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The mammalian Nek1 kinase is involved in primary cilium formation

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Abstract **Recent studies implicate primary cilium (PC) proteins in the etiologies of various polycystic kidney diseases (PKD). NIMA-related kinases (NRKs) are conserved serine/threonine kinases, which are usually defined as 'mitotic kinases'. Murine mutants for the NRKs, *nek1* (*kat* mice) suffer from PKD, suggesting that it may be involved in cilium control. We demonstrated herein that Nek1 is localized to basal body region and that Nek1 overexpression inhibits ciliogenesis in Madin–Darby canine kidney epithelial cells. The number of primary cilia is dramatically reduced in *kat2J* mouse embryonic fibroblasts culture. It is thus hypothesized that Nek1 links cell cycle progression and the PC cycle.**

Keywords: Primary cilium; Polycystic kidney disease; Ciliogenesis; NIMA; Kinase

1. Introduction

The cilium is an ancient and highly conserved eukaryotic organelle projecting from the cell surface. The primary cilium (PC) is a solitary, usually immotile cilium, found on most of vertebrate cells. Recent works demonstrated involvement of the PC in diverse sensory functions including mechano-, chemo- and photosensation, and concomitantly PC dysfunction has been implicated in a plethora of human diseases [1–3]. The most studied 'ciliary disease' is polycystic kidney disease (PKD). PKD is a common human inherited disease, characterized by benign polycystic tumors that are produced by abnormal renal epithelial cell overgrowth, eventually obstructing kidney function. Several studies demonstrated that the proteins mutated in human and murine models of PKD are localized to the cilium, and/or to the basal body [4–8]. Loss of cilia, disruption of the intraflagellar transport, or mis-regulation of ciliary signaling, leads to epithelial cell proliferation and cystogenesis. The mechanistic connection between the ciliary defects and the accelerated cell proliferation is still mainly obscure.

The NIMA-related kinase (NRK) family members are commonly referred as 'mitotic kinases'. In the filamentous fungus *Aspergillus nidulans* the founder kinase, never in mitosis, gene A (NIMA), is indispensable for mitotic entrance. Members of the NRK family are found in almost all eukaryotes, including protists, fungi, plants and animals. The mammalian genome has 10 NRKs, designated Neks, belonging to five ancestral NRK clades (Nek1,3,5; Nek2; Nek4,11; Nek6,7; Nek8,9) [9,10]. Naturally, early research was focused on comparing the functions of the newly identified kinases to fungal NIMA documented mitotic roles, yielding remarkable similarities [9,11,12]. However, recent research, mainly performed in protists, suggests a conserved involvement of the NRKs in ciliogenesis. It has been shown that *Trypanosoma* NRKC is localized to the basal body and inhibits flagella formation following overexpression [13]. Interestingly, overexpression of a *Chlamydomonas* axonemal NRK, Cnk2p, reduces cilium length, while diminution of its levels produces large cells with long flagella [14]. Similarly, in the ciliated *Tetrahymena* different NRK members were localized to specific subsets of cilia, and overexpression of distinct NRKs shortens cilia. It was thus hypothesized that the unequal size of the cilia in different regions of this ciliate is regulated by the specific NRKs expressed at each location [15].

Mutant mice for Nek1 kinase (designated *kat* mice) exhibit pleiotropic effects including slowly progressing PKD, choroid plexus cysts, male sterility, facial dysmorphism and runting [16,17]. The PKD phenotype provides a clue for Nek1 involvement in cilium structure or function. However, no direct connection between Nek1 and the cilium has been reported. In this study, we demonstrate that Nek1 protein is localized to the basal body region and that overexpression of Nek1 inhibits ciliogenesis. In addition, much lower percentage of *kat2J* mouse embryonic fibroblasts (MEF) bear a PC, and frequently a long and branched cilium-like structure appears on *kat2J* MEF. These results thus indicate that the cilium is a major target of Nek1 activity.

2. Materials and methods

2.1. Cell cultures, infection, and mouse embryonic fibroblasts (MEF) derivation

Tet-off Madin–Darby canine kidney (MDCK) cells stably expressing tet-transactivator were cultured in DMEM medium supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics. Recombinant tetracycline-regulated adenovirus expressing full-length Nek7 protein tagged with myc at its N-terminus was produced essentially as described before [18]. Nek1 levels were regulated by the concentration of doxycycline, the amount of virus, and the length of time after infection, to obtain the minimal expression that avoids toxic impacts. MEF were isolated from E13.5 *kat2J*, *wt* and *het* embryos, and in all experiments fibroblast derived from siblings were compared. The MEF were cultured in DMEM medium supplemented with 10% fetal calf serum 2 mM glutamine and antibiotics.

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Abbreviations: MDCK, Madin–Darby canine kidney; MEF, mouse embryonic fibroblasts; NIMA, never in mitosis, gene A; NRK, NIMA-related kinase; PC, primary cilium; PKD, polycystic kidney disease

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2.2. Immunofluorescence staining

For immunofluorescence microscopy, cultures grown on coverslips were fixed in 4% paraformaldehyde for 20 min, blocked with 20% FBS/0.5% Triton X-100 in PBS, and incubated overnight with primary antibodies at 4 °C. The generation of affinity purified Nek1 polyclonal antibodies was described before [19]. Other primary antibodies were purchased from: β-tubulin monoclonal antibodies (DSHB), acetylated α-tubulin monoclonal antibodies and anti-γ-tubulin polyclonal antibodies (Sigma), and myc (Delta Biolabs). Secondary fluorescein isothiocyanate (FITC)-conjugated and rhodamin conjugated antibodies were purchased from Jackson ImmunoResearch. Staining was analyzed using an ApoTome microscope (Zeiss) or a LSM 510 Zeiss laser scanning confocal microscope.

2.3. Western blotting

Cells or tissues were lysed in HNTG buffer (20 mM Hepes at pH 7.5, 150 mM sodium chloride, 10% glycerol, 1% Triton-X, 1 mM EDTA and 1 mM EGTA) for protein extraction. Proteins were run on 8% acrylamide gels, transferred to nitrocellulose membranes and visualized by immunoblotting with anti-Nek1 diluted 1:150 anti-myc diluted 1:500 (DSHB) and anti-actin diluted 1:500 (DSHB).

3. Results

3.1. Nek1 overexpression inhibits ciliogenesis

To explore possible influences of Nek1 on ciliogenesis, we overexpressed Nek1 in MDCK cells. MDCK epithelial cells are commonly used as a model for polarized cells bearing PC. Confluent MDCK cells grow cilia within several days of further culturing. To enable efficient and controlled overexpression, we generated adenovirus vector expressing full length Nek1 downstream of a tetracycline operon (designated myc-Nek1-Ad). The virus was used to infect Tet-off MDCK cells, thus enabling tetracycline-dependent Nek1 expression. Nek1 overexpression (in the absence of tetracycline-analogue) in Tet-off MDCK cells that have just become confluent (before cilia growth) prevented almost entirely cilia formation. The percent of cells bearing a cilium was dropped from 14.6% in uninfected cells to 2.9% in the infected cells (Fig. 1AI). In the presence of doxycycline, the cells did not express detectable levels of Nek1 (Fig. 1B), and the infection had no influence on cilia number (Fig. 1AI), suggesting that the viral infection per-se did not influence ciliogenesis. To examine whether Nek1 overexpression affect existing cilia, Tet-off MDCK cells which already express cilia on their surface were infected with myc-Nek1-Ad for either 16 or 48 h. Nek1 overexpression, but not viral infection by itself, reduced the percentage of cells bearing cilia (Fig. 1AII, III and C–E). As can be seen in Fig. 1D, Nek1 overexpression usually stained a single spot within the overexpressing cells, presumably the centrosome/basal body (see below). It should be noted that cells overexpressing detectable levels of Nek1 (stained by the myc-tag epitope) rarely pre-

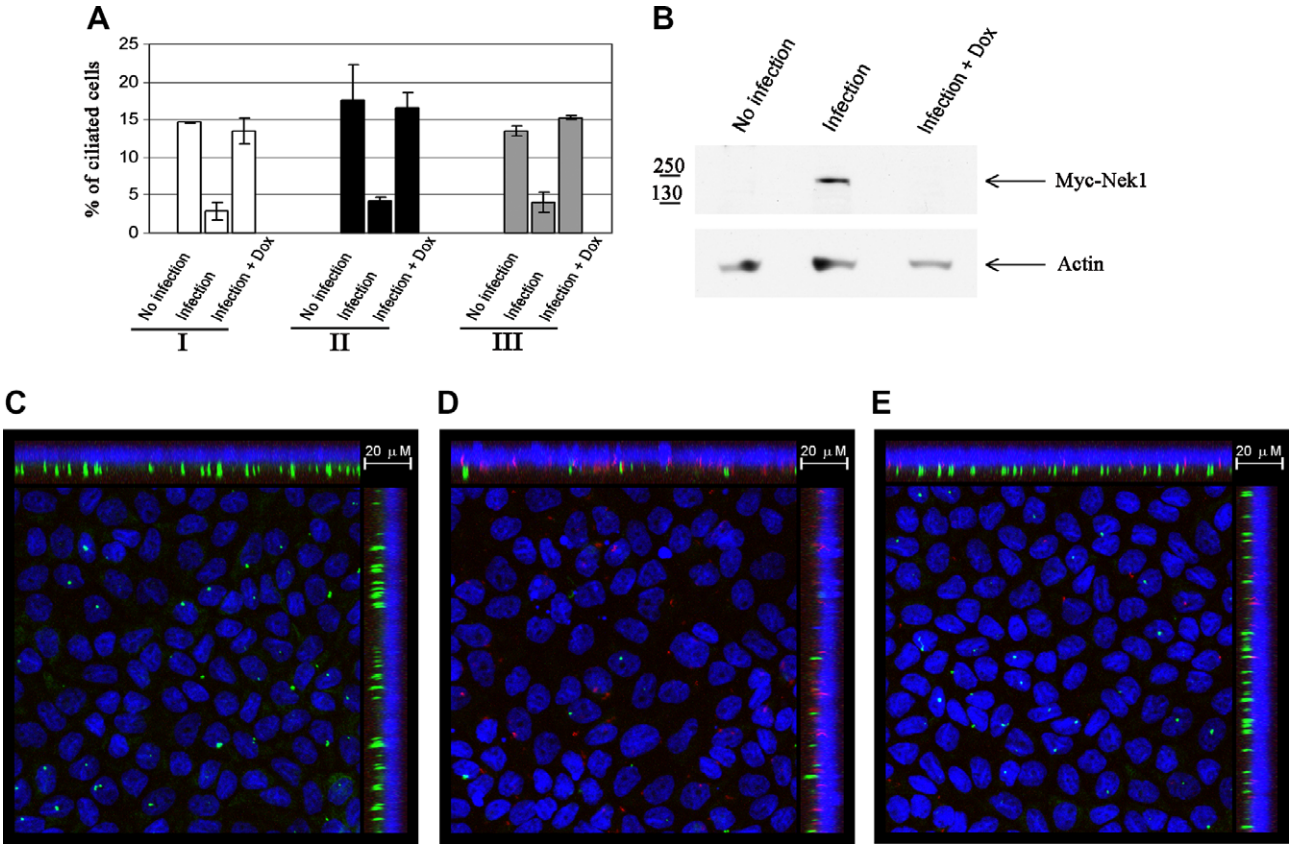

Fig. 1. Nek1 overexpression reduces cilium number. (A) Infection of MDCK cells that have just become confluent for 5 days (I), infection of cells 5 days following confluency for 16 h (II) or 48 h (III). (B) Western blot analysis of MDCK cells infected for 24 h with or without the addition of 100 ng/ml of doxycycline. (C–E) Confluent MDCK cells were grown for additional 5 days (for cilium assembly), infected with Myc-Nek1-Ad for 48 h, fixed and stained with anti-acetylated-tubulin Ab (green), and anti-myc (red). Nuclei were stained by Hoechst 3342 (blue). Vertical serial photos were taken by LSM 510 Zeiss laser scanning confocal microscope. Flattening of all the layers was performed using Carl Zeiss AxioVision LE software. Lateral view is presented on the upper and right side of each picture. (C) No infection. (D) Infection without doxycycline. (E) Infection in the presence of doxycycline.

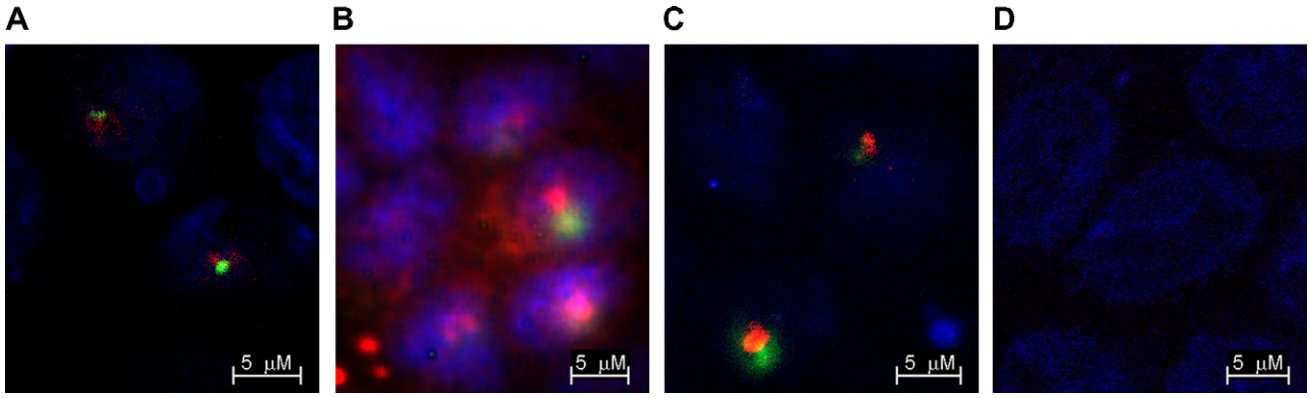

Fig. 2. Nek1 is localized to the basal body. Confluent MDCK cells were grown for additional four days and infected with Myc-Nek1-Ad. Cells were grown for 18 h post-infection, followed by fixation and staining. (A) Red – Ac-tubulin, green – Myc-Nek1, blue – Hoechst nuclear staining. (B–C) Red – Myc-Nek1, green – Ac-tubulin, blue – Hoechst nuclear staining. (D) Uninfected cells stained with secondary Ab only.

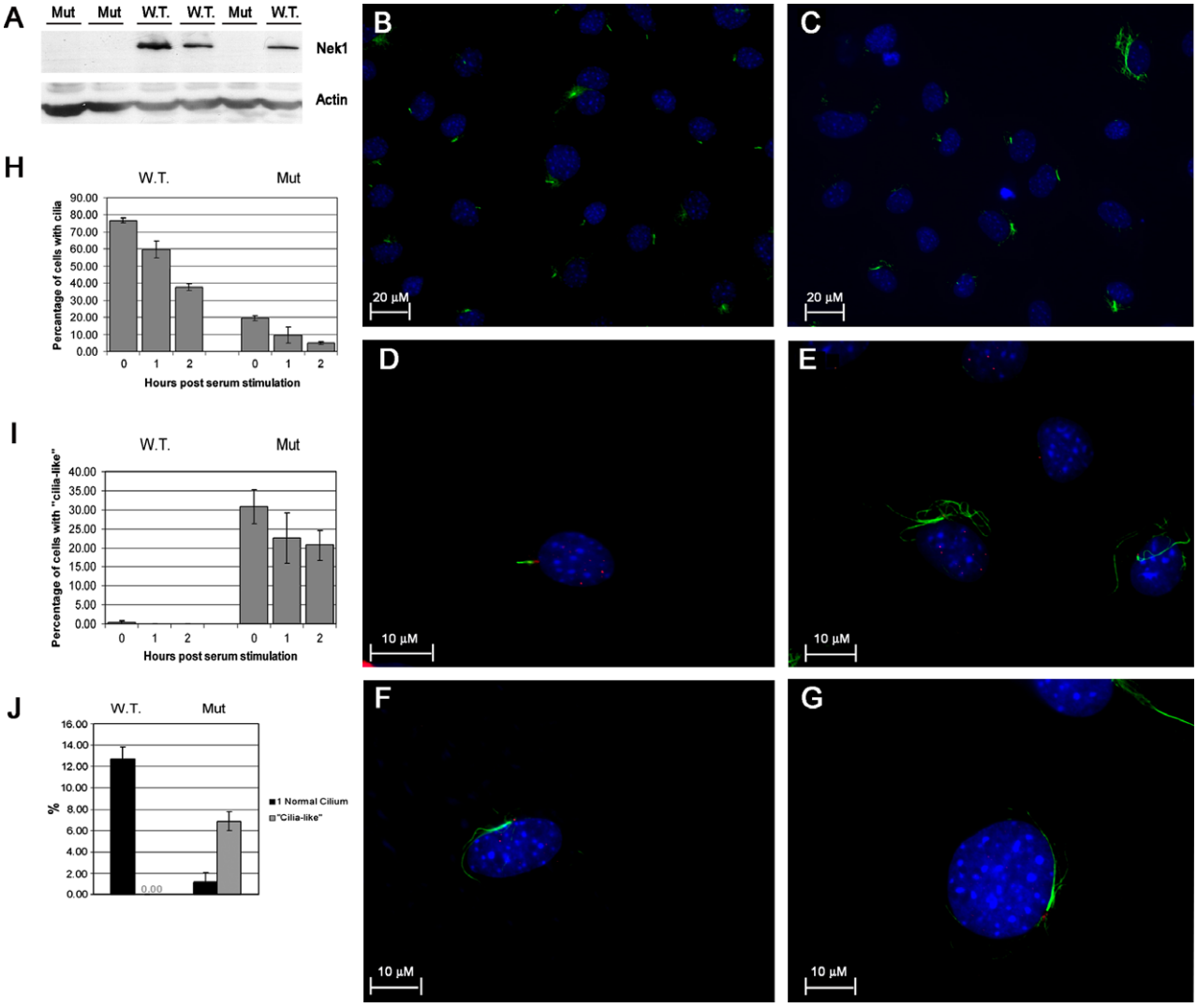

Fig. 3. *kat2J* MEF cells have reduced number of cilia and possess "cilia-like" structure. (A) Genotyping of embryos. Heterozygotes for *nek1* mutation were crossed, and at 13.5 days of embryogenesis MEF were isolated from the trunk, and extracts from the heads were blotted and incubated with affinity-purified antibodies against Nek1. To asses loading, the membrane was blotted with anti-actin antibodies. (B-G) Immunofluorescence detection of cilia. Forty-eight hours post-starvation *wt* MEF cells (B, D) and *kat2J* fibroblasts (C, E–G) were fixed and immunostained with anti-acetylated-tubulin (green) and anti-γ-tubulin (red). Hoechst 3342 counterstained nuclei (blue). (H–I) Number of 'normal' cilia (H) and cilia-like (I) in wt/het and *kat2J* fibroblast following 48 h serum starvation (time 0), and 1 and 2 h post-serum addition. (J) Number of normal and cilia-like projections in non-starved proliferating cultures of *wt/het* and *kat2J* fibroblasts. Data are based on two independent experiments of three wt/het and three mut cultures of MEF.

sented a cilium (Fig. 1D). The results thus suggest that Nek1 is a negative regulator of ciliogenesis in MDCK cells (at least at high levels of expression).

3.2. Nek1 is localized to the basal body region

Nek1 has been localized previously to the centrosome of an inner medullary collecting duct (IMCD-3) cell line [20]. In agreement with this report Nek1 was localized to the centrosome of proliferating MDCK cells infected with myc-Nek1-Ad (not shown). To explore possible association of Nek1 protein with the cilium, the sub-cellular localization of myc-Nek1 was observed in MDCK cells already expressing cilia on their surface following infection with myc-Nek1-Ad for 18 h. As described above, in most infected cells no cilium was apparent. However, in the cases in which both Nek1 and acetylated tubulin were seen within the same cell, Nek1 was localized to an area proximal, adjacent, but distinct from the region stained by the acetylated α-tubulin, indicating localization to the basal body region (Fig. 2).

3.3. MEF from kat2J mice have reduced number of normal cilia and harbor long and branched cilium-like structures

The overexpression experiments suggest that Nek1 inhibits ciliogenesis. To test this supposition, MEFs were produced from E13.5 *kat2J* embryos and their *wt* and heterozygote (*het*) littermates, and genotyped by Western analysis using polyclonal antibodies against Nek1 protein (Fig. 3A). *kat2J* mice have a null mutation in Nek1 kinase [16]. Mammalian fibroblasts tend to grow cilia at the G_0 phase, usually triggered by serum starvation. Following 48 h of serum starvation the percentage of cells bearing cilia was dramatically lower in Nek1 mutants' fibroblasts (20%, compared to 75% in the *wt/het*) (Fig. 3B, C, and H). Intriguingly, using staining for acetylated tubulin, in about 30% of the starved *kat2J* fibroblasts typical long and thick extensions, emanating from a γ-tubulin center, were seen (Fig. 3D–G and I). The extensions usually split into several branches stained by the acetylated tubulin marker, suggesting that they do not serve as functional cilia. Such long structures were very rarely seen in serum-starved *wt* MEF (in less than 1% of the cells; Fig. 3I). In proliferating MEF cultures from *kat2J* mice, the percentage of the cells bearing this extension was much reduced (about 7%) (Fig. 3J), probably representing cells which withdraw from the cell cycle. Similarly, proliferating *wt/het* cultures had about 13% of cells bearing seemingly normal cilia (Fig. 3J).

3.4. Dynamics of cilia disappearance following re-incubation with serum

The acquisition of cilia by mammalian fibroblasts in serum-starved culture is reversible and serum addition results in cilia disappearance. To examine whether cilia absorption pace is affected in the mutants we observed 'normal' cilia loss following serum addition. Cilia loss in the mutants' MEF was faster compared to the wt MEF cells. Following 1 h incubation with serum the number of the wt cilia was reduced by 20%, and following 2 h incubation by additional 38% (compared to 1 h incubation). In the mutants, 1 h incubation with serum reduced the number of cilia by 50% of the initial number, and 2 h incubation reduced the number by additional 50% (Fig. 3H). The presence of the stabilized cilium-like extensions in *kat2J* MEF was also affected by serum addition and by 2 h their percentage was reduced from 30% to 20% of the cells (Fig. 3I).

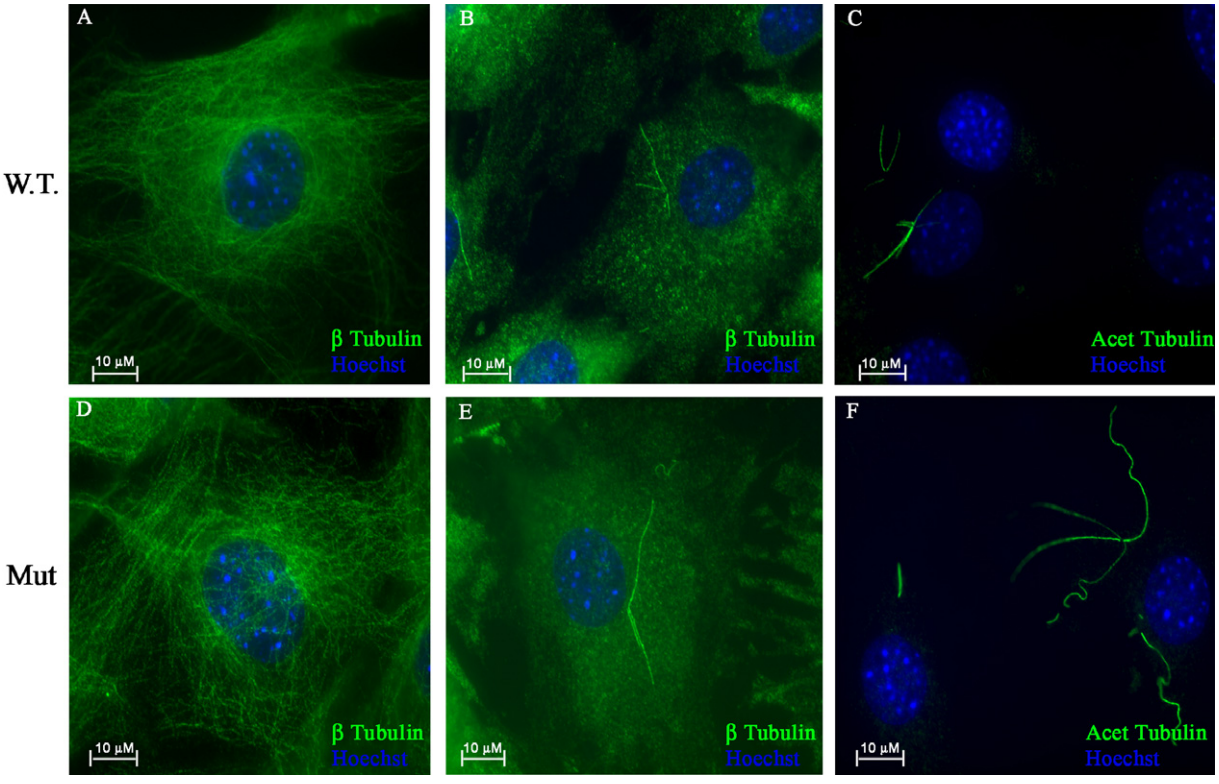

Fig. 4. Microtubule stability is not affected by *nek1* deficiency. MEF were starved for 48 h, and stained with anti-β-tubulin antibodies (A, D). The serum-starved cells were treated with 33 μM nocodozole for 3 hours (B, C, E, and F), fixed and immunostained with antibodies against β-tubulin (B, E) and acetylated tubulin (C, F), both in green. The nuclei were counterstained with Hoechst 3342 (blue). (A–C): *wt/het* MEF, (D–F): *kat2J* MEF.

Several observations suggest that the NRKs are involved in microtubule stability and dynamics (see for reviews [9,12]). Inspection of untreated MEF from *kat2J* and *wt/het* mice did not reveal substantial difference in the microtubule organization in interphase cells (Fig. 4A and D). In addition, wt and mutant cells treated with the depolymerizing drug nocodazole, exhibited similar disappearance of microtubles and stabilized microtubules as revealed by staining with anti-β-tubulin and anti-acetylated-tubulin antibodies, respectively (Fig. 4B, E and C, F).

4. Discussion

A major phenotype of mice devoid of Nek1 is PKD, and a variety of genes mutated in PKD models were implicated in cilium structure and function. However, Nek1 has not been implicated in cilium structure or function. We reported herein that Nek1 is localized to the cilium basal body region, and that in *kat2J* MEF the number of cilia is dramatically reduced. Nek1 has been localized previously to the centrosome [20], and the common usage of the centriole for both centrosome and basal body construction could give a clue for centriolar localization. However, within the centrosome, Nek1 site of residence was localized outside the centriole and the pericentriolar region, and it was suggested to reside in the pericentriolar satellite region of the centrosome [20]. Further work will be necessary to determine the precise localization of Nek1 within the PC.

Interestingly, both Nek1 overexpression (in MDCK cells) and absence of functional Nek1 (in *kat2J* MEF cells) result in reduction in cilia number. One possible explanation is that Nek1 overexpression in MDCK cells perturbed the cell cycle and that the infected cells were stuck in G_1/S or G_2/M. As cilia are preferably made by G_0 arrested cells, cilium number could be affected. However, as Nek1 overexpression was performed in stationary MDCK cells which already withdraw from the cell cycle this option is implausible. To the other side, *kat2J* MEF cells proliferate continuously in culture precluding the option that Nek1 kinase activity reduces ciliogenesis by blocking the cells at a certain phase of the cell cycle. It is thus probable that the ciliogenesis process requires a delicate spatiotemporal activity of Nek1, which is disturbed by robust Nek1 overexpression, as well as by its absence.

In addition to PC loss, *kat* MEF frequently display long, thick and usually branched structures stained by acetylated tubulin antibodies. These structures very rarely appear in wt/het MEF, and their frequency correlate with the frequency of normal cilia, namely in association with serum starvation conditions. Overexpression of several NRK members in protists induced shortening of the (motile) flagella [14,15] whereas their knockdown produces long flagella [14]. These observations indicate a role for this kinase family in the control of ciliary length or disassembly rate, possibly by affecting axonemal microtubule dynamics and stabilization. The long cilia-like protrusions observed in the mutants' MEF are in line with this hypothesis. Nek1 has been shown to bind to the microtubule-dependent motor protein, Kif3A, strengthening the possibility that it influences microtubule-dependent processes.

In vertebrates, cilia are formed on differentiated cells residing at G_0, and re-entrance to the cell cycle is preceded by deciliation. A mechanistic explanation is the usage of the centrosomal elder centriole to assemble the basal body, and vice versa: the need to release the basal centriole in order to establish the mitotic spindle. The localization of Nek1 to the centrosome and to the basal body region and Nek1 effect on cilium formation, combined with the documented involvement of the NRKs in cell cycle control may suggest that Nek1 is involved in the coordination between the cilium and the cell cycle. In addition, the recent recognition that the PC serves as an antenna sensing the extracellular environment makes it an excellent candidate for transmitting information related to cell cycle entrance. As PKD etiologies include disturbances to various structural and sensory ciliary functions, it emphasizes the intimate connection between the cilium and the (uncontrolled) cell cycle, and its utility as a model for dissecting the connections.

Mutant mice for an additional mammalian Nek family member, Nek8, suffer from polycystic kidney (designated *jck*) [21]. Nek8 was localized to the proximal region of the PC [21,22], and lengthening of the cilia in *jck* cystic kidney cells was demonstrated [22]. These characteristics are reminiscence of the Nek1 features represented here. Recent phylogenetic analysis indicates that Nek1 and Nek8 belong to two different ancient clades within the NRK family [10]. Thus, the similar ciliary and PKD phenotypes of Nek1 and Nek8 strengthen the notion that cilium-related functions are ancestral and conserved functions of the Nek kinases.

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