Intraflagellar Transport Proteins Are Essential for Cilia Formation and for Planar Cell Polarity

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ABSTRACT

The highly conserved intraflagellar transport (IFT) proteins are essential for cilia formation in multiple organisms, but surprisingly, cilia form in multiple zebrafish *ift* mutants. Here, we detected maternal deposition of *ift* gene products in zebrafish and found that ciliary assembly occurs only during early developmental stages, supporting the idea that maternal contribution of *ift* gene products masks the function of IFT proteins during initial development. In addition, the basal bodies in multiciliated cells of the pronephric duct in *ift* mutants were disorganized, with a pattern suggestive of defective planar cell polarity (PCP). Depletion of *pk1*, a core PCP component, similarly led to kidney cyst formation and basal body disorganization. Furthermore, we found that multiple *ift* genes genetically interact with *pk1*. Taken together, these data suggest that IFT proteins play a conserved role in cilia formation and planar cell polarity in zebrafish.

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although cilia destined to form early in development show only maintenance defects in ift57hi3417 and ift172hi2211 mutants, cilia programmed to assemble later in development fail to form, providing further support for a conserved role of IFT in cilia formation in zebrafish.

One of the most extensively studied phenotypes associated with ciliary defects is the formation of kidney cysts. Both the canonical and the noncanonical Wnt pathway, or planar cell polarity (PCP) pathway, have been implicated in kidney cyst formation.4,16–21 However, in contrast to the well-established role of cilia in the hedgehog pathway,23,24 the role of cilia in the Wnt pathways is unclear.4,15,23,24 In this study, we demonstrate that, in the kidney duct of embryos at the 16- to 64-cell stage; 20s, samples from embryos at the 20-somite stage.

RESULTS

hi3417 and hi2211 are Zygotic Mutants of ift57 and ift172, Respectively

ift57hi3417 and ift172hi2211 were isolated from an insertional mutagenesis screen in zebrafish.13 Both mutants show similar morphologic phenotypes, including a ventrally curved body axis, kidney cyst formation in the glomerular-tubular region, and dilated kidney ducts (Figure 1, A and B; Supplemental Figure S1; and reference 13). In both mutants, the responsible proviral insertion is located in the 5′ side of the corresponding genes. Reverse transcription-polymerase chain reaction (RT-PCR) with primers spanning the proviral insertion site revealed that the wild-type transcripts are completely absent in each mutant (Figure 1, C and D).

To further verify that inactivation of ift57 and ift172 are responsible for phenotypes seen in hi3417 and hi2211, respectively, we injected mRNA of each gene into embryos from heterozygous carriers of corresponding mutations. Results showed that ift172 mRNA can reduce the frequency of phenotypic embryo from 26 ± 0 to 6 ± 3% (P = 0.01, results from two independent experiments). Similarly, injection of ift57-GFP (GFP, green fluorescent protein) mRNA reduces the percentage of phenotypic embryos from 25 ± 3 to 2 ± 2% (P < 0.001, n = 3). These findings indicate that these two mutants can be rescued by overexpression of their corresponding gene.

In zebrafish, zygotic transcription commences at around the 512-cell stage.25 However, transcripts of a number of cilia-associated genes are maternally deposited and genes can be detected before this stage. To investigate for maternal contribution, we performed RT-PCR on samples from wild-type embryos at multiple developmental stages. The presence of ift57 and ift172 mRNA from embryos at the 4- to 32-cell stage verified that these mRNAs are maternally deposited (Figure 1E).

We further investigated whether IFT proteins can be detected before the maternal-zygotic transition by Western blot analysis. Results show that Ift57 can be readily detected at the 16- to 64-cell stage (Figure 1F). Any Ift57 protein present at this stage could be maternally deposited, translated from maternal mRNA, or both. Regardless of the source, the presence of an IFT protein at such an early stage suggests that it would be difficult to deplete IFT proteins completely with morpholino oligos, including translation-blocking morpholino oligos. To test this notion more directly, we used a mor-

Figure 1. hi3417 and hi2211 are zygotic loss-of-function mutants of ift57 and ift172, respectively. (A and B) A wild-type sibling and a mutant at 4 dpf in side views. Box with dashed line: cyst (in mutant) and the lack of cyst (in wild-type sibling) enlarged in insets. (C and D) Loss of wild-type ift172 and ift57 transcripts in hi2211 and hi3417 mutants at 33 hpf, respectively. Upper panels: RT-PCR with a pair of primers spanning the proviral insertion site. Lower panels: loading controls with a pair of elf1-specific primers using cDNAs serially diluted 1:1, 1:10, 1:100, and 1:1000. (E) RT-PCR time course for ift172 and ift57 using wild-type samples. (F) Western blot against Ift57 (Ift57). β-Tub is used as loading control. β-Tub, β-Tubulin; ctrl, uninjected embryo control; ift57MO, ift57 morphants; wt, wild type; 4-32c, 4- to 32-cell stage; 30%–27h, mixture of samples from 30% epiboly to 27 hpf; 36h, 36 hpf; 50h, 50 hpf; 5d, 5dpf; 16–64 cell, sample from embryos at the 16- to 64-cell stage; 20s, samples from embryos at the 20-somite stage.
pholino oligo against the translation initiation site of ift57. Indeed, a significant quantity of Ift57 protein can still be detected in ift57 morphants, even when they are all phenotypic (Figure 1F).

Cilia Formation Is Affected in ift57 and ift172 Mutants in a Time-Dependent Manner

There are conflicting reports on the status of cilia formation in zebrafish ift mutants. Because cilia formation starts at different time points in different organs, the differences observed may be attributed to the gradual decay of maternally deposited gene products. Early in development, cilia are able to form using maternally contributed gene products, whereas later in development, with the decay of maternal contribution, cilia are unable to form. To test this hypothesis, we followed cilia formation in multiple organs in detail.

Cilia in the neural tube can be detected at 24 hpf (hours post-fertilization) and persist thereafter. We examined cilia formation at 24 hpf in at least 30 embryos from heterozygous carriers for each experiment. No significant difference was seen in any of the embryos, suggesting that cilia are able to form in the neural tube (data not shown). By contrast, at 34 hpf, a stage at which mutants can be reliably identified by their body curvature phenotype, cilia number in both mutants are drastically reduced (Figure 2, A through B'). Interestingly the reduction of cilia number in ift172hi2211 mutants is more pronounced than that in ift57hi3417 mutants (Figure 2, A through B’). Variations in mRNA or protein stability may account for this difference.

The zebrafish pronephric duct contains both single-ciliated cells (SCCs) and multiciliated cells (MCCs). Initially, at 24 hpf, most cells in the pronephric duct display a single cilium. By 2 dpf (days post-fertilization), MCCs can be found in the mid region of the duct, whereas SCCs can be found in both the mid and posterior region of the duct. In both ift57hi3417 and ift172hi2211 mutants, there is no detectable difference in cilia in the pronephric duct at 24 hpf (data not shown). At 2 dpf, cilia in the posterior duct still appear relatively unaffected (Figure 2, C through D’). By contrast, severe ciliary defects can be readily observed in the mid region of the duct (Figure 2, E through F’). A simple explanation is that IFT is specifically involved in building cilia in MCCs, but not in SCCs. Alternatively, this difference may be attributed to the decay of maternally contributed ift gene products between the time of cilia formation in SCCs and MCCs.

To further clarify this picture, we investigated cilia formation in the lateral line organ, where cilia cannot be detected until 2 dpf. In both mutants, cilia formation in the lateral organ is severely affected (Figure 2, G through H’).

We additionally observed disorganized basal bodies in MCCs of the pronephric duct. In wild-type embryos, basal bodies in a MCC are organized into a tight array at the apical side, as shown by $\alpha/H9253$-Tubulin staining (Figure 2, I, J, K, and L). In both ift57hi3417 and ift172hi2211 mutants, basal bodies appeared as disorganized clusters, no longer tightly arrayed (Figure 2, I’, J’, K’, and L’). We further investigated whether the apical localization of the basal body is affected in these mutants by labeling with the apical
marker anti-atypical PKC. Results showed that basal bodies in MCCs are still localized to the apical side of the pronephric duct in both mutants, as evident by clusters in side views at the edge of the lumen (arrows in Supplemental Figure S2).

**Knockdown of pk1 Leads to Disorganization of Basal Bodies in the Kidney Duct and Kidney Cyst Formation**

The PCP pathway has been implicated in kidney cyst formation. In addition, the disorganization of basal bodies in *ift* mutants is suggestive of a PCP defect. We therefore tested whether PCP defects can lead to cyst formation directly by knocking down the expression of a core PCP component, *pk1*, using a morpholino oligo. Injected at 2 ng, *pk1* morphant raised with 1-phenyl-2-thiourea at 3 dpf in side views. (E through H) Basal bodies as shown by anti–cyst (A). (C and D) Pronephric ducts labeled with anti-Cdh17 in side views. Insets: enlarged areas showing a cyst (B) and the lack of disorganization of basal bodies. (A and B) A control embryo and a *pk1* morphant raised with 1-phenyl-2-thiourea at 3 dpf in side views. (E through H) Basal bodies as shown by anti–Cdh17 labeling and cilia by anti-a-Tub labeling. Arrows point to arrayed basal bodies, whereas arrowheads point to clusters of disorganized basal bodies. a-Tub, acetylated Tubulin; ctrlMO, embryos injected with a standard control morpholino; γ-Tub, γ-Tubulin; pk1MO, embryos injected with *pk1* morpholino.

**ift57 and ift172 Genetically Interact with pk1**

Multiple studies provide evidence to support a critical role of the PCP pathway in kidney cyst formation. However, reports on the role of cilia in the PCP pathway are conflicting. We therefore examined for potential genetic interactions between *ift57, ift172, and pk1*. We injected suboptimal doses of the *ift172* morpholino (3.5 ng) and *pk1* morpholino (0.6 ng), either together or with a control morpholino (0.6 and 3.5 ng, respectively). Results showed that the shortened body axis phenotype was found only in embryos that were co-injected with *ift172 and pk1* morpholinos (Figure 4, A through B). Similarly, although a suboptimal dose of *ift57* (0.35 ng) morpholino did not cause shortened body axis, this morpholino injected in combination with the *pk1* morpholino did (Supplemental Figure S3). Interestingly, maternal-zygotic mutants of *ift88* lack a PCP phenotype during gastrulation, raising the possibility that there are functional differences between *ift88* and other *ift* genes. We therefore investigated whether *ift88* genetically interacts with *pk1*. Results showed that a suboptimal dose (1.2 ng) of a previously published *ift88* morpholino synergized with the *pk1* morpholino in inducing the shortened body axis phenotype (Supplemental Figure S3), indicating that *ift88* functions similarly to other *ift* genes in this assay.

To provide more direct support for our hypothesis that *ift* genes genetically interact with *pk1* during CE movement in gastrulation, we analyzed the morphology of paraxial cells in late gastrulae by mosaically expressing a membrane-targeted enhanced GFP (eGFP). As reported previously for wild-type embryos, control embryos injected with a suboptimal dose of either *ift172* morpholino or *pk1* morpholino together with a control morpholino displayed paraxial cells that were elongated along the medial-lateral axis, with a length-to-width ratio (LWR) of 2.6 ± 0.8 and 2.5 ± 0.6, respectively. In contrast, embryos co-injected with suboptimal doses of *ift172 and pk1* morpholinos exhibited a LWR that was significantly reduced to 2.0 ± 0.9 (Figure 4, C through D). Similarly, co-injection of suboptimal doses of *ift57* and *pk1* morpholino reduced the LWR to 1.7 ± 0.5 from 2.6 ± 0.8 and 2.2 ± 0.6 when compared with control embryos injected with *ift57 or pk1* together with a control morpholino, respectively (Figure 4, E through F).

Further support for IFT’s involvement in the PCP pathway comes from a dominant negative construct of *ift172*. Loss-of-function mutants of *ift172* have been identified in *Chlamydo-
ous body axis shortening defect. In a representative experiment, we injected 150 pg of GFP-N1550 is a dominant negative or dominant active form of Ift172, we co-injected 150 pg of GFP-N1550 mRNA together with 2 ng of ift172 morpholino, which by itself does not cause an obvious body axis shortening defect. In a representative experiment, the inclusion of ift172 morpholino increased the percentage of embryos with shortened body axis from 18% to 36%, indicating that GFP-N1550 is a dominant negative form of Ift172.

**DISCUSSION**

The Role of ift Genes in Cilia Assembly in Zebrafish

In both ift57hi3417 and ift172hi2211 mutants, at 1 dpf, cilia are able to form in the neural tube and the pronephric duct and are indistinguishable from cilia in wild-type embryos. A simple interpretation is that ift genes are not required for cilia assembly in zebrafish. As ift genes are highly conserved for ciliary assembly in a wide spectrum of organisms including *Chlamydomonas* and mice, we considered alternative explanations for this difference. In zebrafish, products of many genes are deposited maternally. Therefore, a homozygous null mutant can start its life with a significant quantity of functional products of the mutated gene provided from the mother, masking the function of that mutated gene during early development. Accordingly, we found transcripts of both ift57 and ift172 are supplied maternally. We also detected the presence of Ift57 protein as early as the 16- to 64-cell stage. Interestingly, although maternal ift57 transcripts decay by 33 hpf in mutants (Figure 1D), previous studies indicate that Ift57 protein can be detected at 48 hpf, but not at 4 dpf, in ift57hi3417 mutants. Together, these results suggest that maternally supplied Ift57 protein or transcript can persist for an extensive period of time. Consistent with the gradual degradation of maternal gene products, cilia assembly is initially present on the first day of development in ift57hi3417 and ift172hi2211 mutants, but is lost at later stages of development. Furthermore, although zygotic ift88oval mutants are initially able to form cilia, maternal-zygotic ift88oval mutants are not capable of cilia assembly. Together, these findings suggest that the role for ift genes in cilia assembly is conserved in zebrafish.

The PCP Pathway and Kidney Cyst Formation

PCP refers to the coordinated polarity within a sheet of epithelial cells. It is known that the proper establishment of PCP is regulated by the noncanonical Wnt pathway (for a review, see reference 35). Strikingly, recent studies indicate that the PCP pathway is critical for preventing cyst formation, which is the hallmark of polycystic kidney disease (PKD). Multiple PKD mouse...
mutants show misorientation of cell division axis during kidney development before visible kidney cyst formation. In addition, conditional inactivation of Four-jointed, a gene known to be involved in PCP, leads to kidney cyst formation in mouse. In this study, we demonstrate that inactivation of pk1 can directly lead to kidney cyst formation, providing further support for the involvement of the PCP pathway in preventing kidney cyst formation.

The Role of Cilia in the PCP Pathway

Multiple lines of evidence support an intricate relationship between the cilium and the PCP pathway. First, a number of known PCP pathway components, including Dishevelled, Inturned, and Fuzzy, are required for proper ciliogenesis during Xenopus development. Second, multiple genes have been shown to be involved in both cilia-mediated processes and the PCP pathway. Most directly, inactivation of ift88 in mice leads to CE movement defects during cochlear duct development, a phenotype associated with PCP abnormalities. Furthermore, conditional knockout of ift20 in the mouse kidney leads to misorientation of cell division axis. The critical role of the cilium in PKD pathogenesis, and the close association between PKD and the PCP pathway, is also consistent with a role of cilia in the PCP pathway.

In this study, we show that both ift57 and ift172 mutants display disorganized basal bodies in the kidney duct, similar to that seen in pk1 morphants. We also demonstrate that ift57, ift88, and ift172 genetically interact with pk1 in body axis elongation and the alignment of paraxial cells in later gastrulae. Additionally, we show that overexpression of a dominant negative form of ift172 disrupts the convergence of somites and the hindbrain during early somitogenesis. Together, these data support a functional involvement of IFT genes in the PCP pathway. However, a previous report has indicated that a maternal-zygotic zebrafish mutant of ift88oval showed no obvious defects in convergence extension during gastrulation. On the basis of these results, we propose that during zebrafish gastrulation IFT genes play a minor or redundant role that becomes apparent only when the PCP pathway is already comprised. Additionally, it remains possible that subtle PCP phenotypes exist in maternal-zygotic mutants of ift genes but are not easily detectable. Intriguingly, the PCP phenotypes observed in the mouse cochlear and kidney ducts of ift88 and ift20 mutants may suggest that there exists tissue and stage specificity in the role of IFT genes in this pathway. The genetic interaction between multiple ift genes and a core PCP component provides insight into a novel and intricate relationship between the cilium and planar cell polarity and furthers our understanding of the cilium as a key signaling center for vertebrate cells.

Cloning

Full-length coding sequences of ift57 and ift172 were amplified from a zebrafish cDNA pool and cloned into pCS2 vector. GFP tags were added by PCR cloning. ift172N1550 was generated by PCR cloning.

Microinjection

mMESSAGE mMACHINE kit (Ambion) was used to synthesize capped mRNA. Morpholino oligos were purchased from Genetools. mRNA or morpholino were injected into embryos at the 1-cell stage at indicated concentrations. 5'-CATCCCTCCTCTTTCTTCTCAGTTAC-3' was used to block the translation of ift57 and 5'-GACTCAGGCGAGTTATAAGAAGTGA-3' was used to block the translation of ift172. Previously described morpholino oligos were used to knock down the expression of pk1 (5'-GCCACCGTTATCTCAGCTCCAT-3' and the expression of ift88 (5'-CTGGGACAAATGTCGACATTCTCCAT-3'). A standard oligo (5'-CCTCTTACCTCAGTTACATTATA-3') was injected as a control.

Histologic Analysis

Embryos were fixed in Bouin’s fixative overnight at room temperature, washed three times with PBS, embedded in JB-4 resin from Poly-science, and cut at 4 μm. Slides were then stained with hematoxylin & eosin.

Protein Extraction and Western Blotting

Embryos were manually deyolked following a previously published protocol and then ground in 2X SDS sample buffer (4% SDS, 200 mM dithiothreitol, and 5% β-mercaptoethanol) with a disposable pestle, boiled for 5 minutes, cleared by microcentrifugation at top speed at room temperature for 2 minutes, and run on SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes. Filters were then blotted in PBS buffer with 5% instant milk, incubated sequentially with anti-Hif57 (kindly provided by B. Perkins) at 1:2500 and horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) at 1:5000 to 1:10,000. Signals were then detected with enhanced chemiluminescence detection using the Western Lightning kit from PerkinElmer Life Sciences.

Immunostaining

Immunostaining on fixed embryos was performed as described. Specifically, embryos were fixed in Dent’s fixative. A monoclonal anti-α-tubulin antibody (Sigma) was used at 1:5000. A monoclonal anti-γ-Tubulin antibody (Sigma) was used at 1:200. A rabbit anti-aPKC (Santa Cruz Biotechnology) was used at 1:200. A custom antibody made by Covance against the C-terminal region from amino acid 238 of Arl13b was used at 1:2000. Fluorescence-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1:200. Stained embryos were manually deyolked and flattened in Vectashield Hardset mounting medium (Vector Laboratories). Images were taken using a Nikon Eclipse epifluorescence microscope and a Leica stereoscope. Global contrast and intensity of fluorescence images were adjusted using the Elements software. Images were further cropped in Photoshop.

CONCISE METHODS

Fish Husbandry

Zebrafish were maintained according to standard protocols. All lines were from the Hopkins laboratory and maintained in TAB background.

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RT-PCR
RNA was extracted with trizol reagent (Invitrogen) following manufacturer’s instructions. First strand cDNA was generated with the superscript II RT-PCR system (Invitrogen) and subsequently amplified with PCR. For ift57, primers 5’-AGGATCTGGATGAGGAGGAG-GAAGA-3’ and 5’-GGAATTCGTTTCAATAAGCCTGCGCA-3’ were used. For ift172, primers 5’-AAAACTGAAGTATGCGACCTTAAG-3’ and 5’-GGTCAAGCGCTTGGTTTGA-3’ were used. For elf1, primers 5’-CTTCTAGGCATGACTGTC-3’ and 5’-CGCTAGCATTACCCTCC-3’ were used.

Analysis of Cell Polarity in Late Gastrulae
A previously published protocol, with minor modifications, was followed.41 mRNA encoding a membrane-anchored GFP was injected into one cell of embryos at the 16- to 32-cell stage, which leads to mosaic labeling of cells at later stages. Embryos at the 2- to 5-somite stage were imaged with a Nikon Eclipse epifluorescence microscope and analyzed using the Element software.

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DISCLOSURES
None.

REFERENCES


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