Oncoprotein CIP2A is stabilized via interaction with tumor suppressor PP2A/B56

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Abstract

Protein phosphatase 2A (PP2A) is a critical human tumor suppressor. Cancerous inhibitor of PP2A (CIP2A) supports the activity of several critical cancer drivers (Akt, MYC, E2F1) and promotes malignancy in most cancer types via PP2A inhibition. However, the 3D structure of CIP2A has not been solved, and it remains enigmatic how it interacts with PP2A. Here, we show by yeast two-hybrid assays, and subsequent validation experiments, that CIP2A forms homodimers. The homodimerization of CIP2A is confirmed by solving the crystal structure of an N-terminal CIP2A fragment (amino acids 1–560) at 3.0 Å resolution, and by subsequent structure-based mutational analyses of the dimerization interface. We further describe that the CIP2A dimer interacts with the PP2A subunits B56α and B56γ. CIP2A binds to the B56 proteins via a conserved N-terminal region, and dimerization promotes B56 binding. Intriguingly, inhibition of either CIP2A dimerization or B56α/γ expression destabilizes CIP2A, indicating opportunities for controlled degradation. These results provide the first structure–function analysis of the interaction of CIP2A with PP2A/B56 and have direct implications for its targeting in cancer therapy.

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Introduction

Protein phosphatase 2A (PP2A) is a critical tumor suppressor that normally acts by preventing cellular transformation, whereas its inhibition promotes the various malignant characteristics of human

cancer cells [1,2]. PP2A also regulates various physiological processes. Therefore, further understanding of structural mechanisms of PP2A regulation is highly relevant for various disciplines. In cancer cells, PP2A inhibition results in hyperphosphorylation of a large number of oncogenic drivers and synergizes with other oncogenic events, such as constitutive RAS activity [1,3–5]. Importantly, PP2A complex components are mutated at a relatively low frequency in most types of human cancer. This establishes the reactivation of PP2A as an attractive novel approach in cancer therapy [2,6]. Furthermore, the recent discovery of small molecules and peptides that are capable of restoring PP2A activity in human cancer cell lines provides convincing support to this strategy by demonstrating robust *in vivo* efficacy in preclinical studies [2,7].

PP2A is inhibited in cancer by a group of otherwise unrelated PP2A inhibitor proteins [2,8]. Among them, cancerous inhibitor of PP2A (CIP2A) is the most prevalent oncoprotein. CIP2A is a long-lived protein in cancer cells [9], and its depletion results in inactivation of many oncogenic PP2A targets (e.g., MYC, E2F1, Akt) [6]. Importantly, these effects have been shown to be reversible upon PP2A co-inhibition [6,10–12]. Regarding functional synergism between PP2A inhibition and RAS signaling upon cell transformation, and cell cycle progression [1,3–5], CIP2A overexpression is required for RAS-driven human cell transformation [10]. Moreover, we recently demonstrated significant overlap between CIP2A and RAS-regulated phosphoproteomes [13]. Clinically, CIP2A overexpression is an equally strong predictor of poor survival in TCGA pan-cancer data as KRAS mutation, and corroborating the functional synergism between CIP2A and RAS, the patients with both of these alterations constituted the patient population with clearly the worst outcome [13].

In addition to robust effects of CIP2A depletion by siRNA on malignant cell growth *in vitro* [6], several studies have demonstrated that CIP2A inhibition very potently inhibits xenograft tumor growth of different types of cancer cells [10,14–18]. CIP2A also mediates resistance to many cancer therapeutics [6,9,11,19–21].

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Importantly, even though CIP2A deficiency inhibits MYC activity, and Her2-driven mammary tumorigenesis *in vivo* [11,22], it does not compromise normal mouse development or growth, except for a defect in spermatogenesis [11,22,23]. Notably, high CIP2A protein expression predicts poor patient survival in over a dozen different cancer types [20], and thus, its prognostic and functional relevance equals, or exceeds, that of most oncoproteins that have been traditionally considered important oncogenic drivers. Based on all these data, inhibition of CIP2A protein expression and/or activity could constitute a very efficient cancer therapy strategy without detrimental side effects. However, a lack of structural information for the CIP2A protein has thus far hampered the advancement of this potential cancer therapy target in drug development.

PP2A functions as a protein complex consisting of either a core dimer between the scaffolding A subunit (PR65) and the catalytic subunit PP2Ac, or a trimer in which one of the regulatory B subunits interacts with the AC core dimer [24]. Our current understanding supports the view that different B subunits mediate the substrate specificity of the PP2A trimer [24] and that only a subset of the numerous B subunits are relevant for the tumor suppressor activity of PP2A [25,26]. For example, B56 α mediates PP2A complex recruitment and the PP2A-mediated dephosphorylation of MYC serine 62 [27,28]. Another B56 family protein, B56 γ , functions as a human tumor suppressor [25,26] and negatively regulates Akt kinase phosphorylation [25,29]. CIP2A has been shown to promote phosphorylation and activity of both of these critical PP2A targets [6,9,10,12,16]. However, thus far there has not been any evidence of whether CIP2A would directly bind to any of the numerous PP2A complex components.

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

Results

CIP2A homodimerization

Our understanding of proteins that interact with CIP2A is limited [30]. Therefore, we conducted a yeast two-hybrid (Y2H) analysis with full-length CIP2A as bait, and using commercial Hybrigenics platform with over 80×10^6 prey clones (Fig EV1A). As CIP2A is expressed at a very low level in most normal tissues but is over-expressed in breast cancer [9,11,12], we used a mixed cDNA library from several breast cancer cell lines (T47D, MDA-MB-468, MCF7, BT20). Using Hybrigenics Global Predicted Biological Score (Global PBS[®]) computational platform that scores probability of an interaction to be specific, we surprisingly found CIP2A itself as a very high-confidence interaction partner for full-length CIP2A bait (Fig EV1B). The various CIP2A prey clones that interacted with full-length CIP2A bait are depicted as green bars in Fig 1A. Number of independent interacting CIP2A prey clones allowed selected interaction domain (SID) analysis that delineates the shortest fragment that is shared with all interacting clones, and thus represents a potential region

mediating the CIP2A homodimerization. The SID analysis for interaction between two CIP2A molecules indicated that CIP2A homodimerization is mediated by a region encompassing amino acids 388–558 (Figs 1A and EV1C). Interestingly, structural foldability (flexibility) analysis indicates that this potential homodimerization domain of CIP2A comprises of a well-folded domain that is followed by a flexible linker and a predicted coiled-coil domain that is most likely disordered (Appendix Fig S1). This prediction was supported by notification that based on gel filtration analysis, the C-terminal fragment *per se* tends to aggregate. Also, consistent with an earlier publication [31], full-length CIP2A is not stable enough to be purified from either *E. coli* or insect cells. In contrast, the N-terminal 1–560 fragment of CIP2A spanning the SID could be produced in *E. coli* in large quantities and was relatively stable. Therefore, we focused on the human CIP2A(1–560) fragment to further confirm CIP2A homodimerization.

To biochemically verify CIP2A homodimerization, we used thrombin cleavage to remove the GST tag from GST-CIP2A(1–560)-V5 and used this as prey in a GST pulldown experiment with the parental GST-CIP2A(1–560) protein. Using V5 epitope antibody in Western blot analysis of GST pulldown samples, CIP2A(1–560)-V5 was found to not significantly associate with GST alone, whereas a robust interaction was observed between the two CIP2A fragments (Fig 1B). Cleavage of GST tag from GST-CIP2A(1–560)-V5 in the previous assay excluded the possibility that the observed CIP2A dimerization would be mediated by GST dimerization. However, to further exclude the possibility that interaction was mediated due to dimerization via the affinity tags, CIP2A dimerization was further demonstrated by Coomassie staining of gel after pulldown of CIP2A(1–560) without any tags (Fig 1C). Furthermore, our size-exclusion chromatography-coupled multi-angle light scattering (SEC-MALS) analysis clearly show that purified untagged CIP2A(1–560) has a shape-independent molecular mass of 117.2 kDa in solution, which is in good agreement with the calculated MW of 124.5 kDa for a CIP2A(1–560) dimer (Fig 1D).

Interestingly, in addition to confirming the CIP2A homodimerization indicated by the Y2H assay, the ability to detect dimerization between two different CIP2A proteins in pulldown assays suggests that binding affinity of the dimerization interface may be relatively modest and that there is detectable exchange between interacting monomers. This conclusion is supported by results of microscale thermophoresis (MST) analysis revealing that CIP2A(1–560) homodimerizes with a modest affinity (K_d) of 290 nM (Fig 1E). Whether the C-terminal sequences lacking from CIP2A(1–560) might further stabilize the dimer remains to be studied. On the other hand, many Y2H prey clones of CIP2A that interacted with full-length CIP2A bait contained long stretches of amino acids C-terminally from amino acid 560 (Fig 1A). This clearly indicates that the homodimerization region included in CIP2A(1–560) is functional also in the presence of C-terminal regions of CIP2A. To further verify dimerization of full-length CIP2A containing those C-terminal sequences, we analyzed physical interaction between two differentially epitope-tagged full-length CIP2A proteins in cells by proximity ligation assay (PLA). PLA has been validated recently by numerous studies to detect protein–protein interaction in cultured cells and *in vivo* [22,32]. Here, by using V5 and GFP antibodies coupled with specific PLA probes, we could detect typical PLA dots clearly indicative of interaction between co-transfected CIP2A-V5 and EGFP-CIP2A fusion proteins (Fig 1F, left

Figure 1. Verification of homodimerization of the CIP2A(1–560).

- A Summary of CIP2A prey fragments (green) interacting with full-length CIP2A(1–905) bait (orange) in yeast two-hybrid screen. Original Y2H data are shown in Fig EV1. Number of independent interacting CIP2A prey clones allowed selected interaction domain (SID) analysis that delineates the shortest fragment that is shared with all interacting clones, and thus represents a potential region mediating the CIP2A homodimerization. Gray dotted lines illustrate location of SID across all prey fragments and in full-length CIP2A.
- B Dimerization of CIP2A(1–560) fragment analyzed by GST pulldown. Equal molar amounts of GST and GST-CIP2A(1–560) were incubated with CIP2A(1–560)-V5 fragment for 1 h at 37°C before pulldown.
- C GST-tagged CIP2A(1–560) (90 kDa), but not GST, can pulldown untagged CIP2A(1–560) (60 kDa) in a stoichiometric manner. The SDS-PAGE was stained with Coomassie Blue.
- D SEC-MALS analysis of untagged CIP2A(1–560) on a Superdex 200 Increase 10/300 GL column. The blue curve is the UV absorbance profile, whereas the black line shows the measured molar mass for the major peak. Untagged CIP2A(1–560) has a nominal MW of 62 kDa whereas SEC-MALS chromatogram show shape-independent MW reading at 117.2 kDa which corresponds to the molecular weight of a CIP2A(1–560) dimer.
- E Thermophoresis analysis of interaction between labeled and non-labeled CIP2A(1–560) proteins.
- F Proximity ligation assay (PLA) for interaction between two differently tagged full-length CIP2A proteins. HEK293T cells co-transfected with CIP2A-V5 and EGFP-CIP2A constructs were subjected to PLA with either V5 and GFP antibodies (left panel), or as control with only secondary PLA probes (middle panel). Red dots indicate the association between two CIP2A proteins. As another specificity control, mock-transfected cells were analyzed with PLA including both V5 and GFP primary antibodies. Shown is a representative image from two PLA experiments. Scale bars, 10 μ m.
- G Analysis of endogenous CIP2A dimerization by size-exclusion chromatography of HeLa total cell extract and cytoplasmic extracts. Estimated molecular weights are based on column calibration with standard proteins. Shown is a representative result of three independent experiments. Size difference between CIP2A in different fractions is indicative of post-translational regulation of CIP2A upon complex formation. Gray line in the cytoplasm blot is an artifact from film development and does not affect result interpretation.

panel). On the other hand, only random background PLA signals were observed from non-transfected cells with primary V5 and GFP antibodies, or from CIP2A-V5 and EGFP-CIP2A co-transfected cells subjected to PLA without primary antibodies (Fig 1F).

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

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

Crystal structure of CIP2A(1–560) reveals the homodimerization interface

To date, no structural information about CIP2A is available. In order to gain structural insights into CIP2A dimerization, the CIP2A(1–560) fragment was crystallized, and its crystal structure was determined at 3.0 Å resolution using the selenium-methionine single-wavelength anomalous scattering (SAD) method (Appendix Table S1 and Appendix Fig S2). In the crystal lattice, there are two CIP2A(1–560) molecules, related by a non-crystallographic twofold axis, in each asymmetric unit (Fig 2A). This finding is fully consistent with both Y2H and biochemical data, that CIP2A(1–560) forms a homodimer. Moreover, in the crystal structure, the dimer interface joining two CIP2A(1–560) molecules is located in the C-terminal end of CIP2A(1–560) which also is fully in line with Y2H SID prediction that postulated the dimerization domain to be located in the region 338–558 of CIP2A. Overall, the CIP2A(1–560) dimer structure resembles an oppositely twisted double hook (Fig 2A).

The CIP2A(1–560) monomer is an all-helical protein, with most of the molecule formed by armadillo or armadillo-like repeats (Fig 2B), and can be roughly divided into “tip”, “stem”, and C-dimerization subdomains. The first 185 residues form a “tip”

domain consisting of five shortened armadillo repeats. Following a twist-forming loop, residues 188–505 form the “stem” domain, consisting of atypical armadillo repeats 6–11; residues 507–559 form three helices that are responsible for CIP2A(1–560) dimerization (Fig 2B). Some of the armadillo repeats in the “stem” subdomain display the structural features of HEAT repeats, as revealed by protein folding similarity searches using the Dali server [33]. In addition to the armadillo repeat domains of β -catenin and APC, the atypical HEAT-repeat domain of Wapl is among the closest structural neighbors of the stem subdomain of CIP2A(1–560) (Appendix Table S1 and Appendix Fig S3).

Mutational analysis of CIP2A(1–560) dimerization interface

The dimerization subdomain is formed by the last three helices of CIP2A(1–560) (Fig 3A and B). The last two helices and the loop linking to the previous helix form a relatively flat and highly hydrophobic surface, mediating the homodimerization of CIP2A(1–560) (Fig 3A and B). Formation of this homodimer interface buries an accessible surface area of 1,913 Å², which is typical for specific protein–protein interactions. The two C-terminal ends of the CIP2A(1–560) homodimer are spatially very close to each other, and both point to the “top” side of the twisted double hook (Figs 2A and 3A). The key residues involved in the interaction between CIP2A monomers include V525, L529, L532, L533, L546, and I550 (Fig 3C), and all these residues, with the exception of L533, are evolutionarily conserved across different species (Appendix Fig S4).

To interfere with the CIP2A homodimerization interface, we introduced series of single-point mutations to residues that were directly involved in the interaction between CIP2A monomers, or were predicted to potentially interfere with dimerization, and examined their impact on CIP2A dimerization. All created mutations are depicted in Fig EV2. While some of these CIP2A mutants, especially the ones with multiple mutations, had low solubility that prohibited further *in vitro* test, two soluble single-point mutants, R522D and L533E, repeatedly demonstrated significantly impaired dimerization across six independent assays (Fig 3D and E). L533 is directly involved in the interaction surface between CIP2A monomers (Fig 3C and F). Its substitution by a bulky negatively charged amino

Figure 2. Overall structure of the CIP2A(1–560) dimer.

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

acid is therefore likely to destabilize the dimerization interface. On the other hand, mutation of another conserved residue, arginine 522, to a negatively charged aspartate can be predicted to interfere with dimerization by steric and/or electrostatic clashes with the proximal residues such as E523 (Fig 3F), which also is a strictly conserved residue throughout evolution (Appendix Fig S4). Notably, the mode of interference in dimerization by these mutants was reflected with their potency on reducing pulled-down parental CIP2A(1–560)-V5 protein; L533E inhibited dimerization by up to 70%, whereas R552D being not directly involved in interaction surface caused ~50% inhibition (Fig 3D and E).

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

CIP2A directly interacts with PP2A B56 tumor suppressor subunits

Regardless of functional evidence that PP2A inhibition mediates CIP2A's oncogenic effects [6,10–12], no evidence for direct interaction between CIP2A and any of the PP2A complex components has been demonstrated as yet.

Importantly, in addition to CIP2A homodimerization, we identified PP2A B subunit B56 γ (PPP2R5C) as one of the direct interaction partners of full-length CIP2A by Y2H assay (Fig EV1). On the other hand, Y2H analysis did not reveal direct interaction between CIP2A and scaffolding A subunit, or catalytic C subunit. Direct binding of CIP2A to B56 γ is a very exciting result, as together with B56 α , B56 γ has been shown to be one of the most important tumor suppressor B subunits [25,26]. To verify these results, the CIP2A(1–560) was demonstrated to interact directly with both B56 γ and B56 α in a GST pulldown experiment (Fig 4A). The interaction between CIP2A and B56 γ and B56 α was confirmed by MST analysis, allowing the determination of approximate K_d values for these interactions (Fig 4B). We further verified that full-length CIP2A interacts with B56 α and B56 γ by PLA in HEK293T cells either co-transfected with HA-tagged versions of B56 proteins and CIP2A-V5 (Fig 4C left panel and Appendix Fig S5), or between endogenous CIP2A and B56 (Fig 4C). Control PLA without primary antibodies from parallel samples did not show any background signals (Fig 4C right panel and Appendix Fig S5B).

Biochemical characterization of CIP2A dimerization, including determination of modest affinity (K_d) between monomers, indicated that there most probably exists equilibrium between monomeric

Source data are available online for this figure.

and dimer form of CIP2A(1–560) in solution. Therefore, we wanted to assess whether CIP2A homodimer or monomer form of CIP2A binds to B56 proteins. To this end, recombinant GST or GST-CIP2A(1–560) was incubated with B56 α and the protein complexes were analyzed by size-exclusion chromatography. In the presence of GST alone, both GST and B56 α eluted in separate fractions that, based on column calibration, corresponded to their expected molecular

weights (Fig 4D). This further excludes direct GST tag-mediated binding between B56 α and CIP2A. Consistent with SEC-MALS analyses and other biochemical evidence for CIP2A dimerization, GST-CIP2A(1–560) was mostly eluted in fraction 3 (corresponding to approximate size of 158 kDa; Fig 4D). Importantly, in the presence of GST-CIP2A(1–560), there was a clear shift in elution of B56 α toward fractions 2 and 3, and also CIP2A elution pattern shifted more

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

- A GST-pulldown assay for B56-GST-CIP2A(1-560) interaction. Equal molar amounts are used in all samples. The samples were analyzed by Western blotting using antibodies against GST, B56α, and B56γ. All samples are from the same gel, and black vertical lines indicate where the blot has been cut to remove irrelevant lanes.
- B Thermophoresis analysis of B56-CIP2A(1-560) interactions. CIP2A(1-560) fragment was labeled by NT-647 NHS label.
- C PLA analysis for interaction between endogenous CIP2A and B56α proteins in HEK293T cells. HEK293T cells were analyzed by PLA using antibodies specific for CIP2A or B56α (right panel) and were also analyzed with PLA without primary antibodies (middle panel). As another control, HEK293T cells co-transfected with CIP2A-V5 and HA-B56α constructs were subjected to PLA with V5 and HA antibodies (left panel). Red dots indicate the association between CIP2A and B56α proteins. Shown is a representative image from two PLA experiments. Scale bars, 25 μm.
- D Size-exclusion chromatography analysis of GST-CIP2A(1-560) interaction with B56α. The proteins were incubated together for 1 h at 37°C before the run. As a negative control, B56α was also tested with GST.
- E GST-pulldown assay for interaction between B56α and indicated GST-CIP2A(1-560) WT and dimerization interface mutant proteins. Equal molar amounts of GST and GST-CIP2A(1-560) proteins were incubated with B56α for 1 h at 37°C before pulldown.
- F Quantitation of the Western blot results from (E). Shown is relative B56-binding efficiency of mutants as compared to GST-CIP2A(1-560) WT, quantified as a ratio between B56α and GST-CIP2A(1-560) in pulldown sample. Shown is mean + SEM from four independent B56-binding experiments. Two-sided *t*-test between mutant and WT proteins for their relative CIP2A dimerization $**P < 0.01$. To compare the degree of B56-binding deficiency of R522D and L533E CIP2A(1-560) mutants to the degree of dimerization deficiency, the graph also includes data from Fig 3E.
- G Ratio between observed effects for both dimerization and B56 binding (based on F) was calculated to estimate the degree of contribution of CIP2A dimerization to its maximal B56-binding capacity. Both mutants show comparable degree of impact to B56 binding.

Source data are available online for this figure.

toward fraction 2 corresponding to higher molecular weight complex containing B56α and GST-CIP2A(1-560) dimer (Fig 4D). Based on these results, we rationalized that CIP2A dimerization may make an important contribution to maximal binding to B56α. In order to directly test this, the CIP2A(1-560) dimerization compromised mutant L533E was compared with wild-type CIP2A(1-560) for B56α binding by GST pulldown assay. In line with our hypothesis, L533E mutant showed significantly reduced binding to B56α (Fig 4E and F). Although these data do suggest that CIP2A dimerization may enhance CIP2A binding to B56α, we wanted to further test whether the “weaker” dimerization mutant R522D would also show impaired

B56 binding, and whether the degree of inhibition of dimerization, and B56 binding, would show any correlation between the two mutants. Indeed, also R522D did show weaker binding to B56α than wild-type CIP2A (Fig 4E and F). Importantly, quantification of four independent experiments demonstrated that significantly lowered capacity of dimerization mutants to bind to B56α correlated with their reduced capacity to dimerize (Fig 4E and F). To estimate the contribution of dimerization to maximal B56α binding capacity of CIP2A, we calculated the ratio between observed effects on both dimerization and B56α binding. Notably, both mutants showed comparable ~50% contribution of dimerization

to B56 α binding in our assay conditions (Fig 4G). This supports the conclusion that B56 α binding defect observed with these mutants is caused by similar mechanism, that is, inhibition of dimerization.

Identification of N-terminal B56 binding region of CIP2A

Result that CIP2A dimerization mutants still retain ~50% B56-binding activity (Fig 4G) indicates that other regions of CIP2A might harbor a primary B56 binding site, whereas the role of CIP2A dimerization could be to stabilize B56–CIP2A interaction. To identify such potential additional B56 binding region, we created GST-CIP2A(1–330) protein that does not harbor sequences from SID (Fig 1A), but contains the “tip” sub-domain and N-terminal half of the “stem” sub-domain (Fig 2B). Importantly, whereas again no significant association of GST was found with B56 α , GST-CIP2A(1–330) did show clear interaction (Fig 5A). However, supportive of our conclusion that dimerization increases B56 binding of CIP2A, the GST-CIP2A(1–560), which harbors the dimerization region, showed tighter B56 α binding than GST-CIP2A(1–330) (Fig 5A).

Next, we characterized the regions on CIP2A(1–330) that mediate direct B56 α –CIP2A interaction. Based on an analysis of several N-terminal CIP2A deletion constructs, the minimal region that is required for the B56 α interaction was located between amino acids 159–245 (Fig 5B), which covers the last (fifth) armadillo repeat in the “tip” domain and the first (sixth) repeat in the “stem” domain (Fig 2B). Notably, the same region also mediates interaction between CIP2A and B56 γ (Fig 5C). Next, we modeled the above-identified minimal B56 binding region to CIP2A N-terminal structure, taking also into account the charge distribution. We also assumed that the binding on CIP2A may occur at positively charged areas, since B56 surface is largely negatively charged [34,35]. Strikingly, the “inside” surfaces of CIP2A(1–560) dimer are highly negatively charged (Fig 5D, left panel), indicating that B56 may bind to

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

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

Results above indicate that each N-terminal arm of the double-hook dimer structure of CIP2A contains a B56 binding region. This might facilitate trapping of two B56 proteins by a CIP2A dimer. Alternatively, the two B56 binding regions on one CIP2A dimer could both interact with a single B56 molecule to strengthen the interaction. Co-crystallization of the CIP2A–B56 α complex has been extremely challenging and remains an ongoing effort. Nonetheless, to alternatively dissect between these two possibilities, we analyzed B56 α –CIP2A dimer interaction by incubating together molar equivalent amounts of GST-CIP2A(1–560), B56 α , and the stoichiometry of their interactions was studied by Coomassie staining following GST pulldown. As shown in Fig 5E, intensities of CIP2A dimer and B56 α similar in the analyzed pulldown sample, indicating that each CIP2A dimer can most likely capture two B56 α molecules.

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

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

- A GST-CIP2A(1–330) was compared for B56 α -binding with CIP2A(1–560). The samples were analyzed by Western blotting using antibodies against GST and B56 α . Shown is a representative of three independent experiments with similar results. All samples are from the same gel, and black vertical lines indicate where the blot has been cut to remove irrelevant lanes.
- B, C Mapping of the N-terminal B56 α (B) and B56 γ (C) interaction region in CIP2A by GST-pulldown analysis. Samples were analyzed by Western blotting using antibodies against GST and B56 α or B56 γ .
- D Surface electrostatic potential analysis and potential binding sites for B56. The surface electrostatic potential was calculated using the adaptive Poisson–Boltzmann solver (APBS) module and presented by Pymol. The right panel correlates with the left panel with a ~30° rotation. The potential B56-binding site, predicted based on binding site mapping, surface conservation and charge distribution, is indicated with a yellow oval.
- E Analysis of stoichiometry between CIP2A and B56 binding. Similar molar amounts of GST-CIP2A1–560 and B56 α were incubated together for 1 h at 37°C followed by GST-pulldown analysis and Coomassie staining of SDS–PAGE gel. As CIP2A exists preferentially as a dimer (Fig 1D), the 0.89:1 ratio between CIP2A and B56 in pulldown sample indicate that one CIP2A dimer binds two B56 molecules. All samples are from the same gel, and black vertical lines indicate where the blot has been cut to remove irrelevant lanes.
- F Western blot analysis of protein expression of V5-tagged full-length WT CIP2A(1–905) or L533E and R522D CIP2A mutants from transiently transfected HEK293T cells. All samples are from the same gel, and black vertical lines indicate where the blot has been cut to remove irrelevant lanes.
- G Quantitation of the Western blot results from (F). Shown is mean + SEM, $n = 3$. Two-sided t -test between mutant and WT proteins for their relative expression, $*P < 0.05$, $**P < 0.01$.
- H RT–PCR analysis of CIP2A, β -actin, and GAPDH mRNA expression from transiently transfected HEK293T cells expressing either V5-tagged full-length WT or L533E and R522D mutants. Plotted is mean + SEM from four experiments with duplicate samples.
- I Endogenous CIP2A protein expression in 22RV1 cells transfected with B56 α and B56 γ siRNAs for 72 h.
- J Quantitation of the Western blot results from (I). Shown is mean + SEM, $n = 4$. Two-sided t -test, $**P < 0.01$. All samples are from the same gel, and black vertical lines indicate where the blot has been cut to remove irrelevant lanes.
- K Western blot analysis of pAkt Ser473 protein expression in 22RV1 cells transiently transfected with V5-tagged full-length WT or L533E CIP2A.
- L Quantitation of the Western blot results from (K). Shown is mean + SEM, $n = 3$. Two-sided t -test, $**P < 0.01$.

Source data are available online for this figure.

Figure 5.

Dimerization of CIP2A is important for sustained full-length CIP2A protein expression

To assess the functional impact of CIP2A dimerization and B56 binding in the context of full-length CIP2A, we created CIP2A(1–905) mammalian expression vectors coding for either WT or L533E and R522D mutated V5-CIP2A fusion protein. Intriguingly, as measured using a V5 epitope-specific antibody, both the L533E and R522D mutant full-length CIP2A showed up to 50% lower protein levels as compared to the WT protein in HEK-293 cells (Fig 5F and G). Importantly, inhibition of protein expression of mutants was not due to difference in levels of expression of CIP2A mRNA from transiently transfected cDNA constructs (Fig 5H). Also, it is unlikely that single-point mutation in mutants would cause protein destabilization in solution as thermal unfolding analysis by Prometheus NT.48 (NanoTemper Technologies GmbH), 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 (Fig 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 α or B56 γ with siRNAs also resulted in inhibition of CIP2A protein expression (Figs 5I and J, and EV4D and E), without any impact on CIP2A mRNA expression (Fig 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 [9,16], as compared to WT CIP2A (Fig 5K and L). Similar effect was observed in regulation of another CIP2A target oncoprotein MYC (Appendix Fig S6)

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 in numerous recent studies [10,14–18], 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

PP2A inhibitor proteins have recently emerged as a novel group of human oncoproteins with clinical relevance in various human cancers [2,6]. Among these proteins, CIP2A shows the most prevalent overexpression and is associated with poor patient survival across different types of cancer [20]. The therapeutic effect of inhibition of CIP2A protein expression in tumor growth has been recently validated by numerous studies [10,14–18]. Impact of CIP2A on both oncogenic RAS signaling [10,13,36] and MYC activity *in vivo* [10,12,22], without lack of any detrimental normal tissue homeostasis effects in a CIP2A-deficient mouse model [11,22,23], further illustrated the potential of CIP2A as a future cancer therapy target. However, efforts to target CIP2A for cancer therapy have been thus far hampered by the absence of both a molecular explanation of how CIP2A interacts with PP2A, and by a lack of any 3D structural information about the protein.

Here, we report the first crystal structure of CIP2A, which contain the motifs that are critical for PP2A/B56 binding. Interestingly, by using several independent approaches we demonstrate that CIP2A exists as a homodimer, and this is mediated by a relatively flat and highly hydrophobic surface formed by the last three helices of CIP2A(1–560). Another important discovery reported in this study is the first reported direct interaction between CIP2A and any of the PP2A complex components. Lack of confirmation of direct binding of CIP2A to PP2A proteins has been a significant caveat in our understanding how CIP2A might influence PP2A's tumor suppressor activity. Here, Y2H analysis identified B56 γ as a direct interaction partner for CIP2A, and CIP2A interaction with both B56 γ and B56 α was further validated by several independent approaches. Very importantly, among the all PP2A B subunits, B56 α and B56 γ are the two subunits with the most convincing functional evidence of tumor suppressor activity [25,37]. Moreover, we provide evidence that single-point mutation on CIP2A dimerization domain is sufficient to inhibit both B56 binding and CIP2A's capacity to support pAkt expression. Binding of CIP2A to PP2A via specific B subunits imposes an interesting possible explanation for observations that CIP2A only regulates a fairly restricted number of phosphoproteins [6,13] among thousands of potential target proteins regulated by different PP2A complexes [24,26]. Based on high conservation among all B56 family proteins, we suspect that also they may interact with CIP2A. This, and whether CIP2A interacts with members of other B subunit families than B56, is an important question to be addressed in the future.

Very interestingly, we also provide evidence that CIP2A binding to B56 stabilizes CIP2A protein, further validating that functional relevance of the reported CIP2A–B56 interaction. Interestingly, destabilization of CIP2A upon B56 inhibition is reminiscent of B subunit destabilization upon inhibition of PP2A core complex components [38] and supports the model that CIP2A is an obligate interactor with PP2A/B56. This autoregulatory mechanism for CIP2A stabilization could be a clinically relevant finding, as CIP2A is a very long-lived protein [9] and its high expression associates with poor patient survival in more than 15 different human cancer types [20].

Notably, single-point mutations of conserved residues at dimerization surface impaired both CIP2A dimerization and B56 binding, and we observed a clear positive correlation between these two effects (Fig 4E). Together with high degree of conservation of amino acids mediating CIP2A dimerization, these results strongly indicate that CIP2A dimerization discovered in this study is a biologically relevant mechanism related to PP2A regulation. In the absence of structure of CIP2A–B56 complex, we do not exactly know the molecular basis of how CIP2A dimerization promotes B56 binding. However, based on results that in the context of CIP2A(1–560), single-point mutations that impair dimerization also show decreased B56 binding, we envision that the mechanism may be related to the formation of a novel B56 interaction surface near the CIP2A dimer interface. This mechanism would remotely resemble mechanism how Fbw7 dimerization increases cyclin E binding. In Fbw7 dimer interface, both Fbw7 protomers have one cyclin E binding site, and through dimerization both binding sites become simultaneously accessible to cyclin E, thus leading to increased affinity of Fbw7–cyclin E interaction [39]. Functionally, the most important finding of this study is that a single-point mutation in the CIP2A dimerization interface results in CIP2A protein degradation in cancer cells. This is a very important finding because the therapeutic effects of inhibition of CIP2A protein

expression have been validated by numerous studies [6–8,10–12,21]. Notably, targeted protein degradation has recently gained significant interest as an alternative cancer therapy approach [40,41]. The benefits of drug targeting to induce protein degradation are clear, as such an approach removes any potential activities of the protein as well as any scaffolding functions and results in longer pharmacodynamic effects that are predicted to remain even after drug has been metabolized. We anticipate that more potent target sites for induction of CIP2A degradation will be identified by further dissection of both N-terminal and dimerization domain amino acids critical for CIP2A–B56 binding. Moreover, although we have here determined regions that are sufficient for CIP2A binding to tumor suppressor B56, and demonstrate relevance of CIP2A dimerization in this process, these results do not exclude that the C-terminal sequences, and, for example, post-translation modifications of full-length CIP2A, might also contribute to PP2A binding and regulation. Future work will be thus needed to address why full-length CIP2A can be expressed in cells but not purified in *in vitro* conditions, and whether targeting of C-terminal regions of CIP2A would offer additional benefit in inhibition of CIP2A's oncogenic activities.

In summary, results of this study reveal the first crystal structure of CIP2A—one of the most prevalent human oncoproteins. Our results also provide first insights into how CIP2A interacts with PP2A tumor suppressor subunit B56. In addition to their novelty and biological significance in promoting our understanding of mechanisms of regulation of major cellular serine/threonine phosphatase complex PP2A, these results strongly indicate that the identified N-terminal B56 binding region of CIP2A, together with dimerization domain may serve as potential target regions for cancer therapeutics. We anticipate that these findings will provoke immense interest in developing first series of small-molecule inhibitors toward CIP2A for cancer therapy. These results may also help in understanding mechanisms of PP2A regulation in various other diseases in which PP2A inhibition has pathogenic role.

Materials and Methods

Protein expression and purification for crystallography

The truncated domain of human CIP2A(1–560) was cloned into the pGEX-4T1 vector (GE Healthcare) with an N-terminal GST tag and a TEV cleavage site in between. CIP2A(1–560) was overexpressed in *E. coli* BL21 (DE3) cells (Novagen), grown in LB media. The bacteria cell was cultured at 37°C until OD₆₀₀ reached 0.5–0.7, and then induced by 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16°C overnight. The bacteria pellets were collected and lysed by sonication. The GST fusion protein was first purified by Glutathione Sepharose 4B column. The GST tag was removed by TEV protease at 4°C overnight. The untagged protein was further purified by an ion exchange column (GE Healthcare). The purity of the samples was verified using SDS–PAGE and staining with Coomassie Brilliant Blue. The CIP2A truncated domain was observed as a single band at 60 kDa. The protein was then concentrated for crystallization to 1.5 mg/ml in a buffer containing 20 mM Tris–HCl pH 8.0, 250 mM NaCl, 2 mM DTT. A selenomethionine (SeMet) derivative of the CIP2A truncated domain was expressed in an auto-induction media [42] and purified in the same way as the native protein.

Crystallization, optimization, and data collection

Initial crystals of both the native and the SeMet-substituted CIP2A truncated domain were obtained using the hanging drop vapor diffusion method. Crystals were improved by adding 1% PEG 8000 into the condition consisting of 0.1 M sodium malonate pH 6.0 and 7% PEG 4000. 1 μl of protein solution (1.5 mg/ml) was mixed with 1 μl of reservoir solution and equilibrated over 400 μl 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 ~4 Å resolution. Crystal diffraction quality was improved to ~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 ~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 [43] and Mosflm (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 [44]. 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. The coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 5UFL.

Yeast two-hybrid screen

The yeast two-hybrid screen was performed by Hybrigenics. The full-length CIP2A was used as a bait, and the library in the screen was breast tumor epithelial cells (T47D, MDA-MB-468, MCF7, BT20).

Antibodies

The following primary antibodies were used: CIP2A polyclonal rabbit (pR Ab) [31] or monoclonal mouse (mM Ab) (2G10-3B5) (Santa Cruz sc-80659), PP2A-B56α (23) (mM Ab, Santa Cruz sc-136045) or pR Ab (Upstate Biotechnology 07-334), PP2A-B56γ (N-15) polyclonal goat (pG) Ab (Santa Cruz sc-46459), V5 (Sigma V8012 or Thermo Fisher Scientific E10/V4RR) both mM Ab, GST (B-14) mM Ab (Santa Cruz sc-138), pAkt Ser473 mR Ab (Cell Signaling D9E), β-actin (C4) mM Ab (Santa Cruz Biotechnology sc-47778), GAPDH (6C5) mM Ab (HyTest 5G4-6C5), GFP pR Ab (Life Technologies A-11122), and HA (Y-11) pR Ab (Santa Cruz Biotechnology

sc-805). The following secondary antibodies were used: polyclonal goat anti-mouse immunoglobulins-HRP from Dako (P0447) or from Santa Cruz Biotechnology (sc-2005), polyclonal swine anti-rabbit (P0399) and polyclonal rabbit anti-goat (P0449) immunoglobulins-HRP, both from Dako.

GST-pulldown assays

In all GST-pulldown assays, 10 pmol of each protein was used. The overall volume of each pulldown prep was 200 μ l. The interaction buffer was 50 mM Tris, 150 mM NaCl, 10% glycerol, 0.2% NP-40, 50 μ M ZnSO₄, 2 mM DTT, pH 7.5. The proteins were then incubated 1 h at 37°C or RT as indicated in the figure legends. Next, 5 μ l of GSH agarose (Thermo Scientific) was added in 20 μ l of the interaction buffer and samples were further incubated 1 h at RT in rotation. Thereafter, the samples were washed four times with 250 μ l of ice-cold interaction buffer. The overall washing time was extended at least to 1 h in order to reduce the background. Finally, the samples were centrifuged, the supernatant was carefully discarded, and the resin was resuspended in SDS-PAGE sample buffer, resolved by SDS-PAGE and analyzed by Western blot.

SEC and SEC-MALS

Size-exclusion chromatography for recombinant protein analysis was carried out using Superdex 5/150 column (GE Healthcare). The flow rate was 0.3 ml/min, and the column was operated at RT. The running buffer was 28 mM (Tris pH 7.2, 150 mM NaCl, 0.05% NP-40, 1.25% glycerol, 2 mM DTT). All samples contained 50 pmol of each protein tested. The proteins were first let to form complexes by incubating them in the interaction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 0.2% NP-40, 2 mM DTT) for 1 h at 37°C. The total volume was 120 μ l. The samples were centrifuged at 11,000 g for 5 min before loading to the gel filtration column. In each run, 30 μ l of the sample was injected to column. The total volume of the column is 3 ml. The complex size determination was based on calibration with carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (141 kDa), and beta-amylase (200 kDa) control proteins.

SEC-coupled Multi-angle (laser)-light scattering (SEC-MALS) experiments were performed at RT by loading samples on a 24 ml Superdex 200 Increase size-exclusion column (GE Healthcare) with a TREOS MiniDAWN MALS detector (Wyatt Technology). The buffer used contained 20 mM Tris-HCl (pH 8.0), 275 mM NaCl, and 2 mM DTT.

Analysis of CIP2A dimerization from HeLa cells by size-exclusion chromatography was performed as described previously [45]. The molecular mass standards (Sigma) used to calibrate the column were blue dextran (2,000 kDa) thyroglobulin (669 kDa), apoferritin (443 kDa), b-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

Generation of CIP2A N230E, R522D, and L533E mutants

R522D and L533E mutations were introduced by PCR, using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, TX, USA). As a PCR template, pGEX4T2/CIP2A(1–560) and pcDNA3.1/CIP2A(1–905) V5 were used with the following pairs of

primers, listed sense and antisense, respectively: for R522D, 5'-GAT AATGATGAACAAGTACAGTCTGGACTG-3' and 5'-CTTGTTTCATCA TTATCTGACGTTAAAGCAAAAGC-3', and for L533E, 5'-GAATA TTAGAGGAGGCTGCTCCACTGCCAGA-3' and 5'-GCAGCCTCCTCTA ATATTCTCAGTCCAGACTG-3'. N230E was introduced in pGEX4T2/CIP2A(1–560) by using QuickChange Site-Directed Mutagenesis (QCL-SDM) kit from the same manufacturer and the following set of primers, listed sense and antisense, respectively: 5'-GCTC GAGAGATTCATCAGACTTTTCAACTAATA-3' and 5'-ATGAATCTC TCGAGCATGGAATAGCTTTTC-3'. All constructs were verified by DNA sequencing (Finnish Microarray and Sequencing Centre, Centre for Biotechnology Turku).

Cell culture and Western blotting

22RV1 cells were cultured in RPMI-1640 media (Sigma), and HEK293T and HeLa cells in Dulbecco's modified Eagle's medium (DMEM) (Sigma), both supplemented with 10% (v/v) FBS, 0.5% (v/v) penicillin/streptomycin (10,000 units/10 mg per ml, Sigma), and 2 mM L-glutamine (Biowest). The cells were cultured at 37°C in a humidified incubator under an atmosphere of 5% CO₂ and passaged 2–3 times a week.

All Western blot experiments were performed as described in [10–12] with minor modifications. In all blots where the blot has been cut to remove irrelevant lanes, all samples are from the same gel. Size markers have been indicated for those blots that the information was available.

Analysis of expression of CIP2A WT and L533E mutant in HEK293T cells

HEK293T cells were plated in a 12-well plate format. Cells were transfected using Lipofectamine 2000 (Invitrogen by Thermo Fisher Scientific, IL, USA) or Eugene 6 (Promega) at 3:1, according to the manufacturer's protocol. For 12-well plate scale, 1 μ g of DNA was used. After about 24 h, the growth media was removed, the cells were rinsed twice in cold PBS and then scraped in 100 μ l PBS, mixed with 100 μ l 2 $\times$ SDS-PAGE sample buffer, incubated for 10 min at 95°C and centrifuged at 16,100 g for 15 min. The cleared supernatant (8 μ l in total) was resolved by 4–20% SDS-PAGE and analyzed by Western blot.

For RT-PCR, the cells were plated in a 6-well plate format and transfected with 3 μ g of DNA for 24 h. RNA extraction was done with NucleoSpin RNA kit (Macherey-Nagel). Reverse transcription of the RNA extracts was performed using RNase inhibitor rRNasin (Promega, WI, USA) and M-MuLV RNase H-reverse transcriptase (Finnzymes, Thermo Fisher Scientific, MA, USA). RT-qPCR for CIP2A mRNA was performed on Applied Biosystems 7900HT Fast Sequence Detection System using TaqMan Universal Master Mix II, no UNG (Applied Biosystems, CA, USA), Universal ProbeLibrary probe #69 (Roche Applied Science), and following primer sequences: GAACAGATAAGAAAAGAGTTGAGCATT and CGACCTTCTAATTG TGCCTTTT.

B56 siRNA effects on CIP2A protein expression

The cells were transfected with Oligofectamine (Thermo Fisher Scientific by Life technologies) according to manufacturer's

instructions. 22RV1 cells were seeded in 6- or 12-well plate set-ups so that the confluency at the point of siRNA transfection would be 30–40%. Following siRNAs were used for B56 α knockdown: B56 α -1: UAC CCA UCU GUU ACC ACU CdTdG; B56 α -2: AAG UGU ACG GAA GAU GUU AdGdC (both with symmetrical overhangs, from Sigma); and PP2A-B56 α siRNA (h) (sc-39181 Santa Cruz). For B56 γ knockdown, PP2A-B56 γ siRNA (h) (sc-45847 Santa Cruz) was used.

For B56 siRNA experiment in HeLa cells, following siRNA sequences were used for B56 α knockdown: B56 α -1: UAC CCA UCU GUU ACC ACU CdTdG; B56 α -2: AAG UGU ACG GAA GAU GUU AdGdC. After 72-h transfection, the cells were scraped in ice-cold PBS and snap frozen. Samples were split for Western blotting and RNA extraction by NucleoSpin RNA II kit (Macherey-Nagel). Reverse transcription of the RNA extracts was performed using RNase inhibitor rRNasin (Promega) and M-MuLV RNase H-reverse transcriptase (Finnzymes, Thermo Fisher Scientific). RT-qPCR for CIP2A mRNA was performed on Applied Biosystems 7900HT Fast Sequence Detection System using TaqMan Universal Master Mix II, no UNG (Applied Biosystems), Universal ProbeLibrary probe #69 (Roche Applied Science), and following primer sequences: GAACAGATAAG AAAAGAGTTGAGCATT and CGACCTTCTAATTGTGCCTTT.

Proximity ligation assay (PLA)

HEK293T cells were plated on coverslips in a 12-well plate format. Coverslips were pre-coated with poly-lysine (Sigma-Aldrich), and transfected using Lipofectamine 2000 (Invitrogen by Thermo Fisher Scientific) at 3:1, according to the manufacturer's protocol. The following plasmids were used with the amounts indicated: pEGFPC2-CIP2A(1–905) Flag (1 μ g), pcDNA3.1-CIP2A(1–905) V5 His (3 μ g), pCEP-4HA-B56 α and pCEP-4HA-B56 γ 3 (both 1 μ g). The assay was started about 24 h after transfection. PLA kit from Olink Bioscience was according to manufacturer's instructions. Primary antibodies were diluted in antibody diluent as follows: anti-V5 (E10/V4RR 1:200), anti-GFP (1:500), and anti-HA (1:200), and incubated with the coverslips overnight at 4°C. The slides were analyzed with laser scanning microscope LSM510 META (Carl Zeiss) at 63 $\times$ magnification, and images were processed with Fiji-ImageJ. For studying association of endogenous CIP2A and B56 α , the following primary antibodies were used: anti-CIP2A (mM Ab 2G10-3B5 1:100) and anti-B56 α (pR Ab 07-334 1:50).

Expanded View for this article is available online.

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Author contributions

JW contributed to recombinant CIP2A vector, CIP2A protein purification, crystallization, X-ray diffraction data collection, structural determination, CIP2A dimerization analysis, and manuscript writing. JO helped in recombinant protein vectors for CIP2A, protein interaction studies, Y2H coordination, data analysis, study concept, and manuscript writing. KP contributed to CIP2A mutants, protein interaction studies, structure modeling, functional experiments with CIP2A mutants and B56 siRNA, data analysis, and manuscript writing. ZW helped in crystal structure determination and refinement, CIP2A–B56 interaction using purified proteins. OK contributed to B56 siRNA experiments. TH and GS helped in gel filtration. PMO and ZR contributed to study supervision. WX helped in study supervision, diffraction data collection, data analysis, study concept, and manuscript writing. JW contributed to study supervision, data analysis, study concept, and manuscript writing.

Conflict of interest

The authors declare that they have no conflict of interest.

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