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Research Article

The ciliary rootlet interacts with kinesin light chains and may provide a scaffold for kinesin-1 vesicular cargos

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Abstract

The ciliary rootlet is a large striated fibrous network originating from basal bodies in ciliated cells. To explore its postulated role in intracellular transport, we investigated the interaction between kinesin light chains (KLCs) and rootletin, the structural component of ciliary rootlets. We show here that KLCs directly interact with rootletin and are located along ciliary rootlets. Their interactions are mediated by the heptad repeats of KLCs. Further studies found that these interactions tethered kinesin heavy chains along ciliary rootlets. However, the ciliary rootlet-bound kinesin-1 did not recruit microtubules or move along ciliary rootlets. Additionally, amyloid precursor protein (APP; a kinesin-1 vesicular cargo receptor) and presenilin 1 (a presumed cargo of APP/kinesin-1) were found to be enriched along the rootletin fibers, suggesting that the interaction between ciliary rootlets and kinesin-1 recruits APP and presenilin 1 along ciliary rootlets. These findings indicate that ciliary rootlets may provide a scaffold for kinesin-1 vesicular cargos and, thus, play a role in the intracellular transport in ciliated cells.

Keywords: Rootletin; APP; Presenilin 1; Photoreceptor; Ciliated cells; Vesicular transport and cytoskeleton

Introduction

The ciliary rootlet is a large cytoskeleton in ciliated cells. In a photoreceptor cell, which elaborates an enlarged light-sensing distal cilium known as the outer segment, the ciliary rootlet is tightly bundled and spans the entire length of the cell body. In cells with motile cilia, such as epithelia lining the airways and brain ventricles, ciliary rootlets appear as a fibrous network. Although the ciliary rootlet was discovered as a cellular organelle about a century ago, its biological functions are largely unknown. It has been proposed that the ciliary rootlet may play a role in intracellular transport [1] among other postulated functions. Consistent with this hypothesis, our recent studies suggest that rootletin, the structural component of ciliary rootlets, interacts with kinesin light chain 3 (KLC3) [2].

Kinesins comprise a superfamily of motor proteins, which move their cargos such as proteins and cellular organelles along the microtubule tracks using the energy of ATP hydrolysis. Kinesin-1 (previously referred to as kinesin-I, KHC, N-I and conventional kinesin [3]) is a heterotetramer consisting of two kinesin heavy chains (KHC) and two KLCs. In mice, there are three KHC (Kif5A, Kif5B and Kif5C) [4] and three KLC (KLC1, KLC2 and KLC3) genes [5,6]. Kif5A, Kif5C, and KLC1 are expressed primarily in neurons, while Kif5B, KLC2 and KLC3 are ubiquitously expressed. KHCs have a microtubule-binding domain and a microtubule-dependant ATPase domain responsible for the generation of mechanochemical force [7]. KLCs have conserved motifs of

Abbreviations: KLC, kinesin light chain; KHC, kinesin heavy chain; APP, amyloid precursor protein; JIP, C-Jun N-terminal kinase (JNK)-interacting proteins; HR, heptad repeat; TPR, tetratricopeptide repeat; ODF, outer dense fibers; FRAP, fluorescence recovery after photobleaching; PDI, protein disulphide-isomerase.

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heptad repeats (HR domain) and tandem tetratricopeptide repeats (TPR domain). The HR domain is involved in binding KHC, while the TPR domain is believed to regulate KHC motor activity and bind cargos [8,9].

KLCs bind cargos via cargo receptors. Reported cargo receptors include amyloid precursor protein (APP) [10] and C-Jun N-terminal kinase (JNK)-interacting proteins (JIPs) [11,12]. APP is a transmembrane protein. Deposition of its degradative product (amyloid β-peptide) in brain is a major pathological finding in Alzheimer's disease. Data from KLC1 and APP knockout mice show that both proteins mediate fast anterograde axonal transport of a membrane compartment containing APP processing enzymes such as β-secretase and presenilin 1, suggesting that APP is a vesicular cargo receptor for kinesin-1 [13]. JIPs are scaffolding proteins for the JNK signaling pathway. They link cytoplasmic (dual leucine zipper-bearing kinase) and transmembrane (the Reelin receptor, ApoER2) signaling molecules to kinesin-1. Disruption of kinesin-1 in cultured neurons eliminates the synaptic distribution of JIPs [12]. In addition, a mutation in the *Drosophila* JIP3 homolog, sunday driver, causes accumulation of vesicles in axons of the segmental nerves [11]. Thus, the interaction between JIPs and KLCs may provide motor-driven spatial regulation of cytoplasmic signaling pathways and mediate vesicular transport [14,15].

To study the putative role of ciliary rootlets in intracellular transport, we pursued our earlier finding of an interaction between rootletin and KLC3. Previously, we demonstrated their interaction by a yeast two-hybrid screen and co-expression analysis in cells. Their interaction in vivo was supported by their co-existence in the same compartment of photoreceptor cells [2]. Here, we provide further evidence for the direct interaction of rootletin with other KLCs besides KLC3, the domain of KLCs involved in these interactions, and the functional implication of these interactions. Our studies raise the possibility that ciliary rootlets are a scaffold for kinesin-1 vesicular cargos.

Materials and methods

Plasmids and yeast two-hybrid analysis

A summary of cloned genes and their fragments is listed in Table 1. The full-length rootletin cDNA in the mammalian expression vector pcDNA3.1 was described previously [2]. RootR1 cut from the RootR1/pEGFPC2 plasmid [2] at *Bam*HI/*Eco*RI sites was inserted in the pGBKT7 and pGADT7 vectors (CLONTECH Laboratories, Inc.). The same fragment with the *Bam*HI site filled in was inserted into pGEX4T-1 plasmid (Amersham Biosciences) at the *Not*I/*Eco*RI sites with the *Not*I site filled in. All other cDNAs were amplified by PCR from a retinal cDNA library and cloned into the plasmid vectors shown in Table 1. cDNAs in the pEGFPC2 vector (CLONTECH Laboratories, Inc.) were expressed by transfection into COS-7 cells. cDNAs in the pGBKT7 and pGADT7 vectors were used as bait and prey, respectively, in yeast two-hybrid analysis. cDNAs in pET28a (Novagen) and pGEX4T-1 vectors were used to express proteins in *E. coli*. Yeast two-hybrid analysis was performed as described previously [2].

Table 1
A summary of cloned genes and their fragments used in this study

Fragment/gene	GenBank #	Region (aa)	Vectors
KLC1	AAC27740	2–541	pEGFPC2, pGBKT7, pGADT7, pET28a
KLC1HR	AAC27740	2–168	pGBKT7, pGADT7
KLC1TPR	AAC27740	163–541	pGBKT7, pGADT7
KLC2	AAC27741	2–599	pEGFPC2, pGBKT7, pGADT7, pET28a
KLC2HR	AAC27741	2–196	pGBKT7, pGADT7
KLC2TPR	AAC27741	190–599	pGBKT7, pGADT7
KLC3	NP_666294	2–508	pEGFPC2, pGBKT7, pGADT7, pET28a
KLC3HR	NP_666294	60–156	pGBKT7, pGADT7
KLC3TPR	NP_666294	150–421	pGBKT7, pGADT7
Kif5A	NP_032473	2–1027	pEGFPC2
Kif5C	NP_032475	2–956	pEGFPC2
rootletin	NP_742120	1–2009	pcDNA3.1
RootR1	NP_742120	3–533	pGBKT7, pGADT7, pGEX4T-1
PAT1	NP_080101	2–585	pEGFPC2, pGBKT7, pGADT7, pET28a

Cell culture, transient transfection and fluorescence recovery after photobleaching (FRAP) analysis

COS-7 cells were cultured in DMEM supplemented with 5% fetal bovine serum. Transient transfections were carried out using the Geneshuttle™-40 reagent (Qbiogene, Inc.) according to the manufacturer's instructions. Cells were fixed and immunostained between 24 and 31 h after transfection.

FRAP analysis was performed using a confocal laser scanning microscope (model TCS SP2; Leica) with Leica Confocal software. Cells were grown on plastic plates in complete normal growth medium with 25 mM HEPES. A heating plate was placed on the microscope stage to maintain a constant temperature of 37°C. The bleached regions of the cells were defined manually as about 1.5 μm-wide bars and were bleached for 5 min using full laser power. Rootletin staining of cells immediately fixed after 15-min bleaching demonstrated no damage of the integrity of rootletin fibers from the bleaching (data not shown). In addition, the fully fluorescence recovery of the bleached regions in a short period (about 20 min) suggested that our procedures for photobleaching did not disturb the cell physiology significantly.

Antibodies

A rabbit anti-KLC3 antibody was generated against aa 2–101 of mouse KLC3 and affinity-purified using proce-

dures described previously [2]. The rabbit antibodies to full-length KLC3 [5] and rootletin (Root6 and Root10) [2], chicken antibodies to rootletin (Root6 and Root10) [2], and monoclonal antibody to rhodopsin [16] have been described. Monoclonal anti-KHC (H2), polyclonal anti-APP C-terminus and anti-presenilin-1 N-terminus antibodies were purchased from Chemicon International, Inc. Monoclonal anti-acetylated α-tubulin, monoclonal anti-γ-tubulin, and polyclonal anti-flag antibodies were obtained from Sigma-Aldrich. Monoclonal anti-tubulin (DM1A and DM1B) and anti-KHC antibodies were from Abcam Limited. Polyclonal anti-GFP antibody was from Santa Cruz Biotechnology. Monoclonal anti-PDI antibody was obtained from Affinity Bioreagents. Rabbit anti-KLC1 and anti-KLC2 antibodies were from Dr. L. S. B. Goldstein (University of California San Diego, CA). Secondary antibodies conjugated to Alexa fluorochromes were obtained from Molecular Probes, Inc.

Immunofluorescence

Dissociated photoreceptors were prepared by a brief vortex of dissected mouse retinas in PBS, and the settled buffer was transferred onto poly-lysine-coated glass slides. Both the dissociated photoreceptors and COS-7 cells were fixed in methanol:acetone (19:1) for 10 min at −20°C. Tissue sections were fixed in 4% formaldehyde/PBS for 10 min and permeabilized by 0.2% Triton X-100/PBS for 5 min at room temperature. The subsequent steps of blocking and incubation with primary and secondary antibodies were as described previously [2]. Alexa 488- and Alexa 594-conjugated secondary antibodies were routinely used for double-labeling unless otherwise noted. Stained sections and cells were viewed and photographed on a fluorescent microscope (model 1 × 70; Olympus) equipped with a digital camera (Carl Zeiss MicroImaging, Inc.) or on a confocal laser scanning microscope (model TCS SP2; Leica).

Co-immunoprecipitation and Western blotting

COS cells were homogenized and incubated for 15 min in lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% TritonX-100, 5 mM EDTA, 0.5 mM PMSF, 1× protease inhibitors, and 1 mM DTT). After centrifugation at 18,000 × *g* for 10 min, the supernatant was precleared by incubation with protein G sepharose (Amersham Biosciences) for 1 h. Subsequently, it was incubated with the primary antibody for 3 h and centrifuged at 18,000 × *g* for 10 min. The supernatant was incubated with protein G sepharose for an additional 1 h. After a brief centrifugation at 2000 × *g*, the pellet was washed with lysis buffer three times and then boiled in Laemmli sample buffer. All the procedures were performed at 4°C. A non-immune rabbit IgG served as a negative control. Western blotting was carried out as described previously [2].

Far Western

To prepare the GST-fused RootR1 probe, BL21-codonplus (DE3) cells carrying the RootR1/pGEX4T-1 plasmid were cultured in LB medium/carbenicillin and induced with 1 mM IPTG for 2 h. The pelleted cells were resuspended in TBS (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, and 1× protease inhibitors), and lyzed with the addition of lysozyme and Triton X-100. After centrifugation at 18,000 × *g* for 10 min, the supernatant was used as the probe. The final concentration of GST-fused RootR1 was about 2 μg/ml. GST (Sigma-Aldrich) at a concentration of 12 μg/ml was used on a duplicate blot as a negative control.

To prepare the blot, BL21-codonplus (DE3) cells with PAT1/pET28a (T7-tagged PAT1, a negative control) and KLCs/pET28a (T7-tagged KLCs) plasmids were cultured in LB medium/kanamycin and induced with 1 mM IPTG for 2 h. The cells were lyzed by boiling in Laemmli sample buffer, run on a SDS-PAGE, and transferred to a PVDF membrane. The membrane was blocked with 5% milk for 1 h then incubated with GST-fused RootR1 or GST at 4°C overnight. After washes, bound RootR1 was detected by a guinea pig anti-GST antibody and chemiluminescent reagents (Pierce). To detect the positions and the loading amounts of T7-tagged PAT1 and KLCs, the blot was stripped and re-probed by a HRP-conjugated T7 tag antibody (Novagen).

Results

Rootletin directly interacts with KLCs via their HR domain

Our previous yeast two-hybrid screen found KLC3 to bind the globular domain of rootletin (RootR1) [2]. Therefore, we decided to further investigate the interaction of KLCs with rootletin. Sequence analysis found that both KLC1 and KLC2 had in-frame stop codons in their 5′ untranslated regions. Because these stop codons would preclude the expression of KLC1 and KLC2 as fusion proteins during a yeast two-hybrid screen, we directly examined whether they interacted with rootletin by yeast two-hybrid analyses. We cloned KLC1 and KLC2 devoid of their 5′ untranslated regions and co-transformed each KLC with RootR1 into yeast. Empty vectors and vectors with PAT1, a protein homologous to KLCs with both HR and TPR domains [17] (Fig. 1A), were used as negative controls. Vectors with KLC3 were used as positive controls. The results demonstrated that both KLC1 and KLC2 were able to bind RootR1 independent of the hosting vector configuration (Table 2). However, KLC1 appeared to bind rootletin weakly. To determine which domain of KLCs was involved in the interaction, we cloned both HR and TPR domains from each of the three KLCs (Fig. 1A), and co-transformed each KLC fragment with RootR1 into yeast. The results showed that the HR domain of KLCs mediated

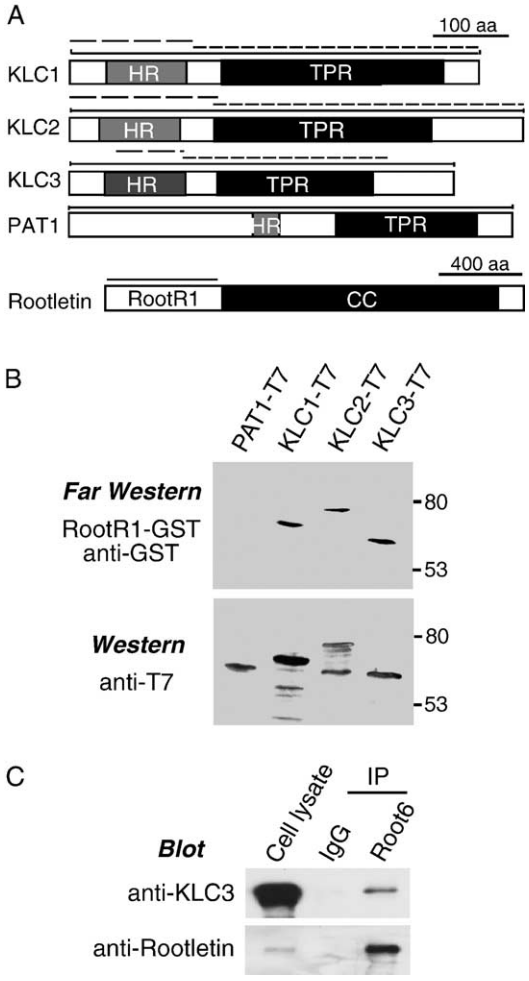

Fig. 1. Rootletin interacts with KLCs shown by several biochemical assays. (A) Schematic diagrams of KLCs, PAT1, rootletin and their fragments (horizontal lines, see Table 1 for details) used in our studies. (B) Far Western analysis demonstrates that rootletin directly binds KLCs. T7-tagged KLCs and PAT1 (a negative control) immobilized on a PVDF membrane were incubated with GST-RootR1 fusion protein or GST (data not shown). Their binding was detected by a subsequent incubation with an anti-GST antibody (upper panel). The same blot stripped and probed with an anti-T7 tag antibody (lower panel) shows the positions and the loading amounts of KLC and PAT1 proteins. (C) KLC3 was co-immunoprecipitated with rootletin from the lysate of COS cells transiently transfected with both rootletin and KLC3. IgG, rabbit immunoglobulin; Root6, rabbit anti-rootletin antibody; IP, immunoprecipitates. Input (cell lysate), 8%.

the interaction (Table 3). To rule out the possibility that rootletin binds KLCs through an intermediate protein present in yeast, we performed far Western analysis using GST-fused RootR1 and T7-tagged KLCs. It was found that GST-fused RootR1 was able to bind KLC1, KLC2, and KLC3, but not PAT1 (Fig. 1B), while control GST did not bind any of them, indicating the direct binding between rootletin and KLCs. To see if the interaction between rootletin and KLCs could take place in mammalian cells, we carried out immunoprecipitation using cells co-transfected with KLC3 and rootletin. KLC3 was detected in the immunoprecipitate of rootletin but not in that of control

Table 2
Interaction of rootletin with KLCs detected by yeast two-hybrid analysis

Bait (pGBKT7)	Prey (pGADT7)	Interaction
RootR1	KLC1	+
RootR1	KLC2	+
RootR1	KLC3	+
RootR1	PAT1	±
RootR1	Empty	–
Empty	KLC1	–
Empty	KLC2	–
Empty	KLC3	–
Empty	PAT1	–
KLC1	RootR1	±
KLC2	RootR1	+
KLC3	RootR1	+
PAT1	RootR1	±
Empty	RootR1	–
KLC1	Empty	–
KLC2	Empty	–
KLC3	Empty	–
PAT1	Empty	–

+, strong interaction as shown by colony growth on selection media after a small amount of inoculation and a short incubation time; ±, weak interaction as shown by colony growth on selection media after a large amount of inoculation and a long incubation time; –, no interaction as shown by no colony growth on selection media after a large amount of inoculation and a long incubation time; Empty, no gene is cloned into the vector.

Table 3
Interaction between rootletin and KLC domains detected by yeast two-hybrid analysis

Bait (pGBKT7)	Prey (pGADT7)	Interaction
RootR1	KLC1HR	+
RootR1	KLC1TPR	–
RootR1	KLC2HR	+
RootR1	KLC2TPR	–
RootR1	KLC3HR	+
RootR1	KLC3TPR	–
Empty	KLC1HR	–
Empty	KLC1TPR	–
Empty	KLC2HR	–
Empty	KLC2TPR	–
Empty	KLC3HR	–
Empty	KLC3TPR	–
KLC1HR	RootR1	+
KLC1TPR	RootR1	–
KLC2HR	RootR1	–
KLC2TPR	RootR1	–
KLC3HR	RootR1	+
KLC3TPR	RootR1	+
KLC1HR	Empty	–
KLC1TPR	Empty	–
KLC2HR	Empty	–
KLC2TPR	Empty	–
KLC3HR	Empty	–
KLC3TPR	Empty	+

+, strong interaction as shown by robust colony growth on selection media; –, no interaction as shown by no colony growth on selection media; Empty, no gene is cloned into the vector. The positive interaction between KLC3TPR/pGBDT7 and RootR1/pGADT7 was false, because KLC3TPR/pGBDT7 was self-activated. The negative interaction between KLC2HR/pGBDT7 and RootR1/pGADT7 was likely false due to the possible self-inhibition of KLC2HR/pGBDT7.

immunoglobin (Fig. 1C), confirming that rootletin and KLC3 formed a complex in cells.

Rootletin and KLCs are co-located in cultured and tissue cells

Because an interaction between two proteins would predict their co-localization in cells, we examined the distribution of rootletin and KLCs in both COS cells and native tissues. We transiently co-transfected COS cells with full-length rootletin and EGFP-tagged KLC1 or KLC2. KLC3 and PAT1 were used as positive and negative controls, respectively. The expression of these recombinant proteins in the cells was confirmed by Western blotting using GFP or rootletin antibodies (data not shown). When KLCs or PAT1 was expressed individually, they existed as different-sized aggregates, probably due to the interaction among their own HR domains. Some of the KLC aggregates were located at the centrosome where endogenous rootletin was concentrated [2]. After co-transfection, a portion of EGFP-tagged KLCs, but not PAT1, was redistributed to the recombinant rootletin fibers (Fig. 2A). We also examined the interaction of recombinant rootletin with endogenous KLC2 and KLC3 in COS cells; KLC1 could not be examined in this experiment because it is expressed only in neurons. In COS cells, both endogenous KLC2 and KLC3 were located at the centrosomal region where the endogenous rootletin was [2]. Additionally, KLC2 projected fine fibers toward the cell periphery, whereas KLC3 was more diffused in the cytoplasm. Upon transfection with rootletin and the formation of rootletin fibrous network, both KLC2 and KLC3 changed their distributions to co-localize with rootletin fibers (Fig. 2B).

To investigate the distribution of endogenous rootletin and KLCs in native tissues, we chose photoreceptor cells, which have robust ciliary rootlets. We prepared dissociated photoreceptor cells from retinas by gentle mechanical disruption. These dissociated photoreceptor cells were detached photoreceptor outer segments with ciliary rootlets attached and fully exposed to reagents. Immunostaining of the dissociated photoreceptor cells showed that both KLC2 and KLC3 were located along ciliary rootlets (Fig. 3). To rule out the possibility that the ciliary rootlets non-specifically bound unrelated antibodies, we stained the dissociated photoreceptor cells with protein disulphide-isomerase (an ER protein in the inner segment), γ-tubulin (a protein in the connecting cilium) and rhodopsin (a protein in the outer segment) antibodies as negative controls. None of them gave signals along the ciliary rootlets. γ-Tubulin and rhodopsin antibodies stained basal bodies and outer segments, respectively, indicating the negative controls worked as expected (Fig. 3). These results demonstrated that at least a portion of KLC2 and KLC3 co-localized with ciliary rootlets in vivo.

The interaction between ciliary rootlets and KLCs recruits KHC but not microtubules along ciliary rootlets

Our findings that KLCs were enriched along the rootlet fibers predicted that a portion of KHCs might be recruited to this location. To assess this issue, we cloned two KHC cDNAs, Kif5A and Kif5C, with EGFP tagged. Transient transfection of these KHCs into COS cells showed that they were localized diffusely in the cytoplasm. Upon co-transfection with rootletin, a significant fraction of the exogenous KHCs re-distributed along the rootletin fibers (Fig. 4A). Endogenous KHC (Kif5B in COS cells) underwent a similar re-distribution upon rootletin transfection. Kif5B in untransfected COS cells appeared as a fibrous network along the microtubules, but a portion of it re-distributed to the recombinant rootletin fibers after the transfection (Fig. 4B). Immunostaining for KHC on the dissociated photoreceptor preparation also revealed the localization of KHC along ciliary rootlets (Fig. 4C). Because the HR domain of KLCs mediates their binding to KHCs, the recruitment of KHCs along ciliary rootlets by KLCs indicated that the HR domain of KLCs was able to bind rootletin and KHCs simultaneously. In addition, comparison of the KLC3 amount in rootletin immunoprecipitates from rootletin/KLC3- and rootletin/KLC3/Kif5C-transfected COS cells indicated that KHCs did not substantially affect the interaction between rootletin and KLCs (Fig. 4D).

KHC binds and moves along microtubules in cells. Therefore, the existence of KHC on ciliary rootlets could bring microtubules to juxtaposition with rootletin fibers. However, double labeling of rootletin and microtubules using several different anti-tubulin antibodies did not show obvious co-alignment between rootletin fibers and microtubules in rootletin-transfected cells (data not shown). Reasoning that over-expression of KLC3 may recruit more KHC and microtubules along the rootletin fibers, we examined COS cells co-transfected with KLC3 and rootletin. Again, no apparent co-alignment between rootletin fibers and microtubules was observed despite the co-localization of KLC3 and rootletin (Fig. 4E). Consistently, tubulin was absent in the rootletin immunoprecipitate from retinal lysate (data not shown).

Kinesin-1 does not migrate along the rootletin fibers shown by FRAP analysis

Ciliary rootlets are long striated fibers originating from basal bodies at the apical end and extending toward the nuclei in ciliated cells. In particular, they span almost the entire cellular length in photoreceptor cells. This feature of ciliary rootlets is similar to that of microtubules. Thus, ciliary rootlets could conceivably act as an alternative moving track for rootlet-bound kinesin-1. Because rootlet-bound kinesin-1 did not appear to associate with microtubules, we reasoned that any detectable movement of

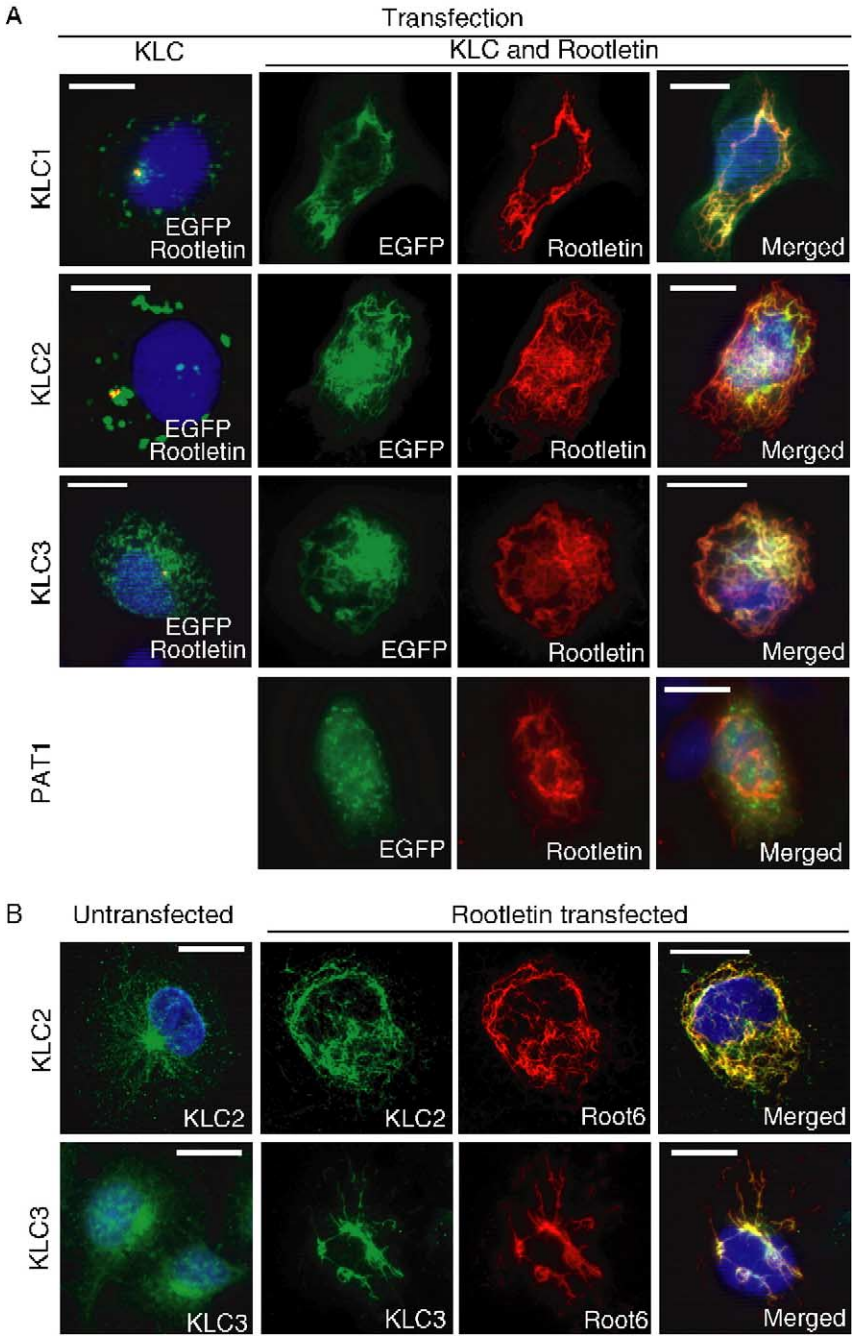

Fig. 2. KLCs are co-localized with rootletin in cultured cells. (A) In cells transfected with EGFP-tagged KLCs, recombinant KLCs (green) were randomly distributed in cytoplasm with a small portion co-localized with the endogenous rootletin (red). In cells co-transfected with rootletin and EGFP-tagged KLCs or PAT1, recombinant KLCs but not PAT1 were recruited to the transfected rootletin fibers. Panels represent the majority of double-transfected cells. (B) Endogenous KLC2 and KLC3 were co-localized with the transfected rootletin in COS cells. This co-localization was seen in about 86% and 87% of rootletin-transfected cells for KLC2 and KLC3, respectively (100 cells were observed each.). In the cells without co-localization, the amount of transfected rootletin was usually extremely low. Cell nuclei were counter stained with Hoechst dye 33342 (blue). Scale bars, 15 μm.

kinesin-1 along ciliary rootlets would support the above notion. Therefore, we examined the movement of rootlet-bound KLC3 on rootletin fibers by FRAP analysis. We photobleached EGFP-tagged KLC3 signals along the rootletin fibers in COS cells co-transfected with KLC3 and rootletin. We then examined whether the bleached regions moved along the rootletin fibers during a period of about 20 min before the bleached regions were fully recovered. We did not detect any migration of the bleached regions along the rootlets (Fig. 5). Because this time frame was long enough for kinesin-1 to move a measurable distance along microtubules, absence of migration suggested that ciliary rootlets were not likely to be the moving tracks for kinesin-1.

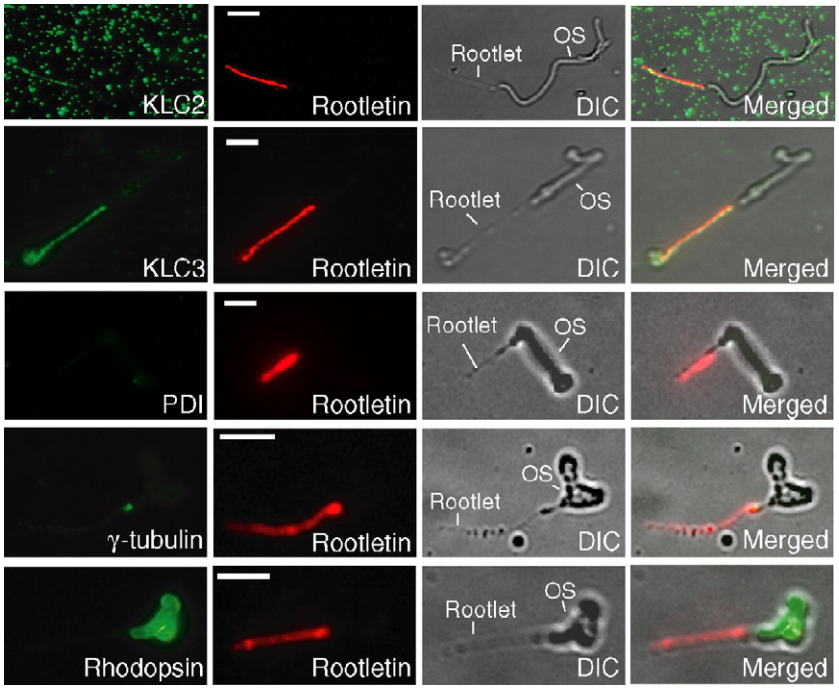

Fig. 3. KLCs are co-localized with rootletin in photoreceptor cells. Double staining of dissociated photoreceptor cells with rootletin and KLC2 or KLC3 antibodies shows that both KLC2 and KLC3 are enriched along the ciliary rootlet. Staining using PDI, γ-tubulin, and rhodopsin antibodies as negative controls, which do not give signals along the ciliary rootlets. The KLC2-positive speckles were resulted from a vesicular/particulate fraction co-sedimented with the dissociated photoreceptors. KLC1 had much more speckles than KLC2, which obscured the observation of KLC1 along the rootlet. OS, photoreceptor outer segment; DIC, differential interference contrast image. Scale bars, 5 μm.

APP and presenilin 1 are enriched along ciliary rootlets

APP is known to bring vesicular cargos to kinesin-1 by associating with KLC1 and KLC2. We therefore investigated whether the interaction between ciliary rootlets and kinesin-1 was able to recruit APP and its cargo proteins to ciliary rootlets. We first examined the distribution of endogenous APP in rootletin-transfected COS cells. Before transfection, APP was located primarily at the Golgi region. After transfection of rootletin, APP was seen overlapping with the rootletin fibers (Fig. 6A). To determine whether APP was also enriched along ciliary rootlets in vivo, we examined APP distribution in photoreceptors by double labeling with rootletin. Co-localization of the two proteins was seen in photoreceptor inner segments on retinal sections (Fig. 6B), and at higher resolution, along the ciliary rootlets in dissociated photoreceptors (Fig. 6C). We also studied the distribution of presenilin 1, a component of APP γ-secretase [18] and the proposed cargo of the APP/KLCs complex [13]. Endogenous presenilin 1 in COS cells was found enriched along the recombinant rootletin fibers compared with a Golgi-like localization in untransfected COS cells (Fig. 6D). Interestingly, no co-localization between rootletin and JIPs, a family of KLCs-interacting proteins [11,12], was detected in the COS cells co-transfected with rootletin and JIPs (JIP2 shown in Fig. 6E). These data suggested that the enrichment of APP and presenilin 1 along ciliary rootlets was specific. Because APP is proposed as a kinesin-1 vesicular cargo receptor with presenilin 1 as one of its cargos, the enrichment of APP and presenilin 1 along the rootletin fibers suggested that at least a subset of kinesin-1 vesicular cargos were located along ciliary rootlets through the association between kinesin-1 and rootletin.

Discussion

In this study, we demonstrate that rootletin associates with kinesin-1 by directly binding the HR domain of KLCs. These interactions, however, appear to differ in their relative strengths. While interactions between rootletin and all three KLCs were detectable by yeast two-hybrid, far Western and co-localization analyses, we were able to detect only KLC3 in the rootletin immunoprecipitate from cell lysates. Consistent with this observation, immunostaining for KLCs on the ciliary rootlet of dissociated photoreceptors gave a strong KLC3 signal and a far weaker one for KLC2. Thus, our data demonstrate that rootletin is able to interact with all three KLCs, with its interaction with KLC3 being the strongest of the three.

We show that the rootlet-bound kinesin-1 does not associate with microtubules or move along the rootlet fibers. It is plausible that the association of rootletin with kinesin-1 mediates the transport of unassembled rootletin to the site of rootlet assembly in vivo. However, this is unlikely to explain the entire physiological significance. Firstly, rootletin interacts with the HR domain of KLCs. If rootletin was merely a cargo of kinesin-1, one would have

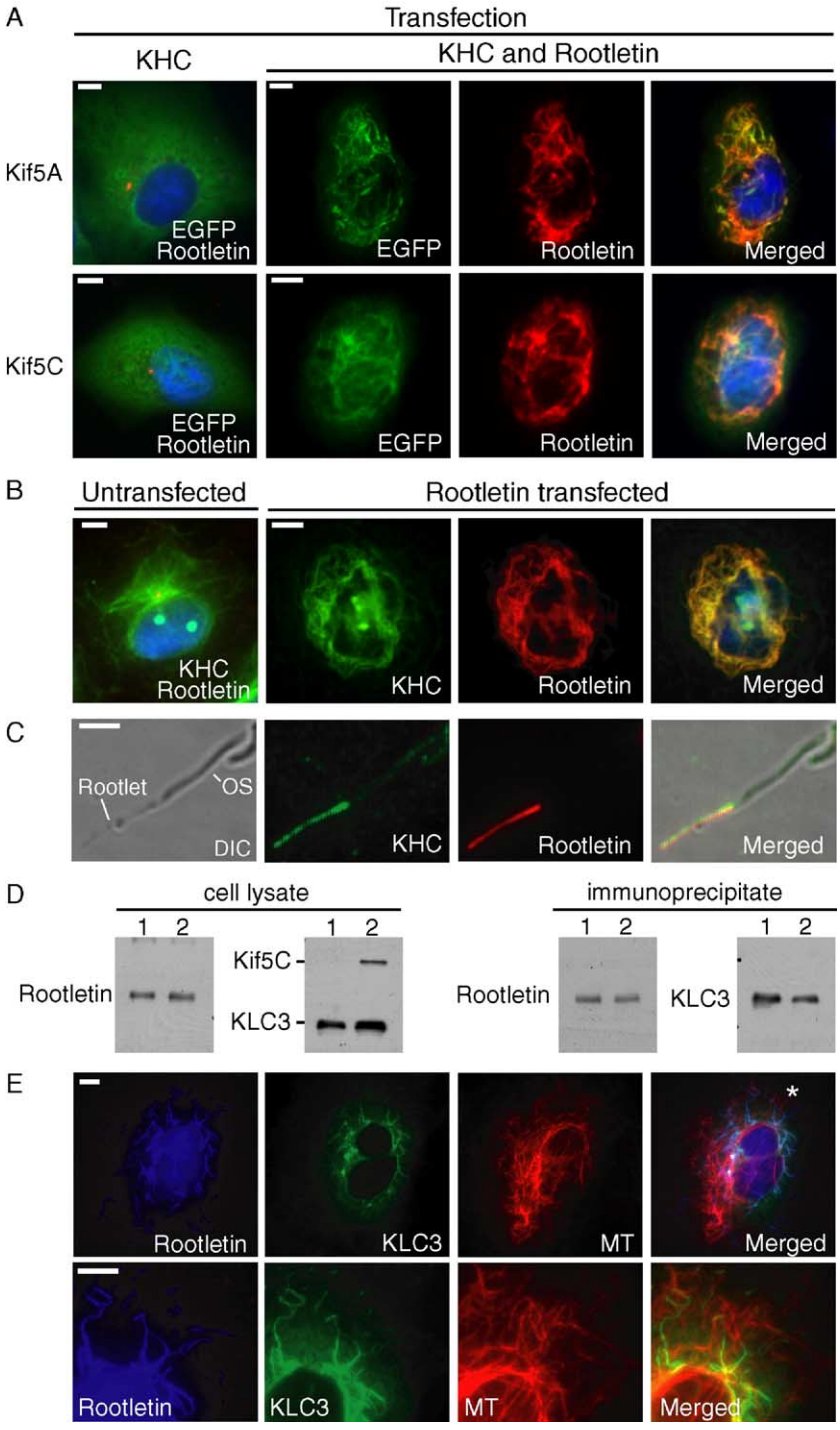

Fig. 4. KHC partially co-localizes with ciliary rootlets, while microtubules do not. (A) EGFP-tagged Kif5A (green) and Kif5C (green) partially co-localize with transfected rootletin (red) in COS cells. (B) Endogenous KHC (green) partially co-localizes with transfected rootletin (red) in COS cells. (C) KHC is located along the ciliary rootlet in dissociated photoreceptor cells. OS, photoreceptor outer segment; DIC, differential interference contrast image. (D) Kif5C expression does not substantially affect the interaction between rootletin and KLC3. COS cells were transfected with either rootletin/EGFP-tagged KLC3 (lane 1) or rootletin/EGFP-tagged KLC3/EGFP-tagged Kif5C (lane 2). Western blotting of cell lysates demonstrates the expression of recombinant proteins. Immunoprecipitation using rootletin antibody pulled down a similar amount of KLC3 in the presence or absence of Kif5C. The amount of rootletin in the immunoprecipitates was used as a control. (E) No co-alignment between rootletin (blue) and microtubules (red) was observed in COS cells co-transfected with full-length rootletin and EGFP-tagged KLC3 (green). The upper panels show a co-transfected cell at a lower magnification. The lower panels show the region of the same cell marked with an asterisk in the upper right panel at a high magnification. The upper merged panel was superimposed with all three left panels. The lower merged panel was superimposed with only microtubules (red) and KLC3 (green). The latter has a similar distribution to rootletin. Panels in parts A and B represent the majority of transfected cells. Nuclear counter stain was by Hoechst dye 33342 (blue) in parts A and B. Scale bars, 5 μm.

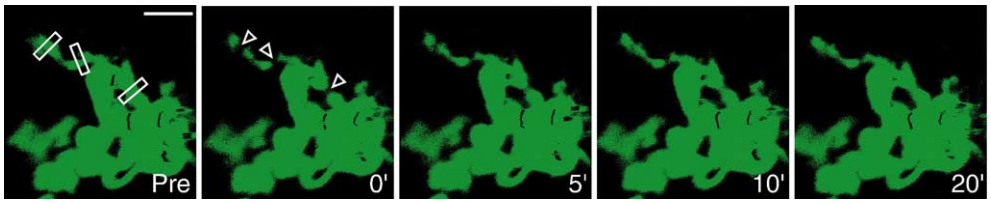

Fig. 5. Kinesin-1 (KLC3) does not migrate along the rootletin fibers. Three bars (arrows), each at 1.5 μm in width, were bleached simultaneously on the individual EGFP-tagged KLC3 fibers in a rootletin/KLC3-co-transfected COS cell. During the time course of fluorescence recovery, the bleached regions did not move along the rootletin fibers. The same result was obtained from three independent COS cells co-transfected with rootletin/KLC3, suggesting that kinesin-1 does not move along the rootletin fibers in vivo. Pre, before photobleaching; numbers of min, the time after photobleaching. Scale bar, 5 μm.

expected it to interact with the cargo-binding TPR domain of KLCs. Secondly, ciliary rootlets are a very stable and static structure comparable to keratin fibers as shown by time-lapse videography and FRAP analysis in cell culture (J Yang, unpublished data). The maintenance of ciliary rootlets may not require a continuously active transport of the

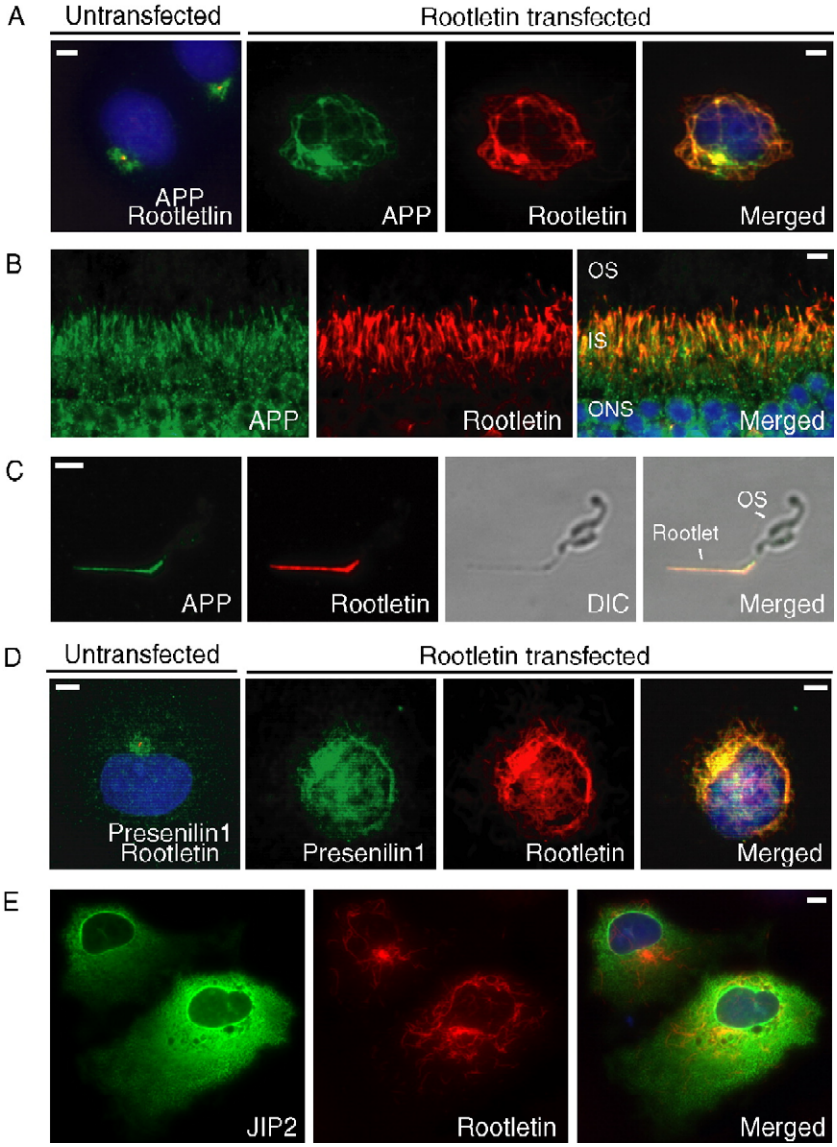

Fig. 6. Kinesin-1 vesicular cargo receptor, APP (green), and its cargo, presenilin 1 (green), are enriched along ciliary rootlets (red). (A) Endogenous APP is recruited along the transfected rootletin fibers in COS-7 cells. (B) APP and rootletin co-localize in the inner segment of photoreceptor cells. (C) APP is enriched along the ciliary rootlet of dissociated photoreceptor cells. (D) Endogenous presenilin 1 is enriched along the transfected rootletin fiber in COS cells. (E) JIP2 (green) is not co-localized with rootletin fibers in COS-7 cells transfected with both rootletin and JIP2. OS, photoreceptor outer segment; IS, photoreceptor inner segment; ONL, photoreceptor nuclear layer; DIC, differential interference contrast image. Nuclear counter stain was by Hoechst dye 33342 (blue). Scale bar, 5 μm.

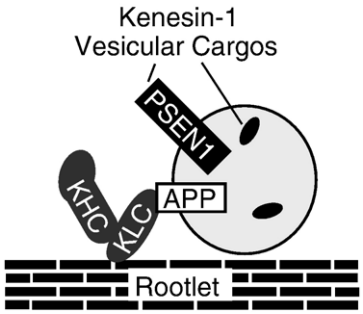

Fig. 7. A schematic diagram illustrating how a ciliary rootlet might serve as a scaffold for kinesin-1 vesicular cargos.

intermediate precursors. Thus, rootletin transport per se cannot explain the large amount of kinesin-1 associating with ciliary rootlets. Thirdly, a kinesin-1 vesicular cargo receptor (APP) and a vesicular cargo (presenilin 1) are recruited along ciliary rootlets. Therefore, the association between kinesin-1 and rootletin appears to execute biological functions in addition to the transport of rootletin monomers/oligomers. Because JIPs are not recruited along rootletin fibers in cells, the available data suggest that ciliary rootlets probably serve as a scaffold for a subset of kinesin-1 vesicular cargos as illustrated in Fig. 7. The subset of kinesin-1 vesicular cargos may be stored, assembled, or/and disassembled along ciliary rootlets in ciliated cells.

Kinesin-1 is a motor protein moving along microtubules toward their plus ends, which are located at the basolateral region in ciliated cells. Therefore, being a proposed scaffold for kinesin-1 vesicular cargos, ciliary rootlets may play a role in the vesicular basolateral transport in these cells, the opposite direction of dynein-mediated transport [19]. APP has been reported to be differentially sorted and delivered in MDCK cells and neurons [20,21]. In MDCK cells, APP is preferentially located on the basolateral cell surface, while in neurons, APP is first delivered to the axon and later appears in the dendrites. Immunostaining of retina with an anti-APP C-terminus antibody showed the distribution of APP from the inner segment to the synaptic end of photoreceptor cells (Fig. 6B), which is equivalent to the basolateral part of epithelial cells. Presenilin 1 previously was shown to be expressed in photoreceptor cells [22]. Thus, the recruitment of APP and presenilin 1 to ciliary rootlets through their association with kinesin-1 may be related to their polarized transport in photoreceptor cells.

In addition to our findings, another study suggesting a role for ciliary rootlets in the vesicular intracellular transport was done by Fariss and colleagues, who found peripherin/rds-positive vesicles accumulating along ciliary rootlets in degenerating photoreceptor cells [1]. Photoreceptor cells are known to have an active intracellular membrane trafficking to support their continuous renewal of outer segments. In the degenerating photoreceptor cells, the accumulated vesicles with outer segment proteins could result from either the defective vesicular intracellular transport to, and/or abnormal endocytosis of, outer segments. Because we did not detect any feature of endosomes in the membranous saccules along the rootletin fibers in cell culture (J Yang, unpublished data), these peripherin/rds-positive vesicles are likely the transport vesicles on route to the outer segments, indicating that intracellular transport occurs around ciliary rootlets.

Several other proteins are known to associate with KLCs besides rootletin, APP and JIPs. They are torsinA [23], glycogen synthase kinase3-binding protein (GBP) [24], vimentin [25,26] and outer dense fiber 1 (ODF1) [27]. APP, JIPs and torsinA bind the TPR domain of KLCs. TorsinA, an early onset dystonia protein, is probably a kinesin-1 cargo during its anterograde transport. GBP binds to an uncharacterized conserved domain of KLCs and is proposed to be a cargo of kinesin-1. Vimentin associates with kinesin-1 through KLCs. This association is assumed to transport the intermediate assembly products of vimentin to its assembly site and maintain an extended filamentous network [28]. ODF1, a unique structural component of sperm tails, was found to bind the HR domain of KLC3, although the physiological significance of this binding is largely unknown. Among these KLC-associated proteins, vimentin and ODF1 are cytoskeletal proteins like rootletin. Though no information is available about the involved KLC domain for vimentin binding, ODF1 interacts the same domain of KLCs as rootletin. Therefore, the association of KLCs with rootletin may have a similar function to its association with vimentin and ODF1. Our finding of ciliary rootlets as a scaffolding site for kinesin-1 vesicular cargos may also provide new insight into the biological function of the association of kinesin-1 with other long cytoskeleton fibers, such as vimentin and ODF1.

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