The roles of intraflagellar transport (IFT) protein 25 in mammalian signaling transduction and flagellogenesis

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Cilium, an organelle with a unique proteome and organization, protruding from the cell surface, generally serves as a force generator and signaling compartment. During ciliogenesis, ciliary proteins are synthesized in cytoplasm and transported into cilia by intraflagellar transport (IFT) particles, where the inner counterparts undergo reverse trafficking. The homeostasis of IFT plays a key role in cilial structure assembly and signaling transduction. Much progress has been made on the mechanisms and functions of IFT; however, recent studies have revealed the involvement of IFT particle subunits in organogenesis and spermatogenesis. In this review, we discuss new concepts concerning the molecular functions of IFT protein IFT25 and how its interactions with other IFT particle subunits are involved in mammalian development and fertility.

Asian Journal of Andrology (2022) 24, 238–242; doi: 10.4103/aja202179; published online: 02 November 2021

Keywords: cilium; hedgehog signaling; IFT25; intraflagellar transport; spermatogenesis

INTRODUCTION

Intracellular transport is driven by molecular motors that move along cytoskeletal tracks, which is responsible for delivering organelles, vesicles, and macromolecular complexes.1 Although specific motors of individual cargos are crucial for executing correct and robust cargo deliveries, generally, they work in concert with distinct protein linkers resulting in multi-subunit complexes.2

Intraflagellar transport (IFT) is a well-studied bidirectional transport of a multi-subunit complex (often referred to as IFT particles or trains) along microtubule-based axoneme between the axoneme and the ciliary membrane.3 This process was initially observed in flagella of green alga Chlamydomonas reinhardtii (C. reinhardtii)4 and since then, it has been shown to be conserved in multiple species, including mammals. IFT plays important roles in the assembly and function of cilia by contributing to several essential processes in motility, sensory reception, and signaling. Defects in IFT and ciliogenesis have been linked to multisystem human disorders such as Bardet–Biedl syndrome (BBS), Joubert syndrome (JBTs), nephronophthisis (NPHP), Meckel syndrome (MKS), and short-rib thoracic dysplasia (SRTD). These disorders are generally referred to as the ciliopathies (Figure 1).3

IFT particles were first purified from the flagellar extracts of C. reinhardtii. These particles were dissociated into two distinct subunit complexes, namely IFT-A and IFT-B, under high ionic strength conditions. A much more complex picture of IFT subunits has been produced through many years of biochemical characterization in ciliate organisms, including mammals.

The IFT-A complex comprises six components (namely IFT43, IFT121, IFT122, IFT139, IFT140, and IFT144) plus the adaptor protein TUB-like protein 3 (TULP3). In a predicted IFT-A model using the visible immunoprecipitation assay, IFT122, IFT140, and IFT144 form the core subcomplex, while the IFT43-IFT121 dimer interacts with IFT139 to form a noncore subcomplex.4 The core and noncore subcomplexes are connected by IFT121. In the mammalian IFT-A complex, TULP3, a member of the Tubby family proteins, is associated with the core subcomplex and probably connects membrane proteins to the IFT-A complex.5 This macromolecular complex is believed to mediate dynein-based delivery of IFT particles from the ciliary tip to the basal body (retrograde trafficking). It is also thought to be involved in the import of membrane proteins across the ciliary gate.5,6 This diffusion and permeability barrier contains transition fibers and the transition zone.

The IFT-B complex is divided into two subcomplexes: the core subcomplex and the peripheral subcomplex. The core subcomplex can be further divided into two parts: the core-1 (B1-1) subgroup composed of five subunits (IFT22, IFT25, IFT27, IFT74, and IFT81) and the core-2 (B1-2) subgroup composed of another five subunits (IFT46, IFT52, IFT56, IFT70, and IFT88). The peripheral subcomplex is composed of six subunits (IFT20, IFT38, IFT54, IFT57, IFT80, and IFT172). Several subunits (i.e., IFT20, IFT38, IFT46, IFT80, and IFT88) were shown to play a crucial role in IFT-B assembly and thereby in ciliary protein trafficking, whereas other subunits (IFT22, IFT25, IFT27, and IFT56) located peripherally in the IFT-B architecture were shown to be dispensable for IFT-B assembly at least in mammalian cells.7–11 IFT-B complex plays an essential role in kinesin-based trafficking of IFT particles from the basal body to the ciliary tip (anterograde trafficking: Table 1).

In addition to the IFT-A and IFT-B complexes, the BBSome, a macromolecular complex, plays an essential role in IFT by contributing...
to ciliary development and signaling.\textsuperscript{16,17} This involves membrane protein trafficking, IFT particle integrity maintenance, and regulation of extracellular signaling vesicle shedding.\textsuperscript{18}

In this review, we discuss the canonical functions of the IFT complex and introduce the novel functions of IFT25 in the mouse model. We outline how IFT25 interacts with other IFT subunits (IFT27 and IFT20). We focus on two main topics: recently emerged molecular functions of IFT25 in hedgehog signaling and the main roles of IFT25 during sperm flagellogenesis.

**IFT25**

Human ortholog of IFT25, Clorf41, was first purified from saline extracts of human term placentas and characterized as a member of small heat shock protein (sHSP), accordingly named as Hspb11, Hsp16.2, and HSPC034.\textsuperscript{19} However, this characterization was not supported by structural chemistry analysis due to lack of canonical HSP feathers in HSPC034, including low percentage of consequence similarity, absence of an alpha-crystallin domain, distinct topology, and aggregation activity, which provide an argument concerning the main biological function of HSPC034.\textsuperscript{20} The key clue to this issue came from systematic mapping of human protein interactome networks. In the yeast two-hybrid system, HSPC034 was found to interact with a known IFT subunit, IFT27,\textsuperscript{21} HSPC034 orthologue (FAP232) also appeared in flagellar proteome of *C. reinhardtii*.\textsuperscript{22} Both studies prompted the characterization of the HPS1.6 orthologue in mammal model organisms. In kidney epithelial cells (IMCD3), green fluorescent protein (GFP)-fused FAP232 was distributed at the ciliary basal body and along axoneme as punctual dots, which is a typical distribution pattern of IFT complex.\textsuperscript{23} In addition, two independent studies concluded that FAP232 orthologue in *C. reinhardtii* was part of IFT-B complex and named it IFT25 after its real molecular weight in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).\textsuperscript{24,25} Most recently, several high-resolution approaches delineated the peripheral location of IFT25 in the IFT-B model.\textsuperscript{6}

The process of characterization established a unique association between IFT25 and another IFT-B1 core subunit, IFT27. This protein serves as a Rab-like small GTPase, the activity of which is modulated upon nucleotide-binding state.\textsuperscript{26} As being lack of typical prenylation motif of Rab proteins, IFT27 is not allowed for membrane association upon GTP or GDP binding. This structural characteristic defines a distinct cellular distribution pattern compared with canonical Rab proteins Rab8 and Rab11, despite high structural similarity. Immunofluorescent microscopy assay showed that IFT27 is located at the ciliary basal body and translocation zone, significantly overlapped with IFT25. In addition, in mammal models, IFT27 was immunoprecipitated by IFT25 antibody in the reciprocal pull-down.\textsuperscript{27} The most compelling evidence of tight association between these two IFT-B subunits came from X-ray crystallographic analyses, in which IFT25/27 heterodimer was found to interact through a conserved interface.\textsuperscript{28} Notable, IFT25 in *C. reinhardtii* was found to stabilize IFT27 by preventing its aggregation, but this requirement for stability is not mutual, as the abundance of IFT25 is not affected by the loss of IFT27.\textsuperscript{11} These data have shown physical and functional interaction between IFT25 and IFT27. Given the specific module of heterodimer, it is not surprised to find that both specific *Ift25* and *Ift27* mutant mice manifest highly similar phenotypes.

**IFT25 AND HEDGEHOG SIGNALING**

The phylogenetic distributions of IFT25 among a variety of model organisms suggest a biological function besides in ciliogenesis. Its gene orthologs are present in ciliate species genome including *C. reinhardtii* and various vertebrates, but not in *Drosophila melanogaster* (*D. melanogaster*) and *Caenorhabditis elegans* (*C. elegans*). These flies and worms (invertebrates) assemble normal cilia in the absence of IFT25. However, this subunit of the IFT complex has been now thought to be involved in hedgehog signaling of mammals. *Ift25*-deficient mice died shortly after birth, accompanied by a series of phenotypes due to hedgehog signaling defects. These include polydactyly, cleft palate, lung isomerism, and structural heart defects, which cannot completely be explained by ciliary structural defects. Photochemistry analysis of embryonic fibroblast (MEF) from *Ift25* mutant mice revealed that Gli2, the transcription factor of the hedgehog pathway, was not trafficked to the ciliary tip in response to pathway activation. Moreover, other hedgehog components, Patched-1 (Pch1) and Smoothened (Smo), were also found trapped in cilia, thus keeping the pathway in an attenuated state.\textsuperscript{29} These data revealed the involvement of IFT25 in coupling hedgehog components to IFT particles, which suggested a separable role of IFT25 in signal transduction upon stimulation. Recently, this suggestion was supported by duplex kidney conformation of *Ift25* and *Ift27* mutant mice, which phenocopy urogenital system phenotypes caused by a Gli3 mutation in murine.\textsuperscript{30}

The molecular interaction of hedgehog components and the IFT25/27 heterodimer remain poorly understood. However, these may involve coupling of the BBsome to IFT-B complex. The BBsome has been proven to associate with trafficking of specific receptors, including Pach1 and Smo, into cilia and exporting membrane proteins from cilia.\textsuperscript{15} This macromolecular complex undergoes dynamic transports between cell body and cilia, which is regulated by BBS-binding protein/ regulator leucine zipper transcription factor like 1 (Lztfl1) and the small GTPase ADP ribosylation factor like GTPase 6 (Arl6). In IFT27-deficient fibroblast cells, Smo abnormally accumulated in cilia, accompanied with ciliary BBsome and Lztfl1 aggregation. In addition, mutations in *Lztfl1* resulted in accumulation of BBsome proteins in cilia, without disturbing the IFT27 distribution. These data indicate that Lztfl1 functions the downstream of the IFT25/27 heterodimer in coupling the BBsome to the IFT particle, which regulates the removal of hedgehog

| Functions of intraflagellar transport protein 25 |
|-------------------------------------------------|
| **Table 1: Components of intraflagellar transport complexes and their activities** |
| **IFT complex** | **IFT-A** | **IFT-B** |
| **Components** | **Core subcomplex** | **Core subcomplex** |
| | IFT122 | IFT22 |
| | IFT140 | IFT25 |
| | IFT144 | IFT27 |
| **Noncore subcomplex** | **Peripheral subcomplex** |
| IFT43 | IFT20 |
| IFT121 | IFT38 |
| IFT139 | IFT54 |
| IFT74 | IFT57 |
| IFT81 | IFT80 |
| IFT8 |
| **Activities** | **Retrograde trafficking:** import of membrane protein across the ciliary gate | **Anterograde trafficking** |

IFT: intraflagellar transport
During hedgehog signaling, the hedgehog components, Ptch1 and Smo, both interact with Lztfl1 and IFT-B (i.e., IFT25/27 heterodimer). IFT: intraflagellar transport; Ptch1: Patched-1; Smo: Smoothened; Lztfl1: leucine zipper transcription factor like 1.

The redundancy of IFT25 in IFT complex assembly and ciliogenesis is well documented. As noted, genes encoding IFT25 homologs are absent from the genomes of D. melanogaster and C. elegans, both of which are ciliate organisms. RNA interference (RNAi)-mediated knockdown of IFT25 exerted no effects on IFT-B complex assembly and flagellar length in C. reinhardtii. Moreover, global Ift25 knockout (KO) mice survived to birth with normal abundance of cilia in tracheal epithelial cells, and no detectable changes were identified in ciliary conformation. However, our study showed that conditional deletion of Ift25 in mouse male germ cells resulted in reduced sperm number and multiple sperm defects, including round heads; short and bent tails, with some tails showing branched flagella and others with frequent abnormal thicknesses; as well as swollen tips of the tail. Transmission electron microscope (TEM) analysis revealed disordered axoneme and microtubule doublets accompanied by disrupted accessory structures, which indicates the crucial role of IFT25 in sperm flagellogenesis. Due to the conserved conformation and composition of axoneme across species, IFT25 may play different roles in the development of cilia/flagella in somatic cells and male germ cells. Although the mechanisms underlying this new concept of IFT25 function remain elusive, these may involve retrograde trafficking in sperm flagella. To support this concept, in Trypanosoma brucei (T. brucei), another flagellar model organism, knockdown of IFT25 using RNAi led to a phenotype similar to that in mouse male germ cells. Notably, depletion of IFT25 induced dilation of unknown molecular composition at the distal flagellar end in both mouse sperm and T. brucei, which is typical for retrograde trafficking defects. Immunofluorescence assay analysis, subsequently, revealed a disruption of IFT-B complex cycling between the cilia and the cell body. This deregulated process prevents IFT-A entry into cilia, which is essential for IFT complex integrity and retrograde IFT.

The distinct function of IFT25 in somatic ciliogenesis and sperm flagellogenesis can be highly associated with protein variants among species and cells. For example, C. reinhardtii IFT25 shares 30%–35% similarity with the human homolog. IFT complex comprises at least 22 proteins, which can result in various compositions and architectures of IFT particles. Furthermore, the general regulation of protein expression at the transcription level and posttranscriptional level produces homologous proteins associated with cell types. These molecular diversities necessitate the structural studies of IFT machinery to reconcile such discrepancies in mammals.

In addition, both IFT25/27 and BSosome are involved in sperm flagellogenesis. The phylogenetic data demonstrate the high conservation of BSosome components (BBS1, BBS4, and BBS8) in multiple species. Mice with global knockout of BBS4 showed normal primary cilia and motile cilia, which is evidenced by the intact structure of cilia in kidney epithelial cells and tracheal cells. However, the disruption of BBS4 resulted in sperm flagellogenesis failure, despite the normal sperm head and acrosome. This phenotype indicates that BSosome is involved in sperm flagellogenesis. Given that IFT25/27 complex is linked to BSosome by Lztfl1, depletion of IFT25 in spermatozoa can disrupt recruitment of cargos to the IFT complex, which will phenocopy the BSosome knockout mouse.

**IFT25 AND IFT27, DIFFERENT ROLES IN SPERM ASSEMBLE**

The popular architecture of IFT machinery is mainly based on the studies of model organisms, especially C. reinhardtii. Whether this architecture and binary protein interactions work in mammalian cells is still uncertain.
The cell type- and species-specific protein isoforms make it more complicated to study interactions between IFT subunits. One example is the functions of IFT25 and IFT27. It has been shown that the two proteins form a complex in *C. reinhardtii*. Even though similar phenotypes were reported in the germ-cell-specific Ift25 and Ift27 knockout mice, differences were discovered. Decreased testicular expression level of IFT20 was found in the germ-cell-specific mutants, but not in the Ift27 mutants. Similarly, sperm lipid raft was missing in the Ift25 mutants, but not in the Ift27 mutants. Given that IFT25 has not been proven to regulate IFT20 expression, this is more possible due to the variable stability, which is reminiscent of the fact that IFT27 is stabilized by combination with IFT25. These data raise a question whether IFT25 stabilizes IFT20 by direct combination, or an indirect approach. To further explore this, structural and biochemical studies will expand our knowledge on the molecular mechanism of IFT in mammals. IFT25 might also be involved in lipid trafficking, but not IFT27.

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