INTRODUCTION

Primary cilia are microtubule-based structures that originate from a centriolar anchor, known as the basal body, and extend from the apical surface of most mammalian cells. Construction of a cilium is initiated during the G1 phase of the cell cycle and cilia continue to grow as cells exit the cell cycle (G0). This complex process involves changes in both the actin and microtubule cytoskeletons and involves membrane traffic from endosomal and Golgi compartments.
Mature primary cilia receive signals for many signaling pathways such as Hedgehog (Hh), Wingless/wnt (Wnt), Notch, receptor tyrosine kinase (RTK), and TGF-β. The importance of cilia is highlighted by the range of diseases caused by defective cilia, collectively known as ciliopathies that result in blindness, obesity, cystic kidney, and psychiatric disorders. In ciliopathies, cilia may be either absent, short, elongated or contain a bulbous distal end. For example, short cilia with bulbous distal tips are caused by the inactivation of retrograde trafficking, while long cilia with bulbous tips are seen in polycystic kidney disease. To better understand ciliopathies and cilia-associated signaling pathways, many studies have now begun to focus on the molecular mechanisms that regulate vesicle traffic to the cilia.

Vesicle trafficking to cilia requires a network of microtubules and actin filaments that vesicles use as tracks to reach the basal body. DIAPH1 is a member of the DIAPH group of formins that function as key regulators of actin nucleation and polymerization, and microtubule stability. The murine homolog of Diaphanous is called mDia, and there are three mouse homologs, mDia1, mDia2, and mDia3 that are orthologs of human DIAPH1, DIAPH3, and DIAPH2, respectively. DIAPH1 is a multi-domain protein consisting of a GTPase-binding domain (GBD) at the N-terminal, flanked by an FH3 domain, which contains diaphanous-inhibitory domain (DID) and dimerization domain (DD). C-terminal to the FH3 domain is the FH1 domain that interacts with profilin and the highly conserved FH2 domain involved in actin polymerization and microtubule stability. At the C-terminus is the autoregulatory DAD domain that interacts with the GBD domain to inactivate DIAPH1. DIAPH1 is activated by binding to the small GTP-binding proteins of the Rho subfamily: RhoA and RhoC. To date, DIAPH1 has not been directly linked with primary cilia function or ciliogenesis, but other formins such as DAAM1 and INF1 have been linked to motile and primary cilia, respectively. Interestingly, DIAPH1 proteins are involved in vesicle trafficking, as DIAPH1 was shown to be involved in the formation of Golgi-derived transport vesicles, and to govern endosome dynamics. In addition, in neurons, DIAPH1 is required for the formation of synaptic endosome-like vacuoles and synaptic vesicle endocytosis is mediated by DIAPH1-dependent actin assembly. Interestingly, DIAPH1 mutations Q778X (nonsense mutation creating 86.5 KDa truncated DIAPH1) and I530S (a mutation in the coiled-coil domain that causes structural instability of DIAPH1), predicted to cause a “loss-of-function,” or at R1204X (a mutation within the C-terminal DAD domain disrupting DID-DAD interaction creating constitutively active DIAPH1), predicted to cause a “gain-of-function” for DIAPH1 in humans, result in disorders such as hearing loss, blindness, microcephaly, and obesity that are similar to phenotypes commonly found in ciliopathy disorders. In addition, DIAPH1 was shown to interact, and co-localize at mitotic spindles, with polycystin-2 protein (PC-2), a protein that is involved in autosomal dominant polycystic kidney disease wherein defective cilia are excessively long. Altogether, these studies raise the untested possibility that the DIAPH1 regulation of the cytoskeleton may also be important in the formation of primary cilia.

Here, we investigated the role of DIAPH1 proteins in ciliogenesis and cilia maintenance. Using siRNA-mediated depletion of DIAPH1, we observed impaired ciliogenesis and reduced cilia length. We specifically targeted DIAPH1 to the basal body using centrin or PACT domains and observed elongation of cilia as well as the formation of bulbous cilia tips. Moreover, targeting DIAPH increased the trafficking of post-Golgi vesicle (IFT20) and early/recycling endosomes to the base of cilia. Our data suggest a model in which DIAPH1 proteins function in regulating actin and microtubule dynamics to coordinate vesicle trafficking to the base of cilia, and consequently regulate ciliogenesis and cilia maintenance.

2 MATERIALS AND METHODS

2.1 Cell culture and transfections

Immortalized human retinal pigment epithelial cells (hTERT-RPE1) (American Tissue Culture Collection) were cultured in DMEM/F12 with 10% FBS and grown at 37°C with 5% CO₂. Human foreskin fibroblast (HFF1) cells (American Tissue Culture Collection, Manassas, Virginia, United States) were cultured in DMEM with 15% FBS and grown at 37°C with 5% CO₂. Human foreskin fibroblast (HFF1) cells were cultured in DMEM/F12 with 10% FBS and grown at 37°C with 5% CO₂. Cells were washed twice in phosphate-buffered saline (PBS) and serum-starved in DMEM/F12 or DMEM overnight to induce the formation of primary cilia. Cells were transfected with constructs and were incubated overnight for 18 hours and serum-starved for 24 hours before they were examined by immunofluorescence staining, live microscopy or immunoprecipitation. For knockdown experiments, cells were electroporated with siRNA and were incubated for 24, 48 or 72 hours and later examined for efficient protein depletion. For overexpression and rescue experiments, hTERT-RPE cells were transfected with Lipofectamine 3000 (Invitrogen, Burlington, Ontario, Canada).

2.2 Plasmid construction and siRNA

For the generation of target proteins to basal bodies, Centrin1 (CETN1)cDNA (generous gift from Laurence Pelletier, Lunenfeld-Tanenbaum Research Institute, Toronto, Ontario, Canada) and PACT cDNA (generous gift from Leonidas Tsiokas, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States) were amplified...
by PCR using EcoRI and XhoI overhangs, and ligated into the pEGFP-C1 vector (Clontech, Mountain View, California, United States) with low-expressing promoter by replacing CMV promoter with a low-expressing Sept2 promoter. The resulting vector encoded GFP-CETN1 or -PACT protein expressed at lower amounts compared to using the CMV promoter. Low-expressing GFP-CETN1-DIAPH1 constructs were made by amplifying DIAPH1 fragments and linearized vector backbone (GFP-CETN1 or GFP-PACT) using PCR. Gibson assembly kit (New England Biolabs, Ipswich, Massachusetts, United States) was used to generate the final constructs. Myc-tagged DIAPH1 (mDia1) and DIAPH3 (mDia2) (generous gift from John Copeland, University of Ottawa, Ottawa, Ontario, Canada), and myc-tagged DIAPH2 (mDia3) (generous gift from Yinghui Mao, Columbia University, New York, New York, United States). siRNA human DIAPH1 were obtained from Dharmaco, Inc. Lafayette, Colorado, United States (CTM-261432:PALOA-00007) or GenePharma Shanghai, China (DIAPH1-homo-2979B), and siRNAs were screened for their ability to knockdown protein by western blotting, and the most efficient siRNA was used for subsequent experiments including western blots.

2.3 | Drug treatments

For IMM01, hTERT-RPE cells were serum-starved for 24 hours and then treated with 100μM of IMM01 (#2406; AXON Medchem LLC, Reston, Virginia, United States) for 2 hours. For Rho inhibitor I, hTERT-RPE cells were serum-starved for 24 hours and then treated for 4 hours or 4 hours with 1.0 μg/mL of Rho inhibitor I, which causes ADP ribosylation of Rho Asn-41 (CT04, Cytoskeleton, Inc., Denver, Colorado, United States).

2.4 | Western blotting

Cell lysates were electrophoresed on an 8% gel to detect DIAPH proteins. Samples were transferred to PVDF-membrane using a standard wet transfer procedure. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.05% Tween 20 (TBST) and incubated with primary and then secondary antibodies for 1 hour each. Between each step, membranes were washed extensively with TBST (three times for 15 minutes at room temperature). The dilutions of rabbit antibodies for western blots were as follows: DIAPH1 at 1:500 (ab133683; Abcam, Cambridge, Massachusetts, United States), and GFP at 1:1000 (A-11122; Invitrogen, Burlington, Ontario, Canada). The dilutions for mouse antibodies were as follows: Myc at 1:1000 (9E10; Covance, Toronto, Ontario, Canada), GAPDH at 1:50000 (AB2302; EMD Millipore, Oakville, Ontario, Canada). The secondary antibodies, which were all horseradish peroxidase (HRP)-conjugated goat) were used at a dilution of 1:5000 to detect either rabbit or mouse primary antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pennsylvania, United States).

2.5 | Immunofluorescence

Cells plated on glass coverslips were either fixed with paraformaldehyde or ice-cold methanol depending on the antibody. Ice-cold methanol was used to examine the basal body localization of GFP-CETN1-fusion proteins. For methanol-fixation, first, the culture media was removed from cells and the cells were washed with phosphate-buffered saline (PBS) and then fixed with ice-cold methanol for 3 minutes. Later, cells were blocked in PBS with 5% fetal bovine serum or horse serum for 20-30 minutes. Cells were then incubated with primary antibodies for 1 hour, washed twice with 1XPBS, and then an appropriate secondary antibody was added for 1 hour at room temperature. Primary antibodies for DIAPH1 (ab11173; Abcam, Cambridge, Massachusetts, United States), Polycystin 2 (PC2) (ab214317; Abcam, Cambridge, Massachusetts, United States), GFP chicken (ab13970; Abcam, Cambridge, Massachusetts, United States), EHD1 (ab201354, and EB1 (ab53358) were purchased from Abcam, Cambridge, Massachusetts, United States. Arl13B (1711-1-AP), IFT20 (13615-1-AP), Rab11 (20220-1-AP), and IFT88 (139671-1-AP) were purchased from Proteintech, Rosemont, Illinois, United States. Antibody for myc (9E10 Sc-40) was purchased from Santa Cruz Biotechnology, Santa Cruz, California, United States. Antibody against basal body Centrin1 (20H5; 04-1624) was purchased from EMD Millipore, Oakville, Ontario, Canada and acetylated tubulin (T7451) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Dynnein 2 HC and Dynnein 2 LIC3 antibodies were a generous gift from Richard Vallee at Columbia University, New York, New York, United States. All fluorescently conjugated goat secondary antibodies (Alexa Fluor 488, 564, and 684 dyes) purchased from Invitrogen (Burlington, Ontario, Canada) were used at 1:500. These were washed three times with PBS. Cells were counter-stained with Hoechst (1:10000; Sigma-Aldrich, Oakville, Ontario, Canada) for DNA. Finally, coverslips were mounted using a fluorescence mounting medium from Dako, Glostrup, Denmark. For PFA-fixation, first cultured media from cells was removed and then cells were washed with PBS. Cells were then fixed in 1XPBS + 2%PFA. After 10 minutes, cells were rinsed in PBS and PFA was inactivated and cells were permeabilized using buffer A (PBS with 25 mM glycine, 25 mM ammonium chloride, and 0.1% Triton X-100) for 10 minutes. Later cells were washed with PBS and blocked in...
PBS with 5% fetal bovine serum or horse serum for 20-30 minutes. The immunostaining procedure was followed as described above.

### 2.6 Microscopy

Cells were imaged using a Quorum spinning disk confocal equipped with EMCCD digital camera (Hamamatsu ImagEM). Images were acquired using 63x oil immersion objective (HCX Plan Apochromat with a numerical aperture of 1.40–0.7; Leica) and the equipment was driven by Volocity acquisition software (Quorum Technologies, Puslinch, Ontario, Canada) and powered by a power Mac G5 (Apple). Cells were imaged using an inverted fluorescence microscope (DMI82, Leica) equipped with a Hamamatsu C9100-12 back-thinned EMCCD camera and Yokogawa CSU 10 spinning disk confocal scan head (with Spectral Aurora Borealis upgrade). Two separate diode-pumped solid-state laser lines were used at 491 nm and 561 nm (Spectral Applied Research Inc, Richmond Hill, Ontario, Canada) along with a 1.5x magnification lens. A 63x/1.4 objective was used with the following emission filters: 515 nm ± 40 and 594 nm ± 40.

### 2.7 Quantification

The length of cilia stained with acetylated tubulin was measured using the line scanning tool in Volocity software, while fluorescence intensity of the specific region of interest was determined using the ROI tool. To measure the intensity of acetylated tubulin along the axoneme of cilia, the line intensity scan tool was used. All the data of fluorescence intensity scans were subtracted from the background. For each experiment, 50 or more cilia or cells are counted. All data were plotted, and $P$ values calculated using Excel.

### 3 RESULTS

#### 3.1 DIAPH1 functions in ciliogenesis and localizes to the ciliary base

Previous studies showed formins DAAM1 and INF1 played roles in ciliogenesis of motile and primary cilia, respectively. Additionally, RhoA has previously been implicated in ciliogenesis. Since DIAPH1 belongs to a subfamily of formins that is regulated by RhoA, and because mutations in DIAPH1 gave clinical phenotypes similar to known ciliopathies, we focused primarily on DIAPH1 and tested its role in ciliogenesis. First, we examined the localization of DIAPH1 in ciliated hTERT-RPE1 relative to the axonemal marker acetylated tubulin and the basal body marker pericentrin. Using confocal microscopy, we observed that DIAPH1 is localized with pericentrin at the base of cilia (Figure 1A). To determine if the localization of DIAPH1 at the cilia base is dependent on Rho signaling, hTERT-RPE1 cells were serum-starved for 24 hours to induce ciliogenesis, and then cells were treated for 2 hours or 4 hours with Rho inhibitor that specifically inhibits RhoA, RhoB, and RhoC, but it will not affect other Rho family proteins such as Rac or Cdc42. Cells were stained with DIAPH1, acetylated tubulin, and phalloidin (Figure 1B). We observed a reduction of DIAPH1 levels at base of cilia after 4 hours of Rho inhibitor treatment in comparison to control, while no change was seen after 2 hours of treatment (Figure1C). To determine the function of DIAPH1 in cilia, two different short interfering RNA (siRNA) sequences were used to knockdown DIAPH1 in hTERT-RPE1 cells (siDIAPH1#1 by Dharmaco; siDIAPH1#2 GenePharma) (Figure 1D-G). Using western blots, we detected endogenous DIAPH1 using human-specific anti-DIAPH1 antibodies, the myc-tagged mouse homolog of DIAPH1 used for rescue, and GAPDH (loading control) (Figure 1D). To confirm that the basal body signal detected by immunostaining was true, depleted cells were stained for DIAPH1 and no signal was detected at the basal body (Figure 1E). Depletion of DIAPH1 impaired ciliation (Figure 1F) and decreased cilia length in hTERT-RPE cells (Figure 1G). Using the mouse homolog of DIAPH1, we were able to rescue both phenotypes (Figure 1D,F,G). Together, our data indicate that Rho signaling plays a role in recruitment on DIAPH1 to base of the cilia and DIAPH1 functions in ciliogenesis and determination of cilia length.

#### 3.2 Requirements for actin and microtubule functions of DIAPH1 in ciliogenesis

Since the depletion of DIAPH1 decreased cilia length, we examined the effect of DIAPH1 depletion on proteins that are involved in trafficking to cilia (Figure 2). Upon the depletion of DIAPH1, there was a dramatic decrease in the levels of Arl13b and PC1 within cilia. Smaller, but still significant decreases in the intraflagellar trains at the distal tip of cilia (as detected with IFT88) were also observed (Figure 2B, C). As well, we detected a decrease in the trafficking of Rab11 and IFT20 to the ciliary base upon the depletion of DIAPH1 (Figure 2C). These decreases were all rescued by DIAPH1 re-expression.

Since DIAPH1 proteins regulate both actin polymerization and microtubule stabilization, we investigated which of these functions were important for ciliogenesis. We created mutant forms of DIAPH1 unable to promote actin polymerization (W767A, I845A, K994A, K999A) or unable to stabilize microtubules DIAPH1 (K989A) and named them
DIAPH1ACT and DIAPH1MT, respectively. Upon the depletion of DIAPH1 in hTERT-RPE cells, neither mutant form was able to rescue the reduction in ciliation (Figure 3B) or cilia length (Figure 3C). As well the mutants were not able to rescue the reduced recruitment of proteins such IFT88, ciliary membrane (PC1 and PC2) or intraflagellar transport (IFT20), indicating that both functions of DIAPH1 are needed for ciliogenesis (Figure 3D). The reduction of IFT88 was more severe in the rescue with DIAPH1ACT mutant vs knockdown alone, perhaps suggesting that the recruitment of these two
markers to the cilia is more dependent on DIAPH1-mediated actin polymerization. Together, DIAPH1 is localized to the base of cilia and its functions in actin polymerization and microtubule stabilization are needed for ciliogenesis and maintenance of cilia length.

3.3 Basal body targeted DIAPH1 affects cilia length and morphology

To complement the depletion studies, we examined the effect of DIAPH1 activation or overexpression on cilia growth. Surprisingly, however, global DIAPH1 activation by treating cells with the DIAPH1 agonist IMM01 after serum starvation resulted in a decrease in cilia length (Figure 4A, B) with a stubby morphology (Figure 4A) likely caused by competition for effectors between cilia-localized and non-localized DIAPH1. Since the global expression of DIAPH1 reduced cilia length (Figure 4C), we assessed the cilia-specific function of DIAPH1 by targeting it directly to the basal body. For this purpose, we fused full-length DIAPH1 to either centrin, a basal body protein or a centrosome-targeting PACT domain, which in both cases allowed localized increases of DIAPH1 at the ciliary base without affecting the DIAPH1 levels elsewhere in the cell (for examples, see Figure 4E, F). GFP-centrin or -PACT tagged constructs (GFP-CETN1, GFP-PACT) were transfected into hTERT-RPE cells and the cells were serum-starved to induce ciliogenesis, and later immunostained, and the length of the cilia was measured. Integrity of the fusion protein was confirmed by western blotting with anti-GFP antibodies (Figure 4D). Cells transfected with GFP-CETN1-DIAPH1 or GFP-PACT-DIAPH1 had cilia that were longer or had a bulbous protrusion at their distal tips, as visualized by confocal microscopy (Figure 4E, F). When stratifying cilia of transfected cells with bulbs vs without, cilia with bulbs were shorter in length than cilia without bulbs or control cilia (Figure 4C). Overall, there was an increase in the percentage of cilia with a bulb upon targeting DIAPH1 to the base, but no increase in bulbous cilia following global overexpression of DIAPH1 (Figure 4G). To confirm that this phenomenon was not specific to hTERT-RPE cells, HFF1 cells were also transfected with GFP-CETN1-DIAPH1 and bulb formation and elongation were again detected (Data not shown). On average the bulbs had twice the diameter of non-bulbous tips, while no differences were detected in the intensity of GFP at the base of cilia with or without bulb. This effect was mediated by the presence of DIAPH1, since there was no change in the cilia length or percentage of ciliation caused by the transfection of GFP-CETN1 alone compared to untransfected cells (Data not shown). We also compared the rescue of DIAPH1 depletion with the global expression of DIAPH1 vs basal-body targeted DIAPH1 (Figure 4H-J). Basal-body targeted DIAPH1 was able to rescue ciliogenesis defects comparable to GFP-DIAPH1 (Figure 4I, J), but cilia were longer when basal-body targeted DIAPH1 was expressed in comparison to GFP-DIAPH1 (Figure 4J). GFP-CENT1 alone was unable to rescue ciliogenesis or cilia length defects (Figure 4I, J).

Elongation of cilia and the formation of bulbous tips when DIAPH1 proteins are targeted to the base could result from increased ciliary trafficking of the membrane and cytoskeletal proteins. Elongation of the axoneme is promoted during ciliary growth by IFT-based transport of tubulin. Since IFT is required for the elongation of the axoneme at the distal tip, we examined IFT88 intensity as a proxy for anterograde trafficking. There was an increase of IFT88 intensity in the cilia of cells transfected with GFP-CETN1-DIAPH1 (Figure 5A-C) in comparison to the control GFP-CETN1. Since DIAPH1 proteins were shown to interact with polycystin-2 (PC2) and since PC1 and PC2 are associated with ciliopathies, we examined the effect of DIAPH1 on PC2 trafficking. There was an increase in the intensity of PC2 in cilia of cells with basal body targeted-DIAPH1, suggesting an increase in the...
FIGURE 2 Decreased trafficking to primary cilia upon the depletion of DIAPH1. A-C, Depletion of DIAPH1 decreases trafficking within cilia. hTERT-RPE cells were transfected with siCtr, siDIAPH1 or siDIAPH1 and the rescue construct myc-DIAPH1 for 48 hours. A, After 48 hours of siRNA treatment, cells were fixed and stained for PC1, Arl13B, and GFP (not shown) or B, cells were fixed and stained for IFT88, EB3, and acetylated tubulin. Scale bars are 1 μm for the represented images. C, Box-and-whisker plots for average fluorescence intensity were measured to determine relative protein levels of Arl13B, PC1, IFT88, IFT20, and Rab11. Error bars represent ±SD of three independent experiments; > 100 cells per experiment. Two-tailed t test analysis was performed to compare siCtr to all samples or siDIAPH1 to the rescue samples, *P < .05, **P < .01, ***P < .005, ****P < .001
trafficking of PC2 to cilia (Figure 5A-C). Increased trafficking in and out of cilia contributes to the maintenance of cilia length, so we investigated the level of dynein proteins (retrograde trafficking) in the elongated cilia. Dynein-2 light (LIC3) and heavy chain (HC) were also increased in elongated cilia (Figure 5A-C), particularly near the base. These data suggest that basal-body targeted DIAPH1 proteins alter both anterograde and retrograde trafficking within elongated cilia.

The balance of anterograde and retrograde IFT controls ciliary architecture and in turn regulates the trafficking of cargo.
proteins. In mouse studies, defects in retrograde trafficking (IFT-B and dynein) have been linked to the formation of bulbous distal ends in primary cilia.\textsuperscript{11,43} Accumulation of IFT88 at the distal tip of bulbous cilia was detected, suggesting that bulbs form at the tip of cilia due to an imbalance in favor of anterograde over retrograde transport (Figure 5D, E). Altogether, it appears that DIAPH proteins regulate cilia length and morphology by affecting transport within cilia.
**FIGURE 4** Basal body-targeted DIAPH regulates cilia length and morphology. A, B, Activation of DIAPH decreases in cilia length. A, B, hTERT-RPE1 cells were serum-starved for 2 hours, and then treated for 2 hours with 100 µM of IMM01 or with DMSO as a negative control. A, Cells were fixed and stained for phalloidin (actin marker), actub, and DAPI. Scale bars are 10 µm for big image and 1 µm for the smaller images. B, Box-and-whisker plots for the cilia length measurement. C-G, Global overexpression of DIAPH or basal body targeted DIAPH1 regulates cilia length. hTERT-RPE1 cells were transfected with either GFP alone, GFP-DIAPH1, GFP-CETN1 (control), GFP-CETN1-DIAPH1, GFP-PACT or GFP-PACT-DIAPH1, and cells were serum-starved for 24 hours. C, Box-and-whisker plots for quantifications of cilia length with or without bulb (D) or lysates were blotted for GFP; to detect protein size and to ensure the integrity of the expressed protein, E, F, Basal body targeted DIAPH1 caused cilia elongation and bulb formation at the tips of the cilia (E) or using confocal images showing cells transfected with GFP-CETN1 or GFP-CETN1-DIAPH1 were fixed and stained for actin or microtubule (cilia marker) and GFP (for detected transfected cells). Cilia are shown in boxes (left panels) and blown up (right panels). Scale bars are 10 µm in the left panel and 1 µm in the right-side panels. F, Confocal images showing hTERT-RPE1 cells were transfected with GFP-PACT or GFP-PACT-DIAPH1 and serum-starved for 24 hours and fixed, stained for GFP and acetylated tubulin. Scale bars are 1 µm. G, Percentage bulb formation on cilia was quantified. H-J, Impairment of ciliation and cilia length by DIAPH1 depletion can be rescued by basal body targeted DIAPH1. hTERT-RPE1 cells were transfected with scrambled control (siCtr) or siRNA targeted toward human DIAPH1 (siDIAPH1) and cells were rescued using wild-type constructs GFP-DIAPH1 or basal body targeted DIAPH1 GFP-CETN1-DIAPH1 or negative control GFP-CETN1. H, Western blots of rescued cells blotted for human-specific DIAPH1, GFP, or GAPDH on the top panel. Relative intensity of DIAPH1/GAPDH is shown below panel. I. I, Quantification of ciliation and cilia length in hTERT-RPE cells treated with GFP-CETN1 or GFP-CETN1-DIAPH1 was performed to compare GFP control to the DIAPH1 with GFP to or to compare CETN1-GFP control to the DIAPH1 with GFP-CETN1 or GFP-PACT control was compared to DIAPH1 with GFP-PACT or to compare siCtr to all samples or siDIAPH1 to the rescue samples. White arrows point toward bulb formation at the tip of the cilia, and green arrows point toward the base of the cilia.

### 3.4 DIAPH1 is required for trafficking cargos to the ciliary base

The observed increase in trafficking within cilia could be due to the accumulation of cargo trafficking to the ciliary base since DIAPH1 has been associated with endosomes and post-Golgi vesicle trafficking.\(^24,25,28\) We, therefore, considered whether targeting DIAPH1 proteins to the basal body would regulate trafficking to the base.

A key player in mediating traffic of post-Golgi vesicles to the ciliary base is IFT20, and we observed an increase in its concentration near the base in cells expressing GFP-CETN1-DIAPH1 (Figure 6A, D). Additionally, we observed an increase in the level of protein markers that are associated with recycling (Rab11) endosomes (Figure 6B, D). This increase in recycling endosome accumulation was also obtained upon targeting DIAPH1 to the ciliary base using a PACT tag (Figure 6E). EHD1, a protein that acts in early endocytic membrane fusion and trafficking of recycling endosomes to the ciliary pocket was also significantly increased (Figure 6C, D). Collectively, these data indicate that DIAPH1 proteins at the base of cilia recruit post-Golgi and endosomal vesicles to the ciliary base.

### 3.5 Actin- and microtubule-dependent functions of DIAPH1 are required for cilia maintenance

To more precisely determine the relative contributions of DIAPH1-mediated actin nucleation vs microtubule stabilization in regulating cilia length, DIAPH1 mutants defective for actin or microtubule functions were examined for their ability to regulate cilia length and traffic vesicles to the ciliary base (Figure 7). hTERT-RPE1 cells were transfected with GFP-CETN1 or GFP-CETN1 fusions of wild type or DIAPH1 ACT or DIAPH1 MT mutants and their effects on cilia formation were determined. The ACT and MT mutants were not as potent as wild-type DIAPH1 at increasing cilia length or bulb formation (Figure 7A-C). These results suggest that both the actin polymerization and microtubule stabilization functions of DIAPH1 proteins play an important part in bulb formation and cilia length maintenance. In addition, we examined if ACT or MT mutants affected trafficking to the base and within cilia. In hTERT-RPE cells, reductions in the trafficking of retrograde (Dynein-2 LIC3) and anterograde (IFT88, PC2) proteins within cilia were detected in the presence of ACT and MT mutant DIAPH1 proteins (Figure 7D). As well, a reduction in IFT20 recruitment to the base of cilia was detected upon targeting DIAPH1 mutants (Figure 7D). However, none of these mutants affected the percentage of ciliated cells (Figure 7E). Collectively, these results demonstrate the requirements of both actin and microtubule functions of DIAPH1 in their effects on post-Golgi/recycling endosome traffic to the ciliary base.

### 3.6 DIAPH1 mutations mimicking diseases in humans disrupt DIAPH1 function in cilia

Since both predicted “gain-of-function” DIAPH1 mutation at R1204X and “loss-of-function” DIAPH1 mutations at Q778X and possibly I530S in humans cause disorders such as hearing loss, blindness, microcephaly, and obesity that are common in ciliopathy disorders, the effects of these DIAPH1 mutations were examined on primary cilia (Figure 8A).
R1204X is a truncation mutation located in the DAD domain that would be expected to disrupt the autoinhibitory DID-DAD interaction, thus creating constitutively active DIAPH1 lacking the c-terminus. R1204X causes autosomal dominant non-syndromic sensorineural hearing loss, DFNA1, characterized by progressive deafness starting in childhood and by morphological abnormalities in the actin-based stereocilia structures of the inner ear, causing them to be short, fused,
FIGURE 5 Changes in anterograde and retrograde trafficking within the cell upon basal body targeted-DIAPH1. A-C, Elongated cilia upon basal body targeted-DIAPH1 show an increase in anterograde and retrograde trafficking. A, hTERT-RPE1 were transfected with GFP-CETN1 or GFP-CETN1-DIAPH1 and serum-starved for 24 hours and later fixed and stained for GFP (not shown) and acetylated tubulin and IFT88 or PC2 or Dynein-2 LIC3 or Dynein-2 HC. Scale bars are 3 μm for each image. B, Line intensity graph of a representative cilium and (C) average intensity for IFT88, PC2, Dynein-2 LIC3 or Dynein-2 HC were determined in cilia upon targeting DIAPH1 to the basal body. D-E, Bulbed cilia upon basal body targeted-DIAPH1 show retrograde trafficking defects. D, hTERT-RPE1 were transfected with GFP-CETN1 or GFP-CETN1-DIAPH1 and serum-starved for 24 hours and later fixed and stained for GFP (not shown) and acetylated tubulin and IFT88. Scale bars are 3 μm for each image. E, Box-and-whisker plots for average intensity of IFT88 at the tip of the cilia with bulb. Error bars represent ± SD of three independent experiments; n = 50 each, *P < .05, **P < .01, ***P < .005, ****P < .001. Two-tailed t test analysis was performed to compare GFP-CETN1 control to the rest of samples.

FIGURE 6 DIAPH1 functions in trafficking vesicles to the base of cilia. A-D, Targeting DIAPH1 to the cilia base using CETN1 increased in trafficking to the ciliary base. hTERT-RPE1 were transfected with alone GFP-CETN1 or GFP-CETN1-DIAPH1. After 24 hours of serum starvation the cells were fixed and stained with antibodies specific for acetylated tubulin, GFP, and (A) IFT20, (B) Rab11, (C) EHD1. Scale bars are 5 μm for each image. D, Quantification of signals at the cilia base were conducted for the fluorescence protein intensity scan. E, Targeting DIAPH1 to the cilia base using PACT increased Rab11 trafficking to the ciliary base. hTERT-RPE were transfected with GFP-PACT or GFP-PACT-DIAPH1, and after 24 hours of serum starvation, fixation, and staining for acetylated tubulin, GFP, and Rab11 (not shown). Box-and-whisker plots for the quantification of Rab11 signal near the cilia base are presented. Error bars represent ± SEM of three independent experiments; n = 50 each, *P < .05, **P < .01, ***P < .005, ****P < .001. Two-tailed t test analysis was performed to compare GFP-CETN1 control to the rest of samples.
elongated or sparse. In the case of the Q778X mutation, homozygous patients with this nonsense mutation in the FH2 domain have microcephaly, blindness, early onset seizure, development delay, and bronchiectasis. This nonsense mutation leads to a truncated 86 kDa protein lacking the carboxyl-terminus bearing the highly conserved region of FH2 that is responsible for DIAPH1’s function in actin polymerization and microtubule stabilization. A third mutation, I530S in the highly conserved coiled-coil domain of DIAPH1, showed non-syndromic sensorineural hearing loss likely due to decreased structural instability of DIAPH1.

We reasoned that mutations such as R1204X, Q778X, and I530S could affect DIAPH1 function in ciliogenesis and cilia maintenance. Using mouse homologs of DIAPH1, we generated GFP tagged constructs with the individual mutations R1204X, Q778X, or I530S. We used these mutants and

**FIGURE 7** DIAPH1 functions trafficking vesicles to the ciliary base is actin- and microtubule dependent. A-C, Decrease in elongation and bulb formation upon targeting DIAPH1 mutants for actin and microtubule to the ciliary base. hTERT-RPE1 cells were transfected with GFP-CETN1, GFP-CETN1-DIAPH1WT, mutant GFP-CETN1-DIAPH1ACT or GFP-CETN1-DIAPH1MT (A) After 24 hours of serum starvation, fixation, and staining for acetylated tubulin, GFP. Scale bars are 3 μm for each image. A green arrow indicates the signal at the basal body. Quantifications of (B) cilia length and (C) percentage of bulb formation were conducted. D-E, DIAPH1 function in vesicle trafficking is actin and microtubule dependent. hTERT-RPE1 cells were transfected with GFP-CETN1, GFP-CETN1-DIAPH1, GFP-CETN1-DIAPH1ACT or GFP-CETN1-DIAPH1MT. After 24 hours of serum starvation, the cells were fixed and stained with antibodies specific for acetylated tubulin, GFP and Dynein-2 LIC3, IFT88, PC2 or IFT20. D, Box-and-whisker plots for the quantification of signals at the cilia were conducted for the fluorescence protein intensity scan. E, Percentage of cells with cilia was quantified. Error bars represent ±SEM of three independent experiments; n = 50 each, *P < .05, **P < .01, ***P < .005, ****P < .001. Two-tailed t test analysis was performed to compare GFP-CETN1 control to all of the samples and as well WT DIAPHs were compared to mutant DIAPH1s. Green arrows point toward the base of cilia.
wild-type DIAPH1 constructs to rescue the phenotype of ciliogenesis and cilia length defects following DIAPH1 depletion. Efficacy of the knockdown was shown by western blot probing with a human-specific anti-DIAPH1 antibody, GFP to detect rescue constructs and GAPDH as a loading control (Figure 8B). We found that none of these mutants were able to rescue the impairment of ciliogenesis upon the depletion of DIAPH1 (Figure 8C), further suggesting that the normal, regulated function of DIAPH1 is required for ciliogenesis. However, some properties associated with DIAPH1 depletion were rescued. For example, DIAPH1 R1204X was able to rescue the reduction of cilia length.
PC2, and IFT88 in cilia (Figure 8C, D). Interestingly, no mutants were able to rescue the reduction of IFT20 levels at the cilium base upon the depletion of DIAPH1 (Figure 8D). Hence, the full function of DIAPH1 is required to achieve the spectrum of properties seen during ciliogenesis.

We generated CETN1-fusion constructs for each of these mutations and tested their effects on ciliation and cilia maintenance. All mutants of DIAPH1 showed cilia abnormalities (bulged or very long), but constitutively active DIAPH1 mutant R1204X showed an increase in extremely long (more than 6 μm long) cilia in comparison to control wild-type DIAPH1 (Figure 9A, B, D), while there was an increase in the disruption of acetylated tubulin patterns in all mutants in comparison to wild-type DIAPH1 (Figure 9C). There was an increase in bulb formation for all mutants in comparison to control GFP-CETN1, but the mutation that caused microcephaly, Q778X (disruption of CC domain) showed an increase in bulb formation (Figure 9B), a decrease in cilia length (Figure 9C, D) and a decrease in the percentage of cells with cilia (Figure 9B) in comparison to wild-type DIAPH1. Furthermore, we observed DIAPH1 mutants Q778A and I530S disrupted trafficking in cilia (Figure 9E). There was a reduction in the level of IFT88 and PC2 proteins at the base of cilia upon targeting DIAPH1 mutants (Q778X and I530S) to the base in comparison to wild-type DIAPH1, indicating the importance of the proper function and structure of DIAPH1 proteins in regulating traffic (Figure 9E). As well, the level of IFT20 was reduced in all mutants of DIAPH1, indicating that the proper function of DIAPH1 is required for IFT20 trafficking (Figure 9E). Moreover, the effects of these mutations on primary cilia suggest that the similarity between the clinical phenotypes caused by DIAPH1 mutations and those seen in classical ciliopathies may be in part rooted in primary cilia defects in human DIAPH1 mutant patients. Given our findings and the requirement for DIAPH in ciliogenesis and cilia maintenance, we can theorize that each mutant DIAPH1 might disrupt a different function of DIAPH1.

**DISCUSSION**

Regulation of the cytoskeleton and of endosomal and post-Golgi membrane traffic has been implicated in ciliogenesis and cilia maintenance. RhoA signaling plays a critical role in ciliogenesis by regulating the actin structures for basal body docking to the plasma membrane and subsequent axoneme growth. One well-characterized RhoA effector is DIAPH1, a formin involved in actin polymerization whose role in ciliogenesis had not been examined. Interestingly, DIAPH1 also interacts with the +TIP microtubule-binding EB proteins and the tumor suppressor adenomatous polyposis coli (APC) to form a tripartite complex involved in stabilizing microtubules. RhoA, and EB proteins have previously been localized to the base of cilia. EB1 was shown to bind to microtubule minus-ends, anchoring them at the basal body and in turn facilitating vesicular trafficking to the base of cilia. APC has also been implicated in cilia function, raising the possibility that DIAPH might function with EB1 and APC in ciliogenesis. Since DIAPH proteins had also previously been shown to participate in endosome and Golgi vesicle trafficking, we reasoned that DIAPH proteins may have several possible roles in ciliogenesis or cilia maintenance. This hypothesis is bolstered by the fact that mutations of DIAPH1 in humans give phenotypes commonly seen in ciliopathies.

Our data indicate that RhoA signaling regulates the recruitment of DIAPH1 to the base of the cilia and siRNA-depletion of DIAPH1 resulted in decreased cilia frequency and length. Depletion of DIAPH1 impairs ciliogenesis, possibly during axonemal elongation since decreases in cilia length were detected following depletion. However, it is also possible that impairment in ciliogenesis upon DIAPH1 depletion could be due to a defect in endocytic recycling, since we detected a decrease in IFT20 and Rab11 accumulation. As well, our findings that neither actin polymerization nor
microtubule stabilization mutants of DIAPH1 rescue ciliation suggests that both functions of DIAPH1 are important in this process. Other studies have shown that changes in either actin or microtubule networks can influence cilia length by changing the level of soluble tubulin⁴⁹ or regulating the trafficking of vesicles to the base of cilia.⁵⁰
Since ciliogenesis consists of multiple steps that include post-Golgi vesicles docking to distal appendages of the M-centriole to create distal appendage vesicles (DAVs), ciliary assembly includes ciliary membrane formation (EHD1, SNAP29, PACSIN1/2) and IF20 recruitment of post-Golgi vesicles containing TZ proteins, and ciliary extension (the Rab8-Rab11 cascade) and elongation,\(^{51,52}\) it is possible that DIAPH1 works at several steps of ciliogenesis. We observed that the level of proteins involved in trafficking proteins to cilia, such as IFT88, membrane proteins (PC1 and PC2), IFT20, and Rab11 were reduced. Thus, the depletion of DIAPH1 appears to decrease the trafficking of vesicles to the ciliary base which eventually leads to decreases of IFT trafficking within cilia and a subsequent decrease in cilia length. Our results are consistent with previous studies indicating that cilia length and maintenance are regulated by IFT through the bidirectional transport that moves cargo along the axonemal microtubules of cilia.\(^{50}\)

To maintain steady-state cilia length, the intrinsically unstable axoneme requires an ongoing supply of building material.\(^{50}\) According to the supply-limitation model, cilia will grow until the cell body pool of ciliary precursors is exhausted. The availability of cargoes, therefore, regulates IFT cargo loading and cilia length.\(^{50}\) Our results are also consistent with this model, where we show basal body targeted DIAPH1 caused elongation or the formation of bulbs at the tips of cilia, probably owing to their ability to traffic vesicles and cargo to the base of the cilia. We observed increases in post-Golgi (IFT20) and early (EEA1) and recycling (Rab11) endosomes at the ciliary base. Coincident with cilia elongation and bulb formation by DIAPH1, we noted increasing IFT (anterograde and retrograde) within cilia and defective retrograde trafficking, respectively. What causes the decrease in retrograde trafficking in cilia with bulbs is not clear, but it is possible that membrane addition exceeds tubulin polymerization; thus, free tubulin levels may be a limiting factor. Shedding of cilia membrane as vesicles that contain IFT and ciliary membrane proteins from the tip of cilia has been previously described to occur as a normal means to downregulate Hh signaling, and this also occurs in cases of defective retrograde traffic (eg, mutant BBS or IFT proteins).\(^{53,54}\) These structures, also known as ectosomes, are also shed from cilia during resorption prior to mitosis; in this case, shedding is known as decapitation.\(^{55}\) Since no change in cilia number was detected in cells expressing basal body targeted DIAPH1, bulb formation was not likely to be due to cilia resorption. Interestingly, ectosome formation involves the actin cytoskeleton,\(^{56}\) and we observed less bulb formation when actin nucleation mutants of DIAPH1 were expressed. Therefore, this may indicate that the presence of bulbs at the tips of cilia facilitates the trafficking of excess cargo from cilia in a manner similar to ectosome formation. Therefore, excess DIAPH1 at the base may favor anterograde over retrograde trafficking of membranous material, resulting in a buildup of membrane and membrane proteins. If cilia can grow longer, this excess will be absorbed, but when cilia length is limited, perhaps by the supply of tubulin dimers, ectosomes form to eliminate the excess material at the distal tip.

Evidence for the role of DIAPH in ciliogenesis is supported by knockout mouse studies.\(^{56}\) Mice lacking both mDia1 and mDia3 displayed impaired tangential migration of neurons, a process regulated by primary cilia in neurons.\(^{56,57}\) Additionally, disruption of the neuroepithelial apical surface and periventricular dysplasia were also detected in mice deleted of mDia1/3. mDia1 knockout mice showed defects in T cell chemotaxis and migration.\(^{58,59}\) Interestingly, homozygous loss of DIAPH1 causes microcephaly in humans,\(^{31}\) and microcephaly is a phenotype frequently seen in ciliopathy patients.\(^{60}\)

The connection between DIAPH1 and ciliopathies is supported by our findings that mutant forms of DIAPH1, when targeted to the basal body, caused pronounced defects in cilia, and some mutations were unable to rescue the impairment of ciliation, cilia length or trafficking upon the depletion of DIAPH1. The “gain-of-function” constitutively active DIAPH1R1204X form behaved like overexpression of wild type, as it was able to rescue the cilia length impairment, intensity of acetylated tubulin, and the reduction in the levels of IFT88 and PC2, but it was unable to rescue the impairment in ciliation frequency and IFT20 level upon DIAPH1 depletion.
Moreover, the expression of the presumed “loss-of-function” forms of DIAPH1 (Q778X and I530D) were not able to rescue ciliogenesis, cilia length or decreases in PC2, IFT88 or IFT20, thereby mimicking the depletion of DIAPH1.

Patients with Q778X suffer from a variety of phenotypes seen in ciliopathies such as microcephaly, blindness, early onset seizure, developmental delay, and bronchiectasis,31,32 which could be due to the lack of functional DIAPH1. I530S located in the coiled-coil domain is another DIAPH1 mutation that causes hearing loss in patients.33 Previous studies showed that the loss of hearing caused by DIAPH1 R1204X mutant (which is constitutively active DIAPH1) or by overexpression of wild-type DIAPH1 or DIAPH3 in mice, resulted from defects in stereociliary bundles.30,61-63 But whether DIAPH3 localizes in cilia and contributes to the functions in cilia is unclear. Stereocilia organization and structure is determined by the kinocilium, and complete loss of the kinocilium results in misformed and misoriented stereocilia bundles as well as mispositioned basal bodies.64,65 As well, cilia-related proteins IFT88 and Kif3 have been shown to be required for proper stereocilia bundle orientation.65,66 It is possible that the defects that were detected in the previous studies in the stereocilia that caused hearing loss upon the overexpression or loss of DIAPH1 might be due in part to the disruption of DIAPH1 expression at the kinocilium. Future studies will be required to determine if sensorineural hearing loss represents a novel subclass of ciliopathy.

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AUTHOR CONTRIBUTIONS

O. Palander and W.S. Trimble designed research. O. Palander performed research. O. Palander and W. S. Trimble analyzed data. O. Palander and W.S. Trimble wrote the paper.

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