Molecular Biology of the Cell
Vol. 17, 1033–1040, February 2006

Rootletin Interacts with C-Nap1 and May Function as a Physical Linker between the Pair of Centrioles/Basal Bodies in Cells

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Submitted October 12, 2005; Revised November 30, 2005; Accepted November 30, 2005
Monitoring Editor: Trisha Davis

Rootletin, a major structural component of the ciliary rootlet, is located at the basal bodies and centrosomes in ciliated and nonciliated cells, respectively. Here we investigated its potential role in the linkage of basal bodies/centrioles and the mechanism involved in such linkages. We show that rootletin interacts with C-Nap1, a protein restricted at the ends of centrioles and functioning in centrosome cohesion in interphase cells. Their interaction in vivo is supported by their colocalization at the basal bodies/centrioles and coordinated association with the centrioles during the cell cycle. Ultrastructural examinations demonstrate that rootletin fibers connect the basal bodies in ciliated cells and are present both at the ends of and in between the pair of centrioles in nonciliated cells. The latter finding stands in contrast with C-Nap1, which is present only at the ends of the centrioles. Transient expression of C-Nap1 fragments dissociated rootletin fibers from the centrioles, resulting in centrosome separation in interphase. Overexpression of rootletin in cells caused multinucleation, micronucleation, and irregularity of nuclear shape and size, indicative of defects in chromosome separation. These data suggest that rootletin may function as a physical linker between the pair of basal bodies/centrioles by binding to C-Nap1.

INTRODUCTION

The ciliary rootlet is a large striated fibrous cytoskeleton in ciliated cells. In a photoreceptor cell, which has a specialized cilium known as the outer segment, the ciliary rootlet is a thick striated bundle originating from the proximal ends of basal bodies and spanning the entire length of cell body. In cells with motile cilia such as epithelial cells lining the respiratory tract, brain ventricles, and oviduct, ciliary rootlets originate from the proximal ends of multiple basal bodies and form a subapical fibrous network. The functions of the ciliary rootlet had been largely unknown until the recent discovery of its major structural component, rootletin (Yang *et al.*, 2002). Genetic knockdown of rootletin expression by >95% in mouse ablates the formation of ciliary rootlets in ciliated cells. Studies of this mouse model suggest that the ciliary rootlet plays a crucial role in maintaining the cellular integrity in ciliated cells (Yang *et al.*, 2005). Additionally, the ciliary rootlet was found to interact with kinesin light chains and was postulated to provide a scaffold for a subset of kinesin-1 vesicles (Yang and Li, 2005).

In nonciliated cells, a miniature ciliary rootlet is located at the centrosome and does not project a fibrous network into the cytoplasm (Yang *et al.*, 2002). Thus, it is not expected to have a meaningful role in either structural support or intracellular trafficking. The centrosome is composed of a pair of centrioles, which are structurally analogous to basal bodies, and pericentriolar materials. During the cell cycles, the centrioles undergo rounds of cohesion and separation. Malfunction in these processes may lead to the development of tumors (Brinkley and Goepfert, 1998; Doxsey, 1998; Salisbury *et al.*, 1999). A previous study indicates that a proteinaceous linker between the pair of centrioles mediates their cohesion (Paintrand *et al.*, 1992), though the identity of this linker remains unknown. Recent studies demonstrate that several proteins, including NIMA-related kinase 2 (Nek2), protein phosphatase 1 (PP1), C-Nap1, dynamin 2, and RanBP1, are involved in centriolar cohesion (Mayor *et al.*, 2000; Meraldi and Nigg, 2001; Di Fiore *et al.*, 2003; Thompson *et al.*, 2004). Among these proteins, C-Nap1, also known as CEP250 (Mack *et al.*, 1998), is a centrosomal protein and the presumed substrate of Nek2 and PP1 (Fry *et al.*, 1998; Helps *et al.*, 2000; Meraldi and Nigg, 2001). It is proposed that the function of C-Nap1 in centriolar cohesion is regulated by its own phosphorylation status, which depends on a balance between Nek2 and PP1 activities. Studies by immunoelectron microscopy found that C-Nap1 was present only at the proximal ends of, but not between, the two centrioles (Mayor *et al.*, 2000). Importantly, C-Nap1 does not appear able to form elongated polymers, a prerequisite were it to act as a physical linker between the centrioles. These data suggest that C-Nap1 might serve as the anchor for, but is not in itself, an intercentriolar linker. By the same token, dynamin 2 and RanBP1, while capable of regulating the process of centriolar cohesion, are unlikely to act as physical linkers.

Rootletin both associates with the proximal ends of basal bodies and forms elongated polymers between them in photoreceptors (Yang *et al.*, 2005), indicating that the miniature ciliary rootlet in nonciliated cells is a candidate centriole linker. In our rootletin knockdown mouse model, aberrant

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05–10–0943) on December 7, 2005.

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RNA splicing at the site of rootletin gene produces a small amount of abnormal rootletin, which localizes correctly to the proximal ends of basal bodies and centrioles (Yang *et al.*, 2005). Hence a putative centriolar function for rootletin in nonciliated cells could not be determined definitively in this genetic model. C-Nap1 is the closest homolog of rootletin in the mammalian genome. Both proteins have extended coiled-coil domains mediating homo- and/or hetero-oligomerization. In this study, we investigated whether these two proteins interacted with each other using biochemical and cellular approaches and present evidence to support the role of the ciliary rootlet as a physical linker between the pair of basal bodies/centrioles in cells.

MATERIALS AND METHODS

Plasmids and Yeast Two-Hybrid Analysis

Mouse rootletin in EGFPC2 vector and its fragments R1-R4 in pGADT7 and pGBKT7 vectors were described previously (Yang *et al.*, 2002). Mouse C-Nap1 fragments (C1, aa 7–656; C2, aa 651-1316; C3, aa 1311–1987; C4, aa 1982–2414; NT, aa 13–1233; CT, aa 1228–2414, accession number, DQ148475) were PCR amplified from kidney, embryo, and lung cDNAs and cloned into EGFPC2 (NT and C4), EGFPC3 (CT), and pGADT7 and pGBKT7 vectors (C1–C4).

Yeast two-hybrid analysis was performed as described previously (Yang *et al.*, 2002). Briefly, rootletin and C-Nap1 fragments in pGBKT7 vector were cotransformed with C-Nap1 and rootletin fragments in pGADT7 vector, respectively. Empty pGBKT7 and pGADT7 vectors were used as negative controls. Cotransformants were grown on both SD-4 (-Leu, -Trp, -Ade, and -His) and SD-2 (-Leu and -Trp) plates. In our experiments, all cotransformants were able to grow on SD-2 plates, indicating a successful cotransformation. The growth on SD-4 plates indicated an existence of interaction between the two cotransformed protein fragments.

Coimmunoprecipitation and Western Blotting

COS cells were homogenized and incubated for ~120 min in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor, and 1 mM DTT). After centrifugation at 18,000 × *g* for 10 min, the supernatant was incubated with the primary antibody for 3 h and then centrifuged at 18,000 × *g* for 10 min. The resulting supernatant was incubated with protein G-Sepharose (Amersham Biosciences, Piscataway, NJ) for an additional 1 h. After a brief centrifugation at 2000 × *g*, the pellet was washed with lysis buffer four times and then boiled in Laemmli sample buffer. All the procedures were performed at 4°C. A nonimmune rabbit IgG served as a negative control. Western blotting was carried out as described previously (Yang *et al.*, 2002).

Antibodies

The rabbit and chicken rootletin (Root6 and Root10) antibodies (Yang *et al.*, 2002) and the rabbit C-Nap1 antibody (R63; Fry *et al.*, 1998) have been described. Monoclonal antibodies against C-Nap1 and Nek2 were from BD Transduction Laboratories (Lexington, KY). Monoclonal anti-γ-tubulin antibody was obtained from Sigma-Aldrich (St. Louis, MO). Secondary antibodies conjugated to Alexa fluorochromes, Hoechst dye 33342, and TOTO-3 were obtained from Molecular Probes (Eugene, OR).

Cell Culture and Transfection

COS-7, HEK 293t, and MDCK cells were cultured in DMEM supplemented with 5% fetal bovine serum. Transient transfections were carried out using the Geneshuttle-40 reagent (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions.

To generate a heterogeneous population of MDCK cells that expressed rootletin at varying levels, MDCK cells were transfected with EGFP-tagged rootletin using the Geneshuttle-40 reagent. Cells carrying the EGFP plasmid were selected in culture media supplemented with 1 mg/ml G418 for 14 d. The obtained cells were maintained in the presence of 1 mg/ml G418 afterward. Expression of rootletin was assessed by Western blotting and EGFP fluorescence under a fluorescent microscope.

Immunofluorescence Microscopy

Dissociated photoreceptors were prepared as described previously (Yang *et al.*, 2002). Cells and photoreceptors were fixed in methanol:acetone (19:1) for 10 min at −20°C. Tissue sections were fixed in 4% formaldehyde/phosphate-buffered saline for 10 min and permeabilized by 0.2% Triton X-100/phosphate-buffered saline for 5 min at room temperature. The subsequent steps of blocking and incubation with primary and secondary antibodies were as described previously (Yang *et al.*, 2002). Alexa 488- and 594-conjugated secondary antibodies were routinely used for tissue double-labeling. Alexa 546- and 633-conjugated secondary antibodies were routinely used for double-labeling of cells transfected with EGFP-tagged proteins. Alexa 488-, 546-, and 633-conjugated secondary antibodies were routinely used for tissue triple-labeling. Stained sections and cells were viewed and photographed on a fluorescent microscope (model 1X70; Olympus, Lake Success, NY) equipped with a digital camera (Carl Zeiss MicroImaging, Thornwood, NY) or on a confocal laser scanning microscope (model TCS SP2; Leica, Deerfield, IL).

Transfected cells were randomly chosen to analyze the phenotype, and their neighboring nontransfected cells were randomly chosen as controls. The two centrosomes with a distance equal or larger than 2 μm in the interphase cells were considered as separated.

Immunoelectron Microscopy

HEK 293t cells were fixed with 4% formaldehyde/phosphate-buffered saline for 30 min, washed with TTBS buffer (Tween/Tris-buffered saline), blocked in 5% goat serum/TTBS for 1 h, and incubated with rabbit anti-rootletin or anti-FKBP8 (FK506-binding protein 8, a negative control) antibody at 4°C overnight. After rinses, cells were incubated with Nanogold goat anti-rabbit antibody, postfixed sequentially with 1% formaldehyde/2.5% glutaraldehyde/0.1 M cacodylate buffer and 2% osmium tetroxide. Cells were silver-enhanced, dehydrated, embedded in Epon, and sectioned at 70-nm thickness.

RESULTS

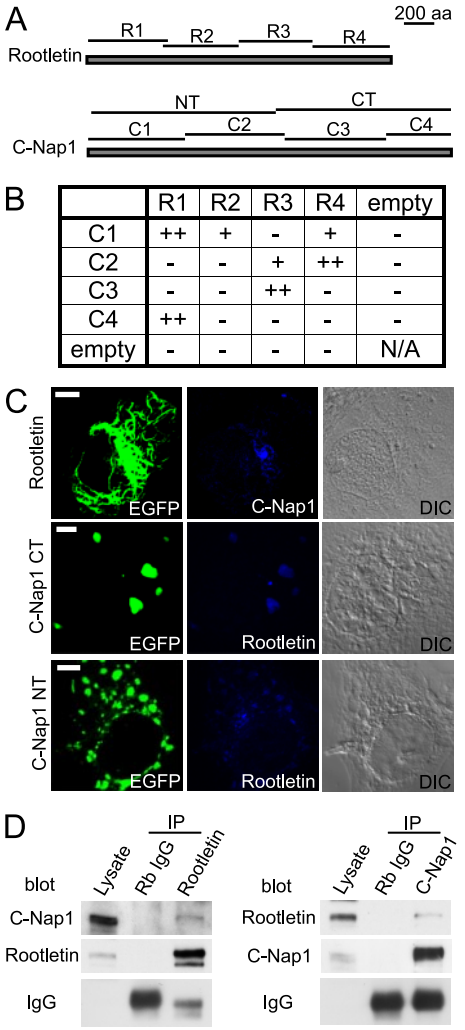
Rootletin Interacts with C-Nap1 at Multiple Regions

We investigated, by yeast two-hybrid analysis, the interaction between rootletin and C-Nap1 and the specific regions of these two proteins mediating the interaction. We divided each of these two proteins into four fragments (R1–R4 for rootletin and C1–C4 for C-Nap1; Figure 1A), and cloned them into both bait (pBKT7) and prey (pADT7) vectors. After cotransformation of rootletin and C-Nap1 fragments into yeast cells, we analyzed the growth of the cotransformants on the selective medium. The results demonstrated that all four rootletin and C-Nap1 fragments were able to bind a region(s) of the their partner proteins (Figure 1B). Thus, rootletin and C-Nap1 were able to interact with each other and their interaction may be mediated by multiple segments of these proteins. Survey of the interacting domains between rootletin and C-Nap1 suggested that they did not simply bind to each other by the lateral interaction along their full lengths, and their binding may be complex, including head-to-head or head-to-tail interactions.

To further investigate the interaction between rootletin and C-Nap1, we transiently transfected COS cells with EGFP-fused rootletin or the fragments of C-Nap1 (Figure 1A). Recombinant rootletin, C-Nap1 CT, and C-Nap1 NT, when transiently expressed, recruited their endogenous partner proteins (Figure 1C), suggesting their interaction in vivo. Interestingly, transient expression of the recombinant proteins appeared to increase the amount of their endogenous partner proteins, suggesting that they may be stabilized through their interactions. Additionally, reciprocal coimmunoprecipitation of endogenous rootletin and C-Nap1 from COS cell lysate (Figure 1D) was also consistent with their interaction in vivo.

Rootletin Overlaps with C-Nap1 at the Proximal Ends of Basal Bodies/Centrioles

C-Nap1 is a known centrosomal protein located at the proximal ends of centrioles in nonciliated cells (Fry *et al.*, 1998; Mayor *et al.*, 2000). To investigate whether and where it was expressed in ciliated cells, we triple-immunostained dissociated photoreceptors with C-Nap1, γ-tubulin, and rootletin antibodies. C-Nap1 was found to be expressed in photoreceptors, where it was restricted to the basal bodies. In contrast, rootletin immunoreactivity was distributed throughout the rootlet (Figure 2A). Given its continuity with the rootlet, which originates from the proximal ends of basal bodies, this staining pattern of C-Nap1 suggest that it is



	R1	R2	R3	R4	empty
C1	++	+	-	+	-
C2	-	-	+	++	-
C3	-	-	++	-	-
C4	++	-	-	-	-
empty	-	-	-	-	N/A

Figure 1. Rootletin interacts with C-Nap1. (A) Schematic diagrams of rootletin and C-Nap1 fragments used in this study. (B) Yeast two-hybrid analysis on the regions of rootletin and C-Nap1 involved in their interaction. ++ and +, same results were obtained regardless of the host vector configuration (prey or bait vector). ++, 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. (C) Exogenous expression of rootletin or C-Nap1 fragments recruits its endogenous partner protein. DIC, differential interference contrast image; Bars, 5 μm. (D) Rootletin and C-Nap1 are coimmunoprecipitated with each other from COS cell lysate. The IgG blots show the amount of antibodies used in the coimmunoprecipitation experiment. Rb IgG, rabbit immunoglobulin, a negative control; IP, immunoprecipitate; Blot, Western blot.

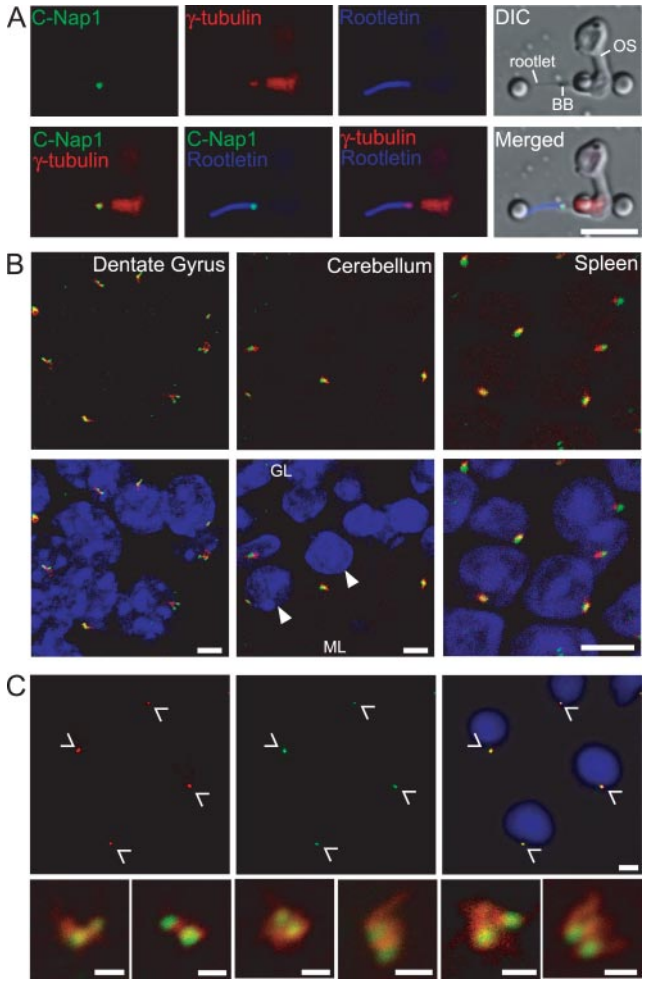


Figure 2. Rootletin and C-Nap1 are partially colocalized in cells shown by confocal laser scanning microscopy. (A) C-Nap1 (green) is located at the basal bodies (γ-tubulin, red) and partially colocalized with rootletin (blue) in dissociated photoreceptors. The staining of γ-tubulin antibody on the outer segment is nonspecific signals. OS, outer segment; BB, basal bodies; DIC, differential interference contrast image. (B) Rootletin (red) is partially colocalized with C-Nap1 (green) in cells from the dentate gyrus, cerebellum, and spleen. Top panels, the superimposed images of rootletin and C-Nap1. Bottom panels, the images in top panels merged with nuclei staining (blue, TOTO-3). Arrowheads, Purkinje cells; GL, granular layer; ML, molecular layer. (C) Rootletin (red) is colocalized with C-Nap1 (green) at the centrosomes (arrows) in nonciliated COS cells. Top panels, a view of several COS cells double-stained with rootletin and C-Nap1. Right is a merged image of rootletin and C-Nap1 staining. Cell nuclei were counterstained with Hoechst dye 33342 (blue). Bottom panels, several superimposed images of rootletin and C-Nap1 at a high magnification. Bars, 5 μm except the ones in 2C, bottom panels, which are 1 μm.

most likely located at the proximal ends of basal bodies, similar to where it is in nonciliated cells.

Rootletin and C-Nap1 are both known as centrosomal proteins in nonciliated cells. However, no comparison on their locations at the centrioles has been conducted. We examined their localization at the centrosome in both tissues and cultured cells by confocal laser scanning microscopy. In the brain, rootletin antibody stained the rudimentary rootlets in a variety of neurons such as granular neurons in the dentate gyrus and Purkinje cells in the cerebellum. Similar to the findings in ciliated cells, C-Nap1 antibody stained only the origins of the rudimentary rootlets (Figure 2B). Colocalization of rootletin and C-Nap1 at the centrioles was also seen in the red pulp of spleen where reticular cells are prevalent (Figure 2B).

To study the colocalization of these two proteins in detail, nonciliated cultured cells were examined. Double staining of rootletin and C-Nap1 in COS cells confirmed that they were both located at the centrosomes (Figure 2C, top panels).

High-magnification images showed the colocalization of rootletin with C-Nap1 at the proximal ends of the two centrioles. Unlike C-Nap1, which was limited to the proximal ends of centrioles, rootletin immunoreactivity extended further and was seen between and around the two centrioles (Figure 2C, bottom panels). These immunostaining data demonstrated that rootletin and C-Nap1 were partially colocalized at the basal bodies/centrioles in cells, supporting their association at those places in vivo.

The Ciliary Rootlet Is Located between and at the Proximal Ends of Centrioles in Nonciliated Cells

Transmission electron microscopy showed unambiguously that the ciliary rootlet, as striated fibers, connected the pair of basal bodies in photoreceptors (Figure 3A), indicating that the rudimentary rootlet in nonciliated cells may also be the fibers connecting the centrioles. To confirm the similarity of the rootlet in both nonciliated cells and photoreceptors, we conducted double-immunostaining with antibodies against rootletin and γ-tubulin (Figure 3B) or C-Nap1 (Figure 2C) in nonciliated cells. The rudimentary rootlet was shown around and between the pair of centrioles in the centrosomes. At an ultrastructural level, immunoelectron microscopy using a rootletin antibody demonstrated that rootletin was located between (Figures 3, C–E) and at the proximal ends (Figure 3, E–G) of the pair of centrioles in nonciliated cells. This localization of the ciliary rootlet relative to the pair of centrioles is consistent with the rootlet being the physical intercentriolar linker in interphase nonciliated cells.

Rootletin and C-Nap1 Undergo Parallel Changes at the Centrioles through the Cell Cycle

C-Nap1 was shown to undergo a cell cycle-related association with the centrioles. Specifically, C-Nap1 dissociates from the centrioles at the beginning of mitosis and reassociates with them at the end of mitosis (Fry *et al.*, 1998; Mayor *et al.*, 2000). If the ciliary rootlet binds to C-Nap1 at the proximal ends of centrioles as a physical intercentriolar linker, rootletin may also undergo the same pattern of changes as C-Nap1 at the centrosome through the cell cycle. Double-immunostaining of COS cells showed that rootletin did change its immunoreactivity in a similar pattern to C-Nap1 during the cell cycle. Both signals decreased sharply at the onset of mitosis and disappeared in anaphase. Later on, they reemerged and reached their maximal levels as cells entered the interphase (Figure 4).

C-Nap1 Fragments Dissociate Rootletin from the Centrioles and, Thus, Separate the Pair of Centrioles in Interphase Cells

The C-terminus of C-Nap1 is known to interact with and probably function as the substrate for the cell cycle-regulated protein kinase Nek2 (Fry *et al.*, 1998). This interaction appears to regulate the centrosomal localization of C-Nap1 through the cell cycle (Mayor *et al.*, 2002). Exogenous expression of the C-terminus of human C-Nap1 has been found to cause centrosome splitting in interphase (Mayor *et al.*, 2000), suggesting that the C-terminal fragment of C-NAP1 acts as a dominant-negative mutant to disrupt the normal function of Nek2 and C-Nap1 at the centrosome. If rootletin, as we hypothesized, acts as the centriolar linker through binding to C-Nap1, its localization and function at the centrosome would be similarly affected by this mutant. To test this, we transfected the C-terminal fragment of mouse C-Nap1, C-Nap1 C4, into COS cells and investigated the distribution of rootletin at the centrosomes. As expected, transient expression of this fragment caused centrosome separation in 55.7%

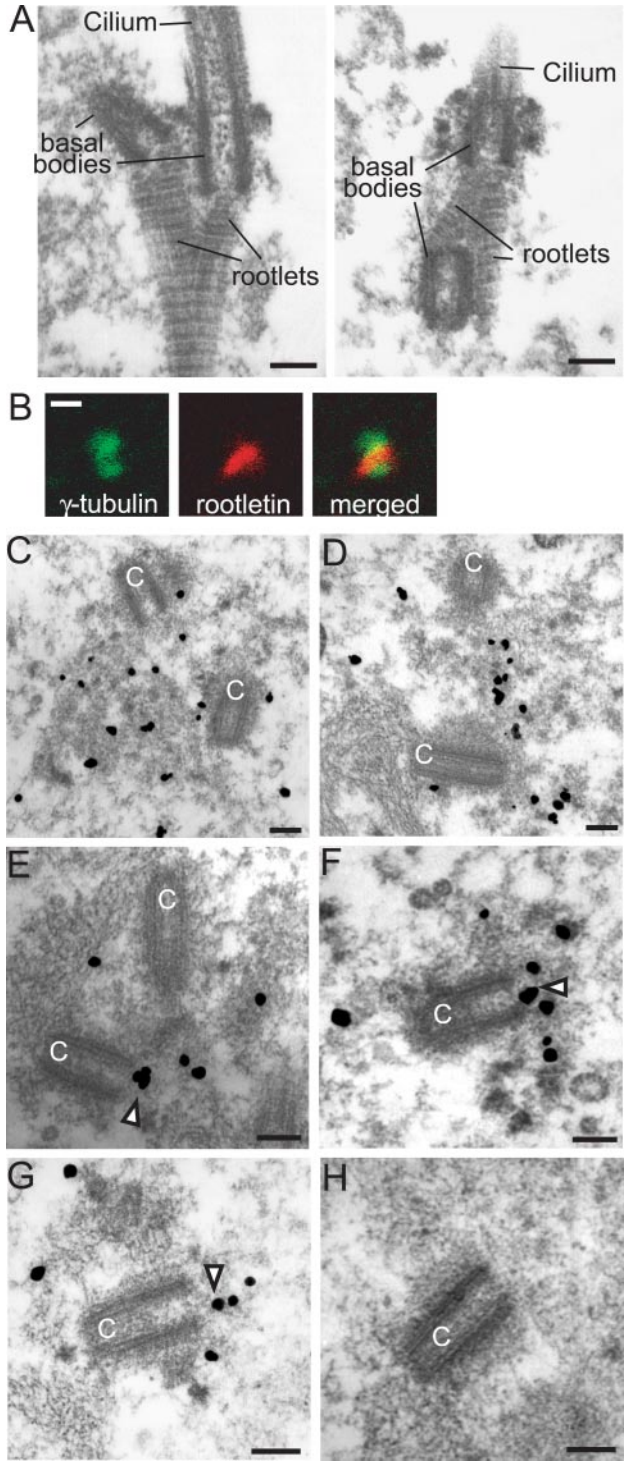


Figure 3. The ciliary rootlet is located between and around the pair of centrioles/basal bodies in cells. (A) The ciliary rootlet originates from and connects the pair of basal bodies in photoreceptors shown by electron microscopy. Bars, 200 nm. (B) Rootletin (red) is located between the pair of centrioles marked by γ-tubulin staining (green) in nonciliated cells. Bar, 1 μm. (C–G) Immunoelectron micrographs of rootletin in HEK 293t cells demonstrate that rootletin is mainly located between the two centrioles (C–E) and at their proximal ends (arrowheads; E–G). The proximal ends of centrioles may be out of the sectioning plate in C and D. FKBP staining (H), as a negative control, does not show signals around the centrioles. Bars, 200 nm. C, centriole.

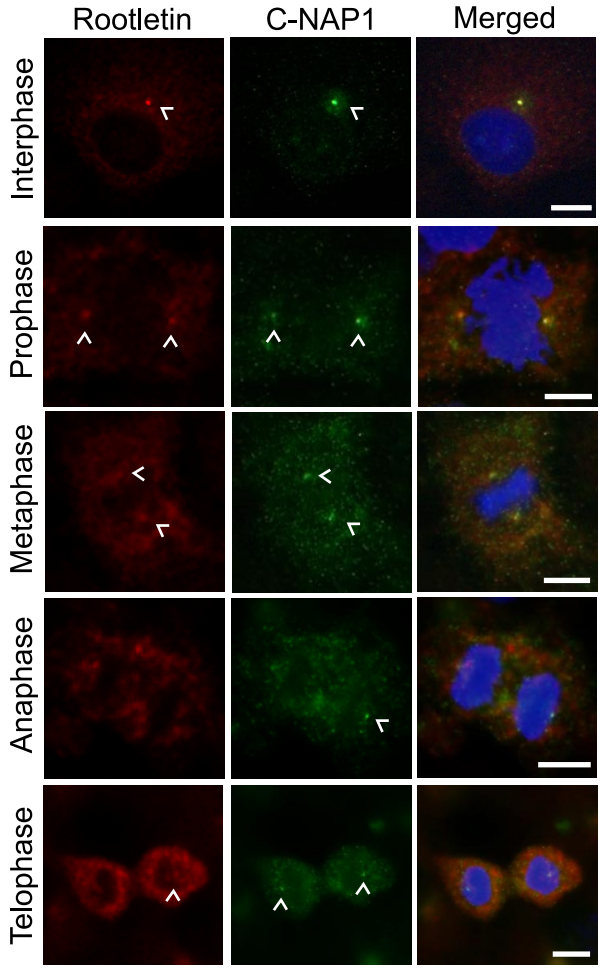


Figure 4. The immunoreactivity of rootletin (red) at the centrioles (arrows) exhibits a similar change pattern to C-Nap1 (green) during the cell cycle. Their immunoreactivities decrease at the onset of mitosis (prophase), disappear during anaphase, reemerge at the telophase, and increase to the highest level at interphase. Nuclei or chromosomes were counterstained with Hoechst dye 33342 (blue). All images were taken at the same conditions. Bars, 5 μm.

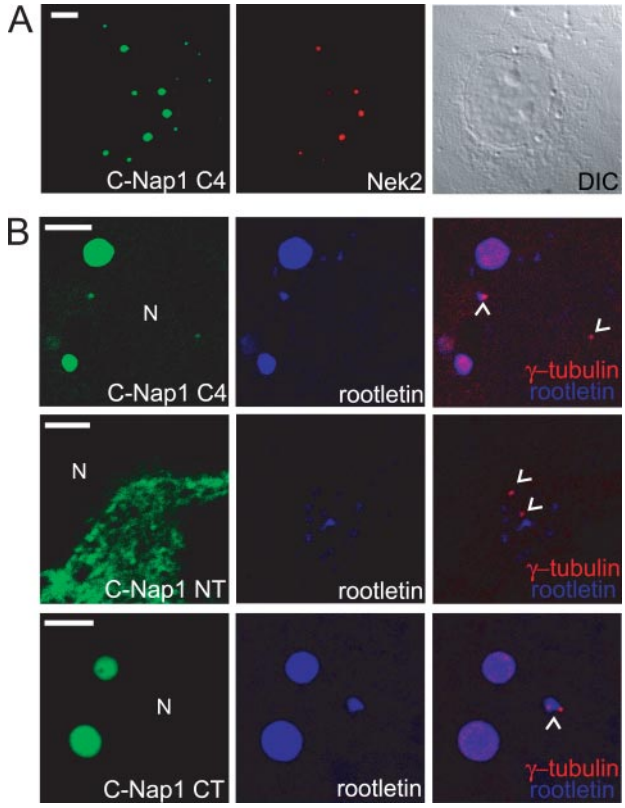


Figure 5. Exogenous expression of C-Nap1 C4 and NT fragments results in dislocation of rootletin at the centrosomes and separation of centrosomes. (A) Exogenous expression of C-Nap1 C4 fragment (green) recruits the endogenous Nek2 (red) to the aggregates of C-Nap1 C4. (B) Exogenous expression of C-Nap1 C4 and NT, but not CT fragments (green) causes dislocation of rootletin (blue) from the centrosomes (γ-tubulin signals, red, arrow heads) and centrosome separation. N, the place of the nucleus; DIC, differential interference contrast image. Bar, 5 μm.

of 88 transfected cells, whereas centrosome separation was only observed in 18.6% of 97 nontransfected cells. In addition, immunostaining of the transfected cells with a Nek2 antibody and a monoclonal antibody against C-Nap1, which did not recognize the C4 region, demonstrated that both endogenous Nek2 (Figure 5A) and C-Nap1 (unpublished data) were recruited from the centrosomes to the C4 aggregates. These confirmed that this fragment acted as a dominant-negative mutant. As a result, rootletin was abnormally distributed at the centrosomes in 68.8% of 32 transfected interphase cells. It was often detached from one or both of the separated centrioles (Figure 5B). Furthermore, the ratio of cells with abnormal rootletin distribution at the centrosome (67.8%) was higher than the ratio of cells with the separated centrioles (55.7%), suggesting that the disconnection of the rudimentary rootlet from the centrosomes is the cause of the centrosome separation.

By yeast two-hybrid analysis C-Nap1 and rootletin interacts with each other at multiple regions. If physiologically relevant, C-Nap1 fragments representing those regions other than C-Nap1 C4 may similarly act as dominant-negative mutants. We therefore examined the interphase cells transiently transfected with other fragments of C-Nap1. In most of C-Nap1 NT-transfected cells, the distribution of rootletin at the centrosomes appeared aberrant. Several tiny pieces of rootletin were found randomly around the centrosomes. Rootletin either completely disappeared from the centrosomes or remained at only one of the centrioles. The pair of centrioles marked by γ-tubulin was separated in 70% of 20 transfected cells (Figure 5B). As a control, only 12.5% of eight nontransfected cells had separated centrosomes. Thus, the expression of C-Nap1 NT fragment, like the expression of C4 fragment, was able to disturb the localization of rootletin at the centrosomes and result in centrosome separation. The effects of the exogenous expression of C-Nap1 C4 and NT fragments on the distribution of rootletin and the separation of the pair of centrioles were specific, because the expression of C-Nap1 CT fragment did not appear to have these effects (Figure 5B).

Overexpression of Rootletin Causes Multinucleation, Micronucleation, and Irregularity of Nuclear Size and Shape in Interphase Cells

If the rudimentary rootlet functionally interacts with C-Nap1 and serves as a physical linker of the pair of centrioles,

then overexpression of rootletin may interfere with centrosomal function during the cell cycle. Indeed, cells overexpressing rootletin were found to have nuclear defects compared with their nontransfected neighboring cells. These defects included multinucleation, micronucleation, and nuclei of large size or with irregular shape. Because the extensive rootletin fibrous networks in transfected cells were not fully dissolved during mitosis (Yang *et al.*, 2002), the observed nuclear defects could have resulted from the physical obstruction of the formation of mitotic spindle and chromosomal segregation by rootletin fibers. To rule out this possibility, we generated a heterogeneous MDCK cell population stably expressing recombinant rootletin. Among these cells, the expression levels of recombinant rootletin were variable but generally low and insufficient to form fibrous networks. Analysis of these MDCK cells found the same nuclear defects (Figure 6, A and B). These data demonstrate that the nuclear defects were not caused by "steric hindrance" from rootletin fibers. Rather, excess rootletin remaining at the spindle poles (Figure 6C) likely interfered with the coordinated dissociation of rootletin and C-Nap1 from the centrosomes. The observed nuclear defects were similar to those found in cells transfected with a mutant form of Nek2A, which loses its activity to stimulate centrosome separation and presumably results in defective chromosome segregation (Faragher and Fry, 2003). Therefore, these data are consistent with the hypothesis that C-Nap1 and rootletin functionally interact and that their coordinated dissociation from the centrosomes is important for the normal mitotic process.

DISCUSSION

Data from this study suggest that, in nonciliated cells, the rudimentary rootlet connects the pair of centrioles through physically binding to C-Nap1 and participates in centrosome cohesion during the cell cycle. We present several lines of evidence in support this conclusion. First, rootletin and C-Nap1 physically interact through multiple regions. These two proteins are coordinately regulated at the centrosome through the cell cycle. Second, ultrastructural examinations demonstrate that the ciliary rootlet links the pair of centrioles/basal bodies in cells. Third, dominant negative mutants of C-Nap1 disturb the distribution of rootletin at the centrosomes and separate the pair of centrioles in interphase cells. Finally, overexpression of rootletin causes nuclear defects similar to those caused by a mutant form of Nek2A, a protein known to regulate C-Nap1.

Based on our findings and available data in the literature, we propose the following model for the rootletin fibers and C-Nap1 to participate together in the linkage of centrioles/basal bodies. In interphase cells, the rootlets connect the proximal ends of the centrioles through binding to C-Nap1. At the beginning of mitosis, C-Nap1 is phosphorylated by Nek2. This phosphorylation induces the departure of C-Nap1 from the centrioles (Mayor *et al.*, 2002), which in turn results in the disconnection of the rootlets from the centrioles. Without the physical intercentriolar linker connected, the two parent centrioles are free to separate and form the spindle poles. At the end of mitosis, C-Nap1 is dephosphorylated by PP1 and again associates with the centrioles. The rootlets are then able to connect the parent and daughter centrioles. When cells exit mitosis and differentiate into ciliated cells, the pair of basal bodies remains connected by a ciliary rootlet. The manner with which rootlets link the pair of basal bodies/centrioles does not fundamentally differ, as illustrated schematically in Figure 7. The key difference

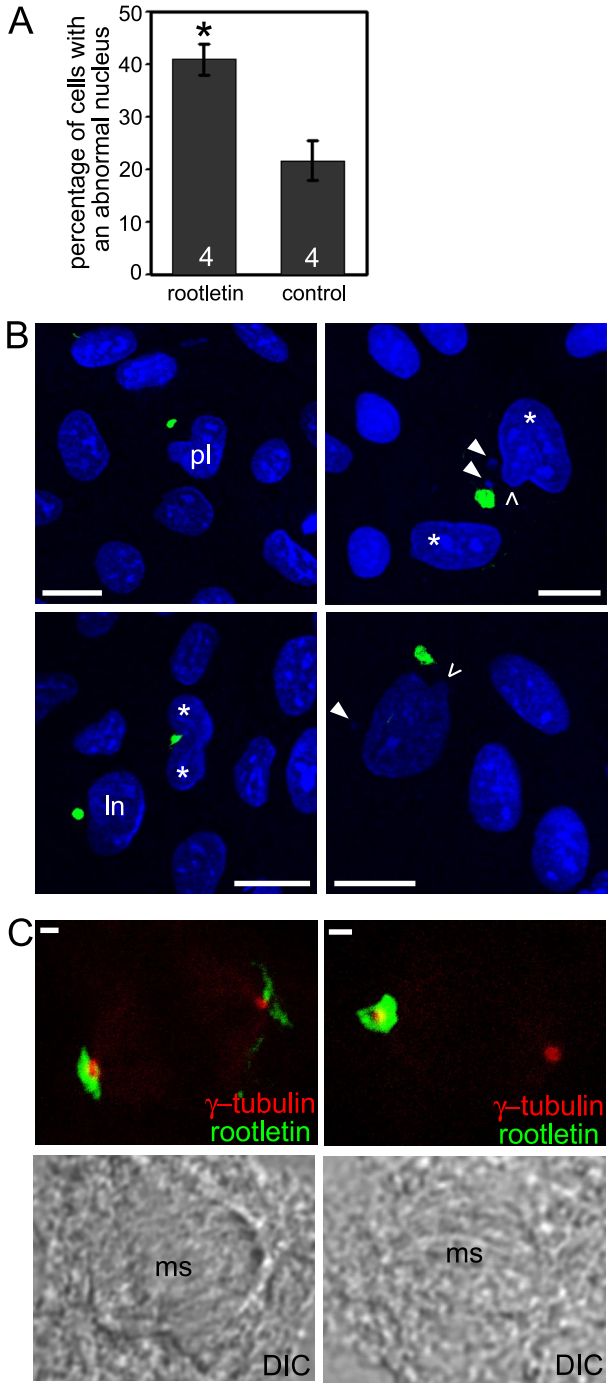


Figure 6. Overexpression of rootletin causes multinucleation, micronucleation, and irregularity of nuclear size and shape. (A) Overexpression of a small amount of rootletin in MDCK cells doubles the percentage of cells with an abnormal nucleus. The analysis was conducted in four independent cell culture chambers. (B) Representative images show the abnormality of nuclei (blue, TOTO-3) in MDCK cells transfected with rootletin (green). The pair of nuclei marked with asterisks is in same cells (multinucleation). Solid arrowheads indicate micronuclei. Empty arrowheads indicate blebs protruding from the nuclei. ln, large nucleus; pl, polylobulated nucleus. Bars, 5 μm. (C) Exogenous rootletin remains attached to the centrioles (spindle poles) in mitotic COS cells. ms, mitotic spindle; DIC, differential interference contrast image; Bars, 1 μm.

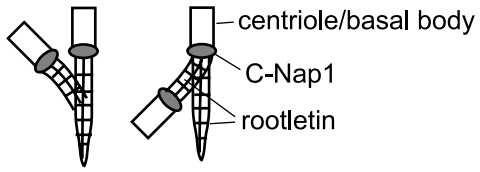


Figure 7. Schematic diagrams illustrate that the ciliary rootlet links the pair of centrioles/basal bodies through binding to C-Nap1 in both nonciliated and ciliated cells.

would be an upregulation of rootletin in cells that develop cilia, which polymerizes to form the large rootlets.

Although the above model is well supported by the available data, there remain many questions. We do not know how other proposed centriolar linker proteins, e.g., the rho-associated protein kinase p160ROCK (Chevrier *et al.*, 2002), might fit in this model. It remains unclear if rootletin polymer undergoes regulated phosphorylation at the centrioles through the cell cycle. The centriolar linkage proposed in this model is rigid and only dissolves during mitosis. We do not know how this model is modified in vivo to allow an active centriolar movement during interphase in some cells (Piel *et al.*, 2000). Ultimately, complete ablation of rootletin expression in an in vivo model is required to determine the role of rootletin in centriole cohesion and the role of centriole cohesion in whole animals.

The observed changes of rootletin immunoreactivity during the cell cycle support the notion that the rudimentary rootlet is a putative intercentriolar linker. In most cells, the two parent centrioles stay close to each other during most of the interphase when they duplicate their daughter centrioles and mature at S and G2 phases. After entry into mitosis, the two parent centrioles are separated from each other. Each forms a spindle pole together with its own daughter centriole. Similarly, the rootletin immunoreactivity is maximal at the centrosome during interphase and decreases sharply at the spindle poles when entering mitosis. The weak rootletin immunoreactivity during mitosis may be due to the separated parent centrioles.

Multinucleation, micronucleation, and the irregularity of nuclear size and shape caused by overexpression of rootletin are features of nuclear defects found in cancer cells. In spite of progress in understanding the molecular changes in cancer cells, the mechanisms underlying these nuclear defects in cancer are not completely clear. In addition, deficiencies in the components of the nuclear matrix, such as A-type lamin and emerin, have been found to result in changes of nuclear shape (Lammerding *et al.*, 2005). Although there are many possible mechanisms involved, we believe that the mechanism underlying the nuclear defects caused by overexpression of rootletin is similar to the one caused by expression of a mutant form of Nek2A, probably the defect in centrosome separation during mitosis.

The expression and localization of C-Nap1 in ciliated cells found in this study indicate that it can provide an anchorage for the ciliary rootlet at the basal bodies through its interaction with rootletin. In ciliated cells, the ciliary rootlet is a large cytoskeleton originating from the proximal ends of basal bodies and extending toward the nuclei. It plays a crucial role in maintaining the cellular integrity as shown by our previous studies (Yang *et al.*, 2002). Thus, the anchorage of the ciliary rootlet to the basal bodies is essential. Loss of C-Nap1 expression would disrupt the formation of functional rootlets in ciliated cells, in addition to centriole cohesion, leading to a similar set of defects as in a rootletin mutant.

While this manuscript was in preparation, an article was published by another group of investigators that had reached a similar conclusion (Bahe *et al.*, 2005). The authors of that study identified the interaction between rootletin and C-Nap1 of human origin by yeast two-hybrid analysis. They demonstrated, by siRNA gene knockdowns and immunolocalization, that rootletin was required for centrosome cohesion. In addition, they propose that rootletin association with the centrosome may be regulated by Nek2-mediated phosphorylation. Our study, in comparison, utilized dominant-negative mutants in the functional analyses of rootletin in centrosome cohesion. Our study also provides a more detailed characterization of the interaction between rootletin and C-Nap1, including the dissection of interacting domains and coimmunoprecipitation. The two studies are largely consistent and complementary. Together, these studies provide strong support for the aforementioned conclusion that rootletin and C-Nap1 act in concert to mediate centrosome cohesion.

ACKNOWLEDGMENTS

We thank Dr. Erich A. Nigg (University of Geneva, Geneva, Switzerland) for the gift of C-Nap1 antibody (R63) and members of the Li laboratory for their helpful suggestions. This work was supported by National Institutes of Health (EY14426 and core grant P30 EY14104), Philip Morris USA, and Philip Morris International.

REFERENCES

Bahe, S., Stierhof, Y. D., Wilkinson, C. J., Leiss, F., and Nigg, E. A. (2005). Rootletin forms centriole-associated filaments and functions in centrosome cohesion. J. Cell Biol. *171*, 27–33.

Brinkley, B. R., and Goepfert, T. M. (1998). Supernumerary centrosomes and cancer: Boveri's hypothesis resurrected. Cell Motil. Cytoskelet. *41*, 281–288.

Chevrier, V., Piel, M., Collomb, N., Saoudi, Y., Frank, R., Paintrand, M., Narumiya, S., Bornens, M., and Job, D. (2002). The Rho-associated protein kinase p160ROCK is required for centrosome positioning. J. Cell Biol. *157*, 807–817.

Di Fiore, B., Ciciarello, M., Mangiacasale, R., Palena, A., Tassin, A. M., Cundari, E., and Lavia, P. (2003). Mammalian RanBP1 regulates centrosome cohesion during mitosis. J. Cell Sci. *116*, 3399–3411.

Doxsey, S. (1998). The centrosome—a tiny organelle with big potential. Nat. Genet. *20*, 104–106.

Faragher, A. J., and Fry, A. M. (2003). Nek2A kinase stimulates centrosome disjunction and is required for formation of bipolar mitotic spindles. Mol. Biol. Cell *14*, 2876–2889.

Fry, A. M., Mayor, T., Meraldi, P., Stierhof, Y. D., Tanaka, K., and Nigg, E. A. (1998). C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. J. Cell Biol. *141*, 1563–1574.

Helps, N. R., Luo, X., Barker, H. M., and Cohen, P. T. (2000). NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. Biochem. J. *349*, 509–518.

Lammerding, J., Hsiao, J., Schulze, P. C., Kozlov, S., Stewart, C. L., and Lee, R. T. (2005). Abnormal nuclear shape and impaired mechanotransduction in emerin-deficient cells. J. Cell Biol. *170*, 781–791.

Mack, G. J., Rees, J., Sandblom, O., Balczon, R., Fritzler, M. J., and Rattner, J. B. (1998). Autoantibodies to a group of centrosomal proteins in human autoimmune sera reactive with the centrosome. Arthritis Rheum. *41*, 551–558.

Mayor, T., Hacker, U., Stierhof, Y. D., and Nigg, E. A. (2002). The mechanism regulating the dissociation of the centrosomal protein C-Nap1 from mitotic spindle poles. J. Cell Sci. *115*, 3275–3284.

Mayor, T., Stierhof, Y. D., Tanaka, K., Fry, A. M., and Nigg, E. A. (2000). The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. J. Cell Biol. *151*, 837–846.

Meraldi, P., and Nigg, E. A. (2001). Centrosome cohesion is regulated by a balance of kinase and phosphatase activities. J. Cell Sci. *114*, 3749–3757.

Paintrand, M., Moudjou, M., Delacroix, H., and Bornens, M. (1992). Centrosome organization and centriole architecture: their sensitivity to divalent cations. J. Struct. Biol. *108*, 107–128.

Piel, M., Meyer, P., Khodjakov, A., Rieder, C. L., and Bornens, M. (2000). The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. J. Cell Biol. *149*, 317–330.

Salisbury, J. L., Whitehead, C. M., Lingle, W. L., and Barrett, S. L. (1999). Centrosomes and cancer. Biol. Cell *91*, 451–460.

Thompson, H. M., Cao, H., Chen, J., Euteneuer, U., and McNiven, M. A. (2004). Dynamin 2 binds gamma-tubulin and participates in centrosome cohesion. Nat. Cell Biol. *6*, 335–342.

Yang, J., Gao, J., Adamian, M., Wen, X. H., Pawlyk, B., Zhang, L., Sanderson, M. J., Zuo, J., Makino, C. L., and Li, T. (2005). The ciliary rootlet maintains long-term stability of sensory cilia. Mol. Cell Biol. *25*, 4129–4137.

Yang, J., and Li, T. (2005). The ciliary rootlet interacts with kinesin light chains and may provide a scaffold for kinesin-1 vesicular cargos. Exp. Cell Res. *309*, 379–389.

Yang, J., Liu, X., Yue, G., Adamian, M., Bulgakov, O., and Li, T. (2002). Rootletin, a novel coiled-coil protein, is a structural component of the ciliary rootlet. J. Cell Biol. *159*, 431–440.