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Early defects in photoreceptor outer segment morphogenesis in zebrafish *ift57*, *ift88* and *ift172* Intraflagellar Transport mutants ☆

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ABSTRACT

Intraflagellar Transport (IFT) refers to a highly conserved process occurring in eukaryotic ciliated structures. In vertebrate photoreceptors, IFT mediates protein trafficking to the outer segments. The IFT particle is a multi-subunit complex and mutations in many individual components causes photoreceptor defects. In zebrafish, mutations in the *ift57*, *ift88*, and *ift172* genes result in retinal degeneration by 5 days post fertilization (dpf). Although the effects of these mutations on photoreceptor survival have been described, early developmental morphogenesis remains poorly understood. We used transmission electron microscopy and immunohistochemistry to examine these mutants at 60, 72, and 96 h post fertilization (hpf) and describe early photoreceptor morphogenesis defects.

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1. Introduction

Vertebrate photoreceptors have a unique morphology consisting of an inner and outer segment linked by a connecting cilium. The tip of the outer segment is shed on a daily basis and is phagocytosed by the retinal pigment epithelium (Young, 1967). To replace this lost material, the photoreceptor inner segments contain cellular organelles that continuously supply material for outer segment maintenance. The connecting cilium that bridges the inner and outer segments possesses a 9 + 0 microtubule structure (Fawcett & Porter, 1954). The connecting cilium can be classified as having three distinct regions; an upper axoneme that is continuous with the outer segment, a middle part that is surrounded by surface membrane and a lower portion that penetrates into the inner segment and attaches to the basal body, which is a centriole-like structure that anchors the connecting cilium in retinas (De Robertis, 1956).

In the initial stage of photoreceptor development, a cilium projects from a bulge of protoplasm of the inner segment (De Robertis, 1956). The apical end of the primitive cilium continues to enlarge and accumulates "morphogenetic material" that consists of short pieces of tubules and vesicles. Primitive disk membranes then replace the morphogenetic material and align transverse to the surface membrane. These morphogenetic observations show that the outer segment is formed as a result of differentiation of the distal portion of the primitive cilia. The basal part of the cilium however, remains undifferentiated and is continuous with the basal body (De Robertis, 1956; Horst, Johnson, & Besharse, 1990). The outer segment hence requires transport of ciliary components, such as membranes and microtubules, and proteins required for structure and phototransduction (Mendez, Lem, Simon, & Chen, 2003; Perkins, Fadool, & Dowling, 2004; Peterson et al., 2003).

Protein trafficking via the connecting cilium is mediated by a process known as Intraflagellar Transport (IFT). IFT was discovered in the unicellular biflagellate organism, *Chlamydomonas*, as the movement of granule like particles along the length of the flagella (Kozminski, Johnson, Forscher, & Rosenbaum, 1993). In adult photoreceptors, IFT proteins localize to the inner segment, basal body region and along the length of the outer segment axoneme (Luby-Phelps, Fogerty, Baker, Pazour, & Besharse, 2008; Pazour et al., 2002). In vertebrates, mutations in the IFT particle affect several tissues such as photoreceptors, kidney, nodal cilia, olfactory neurons and hair cells (Pazour et al., 2002, Tsujikawa & Malicki, 2004).

Mutations in three specific IFT subunits, *ift57*, *ift88*, and *ift172*, have profound effects on the retina (Gross & Perkins, 2008; Krock & Perkins, 2008; Pazour et al., 2002; Tsujikawa & Malicki, 2004). The mouse null mutants *hippi*, *flexo*, and *wimple*, which encode *Ift57*, *Ift88*, and *Ift172*, respectively, cause defects in left-right patterning and Shh signaling. These mutations result in embryonic lethality prior to retinal differentiation, which has prevented analysis of photoreceptor phenotypes in these mutants (Houde et al., 2006; Huangfu et al., 2003). However, a hypomorphic mutation of *Ift88* in mice, the *Ift88*^{Tg737Rpw} allele, is viable and shows gradual loss of outer segments followed by photoreceptor degeneration (Pazour et al., 2002). We previously found that *ift57* mutant zebrafish had short outer segments whereas *ift88* and *ift172* mutants completely

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lack outer segments at 5 days post fertilization (dpf) (Gross et al., 2005; Krock & Perkins, 2008). It is unclear, however, whether the initial stages of outer segment growth, such as primitive cilium projection or assembly of "morphogenetic material," are also affected in zebrafish *ift57* mutants. Detailed ultrastructural analyses of *ift88* and *ift172* mutants at early timepoints have not been described and it is unclear if loss of these subunits prevents all aspects of outer segment formation or if early morphological differentiation occurs but the nascent outer segment rapidly degenerates.

To address these questions, we analyzed the three IFT mutants at 60 hpf, 72 hpf, and 96 hpf, by transmission electron microscopy and immunohistochemistry to chart the progress of photoreceptor morphogenesis and examine the distribution of proteins destined for the outer segment. Our results indicated that outer segments initially formed normally in a subset of *ift57* mutant photoreceptors but further maturation was inhibited. In contrast, outer segment formation was never observed *ift88* and *ift172* mutants. These results reveal subtle differences between various IFT mutants and suggest different factors are necessary for initiating outer segment formation.

2. Materials and methods

2.1. Zebrafish care and maintenance

The zebrafish *oval* locus encodes *ift88* and *oval* mutants contain a nonsense mutation resulting in a premature stop codon identified in exon 11 (Tsujikawa & Malicki, 2004). The *ift88* null mutant zebrafish were a gift from Jarema Malicki. *ift57* and *ift172* mutants were obtained from a retroviral insertional mutagenesis screen (Gross et al., 2005). All fish were maintained in accordance with established procedures (Westerfield, 1995).

2.2. Transmission electron microscopy

All mutants and wild type fish were fixed at 60 hpf, 72 hpf, and 96 hpf in 1% paraformaldehyde, 2.5% glutaraldehyde, and 1% tannic acid. Embryos were processed with osmium tetroxide as secondary fixative, followed by a dehydration series in ethanol and infiltrated with epoxy resin as previously described (Krock & Perkins, 2008). Transverse sections (0.1 μm in thickness) obtained at the optic nerve region were post stained with 2% uranyl acetate and Reynolds lead citrate. Images were collected on a JEOL 1200EX transmission electron microscope and whole images were adjusted for brightness and contrast with Adobe Photoshop.

2.3. Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde at the designated timepoints and processed as described previously (Perkins, Nicholas, Baye, Link, & Dowling, 2005). The dilution of primary antibodies used were as follows: monoclonal 1D1 (1:100), monoclonal ZPR1 (1:200), monoclonal anti-acetylated tubulin (Sigma 1:500), rabbit polyclonal anti-IFT52 (1:3000), and polyclonal anti-IFT88 (1:5000) (Krock & Perkins, 2008). Fluorescent labeled anti-mouse and anti-rabbit secondary antibodies were used at 1:500 dilutions (Invitrogen). DAPI (1:400) was used as a counter stain. Images were collected using an AxioImager fitted with an ApoTome attachment (Zeiss) and processed with Adobe Photoshop.

2.4. SDS page and western blotting

Embryo heads were removed at 60, 72, and 96 hpf and lysis buffer (PBS + 1% Triton + 5 mM EDTA) was added at 3 μl per head. The lysate was homogenized and sonicated prior to dilution in 4× SDS buffer. The solution was boiled at 95 °C for 5 min and centrifuged for 10 min at 13,200 rpm. Samples were loaded on 12% Tris–HCl ready gels (Bio-Rad). Proteins were transferred onto a PVDF membrane and labeled with appropriate antibodies. The primary antibody dilutions used are as follows: Rabbit anti-IFT88 (1:3000), Rabbit anti-IFT52 (1:1500), and mouse anti-acetylated tubulin (1:10,000) (Sigma). Super signal west femto maximum sensitivity substrate (Thermo scientific) and Immunostar HRP substrate Kit (Bio-Rad) were used as secondary antibodies for detection of IFT antibodies and acetylated tubulin, respectively.

3. Results

We analyzed the zebrafish *ift57*$^{hi3417/curly}$, *ift88*$^{tz288b/oval}$, and *ift172*$^{hi2211/moe}$ mutant alleles, which we will refer to as the *ift57*, *ift88*, and *ift172* mutants, respectively. The *ift57* and *ift172* mutations resulted from retroviral insertions into exons near the 5′ end of the gene (Amsterdam et al., 1999), whereas the *ift88* mutation is a nonsense mutation generated by ENU mutagenesis (Malicki et al., 1996). These mutants were described as having defects in ciliated sensory neurons and pronephric cilia (Gross et al., 2005; Sun et al., 2004; Tsujikawa & Malicki, 2004).

Zebrafish carrying homozygous mutations in the *ift57*, *ift88*, and *ift172* genes exhibit photoreceptor cell death (Gross et al., 2005; Krock & Perkins, 2008; Tsujikawa & Malicki, 2004). The 60 hpf timepoint was chosen because the first photoreceptors begin to differentiate at 50–54 hpf (Hu & Easter, 1999; Raymond, Barthel, & Curran, 1995; Raymond, Barthel, Rounsifer, Sullivan, & Knight, 1993) and the first outer segments can be readily observed at 60 hpf (Schmitt & Dowling, 1999). Analysis at 72 and 96 hpf were used to determine any defects in outer segment development as wild type retinas mature (Nawrocki, 1985).

We used transmission electron microscopy to analyze early photoreceptor anatomy and outer segment structure at 60 hpf in all three IFT mutants (Fig. 1). Transmission electron microscopic study of wild type photoreceptor architecture at 60 and 72 hpf has been previously described in detail (Schmitt & Dowling, 1999). Consistent with these previous studies, we observed similar features in wild type embryos at 60 hpf, such as large numbers of mitochondria in the inner segments and sporadic outer segments in the ventral patch (Fig. 1A–C). Wild type photoreceptor outer segments were short but contained loosely stacked disk membranes and connecting cilia were observed (Fig. 1B). We occasionally observed primary cilia extended from the apical surface of the inner segment in central and dorsal regions of the retina, where morphogenesis was just beginning (Fig. 1C). We found outer segments in *ift57* mutants at this timepoint (Fig. 1D–F). These outer segments contained regularly stacked disk membranes and did not differ significantly from wild type. Statistical analysis using a student t-test between wild type and *ift57* mutant embryos revealed no difference in outer segment length at this timepoint (Fig. 4A; $p > 0.05$). In contrast, we never observed organized structures resembling an outer segment in *ift88* or *ift172* mutants (Fig. 1G–L). Interestingly, we occasionally observed disorganized membrane material deep within the inner segment and below the mitochondria (Fig. 1K). In both *ift88* and *ift172* mutants, we also observed disorganized membranes in the apical region of the inner segments (Fig. 1H, I, and L). However, connecting cilia were not detected in *ift88* or *ift172* mutants and these disorganized structures did not resemble outer segments. On rare occasions, we observed parallel arrays of membrane along the lateral membrane of all IFT mutants, which were similar to the ones observed along the lateral membrane for *ift88* mutants at 88 hpf (Tsujikawa & Malicki, 2004).

We next examined the IFT mutants at 72 hpf to look for elongation of outer segments and further photoreceptor differentiation. Wild type larvae at 72 hpf showed an increase in number of outer

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