

# **Structural insights into oncoprotein CIP2A and its stabilization via interaction with PP2A/B56**

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28 **Abstract**

29

30

31 Protein phosphatase 2A (PP2A) is a critical tumor suppressor, inhibition of  
32 which promotes various malignant characteristics of human cancer cells.  
33 PP2A inhibitor protein Cancerous Inhibitor of PP2A (CIP2A) is involved in  
34 progression of most human cancer types by supporting activities of several  
35 critical cancer drivers. Critically, 3D structure of CIP2A has not been solved,  
36 and it remains enigmatic how it interacts with PP2A. Here, we discover by  
37 yeast-two-hybrid assay and subsequent validation experiments that CIP2A  
38 forms a homodimer. CIP2A homodimerization was confirmed by solving the  
39 crystal structure of CIP2A fragment 1-560 at 3.0 Å resolution, and by  
40 subsequent structure-based mutational analysis of dimerization interface. We  
41 further discover that CIP2A dimer interacts with PP2A B56 $\alpha$  and B56 $\gamma$  tumor  
42 suppressor subunits. CIP2A binds to B56 proteins via N-terminal conserved  
43 region, but importantly CIP2A dimerization promotes B56 binding. Intriguingly,  
44 inhibition of either CIP2A dimerization or B56 $\alpha/\gamma$  expression destabilized  
45 CIP2A protein in cancer cells, indicating opportunities for controlled  
46 degradation regulation. Together, these results provide the first structure-  
47 function analysis of CIP2A interaction with the PP2A/B56 tumor suppressor  
48 with direct implications to targeting of CIP2A for cancer therapy.

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51

## 52 **Introduction**

53

54 Protein phosphatase 2A (PP2A) is a critical tumor suppressor that normally  
55 acts by preventing cellular transformation, whereas its inhibition promotes the  
56 various malignant characteristics of human cancer cells (Perrotti & Neviani,  
57 2013, Westermarck & Hahn, 2008). PP2A also regulates various physiological  
58 processes. Thereby, further understanding of structural mechanisms of PP2A  
59 regulation is highly relevant for various disciplines. In cancer cells, PP2A  
60 inhibition results in hyperphosphorylation of a large number of oncogenic  
61 drivers and synergizes with other oncogenic events, such as constitutive RAS  
62 activity (Hahn & Weinberg, 2002, Naetar, Soundarapandian et al., 2014,  
63 Westermarck & Hahn, 2008, Zhao, Gjoerup et al., 2003). Importantly, PP2A  
64 complex components are mutated at a relatively low frequency in most types  
65 of human cancer. This establishes the reactivation of PP2A as an attractive  
66 novel approach in cancer therapy (Khanna, Pimanda et al., 2013b, Perrotti &  
67 Neviani, 2013). Furthermore, the recent discovery of small molecules and  
68 peptides that are capable of restoring PP2A activity in human cancer cell lines  
69 provides convincing support to this strategy by demonstrating robust *in vivo*  
70 efficacy in preclinical studies (Farrell, Allen-Petersen et al., 2014, Perrotti &  
71 Neviani, 2013).

72

73 PP2A is inhibited in cancer by a group of otherwise unrelated PP2A inhibitor  
74 proteins (Lambrecht, Haesen et al., 2013, Perrotti & Neviani, 2013). Among  
75 them, Cancerous inhibitor of PP2A (CIP2A) is the most prevalent oncoprotein.

76 CIP2A is a long-lived protein in cancer cells (Tseng, Liu et al., 2012), and its  
77 depletion results in inactivation of many oncogenic PP2A targets (e.g., MYC,  
78 E2F1, Akt) (Khanna et al., 2013b). Importantly, these effects have been  
79 shown to be reversible upon PP2A co-inhibition (Junttila, Puustinen et al.,  
80 2007, Khanna et al., 2013b, Laine, Sihto et al., 2013, Niemelä, Kauko et al.,  
81 2012). Regarding functional synergism between PP2A inhibition and RAS  
82 signaling upon cell transformation, and cell cycle progression (Hahn &  
83 Weinberg, 2002, Naetar et al., 2014, Westermarck & Hahn, 2008, Zhao et al.,  
84 2003), CIP2A overexpression is required for RAS-driven human cell  
85 transformation (Junttila et al., 2007). Moreover, we recently demonstrated  
86 significant overlap between CIP2A and RAS-regulated phosphoproteomes  
87 (Kauko, 2015). Clinically, CIP2A overexpression is an equally strong predictor  
88 of poor survival in TCGA pan-cancer data as KRAS mutation, and  
89 corroborating the functional synergism between CIP2A and RAS, the patients  
90 with both of these alterations constituted the patient population with clearly the  
91 worst outcome (Kauko et al., 2015).

92

93 In addition to robust effects of CIP2A depletion by siRNA on malignant cell  
94 growth *in vitro* (Khanna et al., 2013b), several studies have demonstrated that  
95 CIP2A inhibition very potently inhibits xenograft tumor growth of different  
96 types of cancer cells (Come, Laine et al., 2009, Junttila et al., 2007, Liu, Qiu  
97 et al., 2016, Liu, He et al., 2014, Ma, Wen et al., 2011, Xue, Wu et al., 2013).  
98 CIP2A also mediates resistance to many cancer therapeutics (Khanna, Kauko  
99 et al., 2013a, Khanna & Pimanda, 2015, Khanna et al., 2013b, Laine et al.,  
100 2013, Lucas, Harris et al., 2015, Tseng et al., 2012). Importantly, even though

101 CIP2A deficiency inhibits MYC activity and Her2-driven mammary  
102 tumorigenesis *in vivo* (Laine et al., 2013, Myant, Qiao et al., 2015), it does not  
103 compromise normal mouse development or growth, except for a defect in  
104 spermatogenesis (Laine et al., 2013, Myant et al., 2015, Ventela, Come et al.,  
105 2012). Notably, high CIP2A protein expression predicts poor patient survival  
106 in over a dozen different cancer types (Khanna & Pimanda, 2015), and thus  
107 its prognostic and functional relevance equals, or exceeds, that of most  
108 oncoproteins that have been traditionally considered important oncogenic  
109 drivers. Based on all this data, inhibition of CIP2A protein expression and/or  
110 activity could constitute a very efficient cancer therapy strategy without  
111 detrimental side effects. However, a lack of structural information for the  
112 CIP2A protein has thus far hampered the advancement of this potential  
113 cancer therapy target in drug development.

114

115 PP2A functions as a protein complex consisting of either a core dimer  
116 between the scaffolding A subunit (PR65) and the catalytic subunit PP2Ac, or  
117 a trimer in which one of the regulatory B subunits interacts with the AC core  
118 dimer (Sents, et al., 2013). Our current understanding supports the view that  
119 different B subunits mediate the substrate specificity of the PP2A trimer  
120 (Sents et al., 2013), and that only a subset of the numerous B subunits are  
121 relevant for the tumor suppressor activity of PP2A (Eichhorn, Creighton et al.,  
122 2009, Sablina, Hector et al., 2010). For example, B56 $\alpha$  mediates PP2A  
123 complex recruitment and the PP2A-mediated dephosphorylation of MYC  
124 serine 62 (Arnold & Sears, 2006, Yeh, Cunningham et al., 2004). Another B56  
125 family protein, B56 $\gamma$ , functions as a human tumor suppressor (Eichhorn et al.,

126 2009, Sablina et al., 2010) and negatively regulates Akt kinase  
127 phosphorylation (Rocher, Letourneux et al., 2007, Sablina et al., 2010). CIP2A  
128 has been shown to promote phosphorylation and activity of both of these  
129 critical PP2A targets (Junttila, Puustinen et al., 2007, Khanna et al., 2013b,  
130 Ma et al., 2011, Niemelä et al., 2012, Tseng et al., 2012). However, thus far  
131 there has not been any evidence of whether CIP2A would directly bind to any  
132 of the numerous PP2A complex components.

133

134 Here, we present the first crystal structure of CIP2A and reveal that CIP2A  
135 binds to PP2A B56 $\alpha$  and B56 $\gamma$  tumor suppressor subunits directly. Both the  
136 CIP2A N-terminal region and CIP2A dimerization contribute to maximal B56  
137 binding. We further show that B56 binding determines CIP2A protein stability  
138 in human cell lines. Together these results provide important insights to poorly  
139 understood oncogenic protein CIP2A, and may help designing approaches for  
140 inhibiting CIP2A protein expression for cancer therapy.

141

## 142   **Results**

143

### 144   **CIP2A homodimerization**

145

146   Our understanding of proteins that interact with CIP2A is limited (Pallai,  
147   Bhaskar et al., 2015). Thereby we conducted a yeast-two-hybrid (Y2H)  
148   analysis with full-length CIP2A as bait, and using commercial Hybrigenics  
149   platform with over  $80 \times 10^6$  prey clones (Figure EV1A). As CIP2A is  
150   expressed at a very low level in most normal tissues but is overexpressed in  
151   breast cancer (Laine et al., 2013, Niemelä et al., 2012, Tseng et al., 2012), we  
152   used a mixed cDNA library from several breast cancer cell lines (T47D, MDA-  
153   MB-468, MCF7, BT20). Using Hybrigenics Global Predicted Biological Score  
154   (Global PBS<sup>®</sup>) computational platform that scores probability of an interaction  
155   to be specific, we surprisingly found CIP2A itself as a very high confidence  
156   interaction partner for full-length CIP2A bait (Figure EV1B). The various  
157   CIP2A prey clones that interacted with full-length CIP2A bait are depicted as  
158   green bars in figure 1A. Number of independent interacting CIP2A prey clones  
159   allowed Selected Interaction Domain (SID) analysis that delineates the  
160   shortest fragment that is shared with all interacting clones, and thus  
161   represents a potential region mediating the CIP2A homodimerization. The SID  
162   analysis for interaction between two CIP2A molecules indicated that CIP2A  
163   homodimerization is mediated by a region encompassing amino acids 388-  
164   558 (Fig. 1A and Figure EV1C). Interestingly, structural foldability (flexibility)  
165   analysis indicates that this potential homodimerization domain of CIP2A  
166   comprises of a well-folded domain that is followed by a flexible linker and a

167 predicted coiled-coil domain that is most likely disordered (Appendix Figure  
168 S1). This prediction was supported by notification that, based on gel filtration  
169 analysis, the C-terminal fragment *per se* tends to aggregate (results not  
170 shown). Also, consistent with an earlier publication (Soo Hoo, Zhang et al.,  
171 2002), full-length CIP2A is not stable enough to be purified from either *E. coli*  
172 or insect cells (results not shown). In contrast, the N-terminal 1-560 fragment  
173 of CIP2A spanning the SID could be produced in *E. coli* in large quantities,  
174 and was relatively stable. Therefore, we focused on the human CIP2A(1-560)  
175 fragment to further confirm CIP2A homodimerization.

176

177 To biochemically verify CIP2A homodimerization, we used thrombin  
178 cleavage to remove the GST tag from GST-CIP2A(1-560)-V5 and used this as  
179 prey in a GST pull-down experiment with the parental GST-CIP2A(1-560)  
180 protein. Using V5 epitope antibody in western blot analysis of GST pull-down  
181 samples, CIP2A(1-560)-V5 was found to not significantly associate with GST  
182 alone, whereas a robust interaction was observed between the two CIP2A  
183 fragments (Fig. 1B). Cleavage of GST tag from GST-CIP2A(1-560)-V5 in the  
184 previous assay excluded the possibility that the observed CIP2A dimerization  
185 would be mediated by GST dimerization. However, to further exclude the  
186 possibility that interaction was mediated due to dimerization via the affinity  
187 tags, CIP2A dimerization was further demonstrated by Coomassie staining of  
188 gel after pull-down of CIP2A(1-560) without any tags (Fig. 1C). Furthermore,  
189 our size-exclusion chromatography-coupled multi-angle light scattering (SEC-  
190 MALS) analysis clearly show that purified untagged CIP2A(1-560) has a  
191 shape-independent molecular mass of 117.2 kD in solution, which is in good

192 agreement with the calculated MW of 124.5 kD for a CIP2A(1-560) dimer (Fig.  
193 1D).

194

195         Interestingly, in addition to confirming the CIP2A homodimerization  
196 indicated by the Y2H assay, the ability to detect dimerization between two  
197 different CIP2A proteins in pull-down assays suggests that binding affinity of  
198 the dimerization interface may be relatively modest, and that there is  
199 detectable exchange between interacting monomers. This conclusion is  
200 supported by results of MicroScale Thermophoresis (MST) analysis revealing  
201 that CIP2A(1-560) homodimerizes with a modest affinity (K<sub>d</sub>) of 290 nM (Fig.  
202 1E). Whether the C-terminal sequences lacking from CIP2A 1-560 might  
203 further stabilize the dimer, remains to be studied. On the other hand, many  
204 Y2H prey clones of CIP2A that interacted with full-length CIP2A bait contained  
205 long stretches of amino acids C-terminally from amino acid 560 (Figure 1A).  
206 This clearly indicates that the homodimerization region included in CIP2A(1-  
207 560) is functional also in the presence of C-terminal regions of CIP2A. To  
208 further verify dimerization of full-length CIP2A containing those C-terminal  
209 sequences, we analyzed physical interaction between two differentially  
210 epitope-tagged full-length CIP2A proteins in cells by proximity ligation assay  
211 (PLA). PLA has been validated recently by numerous studies to detect  
212 protein-protein interaction in cultured cells and *in vivo* (Myant et al., 2015,  
213 Weibrecht, Leuchowius et al., 2010). Here, by using V5 and GFP antibodies  
214 coupled with specific PLA probes, we could detect typical PLA dots clearly  
215 indicative of interaction between co-transfected CIP2A-V5 and EGFP-CIP2A  
216 fusion proteins (Fig. 1F, left panel). On the other hand, only random

217 background PLA signals were observed from non-transfected cells with  
218 primary V5 and GFP antibodies, or from CIP2A-V5 and EGFP-CIP2A co-  
219 transfected cells subjected to PLA without primary antibodies (Fig. 1F).

220 To further establish dimerization of endogenous CIP2A we subjected  
221 HeLa cell extracts to size exclusion chromatography. Consistently with all  
222 other results, this analysis clearly showed that both in whole cell lysate, and  
223 in cytoplasmic soluble fraction, CIP2A monomer (appr. 90 kDa) is a minor  
224 fraction of total cellular CIP2A pool, whereas majority of CIP2A is found on  
225 both dimer (appr. 150-200 kDa), and higher molecular weight complex (appr.  
226 > 440 kDa) (Fig. 1G). This result further supports our conclusions that CIP2A  
227 is an obligate dimer.

228

229 These results reveal that CIP2A homodimerizes, and suggest that the  
230 dimerization is mediated by a region containing amino acids 338-558.

231

### 232 **Crystal structure of CIP2A(1-560) reveals the homodimerization interface**

233

234 To date, no structural information about CIP2A is available. In order to gain  
235 structural insights into CIP2A dimerization, the CIP2A(1-560) fragment was  
236 crystallized, and its crystal structure was determined at 3.0 Å resolution using  
237 the selenium-methionine single-wavelength anomalous scattering (SAD)  
238 method (Appendix Table S1 and Appendix Figure S2). In the crystal lattice,  
239 there are two CIP2A(1-560) molecules, related by a non-crystallographic 2-  
240 fold axis, in each asymmetric unit (Fig. 2A). This finding is fully consistent both  
241 with Y2H data and with biochemical data, that CIP2A(1-560) forms a

242 homodimer. Moreover, in the crystal structure, the dimer interface joining two  
243 CIP2A(1-560) molecules is located in the C-terminal end of CIP2A(1-560)  
244 which also is fully in line with Y2H SID prediction that postulated the  
245 dimerization domain to be located in the region 338-558 of CIP2A. Overall,  
246 the CIP2A(1-560) dimer structure resembles an oppositely-twisted double  
247 hook (Fig. 2A).

248

249       The CIP2A(1-560) monomer is an all-helical protein, with most of the  
250 molecules formed by armadillo or armadillo-like repeats (Fig. 2B), and can be  
251 roughly divided into “tip”, “stem” and C-dimerization subdomains. The first 185  
252 residues form a “tip” domain consisting of 5 shortened armadillo repeats.  
253 Following a twist-forming loop, residues 188-505 form the “stem” domain,  
254 consisting of atypical armadillo repeats 6-11; residues 507-559 form three  
255 helices that are responsible for CIP2A(1-560) dimerization (Fig. 2B). Some of  
256 the armadillo repeats in the “stem” subdomain display the structural features  
257 of HEAT repeats, as revealed by protein folding similarity searches using the  
258 Dali server (Holm & Sander, 1997). In addition to the armadillo repeat  
259 domains of  $\beta$ -catenin and APC, the atypical HEAT-repeat domain of Wapl is  
260 among the closest structural neighbors of the stem subdomain of CIP2A(1-  
261 560) (Appendix Table S1 and Appendix Figure S3).

262

### 263 **Mutational analysis of CIP2A(1-560) dimerization interface**

264

265       The dimerization subdomain is formed by the last three helices of  
266 CIP2A(1-560) (Fig. 3A,B). The last two helices and the loop link to the

267 previous helix to form a relatively flat and highly hydrophobic surface,  
268 mediating the homodimerization of CIP2A(1-560) (Fig. 3A,B). Formation of  
269 this homodimer interface buries an accessible surface area of 1913 Å<sup>2</sup>, which  
270 is typical for specific protein-protein interactions. The two C-terminal ends of  
271 the CIP2A(1-560) homodimer are spatially very close to each other, and both  
272 point to the “top” side of the twisted double hook (Figs. 2A and 3A). The key  
273 residues involved in the interaction between CIP2A monomers include V525,  
274 L529, L532, L533, L546 and I550 (Fig. 3C), and all these residues, with the  
275 exception of L533, are evolutionarily conserved across different species  
276 (Appendix Figure S4).

277         To interfere with the CIP2A homodimerization interface, we introduced  
278 series of single point mutations to residues that were directly involved in in the  
279 interaction between CIP2A monomers, or were predicted to potentially  
280 interfere with dimerization, and examined their impact on CIP2A dimerization.  
281 All created mutations are depicted in Figure EV2. While some of these CIP2A  
282 mutants, especially the ones with multiple mutations, had low solubility that  
283 prohibited further *in vitro* test, two soluble single point mutants, R522D and  
284 L533E, repeatedly demonstrated significantly impaired dimerization across six  
285 independent assays (Fig. 3D and E). L533 is directly involved in the  
286 interaction surface between CIP2A monomers (Fig. 3C and F). Its substitution  
287 by a bulky negatively charged amino acid is therefore likely to destabilize the  
288 dimerization interface. On the other hand, mutation of another conserved  
289 residue, arginine 522, to a negatively charged aspartate can be predicted to  
290 interfere with dimerization by steric and/or electrostatic clashes with the  
291 proximal residues such as E523 (Fig. 3F), which also is a strictly conserved

292 residue throughout evolution (Appendix Figure S4). Notably, the mode of  
293 interference in dimerization by these mutants was reflected with their potency  
294 on reducing pulled-down parental CIP2A(1-560)-V5 protein; L533E inhibited  
295 dimerization by up to 70%, whereas R552D being not directly involved in  
296 interaction surface caused approximately 50% inhibition (Fig. 3D,E).

297       These results reveal that previously unappreciated homodimerization  
298 of CIP2A is mediated by an evolutionary conserved three-helix subdomain  
299 (residues 507-559), which form a planar interaction surface.

300

### 301 **CIP2A directly interacts with PP2A B56 tumor suppressor subunits**

302

303       Regardless of functional evidence that PP2A inhibition mediates  
304 CIP2A's oncogenic effects (Junttila, Puustinen et al., 2007, Khanna et al.,  
305 2013b, Laine et al., 2013, Niemelä et al., 2012), no evidence for direct  
306 interaction between CIP2A and any of the PP2A complex components have  
307 been demonstrated as yet.

308       Importantly, in addition to CIP2A homodimerization, we identified PP2A  
309 B subunit B56 $\gamma$  (PPP2R5C) as one of the direct interaction partners of full-  
310 length CIP2A by Y2H assay (Figure EV1). On the other hand, Y2H analysis  
311 did not reveal direct interaction between CIP2A and scaffolding A-subunit, or  
312 catalytic C-subunit. Direct binding of CIP2A to B56 $\gamma$  is very exciting result, as  
313 together with B56 $\alpha$ , B56 $\gamma$  has been shown to be one of the most important  
314 tumor suppressor B subunits (Eichhorn et al., 2009, Sablina et al., 2010). To  
315 verify these results, the CIP2A(1-560) was demonstrated to interact directly  
316 with both B56 $\gamma$  and B56 $\alpha$  in a GST pull-down experiment (Fig. 4A). The

317 interaction between CIP2A and B56 $\gamma$  and B56 $\alpha$  was confirmed by MST  
318 analysis, allowing the determination of approximate K<sub>d</sub> values for these  
319 interactions (Fig. 4B). We further verified that full-length CIP2A interacts with  
320 B56 $\alpha$  and B56 $\gamma$  by PLA in HEK293T cells either co-transfected with HA-  
321 tagged versions of B56 proteins and CIP2A-V5 (Fig. 4C left panel and  
322 Appendix Figure S5A), or between endogenous CIP2A and B56 (Fig. 4C).  
323 Control PLA without primary antibodies from parallel samples did not show  
324 any background signals (Fig. 4C right panel and Appendix Figure S5B).

325

326 Biochemical characterization of CIP2A dimerization, including  
327 determination of modest affinity (K<sub>d</sub>) between monomers, indicated that there  
328 most probably exists equilibrium between monomeric and dimer form of  
329 CIP2A(1-560) in solution. Therefore, we wanted to assess whether CIP2A  
330 homodimer or monomer form of CIP2A bind to B56 proteins. To this end,  
331 recombinant GST or GST-CIP2A(1-560) were incubated with B56 $\alpha$  and the  
332 protein complexes were analyzed by size exclusion chromatography. In the  
333 presence of GST alone, both GST and B56 $\alpha$  eluted in separate fractions that,  
334 based on column calibration, corresponded to their expected molecular  
335 weights (Fig. 4D). This further excludes direct GST-tag mediated binding  
336 between B56 $\alpha$  and CIP2A. Consistent with SEC-MALS analyses and other  
337 biochemical evidence for CIP2A dimerization, GST-CIP2A(1-560) was mostly  
338 eluted in fraction 3 (corresponding to approximate size of 158 kDa; Fig. 4D).  
339 Importantly, in the presence of GST-CIP2A(1-560), there was a clear shift in  
340 elution of B56 $\alpha$  toward fractions 2 and 3, and also CIP2A elution pattern  
341 shifted more towards fraction 2 corresponding to higher molecular weight

342 complex containing B56 $\alpha$  and GST-CIP2A(1-560) dimer (Fig. 4D). Based on  
343 the results, we rationalized that CIP2A dimerization may make an important  
344 contribution to maximal binding to B56 $\alpha$ . In order to directly test this, the  
345 CIP2A(1-560) dimerization compromised mutant L533E was compared with  
346 wild-type CIP2A(1-560) for B56 $\alpha$  binding by GST pull-down assay. In line with  
347 our hypothesis, L533E mutant showed significantly reduced binding to B56 $\alpha$   
348 (Fig. 4E,F). Although this data do suggest that CIP2A dimerization may  
349 enhance CIP2A binding to B56 $\alpha$ , we wanted to further test whether the  
350 “weaker” dimerization mutant R522D would also show impaired B56 binding,  
351 and whether degree of inhibition of dimerization, and B56 binding, would show  
352 any correlation between the two mutants. Indeed, also R522D did show  
353 weaker binding to B56 $\alpha$  than wild-type CIP2A (Fig. 4E,F). Importantly  
354 quantification of four independent experiments demonstrated that significantly  
355 lowered capacity of dimerization mutants to bind to B56 $\alpha$  correlated with their  
356 reduced capacity to dimerize (Fig. 4E,F). To estimate the contribution of  
357 dimerization to maximal B56 $\alpha$  binding capacity of CIP2A, we calculated the  
358 ratio between observed effects on both dimerization and B56 $\alpha$  binding.  
359 Notably, both mutants showed comparable approximately 50% contribution of  
360 dimerization to B56 $\alpha$  binding in our assay conditions (Fig. 4G). This supports  
361 the conclusion that B56 $\alpha$  binding defect observed with these mutants is  
362 caused by similar mechanism, i.e. inhibition of dimerization.

363

#### 364 **Identification of N-terminal B56 binding region of CIP2A**

365

366 Result that CIP2A dimerization mutants still retain ~50% B56-binding activity  
367 (Fig. 4G), indicates that other regions of CIP2A might harbor a primary B56  
368 binding site, whereas the role of CIP2A dimerization could be to stabilize B56-  
369 CIP2A interaction. To identify such potential additional B56 binding region, we  
370 created GST-CIP2A(1-330) protein that does not harbor sequences from SID  
371 (Fig. 1A), but contains the “tip” sub-domain and N-terminal half of the “stem”  
372 sub-domain (Fig. 2B). Importantly, whereas again no significant association of  
373 GST was found with B56 $\alpha$ , GST-CIP2A(1-330) did show clear interaction (Fig.  
374 5A). However, supportive of our conclusion that dimerization increases B56  
375 binding of CIP2A, the GST-CIP2A(1-560), which harbors the dimerization  
376 region, showed higher B56 $\alpha$  binding than GST-CIP2A(1-330) (Fig. 5A).  
377  
378 Next, we characterized the regions on CIP2A(1-330) that mediate direct  
379 B56 $\alpha$ -CIP2A interaction. Based on an analysis of several N-terminal CIP2A  
380 deletion constructs, the minimal region that is required for the B56 $\alpha$  interaction  
381 was located between amino acids 159 to 245 (Fig. 5B), which covers the last  
382 (fifth) armadillo repeat in the “tip” domain and the first (sixth) repeat in the  
383 “stem” domain (Fig. 2B). Notably, the same region also mediates interaction  
384 between CIP2A and B56 $\gamma$  (Fig. 5C). Next, we modeled the above identified  
385 minimal B56 binding region to CIP2A N-terminal structure, taking also into  
386 account the charge distribution. We also assumed that the binding on CIP2A  
387 may occur at positively charged areas, since B56 surface is largely negatively  
388 charged (Cho, Morrone et al., 2007, Xu, Xing et al., 2006). Strikingly, the  
389 “inside” surfaces of CIP2A(1-560) dimer are highly negatively charged (Fig.  
390 5D, left panel), indicating that B56 may bind to positively charged outer

391 surface of CIP2A molecules (right panel of Fig. 5D, which correlates with the  
392 left panel with a ~30° rotation).

393 Indeed, the N-terminal CIP2A binding region between residues 159 to  
394 245, forms a positively charged surface (Fig. 5D, yellow oval). Notably, this  
395 region also represents, together with dimerization interface, the most  
396 conserved area on CIP2A surface (Figure EV3), suggesting that CIP2A-B56  
397 binding is a conserved feature in evolution. One of the strictly conserved  
398 amino acids at the center of the positively charged interaction region is N230,  
399 which structurally points out from the surface of CIP2A (Figure EV4A). In  
400 support of importance of this region in mediating B56 interaction, exchanging  
401 N230 to negatively charged glutamic acid (N230E) significantly inhibited  
402 CIP2A binding to B56 $\alpha$  (Figure EV4B).

403

404 Results above indicate that each N-terminal arm of the double hook  
405 dimer structure of CIP2A contains a B56 binding region. This might facilitate  
406 trapping of two B56 proteins by a CIP2A dimer. Alternatively, the two B56  
407 binding regions on one CIP2A dimer could both interact with a single B56  
408 molecule to strengthen the interaction. Co-crystallization of the CIP2A-B56 $\alpha$   
409 complex has been extremely challenging and remains an ongoing effort.  
410 Nonetheless, to alternatively dissect between these two possibilities, we  
411 analyzed B56 $\alpha$ -CIP2A dimer interaction by incubating together molar  
412 equivalent amounts of GST-CIP2A(1-560), B56 $\alpha$ , and the stoichiometry of  
413 their interactions was studied by Coomassie staining following GST pull-down.  
414 As shown in figure 5E, intensities of CIP2A dimer and B56 $\alpha$  similar in the

415 analyzed pull-down sample, indicating that each CIP2A dimer can most likely  
416 capture two B56 $\alpha$  molecules.

417

418 Together these results provide first evidence that CIP2A directly binds  
419 to a PP2A complex component. Importantly, the PP2A proteins that CIP2A  
420 were found to interact with are the two best-characterized tumor suppressor  
421 components of PP2A, B56 $\alpha$  and B56 $\gamma$ . Furthermore, by using mutants created  
422 via structure-directed mutagenesis, we provide evidence for co-operation in  
423 B56 binding between N-terminal region of CIP2A, and CIP2A dimerization.

424

#### 425 **Dimerization of CIP2A is important for sustained full-length CIP2A** 426 **protein expression**

427

428 To assess the functional impact of CIP2A dimerization and B56 binding  
429 in the context of full-length CIP2A, we created CIP2A(1-905) mammalian  
430 expression vectors coding for either WT or L533E and R522D mutated V5-  
431 CIP2A fusion protein. Intriguingly, as measured using a V5 epitope-specific  
432 antibody, both the L533E and R522D mutant full-length CIP2A showed up to  
433 50% lower protein levels as compared to the WT protein in HEK-293 cells (Fig.  
434 5F and G). Importantly inhibition of protein expression of mutants was not due  
435 to difference in levels of expression of CIP2A mRNA from transiently  
436 transfected cDNA constructs (Fig. 5H). Also, it is unlikely that single point  
437 mutation in mutants would cause protein destabilization in solution as thermal  
438 unfolding analysis by Prometheus NT.48 (NanoTemper Technologies GmbH),  
439 showed identical melting point for recombinant WT and L533E proteins, and

no indications of difference in protein folding of L533E compared to the WT protein (Figure EV4C). Notably, loss of CIP2A protein stability by L533E and R522D mutation may be directly linked to its impaired B56 binding capacity, as depletion of either B56 $\alpha$  or B56 $\gamma$  with siRNAs also resulted in inhibition of CIP2A protein expression (Fig. 5I, J and Figure EV4D, E), without any impact on CIP2A mRNA expression (Figure EV4F). Importantly, the effects of L533E mutant on CIP2A protein expression was validated in another cell line (22RV1) with low endogenous CIP2A levels (Fig. 5K). Furthermore, inhibition of L533E mutant expression correlated very well with significantly lower capacity to support expression of a well-established CIP2A target pAkt (Ma et al., 2011, Tseng et al., 2012), as compared to WT CIP2A (Fig. 5K,L).

Together these results establish functional relevance for CIP2A dimerization, and B56 binding, discovered in this study. As functional consequences of high CIP2A protein expression on tumorigenesis are very well established on numerous recent studies (Come et al., 2009, Junttila, Puustinen et al., 2007, Liu et al., 2016, Liu et al., 2014, Ma et al., 2011, Xue et al., 2013), it is conceivable that targeting of CIP2A binding to B56 could constitute a first structure-based strategy for therapeutic inhibition of CIP2A protein stability and activity.

## **Discussion**

465 PP2A inhibitor proteins have recently emerged as a novel group of human  
466 oncoproteins with clinical relevance in various human cancers (Khanna et al.,  
467 2013b, Perrotti & Neviani, 2013). Among these proteins, CIP2A shows the  
468 most prevalent overexpression and is associated with poor patient survival  
469 across different types of cancer (Khanna & Pimanda, 2015). The therapeutic  
470 effect of inhibition of CIP2A protein expression in tumor growth has been  
471 recently validated by numerous studies (Come et al., 2009, Junttila, Puustinen  
472 et al., 2007, Liu et al., 2016, Liu et al., 2014, Ma et al., 2011, Xue et al., 2013).  
473 Impact of CIP2A on both oncogenic RAS signaling (Junttila, Puustinen et al.,  
474 2007, Kauko et al., 2015, Mathiasen, Egebjerg et al., 2012), and MYC activity  
475 *in vivo* (Junttila, Puustinen et al., 2007, Myant et al., 2015, Niemelä et al.,  
476 2012), without lack of any detrimental normal tissue homeostasis effects in a  
477 CIP2A-deficient mouse model (Laine et al., 2013, Myant et al., 2015, Ventela  
478 et al., 2012), further illustrates the potential of CIP2A as a future cancer  
479 therapy target. However, efforts to target CIP2A for cancer therapy have been  
480 thus far hampered by the absence of both a molecular explanation of how  
481 CIP2A interacts with PP2A, as well as by a lack of any 3D structural  
482 information about the protein.

483

484 Here, we report the first crystal structure of CIP2A, which contain the motifs  
485 that are critical for PP2A/B56 binding. Interestingly, by using several  
486 independent approaches we demonstrate that CIP2A exists as a homodimer,  
487 and this is mediated by a relatively flat and highly hydrophobic surface formed  
488 by the last three helices of CIP2A(1-560). Another important discovery  
489 reported in this study is the first reported direct interaction between CIP2A

490 and any of the PP2A complex components. Lack of confirmation of direct  
491 binding of CIP2A to PP2A proteins has been a significant caveat in our  
492 understanding how CIP2A might influence PP2A's tumor suppressor activity.  
493 Here Y2H analysis identified B56 $\gamma$  as a direct interaction partner for CIP2A,  
494 and CIP2A interaction with both B56 $\gamma$  and B56 $\alpha$  was further validated by  
495 several independent approaches. Very importantly, among the all PP2A B  
496 subunits, B56 $\alpha$  and B56 $\gamma$  are the two subunits with the most convincing  
497 functional evidence of tumor suppressor activity (Arnold & Sears, 2008,  
498 Sablina et al., 2010). Moreover, we provide evidence that single point  
499 mutation on CIP2A dimerization domain is sufficient to inhibit both B56  
500 binding, and CIP2A's capacity to support pAkt expression. Binding of CIP2A  
501 to PP2A via specific B subunits imposes an interesting possible explanation  
502 for observations that CIP2A only regulates a fairly restricted number of  
503 phosphoproteins (Kauko et al., 2015, Khanna et al., 2013b) among thousands  
504 of potential target proteins regulated by different PP2A complexes (Eichhorn  
505 et al., 2009, Sents et al., 2013). Based on high conservation among all B56  
506 family proteins, we suspect that also they may interact with CIP2A. This, and  
507 whether CIP2A interacts with members of other B subunit families than B56 is  
508 an important question to be addressed in the future.

509       Very interestingly, we also provide evidence that CIP2A binding to B56  
510 stabilizes CIP2A protein, further validating that functional relevance of the  
511 reported CIP2A-B56 interaction. Interestingly, destabilization of CIP2A upon  
512 B56 inhibition is reminiscent of B subunit destabilization upon inhibition of  
513 PP2A core complex components (Silverstein, Barrow et al., 2002), and  
514 supports the model that CIP2A is an obligate interactor with PP2A/B56. This

515 autoregulatory mechanism for CIP2A stabilization could be clinically relevant  
516 finding, as CIP2A is a very long-lived protein (Tseng et al., 2012) and its high  
517 expression associates with poor patient survival in more than 15 different  
518 human cancer types (Khanna & Pimanda, 2015).

519

520       Notably, single point mutations of conserved residues at dimerization  
521 surface impaired both CIP2A dimerization and B56 binding, and we observed  
522 a clear positive correlation between these two effects (Fig. 4E). Together with  
523 high degree of conservation of amino acids mediating CIP2A dimerization,  
524 these results strongly indicate that CIP2A dimerization discovered in this  
525 study is a biologically relevant mechanism related to PP2A regulation. In the  
526 absence of structure of CIP2A-B56 complex, we do not exactly know the  
527 molecular basis of how CIP2A dimerization promotes B56 binding. However,  
528 based on results that in the context of CIP2A(1-560), single point mutations  
529 that impair dimerization also show decreased B56 binding, we envision that  
530 the mechanism may be related to the formation of a novel B56 interaction  
531 surface near the CIP2A dimer interface. This mechanism would remotely  
532 resemble mechanism how Fbw7 dimerization increases Cyclin E binding. In  
533 Fbw7 dimer interface, both Fbw7 protomers have one Cyclin E binding site,  
534 and through dimerization both binding sites become simultaneously  
535 accessible to Cyclin E, thus leading to increased affinity of Fbw7-Cyclin E  
536 interaction (Davis, Welcker et al., 2014). Functionally, the most important  
537 finding of this study is that a single point mutation in the CIP2A dimerization  
538 interface results in CIP2A protein degradation in cancer cells. This is a very  
539 important finding because the therapeutic effects of inhibition of CIP2A protein

540 expression have been validated by numerous studies (Farrell et al., 2014,  
541 Junttila, Puustinen et al., 2007, Khanna et al., 2013b, Laine et al., 2013,  
542 Lambrecht et al., 2013, Lucas et al., 2015, Niemelä et al., 2012). Notably,  
543 targeted protein degradation has recently gained significant interest as an  
544 alternative cancer therapy approach (Ablain, Nasr et al., 2011, Ray, Cuneo et  
545 al., 2015). The benefits of drug targeting to induce protein degradation are  
546 clear, as such an approach removes any potential activities of the protein as  
547 well as any scaffolding functions and results in longer pharmacodynamic  
548 effects that are predicted to remain even after drug has been metabolized. We  
549 anticipate that more potent target sites for induction of CIP2A degradation will  
550 be identified by further dissection of both N-terminal and dimerization domain  
551 amino acids critical for CIP2A-B56 binding. Moreover, although we have here  
552 determined regions that are sufficient for CIP2A binding to tumor suppressor  
553 B56, and demonstrate relevance of CIP2A dimerization in this process, these  
554 results do not exclude that the C-terminal sequences, and for example post-  
555 translation modifications of full-length CIP2A, might not also contribute to  
556 PP2A binding and regulation. Future work will be thus needed to address why  
557 full-length CIP2A can be expressed in cells but not purified in *in vitro*  
558 conditions, and whether targeting of C-terminal regions of CIP2A would offer  
559 additional benefit in inhibition of CIP2A's oncogenic activities.

560

561 In summary, results of this study reveal the first crystal structure of CIP2A -  
562 one of the most prevalent human oncoprotein. Our results also provide first  
563 insights into how CIP2A interacts with PP2A tumor suppressor subunit B56. In  
564 addition to their novelty and biological significance in promoting our

565 understanding of mechanisms of regulation of major cellular serine/threonine  
566 phosphatase complex PP2A, these results strongly indicate that the identified  
567 N-terminal B56 binding region of CIP2A, together with dimerization domain  
568 may serve as potential target regions for cancer therapeutics. We anticipate  
569 that these findings will provoke immense interest in developing first series of  
570 small molecule inhibitors towards CIP2A for cancer therapy. These results  
571 may also help in understanding mechanisms of PP2A regulation in various  
572 other diseases in which PP2A inhibition has pathogenic role.

573  
574

## 575 **Materials and methods**

### 576

### 577 **Protein expression and purification for crystallography**

578 The truncated domain of human CIP2A(1-560) was cloned into the pGEX-4T1  
579 vector (GE Healthcare) with an N-terminal GST tag and a TEV cleavage site  
580 in between. CIP2A(1-560) was over-expressed in *E. coli* BL21 (DE3) cells  
581 (Novagen), grown in LB media. The bacteria cell was cultured at 37 °C until  
582 O.D.600 reached 0.5-0.7, and then induced by 0.2 mM Isopropyl  $\beta$ -D-1-  
583 Thiogalactopyranoside (IPTG) at 16 °C overnight. The bacteria pellets were  
584 collected and lysed by sonication. The GST fusion protein was first purified by  
585 Glutathione Sepharose 4B column. The GST tag was removed by TEV  
586 protease at 4°C overnight. The untagged protein was further purified by an ion  
587 exchange column (GE Healthcare). The purity of the samples was verified  
588 using SDS-PAGE and staining with Coomassie Brilliant Blue. The CIP2A  
589 truncated domain was observed as a single band at 60 kDa. The protein was  
590 then concentrated for crystallization to 1.5 mg/ml in a buffer containing 20 mM  
591 Tris·HCl pH 8.0, 250 mM NaCl, 2 mM DTT. A selenomethionine (SeMet)  
592 derivative of the CIP2A truncated domain was expressed in an auto-induction  
593 media (Studier, 2005) and purified in the same way as the native protein.

### 594 **Crystallization, optimization and Data Collection**

595 Initial crystals of both the native and the SeMet-substituted CIP2A truncated  
596 domain were obtained using the hanging drop vapor diffusion method.  
597 Crystals were improved by adding 1% PEG 8000 into the condition consisting  
598 of 0.1 M sodium malonate pH 6.0 and 7% PEG 4000. 1  $\mu$ l of protein solution  
599 (1.5 mg/ml) was mixed with 1  $\mu$ l of reservoir solution and equilibrated over  
600 400  $\mu$ l reservoir solution at room temperature (RT). Diamond-shaped crystals

usually grew to their full sizes in a few days. After an optimization of cryo-protection conditions, best crystals diffracted to  $\sim 4$  Å resolution. Crystal diffraction quality was improved to  $\sim 3.6$  Å resolution by careful dehydration of crystals. To further improve the diffraction, we carried out a temperature-gradient screening. CIP2A crystals were sealed in foam boxes under different soaking conditions and transferred into a 4°C cold-room for slow cooling-down, and crystals were equilibrated at 4°C for different time periods. Crystals of Se-Met-substituted CIP2A soaked at 4°C for  $\sim 2$  weeks gave dramatically better diffractions (better than 3 Å resolution) than shorter soakings. Crystals were frozen by liquid nitrogen. Crystal diffraction data sets were collected at the Advanced Light Source (ALS), beamlines 8.2.1 and 8.2.2. Diffraction data sets were processed by HKL2000 (Otwinowski & Minor, 1997) and Mosfilm (Leslie and Powell 2007).

#### **Structure determination and refinement**

The structure was determined by single-wavelength anomalous dispersion (SAD) using one 3.5 Å data set collected at wavelength 0.97945 Å. The selenium sites and the initial phases were determined by PHENIX (Adams, Afonine et al., 2010). Twelve selenium sites were found in one asymmetric unit. The experimental electron density map clearly showed the presence of two CIP2A molecules in one asymmetric unit, allowing the tracing of a model of the C-terminal half of the protein. In the crystal lattice, the N-terminal half of CIP2A(1-560) molecules have much high B factors than the C-terminal half, and density for this part did not allow us to build loop residues between armadillo repeat helices.

#### **Thermophoresis**

626 The analysis was carried out by Monolith NT.115 instrument and NT.115  
627 hydrophilic capillaries. For the analysis, GST-CIP2A (1-560) and GST proteins  
628 were labelled with NT-647 dye by using Monolith protein labeling kit (Red  
629 NHS). The instrument was pre-warmed to 37 °C before the analysis. Each  
630 sample set was analyzed twice by using red laser with 20% and 40% led  
631 power, respectively.

### 632 **Yeast-two-hybrid screen**

633 The yeast-two-hybrid screen was performed by Hybrigenics. The full-length  
634 CIP2A was used as a bait and the library in the screen was Breast Tumor  
635 Epithelial Cells (T47D, MDA-MB-468, MCF7, BT20).

### 636 **Antibodies**

637 The following primary antibodies were used: CIP2A polyclonal Rabbit (pR Ab)  
638 (Soo Hoo et al., 2002) or monoclonal Mouse (mM Ab) (2G10-3B5) (Santa  
639 Cruz sc-80659), PP2A-B56- $\alpha$  (23) (mM Ab, Santa Cruz sc-136045) or pR Ab  
640 (Upstate Biotechnology 07-334), PP2A-B56- $\gamma$  (N-15) polyclonal Goat (pG) Ab  
641 (Santa Cruz sc-46459), V5 (Sigma V8012 or Thermo Fisher Scientific  
642 E10/V4RR) both mM Ab, GST (B-14) mM Ab (Santa Cruz sc-138), pAkt  
643 Ser473 mR Ab (Cell Signaling D9E),  $\beta$ -Actin (C4) mM Ab (Santa Cruz  
644 Biotechnology sc-47778), GAPDH (6C5) mM Ab (HyTest 5G4-6C5), GFP pR  
645 Ab (Life technologies A-11122) and HA (Y-11) pR Ab (Santa Cruz  
646 Biotechnology sc-805). The following secondary antibodies were used:  
647 Polyclonal Goat Anti-Mouse Immunoglobulins-HRP from Dako (P0447) or  
648 from Santa Cruz Biotechnology (sc-2005), Polyclonal Swine Anti-Rabbit  
649 (P0399) and Polyclonal Rabbit Anti-Goat (P0449) Immunoglobulins-HRP,  
650 both from Dako.

651 **Protein purification for CIP2A dimerization and PP2A-binding assays**

652 CIP2A(1-560) V5His and B56 $\alpha$  were cloned into the pGEX-4T-1 vector  
653 containing Thrombin and TEV cleavage sites, respectively. GST-CIP2A(1-  
654 560) WT, -R522D and -L533E were cloned into the pGEX-4T-2 vector. The  
655 proteins were produced in *E. coli* BL21 strain induced by 0.2 mM IPTG when  
656 the O.D.<sub>600</sub> was 0.6-0.8. The cells were then incubated at 23 °C and  
657 harvested after 4 h. The bacteria pellets were collected and lysed by  
658 sonication in a buffer containing 100 mM Tris HCl pH 8, 300 mM NaCl, 0.1%  
659 Triton X-100, 2 mM DTT, 20 mg lysozyme/ 150 ml lysis buffer and 1 x  
660 Protease Inhibitor tablet EDTA-free (Pierce). For some purifications, NaCl was  
661 increased to 500 mM and DTT to 5 mM, to try to increase the overall sample  
662 purity. Clarified lysate was incubated with GSH agarose at 4°C for at least 1-  
663 2h. The beads were washed extensively with the same buffer listed above,  
664 but omitting lysozyme. The bound material was eluted in a buffer with 20 mM  
665 reduced GSH and 200 mM NaCl. The eluted fractions were analyzed by SDS-  
666 PAGE and staining in Coomassie Brilliant Blue (Invitrogen). The pulled  
667 fractions were finally stored in a buffer containing 20 mM Tris HCl pH 8, 150  
668 mM NaCl, 0.05 % Triton x-100, 10 % glycerol and 2 mM DTT.

669 From CIP2A(1-560) V5His, the GST tag was removed by adding Thrombin  
670 (GE Healthcare) and incubating overnight at 4°C. Non-digested protein and  
671 GST were collected with GSH agarose and Thrombin was inactivated with 1  
672 mM PMSF. The sample was centrifuged and collected supernatant containing  
673 CIP2A(1-560) V5His was transferred into a clean tube, dialyzed as above and  
674 flash-frozen to -80°C. From B56 $\alpha$ , the GST tag was removed by adding  
675 AcTEV (Life technologies) and incubating overnight at 4°C. Simultaneously,

676 GSH agarose and NiNTA slurry were added to collect non-digested protein  
677 and GST and TEV, respectively. Incubation was allowed overnight at 4°C with  
678 gentle rocking. Next, the sample was processed as above.

#### 679 **GST pulldown assays**

680 In all GST pulldown assays, 10 pmol of each protein was used. The overall  
681 volume of each pulldown prep was 200 µl. The interaction buffer was 50 mM  
682 Tris, 150 mM NaCl, 10% glycerol, 0.2% NP-40, 50 µM ZnSO<sub>4</sub>, 2 mM DTT, pH  
683 7.5. The proteins were then incubated 1 h at 37°C or RT as indicated in the  
684 figure legends. Next, 5 µl of GSH agarose (Thermo Scientific) was added in  
685 20 µl of the interaction buffer and samples were further incubated 1 h at RT in  
686 rotation. Thereafter, the samples were washed four times with 250 µl of ice  
687 cold interaction buffer. The overall washing time was extended at least to 1 h  
688 in order to reduce the background. Finally the samples were centrifuged, the  
689 supernatant was carefully discarded and the resin was resuspended in SDS-  
690 PAGE sample buffer, resolved by SDS-PAGE and analyzed by Western blot.

#### 691 **SEC and SEC-MALS**

692 Size exclusion chromatography for recombinant protein analysis was carried  
693 out using Superdex 5/150 column (GE Healthcare). The flow rate was 0.3  
694 ml/min and the column was operated at RT. The running buffer was 28 mM  
695 Tris pH 7.2, 150 mM NaCl, 0.05% NP-40, 1.25% glycerol, 2 mM DTT). All  
696 samples contained 50 pmol of each protein tested. The proteins were first let  
697 to form complexes by incubating them in the interaction buffer (50 mM Tris pH  
698 7.5, 150 mM NaCl, 5% glycerol, 0.2% NP-40, 2 mM DTT) for 1 h at 37 °C.  
699 The total volume was 120 µl. The samples were centrifuged at 11,000 g for 5  
700 min before loading to the gel filtration column. In each run, 30 µl of the sample

701 was injected to column. The total volume of the column is 3 ml. The complex  
702 size determination was based on calibration with carbonic anhydrase (29 kDa),  
703 bovine serum albumin (66 kDa), alcohol dehydrogenase (141 kDa) and beta  
704 amylase (200 kDa) control proteins.  
705 SEC-coupled Multi-angle (laser)-light scattering (SEC-MALS) experiments  
706 were performed at RT by loading samples on a 24 mL Superdex 200 increase  
707 size exclusion column (GE Healthcare) with a TREOS MiniDAWN MALS  
708 detector (Wyatt Technology). The buffer used contained 20 mM Tris-HCl (pH  
709 8.0), 275 mM NaCl and 2 mM DTT.

710 Analysis of CIP2A dimerization from Hela cells by size-exclusion  
711 chromatography was performed as described previously (Sarek, Jarviluoma et  
712 al., 2006). The molecular mass standards (Sigma) used to calibrate the  
713 column were blue dextran (2,000 kDa) thyroglobulin (669 kDa), apoferritin  
714 (443 kDa), b-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine  
715 serum albumine (66 kDa) and carbonic anhydrase (29 kDa).

#### 716 **Generation of CIP2A N230E, R522D and L533E mutants**

717 R522D and L533E mutations were introduced by PCR, using QuickChange II  
718 XL Site-Directed Mutagenesis Kit (Agilent Technologies, TX, USA). As a PCR  
719 template, pGEX4T2/ CIP2A(1-560) and pcDNA3.1/ CIP2A(1-905) V5 were  
720 used with the following pairs of primers, listed sense and antisense,  
721 respectively: for R522D, 5'-GATAATGATGAACAAGTACAGTCTGGACTG-3'  
722 and 5'-CTTGTTTCATCATTATCTGACGTTAAAGCAAAAGC-3', and for L533E,  
723 5'-GAATATTAGAGGAGGCTGCTCCACTGCCAGA-3' and 5'-  
724 GCAGCCTCCTCTAATATTCTCAGTCCAGACTG-3'. N230E was introduced  
725 in pGEX4T2/ CIP2A(1-560) by using QuickChange Site-Directed Mutagenesis

726 (QCL-SDM) kit from the same manufacturer and the following set of primers,  
727 listed sense and antisense, respectively: 5'-  
728 GCTCGAGAGATTCATCAGACTTTTCAACTAATA-3' and 5'-  
729 ATGAATCTCTCGAGCATGGAATAGCTTTTC-3'. Total volume of the PCR  
730 reaction was 25 µl. The PCR program was as follows: 1 min at 95°C (one  
731 cycle), 1 min at 95°C- 1 min at 58°C- 5 min at 68°C (18 cycles), followed by  
732 10 min final extension at 68°C and storage at 4°C. Next, 0.5 µl of DpnI  
733 enzyme was added to each 25 µl PCR mix and incubated for about 2h at  
734 37°C. All constructs were verified by DNA sequencing (Finnish Microarray and  
735 Sequencing Centre, Centre for Biotechnology Turku).

#### 736 **CIP2A dimerization and B56α-binding assays with CIP2A mutants**

737 General conditions were slightly modified from described above in the GST  
738 pulldown assays section. Reaction volume was reduced to 150 µl. GST-  
739 CIP2A(1-560) variants were pre-incubated in reaction buffer at RT for 30 min,  
740 and then B56α was added (10 pmol). Reaction was allowed to proceed for 1h  
741 at 37°C. In the interaction buffer for B56α-assay, NP-40 was substituted with  
742 chemically equivalent Igepal (Sigma, Steinheim, Germany) and 50 µM zinc  
743 ions, from 100 mM stock solution of zinc acetate (Fluka Chemika, Steinheim,  
744 Germany), were included only in the washing buffer. Washes following  
745 incubation with GSH agarose were conducted for 2h at 4°C.

746 Dimerization reaction was conducted for 1h at 37°C. After 1h RT incubation  
747 with GSH agarose, the beads were washed four times with reaction buffer (as  
748 listed above but w/o  $\text{Zn}^{2+}$ ) for 1h total at 4°C.

749 The bound material was eluted in 30-35 µl of 2 x SDS-PAGE sample buffer  
750 for 10 min at 95°C and the recovered supernatant was analyzed by Western

751 blot. Typically, 5 µl of the eluted material and of the inputs were resolved by 4-  
752 20% SDS-PAGE (Mini-PROTEAN TGX Gels, BIO-RAD, USA).

### 753 **Cell culture**

754 22RV1 cells were cultures in RPMI-1640 media (Sigma), and HEK293T and  
755 HeLa cells in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), both  
756 supplemented with 10% (v/v) FBS, 0.5% (v/v) penicillin/streptomycin (10,000  
757 units/ 10 mg per ml, Sigma) and 2 mM L-Glutamine (Biowest). The cells were  
758 cultured at 37°C in a humidified incubator under an atmosphere of 5% CO<sub>2</sub>  
759 and passaged 2-3 times a week.

### 760 **Analysis of expression of CIP2A WT and L533E mutant in HEK293T cells**

761 HEK293T cells were plated in a 12 well-plate format. Cells were transfected  
762 using Lipofectamine 2000 (Invitrogen by Thermo Fisher Scientific, IL, USA) or  
763 Eugene 6 (Promega) at 3:1, according to the manufacturer's protocol. For 12  
764 well-plate scale, 1 µg of DNA was used. After about 24h, the growth media  
765 was removed, the cells were rinsed twice in cold PBS and then scraped in  
766 100 µl PBS, mixed with 100 µl 2 x SDS-PAGE sample buffer, incubated for 10  
767 min at 95°C and centrifuged at 13,200 rpm for 15 min. The cleared  
768 supernatant (8 µl in total) was resolved by 4-20% SDS-PAGE and analyzed  
769 by Western blot.

770 For RT-PCR, the cells were plated in a 6 well-plate format and transfected  
771 with 3 µg of DNA for 24h. RNA extraction was done with NucleoSpin RNA kit  
772 (Macherey-Nagel). Reverse transcription of the RNA extracts was performed  
773 using RNase inhibitor rRNasin (Promega, WI, USA) and M-MuLV RNase H-  
774 reverse transcriptase (Finnzymes, ThermoFisher Scientific MA, USA). RT-  
775 qPCR for CIP2A mRNA was performed on Applied Biosystems 7900HT Fast

776 Sequence Detection System using TaqMan Universal Master Mix II, no UNG  
777 (Applied Biosystems, CA, USA), Universal ProbeLibrary probe #69 (Roche  
778 Applied Science), and following primer sequences:  
779 GAACAGATAAGAAAAGAGTTGAGCATT and  
780 CGACCTTCTAATTGTGCCTTTT.

781  
782 **Analysis of pAkt Ser473 protein expression in 22RV1 cells**

783 22RV1 cells were plated in a 12 well-plate format. Cells were transfected  
784 using Lipofectamine 3000 (Invitrogen by Thermo Fisher Scientific) at 3:1,  
785 following manufacturer's instructions. For transfection, cells were placed in  
786 Optimem, which was removed about 6-7 hours after transfection. The amount  
787 of DNA used for transfection was 1 µg. About 24h post-transfection, the cells  
788 were lysed for Western blotting.

789 **B56 siRNA effects on CIP2A protein expression**

790 The cells were transfected with Oligofectamine (ThermoFisherScientific by  
791 Life technologies) according to manufacturers instructions. 22RV1 cells were  
792 seeded in 6 or 12 well-plate set-ups so that the confluency at the point of  
793 siRNA transfection would be 30-40%. Following siRNAs were used for B56α  
794 knockdown: B56α-1: UAC CCA UCU GUU ACC ACU CdTdG; B56α-2: AAG  
795 UGU ACG GAA GAU GUU AdGdC (both with symmetrical overhangs, from  
796 Sigma) and PP2A-B56-α siRNA (h) (sc-39181 Santa Cruz). For B56γ  
797 knockdown, PP2A-B56-γ siRNA (h) (sc-45847 Santa Cruz) was used.

798

799 For B56 siRNA experiment in HeLa cells following siRNA sequences were  
800 used for B56α knockdown: B56α-1: UAC CCA UCU GUU ACC ACU CdTdG;  
801 B56α-2: AAG UGU ACG GAA GAU GUU AdGdC. After 72h transfection, the

802 cells were scraped in ice cold PBS and snap frozen. Samples were split for  
803 Western blotting and RNA extraction by NucleoSpin RNA II kit (Macherey-  
804 Nagel). Reverse transcription of the RNA extracts was performed using  
805 RNase inhibitor rRNasin (Promega) and M-MuLV RNase H- reverse  
806 transcriptase (Finnzymes, ThermoFisher). RT-qPCR for CIP2A mRNA was  
807 performed on Applied Biosystems 7900HT Fast Sequence Detection System  
808 using TaqMan Universal Master Mix II, no UNG (Applied Biosystems),  
809 Universal ProbeLibrary probe #69 (Roche Applied Science), and following  
810 primer sequences: GAACAGATAAGAAAAGAGTTGAGCATT and  
811 CGACCTTCTAATTGTGCCTTTT.

812  
813 **Proximity Ligation Assay (PLA)**

814 HEK293T cells were plated on coverslips in a 12 well-plate format. Coverslips  
815 were pre-coated with poly-lysine (Sigma-Aldrich), and transfected using  
816 Lipofectamine 2000 (Invitrogen by Thermo Fisher Scientific) at 3:1, according  
817 to the manufacturer's protocol. The following plasmids were used with the  
818 amounts indicated: pEGFPC2-CIP2A(1-905) Flag (1 µg), pcDNA3.1-CIP2A(1-  
819 905) V5 His (3 µg), pCEP-4HA-B56α and pCEP-4HA-B56γ3 (both 1 µg). The  
820 assay was started about 24h after transfection. PLA kit from Olink Bioscience  
821 was used. Cells were fixed with 4% PFA/ PBS for 15 min at RT, followed by  
822 three-5 min washes in PBS with gentle agitation. Cells were permeabilized  
823 with ice-cold methanol for 10 min at -20°C, followed by two short and one 5  
824 min-wash in PBS. Next, they were blocked in blocking solution for 30 min at  
825 37°C in foil-covered PLA dish. Primary antibodies were diluted in antibody  
826 diluent as follows: anti-V5 (E10/V4RR 1:200), anti-GFP (1:500) and anti-HA  
827 (1:200), and incubated with the coverslips overnight at 4°C. Next the

coverslips were washed two times in Buffer A, and then incubated with mouse and rabbit probes diluted 5:1 in antibody diluent for 1h at 37°C. After washing two times in Buffer A PLA reaction was performed according manufacturers instructions. The slides were analyzed with laser scanning microscope LSM510 META (Carl Zeiss) at 63 x magnification and images were processed with Fiji-ImageJ.

For studying association of endogenous CIP2A and B56 $\alpha$ , the following primary antibodies were used: anti-CIP2A (mM Ab 2G10-3B5 1:100) and anti-B56 $\alpha$  (pR Ab 07-334 1:50).

### **Thermal stability profiling**

Thermal stability measurements were conducted by using Prometheus NT.48 (Nanotemper Technologies). For this purpose, GST CIP2A(1-560) WT and L533E mutant were used at 10  $\mu$ l volume per capillary. Samples were measured 75 min at 1°C/min in the temperature range from 20°C to 95°C. The temperature-dependent protein unfolding was measured by label-free (UV-excited, at 280 nm) real time monitoring of tryptophane (Trp) fluorescence emission maximum shift from 330 (folded) to 350 nm (unfolded) wavelengths. Protein unfolding transition point, where half of the protein is in unfolded state, was determined by plotting the ration of fluorescence intensities (F350/F330) over temperature. Melting temperature (T<sub>m</sub>) was calculated by determining the maximum of the first derivative of the fluorescence maximum shift signal.

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865

#### 866 **Conflicts of interest**

867 The authors declare that they have no conflict of interest

#### 868 **Supplementary materials**

869 Supplemental information includes 9 figures and 1 table

870

871

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1072 **Figure legends**

1073

1074 **Figure 1. Verification of homodimerization of the CIP2A(1-560)**

1075 **A)** Summary of CIP2A prey fragments (green) interacting with full-length  
1076 CIP2A (1-905) bait (orange) in yeast-two-hybrid screen. Original Y2H data is  
1077 shown in Figure EV1. Number of independent interacting CIP2A prey clones  
1078 allowed Selected Interaction Domain (SID) analysis that delineates the  
1079 shortest fragment that is shared with all interacting clones, and thus  
1080 represents a potential region mediating the CIP2A homodimerization. Grey  
1081 dotted lines illustrate location of SID across all prey fragments and in full-  
1082 length CIP2A. **B)** Dimerization of CIP2A(1-560) fragment analyzed by GST  
1083 pulldown. Equal molar amounts of GST and GST-CIP2A(1-560) have been  
1084 incubated with CIP2A(1-560)-V5 fragment for 1 h at 37 °C before pull-down.  
1085 **C)** GST-tagged CIP2A (90 kDa), but not GST, can pull-down untagged CIP2A  
1086 (60 kDa) in a stoichiometric manner. The SDS-PAGE was stained with  
1087 Coomassie Blue. **D)** SEC-MALS analysis of untagged CIP2A(1-560) on a  
1088 Superdex 200 increase 10/300 GL column. The blue curve is the UV  
1089 absorbance profile; whereas the black line shows the measured molar mass  
1090 for the major peak. Untagged CIP2A(1-560) has a nominal MW of 62 kD  
1091 whereas SEC-MALS chromatogram show shape-independent MW reading at  
1092 117.2 kD which corresponds to molecular weight of CIP2A(1-560) dimer. **E)**  
1093 Thermophoresis analysis of interaction between labeled and non-labeled  
1094 CIP2A(1-560) proteins. **F)** Proximity ligation assay (PLA) for interaction  
1095 between two differently tagged full-length CIP2A proteins. HEK293T cells co-  
1096 transfected with CIP2A-V5 and EGFP-CIP2A constructs were subjected to

1097 PLA with either V5 and GFP antibodies (left panel), or as control with only  
1098 secondary PLA probes (middle panel). Red dots indicate for association  
1099 between two CIP2A proteins. As another specificity control, mock transfected  
1100 cells were analyzed with PLA including both V5 and GFP primary antibodies.  
1101 Shown is a representative image from two PLA experiments. **G)** Analysis of  
1102 endogenous CIP2A dimerization by size-exclusion chromatography of HeLa  
1103 cell total cell extract and cytoplasmic extracts. Estimated molecular weights  
1104 are based on column calibration with standard proteins. Shown is a  
1105 representative result of three independent experiments. Size difference  
1106 between CIP2A in different fractions is indicative of post-translational  
1107 regulation of CIP2A upon complex formation.

1108

1109 **Figure 2. Overall structure of the CIP2A(1-560) dimer**

1110 **A)** Overall structure of the CIP2A(1-560) dimer. Two views of the crystal  
1111 structure are related by a 90 degree rotation. Positions of N- and C-termini are  
1112 labeled. **B)** Separated view of a CIP2A(1-560) monomer, in three orthogonal  
1113 views. Positions of the three subdomains are boxed.

1114

1115 **Figure 3. Mutations at the dimer interface of CIP2A negatively affect its**  
1116 **dimerization efficiency**

1117 **A)** Detailed interactions in the CIP2A dimer interface mediated by the three  
1118 helices of C-dimerization domain. The two CIP2A molecules are shown in  
1119 blue and green. **B)** Alternative image of dimer interface in which one CIP2A  
1120 monomer is shown in space-filled model. The structural image was generated  
1121 by Pymol. **C)** Positions of key hydrophobic residues in the CIP2A homo-

1122 dimerization interface are shown in a “peeled-apart” view. **D)** Dimerization of  
1123 indicated GST-CIP2A(1-560) WT and mutant proteins analyzed by GST  
1124 pulldown. Equal molar amounts of GST and GST-CIP2A(1-560) proteins were  
1125 incubated with CIP2A(1-560)-V5 fragment for 1 h at 37 °C before pulldown.  
1126 Samples were analyzed by Western blot using V5 and GST antibodies.  
1127 Representative image from six experiments is shown. **E)** Quantification of  
1128 effects of dimerization interface point mutations on CIP2A dimerization.  
1129 Western blot representative result is shown in D). Shown is relative  
1130 dimerization efficiency of indicated CIP2A mutants as compared to GST-  
1131 CIP2A(1-560) WT, quantified as a ratio between CIP2A(1-560)-V5 and GST-  
1132 CIP2A(1-560) in pull-down sample. Shown is mean + S.E.M. from six  
1133 independent experiments. Two-sided t-test between mutant and WT proteins  
1134 for their relative CIP2A dimerization \*\*  $p < 0.01$ . **F)** CIP2A-dimer interface with  
1135 R522D and L533E mutations. The helices of two monomeric CIP2A units are  
1136 shown in green and blue. Residues at the dimer interface are shown as sticks  
1137 in magenta. R522 and L533 which are substituted for D and E, respectively,  
1138 are shown as red sticks and indicated by red text. E523 which might  
1139 contribute to disrupting the dimer interface by creating electrostatic repulsions  
1140 with R522D mutant of CIP2A are shown as black sticks. The structure was  
1141 generated in Pymol.

1142

1143 **Figure 4. Role of dimerization on direct interaction between CIP2A and**  
1144 **B56 PP2A regulatory subunits**

1145 **A)** GST pulldown assay for B56 – GST-CIP2A(1-560) interaction. Equal molar  
1146 amounts are used in all samples. The samples were analyzed by Western

1147 blotting using antibodies against GST, B56 $\alpha$  and B56 $\gamma$ . **B)** Thermophoresis  
 1148 analysis of B56 - CIP2A(1-560) interactions. CIP2A(1-560) fragment was  
 1149 labeled by NT-647 NHS label. **C)** PLA analysis for interaction between  
 1150 endogenous CIP2A and B56 $\alpha$  proteins in HEK293T cells. HEK293T cells  
 1151 were analyzed by PLA using antibodies specific for CIP2A or B56 $\alpha$  (right  
 1152 panel), and were also analyzed with PLA without primary antibodies (middle  
 1153 panel). As another control, HEK293T cells co-transfected with CIP2A-V5 and  
 1154 HA-B56 $\alpha$  constructs were subjected to PLA with V5 and HA antibodies (left  
 1155 panel). Red dots indicate for association between CIP2A and B56 $\alpha$  proteins.  
 1156 Shown is a representative image from two PLA experiments. **D)** Size  
 1157 exclusion chromatography analysis of GST-CIP2A(1-560) interaction with  
 1158 B56 $\alpha$ . The proteins were incubated together 1 h at 37 °C before the run. As a  
 1159 negative control, B56 $\alpha$  was also tested with GST. **E)** GST-pulldown assay for  
 1160 interaction between B56 $\alpha$  and indicated GST-CIP2A(1-560) WT and  
 1161 dimerization interface mutant proteins. Equal molar amounts of GST and  
 1162 GST-CIP2A(1-560) proteins were incubated with B56 $\alpha$  for 1 h at 37 °C before  
 1163 pulldown. **F)** Quantitation of the Western blot results from E). Shown is  
 1164 relative B56-binding efficiency of mutants as compared to GST-CIP2A(1-560)  
 1165 WT, quantified as a ratio between B56 $\alpha$  and GST-CIP2A(1-560) in pull-down  
 1166 sample. Shown is mean + S.E.M. from four independent B56-binding  
 1167 experiments. Two-sided t-test between mutant and WT proteins for their  
 1168 relative CIP2A dimerization \*\*  $p < 0.01$ . To compare the degree of B56-  
 1169 binding deficiency of R522D and L533E CIP2A(1-560) mutants to the degree  
 1170 of dimerization deficiency, the graph also includes data from figure 3E. **G)**  
 1171 Ratio between observed effects for both mutants on both dimerization and

1172 B56 binding (based on F) was calculated to estimate the degree of  
1173 contribution of CIP2A dimerization to its maximal B56-binding capacity. Both  
1174 mutants show comparable degree of impact to B56 binding.

1175

1176 **Figure 5. Mapping of N-terminal B56-binding region in CIP2A**

1177 **A)** GST-CIP2A(1-330) was compared for B56 $\alpha$ -binding with CIP2A(1-560).  
1178 The samples were analyzed by Western blotting using antibodies against  
1179 GST and B56 $\alpha$ . Shown is a representative of three independent experiments  
1180 with similar results. **B,C)** Mapping of the N-terminal B56 $\alpha$  (B) and B56 $\gamma$  (C)  
1181 interaction region in CIP2A by GST pulldown analysis. samples were  
1182 analyzed by Western blotting using antibodies against GST and B56 $\alpha$  or B56 $\gamma$ .  
1183 **D)** Surface electrostatic potential analysis and potential binding sites for B56.  
1184 The surface electrostatic potential was calculated using the Adaptive Poisson-  
1185 Boltzmann Solver (APBS) module and presented by Pymol. The right panel  
1186 correlates with the left panel with a  $\sim 30^\circ$  rotation. The potential B56-binding  
1187 site, predicted based on binding site mapping, surface conservation and  
1188 charge-distribution, is indicated with a yellow oval. **E)** Analysis of  
1189 stoichiometry between CIP2A and B56 binding. Similar molar amounts of  
1190 GST-CIP2A1-560 and B56 $\alpha$  were incubated together for 1 h at 37 °C followed  
1191 by GST pulldown analysis and Coomassie staining of SDS-PAGE gel. As  
1192 CIP2A exists preferentially as a dimer (Fig. 1D), the 0.89:1 ratio between  
1193 CIP2A and B56 in pull-down sample indicate that one CIP2A dimer binds two  
1194 B56 molecules. **F)** Western blot analysis of protein expression of V5 tagged  
1195 full-length WT CIP2A(1-905) or L533E and R522D CIP2A mutants from  
1196 transiently transfected HEK293T cells. **G)** Quantitation of the Western blot

1197 results from F). Shown is mean + S.E.M, n=3. Two-sided t-test between  
 1198 mutant and WT proteins for their relative expression \*  $p < 0.05$ , \*\*  $p < 0.01$ . **H)**  
 1199 RT-PCR analysis of CIP2A,  $\beta$ -Actin and GAPDH mRNA expression from  
 1200 transiently transfected HEK293T cells expressing either V5 tagged full-length  
 1201 WT or L533E and R522D mutants. Plotted is mean + S.E.M. from four  
 1202 experiments with duplicate samples. **I)** Endogenous CIP2A protein expression  
 1203 in 22RV1 cells transfected with B56 $\alpha$  and B56 $\gamma$  siRNAs for 72 hours. **J)**  
 1204 Quantitation of the Western blot results from I). Shown is mean + S.E.M, n=4.  
 1205 Two-sided t-test \*\*  $p < 0.01$ . **K)** Western blot analysis of pAkt Ser473 protein  
 1206 expression in 22RV1 cells transiently transfected with V5 tagged full-length  
 1207 WT or L533E CIP2A. **L)** Quantitation of the Western blot results from K).  
 1208 Shown is mean + S.E.M., n=3. Two-sided t-test \*\*  $p < 0.01$ .

1209

## 1210 **Expanded View Figure Legends**

1211

1212 **Figure EV1.** A) Summary of screen parameters and description of Global  
 1213 PBS classification for confidence of interactions observed in the screen. B)  
 1214 List of CIP2A (KIAA1524) and B56g (PPP2R5C) prey clones found to interact  
 1215 with full-length CIP2A bait in Y2H screen. C) Description of selected  
 1216 interaction domain (SID) determination and graphical representation of SID for  
 1217 both CIP2A (KIAA1524) and B56g (PPP2R5C) interaction with full-length  
 1218 CIP2A bait. The SID is depicted in relation to predicted structural domains of  
 1219 both proteins (other colour bars).

1220 **Figure EV2.** Screening for mutants of CIP2 deficient for dimerization. The  
 1221 helices of two monomeric CIP2A units are shown in light grey and blue.

1222 Mutated residues are shown as red sticks. The following mutants were  
1223 generated and tested: L529A, L532A, L529A L532A (2A), L529A L532A  
1224 L533A (3A), R522D, Q526E, L529E, L533E, R522D Q526E, Q526E L529E  
1225 L533E (3E). The structure was generated in RasWin.

1226 **Figure EV3.** Conservation of potential B56 binding site on CIP2A(1-560)  
1227 between aa. 159-245 (yellow oval). Conservation analysis was made based  
1228 on ten CIP2A sequences from representative species (see also Appendix  
1229 Figure S4). The darker color indicates higher conservations.

1230 **Figure EV4.** Structure-function relationship between CIP2A-B56 interaction  
1231 and CIP2A protein stability. A) CIP2A monomers are shown in green and  
1232 cyan. Minimal region on CIP2A required for B56 $\alpha$  binding, 159-245, is in  
1233 magenta. N230 residue from this region is shown as blue stick. It is pointed  
1234 away from the CIP2A surface and is in the middle of a positively charged and  
1235 conserved patch (see Figure EV3). The figure was made in Pymol. B)  
1236 Impaired interaction of GST-CIP2A(1- 560) N230E mutant with B56 $\alpha$ .  
1237 Quantitation is mean + S.E.M. from 4 independent experiments.  $p = 0.01$ , t-  
1238 test. C) Thermal unfolding analysis by Prometheus NT.48 shows comparable  
1239 melting profile for recombinant CIP2A(1-560) WT and L533E mutant,  
1240 indicating similar protein folding. D) Inhibition of B56 $\alpha$  destabilizes CIP2A  
1241 protein in HeLa cells. Shown is Western blot analysis from HeLa cells 72h  
1242 after transfection. E) Quantification of relative CIP2A protein levels from D).  
1243 Shown is mean + S.D. from 3 independent experiments. F) RNAi-mediated  
1244 depletion of B56 $\alpha$  does not impact endogenous CIP2A mRNA expression.  
1245 The analysis is done from parallel samples to those used in D).

1246

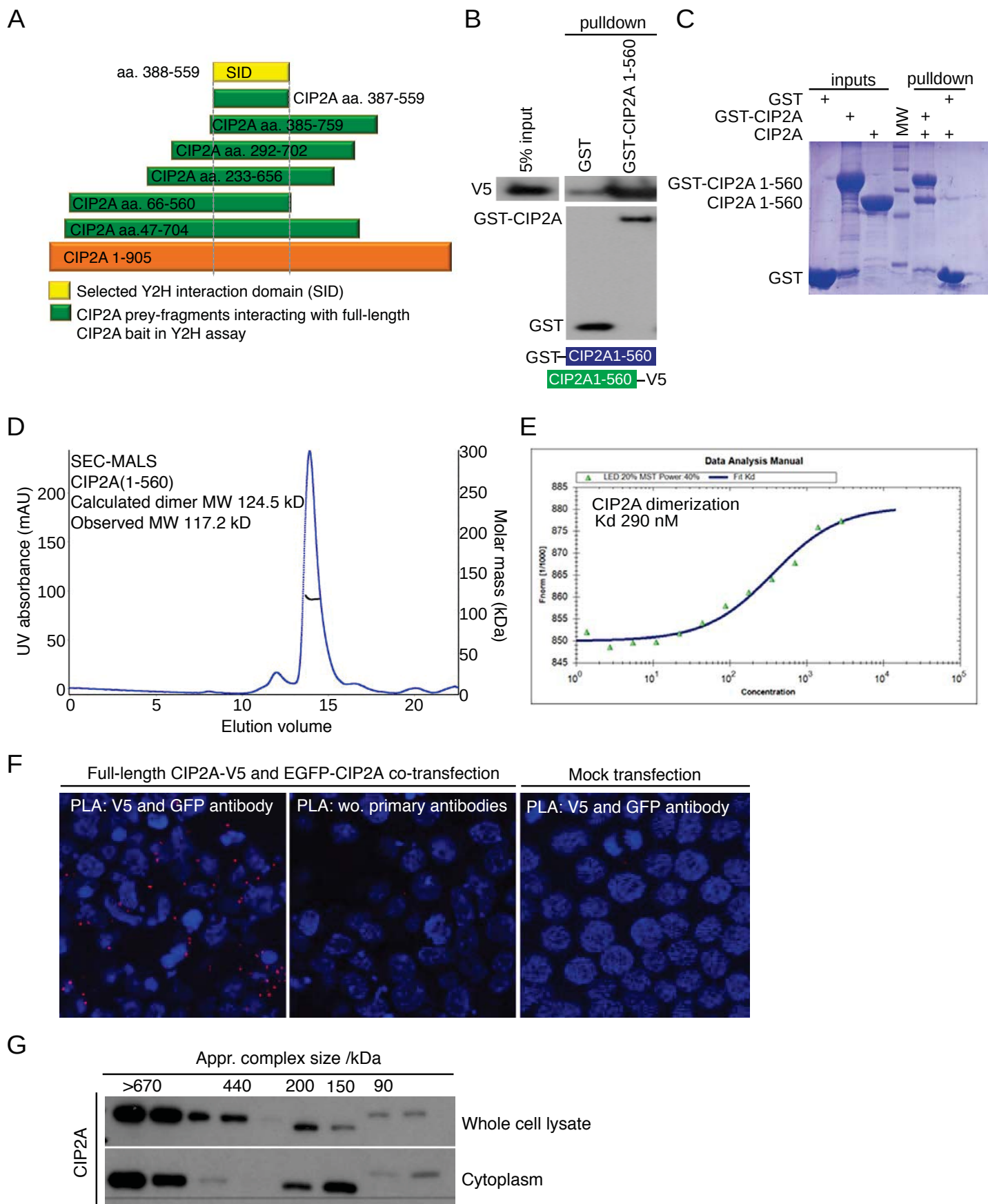


Figure 1

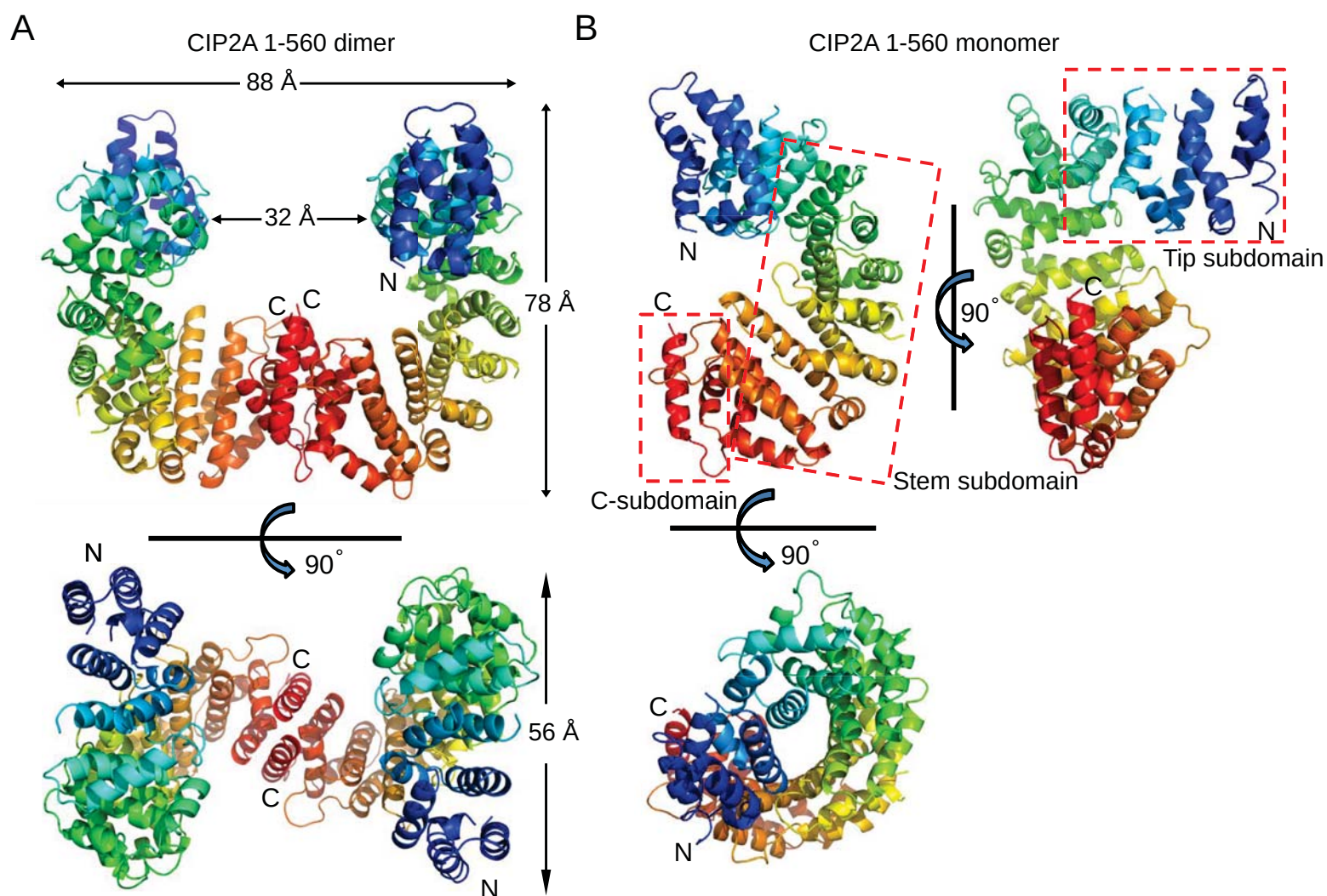


Figure 2

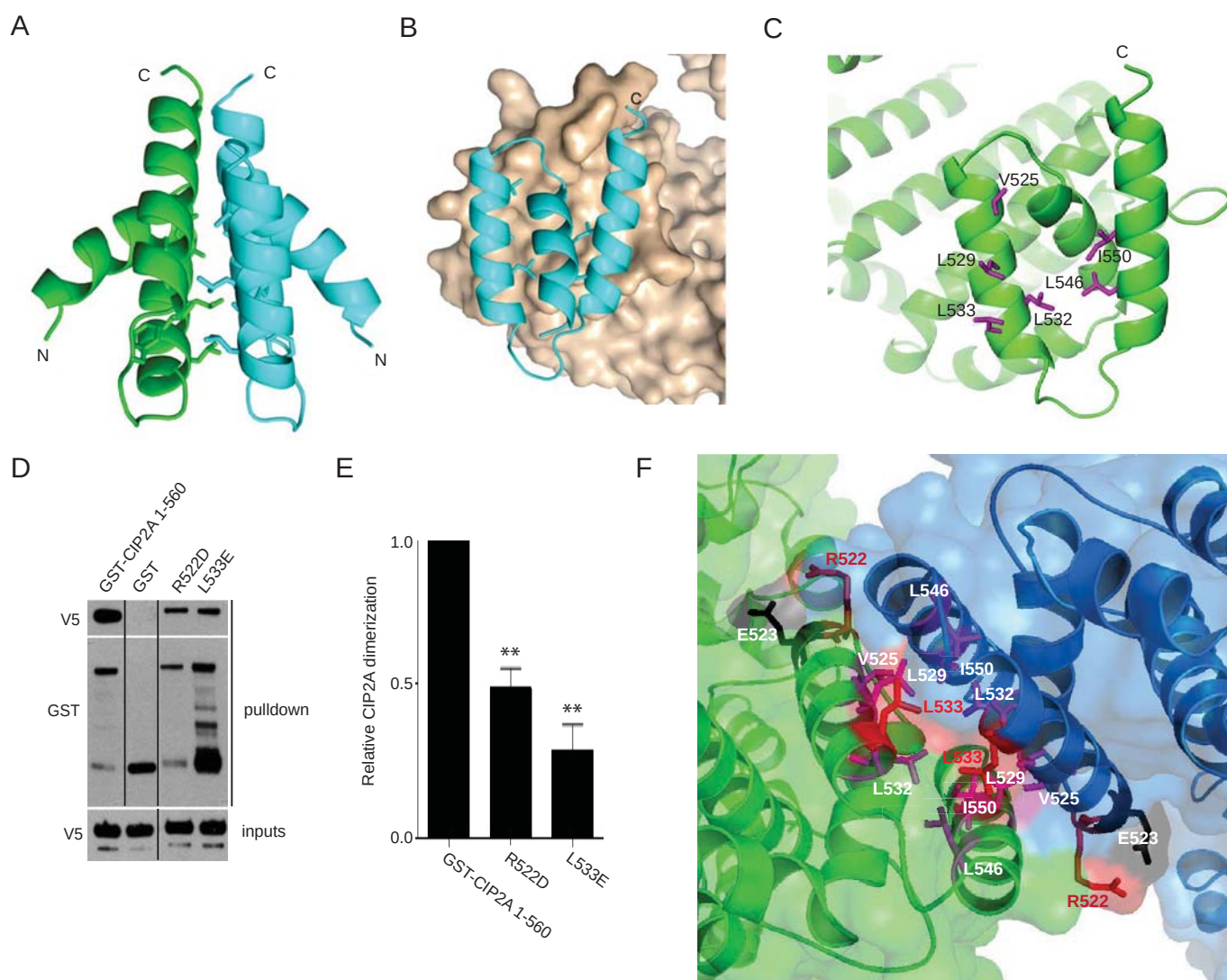


Figure 3

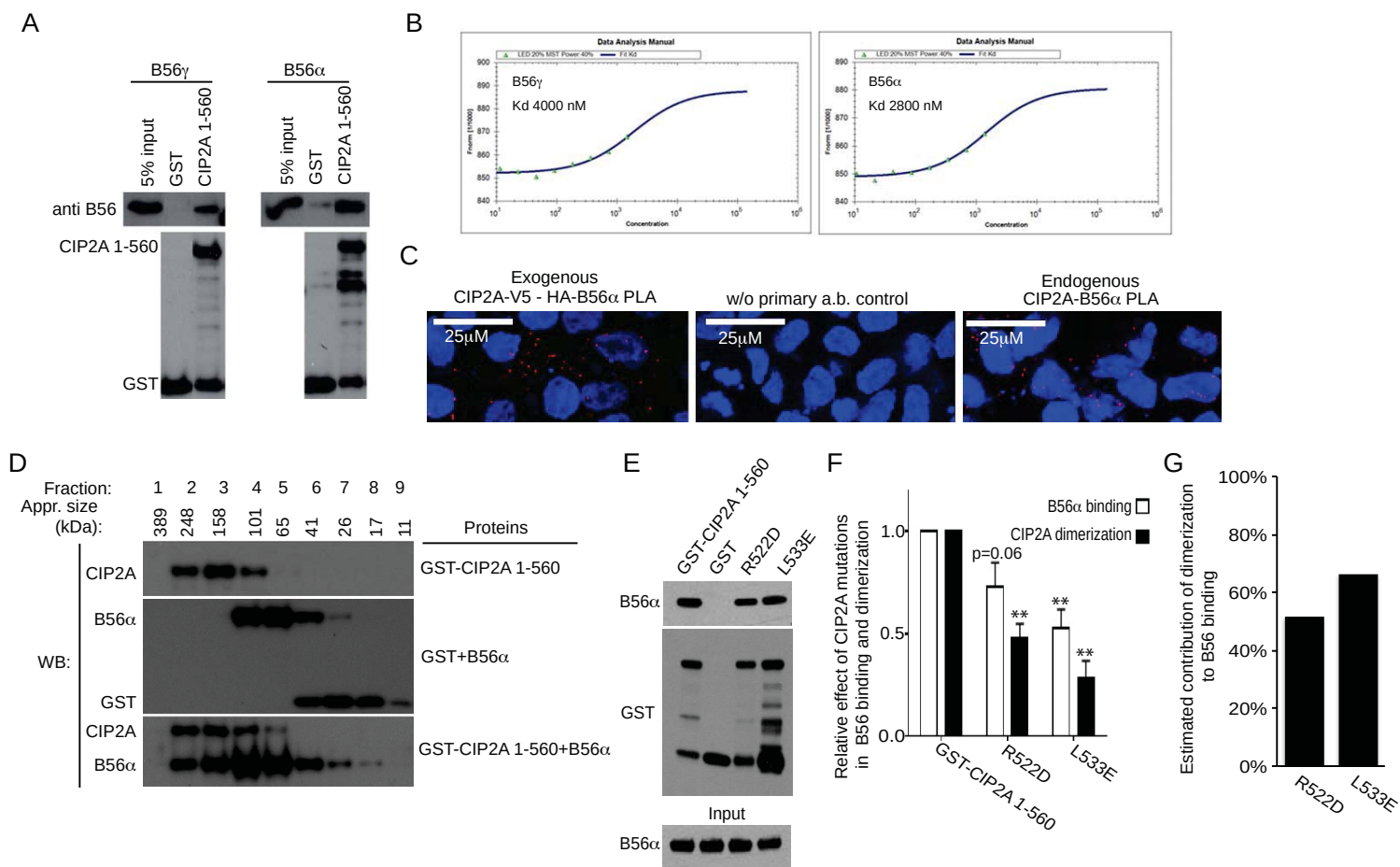
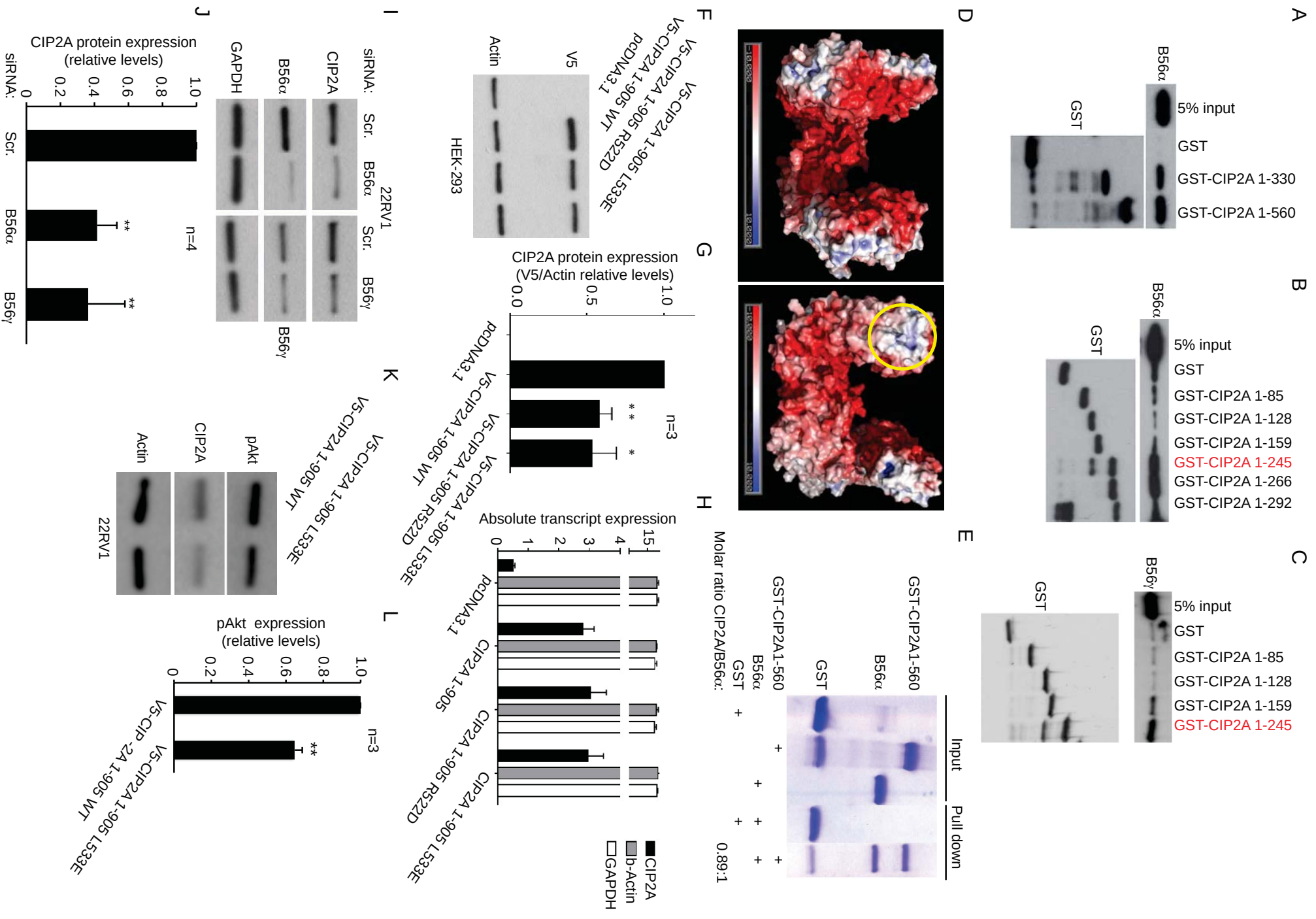


Figure 4



## Figure 5

## Expanded View Figure Legends

Figure EV1. A) Summary of screen parameters and description of Global PBS classification for confidence of interactions observed in the screen. B) List of CIP2A (KIAA1524) and B56g (PPP2R5C) prey clones found to interact with full-length CIP2A bait in Y2H screen. C) Description of selected interaction domain (SID) determination and graphical representation of SID for both CIP2A (KIAA1524) and B56g (PPP2R5C) interaction with full-length CIP2A bait. The SID is depicted in relation to predicted structural domains of both proteins (other colour bars).

Figure EV2. Screening for mutants of CIP2 deficient for dimerization. The helices of two monomeric CIP2A units are shown in light grey and blue. Mutated residues are shown as red sticks. The following mutants were generated and tested: L529A, L532A, L529A L532A (2A), L529A L532A L533A (3A), R522D, Q526E, L529E, L533E, R522D Q526E, Q526E L529E L533E (3E). The structure was generated in RasWin.

Figure EV3. Conservation of potential B56 binding site on CIP2A(1-560) between aa. 159-245 (yellow oval). Conservation analysis was made based on ten CIP2A sequences from representative species (see also Appendix Figure S4). The darker color indicates higher conservations.

Figure EV4. Structure-function relationship between CIP2A-B56 interaction and CIP2A protein stability. A) CIP2A monomers are shown in green and cyan. Minimal region on CIP2A required for B56 $\alpha$  binding, 159-245, is in magenta. N230 residue from this region is shown as blue stick. It is pointed away from the CIP2A surface and is in the middle of a positively charged and conserved patch (see Figure EV3). The figure was made in Pymol. B) Impaired interaction of GST-CIP2A(1- 560) N230E

mutant with B56 $\alpha$ . Quantitation is mean + S.E.M. from 4 independent experiments.  $p = 0.01$ , t-test.

C) Thermal unfolding analysis by Prometheus NT.48 shows comparable melting profile for recombinant CIP2A(1-560) WT and L533E mutant, indicating similar protein folding. D) Inhibition of B56 $\alpha$  destabilizes CIP2A protein in HeLa cells. Shown is Western blot analysis from HeLa cells 72h after transfection. E) Quantification of relative CIP2A protein levels from D). Shown is mean + S.D. from 3 independent experiments. F) RNAi-mediated depletion of B56 $\alpha$  does not impact endogenous CIP2A mRNA expression. The analysis is done from parallel samples to those used in D).

A

Results Summary

homo sapiens - CIP2A  
vs Human Breast Tumor Epithelial Cells\_RP1

Thu, Mar 24, 2011 - 02:43 PM

Screen Parameters

|                         |                                             |
|-------------------------|---------------------------------------------|
| Nature                  | cDNA                                        |
| Reference Bait Fragment | homo sapiens - CIP2A (1-905) ; hgx2736v1    |
| Prey Library            | Human Breast Tumor Epithelial Cells_RP1     |
| Vector(s)               | pB27 (N-LexA-bait-C fusion)                 |
| Processed Clones        | 45 (pB27) + 142 (pB27)                      |
| Analyzed Interactions   | 35.4 millions (pB27) + 54.4 millions (pB27) |
| 3AT Concentration       | 0.0 mM (pB27) + 0.0 mM (pB27)               |

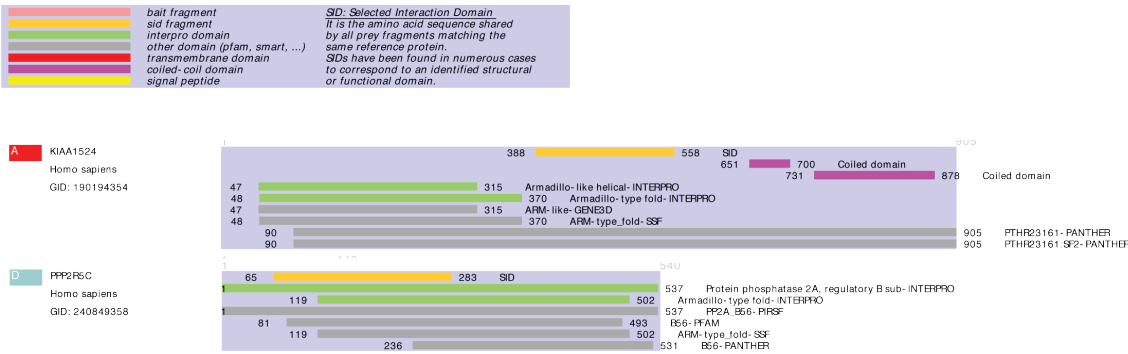
Global PBS®

| Global PBS (for Interactions represented in the Screen) |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Nb | %     |
|---------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|-------|
| <b>A</b>                                                | Very high confidence in the interaction                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | 4  | 10.3% |
| <b>B</b>                                                | High confidence in the interaction                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 4  | 10.3% |
| <b>C</b>                                                | Good confidence in the interaction                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 2  | 5.1%  |
| <b>D</b>                                                | Moderate confidence in the interaction<br>This category is the most difficult to interpret because it mixes two classes of interactions :<br>- False-positive interactions<br>- Interactions hardly detectable by the Y2H technique (low representation of the mRNA in the library, prey folding, prey toxicity in yeast)                                                                                                                                                                                 | 21 | 53.8% |
| <b>E</b>                                                | Interactions involving highly connected prey domains, warning of non-specific interaction. The threshold for high connectivity is 10 for screens with Human, Mouse, Drosophila and Arabidopsis and 6 for all other organisms. They can be classified in different categories:<br>- Prey proteins that are known to be highly connected due to their biological function<br>- Proteins with a prey interacting domain that contains a known protein interaction motif or a biochemically promiscuous motif | 7  | 17.9% |
| <b>F</b>                                                | Experimentally proven technical artifacts                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | 1  | 2.6%  |

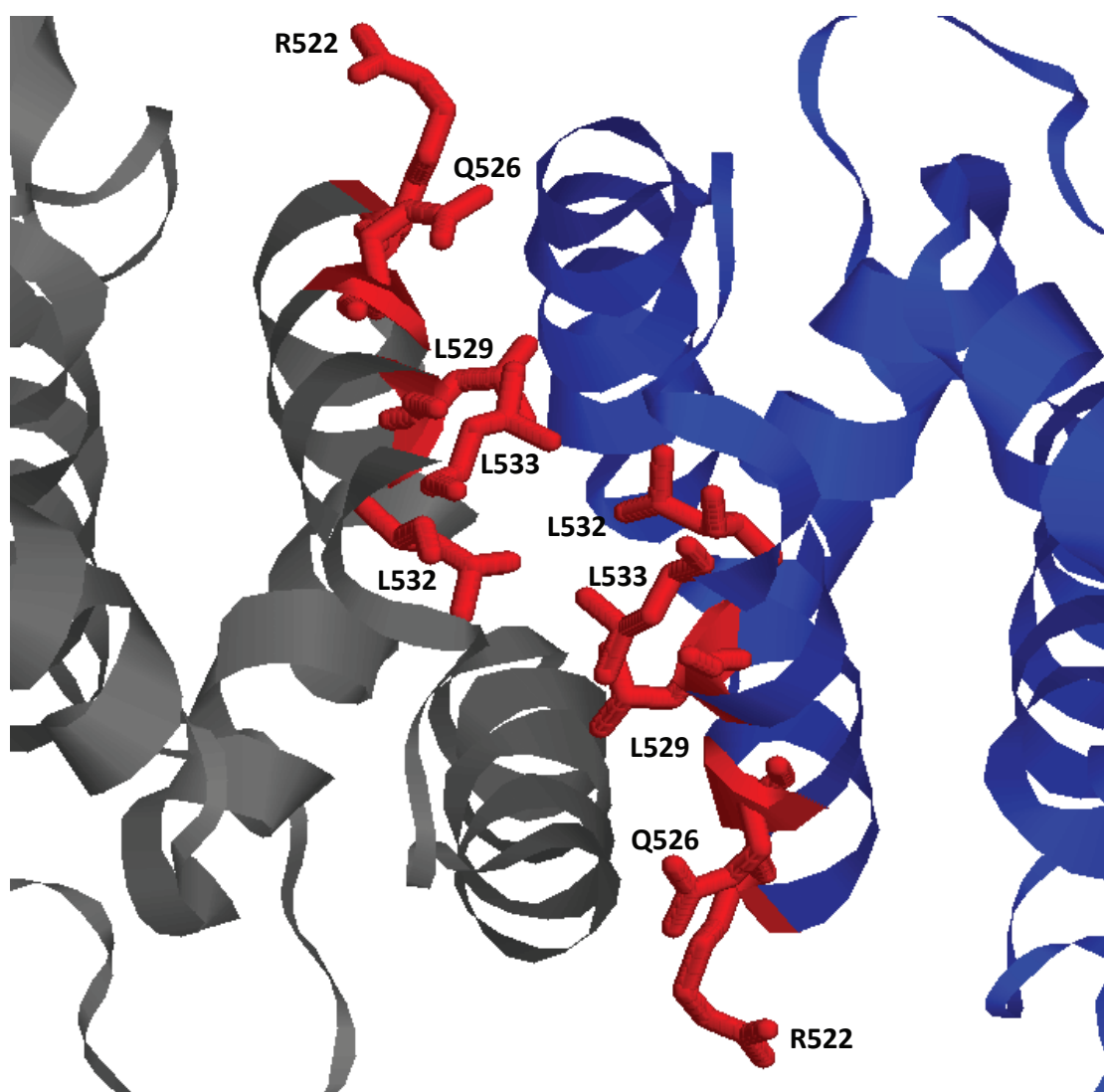
B

| Clone Name | Type Seq | Gene Name (Best Match)  | Start..Stop (nt) | Frame | Sense | %Id 5p | %Id 3p | PBS      |
|------------|----------|-------------------------|------------------|-------|-------|--------|--------|----------|
| pB27_B-119 | 5p/3p    | Homo sapiens - KIAA1524 | 141..2111        | IF    |       | 98.9   | 99.1   | <b>A</b> |
| pB27_B-145 | 5p/3p    | Homo sapiens - KIAA1524 | 141..2111        | IF    |       | 98.0   | 99.0   | <b>A</b> |
| pB27_B-69  | 5p/3p    | Homo sapiens - KIAA1524 | 198..1680        | IF    |       | 99.6   | 99.4   | <b>A</b> |
| pB27_A-19  | 5p/3p    | Homo sapiens - KIAA1524 | 699..1696        | IF    |       | 98.3   | 97.3   | <b>A</b> |
| pB27_B-9   | 5p/3p    | Homo sapiens - KIAA1524 | 699..1696        | IF    |       | 97.9   | 99.7   | <b>A</b> |
| pB27_B-116 | 5p/3p    | Homo sapiens - KIAA1524 | 876..2106        | IF    |       | 98.5   | 98.9   | <b>A</b> |
| pB27_B-128 | 5p/3p    | Homo sapiens - KIAA1524 | 876..2106        | IF    |       | 98.7   | 97.8   | <b>A</b> |
| pB27_A-26  | 5p/3p    | Homo sapiens - KIAA1524 | 876..2106        | IF    |       | 98.5   | 99.9   | <b>A</b> |
| pB27_A-24  | 5p/3p    | Homo sapiens - KIAA1524 | 876..2106        | IF    |       | 97.4   | 98.6   | <b>A</b> |
| pB27_B-104 | 5p/3p    | Homo sapiens - KIAA1524 | 1155..2277       | IF    |       | 98.0   | 99.3   | <b>A</b> |
| pB27_A-20  | 5p/3p    | Homo sapiens - KIAA1524 | 1155..2277       | IF    |       | 98.6   | 97.3   | <b>A</b> |
| pB27_A-32  | 5p/3p    | Homo sapiens - KIAA1524 | 1155..2277       | IF    |       | 99.4   | 99.6   | <b>A</b> |
| pB27_B-45  | 5p/3p    | Homo sapiens - KIAA1524 | 1161..1676       | IF    |       | 100.0  | 100.0  | <b>A</b> |
| pB27_B-130 | 5p/3p    | Homo sapiens - PPP2R5C  | 192..850         | IF    |       | 100.0  | 98.8   | <b>D</b> |
| pB27_B-22  | 5p/3p    | Homo sapiens - PPP2R5C  | 192..850         | IF    |       | 99.8   | 99.7   | <b>D</b> |
| pB27_B-102 | 5p/3p    | Homo sapiens - PPP2R5C  | 192..850         | IF    |       | 99.7   | 99.7   | <b>D</b> |
| pB27_B-122 | 5p/3p    | Homo sapiens - PPP2R5C  | 192..850         | IF    |       | 99.8   | 96.7   | <b>D</b> |

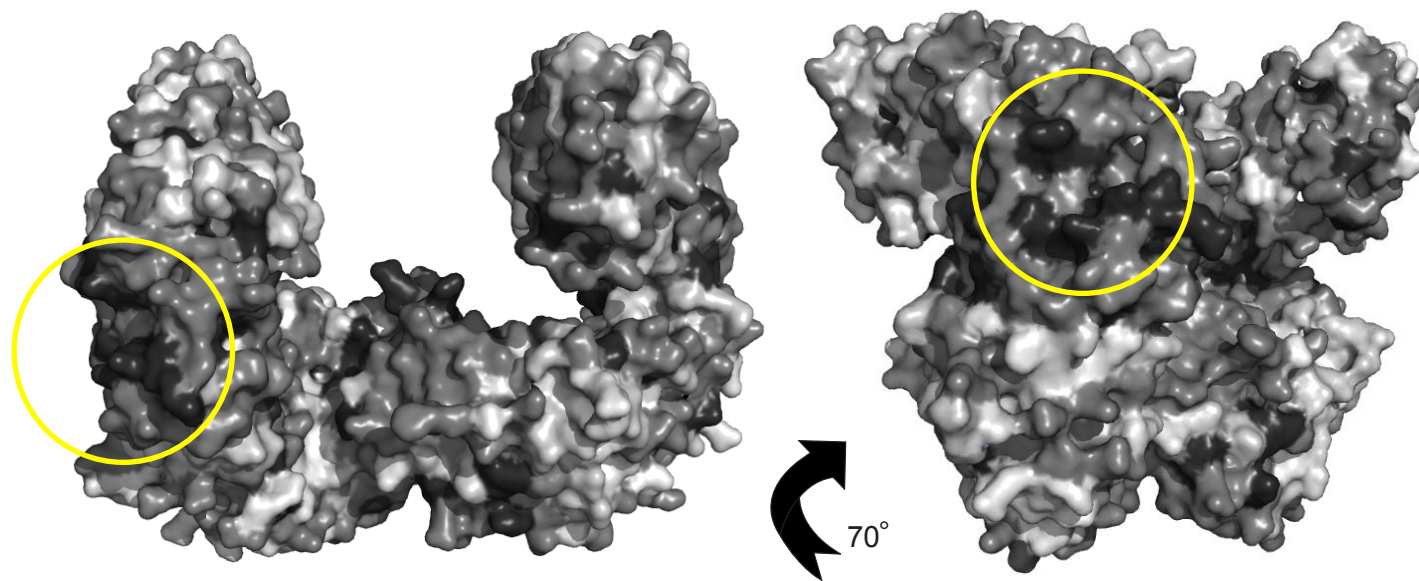
C



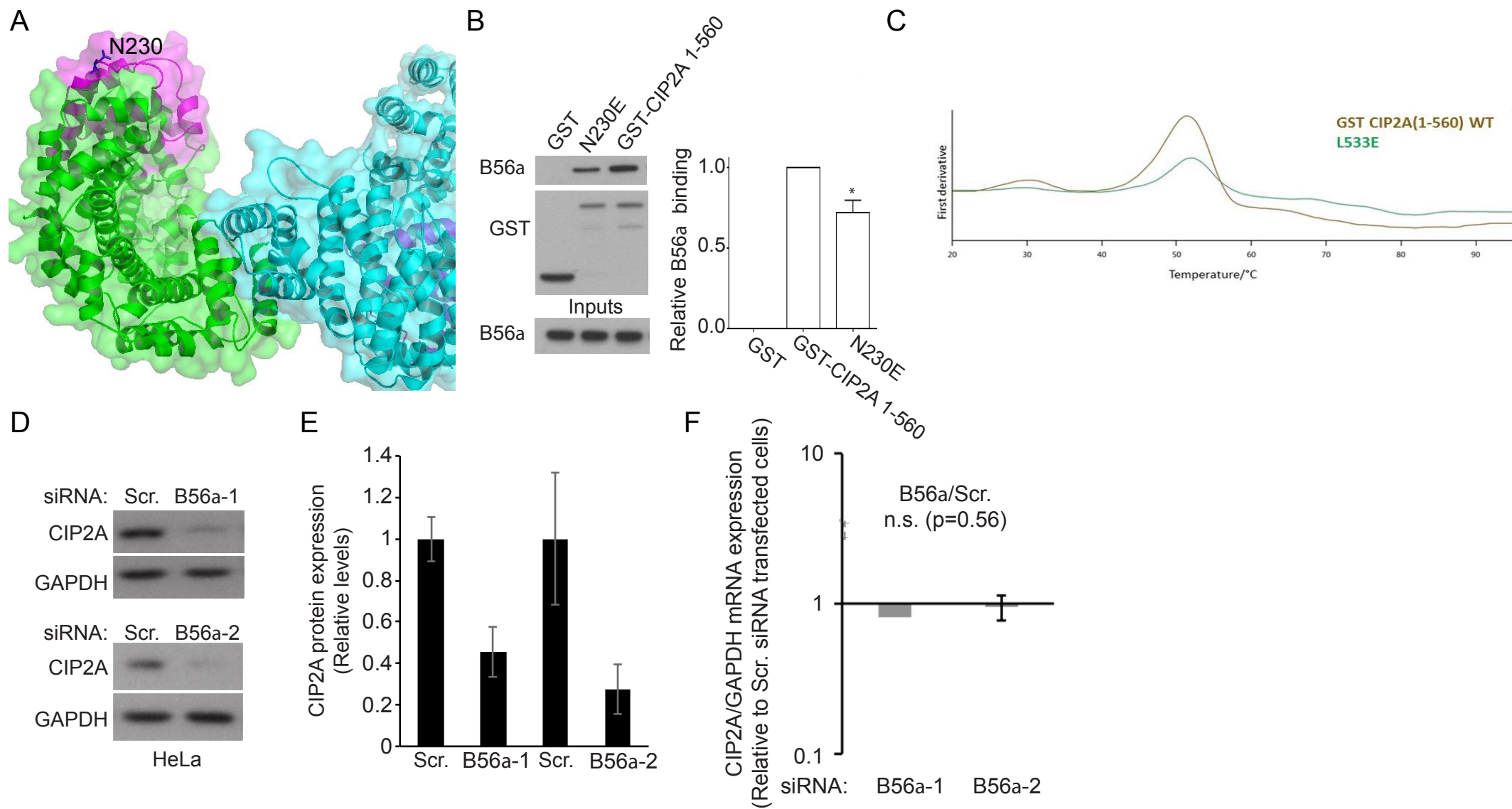
Expanded view 1



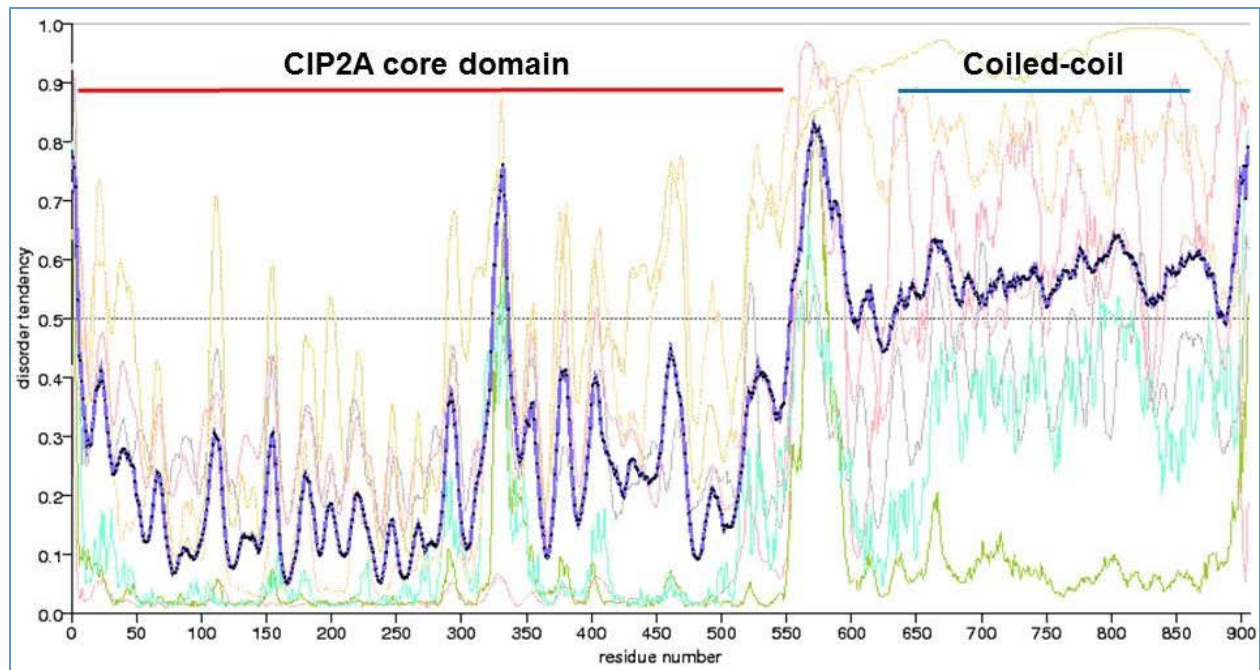
Expanded view 2



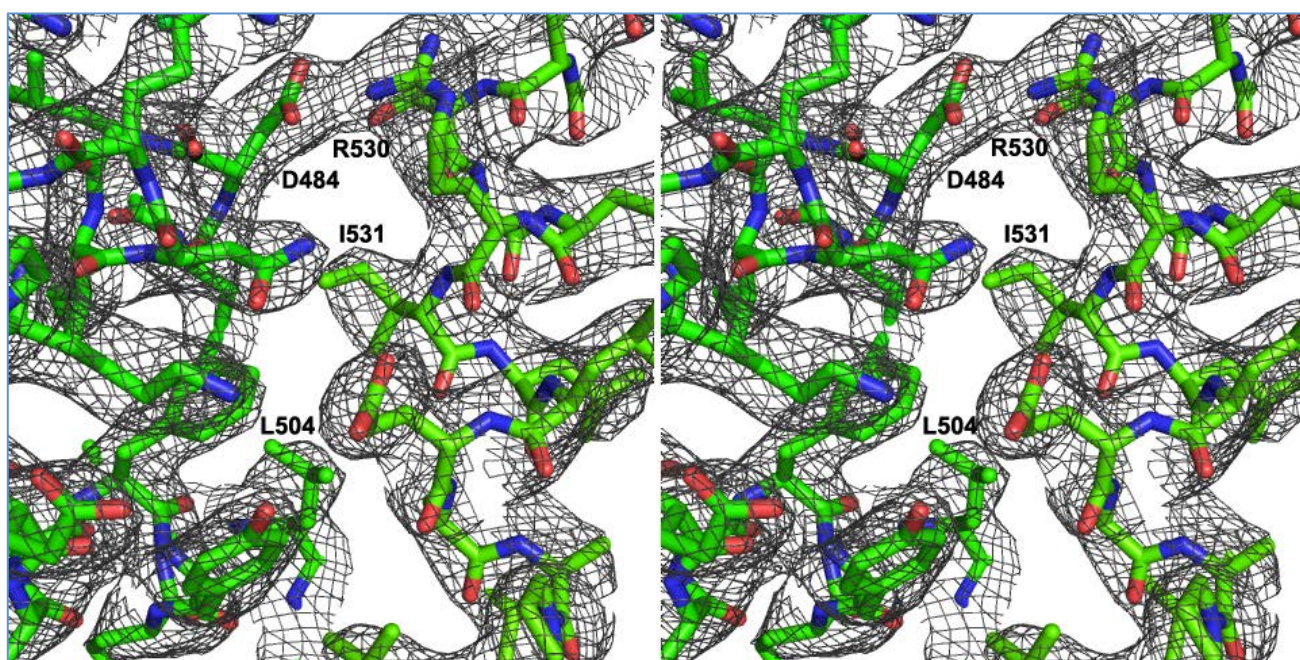
Expanded view 3



Expanded view 4

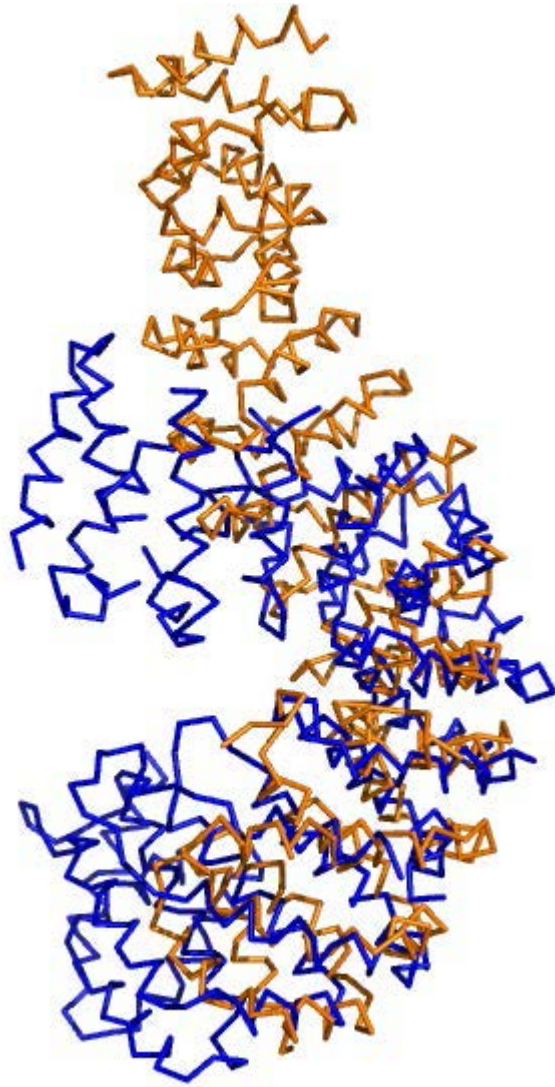


**Appendix Figure S1.** Folding propensity of human CIP2A. The X-axis corresponds to residue number 1-905. The Y axis is the disorder tendency for each residue. The blue curve is the average result from six different programs, as summarized by the metaPrDOS server. Higher values indicate higher disorder propensity. This prediction is consistent with our biochemical analysis that CIP2A(1-560) forms the folded core domain, whereas the rest of CIP2A, including the predicted coiled-coil region, is most likely disordered.

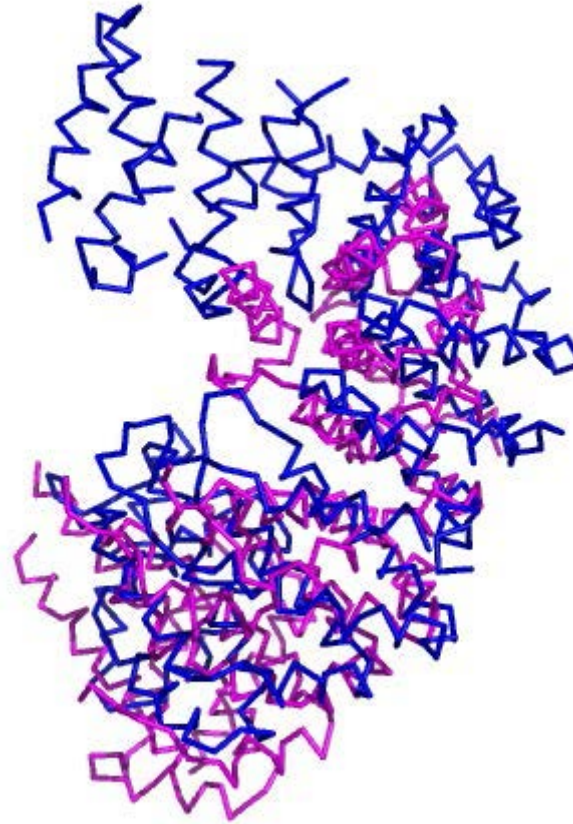


**Appendix Figure S2.** Stereo view of 2Fo-Fc electron density map, contoured at  $1\sigma$ , showing the joint area of the stem and C-dimerization domains. While D484 and L504 are parts of the stem domain, R530 and I531 belong to the dimerization domain.

A

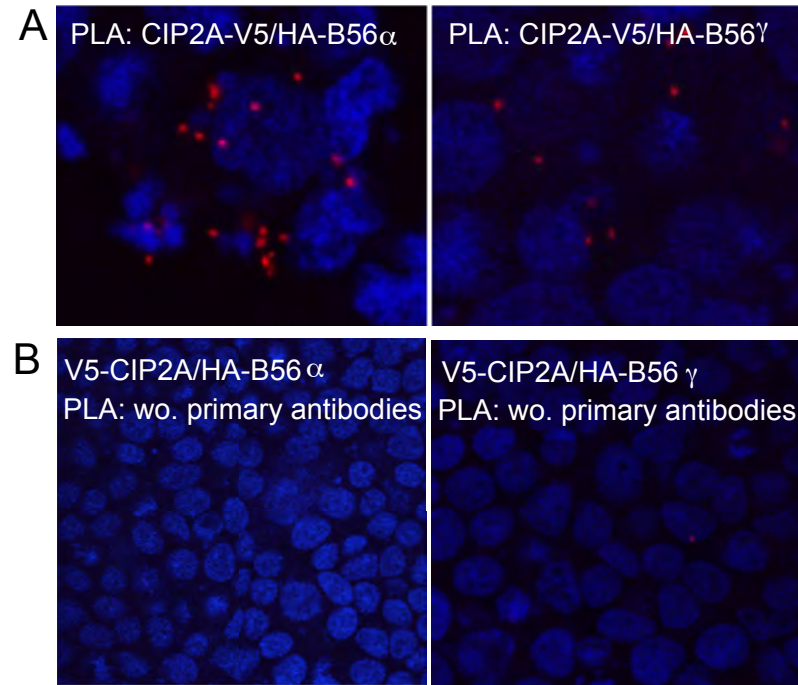


B



**Appendix Figure S3.** The CIP2A 3D structure is distinct from all known protein structures. No known homodimer structure folds in a similar shape. Based on 3D structure comparison carried out by the Dali server (Appendix Table S1), the two most similar 3D structures of CIP2A are these of  $\beta$ -catenin (PDB ID: 1TH1, an armadillo-repeat protein) and Wapl (PDB ID: 4K6J, a HEAT-repeat protein). The CIP2A structure (in blue) is superimposed with these of  $\beta$ -catenin (orange, panel A) and Wapl (pink, panel B).





**Appendix Figure S5.** A) PLA for interaction between full-length V5-tagged CIP2A protein and HA-tagged B56 $\alpha$ , and - $\gamma$ 3. HEK293T cells co-transfected with CIP2A-V5 and HA-tagged B56 constructs were subjected to PLA with V5 and HA antibodies. Red dots indicate for association between CIP2A and B56 proteins. Shown is representative image from two PLA experiments with similar results. B) Specificity of PLA reaction to CIP2A-B56 interaction cells transfected with CIP2A-V5 and HA-tagged B56 constructs were subjected to PLA without V5 and HA antibodies. A) and B) panels are shown with different magnification to better illustrate the positive PLA signals in A).