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PPP1R42, a PP1 binding protein, regulates centrosome dynamics in ARPE-19 cells

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Abstract

Background—The centrosome is the primary site for microtubule nucleation in cells and orchestrates reorganization of the microtubule cytoskeleton during the cell cycle. The activities of the centrosome must be closely aligned with progression of the cell cycle; misregulation of centrosome separation and duplication is a hallmark of cancer. In a subset of cells, including the developing spermatid, the centrosome becomes specialized to form the basal body thereby supporting growth of the axoneme in morphogenesis of cilia and flagella, structures critical for signaling and motility. Mammalian spermatogenesis is an excellent model system to investigate the transformations in cellular architecture that accompany these changes including formation of the flagellum. We have previously identified a leucine rich repeat protein (PPP1R42) that contains a protein phosphatase-1 (PP1) binding site and translocates from the apical nucleus to the centrosome at the base of the flagellum during spermiogenesis. In this manuscript we examine localization and function of PPP1R42 in a ciliated epithelial cell model as a first step in understanding the role of this protein in centrosome function and flagellar formation.

Results—We demonstrate that PPP1R42 localizes to the basal body in ARPE-19 retinal epithelial cells. Colocalization and co-immunoprecipitation experiments further show that PPP1R42 interacts with γ -tubulin. Inhibition of PPP1R42 with small interfering RNAs (siRNAs) causes accumulation of centrosomes indicating premature centrosome separation. Importantly, the activity of two signaling molecules that regulate centrosome separation, PP1 phosphatase and NEK2 kinase, changes when PPP1R42 is inhibited: PP1 activity is reduced with a corresponding increase in NEK2 activity.

Conclusions—We have identified a role for the PP1-binding protein, PPP1R42, in centrosome separation in ciliated ARPE-19 cells. Our finding that inhibition of PPP1R42 expression increases the number of centrosomes per cell is consistent with our model that PPP1R42 is a positive regulator of PP1. PPP1R42 depletion reduces the activity of PP1 leading to activation of NEK2, the kinase responsible for phosphorylation of centrosomal linker proteins promoting centrosome separation. This work identifies a new molecule localized to the centrosome and basal body with a role in the complex signaling network responsible for controlling centrosome activities.

Keywords

centrosome; polarization; PP1; PPP1R42; TLRR

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Authors' contributions

RW carried out the experiments with cultured cells including indirect immunofluorescence, siRNA inhibition and immunokinase assays. ND conducted the PPP1R42 co-immunoprecipitation and mitotic index experiments. AOS conceived the experiments and drafted the manuscript. RW and ND contributed to writing the manuscript.

Background

The centrosome, or microtubule organization center (MTOC), plays a critical role in the dramatic restructuring of microtubule arrays required during the cell cycle (Nigg and Raff, 2009; Bornens, 2012). In dividing cells, centrosome behavior is tightly regulated and linked to progression through the cell cycle; centrosomes duplicate at S phase and separate to form the spindle poles at metaphase. Following division, cells can either continue dividing given favorable growth conditions, or enter G₀, if nutrients are lacking or if the cell commits to differentiate. In the latter case, the centrosome migrates to the plasma membrane and transforms into a basal body to give rise to a primary cilium. This process is analogous to the developmental program involved in biogenesis of the flagellum in differentiation of male germ cells. Mammalian spermatogenesis is a highly regulated developmental process that transforms the nonpolar spermatogonium into a highly polarized spermatozoon (Clermont, 1972). This transition is accompanied by truly remarkable changes in the microtubule cytoskeleton including formation of the flagellum.

We have recently identified and characterized a leucine-rich repeat protein (which we termed testis leucine rich repeat, TLRR) which has been recently renamed PPP1R42 (Wang and Sperry, 2008; Wang et al., 2010; Wang and Sperry, 2011). PPP1R42 is highly expressed in the testis and we have shown that it first appears near the apical aspect of the nucleus of elongating spermatids and then translocates to the basal body associated with the sperm flagella (Wang and Sperry, 2008; Wang et al., 2010). PPP1R42 contains a binding site for protein phosphatase-1 (PP1) and we have demonstrated previously that these two proteins reside in the same complex in the testis (Wang and Sperry, 2008; Wang et al., 2010; Wang and Sperry, 2011). Additionally, this complex displays phosphatase activity that is at its highest level when spermatocytes transition to spermatids (Wang and Sperry, 2011).

PP1 is a multifunctional enzyme that regulates a wide range of cellular processes, from glycogen metabolism to progression through the cell cycle (reviewed in Ceulemans and Bollen, 2004). This versatility reflects interaction of the catalytic domain of PP1 with numerous regulatory subunits that modify its activity through activation, inhibition, or intracellular targeting (reviewed in Cohen, 2002). PP1 participates at several distinct points in the cell cycle including the G₂/M and G₁/S transitions through interaction with regulatory subunits specific to these control points in the cell cycle (reviewed in Trinkle-Mulcahy and Lamond, 2006; Kolupaeva and Janssens, 2012). An important milestone in preparation for chromosome segregation is the separation of centrosomes to form the poles of the mitotic spindle. PP1 plays a central regulatory role in this process through its interaction with and dephosphorylation of NEK2 (NIMA-related kinase 2) (Helps et al., 2000; Mi et al., 2007). The NEK2 kinase is localized to the centrosome and functions to phosphorylate centrosomal linker proteins leading to their displacement from the centrosome, triggering centrosome separation (Fry et al., 1998a; Fry et al., 1998b; Bahe et al., 2005). PP1 inactivates NEK2 by dephosphorylation to inhibit its kinase activity, thereby counteracting centrosome separation (Helps et al., 2000; Mi et al., 2007).

Our identification of a PP1 binding protein at the centrosome of the developing spermatid supports the hypothesis that PPP1R42 participates in the activities and dynamics of the spermatid centrosome through regulation of PP1 activity at the centrosome (Wang et al., 2010). As a first step to address this role for PPP1R42, we have examined its function in a ciliated cell model, the retinal pigmented epithelial cell line ARPE-19. In this manuscript we describe the localization of PPP1R42 to the centrosome and basal body of primary cilia in ARPE-19 cells and the effect of PPP1R42 inhibition on centrosome dynamics and on the

signaling molecules involved. We propose that PPP1R42 acts upstream of the NEK2 kinase to suppress centrosome separation.

Results

PPP1R42 is localized at the basal body of primary cilia in ciliated epithelial cells

We have demonstrated previously that PPP1R42 is located near the basal body of the sperm flagellum of elongating spermatids (Wang and Sperry, 2008). In order to determine whether this localization to the centrosome is conserved in other ciliated cell types, we investigated the location of PPP1R42 in the human retinal epithelial cell line, ARPE-19, using an antibody to the human ortholog of mouse PPP1R42. This antibody reacts with a protein of the same size in mouse testis lysate to that of the original antibody we prepared against the mouse protein (Figure 1A) (Wang and Sperry, 2008). Two reactive higher molecular weight bands were apparent that disappear when the samples are incubated with urea, indicative of protein aggregation in SDS (unpublished results). ARPE-19 cells develop primary cilia upon confluence and acquisition of polarity. In postconfluent cells costained for PPP1R42 and acetylated tubulin (a marker for primary cilia), PPP1R42 is located at the base of these structures (Figure 1B, arrows; inset, arrow). We also stained the cells with an antibody to claudin-1, a component of junctional complexes, to confirm that cells were near polarization under our culture conditions (Figure 1C). Our conditions to induce cilia formation in ARPE-19 cells mimic procedures used successfully by others (Wang and Brautigan, 2008). The negative control incubated with anti-acetylated tubulin but without the primary antibody to PPP1R42 displayed no specific staining (Figure 1D).

PPP1R42 colocalizes with γ -tubulin in ciliated epithelial cells

In order to confirm that PPP1R42 is associated with the basal body of ARPE-19 cells, we colocalized this protein with γ -tubulin, a resident protein of the basal body/centrosome. PPP1R42 and γ -tubulin colocalize in ARPE-19 cells at the base of primary cilia consistent with localization of this protein to basal bodies in this cell type (Figure 2A-C). We further verified the association of PPP1R42 with γ -tubulin by co-immunoprecipitation. PPP1R42 associates with γ -tubulin in complexes immunoprecipitated with antibodies to PPP1R42 (R42, Figure 2D). The relationship between gamma tubulin and PPP1R42 in spermatids and cultured cells supports the hypothesis that PPP1R42 performs a conserved function at the centrosome/basal body.

Inhibition of PPP1R42 causes an increase in centrosome number

We next sought to determine whether depletion of PPP1R42 in ARPE-19 cells affected the behavior of centrosomes and/or formation of primary cilia in these cells. In order to determine whether the reduction of PPP1R42 affected the dynamics of the centrosome in these cells, we reduced PPP1R42 by more than 90% compared to control values (Figure 3A). In these cells, we observed a ten-fold increase in the number of cells with greater than 2 centrosomes (Figure 3B). Most affected cells had 4 centrosomes per cell (Figure 3C, D), and a reproducible percentage of cells (2%) had greater than 4 centrosomes per cell, some having as many as 6-8 centrosomes (arrows, Figure 3E, F). This high number of centrosomes was never observed in control cells. A range of centrosome distribution patterns was observed in affected cells with some cells displaying dispersed centrosomes (Supplementary Figure 1). We did not observe a significant difference in cilia number or morphology in knockdown cells. However, we did observe that affected cells appeared to have larger nuclei, suggestive of endoreduplication (compare cells indicated by arrowheads, Figure 3C, E). We quantitated this effect and found that the difference in nuclear size between affected and unaffected cells was significant at $p=0.02$ (Figure 3G, H).

PPP1R42 inhibition causes a block at the G₂/M boundary and an increase in cell death

Prior studies have found a relationship between centrosome over duplication and cell cycle disruption (Loncarek et al., 2010; Steere et al., 2011). Therefore, it is likely that the accumulation of centrosomes in PPP1R42 knockdown cells would be associated with interruption in G₂ where centrosomes duplicate and separate. We analyzed a cycling population of cells; either transfected with the control nontargeting siRNA or the PPP1R42 knockdown siRNA and measured the percentage of cells in each phase of the cell cycle with flow cytometry. The percentage of cells in the G₁ phase of the cell cycle was reduced, but not significantly; however, the 77% increase in cells in G₂ was statistically significant (Figure 4A). In our flow cytometry experiments, we observed an increase in cells in the Sub G₁ fraction suggestive of an increase in cell death in treated cells (unpublished data). In order to confirm these findings and determine whether the increase in cell death was due to increased apoptosis, we used Annexin V staining to detect apoptotic cells. PPP1R42 knockdown caused a significant increase in dead cells (200%, Figure 4B) and a reproducible but not statistically significant increase in apoptotic cells (unpublished data).

The observed increase in G₂ cells in cells depleted for PPP1R42 is consistent with a potential G₂/M block in treated cells (Figure 4A). To confirm this finding, we measured the mitotic index of a population of synchronized cells followed by treatment with either knockdown or control siRNA. The percentage of cells in mitosis, as measured by phospho-histone H3 reactivity, was significantly reduced in PPP1R42 depleted cells (Figure 4C, D). These findings support the proposal that PPP1R42 performs an essential function as cells enter mitosis and is consistent with an important role at the centrosome.

PPP1R42 knockdown results in reduction in PP1 protein and increased phosphorylation of PP1

PP1 is a key regulatory molecule in many cellular processes including centrosome separation (Helps et al., 2000; Mi et al., 2007). We predict that if PPP1R42 positively regulates PP1 activity, depletion of PP1 with siRNA will reduce activity of PP1, either by reduction in the total amount of protein or by hyperphosphorylation in PPP1R42 knockdown cells. Therefore we examined the level of PP1 protein and its phosphorylation status in cells after PPP1R42 depletion. The level of PP1 protein was reduced reproducibly by approximately five-fold in treated cells (Figure 5A, B). We next probed protein from control and siRNA treated cells with an antibody specific for phosphorylated and inactive PP1 (phosphorylated at threonine 320). Although phospho-PP1 represents a relatively small percentage of total PP1, we were able to detect a reproducible increase in phospho-PP1 in cells depleted for PPP1R42. (Figure 5C, D). These data support the proposal that PPP1R42 is a positive regulator of PP1 activity because its reduction decreases the amount of active PP1.

NEK2 activity is increased when PPP1R42 is depleted

A balance of phosphorylation/dephosphorylation governs separation of centrosomes prior to mitosis (Meraldi and Nigg, 2001). PP1 at the centrosome dephosphorylates and inactivates NEK2 thereby inhibiting its ability to induce centrosome separation (Helps et al., 2000; Mi et al., 2007). We next wanted to determine whether NEK2 activity is affected by PPP1R42 depletion. We predict that reduction in active PP1 in PPP1R42 knockdown cells will result in activated NEK2 thereby leading to premature centrosome separation. NEK2 was immunoprecipitated from control and knockdown cell lysates and its kinase activity measured. NEK2 activity increased 6-fold in cells depleted for PPP1R42 (Figure 6A, B). Similar to the situation with PP1 after knockdown, we observed a reduction in Nek2 in treated cells, but not to the extent of PP1 (unpublished data) This finding is consistent with

the model that PPP1R42 activates PP1 to negatively regulate NEK2 thereby suppressing centrosome separation.

Discussion

PPP1R42 is a PP1 regulatory protein expressed at high levels in the testis (Wang and Sperry, 2008). We have shown previously that PPP1R42 forms a complex with PP1 in male germ cells and this complex is at its highest level in spermatocytes and spermatids (Wang and Sperry, 2011). Importantly, PPP1R42 is found near the centrosome of elongating spermatids (Wang et al., 2010). In the current study we demonstrate that PPP1R42 is also associated with the basal body/centrosome in the ciliated cell line ARPE-19, suggestive of a conserved role for this protein in centrosome function. However, we did observe a potentially significant difference between staining in the two cell types, which may reflect important differences in the function and fate of the centrosome in these two cellular contexts. In rodent spermatogenesis, the centrosome degenerates and γ -tubulin is lost from sperm (Manandhar et al., 1998). Interestingly, staining for γ -tubulin in elongating spermatids is more diffuse than that seen in ARPE-19 cells (Wang et al., 2010), which may reflect the ultimate destruction of this organelle and therefore the observed difference in PPP1R42 staining in spermatids compared to ARPE-19 cells.

PP1 is also a component of the centrosome and regulates centrosome separation at G₂/M through interaction with NEK2 (Andreassen et al., 1998; Helps et al., 2000; Mi et al., 2007). We hypothesize that PPP1R42 positively regulates the function of PP1 at the centrosome, which in turn negatively regulates NEK2 (Figure 7). Our finding that knockdown of PPP1R42 in ARPE-19 cells causes a ten-fold increase in the number of cells with greater than two centrosomes supports the proposal that PPP1R42 is an activator of PP1 at the centrosome. According to this model, reduction in PPP1R42 levels following siRNA inhibition decreases levels of active, dephosphorylated PP1, thereby resulting in hyperphosphorylation and/or increased levels of NEK2 promoting centrosome separation. Indeed, we show that NEK2 kinase activity is increased when PPP1R42 is reduced. This shifts the balance toward phosphorylation of centrosomal linkage proteins required for centrosome separation.

In addition to increased phosphorylation of PP1 when PPP1R42 is depleted, we detected a significant decrease in total levels of PP1 protein consistent with a potential role for PPP1R42 in stabilization of PP1. Such a role has also been demonstrated for the PP1 regulatory subunit, Inh-2, that is a chaperone for PP1 (Alessi et al., 1993; MacKintosh et al., 1996).

Additional PP1 regulatory subunits have been found at the centrosome including PPP1R2 and PPP1R11 (formerly known as Inh-2 and Inh-3) (Eto et al., 2002; Huang et al., 2005). Our data represent the first description of an activating subunit of PP1 identified at the centrosome. PPP1R2 forms a complex with PP1 and NEK2 to release the inhibition of NEK2 by PP1 and cause centrosome splitting (Eto et al., 2002). Our results support an opposing role for PPP1R42 in suppressing centrosome separation. Other investigators have demonstrated that binding of regulatory subunits to PP1 is mutually exclusive including that of PPP1R2 (Egloff et al., 1997; Connor et al., 2000); reviewed in (Aggen et al., 2000). Therefore, we do not expect that PPP1R42 is part of the PP1/NEK2/Inh-2 complex. Instead, we propose that PPP1R42 may serve a complementary role in the double negative feedback loop between NEK2 and PP1 by displacing an inhibitory subunit.

Cells knocked down for PPP1R42 accumulate in G₂, which may reflect a block in mitosis due to disruption of centrosome activities at the G₂/M boundary. Indeed, knockdown cells

did not enter mitosis at the rate of their control counterparts. These observations support the proposal that PPP1R42 is required for cells to enter mitosis and are consistent with a role for this protein in centrosome separation prior to spindle formation.

Interference with PP1 activity is expected to have pleiotropic effects on cells, reflecting its regulation of multiple events important for cell cycle progression (reviewed in Berndt, 1999; Ceulemans and Bollen, 2004). In addition to its involvement in centrosome separation as described here, PP1 participates in chromosome dynamics during M phase (Emanuele et al., 2008; Kim et al., 2010; Qian et al., 2011), mitotic exit (Wu et al., 2009), and cytokinesis (Cheng et al., 2000; Trinkle-Mulcahy et al., 2003; Wang et al., 2008). PP1 also plays an important role at several control points in the cell cycle. The retinoblastoma (Rb) protein, a regulator of mitotic exit as well as the G₁/S transition, is a substrate for PP1. Inhibition of PP1 causes cell cycle arrest at S phase (Alberts et al., 1993) while overexpression results in G₁ arrest (Berndt et al., 1997). These multiple roles of PP1 in the cell cycle may explain the striking increase in cell death we observe in PPP1R42 knockdown cells, and suggests multiple roles for this protein in regulation of PP1 function.

There are three PP1 genes, PP1 α , PP1 β and PP1 γ , which encode four isoforms. PP1 isoforms have specific functions in the events of the cell cycle and likely interact with different regulatory subunits to perform these distinct functions. PP1 β , not α or γ , is required for NEK2 mediated centrosome splitting (Mi et al., 2007); therefore, we predict that PPP1R42 interacts with PP1 β due to the effect of PPP1R42 knockdown on centrosome behavior. PP1 α also regulates phosphorylation of Rb in G₁ (Berndt et al., 1997). The gamma isoform of PP1 is involved in regulation of cytokinesis (Cheng et al., 2000). PP1 isoforms are also differentially expressed in the testis, suggestive of specific roles during spermatogenesis (Chakrabarti et al., 2007). PP1 α is located in the nucleus of spermatocytes and round spermatids implicating it in nuclear functions in these cells (Shima et al., 1993). Furthermore, mice deleted for the PP1 α gene are infertile with severely disrupted sperm development including defects in meiosis with an increase in polyploidy (Varmuza et al., 1999). These abnormalities are consistent with a role for PP1 in both cell division and flagellar development and suggest that PP1 isoforms interact with distinct regulatory subunits during male germ cell development. Further experiments are currently underway in our laboratory to determine whether PPP1R42 binding to PP1 is isoform specific.

The centrosome is a versatile organelle, nucleating microtubules at interphase, promoting spindle formation at metaphase, and acting as a basal body supporting growth of the axoneme of cilia and flagella in differentiated cells (reviewed in Kobayashi and Dynlacht, 2011). Coordination of centrosome activities with the cell cycle involves a complex network of kinases and phosphatases. PP1 is located at the intersection of several signaling pathways governing centrosome behavior in cycling and quiescent cells. Aurora A, a centrosome associated kinase essential for mitotic progression, is inactivated by PP1 and Aurora A, in turn, negatively regulates PP1 (Katayama et al., 2001). Interestingly, Aurora A participates in regulation of both mitosis and cilia formation. Activation of Aurora A causes disassembly of cilia (Pugacheva et al., 2007). Therefore, increased PP1 activity mediated by PPP1R42 is predicted to result in lengthened cilia. NEK2 kinase is also localized at the centrosome and negatively regulated by PP1. Like Aurora A, NEK2 is implicated in cilia resorption (Spalluto et al., 2012). It is thought that cilia disassembly is necessary to free the basal body to participate in events leading to cell division. By regulation of PP1, PPP1R42 acts at a critical point in the regulatory switch between cell division and differentiation mediated by the centrosome.

We demonstrate here that PPP1R42 is localized to the basal body/centrosome of ciliated ARPE-19 cells, analogous to its localization in elongating spermatids. The data presented

here support the hypothesis that PPP1R42 regulates centrosome separation through its interaction with PP1, a known regulator of NEK2 and of centrosome separation. Future experiments are planned to extend these studies to investigate the role of PPP1R42 beyond cultured cells to polarized male germ cells.

Materials and Methods

Cell Culture and siRNA transfection

Human retinal pigmented epithelial cells (ARPE-19) were obtained from American Type Tissue Collection (Manassas, VA) and grown in DMEMF12 media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin as directed by the supplier. Inhibition of *Ppp1R42* in ARPE-19 cells was performed using the ON-TARGETplus SMARTpool siRNA LOC286187 from Dharmacon/Thermo Scientific (Rockford, IL). The siRNAs represented in the pool are listed in Table 1. Cells were transfected using the Lipofectamine-RNAiMAX reagent (Invitrogen; Carlsbad, CA) according to manufacturer's recommendations. Briefly, cells were transfected for either 48 hours or 72 hours with a final concentration of 20 μ M siRNA. Transfection conditions were optimized and validated using the FITC-lamin positive control from Dharmacon/Thermo Scientific (Rockford, IL). Following incubation, knockdown was confirmed by quantitative RT-PCR and western blot.

Indirect Immunofluorescence

PPP1R42 was detected in cultured cells essentially as previously described (Wang et al., 2010). ARPE-19 cells, either untreated or treated with siRNAs, were grown to approximately 70% confluence on coverslips, fixed and permeabilized with methanol at -20°C for 10 minutes, and then nonspecific sites were blocked by incubation in 3% BSA in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100) for 30 minutes. The cells were incubated with PPP1R42 polyclonal antibody directed against the human ortholog (1:200; HPA028628; Sigma Aldrich; St. Louis, MO) in TBST overnight at 4°C followed by detection with a Texas Red-conjugated donkey anti-rabbit secondary antibody (1:100; Jackson ImmunoResearch Laboratories; West Grove, PA). α -tubulin was detected with a FITC-conjugated monoclonal anti- α -tubulin antibody (1:50; F2168; Sigma Aldrich; St. Louis, MO), β -tubulin detected with mouse anti- β -tubulin (1:25; T6557; Sigma Aldrich; St. Louis, MO), and acetylated tubulin detected with mouse anti-acetylated tubulin (1:100; T6793; Sigma Aldrich; St. Louis, MO). Claudin staining was used as a measure of cell polarization and claudin-1 was detected with a monoclonal antibody (Invitrogen; 2H1020; Carlsbad, CA). DNA was stained with DAPI incorporated into the mounting media (Vector Laboratories; Burlingame, CA).

The intracellular localization of proteins was observed with a Nikon E600 fluorescence microscope, Pan Fluor 100 $\times$ objective (N.A. 0.5-1.3) or Pan Fluor 40 $\times$ objective (N.A. 0.75), fit with appropriate filters and images captured with an Orca II CCD camera, model C4742-95 (Hamamatsu; Bridgewater, NJ) and Metamorph image analysis and acquisition software (Universal Imaging Corporation; Downingtown, PA). Images were exported to Photoshop (Adobe; San Jose, CA) and only linear adjustments to brightness and/or contrast were performed.

Western Blot

All use of animals was approved by the East Carolina University Brody School of Medicine Institutional Animal Care and Use Committee (protocol #W179c) in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Mouse testis extract was prepared as previously described (Wang and Sperry, 2011). Briefly, decapsulated testes from adult or postnatal mice were homogenized in buffer (10 mM Tris-

HCl, pH 7.0, 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine-HCl, 150 mM PMSF, and 0.1% (v/v) β -mercaptoethanol), supplemented with mammalian protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO), and centrifuged at $16,000 \times g$ to remove cellular debris. Protein samples were separated by polyacrylamide gel electrophoresis (PAGE) through 10% acrylamide gels or precast 4-20% acrylamide gels (Thermo Scientific; Rockford IL) followed by transfer from the gel matrix to PVDF membrane (BioRad Laboratories; Hercules CA) in Towbin transfer buffer. ARPE-19 total cell lysate was prepared by repeated freeze/thaw of cultured cells in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 1% NP-40 supplemented with protease and phosphatase inhibitors. PPP1R42 was detected on membranes using anti-human PPP1R42 antibody. Other antibodies used for western blot in these experiments were anti-actin (1:3,000; clone AC-15; Sigma Aldrich, St. Louis, MO), anti-phospho-PP1 (Thr320) (1:200; sc443; Cell Signaling; 2581; Danvers, MA), anti-PP1-FL18 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and anti- α -tubulin (Sigma-Aldrich, St. Louis, MO). Immune complexes bound to the membrane were detected with horseradish peroxidase-conjugated donkey secondary antibody (Jackson ImmunoResearch Inc.; West Grove, PA) diluted 1:40,000 in TTBS (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 5% milk and developed with enhanced chemiluminescent reagent as described by the manufacturer (Thermo Scientific; Rockford, IL). In each figure, protein lanes were taken from the same blot and, if separated in the original blot, this is indicated by a white line in the figure.

Co-immunoprecipitation

Protein complexes were collected by immunoprecipitation using the TrueBlot protocol according to the manufacturer's instructions (eBiosciences; San Diego, CA). Briefly, affinity purified antibody to PPP1R42 was incubated with precleared cell lysate (1 mg protein) followed by anti-rabbit IgG beads. After transfer to membrane, immunoprecipitated proteins were detected with anti- α -tubulin (1:500; Sigma-Aldrich; St. Louis, MO), and TrueBlot anti-rabbit IgG HRP (eBiosciences; San Diego, CA). Use of the TrueBlot secondary prevents detection of IgG heavy chain. Negative control for coimmunoprecipitation was a nonspecific antibody (anti-FLAG; Sigma Aldrich; St. Louis, MO) bound to beads.

Immunokinase Assay

NEK2 was immunoprecipitated from ARPE-19 cell lysates and immune complexes bound to Sepharose beads were washed 3 times with lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 1% NP-40) and twice with kinase buffer (5mM MOPS, pH 7.2, 2.5 mM B-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 5mM MgCl₂, 0.05 mM DTT). Immune complexes were resuspended in 25 μ l kinase buffer containing 10 μ g myelin basic protein as substrate (Sigma Aldrich; St. Louis, MA), 50 mM ATP with 1 μ Ci ³²P-gATP. Reactions were incubated at 30°C for 15 minutes, the immune complexes collected by centrifugation, washed four times with lysis buffer, resuspended in SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Statistical Analysis

The data for centrosome quantitation, flow cytometry, and mitotic index were expressed as mean $\pm$ SEM. The differences between the two groups were analyzed using the unpaired Student's t test. A p value of <0.05 was considered significant.

Flow Cytometry

For cell cycle analysis by flow cytometry, cells transfected for 72 hours with either the knockdown siRNA pool or the nontargeting control pool were stained with propidium iodide. Briefly, cells were trypsinized, fixed in 70% ethanol, washed in PBS, and collected

by centrifugation. Cells were then stained with 50 µg/ml propidium iodide in the presence of RNaseA. Samples were counted with a FACScan flow cytometer (Becton Dickinson; Franklin Lakes, NJ) and data was analyzed with CellQuest, version 3.3 (BD Biosciences; San Jose, CA) and ModFit LT, version 3.1 (Verity Software House, Inc.; Topsham, ME) software packages. Apoptosis in transfected cells was measured using the Annexin V-FITC apoptosis detection kit (Calbiochem; Billerica, MA) according to the manufacturer's directions.

Mitotic Index

ARPE-19 cells were cultured to 90-100% confluency, washed with PBS 3 times and serum starved in DMEM-F12, 1% Pen/strep for 24 hours to arrest cells in G₀. Serum was then added to the culture media for 24 hours. PPP1R42 message was depleted using *PPP1R42* siRNA pool (Dharmacon/Thermo Scientific; Rockford, IL) as described above and cells were cultured on coverslips for 48 hours. Cells were prepared for immunofluorescence as described above then stained using phospho-histone H3 polyclonal antibody (1:50; Cell Signaling Technology; Danvers, MA) detected with a Texas Red conjugated donkey anti-rabbit secondary antibody (1:100; Jackson ImmunoResearch Laboratories; West Grove, PA). The negative control did not include primary antibody. Coverslips were mounted using Vectashield containing DAPI (Vector Laboratories; Burlingame, CA). Cells were viewed with immunofluorescence microscopy and phospho-histone H3 positive cells and total cells counted and data analyzed for statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MTOC	microtubule organizing center
PP1	protein phosphatase-1
PPP1R2	protein phosphatase-1 regulatory subunit 2
PPP1R11	protein phosphatase-1 regulatory subunit 11
PPP1R42	protein phosphatase-1 regulatory subunit 42
ARPE-19	retinal pigment epithelial-19
NEK2	Never in Mitosis (Nim) related kinase-2
TLRR	testis leucine rich repeat
siRNA	small interfering RNA

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Figure 1. PPP1R42 localizes to the basal body of primary cilia in ARPE-19 cells.

(A) Adult mouse testis total tissue lysate (mTestis) or ARPE-19 total cell lysate (200 μ g) were separated by PAGE, proteins transferred to PVDF membrane, and probed with an antibody against the human ortholog of mouse PPP1R42 (anti-hPPP1R42) or the rabbit antibody raised against a peptide epitope of the mouse protein (anti-mPPP1R42). The molecular weight marker is listed to the left. (B) ARPE-19 cells were fixed and stained with antibodies to human PPP1R42 (red), and acetylated tubulin (green). Arrows indicate PPP1R42 positive staining at the base of cilia. An individual cell is enlarged in the inset at upper right to better illustrate PPP1R42 staining. (C) ARPE-19 cells were stained with an antibody to claudin-1 (red). (D) ARPE-19 cells were fixed and stained with acetylated tubulin in the absence of the PPP1R42 primary antibody as negative control. DAPI was used to stain nuclei (blue). Bar indicates 10 μ m.

Figure 2. PPP1R42 colocalizes with γ -tubulin in ARPE-19 cells.

ARPE-19 cells were fixed and triple stained with PPP1R42 (red), γ -tubulin (green) and DAPI (blue). (A) The merged image from all three channels, (B) the green channel corresponding to γ -tubulin staining, and (C) the red channel representing PPP1R42 staining. Size bar indicates 10 μ m. (D) PPP1R42 was immunoprecipitated with the anti-PPP1R42 antibody (R42) and the blot probed with antibodies to R42 (left panel) or to γ -tubulin (right panel). Immunocomplexes were detected using the Trueblot secondary antibody to prevent detection of IgG heavy chains (eBiosciences; San Diego, CA). Negative control for coimmunoprecipitation was an unrelated rabbit antibody bound to beads (-C).

Figure 3. Inhibition of PPP1R42 causes an increase in centrosome number in ARPE-19 cells.

(A) Protein harvested from ARPE-19 cells transfected with the inhibiting siRNA pool (KD), or a non-targeting siRNA (OT) was blotted for PPP1R42. Actin was used as a loading control. (B) The number of centrosomes per cell was counted in 3,833 cells (862 knockdown, 1,032 non-targeting, and 1,939 untreated). PPP1R42 depletion caused an approximately 10-fold increase in the number of cells containing more than 2 centrosomes (indicated on the y-axis) compared to cells transfected with the control siRNA. Examples of cells harboring supernumerary centrosomes are shown in panels C-F. Centrosomes were stained with an antibody to γ -tubulin (green) and DNA with DAPI (blue). The arrows in E and F indicate 6-8 centrosomes per cell. This number of centrosomes was never observed in control cells. The arrowheads in C and E compare the nuclei of cells containing multiple centrosomes with normal cells; cells with extra centrosomes often appear to have larger nuclei. The size of nuclei in treated and control cells was quantitated and is displayed as a bar graph (G) and scatter plot (H). The bar indicates 10 μ m.

Figure 4. PPP1R42 inhibition causes a block at the G₂/M boundary and an increase in cell death.

(A) Graphical representation of the percentage of cells in each stage of the cell cycle as determined by flow cytometry. The difference in cells in G₂ between knockdown and control cells was significant at $p=0.004$. (B) Percentage dead cells detected by flow cytometry after Annexin V staining. Asterisks indicate significance at $p=0.03$; the experiment was repeated 9 times. (C) ARPE-19 cells, treated with the control (off target, left panel) or the knockdown (right panel) siRNA, were stained with an antibody to the phosphorylated form of histone H3 along with DAPI to stain the nucleus. Bar indicates 20 μ m. (D) Percentage of cells in mitosis as determined by phospho-H3 staining. 1,846 cells were counted in a total of 3 separate experiments (906 off target, 904 knockdown); asterisks indicate significance at $p=.005$.

Figure 5. Depletion of PPP1R42 causes a decrease in PP1 protein level and an increase in phosphorylation.

ARPE-19 cells were treated with *Ppp1R42* knockdown siRNA or control siRNA for 48 hours and total cell lysate prepared. (A) 25 μ g protein was separated by SDS-PAGE, transferred to membrane and probed with antibodies to the indicated proteins. PP1-FL18 recognizes all PP1 isoforms. (B) Semi-quantitative analysis of PP1 protein expression in treated and control cells is illustrated graphically. (C) 50 μ g protein was separated by SDS-PAGE, transferred to membrane and probed with the indicated antibodies. p-PP1 represents PP1 phosphorylated at Thr320. (D) Graphical illustration of semi-quantitative analysis of phospho-PP1 expression in treated and control cells. Molecular weight markers are listed to the left in panels A and C. Each experiment was repeated 3 times and representative blots are shown.

Figure 6. NEK2 activity is increased after PPP1R42 depletion.

NEK2 was immunoprecipitated from total cell lysate prepared from cells treated with either off target (OT), knockdown (KD) siRNA, or left untreated (UT). (A) The kinase activity present in the immunoprecipitates was assayed according to Materials and Methods using myelin basic protein as substrate for phosphorylation then visualized by autoradiography. (B) A graphical representation of semi-quantitative analysis of kinase activity in control and treated cells. Each experiment was repeated 3 times and a representative blot is shown.

Figure 7. Model for PPP1R42 regulation of PP1 function in centrosome separation. NEK2 is a pivotal molecule in regulation of centrosome separation through its phosphorylation of centrosomal linker proteins causing their disengagement (reviewed in (Mardin and Schiebel, 2012)). PP1 deactivates NEK2 through dephosphorylation and therefore PP1 activity inhibits centrosome separation (represented by red circles). PP1 and NEK2 activities are reciprocally regulated through a negative feedback loop of phosphorylation/dephosphorylation. Our results support the model outlined here where PPP1R42 is an activator of PP1. Decreased PPP1R42 reduces the activity of PP1 with a corresponding increase in the level of phosphorylated and active Nek2 thereby stimulating centrosome separation in PPP1R42 knockdown cells.

Table 1
Sequence of siRNAs used in this study

The nucleotide sequences of the four siRNA present in the knockdown pool are depicted.

siRNA	Sequence
1	CGACUGACCUUGGAUCUAA
2	CCACAAAUCUGACCCACUU
3	CCAAAUCACUGGGUACAUA
4	UAGAAGGUUUAGAAGGAUU