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# ASSOCIATION OF THE TENSIN N-TERMINAL PTP DOMAIN WITH ALPHA ISOFORM OF PROTEIN PHOSPHATASE-1 IN FOCAL ADHESIONS.

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Running Title: Tensin and PP1 in focal adhesions.

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**Focal adhesions attach cultured cells to the extracellular matrix and we found endogenous protein phosphatase-1 alpha isoform (PP1 $\alpha$ ) localized in adhesions across the entire area of adherent fibroblasts. However, in fibroblasts migrating into a scrape wound or spreading after replating PP1 $\alpha$  did not appear in adhesions near the leading edge but was recruited into other adhesions coincident in time and space with incorporation of tensin. Endogenous tensin and PP1 $\alpha$  co-precipitated from cell lysates with isoform-specific PP1 antibodies. Chemical cross-linking of focal adhesion preparations with Lomant's reagent demonstrated molecular proximity of endogenous PP1 $\alpha$  and tensin, while neither focal adhesion kinase nor vinculin were cross-linked and co-precipitated with PP1 $\alpha$ , suggesting distinct spatial sub-domains within adhesions. Transient expression of truncated tensin showed the N terminal 360 residues, which comprise a PTP domain, alone were sufficient for isoform-selective co-precipitation of co-expressed PP1 $\alpha$ . Human prostate cancer PC3 cells are deficient in tensin relative to fibroblasts and have fewer, mostly peripheral adhesions. Transient expression of GFP-tensin in these cancer cells induced formation of adhesions and recruited endogenous PP1 $\alpha$  into those adhesions. Thus, the PTP domain of tensin exhibits isoform-specific association with PP1 $\alpha$  in a restricted spatial region of adhesions that are formed during cell migration.**

Focal adhesions link force-generating elements of the actin cytoskeleton to the extracellular matrix. These adhesions are assembled initially in response to integrin engagement as cells protrude their leading edge in the process of spreading and migration (1-3). These initial adhesions contain focal adhesion kinase, paxillin, vinculin and other proteins (4,5). Phosphorylation of these proteins on Tyr residues accompanies the assembly of these adhesions and P-Tyr immunostaining is concentrated at these sites (6). Focal adhesions dynamically exchange protein components as they connect to F-actin filaments and develop contractile force for cell locomotion. Adhesions that produce actomyosin-dependent movement of fibronectin receptors have been called "fibrillar adhesions" (7) to distinguish them from adhesions near the leading edge of the cell. These adhesions are relatively enriched in tensin, a protein that has a protein Tyr phosphatase (PTP) domain related to PTEN (phosphatase and tensin1 homologue on chromosome 10), a Src homology 2 (SH2) domain and a phosphotyrosine binding (PTB) domain (8,9). The overall process of focal adhesion maturation can be followed either in cells migrating on 2D surfaces, or following replating onto matrix-coated surfaces as cells spread. It is now appreciated that focal adhesions are dynamic structures rapidly exchanging constituent proteins (10). Current work on dynamics of focal adhesions suggests that Tyr phosphorylation is a major requirement for their turnover. However, results also show Ser/Thr phosphorylation has a role in the assembly and disassembly of focal adhesions (11). Accumulating data show that focal

adhesion proteins are phosphorylated at many Ser and Thr residues (<http://www.cellmigration.org>) and understanding the control of and the functional consequences of these modifications is a challenge for future work. One step will be defining which Ser/Thr kinases and phosphatases are in focal adhesions and the basis for their localization.

Our interest in Ser/Thr phosphorylation of myosin and focal adhesion proteins led us some years ago to find protein Ser/Thr phosphatase-1 (PP1) in focal adhesions of adherent fibroblasts (12). There are three predominant PP1 isoforms, named alpha, delta (*a.k.a.* beta) and gamma-1 that are widely expressed in somatic cells, with nearly identical catalytic domains of about 300 residues, plus 20-30 C terminal residues that are distinctive between isoforms (13). These C terminal sequences have been used to prepare isoform-specific antibodies to study the cellular distribution of individual isoforms (14,15). One example of isoform specificity is the binding of the PP1delta isoform to MYPT1 and G<sub>M</sub> regulatory subunits (16,17). The PP1delta isoform also has been reported to associate directly with focal adhesion kinase (FAK), which might account for its localization in focal adhesions (18). More recent study indicates that PP1 and GSK3 react with different Ser phosphorylation sites in FAK (18). In this study we used isoform-specific antibodies to visualize the alpha isoform of PP1 in focal adhesions. During cell migration or cell spreading the PP1 $\alpha$  was not seen in focal adhesions near the leading edge, but it was in other adhesions and co-localized with tensin. We co-precipitated tensin and PP1 $\alpha$  and chemically cross-linked these proteins in preparations of focal adhesions made by detergent extraction of adherent cells. Further, the N-terminal domain of tensin, which is the domain that resembles a protein Tyr phosphatase (PTP) (8), is the basis for its relationship to PTEN protein, and is one of two regions that mediates tensin binding to adhesions (21) is shown to be sufficient for isoform-specific association with PP1 $\alpha$ . The results reveal a new spatial domain within cells where PP1 $\alpha$  is targeted, where it can potentially

alter the Ser/Thr phosphorylation of select focal adhesion proteins.

## Materials and Methods

### *Reagents*

Antibodies against vinculin, paxillin and tensin were purchased from Sigma (St. Louis, MO), BD Transduction Laboratories (San Diego, CA) and Chemicon-Upstate (Temecula, CA), respectively. Chemicals not otherwise specified were purchased from Fisher Scientific. Rabbit polyclonal antibody against a C-terminal 11-residue peptide of human PP1alpha with an N terminal Cys added was affinity purified using the SulfoLink Coupling Gel Protocol (Pierce, Rockford, IL) from serum recovered after immunization with peptide conjugated to keyhole limpet hemocyanin. Anti-PP1delta antibody was produced in parallel. The cDNAs of PP1alpha and PP1delta were cloned by RT-PCR method using cDNA libraries of human placenta and pig aorta, respectively, and confirmed by automated DNA sequencing. Amino acid sequence of pig PP1delta is identical to other mammalian PP1delta (accession No AB016735). GFP-tensin vector was characterized previously (19).

### *Cell Culture and Microscopy*

Rat embryo fibroblasts (REF52) and COS7 cells were maintained in Dulbecco's modified Eagle medium (DMEM, GibcoBRL) supplemented with 10% newborn calf serum (NCS, GibcoBRL) in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. Human prostate carcinoma cells (PC3) were grown in DMEM supplemented with 10% fetal bovine serum (FBS, GibcoBRL). Cells on coverslips were rinsed with PBS (phosphate buffered saline), fixed and permeabilized with -20°C methanol for 2 min, rinsed twice again with PBS, and incubated in a 3% BSA (bovine serum albumin) in PBS solution for one hr at room temperature. Primary antibodies diluted in 3% BSA-PBS were incubated with the specimen for at least 1 h at room temperature or overnight at 4°C. After washing for 5 min with PBS three times, the appropriate secondary antibodies (goat anti-rabbit Rhodamine or goat anti-mouse TRITC) diluted in 3% BSA in PBS were incubated with the specimen. Cover slips were then washed as

above and mounted onto glass slides with 10  $\mu$ l of Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Staining without primary antibody was done with parallel specimens side-by-side. In each case this yielded a blank image, as a negative control. Digital images of fixed cells were captured using OpenLab software (Improvision, Lexington, MA) with a Nikon Microphot-SA epifluorescence microscope equipped with a Nikon Plan Apo 60x/1.4 oil immersion objective; filter sets for FITC, Texas Red, and DAPI fluorophores; and a Hamamatsu Orca II digital camera. Raw digital images (LIF files) were converted to 8-bit TIFF files in OpenLab and further processed (contrast-enhanced, pseudo colored, and merged) using Photoshop 5.5 software (Adobe).

#### *Cell Migration and Replating*

REF52 cells were replated onto fibronectin-coated coverslips and incubated at 37 °C for 30 min, 60 min, or 5 h. Cells were fixed with methanol and stained with a mixture of rabbit polyclonal anti-PP1-alpha (green) and one of the following mouse monoclonal antibodies to known focal adhesion proteins: anti-tensin, anti-paxillin, and anti-vinculin (red). Cells were visualized and images processed as above. For 2D migration REF52 cells were grown to 100% confluence on fibronectin-coated coverslips and the monolayer “wounded” with a razor blade to remove cells from a stripe across the culture. Cells were allowed to migrate into the wound for 8 hr before fixation with methanol and double staining with rabbit anti-PP1alpha and monoclonal mouse anti-vinculin.

#### *Expression and Immunoblotting of HA-PP1alpha and delta*

COS7 cells in 35 mm dishes were transfected with 3.5  $\mu$ g of HA<sub>3</sub>-PP1alpha or HA<sub>3</sub>-PP1delta in the pRK7 vector using EscortV transfection reagent (Sigma), following manufacturer’s protocol. After a 24-h transfection, cells were lysed with 50  $\mu$ l of Laemmli sample buffer. The samples were boiled for 10 min and were subjected to 10% SDS-PAGE plus immunoblotting. Triplicate replica membranes were prepared and stained in parallel with anti-HA, anti-PP1alpha and anti-PP1delta.

#### *Immunoprecipitation and Immunoblotting.*

For immunoprecipitation a 100 mm dish of confluent rat aorta smooth muscle cells were lysed on ice for 10 min in 1.0 ml of 50 mM MOPS, pH 7.0, 0.1 M NaCl, 1 mM EGTA, 5% glycerol, 0.1 % Triton X-100, 0.3% 2-mercaptoethanol, 0.4 mM PefaBloc. The lysate was centrifuged at 20,000 x g for 20 min and the supernatant split in half; half was mixed 20  $\mu$ l of anti-PP1alpha antiserum, the other half with 20  $\mu$ l of pre-immune serum and 15  $\mu$ l of a 50% slurry of Protein A-agarose beads added to both samples. After incubation overnight at 4°C the beads were washed 4X by centrifugation with 0.15 ml of the lysis buffer, and proteins were eluted with SDS sample buffer and separated by SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes (Biorad 0.22  $\mu$ m) that were blocked with 5% non-fat milk in TBS plus 0.1 % Tween 20 (TBS-T). Primary and secondary antibodies were diluted with the blocking buffer. The immunocomplexes were detected using SuperSignal West Pico Luminol/Enhancer Solution (Pierce, Rockford, IL) and X-ray film (Kodak X-omat).

#### *Cell Permeabilization, Cross-linking and Immunoprecipitation of PP1 Complexes*

REF52 cells were grown to between 75-95% confluency in 10 cm dishes, rinsed with PBS and permeabilized with 0.1% Triton X-100 and 5 mM MgCl<sub>2</sub> in PBS for 0.5 min at room temperature. The chemical cross linker Lomant’s reagent, dithiobis[succinimidylpropionate] (DSP, Pierce Chem. Co.) dissolved in dimethyl sulfoxide (DMSO) was combined with PBS to a final concentration of 4  $\mu$ g/ml and incubated with the cytoskeletons for 30 min at room temperature. Quenching of the reaction used 50 mM NH<sub>4</sub>Cl in PBS for 10 min at room temperature. Samples were scraped from the dish in RIPA Buffer (50 mM MOPS, pH 7.5, 1 % Igepal CA-630 (a.k.a. NP-40), 0.25% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 mM beta-glycerol phosphate and 1 mM PefaBloc). After sonication for 30 sec the suspension was centrifuged for 10 min at 13,800 rpm at 4 °C. The pellet was resuspended in RIPA Buffer and aliquots taken from both pellet and supernatant

(RIPA Insoluble and RIPA Extract). The RIPA extract was divided into two aliquots. Rabbit pre-immune or rabbit anti-PP1alpha serum coupled to protein A resin (Sigma P-3476) was incubated with the extract overnight at 4 °C. The resin was pelleted by centrifugation at 8,000 rpm for 2 min and the supernatant taken as sample for immunoblot analysis. Resin was washed 3x with RIPA Buffer then 2X SDS sample buffer was added to the resin. Samples were boiled for 10 min and subjected to 4-15% gradient SDS-PAGE using precast gels (BioRad), followed by immunoblotting.

*Tensin and PP1 Co-expression and Pull Down*  
Either COS7 or HEK293 cells were seeded at 30% confluence and plated overnight, then transfected using calcium phosphate with 5 µg of pTriEx4 plasmid (Novagen) encoding tensin plus 0.5 µg of pKR7 plasmid encoding HA<sub>3</sub>-PP1alpha. After 24-48 hr cells were washed with PBS and lysed in buffer containing 50 mM MOPS, pH 7.0, 0.1 M NaCl, 1 mM EGTA, 5% glycerol, 0.1% Tween 20, 0.1% 2-mercaptoethanol and 0.8 mM Pefabloc. An extract was recovered as the supernatant after 15 min centrifugation at 13,800 rpm and a sample reserved before adsorption to S-protein beads (Novagen) for 1 hr. The beads were washed three times by centrifugation with the lysis buffer and the proteins eluted with SDS sample buffer and boiling. Proteins were resolved by SDS-PAGE using a double-layered gel that was 10% in the bottom half and 6% in the top half, to afford resolution of both the tensin proteins and the PP1. Proteins were transferred onto nitrocellulose submerged in transfer buffer by overnight electrophoresis at 50V and 120 mA. Filters were blocked with 5% non-fat milk and immunoblotted with HRP-conjugated S-probe (Novagen) at 1:10,000 or anti-HA at 1:10,000 followed by anti-mouse-HRP at 1:10,000 dilution and developed as described above.

## RESULTS

### *Dynamic Recruitment of Protein Phosphatase-1 (PP1) into Focal Adhesions.*

Rat embryo fibroblasts (REF52 cells) grown on fibronectin-coated coverslips develop focal adhesions across the entire bottom of the cell and these adhesions contain both vinculin and PP1alpha, based on immunofluorescent staining after fixation (Figure 1A, B). By comparison REF52 fibroblasts showed localization of the PP1delta isoform primarily along actin stress fibers (Figure 1C, D). The specificity of these anti-PP1alpha and anti-PP1delta antibodies was demonstrated by selective immunostaining of the respective epitope-tagged PP1 isoforms expressed in COS7 cells (Figure 1E), using anti-HA staining (top panel) to show that equivalent amounts of the PP1 proteins were in each lane. The results demonstrate differential localization of these PP1 isoforms in fibroblasts and isoform-specific targeting of PP1alpha to focal adhesions. Focal adhesions are dynamic structures that undergo reorganization in migrating cells. Polarized migration of cells occurs when confluent monolayers are “wounded” to scrape away cells. Migrating REF52 cells at the edge of the wound formed protrusions with prominent adhesions behind the leading edge that stained for vinculin (Figure 2, arrows in bottom panel) but not PP1alpha (compare Figure 2, upper panel). PP1alpha was localized in focal adhesions in the same cell, but only in those adhesions further away from the front of the cell nearer to the center of the cell (arrows in top panel, Fig. 2). Vinculin served as a marker for focal adhesions across the entire area of the cell, whether or not the adhesions stained for PP1alpha (Fig. 2, merged image). Because PP1alpha was not detected in adhesions near the leading edge but in other adhesions in the same cell we suspected it was being dynamically recruited into adhesions during cell migration.

To test this hypothesis REF52 cells were taken into suspension and replated onto fibronectin-coated coverslips. At various times cells were fixed and focal adhesions were visualized by immunofluorescent staining of endogenous proteins (Figure 3). The three larger images are merged from the smaller

images at upper left of cells stained for PP1alpha (green) and the images at upper right for vinculin, P-Tyr and tensin (red/orange, top to bottom). Adhesions around the perimeter of the cells nearest to the leading edge contained vinculin and P-Tyr proteins, but did not stain for PP1alpha, as seen in the outermost red/orange adhesions in the merged images (Figure 3A, B). In these same cells other adhesions localized nearer the center of the cells were double immunostained for vinculin and PP1alpha (Figure 3A), or separately for P-Tyr and PP1alpha (Figure 3B). These double positive adhesions appear yellowish-green in the merged images. Thus, in both cells migrating into a wound (Fig. 2, above) and cells spreading after replating (Fig. 3) there were two populations of adhesions: peripheral adhesions without PP1alpha and other, more central focal adhesions that contained PP1alpha. This differential distribution of PP1alpha relative to vinculin or P-Tyr (or paxillin, data not shown) was a transient phenomenon only seen while cells were actively spreading. At later times after cells were spread and stationary ( $t > 4$  h) all the focal adhesions were double stained for both vinculin and PP1alpha, as seen in Figure 1A,B.

Tensin is a protein previously localized in a particular subset of focal adhesions called “fibrillar adhesions” that develop from peripheral adhesions in the course of cell migration (7). Prompted by the differential PP1alpha localization in adhesions of migrating and spreading cells we immunostained for endogenous tensin and found co-localization with PP1alpha in spreading, migrating and static cells (Fig. 3C). There was full overlap of staining for PP1alpha and tensin, even as early as 30 min after replating. Furthermore, throughout the process of cell spreading, in specimens fixed and stained at various times from 30 min to 4 hr, we observed spatial coincidence of tensin and PP1alpha in adhesions. Similar observations were made during spreading of rat aorta smooth muscle cells after replating (not shown), so the phenomenon was not unique to REF52 fibroblasts. We found tensin and PP1alpha are recruited together at the same time into the same sites during dynamic remodeling of focal adhesions in migrating cells.

#### *Spatial Proximity of Tensin and PP1alpha in Focal Adhesions.*

Because of the limited spatial resolution of light microscopy it is not possible to determine whether co-localization of endogenous tensin and PP1alpha by immunofluorescent staining is due to interaction between the proteins. Therefore, we immunoprecipitated PP1alpha from extracts of rat smooth muscle cells and by immunoblotting found co-precipitation of a fraction of tensin from the lysate (Figure 4). The co-immunoprecipitation of tensin was specific for PP1alpha antiserum relative to pre-immune serum, as a negative control. As an additional control for specificity we immunoprecipitated PP1delta from the same extracts and did not recover any detectable tensin (not shown). The results indicated that endogenous PP1alpha and tensin in fibroblasts were together in a complex that was stable enough to be immunoprecipitated.

For additional evidence of endogenous tensin-PP1alpha association we optimized conditions to permeabilize REF52 cells without dissociating all the PP1alpha from focal adhesions, using minimal time and low concentration of Triton X-100 detergent (Figure 5A). Focal adhesions are complex, relatively insoluble protein assemblies, like the cytoskeleton itself, so one faces the experimental challenge of solubilizing these assemblies for analysis while attempting to preserve protein-protein interactions between specific constituents. We resorted to chemical cross-linking of the focal adhesion preparations to identify near-neighbor interactions. Different reaction times and a range of conditions were tested to establish the highest concentration of Lomant’s reagent, dithiobis[succinimidyl]propionate] (DSP) that could be used without diminishing immunostaining of the PP1-alpha in the preparations. Using this optimized protocol we solubilized the PP1alpha complexes from DSP cross-linked preparations with a buffer containing both dodecylsulfate and Triton X-100 and then immunoprecipitated with anti-PP1alpha antibodies. Cross-links between PP1alpha and its neighbors were cleaved by chemical reduction of the central disulfide bridge in the

reagent and the separate proteins were resolved by SDS-PAGE. The amount of PP1alpha recovered at each individual step of the protocol was assayed by immunoblotting (Fig. 5B). These results showed that essentially all the PP1alpha complexes in the preparations were solubilized (lane 1, extract vs. lane 2, residual pellet). Most of the PP1alpha complexes were recovered in the specific anti-PP1alpha immunoprecipitate (lane 7), clearing the remaining extract (lane 5). The anti-PP1alpha precipitation was compared to a non-immune control precipitate, where the complexes remained in the supernatant (lane 4) and were not concentrated in the immunoprecipitated pellet (lane 6). These data demonstrated selective immunoprecipitation of chemically cross-linked PP1alpha complexes that were solubilized from Triton X-100-resistant cytoskeletons of REF52 fibroblasts.

We analyzed these PP1alpha complexes for recovery of focal adhesion proteins by immunoblotting (Fig. 5C). Both PP1alpha and tensin were concentrated and greatly enriched in the cross-linked immunocomplexes, relative to the amounts of these respective proteins in the permeabilized cells (input lane). Control precipitations with preimmune serum (Fig. 5C, lane labeled PreIm) did not enrich either tensin or PP1alpha, showing the specificity of immunoprecipitation. In contrast, focal adhesion kinase (FAK) was not detected in the anti-PP1alpha immunoprecipitates but was present in the extracts of the cytoskeletons (Fig. 5C). Some vinculin co-immunoprecipitated with PP1alpha, not pre-immune serum, however, this represented only a miniscule fraction of vinculin in the input fraction where it was very prominently stained. Thus, despite its relative abundance in cytoskeletons, vinculin was not efficiently cross-linked to PP1alpha. Our interpretation was that vinculin and PP1alpha do not exist in molecular proximity to one another in focal adhesions. As an additional control we immunoblotted for actin, but it was not detected in the specific anti-PP1alpha immunoprecipitates (not shown), so binding to F-actin could not account for an indirect recruitment of tensin to PP1alpha. The specific co-precipitation following chemical crosslinking showed that endogenous PP1alpha and tensin

were in molecular proximity to one another in focal adhesions.

#### *Tensin N-Terminal Domain Mediates Association with PP1*

In addition to examination of endogenous proteins we used over-expression of tagged tensin and tagged PP1alpha in COS7 cells to test for which region of tensin was necessary or sufficient to mediate association with PP1alpha. Full length tensin (1735 residues) was fused with a His<sub>6</sub>-S-peptide tag at the N terminus and from this construct a series of truncated fusion proteins containing tensin residues 1-360, 1-740, 1-1462 were created by introducing stop codons by site-directed mutagenesis. These proteins were co-expressed with HA-tagged PP1alpha and protein complexes were pulled down from cell extracts using beads conjugated with S-protein. The bound proteins were resolved by SDS-PAGE and analyzed by staining with HRP-conjugated S-protein and immunoblotting for the HA epitope tag. The three truncated tensin His-S tagged proteins were expressed and migrated at the expected sizes in SDS-PAGE. Despite efforts to express nearly comparable amounts of the His-S-tensin proteins the full-length wild-type (WT) tensin was barely detected in pull-downs, and we suggest this is probably due to a combination of factors, including low level of expression, limited solubilization, and resistance to electrotransfer during immunoblotting. Cells expressing HA-PP1alpha but no S-peptide tensin fusion protein were used to prepare control extracts to show no HA-PP1alpha bound to S-protein beads (Fig. 6A, BLK lane). Even the trace amounts of the full-length, wild-type (WT) tensin effectively pulled down HA-PP1alpha (right lane). Truncations of tensin from the C-terminus did not much reduce pull-down of HA-PP1alpha. Even the shortest protein that contained only residues 1-360 of tensin pulled-down HA-PP1alpha in this assay. We concluded that the N terminal residues 1-360 in tensin were sufficient to provide stable association with HA-PP1alpha.

Specificity of tensin for the endogenous alpha vs. the delta isoform of PP1 was confirmed in pull-down assays with over-expressed proteins (Fig. 6B). A tensin S-peptide fusion protein with residues 1-740 was

expressed alone as a control (Fig. 6B, left lanes) or co-expressed with either HA-PP1alpha (center lanes) or HA-PP1delta (right lanes). Immunoblots with anti-HA show about equal levels of over-expressed HA-PP1alpha and HA-PP1delta (Fig. 6B, upper left). Complexes recovered on S-protein beads (right panels) were analyzed for PP1 recovery by anti-HA immunoblotting for the epitope tag on both isoforms (upper panel) and for recovery of the tagged tensin (lower panel). Only HA-PP1alpha, not HA-PP1delta, associated with the tensin 1-740 fusion protein (Fig. 6B, upper right panel). These results confirm that the N terminal half of tensin is sufficient to mediate association with PP1 and dictate isoform specificity.

Binding of PP1 to regulatory subunits involves [R/K]VxF motifs in the subunits that interact with a cleft on the backside of PP1 opposite the active site. Tensin has six VxF motifs, three in the N terminal 360 residues and three in the C terminal 300 residues. Deletion of the C terminal region of tensin did not eliminate association with PP1, indicating these VxF motifs were not strictly required. However, there could be multiple sites in tensin for association with PP1, therefore we further tested the N terminal tensin domain residues 1-360 vs. the C terminal domain, residues 1401-1735 (Fig. 6C). Single transfection to express HA-PP1alpha served as control (Fig. 6C, left lanes) compared to dual transfection to express HA-PP1alpha plus S-peptide fusions with tensin N terminal (center lanes) or C terminal (right lanes) domains. Immunoblotting the cell extracts (left panels) demonstrated expression levels of HA-PP1alpha were the same in control and both experimental samples. Detection with S-protein conjugated to HRP showed that both the expression levels and recovery by pull-down of S-peptide tensin(1401-1735) were higher than the levels of S-peptide tensin(1-360) (Fig. 6C, lower panels). Only the N terminal domain of tensin (residues 1-360) associated with HA-PP1alpha, even when higher levels of the C terminal domain were compared. We have mutated individually two of the VxF motifs in the 1-360 region replacing the F with A, however these point mutations did not abrogate pull down of co-expressed HA-PP1alpha (not shown).

#### *GFP-Tensin Recruits PP1 to Focal Adhesions in PC3 Prostate Cancer Cells*

Tensin is a key component of focal adhesions, but certain human prostate carcinoma cell lines (PC3), have been reported to be essentially devoid of tensin. Indeed, we detected only trace levels of tensin protein by immunoblotting cell extracts of PC3 cells, in contrast to intense staining of tensin in an equivalent amount of total protein from REF52 cells, on the same blot (Figure 7A, top panel). As loading controls we showed that these cell extracts had essentially identical amounts of endogenous PP1alpha and vinculin by immunoblotting with specific antibodies (Figure 7A). We noticed that PC3 cells had slightly higher levels of FAK relative to REF52 cells (Figure 7A, lower panel). With anti-tensin antibody PC3 cancer cells showed dim and diffuse cytoplasmic staining barely above background (Figure 7B, upper panel) consistent with the absence of the protein on immunoblots. Immunostaining for endogenous PP1alpha in these cells showed it was located in the nucleus and was diffuse in the cytosol, but not in distinct focal adhesions (Fig. 7B, center panel). There was some staining for PP1alpha along the perimeter of the cell. Focal adhesions in PC3 cells were immunostained with anti-vinculin, which exposed a ring of short, broad adhesions on the outermost edge of the cells (Fig. 7B, bottom panel). The PP1alpha staining did not overlap with these peripheral adhesions. Thus, low levels of endogenous tensin in human PC-3 prostate cancer cells was correlated with only a few focal adhesions at the perimeter of the cell and diffuse cytoplasmic distribution of endogenous PP1alpha. These cancer cells provided a near-null background for transient over expression of ectopic tensin.

Would tensin induce formation of more focal adhesions in human cancer cells and also recruit PP1 alpha to those focal adhesions? We tested this idea by transiently transfecting PC3 cells with a plasmid encoding GFP-tensin. The GFP-tensin expressed at low levels (Fig. 7C, right hand cell) was localized into a few adhesions around the perimeter of the cell and these same adhesions recruited endogenous PP1alpha (Fig. 7C, lower panel). The GFP-tensin co-localized with vinculin in these



peripheral adhesions, confirmed with immunofluorescent double staining of fixed cells (not shown). At higher levels of expression the GFP-tensin was incorporated into adhesions along the perimeter, and also induced the formation of new adhesions across the entire area of the cell (Figure 7C, left cell). Importantly, endogenous PP1alpha became co-localized with GFP-tensin in both the perimeter and interior adhesions (Fig. 7C, lower panel, arrows). In addition there were green fluorescent foci in the cytoplasm of these high expressing cells that were more diffuse and circular in appearance, which distinguished them from adhesions. These could be merely cytoplasmic protein aggregates, but were sites for recruitment of endogenous PP1alpha that was immunostained coincident with the GFP-tensin. Endogenous PP1delta was not recruited or localized in the adhesions or foci in PC3 cells expressing GFP-tensin (not shown), consistent with the isoform specificity of tensin. If GFP was expressed in these cells as a control it did not localize into adhesions, or induce formation of additional adhesions or cytoplasmic foci, and did not result in re-localization of endogenous PP1alpha. Cells over-expressing GFP-tensin were tested for GFP emission through the rhodamine filter at the same exposure times as used for staining PP1 to insure that the images of PP1alpha were not due to fluorescence from GFP-tensin (bleed-through). These experiments demonstrated that expression of GFP-tensin in PC3 cells was sufficient to induce formation of new adhesions and cytoplasmic foci that specifically recruited endogenous PP1alpha.

## DISCUSSION

In this study we demonstrate that fibroblasts in a wound-healing model or replated on fibronectin to provoke cycling of focal adhesions revealed recruitment of tensin and PP1alpha together in time and space, distinct from the recruitment of vinculin and paxillin, well-recognized components of focal adhesions. Focal adhesions are now appreciated as dynamic structures, constantly remodeled by a cycle of formation, maturation and dispersal. Initial adhesions formed at the leading edge of migrating or spreading cells mature and change composition

by release and recruitment of protein components. Studies of this process have highlighted the striking differences in composition between early adhesions that are rich in the P-Tyr proteins vinculin and paxillin, but lack tensin, and adhesions that are tensin-rich but have little detectable P-Tyr (20,21). Our working hypothesis is that tensin recruits PP1 into focal adhesions to catalyze selective dephosphorylation of Ser/Thr in nearby proteins. It is possible and even likely that tensin itself is a substrate of PP1alpha, because it is reported to be phosphorylated on Ser and Thr, as well as Tyr residues (22). Which focal adhesion proteins are the substrates of PP1 and how their phosphorylation state affects adhesion dynamics or cell migration are complex unanswered questions. Though many focal adhesion proteins undergo Ser/Thr phosphorylation, the effects of phosphorylation on function is not understood and generally under-studied. One recent idea is that Ser/Thr phosphorylation regulates rates of focal adhesion assembly-disassembly (11), a promising direction for new studies.

Tensin and PP1alpha were selectively co-precipitated from cell extracts and from chemically cross-linked focal adhesions, showing these proteins are associated together in complexes. Chemical cross-linking with Lomant's reagent (DSP) forms spans ~12 Å end to end. This distance is considerably less than the diameter of the cross-linked proteins themselves, such as PP1alpha which is 50 x 35 x 35 Å (23) suggesting that tensin and PP1 are within close molecular proximity in adhesions. It was remarkable that neither FAK, nor vinculin nor actin was cross-linked to PP1alpha in focal adhesions considering the abundance of these focal adhesion components and the attachment of actin filaments to tensin. We took the crosslinking results as evidence for distinct spatial domains within focal adhesions. We imagine that tensin-PP1alpha complexes are separate from complexes that contain FAK or vinculin.

We favor the hypothesis that the tensin N-terminal domain directly binds PP1alpha and functions as a regulatory subunit. Many PP1 regulatory subunits act as targeting proteins and directly bind PP1 with a canonical RVxF motif for primary interaction. Tensin has at least six

sequences that conform to this motif, distributed in the N-terminal and C-terminal domains. By making truncated versions of tensin, expressing them in cells and testing for co-precipitation of co-expressed PP1alpha we found that the N terminal domain (residues 1-360) alone is sufficient for tensin to associate with PP1. This domain is related in sequence to PTP and PTEN and alone can target GFP to focal adhesions (21). The multiple VxF motifs in the C terminus of tensin might function to bind PP1, separately from the N terminus, but a fusion protein with the tensin C terminal domain did not associate with PP1, arguing that the VxF motifs in this region are not used to bind PP1. However, we are mindful that a lack-of-function result in the assay is not conclusive. Perhaps modification (e.g. phosphorylation) or association with other factors might be required for PP1 binding to the C terminal domain, and full-length tensin might be required for activation of the C terminal domain. We do know the N terminal domain associates with PP1alpha not PP1delta and mutation of either one of two VxF motifs in this region did not eliminate association in a pull-down assay (not shown). Additional experiments are required to map residues in tensin(1-360) that are required for PP1alpha association and to test for direct binding with purified recombinant proteins. As yet we cannot exclude participation of a scaffold protein that binds tensin and PP1alpha together in a complex, but at least such a protein must be specific for the PP1alpha isoform.

Transiently expressed GFP-tensin appears in focal adhesions and foci in human cancer PC3 cells otherwise defiant in tensin. Our results are consistent with previous reports that GFP-tensin localizes into focal adhesions in NIH3T3 cells (19). In the living cells visualized without fixation these adhesions were well-formed, elongated structures as seen in fibroblasts, and were evenly distributed across the cell. We noted that after fixation the peripheral adhesions were unchanged in appearance, but adhesions toward the center of the cell area were lost or became more diffuse foci. This offers a clue that perhaps the stability or organization of the focal adhesions induced by GFP-tensin in these cancer cells was different from native focal adhesions in fibroblasts. The

sensitivity to fixation was not seen in fibroblasts, even those expressing GFP-tensin, so it was not just because of the over-expression, but more likely due to the composition and/or modifications of focal adhesion proteins in the cancer cells. These GFP-tensin-induced focal adhesions did recruit endogenous PP1alpha but not PP1delta, again revealing isoform-specific targeting of the phosphatase in living cells.

It has become evident that focal adhesions physically recruit and are modified by multiple Ser/Thr phosphatases that thereby have effects on cell migration. PP2A is co-precipitated with the focal adhesion protein paxillin and in metastatic mouse melanoma BL6 cells a truncated version of a PP2A regulatory B subunit impairs targeting of the phosphatase to focal adhesions and prevents specific dephosphorylation of paxillin (24). Reduction in PP2A levels by antisense oligonucleotides increased paxillin phosphorylation and cell motility (25). Cytostatin, a specific chemical inhibitor for protein phosphatase 2A (PP2A), suppresses cell adhesion to extracellular matrix (26). Other results have shown PP1delta localizes in focal adhesions of rat smooth muscle cells and HeLa cells, fibroblasts, endothelial cells and keratinocytes (27). This localization possibly involves direct interaction of the PP1delta isoform with the C-terminal domain of FAK (FRNK region). PP1delta is proposed to mediate dephosphorylation of Ser in FAK when cells are released from mitosis (28). Our results may be complementary, not necessarily conflicting with the reported PP1delta-FAK interaction. It is possible PP1alpha and delta isoforms may both function in regulation of focal adhesions by assembly into different complexes with different partners, i.e. tensin or FAK. Results here show specific interaction and cross-linking of PP1alpha with tensin, but not with FAK. Interaction with regulatory subunits spatially restricts PP1 within cells, as well as alters substrate specificity (29) and determines sensitivity to inhibitor proteins (30). Therefore we presume that PP1alpha binds to the PTP domain of tensin to selectively regulate a specific subset of phosphoproteins in focal adhesions. Although dozens of possible Ser/Thr phosphorylation sites are predicted and now found in focal adhesion proteins (31) not much

is known about their effects on function or response to signaling, subjects for further study.

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## FIGURE LEGENDS

**Figure 1.** *Differential Localization of PP1alpha and PP1delta in rat embryo fibroblasts.* REF52 cells grown on fibronectin-coated coverslips were fixed with methanol and stained with rabbit anti-PP1alpha (A) or anti-PP1delta (C) and with mouse monoclonal anti-vinculin antibodies (B) or rhodamine-conjugated phalloidin (D). Cells were visualized by fluorescence microscopy with fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse secondary antibodies. (E) The cDNA for PP1alpha and for PP1delta were transiently expressed in COS7 cells that were dissolved directly in SDS sample buffer and immunoblotted with anti-HA as a loading control. The samples also were immunoblotted with either anti-PP1-alpha (center panel) or anti-PP1-delta (lower panel). Differential staining of the PP1 alpha and delta isoforms demonstrates the specificity of the antibody preparations.

**Figure 2.** *Focal adhesions in migrating fibroblasts.* REF52 cells were grown to 100% confluence on fibronectin-coated coverslips and “wounded” to scrape away cells. Cells migrated into the wound area (from left to right) for 8 hr before fixation and double staining as described for Figure 1, with anti-PP1alpha (top) and anti-vinculin (bottom) antibodies. Arrows highlight different populations of focal adhesions that stained for both proteins (top panel), or those that stained for vinculin but not PP1 (bottom panel). Immunofluorescent images were merged (center), with yellow revealing overlap. We note that these experiments were restricted to methanol fixation. Paraformaldehyde fixation gives higher contrast and better defined images of focal adhesions, but poor immunostaining of endogenous PP1.

**Figure 3.** *Localization of PP1alpha and Focal Adhesions in Spreading Fibroblasts.* REF52 cells were suspended and replated onto fibronectin-coated coverslips that were incubated at 37°C for 30 to 60 min, fixed with methanol and stained with a mixture of rabbit anti-PP1alpha (upper left panels, green) and one of the monoclonal antibodies to vinculin (A), P-Tyr (B) or tensin (C). Immunofluorescent images were merged (larger panels) to show that the outermost adhesions were stained with vinculin (red in A) or P-Tyr (orange in B) but did not co-stain for PP1, whereas all the focal adhesions co-stained with tensin and PP1 (C).

**Figure 4.** *Co-immunoprecipitation of Endogenous Tensin with PP1alpha.* Extracts of rat aorta smooth muscle cells were precipitated in parallel as described under Materials & Methods with rabbit anti-PP1alpha serum or pre-immune rabbit serum as a control and the precipitates analyzed by immunoblotting. The tensin and PP1alpha proteins in 2% of the cell extract used for precipitation were identified on the same immunoblots (left lane labeled “Input”).

**Figure 5.** *Isolation of Chemically Crosslinked PP1alpha-Tensin Complexes from Focal Adhesions.* (A) REF52 cells were permeabilized with low concentrations of Triton X-100 and stained with rabbit anti-PP1-alpha antiserum to show preservation of focal adhesions and exposure of the epitope. (B) The permeabilized cell preparations were chemically cross-linked with Lomant’s reagent, solubilized with RIPA buffer and analyzed by immunoblotting for PP1alpha. The RIPA extract of the cross-linked

cytoskeleton (lanes 1 and 3) was compared to the insoluble residue (pellet) after extraction (lane 2) and the supernatants (lanes 4 and 6) and pellets (lanes 5 and 7) from preimmune control and anti-PP1alpha immunoprecipitations, respectively. This shows concentration of solubilized PP1alpha complexes by specific immunoprecipitation (lane 7). (C) The chemically cross-linked complexes recovered by preimmune serum and PP1alpha immunoprecipitations were analyzed by immunoblotting, in comparison to the extract of cross-linked cytoskeletons (Input). Immunoblotting used rabbit anti-PP1alpha, mouse anti-tensin, mouse anti-FAK and mouse anti-vinculin, as described in Materials & Methods. These results were reproduced in three independent experiments.

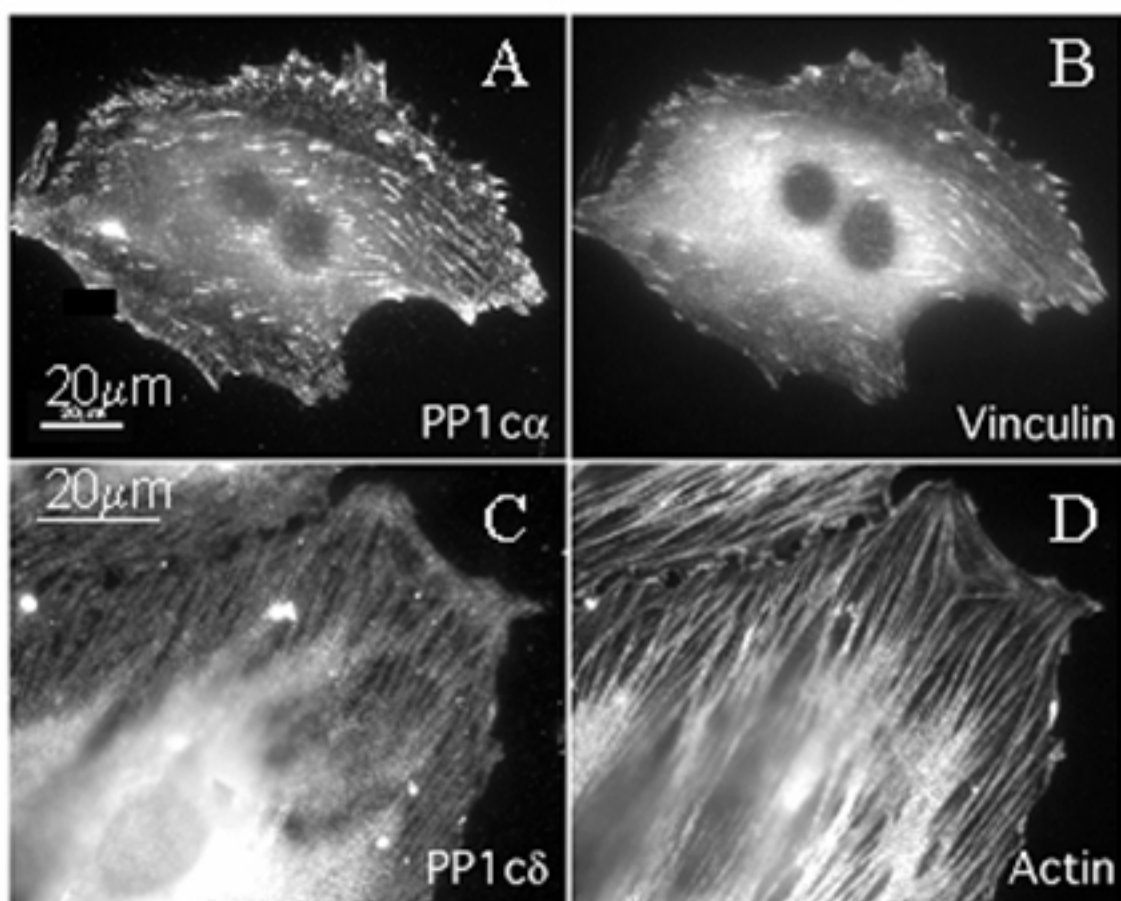
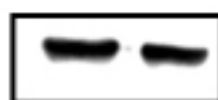
**Figure 6.** *Isoform-Specific Association of PP1alpha with the N terminal Domain of Tensin.* (A) Full length wild-type (WT) tensin tagged at the N terminus with His<sub>6</sub>-S-peptide and versions truncated by stop codons after residues 1462, 740 or 360 were transiently expressed in COS7 cells. Control cells were transfected to express HA<sub>3</sub>-PP1alpha and experimental cells were co-transfected to express tensin plus HA<sub>3</sub>-PP1alpha. The levels of HA-PP1alpha in the cell extracts were compared by anti-HA immunoblotting (top frame). Complexes were pulled-down on S-protein beads and analyzed for S-peptide tensin and for HA<sub>3</sub>-PP1alpha. (B) Cells were transfected to express His<sub>6</sub>-S-peptide-tensin (1-740) alone or with co-expressed HA<sub>3</sub>-PP1alpha or HA<sub>3</sub>-PP1delta. The levels of the proteins in the extracts are shown by staining for HA or S-peptide (left panels) and the recovery of the proteins pulled-down on S-protein beads is shown in the right panels. (C) HA<sub>3</sub>-PP1alpha was expressed in COS7 cells alone or with either tensin residues 1-360 or 1401-1735 fused to His<sub>6</sub>-S-peptide. Pull down assay was conducted and analyzed as described in B, above.

**Figure 7.**

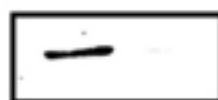
*Expression of GFP-Tensin in Tensin-deficient Human Prostate Cancer PC3 Cells.* (A) Whole cell extracts of rat REF52 cells and human prostate cancer PC3 cells were analyzed by immunoblotting the same amount of total protein with antibodies against (top to bottom) tensin (200 kDa), PP1alpha (37 kDa), vinculin (116 kDa) and FAK (125 kDa). (B) Human prostate cancer cells were grown on fibronectin-coated coverslips, fixed and stained with either mouse anti-tensin (top panel), rabbit anti-PP1alpha (center panel), or mouse anti-vinculin (lower panel). Proteins were visualized with Oregon Green 488-conjugated anti-rabbit and rhodamine-conjugated anti-mouse secondary antibodies. Images with identical exposure times were processed in Photoshop 5. (C) PC3 cells grown on coverslips were transfected with a plasmid encoding GFP-Tensin and incubated overnight at 37°C. Cells were fixed with methanol and images acquired for the localization of the GFP fluorescence by direct fluorescence (upper panel) and localization of the PP1alpha by immunofluorescence with rhodamine-conjugated anti-rabbit secondary antibody (bottom panel).

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**E**HA<sub>9</sub>-PP1α    HA<sub>9</sub>-PP1δ

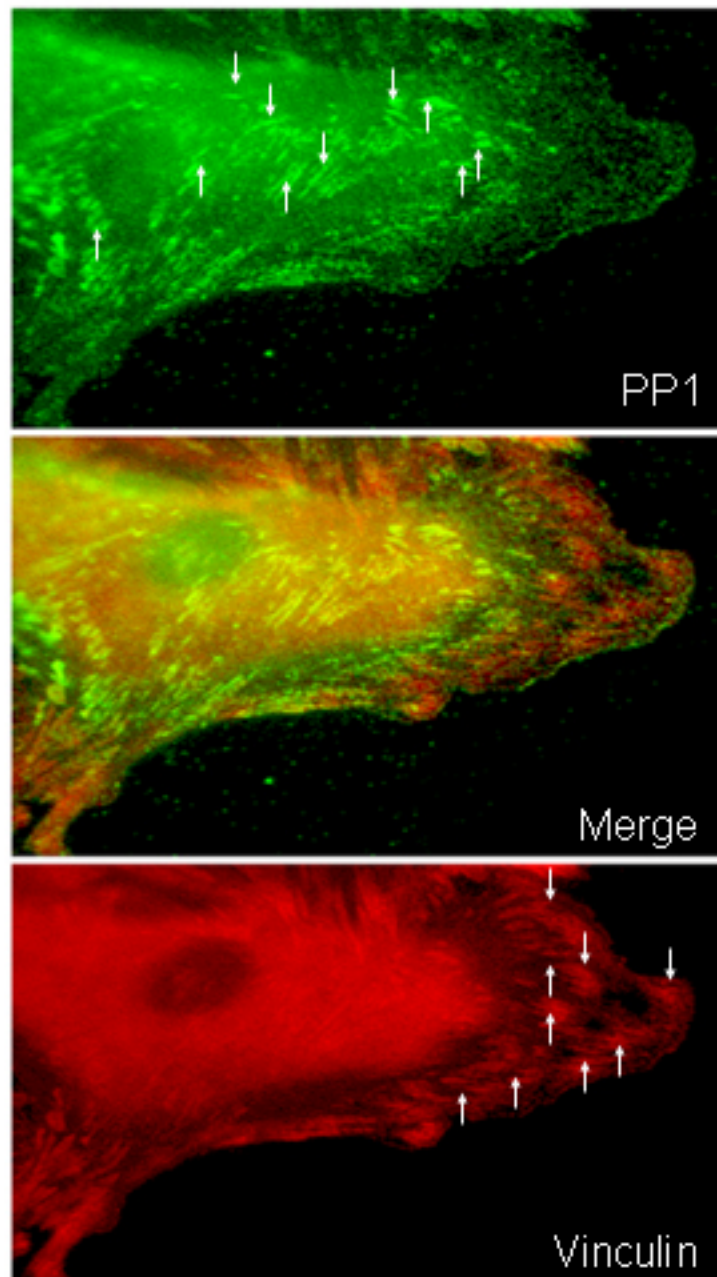
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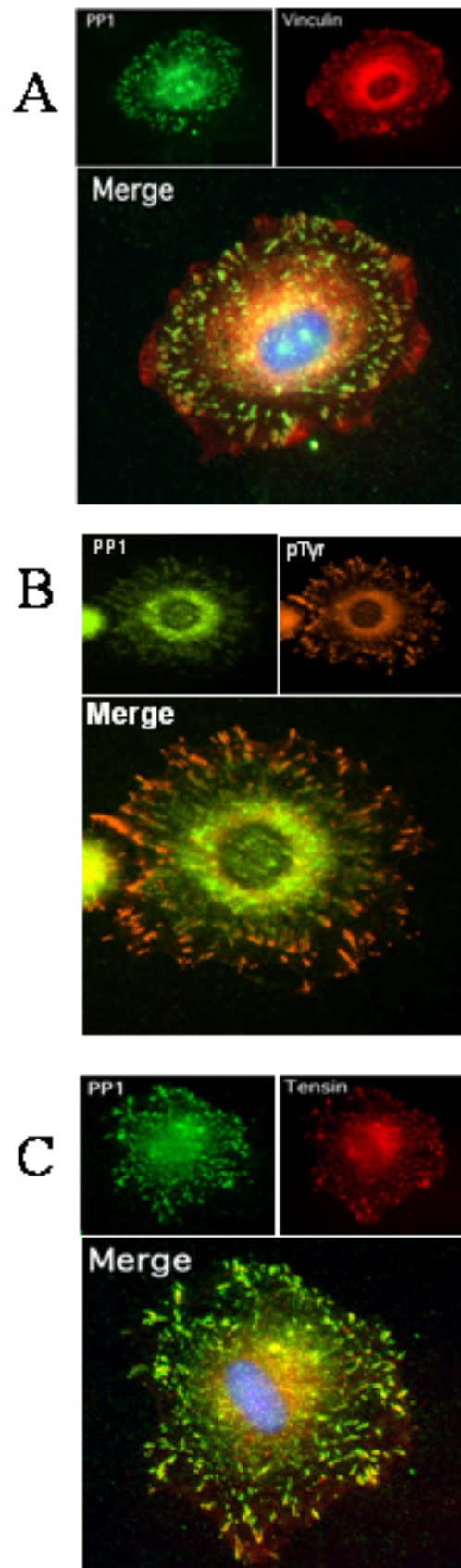


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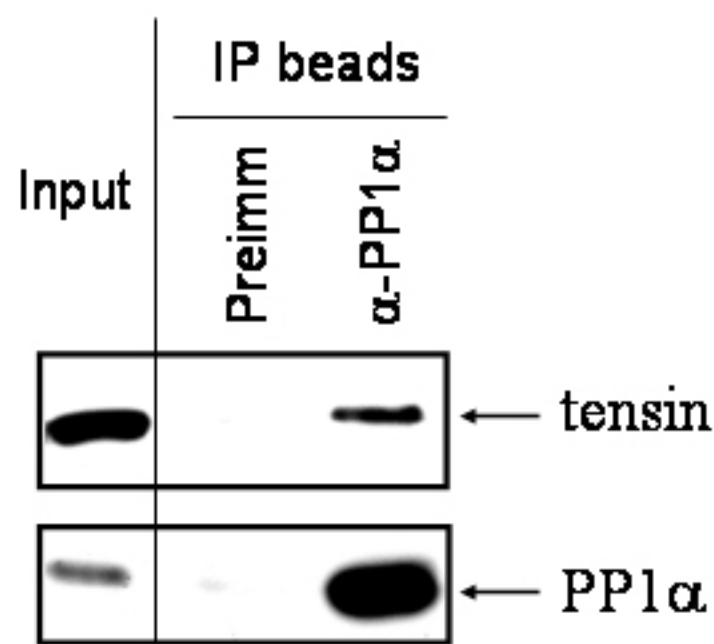


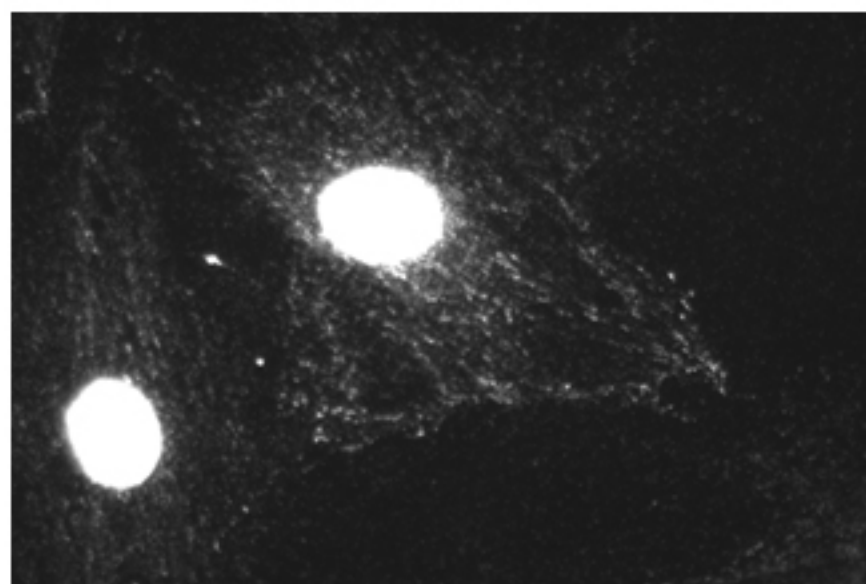
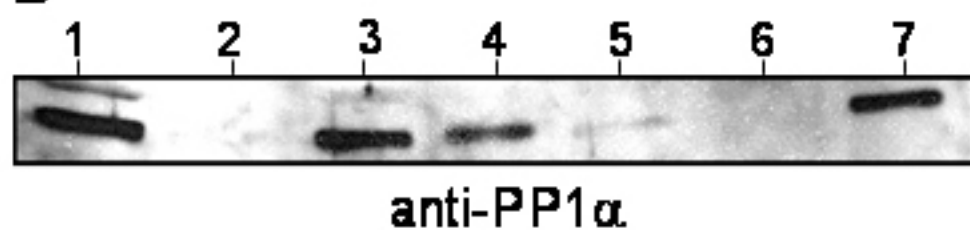
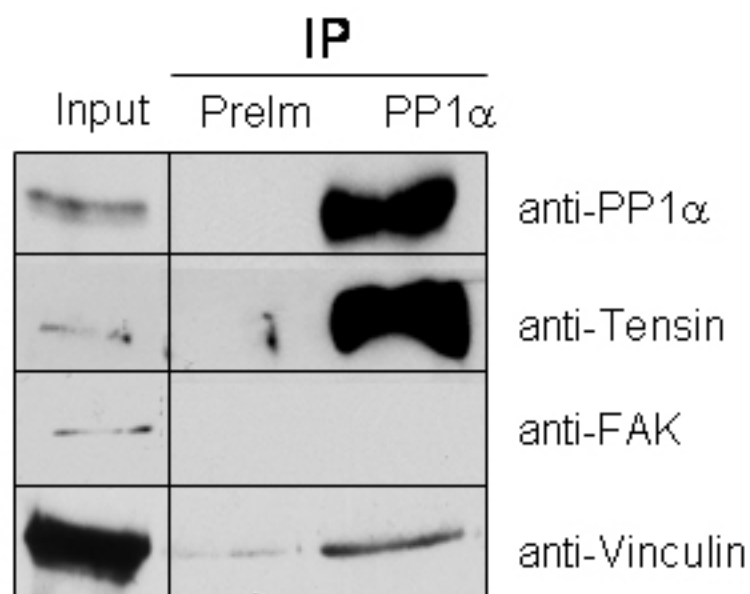
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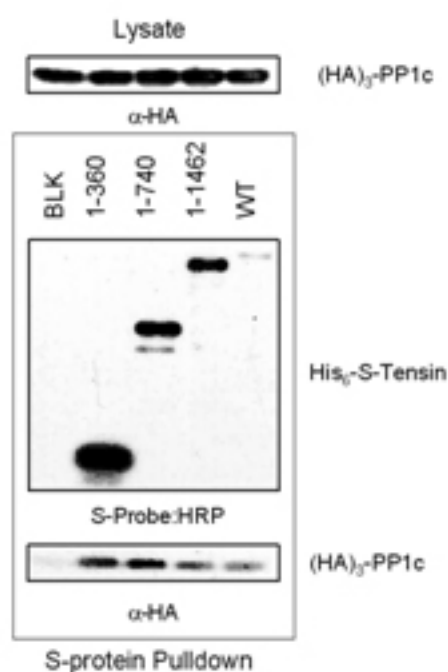
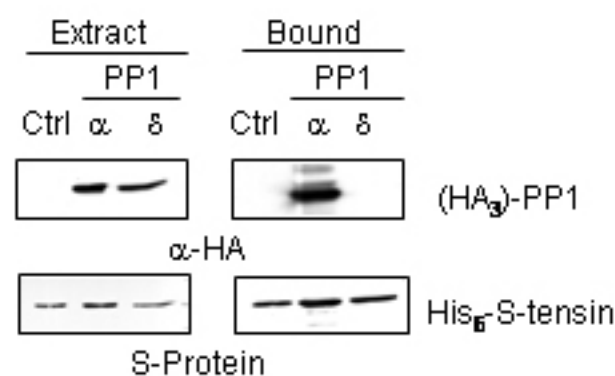
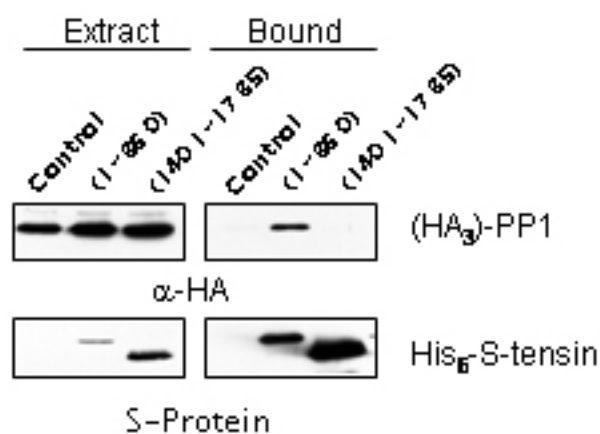


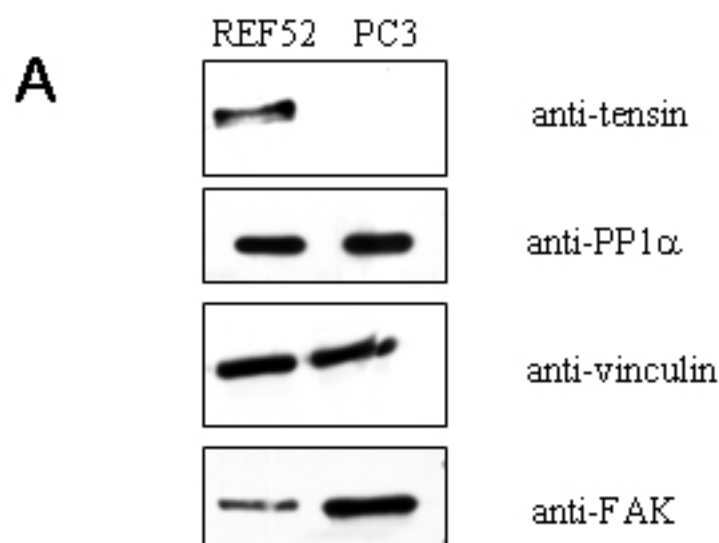
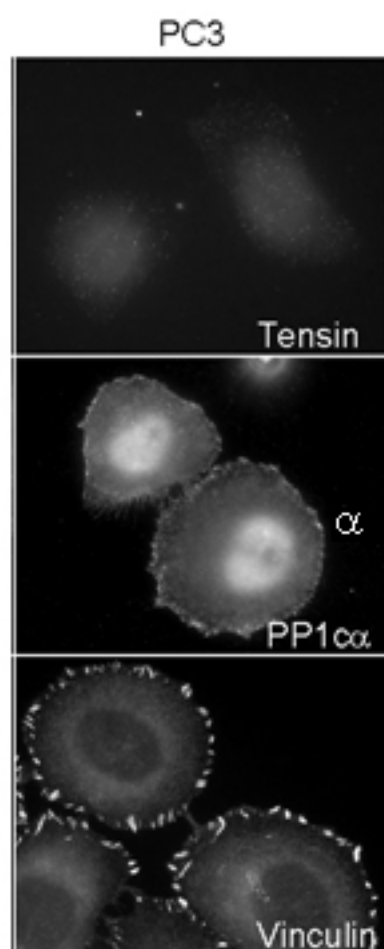






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