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OVEREXPRESSION OF SPONGE BHLH MRNA IN XENOPUS LAEVIS DISRUPTS INNER EAR NEUROSENSORY DEVELOPMENT

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A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Biology

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Lori C. Adams
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Approval Seal Here

Abstract

To uncover the steps necessary to restore hearing loss in a more superior way than is currently available, it is necessary to obtain a more complete understanding of the genetic and molecular mechanisms of vertebrate mechanosensory development. Attempts to fully restore hearing are currently focused on manipulating adult ear cells in model organisms using specific basic helix-loop-helix (bHLH) genes. The main purpose of our study was to better understand the development of inner ear neurosensory pathfinding ability and determine if neuronal projections are controlled by diffusible cues from the hindbrain, or by cues from the neurons themselves. To address this we overexpressed an ancestral proneuronal bHLH gene from the sponge *Amphimedon queenslandica* in the two-cell stage embryo of *Xenopus laevis.* Following this we performed ear transplants from the injected animals to control animals as well as from control animals to the injected. It was found that when transplanting from an injected animal to a control the pathfinding ability of the neurons in the inner ear was derailed in the same way that it is derailed in an injected animal prior to transplantation. When transplanting from the control animal to an injected the inner ear neurons were not derailed. These findings suggest that cues from the hindbrain are not responsible for the pathfinding ability of inner ear afferents, but rather the neurons themselves affect the pathfinding ability. From this we determined that the overexpression of *AmqbHLH* affects the neurons ability to pathfind, likely due to a malfunction of the wnt/PCP pathway component, Frizzled.

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TABLE OF CONTENTS

Introduction

The development of the vertebrate inner ear begins with a placodal thickening of the surface ectoderm. Following the initial thickening of the ectoderm, the newly formed otic placode invaginates, proliferates, and diversifies to become a distinct sensory component of the peripheral nervous system, forming the otic vesicle and ultimately the vertebrate ear (Ladher et al., 2010). Important in these early progressive steps is a number of signaling events by surrounding germ layer derivatives (Fritzsch et al., 2010). In particular, suppression of Bone morphogenetic protein 4 (Bmp4) and expression of Fibroblast growth factor (Fgf) is an essential step in the placode induction involved in inner ear development (Abelló et al., 2010), but also the ex[pression of multiple transcription factors such as Sox2 and various basic Helix-Loop-Helix genes. The adult ear is functionally responsible for controlling angular acceleration, linear acceleration, and hearing. These functions are made possible by the formation of distinct cell types in a distinct neuroepithelium.

The vertebrate inner ear is composed of four cell types: sensory neurons, epithelial mechanoreceptor cells (hair cells), hair cell support cells and otic epithelium (Moore, 2015). The formation of these cell types and their subsequent function is of interest to understand the complexity by which the ear operates locally and with the central nervous system. The number, anatomical position, and cell type of the neurosensory cells that form have been found to be determined by upstream basic helix-loop-helix (bHLH) regulatory genes (Fritzsch et al., 2010). The Delta/Notch pathway is among the important events that regulates neurogenesis during inner ear development. Notch signaling regulates the expression of potent bHLH transcription factors which act to differentiate cell type specific programs of gene expression. The transcription factors that have been identified as important for neurosensory development include three bHLH

genes: *Neurogenin 1 (Neurog1)* for the induction of neurons, *Neuronal differentiation 1 (Neurod1)* for differentiation of sensory neurons, and *Atonal 1 (Atoh1)* for hair cell differentiation (Fritzsch et al., 2010). The upregulation of these proneural bHLH genes are considered to be the start of neurogenic/neurosensory cellular differentiation (Ladher et al., 2010).

The developmental pathways that mediate ear formation and cellular differentiation reveal the mechanism behind the evolution of neurosensory systems (Fritzsch et al., 2010). Neurosensory development in the ear is an evolutionarily conserved process. By study of this evolution, valuable insight into ear morphogenesis through networks of genetic interactions can be understood. One of the known driving forces for evolutionary change is gene duplication, which could lead to changes in the protein sequence or changes in the expression of the genes (Ohno, 2013). Most commonly, gene duplication leads to loss of function, however, evolutionary novelties are able to occur and gene duplications can potentially give rise to speciation events (Lynch and Conery, 2000).

Many genes show duplication in vertebrates relative to their single celled ancestor. Genes of interest include the cell fate decision making genes involved in encoding the bHLH transcription factors responsible for the differentiation of neurons and hair cells in the inner ear (Pan et al., 2012). *Neurog1, Neurod1,* and *Atoh1* are all a part of the proneural *atonal* family of bHLH transcription factors (Fritzsch et al., 2010), and data suggests that the *atonal*-like family of bHLH genes originated with the metazoans (Richards et al., 2008). Sponges lack neurosensory epithelia, however, they possess a single *atonal*-like bHLH gene (Richards et al., 2008). In triploblasts, the *atonal* family of bHLH genes underwent duplication and diversification events allowing for the assignment of novel functions for each member of the *atonal* family in

vertebrates (Pan et al., 2012). While invertebrates retain a single *atonal* gene and generate single mechanosensory cells, vertebrates, by using the different *atonal* family members, can generate a situation where individual bHLH factors segregate to separate neural and sensory precursors (Fritzsch and Elliott, 2017). In vertebrates, inner ear neurons depend on *Neurog1* and hair cells depend on *Atoh1* for their differentiation (Fritzsch et al., 2010). It is evident that the evolutionary split of single neurosensory cells into neurons and sensory cells was made possible by duplicating *Atoh1* and *Neurog1* with *Neurod1* being expressed in both and providing both with a negative feedback loop (Fritzsch and Elliott, 2017). This suggests a clonal relationship between some neurons and hair cells and that the sensory precursor cells may undergo an additional round of cell division in order to give rise to the neurons through *Neurog1* expression and to the hair cells through *Atoh1* expression (Fritzsch and Beisel, 2004). Further study of the evolution of bHLH genes allows insight into the genetic and molecular interactions that drive development.

A more complete understanding of the genetic and molecular mechanisms of vertebrate mechanosensory development is of utmost importance as it can uncover the steps necessary to restore a hearing organ (Fritzsch et al., 2010). Sensorineural hearing loss affects over 200 million people worldwide, including nearly half of individuals over the age of 65 (Pan et al., 2012). Studies have shown that older individuals with hearing loss are more susceptible to dementia (Lin et al., 2011), as well as depression due to feelings of social isolation from the lack of ability to communicate through language (Mener et al., 2013). While hearing-aids and cochlear implants can restore some hearing, being able to rebuild a lost hearing organ would be superior. Attempts to fully restore hearing currently are focused on the manipulation of proliferation of adult ear cells in model organisms by using specific genes (Kopecky and Fritzsch, 2011).

The sponge *Amphimedon queenslandica,* which has no neurons and lacks a nervous system, is of particular interest in studies aimed toward understanding of molecular interactions needed to be understood for hearing restoration (Richards et al., 2008). *A. queenslandica* possesses an atonal-like bHLH gene, *AmqbHLH*, that provides developmental mechanisms similar to neurogenesis in bilaterians in the expression likely of primitive sensory cells in its epithelium. *AmqbHLH1* was discovered to act in a proneural manner when expression was induced in metazoan species *Drosophila* and *Xenopus* (Richards et al., 2008). Introducing bHLH genes from a species with no true nervous system to a species with a complex nervous system provides a framework for study of interactions between genes and their associated products during development. Overexpression of each of the proneural bHLH genes, *Neurod1* (Lee et al., 1995), *Neurog1* (Ma et al., 1996), and *Atoh1* (Kim et al., 1997) as well as the *atonal*-like *AmqbHLH1* (Richards et al., 2008) has been induced in the frog, *Xenopus laevis,* to assess their function. The overexpression of any of thesepro-neural bHLH genes results in the formation of ectopic neurons in ectodermal progenitors normally destined for nonneuronal fates. This conversion of non-neuronal ectoderm into neurons demonstrates that members of the proneural bHLH family have the ability to convert non-neuronal ectoderm into neurons. This contrasts with their function in normal neuronal development. During the normal developmental progression of neuronal induction and formation of neuronal precursors, bone morphogenetic proteins (BMP), *Bmp4* in particular, must be downregulated and fibroblast growth factors (Fgfs) must be upregulated. Conversion of non-neuronal ectoderm into neurons could be due to interactions with BMPs and Fgfs supporting cell development in the ectoderm or simply override the antagonistic ability of BMP4 to block neuronal development. Indeed, evidence already shows that *Neurod1* can negatively regulate expression of *Bmp4* that inhibits neurogenesis in the

ectoderm (Bond et al., 2012), therefore, bHLH gene expression may interact with these factors in other ways that does not require the prior downregulation of BMP4.

Thus, while previous studies on overexpression of the proneural bHLH genes in *Xenopus* were instrumental in demonstrating the neurogenic capabilities of these genes, more information is needed in order to understand the molecular role in development that the proneural genes specifically play for long term neurosensory stability that should last a lifetime for a given hair cell or neuron. Among the unresolved issues of previous neuronal induction work is the long time differentiation and connection to the CNS of the bHLH gene mediated transformation of ectodermal cells. In particular, further investigations on the connections made by these neruons and comparisons with inner ear sensory neurons have not been performed. Normally developing inner ear sensory neurons are able to project centrally and peripherally correctly before their target cells begin to differentiate (Fitzsch et al., 2005). The correct targeting of auditory afferents is possible without hair cells or auditory nuclei, indicating that other mechanisms guide the afferents. These mechanisms may be related to known pathfinding molecules released from the hindbrain (Fritzsch and Elliott., 2017). Interestingly, transplanted ears near the hindbrain in *Xenopus* have shown to project directly to the correct vestibular nuclei, supporting the idea that diffusible factors released from the hindbrain play a role in the pathfinding process (Elliott et al., 2014). By transplanting ears, the pathfinding substrate is disrupted, however, the neurons can still end up in precisely the right area

While studies have shown the ability of transplanted ears to make the correct neuronal projections, it is unknown how overexpression of certain bHLH genes affect the ability of the neurons to use the diffusible factors from the hindbrain to make central projections. Previous data demonstrated central projections in the developing inner ear afferents being derailed in

mutants of certain transcription factors such as *Neurod1* (Jahan et al., 2010). We proposed that overexpression of a different bHLH gene, *AmqbHLH1,* in *Xenopus laevis* even at a low concentration would be enough to create a disorganized central projection following transplant of the affected ear to a control animal. Our data reveal that overexpression of *AmqbHLH1* interrupts the signals from the hindbrain leading to abnormal central projections.

Materials and Methods

Animals:

Xenopus laevis embryos were obtained though induced ovulation of females using an injection of human chorionic gonadotropin. Following injection the females were squeezed to pressure eject fresh eggs. Eggs were fertilized with a sperm suspension in 4ml 0.3X Marc's Modified Ringer's Solution (MMR) for 4 minutes, after which the embryos were flooded with 0.1X MMR solution. About 20 minutes following fertilization the MMR solution was drained of and 2% cysteine was added to the embryos in order to remove the jelly coats. Once the coats were effectively removed the cysteine was poured off and the embryos were washed with 0.1X MMR. Embryos were kept at 18ºC in 90 mm Petri dishes containing 0.1X MMR until they reached the two-cell stage at which point injections were performed (see below). About 25 injected animals were kept in one 90 mm Petri dish while 25 uninjected control animals were kept in a separate dish.

AmqbHLH1 mRNA Injections:

Embryos were placed into a Ficoll solution (2% Ficoll 400, GE/Pharmacia, in 0.5X MMR) in order to expand the plasma membrane to the outer membrane. They were then injected with 25pg of *AmqbHLH1* mRNA at the two-cell stage into both the left and right blastomeres using a 3nl/sec calibrated glass needle controlled by a Pico-Injector (Harvard Apparatus, Hollison, MA). The embryos were injected into both cells at the two-cell stage, resulting in treatment of the entire embryo. One half of the animal was used to examine the effect of sponge bHLH genes on development of the ear, while the other half of the animal had its ear transplanted to a control animal and subsequently received a control ear transplant. Following

injections the embryos remained in Ficoll solution overnight. Ficoll solution was then replaced with 0.1X MMR while the embryos were allowed to grow.

Hair cell to ear volume correlation measurements:

Images of *AmqbHLH* injected animals were taken using a confocal microscope, which took cross sectional scans (Leica). Using Lecia software, images were added together to make one z-series max image. The images show tubulin staining, myoVI staining, and Hoechst staining. Measurements of ear length were done using the largest anterior-posterior axis defined by the Hoechst stained nuclei surrounding the outer edge of the ear for both injected ears and uninjected ears. The height of the ear was measured by scanning through the z-sections and measuring where hair cell formation began and stopped. Prior studies identified the hair cell distribution by looking at the number of distinct sensory patches and the patterns of innervation. Further analysis of counting the individual hair cells present in each sensory patch was performed.

Ear Transplantations:

All transplantations were performed in 1X MMR pH 7.6-7.8, diluted from 10x stock (1M NaCl, 18 mM KCl, 20mM $CaCl_2$, 10 mM $MgCl_2$, 150 mM Hepes). Otic placodes from the right side of stage 25-27 embryos were removed and transferred from *AmqbHLH1* injected animals to control animals and vice versa. Embryos were kept in 1xMMR for about 10-15 minutes to promote healing before transfer to 0.1X MMR. Healing was confirmed visually as a fusion of the ectoderm superficial to the otocyst with the ectoderm of the insertion site.

Animals were reared and the transplantation was checked daily for continued growth. The animals were classified by three categories for evaluation:

a) sponge ear on sponge injected animal,

b) sponge ear on control animal,

c) control ear on sponge injected animal. Animals were kept in 0.1X MMR at room temperature until time of fixation at St 46.

Fixation, imaging, and Lipophilic dye label:

Animals were allowed to grow until stage 46 at which point they were anesthetized in 0.02% Benzocaine and fixed in 10% paraformaldehyde (PFA) overnight. Following fixation, images were taken of the dorsal, ventral and lateral axes of the animals for comparative phenotypic data using a camera mounted to a dissecting microscope (Leica). This was done to observe which animals formed a reduced ear phenotype.

Lipophilic dye-soaked filter paper (Fritzsch et al., 2005) was used to backfill from the spinal cord to the transplanted and control ears. In preparation of injection, the lower jaw was removed from each animal to give clear access to the injection sites. Small pieces of dye-soaked filter paper were injected into the ear vesicle as well as slightly caudal to the ear to label for the trigeminal nerve and lateral line using Green on control side and Red on transplant side (Figure 1). The animals were kept in 0.4% PFA at 36^{o}C overnight to allow for dye diffusion. Finally, the hindbrains were removed from the animals, cut into hemisections, and were mounted on a slide in glycerol. Images were taken with a Leica TCS SPE confocal microscope.

Results

Classification of embryos:

Following the ear transplants the embryos were examined for phenotypes based on the degree of development of the ear. Three distinct phenotypes were observed: no ear formation, empty vesicle formation, or a reduced ear. The animals best suited for this study were those with the reduced ear phenotype as they will still form somewhat organized arrangements of neurons and hair cells. The animals with the reduced ear phenotype were sorted out and labeled with lipophilic dye (figure 1).

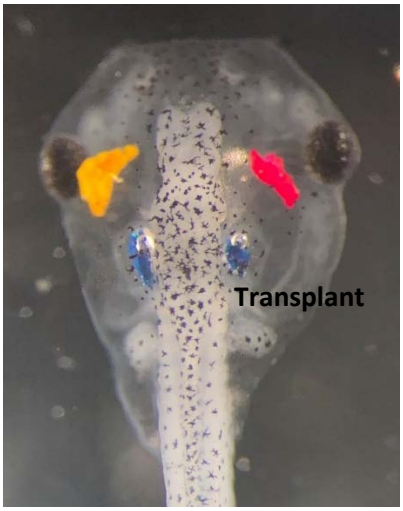

Figure 1. Stage 46 *Xenopus laevis* embryo following lipophilic dye injections. The right side of the animal is the side with the transplanted ear. Following removal of the lower jaw, blue dye was inserted in the ear vesicles, red dye and yellow dye were inserted just caudal to the ears on the transplanted side and control side respectively.

Examination of inner ear central projections

To examine the patterns of central afferent projections of the inner ear following *AmqbHLH* injection and subsequent transplant, the neurons were labeled by lipophilic dye applications. Animals with control ears receiving no *AmqbHLH* injection developed inner ear projections with sharp boundaries to the lateral line (dorsal) and trigeminal (V) nerve projections (figure 2A). Animals injected with *AmqbHLH* with reduced ear phenotype showed varying degrees of disorganization on the non-transplant side (figure 2B), and also showed aberrant motor neuron formation in the brain (figure 2C). Specifically, facial branchial motoneurons projected to the trigeminal nerve and were overlapping with inner ear efferent neurons, also derived from facial branchial motoneurons (Simmons et al., 2011).

Animals receiving injections of *AmqbHLH* had their right ears transplanted to control animals that did not receive any injection. The right control ears were also transplanted into the injected animal. Control ears transplanted to injected animals did not show any disorganization of inner ear afferents into the lateral line (figure 3 A-B). When the ear was transplanted from the injected animal to the control, however, the sensorineural afferents appeared to be disorganized as shown by projection of inner ear neurons into the lateral line (figure 3 C-D).

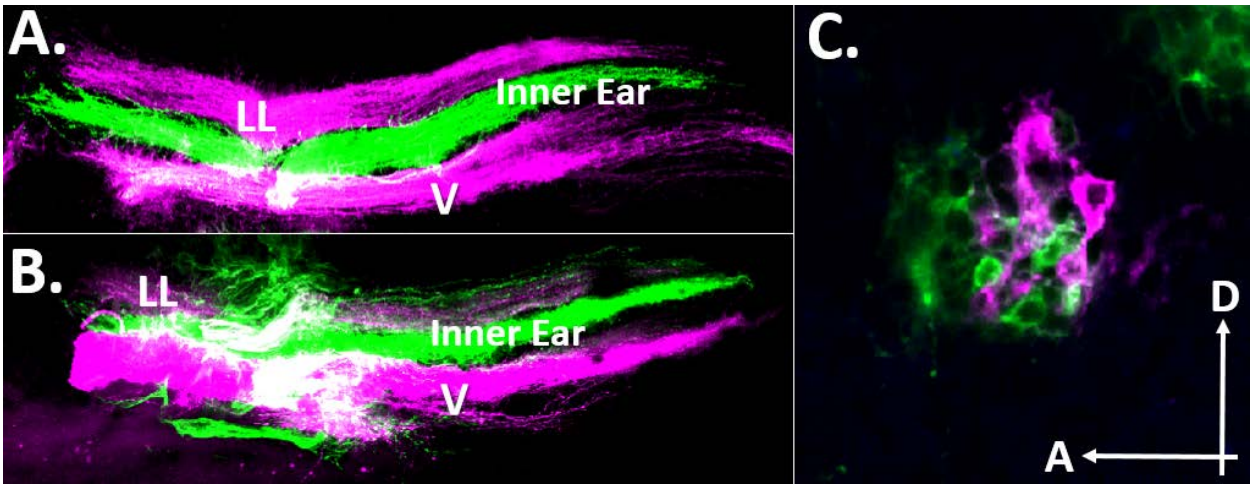

Figure 2. Inner ear central projections in stage 46 *Xenopus laevis*. (A) Projections from control animal with no injection of *AmqbHLH*. **(B)** Projections from non-transplanted ear side (left) of an animal receiving *AmqbHLH* injection. **(C)** Motor neuron formation found in the same animal as in B. Note that trigeminal/lateral line, inner ear cells all overlap. LL=lateral line, V=Trigeminal cranial nerve projection.

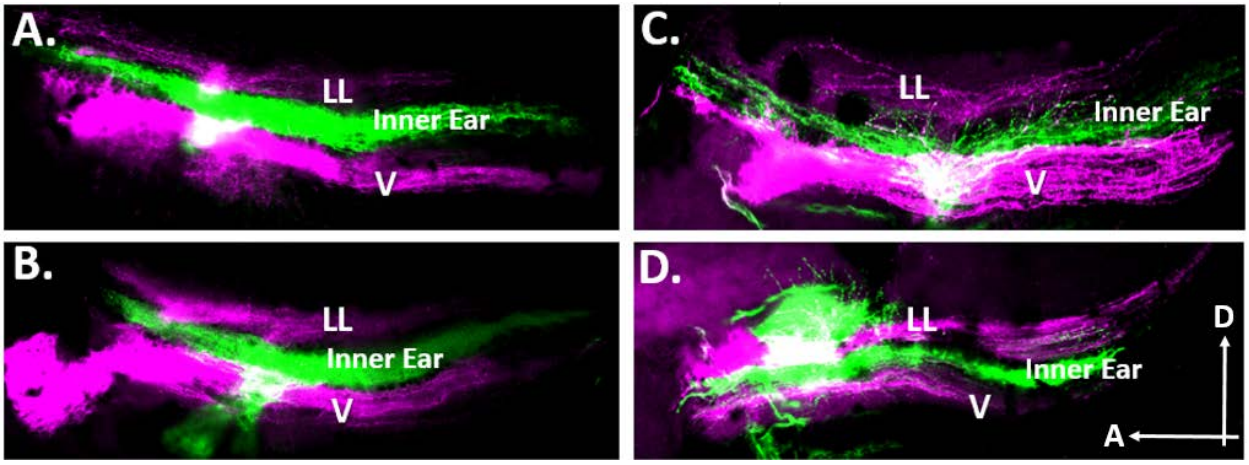

Figure 3. Inner ear central projections in stage 46 *Xenopus laevis*. (A-B) Projections from right side of *AmqbHLH* injected animals receiving transplanted control ears. **(C-D)** Projections from right side of control animal receiving transplanted *AmqbHLH* exposed ears. LL=lateral line, V=Trigeminal cranial nerve

Discussion

In this study we aimed to determine how overexpression of the ancestral sponge *AmqbHLH* gene would affect the ability of the neurons in the inner ear to use the diffusible factors from the hindbrain to make central projections. We performed transplants in order to determine changes in the phenotypes of our frogs were due to effects in the hindbrain or the inner ear afferents. Our data showed that transplants of an ear from a control animal to an animal with overexpression of *AmqbHLH* demonstrated normal projections. This means that overexpression of the sponge bHLH genes in the brain does not affect the navigation of the inner ear neurons. However, when transplanting an ear from an animal that received overexpression of *AmqbHLH* to a control animal, neurons could be traced from the ear entry point going into the territory of the lateral line. Thus, overexpression of *AmqbHLH* at low concentrations in the afferents is enough to subtly derail the organization of central projections of the inner ear.

The phenotypic results from this study are similar to the results from studies of *Neurod1,* Fzd3, and Prickle1 mutants. Mutations in the Wnt/PCP pathway component, prickle, has shown to result in the misrouting of afferents (Duncan, Elliott, et al., in preparation). Fzd3 plays a role in axon guidance in other systems and is expressed in the inner ear. In mice with mutant Fzd3, disorganization of the neural central projections occurs due to Wnt signaling being altered and affecting afferent pathfinding (Yang et al., 2017). Further work is needed to verify that the projection phenotype in our treated frog ears relates to the emerging idea of Wnt/Fzd signaling in this inner ear afferent sorting process.

Another finding from this study was the expression of motor neurons in the central projections of the inner ear of animals injected with *AmqbHLH.* This phenotype was purely a result of overexpression of the bHLH genes as no transplant was performed. Although this was

not expected, it is not surprising given that previous studies have shown motor neuron expression in the hindbrain following overexpression of bHLH genes (Fritzsch et al., 2017). We already know there is a defect in some motor neurons with sponge injections leading to the aberrant expression of Mauthner cells.

Our study was limited in that we only performed injections at one concentration of *AmqbHLH.* Since we know that higher levels of sponge bHLH genes give rise to more affected ear phenotypes, future studies should focus on testing the dose-response of *AmqbHLH* injections and the effects it has on transplants of ears. Understanding how these genes can derail the central projections of the inner ear is essential to understanding the mechanisms underlying loss of hearing, and thus can provide insight as to how a hearing organ can be restored.

Literature Cited

Bond, A.M., Bhalala, O.G., Kessler, J.A., 2012. The Dynamic Role of Bone Morphogenetic Proteins in Neural Stem Cell Fate and Maturation. Developmental neurobiology 72, 1068-1084.

Elliott, K.L., Houston, D.W., DeCook, R., Fritzsch, B., 2015. Ear manipulations reveal a critical period for survival and dendritic development at the single-cell level in Mauthner neurons. Developmental Neurobiology 75, 1139-1351.

Fritzsch, B., Beisel, K.W., 2004. Keeping sensory cells and evolving neurons to connect them to the brain: molecular conservation and novelties in vertebrate ear development. Brain Behav Evol 64, 182-197.

Fritzsch, B., Eberl, D. F., & Beisel, K. W. (2010). The role of bHLH genes in ear development and evolution: revisiting a 10-year-old hypothesis. *Cellular and Molecular Life Sciences* , 3089-3099

Fritzsch, B., Elliott, K.L., 2017. Gene, cell, and organ multiplication drives inner ear evolution. Developmental Biology 431, 3-15.

Jahan, I., Kersigo J., Pan, N. (2010). *Neurod1* regulates survival and formation of connections in mouse ear and brain. *Cell Tissue Res* 341:95-110

Kim, P., Helms, A.W., Johnson, J.E., Zimmerman, K., 1997. XATH-1,a Vertebrate Homolog ofDrosophila atonal,Induces Neuronal Differentiation within Ectodermal Progenitors. Developmental Biology 187, 1-12

Kopecky B, Fritzsch B. 2011. Regeneration of Hair Cells: Making Sense of All the Noise.. Pharmaceuticals (Basel). 4(6):848-879.

Ladher R. K., O'neill P., Begbie J. (2010). From shared lineage to distinct functions: the development of the inner ear and epibranchial placodes. *Development* 137 1777–1785 10.1242/dev.040055

Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H (1995) Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 268:836–844

Lin FR, Metter EJ, O'Brien RJ, Resnick SM, Zonderman AB, Ferrucci L. Hearing Loss and Incident Dementia. *Arch Neurol.* 2011;68(2):214–220. doi:10.1001/archneurol.2010.362

Lynch, M., & Conery, J. (2000). The Evolutionary Fate and Consequences of Duplicate Genes. Science, 1151-1154.

Ma, Q., Kintner, C., Anderson, D.J., 1996. Identification of neurogenin, a Vertebrate Neuronal Determination Gene. Cell 87, 43-52.

Mener, D. J., Betz, J., Genther, D. J., Chen, D., & Lin, F. R. (2013). Hearing Loss and Depression in Older Adults. *Journal of the American Geriatrics Society*, *61*(9), 1627–1629.

Moore, K.L., Persaud, T.V.N. & Torchia, M.G. (2015). *The developing human: clinically oriented embryology* (10th ed.). Philadelphia: Saunders.

Ohno, Susumu (2013). Sex Chromosomes and Sex-linked genes. Duarte, California: City of Hope Medical Center

Pan, N., Kopecky, B., Jahan, I., & Fritzsch, B. (2012). Understanding the evolution and development of neurosensory transcription factors of the ear to enhance therapeutic translation. *Cell and Tissue Research*, *349*(2), 415–432.

Richards, G. S., Simionato, E., Perron, M., Adamska, M., Vervoort, M., & Degnan, B. M. (2008). Sponge Genes Provide New Insight into the Evolutionary Origin of the Neurogenic Circuit. *Current Biology*, 1154-1161.

Simmons D., Duncan J., de Caprona D. C., Fritzsch B. (2011). Development of the inner ear efferent system, in Auditory and Vestibular Efferents, eds Ryugo K. D., Fay R. R., Popper A. N., editors. (NewYork, NY: Springer;), 187–216.

Yang T, Kersigo J, Wu S, Fritzsch B, Bassuk AG (2017) Prickle1 regulates neurite outgrowth of apical spiral ganglion neurons but not hair cell polarity in the murine cochlea. PLOS ONE 12(8): e0183773.