The ciliopathy-associated protein homologs RPGRIP1 and RPGRIP1L are linked to cilium integrity through interaction with Nek4 serine/threonine kinase

Karlien L.M. Coene1,2, Dorus A. Mans1,2, Karsten Boldt5,6, C. Johannes Gloeckner5,6, Jeroen van Reeuwijk1,2, Emine Bolat1, Susanne Roosing1,2, Stef J.F. Letteboer1, Theo A. Peters3,7, Frans P.M. Cremers1,2, Marius Ueffing5,6 and Ronald Roepman1,2,4,*

1Department of Human Genetics, 2Nijmegen Centre for Molecular Life Sciences, 3Department of Otorhinolaryngology and 4Institute for Genetic and Metabolic Disease, Radboud University Nijmegen Medical Centre, 6500 HB Nijmegen, The Netherlands, 5Division of Experimental Ophthalmology and Medical Proteome Center, Center of Ophthalmology, University of Tübingen, D-72074 Tübingen, Germany, 6Department of Protein Science, Helmholtz Zentrum München, German Research Center for Environmental Health, D-85764 Neuherberg, Germany and 7Donders Institute for Brain, Cognition and Behaviour, 6500 HB Nijmegen, The Netherlands

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Recent studies have established ciliary dysfunction as the underlying cause of a broad range of multi-organ phenotypes, known as ‘ciliopathies’. Ciliopathy-associated proteins have a common site of action in the cilium, however, their overall importance for ciliary function differs, as implied by the extreme variability in ciliopathy phenotypes. The aim of this study was to gain more insight in the function of two ciliopathy-associated protein homologs, RPGR interacting protein 1 (RPGRIP1) and RPGRIP1-like protein (RPGRIP1L). Mutations in RPGRIP1 lead to the eye-restricted disease Leber congenital amaurosis, while mutations in RPGRIP1L are causative for Joubert and Meckel syndrome, which affect multiple organs and are at the severe end of the ciliopathy spectrum. Using tandem affinity purification in combination with mass spectrometry, we identified Nek4 serine/threonine kinase as a prominent component of both the RPGRIP1- as well as the RPGRIP1L-associated protein complex. In ciliated cells, this kinase localized to basal bodies, while in ciliated organs, the kinase was predominantly detected at the ciliary rootlet. Down-regulation of NEK4 in ciliated cells led to a significant decrease in cilium assembly, pointing to a role for Nek4 in cilium dynamics. We now hypothesize that RPGRIP1 and RPGRIP1L function as cilium-specific scaffolds that recruit a Nek4 signaling network which regulates cilium stability. Our data are in line with previously established roles in the cilium of other members of the Nek protein family and define NEK4 as a ciliopathy candidate gene.

INTRODUCTION

Recent studies have highlighted the importance of cilia for several biological processes, such as locomotion, cell proliferation and developmental signaling (1). Genetic defects that lead to disruption of cilium function or formation have been associated with a broad spectrum of human diseases, collectively named ciliopathies (2). Although the list of ciliopathy genes keeps increasing, it often remains elusive how their encoded proteins contribute to a functional cilium. We have previously identified the protein homologs RPGR-interacting protein 1 (RPGRIP1) and RPGRIP1-like protein (RPGRIP1L), that are both associated with ciliopathies; RPGRIP1 mutations lead to Leber congenital amaurosis
fetuses (5), while liver and limb malformations observed in human MKS mice die at mid-gestation and recapitulate the brain, kidney, and orofacial abnormalities, or in the lethal Meckel syndrome [MKS5 (MIM 611561)] (5). RPGRIP1 was identified as an interacting protein of RPGR (retinitis pigmentosa GTPase regulator). Defects in RPGR cause X-linked retinitis pigmentosa [RP3 (OMIM 300029)] (6,7). RPGR and RPGRIP1 co-localize in connecting cilia of photoreceptors, and Rpgrip1 knockout mice show early-onset retinal degeneration (8–10). As RPGR mislocalized to inner segments of photoreceptors of these mice, RPGRIP1 was proposed to function as an anchor for RPGR in the connecting cilium (9). RPGRIP1 also links to nephrocystins via nephrocystin-4, a protein involved in nephronophthisis [NPHP4 (MIM 606966)] (11) and the retinal–renal Senior–Løken syndrome [SLSN4 (MIM 606996)] (12). The RPGRIP1-nephrocystin-4 interaction is disrupted by disease-associated mutations in either interactor (13). RPGRIP1L is the only homolog of RPGRIP1, based on 29% amino acid identity. Like RPGRIP1, RPGRIP1L interacts with RPGR and nephrocystin-4 and localizes to basal bodies of cilia in brain, retina and kidney (4,14). Rpgrip1L−/− (Ftm) mice die at mid-gestation and recapitulate the brain, kidney, liver and limb malformations observed in human MKS fetuses (5), while Nphp4−/− mice only recapitulate an early-onset retinal degeneration, resembling LCA, without renal failure (15). From these data, it is clear that RPGRIP1, RPGRIP1L and their protein partners play a crucial role in ciliary homeostasis, especially regarding the photoreceptor sensory cilium (16).

In order to gain more insight in the exact function that these homologs fulfill, we scrutinized their protein networks by affinity proteomics. We identified a new, prominent member of both the RPGRIP1- and the RPGRIP1L-associated protein complex, the serine/threonine kinase Nek4. Nek4 belongs to the never in mitosis A (NIMA)-related protein kinase family, which counts 11 members in mammals, based on homology of their N-terminal serine/threonine kinase domains. Nek1 and Nek8 have a ciliary function, as mutations in the corresponding genes lead to polycentric kidney disease and craniofacial abnormalities in murine models (17,18). In humans, NEK1 mutations cause short-rib polydactyly syndrome type Majewski [SRPS (MIM 263520)], an embryonic-lethal osteochondrodysplasia which is now also recognized as a ciliary disorder (19). Although Nek4 is listed in the ciliary proteome database as part of both the Chlamydomonas flagellar proteome and the mouse photoreceptor proteome (20–22), a specific role for Nek4 in the cilium has not yet been shown. We now demonstrate that Nek4 prominently decorates the base of the cilium and associates with ciliary rootlets, structures that are essential for ciliary stability, in tissues that are prominently affected in ciliopathies (photoreceptors, kidney and brain). We propose a mechanism in which RPGRIP1 and RPGRIP1L function as scaffolds that recruit Nek4, its substrates and its regulators to their active site in the cilium, thus maintaining cilium integrity.

RESULTS
Tandem affinity purification studies identify Nek4 as a member of both the RPGRIP1L- as well as the RPGRIP1-associated protein complex

To investigate the protein complexes that are associated with RPGRIP1 and RPGRIP1L, we have used the Strep-tag II/FLAG tandem affinity purification (SF-TAP) method (23). For both homologs, the protein fragment that encompasses the C2 domain until the C-terminal end of the protein (C2-end) was fused to an N- or C-terminal Strep/FLAG-tag (NTAP or CTAP) and expressed in human embryonic kidney 293T (HEK293T) cells. The encoded fusion proteins localized to their native site of expression; for RPGRIP1 this was the basal body, while RPGRIP1L localized to the transition zone (Supplementary Material, Fig. S1). Mass spectrometric (MS) analysis of the purified protein complexes identified a total of 46 unique, co-purified proteins in the RPGRIP1 experiments, while 21 were identified for RPGRIP1L (Supplementary Material, Table S1). Only proteins that were found in two replicate TAP experiments were taken into account, while proteins that were also detected in the negative control sample were considered to be false positives. The previously reported interactor RPGR was present in the purified protein network for both RPGRIP1 as well as for RPGRIP1L, and also the known RPGRIP1L interactor NPHP4 was identified (4,6,14). Cross-referencing with the ciliary proteome database (20) pointed to a significant over-representation of ciliary proteins in the isolated protein complexes (Table 1), which is fully in line with their ciliary role. Strikingly, the TAP experiments identified Nek4 serine/threonine kinase (UniProt ID P51957) as a prominent member of both the RPGRIP1- and the RPGRIP1L-associated protein complex; MS analysis rendered 55% sequence coverage for Nek4 in the RPGRIP1L co-purified complex, while in the RPGRIP1-TAP experiment, a level of 23% sequence coverage was reached (Supplementary Material, Table S1). These high levels of Nek4 sequence coverage indicate that this protein is a stable and prominent part of the protein complexes of both homologs. In total, the identified interactorome of RPGRIP1 and RPGRIP1L shared seven proteins: Nek4, RPGR, RPL23, SLC25A5 and three heat shock proteins. Expression of heat shock proteins could be induced non-specifically by the over-expression of large amounts of exogenous proteins upon transient transfection. The shared interactor SLC25A5 is a mitochondrial protein that is associated with X-linked RP (XLRP), as its expression was found to be down-regulated in retinas of 16-week-old dogs with XLRP due to a RPGRORF15 microdeletion (24). However, the level of sequence coverage in the MS analysis was lower for SLC25A5 compared with that of Nek4, suggesting that this protein may be less abundantly present in the RPGRIP1/RPGRIP1L-associated protein complexes. An additional TAP experiment using full-length RPGRIP1 fused to a C-terminal TAP-tag as bait again confirmed the abundant presence of Nek4 in the associated protein network. Therefore, we focused our attention on the prominent interaction with Nek4.
Validation of the Nek4-RPGRIP1 and Nek4-RPGRIP1L interaction

To validate our SF-TAP studies, we first confirmed endogenous expression of NEK4 mRNA in HEK293T cells using quantitative PCR (qPCR, data not shown). Then, we validated the results of the TAP assays of NTAP-RPGRIP1(2-end) and NTAP-RPGRIP1L(2-end) by western blotting of TAP-eluates using a specific anti-Nek4 antibody. Indeed, Nek4 could be detected in the purified protein complexes of both homologs (Fig. 1A).

To further assess the interaction between Nek4 and RPGRIP1/RPGRIP1L, we performed both GST pull-down and co-immunoprecipitation assays. In the pull-down assay, GST-fused RPGRIP1 and RPGRIP1L efficiently pulled down 3xFLAG-Nek4 from lysates of transfected COS-1 cells (Fig. 1B). Nek4 did not significantly co-purify with unfused GST. We further evaluated the binding of Nek4 with RPGRIP1 and RPGRIP1L in cellulo by co-immunoprecipitation of epitope-tagged proteins from COS-1 cells. When 3xFLAG-RPGRIP1 and 3xFLAG-RPGRIP1L were immunoprecipitated via anti-FLAG affinity purification, 3xHA-Nek4 co-precipitated, while no significant co-precipitation of 3xHA-Nek4 could be observed, confirming the specificity of the detected interaction (Fig. 1C, panels 1–4). Specificity was further confirmed by including the unrelated 3xFLAG-p63 protein in the assay, which failed to co-precipitate with Nek4, while the robustness of the binding was confirmed in reciprocal assays, using anti-HA affinity purification for immunoprecipitation (Fig. 1C, panels 5–8). Similar co-immunoprecipitation assays with full-length RPGRIP1 further confirmed these results (Supplementary Material, Fig. S2).

Next, we performed additional pull-down assays from bovine retinal lysates to validate the interaction of Nek4 with RPGRIP1 and RPGRIP1L in retinal cells. GST-fused RPGRIP1 and RPGRIP1L were immobilized on glutathione beads and incubated with bovine retinal extracts. Two specific anti-Nek4 immunoreactive bands at ~90 and 100 kDa could be detected in the isolated protein complexes, although the current annotation of the cow genome lists only a single Nek4 isoform (Fig. 1D). The 90 kDa isoform showed higher binding affinity to RPGRIP1 and RPGRIP1L than the 100 kDa isoform, possibly indicating preference for interaction with the 90 kDa isoform in the retina. Unfused GST showed no capacity to pull down endogenous Nek4 from bovine retinal extracts. This experiment shows that the Nek4-RPGRIP1 and Nek4-RPGRIP1L interactions that we initially discovered in HEK293T cells are also relevant for the retina in vivo, further strengthening the biological significance of these interactions.

As a final confirmation of the validity of the interactions with Nek4, and to evaluate whether the interactions were direct or indirect, we conducted a yeast two-hybrid screen utilizing a human retinal oligo-dT primed cDNA library, using full length and three fragments of Nek4 as baits. Only the screen with one of the fragments (Nek4<sup>560–841</sup>) as bait resulted in the identification of three positive clones. Excitingly, all preys encoded fragments of the C-terminal region of RPGRIP1: two clones encoded amino acids 882–1286 of RPGRIP1 and one clone encoded amino acids 1028–1286 of RPGRIP1. The Nek4-RPGRIP1 interaction could be confirmed in a dedicated yeast two-hybrid assay (Supplementary Material, Fig. S3). Even though RPGRIP1L did not come forward as a Nek4 interactor from the yeast two-hybrid screen, we included this protein in the dedicated yeast two-hybrid assay and could also confirm direct interaction of RPGRIP1L with Nek4 (Supplementary Material, Fig. S3).

Nek4 co-localizes with RPGRIP1 and RPGRIP1L at the basal body and is located at the ciliary rootlet

The subcellular localization of Nek4 in ciliated cells was assessed by immunocytochemistry. We expressed Nek4 fused to an enhanced cyan fluorescent protein (eCFP) or monomeric red fluorescent protein (mRFP) tag in immortalized human retinal pigment epithelium cells (hTERT-RPE1) and counterstained with the basal body/cilium marker GT335 (Supplementary Material, Fig. S4). eCFP- and mRFP-Nek4 localized diffusely throughout the cytoplasm, but showed significant concentration at the basal body region.

Staining of ciliated hTERT-RPE1 cells with anti-Nek4 in combination with GT335 revealed that also endogenous Nek4 concentrates at the base of the cilium (Fig. 2A). Triple staining of α-Nek4, α-RPGRIP1L and GT335 showed RPGRIP1L localization at the transition zone, while Nek4 envelopes both centrioles (Fig. 2B). Even though no exact co-localization was observed, Nek4 and RPGRIP1L both proved to be exclusively present at the ciliary base. The overlapping co-localization of RPGRIP1 with RPGRIP1L indirectly showed that RPGRIP1 is present in close proximity to Nek4 in ciliated cells as well (Fig. 2C).
In rat photoreceptor cells, Nek4 localized to thread-like structures which traversed the inner segment up to the connecting cilium (Fig. 3A). This localization pattern resembles staining for rootletin, the main component of the ciliary rootlet that maintains structural stability of the (photoreceptor) cilium (25, 26). Co-staining using an α-rootletin antibody showed that Nek4 is present in these complexes. No Nek4 could be detected in the untransfected negative control sample, which was simultaneously processed in the TAP experiment.

**Figure 1.** Analysis of the interaction between Nek4 and RPGRIP1 and RPGRIP1L. (A) An α-Nek4 immunoblot of the RPGRIP1- and RPGRIP1L-associated protein complexes, which were purified via the SF-TAP procedure, showed that Nek4 is present in these complexes. No Nek4 could be detected in the untransfected negative control sample, which was simultaneously processed in the TAP experiment. (B) GST-RPGRIP1RID, GST-RPGRIP1C2-end, GST-RPGRIP1LRD, and GST-RPGRIP1L2-end (10% input shown in panel 1; pull-down assay shown in panel 2) efficiently pulled down 3xFLAG-Nek4 (10% input shown in panel 1; pull-down assay shown in panel 2). Unfused GST failed to pull down 3xFLAG-Nek4. In the pull-down experiments with GST-RPGRIP1C2-end and GST-RPGRIP1L2-end, the bands observed for 3xFLAG-Nek4, which run at ~100 kDa, appear shifted due to the presence of the GST-fusion protein at a similar height. (C) 3xHA-Nek4 co-precipitated with 3xFLAG-RPGRIP1C2-end and 3xFLAG-RPGRIP1L2-end, whereas 3xHA-Nek8 did not (5% of protein input shown in panels 1 and 2; FLAG-immunoprecipitates in panel 3 and co-precipitation in panel 4). Interaction specificity was further confirmed by including the unrelated 3xFLAG-p63 protein in the assay, which failed to co-precipitate with Nek4. Panels 5–8 show similar results for anti-HA immunoprecipitation. (D) Endogenous Nek4 (two isoforms of 90 kDa and 100 kDa, respectively) could be pulled down from bovine retinal lysates by GST-RPGRIP1C2-end and GST-RPGRIP1L2-end (α-Nek4 immunoblot shown in panel 2). Unfused GST was not able to pull down endogenous Nek4. Panel 1 shows 10% of GST-fusion protein used in the pull-down assays.
present in concentrated patches along the photoreceptor rootlet (Fig. 3B). Partial co-localization of Nek4 with RPGRIP1L could be observed at the point where the apical site of the rootlet connects to the base of the ciliary axoneme (Fig. 3C). Co-staining of RPGRIP1 and RPGRIP1L indirectly showed that RPGRIP1 also partly co-localizes with Nek4 in photoreceptor cells (Fig. 3D).

In rat kidney tubules, Nek4 was present in cilium-like structures that originated from GT335-stained basal bodies (Fig. 4A). Co-staining with an α-rootletin antibody confirmed that Nek4 was present in the ciliary rootlet of kidney tubules (Fig. 4B), and co-localization with RPGRIP1L could be observed on the distal end of the rootlet (Fig. 4C). Similar stainings were also performed on sections of rat brain. RPGRIP1L was previously described to be present in foci on cells lining the ventricles of the brain, representing the basal bodies of ependymal cilia in the choroid plexus (4). However, Nek4 could not be observed at the rootlets of these cilia, but localized diffusely throughout the cytoplasm of the ventricular cells (Fig. 5A). In the striatum, which is positioned adjacent to the ventricles, we did detect Nek4 specifically in the ciliary rootlet in several ciliated organs in vivo.

**NEK4 is expressed in ciliated human tissues**

To evaluate NEK4 expression in ciliated human organs, we analyzed NEK4 mRNA expression in several ciliated and non-ciliated human tissues by quantitative PCR (qPCR, primer sequences given in Supplementary Material, Table S2). GUSB was used as a reference gene for baseline expression (27). Compared with the lowest expression in placenta, the highest NEK4 expression level was measured in the brain, followed by the testis, heart and retina (Fig. 6). Expression in kidney was detected as well, although at a lower level. A comparable expression profile was observed for RPGRIP1L, whereas RPGRIP1 expression was limited to testis and retina (4).

**Nek4 is involved in cilium assembly**

As we discovered that Nek4 is a ciliary protein, we assessed a possible role for Nek4 in cilium assembly. Nek4 was previously included in a genome wide RNAi screen for modulators of ciliogenesis and ciliary length by Kim et al. (28), in which single siRNAs were used. However, this study found ambiguous results for the effect of NEK4 knock down on cilium assembly. We chose a different strategy and knocked down NEK4 in ciliated hTERT-RPE1 cells using a combined pool of three small interfering RNAs (siRNAs) specifically targeting NEK4 (Invitrogen, siRNA sequences given in Supplementary Material, Table S3). Efficient knock-down could be shown by qPCR (data not shown). Depletion of NEK4 expression resulted in a significant decrease in ciliated cell numbers in two independent experiments ($\chi^2$-test, $P \leq 0.005$, Fig. 7A).
As Nek4 belongs to a family of cell-cycle-related kinases, we tested whether the cilium assembly defect induced by NEK4 knock-down might be due to altered cell cycle progression. If cells are blocked in mitosis, they are unable to enter the G0-phase of the cell cycle, during which cilia are usually assembled. Mitotic arrest would therefore prevent the formation of cilia indirectly. To assess this possibility, we performed flow cytometry (FACS) experiments to analyze cell-cycle profiles of NEK4 siRNA-treated versus non-targeting siRNA-treated hTERT-RPE1 cells. We also included PLK1 siRNA-treatment in this experiment as a positive control (siRNA sequences given in Supplementary Material, Table S3) (29). In two independent experiments, we did not observe any differences in mitotic profiles for NEK4 siRNA-treated cells versus non-targeting siRNA-treated cells, while PLK1 siRNA-treatment caused a significant block in cell-cycle progression (Fig. 7B). These results were in agreement with the findings of Doles and Hemann (30) from comparable NEK4 siRNA experiments, and indicate that the NEK4 siRNA-induced cilium-disassembly effect is not caused by cell-cycle impairment. Nek4 apparently has a function in maintaining cilium stability in hTERT-RPE1 cells, but does not influence progression of the cell cycle, in contrast to other ‘mitotic kinases’ belonging to the Nek-family.

**DISCUSSION**

In this study, we applied a proteomic approach to gain more insight in the function of RPGRIP1 and RPGRIP1L, two homologous proteins associated with the ciliopathies LCA, and JS and MKS, respectively. We discovered that RPGRIP1 and RPGRIP1L share a prominent common interactor, the serine/threonine kinase Nek4. Nek4 is an unexplored member of
the NIMA-related protein kinase family, which counts 11 members in mammals, based on their homologous N-terminal serine/threonine kinase domains (31). The founder kinase of this family is never in mitosis gene A (NIMA), which is crucial for Aspergillus Nidulans mitotic entrance, hence its name (32). Also in mammals, Nek2, Nek6, Nek7 and Nek9 were shown to play a role in cell-cycle regulation (31). Nek4 has highest homology to Nek11, which is required for DNA damage-induced G2/M arrest (33). A recent publication by Doles and Hemann (30) identified Nek4 in an RNAi-based screen for mediators of response to the chemotherapeutic agent Taxol, suggesting a role for Nek4 in microtubule polymerization. Recent studies, however, have revealed that the cilium can also be a site of action for Nek proteins. In several protists, such as Tetrahymena, Chlamydomonas and Trypanosoma, Nek proteins appear to regulate cilium length (34–36). In mammals, a cilium function has been established for the Nek-family members Nek1 and Nek8. Nek1 mutant mice (kat/kat-2J) show male sterility, facial dysmorphism and polycystic kidney disease, which are all human ciliopathy hallmarks (18,37). Interestingly, a recent study identified NEK1 mutations in human fetuses with SRPS, a lethal ciliopathy that primarily affects bone development, but also features polydactyly, renal abnormalities and cardiac defects, recapitulating the Nek1 mouse mutant phenotype (19). Nek8 mutant mouse and zebrafish display cystic kidney disease (38). Screening a cohort of 588 nephronophthisis patients for NEK8 mutations resulted in the identification of a single homozygous missense change and two heterozygous missense changes (39). Bioinformatic analysis revealed that there is a positive correlation between the number of NEK genes in the genome of a particular organism and whether or not this organism has ciliated cells (40). Organisms in which ciliated cells can still divide because their centrioles can serve as both ciliary basal bodies and as spindle poles in the cell cycle have a significant increase in the number of NEK genes present in their genome. All of these studies point to the importance of Nek family members for the correct functioning of the cilium. The association of specifically Nek4 with two major ciliopathy-associated protein homologs suggests that this Nek protein plays a similar pivotal role in the cilium.

The interaction of RPGRIP1 and RPGRIP1L with Nek4 was confirmed by different lines of evidence. Our
immunohistochemistry results showed that the subcellular localization of Nek4 is confined to the ciliary rootlet and partially overlaps with the transition zone of the cilium, where both RPGRIP1 and RPGRIP1L are present. The rootlet is a cytoskeleton-like structure that originates from the basal body and extends towards the cell nucleus. The major component of the ciliary rootlet in all ciliated cells is the rootletin protein (26). Rootletin mutant mice lack rootlets in ciliated cells but do not show major ciliopathy phenotypes during early stages of life (25,26). Starting at an age of 18 months, however, degeneration of photoreceptors is apparent in these mutant mice. This indicates that the rootlet is not required for the development, but for the long-term stability of photoreceptor cells. Bahe et al. (41) showed that rootletin also has a function in centrosome cohesion, in collaboration with its interactor C-Nap1. Both proteins are targets for phosphorylation by Nek2 kinase, a modification that regulates their association with the centrosome. As observed for many cellular processes, it is likely that regulation of centrosome cohesion depends on the overall balance of kinase- versus phosphatase-activities (42). The presence of Nek4 at the rootlet might indicate that Nek4 joins Nek2 in this regulatory mechanism, however, over-expression or down-regulation of NEK4 did not lead to centrosome splitting (data not shown), which points to a different role for Nek4 at the ciliary rootlet.

Because Nek4 is a serine/threonine kinase, a logical hypothesis would be that its interactors RPGRIP1 and RPGRIP1L are targets for phosphorylation. So far, no specific kinase substrates have been reported for human Nek4. For the murine counterpart of Nek4 (Stk2), general phosphorylation targets were investigated by Hayashi et al. (43). This study showed that α- and β-casein, phosvitin and MAP2 are Stk2 substrates. Our assessment of phosphorylation of RPGRIP1 and RPGRIP1L by Nek4 using kinase assays, however, was negative (Supplementary Material, Fig. S5). In addition, even though Nek4 was part of the RPGRIP1- and RPGRIP1L-associated complexes purified by TAP, MS analysis did not show a significant amount of phosphorylated peptides for RPGRIP1 (5 out of 129), nor for RPGRIP1L (none). A mobility shift assay, in which RPGRIP1 or RPGRIP1L was co-expressed with either wild-type or kinase-dead Nek4 (Nek4K35R, mutations in the analogous lysine residue inactivate kinase function in Nek1, Nek2 and Nek11) (44–46) in HEK293T cells, showed no phosphorylation-induced band shift for either homolog (Supplementary Material, Fig. S6). These results suggest that RPGRIP1 and RPGRIP1L are no substrates for Nek4 phosphorylation, although these proteins could still contribute to Nek4 kinase function in a different capacity, for example as scaffolds.

Scaffolds are proteins that facilitate the assembly of signaling complexes, including the kinase, its substrates, positive and negative allosteric regulators, and even other kinases and phosphatases, at the correct cellular location (47). Kinase activity is not just determined by substrate specificity, but also by the specific time point and cellular location at which a kinase is assembled into a functional complex. Scaffolds can provide a platform for kinase activity at a specific cellular site, and some scaffolds are even actively transported along microtubules to reach their site of action (47). 

Figure 5. Subcellular localization of Nek4 in rat brain. (A) Nek4 (red, upper panel in magnifications) localizes to rootlets (green, middle panel in magnifications) in the striatum, indicated with the full arrow (region shown in left magnifications). In ventricular cells, Nek4 localizes diffusely throughout the cytoplasm, designated by the open arrow (region shown in right magnification). (B) When Nek4 (red, upper panel in magnifications) was counterstained with RPGRIP1L (green, middle panel in magnifications), RPGRIP1L could be observed at the distal end of the rootlet, which was again stained by α-Nek4 in the striatum. This region is appointed by the full arrow (region shown in the left magnification). The right magnification shows an enlargement of the staining in the ventricular cells, where RPGRIP1L is present in the transition zone of the ependymal cilia (indicated with the open arrow). The bottom panel of the magnifications shows an overlay. In all images, nuclei are stained with DAPI (Scale bars: 10 μm).
example of a serine/threonine kinase pathway that is regulated by scaffolds is the well-known mitogen-activated protein kinase (MAPK) pathway (48). As we show that NEK4 knockdown negatively influences cilium assembly, we propose that Nek4 is part of a functional signaling network at the rootlet/basal-body region of the cilium, which regulates cilium stability. Within this network, RPGRIP1 and RPGRIP1L could fulfill the role of cilium-specific Nek4 scaffolds, thereby indirectly contributing to Nek4 kinase function. Recent data support a potential scaffold function for RPGRIP1L, as the C. elegans orthologous protein performs a central role in anchoring other MKS and NPHP module proteins at the ciliary transition zone, which is required for basal body docking and formation of the ciliary gate (49).

RPGRIP1 is mainly expressed in testis and retina, while RPGRIP1L is more ubiquitously expressed (4). This is in agreement with the different phenotypes observed for mutations in RPGRIP1 and RPGRIP1L, as mutations in RPGRIP1 cause congenital blindness (3), while loss of RPGRIP1L affects multiple organs, leading to the severe Joubert (4,5) or the lethal Meckel syndrome (5). Considering its ubiquitous expression, RPGRIP1L could have a general scaffold function in the ciliary transition zone, providing a platform for the assembly of regulating networks, containing Nek4 among other proteins. The restriction of RPGRIP1 expression to testis and retina could indicate that the RPGRIP1 protein has a more specialized scaffold function in cilia of these organs. The sensory cilia of the retina, which are the photoreceptor outer segments, are highly sensitive to mechanical stress as they are connected to the inner segment only by a thin cellular bridge, the so-called connecting cilium. This structure is equivalent to the transition zone of primary cilia. Considering the vulnerability of photoreceptors to mechanical trauma, it is evident that, apart from structural support by the ciliary rootlet, tight regulatory mechanisms are required to maintain cilium stability. A Nek4-associated signaling network, assembled on RPGRIP1 as a connecting cilium-specific platform, would fit this role perfectly. The dissection of the constituents of these signaling networks, including substrates, regulators and possibly other kinases and phosphatases, will be a challenging task that will require both sensitive proteomic techniques as well as complex computational modeling. Identification of direct Nek4-phosphorylation targets in the cilium will be a first step in this elaborate process.

Even though the exact composition and function of ciliary regulatory networks remain elusive, this study takes the first steps in unraveling these processes. We show that the ciliary protein homologs RPGRIP1 and RPGRIP1L interact with Nek4 kinase, and potentially function as scaffolds that provide a platform on which cilium-specific kinases, regulators and substrates can be assembled. Our siRNA data indicate that Nek4 is important for cilium assembly and/or maintaining cilium stability. Taken together, this study provides substantial evidence to consider NEK4 as a ciliopathy candidate gene. Mutational screening of 10 retinal ciliopathy patients, preselected based on homozygosity mapping data from a cohort of 500 non-syndromic retinal dystrophy patients (S.R., unpublished data), identified one homozygous NEK4 missense change (c.767T>C, p.L256P) in a Pakistani cone–rod dystrophy patient (Supplementary Material, Fig. S7A). Polyphen predicted this variant to be possible damaging, however, it did not lead to ciliary mislocalization nor to loss of interaction with RPGRIP1 and RPGRIP1L (Supplementary Material, Fig. S7B and C), therefore the pathogenic potential of this variant is questionable. The severe phenotype caused by
homozygous NEK1 mutations (19) and the fact that only heterozygous NEK8 missense mutations have been identified so far (38), may indicate that patients at the severe end of the ciliopathy spectrum should be screened for NEK4 nonsense defects.

**MATERIALS AND METHODS**

**DNA constructs**

Entry clones encoding full-length Nek4 of 841 amino acids [NCBI Reference Sequence: NM_003157 (gene); P51957 (protein)] were generated by PCR using a NEK4 cDNA IMAGE clone (GenBank EF560744.1) as template. Also the NEK4 entry clones encoding amino acids 1–300, amino acids 293–566 or amino acids 560–841 were generated by PCR from this NEK4 cDNA IMAGE clone. Expression constructs were created from entry clones with Gateway technology (Invitrogen, Leek, The Netherlands) according to the manufacturer’s instructions. RPGRIP1 and RPGRIP1L constructs were generated as previously described (4,13). In short, for both proteins, ‘C2-end’ constructs encoded a fragment spanning from the first C2 domain to the C-terminus of the protein (RPGRIP1C2-end, amino acids 611–1286, and RPGRIP1L C2-end amino acids 591–1235 of isofrom Q68CZ1-2). The RPGRIP1Rid construct encoded the RPGR-interacting domain (Rid, amino acids 905–1286) and the RPGRIP1L RID construct encoded the RPGRIP1L protein fragment homologous to the RPGRIP1 Rid (amino acids 897–1235 of isofrom Q68CZ1-2). The RPGRIP1 full-length construct was kindly provided by Prof. Gerd Walz (University of Freiburg, Freiburg, Germany). Entry clones for the kinase dead Nek4 K35R mutant and the L256P variant were generated by site-directed mutagenesis PCR on the wild-type plasmid. Primers are available upon request. The sequence of all entry clones was verified by nucleotide sequencing.

**Antibodies**

Polyclonal rabbit-α-Nek4, raised against the C-terminal FDREVRLREHMGEKYTT peptide, was obtained from Abnova (Jhongli, Taiwan). Antibody specificity was confirmed on a western blot of 3xFLAG-Nek4 and 3xFLAG-Nek8 lysates (Supplementary Material, Fig. S8A), on which the antibody showed reactivity to 3xFLAG-Nek4, but not to 3xFLAG-Nek8. Pre-incubation of the Nek4 antibody with a GST-fused C-terminal Nek4 fragment (GST-Nek4560 – 841) also led to complete loss of specific signal in immunohistochemistry of rat retinal sections (Supplementary Material, Fig. S8B). Human-α-rootletin antibody was purchased from Novus Biologicals (Cambridge, UK). Affinity purified guinea pig-α-RPGRIP1L (SNC040) was previously described by Arts et al. (4). Rabbit-α-RPGRIP1 was kindly donated by Dr Paulo Ferreira (Duke Eye Center, Durham, NC, USA). Dr Carsten Janke (CNRS Centre de Recherches en Biochimie Macromoléculaire, Montpellier, France) provided the α-polyglutamyalted tubulin antibody (GT335).

**Tandem affinity purification**

HEK293T (human embryonic kidney) cells were transfected for 48 h with constructs that encoded RPGRIP1C2-end or RPGRIP1L C2-end fused to an SF-TAP-tag (23). Effectene (Qiagen, Venlo, The Netherlands) was used as transfection reagent. After 48 h, transfected cells were lysed in lysis buffer containing 30 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet-P40, protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 20 min at 4°C. After sedimentation at 10 000 g, the cleared supernatant was incubated for 2 h at 4°C with Strep-Tactin superfllow (IBA, Göttingen, Germany). Subsequently, the resin was washed three times in wash buffer (30 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Nonidet-P40) supplemented with phosphatase inhibitors. Protein complexes were eluted with 2 mM desthiobiotin (IBA) in TBS. For the second purification step, the eluates were transferred to anti-FLAG M2 agarose (Sigma-Aldrich) and incubated for 2 h at 4°C. The FLAG-beads were washed three times with wash buffer and subsequently, proteins were eluted with FLAG peptide (200 μg/ml, Sigma-Aldrich) in TBS. Then, % of
the sample was separated by SDS–PAGE and stained with silver according to standard protocols. The rest of the sample was subjected to protein precipitation with chloroform and methanol. Protein precipitates were stored at –80°C.

Mass spectrometry
Prior to LC-MS/MS analysis, protein precipitates were subjected to trypic proteolysis as described by Gloeckner et al. (50). Digested samples were loaded in the UltiMate 3000 nano HPLC system. Peptides were eluted from the trap column onto the analytical column using an acetonitrile gradient. The eluted peptides were analyzed by mass spectrometry (LTQ Orbitrap XL, Thermo Fisher Scientific, Waltham, USA). Tandem mass spectra were extracted by Bioworks Browser (Thermo Fisher Scientific) version 3.3.1. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Sequest (version SRF v. 5, ThermoFinnigan, San Jose, CA). Sequest was set up to search the human subset of the Uniref100 database (Release 2008_02, 263932 entries), assuming trypsin as the digestion enzyme. Sequest was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 10 PPM. Oxidation of methionine, iodoacetamide derivative of cysteine and phosphorylation of serine, threonine and tyrosine were specified in Sequest as variable modifications. The Sequest result files were loaded in Scaffold (version Scaffold_2.02.01, Proteome Software Inc., Portland, OR) to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >80% probability as specified by the Peptide Prophet algorithm (51). Protein identifications were accepted if they could be established at >99% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (52). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

GST pull-down
The ‘C2-end’ and ‘RID’ fragments of RPGRIP1 and RPGRIP1L, described in the preceding paragraph ‘DNA Constructs’, were cloned into pDEST15 (Gateway cloning system, Invitrogen). For the creation of GST-fusion proteins, BL21-DE3 cells were transformed with pDEST15 constructs. Cells were induced overnight with 0.5 mM IPTG at 30°C and subsequently lysed in STE buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet-P40 and 1 mM sodium-orthovanadate], supplemented with complete protease inhibitor cocktail (Roche Diagnostics). Lysates were incubated with glutathione–sepharose 4B beads (Amersham Biosciences). After incubation, supernatant was loaded on a NuPAGE 4–12% Bis–Tris SDS–PAGE gel by staining with SimplyBlue SafeStain (Invitrogen). Beads with bound GST-fusion proteins were incubated for 2.5 h at 4°C with lysates of COS-1 cells expressing 3xFLAG-tagged Nek4. After incubation, beads with bound protein complexes were washed in lysis buffer. Then beads were taken up in 4× NuPAGE Sample Buffer and heated for 10 min at 70°C. Beads were precipitated by centrifugation and supernatant was loaded on a NuPAGE Novex 4–12% Bis–Tris SDS–PAGE gel. The presence of 3xFLAG-Nek4 in complex with GST-RPGRIP1 or GST-RPGRIP1L was assessed by immunoblotting, followed by staining with a monoclonal mouse α-FLAG primary antibody (Sigma-Aldrich) and goat-α-mouse couple to IRDye800 (Rockland Immunochemicals, Gilbertsville, PA, USA) as a secondary antibody. Fluorescence was analyzed on a Li-Cor Odyssey 2.1 infrared scanner.

For the pull-down experiment from bovine retinal extracts, retinas were dissected from fresh bovine eyes, obtained from the local slaughterhouse. Retinas were lysed by sonification for 1 min in lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet-P40 and 1 mM sodium-orthovanadate], supplemented with complete protease inhibitor cocktail (Roche Diagnostics). Retinal extracts were incubated overnight at 4°C with the previously described RPGRIP1- and RPGRIP1L-GST-fusion proteins, which were immobilized on glutathione–sepharose 4B beads (Amersham Biosciences). After overnight incubation, samples were washed three times with lysis buffer. The beads with associated protein complexes were processed as previously mentioned. Immunoblotting with α-Nek4 antibody (1:150, Abnova) was performed to assess interaction of endogenous Nek4 with RPGRIP1 and RPGRIP1L.

Co-immunoprecipitation
3xHA-Nek4 was expressed in combination with either 3xFLAG-RPGRIP1C2-end or 3xFLAG-RPGRIP1L C2-end in HEK293T cells. As a control for specificity, 3xHA-Nek8 was co-expressed with 3xFLAG-RPGRIP1C2-end and 3xFLAG-RPGRIP1L C2-end. After 24 h of expression, cells were lysed on ice in lysis buffer [50 mM Tris–HCL (pH 7.5), 150 mM NaCl, 0.5% Triton X-100] supplemented with complete protease inhibitor cocktail (Roche Diagnostics). Lysates were incubated with α-FLAG M2-agarose from mouse (Sigma-Aldrich) for 5 h at 4°C. After incubation, beads with bound protein complexes were washed in lysis buffer. Then beads were taken up in 4× NuPAGE Sample Buffer and heated for 10 min at 70°C. Beads were precipitated by centrifugation and supernatant was run on a NuPAGE Novex 4–12% Bis–Tris SDS–PAGE gel. The interaction of 3xHA-Nek4 with 3xFLAG-RPGRIP1C2-end or 3xFLAG-RPGRIP1L C2-end was assessed by immunoblotting, followed by staining with monoclonal mouse α-HA or α-FLAG (Sigma-Aldrich) as a primary antibody and goat-α mouse R DY e800 (Rockland Immunochemicals) as a secondary antibody. Fluorescence was analyzed on a Li-Cor Odyssey 2.1 infrared scanner.

Immunocytochemistry
hTERT–RPE1 cells (kindly provided by Prof. Uwe Wolfum, Johannes-Gutenberg Universität, Mainz, Germany) were cultured as previously described (53), seeded on coverslips and serum starved (0,2% FCS) for 24 h prior to fixation. Ciliated
cells were fixed in icecold methanol for 10 min, treated with 1% Triton X-100 in PBS for 5 min and blocked in 2% BSA in PBS for 20 min. Fixed cells were stained for 1 h with rabbit α-Nek4 (1:100, Abnova), monoclonal mouse α-polyglutamyalted tubulin (GT335, 1:1500), rabbit α-γ-tubulin (1:500, Sigma-Aldrich), rabbit α-RPGRIP1 (1:500) or with affinity purified guinea-pig α-RPGRIP1 (1:1500). Coverslips were then washed in PBS and stained for 45 min with Alexa Fluor 405-, 488- or 568-conjugated secondary antibodies (1:500, Molecular Probes, Leiden, The Netherlands). Coverslips were washed again with PBS and briefly with mQ water before being mounted in Vectashield with or without DAPI (Vector Laboratories, Burlington, CA, USA). For stainings in which three primary antibodies were combined, Vectashield without DAPI was used. For siRNA-experiments, immunofluorescence was assayed with a Zeiss LSM510 confocal microscope (Zeiss, Oberkochen, Germany). Samples for immunocytochemistry were analyzed on a Zeiss Axio Imager Z1 fluorescence microscope (Zeiss), equipped with a 63× objective lens. Optical sections were generated through structured illumination by inserting an ApoTome slider into the illumination path. Images were processed using Axiovision 4.3 (Zeiss), Adobe CS4 Photoshop (Adobe Systems, San Jose, CA, USA) and ImageJ software.

**Immunohistochemistry of rat retinal, renal and brain cryosections**

Unfixed eyes, kidneys and brains of 1-month-old Wistar rats were harvested and frozen in melting isopentane. Seven micrometer cryosections were cut and treated with 0.01% Tween in PBS and subsequently blocked in blocking buffer (0.1% ovalbumin and 0.5% fish gelatin in PBS). After the blocking step, the cryosections were incubated overnight with combinations of primary antibodies diluted in blocking buffer. Concentrations of primary antibodies were as described in the paragraph ‘Immunocytochemistry’, human-α-rooletin was used in a 1:1500 dilution. Alexa Fluor 488- and 568-conjugated secondary antibodies were also diluted in blocking buffer and incubated in a dark environment for 1 h. Staining of cell nuclei was performed with DAPI (1:8000). Prolong Gold Anti-fade (Molecular Probes) was used for embedding the sections. Pictures were made with a Zeiss Axio Imager Z1 fluorescence microscope (Zeiss), equipped with a 63× objective lens and an ApoTome slider. Images were processed using Axiovision 4.3 (Zeiss) and Adobe CS4 Photoshop (Adobe Systems). Procedures followed were in accordance with the ethical standards of the responsible committee on animal experimentation.

**Yeast two-hybrid assay**

The GAL4-based yeast two-hybrid system (HybriZAP, Stratagene, La Jolla, USA) was used for the identification of protein interaction partners of Nek4. Constructs encoding full-length or fragments of Nek4 (see paragraph ‘DNA constructs’) fused to a DNA-binding domain (GAL4-BD) were used as baits to screen a human oligo-dT primed retinal cDNA library. The yeast strain PJ69-4a, which carried the HIS3 (histidine), ADE2 (adenine), MEL1 (α-galactosidase) and LacZ (β-galactosidase) reporter genes, was used as a host. Interactions were analyzed by assessment of reporter gene activation based on growth on selective media (HIS3 and ADE2 reporter genes), α-galactosidase colorimetric plate assays (MEL1 reporter gene) and β-galactosidase colorimetric filter lift assays (LacZ reporter gene).

**siRNA experiments**

hTERT-RPE1 cells were seeded on coverslips and transfected with three pooled Silencer Select siRNAs (Invitrogen; 15 nM final siRNA concentration) using Hiperfect (Qiagen), according to manufacturer’s instructions. Non-targeting siRNA (SiSel_NC1, S813, Invitrogen; 15 nM final siRNA concentration) was included as a negative control. The targeting sequence for each siRNA is listed in Supplementary Material, Table S3. Twenty-four hours post-transfection, primary cilium formation was induced by serum starvation (0.2% FCS) for 48 h. Subsequently, cells were harvested for either knockdown analysis or immunocytochemistry. siRNA-treated cells were stained with GT335, which marks the basal body and the ciliary axoneme, and with α-γ-tubulin, which only marks the basal body. In two independent experiments, 100 cells were scored for the presence of cilia using ImageJ software, which assigned cilia if the GT335 signal exceeded the γ-tubulin signal.

**Quantitative PCR**

Total RNA from HEK293T and siRNA-treated hTERT-RPE1 cells was isolated using Trizol (Invitrogen), according to manufacturer’s instructions. One microgram of total RNA was transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Veenendaal, The Netherlands), according to the manufacturer’s protocol. SYBR Green-based real-time quantitative PCR (qPCR) analysis was performed on a 7900 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Primers were developed by the Primer3 program (54) and validated as described before (27). PCR products encompassed at least one exon–exon junction. GUSB was used as reference gene. qPCR quantifications were performed in duplicate on the equivalent of 400 pg/ml input of total RNA in the first-strand synthesis and included a reverse-transcriptase control. Values for experimental threshold cycles (Ct) were within the validated linear range of the primers. Melt curves showed presence of a single PCR product per reaction. All water controls were negative. Difference in NEK4 expression between two tissues was calculated by the comparative Ct or 2-ΔΔCt method (55,56). The adult human cDNA panel was kindly provided by Dr Arjan de Brouwer (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands).

**FACS analysis**

Flow cytometric analysis was performed on a Becton Dickinson FACScan cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Unsynchronized siRNA-treated cells were harvested...
and fixed in 70% ethanol. Prior to cytometric analysis, cells were stained with propidium iodide (PI) to label DNA-content. The PI signal was measured in the FL-3 channel. From the cell-cycle profiles, the percentages of cells with 2N (G1/S phase) or 4N (G2/M phase) DNA content were estimated.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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