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TULP3 is required for localization of membrane-associated proteins ARL13Band INPP5E to primary cilia（TULP3 は膜結合タンパク質 ARL13B および INPP5E の一次繊毛局在に必要である）

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TULP3 is required for localization of membrane-associated proteins ARL13B and INPP5E to primary cilia

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ABSTRACT

The primary cilia are known as biosensors that transduce signals through the ciliary membrane proteins in vertebrate cells. The ciliary membrane contains transmembrane proteins and membrane-associated proteins. Tubby-like protein 3 (TULP3), a member of the tubby family, has been shown to interact with the intraflagellar transport-A complex (IFT-A) and to be involved in the ciliary localization of transmembrane proteins, although its role in the ciliary entry of membrane-associated proteins has remained unclear. Here, to determine whether TULP3 is required for the localization of ciliary membrane-associated proteins, we generated and analyzed TULP3-knockout (KO) hTERT RPE-1 (RPE1) cells. Immunofluorescence analysis demonstrated that ciliary formation was downregulated in TULP3-KO cells and that membrane-associated proteins, ADP-ribosylation factor-like 13B (ARL13B) and inositol polyphosphate-5-phosphatase E (INPP5E), failed to localize to primary cilia in TULP3-KO cells. These defects in the localization of ARL13B and INPP5E in TULP3-KO cells were rescued by the exogenous expression of wild-type TULP3, but not that of mutant TULP3 lacking the ability to bind IFT-A. In addition, the expression of TUB protein, another member of the tubby family whose endogenous expression is absent in RPE1 cells, also rescued the defective ciliary localization of ARL13B and INPP5E in TULP3-KO cells, suggesting that there is functional redundancy between TULP3 and TUB. Our findings indicate that TULP3 participates in ciliogenesis, and targets membrane-associated proteins to primary cilia via binding to IFT-A.

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1. Introduction

Primary cilia are hair-like organelles observed in most vertebrate cells [1]. They contain the microtubular axoneme wrapped by a membrane that is continuous with the plasma membrane. The axoneme of primary cilia has a ring of nine outer microtubule doublets and acts as a scaffold for multiple protein complexes, including intraflagellar transport (IFT) particles [1]. The IFT particle is composed of complex A (IFT-A) and complex B, and transports proteins required for the assembly and function of cilia [2]. Transmembrane proteins and membrane-associated proteins are concentrated at the membrane of primary cilia [3]. Ciliary transmembrane proteins include a subset of G-protein-coupled receptors (GPCRs) [4], the TRP-channel family proteins polycystin-1/2 [5], and the single-pass transmembrane protein fibrocystin [6]. They play key roles in sensing extracellular stimuli and in signal transduction [4]. Ciliary membrane-associated proteins include ADP-ribosylation factor-like 13B (ARL13B) [7,8] and inositol polyphosphate-5-phosphatase E (INPP5E) [9]. ARL13B, a small GTPase, is known for its role in Sonic Hedgehog signaling [7] and required for the ciliary targeting of INPP5E [10]. INPP5E converts phosphatidylinositol (4,5)-bisphosphate (PIP_2) of the ciliary membrane into phosphatidylinositol 4-phosphate in the ciliary compartment [11,12]. Palmitoylation of ARL13B [13] and prenylation of INPP5E [10] have been shown to occur in their association with the membrane of primary cilia.

Impairment of the function of cilia causes ciliopathies such as Joubert syndrome and Bardet–Biedl syndrome [1]. In humans, mutations of the ARL13B and INPP5E genes have been identified to cause Joubert syndrome, which features midbrain-hindbrain malformation, congenital cerebellar ataxia, and mental retardation

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[9,14]. On the other hand, Bardet–Biedl syndrome is characterized by obesity, retinal degeneration, renal abnormalities, and polydactyly [15]. Similar to the phenotype of Bardet–Biedl syndrome, spontaneous "tubby" mice, in which the Tub gene is mutated, also show obesity and retinal dystrophy [16,17]. In tubby mice, feeding-related GPCRs such as somatostatin receptor 3 (Sstr3), melanin-concentrating hormone receptor 1 (Mchr1), and the neuropeptide Y receptor Y2 fail to localize at the neuronal primary cilia [18,19]. Tub and four tubby-like proteins (Tulp1–4), which were also subsequently identified, share the carboxyl-terminal "tubby" domain and are collectively referred to as tubby family proteins [20]. In humans, mutations in TUB has been shown to be associated with obesity and retinal dystrophy [21,22].

In tubby family proteins, only TULP3 is ubiquitously expressed in most tissues [23]. Disruption of mouse Tulp3 is embryonically lethal, suggesting that TULP3 is essential for mammalian development [24]. In both TULP3 and TUB, the carboxy-terminal tubby domain interacts with the cytoplasmic side of the plasma membrane by binding to PIP_2 [25,26], while the amino-terminal domain interacts with IFT-A [26]. Via these interactions with IFT-A and PIP_2, TULP3 functions in the ciliary entry of MCHR1 [26]. Further, TULP3 is involved in the ciliary localization of at least 16 transmembrane proteins, including GPR161, an orphan GPCR known to regulate hedgehog signaling [27], SSRT3, MCHR1, fibrocystin, and polycystin 1/2 [23]. Meanwhile, it has remained unclear whether TULP3 is required for ciliary localization of the membrane-associated proteins ARL13B and INPP5E. In contrast, the disruption of IFT-A subunits causes mislocalization of ciliary proteins including ARL13B and INPP5E in mammalian cultured cells and in mice [28–31]. Therefore, in this study, we investigate the impact of the ablation of TULP3 on the localization of ciliary membrane-associated proteins in hTERT RPE-1 (RPE1) cells, a human retinal pigment epithelial cell line. We show the mislocalization of not only GPR161 but also ARL13B and INPP5E in TULP3-knockout (KO) cells. Previous studies showed that the formation of cilia is unaffected by TULP3 knockdown in RPE1 cells [26]. In this study, we describe defective ciliogenesis in TULP3-KO RPE1 cells.

2. Materials and methods

Plasmids. For an expression vector, the immediate early promoter of cytomegalovirus, a cloning site, the IRES sequence, and the cDNA for tdTomato fused with three nuclear localization signal (NLS) [32] were tandemly arranged. It can express both the gene of interest and the nuclear tdTomato. We assign this vector IRES-tdTomato-NLSx3. Human TULP3 and TUB cDNAs were cloned into the IRES-tdTomato-NLSx3 vector. Constructs of human TULP3 mutants, N-mut TULP3 (R24A, K27A, L28A, Q31A, R32A, L34A) [26], C-mut TULP3 (K268A, R270A) [25,26], and NC-mut TULP3 (R24A, K27A, L28A, Q31A, R32A, L34A, K268A, R270A) [26], were generated by inverse PCR using mutation primers and the TULP3 construct as a template.

Cell culture. RPE1 cells (ATCC, CRL-4000, Manassas, VA, USA) were maintained in DMEM/F-12 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1% GlutaMAX (Gibco), and 0.01 mg/ml Hygromycin B (Sigma, St. Louis, MO, USA). Cells were seeded on 4 well chamber slides at a density of 0.75×10^5 cells/well, and cultured in a CO_2 incubator for 48 h. Subsequently, cells were starved for 24 h in the medium without serum for ciliation. For rescue experiments, cells were transfected with indicated plasmids using FuGENE HD (Promega, Madison, WI, USA) 24 h after seeding.

Generation of TULP3 knockout RPE1 cell lines using the CRISPR/Cas9 system. Two different single-guide RNA (sgRNA) sequences, GGAGTATGACAGTTCACCAA (sgRNA1) and GCTGCACCATAAATGGCTCA (sgRNA2), targeting exon 3 of the human TULP3 gene were inserted into pSpCas9(BB)-2A-Puro (PX459) V2.0 vector, a gift from Feng Zhang (Addgene plasmid # 62988) [33], using BbsI restriction sites. RPE1 cells were plated on 24-well plate at a density of 1.25×10^5 cells/well and were transfected with 500 ng of PX459 containing the TULP3 sgRNA sequence using ViaFect (Promega). After 6 h of transfection, about one of six cells were replated on 15 cm dish and selected in medium containing puromycin (15 μg/ml) for 72 h. Subsequently, the cells were maintained in puromycin free medium until establishment of colonies. Then individual colonies were isolated and expanded, and the genomic DNA was extracted to analyze the sequence of CRISPR targeting sites. Two cell lines (#1 from sgRNA1 and #2 from sgRNA2) with biallelic InDel mutations in exon 3 of the TULP3 gene were selected for further experiments.

Immunofluorescence analysis. Samples were fixed with 4% paraformaldehyde in phosphate buffer (Nacalai tesque) at room temperature (RT) for 15 min and permeabilized with 0.2% Triton X-100 (Sigma) in 10 mM phosphate buffered saline (PBS) with 10 mg/ml bovine serum albumin (Sigma), 2% goat serum (Gibco), and 0.02% sodium azide (Sigma). The permeabilization buffer without Triton X-100 was used for antibody dilution, and 10 mM PBS was used for washes. Samples were incubated with primary antibodies at 4 °C overnight, and with secondary antibodies at RT for 1 h. For INPP5E detection, cells were permeabilized with the primary and secondary antibody solution containing with 0.1% saponin (Nacalai tesque) [34]. The following antibodies were used: mouse monoclonal anti-acetylated α-tubulin (sc-23950, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100), rabbit anti-Glu-tubulin (detyrosinated form of the α-tubulin, AB3201, Millipore, Billerica, MA, USA, 1:200), rabbit anti-ARL13B (SAB2700707, Sigma, 1:500), rabbit anti-INPP5E (17797-1-AP, Proteintech, Rosemount, IL, USA, 1:500) and rabbit anti-GPR161 (13398-1-AP, Proteintech, 1:200) for primary antibodies. Alexa Fluor 594, Alexa Fluor 405 or Alexa Fluor 488-conjugated goat anti-mouse IgG, and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA, 1:500) for secondary antibodies. For visualization of DNA, the samples were stained with 10 μg/ml Hoechst 33342 (Invitrogen) for 1 min. The samples were observed with a fluorescence microscope (Zeiss LSM880, Oberkochen, Germany). Image analysis was carried out with the NIH ImageJ.

Western blot analysis. Western blotting was performed as previously described [35]. Briefly, RPE1 cells were lysed in PBS containing 1% Nonidet P-40 (Wako, Osaka, Japan) and protease inhibitors on ice, followed by centrifugation at 15,000*g* for 15 min. The supernatant was subjected to SDS-PAGE, and transferred to a PVDF membrane. After blocking with 5% skimmed milk in PBS containing 0.5% Tween 20 (PBST) the membrane was incubated with rabbit anti-TULP3 (13837-1-AP, Proteintech, 1:2000), diluted with 1% skimmed milk in PBST overnight at 4 °C. After being washed with PBST, the membrane was incubated with HRP-conjugated anti-rabbit IgG antibody (#7074, Cell Signaling Technology, Danvers, MA, USA, 1:6000) at RT for 1 h. The bands were visualized by chemiluminescence using an ECL kit (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis. Differences between groups were examined for statistical significance using one-way ANOVA with Tukey's post hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Lack of TULP3 downregulates ciliary formation

We disrupted the TULP3 gene in RPE1 cells using the CRISPR/Cas9 system. We obtained two independent TULP3-KO cell lines (#1 and #2) using two different sgRNAs targeting exon 3 of the

TULP3 gene. The absence of TULP3 protein expression in TULP3-KO cell lines was confirmed by western blot analysis using TULP3 antibody (Fig. 1A).

Primary cilia in TULP3-KO cell lines and parental RPE1 cells were labeled by immunofluorescence using acetylated α-tubulin (Ac-tubulin) and Glu-tubulin as ciliary markers after 24 h of serum starvation (Fig. 1B). Then, the occurrence and length of primary cilia were analyzed. The number of cells lacking cilia or having only short ones (<2 μm) was greater and the number of cells having long cilia (≥2 μm) was smaller in the TULP3-KO cell lines than in the parental RPE1 cells (Fig. 1C). On average, the primary cilia were significantly shorter in the TULP3-KO cell lines than in the parental

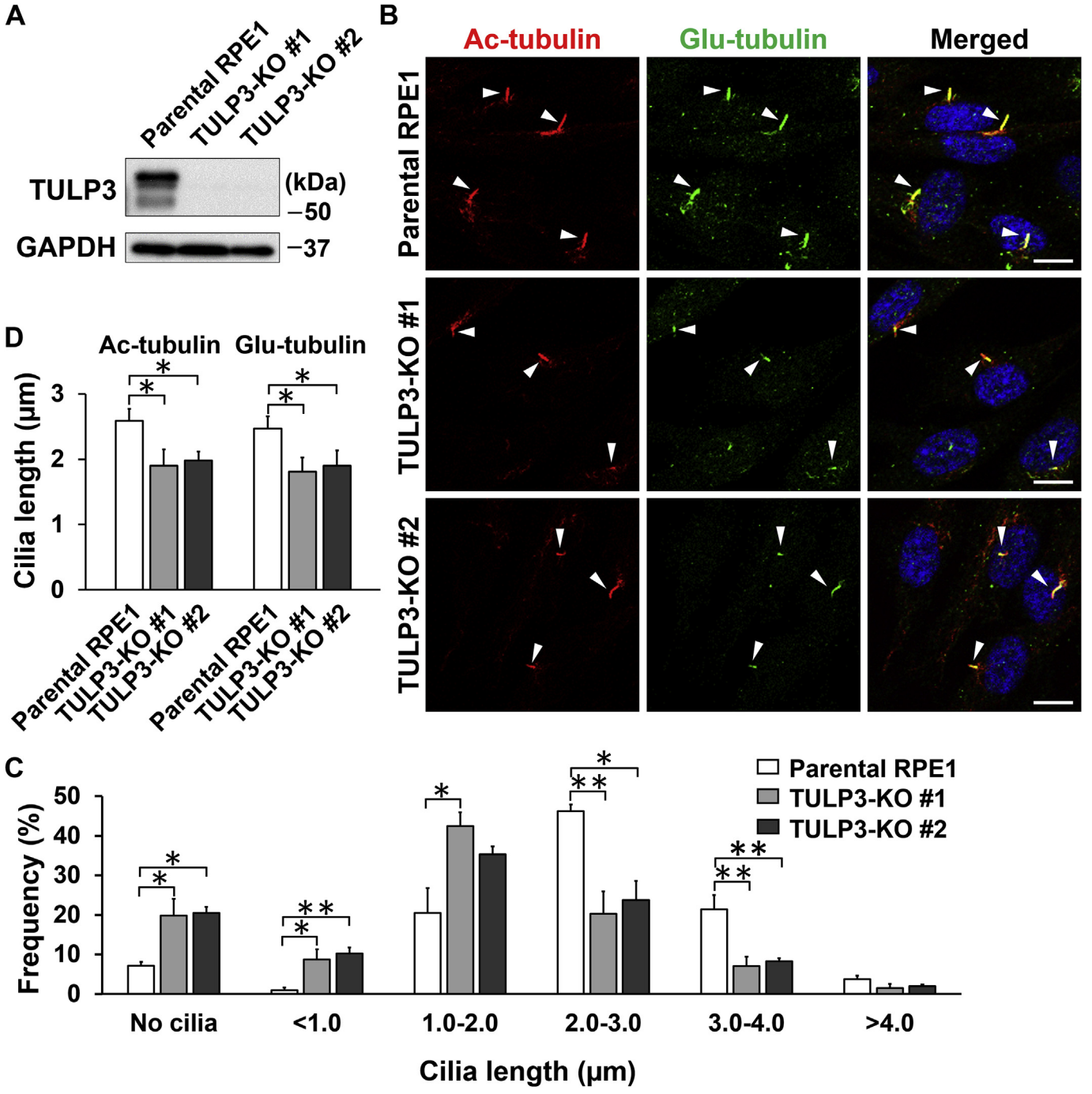

Fig. 1. Abrogation of TULP3 downregulates the formation of cilia in RPE1 cells. (A) Establishment of TULP3-knockout (KO) RPE1 cells using the CRISPR/Cas9 system. Western blot analysis using TULP3 antibody confirmed the absence of TULP3 expression in TULP3-KO cell lines #1 and #2. GAPDH expression served as a loading control. (B) After 24 h of serum starvation, the primary cilia of parental RPE1 and TULP3-KO cell lines were visualized by staining with antibodies against markers of cilia, acetylated α-tubulin (Ac-tubulin, red), and Glu-tubulin (green). In merged images, nuclei stained with Hoechst 33342 (blue) are shown. Arrowheads mark primary cilia. Scale bars represent 10 μm. (C) The lengths of cilia labeled with antibody to acetylated α-tubulin were measured. The distribution of the lengths of cilia is presented as a histogram. Data are presented as mean ± SE of three independent experiments. (D) The lengths of Ac-tubulin-positive or Glu-tubulin-positive cilia were scored. Data are presented as mean ± SE of three independent experiments. At least 76 cells were analyzed in each condition in (C) and (D). $^{*}P < 0.05$; $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cells (Fig. 1D). Our data revealed that ciliary formation and length were downregulated by the abrogation of TULP3.

3.2. Ciliary localization of ARL13B and INPP5E is abolished by absence of TULP3

Whether TULP3 is required for the ciliary localization of the membrane-associated proteins ARL13B and INPP5E was investigated using our TULP3-KO cell lines. First, the ciliary localization of endogenous GPR161 was detected in parental RPE1 cells (Figs. S1A and B). As expected, it was hardly recognized in TULP3-KO cell lines (Figs. S1A and B), which is in agreement with a previous finding in TULP3-knockdown cells [23]. Next, the endogenous ARL13B protein was detected in nearly all cilia of parental RPE1 cells (Fig. 2A and B). In contrast, almost none of TULP3-KO cells #1 and #2 displayed detectable ciliary localization of ARL13B (Fig. 2A and B). Similarly, ciliary localization of endogenous INPP5E was found in parental RPE1 cells, but it was hardly observed in TULP3-KO cells (Fig. 2C and D).

3.3. Binding of TULP3 to IFT-A, but not to PIP_2, is necessary for ciliary localization of ARL13B and INPP5E

We investigated whether the malformation of cilia in TULP3-KO cells can be rescued by the exogenous expression of TULP3. We found that the expression of TULP3 in TULP3-KO cells recovered the formation and length of cilia (Fig. 3A–C).

We next investigated whether the defective ciliary localization in TULP3-KO cells can be rescued by the exogenous expression of wild-type (WT) and mutant forms of TULP3 as well as TUB. To investigate the necessity of IFT-A- and PIP_2-binding properties of TULP3 for ciliary localization, we employed the following mutant

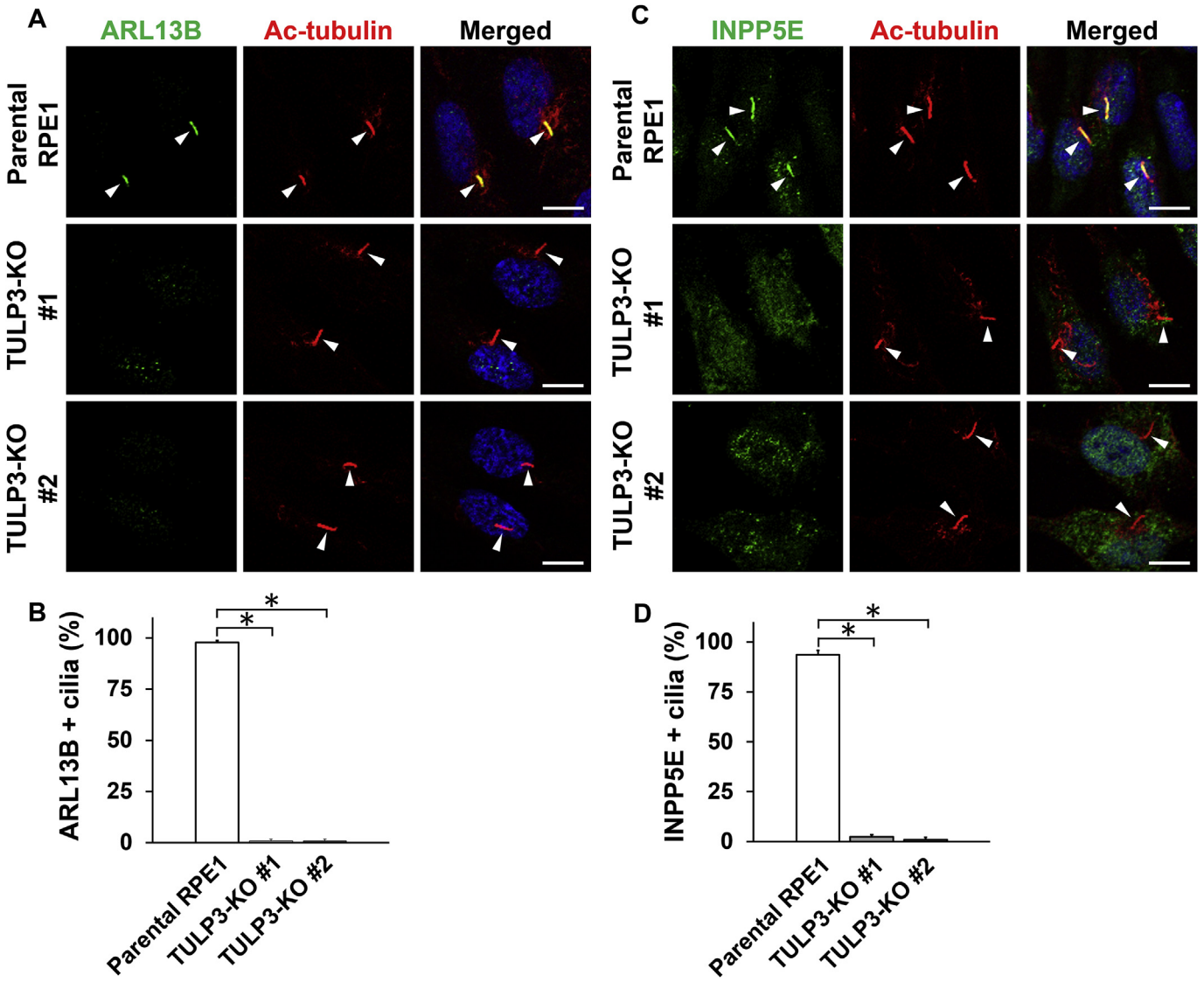

Fig. 2. Absence of TULP3 causes delocalization of membrane-associated proteins ARL13B and INPP5E from primary cilia. (A) Endogenous ARL13B was immunolabeled in parental RPE1 cells, and TULP3-KO cell lines #1 and #2 after 24 h of serum starvation. Cells were costained with anti-Ac-tubulin antibody (red) and with anti-ARL13B antibody (green). In merged images, nuclei stained with Hoechst 33342 (blue) are shown. (B) Quantification of the proportion of cells with ARL13B-positive (+) cilia. Data are presented as mean ± SE of three independent experiments. (C) Endogenous INPP5E was labeled in parental RPE1 cells and TULP3-KO cell lines after 24 h of serum starvation. Cells were stained with anti-Ac-tubulin antibody (red) and with anti-INPP5E antibody (green). (D) Quantification of the proportion of cells with INPP5E-positive (+) cilia. Data are presented as mean ± SE of three independent experiments. Scale bars represent 10 μm, and arrowheads mark primary cilia in (A) and (C). At least 100 cells were analyzed in each condition in (B) and (D). $^*P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

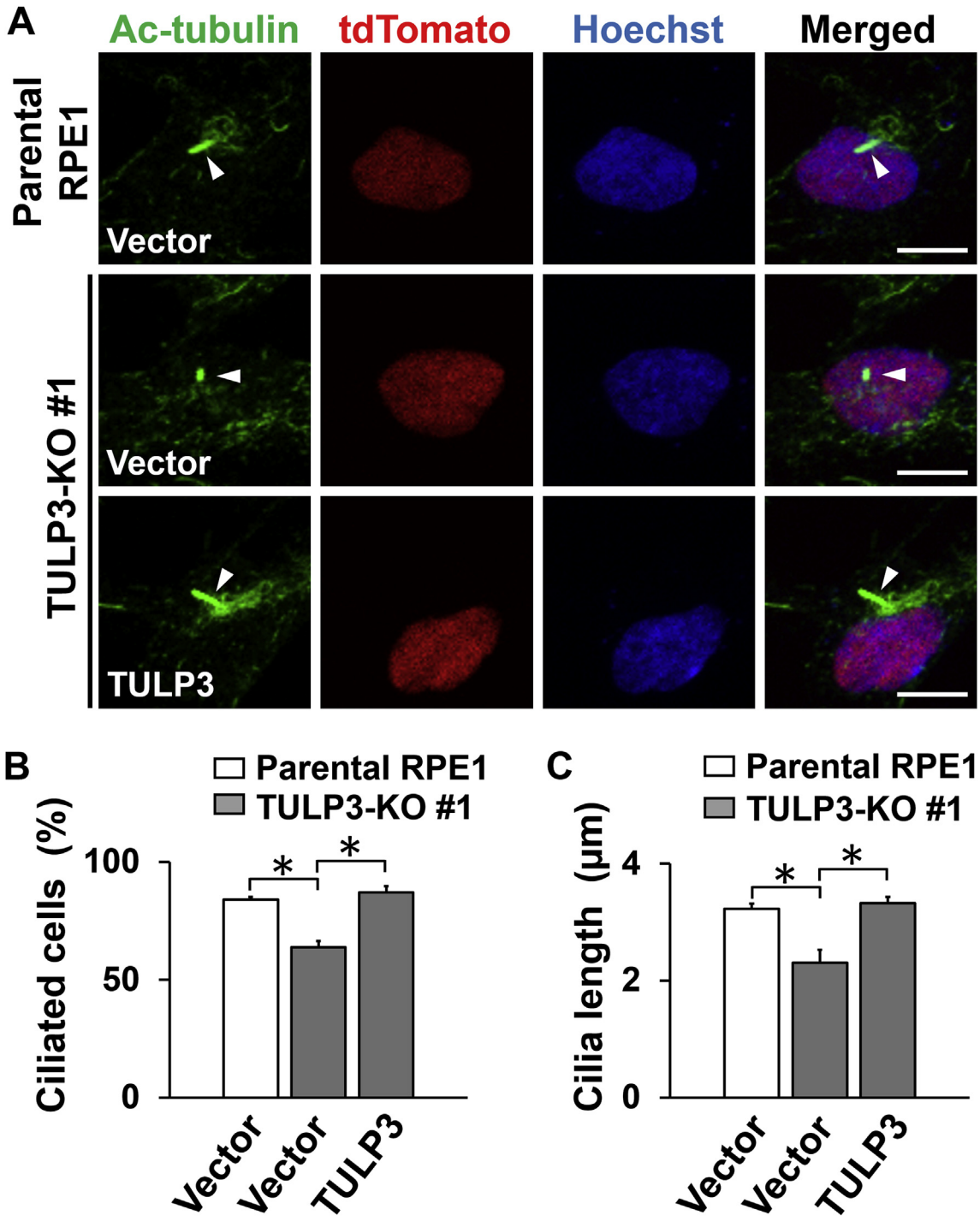

Fig. 3. Expression of TULP3 rescues the defective formation of cilia in TULP3-KO cells. (A) Parental RPE1 and TULP3-KO #1 cells were transfected with empty plasmid (Vector) or cDNA encoding TULP3. The plasmids express both the gene of interest and nuclear tdTomato, and thus the transfected cells are identified by tdTomato expression (red) in the nucleus. After 24 h of serum starvation, primary cilia of parental RPE1 and TULP3-KO cells were visualized by staining with anti-Ac-tubulin antibody (green). Nuclei were stained with Hoechst 33342 (blue). Scale bars represent 10 μm, and arrowheads mark primary cilia. (B, C) Quantification of the proportion of ciliated cells (B) and primary cilium length (C). Data are presented as mean ± SE of three independent experiments. At least 100 cells and 33 cells were analyzed in each condition in (B) and in (C), respectively. $^{*}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

forms of TULP3 utilized in previous studies [25,26]: amino-terminal mutant TULP3 (N-mut TULP3), which cannot bind to IFT-A; carboxyl-terminal mutant TULP3 (C-mut TULP3), which cannot bind to PIP_2; and amino-/carboxyl-terminal mutant TULP3 (NC-mut TULP3), which can bind to neither IFT-A nor PIP_2. Since the TUB protein, another member of the tubby family whose expression is absent in RPE1 cells [23], participates in the ciliary localization of GPCRs [18,19], its expression was also tested in this rescue experiment. The expression of WT, C-mut TULP3, and TUB in TULP3-KO cells recovered the ciliary localization of GPR161, while the expression of N-mut and NC-mut TULP3 failed to rescue the defective ciliary localization of GPR161 (Figs. S2A and B). Similarly,

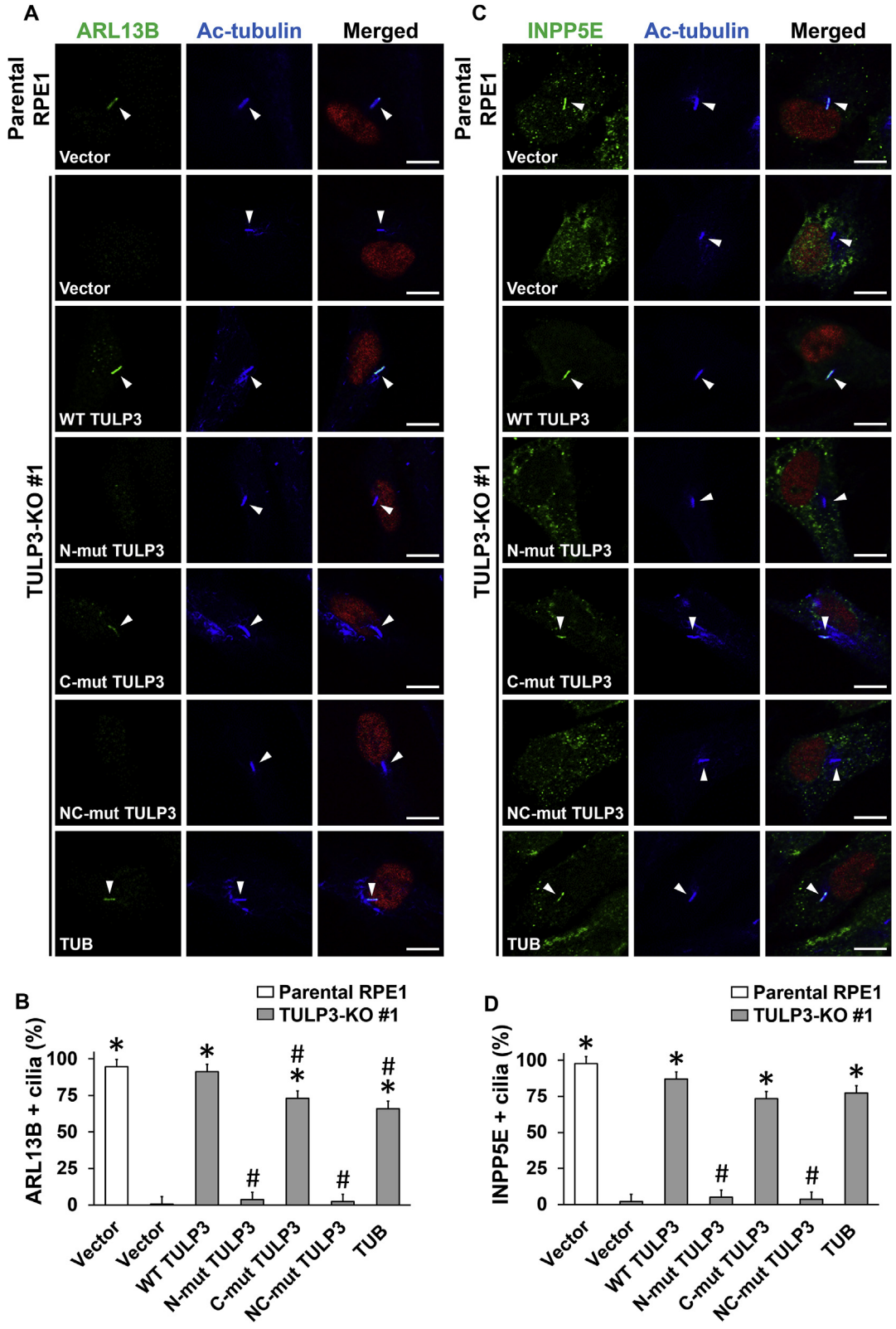

Fig. 4. Expression of TULP3 or TUB rescues the defective localization of ciliary ARL13B and INPP5E in TULP3-KO cells. (A) Parental RPE1 and TULP3-KO #1 cells were transfected with WT TULP3, N-mut TULP3 that cannot bind to IFT-A, C-mut TULP3 that cannot bind to PIP_2, NC-mut TULP3 that can bind to neither IFT-A nor PIP_2, or TUB. The transfected cells were identified by tdTomato (red) in the nucleus. After 24 h of serum starvation, cells were costained by antibodies to Ac-tubulin (blue) and to ARL13B (green). (B) Quantification of the proportion of cells with ARL13B-positive (+) cilia. Data are presented as mean ± SE of three independent experiments. (C) Parental RPE1 and TULP3-KO #1 cells were transfected with the plasmids indicated in each panel. After 24 h of serum starvation, cells were stained by antibodies to Ac-tubulin (blue) and to INPP5E (green). (D) Quantification of the proportion of cells with INPP5E-positive (+) cilia. Data are presented as mean ± SE of three independent experiments. Scale bars represent 10 μm, and arrowheads mark primary cilia in (A) and (C). At least 100 cells were analyzed in each condition in (B) and (D). $^{*}P < 0.01$ compared with TULP3-KO #1 cells transfected with vector; $^{\#}P < 0.01$ compared with TULP3-KO #1 cells transfected with WT TULP3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the expression of WT, C-mut TULP3, and TUB recovered the ciliary localization of ARL13B, while the expression of N-mut and NC-mut TULP3 failed to rescue the defective localization of ARL13B (Fig. 4A and B). Furthermore, WT, C-mut TULP3, and TUB expression rescued the defective localization of INPP5E, while N-mut and NC-mut expression failed to restore the INPP5E localization (Fig. 4C and D). In these experiments C-mut TULP3 and TUB expression recovered the localization of ciliary membrane proteins less efficiently than WT TULP3. Our findings indicate that not the PIP_2-binding property but the IFT-A-binding property of TULP3 is necessary for the proper localization of ciliary membrane proteins GPR161, ARL13B, and INPP5E. They also suggest that there is functional redundancy between TULP3 and TUB for the localization of these ciliary proteins.

4. Discussion

Our results show that the lack of TULP3 protein downregulates the formation of cilia and their length in RPE1 cells. The discrepancy between the TULP3 knockdown experiment in which ciliogenesis was unaffected [26] and our experiment using TULP3-KO cells might be explained by the TULP3 that remained unaffected after RNA interference being sufficient for the normal formation of primary cilia in RPE1 cells.

Intriguingly, our data indicated that TULP3 is required not only for the ciliary localization of transmembrane proteins but also for ciliary entry of the membrane-associated proteins ARL13B and INPP5E. Using the dominant-negative forms of TULP3, Mukhopadhyay and colleagues suggested that both IFT-A and PIP_2 binding properties of TULP3 regulate the trafficking of MCHR1 to primary cilia [26]. However, the present results suggest that the localization of endogenous GPR161 depends on the IFT-A-binding property but not the PIP_2-binding property of TULP3. It is possible that both of these properties are required for the ciliary localization of MCHR1, but not that of GPR161. Moreover, our results suggest that not the PIP_2-binding property but the IFT-A-binding property of TULP3 is necessary for the ciliary localization of ARL13B and INPP5E.

The disruption of IFT144/Wdr19, IFT140, and IFT122, IFT-A subunits that form the core subcomplex of IFT-A [26,30,36], causes defective ciliogenesis and mislocalization of ciliary proteins. KO of IFT122 causes a severe defect of ciliogenesis in RPE1 cells [31]. The expression of ciliopathy-associated mutant forms of IFT122 in IFT122-KO cells was also reported to impair ciliary localization of the membrane-associated proteins ALR13B and INPP5E [31]. In addition, Ift122-mutant mouse embryos were reported to have short cilia in primitive nodes [37]. KO of IFT144 results in defective ciliogenesis in RPE1 cells [30]. Short cilia and the absence of ciliary Arl13b have also been reported to occur in the neural tubes of Ift144-mutant mouse embryos [28]. Moreover, in Ift140-mutant mouse embryos, epithelial cilia were shown to exhibit morphological defects [38]. The IFT140-KO RPE1 cells that we established also display a severe defect in ciliogenesis [Han et al., unpublished data]. Meanwhile, the ablation of IFT121/Wdr35 and IFT139/Ttc21b, IFT-A subunits that form the peripheral subcomplex of IFT-A with IFT43 [30], causes defective ciliary localization of ALR13B and INPP5E in RPE1 cells [29,30]. Notably, using a visible immunoprecipitation assay, Hirano and colleagues demonstrated that interaction occurs between TULP3 and the IFT-A core subcomplex and that a complex composed of TULP3 and six IFT-A subunits subsequently forms [30]. The present study revealed that TULP3-KO cells display a phenotype similar to those of cultured cells with disrupted IFT-A subunits and of mice harboring mutations of IFT-A subunits, providing insight into the role of the functional interplay between TULP3 and IFT-A in regulating the assembly of cilia and the ciliary localization of membrane-associated proteins.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.12.109.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.12.109.

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