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SHORT REPORT

PDGFRβ and oncogenic mutant PDGFRα D842V promote disassembly of primary cilia through a PLCγ- and AURKA-dependent mechanism

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ABSTRACT

Primary cilia are microtubule-based sensory organelles projecting from most quiescent mammalian cells, which disassemble in cells cultured in serum-deprived conditions upon re-addition of serum or growth factors. Platelet-derived growth factors (PDGF) are implicated in deciliation, but the specific receptor isoforms and mechanisms involved are unclear. We report that PDGFRβ promotes deciliation in cultured cells and provide evidence implicating PLCγ and intracellular Ca^{2+} release in this process. Activation of wild-type PDGFRα alone did not elicit deciliation. However, expression of constitutively active PDGFRα D842V mutant receptor, which potently activates PLCγ (also known as PLCG1), caused significant deciliation, and this phenotype was rescued by inhibiting PDGFRα D842V kinase activity or AURKA. We propose that PDGFRβ and PDGFRα D842V promote deciliation through PLCγ-mediated Ca^{2+} release from intracellular stores, causing activation of calmodulin and AURKA-triggered deciliation.

KEY WORDS: Aurora A kinase, PLCγ, Platelet-derived growth factor receptor, Deciliation, Gastrointestinal stromal tumor, Primary cilia

INTRODUCTION

Primary cilia comprise a microtubule axoneme enclosed by a bilayer lipid membrane enriched for specific receptors, such as receptor tyrosine kinases (RTKs) PDGFRα (Schneider et al., 2005) and IGF-1R (Zhu et al., 2009). The axoneme arises from the centrosomal mother centriole, which transforms into a basal body during the G1 or G0 phase of the cell cycle (Kobayashi and Dynlacht, 2011). Upon cell cycle re-entrance, the cilium disassembles, allowing centrioles to duplicate to form mitotic spindle poles (Quarmby and Parker, 2005).

Emerging evidence has linked defective ciliary disassembly to cancer, and several deciliation factors are known cell cycle regulators that are implicated in tumor growth (Pan et al., 2013; Seeger-Nukpezah et al., 2013). Central amongst these is AURKA, which is activated by most other known deciliation factors (Pan et al., 2013; Seeger-Nukpezah et al., 2013). AURKA has been implicated in deciliation in *Chlamydomonas* (Pan et al., 2004) and retinal pigment epithelial (RPE)1 cells, where AURKA has been shown to be activated by HEF1 to promote activation of HDAC6, causing deacetylation and destabilization of axonemal microtubules (Pugacheva et al., 2007), although the importance of HDAC6 for deciliation has been questioned (Goto et al., 2013). Subsequent reports have confirmed the role of AURKA in deciliation and identified additional factors that regulate its activity, including calmodulin (CaM) (Plotnikova et al., 2012), PLK1 (Lee et al., 2012), Pitchfork (Kinzel et al., 2010), Trichoplein (Inoko et al., 2012) and Peroxiredoxin 1 (Gong et al., 2014).

In serum-deprived, ciliated RPE1 and NIH3T3 cells, deciliation occurs in two waves – at the G0–G1 transition within 2 h of re-adding serum or growth factors, and at the G1–S transition (approximately 18 h post serum), which is serum- and/or growth-factor-independent (Pugacheva et al., 2007; Spalluto et al., 2013; Tucker et al., 1979). Serum-induced deciliation has been suggested to involve PDGF signaling (Pugacheva et al., 2007; Tucker et al., 1979), and one study indicates that PDGF-AA, which specifically activates homodimeric PDGFRα (Andrae et al., 2008), induces partial deciliation in RPE1 cells (Yeh et al., 2013), presumably because additional growth factors like IGF-1 are also required (Bielas et al., 2009; Yeh et al., 2013). The mechanism by which PDGF promotes deciliation is unclear, although PI3K has been implicated in the deciliation in fibroblasts that can be induced by a combination of PDGF-AA and IGF-1 (Bielas et al., 2009). Interestingly, increased expression or activation of PDGFRα or PDGFRβ has been linked to cancer (Andrae et al., 2008). For example, the constitutively active PDGFRα D842V mutant accounts for approximately 5% of all gastrointestinal stromal tumors (GISTs), and drugs targeting PDGFRα and related RTKs are frequently used to treat GISTs (Corless et al., 2011; Kudo, 2011; Pietras et al., 2003). However, it is unclear whether the oncogenic potential of PDGFRα D842V is linked to ciliary defects. Here, we investigated the involvement of PDGFRα, PDGFRβ and PDGFRα D842V in deciliation in cultured cells.

RESULTS AND DISCUSSION

PDGFRβ promotes deciliation in cultured cells

To investigate the role of PDGFRα and PDGFRβ in deciliation, NIH3T3 cells were deprived of serum for 48 h to induce ciliogenesis and incubated for 10 h with specific ligands, and the percentage of ciliated cells was quantified with immunofluorescence microscopy using antibodies against ARL13B and acetylated tubulin. This analysis showed that the ligand PDGF-DD – specific for homodimeric PDGFRβ (Fredriksson et al., 2004) – potently promoted deciliation, whereas PDGF-AA – specific for homodimeric PDGFRα (Fredriksson et al., 2004) – as well as IGF-1 and EGF failed to induce significant deciliation under these conditions (Fig. 1A). PDGF-DD also caused significant deciliation in RPE1 cells

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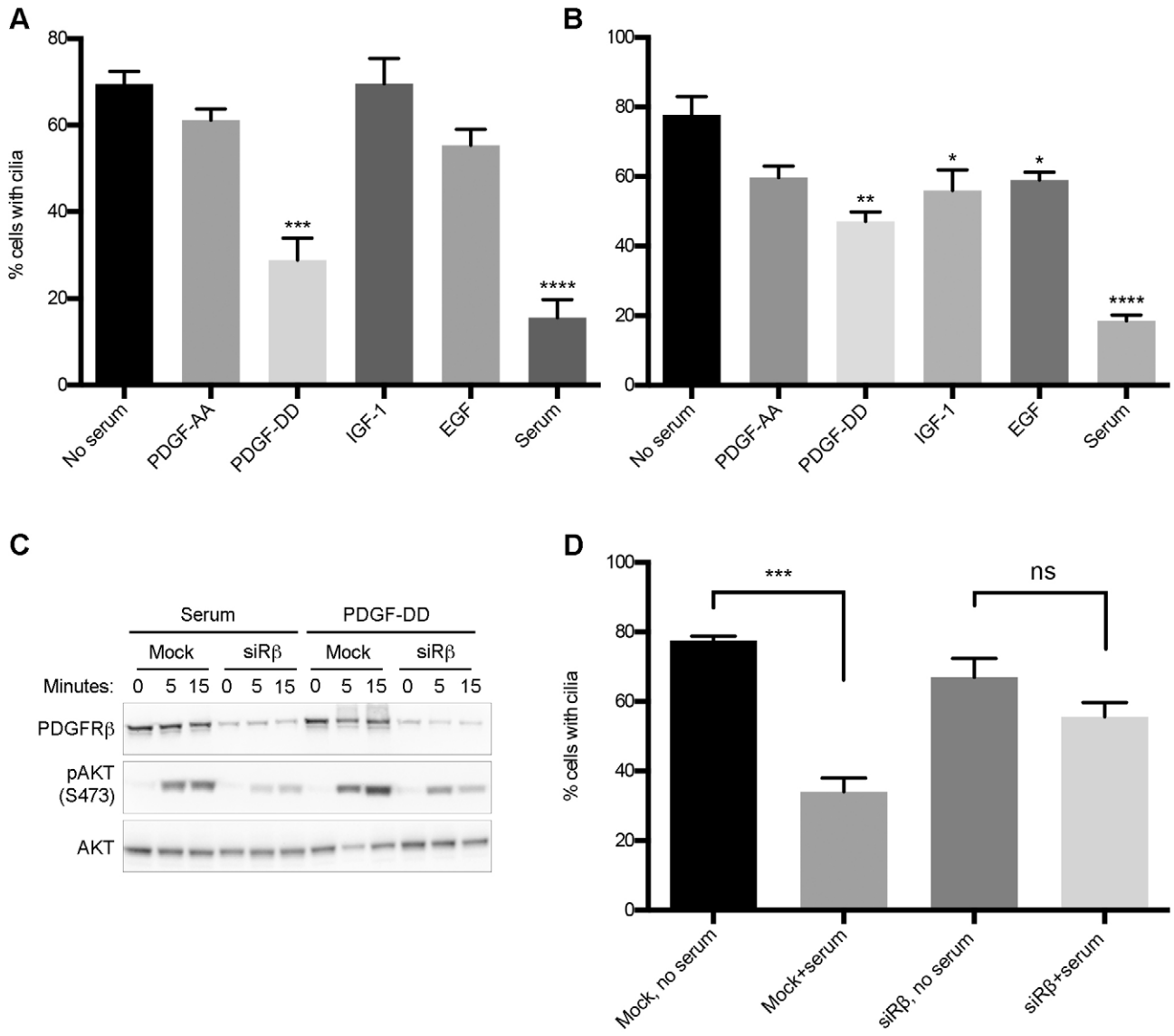

Fig. 1. Involvement of PDGFRβ in ciliary disassembly. (A) NIH3T3 cells or (B) RPE1 cells were serum-deprived (48 h) and incubated for 10 h with serum or 100 ng/ml of ligand, as indicated. Cilia were quantified by using immunofluorescence microscopy with antibodies against ARL13B and acetylated tubulin (n=3; >50 cells counted per condition). (C) Western blot of RPE1 cells that had been transfected with siRNA against PDGFRβ (siRβ). At 24 h post transfection, cells were serum-deprived (48 h) and incubated with serum or PDGF-DD (100 ng/ml) for the indicated times. (D) Quantification of cilia (as described in A) in serum-deprived mock-transfected or PDGFRβ-depleted RPE1 cells before (no serum) or 10 h after serum re-addition. ****P≤0.0001, ***P≤0.001, **P≤0.01, *P≤0.05 (see Materials and Methods for statistical tests). pAKT, phosphorylated AKT.

(Fig. 1B), but not as efficiently as in NIH3T3 cells (Fig. 1A) or in RPE1 cells that had been treated with serum (Fig. 1B), probably because additional growth factors, such as IGF-1, mediate deciliation in RPE1 cells (Yeh et al., 2013). Accordingly, modest deciliation was observed in RPE1 cells that were treated with IGF-1 or EGF (Fig. 1B). Thus, activation of PDGFRβ–PDGFRβ homodimers promotes deciliation in NIH3T3 cells and RPE1 cells, whereas activation of PDGFRα–PDGFRα homodimers does not. We cannot exclude that activation of PDGFRα or other RTKs causes deciliation under different experimental conditions – e.g. upon simultaneous activation of multiple receptors.

To substantiate the role of PDGFRβ in deciliation, we depleted the receptor from RPE1 cells with small interfering (si)RNA, which we confirmed by using a ligand stimulation assay and subsequent western blotting with antibodies against PDGFRβ and AKT phosphorylated at residue S473 (Fig. 1C), and investigated by using immunofluorescence microscopy whether serum-deprived cells could deciliate following serum re-addition for 10 h. Depletion of PDGFRβ did not significantly affect the ability of cells to form cilia but markedly impaired serum-induced deciliation (Fig. 1D). We conclude that PDGFRβ is essential for serum-induced ciliary disassembly in RPE1 cells.

PLCγ is required for deciliation in RPE1 cells

Next, we investigated the pathway involved in PDGFRβ-dependent serum-induced deciliation. PDGFRα and PDGFRβ signal through MEK1/2–ERK1/2, PI3K–AKT and PLCγ (also known as PLCG1) pathways (Andrae et al., 2008), but PDGFRβ activates PLCγ more potently than PDGFRα (Eriksson et al., 1995). Inhibitors against AKT1 and AKT2 (Akti1/2) or MEK1/2 (U0126) did not significantly affect serum-induced deciliation in RPE1 cells (supplementary material Fig. S1), whereas PLCγ inhibitor

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U73122 completely abolished serum-induced deciliation in these cells (Fig. 2A), similar to AURKA or CaM (W13) inhibition (Fig. 2A) (Plotnikova et al., 2012; Pugacheva et al., 2007). Thus, PLCγ activity is important for serum-induced deciliation in RPE1 cells. Consistently, western blot analysis confirmed that serum, PDGF-DD or EGF caused phosphorylation of PLCγ at residue Y783, whereas the effects of PDGF-AA and IGF-1 on PLCγ Y783 phosphorylation were undetectable (Fig. 2B). PDGF-AA stimulation also failed to induce phosphorylation of S473 on AKT (Fig. 2B), probably because PDGFRα is expressed at low levels in RPE cells (Lei et al., 2011).

PLCγ catalyzes formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol from phosphatidylinositol 4,5-bisphosphate, which respectively lead to activation of IP_3 receptors and release of Ca^{2+} from intracellular stores, as well as to activation of PKC (Berridge, 1993). Intracellular Ca^{2+} release triggers deciliation in *Chlamydomonas* (Quarmby and Hartzell, 1994; Quarmby et al., 1992) and mammalian cells (Plotnikova et al., 2012; Tucker et al., 1979) by activating CaM and thereby AURKA (Plotnikova et al., 2012). We hypothesized that PDGFRβ promotes deciliation by activating PLCγ-mediated intracellular Ca^{2+} release, in turn leading to activation of CaM and AURKA-triggered deciliation. Indeed, PDGFRβ-depleted RPE1 cells, which fail to undergo serum-induced deciliation (Fig. 1D), that were treated with the Ca^{2+} ionophore ionomycin deciliated to the same extent as mock-transfected control cells (Fig. 2C). Thus, forced release of Ca^{2+} from intracellular stores rescued the deciliation phenotype of PDGFRβ-depleted RPE1 cells.

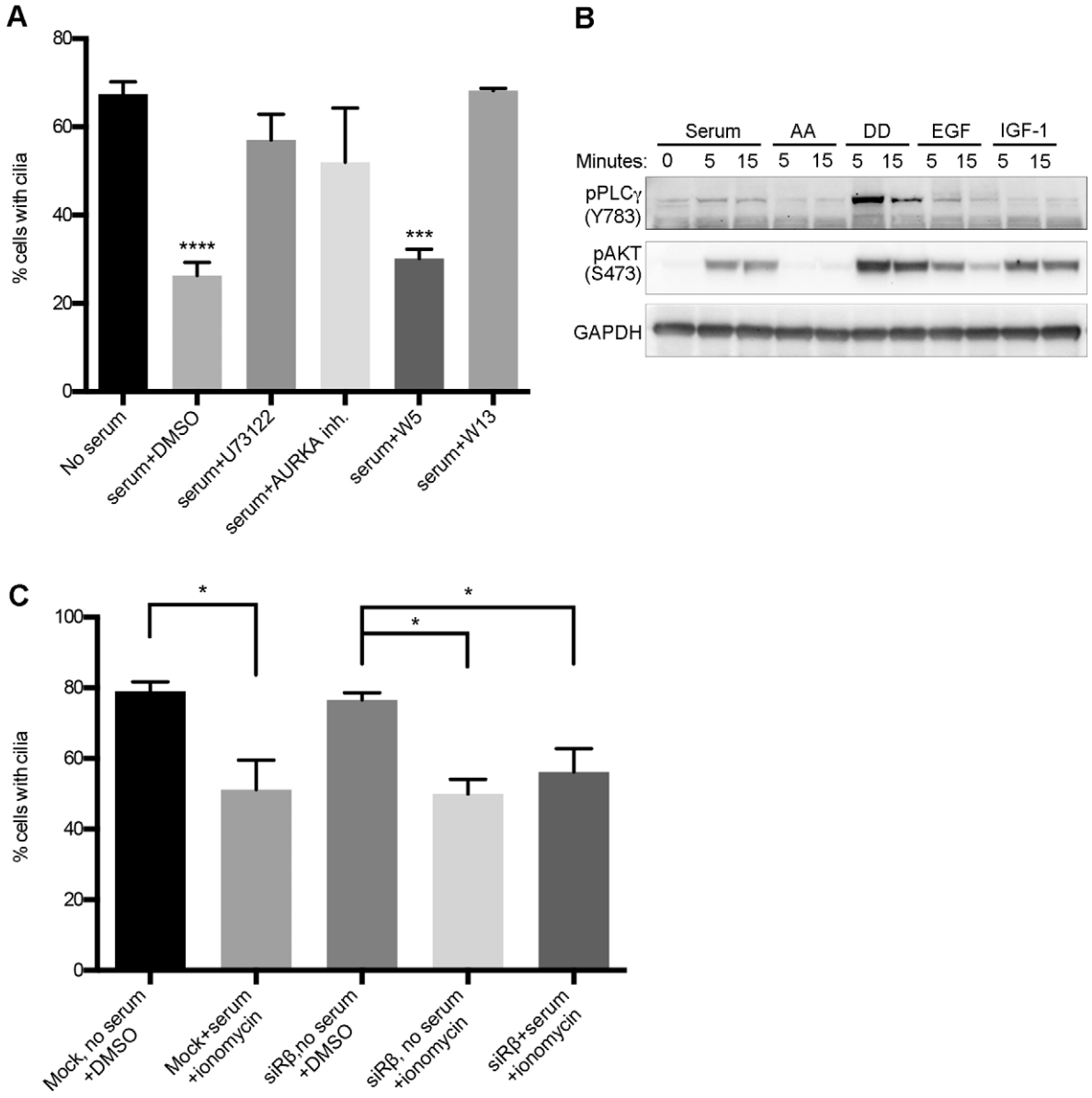

Fig. 2. PDGFRβ promotes deciliation through a Ca^{2+}-dependent mechanism. (A) Serum-deprived (48 h) RPE1 cells were incubated in serum-containing medium for 8–10 h with inhibitors against PLCγ (5 µM U73122), AURKA (0.5 µM AURKA inhibitor III, AURKA inh.) or calmodulin (50 µM W13). W5 (50 µM) is a negative control for W13. Cilia were quantified as described in Fig. 1A. (B) Western blot of serum-deprived (48 h) RPE1 cells that had been stimulated with serum or the indicated RTK ligands (100 ng/ml) for the indicated times. (C) Quantification of cilia in serum-deprived (48 h) mock-transfected or PDGFRβ-depleted RPE1 (siRβ) cells incubated without or with serum plus DMSO or 1 µM ionomycin for 4 h. ****$P \leq 0.0001$, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$ (see Materials and Methods for statistical tests). AA, PDGF-AA; DD, PDGF-DD; pAKT, phosphorylated AKT; pPLCγ, phosphorylated PLCγ.

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Expression of constitutively active PDGFRα D842V impairs ciliation in RPE1 cells

The oncogenic PDGFRα D842V mutant, which is constitutively active owing to conformational changes in the ATP-binding pocket (Corless et al., 2011; Olson and Soriano, 2009), potently activates PLCγ compared to wild-type PDGFRα (Bahlawane et al., 2015; Olson and Soriano, 2009). To confirm the importance of PLCγ signaling in deciliation, we investigated whether expression of PDGFRα wild-type (WT) and the mutant D842V affects ciliation in RPE1 cells. GFP-tagged versions of the receptors (WT–GFP and D842V–GFP) were expressed in cells, which were serum-deprived for 12 h with or without RTK inhibitors AG1296 (selective for PDGFR) and imatinib (selective for PDGFR, BCR-Abl and KIT), and cells were analyzed by western blotting with antibodies against GFP and PDGFRα phosphorylated at Y754 to assess fusion protein expression and functionality. Antibodies against phosphorylated Y754 have been used previously to demonstrate the autophosphorylation and activity of PDGFRα D842V (Heinrich et al., 2012; Moenning et al., 2009) and appear to be specific for PDGFRα–PDGFRβ heterodimers (Rupp et al., 1994). Blotting for GFP showed that WT–GFP migrated as three bands on the gel, corresponding to mature, partially glycosylated (high-mannose) and unglycosylated forms, whereas D842V–GFP was primarily in the high-mannose and unglycosylated forms (Fig. 3A), as reported previously (Bahlawane et al., 2015). The blot of phosphorylated PDGFRα (at Y754) revealed that WT–GFP (mature and high-mannose forms) was moderately phosphorylated, presumably owing to receptor clustering, and this phosphorylation was completely inhibited by AG1296 or imatinib (Fig. 3A). By contrast, D842V–GFP (high-mannose form) was strongly phosphorylated and was fully (AG1296) or partially (imatinib) resistant to inhibition. These results are fully compatible with data published previously (Bahlawane et al., 2015; Corless et al., 2011; Heinrich et al., 2012) and confirm that heterologously expressed WT–GFP and D842V–GFP behave as expected. In addition to results obtained using the antibody against phosphorylated PDGFRα (at Y754) (Fig. 3A), which point to autophosphorylated PDGFRα–PDGFRβ heterodimers (Rupp et al., 1994), we obtained similar results with an antibody against phosphorylated PDGFRα (at Y720) (data not shown), recognizing autophosphorylated PDGFRα–PDGFRα (Schneider et al., 2005). This suggests that the heterologously expressed WT–GFP and D842V–GFP fusion proteins might engage in both homodimeric (exogenous PDGFRα–PDGFRα) and heterodimeric (exogenous PDGFRα and endogenous PDGFR β) complexes in the cells, although further work is required to confirm this.

Endogenous PDGFRα localizes to the primary cilium in mouse fibroblasts and ciliary PDGFRα–PDGFRα signaling activates

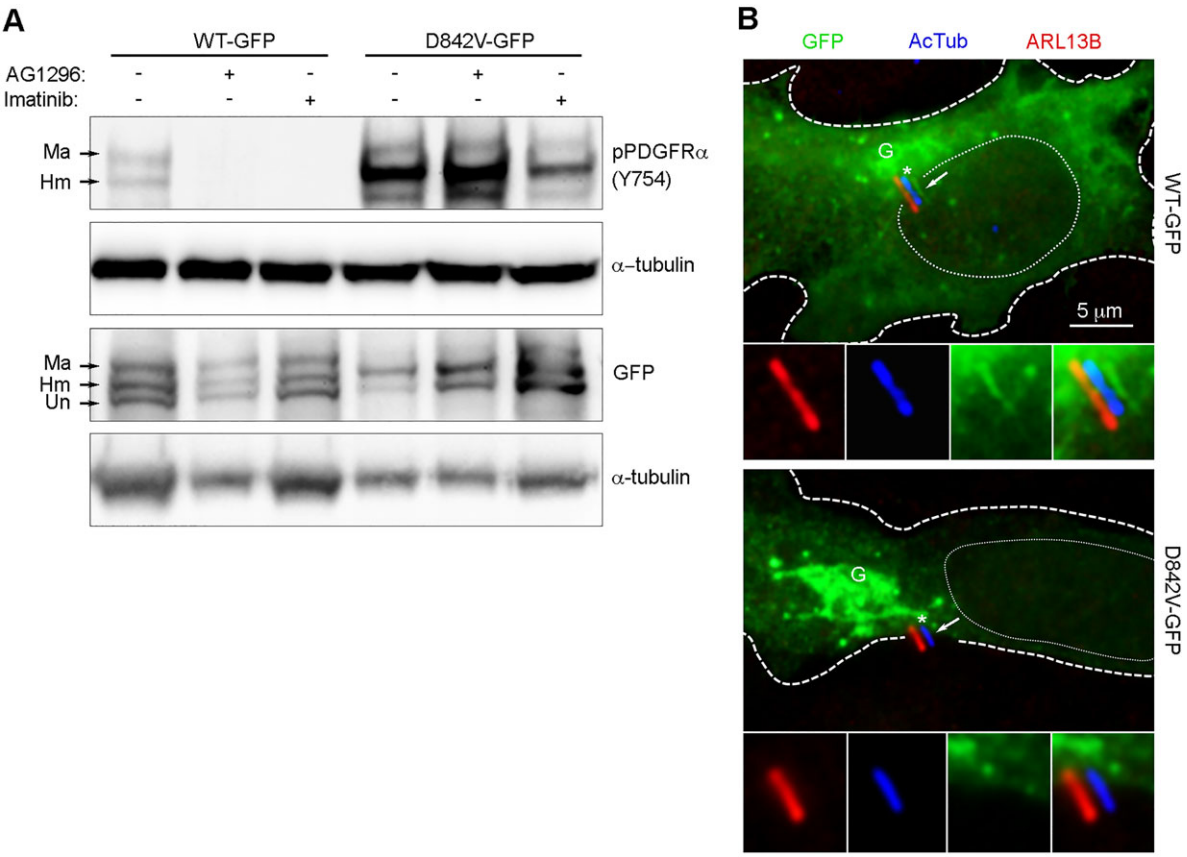

Fig. 3. Expression of WT–GFP and D842V–GFP in RPE1 cells. (A) Cells were transfected with plasmids encoding WT–GFP or D842V–GFP, incubated for 24 h in serum-containing medium followed by 12 h in medium without serum±10 μM AG1296 or imatinib. Cells were analyzed by western blotting using antibodies against the indicated proteins. Bands corresponding to mature (Ma), partially glycosylated (high-mannose, Hm) and unglycosylated (Un) GFP-tagged receptor are marked (Bahlawane et al., 2015). (B) Immunofluorescence microscopy analysis of serum-starved (12 h) RPE1 cells expressing the indicated GFP fusions using antibodies against GFP (green), ARL13B (red) and acetylated tubulin (AcTub, blue). Green, blue and red images were merged and shifted to assess colocalization of GFP-tagged receptors and ciliary markers. Arrow, cilium; asterisks, ciliary base; G, Golgi. Smaller images show enlarged views of the primary cilium region of these images. Dashed lines outline the nucleus, bold dashed lines outline the cell periphery. pPDGFRα, PDGFRα phosphorylated at the indicated residue.

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MEK1/2–ERK1/2 and AKT at the ciliary base to regulate directional cell migration (Clement et al., 2013; Schneider et al., 2010). Accordingly, immunofluorescence microscopy analysis of transfected RPE1 or NIH3T3 cells, which were serum-deprived for 12 or 24 h to promote ciliogenesis, showed that WT–GFP localized to the primary cilium and Golgi, marked by using antibodies against ARL13B or acetylated tubulin and IFT20 (Fig. 3B; supplementary material Fig. S2A). D842V–GFP localized to the Golgi in a similar manner, as reported previously (Bahlawane et al., 2015), but was not detected in cilia of the few ciliated cells observed (Fig. 3B). Indeed, most of the D842V–GFP-expressing cells examined lacked cilia (Fig. 4A), and this was not the result of over expression of the fusion protein because the average cellular expression level of D842V–GFP was approximately fourfold lower than that for WT–GFP, and their transfection efficiencies were similar (supplementary material Fig. S2B). Staining with an antibody against retinoblastoma protein (Rb) that was phosphorylated at serine residues 807 and 811 confirmed that the serum-deprived D842V–GFP-expressing cells were in growth arrest, indicating that absence of cilia was not secondary to cell cycle defects (supplementary material Fig. S3). RTK inhibitors AG1296 or imatinib did not significantly affect ciliation in D842V–GFP-expressing cells (Fig. 4A), whereas crenolanib, a potent inhibitor of PDGFRα D842V kinase activity (Fig. 4C) (Heinrich et al., 2012), restored ciliation of D842V–GFP-expressing cells to that of controls (Fig. 4B). This suggests that D842V–GFP impairs ciliation in RPE1 cells through the kinase activity of the mutant receptor.

AURKA inhibition restores ciliation in D842V–GFP-expressing cells

Because PLCγ is important for deciliation in RPE1 cells (Fig. 2A) and is potently activated by PDGFRα D842V (Bahlawane et al.,

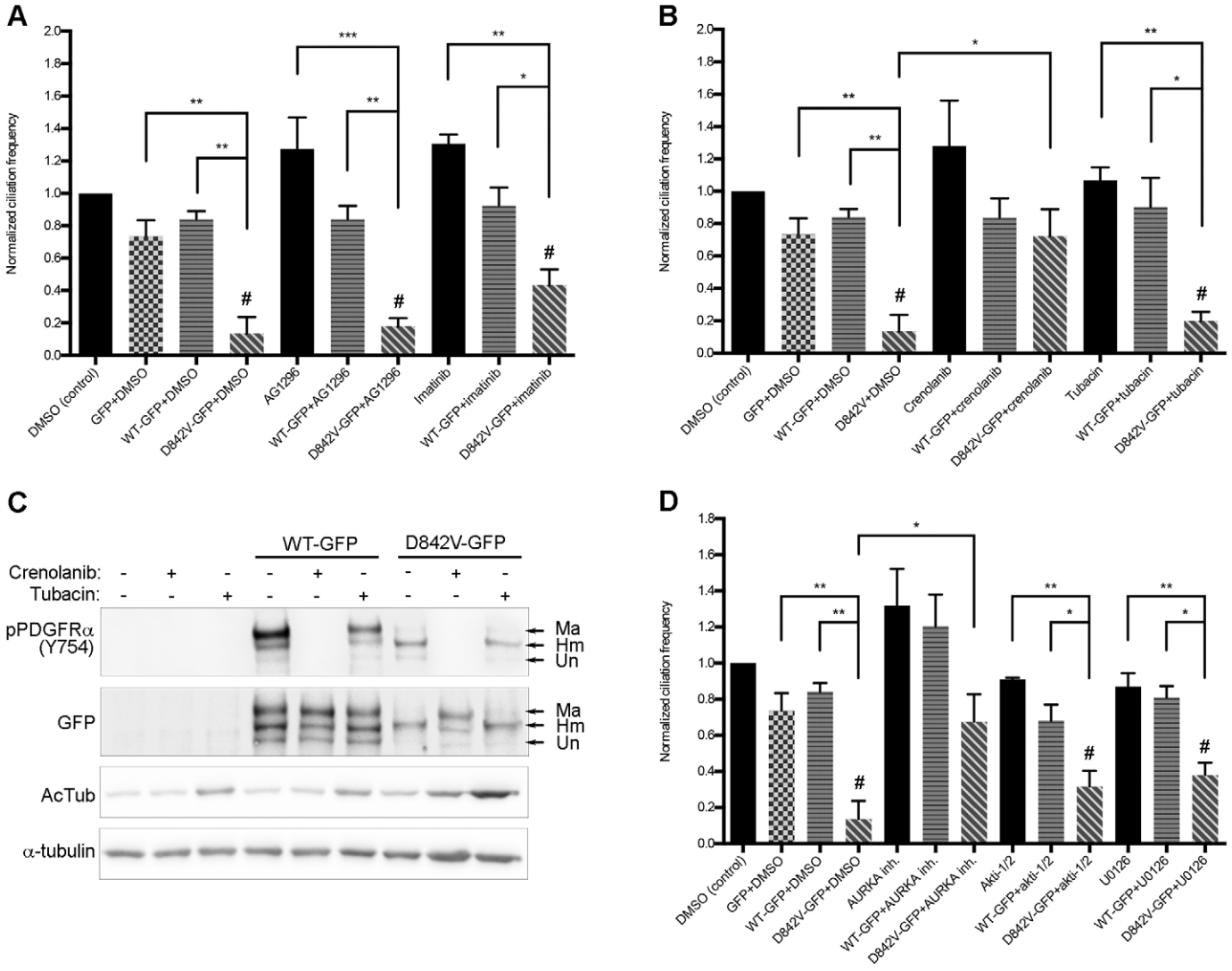

Fig. 4. Expression of D842V–GFP leads to AURKA-dependent absence of cilia. Untransfected RPE1 cells (black columns) or cells expressing GFP, WT–GFP or D842V–GFP were incubated for 12 h in serum-free medium supplemented with 10 µM of the RTK inhibitors AG1296 or imatinib (A), or 1 µM crenolanib or 2 µM HDAC6 inhibitor (tubacin) (B) before being subjected to immunofluorescence microscopy analysis with antibodies against ARL13B and acetylated tubulin (AcTub). Cilia were quantified in blinded experiments (n=3; >50 cells counted per condition), and the numbers were normalized to those of untransfected cells that had been treated with DMSO (control). (C) Western blot corresponding to the cells analyzed in B. (D) Cells were analyzed as described in A and B, but in the presence of 0.5 µM AURKA inhibitor III (AURKA inh.), 3 µM AKT inhibitor (Akti-1/2) or 3 µM MEK1/2 inhibitor (U0126). ****$P\leq0.0001$, ***$P\leq0.001$, **$P\leq0.01$, *$P\leq0.05$ (see Materials and Methods for statistical tests). #Not significantly different from each other. pPDGFRα, PDGFRα phosphorylated at the indicated residue.

2015; Olson and Soriano, 2009), we asked whether PLCγ inhibition affects ciliation in D842V–GFP-expressing RPE1 cells. Unfortunately, expression of D842V–GFP combined with incubation in serum-free medium containing PLCγ inhibitor (U73122) caused cell death. However, western blot analysis confirmed that D842V–GFP activated PLCγ, as judged by examining phosphorylation at residue Y783 (supplementary material Fig. S2C). Furthermore, inhibition of AKT and MEK1/2, two major kinases that are activated by PDGFRα (Andrae et al., 2008), did not significantly affect ciliation in D842V–GFP-expressing cells (Fig. 4D; supplementary material Fig. S2D). By contrast, inhibition of AURKA significantly restored the ciliation of D842V–GFP-expressing cells (Fig. 4D). We did not observe any effect of HDAC6 inhibition (tubacin) on the ciliation frequency of D842V–GFP-expressing cells (Fig. 4B,C), suggesting that D842V–GFP affects ciliation independently of HDAC6.

Conclusions

Collectively, our results suggest that PDGFRβ and PDGFRα D842V promote deciliation by activating PLCγ, which causes intracellular release of Ca^{2+} and activation of CaM and AURKA (Plotnikova et al., 2012). Notably, chimeric mice lacking endogenous PDGFRβ display renal cysts and glomerulosclerosis (Klinghoffer et al., 2001), phenotypes associated with defective intracellular Ca^{2+} signaling (Kuo et al., 2014) and ciliopathies (Hildebrandt et al., 2011). Because PDGFRα D842V and AURKA expression is linked to GIST (Corless et al., 2011; Yeh et al., 2014), and precursors of GIST cells are ciliated (Castiella et al., 2013), it will be interesting to study whether GIST cells display ciliary defects that are caused by elevated PLCγ and AURKA activity.

MATERIALS AND METHODS

Antibodies

For western blot, primary antibodies were (dilutions in parenthesis): rabbit anti-AKT (1:500) that recognizes all three AKT isoforms, rabbit anti-phosphorylated-AKT at S473 (1:500), rabbit anti-ERK1/2 (1:500), rabbit anti-phosphorylated-ERK1/2 at T202 and Y204 (1:500), rabbit anti-GAPDH (1:2000); rabbit anti-phosphorylated-PLCγ1 at Y783 (1:500) from Cell Signaling Technology; mouse anti-α-tubulin (1:5000) from Sigma-Aldrich; rabbit anti-PDGFRα (1:200) from Abcam; rabbit anti-GFP (1:500), rabbit anti-phosphorylated-PDGFRα at Y754 or -PDGFRα at Y720 (1:200), rabbit anti-PDGFRβ (1:200), rabbit anti-phosphorylated-PDGFRβ at Y857 (1:200) from Santa Cruz Biotech. Secondary antibodies for western blotting were horseradish-peroxidase-conjugated goat anti-mouse and swine anti-rabbit antibodies (1:4000) from Dako. For immunofluorescence microscopy analysis, primary antibodies were (dilutions in parenthesis): mouse anti-acetylated-α-tubulin (1:2000) from Sigma-Aldrich; rabbit anti-ARL13B (1:1000) from ProteinTech; chicken anti-GFP (1:2000) from Abcam; rabbit anti-phosphorylated-Rb at S807 and S811 (1:200) from Cell Signaling Technology. Rabbit polyclonal antibody against IFT20 (1:500) was from Dr Gregory Pazour (University of Massachusetts Medical School, Worcester, MA) (Follit et al., 2006). Secondary antibodies for immunofluorescence microscopy (all from Invitrogen and diluted 1:600) were AlexaFluor350-conjugated donkey anti-mouse or donkey anti-rabbit; AlexaFluor488-conjugated donkey anti-mouse and goat anti-chicken; AlexaFluor568-conjugated donkey anti-mouse and donkey anti-rabbit.

Ligands and inhibitors

Akti-1/2 and U0126 were from VWR; growth factors from R&D systems; ionomycin, PLCγ inhibitor U73122 and AURKA inhibitor III (Cyclopropanecarboxylic acid {3-[4-(3-trifluoromethyl-phenylamino)-pyrimidin-2-ylamino]-phenyl}-amide) from Sigma; calmodulin inhibitor W13 and W5 from Calbiochem. Tubacin was from Dr Stuart Schreiber (Broad Institute of Harvard & MIT, Cambridge, MA) (Haggarty et al., 2003). Stocks of ligands and inhibitors were prepared in DMSO.

Molecular biology procedures

Mouse *Pdgfra* was PCR-amplified from a cDNA clone (IMAGE ID 5704645) and cloned into pEGFP-N1 (Clontech) to create WT-GFP. D842V-GFP was generated from WT-GFP using mutated primers and standard cloning procedures. Plasmids were sequenced at Eurofins MWG Operon.

Cell culture and transfection

Cells were cultured as described previously (Schneider et al., 2005; Schrøder et al., 2011). Transfection of RPE1 cells with plasmids was performed using FuGENE® 6 (Promega). For siRNA transfection, cells were seeded to 40% confluence and transfected with *PDGFRB*-specific (5′-AAUGAUGCCGAGGAACUAUUCAU-3′) or mock siRNA (5′-UAAUGUAUUGGAAGGCAUA-3′) using DharmaFECT (Dharmacon).

SDS-PAGE and western blot analysis

SDS-PAGE and western blotting were performed as described previously (Christensen et al., 2001; Schrøder et al., 2011), except that secondary antibodies were conjugated to horseradish peroxidase, and blots were developed with the FUSION-Fx chemiluminescence system (Vilber Lourmat). Images were processed in Adobe Photoshop CS6.

Immunofluorescence microscopy and statistical analyses

Procedures for immunofluorescence microscopy have been described previously (Schneider et al., 2005; Schrøder et al., 2011). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Significance was calculated using data from three independent experiments and Student's *t*-test (when comparing two groups) or one-way ANOVA followed by Tukey's post-hoc test. Error bars denote s.e.m. *P*-values: ****$P \leq 0.0001$, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

B.S.N. and R.R.M. performed most experiments and analyzed results. F.M.S. generated recombinant plasmids and provided data for Fig. 1A and supplementary material Fig. S2A. All authors conceived and planned experiments. B.S.N., L.B.P. and S.T.C. made figures. L.B.P. wrote the manuscript with input from all authors.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.173559/-/DC1

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