B55α bound to microcystin-LR[9] (MC-L complex, protein subunits in gray, microcystin-LR as salmon sticks) on the structure of the PP2A/B55α complex with IER5. In the IER5 complex, the B55α subunit is purple, the catalytic subunit is wheat, the scaffolding subunit is green, and IER5 is cyan. Helices are depicted as solid cylinders. Alignment was performed on the B55α subunit. Note the increased curvature of the scaffolding subunit and the 22 Å

displacement of its C terminus in the IER5 structure relative to the microcystin-LR structure. B, Comparison showing the different binding modes of IER5, ARPP19 and FAM122A when bound to PP2A/B55α[11]. Structures are shown in cartoon representation with a transparent surface. The three PP2A subunits are colored as in (A), with IER5 in cyan, ARPP19 in blue, and FAM122A in red. Structures were aligned on the B55α subunit. C, Surface representations of B55α (purple) with bound IER5-N50 (cyan) shown in cartoon representation. On each copy, surface residues of B55α important for IER5-N50 binding or substrate recruitment are painted a different color from left to right: IER5-N50, pink surface; p107[25], blue surface; PRC1[26], yellow surface; pTau[9], green surface.

Supplementary Fig. 5, related to Figs. 1 and 2. A, Size exclusion chromatogram of the purified PP2A/B55α complex on a Superdex 200 column. An SDS-PAGE gel of the purified complex is shown to the right of the chromatogram. The peak at approximately 20 mL elution volume corresponds to the FLAG peptide. B, SDS-PAGE gels of purified MBP-fusions for IER5-N50, IER5-N50 K17E, IER5-FL and IER5-FL K17E.

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776 **Table S1, related to Fig. 5. Predictions of complex structures between IER/SERTAD**

777 **superfamily proteins and PP2A/B55α.** Structures of complexes between IER, SERTAD, and

778 CDCA4 proteins with PP2A/B55α heterotrimers were predicted using alphafold2[31] (Fig. 5) and
779 scored using predictomes[56]. Each column shows a metric used to score the predictive value
780 of the model. Average models indicate the mean number of interface contacts observed for
781 the five models that were generated. The maximum (max) number of models counts how many
782 models share one or more of the predicted contacts in other models. Predicted Dockq
783 (pDOCKq) is a metric that incorporates the alphafold2 pLDDT scores across the predicted
784 protein-protein interaction interface[57]. Predicted local distance difference test (pLDDT), and
785 predicted alignment error (PAE) are alphafold2 metrics[31].

Protein	average models	max number of models	best model	best model pDockQ	best model pLDDT	best model PAE
SRTD4	4.8	5	4	0.713	87.7	1.8
SRTD2	4.7	5	4	0.72	90	1.6
STD1	4.7	5	4	0.722	91.5	1.4
CDCA4	4.6	5	4	0.715	89.9	1.6
IER5	4.5	5	4	0.714	88.1	1.6
IER5L	4.4	5	4	0.714	88.5	1.4
IER2	4.4	5	4	0.713	87.7	1.7
SRTD3	4.4	5	3	0.714	88.1	1.7

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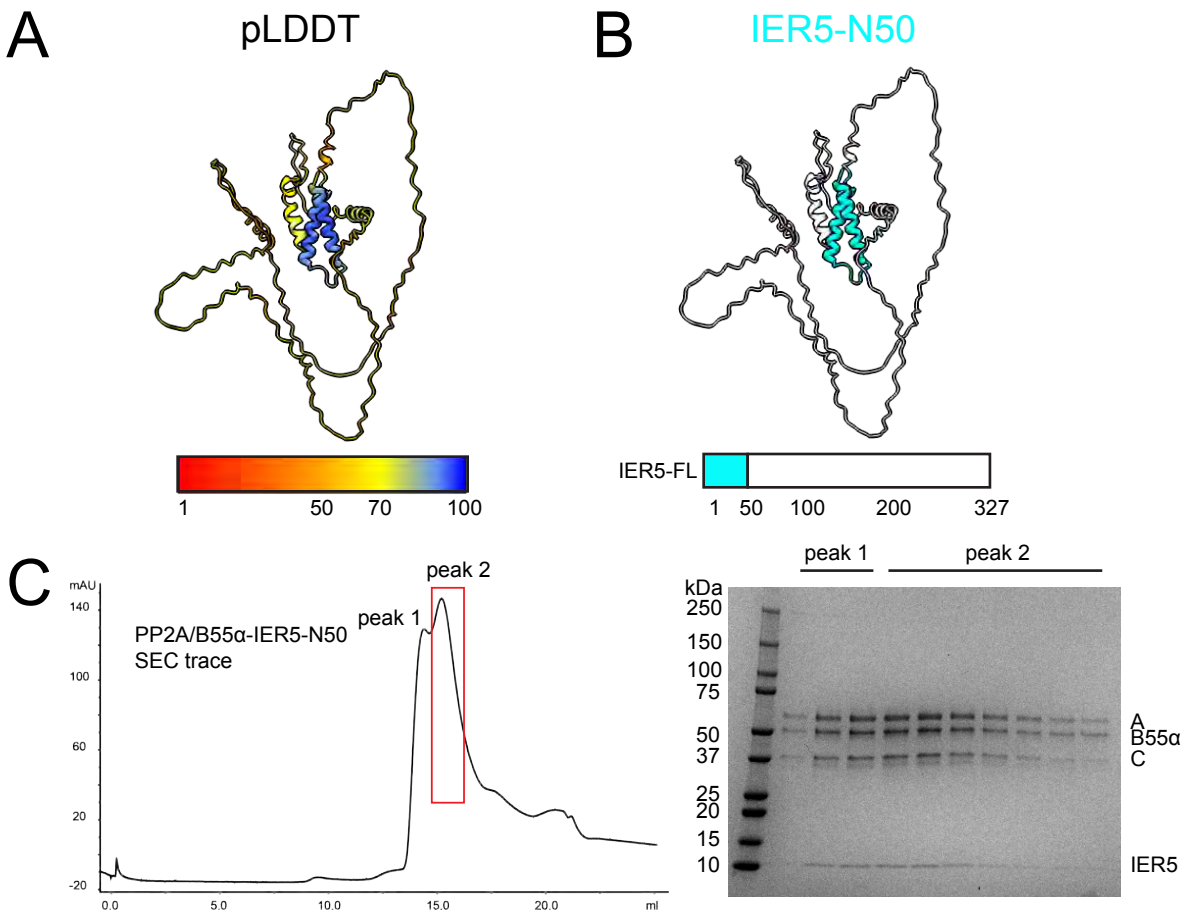

A
pLDDT
1
50
70
100
B
IER5-N50
IER5-FL
1
50
100
200
327
C
PP2A/B55α-IER5-N50
SEC trace
peak 1
peak 2
mAU
140
100
60
20
-20
0.0
5.0
10.0
15.0
20.0
ml
peak 1
peak 2
kDa
250
150
100
75
50
37
25
20
15
10
A
B55α
C
IER5

5383 micrographs

patch motion correction
patch CTF correction

Particle picking
3.95M particles

blob picker

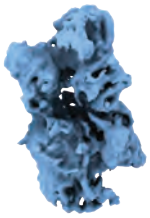
2D classification → 2.77 M junk particles

ab initio (3 classes)
100k particles

heterogeneous refinement → junk class
297k particles

monomer class
552k particles

dimer class
317k particles

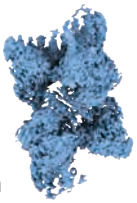


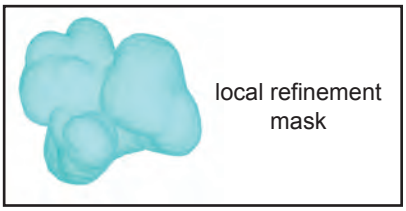
non-uniform refinement (3.78 Å)
local motion correction
non-uniform refinement (3.67 Å)

monomer map (C1)

non-uniform refinement (3.88 Å)
local motion correction
non-uniform refinement (3.61 Å)
non-uniform refinement [C2] (3.44 Å)
local motion correction
non-uniform refinement x 2 [C2] (3.17 Å)

dimer map (C2)





symmetry expansion
630k particles

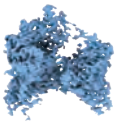
masked non-uniform refinement (3.82 Å)

map as input

local refinement (3.04 Å)

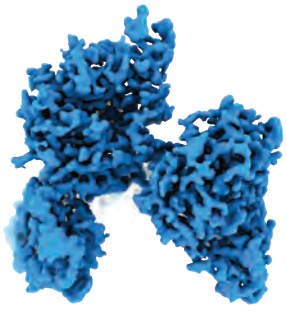
monomer particles aligned to symmetry expanded dimer map

symmetry expanded dimer map

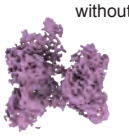


combine particles

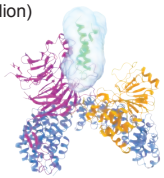
local refinement

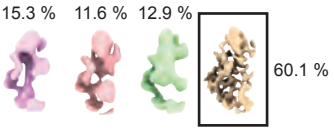


Masked 3D classification without alignment (Relion)



combined map
3.09 Å



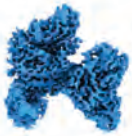


99k particles

738k particles

Particle subtraction on alternate copy of PP2A-IER5

Local refinement on alternate copy of PP2A-IER5

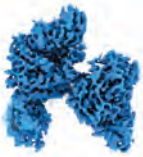


cryosieve and local refinement

particles

deepemhanced

67k particles



rebalance orientations and local refinement

local refinement

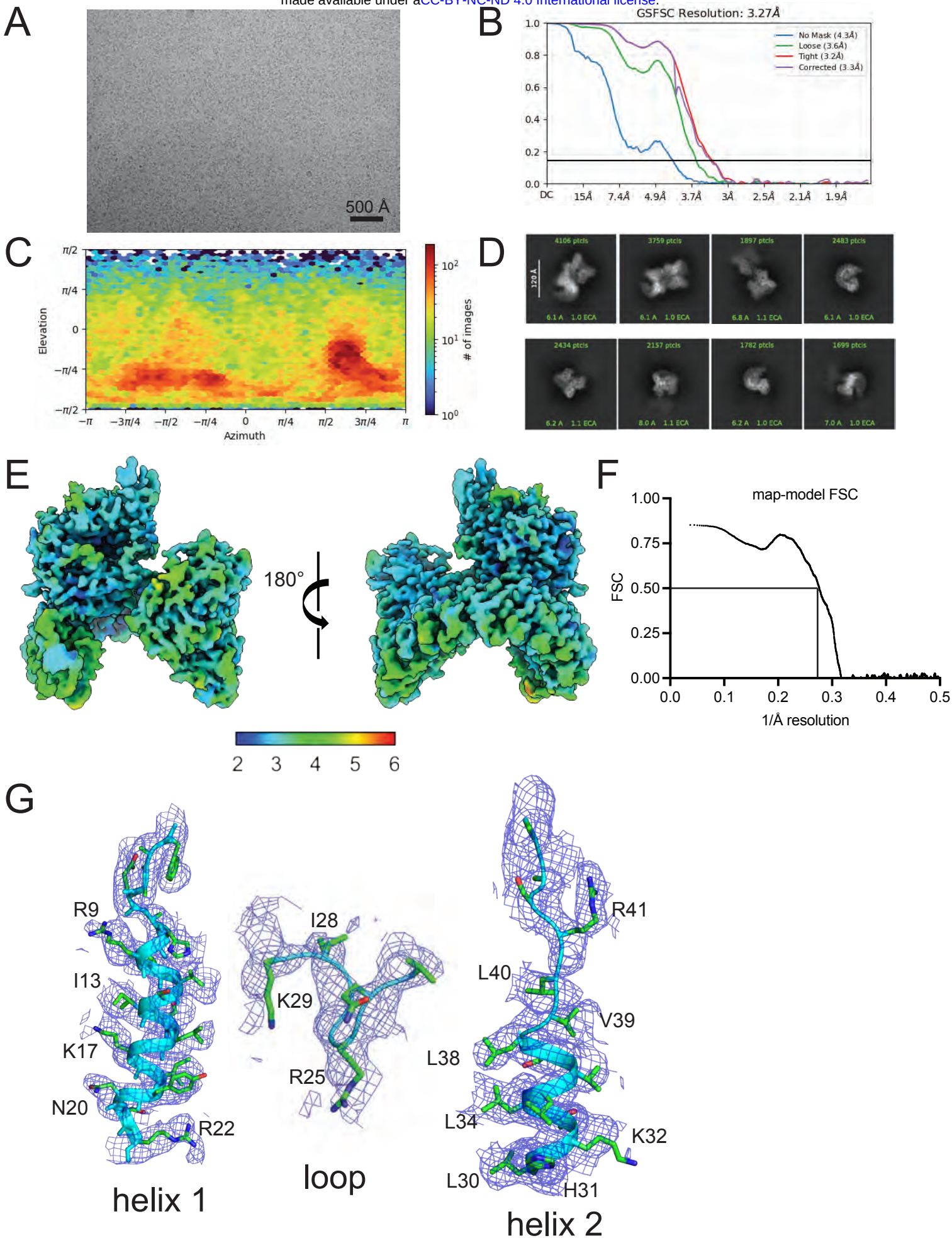
Cryosieved map
3.14 Å

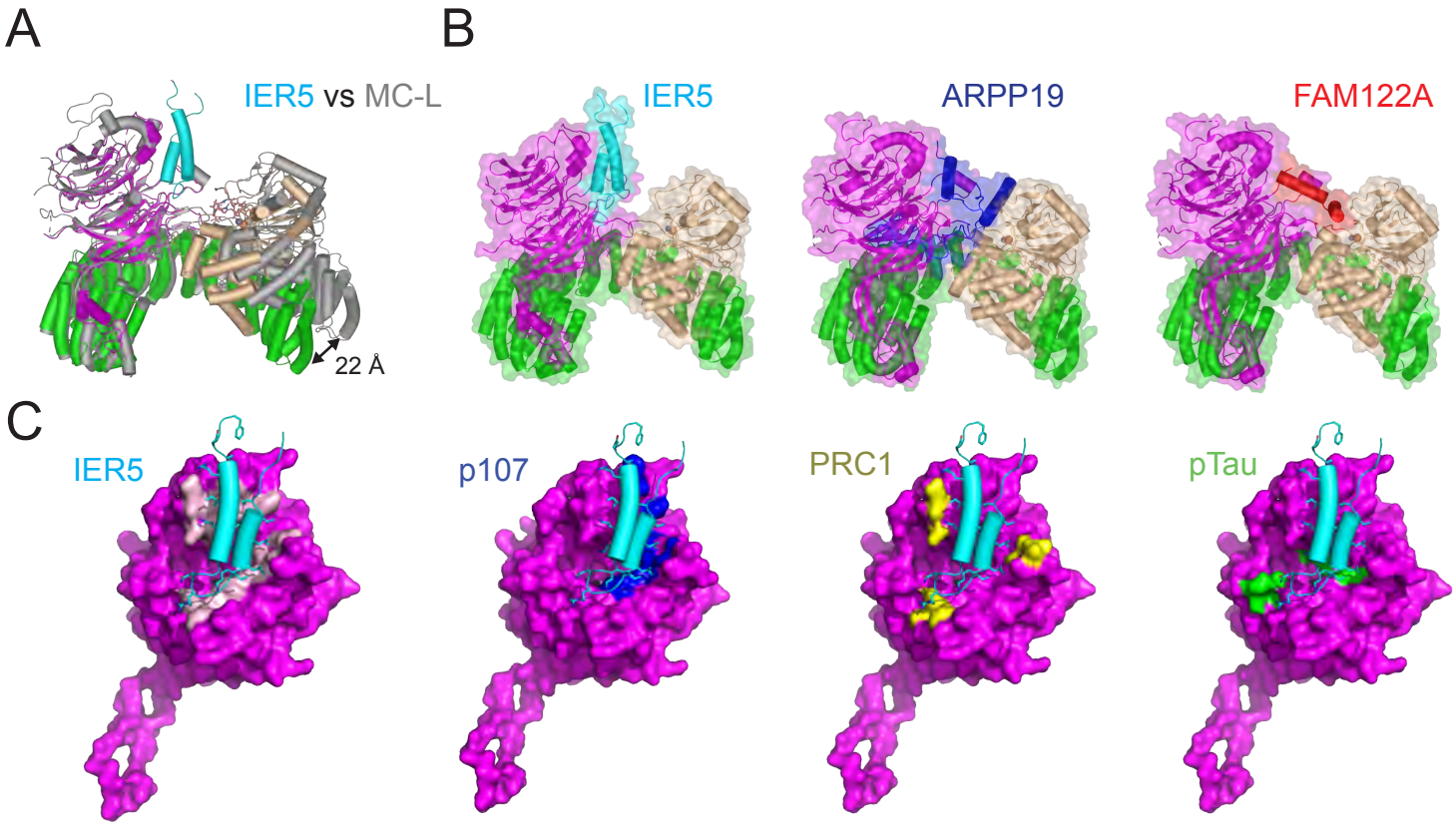
IER5 focused map
3.15 Å

alignments

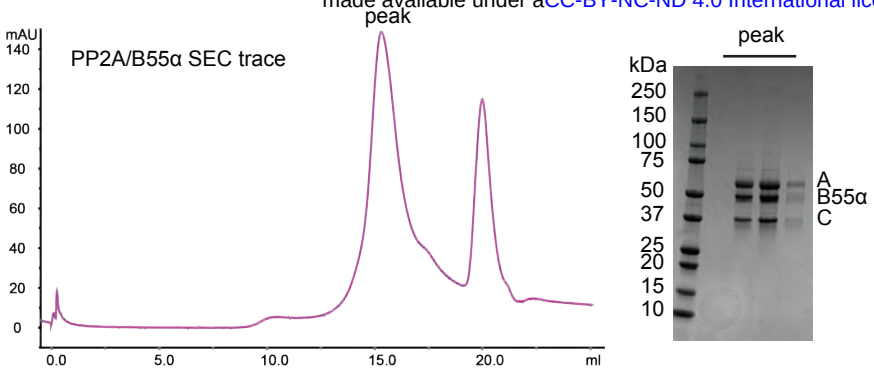
3.27 Å

3.18 Å





A



B