



- (51) **International Patent Classification:**
C07K 14/82 (2006.01) *G06F 19/16* (2011.01)
- (21) **International Application Number:**
PCT/FI2016/050445
- (22) **International Filing Date:**
17 June 2016 (17.06.2016)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
62/181,535 18 June 2015 (18.06.2015) US
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- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

- *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title:** CRYSTAL STRUCTURES OF CIP2A

(57) **Abstract:** The present invention relates to crystalline CIP2A and in particular to the use of structural information of crystalline human CIP2A for ligand and/or inhibitor identification, design and production, as well as *in silico* and *in vitro* screening assays for detection of such ligands and/or inhibitors.



CRYSTAL STRUCTURES OF CIP2A

FIELD OF THE INVENTION

The present invention relates to crystalline CIP2A and in particular to the use of structural information of crystalline human CIP2A for ligand and/or inhibitor identification, design and production, as well as *in silico* and *in vitro* screening assays for detection of such ligands and/or inhibitors.

BACKGROUND OF THE INVENTION

Human cell transformation and cancer progression is driven by a combination of hyperactive oncogenic signaling and the inhibition of critical tumor suppressors. Therefore, efficient cancer therapies should aim to restore the physiological activities of both of these mechanisms. However, although inhibition of hyperactivated oncogenic kinases by small molecule inhibitors is an established cancer treatment modality, there are no approved cancer therapies to date based on the reactivation of inhibited tumor suppressors.

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. 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. Importantly, in contrast to another important tumor suppressor, p53, which is genetically inactivated in a large number of human cancers and is thus refractory to reactivation by most approaches, PP2A complex components are mutated at a relatively low frequency in most types of human cancer. This establishes the reactivation of PP2A as a very attractive novel approach in cancer therapy. 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.

A number of recent studies have demonstrated that PP2A is inhibited in cancer by a group of otherwise unrelated PP2A inhibitor proteins. Among them, Cancerous inhibitor of PP2A (CIP2A) is the most prevalent oncoprotein and has been shown to drive the malignant growth of numerous human cancer cell lines. Depletion of CIP2A in cancer cells results in dephosphorylation of many oncogenic PP2A targets (e.g., MYC, E2F1, Akt), and these effects have been shown to be reversible upon PP2A co-inhibition. Regarding functional

synergism between PP2A inhibition and RAS signaling, CIP2A overexpression is required for RAS driven human cell transformation, and extensive overlap between RAS and CIP2A-regulated phosphoproteomes has been recently discovered. Nonetheless, the molecular basis of how CIP2A inhibits PP2A activity
5 towards these critical targets remains enigmatic.

In vivo, RNAi-mediated depletion of CIP2A expression potently inhibits xenograft tumor growth, and we recently demonstrated reduced Her2-driven mammary tumorigenesis in a CIP2A-deficient genetic mouse model. More recently, this mouse model was found to be totally resistant to develop-
10 ment of another breast cancer subtype. Importantly, CIP2A deficiency does not compromise normal mouse development or growth, except for a defect in spermatogenesis. As CIP2A is overexpressed in more than 60% of most types of human cancer and predicts poor patient survival in over dozen different cancer types, its prognostic and functional relevance equals, or exceeds, that of
15 most oncoproteins that have been traditionally considered important oncogenic drivers. For example, comparison of clinical relevance of CIP2A overexpression and KRAS mutation across the Cancer Genome Atlas (TCGA) pan-cancer data revealed that these alterations have similar prognostic role in colon cancer patients, and patients with both of these alterations represent the patient
20 population with clearly the worst prognosis. Indeed, studies to date indicate that inhibiting CIP2A protein expression may constitute a very efficient cancer therapy strategy without detrimental side effects.

PP2A functions as a protein complex consisting of either a core dimer between the scaffolding A subunit (PP2R1A, PP2R1B) and the catalytic subunit PP2Ac (PPP2CA, PP2CB) or a trimer in which one of the numerous
25 regulatory B subunits (PPP2R2A, PPP2R2B, PPP2R2C, PPP2R2D; PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, PPP2R5E; PPP2R3A, PPP2R3B, PPP2R3C; STRN, STRN3, STRN4) interacts with the AC core dimer. The binding of the B subunits to the core dimer is a highly regulated process, the details of which yet remain to be fully understood; however, it has
30 been shown that different B subunits mediate the substrate specificity of the PP2A trimer. Our current understanding supports the view that only a subset of the numerous B subunits are relevant for the tumor suppressor activity of PP2A, which is likely based on their selective binding to certain oncogenic
35 PP2A target phosphoproteins. For example, B56 α (PPP2R5A) binds directly to the PP2A target MYC and mediates PP2A complex recruitment and the PP2A-

mediated dephosphorylation of MYC serine 62. CIP2A has been shown to inhibit this process, though the PP2A sub-complex selectivity of CIP2A remains to be mechanistically explained. Understanding the mechanism by which CIP2A can inhibit PP2A tumor suppressor activity without affecting the activity of all PP2A complexes is also a relevant question in light of understanding why the inhibition of CIP2A does not have detrimental effects on mouse viability, which is observed with the inhibition of another PP2A inhibitory protein: PME-1. In addition to the uncertainty regarding the mechanism by which CIP2A inhibits PP2A activity, a lack of structural information for the CIP2A protein has thus far hampered the advancement of this potential cancer therapy target in drug development.

BRIEF DESCRIPTION OF THE INVENTION

In one aspect, the present disclosure provides crystal structures of CIP2A, the crystal structures being present on space group $P6_5$ with two CIP2A molecules in one asymmetric unit.

In another aspect, the present disclosure provides a method of identifying a compound that interacts with CIP2A. Said method comprises the steps of:

a) providing X-ray diffraction data of crystalline CIP2A, capable of displaying a three-dimensional representation of CIP2A when read by an appropriate machine and processed by a computer program for determining molecule structures,

b) performing rational drug design or *in silico* screening of a compound library on the basis of the three-dimensional representation of CIP2A, and

c) identifying a potential compound that interacts with CIP2A.

In a further aspect, the present disclosure provides a method of preparing the present crystal of CIP2A. Said method comprises the steps of:

a) providing a solution of CIP2A in a suitable buffer, such as 20 mM Tris-HCl, pH 8.0, 250 mM NaCl and 2 mM dithiothreitol,

b) mixing the solution with a crystallization solution comprising 7% (w/v) polyethylene glycol (PEG 4000), 0.1 M sodium malonate, pH 6.0, and

c) incubating the mixture under conditions to promote hanging drop vapour diffusion for a time sufficient to produce the crystal of CIP2A.

In a still further aspect, the present disclosure provides use of the

structure of the present crystal of CIP2A in modelling a relevant molecule which interferes with the interaction of CIP2A with PP2A proteins.

Other objectives, aspects, embodiments, details and advantages of the present invention will become apparent from the following figures, detailed
5 description, examples, and dependent claims.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following the invention will be described in greater detail by means of preferred embodiments with reference to the attached drawings, in which

10 Figure 1A illustrates the 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.

Figure 1B illustrates a separated view of a CIP2A(1-560) monomer in three orthogonal views. Positions of the three subdomains are boxed.

15 Figures 2A to 2D demonstrate the verification of homodimerization of full-length CIP2A.

Figure 2A shows a summary of selected CIP2A interacting proteins found in the yeast-two-hybrid screen. The yellow bar shows the areas mediating the interaction. These areas are based on the fragments being able to in-
20 teract with full-length CIP2A.

Figure 2B demonstrates the dimerization of CIP2A(1-560) fragment as 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.

Figure 2C demonstrates size exclusion chromatography analysis of
25 GST-CIP2A(1-560) and GST. The samples were incubated 1 h at 37 °C before the run. The chromatography step was carried out at RT.

Figure 2D demonstrates thermophoresis analysis of CIP2A(1-560) dimerization.

Figures 3A to 3C illustrate direct interactions between CIP2A and
30 members of the B56 family of PP2A regulatory subunits.

Figure 3A demonstrates a GST pulldown assay for B56 – GST-CIP2A(1-560) interaction. Equal molar amounts were used in all samples. The proteins were incubated 1 h at 37 °C.

Figure 3B demonstrates a thermophoresis analysis of B56 -
35 CIP2A(1-560) interactions. CIP2A(1-560) fragment was labeled by NT-647

NHS label.

Figure 3C demonstrates a size exclusion chromatography analysis of GST-CIP2A(1-560) interaction with B56 α . The proteins were incubated together 1 h at 37 °C before the run. As a negative control, B56 α was also tested with GST. The chromatography step was carried out at RT.

Figures 4A to 4C illustrate direct binding of CIP2A to either B56 or PP2Ac. For each experiment represented in these figures, equal molar amounts of proteins were used, and the proteins were incubated 1 h at 37 °C, unless otherwise indicated.

Figure 4A demonstrates the mapping of the B56 interaction site in CIP2A by GST pulldown analysis. Equal molar amounts of each fragment and GST were incubated with B56 α for 1 h at 37 °C.

Figure 4B demonstrates the mapping of the PP2Ac interaction site in CIP2A by GST pulldown analysis. Equal molar amounts of each fragment and GST were incubated with B56 α for 1 h at 37 °C.

Figure 4C demonstrates the conservation of CIP2A(1-560) dimer interface based on ten CIP2A sequences from representative species. The darker color indicates higher conservations.

Figures 5A to 5F illustrate that CIP2A dimerization interface mutagenesis results in a CIP2A dimer that is deficient in B56 binding.

Figure 5A shows the CIP2A(1-560) homodimerization interface. This interface is mostly formed by hydrophobic residues in the last two helices of CIP2A(1-560). The positions of the C-termini were labeled.

In Figure 5B, positions of key hydrophobic residues in the CIP2A homo-dimerization interface are shown in a “peeled-apart” view.

Figure 5C demonstrates an analysis of mutational effects by GST pulldown assay for B56 α – GST-CIP2A(1-560) interaction. Equal molar amounts were used in all samples. Representative result is shown.

Figure 5D demonstrates quantitation of the Western blot result. Shown is mean values + S.E.M from three independent experiments.

Figure 5E illustrates analysis of mutational effects by GST pulldown assay for CIP2A(1-560) dimerization. Equal molar amounts were used in all samples.

Figure 5F demonstrates a thermophoresis analysis of the mutational effect on CIP2A(1-560) dimerization. The CIP2A(1-560) WT fragment was labeled by NT-647 NHS label.

Figures 6A to 6D demonstrate that mutations at the dimer interface of CIP2A negatively affect its dimerization efficiency and B56 binding.

Figure 6A illustrates dimerization of indicated GST-CIP2A(1-560) WT and mutant proteins analyzed by GST pulldown. Equal molar amounts of
5 GST and GST-CIP2A(1-560) proteins were incubated with CIP2A(1-560)-V5 fragment for 1 h at 37 °C before pulldown.

Figure 6B illustrates a GST-pulldown assay for interaction between B56 α and indicated GST-CIP2A(1-560) WT or dimerization interface mutant proteins. Equal molar amounts of GST and GST-CIP2A(1-560) proteins were
10 incubated with B56 α for 1 h at 37 °C before pulldown.

Figure 6C shows quantitation of the Western blot results from Figure 6A and 6B. Shown is relative B56 binding efficiency of mutants as compared to GST-CIP2A(1-560) WT, quantified as a ratio between B56 α and GST-CIP2A(1-560) in pull-down sample. Shown is mean + S.E.M from four inde-
15 pendent B56-binding experiments. 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 quantitation of relative CIP2A dimerization efficiency from Figure 6A.

In Figure 6D, the ratio between observed effects for both mutants on
20 both dimerization and B56 binding (based on Figure 6C) was calculated to estimate the degree of contribution of CIP2A dimerization to its maximal B56 binding capacity. Both mutants showed comparable degree of impact to B56 binding.

Figures 7A to 7I demonstrate that a full length CIP2A 3A dimeriza-
25 tion interface mutant is degraded in cells and loses its oncogenic activity.

Figure 7A shows an RT-PCR analysis of CIP2A mRNA expression from retrovirally infected NIH3T3 cells expressing either V5 tagged full-length WT or 3A CIP2A mutant.

Figure 7B shows a Western blot analysis of protein expression of V5
30 tagged full-length WT or 3A CIP2A mutant from retrovirally infected NIH3T3 cells. Shown is a representative blot of numerous independent experiments with similar results.

Figure 7C shows a Western blot analysis of protein expression of
35 V5 tagged full-length WT or 3A CIP2A mutant from transiently transfected HeLa cells. Shown is a representative blot of numerous independent experi-

ments with similar results.

Figure 7D shows an endogenous CIP2A protein expression in HeLa cells transfected with two independent B56 α siRNA for 72 hours. Shown is a representative result of three independent experiments.

5 Figure 7E demonstrates an analysis of CIP2A mRNA expression in HeLa cells transfected with two independent B56 α siRNAs for 72 hours. Shown is expression difference relative to Scr. siRNA transfected cells.

Figure 7F is a representative figure of cell densities of NIH-3T3 cells stably transduced with either WT CIP2A or the CIP2A-3A mutant compared at day 5 after seeding equal number of cells at low confluence. Scale bar is 1000 μ M.

Figure 7G demonstrates protein expression of V5 tagged CIP2A WT and 3A mutant in NIH-3T3 cells after 48h of serum starvation (+). CIP2A antibody recognizes the exogenous CIP2A in the absence of endogenous CIP2A expression in these cells.

Figure 7H illustrates the effect of serum starvation on cell viability of stably transduced NIH-3T3 clones expressing either CIP2A WT or the 3A mutant. Shown is the mean reduction in cell viability as compared to normal culture medium + S.E.M of 3 experiments with 6 replicate samples.

20 Figure 7I illustrates increased caspase 3 activity in CIP2A 3A mutant expressing cells after 72h of serum starvation. Shown are the mean values + S.E.M of 2 experiments with 6 replicate samples.

Figures 8A to 8E illustrate the functional impact of L533E and R522D mutations.

25 Figure 8A shows a Western blot analysis of protein expression of V5 tagged full-length WT or L533E and R522D CIP2A mutants from transiently transfected HEK293T cells. Shown is a representative blot for relative V5/ β -Actin from three independent experiments with similar results.

Figure 8B shows the quantitation of the Western blot results from A. Shown is mean + S.E.M.

Figure 8C is an 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 mutant. Plotted is mean + S.E.M. from four experiments with duplicate samples.

35 Figure 8D shows a Western blot analysis of c-MYC protein expression in 22RV1 cells transiently transfected with V5 tagged full-length WT or

L533E CIP2A. Shown are representative blots from four independent experiments with similar results.

Figure 8E shows the quantitation of the Western blot results from D. Shown is mean + S.E.M.

5 Figure 9 illustrates the folding propensity of human CIP2A. The X-axis corresponds to residue numbers 1-905. The Y axis is the disorder tendency for each residue. The darkest curve is the average result from 6 different programs, as summarized by the metaPrDOS server. Higher values indicate higher disorder propensity. This prediction is consistent with the present biochemical analysis that CIP2A(1-560) forms the folded core domain, whereas
10 the rest of CIP2A including the predicted coiled-coil region is most likely disordered.

Figure 10 is a stereo view of 2Fo-Fc electron density map, contoured at 1σ , showing the joint area of the stem and C-dimerization domains.
15 While D484 and L504 are parts of the stem domain, R530 and I531 belong to the dimerization domain.

Figure 11 demonstrates that 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 (Table
20 2), the two most similar 3D structures to CIP2A are β -catenin (PDB ID: 1TH1; an armadillo-repeat protein) and Walp (PDB ID: 4K6J; a HEAT-repeat protein). The CIP2A structure (in black) is superimposed with the structure of β -catenin (grey, panel A) and Walp (grey, panel B), respectively.

Figure 12 confirms CIP2A dimerization by CIP2A(1-560) cross pull-down and SEC analysis. Figure 12A shows that GST-tagged CIP2A (90KD),
25 but not GST, can pull-down untagged CIP2A (60KD) in a stoichiometric manner. The SDS-PAGE was stained with Coomassie Blue. Figure 12B shows SEC analysis of untagged CIP2A(1-560), which has a nominal MW of 62kD. Positions of 150 kD and 66 kD MW markers are marked. Inlet is the Coomassie
30 Blue-stained SDS-PAGE of the CIP2A peak.

Figure 13 indicates that PP2A interaction results obtained with B56 α can be generalized to another B56 family tumor suppressor B56 γ (PPP2R5C) and that similar to B56 α , the minimal region that mediates CIP2A interaction with B56 γ is located between amino acids 159 to 245.

35 Figure 14 shows surface electrostatic potential analysis and potential binding sites for C α and B56. The surface electrostatic potential was calcu-

lated using the APBS module and presented by Pymol. On the basis of the “front” view, it is clear that the “inner” surfaces of the CIP2A twisted double hook shape is highly negatively charged. The view with $\sim 75^\circ$ rotation indicates the potential B56 and C α binding sites, speculated based on both surface conservation and charge-distribution, by white and grey ovals, respectively.

Figure 15 is an illustration of detailed interactions in the CIP2A dimer interface mediated by the three helices of C-dimerization domain. In Figure 15A, hydrophobic interactions are shown in two orientations. The two CIP2A molecules are shown in lighter and darker grey, respectively. In the second orientation, one CIP2A is shown in space-filled model. In Figure 15B, shown are the hydrogen bonds formed between the two CIP2A subunits.

Figure 16 is an illustration of a structure model of conformation change in the CIP2A-3A dimer superimposed with CIP2A homodimer. The CIP2A-3A mutant dimer structure in which WT CIP2A is shown in light grey (left side) and 3A mutant CIP2A in dark grey (right side), respectively, was simulated by the Rosetta package. In the final model 3A mutant (right side) was superimposed into the WT CIP2A (right side).

Figure 17 shows that transiently transfected CIP2A WT and 3A mutant cDNAs produce equal levels of CIP2A transcripts. Shown is a relative increase in expression as compared to pCDNA vector transfected Hela Cells 24h after transfection. Shown is mean+S.D. from 2 biological replicate experiments.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure provides crystal structures of CIP2A. Preferably, the crystals are of sufficient quality to allow the determination of the three-dimensional X-ray diffraction structure to a resolution of about 3 Å to about 4 Å, preferably 3Å. The disclosure also relates to methods of preparing and crystallizing CIP2A. The information derived from the crystal structures can be used to analyse and modify CIP2A activity as well as to identify compounds that interfere with the binding of CIP2A to PP2A proteins.

Crystallization requires abundant amounts of the polypeptide to be crystallized in a relatively homogeneous form. To this end, a variety of host-expression vector systems may be employed for recombinant expression of CIP2A as is readily apparent to a skilled person. Suitable vectors include, but are not limited to, modified viruses, bacteriophages, plasmids and plasmid de-

rivatives, e.g. pGEX vectors. Depending on the vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. However, the vector system and the regulatory elements chosen must be compatible with the host cell selected for expression. Typical non-limiting examples of suitable expression hosts include mammalian cells (e.g. CHO cells), insect cells, yeast cells, and bacteria (e.g. *E. coli*).

In some embodiments it may be advantageous to produce a derivative of CIP2A wherein selenomethionine is incorporated into the CIP2A polypeptide in place of methionine. Depending on the host system employed, those skilled in the art can easily select a suitable protocol by which the host cells are induced to incorporate selenomethionine into proteins by suppression of methionine biosynthesis.

A CIP2A encoding vector may be introduced into the desired host cells by any suitable method known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, calcium phosphate precipitation, lipofection, or use of a gene gun.

For use in the present invention, a variety of CIP2A polypeptides may be utilized. Such polypeptides include full-length CIP2A consisting of amino acid residues 1-905 of SEQ ID NO:1, and any truncated form thereof. Owing to a poor stability of full-length CIP2A when produced in either *E. coli* or insect cells, as well as aggregation of the C-terminal fragment spanning residues 561-905 of SEQ ID NO:1, a preferred CIP2A polypeptide for crystallization purposes is the truncated CIP2A polypeptide of amino acid residues 1-560 of SEQ ID NO:1. Other preferred fragments of CIP2A for crystallization purposes include, but are not limited to, amino acid residues 1-245 of SEQ ID NO:1, amino acids 159-245 of SEQ ID NO:1, amino acid residues 232-560 of SEQ ID NO:1, 265-560 of SEQ ID NO:1, 293-560 of SEQ ID NO:1, and 331-560 of SEQ ID NO:1. However, as is apparent to those having skill in the art, the exact amino acid boundaries may vary within the practical limits of the disorder propensity of CIP2A (Figure 9).

The CIP2A amino acid sequence may be altered from the naturally occurring sequence by any amino acid substitutions, insertions, deletions, and the like in any number of combinations in order to produce a conformationally modified or dimerization deficient CIP2A polypeptide for crystallization purposes. Preferred mutations include, but are not limited to L529A, L532A, L533A,

R522D, and L533E. Mutations L529A, and L533A appear to interfere with CIP2A conformation but not prevent CIP2A dimerization, whereas mutations R522D and L533E appear to disrupt the dimerization.

Prior to crystallization, purification steps are employed to ensure
5 that CIP2A is isolated in a relatively homogeneous state. In general, the higher the purity, the better the likelihood of success in subsequent crystallization steps. Non-limiting examples of typical purification methods include the use of centrifugation, partial fractionation using salt or organic compounds, dialysis, conventional column chromatography (such as ion-exchange, molecular sizing
10 chromatography etc.), high performance liquid chromatography (HPLC), and gel electrophoresis methods.

Any crystallization technique known to those skilled in the art may be employed to obtain crystals of the present invention. Commonly used techniques include batch crystallization, vapour diffusion (either by sitting drop or
15 hanging drop) and dialysis. In each of these methods, it is important to promote continued crystallization after nucleation by maintaining a supersaturated solution. In some instances, seeding of the crystals is required to obtain X-ray quality crystals.

Generally, the crystals of the invention are grown by dissolving substantially pure CIP2A polypeptide in an aqueous buffer containing a precipitant
20 at a concentration just below that necessary to precipitate the protein. Water is removed by controlled evaporation to produce precipitating conditions, which are maintained until crystal growth ceases.

In a preferred embodiment of the invention, CIP2A crystals are grown by the hanging drop technique. In this method, an initial polypeptide mixture is created by adding a precipitant to concentrated polypeptide solution. The resulting precipitant concentration is about half that required for crystallization. The polypeptide/precipitant solution is then placed on a glass slide which is inverted and allowed to equilibrate in a sealed container with a larger aqueous
30 reservoir having a precipitant concentration optimal for producing crystals. The difference in the precipitant concentrations causes the protein solution to have a higher vapor pressure than the reservoir solution. Since the container is sealed, water from the polypeptide mixture transfers to the reservoir solution resulting in the crystallization of the peptide. Usually, the sealed container is
35 allowed to stand for about 1 to 6 weeks until crystals grow.

As is described in Example 2 below, it has been found that hanging

drops containing 1.0 μ l of CIP2A polypeptide (1.5 mg/ml in 20 mM Tris-HCl, pH 8.0, 250 mM NaCl and 2 mM dithiothreitol) and 1.0 μ l of reservoir solution (7% w/v polyethylene glycol (PEG 4000), 0.1 M sodium malonate, pH 6.0) equilibrated over 400 μ l of reservoir buffer at room temperature provide diamond shaped crystals in a few days.

Of course, those having skill in the art will recognize that the above-described crystallization conditions can be varied. Such variations may be used alone or in combination, and include, for instance, variations in polypeptide concentrations, Tris-HCl concentrations, pH ranges, and temperature ranges.

Once a crystal of the present disclosure is grown, it is exposed to an intense beam of X-rays produced by a conventional X-ray source (such as a sealed tube or a rotating anode) or a synchrotron source. Methods of crystal data characterization include, but are not limited to, precision photography, oscillation photography and diffractometer data collection. In Example 2 below, diffraction data sets were collected at beamlines 8.2.1 and 8.2.2 of the Advanced Light Source (ALS) using a ADSC Q315R detector.

Data processing and reduction can be carried out using programs such as DENZO, XDISPLAYF, and SCALEPACK of the HKL2000 program suite, as well as XDS and MOSFLM.

Methods of obtaining the three-dimensional structure, as well as the atomic coordinates, of a crystalline polypeptide on the basis of diffraction data are well-known in the art. For example, single-wavelength anomalous dispersion (SAD) or multi-wavelength anomalous dispersion (MAD) may be used for structure elucidation. Non-limiting examples of software suitable for structure determination and refinement include programs of the PHENIX program suite, as well as AMORE, COOT, and REFMAC of the CCP4i program suite.

Structural coordinates of a crystalline composition of this invention may be stored in a machine-readable form on a machine-readable storage medium, e.g. a computer hard drive, random-access memory (RAM), read-only memory (ROM e.g. CDROM), or a diskette, either local to a computer or remote (e.g. a networked storage medium, including the internet), for display as a three-dimensional shape or for other uses involving computer-assisted manipulation of, or computation based on, the structural coordinates or the three-dimensional structures they define. For example, data defining the three dimensional structure of CIP2A, or portions or structurally similar homologues

thereof, may be stored in a machine-readable storage medium, and may be displayed as a graphical three-dimensional representation of the protein structure, typically using a computer capable of reading the data from said storage medium and programmed with instructions for creating the representation from
5 such data. Such data may be used for a variety of purposes, such as the elucidation of other related structures and drug discovery.

Initially, the present crystalline CIP2A(1-560) fragment diffracted up to a resolution of about 4.0 Å. By careful dehydration of the crystals, diffraction quality was improved to a resolution of about 3.6 Å. Finally, the crystal struc-
10 ture of CIP2A(1-560) was determined at 3.0 Å resolution by altering the crystallization conditions and using selenium-methionine single-wavelength anomalous scattering (SAD) method.

The three-dimensional crystal structure revealed that CIP2A exists as a homodimer and that homodimerization is mediated by a relatively flat and
15 highly hydrophobic surface formed by the last three helices of CIP2A(1-560). To be more specific, amino acid residues 507-559 of SEQ ID NO: 1 appear to be responsible for CIP2A(1-560) dimerization. Additionally, the N-terminal region seems to be particularly important for B56 α binding. More specifically, amino acids 159-245 of SEQ ID NO:1 appear to contain features required for
20 B56 α binding, with 1-245 fragment showing higher efficiency than fragment 1-159 of SEQ ID NO:1. Narrowing down the region further was not possible because fragments between 159-245 proved to be unstable.

The refined three-dimensional CIP2A structure according to the present invention is represented by the structure determination statistics given in
25 Table 1 below. The crystalline CIP2A may form in the space group $P6_5$ with the unit cell dimensions of $\pm 5\%$ of those presented in Table 1. In one embodiment, the crystalline CIP2A may form in the space group $P6_5$ with the unit cell dimensions of $a = b = 153.28 \text{ \AA}$, $c = 105.44 \text{ \AA}$, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$. In another embodiment, the crystalline CIP2A may form in the space group $P6_5$ with the
30 unit cell dimensions of $a = b = 154.61 \text{ \AA}$, $c = 105.68 \text{ \AA}$, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$. However, the present invention contemplates CIP2A crystals, which may form in any space group including, but not limited to, $P6_5$.

Table 1. Diffraction Data and Refinement		
Dataset	SeMet-CIP2A	Native-CIP2A
<i>Crystal Parameters</i>		
Space group	P6 ₅	P6 ₅
Cell dimensions	a=b=154.61 Å, c=105.68 Å, $\alpha=\beta=90^\circ$, $\gamma=120^\circ$	a=b=153.28 Å, c=105.44 Å, $\alpha=\beta=90^\circ$, $\gamma=120^\circ$
Cell content	2 protein molecules/AU	2 protein molecules/AU
Solvent content	58.2%	57.4%
<i>Data Collection</i>		
Wavelength	0.97945 Å	0.97945 Å
Resolution	50 Å -3.5 Å (3.61 Å -3.5 Å)	50.0 Å - 3.0 Å (3.11 Å - 3.0 Å)
Unique reflections	19,639	28,364
R _{sym}	6.6% (84.3%)	7.5% (96.9%)
Average I/ σ	55.0 (3.6)	25.4 (2.3)
Completeness	98.7% (99.3%)	99.9% (100%)
Redundancy	15.2 (15.3)	7.4 (6.4)
Se sites (found/total)	12/16	
<i>Refinement</i>		
R _{work} /R _{free}		24.1% / 27.2%
Average B factor		81.8 Å ²
Rmsd bond ideality		0.0049 Å
Rmsd angle ideality		0.8504°
Ramachandran favored		95.2%
Protein residues		1035
Water		0
AU, asymmetric unit; rmsd, root-mean-square deviation. Values in parentheses indicate the corresponding statistics in the highest-resolution shell.		

The crystals of the invention, and particularly the refined three-dimensional structure obtained therefrom, have a wide variety of uses including, but not limited to, identifying molecules that bind to various native or mutated domains of CIP2A. The three-dimensional structure is particularly useful for identifying inhibitors which target the homodimerization domain as an approach towards developing new therapeutic agents with PP2A-inhibiting activity. Moreover, the 3D structure may be utilized not only for developing molecules that would target the dimerization interface directly, but also other regions of CIP2A, particularly regions that appear to mediate PP2A binding, such as the N-terminal region.

As used herein, the terms “PP2A proteins” and “components of the PP2A complex”, or any equivalent expressions thereof, refer to PP2R1A,

PP2R1B; PPP2CA, PP2CB; PPP2R2A, PPP2R2B, PPP2R2C, PPP2R2D; PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, PPP2R5E; PPP2R3A, PPP2R3B, PPP2R3C; STRN, STRN3, or STRN4 either alone or in any combination thereof. Preferred PP2A proteins include, but are not limited to, 5 PPP2R5A (B56 α), PPP2R5D (B56 δ), PPP2R5C (B56 γ), and PPP2CA (PP2Ac) either alone or in any combination thereof.

As is described in the Examples below, subtle interference with dimerization surface by mutations revealed that dimerization surface conformation is critical for CIP2A to bind to B56 α . Mutations of critical amino acids at 10 the interaction interface may block substrate binding with or without disrupting dimerization.

The present *in vitro* biochemical interaction analyses demonstrate for the first time direct interaction between CIP2A and any of the PP2A protein complex components. It is demonstrated that CIP2A binds to B56, resulting in 15 the formation of the high molecular weight CIP2A-B56 complex. However, CIP2A also binds directly to PP2A catalytic subunit PP2Ac.

CIP2A is a very promising protein target for cancer therapy, and the results disclosed herein provide a structural rationale for the development of inhibitors that target the CIP2A homodimerization interface. Importantly, although CIP2A homodimerization is mediated by several intermolecular interactions, the dissociation constant for this interaction is approximately 290 nM, making its disruption by small molecules feasible. Moreover, there are very clear effects on B56 binding of minor structural changes in the dimerization interface, and together with the intrinsic disorder of the "stem" region, this indicates that CIP2A might be susceptible to targeting by allosteric modulators 25 without a need for breaking the dimer.

Therapeutically, the most striking finding of the present disclosure is that a small structural perturbation in the CIP2A dimerization interface results in CIP2A protein degradation in cancer cells. In general, the benefits of drug 30 targeting to induce protein degradation are also 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.

Thus, one aspect of the present invention includes an *in silico* 35 method of using a crystal of the present invention in a drug screening assay. Any existing library of potential ligands of CIP2A can be examined through the

use of computer modelling using a docking program such as GRAM, DOCK, AUTODOCK, MOE-DOCK or FLEXX. This procedure can include, for example, computer fitting of potential ligands, preferably antagonists of CIP2A, to the homodimerization domain or the N-terminal PP2A-binding region of CIP2A to determine how well the shape and the chemical structure of the potential lig-
5 and will interfere with CIP2A interaction with PP2A proteins. Clinically relevant potential drugs need not break the CIP2A dimer in order to interfere with the CIP2A interaction with PP2A proteins but, in some embodiments, they may well cause the breaking of the CIP2A dimer.

10 In some embodiments, crystal structures of CIP2A with mutated homodimerization domains, such as those comprising one or more mutations of L529A, L532A, L533A, L533E and R522D, may be used for counter-screening potential anti-cancer drugs. In such cases a potential anti-cancer drug would be such that it is able to bind to a non-mutated CIP2A but not to a
15 mutated CIP2A.

Docking algorithms can also be used to verify interactions with ligands designed *de novo*. As used herein, “*de novo* compound design” refers to the process wherein the three-dimensional structure of CIP2A is used as a platform or basis for the rational design of compounds that will interact with
20 CIP2A, preferably with the domains that affect CIP2A binding to PP2A proteins.

Computer programs can also be employed to estimate the attraction, repulsion, and steric hindrance between the potential ligand and CIP2A. Generally, the tighter the fit (e.g. the lower the steric hindrance, and/or the
25 greater the attractive force) the more potent the potential drug will be since these properties are consistent with a tighter binding constant.

After selecting a potential drug by *in silico* computer modelling or *de novo* compound design, the potential drug may be produced and contacted with CIP2A, or a fragment thereof, in order to detect its ability to bind CIP2A
30 and, preferably interfere with CIP2A binding to PP2A proteins. In a particular embodiment either the potential drug or the CIP2A or fragment thereof is labeled. In another embodiment the CIP2A or fragment thereof is bound to a solid support. Non-limiting examples of high throughput techniques for assessing the binding of the potential drug to CIP2A include microscale thermophoresis
35 (MTS) and fluorescence-based thermal shift (FTS) assay. Non-limiting examples of high throughput techniques for determining whether or not CIP2A inter-

actions with PP2A proteins include isothermal calorimetry (ITC), surface plasmon resonance (SPR), microscale thermophoresis, fluorescence resonance energy transfer (FRET) and amplified luminescent proximity homogeneous assay screen (AlphaScreen, Perkin Elmer). In a particular embodiment either
5 CIP2A or PP2A proteins are labeled. In another embodiment the CIP2A or PP2A proteins are bound to a solid support.

In a preferred embodiment, the method further comprises growing a supplemental crystal containing a protein-drug complex formed between the CIP2A and the candidate drug.

10 Another aspect of the present invention relates to the identification of small molecules that inhibit CIP2A interaction with PP2A proteins in a high throughput screening assay. In some non-limiting embodiments, interaction conditions for such assays may not contain any divalent cation chelators such as EDTA, EGTA, and the like. In some cases, interaction studies should not be
15 carried out at the room temperature unless the interaction conditions comprise zinc. Thus, if zinc is not included, a reaction temperature of 37°C may be beneficial for carrying out an interaction reaction between CIP2A and PP2A proteins. On the other hand, if zinc is included, said interaction reaction may be successfully carried out at the room temperature. However, in some other em-
20 bodiments, the reaction may be successfully carried at the room temperature even if zinc is not included in the interaction conditions.

In an embodiment of the above aspect, CIP2A and PP2A proteins are allowed to interact in a buffer containing 50 mM Tris, 150 mM NaCl, 10% glycerol, 0.2% Igepal, 2 mM DTT, pH 7.5 for 1 hour at 37°C followed by addi-
25 tion of GSH agarose (available e.g. from Thermo Scientific) and further incubation for 1 hour at 37°C in rotation.

In another embodiment of the above aspect, CIP2A and PP2A proteins are allowed to interact in a buffer containing 50 mM Tris, 150 mM NaCl, 10% glycerol, 0.2% NP-40, 50 µM ZnSO₄, 2 mM DTT, pH 7.5 for 1 hour at the
30 room temperature followed by addition of GSH agarose and further incubation for 1 hour at the room temperature in rotation. Preferably, the GSH agarose is added in a volume of 5 µl in 20 µl of the interaction buffer. After incubations, the reactions are washed with a suitable solution a sufficient number of times. Preferably, ice cold interaction buffer, e.g. in an amount of 250 µl, is used for
35 the washings which are repeated a couple of times, such as four times. The overall washing time may be extended, for instance, to about 1 hour in order to

reduce the background signal. As is apparent to those skilled in the art, the exact conditions used for carrying out the interaction reaction between CIP2A and PP2A proteins are not limited to those mentioned above but may be varied as generally known in the art.

5 After formation of an interaction between CIP2A and PP2A proteins as set forth above, a candidate small molecule compound is added and its effect on said interaction is determined by any suitable method including, but not limited to, isothermal calorimetry (ITC), surface plasmon resonance (SPR), microscale thermophoresis, fluorescence resonance energy transfer (FRET)
10 and amplified luminescent proximity homogeneous assay screen (AlphaScreen, Perkin Elmer). In a particular embodiment either CIP2A or PP2A proteins are labeled. In another embodiment the CIP2A or PP2A proteins are bound to a solid support.

 It will be obvious to a person skilled in the art that, as technology
15 advances, the inventive concept can be implemented in various ways. The invention and its embodiments are not limited to the examples described above but may vary within the scope of the claims.

EXAMPLES

Example 1

20 **Protein expression and purification**

 Full-length CIP2A is not stable enough to be purified from either *E. coli* or insect cells for crystallization purposes (results not shown). According to structural foldability (flexibility) analysis, the C-terminal fragment spanning residues 561-905 is predicted to be intrinsically disordered (Fig. 9). Furthermore,
25 based on gel filtration, the C-terminal fragment per se tends to aggregate (results not shown). However, as the N-terminal 560 residues of CIP2A likely exhibit an ordered structure (Fig. 9), we focused on a 3D structure analysis of the CIP2A(1-560) fragment.

 The truncated domain of human CIP2A (residues 1-560) was cloned
30 into the pGEX-4T1 vector (GE Healthcare) with an N-terminal GST (glutathione-S-transferase) tag and a TEV cleavage site in between. CIP2A(1-560) was over-expressed in *E. coli* BL21 (DE3) cells (Novagen), grown in LB media. The bacteria cell was cultured at 37 °C till O.D.600 reaches 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 at 4°C overnight. Then the proteins were further purified by a cation exchange column on FPLC (GE Healthcare). The
5 purity of samples was verified using SDS-PAGE stained with Coomassie Brilliant Blue. The CIP2A truncated domain was observed as a single band at 60 kDa. The protein was then concentrated to 1.5 mg/ml in a buffer containing 20 mM Tris·HCl pH 8.0, 250 mM NaCl, 2 mM DTT for crystallization. A selenomethionyl (SeMet) derivative of the CIP2A truncated domain was expressed in an
10 auto-induction media (F. William Studier, 2005) and purified in the same way as the native protein.

EXAMPLE 2

Crystallization, optimization and Data Collection

Initial crystals of both the native and the SeMet-substituted CIP2A
15 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. Diamond-shaped crystals usually
20 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
25 equilibrated at 4°C for different time periods. Amazingly, 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
30 Light Source (ALS), beamlines 8.2.1 and 8.2.2. Diffraction data sets were processed by HKL2000 (Otwinowski and Minor, 1997) and Mosflm (Battye et al. 2011).

Structure determination and refinement

The structure was determined by single-wavelength anomalous dis-

person (SAD) using one data set collected at wavelength 0.9793 angstrom, which was also used for refinement. The selenium sites and the initial phases were determined by PHENIX (Adams et al., 2010). Fourteen selenium sites were found in one asymmetric unit (there are 8 Met residues in each CIP2A(1-560) fragment, including the N-terminal Met that was not found by PHENIX). The experimental electron density map clearly showed the presence of two CIP2A molecules in one asymmetric unit. The structural model was gradually improved using iterative cycles of manual rebuilding with the program COOT (Emsley et al., 2010) and refinement with Refmac5 of CCP4 6.1.2 Program Suite (CCP4, 1994). In the crystal lattice, the “tip” domain of CIP2A(1-560) has an average B-factor of $97.77 \text{ \AA}^2$, while the stem and dimerization domains have an average B-factor of $66.39 \text{ \AA}^2$, and weak electron densities for the tip domain part did not allow us to build some loop residues between armadillo repeat helices.

The structural model revealed that in the crystal lattice, there are two CIP2A(1-560) molecules, related by a non-crystallographic 2-fold axis, in each asymmetric unit as illustrated in Figure 1A. This finding is consistent with the biochemical data set forth in the Examples below. The C-terminal ends of two CIP2A(1-560) molecules join to form a stable dimer interface. Overall, the CIP2A(1-560) dimer structure resembles an oppositely-twisted double hook.

The CIP2A(1-560) monomer is an all-helical protein, with most of the molecules formed by armadillo or armadillo-like repeats (Fig. 1B), and can be roughly divided into “tip”, “stem” and C-dimerization domains. The first 185 residues form a “tip” domain consisting of 5 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. 1B). Some of the armadillo repeats in the “stem” domain display the structural features of HEAT repeats, as revealed by protein folding similarity searches using the Dali server (Holm and Sander, 1997). In addition to the armadillo repeat domains of β -catenin and APC, the atypical HEAT-repeat domain of Walp is among the closest structural neighbors of the stem subdomain of CIP2A(1-560) (Fig. 11 and Table 2).

Table 2. Dali Search Summary

No:	Chain	Z	rmsd	dali	nres	%id	PDB	Description
1:	4k6j-B	19.1	5.1	344	471	12	PDB	MOLECULE: WINGS APART-LIKE PROTEIN
HOMOLOG;								
2:	1jpp-B	19.0	13.3	409	502	13	PDB	MOLECULE: BETA-CATENIN;
3:	1jpw-C	18.9	12.9	418	502	13	PDB	MOLECULE: BETA-CATENIN;
4:	1jpw-B	18.8	13.0	418	502	13	PDB	MOLECULE: BETA-CATENIN;
5:	1v18-A	18.7	12.7	430	507	13	PDB	MOLECULE: BETA-CATENIN;
6:	3nmw-B	18.7	4.9	285	325	13	PDB	MOLECULE: APC VARIANT PROTEIN;
7:	3nmw-A	18.7	4.9	284	326	13	PDB	MOLECULE: APC VARIANT PROTEIN;
8:	1th1-A	18.5	10.6	385	514	15	PDB	MOLECULE: BETA-CATENIN;
9:	1i7x-A	18.5	13.3	427	522	13	PDB	MOLECULE: BETA-CATENIN;
10:	3nmw-C	18.5	4.9	283	323	12	PDB	MOLECULE: APC VARIANT PROTEIN;
11:	3bct-A	18.3	12.8	418	457	13	PDB	MOLECULE: BETA-CATENIN;
12:	1jpw-A	18.2	13.2	425	502	13	PDB	MOLECULE: BETA-CATENIN;
13:	3nmw-B	18.1	5.3	283	331	12	PDB	MOLECULE: APC VARIANT PROTEIN;
14:	3nmw-A	18.1	5.3	284	331	13	PDB	MOLECULE: APC VARIANT PROTEIN;
15:	3qhe-A	18.1	5.2	284	323	12	PDB	MOLECULE: ADENOMATOUS POLYPOSIS COLI;
16:	2jdg-B	18.0	7.7	366	426	12	PDB	MOLECULE: IMPORTIN ALPHA-1 SUBUNIT;
17:	3t7u-A	17.8	5.3	279	309	13	PDB	MOLECULE: ADENOMATOUS POLYPOSIS COLI;
18:	3t7u-B	17.8	5.3	279	311	14	PDB	MOLECULE: ADENOMATOUS POLYPOSIS COLI;
19:	4k6j-A	17.7	5.3	352	497	13	PDB	MOLECULE: WINGS APART-LIKE PROTEIN
HOMOLOG;								
20:	3qhe-C	17.7	5.1	278	318	13	PDB	MOLECULE: ADENOMATOUS POLYPOSIS COLI;

The dimeric structure of mutated CIP2A(1-560) containing three leucine to alanine substitutions at the homodimerization interface, namely L529A, L532A, and L5333A was modeled by M-ZDOCK (Pierce et al., 2014) with 2-fold symmetry, which was followed by full-atom docking by Rosetta (Gray et al., 2003).

EXAMPLE 3.**Experimental procedures for biochemical analysis****10 Protein expression and purification for biochemical analysis**

CIP2A constructs used in interaction experiments were cloned into the pGEX-4Tt-2 vector containing Thrombin cleavage site. The CIP2A deletion constructs were cloned individually by generating them by PCR. The B subu-

nits and PR65 into the pGEX vector containing TEV cleavage site. The proteins were produced in *E. coli* BL21 strain induced by 0.2 mM Isopropyl β -D-1-Thiogalactopyranoside (IPTG) when the O.D.600 was 0.6-0.8. The cells were then shaken at 23 °C and harvested after 3.5 h. The bacteria pellets were collected and lysed by sonication. The GST fusion protein was purified by Glutathione Sepharose 4B column. From the B subunits the GST tag was removed by TEV cleavage at 4 °C overnight. In dimerization assay the GST tag was also removed from CIP2A(1-560) by thrombin at RT overnight. The glutathione containing buffer was replaced by interaction buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 0.2% NP-40, 2 mM DTT) using dialysis at 4 °C overnight. The purified proteins were stored at -80 °C. The PP2A catalytic subunit PP2Ac was produced as a baculoviral protein as described in (Cho and Xu, 2007). PP2A trimers and dimers were formed by incubating the A, B and C subunits in 1:1:1 stoichiometry (or 1:1 in case of AC dimer) for 1 h at room temperature. The complex formation is very efficient for the trimer based on gel filtration analysis. In some experiments commercially available PP2A dimer purified from reticulocytes (Millipore) was used.

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).

GST pulldown assays

In all GST pulldown assays, 10 pmol of each protein was applied, except in the experiment of the Figure 4E in which ~5 μ M GST-CIP2A(1-560) and excessive B56 α /PP2Ac were used as indicated in the Figure. The overall volume of each pulldown prep is 200 μ l. The interaction buffer is 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. Then 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 spun down and the supernatant is carefully removed and discarded and the resin was suspended in SDS-PAGE sample buffer and analyzed by Western blot. The antibodies used for

the detection are B56 α (Santa Cruz sc-136045), B56 δ Santa Cruz (sc-271363), B56 ϵ Santa Cruz (sc-376176), B56 γ (Santa Cruz sc-46459), PP2Ac (Cell Signalling #2038S), Anti-V5 (Sigma V8012), Lamin A/C (Santa Cruz sc-20681), GST (Santa Cruz sc-138).

5 **Size exclusion chromatography**

Size exclusion chromatography was carried out using Superdex 5/150 column (GE Healthcare). The flow rate was 0.3 ml/min and the column was operated at room temperature. The running buffer was 28 mM Tris, 150 mM NaCl, 0.05% NP-40, 1.25% glycerol, 2 mM DTT, pH 7.2). 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, 150 mM NaCl, 5% glycerol, 0.2% NP-40, 2 mM DTT, pH 7.5) for 1 h at 37 °C. The total volume was 120 μ l. The samples were centrifugated briefly 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.

Thermophoresis

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

Retroviral expression of full-length CIP2A constructs

A day before infection, 30,000 NIH3T3 cells were plated in each well of a 6-well plate. Next day, the 48 h virus preparation was warmed to 37 °C and mixed with polybrene to final concentration of 8 μ g/ml. The culture media was removed and replaced with 3 ml of the virus preparation. On third day, the media was replaced with 3 ml of 72 h virus preparation treated the same way as in the previous step. On the next day the cells were trypsinized and 1/8 of the cells were replated on another 6-well plate. Puromycin selection was started on the following day with 1 μ g/ml of puromycin and continued for 3 days when all control cells were found dead.

Analysis of expression of CIP2A WT and mutant proteins from mammalian cells

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. After about 24h, 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 x SDS-PAGE sample buffer, incubated for 10 min at 95°C and centrifuged at 13,200 rpm for 15 min. The cleared supernatant (8 μ l) was resolved by 4-20% SDS-PAGE and analyzed by Western blot with the following antibodies: V5 (as above, used at 1:2,000, with secondary anti-mouse at 1:2,000) and β -Actin (Santa Cruz Biotechnology, C-4, mMAb, used at 1:5,000, with secondary anti-mouse at 1:5,000). For quantification, for each sample, the signal from the V5 was first normalized against β -Actin. Next, the value for WT CIP2A(1-905) was set as 1 and the value for the CIP2A mutant was adjusted accordingly.

For RT-PCR, the cells were plated in a 6 well-plate format. 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, ThermoFisher 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 (SEQ ID NO:4) and CGACCTTCTAATTGTGCCTTTT (SEQ ID NO:5).

Analysis of c-MYC protein expression in 22RV1 cells transiently expressing CIP2A WT or L533E mutant

22RV1 cells were plated in a 12 well-plate format. Cells were transfected using Lipofectamine 3000 (Invitrogen by Thermo Fisher Scientific) or Eugene 6 (Promega) at 3:1, according to the manufacturer's protocol. For transfection, cells were placed in Optimem, which was removed about 6-7 hours after transfection. About 24h post-transfection, the cells were lysed as above. In this case, they were mixed with 20 μ l 6 x SDS-PAGE sample buffer and 12 μ l was resolved by 4-20% SDS-PAGE and analyzed by Western blot with the following antibodies: CIP2A (Santa Cruz Biotechnology, 2G10-3B5,

mMAb, used at 1:1,000), β -Actin (as above), with secondary anti-mouse at 1:5,000, and c-MYC (abcam, Y69, polyclonal Rabbit Ab), with secondary anti-rabbit at 1:2,000. For quantification, for each sample, the signal from the c-MYC was first normalized against β -Actin. Next, the value for pcDNA3.1 vector control was set as 1 and the values for the CIP2A variants were adjusted accordingly. Data was plotted with GraphPad Prism6 showing mean + S.E.M. from four experiments.

siRNA and transient transfection experiments

For B56 siRNA experiment the cells were transfected in with Oligo-fectamine (Life technologies) using manufacturer's protocol scaled by a factor of 7.5 for 10cm plates. Following siRNA sequences with symmetrical overhangs were used for B56 α knockdown: B56 α -1:UAC CCA UCU GUU ACC ACU CdTdG (SEQ ID NO:2), B56 α -2: AAG UGU ACG GAA GAU GUU AdGdC (SEQ ID NO:3). After 72h 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, ThermoFisher). 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: GAACAGATAAGAAAAGAGTT-GAGCATT (SEQ ID NO:4) and CGACCTTCTAATTGTGCCTTTT (SEQ ID NO:5).

To assess stability and functional effects of the 3A mutant of CIP2A, HeLa cells were transfected by using Eugene HD either with empty pcDNA3.1 vector, wild type V5-CIP2A WT or 3A mutant vector and cells were harvested on SDS buffer 2-3 days after transfection. Western blot analysis was performed with following antibodies: V5 (mouse monoclonal, R960-25; Invitrogen), GAPDH (mouse monoclonal, 5G4-6C5; Hytest).

Preparation of mammalian and retroviral expression plasmids

Full-length CIP2A was cloned in pcDNA3.1/V5-His TOPO vector. The reverse primer used to amplify CIP2A sequence was designed to be in frame with the vector, so it did not contain stop codon, or any additional sequence at the 5' end. The forward primer carried also an EcoRI site at the 5'

end. This allowed CIP2A sequence together with C-terminal tags to be cut out from the vector as an EcoRI-PmeI fragment. This fragment was recloned into the pBabe vector at for retroviral expression. The restriction sites used were EcoRI and Sall. To make the latter site compatible with PmeI site ligation it was filled with T4 polymerase in order to produce blunt end. Mutagenesis was done by Genscript or by 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. All constructs were verified by DNA sequencing (Finnish Microarray and Sequencing Centre, Centre for Biotechnology Turku).

Preparation of retroviruses

Retroviruses were produced from the pBabe constructs by using Phoenix ecotropic packing cells. The cells were cultured in DMEM medium containing 10% FBS, 1% Pen-Strep, 1% Glutamine and 1% non-essential amino acids. On first day 1 million cells were plated on 10 cm plate. Plasmid transfection was done on the next day using 24 µg of plasmid and 72 µl of Eugene HD transfection reagent (Promega) according to the protocol of the Eugene HD. On day 3, 4 ml of fresh media was added. On day 4, the media was collected and replaced with fresh media. The 48 h virus preparation was filtered using 0.45 µm filters, aliquoted and stored at -80°C. On day 5 the 72 h virus preparation was harvested as above.

EXAMPLE 4

Verification of CIP2A dimerization

CIP2A dimerization observed by crystallization was confirmed independently by a yeast-two-hybrid (Y2H) analysis with full-length CIP2A (Fig. 2A). As CIP2A is expressed at a very low level in most normal tissues but is overexpressed in breast cancer, we used a mixed cDNA library from several breast cancer cell lines (T47D, MDA-MB-468, MCF7, BT20). An analysis of overlapping clones that interacted with full-length CIP2A revealed that CIP2A homodimerization is mediated by a region encompassing amino acids 338-558 (Fig. 2A; SID), which is fully consistent with the structure of the CIP2A homodimer (Fig. 1A). To further confirm CIP2A homodimerization, we used thrombin cleavage to remove the GST tag from GST-CIP2A(1-560) and used this as prey in a GST pull-down experiment with the parental GST-CIP2A(1-

560) protein. CIP2A(1-560) did not significantly associate with GST alone, whereas a robust interaction was observed between the two CIP2A fragments (Fig. 2B and S4A). Next, a gel filtration analysis was performed to confirm these findings. GST-CIP2A(1-560) has an expected monomer molecular weight of 90 kDa. However, the protein mostly eluted in fraction 3, and based on gel filtration column calibration, fraction 3 contains proteins that are approximately 160 kDa (Fig. 2C). To exclude the possibility that high molecular weight complex formation was due to dimerization via the GST moieties, CIP2A dimerization was further confirmed by SEC analysis of untagged CIP2A(1-560) (Fig. 10B). Finally, MicroScale Thermophoresis (MST) analysis revealed that CIP2A homodimerizes with an approximate affinity (Kd) 290 nM (Fig. 2D).

Together with the structural analysis (Fig. 1), these results reveal a previously unappreciated homodimerization of full-length CIP2A (Fig. 2A) mediated by the last three helices of CIP2A(1-560), which form a planar interaction motif.

EXAMPLE 5

Direct interaction between CIP2A and B56

In addition to CIP2A, B subunit B56 γ (PPP2R5C) was one of the direct interaction partners that were identified by Y2H to bind to full-length CIP2A (Fig. 2A). This is very interesting, as B56 γ has been shown to be one of the most important tumor suppressor B subunits. To verify these exciting results, the CIP2A(1-560) was demonstrated to interact directly with B56 γ in a GST pull-down experiment (Fig. 3A). Interestingly, when other members of the B56 family were tested, CIP2A was found to also interact with B56 α and very prominently with B56 δ but not with B56 ϵ (Fig. 3A). The selectivity among B56 family members was verified by MST analysis, allowing the determination of approximate Kd values for these interactions (Fig. 3B).

In addition to B56 γ B56 α functions as a tumor suppressor by directing the PP2A complex to MYC. As CIP2A has been verified in several independent studies to inhibit PP2A-mediated MYC dephosphorylation, we used B56 α as the representative member of the B56 family in all subsequent interaction analysis. To confirm that B56 proteins interact with the CIP2A dimer, we pre-incubated recombinant GST or GST-CIP2A with B56 α and analyzed the gel filtration elution patterns of both B56 α and CIP2A by western blotting. In the presence of GST alone, B56 α eluted in fractions 4 and 5, whereas in the

presence of GST-CIP2A, there was a clear shift toward higher molecular weight complexes containing the CIP2A dimer (Fig. 3C).

EXAMPLE 6

Analysis of the B56 and PP2Ac interaction regions of CIP2A

5 Next we characterized the regions on CIP2A that mediate its direct interactions with B56 α and B56 γ . The CIP2A(1-560) fragment showed stronger binding to CIP2A than the N-terminal fragment CIP2A(1-330). Based on an analysis of several additional N-terminal CIP2A deletion constructs, the minimal region that mediates the B56 α interaction is located between amino acids
10 159 to 245 (Fig. 4A), which covers the last (fifth) armadillo repeat in the "tip" domain and the first (sixth) repeat in the "stem" domain (Fig. 1B). Importantly, this is a very well conserved region in CIP2A(1-560) protein sequence between different species and forms the most conserved area on CIP2A surface (Fig. 4C), suggesting that B56 binding is a conserved feature in evolution. This is
15 also supported by the finding that the same region mediates interaction between CIP2A and B56 γ Fig. 13. Interestingly, unlike for B56, both the CIP2A(1-330) and (1-560) fragments showed equal binding to PP2Ac, and the interaction between CIP2A and PP2Ac appears to be mainly mediated by the third armadillo repeat between amino acids 85 and 128 (Fig. 4B).

20 Next we modeled the above identified minimal binding regions to CIP2A structure, taking also into account the charge distribution and assuming that the binding on CIP2A may occur at positively charged areas since both B56 and PP2Ac surfaces are largely negatively charged. Strikingly, the "inside" surfaces of CIP2A(1-560) dimer are highly negatively charged (Fig. 14), indicating that B56 and PP2Ac may bind to positively charged outer surface of
25 CIP2A molecules. Since B56 binding to CIP2A might cause a slight steric hindrance for simultaneous PP2Ac binding, we speculate that B56 and PP2Ac could bind to two neighboring outer surface areas of CIP2A which are both positively charged (Fig. 14). Our efforts to confirm this assumption by crystallization of the CIP2A-B56 and CIP2A-PP2Ac complexes have not been successful so far.

30 Together these results show that whereas PP2Ac interaction is mediated by N-terminal armadillo domain, maximal binding of B56 seem to involve multiple regions. Moreover, as the region between residues 330 and 560
35 strongly contributes to both B56 binding (Fig. 4A), and mediates CIP2A dimeri-

zation (Fig. 1A and 2A), this suggests that CIP2A dimerization may be relevant for binding to B56 to a CIP2A dimer.

EXAMPLE 7

Mutational analysis of the CIP2A(1-560) dimerization interface

5 The dimerization subdomain is formed by the last three helices of CIP2A(1-560) (Fig. 5A). The last two helices and the loop link to the previous helix to form a relatively flat and highly hydrophobic surface, mediating the homodimerization of CIP2A(1-560) (Fig. 5A and Fig. 15A). Formation of this homodimer interface buries an accessible surface area of 1913 Å², which is
10 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 (Fig. 5A and 15A). The key residues involved in the interaction between CIP2A monomers include V525, L529, L532, L533, L546 and I550 (Fig. 5B and 15B), and all these residues,
15 with the exception of L533, are evolutionarily conserved across different species.

 To interfere with the CIP2A homodimerization interface, we mutated three leucines (L529, L532 and L533) to alanines, collectively referred to as the 3A mutant, and examined the impact on direct binding to B56α. These
20 three residues form a cluster, and modeling of their mutation to alanines indicated local sidechain conformational changes only. Intriguingly, the mutation of L529 significantly decreased CIP2A(1-560) binding to B56α; furthermore, the combination of all three mutations nearly abolished B56α binding (Fig. 5C and D, (CIP2A1-560-3A)). Interestingly, despite the loss of B56 binding affinity, nei-
25 ther the L529A nor the 3A mutants showed any defect in CIP2A dimerization capacity, as measured by either GST pull-down assay (Fig. 5E) or MST analysis (Fig. 5F). This indicates that the introduced mutations do not prevent CIP2A dimerization but may cause sufficient conformational change in CIP2A dimer so that it disrupts B56α binding. This hypothesis is supported by structure
30 model of CIP2A-3A dimer indicating for a clear conformational shift as compared to WT dimer (Fig. 16).

 In addition to 3A mutants, which caused B56 binding defect without clear impact on CIP2A dimerization, we identified two soluble single point mutants, R522D and L533E, that repeatedly demonstrated significantly impaired
35 dimerization across six independent assays (Fig. 6A and C). L533 is directly

involved in the interaction surface between CIP2A monomers. Its substitution by a bulky negatively charged amino 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, which also is a strictly conserved residue throughout evolution. 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 approximately 50% inhibition (Fig. 6A and C).

In order to directly test the impact of CIP2A dimerization on B56 binding, the CIP2A(1-560) dimerization compromised mutant L533E was compared with wild-type CIP2A(1-560) for B56 α binding by GST pull-down assay. In line with our hypothesis, L533E mutant showed significantly reduced binding to B56 α (Fig. 6B and C). Although this data do suggest that CIP2A dimerization may enhance CIP2A binding to B56 α , we wanted to further test whether the “weaker” dimerization mutant R522D would also show impaired B56 binding, and whether degree of inhibition of dimerization, and B56 binding would show any correlation between the two mutants. Indeed, also R522D did show weaker binding to B56 α than wild-type CIP2A (Fig. 6B and C). Importantly quantification of four independent experiments demonstrated that significantly lowered capacity of dimerization mutants to bind to B56 α correlated with their reduced capacity to dimerize (Fig. 6B and C). To estimate the contribution of dimerization to maximal B56 α binding capacity of CIP2A, we calculated the ratio between observed effects on both dimerization, and B56 α binding. Notably, both mutants showed comparable approximately 50% contribution of dimerization to B56 α binding in our assay conditions (Fig. 6D). This supports the conclusion that B56 α binding defect observed with these mutants is caused by similar mechanism, i.e. inhibition of dimerization.

EXAMPLE 8

A CIP2A dimerization interface mutant is degraded in cells and loses its oncogenic activity

To assess the functional impact of the loss of B56 binding via CIP2A dimerization interface mutations, we created full-length CIP2A mammalian ex-

pression vectors coding for either wild-type or the 3A mutated protein. It has been demonstrated that CIP2A overexpression promotes the proliferation and RAS-driven malignant transformation of NIH-3T3 cells. Therefore, these otherwise CIP2A-negative cells were infected with retroviral CIP2A WT or 3A constructs or with the pBabe control vector. As shown in Fig. 7A, RT-PCR revealed that both the CIP2A WT and 3A constructs produced equal amounts of the transcript. However, repeated experiments showed dramatically lower protein expression levels in the 3A mutant compared to the WT protein, as measured using a V5 epitope-specific antibody (Fig. 7B). To assure that the difference in recombinant protein expression by either WT- or 3A-encoding cDNAs was not a clonal effect or specific to untransformed NIH-3T3 cells, human HeLa cells were transiently transfected with mammalian expression constructs, and the expression of recombinant CIP2A proteins was assayed at 72 h after transfection. As observed in NIH-3T3 cells, the 3A mutant displayed very low protein expression in HeLa cells (Fig. 7C), even though the WT and 3A transgenes were quantitatively expressed at the same level (Fig. 17).

Results above indicate that CIP2A binding to B56 α may not only disrupt functional PP2A complex but also autoregulate CIP2A protein expression. To test this interesting hypothesis, B56 α expression was inhibited in HeLa cells by two independent siRNAs, and both CIP2A protein and mRNA expression was studied after 72 h. Although B56 α depletion did induce robust inhibition of endogenous CIP2A protein expression (Fig. 7D), neither of the B56 α siRNAs had a significant impact on CIP2A mRNA expression (Fig. 7E).

The low expression of the CIP2A-3A mutant protein resembles the situation in which CIP2A expression is inhibited in cancer cells by RNAi, genetic deletion, or by various chemicals which has been demonstrated in numerous studies to inhibit cell survival and malignant growth. NIH-3T3 cells do not endogenously express CIP2A, making them a very suitable model to test whether the CIP2A-3A mutant has lost its potential to drive cell survival. Indeed, when the cell densities of NIH-3T3 cells stably transduced with either WT CIP2A or the CIP2A-3A mutant were compared at day 5 after seeding the cells at low confluence, there were clearly fewer cells in the CIP2A-3A mutant plates (Fig. 7F). To directly compare the capacity of WT CIP2A or the CIP2A-3A mutant to support cell viability, we examined the effects of serum starvation of stably transduced NIH-3T3 clones on CIP2A protein expression, cell viability, and apoptosis induction. Serum starvation for 48 h inhibited expression of both WT

and 3A mutant protein, though the lower expression of the 3A mutant under these conditions correlated with a significantly decreased capacity of the mutant to support cell viability in the first 24 h (Fig. 7G and H), after which ATP-dependent oxygen consumption in both clones fell below the detection limit. However, the lower expression of the 3A mutant at 72 h of serum starvation manifested in significantly increased levels of apoptosis induction, as measured by an indirect caspase 3 activity assay (Fig. 7I).

EXAMPLE 9

Functional analysis of CIP2A dimerization disrupting mutations

To assess the functional impact of CIP2A mutations L533E and R522D, that disrupt CIP2A dimerization, we created CIP2A(1-905) mammalian expression vectors coding for either WT or L533E and R522D mutated V5-CIP2A fusion protein. Similarly to CIP2A-3A mutants, 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. 8A and B). 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. 8C). Importantly, the effects of L533E mutant on CIP2A protein expression was validated in another cell line (22RV1) with low endogenous CIP2A levels (Fig. 8D). Furthermore, L533E mutant showed significantly lower capacity to support expression of well-established CIP2A and B56 α target MYC, as compared to WT CIP2A (Fig. 8D, 8E).

Together these results establish relevance for CIP2A dimerization and B56 α binding discovered in this study. As functional consequences of CIP2A-mediated PP2A inhibition on malignant cell growth and tumorigenesis are very well established on numerous recent studies (Junttila et al., 2007, Khanna et al., 2013, Laine et al., 2013), it is conceivable that targeting of CIP2A regions that contribute to both CIP2A dimerization, and B56 binding could constitute a first structure-based strategy for therapeutic CIP2A inhibition.

EXAMPLE 10

Discussion

Inhibition of PP2A tumor suppressor activity is a prerequisite for

human cell transformation and mediates cancer drug resistance. Thus, understanding the molecular mechanisms by which PP2A function is inhibited is of crucial importance to cancer biology. Except for the relatively infrequent genetic and epigenetic inhibition of PP2A B subunits, most of the oncogenic mechanisms for inhibiting PP2A activity identified thus far affect the catalytic activity of PP2Ac. Indeed, this is the mechanism of various natural PP2A inhibitor compounds, such as okadaic acid, the viral inhibitor protein SV40 small-t, and two other well-characterized PP2A inhibitor proteins, SET and PME-1. We present here evidence for CIP2A binding to B56, resulting in formation of the high molecular weight CIP2A-B56 complex. Importantly, CIP2A does not bind to all B56 family PP2A B subunits, and its binding affinity to members of the B56 family can differ greatly. Among the B56 family members that CIP2A can displace from the PP2A trimer are B56 α and B56 γ which are the two PP2A B subunits with the most convincing functional evidence of tumor suppressor activity. We postulate that the binding affinity differences between different B subunits creates a hierarchy between PP2A complexes that are subject to CIP2A-mediated inhibition, and this may explain why CIP2A only regulates a fairly restricted number of PP2A target phosphoproteins. These conclusions are supported by our unpublished phosphopeptide mass spectrometry analysis of phosphorylation events regulated either by CIP2A, PME-1 or SET. In this dataset, CIP2A regulates a characteristic combination of phosphorylation sites that is more limited than PME-1 or SET phosphoproteomes. The lack of general inhibition of PP2A activity is also supported by the absence of viability defects in a CIP2A-deficient mouse model compared to PME-1-knock-out models.

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CLAIMS

1. A crystal of CIP2A, the crystal being present on space group $P6_5$ with two CIP2A molecules in one asymmetric unit.

2. The crystal according to claim 1, wherein said space group has
5 unit cell dimensions of $\pm 5\%$ of $a = b = 153.28 \text{ \AA}$, $c = 105.44 \text{ \AA}$, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$, or $\pm 5\%$ of $a = b = 154.61 \text{ \AA}$, $c = 105.68 \text{ \AA}$, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$.

3. The crystal according to claim 1 or 2, wherein the CIP2A comprises an amino acid sequence set forth in SEQ ID NO:1.

4. The crystal according to any preceding claim, wherein the CIP2A
10 comprises amino acids 1-905 of SEQ ID NO:1, amino acids 1-560 of SEQ ID NO:1, amino acids 1-245 of SEQ ID NO:1, amino acids 159-245 of SEQ ID NO:1, amino acids 232-560 of SEQ ID NO:1, amino acids 265-560 of SEQ ID NO:1, amino acids 293-560 of SEQ ID NO:1, amino acids 331-560 of SEQ ID NO:1, or amino acids 507-559 of SEQ ID NO:1.

15 5. The crystal according to any preceding claim, wherein the homodimerization domain of CIP2A comprises one or more mutations, preferably selected from the group consisting of L529A, L532A, L533A, R522D, and L533E.

20 6. A method of identifying a compound that interacts with CIP2A, comprising the steps of:

a) providing X-ray diffraction data of crystalline CIP2A, capable of displaying a three-dimensional representation of CIP2A when read by an appropriate machine and processed by a computer program for determining molecule structures,

25 b) performing rational drug design or *in silico* screening of a compound library on the basis of the three-dimensional representation of CIP2A, and

c) identifying a potential compound that interacts with CIP2A.

30 7. The method according to claim 6, wherein said drug design is targeted to a homodimerization domain of CIP2A or an N-terminal PP2A-binding region of CIP2A.

8. The method according to claim 6 or 7, wherein said interaction of the potential compound with CIP2A prevents CIP2A from interacting with PP2A proteins.

35 9. The method according to any one of claims 6 to 8, further com-

prising the steps of:

- d) contacting said potential compound with CIP2A, and
- e) determining whether or not said potential compound prevents CIP2A from binding to PP2A proteins or causes the breakage of CIP2A complex with PP2A proteins.

5 10. A method of preparing the crystal of CIP2A according to any one of claims 1 to 4, which comprises the steps of:

- a) providing a solution of CIP2A in a suitable buffer, such as 20 mM Tris-HCl, pH 8.0, 250 mM NaCl and 2 mM dithiothreitol,
- 10 b) mixing the solution with a crystallization solution comprising a precipitating agent, preferably with a crystallization solution comprising 7% (w/v) polyethylene glycol (PEG 4000), 0.1 M sodium malonate, pH 6.0, and
- c) incubating the mixture under conditions to promote hanging drop vapour diffusion for a time sufficient to produce the crystal of CIP2A.

15 11. Use of the structure of the crystal of CIP2A according to any one of claims 1 to 4, in modelling a relevant molecule which interferes with the interaction of CIP2A with PP2A proteins.

20 12. Use of CIP2A and PP2A proteins for performing a high throughput screen to identify molecules which interfere with the interaction of CIP2A with PP2A proteins.

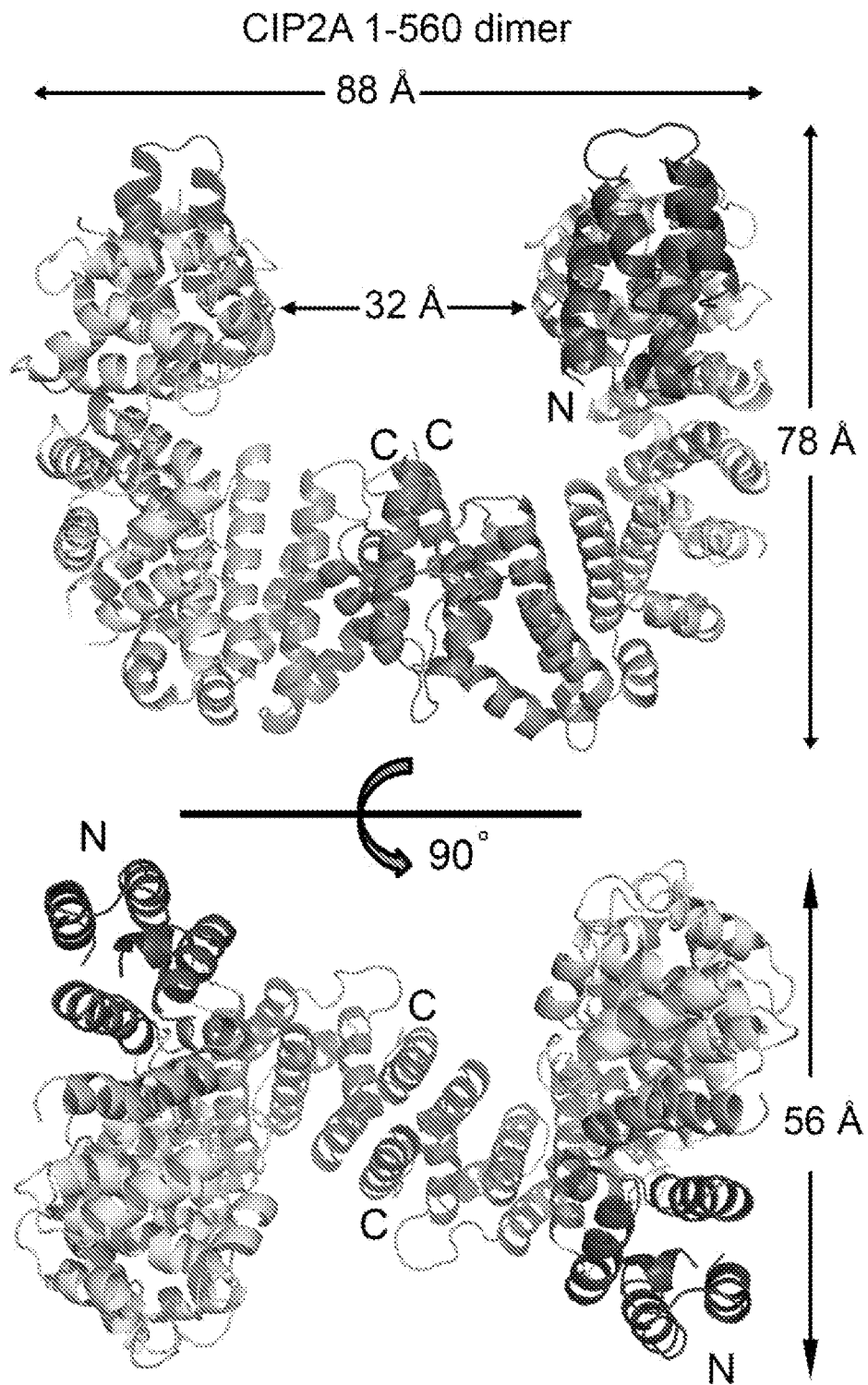


Figure 1A

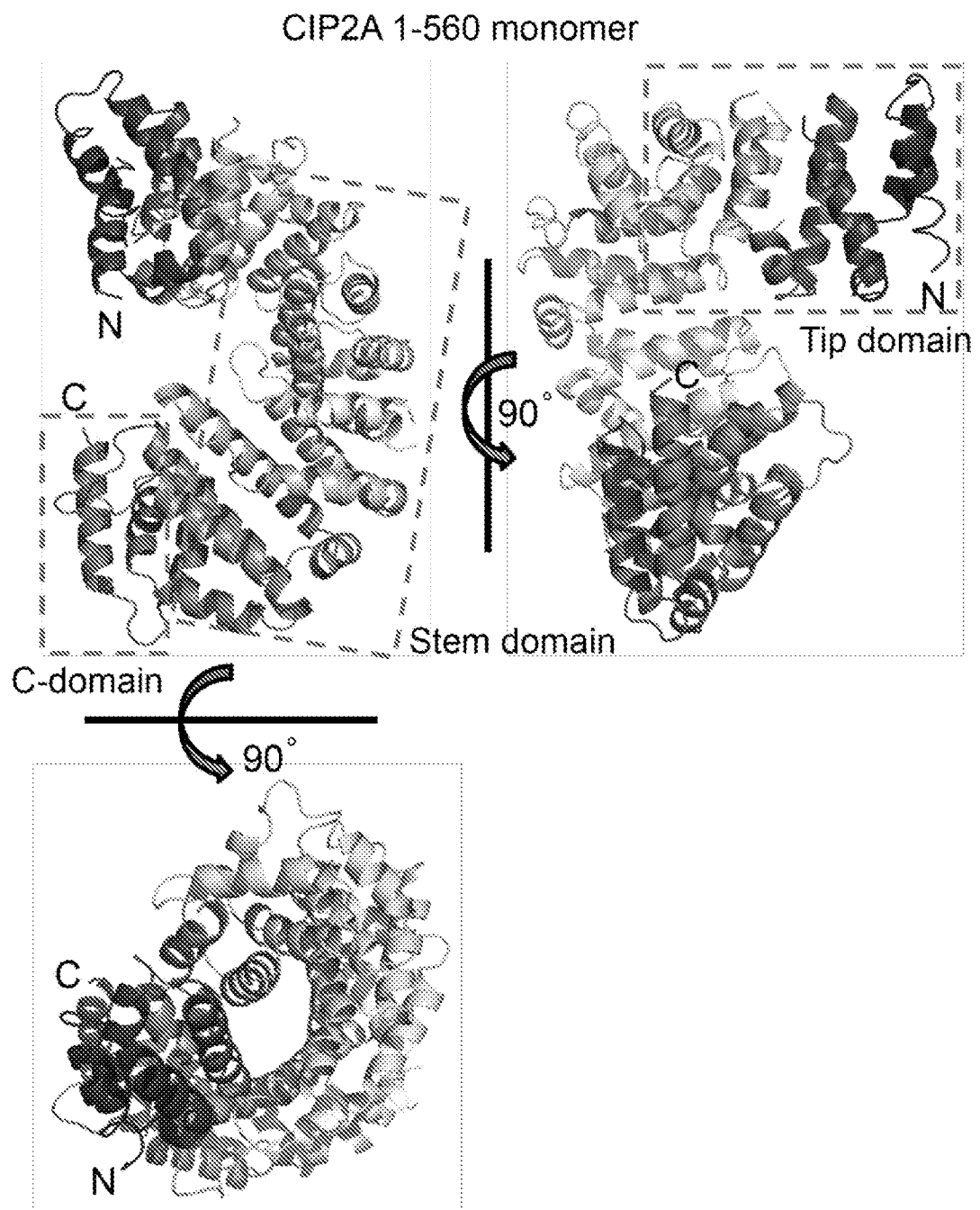


Figure 1B

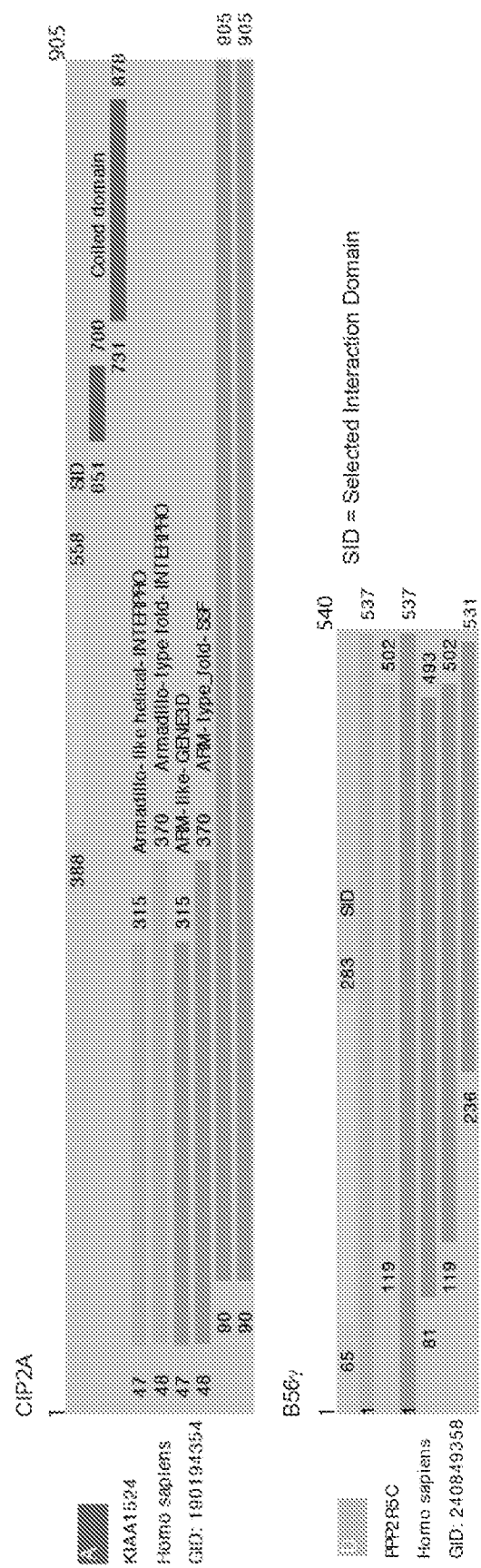


Figure 2A

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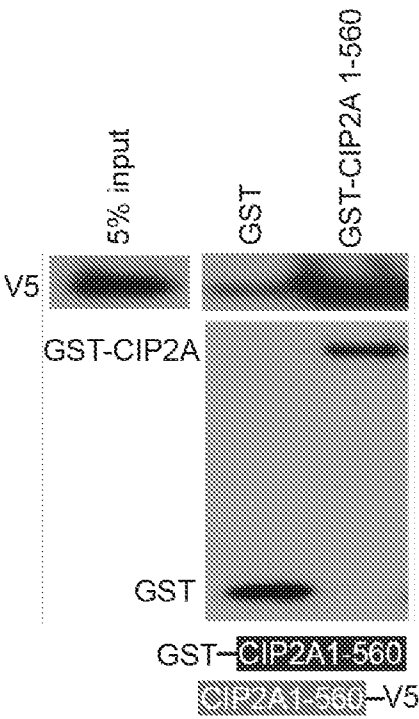


Figure 2B

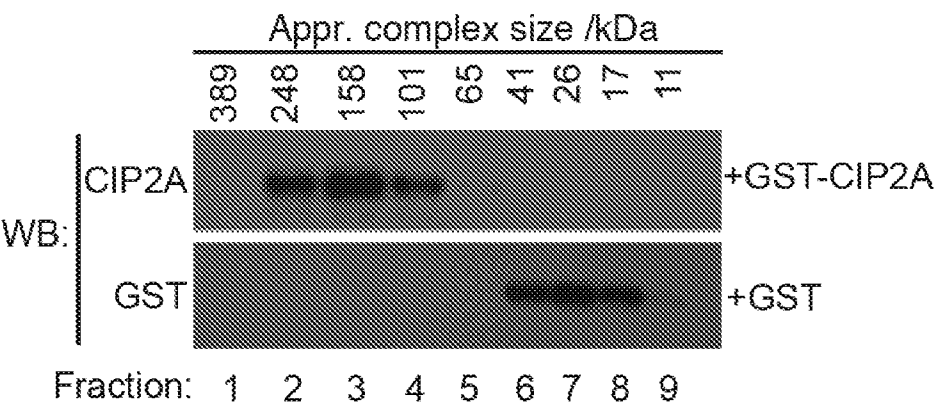


Figure 2C

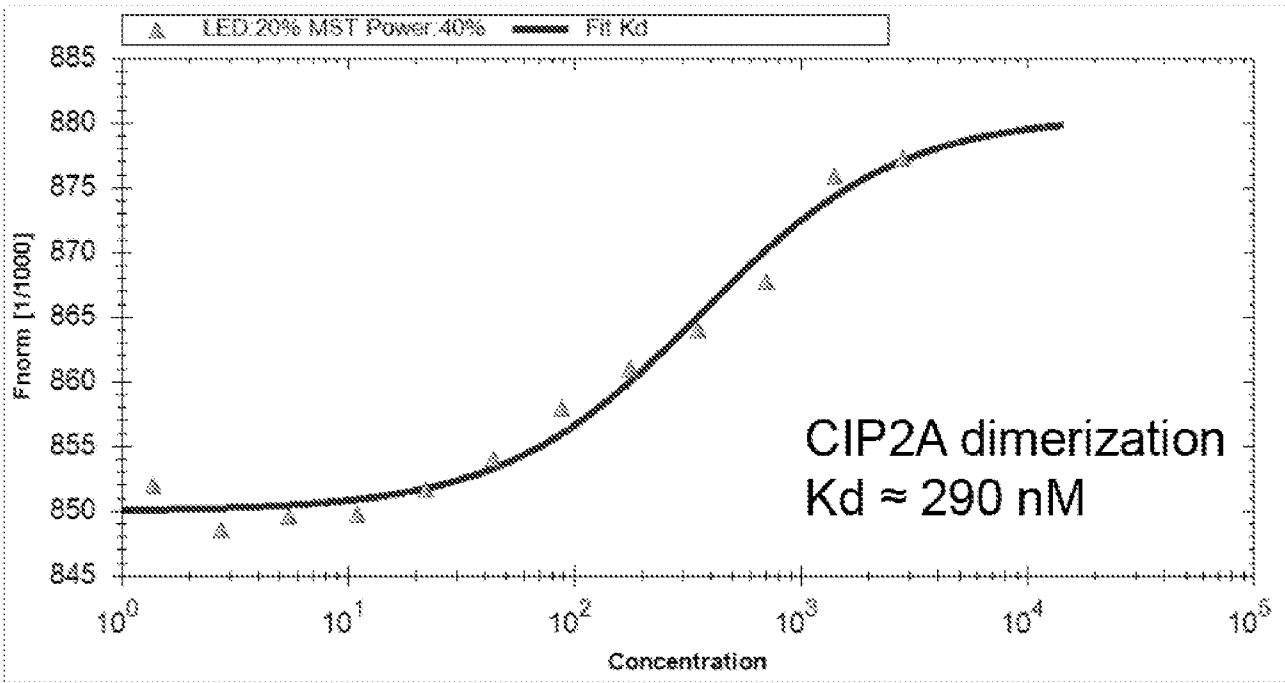


Figure 2D

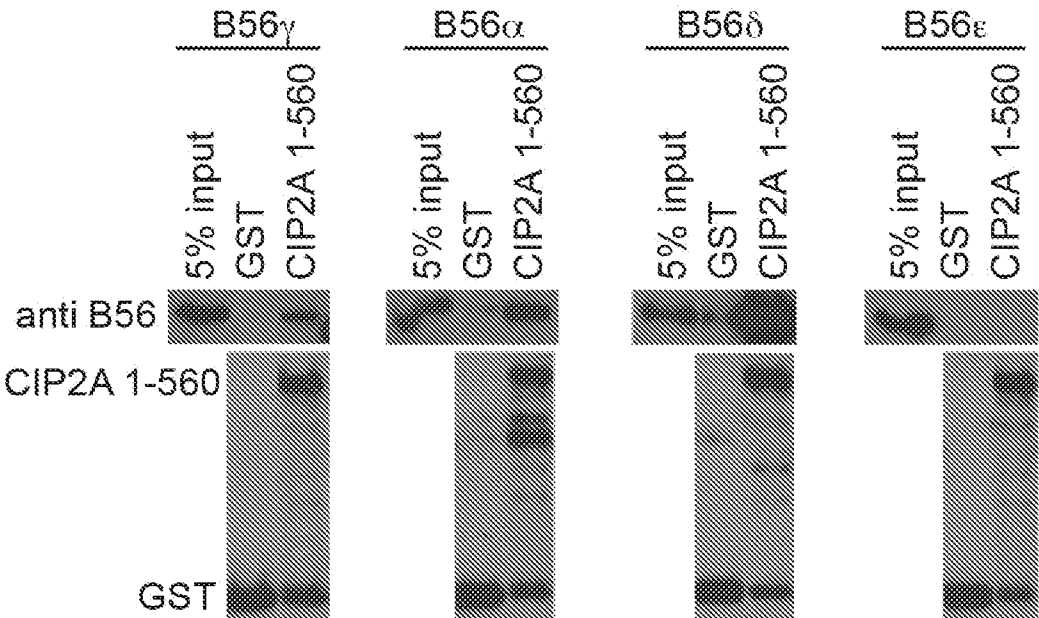


Figure 3A

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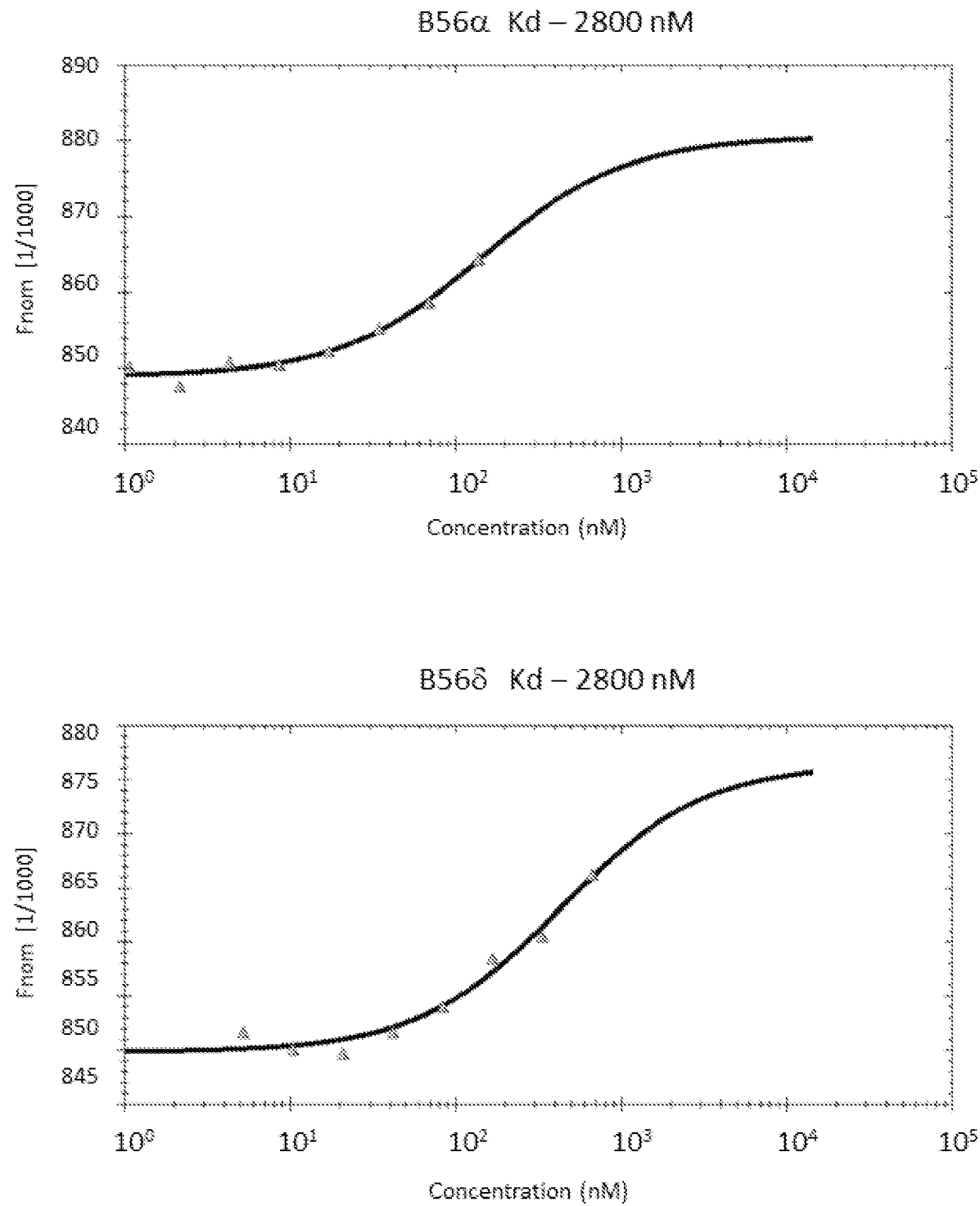


Figure 3B

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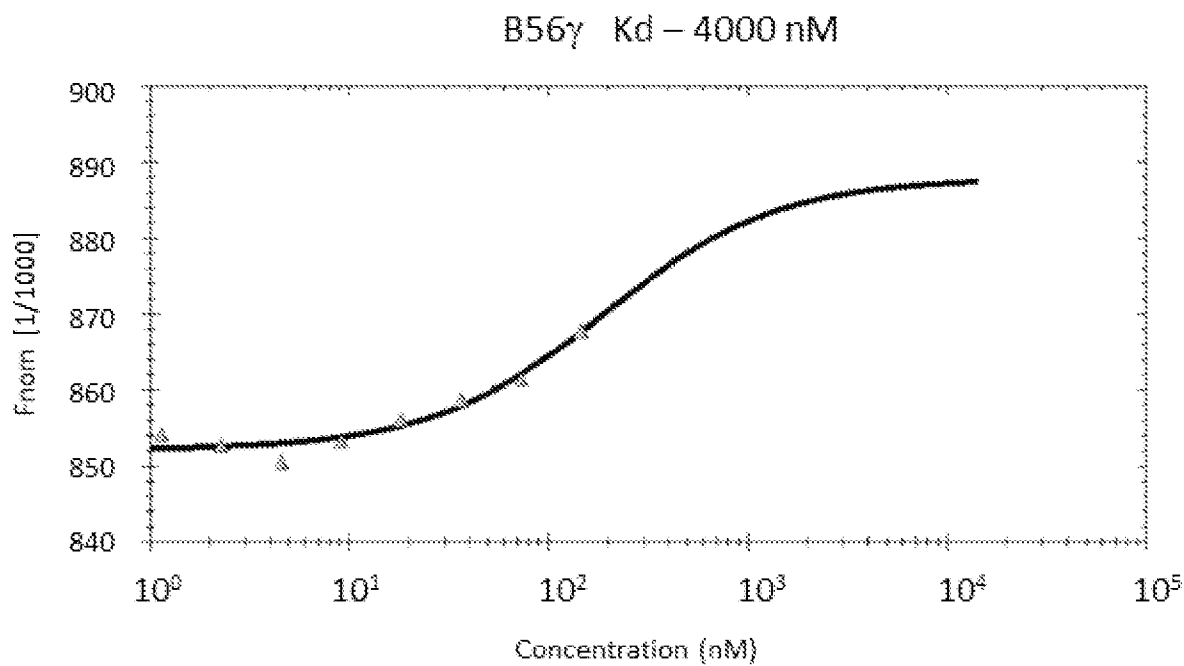
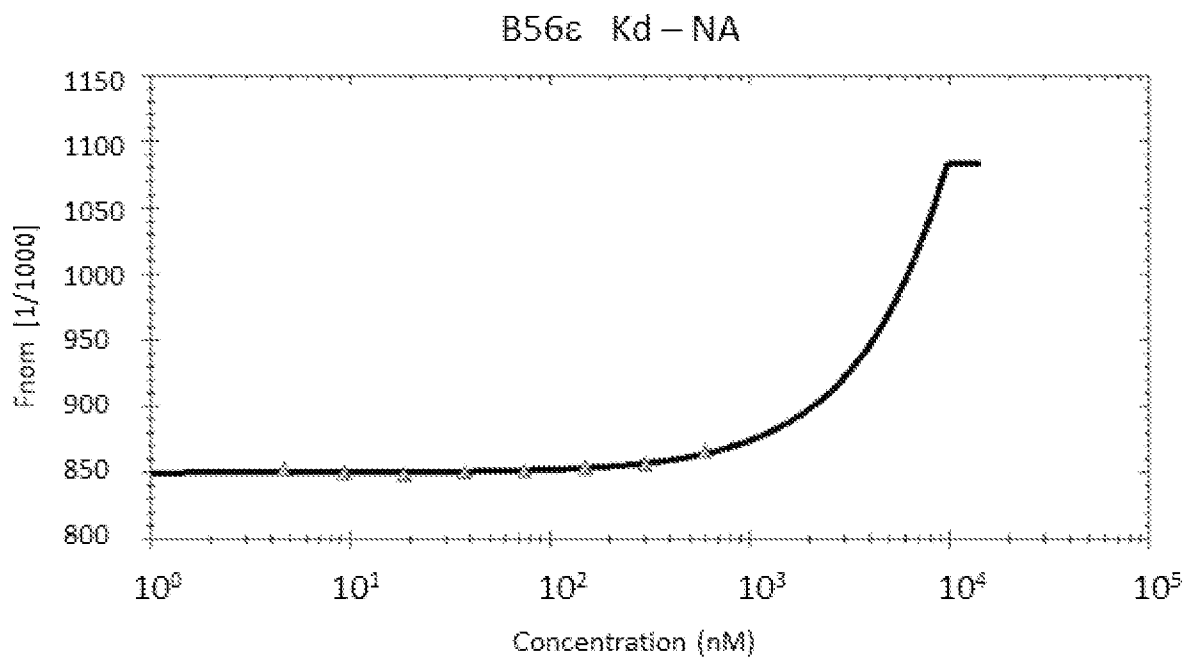


Figure 3B cont.

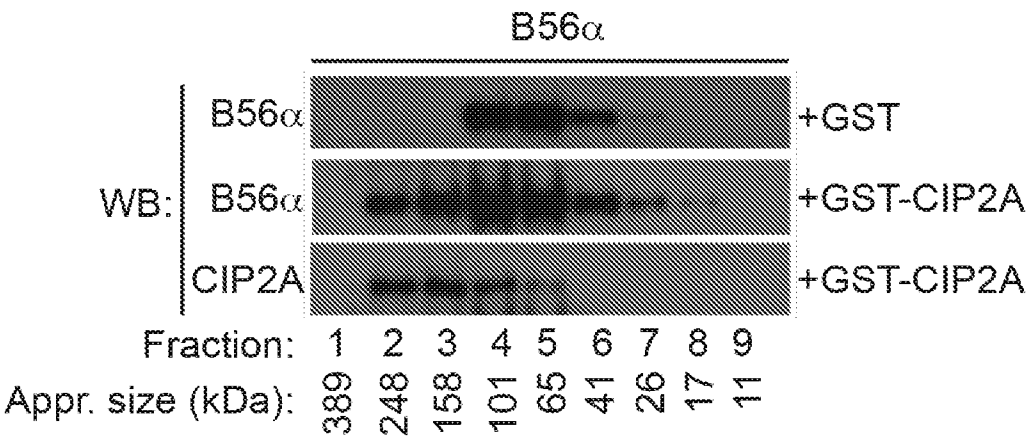


Figure 3C

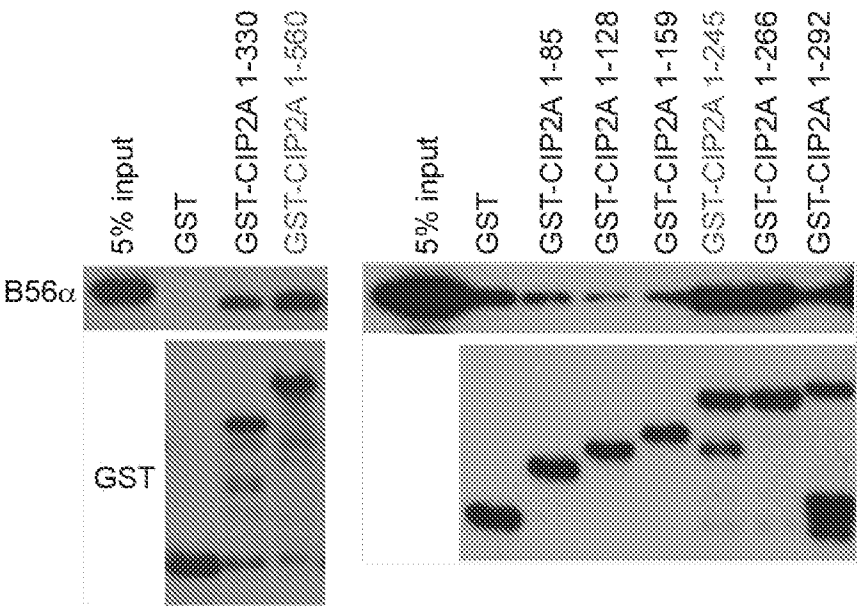


Figure 4A

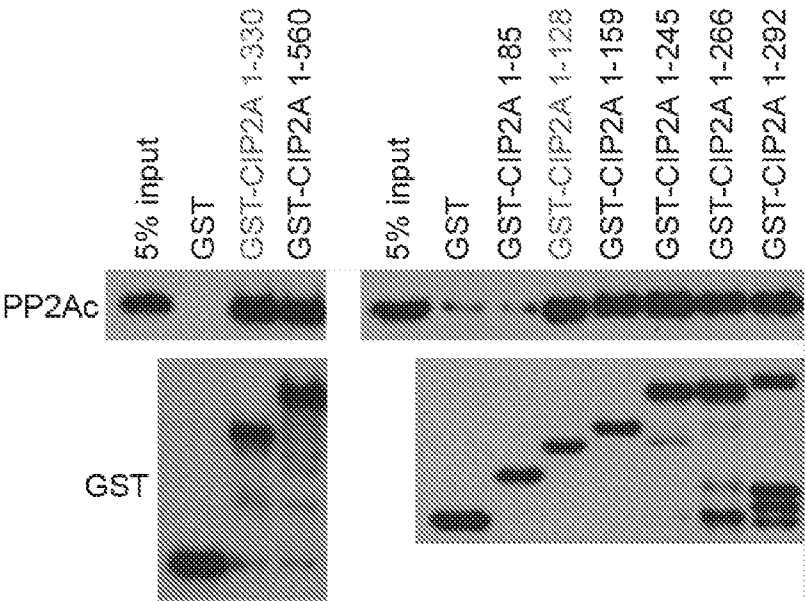


Figure 4B

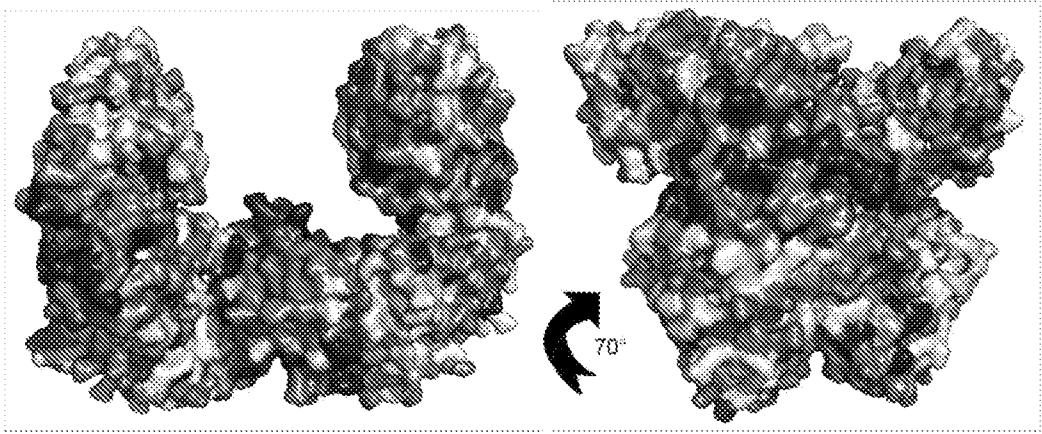


Figure 4C

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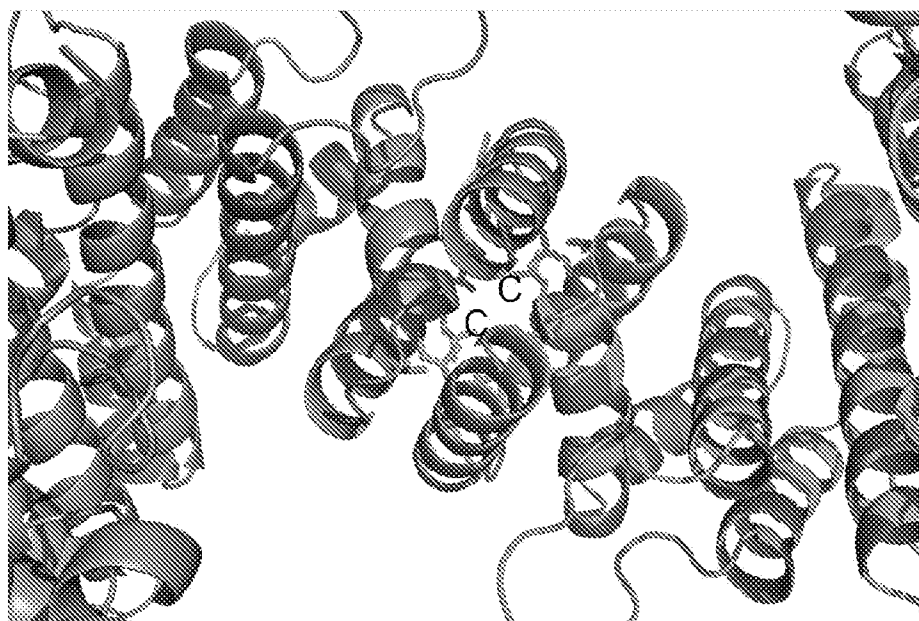


Figure 5A

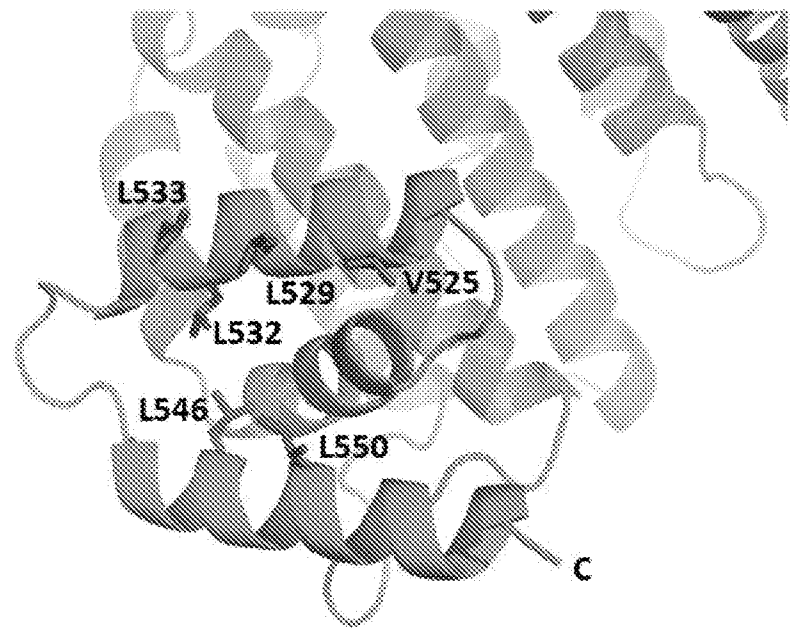


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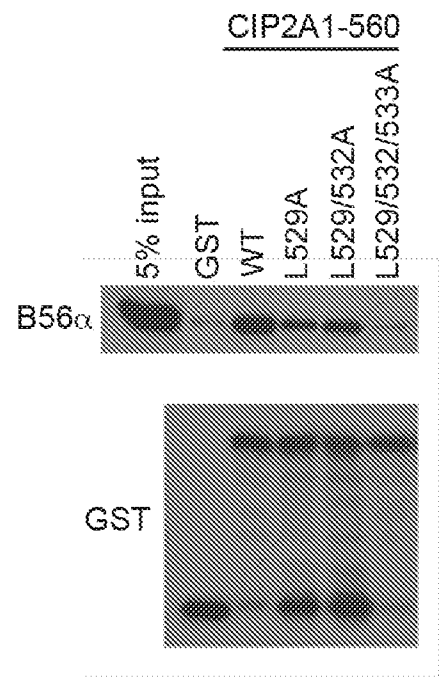


Figure 5C

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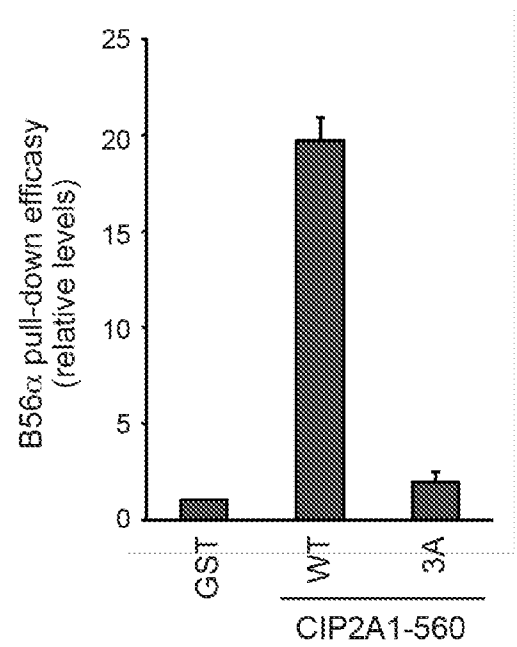


Figure 5D

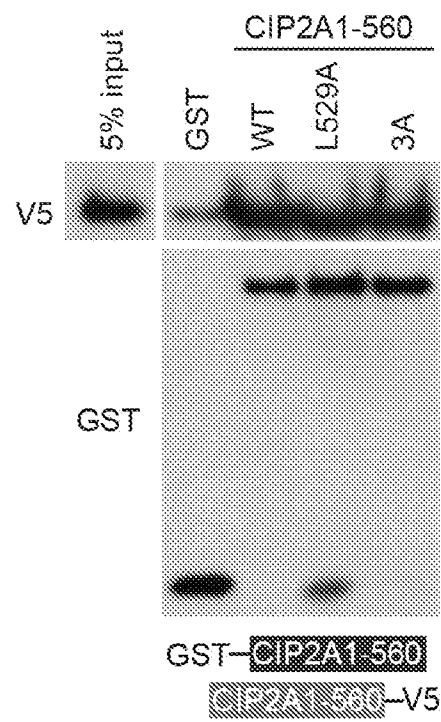


Figure 5E

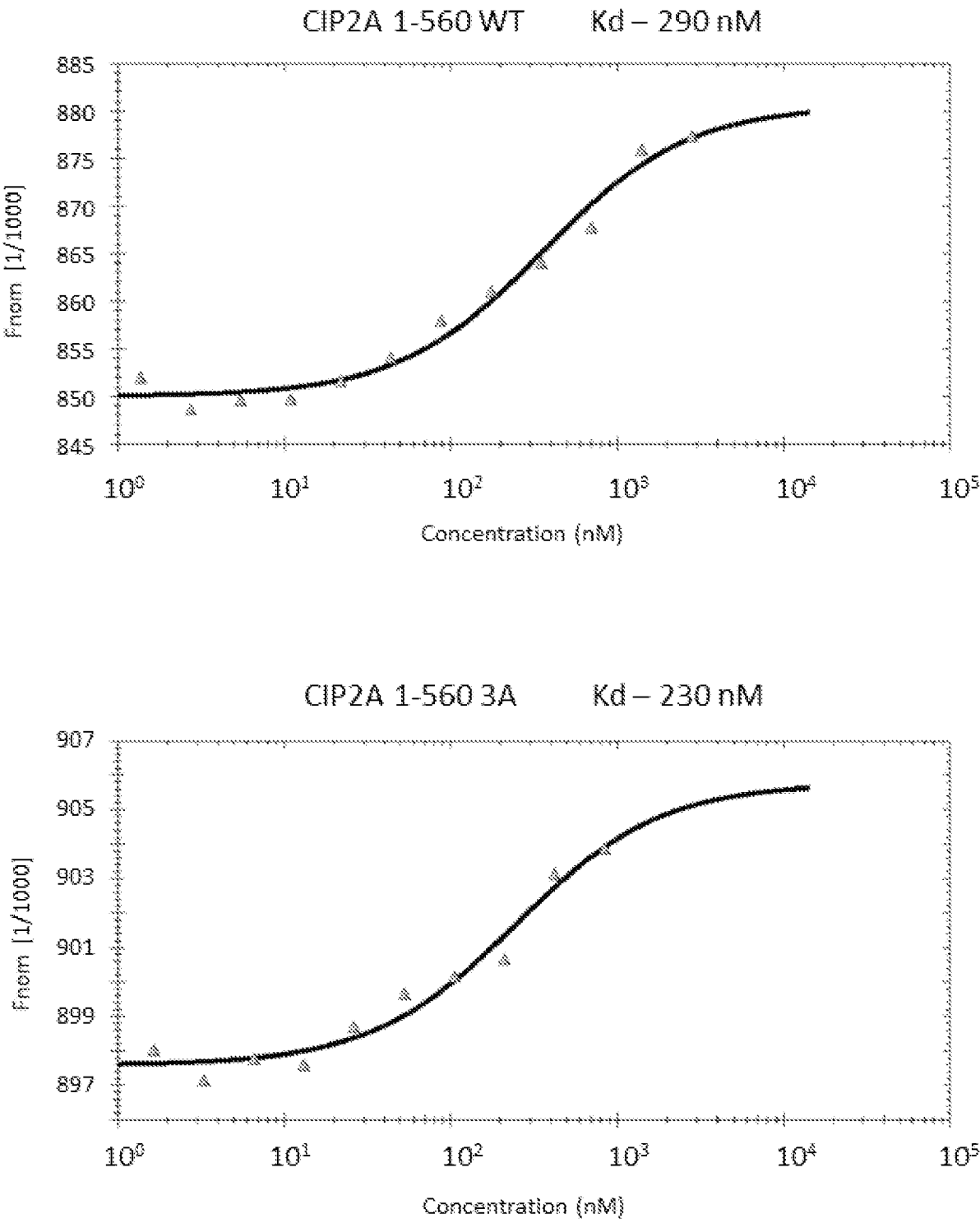


Figure 5F

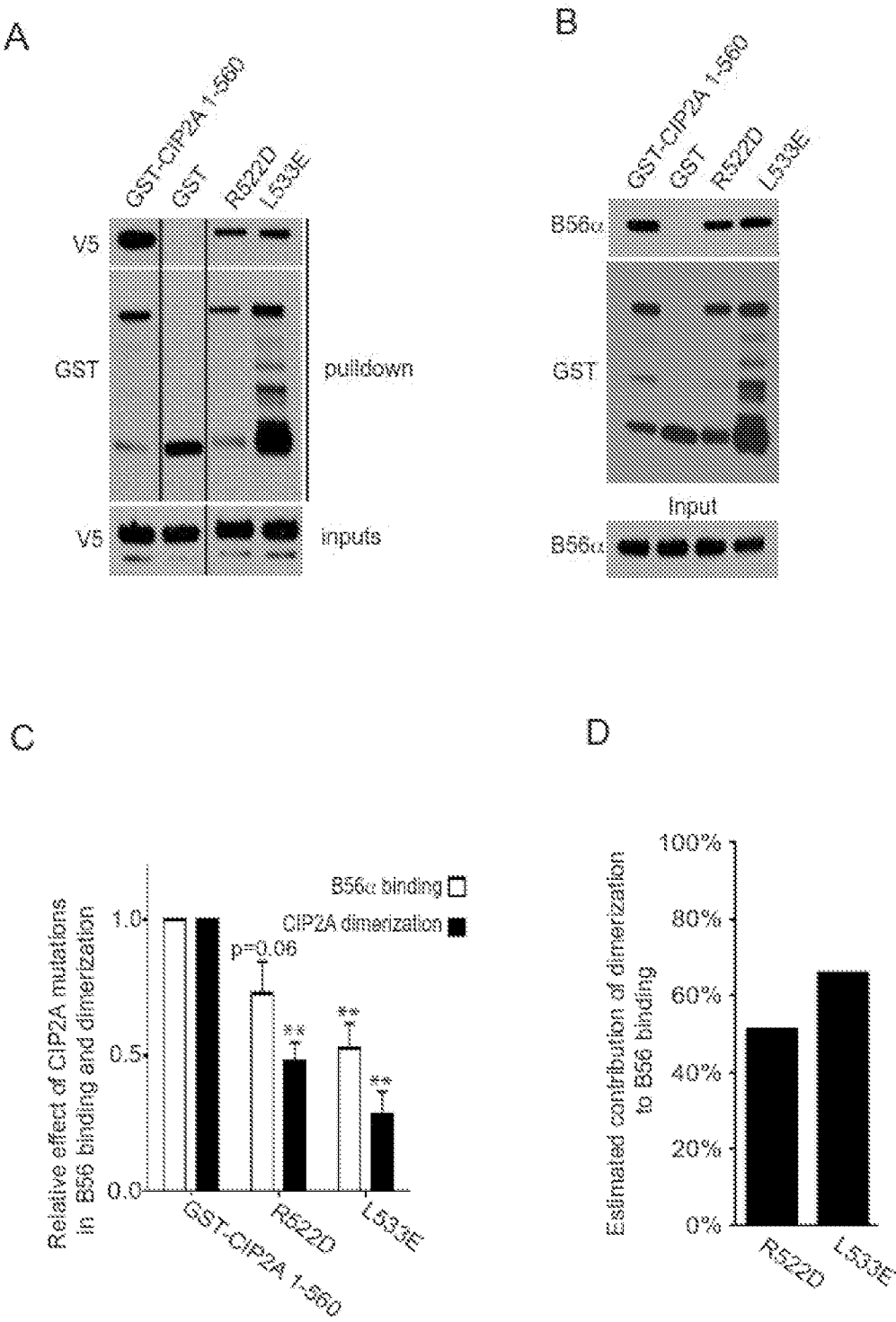


Figure 6

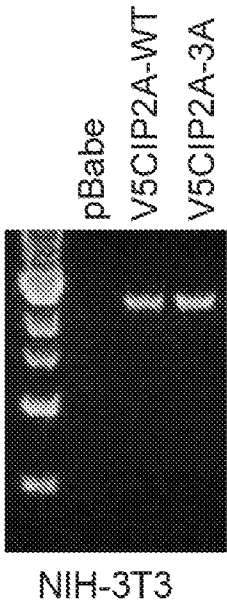


Figure 7A

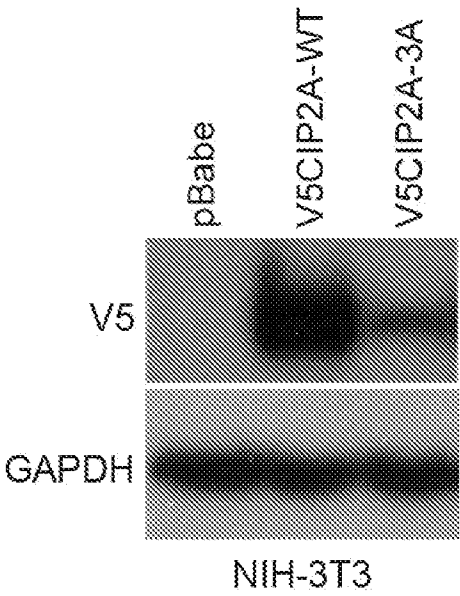


Figure 7B

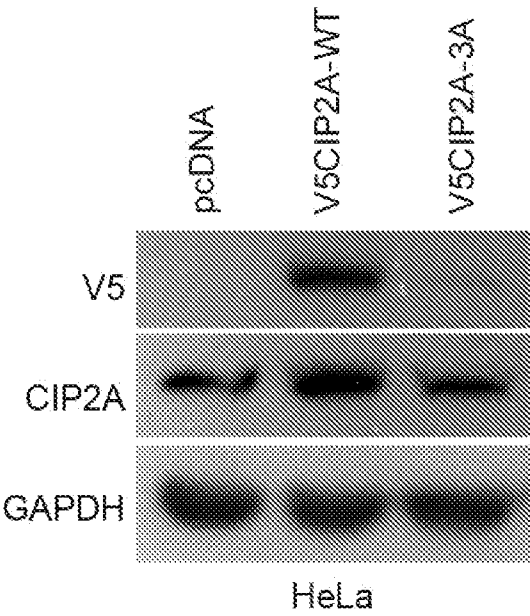


Figure 7C

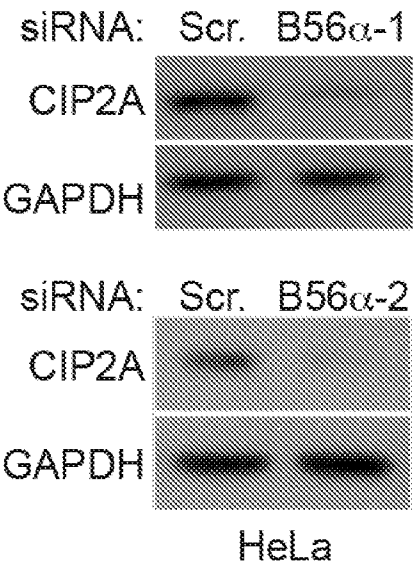


Figure 7D

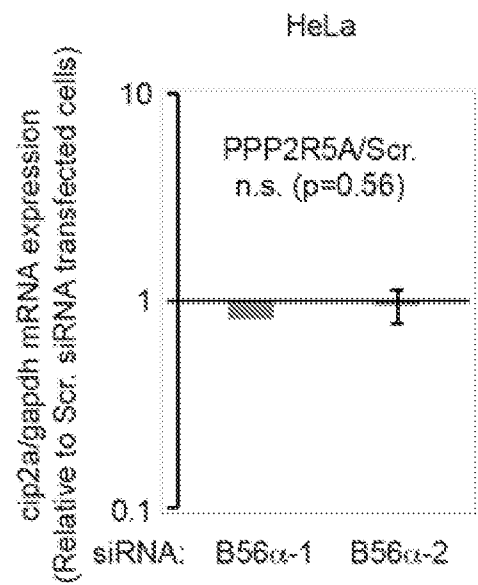


Figure 7E

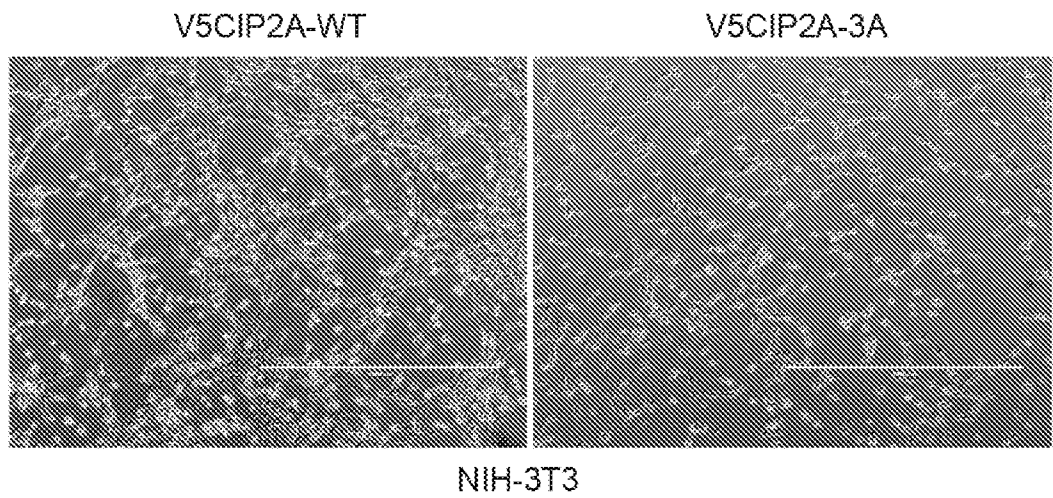


Figure 7F

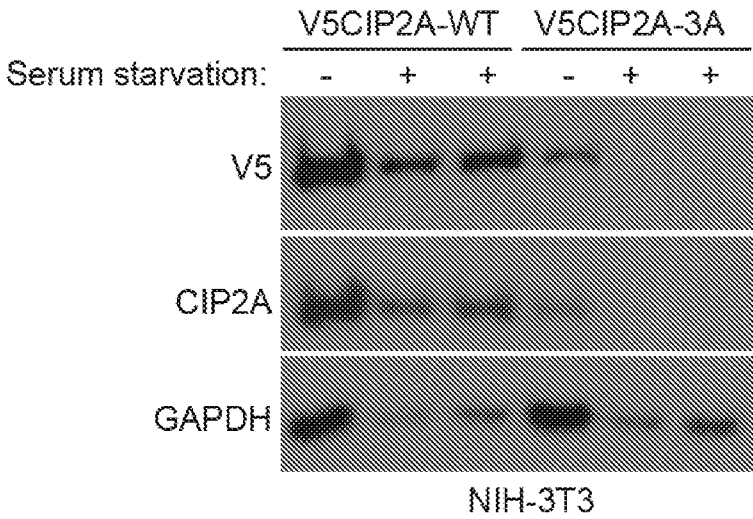


Figure 7G

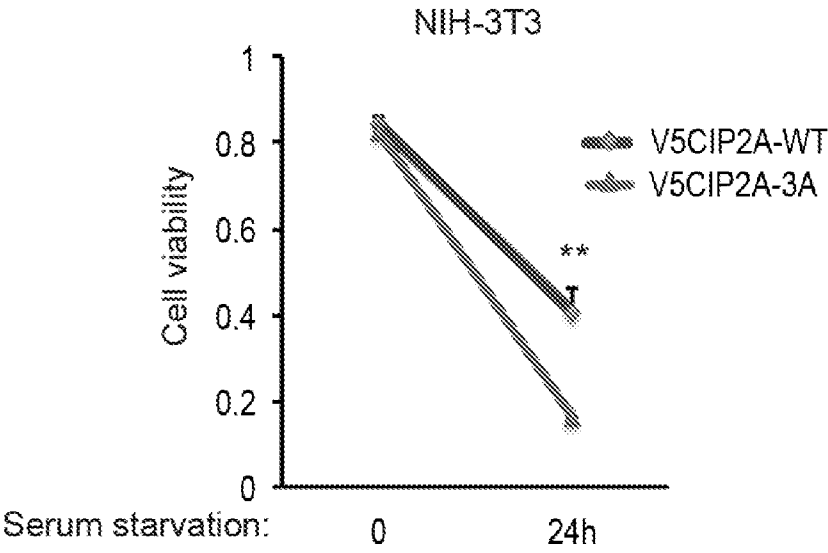


Figure 7H

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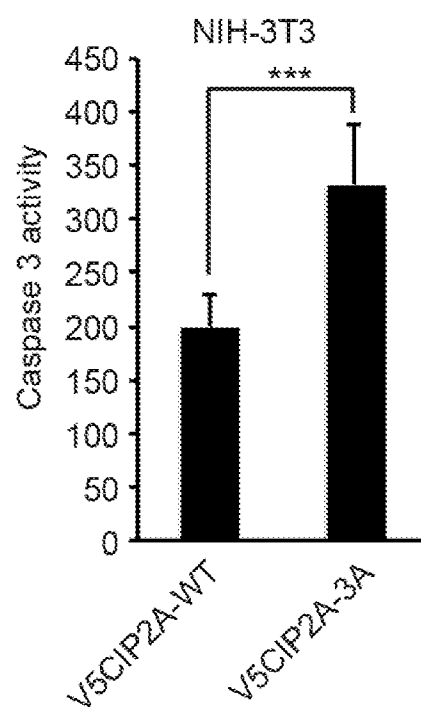


Figure 7I

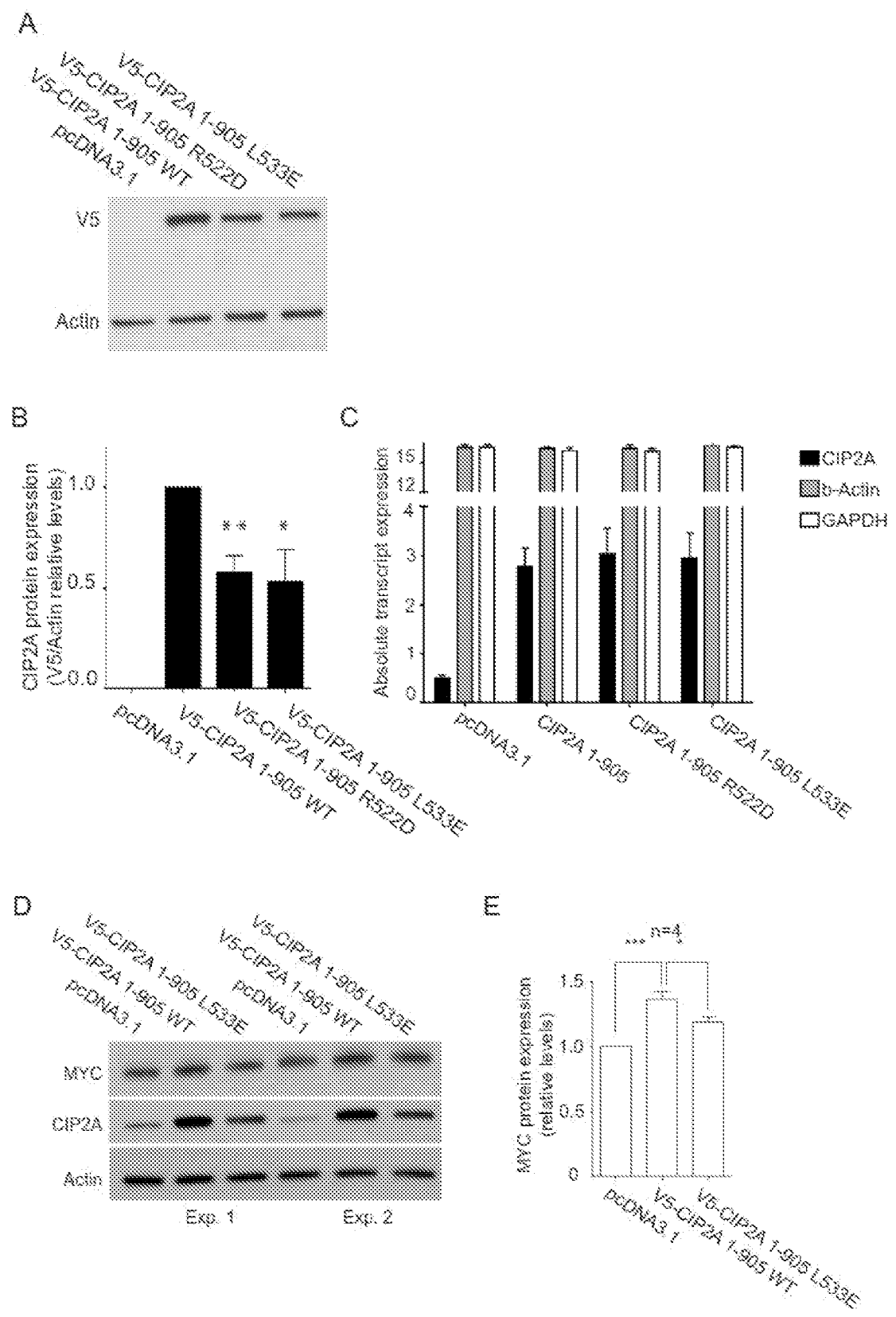


Figure 8

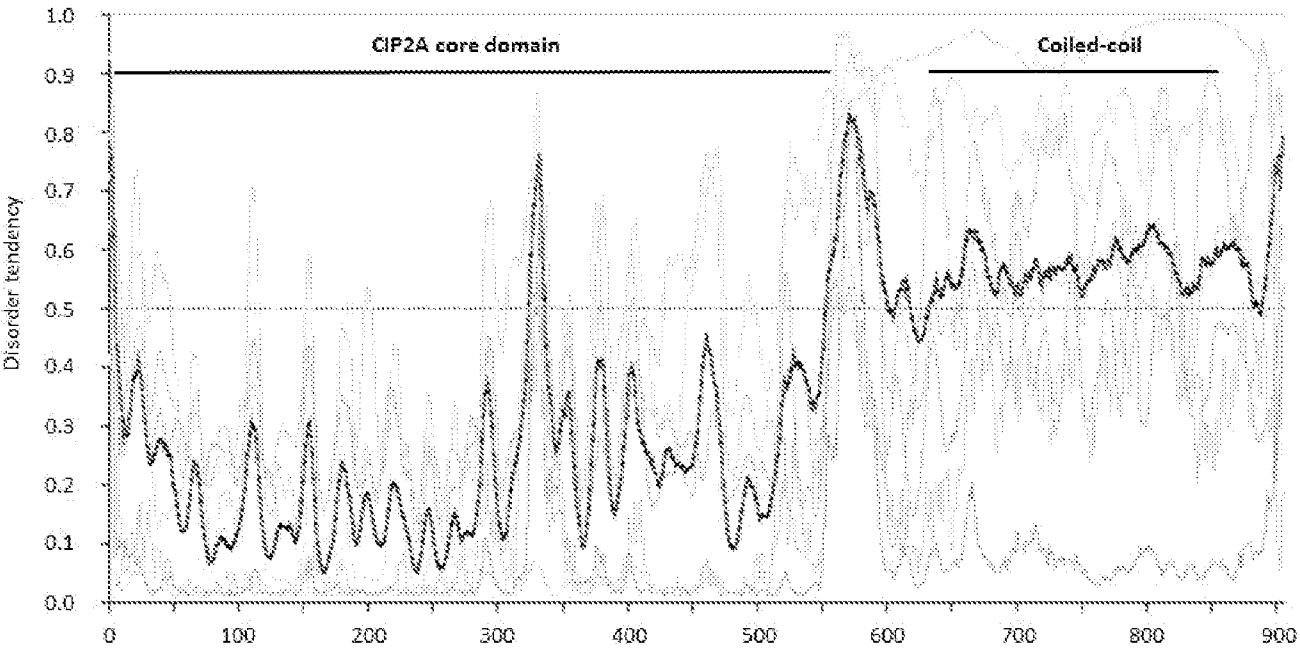


Figure 9

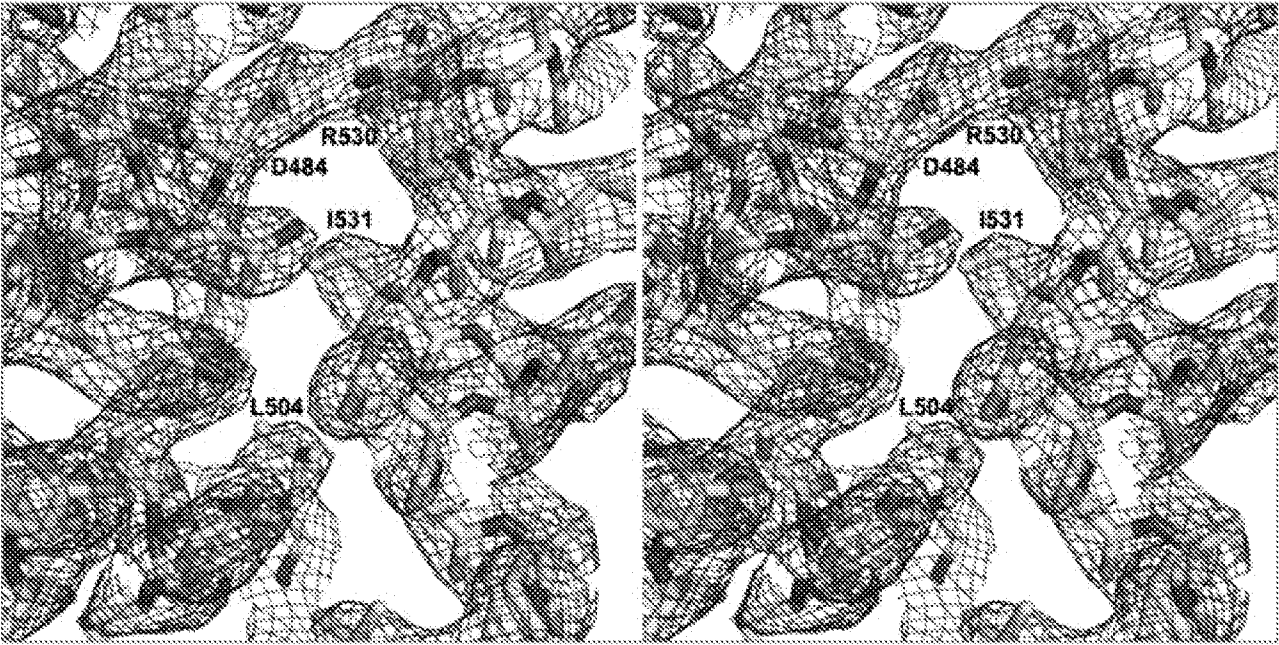


Figure 10



Figure 11A



Figure 11B

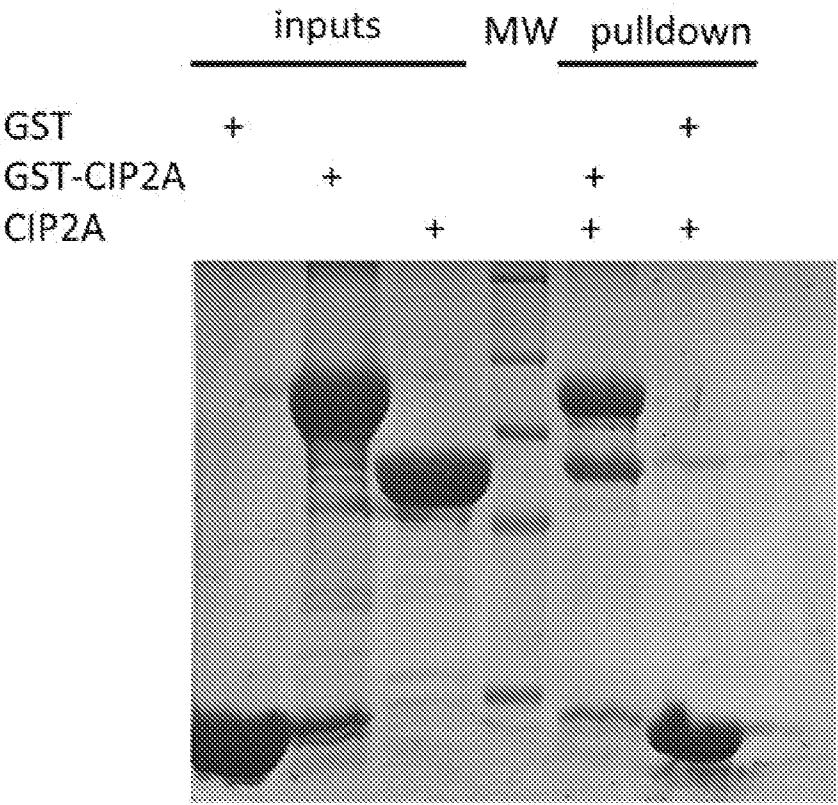


Figure 12A

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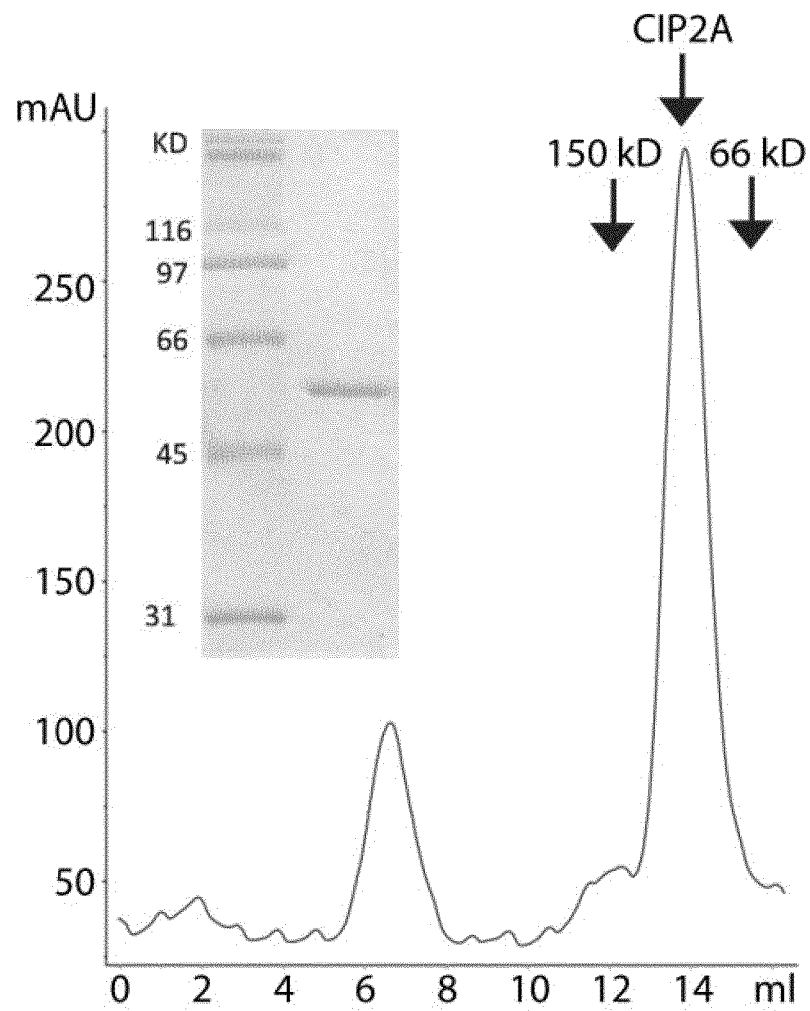


Figure 12B

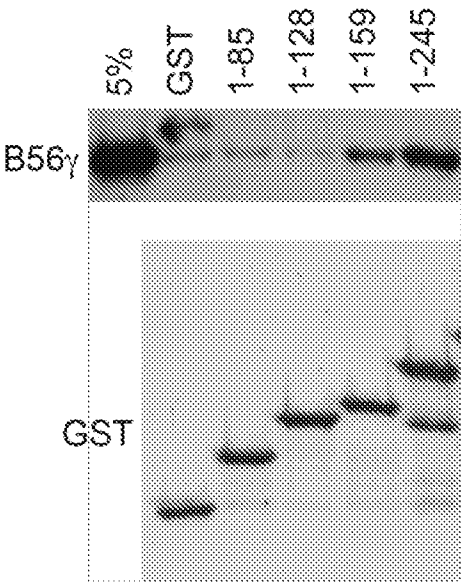
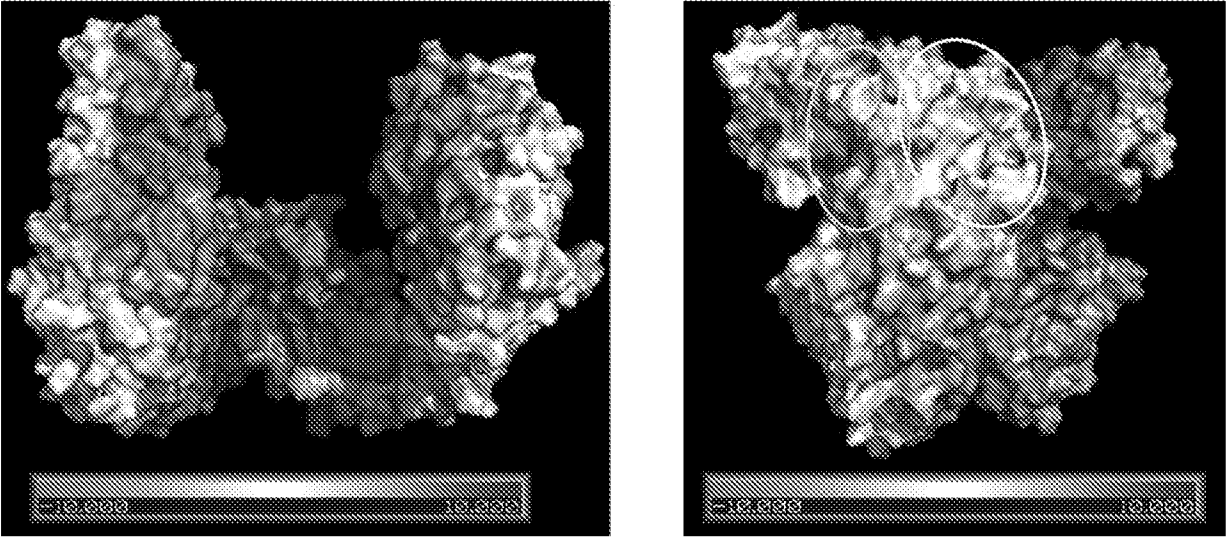


Figure 13



The "front" view

~70° rotation from the "front" view

Figure 14

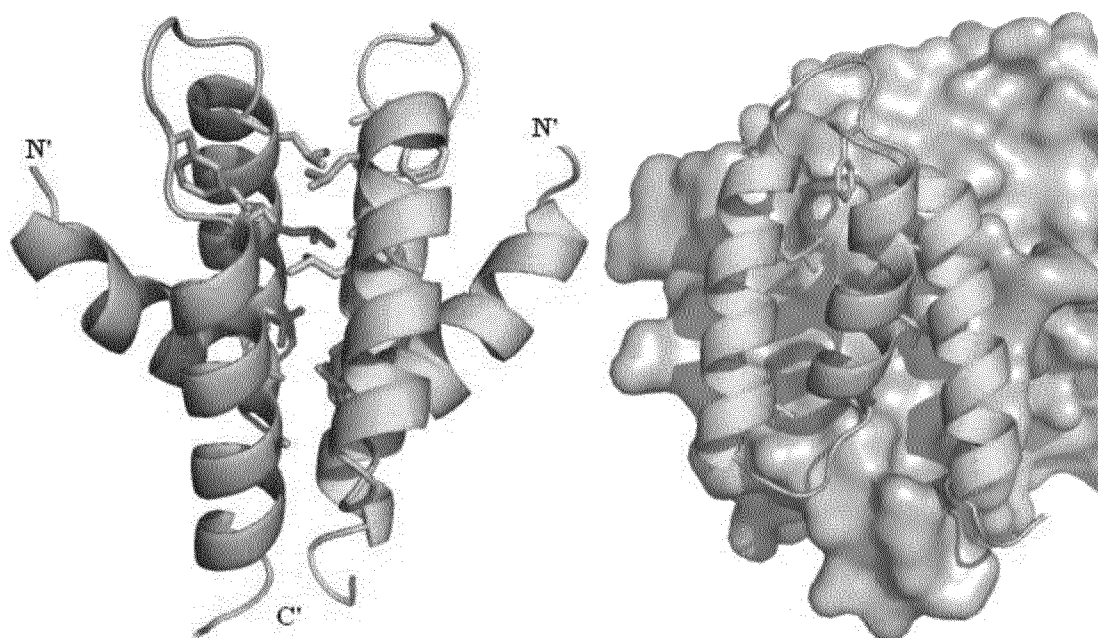


Figure 15A

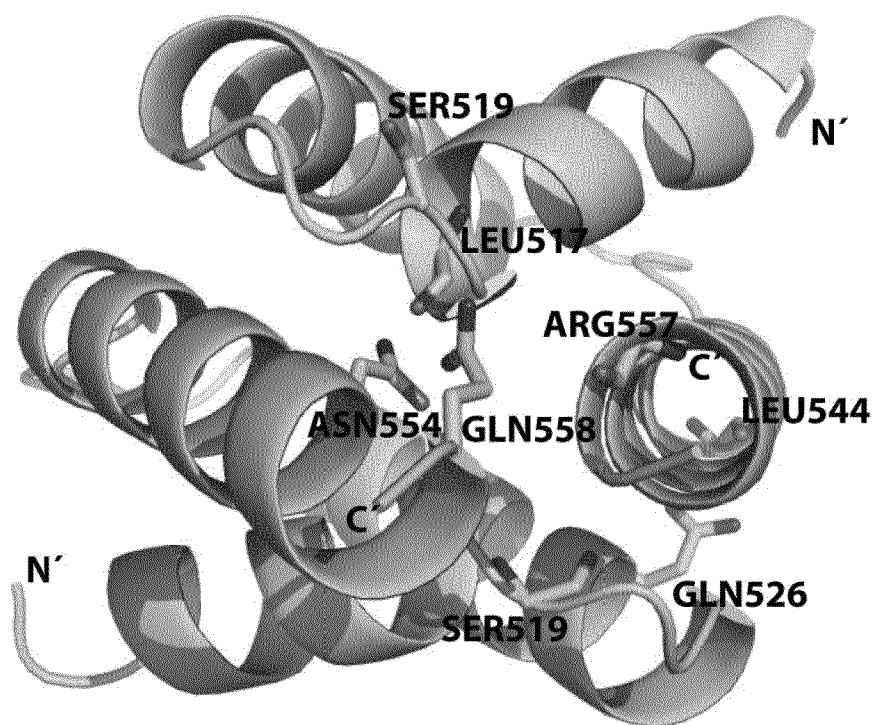


Figure 15B

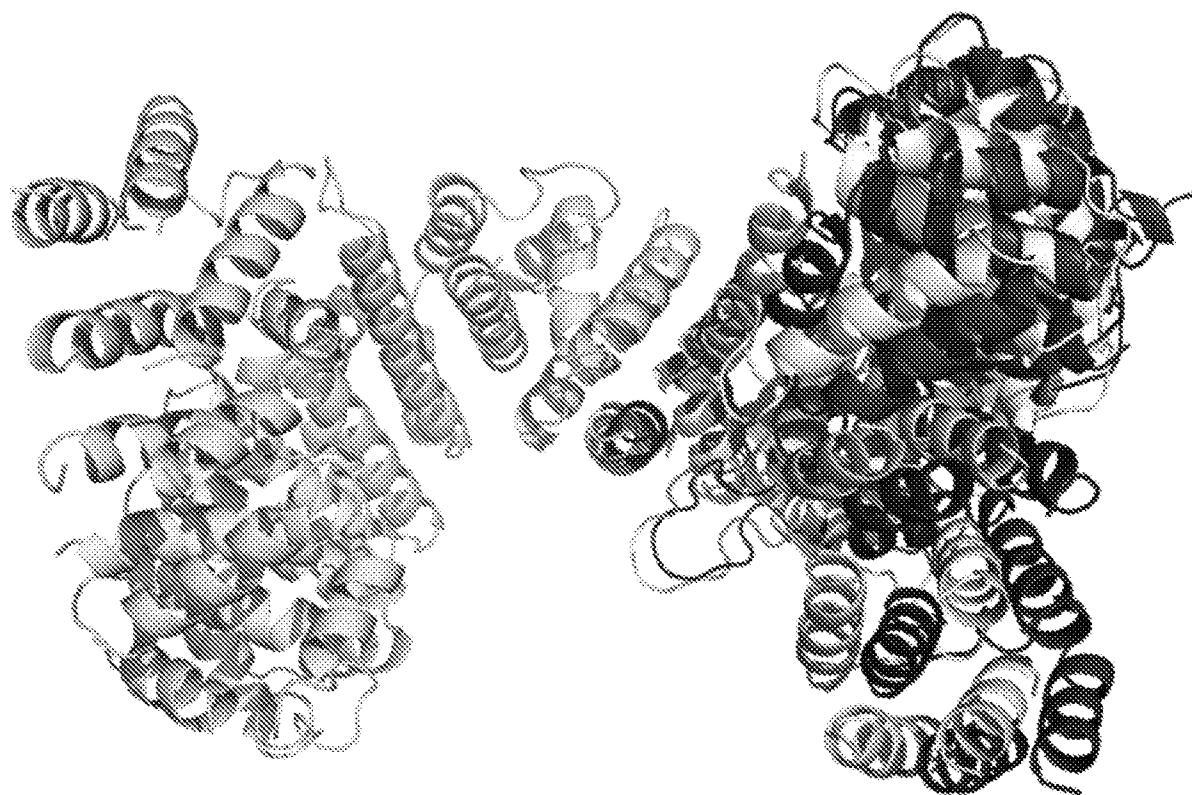


Figure 16

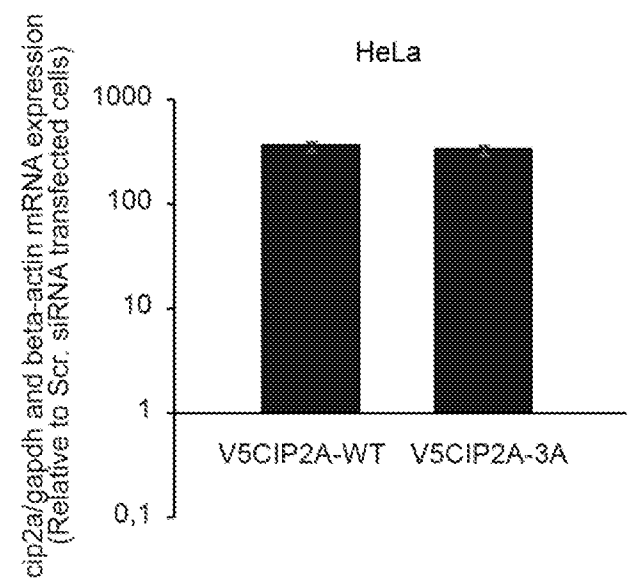


Figure 17

INTERNATIONAL SEARCH REPORT

International application No

PCT/FI2016/050445

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/82 G06F19/16
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K G06F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2009/108745 A1 (UNIV PRINCETON [US]; SHI YIGONG [US]; CHEN YU [US]; XU YANHUI [CN]) 3 September 2009 (2009-09-03) the whole document	1-12
X	WO 2007/104835 A1 (WESTERMARCK JUKKA [FI]; PUUSTINEN PIETRI [FI]; JUNTILA MELISSA [US]) 20 September 2007 (2007-09-20)	12
Y	The query sequence SEQ ID NO:1 has 99.87 % identity (100.00 % similarity) over 746 positions in a common overlap (range (q:s): 1-746:160-905) with subject GSP:ALJ27803 (length: 905) from W02007104835-A1 published on 2007-09-20.; the whole document	1-11



Further documents are listed in the continuation of Box C.



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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search

20 September 2016

Date of mailing of the international search report

04/10/2016

Name and mailing address of the ISA/

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Authorized officer

Vix, Olivier

INTERNATIONAL SEARCH REPORT

International application No

PCT/FI2016/050445

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MELISSA R. JUNTILA ET AL: "CIP2A Inhibits PP2A in Human Malignancies", CELL, vol. 130, no. 1, 1 July 2007 (2007-07-01), pages 51-62, XP055303723, US ISSN: 0092-8674, DOI: 10.1016/j.cell.2007.04.044 the whole document	1-12
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International application No

PCT/FI2016/050445

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Y	EP 2 239 577 A1 (VERENIGING VOOR CHRISTELIJK HOGER ONDERWIJS WETENSCHAPPELIJK ONDERZOEK) 13 October 2010 (2010-10-13) The query sequence SEQ ID NO:1 has 100.00 % identity (100.00 % similarity) over 746 positions in a common overlap (range (q:s): 1-746:160-905) with subject GSP:AYK69369 (length: 905) from EP2239577-A1 published on 2010-10-13.; the whole document	1-12
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INTERNATIONAL SEARCH REPORT

International application No

PCT/FI2016/050445

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Danilo Perrotti: "Review Protein phosphatase 2A: a target for anticancer therapy", The Lancet oncology, vol.14, nr. 6, 1 May 2013 (2013-05-01), pages e229-e238, XP055301035, Retrieved from the Internet: URL:http://www.sciencedirect.com/science/article/pii/S1470204512705582 [retrieved on 2016-09-08] see page e231-e233; the whole document	1-12
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X,P	KÄTHE M. DAHLSTRÖM ET AL: "3D model for Cancerous Inhibitor of Protein Phosphatase 2A armadillo domain unveils highly conserved protein-protein interaction characteristics", JOURNAL OF THEORETICAL BIOLOGY., vol. 386, 21 September 2015 (2015-09-21), pages 78-88, XP055303084, GB ISSN: 0022-5193, DOI: 10.1016/j.jtbi.2015.09.010 the whole document	1-12

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Information on patent family members

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