Biochemical and Biophysical Research Communications 373 (2008) 119–124

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Domain architecture of the atypical Arf-family GTPase Arl13b involved in cilia formation

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ARTICLE INFO

Article history:
Received 28 May 2008
Available online 11 June 2008

Keywords:
Cilia
Arl13b
Arf
Arl
Ras
Small GTPase
Coiled-coil

ABSTRACT

Arl13b is an atypical Arf/Arl-family GTPase consisting of an extending large C-terminal region (C domain) and Arf-homologous GTP-binding motifs in the N terminus (N domain). Although Arl13b appears to be involved in cilia formation, its precise function and roles of the domains remain unknown. Here, we show the unique domain architecture of Arl13b by analyzing the relationship between its biochemical properties and cilia formation. Arl13b binds guanine nucleotides and specifically localizes to cilia. The ciliary localization of Arl13b requires both N and C domains but is independent of its guanine nucleotide-binding ability. Arl13b is capable of self-associating via N domain, and overexpression of N domain inhibits not only cilia formation but also the maintenance of pre-generated cilia. These findings suggest that N and C domains of Arl13b cooperatively regulate its ciliary localization and that N domain-dependent self-association of Arl13b may be important for its function in cilia biogenesis.

Cilia are slender microtubule-based subcellular organelles that project on most mammalian cells [1–4]. Motile and non-motile cilia are involved in generation of fluid flow and sensing of chemical or physical extracellular environments, respectively. Previous studies have revealed that cilia play crucial roles in vertebrate development and tissue homeostasis, and that ciliary dysfunctions underlie numerous human disorders [5–9].

Arl13b is a member of the Arf (ADP-ribosylation factor)/Arl (Arf-like)-family small GTPases. However, this GTPase has a quite unique sequence different from typical small GTPases: Arl13b consists of not only guanine nucleotide-binding motifs located at the N terminus (N domain) but also an extended C-terminal region (C domain) that contains a coiled-coil motif and a proline-rich region. Interestingly, recent genetic screenings in zebrafish and mice have identified mutations in Arl13b gene, which lead to defects in kidney and nodal cilia formation, respectively [10,11]. However, its precise function and significances of its multiple domains in cilia formation remain totally unknown. In the present study, we show a functional analysis of the domain architecture of Arl13b. Our findings suggest that ciliary localization of Arl13b is cooperatively regulated by N and C domain, and self-interaction of Arl13b via N domain may be important for its function(s) not only in cilia formation but also in maintenance of cilia.

Materials and methods

Plasmid construction. pFlag-C-CMV5 was derived from pCMV5-Flag by modification of a position of Flag tag sequence, and pEGFP-C-CMV5 was derived from pFlag-C-CMV5 by replacing the Flag sequence to EGFP one [12]. To construct pEGFP-C-CMV5-Arl13b and pFlag-C-CMV5-Arl13b, human Arl13b cDNA without stop codon was subcloned into the region between HindIII and SalI sites of pEGFP-C-CMV5 and pFlag-C-CMV5, respectively. Arl13b-deletion mutants were made by PCR and subcloned into the region between HindIII and SalI sites of pEGFP-C-CMV5. T35N and N130I mutants were generated by one-day mutagenesis method [13]. To obtain pGEX6P1-Arl13b, human Arl13b cDNA corresponding to amino acids 20–428 was inserted into the region between BamHI and SalI sites of pGEX6P1 (GE healthcare).

Cell culture and transfection. HEK293T and RPE1 cells (hTERT-RPE1, Clontech) were cultured in Dulbecco's modified Eagle's medium (DMEM) and DMEM/F12 [1:1] (Invitrogen), respectively, supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C. DNA was transfected into cells using LipofectAMINE 2000 (Invitrogen) or FuGENE6 (Roche). To promote cilia formation, the cells were cultured for 48 h in a starvation medium consisting of DMEM/F12 [1:1] and 0.1% (w/v) BSA.

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doi:10.1016/j.bbrc.2008.06.001

Production of anti-human Arl13b polyclonal antibody. To generate an anti-human Arl13b polyclonal antibody, C-terminal peptide (CAVPQRPNSDAHDVIS) of human Arl13b was conjugated with keyhole limpet hemocyanin and injected into rabbits. The antiserum was affinity-purified with the antigen peptide using SulfoLink Kit (PIERCE) according to the manufacturer's instructions.

Immunostaining and microscopy of RPE1 cells. RPE1 cells were cultured on a coverglass (15-mm diameter) and washed three times with PBS, followed by fixation with 4% paraformaldehyde in PBS for 15 min. After permeabilization with 0.2% Triton X-100 in PBS for 10 min, the cells were maintained with a blocking solution consisting of 5% bovine serum albumin in Tris-buffered saline for 30 min and further incubated with the above anti-hArl13b polyclonal antibody and/or an anti-acetylated tubulin monoclonal antibody (Sigma, 1:500 dilution) in the blocking solution at 37 °C for 2 h. The cells were washed three times with PBS and incubated with an Alexa-488/568-conjugated secondary antibody (Molecular Probes) and 4′,6′-diamidino-2-phenylindole (DAPI) for 30 min in the blocking solution. After washed three times with PBS, the coverglass was mounted onto a glass slide in Permafluor mounting medium (Immunon) and viewed on an Axio Imager.M1 (ZEISS) equipped with a AxioCam HRm camera (ZEISS).

Expression and purification of recombinant Arl13b proteins. GST-Arl13b protein was produced in *Escherichia coli* BL21-CodonPlus (DE3) (STRATAGENE) containing the pGEX6P1-Arl13b by incubating with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 20 °C for 16 h. The cells were resuspended in the buffer A consisting of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% (w/v) Triton X-100, 2 μg/ml of aprotinin, and 2 μg/ml of leupeptin. The mixture was sonicated for 5 min on ice and centrifuged at 100,000*g* for 60 min at 4 °C. The clear supernatant was incubated with Glutathione Sepharose 4B (GE healthcare) at 4 °C for 2 h. After washing the resin three times with the buffer A containing 0.1% Triton X-100 but lacking protease inhibitors, GST-Arl13b was eluted from the resin with the same buffer containing 15 mM glutathione. The eluted proteins were applied to a PD-10 column (GE healthcare) to eliminate glutathione.

Guanine nucleotide-binding assay. Guanosine 5′-(γ-thio) triphosphate (GTPγS)-binding assay was performed by the filter method as described previously [14,15]. Purified GST-Arl13b protein (approximately 2 pmol of alive GTPγS-binding activity) was incubated with the indicated concentrations of [^{35}S]GTPγS (0.23 KBq/pmol) at 25 °C in the total volume of 40 μl of a reaction mixture consisting of 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM Na–EDTA, 6 mM $MgCl_2$, 1 mM DTT, 0.1% (w/v) Triton X-100, 3 mM dimyristoyl *L*-α-phosphatidylcholine (DMPC), and 0.5 mM adenosine 5-(β,γ-imido)triphosphate. After incubation for the indicated times, samples were diluted with 2 ml of an ice-cold buffer (20 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, and 100 mM NaCl) and filtered through a nitrocellulose membrane (0.45-μm pore size, Advantech). The membrane was washed four times with 2 ml of the ice-cold buffer and dried at 68 °C. Radioactivity retained on the membrane was determined by a liquid scintillation counter.

Immunoprecipitation assay. HEK293T cells that had been cultured in 35-mm dish for 48 h after transfection with pFLAG-C-CMV5-Arl13b and pEGFP-C-CMV5-Arl13b plasmids were lysed with 1 ml of the ice-cold buffer B consisting of 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1% (w/v) NP-40, 2 μg/ml of aprotinin, and 2 μg/ml of leupeptin. The cell lysates were centrifuged at 15,000 rpm for 20 min at 4 °C, and the supernatants were precleared with 20 μl of Sepharose 4B resin, followed by immunoprecipitation with anti-FLAG M2-Agarose affinity gel (Sigma). After washing the resin three times with 1 ml of the buffer B containing 0.1% NP-40 but lacking protease inhibitors, immunoprecipitated proteins were eluted by SDS–PAGE sample buffer from the resin and subjected to immunoblot analysis with anti-Flag monoclonal (M2, Sigma) and anti-GFP polyclonal (Clontech) antibodies.

Results

Characterization of Arl13b as a guanine nucleotide-binding protein

Arl13b has well-conserved guanine nucleotide-binding motifs in its N-terminus (see Fig. 2A later). Thus, we first examined whether Arl13b protein actually binds guanine nucleotides *in vitro*. Since recombinant Arf-family GTPases expressed in bacteria are often difficult to be purified as a stable form due to the hydrophobic properties of their N-terminal regions, we purified recombinant GST-Arl13b as a truncated form lacking its N-terminal 19 amino acids (Fig. 1A). Fig. 1B shows that a non-hydrolyzable GTP analogue, GTPγS, associated with the N-terminal truncated GST-Arl13b in a time-dependent manner at 1 mM Mg^{2+}. GTPγS-binding to GST-Arl13b required the physiological concentrations (mM order) of Mg^{2+}, and the binding was completely abolished at 1 μM Mg^{2+} (not shown). As shown in Fig. 1C, GTPγS bound to GST-Arl13b with the apparent K_a value of approximately 20 μM. These results indicate that Arl13b behaves as a typical Arf-family small GTPase, though its affinity for GTPγS is slightly lower than those of other Arf-family members [16,17].

Arl13b localizes to cilia independent of its guanine nucleotide-binding ability

Arl13b localizes to cilia in many tissues of mice [10]. RPE1 cells are derived from human retinal pigment epithelial cells, which can project primary cilia when cultured in a starvation medium. We therefore developed a specific antibody against human Arl13b to investigate the subcellular localization of endogenous Arl13b in RPE1 cells. As shown in Fig. 1D–G, the Arl13b signal strongly colocalized with acetylated tubulin (a cilia marker), indicating that endogenous Arl13b localizes primarily to cilia in RPE1 cells. We next investigated whether guanine nucleotide-binding to Arl13b is required for the ciliary localization. For the analysis, we investigated the subcellular localization of ectopically expressed wild-type and point mutants of Arl13b-EGFP. As shown in Fig. 1H, wild-type Arl13b-EGFP similarly localized to cilia as endogenous Arl13b in RPE1 cells. Interestingly, Arl13b/T35N and Arl13b/N130I mutants, both of which are supposed to be a nucleotide-free form based on the previous studies in other small GTPases [18–22], also localized to cilia (Fig. 1I–J). These results suggest that the ciliary localization of Arl13b is independent of its guanine nucleotide-binding ability.

Ciliary localization of Arl13b is abolished by its domain deletion

We next investigated which domain in Arl13b is responsible for its ciliary localization. Mammalian Arl13b is an atypical Arf/Arl-family GTPase with a long C-terminally extending region that contains a coiled-coil motif and a proline-rich region (PRR). Therefore, we expressed various deletion mutants of Arl13b and analyzed their subcellular localization in RPE1 cells: A schematic representation of the deletion mutants is shown in Fig. 2A. Each deletion mutant was expressed as a C-terminally EGFP-fusion protein in RPE1 cells, and the cells were subjected to immunostaining with the anti-acetylated tubulin antibody. As shown in Fig. 2C, N domain, which contains the entire GTP-binding motifs but lacks the extending C-terminus region, failed to localize to cilia: It displayed diffusible cytoplasmic localization. In addition, both C domain and PRR also displayed cytoplasmic localization and did not localize to cilia (Fig. 2D–E). These results indicate that none of the previously iden-

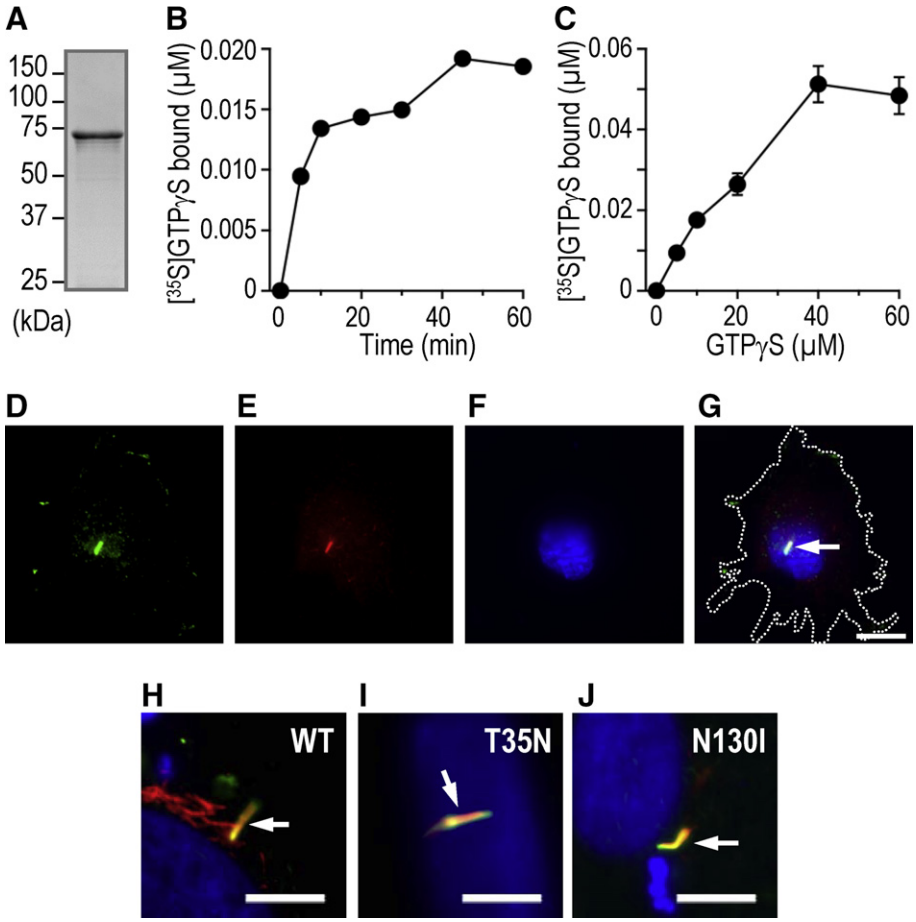

Fig. 1. Biochemical properties and ciliary localization of Arl13b. (A) The purified GST-Arl13b used for *in vitro* GTPγS-binding assay was separated by SDS–PAGE and stained with Coomassie brilliant blue. (B and C) GST-Arl13b (approximately 2 pmol of alive GTPγS-binding activity) was incubated with 5 μM (B) or the indicated concentrations (C) of [^{35}S]GTPγS for the indicated times (B) or 1 h (C) in the presence of 1 mM Mg^{2+}, and the amounts of [^{35}S]GTPγS bound to GST-Arl13b were estimated. Each data represents the mean and standard errors of triplicate determinations. (D–G) Analysis of subcellular localization of endogenous Arl13b in ciliated RPE1 cells. RPE1 cells, which had been cultured in the starvation medium for 48 h to project cilia, were immunostained with anti-hArl13b (D) and anti-acetylated tubulin (E) antibodies. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (F), and a merged image is shown in (G). An arrow indicates a cilium. Scale bar: 10 μm. (H–J) Ciliary localization of wild-type and point mutants of Arl13b. RPE1 cells were transiently transfected with plasmid vectors for expressing the C-terminally EGFP-fused wild-type (WT) and point mutants (T35N and N130I) of Arl13b, and further incubated in the starvation medium for 48 h to generate cilia. The cells were immunostained with the anti-acetylated tubulin antibody (red), and the nuclei were stained with DAPI (blue). The green signals represent Arl13b-EGFP. Arrows indicate cilia. Scale bars: 5 μm.

tified domains constitutes the critical region for ciliary localization of Arl13b and that multiple regions might be involved in its localization.

Self-association of Arl13b through its N domain containing GTP-binding motifs

Coiled-coil motif is a structural domain that mediates protein oligomerization [23]. Since a coiled-coil motif exists in the middle region of Arl13b, we investigated whether Arl13b has ability to self-associate dependent on the motif. To this end, 293T cells were co-transfected with Arl13b-Flag and Arl13b-EGFP expression vectors. Lysates from the transfectants were subjected to immunoprecipitation using an anti-Flag antibody, and the immunoprecipitated proteins were analyzed by western blotting with an anti-GFP antibody. As shown in Fig. 3, wild-type Arl13b-EGFP was detected in the immunoprecipitated fraction containing Arl13b-Flag, indicating that Arl13b-EGFP associates with Arl13b-Flag. We further investigated which region of Arl13b is responsible for the association by using deletion mutants of Arl13b-EGFP. Surprisingly, N domain of Arl13b-EGFP was, but C domain containing the coiled-coil was not, co-precipitated with Arl13b-Flag. These findings suggest that Arl13b is capable of self-associating via its N domain in living cells, though we cannot totally rule out the possibility that other cellular component(s) might mediate the association.

Inhibition of cilia formation and its maintenance by overexpression of Arl13b/N domain

During the course of this study using deletion mutants of Arl13b, we noticed that the ectopic expression of Arl13b/N domain reduced the number of ciliated cells. We thus examined the effects of each Arl13b domain on cilia formation by over-expressing the various mutants in RPE1 cells. The RPE1 cells, after being transfected with plasmid vectors to express EGFP-fused Arl13b proteins, were subsequently allowed to project primary cilia by incubation in the starvation medium. Quantitative analysis of cilia formation was performed by counting the number of ciliated cells among GFP-positive cells. As shown in Fig. 4A, the efficiency of cilia formation was not affected by the expression of wild-type or C domain of Arl13b, whereas it was significantly reduced in cells expressing N domain. Although previous studies using Arl13b genetic mutants have shown that Arl13b is essential for normal cilia formation [10,11], it remains unknown whether Arl13b is required also for the maintenance of cilia. We thus overexpressed Arl13b deletion mutants in preciliated RPE1 cells and scored the cilia formation

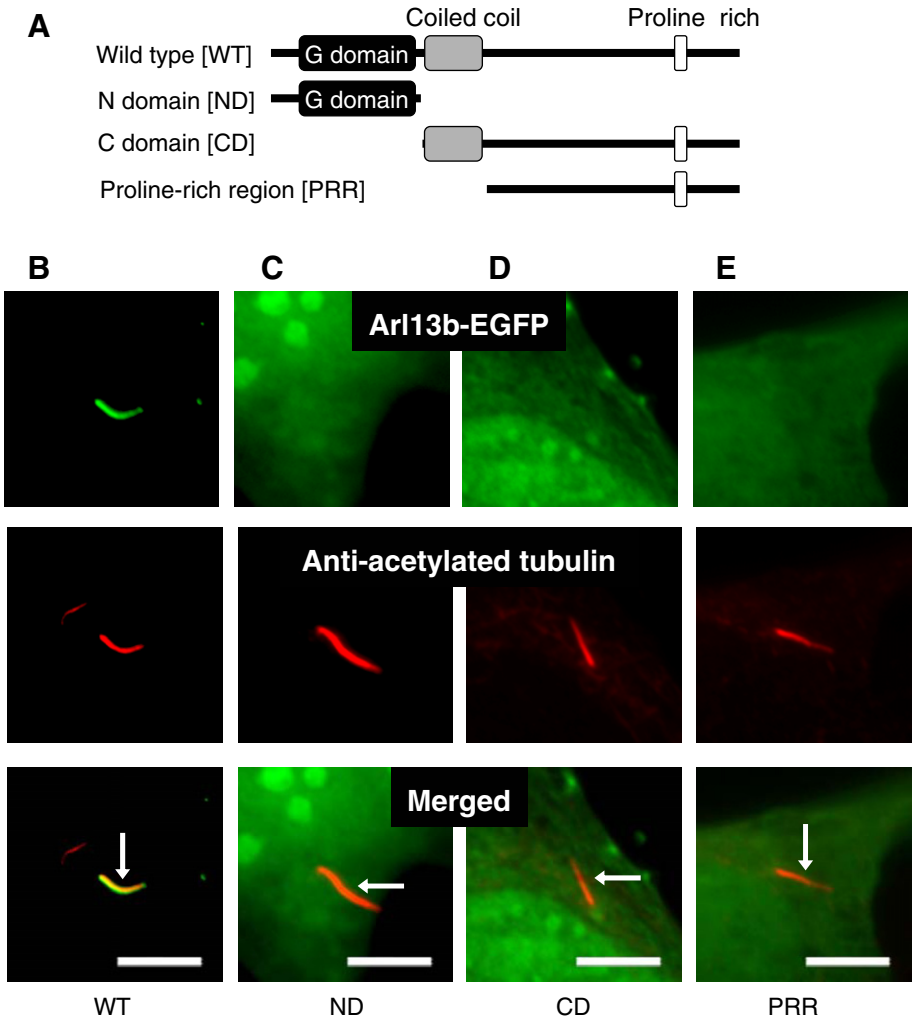

Fig. 2. Ciliary localization of Arl13b requires both its N and C domains in RPE1 cells. (A) Schematic representation of Arl13b deletion mutants used in this study. The regions corresponding to the amino acid 1–190, 193–428, and 245–428 are referred to as N domain, C domain and PRR (proline-rich region), respectively. GTP-binding motifs (G domain), coiled-coil and proline-rich regions are boxed in black, grey and while, respectively. (B–E) RPE1 cells were transfected with plasmid vectors for expressing the C-terminally EGFP-fused wild-type and deletion mutants of Arl13b. The cells were incubated and immunostained as described in Fig. 1. Arrows indicate cilia. Scale bars: 5 μm.

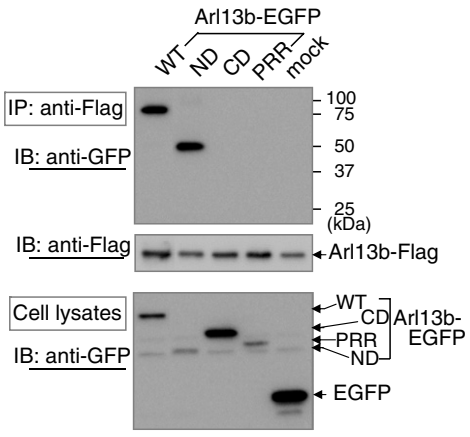

Fig. 3. Self-association of Arl13b through its N domain. 293T cells were co-transfected with two expression vectors, pFlag-C-CMV5-Arl13b wild-type (WT) and each one of the pEGFP-C-CMV5-Arl13b mutants (see Fig. 2A), and further incubated. The cell lysates were immunoprecipitated with an anti-Flag monoclonal antibody. The immunocomplexes were subjected to western blotting with the anti-GFP (top) and anti-FLAG (middle) antibodies. The lysates were also subjected to Western blotting with the anti-GFP antibody (bottom).

using the same strategy. As shown in Fig. 4B, the ratio of ciliated cells was significantly reduced by the overexpression of N domain of Arl13b, suggesting that the overexpression impairs the maintenance of preformed cilia.

Discussion

In the present work, we first characterized biochemical properties of Arl13b, whose structure is quite unique in terms of the presence of an extended C-terminal region in addition to well-conserved GTP-binding motifs in the N terminus. We confirmed that Arl13b actually associates with GTPγS in *in vitro* assay. We also found that possible two nucleotide-free mutants (T35N and N130I) are capable of localizing to cilia as well as wild-type Arl13b. A putative *C. elegans* ortholog (Y37E3.5/ARL-13) of Arl13b has been shown to localize to cilia of sensory neurons [24]. Interestingly, the G domain of *C. elegans* ARL-13 lacks a consensus motif (G4 motif) required for binding to guanine base, indicating that *C. elegans* ARL-13 may be a guanine nucleotide-free form. These observations, together with our findings of the ciliary localization of nucleotide-free Arl13b mutants, suggest that the guanine nucleotide binding to Arl13b is not critically required for its ciliary localization. We found that all deletion mutants used in this study do

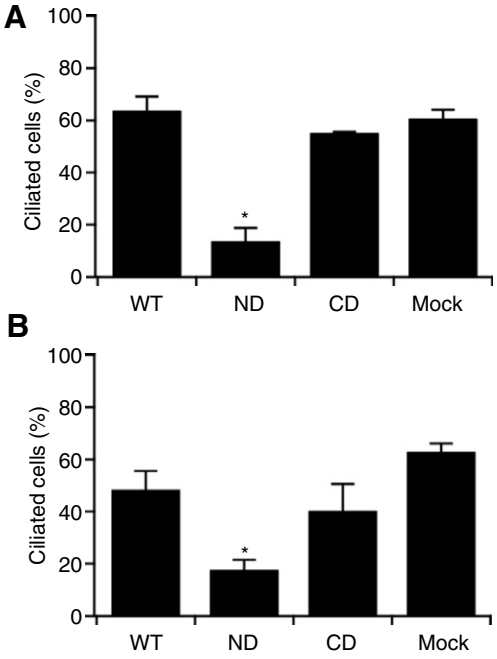

Fig. 4. Overexpression of the Arl13b N-domain inhibits cilia formation in RPE1 cells. (A) RPE1 cells were transfected with each one of expression vectors producing the C-terminally EGFP-fused wild-type and the deletion mutants of Arl13b, and further incubated in the starvation medium for 48 h to generate cilia. The cells were immunostained with the anti-acetylated tubulin antibody, and the number of cells forming primary cilia was counted. The data are expressed as percentages of the total cells expressing EGFP. (B) RPE1 cells, after being incubated in the starvation medium for 48 h to generate cilia, were transfected with the expression vectors encoding the C-terminally EGFP-fused Arl13b. The cells were further incubated in the starvation medium for another 48 h and subjected to the counting of cilia-forming cells as described above. More than 50 cells were counted for each experiment, and the data from three independent results are shown. Error bars indicate SEM. Statistical analyses were performed by Student's *t*-test. $^{*}p < 0.01$, comparing ND with WT or Mock.

not localize to cilia but display cytoplasmic localization. Future analysis using more limited deletion and/or point mutants of Arl13b may be helpful in determining the critical region required for the ciliary localization of Arl13b.

We have shown that Arl13b has ability to self-associate via its N domain containing GTP-binding motifs. Interestingly, overexpression of Arl13b/N domain significantly reduced not only cilia formation but also its maintenance in RPE1 cells. Considering that the overexpressed N domain localizes in cytoplasm, the N domain might trap the endogenous Arl13b in cytosol to prevent its ciliary localization, thereby inhibiting function(s) of Arl13b in cilia formation. Alternatively, the self-association of Arl13b might be important for its function. Several small GTPases form homodimers or multimers, and self-association of these proteins appears to be important for their functions [12,25–28]. Thus, it is tempting to speculate that the self-association of endogenous Arl13b may be abrogated by the overexpressed N domain, resulting in the inhibition of Arl13b function(s).

Formation and maintenance of cilia depend on specialized microtubule-associated transport machinery, termed intraflagellar transport (IFT) [29–32]. IFT bi-directionally conveys several cargoes, such as receptors, channels, and signaling molecules, from basal body to ciliary tip. We have suggested that Arl13b is necessary for both the formation and maintenance of cilia in RPE1 cells. Considering that most Arf/Arl-family small GTPases act in diverse intracellular trafficking pathways [33,34], Arl13b may be engaged in the regulation of IFT. Arl13b has an extending C domain containing a coiled-coil and a PRR, which can mediate protein–protein interaction. Given that the extending C domain is required for ciliary localization of Arl13b, C domain may mediate interaction of Arl13b with IFT complex and/or motor proteins. Identification of binding partner(s) to C domain would give a clue to clarify the function role(s) of Arl13b in cilium biogenesis.

Acknowledgments

This work was supported in part by research grants (to T.K., K.K., and T.K.) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, the Japan Society for the Promotion of Science (JSPS), the Astellas Foundation for Research on Metabolic Disorders, and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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