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Cog4 is required for protrusion and extension of the epithelium in the developing semicircular canals

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ABSTRACT

The semicircular canals in the inner ear sense angular acceleration. In zebrafish, the semicircular canals develop from epithelial projections that grow toward each other and fuse to form pillars. The growth of the epithelial projections is driven by the production and secretion of extracellular matrix components by the epithelium. The conserved oligomeric Golgi 4 protein, Cog4, functions in retrograde vesicle transport within the Golgi and mutations can lead to sensory neural hearing loss. In zebrafish *cog4* mutants, the inner ear is smaller and the number of hair cells is reduced. Here, we show that formation of the pillars is delayed and that secretion of extracellular matrix components (ECM) is impaired in *cog4*$^{-/-}$ mutants. These results show that Cog4 is required for secretion of ECM molecules essential to drive the growth of the epithelial projections and thus regulates morphogenesis of the semicircular canals.

1. Introduction

The inner ear is composed of the cochlea, for auditory sensation, and the vestibular apparatus essential for balance. In the vestibular apparatus, the vestibule and the semicircular canals detect linear and angular acceleration, respectively. The size and formation of semicircular canals differ among species (Alsina and Whitfield, 2017). In zebrafish, formation of the semicircular canals starts with epithelial projections of the otic vesicle that extend toward each other (Haddon and Lewis, 1996; Waterman and Bell, 1984). Opposite projections ultimately contact one another and fuse to form the pillars (Haddon and Lewis, 1996; Waterman and Bell, 1984) that later differentiate into semicircular canals.

In *Xenopus laevis*, scanning electron microscopy and Alcian blue staining showed that the epithelial projections are filled with extracellular matrix (ECM) and that the ECM drives their growth (Haddon and Lewis, 1991). Injection of hyaluronidase into the epithelial protrusions led to their collapse, indicating that hyaluronan is a major component of the ECM (Haddon and Lewis, 1991). In addition, Geng and colleagues showed that the epithelial projections express several markers of the ECM during their growth, such as hyaluronan, chondroitin sulfate proteoglycan, and collagen type II (Geng et al., 2013).

A critical step in making the ECM is secretion of its components. The Conserved oligomeric Golgi (COG) complex is composed of eight proteins, COG1-8, distributed between two lobes, A and B. The COG complex functions in retrograde vesicle transport within the Golgi, particularly vesicle tethering (Miller and Ungar, 2012). Physiologically, the COG proteins participate in sorting glycosylation enzymes to maintain glycosylation homeostasis (Miller and Ungar, 2012). Thus, defects in COG proteins lead to aberrant glycoconjugate synthesis, protein sorting, and protein secretion. The COG4 protein is a subunit of lobe A. In humans, *COG4* mutations lead to congenital disorders of glycosylation type IIJ. Patients affected with this disease present with a range of symptoms including dysmorphia, microcephaly, developmental delay, hypotonia, seizures, failure to thrive, coagulopathy, liver cirrhosis, and nystagmus (Ng et al., 2011; Reynders et al., 2009; Richardson et al., 2009). Recently, a mutation in COG4 was associated with Saul-Wilson syndrome, a rare disease characterized by dysmorphia and developmental delay (Ferreira et al., in press). Some patients with this mutation also display sensorineural hearing loss. In zebrafish mutants for *cog4*, we found phenotypes similar to the symptoms of human Saul-Wilson syndrome. *cog4*$^{-/-}$ mutants are shorter and display smaller jaws, due to improper stacking of chondrocytes, stubby pectoral fins, and smaller eyes and ears (Ferreira et al., in press).

Here, we investigated the mechanism that gives rise to the inner ear phenotype in zebrafish *cog4* mutants. We found that the semicircular canals do not develop correctly in *cog4*$^{-/-}$ mutants. Extension of the epithelial projections that form the pillars is delayed. We show that this delay is due to defective secretion of ECM components.

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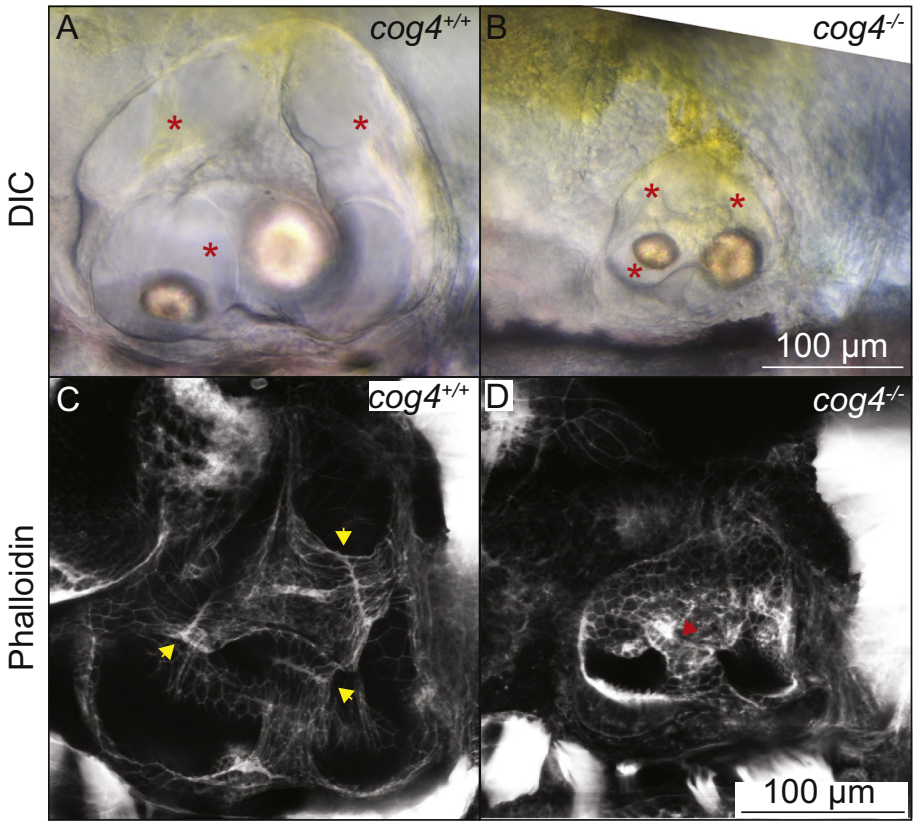

Fig. 1. The pillars do not form properly in *cog4*$^{-/-}$ mutants. Live images of the inner ear of *cog4*$^{+/+}$ sibling (A, n = 34 larvae) and *cog4*$^{-/-}$ mutant larvae (B, n = 15 larvae). Red stars indicate the semicircular canals. Phalloidin staining of the inner ear of *cog4*$^{+/+}$ sibling (C, n = 24 larvae) and *cog4*$^{-/-}$ mutant larvae (D, n = 25 larvae). Yellow arrowheads indicate the fusion plates that form in 100% (24 out of 24) of wild-type siblings (C). Red arrowhead points to the malformed pillar. One or more malformed pillars are observed in 84% (21 out of 25) of homozygous mutant larvae (D). Anterior to the left and dorsal to the top. 5 dpf.

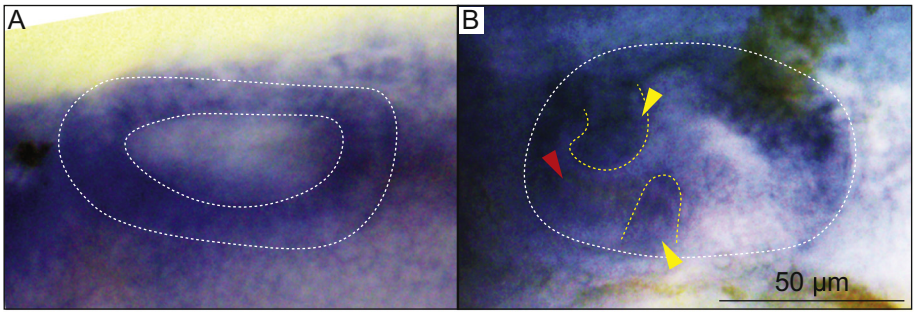

Fig. 2. *cog4* is differentially expressed during inner ear morphogenesis. *In situ* hybridization of *cog4* in the ear at 28 hpf (A, n = 18 embryos), and 52 hpf (B, n = 18 larvae). Dorsal view, anterior to the left (A). Lateral view, anterior to the left (B). Yellow arrowheads indicate the anterior and ventral projections. Red arrowhead indicates the hair cells of the anterior macula (B). The inner ear is outlined in white (A, B) and the epithelial projections in yellow (B).

2. Experimental procedures

2.1. Zebrafish maintenance and staging

Wild-type ABCxTu and heterozygous mutant *cog4*$^{b1312/+}$ (Ferreira et al., in press) adult zebrafish were maintained as previously described (Westerfield, 2007). The *cog4*^{b1312} allele is a 13 bp deletion in exon 12 of *cog4*. The mutation introduces a frameshift followed by an early stop codon (Ferreira et al., in press). Complementation tests with another allele of *cog4* (Ferreira et al., in press) indicate that this allele is a null allele. Embryos and larvae were staged according to the standard staging series (Kimmel et al., 1995). Siblings are defined as a mix of homozygous WT and heterozygous mutants generated by incrosses of heterozygous mutant adults. All experimental procedures were approved by the local IACUC.

2.2. In situ hybridization and histology

The procedure followed the previously published protocol (Thisse and Thisse, 2008). Larvae were hybridized with a digoxigenin labeled RNA probe spanning a 736 bp coding sequence between exons 3 and 8 of *cog4* (Ferreira et al., in press). Stained larvae were embedded in 1% agarose, 0.5% agar, and 5% sucrose medium and 16 µm cryosections were cut.

2.3. Immunolabeling, phalloidin staining, Alcian blue staining

Wholemount larvae were stained following our previously published protocol (Blanco-Sanchez et al., 2014) with minor modifications. 52 h post-fertilization (hpf) and 5 days post-fertilization (dpf) larvae were fixed in BT fix overnight and permeabilized with proteinase K (10 µg/ml) for 30 min or 1 h, respectively. Primary antibodies were mouse anti-Collagen type II (Developmental Studies Hybridoma Bank, II-II6B3; 1:200). Secondary antibodies were goat anti-mouse Alexa-Fluor-568-conjugated (Vector Laboratories, 1:200). Phalloidin staining was performed as previously described (Blanco-Sanchez et al., 2014). Alcian blue staining was performed as previously described (Walker and Kimmel, 2007).

2.4. Drug treatment

Brefeldin A (Sigma; BFA) was suspended in ethanol at a stock