DETERMINING THE MEMBRANE TOPOLOGY OF TRANSMEMBRANE O-METHYLTRANSFERASE IN ZEBRAFISH HAIR CELLS USING A SPLIT GFP SYSTEM

by

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Abstract

Mechanosensory hair cells are used by the auditory and vestibular systems to convert mechanical vibrations into electrical signals. Given their specialized function and morphology, hair cells express a unique set of genes for which, in some cases, little is known about their protein function. One such gene is called *transmembrane o-methyltransferase (tomt).* Mutations in *tomt* have shown it causes non-syndromic deafness in humans, mice, and zebrafish. Tomt is a transmembrane protein localized in the hair cell secretory pathway where it facilitates the trafficking of Tmc1 and Tmc2, which are pore-forming subunits of the hair cell mechano-electrical transduction channel. However, the membrane topology and functional domains of Tomt have not been clearly defined. We will experimentally determine the membrane topology of Tomt in zebrafish hair cells using the split GFP system.

Green fluorescent protein (GFP) is an eleven-stranded beta-barrel that emits green light when excited by blue light photons. The GFP protein can be split into self-assembling fragments consisting of beta strands 1-10 and beta strand 11. The two parts can reconstitute the GFP chromophore. Transgenic lines of fish expressing GFP1-10 in either the cytoplasm or the lumen of the endoplasmic reticulum were created. We tested the functionality of the cytoplasmic and luminal GFP1-10 lines by adding C-terminal GFP11 tags to proteins of known topology or subcellular localization. If the GFP11 tag is localized to the same subcellular compartment as the GFP1-10, then we expect to observe GFP fluorescence. After confirming that the cytoplasmic and luminal GFP1-10 transgenes function as expected, Tomt can be assayed. The GFP11 tag will be fused to the Tomt C-terminus and injected into transgenic fish expressing cytoplasmic or luminal GFP1-10. If Tomt is a type I (C-terminus in the cytoplasm) protein, then we should observe reconstituted GFP only in the cytoplasmic GFP1-10 fish. Conversely, if TOMT is a type II (C-terminus in the lumen) protein, then we expect to see reconstituted GFP in the luminal GFP1-10 line only. Determining the topology of Tomt will help to define its functional domains and provide information on how Tomt facilitates TMC protein folding or trafficking.

Introduction

Hair cells are found in the auditory, vestibular and lateral line sensory systems of vertebrates. These mechanosensory cells convert mechanical vibrations into electrical signals sent to the brain as sensory information. The lateral line system in aquatic animals allows organisms to detect movements in the water. In terrestrial animals, the vestibular system is responsible for sensing changes of the head position and ultimately works to establish balance in the organism. Hairs cells are also present in the auditory system and are responsible for hearing. In mammals, the hair cells are located in the cochlea in the inner ear and convert the pressure waves caused by sound waves into auditory information.

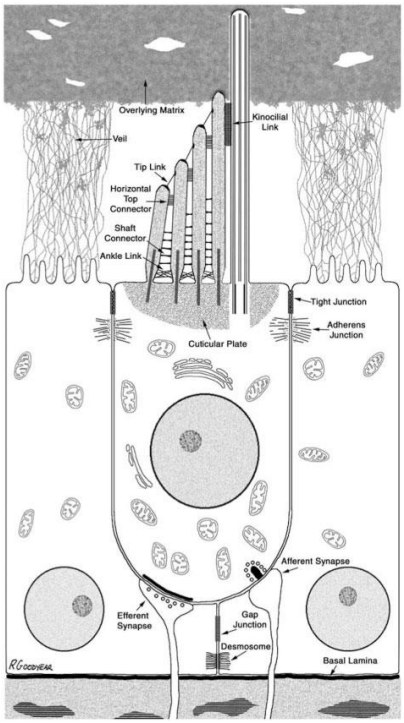

Figure 1. Hair cell structure depicting the hair cell bundle and tip links. (Goodyear et al. 2006)

This ability to convert mechanical information to sensory information is due to the structure of the hair cells, seen in Figure 1 from Goodyear et al. (2006). Each hair cell has connected stereocilia known as the hair cell bundle. The hair bundle is arranged in a staircase formation, with tip links made of cadherin, a type of transmembrane protein that bind cells together connecting the rows of the bundle. Cadherin-23 (CDH23) and Protocadherin-15 (PCDH15) make up the length of the tip link. CDH23 forms the upper part and PCDH15 forms the lower portion (Siemens et al. 2004; Kazmierczak et al. 2007; Müller 2008; Alagramam et al. 2011). When the hair bundle is disturbed by a positive mechanical stimulus, the bundle deflects towards the tallest stereocilia in the staircase array. This deflection of the bundle tensions the tip links, causing mechanically-gated ion channels in the stereocilia to open, allowing ions such as K^+ and Ca^{2+} to enter into the hair cells (Corey and Hudspeth 1979). These channels are located at the tips of the stereocilia in the area where the cadherin links are located. The tip links act as a gate to open the channels when disturbed and the influx of ions establishes a concentration gradient. The ions are able to transduce the mechanical stimuli into sensory information via the afferent synapse located at the base of the hair cell. The afferent synapse releases the neurotransmitter glutamate in proportion to the amount of Ca^{2+} influx in the cell (Glowatzki et al. 2008; Rutherford and Pangrsic 2012; Fettiplace 2017). The glutamate is released with almost no delay and the sensory information is transmitted to the central nervous system and brain.

While we have gained much information about how hair cells and the apical hair bundle operate, questions remain as to how these specialized cellular and molecular structures form. In particular, the molecular and genetic mechanisms of MET complex assembly are relatively unknown. Vertebrate hair cells express a unique set of genes for which, in some cases, little or nothing is known about their protein function. One important gene that we know little about is called *transmembrane o-methyltransferase* (*tomt*). Mutations in the gene *transmembrane O-methyltransferase* (*TOMT* / *LRTOMT2*) cause non-syndromic deafness in humans, mice, and zebrafish. *tomt* is expressed specifically in sensory hair cells and the TOMT protein is localized in the hair cell secretory pathway. TOMT facilitates the apical trafficking of transmembrane channel-like proteins TMC1 and TMC2, which are the putative pore-forming subunits of the hair cell mechano-electrical transduction (MET) channel (Erickson et al. 2017; Cunningham et al. 2017; Du et al. 2008; Ahmed et al. 2008).

Structurally, TOMT is predicted to have an N-terminal transmembrane domain (TMD) and "linker" region, followed by an O-methyltransferase (O-MT) domain at its C-terminus. Genetic evidence suggests that the O-MT domain is playing a non-enzymatic role in regulating MET channel assembly. However, preliminary results suggest that both the N-terminal and O-MT domains are required together for TOMT's function. Co-immunoprecipitation experiments show that mouse TOMT and TMC1 can directly interact in cultured cells. However, it is not clear which regions of the proteins are involved in this interaction, nor have TOMT’s functional domains been identified.

TOMT is predicted to be a single-pass transmembrane domain protein. Transmembrane domain proteins (TMD) are types of integral membrane proteins that are located in the cell membrane and organelle membranes within the cell. The TMD membrane-spanning domain consists of hydrophobic amino acid residues arranged as one or more alpha-helixes or as multiple beta-strands across the membrane. TMD's perform a variety of functions including signal transduction, formation of channels, cell-to-cell communication, and others. The topology of TMD proteins are described according to the subcellular localization of their N- and C-terminal ends. While there are six classes of TMD proteins, there are only 2 classes of single-pass TMD proteins: Type I are oriented with the C-terminus in the cytoplasmic space and the N-terminus in the lumen of the ER/Golgi, while Type II are oriented with the N-terminus in the cytoplasmic space and the C-terminus in the lumen of the ER/Golgi. Understanding the topology of the membrane is important to define the functional domains and determining protein function and interactions.

As a step towards clearly defining the functional domains in TOMT, we will experimentally determine the membrane topology of TOMT in zebrafish hair cells using the split GFP system (Xie et al. 2017; Kamiyama et al. 2016; Cabantous, Terwilliger, and Waldo 2005). GFP (green fluorescent protein) is an eleven stranded beta-barrel with an alpha helix running through the center that emits green light when excited by photons of blue light. The GFP protein can be split into self-assembling fragments consisting of beta-strands 1-10 and a separate beta-strand 11. These two parts have the ability to reconstitute the GFP chromophore (Seong-In et al. 2015). This split GFP system can be used to determine the membrane topology of a protein. By fixing the GFP11 tag to the C-terminus of the protein and expressing the complementary GFP1-10 protein in either the cytoplasm or lumen of the ER, it is possible to determine the orientation of the protein across the membrane. If the tag is attached to the C-terminus and illumination is seen in the Cyto-GFP1-10, then the protein is Type I (Figure 2). If fluorescence is observed in the Lumen-GFP1-10, then the protein is Type II (Figure 3).

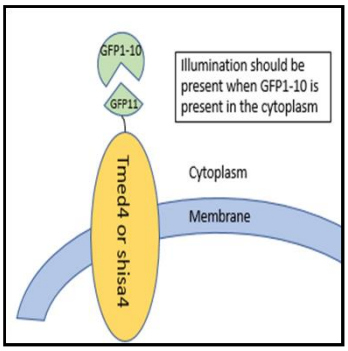

Figure 3: The predicted GFP fluorescence of the Type I control proteins.

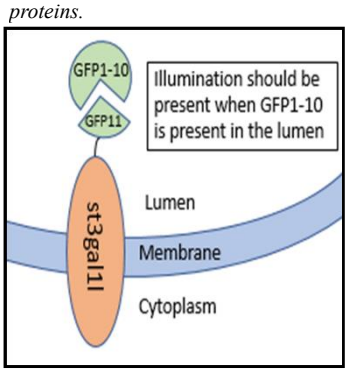

Figure 3: The predicted GFP fluorescence of the Type II control protein.

Results

To validate the use of the split GFP system, GFP11x7 was fused to the C-terminus of proteins of known topology or subcellular localization. G3bp1 has been experimentally identified as a cytoplasmic protein (Parker et al. 1996). Tmed4, a member of the p24 family, has been identified as a Type I protein (Luo and Wang, 2007). Shisa4 has been identified as a Type I protein (Pei & Grishin, 2011). St3gal1l has been experimentally identified as a Type II protein (Paulson and Colley, 1989). Progeny of stable transgenic fish lines expressing either Cyto-GFP1-10 or Lumenal-GFP1-10 were injected with the construct expressing the GFP11 counterpart. If the GFP11 tag is localized to the same subcellular compartment as the GFP1-10, then we expect to observe GFP fluorescence. Fish larvae were live-imaged five days post injection at two locations: the inner ear and neuromasts. GFP signals were observed in the Lumenal-GFP1-10 larvae when St3gal1l-GFP11x7 was present and in the Cyto-GFP1-10 larvae when G3bp1-GFP11x7 was present (Figure 4). Artifacts caused by the GFP1-10 tag or GFP11x7 tag not localizing to the correct subcellular locations were also imaged and recorded separately (Figure 5). These imaging results experimentally confirmed the topology of the control proteins, identifying G3bp1 as a cytoplasmic protein and St3gal1l as a Type II TMD protein. These results validated the split GFP system as a tool to identify the topology of a protein in zebrafish hair cells.

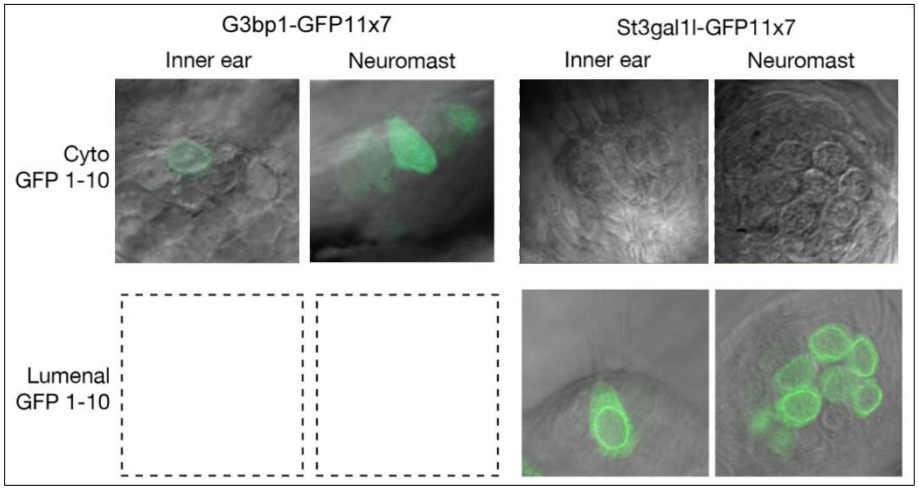

Figure 4: Live cell imaging using control proteins. GFP fluorescence is present when both the GFP1-10 and GFP11 fragments localize to the same subcellular compartment.

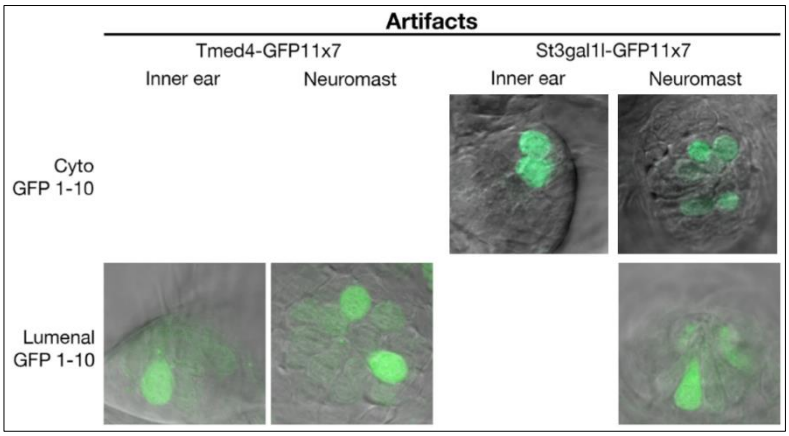

Figure 5: Recorded artifacts from injected Cyto-GFP1-10 and Lumenal-GFP1-10 lines.

Discussion

In this study we experimentally confirmed that the split GFP system (Xie et al. 2017; Kamiyama et al. 2016; Cabantous, Terwilliger, and Waldo 2005) can be used to determine protein topology in zebrafish hair cells. The known topology of two proteins, G3bp1 (Type 1) and St3gal1l (Type II), was confirmed using the split GFP system. We originally planned to use constructs for Shisa4, a type I protein (Pei & Grishin, 2011), and Tmed4 in the split GFP system, however, neither of these constructs reconstituted the GFP protein when injected into the Cyto-GFP1-10 lines. Tmed4-GFP11x7 did produce artifacts in the Lumenal-GFP1-10 lines, indicating the construct could reconstitute the GFP protein, though it did not produce results in the Cyto-GFP1-10 line. Due to the lack of results with the Shisa4-GFP11x7 and Tmed4-GFP11x7 constructs, we chose to use a cytoplasmic protein, G3bp1, that could better localize to the Cyto-GFP1-10. In the Lumenal-GFP1-10fish line, we observed GFP artifacts that may be due the GFP1-10 tag not localizing in the correct location within the cell. An endoplasmic reticulum signal peptide was added to the Lumen-GFP1-10 construct to localize and retain the tag in the ER lumen. It is possible that, despite the signal peptide, that not all of the Lumen-GFP1-10 localized to the ER lumen, causing the observable artifacts in Figure 5.

Unfortunately, the main goal of the project, to determine the topology of the Tomt protein using the split GFP system, was unable to be completed due to unforeseen circumstances. However, the data gathered during the course of the project provides promising information on the use of the split-GFP system. Given the results provided by the control experiments, we are confident that by injecting the Tomt-GFP11x7 into the transgenic fish lines we can identify Tomt has either a Type I or Type II protein. Determining the topology of Tomt will help to define its functional domains and provide information on how Tomt facilitates TMC protein folding or trafficking in hair cells.

Materials and Methods

Transgenic Lines and Tol2 Gateway Cloning

Transgenic fish were generated using the Tol2 Gateway system as described by Kwan et al. (2007). The Tol2 Gateway cloning system was used to make constructs to express GFP1-10 in the cytoplasm or lumen of the endoplasmic reticulum (Cyto-GFP1-10 and Lumen-GFP1-10) with RFP (red fluorescent protein) expressed in the lens. The RFP expression served as a transgenesis marker as GFP1-10 is not visible until its complementary GFP11 counterpart is present. Generation of constructs is illustrated in Figure 6, a modified figure from Kwan et al. (2007).

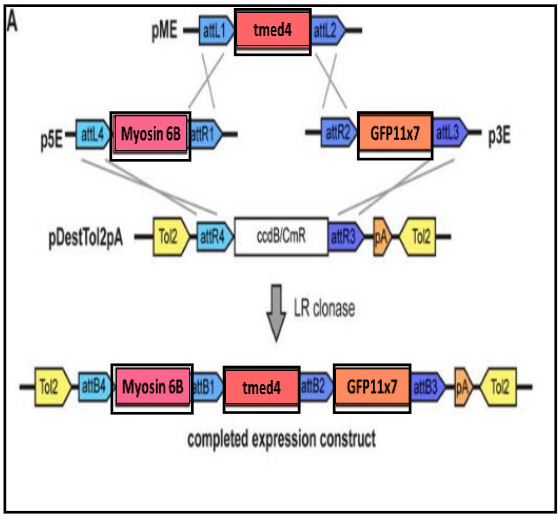

Figure 6: Visual of the Tol2 system used to make constructs.

Constructs were inserted into bacterial plasmids to create vectors with the construct to insert into the fish. Fish were injected with the GFP1-10 with RFP expression construct and raised to adulthood. To localize the Lumen-GFP1-10 transgenic construct to the endoplasmic reticulum lumen, an endoplasmic reticulum signal peptide was added to GFP1-10 to localize the GFP tag to the lumen and retain it within the ER. Founder fish were identified by interbreeding fish injected with GFP1-10 and screening the offspring for RFP expression in the eye 3 days after breeding. Founder fish were used to generate stable transgenic lines of Lumen-GFP1-10 and Cyto-GFP1-10. A list of the constructs used in the split GFP system and predicted results is provided in Table 1.

Imaging

To image the fish, larvae were anesthetized with E3 plus 0.015% MS-222 and laterally mounted on a microscope slide with low-melting point agar. Mounted larvae were imaged using a confocal microscope with 40x water lens.

Transgenic Line	Construct Injected	Predicted Topology/Sub cellular Localization	Predicted GFP Reconstitution	Interpretable Results
Cyto-GFP1-10 (GE;myo6b:sp-GFP1-10-KDEL)	GH; myo6b:shisa4-GFP11x7	Type I	Yes	No
	GH; myo6b:tmed4-GFP11x7	Type I	Yes	No
	GH; myo6b:g3bp1-GFP11x7	Cytosol	Yes	Yes
	GH; myo6b:st3gal1l-GFP11x7	Type II	No	Yes
Lumen-GFP1-10 (RE;myo6b:GFP1-10-P2A-sfCherry1-10)	GH; myo6b:tmed4-GFP11x7	Type I	No	No
	GH; myo6b:st3gal1l-GFP11x7	Type II	Yes	Yes

Table 1: Transgenic lines and constructs used in this study with predicted results for the injections performed.

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