HHS Public Access

Author manuscript

Cytoskeleton (Hoboken). Author manuscript; available in PMC 2022 May 26.

Published in final edited form as:

Cytoskeleton (Hoboken). 2010 February ; 67(2): 112–119. doi:10.1002/cm.20428.

Inv Acts as a Molecular Anchor for Nphp3 and Nek8 in the Proximal Segment of Primary Cilia

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Abstract

A primary cilium is an antenna-like structure extending from the surface of most vertebrate cells. It is structurally divided along its vertical axis into sub-compartments that include the ciliary tip, the shaft, the ciliary necklace segment, the transitional zone and the basal body. We recently discovered that the shaft of the primary cilia has a distinct molecular compartment, termed the "Inv compartment", which is characterized by the accumulation of Inv at the base of primary cilia. *Inv* was discovered as a causative gene in *inv* mutant mice. It was later found to be responsible for the infantile type of nephronophthisis (NPHP2). Nephronophthisis (NPHP) is an autosomal recessive kidney disease. Nine causative genes have been identified, with all examined products thought to function in cilia, basal body and/or centrioles. However, their exact intra-ciliary localization and relationship have not been clear. Here, we report that products of *Nphp3* and *Nek8* (the mouse orthologs of the causative genes for NPHP3 and NPHP9, respectively) localize to the Inv compartment. We also show that Inv is essential for the compartmental localization of Nphp3 and Nek8, whereas localization of Inv does not require Nphp3 or Nek8. Nphp1 and Nphp4 also localize at the proximal region of the cilium, but not in Inv compartment. Our results indicate that Inv acts as an anchor for Nphp3 and Nek8 in the Inv compartment, and suggest that Inv compartment is a candidate site for intra-ciliary interaction of Inv, Nphp3 and Nek8.

Keywords

primary cilia; Inv; Inversin; NPHP; Nek8

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Additional Supporting Information may be found in the online version of this article.

Introduction

A primary cilium is an antenna-like structure extending from the surface of most vertebrate cells. The primary cilium is structurally divided along its vertical axis into sub-compartments that include the ciliary tip, the shaft, the ciliary necklace segment, the transitional zone and the basal body [Wheatley, 1967; Gilula and Satir, 1972; Wheatley, 1995]. These ciliary sub-compartments are clearly observed by electron microscopy, and some intra-ciliary molecules are detected in the sub-compartments [Deane et al., 2001; Kubo et al., 2008]. In addition to these sub-compartments, we have recently reported that the primary cilium has a distinct molecular compartment within the proximal segment of ciliary shaft that was not previously recognized by morphological examination. This molecular compartment is revealed by Inv localization, thus we named this segment as the “Inv compartment” [Shiba et al., 2009].

The *inv* (inversion of embryonic turning) mouse mutant shows *situs inversus* associated with multiple renal cysts [Yokoyama et al., 1993; Mochizuki et al., 1998; Morgan et al., 1998]. Mutation in the *INVS* gene in human was later found to cause nephronophthisis type 2 (NPHP2) [Otto et al., 2003]. Nephronophthisis is characterized by chronic tubulointerstitial nephritis with various size renal cysts [Hildebrandt and Zhou, 2007]. The disease is the most common genetic cause of chronic renal failure in childhood. Nephronophthisis is considered to be a ciliopathy, as the proteins encoded by the causal genes are localized in cilia, basal body and/or centrioles [Mollet et al., 2002; Otto et al., 2005; Fliegauf et al., 2006; Arts et al., 2007; Attanasio et al., 2007; Delous et al., 2007; Otto et al., 2008; Sohara et al., 2008; Shiba et al., 2009]. Inv has been reported to co-immunoprecipitate with Nphp1 and Nphp3 [Otto et al., 2003; Bergmann et al., 2008]. Nphp1 is reported to bind with Nphp3 and Nphp4 [Mollet et al., 2002; Olbrich et al., 2003]. Thus, it has been speculated that the Nphps form a complex and function in a closely related pathway whose disruption leads to a common phenotype, renal cyst formation. Since Inv specifically localizes to the Inv compartment of primary cilia, the compartment is a likely site for Nphp interaction. Nek8, a causative gene for NPHP9 [Otto et al., 2008], is localized at a base of the primary cilia [Mahjoub et al., 2005; Sohara et al., 2008], possibly in the Inv compartment. However, not only relationships between these Nphps but also intraciliary function of Inv remain elusive.

In the present study, we have examined detailed intra-ciliary localization of Nphp1, Nphp3, Nphp4, and Nek8 in mouse renal epithelial cells (Dai1 cells) and their relationships. Our present study showed that the Inv compartment of the primary cilia is composed of multiple molecules and that Inv acts as an anchor for other Nphp proteins.

Materials and Methods

Reagents

Anti-acetylated α-Tubulin antibody (clone 6-11B-1) and anti-γ-Tubulin (clone GTU-88) from Sigma (St Louis, MO) and cell culture supplements from Invitrogen (Carlsbad, CA) were used. Restriction enzymes were purchased from Takara (Sigma, Japan), and the pEGFP vectors and pmKO2 expression vectors were purchased from Clontech (Mountain View,

CA). Unless otherwise stated, all other chemicals were purchased from Sigma (St Louis, MO) or Wako Pure Chemical (Osaka, Japan).

Cell Culture

Wild-type mouse renal epithelial cell line (Dai1 cells) and *Inv* mutant cell line (Dai2 cells) were grown in the medium described previously [Shiba et al., 2009]. Briefly, cells were cultured on glass coverslips coated with human collagen IV (50 mg/ml) at 33°C in D-MEM/F-12 medium containing 0.5% fetal bovine serum, 100 μM MEM non-essential amino acid solution, 5 mg/l insulin, 5 μg/l sodium selenite, 5 μg/ml transferrin, 400 mg/l dexamethasone, 10 ng/ml epidermal growth factor, 5 pg/ml 3,3,5-triido-l-thyronine, 10,000 U/l penicillin, 100 mg/l streptomycin, and 250 μg/l Fungizone®.

Preparation of Fusion Protein Constructs

A full-length *Inv*-encoding cDNA (1–1062) and GFP-tagged full length *Inv* (1–1062) construct has been described previously. Full-length *Nphp1* (Accession NM_016902), *Nphp3* (Accession NM_028721) and *Nphp4* (Accession NM_153424) were subcloned into pEGFP-N or pmKO2-N vectors (Clontech, Mountain View, CA) using PCR-based methods.

Transfections

Transfections were performed using Gene juice (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, the day before transfection, cells were plated at sub-confluent density on glass dishes. Cells were incubated overnight in 1000 μl culture medium and 50 μl serum-free medium that contained 5 μl of Gene juice and 0.5 μg of expression construct DNA per well.

Immunofluorescence Microscopy

Cells were fixed in ice-cold methanol/acetone [1:1] for 10 min, permeabilized in 0.1% Triton X-100 for 20 min, quenched in PBS (137 mM NaCl, 2.6 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) with 1% BSA for 1 h at room temperature. Cells were then incubated with primary antibodies at room temperature for 2 h (mouse anti-acetylated α-tubulin (clone 6-11B-1) [1:2000], mouse anti-γ-tubulin (clone GTU-88) [1:2000], rabbit anti-Inv (90086) [1:500], mouse anti-Myc [1:2000], rabbit anti-Nek8 [1:1000]. Cells were washed in PBS and incubated with Alexa350, 488 or 555-conjugated goat anti-mouse or rabbit IgG for 1 h. Fluorescence was visualized on an IX70 microscope with U-MWU2 (BP 330–385 nm, BA 420 nm), U-MGFPHQ (BP 460–480 nm, BA 495–540 nm) and U-MWIG2 (BP 520–550 nm, BA 580 nm) filters with a xenon light source. Digital images were processed by MetaMorph.

Results

Nphp3 and Nek8, but not Nphp1 or Nphp4, Localize to the Inv Compartment

We first examined Nphp3 (the causative gene product of *NPHP3* [Olbrich et al., 2003]) to determine if the protein is localized in Inv compartment. The Inv compartment, which is not detected morphologically, is defined by Inv localization (Fig. 1A, a top panel) [Shiba et

al., 2009]. While Nphp3 has been considered likely to be cilial-localized, this has not been proven. We generated GFP-tagged *Nphp3* construct, and transfected the fusion-construct into Dai1 cells (an immortalized mouse renal epithelial cell). GFP signal was observed at the base of primary cilia (100%, $n = 38$), but did not overlap with γ-tubulin staining (Fig. 1A, a middle panel), indicating that Nphp3 localize in the primary cilia and not in basal bodies. Furthermore, the GFP signal perfectly overlapped with Inv localization, indicating that Nphp3 localizes to the Inv compartment (Fig. 1B). In addition to the GFP-tagged Nphp3, Myc-tagged Nphp3 was also detected at the Inv compartment (Supporting Information Fig. S1).

As previously reported [Sohara et al., 2008], Nek8 was also detected at the base of primary cilia (100%, $n = 32$), and its localization did not overlap with γ-tubulin immunostaining (Fig. 1A, a bottom panel). Notably, Nek8 localization overlapped with Inv signal, indicating that Nek8 also localizes to the Inv compartment (Fig. 1C). The results show that Inv compartment is composed of at least three molecules, Inv, Nphp3 and Nek8.

Nphp1 has been reported to co-immnoprecipitate with Inv [Otto et al., 2003] and Nphp4 [Mollet et al., 2005]. We transfected GFP-tagged *Nphp1* and *Nphp4* constructs into Dai1 cells. GFP signals were detected at the base of the primary cilium (acetylated-tubulin signals), but did not overlap with γ-tubulin staining (Fig. 2A). They did not overlap with the Inv compartment (Fig. 2A). To see that Nphp1 and Nphp4 are co-localized, we generated mKO2 (red)-tagged full-length constructs of *Nphp1* or *Nphp4*. The localization of GFP-tagged *Nphp1* and mKO2-tagged *Nphp4* signals (and vice versa) completely overlapped (Fig. 2B). The localizations of Nphp1 and Nphp4 were not changed in *Inv* mutant cells (Dai2 cells) (Fig. 2C).

Inv Compartment Localization of Nphp3 and Nek8 is Dependent on Inv

We then studied if Inv expression is required for intra-ciliary localization of Nphp3 and Nek8. We transfected the *Nphp3*-GFP fusion construct to *Inv* mutant cells (Dai2 cells). Nphp3-GFP transfected cell were identified by cytoplasmic GFP signal. We found two types of cells in Nphp3-GFP transfected cells: one has faint and patchy GFP signals along the entire length of primary cilia, and the other has extremely faint or no detectable Nphp3-GFP signal in the primary cilia (Fig. 3A, top and middle panels). The cells that have the faint Nphp3-GFP signals in the primary cilia are 61.4% of the examined cells ($n = 83$) (Fig. 3A, a top panel). The specific localization of Nphp3 to the Inv compartment was completely lost in *Inv* mutant cells in both of cells ($n = 83$). Nek8 localization to the primary cilia was also completely lost in all the observed *Inv* mutant cells ($n = 51$) (Fig. 3A, a bottom panel).

Transfection of GFP-tagged full-length *Inv* construct into *Inv* mutant cells rescued the Nphp3 localization to the Inv compartment in 94.1% of the transfected cells ($n = 34$) (Fig. 3B, a top panel) and the Nek8 localization in the Inv compartment (100%, $n = 40$) (Fig. 3B, a bottom panel). Transfection of Inv (1–1002) into *Inv* mutant cells was unable to recover the Inv compartment localization of Nphp3 (Supporting Information Fig. S2 upper panel). The signal patterns of Nphp3 in *Inv* mutant cells co-transfected with and without Inv (1–1002) are identical. We observed the faint ciliary Nek8 signals in Inv (1–1002) transfected cells along entire cilia, but the signal was not confined to the Inv compartment

when transfected Inv (1–1002) in *Inv* mutant cells (Supporting Information Fig. S2 lower panel).

Localization to Inv Compartment of Inv C-Terminus is Independent of Nphp3 and Nek8 Localization

We then examined if the intraciliary behavior of Inv, Nphp3 and Nek8 are interdependent. We transfected *Inv* C-terminus construct (amino acids 742–1062) into *Inv* mutant cells. Localization of Nphp3 is detected by co-transfection with mKO2-tagged full-length construct of *Nphp3*. Nek8 is detected with the anti-Nek8 antibody. As previously reported, *Inv* C-terminus (amino acids 742–1062) localizes to the Inv compartment (Fig. 3B). However, Nphp3 and Nek8 localization to the Inv compartment was not observed in *Inv* mutant cells with co-transfected *Inv* C-terminal construct (amino acids 742–1062) (3.3%, n = 30 and 0%, n = 36, respectively) (Fig. 3B).

Discussion

The present study has revealed two novel findings about the Inv compartment (Fig. 4). First, not only Inv but also Nphp3 and Nek8 accumulate in the Inv compartment of the primary cilia, suggesting that the Inv compartment is composed of multiple molecules. Second, Inv acts as the intra-ciliary molecular anchor for Nphp3 and Nek8. This is the first evidence for intra-ciliary Inv function. Failure to localize in the Inv compartment of these molecules precludes molecular function, which may result in renal cyst development.

Nphp3 has been reported to be co-immunoprecipitated with Inv [Bergmann et al., 2008], but co-localization was not shown. The present findings indicate that the Inv compartment is the candidate site where Nphp3/Inv complex is formed. Faint and ununiform Nphp3-GFP signals were detected along the entire length of primary cilia in *Inv* mutant cells, and some cells have extremely faint or no detectable Nphp3-GFP signal in the primary cilia (Fig. 3A). These results suggest that Nphp3 can localize to the primary cilia without Inv, but it may be unstable in the absence of Inv. Nphp3 may be able to move alone to the cilia but not the Inv compartment, when exogenously expressed. Although NPHP3 was considered to cause an adolescent form of nephronophthisis, mutations in NPHP3 were recently reported in infantile nephronophthisis [Tory et al., 2009]. Our results provide molecular explanation of the phenotypic similarity between Nphp3 and *inv* mutant mice. To maintain proper renal tubular integrity may require either co-localization of Inv and Nphp3 in Inv compartment or accumulation of sufficient Nphp3 in Inv compartment.

The previous report suggested that Nek8 is likely localized at the Inv compartment. However, a relationship between Inv and Nek8 was not determined. In *jck* mice, which carry a *Nek8* gene with a missense mutation in its RCC domain, full-length protein is made, but localizes to the entire length of primary cilium [Sohara et al., 2008]. Strict Inv compartment localization of Nek8 may be required for proper Nek8 function. Of additional note is the finding that mice carrying a null mutation in Nek8 have left-right asymmetry defects (Manning and Beier, unpublished), supporting the likelihood that there are functional interactions with Inv. Nek8 is a serine/threonine kinase, but its targets (substrates) are poorly understood [Liu et al., 2002; Bowers and Boylan, 2004]. Loss of ciliary Nek8 in

Inv mutant cells may reduce the interaction between Nek8 and its targets in the primary cilia. Alternatively, Nek8 may be isolated from cytoplasm by accumulating to the primary cilia in wild-type cells to avoid interacting for its cytoplasmic targets that promote cell cycle progression. In either case, the ectopic Nek8 localization may influence the proper interaction between Nek8 and the substrates. Identifying the Nek8 substrates will reveal the connection between loss of ciliary Nek8 localization and aberrant cell cycle progression leading to renal cyst formation in a future study.

Nphp3 and Nek8 localization in the compartment were lost in *Inv* mutant cells. Introduction of the full-length Inv rescued the Inv compartment localization of both Nphp3 and Nek8. Therefore, an Inv function seems to tether and/or stabilize Nphp3 and Nek8 in the Inv compartment of the primary cilia. *Inv* C-terminal construct [Inv (742–1062)] targeted to the Inv compartment, but could not rescue the compartment localization of Nphp3 and Nek8. The result indicates that the N-terminal region (amino acids 1–741), in which 15 successive repeats of the ankyrin motif (amino acids 41–550) of Inv resides [Mochizuki et al., 1998], is necessary for localization of Nphp3 and Nek8 to the Inv compartment. Since the ankyrin motif is known as a protein-protein interaction motif, the motif in Inv may interact with Nphp3 and Nek8. Inv compartment localization of *Inv* C-terminal construct [*Inv* (742–1062)] does not require the compartment localization of Nphp3 and Nek8, suggesting that the transportation machinery for Inv is independent from Nphp3 and Nek8. Nphp3 and Nek8 are transported at the same time or after Inv localization in the Inv compartment. The results support the idea that Inv is an intraciliary molecular anchor for Nphp3 and Nek8.

We have previously reported that full-length Inv (1–1062) localized to the Inv compartment, but that Inv (1–1002) localized entire length of primary cilia with faint patchy staining [Shiba et al., 2009]. Transfection of Inv (1–1002) did not recover the compartment localization of Nphp3 and Nek8. Although no Nek8 signals were observed in *Inv* mutant cells, we found quite faint Nek8 signals in some Inv (1–1002) transfected cells. The signals are not confined to the Inv compartment but observed along entire cilia. Nek8 can be transported by Inv to the cilia.

Poycystin-1 and polycystin-2 are the causal gene products for ADPKD (autosomal dominant polycystic kidney disease). They bind each other and cooperate as the fluid flow sensor in the primary cilia [Hanaoka et al., 2000; Nauli et al., 2003]. Bardet-Biedl syndrome (BBS) is a systemic disorder, and BBS proteins compose a complex (BBSome) required for ciliogenesis [Nachury et al., 2007]. Most Nphps localize to the primary cilia or basal body [Mollet et al., 2002; Otto et al., 2005; Fliegauf et al., 2006; Arts et al., 2007; Attanasio et al., 2007; Delous et al., 2007; Otto et al., 2008; Sohara et al., 2008; Shiba et al., 2009]. Nphp1 and Nphp4 are not localized in the Inv compartment, although a previous report showed that Inv is co-immunoprecipitated with Nphp1 [Otto et al., 2003]. Nphp1 and Nphp4 are localized in the transition zone or ciliary necklace region. Nphp1 and Nphp4 localization were not changed in *Inv* mutant cells. Our results suggest that the primary cilia are not the site for interaction between Nphp1 and Inv. Inv may interact with Nphp1 and Nphp4 when Inv is transported into the primary cilia or other places. To date, nine NPHP genes have been identified. Our results suggest that Nphps form at least two groups: one is Inv, Nphp3 and

Nek8, the other is Nphp1 and Nphp4 (Fig. 4). Interaction of these groups may make Nphp signaling pathway for maintaining renal tubular architecture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work reported here was supported by Grants-in-Aid for Young Scientists from the Ministry of Education, Science, Sports, and Culture (21790187) to D.S. This study was also supported by a grant from the Genome Network Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan to H.K.

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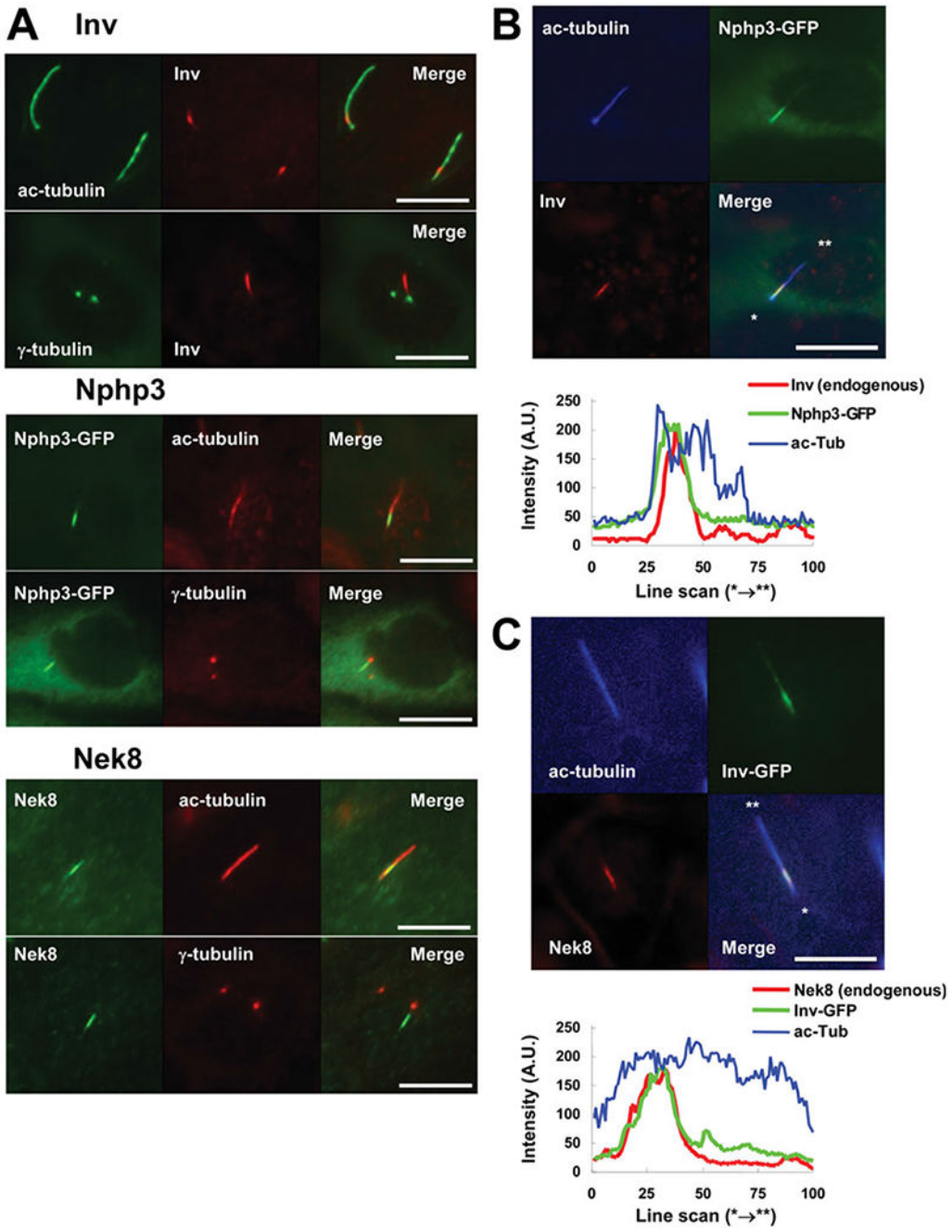

Fig. 1. Inv, Nphp3, and Nek8 localization in the Inv compartment.

A: Localization of Inv, Nphp3, and Nek8 in wild-type mouse renal epithelial cells (Dai1 cells). Inv and Nek8 are detected with the anti-Inv antibody and the anti-Nek8 antibody, respectively. Localization of Nphp3 is detected by transfection with GFP-tagged full-length construct of *Nphp3*. The Inv signal (green) is detected at the base of the primary cilium (ac-tubulin; red), but not in the basal body area (γ-Tubulin; red), that is Inv compartment (a top panel). Nphp3 (a middle panel; green) and Nek8 (a bottom panel; green) are also detected at the base of primary cilia. Scale bars = 10 μm. **B**: Nphp3 localizes to the Inv

compartment. Nphp3 signal (green) is completely overlapped with Inv signal (red). Primary cilia are stained with the anti-acetylated α-Tubulin antibody (ac-tubulin; blue). Scale bar = 10 μm. Line scan of the fluorescent signal from "*" to "**" is also shown (bottom). A.U., arbitrary units. **C**: Nek8 localizes to the Inv compartment. Nek8 signal (red) is completely overlapped with Inv signal (GFP-tagged full-length *Inv*). Primary cilia are stained with the anti-acetylated α-Tubulin antibody (ac-tubulin; blue). Scale bar = 10 μm. Line scan of the fluorescent signal from "*" to "**" is also shown (bottom). A.U., arbitrary units.

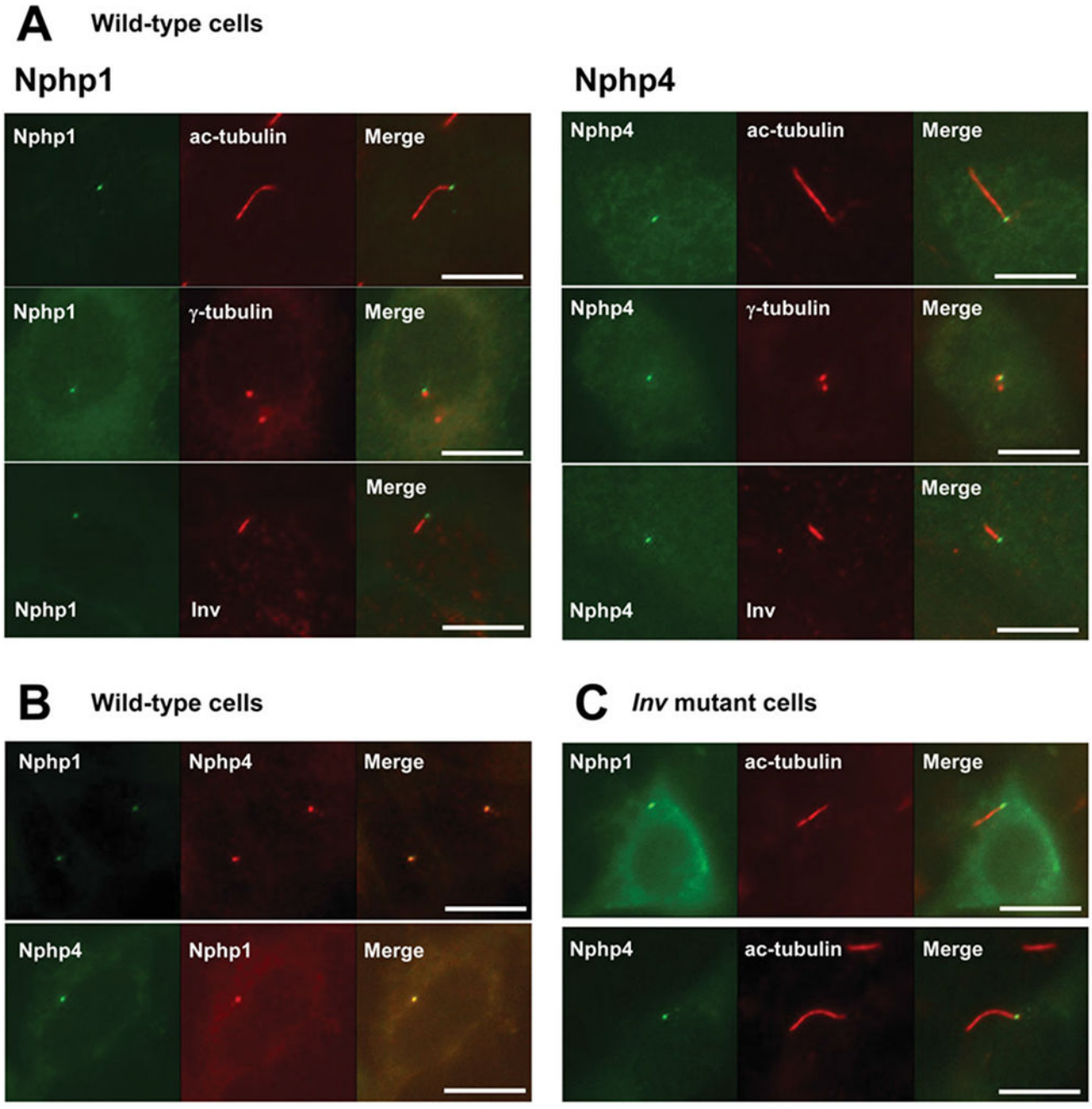

Fig. 2. Localization of Nphp1 and Nphp4.

A: Localization of Nphp1 and Nphp4 are different from Inv compartment. Localization of Nphp1 and Nphp4 are observed using wild-type mouse renal epithelial cells (Dai1 cells). Cells were transfected with GFP-tagged full-length constructs of *Nphp1* or *Nphp4*. Both Nphp1 and Nphp4 signals are detected at around the transition zone of primary cilia (ac-tubulin; red), and not overlapped with the basal body (γ-Tubulin; red). Localization of Nphp1 and Nphp4 (green) are different from Inv compartment (Inv; red) (bottom panels). Scale bars = 10 μm. **B:** Co-localization of Nphp1 and Nphp4 in wild-type mouse renal

epithelial cells (Dai1 cells). Cells are transfected with GFP (green) or mKO2 (red)-tagged full-length constructs of *Nphp1* or *Nphp4*. Both Nphp1 and Nphp4 signals overlap each other. Scale bars = 10 μm. **C**: Localization of Nphp1 and Nphp4 are not changed in *Inv* mutant cells. Localization of Nphp1 and Nphp4 are observed using *Inv* mutant cells (Dai2 cells). Cells were transfected with GFP-tagged full-length constructs of *Nphp1* and *Nphp4*. Both Nphps are detected at the base of primary cilia (ac-tubulin; red), and these signals are consistent with the results obtained from wild type cells (Dai1 cells). Scale bars = 10 μm.

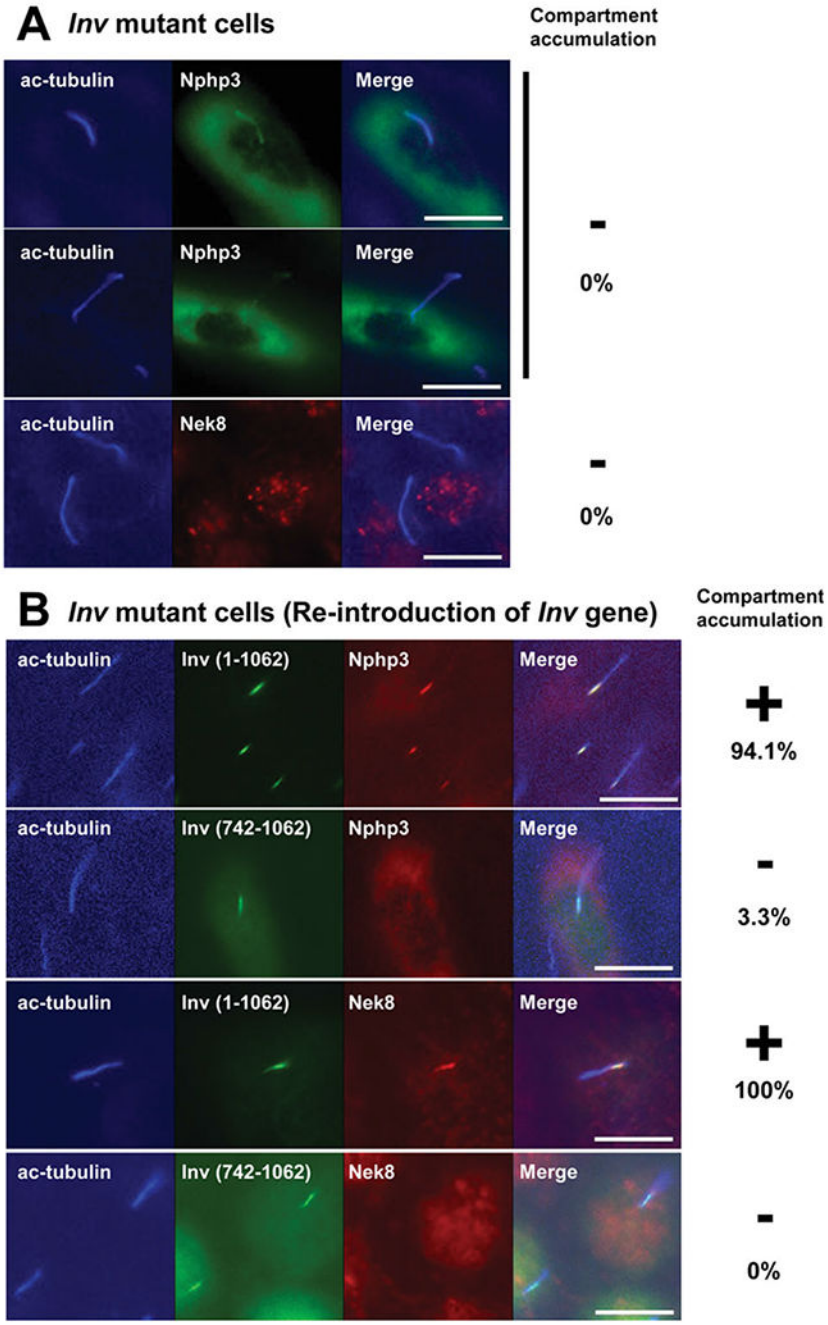

Fig. 3. Nphp3 and Nek8 localization to the Inv compartment are dependent on Inv.

A: Localization of Nphp3 and Nek8 in *Inv* mutant cells (Dai2 cells) are different from those in wild-type cells (Dai1 cells). Nphp3-GFP transfected cells are identified with cytoplasmic GFP signal. Faint and patchy ciliary localization of Nphp3 is observed along entire length of primary cilia in *Inv* mutant cells (a top panel), and some cells have extremely faint or no detectable Nphp3 signals in the primary cilia (a middle panel). Nek8 localization to the primary cilia is not detected in *Inv* mutant cells (a bottom panel). Primary cilia are stained with the anti-acetylated α-Tubulin antibody (ac-tubulin; blue). Results of the localization of

Nphp3 and Nek8 to the Inv compartment are summarized on the right (−, absent). Scale bars = 10 μm. **B:** Inv regulates localization of Nphp3 and Nek8 to the Inv compartment. Introduction of a full-length *Inv* construct, Inv (1–1062), into *Inv* mutant cells recovers Nphp3 and Nek8 localization to the Inv compartment. Localization of Nphp3 is detected by co-transfection with mKO2-tagged full-length construct of *Nphp3*. Nek8 is detected with the anti-Nek8 antibody. A C-terminal *Inv* construct, Inv (742–1062), localizes to the *Inv* compartment, but it does not rescue Nphp3 and Nek8 localization. Results of the localization of Nphp3 and Nek8 to the Inv compartment are summarized on the right (−, absent; +, present). Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

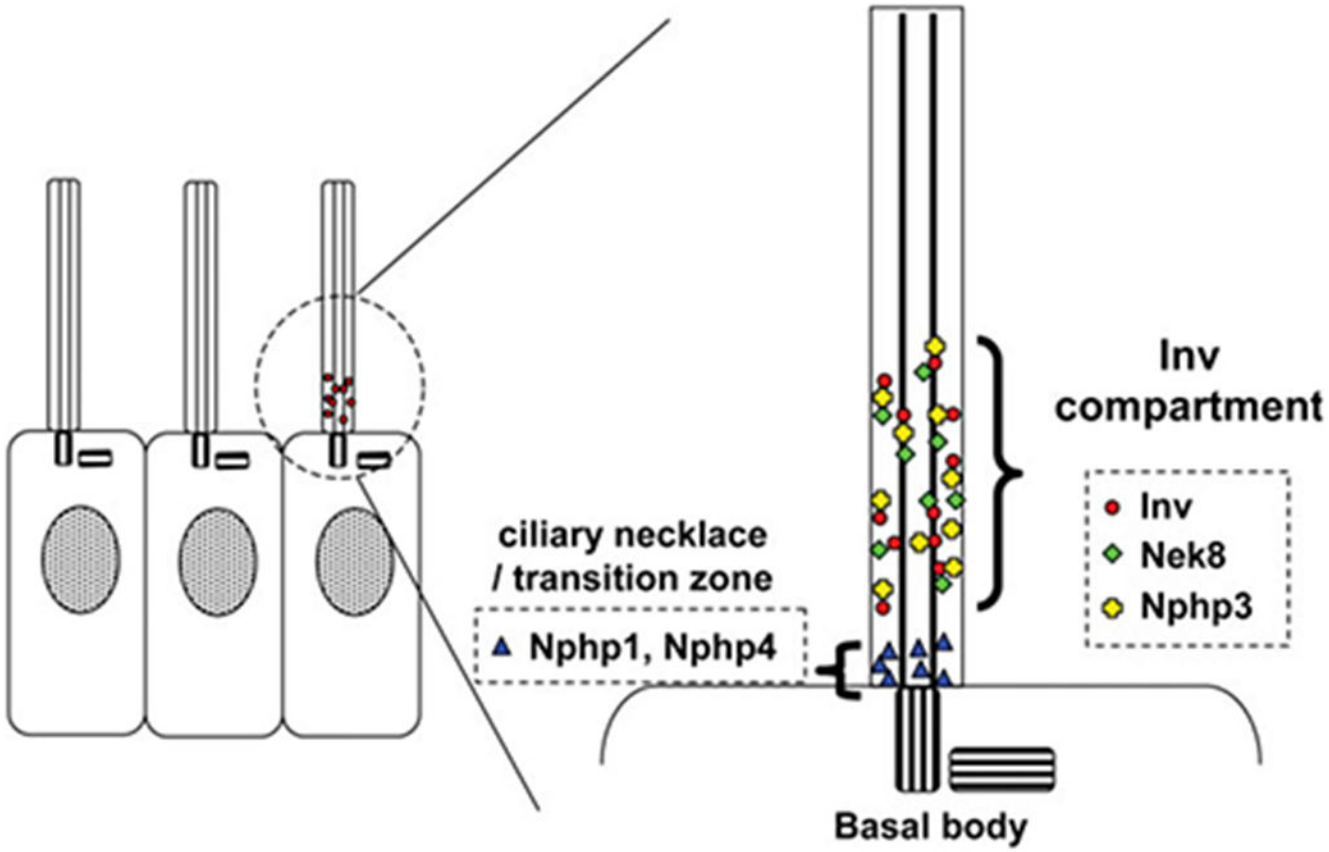

Fig. 4. Classification of Nphps according to Inv compartment localization.

A schematic representation of Inv compartment and Nphps in the primary cilium. Inv, Nphp3, and Nek8 localize at the proximal segment of the primary cilia termed the “Inv compartment”. Inv compartment is a candidate site of intraciliary interaction of these molecules. Nphp1 and Nphp4 are not detected in the Inv compartment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]