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Pericentrin forms a complex with intraflagellar transport proteins and polycystin-2 and is required for primary cilia assembly

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Primary cilia are nonmotile microtubule structures that assemble from basal bodies by a process called intraflagellar transport (IFT) and are associated with several human diseases. Here, we show that the centrosome protein pericentrin (Pcnt) colocalizes with IFT proteins to the base of primary and motile cilia. Immunogold electron microscopy demonstrates that Pcnt is on or near basal bodies at the base of cilia. Pcnt depletion by RNA interference disrupts basal body localization of IFT proteins and the cation channel polycystin-2 (PC2), and inhibits primary cilia assembly in human epithelial cells. Conversely, silencing of IFT20 mislocalizes Pcnt from basal bodies and inhibits primary cilia assembly. Pcnt is found in spermatocyte IFT fractions, and IFT proteins are found in isolated centrosome fractions. Pcnt antibodies coimmunoprecipitate IFT proteins and PC2 from several cell lines and tissues. We conclude that Pcnt, IFTs, and PC2 form a complex in vertebrate cells that is required for assembly of primary cilia and possibly motile cilia and flagella.

Introduction

Centrosomes serve as microtubule-organizing centers in interphase and mitotic cells and play a role in cytokinesis and cell cycle progression (Doxsey, 2001). They are also the precursors of primary cilia, nonmotile sensory organelles found on most vertebrate cells. Ciliary dysfunctions are associated with several human diseases (Pazour and Rosenbaum, 2002; Rosenbaum and Witman, 2002). Primary cilia in vertebrate cells appear to arise from the mother centriole of the centrosome within a membrane sheath, which forms from cytoplasmic vesicles and ultimately fuses with the plasma membrane (Sorokin, 1968). The intimate relationship between the centrosome and the primary cilium suggests that functions and components may be shared between these structures.

Primary cilia assembly occurs by a process called intraflagellar transport (IFT) (Kozminski et al., 1993; Pazour and Rosenbaum, 2002; Rosenbaum, 2002; Rosenbaum and Witman, 2002; Han et al., 2003). Interference with IFT protein function results in loss or reduction of primary cilia (Pazour et al., 2000, 2002a; Pazour and Rosenbaum, 2002). Primary cilia possess cation channels and receptors that appear to activate signal transduction pathways that control cellular function (Pazour and Rosenbaum, 2002; Pazour et al., 2002a; Pazour and Witman, 2003). Polycystin-2 (PC2) is a calcium-selective channel on primary cilia associated with polycystic kidney disease (Somlo and Ehrlich, 2001). It appears to be activated by mechanical movement of primary cilia in response to fluid flow (Nauli et al., 2003), and controls the assembly of primary cilia (Thomson et al., 2003; Watnick et al., 2003). However, little is known about the mechanism by which IFT proteins and PC2 are organized at the centrioles/basal bodies (terms used interchangeably).

A role for centrosome proteins in primary cilia formation has recently been established. Mutants of a *Drosophila* protein that shares homology with the vertebrate centrosome proteins pericentrin (Pcnt) (Flory and Davis, 2003; Zimmerman et al., 2004) and AKAP450 (Keryer et al., 2003) disrupt for-

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Abbreviations used in this paper: DIC, differential interference contrast; IFT, intraflagellar transport; PC2, polycystin-2; Pcnt, pericentrin; RPE1, retinal pigmented epithelial cell line 1; siRNA, small interfering RNA.

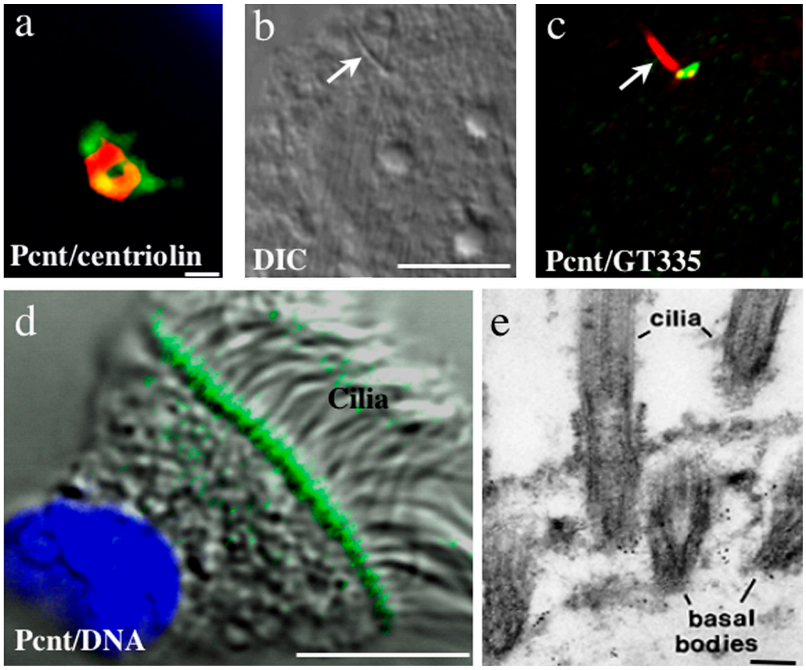

Figure 1. **Pcnt localizes to centrioles and basal bodies.** (a) Immunofluorescence image of a centrosome in RPE1 cells costained for Pcnt (green) and centriolin (red; bar, 1 μm). (b and c) DIC (b) and immunofluorescence (c) images of a primary cilium (arrow) in RPE1 cell stained for Pcnt (green) and centrioles/primary cilium (GT335, red). Bar, 5 μm for b and c. (d) Immunofluorescence image of a ciliated epithelial cell from mouse trachea showing Pcnt (green) at the base of motile cilia (DIC; bar, 5 μm). (e) Immunogold electron microscopic image of a ciliated cell (as in d) after incubation with antibodies to Pcnt and secondary antibodies bound to 5-nm gold (bar, 250 nm).

mation of mechanosensory and chemosensory cilia (Martinez-Campos et al., 2004). *Drosophila* mutants that affect IFT also disrupt formation of *Drosophila* sensory cilia (Han et al., 2003). However, the molecular mechanism by which centrosomes and centrosome proteins modulate primary cilia assembly has not been determined. In this report, we show that Pcnt forms a complex with IFT proteins and PC2 in vertebrate cells and tissues, and that Pcnt depletion by small interfering RNAs (siRNAs) disrupts centriole association of IFTs and PC2 and inhibits primary cilia formation.

Results and discussion

In this work, we have studied a larger isoform of Pcnt using specific siRNAs and antibodies unless otherwise noted (Flory and Davis, 2003; Zimmerman et al., 2004). Immunofluorescence imaging demonstrated that Pcnt partially overlapped with centriolin, a protein associated with the mother centriole at centrosomes (Fig. 1 a) (Gromley et al., 2003). In addition, Pcnt associated with both centrioles at the base of primary cilia (Fig. 1, b and c) and motile cilia (Fig. 1 d). Higher resolution immunogold EM demonstrated that Pcnt was on or near the centrioles of motile cilia (Fig. 1 e).

To test the role of Pcnt in cilia organization, we depleted protein levels by siRNA. We observed a 75–90% reduction in protein levels and a dramatic reduction in centrosome levels of Pcnt in most cells (Fig. 2, a–c; arrow in c) when compared with cells treated with control siRNAs targeting lamins A/C (Fig. 2, a and b) or cells that did not respond to siRNA treatment (Fig. 2 c, bottom cell). In contrast, centrosome localization of γ-tubulin was only slightly affected under these conditions (Fig. 2 c, top cell). Primary cilia were induced in retinal pigmented epithelial cells (RPE1) treated with siRNAs targeting Pcnt or lamin A/C. Cilia were detected with antibodies to polyglutamylated tubulins (GT335; Gromley et al., 2003) and by differential interference contrast (DIC) microscopy. In most cells treated with siRNAs targeting Pcnt, primary cilia failed to assemble (Fig. 2, e, g, and h), whereas control cells treated with siRNAs targeting lamin or ninein assembled normal full-length primary cilia (Fig. 2, d, f, and h; unpublished data).

To address the mechanism of ciliary loss in cells with reduced Pcnt, we examined centriole function, structure, and composition. Consistent with previous results from our group and others (Dammermann and Merdes, 2002; Martinez-Campos et al., 2004; Zimmerman et al., 2004), we found that microtubule organization and nucleation were not significantly disrupted (unpublished data). In addition, centriole ultrastructure was normal (Fig. 2, i–k; $n = 45$ centrosomes). Centrioles were sometimes separated (Fig. 2, e and g), but this was also observed after functional abrogation of proteins that did not affect primary cilia (e.g., ninein; unpublished data).

Because vertebrate primary cilia formation and function requires IFT proteins (Murcia et al., 2000; Pazour et al., 2000) and the cation channel PC2 (Somlo and Ehrlich, 2001; Pazour et al., 2002b; Rosenbaum and Witman, 2002; Nauli et al., 2003), we reasoned that Pcnt might cooperate with these proteins in primary cilia organization. To test this, we first determined the precise localization of these proteins. IFT57 and IFT88 localized primarily to the distal end of the mother centriole near the base of the primary cilium and to the tips and in spots along the length of primary cilia (Fig. 3, a and b). Localization of these IFTs to the distal portion of the mother centriole was consistent with known sites of IFT protein localization in *Chlamydomonas reinhardtii* (Cole et al., 1998; Deane et al., 2001). IFT20 was found on the proximal portion of mother centriole and the lateral aspect of the daughter centriole (Fig. 3 c), an area thought to be involved in interconnecting the two centrioles. PC2 localized primarily to the mother centriole underlying the primary cilium (Fig. 3 d). In mouse tracheal epithelial cells, IFT proteins partially localized with Pcnt to sites at the base

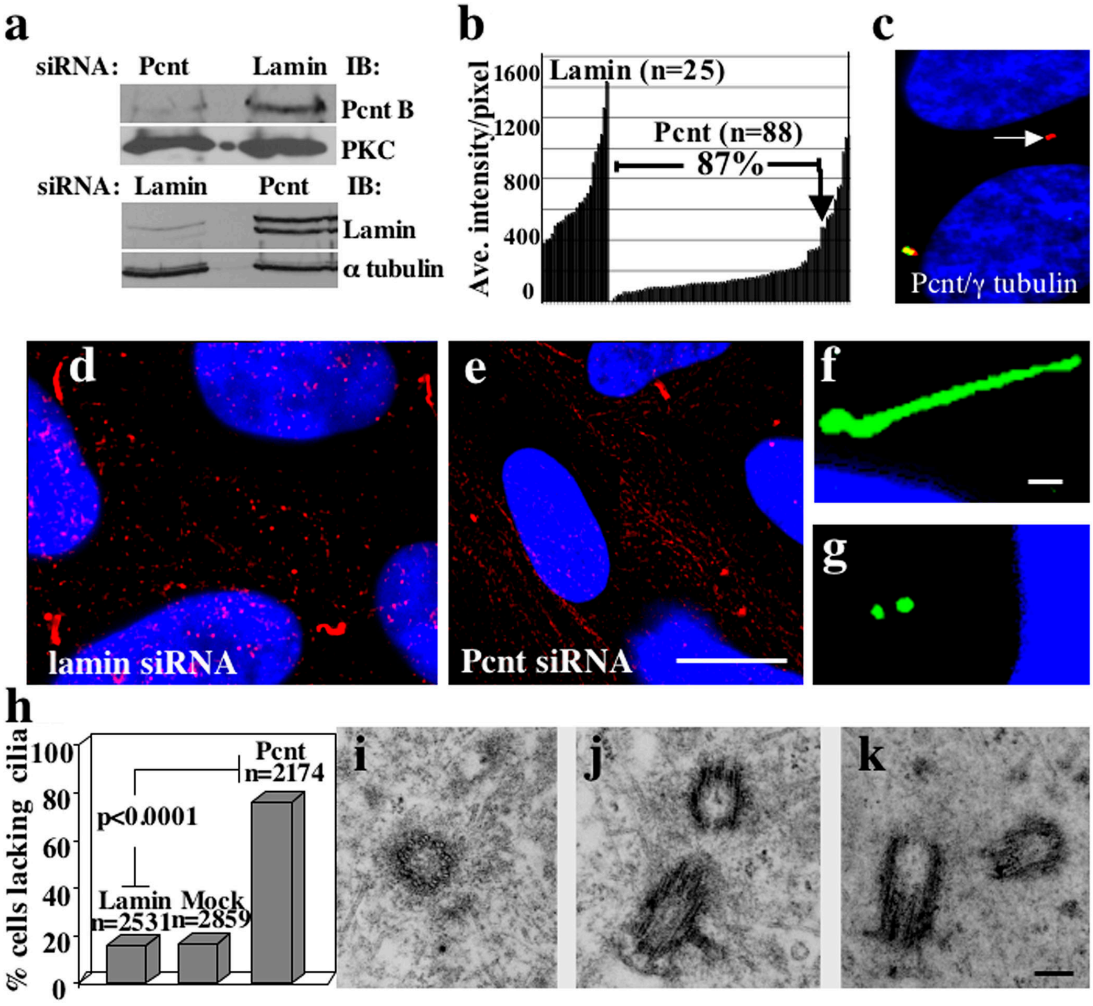

Figure 2. **Pcnt silencing inhibits primary cilia formation.** (a) Pcnt and lamin protein levels (Western blot) after siRNA as indicated. α-Tubulin or PKC, loading controls. (b) Fluorescence intensity of individual centrosomes (bars) after treatment with siRNAs targeting Pcnt or lamin. Centrosomal Pcnt is reduced to levels below the lowest control levels (lamin) in 87% of cells. (c) Immunofluorescence image of RPE1 cells after Pcnt silencing showing reduced centrosomal Pcnt in one cell (green, arrow) and normal level in the other. γ-Tubulin (red) is not significantly affected. Low (d and e) and high (f and g) magnification immunofluorescence images of cilia and centrioles stained with GT335 after treatment with Pcnt (e and g) or lamin (d and f) siRNAs. Bar in e, 5 μm (for d and e); bar in f, 1 μm (for f and g). DNA, blue. (h) Graph showing percentage of cells that lack cilia after treatment with indicated siRNAs. Bars represent average of three experiments. P value, standard *t* test. (i–k) Electron micrographs showing centriole structure in cells with reduced Pcnt. Bar in k, 200 nm (for i–k).

of the motile cilia where basal bodies are found (Fig. 3 e, IFT20).

Next, we addressed the centriolar anchoring mechanism of Pcnt, IFTs, and PC2. We found that Pcnt was dependent on IFT proteins for localization to basal bodies using cells that stably express siRNAs targeting IFT20. These cells showed reduced centriolar IFT20 and lacked primary cilia (Fig. 3, g–g′′ and h) compared with cells of the parent line (Fig. 3, f–f′′ and h). In cells with reduced centriole-associated IFT20, we observed a similar reduction in Pcnt levels (Fig. 3 g, g,′′ and i). In a reciprocal experiment, we found that IFTs and PC2 were dependent on Pcnt for centriole localization. Pcnt localized to both centrioles at the base of cilia, partially colocalized with IFT proteins (Fig. 4 a′′, IFT57) and totally overlapped with PC2 (Fig. 4 c′′). Pcnt silencing reduced the levels of centriolar Pcnt (Fig. 4 b, b′′, c, and c′′; top cell), IFT57 (Fig. 4, b′–b′′), IFT20, IFT88 (unpublished data), and PC2 (Fig. 4, c′–c′′; top cell). In contrast, adjacent nontransfected cells or cells treated with lamin siRNAs had robust staining for IFT57 and PC2 (Fig. 4, a′–a′′ and c′–c′′; bottom cell). These results show that Pcnt and IFTs are codependent in their localization to basal bodies of primary cilia.

Previous reports showed that IFT protein complexes and Pcnt complexes had similar S values on sucrose gradients (17–18S; Dictenberg et al., 1998; Pazour et al., 2002a). To determine whether Pcnt interacted with IFT proteins, we isolated IFT complexes by a multistep procedure (San Agustin and Witman, 2001) and found that Pcnt A and B cofractionated with IFT88 in the final gel filtration column (Fig. 5 a). Based on recent data showing that IFT71 is present on centrosomes and spindle poles (Iomini et al., 2004), we analyzed centrosome preparations (Doxsey et al., 1994) for the presence of IFT proteins. IFT88 (Fig. 5 b) and other IFT proteins (unpublished data) were present in pooled fractions containing centrosome proteins (γ-tubulin, Pcnt), but not in pooled fractions lacking centrosomes. Moreover, our immu-

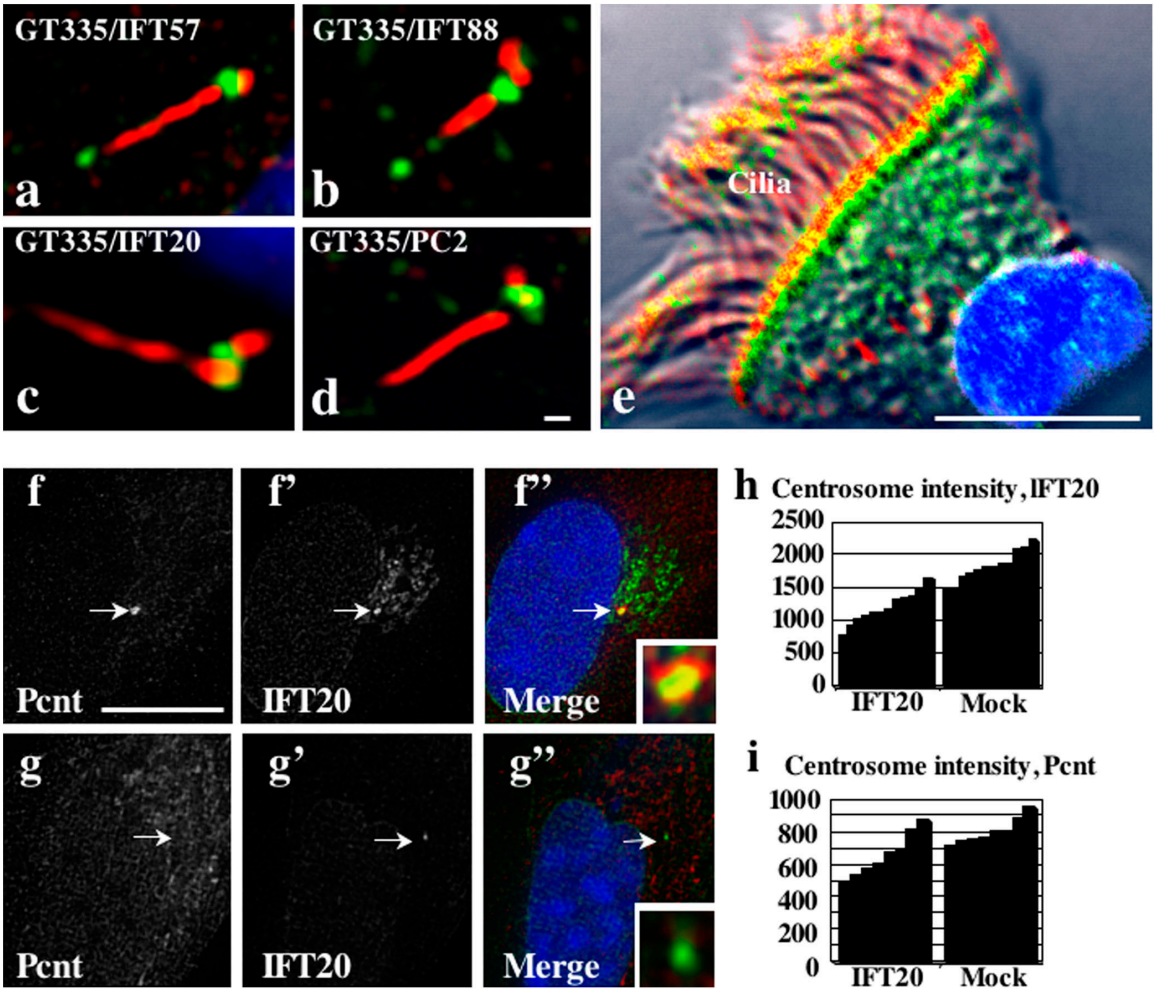

Figure 3. **Localization of IFT proteins and PC2, and mislocalization of Pcnt in cells with reduced IFT20.** (a–d) RPE1 cells stained for IFT57, IFT88, IFT20, and PC2 (green) and for basal bodies/cilia (GT335, red). Bar in d, 1 μm. (e) Pcnt (green) partially colocalizes with IFT20 (red) at the base of motile cilia (seen by DIC) in mouse epithelial cells. DNA, blue. Bar, 5 μm. (f–g′′) Untreated RPE1 cells (f–f′′) or RPE1 cells stably expressing siRNA targeting IFT20 (g–g′′) showing centrosomal levels of IFT20 (f′ and g′), Pcnt (f and g; bar, 5 μm), or merge (f′′ and g′′). Pcnt, red, IFT20, green, DNA, blue at arrows. Insets, enlargements of f′′ and g′′. (h and i) Fluorescence intensity of IFT20 (h) and Pcnt (i) at individual centrosomes (bars) in cells stably expressing IFT20 siRNA or mock, as indicated below graph.

nofluorescence imaging showed that IFT20, 57, 88, and PC2 were abundant at centrosomes in interphase cells and spindle poles during mitosis in RPE1 cells (unpublished data).

Immunoprecipitation of Pcnt using antibodies (that recognize both small, Pcnt A and large, Pcnt B isoforms) raised to two independent domains pulled down endogenous IFT88 from two ciliated cell lines (Fig. 5 c, two top panels); endogenous IFT57 from testes and ciliated cells (unpublished data), and ectopically expressed GST-GFP-IFT20 (Fig. 5 c, bottom) and endogenous PC2 from mitotic cells (Fig. 5 d). We observed no coimmunoprecipitation of any IFT protein or PC2 when Pcnt antibody was omitted (Fig. 5 d; Bd) or substituted with a nonimmune IgG (Fig. 5, c and e; IgG). In reciprocal experiments we found that PC2 immunoprecipitation pulled down endogenous IFT57 from ciliated cells (Fig. 5 e) and that IFT57 pulled down IFT88 (Fig. 5 c). Together, these biochemical data suggest that Pcnt, PC2, and IFT proteins form a complex in the cytoplasm of vertebrate cells.

The data in this manuscript show that Pcnt binds IFT proteins and PC2 and is required for primary cilia formation in human cells. This suggests a model in which Pcnt recruits protein complexes involved in cilia assembly and calcium signaling to centrioles at the base of primary cilia (and perhaps flagella). Because *Drosophila* Pcnt/AKAP450 and IFT were shown separately to function in primary cilia assembly (Han et al., 2003; Martinez-Campos et al., 2004), it is possible that Pcnt has a conserved function in IFT organization during cilia formation in both *Drosophila* and vertebrate cells.

IFT does not appear to play a role in assembly or function of *Drosophila* sperm flagella (Han et al., 2003) as seen in other organisms (Rosenbaum and Witman, 2002). Thus, it is unlikely that defects in flagellar motility in *Drosophila* Pcnt/AKAP450 mutants (Martinez-Campos et al., 2004) are a consequence of disruption of the Pcnt–IFT interaction. However, both vertebrate Pcnt (Dictenberg et al., 1998; Takahashi et al., 2002; Zimmerman et al., 2004) and *Drosophila* Pcnt/AKAP450 (Kawaguchi and Zheng, 2003) interact with complexes containing γ-tubulin, and γ-tubulin has recently been shown to be required for flagellar motility in trypanosomes (McKean et al., 2003). Thus, it is possible that disrup-

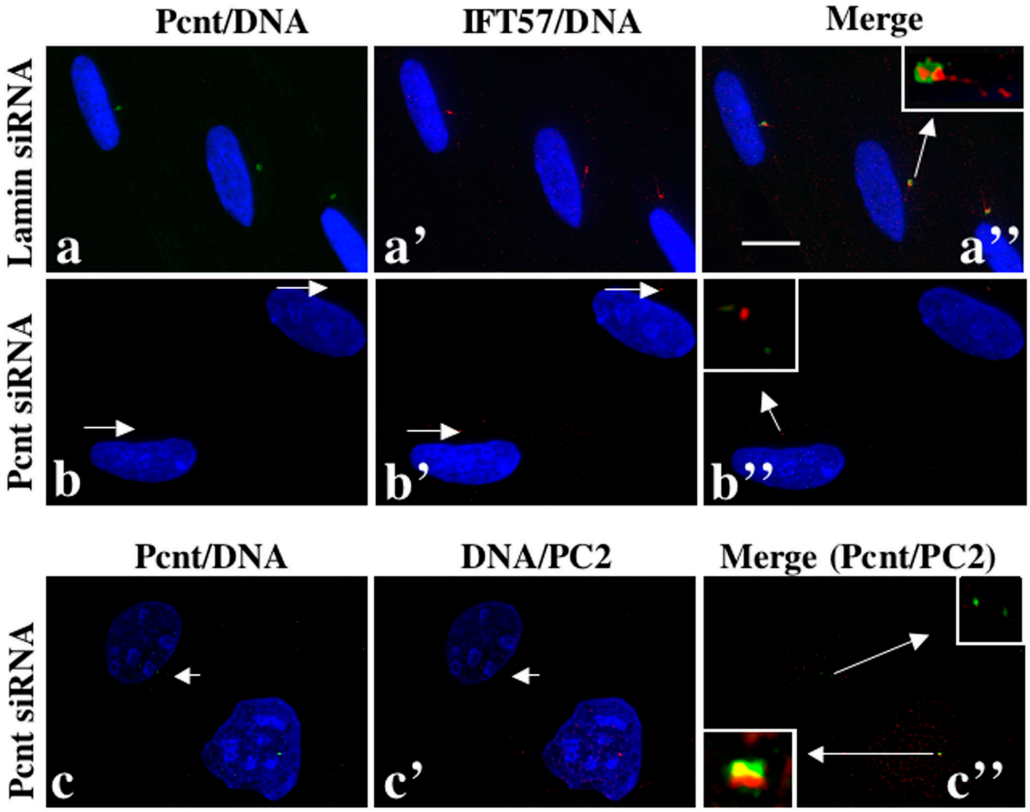

Figure 4. **Pcnt colocalizes to basal bodies with IFT proteins and PC2, and Pcnt silencing mislocalizes IFT proteins and PC2 from basal bodies and centrosomes.** (a–c'') IFT57 (a–b'' red) and PC2 (c–c'', red) are mislocalized from basal bodies in RPE1 cells with reduced Pcnt (b–b'', arrows; c–c'', green, small arrows), but not in RPE1 cells treated with lamin siRNAs (a–a''; bar, 10 μm) or in the cell with control level of Pcnt (c and c'', bottom). Insets: higher magnification of a'', b'', and c'' as indicated by arrows. DNA, blue.

tion of the interaction between Pcnt/AKAP450 and γ-tubulin complexes could account for lack of motility in *Drosophila* flagella. Another possibility is that the observed structural alterations in centrioles in spermatocytes from *Drosophila* Pcnt/AKAP450 mutants (Martinez-Campos et al., 2004) could contribute to defects in both cilia and flagella. However, in this work we did not detect changes in centriole structure in cells depleted of Pcnt. Given the recent findings that Pcnt and other centrosome proteins are integral components of cilia and flagella and that IFT proteins and PC2 are integral components of centrosomes and spindle poles (Iomini et al., 2004; unpublished data), it is likely that perturbation of proteins in one of these compartments affects the function of the other. Because defects in centrosomes and spindle poles are well documented in cells with abrogated Pcnt and Pcnt orthologues, they could also contribute to defects in centrosome derivatives such as cilia and flagella. Moreover, Pcnt and IFT proteins require molecular motors to mediate transport events, so it is possible that they utilize some of the same components to accomplish these functions (Zimmerman and Doxsey, 2000). It is clear from this discussion that a better understanding of the precise role of Pcnt in cilia and flagella assembly/function will require additional studies.

On a final note, it is interesting that centrosomes in *Drosophila* Pcnt/AKAP450 mutants are disorganized but appear to assemble normal mitotic spindles (Martinez-Campos et al., 2004). It is possible that residual functional protein remaining in *Drosophila* mutants is sufficient for spindle function. However, recent results from vertebrate cells indicate that there are several forms of Pcnt (Flory and Davis, 2003), and that a smaller form of the protein is required for spindle organization and function, possibly through its role in anchoring γ-tubulin complexes or IFT proteins at spindle poles (Zimmerman et al., 2004). A larger Pcnt isoform that shares homology with *Drosophila* Pcnt/AKAP450 does not have a dramatic effect on spindle organization (Zimmerman et al., 2004). It is likely that the multiple Pcnt isoforms contribute to a multitude of cellular functions.

Materials and methods

Cells, siRNAs, IFT isolation, and primary cilia formation

Cells used in this work, RPE1 (Morales et al., 1999), a mouse inner medullary collecting duct (IMCD3), primary cells isolated from Tg737 wild-type mouse (488) (Pazour et al., 2000), and freshly isolated primary mouse trachea cells were grown as described in American Type Culture Collection. Trachea dissected from mice in PBS were opened and scraped with a wooden applicator stick. Released ciliated epithelial cells were spun onto coverslips and fixed in −20°C methanol. siRNAs (21-nt; Dharmacon Research, Inc.) targeting Pcnt B (GenBank/EMBL/DDBJ accession no. XM_036857; nt 301–319), Pcnt A/B, or ninein (Dammermann and Merdes, 2002) and lamin A/C (Gromley et al., 2003) were delivered to cells at 200 nM (Oligofectamine; Invitrogen). We also used a stable RPE1 cell line expressing IFT20-specific siRNAs (5′-GGAAGAGTGCAAAGACTTT-3′; Follit and Pazour, in preparation). IFT protein fractions were prepared as described previously (San Agustin and Witman, 2001) using additional protease A inhibitors (Complete Mini tablets; Roche) in lysis buffer. Primary cilia were induced after siRNA treatment (72 h) by culturing RPE1 cells in medium with 0.25% serum and siRNAs for 48 h and were identified using GT335 antibody and DIC microscopy.

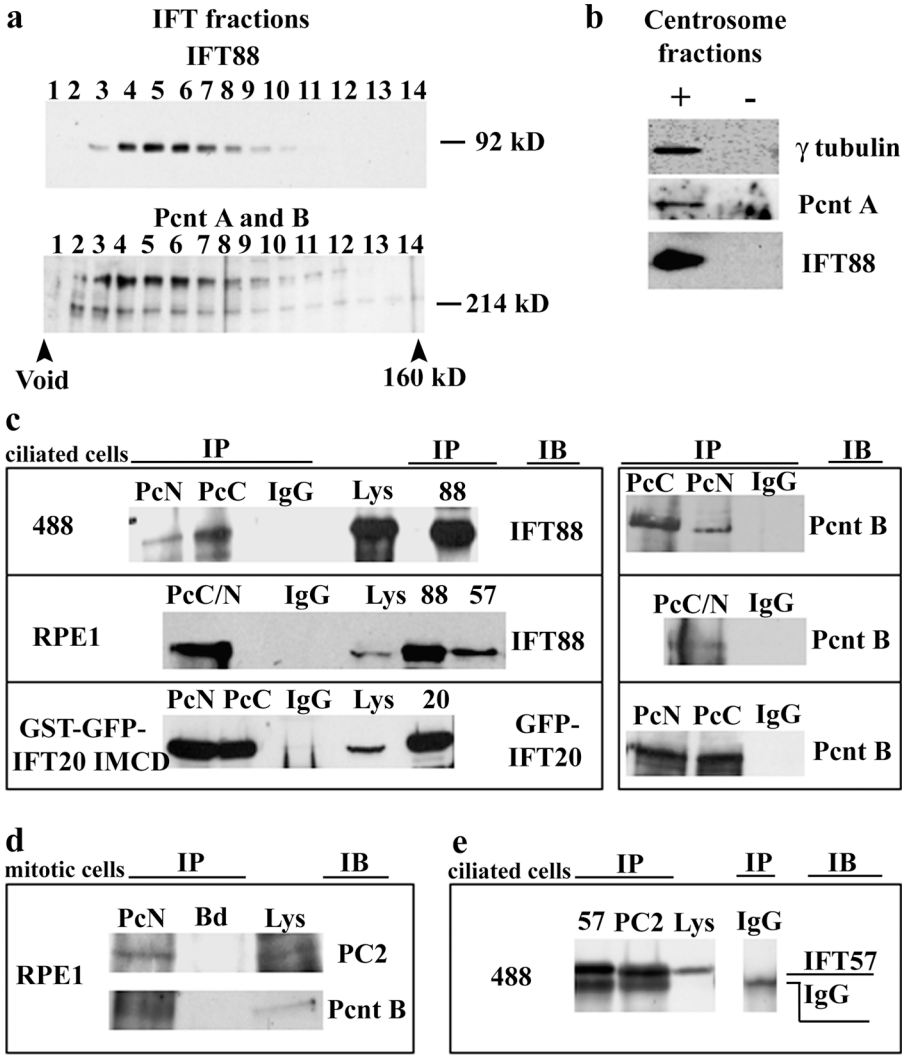

Figure 5. **Pcnt interacts with proteins involved in cilia assembly and function.** (a) Pooled IFT fractions from a sucrose gradient from mouse testes were applied to an FPLC column and fractions were loaded on SDS gels and probed with Pcnt antibodies or IFT88 antibody. (b) Pooled peak centrosome fractions from sucrose gradients (+) containing γ-tubulin, Pcnt, and IFT88 as indicated and pooled noncentrosome fractions (−). (c, top) Pcnt NH_2- and COOH-terminal antibodies (PcN, PcC) independently immunoprecipitated endogenous IFT88 from lysates of ciliated 488 cells. IgG, nonimmune rabbit IgG, lysates (Lys) showing IFT88 at right. Pcnt immunoprecipitation confirmed (right). (c, middle) PcC/N immunoprecipitated IFT88 from ciliated RPE1 cells, as did antibodies to IFT88 and IFT57 but not rabbit IgG. (c, bottom) PcN and PcC pull down a GST–GFP–IFT20 fusion protein from a cell line stably overexpressing the protein, as does a glutathione column (IFT20), but not nonimmune IgG. Blots were probed with anti-GFP antibodies, immunoprecipitation with Pcnt (right); IB, immunoblot antibody. (d) PcN immunoprecipitated PC2 from mitotic RPE1 cells, whereas beads alone did not (Bd). Pcnt immunoprecipitation confirmed by immunoblot (Pcnt B). (e) PC2 antibody, but not rabbit IgG, immunoprecipitated IFT57 from ciliated 488 cells. IFT57, top band. Antibody heavy chain, bottom band.

Immunofluorescence, EM, and RT-PCR

Cells were prepared for immunofluorescence, imaged, deconvolved (MetaMorph; Universal Imaging Corp.), and displayed as two-dimensional projections of three-dimensional reconstructions to visualize the entire cell volume as described in Gromley et al. (2003). We used methanol as fixative, then confirmed using formaldehyde fixation as previously shown (Dictenberg et al., 1998). Immunogold EM was performed as described previously (Doxsey et al., 1994). RT-PCR for amplification of Pcnt B (forward primer 5′-AACACTCTCCATGATTGCCC-3′ and reverse 5′-TACCCTCCCAATCTTTGCTG-3′) and α-tubulin was performed as described previously (Gromley et al., 2003).

Immunoprecipitation, Western blotting, and antibodies

Cells were lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 10 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA, 150 mM NaCl, 1% IGEPAL CA-1630, and Complete Mini tablets. Testes lysates were prepared as described previously (San Agustin and Witman, 2001). Antibodies were added to freshly prepared cell extracts and were incubated at 4°C overnight. Protein A/G Plus-Agarose (Santa Cruz Biotechnology, Inc.) or Glutathione Sepharose 4B (Amersham Biosciences) was washed in lysis buffer, added to the cell extracts, and incubated for 2 h at 4°C. The beads were washed and resuspended in sample buffer. 5% SDS-PAGE gels were used to detect Pcnt and PC2, and 10% gels to detect IFTs. Controls included cell extracts incubated with rabbit IgG or beads alone. No bands were seen with control IgGs under any of these conditions or when control IgGs were used at concentrations >10-fold higher than experimental samples. Cell extracts used in this work for the Pcnt IFT interactions came from cells grown in 0.25% serum for 48 h to induce cilia formation. We used affinity-purified antibodies against the NH_2 and COOH termini of Pcnt A/B (PcN, PcC; Doxsey et al., 1994; Dictenberg et al., 1998), Pcnt B (a gift of T. Davis, University of Washington, Seattle, WA; Flory et al., 2000), IFT proteins (Pazour et al., 2002a), PC2 (Scheffers et al., 2002), centriolin (Gromley et al., 2003), GT335 (a gift of P. Denoulet, Université Pierre et Marie Curie, Paris, France; Wolff et al., 1992), α-tubulin (Sigma-Aldrich), and lamin A/C (Cell Signaling Technology).

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