Retinoic Acid Signaling in Late Vestibular Development of the Inner Ear
and
Techniques for Visualizing the Zebrafish Inner Ear

by

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Abstract

The vertebrate inner ear is a labyrinthine sensory organ responsible for perceiving sound and body motion. A wide range of disorders of the inner ear can arise from congenital disorders, necessitating the identification of molecular components or pathways involved in its development to better understand the causative factors behind inner ear dysfunction. However, while a considerable amount work has been invested in understanding the auditory system, there is a dearth of knowledge concerning the complex developmental programme behind the apparatuses of the inner ear responsible for vestibular function. This applies particularly to our understanding of the diverse roles independent genes or pathways play at later stages of otic development, an area of research that also suffers from a lack of biological tools that can be utilized to analyze inner ear structure and function.

Previous research has identified key cell signaling pathways involved with inner ear development. However, the early disruption of these pathways often produces profound phenotypes that obscure or hinder the manifestation of those that might appear later, complicating our analysis of their roles in late otic development. This applies to the Retinoic Acid (RA) signaling pathway, the disruption of which can generate robust disease phenotypes that preclude the analysis of its involvement in the later development of key vestibular structures. Therefore, using zebrafish as a model organism, we observed for changes to inner ear morphogenesis following late exposure to an RA agonist or antagonist. In doing so, we identified novel RA-responsive transcriptional targets in the inner ear and defined a role for this pathway in semi-circular canal morphogenesis and otolith maintenance. Our work has identified unique roles for this pathway at later stages of development, signifying that the disease phenotypes observed in human patients or other model

organisms are not solely the result of early disruptions to RA signaling prior to or at the onset of otic development.

In trying to identify biological tools that could be used in the analysis of later stages of inner ear development, we performed a screen of transgenic zebrafish lines at the National Institute of Genetics in Japan. At this institute, the Kawakami laboratory has utilized the Tol2 transposon system to generate various gene or enhancer trap lines expressing a zebrafish-optimized form of yeast Gal4 in various embryonic and larval tissues. Through our screen, we identified six lines with unique otic expression patterns that can be used to drive the expression of a visual reporter or other genetic constructs within the ear. Each line can therefore be used in future research to visualize and measure distinct vestibular features of the inner ear or manipulate target gene expression in these structures.

Preface

This thesis is an original work by Kacey Mackowetzky. This study, of which this thesis is a part, has received the necessary research ethics approval from the University of Alberta Animal Policy and Welfare Committee. The author of this thesis has met the Canadian Council on Animal Care (CCAC) mandatory training requirements for animal users on the Care and Use of Animals in Research, Teaching, and Testing.

A portion of Chapter 1 is in revision at the *Journal of Anatomy*:

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Kacey Mackowetzky wrote the main body of this manuscript, the information presented in Table 1, and the figure legends for Figures 1-4. Kevin Yoon drew the illustrations for Figures 2-4 and edited the manuscript. Emily Mackowetzky drew the illustration for Figure 1. Andrew J. Waskiewicz was the supervisory author and edited the manuscript.

In Chapter 3, the equipment and software utilized in the behavioral assay was provided by Dr. Keith Tierney, with Danielle Philibert providing the necessary training. Hayley Todesco and Jainil Doshi contributed to this assay by editing and confirming the larval traces generated by Noldus.

In both Chapter 3 and 4, the enhancer and gene trap GFF transgenic lines were developed by the Kawakami laboratory at the National Institute of Genetics, Japan. They were provided to us following an in-person screen by the author at their facility.

Unless otherwise specified, the data presented in this thesis is the author's original work.

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List of Common Symbols, Nomenclatures, and Abbreviations

ºC	Degrees Celsius
a	Anterior protrusion
ac	Anterior crista
ACUC	Alberta's Animal Care and Use Committee
AD	Autosomal dominant
ADH	Alcohol dehydrogenase
ALDH/RALDH	Aldehyde (Retinaldehyde) dehydrogenase
all	Anterior lateral line ganglia
ap	Anterior protrusion
AP	Anteroposterior
apl	Anterior pole
AR	Autosomal recessive
ARAT	Acyl-CoA:retinol acyltransferase
av	Anteroventral otic vesicle
BCIP	5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt solution
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CCAC	Canadian Council for Animal Care
CCMS	Cerebrocostomandibular syndrome
CCTV	Closed-circuit television
CHARGE	Coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, and ear abnormalities syndrome
CRABP	Cellular retinoic acid-binding protein
CRBP	Cellular retinol-binding protein
CSD	Cochleosaccular degeneration
DEAB	N,N-diethylaminobenzaldehyde
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
dls	Dorsolateral septum

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DV	Dorsoventral
dpf	Days post fertilization
ed	Endolymphatic duct
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EM	Embryo media
FGF	Fibroblast growth factor
Flp	Flippase recombinase
FRT	Flippase recognition target
GFF/Gal4FF	A zebrafish-optimized form of Gal4
GFP	Green fluorescent protein
h	Hours
hpf	Hours post fertilization
kb	Kilobase
l	lateral protrusion
LADD	Lacrimo-auriculo-dento-digital syndrom
lc	lateral crista
lp	lateral protrusion
LRAT	Lecithin:retinol acyl-transferase
M	Molar
MFDGA	Mandibulofacial dysostosis type Guion-Almeida
mg	Milligram
min	Minutes
ml/mL	Millilitre
mm	Milimeters
mM	Millimolar
mRNA	Messenger ribonucleic acid
MS222	Tricaine methanesulfonate/ethyl 3-aminobenzoate methanesulfate
N	Number (total) of individuals or cases in a population
NCOA/SRC	Nuclear receptor co-activators

NCOR	Nuclear receptor co-repressors
NBT	4-nitro blue tetrazolium chloride solution
ng	Nanogram
NIG	National Institute of Genetics (Mishima, Japan)
nM	Nanomolar
n.s.	Not (statistically) significant
NSD	Neuro-sensory competent domain
OMIM	Online mendelian inheritance in man
OP	Otic placode
os	saccular otolith
OTX	Orthodenticle homeobox 1
ou	utricular otolith
OV	Otic vesicle (otocyst)
p	posterior protrusion
pc	posterior crista
PBS	Phospho-buffered saline
PBSDTT	PBST + 1% DMSO + 0.1% Triton X-100
PBST	PBS + 0.1% Tween-20
PCR	Polymerase chain reaction
PCWH	Peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome, and Hirschsprung disease
PFA	Paraformaldehyde
PDGF	Platelet-derived growth factors
PKA	Protein kinase A
pp	posterior protrusion
PPR	Pre-placodal region
Q	Q-value (from the Tukey Method), obtained by subtracting the smallest mean from the largest and dividing this product by the overall group standard error of the mean; indicates statistical significance between a pair
QT	Q-T interval on an electrocardiogram
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element

RBP	Retinol binding protein
RE	Restriction endonuclease
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
RNAse	Ribonuclease
RXR	Retinoid x receptor
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
s	Saccule
SIX	Sino oculis homeobox
SSC	Saline sodium citrate
SD	Standard deviation
sec	Seconds
SHH	Sonic hedgehog
t	T-value, which measures the size of the difference in relation to the variation in the sample data
TL	Tüpfel long fin
Tm	Melting temperature
u	Utricle
UAS	Upstream activation sequence
UDP	Uridine diphosphate
um	Utricular macula
v	Ventral protrusion
vp	Ventral protrusion
VP16	Herpes simplex virus protein vmw65
WNT	Wingless-related integration site
ZIRC	Zebrafish International Resource Center
µM	Micromolars
µl	Microlitres

List of Gene Abbreviations

Aldh1a2, Raldh2	Aldehyde dehydrogenase 1 family, member A2
Aldh1a3, Raldh3	Aldehyde dehydrogenase 1 family, member A3
Atoh1	Atonal BHLH transcription factor 1
Atp2b1a	ATPase plasma membrane Ca2+ transporting 1a
Atp2b2	ATPase plasma membrane Ca2+ transporting 2
Bcdo2	β,β-carotene-9′,10′-dioxygenase
Bcmo1	β,β- carotene-15,15′-monooxygenase
Bmp2b	Bone morphogenetic protein 2b
Bmp4	Bone morphogenetic protein 4
Cdh2	Cadherin 2, type 1, N-cadherin (neuronal)
Cdh23	Cadherin-related 23
Chd7	Chromodomain helicase DNA-binding protein 7
Chsy1	Chondroitin sulfate synthase
Clic5	Chloride intracellular channel 5
Coch	Coagulation factor C homology, cochlin
Col2a1a	Collagen, type II, alpha 1a
Cyp1b1	Cytochrome P450, family 1, subfamily B, polypeptide 1
Cyp26a1	Cytochrome P450, family 26, subfamily A, polypeptide 1
Cyp26b1	Cytochrome P450, family 26, subfamily B, polypeptide 1
Cyp26c1	Cytochrome P450, family 26, subfamily C, polypeptide 1
Cyp26d1	Cytochrome P450 , family 26, subfamily D, polypeptide 1
Cx30.3	Connexin 30.3
Dl1	Delta-like 1
Dlx3b	Distal-less homeobox 3b
Dlx4b	Distal-less homeobox 4b
Dlx5	Distal-less homeobox 5
Dlx6	Distal-less homeobox 6
Eftud2	Elongation factor Tu GTP-binding domain-containing 2
Epha2	Ephrin receptor a2
Ephb2	Ephrin receptor b2

Esrp1	Epithelial splicing regulatory protein 1
Eya1	Eya transcription coactivator and phosphatase 1
Fgf3	Fibroblast growth factor 3
Fgf8	Fibroblast growth factor 8
Fgf10	Fibroblast growth factor 10
Fgfr2	Fibroblast growth factor receptor 2
Foxg1	Forkhead box G1
Foxil	Forkhead box I1
Gata3	GATA binding protein 3
Gbx2	Gastrulation brain homeobox 2
Gjb2	Gap junction protein beta-2
Gjb6	Gap junction protein beta-6
Gli2	GLI family zinc finger 2
Gli3	GLI family zinc finger 3
Gpr126, Adgrg6	Adhesion G protein-coupled receptor G6
Grh12	Grainyhead transcription factor 12
Has3	Hyaluronan synthase 3
Hapln1a	Hyaluronan and proteoglycan link protein 1a
Hapln3	Hyaluronan and proteoglycan link protein 3
Hmx2	H6 family homeobox 2
Hmx3	H6 family homeobox 3
Hoxa1	Homeobox A1
Hoxa2	Homeobox A2
Jag1	Jagged canonical Notch ligand 1
Kcne1	Potassium voltage-gated channel subfamily E regulator subunit 1
Kcnq1	Potassium voltage-gated channel subfamily Q member 1
Lmo4	LIM domain only 4
Lmx1a	LIM homeobox transcription factor 1, alpha
Lrp2	Low density lipoprotein receptor-related protein 2
Myh9	Myosin, heavy chain 9, non-muscle
Myo7a	Myosin VIIA
Ncs1a	Neuronal calcium sensor 1a
Neurod1	Neuronal differentiation 1

Neurog1	Neurogenin 1
Nr3b2/Esrrb	Estrogen related receptor beta
Ntn1	Netrin 1
Oc90	Otoconin 90
Otog	Otogelin
Otogl	Otogelin-like
Otol1	Otolin 1
Otop	Otopetrin
OtxA	Orthodenticle homeobox A
Otx1	Orthodenticle homeobox 1
Otx1b	Orthodenticle homeobox 1b
Otx2	Orthodenticle homeobox 2
Ptcha	Patched A
Pax2	Paired box 2
Pax2a	Paired box 2a
Pax8	Paired box 8
Pcdh15	Protocadherin-related 15
Pou3f4	POU domain, class 3, transcription factor 4
Raldh1	Aldehyde dehydrogenase 1
Rarαa	Retinoic acid receptor, alpha a
Rarαb	Retinoic acid receptor, alpha b
Rarγa	Retinoic acid receptor, gamma a
Rarγb	Retinoic acid receptor, gamma b
Rdh10	Retinol dehydrogenase 10
Ripply3	Ripply transcriptional repressor 3
Rps26	Ribosomal protein S26
Rxrαa	Retinoid x receptor, alpha a
Rxrαb	Retinoid x receptor, alpha b
Rxrβa	Retinoid x receptor, beta a
Rxrβb	Retinoid x receptor, beta b
Rxrγa	Retinoid x receptor, gamma a
Rxrγb	Retinoid x receptor, gamma b
Sema3e	Semaphorin 3E

Shh	Sonic hedgehog signaling molecule
Six1	SIX homeobox1
Slc12a2	Solute carrier family 12, member a2
Slc26a4	Solute carrier family 26, member a4
Smo	Smoothened, frizzled class receptor
Snrpb	Small nuclear ribonucleoprotein polypeptides B and B1
Snx10	Sorting nexin 10
So	Sine oculis
Sox2	SRY-box transcription factor 2
Sox10	SRY-box transcription factor 10
Stm	Starmaker
Tecta	Tectorin alpha
Tectb	Tectorin beta
Tbx1	T-box transcription factor 1
Tbx10a	T-box transcription factor 10a
Tfap2a	Transcription factor AP-2 alpha
Ugdh	UDP-glucose 6-dehydrogenase
Ush1c	Usher syndrome 1c, harmonin
Vcana	Versican a
Vcanb	Versican b
Wnt1	Wingless-type MMTV integration side family, member 1
Wnt3a	Wingless-type MMTV integration side family, member 3A

Chapter 1: Introduction

A portion of Chapter 1 (part 1.1) is in revision with the *Journal of Anatomy*: Mackowetzky, K., Yoon, K. H., Mackowetzky, E., Waskiewicz, A. J., 2021. Development and evolution of the vestibular apparatuses of the inner ear.

1.1 Review of the development and evolution of the vestibular apparatuses of the inner ear

1.1.1 Introduction

Our sensory organs provide us with a way to interact with the world by detecting chemical or physical stimuli, either internally or within our environment, and transducing these signals along neural circuits to the brain for processing and response determination (Vosshall & Carandini, 2009). The inner ear is one of the most fascinating sensory organs because it is responsible for two key systems of perception, the auditory system for perceiving sound and the vestibular system for sensing balance and spatial orientation ("Auditory and Vestibular Systems," 2005). Research into both systems is essential for understanding the diseases that impact inner ear function, but as 1-2 out of every 1,000 children born in the United States suffer from a hearing impairment, this has attracted a greater deal of research into the auditory system (Centers for Disease Control and Prevention, 2017). However, the necessity for research into the vestibular system remains, given that approximately 3.3 million (5.2%) children between the ages of 2-17 in the United States suffer from some form of vestibular dysfunction, as characterized by delayed motor development or chronic dizziness and balance issues (C.-M. Li, Hoffman, Ward, Cohen, & Rine, 2016). Head and neck injuries resulting in lesions to the inner ear and its internal circuit to the brain and congenital disorders whose symptoms do not present until later in life can also result in vestibular dysfunction, usually in the form of bouts of vertigo, the illusion of spinning or tilting, either of the environment or oneself (Gurr & Moffat, 2001; Kolev & Sergeeva, 2016). This illusory perception of motion confers physical risk, although sensory impairments associated with the inner ear also have an impact on mental health. Notably, disabilities related to inner ear dysfunction, particularly those that are acute, also contribute to a higher incidence of anxiety and depression (Yuan, Yu, Shi, Ke, & Zhang, 2015). Therefore, to combat health-related issues caused by vestibular disorders,

research into the development of the inner ear is essential for treating patients suffering from inner ear dysfunction and identifying preventative methods for birth defects associated with the vestibular system.

A growing body of work invested in comprehending the complex developmental programme behind the vestibular apparatuses of the inner ear has provided an extensive foundation for understanding diseases involved in this system. In fact, recent research in animal models has already helped to identify the genetic factors behind some disorders that include vestibular dysfunction, such as Branchio-oto-renal syndrome, Pendred syndrome, Jervell and Lang-Nielsen syndrome, Delpire-McNeill syndrome, and Usher syndrome (Table 1.5.1)(Ceruti, Stinckens, Cremers, & Casselman, 2002; Kulkarni, Rajput, Raglan, Abrams, & Bitner-Glindzicz, 2012; McNeill et al., 2020; Stinckens et al., 2001; Weil et al., 1996). Therefore, the purpose of this review is to give an overview of what is known about inner ear anatomy and function and to summarize the exciting research that has already been conducted on the development and evolution of this intricate sensory organ.

1.1.2 Anatomy and Function of the Inner Ear Vestibular System

The inner ear is a complex, labyrinthine structure situated within the temporal bone on each side of the head (Figure 1.6.1)(Bruss & Shohet, 2019). In humans, it is composed of a central vestibule connected to the cochlea on one side, which houses the auditory machinery of the ear, and three orthogonally situated semi-circular canals on the other. A series of mosaic patches—composed of mechanoreceptive hair cells and their support cells housed within the central vestibule and the semi-circular canals—represent the vestibular sensory organs of the ear.

The first of these vestibular organs, the semi-circular canals, each terminate in a sac that houses an sensory epithelium, or 'crista,' composed of hair cells and support cells sheathed by a gelatinous mass (Mescher, 2010). This gelatinous mass, known as cupula, extends from each sensory epithelia to the ampulla, a bulge situated directly across from an epithelium within a given canal. The hair cells within this epithelium are covered by stereocilia, mechanoreceptors that, when deflected, trigger the release of a chemical transmitter to the nerve terminals synapsed to the base of these cells (Barrett, Barman, Boitano, & Brooks, 2012). The deflection of these stereociliary bundles in the semi-circular canals is triggered in a unique way during angular, or 'rotational,' accelerations of the head. When the head turns, the inner ear moves with it, but the endolymph, a potassium-rich fluid contained within the inner ear, remains inert. Because this fluid remains stationary, it exerts force against the cupula, deflecting the stereocilia within to sense the speed of rotation relative to the axis of the semi-circular canal.

The second group of vestibular organs are known as the maculae, two sensory epithelia positioned such that one, the saccule, is oriented vertically within the vestibule, while the other, the utricle, is oriented horizontally (Purves et al., 2001). As such, they typically detect motion in the vertical and horizonal planes, respectively, although their anatomy and function differ from that of the semi-circular canals. Instead of detecting angular rotations, these sensory organs are responsible for detecting linear accelerations and the position of the head relative to gravity. Additionally, although the hair cells within the maculae are similarly covered by a heavy, gelatinous mass, it is embedded with small, calcium carbonate crystals known as otoliths or otoconia. This otolithic membrane moves according to the direction of gravity, such that when the head is tilted, the membrane shifts, bending the stereocilia of the underlying hair cells (Hain & Helminski, 2007).

Another important, though non-sensory, component of the vestibular system is the endolymphatic duct, which extends dorsally from the utricular and saccular ducts housed within the central vestibule and through the vestibular aqueduct to connect with the endolymphatic sac, which is partially contained between the bony labyrinth surrounding the ear and the posterior cranial fossa (Lo et al., 1997). While endolymph is produced primarily by the various secretory cells surrounding the maculae, this endolymphatic duct and sac system is predominantly responsible for regulating inner ear pressure by either decreasing it through the resorption of endolymph or by increasing it through the production of proteoglycans to attract and bind water (Wackym et al., 1987).

1.1.3 Overview of Development of the Vestibular Structures of Inner Ear

While vestibular disorders can arise from physical trauma to the head or neck (Kolev & Sergeeva, 2016), they can also occur from the improper development of the vestibular structures previously described. As such, understanding the developmental programme of the inner ear and identifying the genes involved in this process can be informative for elucidating the mechanisms behind inner ear dysfunction, whether they appear at birth or manifest later in life. This in turn allows for the identification of potential targets for gene therapy and the development of chemical therapeutic agents for preventative strategies and treatment of vestibular defects.

The aim of the following sections is to discuss some of the most important discoveries already made in animal models concerning the developmental programme of the inner ear. It will outline the first step of inner ear development, the induction of the otic placode, which is the source of all inner ear cell types, and the subsequent formation of the otic vesicle, a closed off portion of the neural ectoderm that is subjected to signaling cues that pattern, or confer an identity on, the

various otic cell types that will eventually arise in this sensory organ (Figure 1.6.2). These sections will also provide a summary description of how the vestibular structures are thereby formed, highlighting genes that have also been identified in human patients suffering from vestibular disorders, some of which can be found in Table 1.

1.1.4 The Otic placode and Formation of the Otic Vesicle

Important discoveries have already been made concerning the developmental plan of the inner ear leading up to the induction of an ectodermal thickening known as the otic placode. Studies in zebrafish have demonstrated that Bone Morphogenetic Protein (BMP) signaling in the late blastula and early gastrula stages of development establishes the competence of the pre-placodal region (PPR) within the ectoderm at the neural plate border where the otic placode originates by inducing the expression of the inner ear marker *foxi1* and other transcription factors (Figure 1.6.2)(Kwon, Bhat, Sweet, Cornell, & Riley, 2010). It is also known that the PPR does not expand from the head into the embryonic trunk due to canonical WNT signaling, which instead promotes neural crest formation posterior to this placodal region (Litsiou, Hanson, & Streit, 2005). Subsequently, PPR specification is co-operatively achieved by the dorsal expression of Fibroblast Growth Factors (FGF) and Platelet-derived Growth Factors (PDGF), which promote the expression of BMP-antagonists. Studies using the aquatic frog *Xenopus* have unveiled another key player for PPR specification, the Retinoic Acid (RA) signaling pathway, which both restricts the posterior domain of *fgf8* expression in the PPR and induces the expression of the posteriorly restricted PPR genes *tbx1* and *ripply3* (Arima et al., 2005; Janesick, Shiotsugu, Taketani, & Blumberg, 2012; Shiotsugu et al., 2004).

Following PPR induction, this region is segregated into several domains fated for the placodal development of different sensory tissues, such as the Otic/Epibranchial Precursor Domain that gives rise to both the otic and epibranchial placodes (Figure 1.6.2)(Freter, Muta, Mak, Rinkwitz, & Ladher, 2008; Ohyama, Mohamed, Taketo, Dufort, & Groves, 2006; Gerhard Schlosser & Ahrens, 2004). This entire domain is defined in zebrafish by the ectodermal expression of the Pax transcription factors, *pax2a* and *pax8*, although the subsequent gradient expression of *pax2a* within this region during somitogenesis further defines the individual placodal fates in zebrafish alone; lower levels favour an epibranchial fate, while higher levels direct an otic fate through the promotion of canonical WNT signaling (McCarroll et al., 2012; Ohyama et al., 2006; Gerhard Schlosser & Ahrens, 2004). However, in *Xenopus* and chick, otic placodal fate relies instead on the expression of *Gbx2* (Steventon, Mayor, & Streit, 2012). It is also important to note that, while various cell fates are not specified until later patterning of the ear, *foxi1*, which enables the expression of *pax8* at this time, provides otic cells with the competence to embark on a neuronal fate, and *dlx3b*/*4b*, which subsequently enable *pax2a* activation, then promote sensory fate while restricting neuronal fate (Hans, Irmscher, & Brand, 2013; Hans, Liu, & Westerfield, 2004). Other factors implicated in otic induction that are highly conserved across vertebrates include Fgf3 and Fgf8, with additional FGFs participating in this process in a species dependent manner (Alvarez et al., 2003; Freter et al., 2008; Ladher, Wright, Moon, Mansour, & Schoenwolf, 2005; Léger & Brand, 2002; D. Liu et al., 2003; Mansour, Goddard, & Capecchi, 1993; Maroon et al., 2002; K. Martin & Groves, 2006; Maulding, Padanad, Dong, & Riley, 2014; Padanad, Bhat, Guo, & Riley, 2012; B.-Y. Park & Saint-Jeannet, 2008; B. T. Phillips, Bolding, & Riley, 2001; Wright & Mansour, 2003). While the loss of activity from any one FGF gene does not appear to

prevent otic induction, impairing at least two members of this family of signalling molecules will result in a complete loss or deficiency of otic tissues.

Once the otic placode is induced, it invaginates into an otic cup in amniotes (Figure 1.6.2A) or cavitates in fish (Figure 1.6.2B) to then form the otic vesicle/otocyst (Kaufman, 1992; Maroon et al., 2002; G. Schlosser & Northcutt, 2000). While the molecular mechanisms behind its cavitation in fish are yet unknown, it is understood that the invagination of this vesicle in amniotes first requires an expansion at the basal aspect of the otic cells, followed by an apical constriction (Sai, Yonemura, & Ladher, 2014). Both stages are driven by the activation of myosin-II, although it triggers the depolymerization of actin basally, in response to localized FGF signaling, and actomyosin contraction apically, which is a RhoA-dependent process (Sai & Ladher, 2008; Sai et al., 2014).

1.1.5 Axial Patterning of Inner Ear

During and after vesicle formation, otic cells will take on a neural, sensory, or non-sensory cell fate. Cells destined to take on a neural or sensory fate originate in the neural-sensory-competent domain (NSD) that originates within the anterior region of the developing vesicle (Fekete & Wu, 2002). Cells within this region that express *Neurog1* are specified to become neuroblasts, which delaminate from the vesicle ventrally, form a transient amplifying population, and give rise to the afferent neurons of the vestibulocochlear (VIII) cranial ganglion, whereas cells that instead express *NeuroD1* are later fated to give rise to hair cells (Sapède, Dyballa, & Pujades, 2012). The posterior region of the otic vesicle gives rise to predominantly non-sensory tissues, although the posterior crista is eventually situated within this area. When considering what factors determine the anteroposterior asymmetry of the ear and direct differential cell fates along this axis,

the RA and FGF signaling pathways have been identified as key factors (Bok et al., 2011; Maier & Whitfield, 2014). Studies in zebrafish and chick show that the expression of *fgf3* in the hindbrain confers an anterior identity within the otic placode, while RA signaling, induced by the expression of *aldh1a3* in the head mesenchyme posteroventral to the placode, promotes the posterior expression of *tbx1*, a negative regulator of neurogenesis within the ear (Figure 3A)(Bok et al., 2011; Maier & Whitfield, 2014; Radosevic, 2011; Radosevic, Robert-Moreno, Coolen, Bally-Cuif, & Alsina, 2011; Raft, Nowotschin, Liao, & Morrow, 2004).

Once the otic vesicle is formed, FGF and RA signaling continue to maintain anteroposterior patterning of the ear (Maier & Whitfield, 2014). Both within the anterior aspect of the vesicle and directly ventral to it, the expression of FGFs in zebrafish induce the expression of *aldh1a3* within the posterior region of the NSD to create a localized source of RA signaling (Figure 1.6.3A). At this point, FGF and RA create a negative feedback loop that regulates the development of sensory hair cells. More precisely, high FGF levels of signaling in the anterior otic vesicle promote the expression of *otx1b*, which restricts neurogenesis and promotes the development of structural cells, with lower levels allowing for the development of sensory cells. Concurrently, RA signaling both restricts *otx1b* expression posteriorly to permit the development of sensory cells and instead promotes *tbx1* expression to allow for the maturation of otic neuroblasts. However, the FGF and RA signaling pathways are not the only players in anteroposterior patterning and neurogenesis. Sonic Hedgehog (SHH) signaling is also believed to be involved in this process, as murine $Shh^{-/-}$ mutants display reduced *Neurog1* and *Neurod1* expression, although the underlying mechanism by which this pathway regulates these genes is not yet well understood (M. M. Riccomagno, Martinu, Mulheisen, Wu, & Epstein, 2002).

Patterning of the inner ear is not limited to the anteroposterior axis; in fact, patterning along the dorsoventral axis is crucial for promoting the development of vestibular versus auditory structures of the inner ear. Of course, identities along the dorsoventral axis are shifted more along the anteroposterior axis in fish—for example, the auditory organ of the amniote inner ear, the cochlea, is situated more ventrally than the auditory organ of the zebrafish inner ear, the saccule, which is more posterior—but the same signaling factors are implicated in conferring similar otic identities regardless of the ultimate 'orientation' of the dorsoventral axis (Bok, Bronner-Fraser, & Wu, 2005; K. L. Hammond, Loynes, Folarin, Smith, & Whitfield, 2003; W. Liu et al., 2002; M. M. Riccomagno et al., 2002; Martin M. Riccomagno, Takada, & Epstein, 2005; Waldman, Castillo, & Collazo, 2007). Sonic Hedgehog (SHH), secreted from the floor plate and notochord, has been identified as a key regulator of dorsoventral identities in many species (though 'anteroposterior' identities in fish), as mediated by opposing gradients of the Gli3 repressor within the dorsal domain of the otic epithelium and Gli2/3 activators ventrally (Figure 1.6.3B)(Bok et al., 2007; Brown & Epstein, 2011; K. L. Hammond et al., 2003; Hartwell et al., 2019). An absence of SHH signaling during inner ear development, as achieved in mouse embryos mutant in *Smo*, an essential transducer of this pathway, results in a wide range of morphological abnormalities later in otic development (Brown & Epstein, 2011). These abnormalities include missing ventral otic derivatives, such as the saccular macula and cochlear duct, and the malformation or absence of dorsal derivatives, such as the utricular macula, endolymphatic duct, and semi-circular canals. Complimentary to this data, ectopic SHH signaling within and around the inner ear results in a complete absence of vestibular structures and a malformed cochlear duct (M. M. Riccomagno et al., 2002). Through these studies, it has been determined that SHH is required for specifying otic cells within the ventral region that would normally express the ventral markers *Otx1, Otx2*, and

Pax2, as *Shh*$^{-/-}$ mutants display the reduced or absent expression of these genes. Ectopic SHH signaling has demonstrated that it also regulates cochlear formation through *Brn4* and *Tbx1* expression in the periotic mesenchyme, expanding the former ventrally and the latter dorsally, and through *Pax2* expression in the otic epithelium, which is expanded from the ventromedial region to throughout the vesicle.

However, SHH does not act alone in determining dorsoventral identities. The canonical WNT signaling pathway has also been implicated as a key player in specifying dorsal otic fates, with a loss of signaling during otic development resulting in missing vestibular structures, although due to the fact that some cells populating the cochlea are presumed to be exposed to WNT signals early on in inner ear development, a truncation of the cochlear duct is also observed (Martin M. Riccomagno et al., 2005). As to how WNT specifies dorsal fates, this is accomplished through the secretion of the canonical WNT ligands *Wnt1* and *Wnt3a* from the dorsal hindbrain (Figure 1.6.2B). In *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ mutants, there is a loss of *Dlx5*, *Dlx6*, and *Gbx2* expression in the dorsal region. Conversely, increased WNT signaling, as achieved through lithium chloride treatment, results in the ventral expansion of these genes in wild type embryos and rescued expression in *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ mutants. When taking into consideration any possible cross-talk between SHH and WNT at this stage of development, the observed dorsal expansion of the SHH effector *Gli1* in *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ mutants suggests that WNT acts to restrict SHH signaling to the ventromedial region of the ear. However, the failed restriction of SHH signaling with lithium chloride treatment in these mutants implies that WNT does not act alone in this process.

More recently, the BMP pathway has been suggested to aid in dorsal specification, as seen with the loss of one or more semi-circular canals with reduced signaling in chick embryos, as well as the restriction of SHH in the ear (Figure 1.6.3B)(Ohta & Schoenwolf, 2018; Ohta, Wang,

Mansour, & Schoenwolf, 2016). BMP ligands expressed in the dorsal hindbrain and otic epithelium promote the expression of the dorsal markers *Hmx3*, through the non-canonical activation of cAMP-dependent Kinase A (PKA), and *Dlx5*, through the canonical Smad-dependent pathway (Ohta et al., 2016). Because the PKA phosphorylation of Gli2/3 results in the processing of Gli repressors, non-canonical BMP signaling can negatively regulate SHH through the reduced expression of the SHH target gene *Otx2* via the overexpression of the BMP ligand *Bmp4*. Additionally, loss of BMP signaling in the otic epithelium results in the expansion of both *Otx2* and *Pax2* expression. Together, this suggests that there is an intricate network of cross-talk between these pathways, where SHH acts as a ventralizing agent that favors the development of the auditory structures of the inner ear while WNT and BMP act as dorsalizing agents that predominantly promote the development of more vestibular structures.

The final axis of the inner ear is the mediolateral, although little is known about how it is established. Research in chick embryos show that some of the genes already discussed, such as *Pax2* and *Gbx2*, have distinct dorsomedial expression patterns in the ear (Brigande, Kiernan, Gao, Iten, & Fekete, 2000; Choo et al., 2006). Interestingly, the *Pax2* expression domain is thought to mark one side of the medial-lateral boundary of the inner ear, on the other side of which we see the ventrolateral expression of *SOHo* (Brigande et al., 2000). In fact, the dorsal boundary between the expression pattern of these two genes marks the location of the endolymphatic duct's outgrowth, and $Pax2^{-/-}$ mutants display malformed endolymphatic ducts that are fused with the common crux, the branching point between the anterior and posterior semi-circular canals, and which are often not properly restricted from the saccular compartment (Brigande et al., 2000; Burton, Cole, Mulheisen, Chang, & Wu, 2004). $Gbx2^{-/-}$ mutants also display abnormalities of this channel, most commonly in the form of an absent endolymphatic duct (Choo et al., 2006). Less is

known about how lateral structures of the ear are specified, but fate-mapping studies in chick through the injection of fluorescent carbocyanine dyes into the cells of the rim of the otic cup suggest that the establishment of the mediolateral axis depends on the establishment of the other axes first (Brigande et al., 2000). This research suggests that while the otic vesicle/otocyst is forming, the lateral region of the otic vesicle that gives rise to the canals first originates in the dorsal region.

1.1.6 Vestibular Neuronal and Sensorial Development

While most genes involved in the specification of different cell fates appear once patterning of the otic vesicle is complete, neuronal specification occurs as early as the formation of the Otic/Epibranchial Precursor Domain, with the early expression of *foxi1* imparting the competence for neuronal cell fate and *dlx3b* and *dlx4b* subsequently promoting sensory fate (Hans et al., 2013; Hans et al., 2004). In mouse embryos, once patterning of the otic vesicle is complete and the NSD is defined by FGF and RA signaling, it is within this region that the expression of the additional neuronal and sensory markers *Eya1*, *Six1*, and *Sox2* overlap (Kiernan et al., 2005; P. X. Xu et al., 1999; W. Zheng et al., 2003; D. Zou et al., 2008). Both human *EYA1* and *SIX1* have been implicated in Branchio-oto-renal syndrome, which is usually characterized by hearing impairments and kidney and urinary tract malformations but can also include vestibular abnormalities, such as a truncated posterior semi-circular canal or an enlarged endolymphatic sac and duct (Abdelhak et al., 1997; Ceruti, Stinckens, Cremers, & Casselman, 2002; Melnick, Bixler, Nance, Silk, & Yune, 1976; Ruf et al., 2004). Studies in mice models have shown that loss of either otic *Eya1* or *Six1* results in the decreased expression of the sensorineural markers *NeuorG1* and *NeuroD1* and that their co-overexpression is capable of inducing neurogenesis in the ear (M.

Ahmed, Xu, & Xu, 2012). *Sox2* has also been determined to be required for the expression of both *NeuroG1* and *NeuroD1* and is engaged in an inhibitory feedback loop with these genes, ultimately controlling the number of neuroblasts that give rise to the afferent neurons of the VIII cranial ganglion (Evsen, Sugahara, Uchikawa, Kondoh, & Wu, 2013; Steevens, Sookiasian, Glatzer, & Kiernan, 2017). It is also possible that *Sox2* accomplishes this by interacting with either *Eya1* or *Six1*, as they have already been shown to co-operatively promote the expression of other otic markers in cochlear cell cultures (M. Ahmed et al., 2012). This includes *Atoh1*, which initiates hair cell differentiation in *NeuroD1* positive cells (P. Chen, Johnson, Zoghbi, & Segil, 2002; N. Pan et al., 2011). It is important to also note that Notch signaling plays an important role within this region for determining hair cell and support cell fates (W. Pan et al., 2013; Petrovic et al., 2014). Initially, as directed through the Notch ligand Jag1, Notch-mediated lateral induction induces *Sox2* expression to specify the prosensory domain and prevent early hair cell differentiation. Once *Atoh1* is expressed, it in turn induces *Dl1*, another Notch ligand. This creates a competition between Di1 and Jag1 for Notch, where the former favors the development of support cells and the latter the development of hair cells, creating a latticed pattern of the two cell types within this sensory domain.

Knowing that the NSD gives rise to the various sensory epithelia found in the mature inner ear, it is important to recognize that mediolateral patterning plays a role in how the neuroblasts in the pre-segregated domain are determined to form ganglia of either the vestibular or auditory branches of the VIII cranial nerve. It has been discovered that otic cells that express *Fgf3* within the anterolateral region of this domain delaminate first and generate vestibular neurons (Bell et al., 2008; Koo et al., 2009). Meanwhile, cells that express *Gata3* and *Lmx1a* within the medial region delaminate later and generate auditory neurons (Koo et al., 2009; Koundakjian, Appler, &

Goodrich, 2007; Lawoko-Kerali et al., 2004). However, another possible player in the process is *Myo7A*, which encodes the unconventional myosin VIIA, a critical motor protein that in chick embryos is expressed in differentiating vestibular neurons before subsequently being restricted to the vestibular afferents of the cristae (K. Nguyen, A. L. Hall, & J. M. Jones, 2015). It's possible that *Myo7A* therefore acts in the innervation of these afferents or axonal migration and pathfinding, although most research into this gene has focused on the role it plays later in organizing the stereocilia bundles of vestibular and auditory hair cells and, therefore, its connection to Usher Syndrome type 1 in human patients (Ernest et al., 2000; Gibson et al., 1995; K. Nguyen et al., 2015; Weil et al., 1996).

Further studies have demonstrated that NSD separation into the individual sensory epithelia requires restriction of *Lmx1a* expression to the non-sensory domain in mice (Nichols et al., 2008). As for the separation between more distinct regions of the initial sensory domain, *Fgf10* appears to play an important role in the separation of the cristae, as mutants lack a posterior crista, only producing reduced and malformed anterior and lateral cristae (Pauley et al., 2003). Another player is *Foxg1*, whose null mutants often have fused anterior and lateral cristae (Hwang, Simeone, Lai, & Wu, 2009). *Otx1* also seems to play a role in cristae separation, as null mutants in mice and zebrafish lack a lateral crista, as well as a lateral canal (Fritzsch, Signore, & Simeone, 2001; Katherine L. Hammond & Whitfield, 2006; H. Morsli et al., 1999). However, these mutants also possess a fused utricle and saccule, which additionally implicates *Otx1* in the proper segregation of the two maculae.

1.1.7 Semi-circular Canal Development

The cells that give rise to the semi-circular canals, which are responsible for detecting angular accelerations of the head, originate in a lateral region of the vesicle adjacent to where the presumptive cristae will form (Brigande et al., 2000; W. Chang, J. V. Brigande, D. M. Fekete, & D. K. Wu, 2004). In fact, the cristae are likely responsible for inducing the formation of the canal ducts through the BMP and FGF signaling pathways, as *Bmp4* in mice, chick, and zebrafish is essential for regulating *Bmp2* and *Dlx5*, two key players in canal formation, and *Fgf10*$^{-/-}$ mutants in chick lack all three canals (Weise Chang, John V. Brigande, Donna M. Fekete, & Doris K. Wu, 2004; Chang et al., 2008; Katherine L. Hammond et al., 2009; Omata et al., 2007). *Fgf3* has also been identified as another FGF involved in canal morphogenesis, as knockouts in mice occasionally lack a posterior canal (Mansour et al., 1993; Pauley et al., 2003).

Although there are morphological variations in how the canals are formed across vertebrates, the end result is three semi-circular canals situated at right angles relative to one another. In zebrafish and *Xenopus*, we see an outgrowth of protrusions that bulge into the vesicle and fuse at a central point to form the pillars around which the three canals are formed (Figure 1.6.4A)(Paterson, 1949; Robert Earle Waterman & Danny H Bell, 1984). In both of these species, glycosaminoglycan hyaluronan production is essential for these outgrowths, although other factors discovered in zebrafish have also been implicated in the formation of these protrusions, such as *atropin2*, *atp1a1a.2*, *atp2b1a*, *cdh2*, *grhl2*, *fgf8*, *ncs1a*, and *otx1* (Asai et al., 2006; Babb-Clendenon et al., 2006; Blasiole et al., 2006; Blasiole et al., 2005; Busch-Nentwich, Söllner, Roehl, & Nicolson, 2004; Cruz, Shiao, Liao, Huang, & Hwang, 2009; Haddon & Lewis, 1991; Katherine L. Hammond & Whitfield, 2006; Han et al., 2011; Neuhauss et al., 1996). More recently, *gpr126*, an adhesion G protein-couple receptor gene expressed in the protrusions, has been identified as an

important regulator of genes that encode extracellular matrix core proteins or are involved with modifying the extracellular matrix, such as *chsy1*, *has3*, *hapln1a, hapln3*, *ugdh*, and *vcana* (Geng et al., 2013). Changes to the extracellular matrix can understandably impact the proliferation or migration of cells within a given tissue, directly affecting tissue morphology, although they could also contribute to signal transduction processes. Future research could therefore focus on determining what signaling pathways might direct or be directed by such changes to the extracellular matrix during semi-circular canal morphogenesis.

In amniotes, morphogenesis of the semi-circular canals begins with the formation of two out-pouches, one vertical, situated dorsally, and one horizontal, which forms laterally around the middle of the otocyst (Bissonnette & Fekete, 1996; Fekete, 1999; Kopecky, Johnson, Schmitz, Santi, & Fritzsch, 2012). The canonical WNT signaling pathway is believed to play an essential role leading up to this stage by promoting cell proliferation and repressing apoptosis, likely ensuring that a sufficient number of cells are available for the formation of these out-pouches (Noda et al., 2012). With the ventral out-pouch, opposing epithelial extend toward one another to form a fusion plate, which fuses and then is resorbed through apoptosis to create two canals along the rim, the anterior and posterior, which are connected centrally at the common crus (Figure 1.6.4B). The horizontal out-pouch undergoes a similar process of fusion and resorption to give rise to the lateral canal. Despite the division between horizontal canal formation and that of the anterior and posterior canals in amniotes, *Lmo4* in mouse embryos appears to be responsible for the outgrowth of both pouches by regulating cell proliferation and maintaining the expression of many players in canal morphogenesis, including *Bmp4*, *Fgf10*, *Gata3*, and *Dlx5* (Deng, Pan, Xie, & Gan, 2010). Here also, the canonical WNT signaling pathway has been identified as an important factor behind semi-circular morphogenesis for its role in inducing the expression of *Netrin1*, which

induces fusion and resorption of the fusion plate by promoting periotic mesenchymal proliferation to push the opposing epithelial together and the detachment of the fusion plate from the basement membrane (Noda et al., 2012; Salminen, Meyer, Bober, & Gruss, 2000).

1.1.8 Production and maintenance of endolymph

During its development, the otic vesicle is filled with endolymph, a fluid that is essential for sensory hair cell function and proper otoconia composition. With respect to hair cell function, the endolymph has a high concentration of potassium, which flows through the transducer channels of these cells with the deflection of their stereocilia and, thereby, triggers their depolarization (Battey, 2000; Bosher & Warren, 1968). Upon depolarization, the hair cells release neurotransmitters at their synapses with the afferent neurons of the vestibulocochlear nerve, the first step in triggering the perception of motion or sound. Some genes in human patients with auditory or vestibular impairments, such as those suffering from Jervell and Lang-Nielsen syndrome and Delpire-McNeill syndrome, have been implicated in controlling the composition or homeostasis of endolymph (Kulkarni, Rajput, Raglan, Abrams, & Bitner-Glindzicz, 2012; McNeill et al., 2020). Mutations in animal models often resulting in reduced endolymphatic volume and potential, commonly leading to a shrinkage or collapse of the membranous labyrinth and hair cell death (Blasiole et al., 2006; Casimiro et al., 2001; Cruz et al., 2009; Delpire, Lu, England, Dull, & Thorne, 1999; Dixon et al., 1999; Flagella et al., 1999). These genes include *Slc12a2*, which encodes the basal Na-K-1Cl co-transporter that pumps potassium from the hair cells into the homologous epithelial 'dark' cells surrounding the vestibular sensory epithelia, and both *Kcnq1* and *Kcne1*, the subunits of KvLQT1, a channel on the apical side of dark cells

responsible for returning potassium to the endolymph (Blasiole et al., 2006; Casimiro et al., 2001; Cruz et al., 2009; Delpire et al., 1999; Dixon et al., 1999; Flagella et al., 1999).

Dark cells, like the marginal cells of the cochlea, are also responsible for producing endolymph during otic development, although there appears to be some variation in the composition and function of endolymph-producing cells surrounding the various sensory epithelia between species (Dohlman, 1965; Harada, Tagashira, & Hirakawa, 1989; Hommerich, 1990; Kimura, Lundquist, & Wersaell, 1964; Villegas, Merker, Helling, Clarke, & Scherer, 2001; Wangemann, 1995). However, one gene that has been found to be essential for the development and function of both dark and marginal cells is the estrogen-related receptor beta, *Nr3b2* (J. Chen & Nathans, 2007; Wangemann, 1995). In *Nr3b2*$^{-/-}$ mouse mutants, these cells lack multiple ion channels and transporters and possess the characteristic narrowing of the membranous labyrinth of the semi-circular canals normally found with improper endolymph composition or homeostasis (J. Chen & Nathans, 2007).

1.1.9 Otoconia (otolith) formation and tethering

To enable the detection of gravity and linear accelerations, the maculae rely on a collection of calcium carbonite crystallites embedded in a gelatinous matrix of proteoglycans and proteins that overlay the hair cells (Ross & Pote, 1984). These bio-mineral structures are called otoliths in fish and otoconia in most other vertebrates, and while there are structural differences between the two, there is a significant overlap in the genes responsible for otoconia and otolith formation. Some of these shared genes are involved in endolymph composition, which has a direct effect on the composition of these bio-mineral structures, impacting their size and shape. These include the anion transporter Pendred, which is encoded by *Slc26a4*, and those encoding the enzyme Carbonic Anhydrase, which are together responsible for maintaining an alkaline endolymphatic pH,

primarily through the production or transport of HCO_3^- (L. A. Everett et al., 2001; Kido et al., 1991; H. M. Kim & Wangemann, 2010, 2011; Nakaya et al., 2007; Tsujikawa et al., 1993). Human patients with mutations in *SLC26A4* suffer from the characteristic enlarged vestibular aqueduct associated with Pendred syndrome, which typically results in progressive hearing loss and often episodic vertigo (Stinckens et al., 2001). Other genes encode proteins that are responsible for the mobilization of calcium, such as *Otop1*, which also participates in protein secretion, and *Atp2b2*, a calmodulin-sensitive plasma membrane calcium-ATPase that increases calcium concentration around the maculae (Blasiole et al., 2006; Hughes, Saito, Schlesinger, & Ornitz, 2007; E. Kim et al., 2010; E. Kim et al., 2011; Kozel et al., 1998). The loss of either of these genes in animal models results in the complete absence of otoliths or otoconia (Blasiole et al., 2006; Hughes et al., 2007; H. M. Kim & Wangemann, 2010, 2011; Kozel et al., 1998).

Other key players in otoconia or otolith formation are the proteins that make up the otolithic membrane, which are collectively referred to as otoconins, although there is some variation in the role each gene plays between species. These encode, for example: Oc90, which recruits calcium and other otoconial matrix components to facilitate crystal nucleation and growth; Otolin-1, a collagen that interacts with Oc90 to act as a scaffold in the same processes; and keratan sulfate proteoglycans, which interact with both Oc90 and Otolin-1, as well as other proteoglycans and collagens, to attract calcium (Deans, Peterson, & Wong, 2010; Killick & Richardson, 1997; Lu et al., 2010; Petko, Millimaki, Canfield, Riley, & Levenson, 2008; Xu et al., 2010; H. Yang et al., 2011; X. Zhao, Yang, Yamoah, & Lundberg, 2007). Null mutants in mice and zebrafish in these genes often result in lost or greatly reduced otoconia or otoliths (Deans et al., 2010; Killick & Richardson, 1997; Lu et al., 2010; Murayama, Herbomel, Kawakami, Takeda, & Nagasawa, 2005; Petko et al., 2008; Xu et al., 2010; H. Yang et al., 2011; X. Zhao et al., 2007).

Another important step in otoconia or otolith formation is the tethering of these bio-crystals to their underlying sensory epithelia, a task shared by various glycoproteins. Two such glycoproteins are Otogelin, which is collagenous, and Otogelin-like, which is non-collagenous, and which together are necessary not only for the anchoring of the otolithic membrane to the maculae but also for the anchoring of the cupula to the crista of the semi-circular canals (Bonnet et al., 2013; Cohen-Salmon, El-Amraoui, Leibovici, & Petit, 1997; M. C. Simmler, M. Cohen-Salmon, et al., 2000; M. C. Simmler, I. Zwaenepoel, et al., 2000; Yariz et al., 2012). Other important factors for proper tethering include *α-Tectorin* and *β-tectorin*, which each encode non-collagenous glycoproteins that are expressed solely in the macular sensory epithelia (Legan, Rau, Keen, & Richardson, 1997; Rau, Legan, & Richardson, 1999). In mouse and zebrafish studies, null mutants of *α-Tectorin* have scattered otoconia and reduced otolithic membrane, whereas *β-tectorin* have fewer or fused otoliths (Legan et al., 2000; C. H. Yang et al., 2011).

1.1.10 Evolution of the vertebrate inner ear

To understand the development of an organ, it is important to consider the changes that it has undergone over the course of its evolution. For example, though considered a vertebrate invention, precursors to many inner ear components have been found in species of the Tunicata (Urochordata) subphylum, which possess mechanoreceptor cells embedded in a gelatinous material similar to the hair cells embedded in cupula within the cristae of the vertebrate semi-circular canals (Bone & Ryan, 1978). These cupular organs possess cytologically similar support cells to those found in vertebrates, and their mechanoreceptor cells possess a comparable morphology of microvilli surrounding a non-motile cilium to the stereocilia surrounding the primary kinocilia of vertebrate hair cells, although the base of these non-vertebrate sensory cells

are lacking afferent or efferent synapses. However, these mechanoreceptors are still capable of responding to near-field vibrations, triggering the protective closure of their siphons and retraction of their bodies. Additional similarities to the vertebrate inner ear, as found in ascidian species of the Tunicata, are that these sensory epithelia are found within an atrium that invaginates from the atrial primordia adjacent to the brain, much in the same way the vertebrate otic placode gives rise to the otic vesicle (H. Wada, Saiga, Satoh, & Holland, 1998). This implies that the inner ear is in fact a chordate invention, as even early Cyclostomata were found to have distinct semi-circular canals (Wever, 1974). By looking to the Agnatha, the sister group of jawed vertebrates, we can perhaps further understand how the inner ear more recently changed over the course of evolution to produce the intricate labyrinth gnathostomes possess today.

Currently, the Agnatha have provided us with some clues concerning the anatomical evolutionary origin of the inner ear. Instead of the three orthogonally-oriented canals found in gnathostomes, both Ostracoderms, which were the armored jawless fish of the Paleozoic, and lampreys possess two canals, an anterior and posterior, suggesting that the lateral canal is a more recent acquisition (Higuchi et al., 2019; Mazan, Jaillard, Baratte, & Janvier, 2000). Hagfish, on the other hand, posses one semi-circular canal that was first predicted to have formed around a solitary protrusion. While this initially suggests that the one-canal morphology is an ancestral trait, hagfish posses two ampullae; following the 'one ampulla per canal' theory, this could instead propose a two-canal situation in our common ancestor, one of which hagfish possibly lost over the course of evolution. Even though we are uncertain about the true ancestral state, a recent comparative analysis between extant cyclostomes has reinforced what we know about the highly conserved molecular mechanisms behind inner ear development (Higuchi et al., 2019). For example, in lampreys, it has been discovered that *Tbx1/10A* is expressed in the posterior domain

of the otic vesicle, where it plays a role in specifying the anteroposterior axis of the ear by suppressing genes involved in the neurogenesis and de-epithelialization of vestibular ganglionic cells, much like its homolog in jawed vertebrates, *TBX1* (Higuchi et al., 2019; Radosevic, 2011; Radosevic et al., 2011; Raft et al., 2004). Additionally, dorsoventral specification of the lamprey ear is reliant on Hedgehog signaling, much as it is in gnathostomes, with the expression of *patched A* originating in the ventral region of the vesicle (Higuchi et al., 2019). However, why cyclostomes failed to develop a lateral canal has yet to be determined. It was once assumed that the OTX family of transcription factors were responsible for lateral canal development, as mice mutant in *Otx1* are missing this third canal, but the expression of *OtxA* within the ventral domain of cyclostomes suggests that these transcription factors alone can not account for the evolution of this canal (Acampora et al., 1998; Higuchi et al., 2019; H. Morsli et al., 1999).

Beyond their use in comparative anatomical and molecular analyses, studies of the inner ear of the Agnatha also provide valuable insight into the evolution of motor behaviors. For example, one question that the Agnatha allows us to address is whether three orthogonally-oriented semi-circular canals are required for the detection of rotational accelerations on all three axes. To reiterate, lampreys posses only two canals, lacking the lateral canal found in gnathostomes that is responsible for detecting horizontal accelerations of the head (Maklad, Reed, Johnson, & Fritzsch, 2014). Does this then imply that they are unable to sense horizonal movement to some degree? Current analyses of their locomotion, a rhythmic undulation of the head and body in the horizontal plane, suggests that they would require the perception of horizontal accelerations to move efficiently. Additionally, while the innervation of the lamprey eye is unique, they posses caudal and rostral recti muscles, implying that they should be capable of the vestibulo-ocular reflex (i.e. stabilization of the eye) during horizontal movements of the head (Cohen, 1974; Maklad et al.,

2014). In fact, a more in-depth analysis of the electrophysiological response of their vestibulocochlear nerve shows that stimuli in all planes, including the horizontal, elicit a response, even though the response from each of its two ampullae is somewhat modified from what is observed in higher vertebrates (Maklad et al., 2014). To summarize, this supports the idea that lamprey are able to perceive and react to stimuli on the horizontal plane, despite lacking a lateral canal.

Given that three unique canals are not required for sensing motion in a three-dimensional space, this gives rise to the question of how other changes in inner ear anatomy translate to differences in behavior. Studies have shown that the 'radius of curvature' of a canal, and therefore its overall size, plays a significant role in the agility of a species (Spoor et al., 2007). For example, it is found that primates and other mammals, such as galagos and tarsiers, possess a large canal radius relative to body mass, enabling them to move faster and with less circumspection than other mammals, such as extant sloths, which are well known for their slow locomotion (Billet, Germain, Ruf, de Muizon, & Hautier, 2013; Spoor et al., 2007). This correlation extends beyond mammals, such that when comparing fish with land-based vertebrates of the same size, a much greater curvature to body mass ratio is found, corresponding to a greater sensitivity to angular accelerations (G. M. Jones & Spells, 1963). However, the ears of these examples all possess an orthogonal three-canal system. When considering a species such as the hagfish, which have only a single vertical canal, it is interesting to see what other compensations need to be made to inner ear anatomy to allow for efficient movement (Higuchi et al., 2019; McVean, 2009) Hagfish also make a fascinating research subject for this topic of study because they do not have functional eyes, nor do they have a conventional 'neck,' which means the degree to which their canal needs to measure angular accelerations is not reliant on their ability to stabilize their gaze during motion

(McVean, 2009). However, electrophysiological analyses indicate that hagfish can still detect rotational accelerations and on more than one axis, although with less sensitivity, especially on the horizonal plane. One reason their single canal detects accelerations on multiple axes could be because, unlike other fish, the internal radius of their canal is much larger than its radius of curvature. However, this anatomical difference might actually exist to compensate for the lack of cupula overlying the ampullae in the hagfish ear. Presently, it is thought that the cupula serves to stabilize and protect the hair cell bundles of the ampullae (Dohlman, 1980). During an acceleration, this cupula is displaced, which bends the embedded hair cells and triggers a response. It is therefore hypothesized that a canal's curvature of radius must be large enough to reduce the time required for the cupula to return to its resting position and, therefore, allow the embedded hair cells to detect the next acceleration. In hagfish, this requirement for a large radius of curvature might simply not exist due to the lack of cupula (McVean, 1991). Altogether, while these studies have given us an idea of how inner ear anatomy affects the motor capabilities of a species, there is still a great deal we can learn about how the diverse assortment of species with a rudimentary inner ear system, or no inner ear at all, perceive and respond to motions of the head and body.

1.1.11 Concluding remarks

A great deal of research has been invested in understanding how the inner ear develops and how its developmental programme has evolved over the course of vertebrate history. This has aided the medical field considerably by providing insights into how mutant variants of human genes in animal models impact both their independent cellular function and the overall function of the inner ear. However, while many genetic factors have already been identified and investigated, there are still gaps in our knowledge concerning all the players involved in otic development and

how they interact with other unknown or known factors. Nevertheless, headway has already been made in restoring vestibular function through gene editing tools in some animal models of vestibular diseases. This includes the intraperitoneal injection of antisense oligonucleotides to block the defective cryptic splice site of *Ush1c* or the supplemental knock-in of this gene with synthetic adeno-associated viral vectors to rescue vestibular function in mice models of Usher syndrome (Delmaghani & El-Amraoui, 2020; J. J. Lentz et al., 2013; B. Pan et al., 2017). While it remains to be seen how soon these techniques can be applied to human patients, continuing research into the developmental biology of the inner ear is essential in identifying new targets for this promising area of therapeutics.

1.2 Zebrafish as a model for inner ear development

Since their proposal as a model organism by George Streisinger in the 1970s and 1980s, zebrafish (*Danio rerio*) have been utilized in the biological sciences to elucidate many shared developmental, genetic, and behavioral processes in vertebrates (G. Streisinger, 1984; George Streisinger, Walker, Dower, Knauber, & Singer, 1981). The external fertilization of the zebrafish makes it an ideal model for the preservation of its mother for future experimentation and for the easy observation and genetic manipulation of embryogenesis, which is aided by the embryo's robustness, large size, and early optical transparency (Dahm, 2006). Additionally, its embryonic development is rapid, with the formation of the head, trunk muscles, and other organs beginning as early as 19 hpf. By 4-5 dpf, the main and posterior chambers of its swim bladder inflate, allowing for the observation of complex swimming behaviors, although earlier reflexes, such as burst swimming for escaping predators, are already evident within the first two days of embryogenesis (reviewed in Kalueff et al., 2013; Winata et al., 2009).

While differences exist in the roles of some genes between zebrafish and mammals, this is an advantage in some respects when considering research into therapeutics for human disorders (Jurynec et al., 2008; Rederstorff et al., 2011). For example, zebrafish have the ability to regenerate their hearts, spinal cord, hair cells, retinal neurons, and photoreceptors, which has aided in the discovery of genetic factors involved with the reconstruction of these cell types and tissues without scarring post-injury (Bernardos, Barthel, Meyers, & Raymond, 2007; Goldshmit et al., 2012; Head, Gacioch, Pennisi, & Meyers, 2013; Lush & Piotrowski, 2014; Poss, Wilson, & Keating, 2002; Steiner, Kim, Cabot, & Hudspeth, 2014). However, even with their differences to mammals, zebrafish are still a powerful model organism for inner ear development given the similar anatomy and function of their vestibular apparatuses to that of other vertebrates (Abbas & Whitfield, 2010; Baxendale & Whitfield, 2014). Already, they have contributed to our understanding of several aspects of vertebrate otic development, such as: the establishment of the pre-placodal region of the ectoderm and otic/epibranchial precursor domain; anteroposterior patterning of the otic vesicle; neurogenesis; semi-circular canal development; and otoconia/otolith formation and tethering (Katherine L. Hammond & Whitfield, 2006; Kwon et al., 2010; Maier & Whitfield, 2014; McCarroll et al., 2012; Murayama et al., 2005; Ohyama et al., 2006; Petko et al., 2008; Riley & Moorman, 2000; R. E. Waterman & D. H. Bell, 1984; Tanya T Whitfield et al., 1996; C. H. Yang et al., 2011). Therefore, there is still a great deal we can learn about inner ear development by looking to zebrafish as a model organism.

1.3 Retinoic acid synthesis in the ear and its signaling pathway

Retinoic acid (RA) is a small, polar morphogen derived from vitamin A (all-*trans* retinol) that exists in many forms in the body, although all-*trans*-RA is the primary ligand utilized in

development as a regulator of gene transcription, while 11-*cis*-RA acts as a vital chromophore that mediates dim-light vision in the vertebrate eye (Cunningham & Duester, 2015; Kono, Goletz, & Crouch, 2008). The metabolism of RA begins with the absorption of all-*trans* retinol in the small intestine, where it is esterified into retinyl ester by acyl-CoA:retinol acyltransferase (ARAT) and lecithin:retinol acyl-transferase (LRAT) prior to its transportation through the bloodstream to the liver for storage, though the liver is also capable of synthesizing retinyl esters (Batten et al., 2004; Fortuna, Trugo, & Borojevic, 2001; Helgerud, Petersen, & Norum, 1982; MacDonald & Ong, 1988). When RA is required elsewhere in the body, these esters can be re-hydrolyzed into retinol and bound to retinol binding protein (RBP) for transport to target tissues (Bashor & Chytil, 1975; Muto & Goodman, 1972; Ong & Chytil, 1975). At this point, they can be stored again intracellularly either through re-esterification via LRAT or by being bound to cellular retinol-binding protein (CRBP), which prevents the non-specific oxidation of retinol (Ong & Chytil, 1976; Penzes & Napoli, 1999).

CRBP is also necessary for presenting all-*trans* retinol to Retinol Dehydrogenase 10 (RDH10), a member of the short-chain dehydrogenase/reductase family that oxidizes retinol esters into all-*trans* retinal (retinaldehyde) during prenatal development (G. Duester, 2000; Penzes & Napoli, 1999; Sandell et al., 2007). Members of the alcohol dehydrogenase (ADH) family are also capable of synthesizing all-*trans* retinal from all-*trans* retinol; however, this is accomplished in the absence of CRBP, and the ADH family has only been implicated in essential RA metabolism during postnatal development and growth (Deltour, Foglio, & Duester, 1999; Kedishvili et al., 1998; Molotkov, Deltour, Foglio, Cuenca, & Duester, 2002). Once all-*trans* retinal is made available to the cell, it is finally oxidized into RA primarily by retinaldehyde dehydrogenases (RALDH; also known as ALDH), which are active in specific spatiotemporal domains and whose

targeted deletions result in distinct developmental defects, highlighting them as the rate-limiting enzymes in RA synthesis (Gregg Duester, Mic, & Molotkov, 2003). Studies in mice have identified Raldh1, Raldh2, and Raldh3 as the essential players in mammalian RA metabolism, although Raldh2 plays a dominant role, with mouse *Raldh2* mutants recapitulating widespread vitamin A deficiency phenotypes (Karen Niederreither, Subbarayan, Dollé, & Chambon, 1999; Karen Niederreither, Vermot, Fraulob, Chambon, & Dollé, 2002). Further studies have found that Raldh2 is required for the production of almost all embryonic RA and plays an essential role in somitogenesis, forelimb development, and organogenesis of the kidneys, heart, and lungs, whereas Raldh1 and Raldh3 are primarily required for the development of sensory tissues (Dupé et al., 2003; Fan et al., 2003; Mic, Haselbeck, Cuenca, & Duester, 2002; Karen Niederreither et al., 1999; Karen Niederreither et al., 2002; K. Niederreither, Vermot, Schuhbaur, Chambon, & Dollé, 2002). Notably, *Raldh2* expression in the somites is thought to contribute RA for the regulation of target genes relevant to early otic development that are expressed either within the otocyst or the hindbrain (Gale, Zile, & Maden, 1999; Karen Niederreither, Vermot, Schuhbaur, Chambon, & Dollé, 2000). With respect to the later spatiotemporal expression of *Raldh* genes within the inner ear itself, all three are detected within the otic epithelium, both in the vestibular and cochlear regions (Raymond Romand, Dollé, & Hashino, 2006; Raymond Romand et al., 2004). As to the vestibular expression of *Raldh1*, it is observed in the semicircular canals, common crus, endolymphatic duct, and the lateral border of the utricle (Raymond Romand et al., 2006; Raymond Romand et al., 2004). *Raldh2* is found within the epithelium adjacent to the cristae, presumably the dark cells of the lateral canal, and the non-sensory membranes overhanging the saccule (Raymond Romand et al., 2006; Raymond Romand et al., 2004). Finally, *Raldh3* is expressed

within the endolymphatic duct and the lateral canal, also presumably within the dark cells (Raymond Romand et al., 2006; Raymond Romand et al., 2004).

Zebrafish possess only two retinaldehyde dehydrogenase orthologs, *aldh1a2* and *aldh1a3*, with *aldh1a2* serving as the main producer of RA during embryogenesis while *aldh1a3* expression presents later in development and in more localized regions of the embryo (Cañestro, Postlethwait, Gonzàlez-Duarte, & Albalat, 2006; Silke Pittlik, Domingues, Meyer, & Begemann, 2008; Xi, Yue, & Yang, 2015). During early zebrafish development, *aldh1a2* is first detected at 12 hpf in the paraxial mesoderm and by 15 hpf within the developing somites and head mesenchyme posteroventral to the otic placode (Begemann, Schilling, Rauch, Geisler, & Ingham, 2001; Grandel et al., 2002; Maier & Whitfield, 2014; S. Pittlik & Begemann, 2012). Expression of *aldh1a2* is subsequently found in other developing tissues, although it persists adjacent to the otic vesicle only until 19 hpf, at which point its expression is greatly reduced (Begemann et al., 2001; Grandel et al., 2002; Maier & Whitfield, 2014). However, *aldh1a2* is not the sole producer of RA for the developing ear. As *aldh1a2* expression decreases, *aldh1a3* expression appears around 19 hpf within the anteroventral region of the otic vesicle (Maier & Whitfield, 2014; Silke Pittlik et al., 2008). It persists in this region until 36 hpf, presumably in the anterior macula (Silke Pittlik et al., 2008). *aldh1a3* expression again appears at 48 hpf around the cristae of the semi-circular canals, within the utricular macula, and within a region of the dorsal otic epithelium that marks the endolymphatic duct. Unlike in mice, only one retinaldehyde dehydrogenase, *aldh1a3*, appears to be responsible for providing the zebrafish inner ear with RA during later stages of otic development.

There are other genes involved with RA synthesis, although their contribution to this process during otic development in zebrafish and other model organisms is unclear. This includes

cyp1b1 (*Cytochrome p450, family 1, subfamily B, polypeptide 1*), which is expressed in the ventral region of the inner ear in what appears to be either within or just ventral to the cristae, although it is involved in metabolic processes not limited to RA synthesis and no mutants for this gene have been observed for otic phenotypes (Chambers, Wilson, Maden, & Lumsden, 2007; Murray, Melvin, Greenlee, & Burke, 2001; Ramos, 1999; Sugden, Leonardo-Mendonça, Acuña-Castroviejo, & Siekmann, 2017). Other enzymes involved in RA metabolism are bmco1 (β,β-carotene-15,15′-monooxygenase) and bcdo2 (β,β-carotene-9′,10′-dioxygenase), although the spatiotemporal expression patterns of each gene are far removed from the developing ear (Levi, Ziv, Admon, Levavi-Sivan, & Lubzens, 2012; Saunders et al., 2019).

Following the synthesis of RA, its distribution during embryogenesis is controlled by the Cytochrome p450 subfamily 26 (Cyp26) enzymes, which hydroxylate RA and target it for degradation (Fujii et al., 1997; J. A. White et al., 1996). Zebrafish possess four genes belonging to this subfamily, *cyp26a1*, *cyp26b1*, *cyp26c1*, and *cyp26d1*, although only the first three are found in mice (Fujii et al., 1997; Gu et al., 2006; MacLean et al., 2001; Tahayato, Dollé, & Petkovich, 2003; J. A. White et al., 1996). In mice, all *Cyp26* genes are expressed within the vestibular region of the inner ear. *Cyp26A1* expression is evident first in the ventrolateral otic epithelium and adjacent periotic mesenchyme, particularly surrounding the developing anterior and posterior semi-circular canals (de Roos et al., 1999; Fujii et al., 1997; Raymond Romand et al., 2006; Raymond Romand et al., 2004; Tahayato et al., 2003). Later, it is restricted to the endolymphatic duct (Raymond Romand et al., 2004). *Cyp26B1* is first found in two small isolated areas of the medial and lateral otic epithelium and subsequently in the periotic mesenchyme around the horizontal canal (Raymond Romand et al., 2006). Later in development, it is found within the cristae and the support cells of the maculae (Raymond Romand et al., 2004). *Cyp26C1* expression

is restricted to the ventral otic epithelium and surrounding mesenchyme (Raymond Romand et al., 2006; Tahayato et al., 2003). The only *cyp26* genes expressed within or around the developing zebrafish ear are *cyp26b1*, *cyp26c1*, and *cyp26d1*. At 48hpf, *cyp26b1* is evident in the what appears to be the dorsal otic vesicle (Q. Zhao, Dobbs-McAuliffe, & Linney, 2005). Both *cyp26c1* and *cyp26d1* are expressed much earlier; *cyp26c1* is evident in the head mesenchyme anterior and ventrolateral to the otic vesicle from 19-26 hpf, and *cyp26d1* is evident in the dorsal otic vesicle from 25-42 hpf (Gu, Xu, Wang, Gao, & Zhao, 2005; Maier & Whitfield, 2014). *cyp26c1* is again evident at 48 hpf in the periotic mesenchyme around the ventral aspect of the ear (Bohnsack & Kahana, 2013). Each of these genes is also expressed in the rhombomeres of the hindbrain, a tissue from which many markers are known to regulate otic development (Gu et al., 2005; Kwak, Phillips, Heck, & Riley, 2002; Lecaudey et al., 2007; Maier & Whitfield, 2014; Q. Zhao et al., 2005). Over the course of inner ear development, *cyb26a1* is expressed in the pharyngeal pouches ventral to the ear, but whether it partakes in otic development from this distance is unknown (Isken et al., 2008; van der Velden, Wang, Querol Cano, & Haramis, 2013).

Once RA is made available in the cell, it is transported into the nucleus by cellular retinoic acid-binding protein (CRABP) and presented to retinoic acid receptor (RAR) and retinoid X receptor heterodimers (RXR) (Aström et al., 1991; A. Budhu, Gillilan, & Noy, 2001; A. S. Budhu & Noy, 2002; Cai et al., 2012; Delva et al., 1999). These heterodimers are bound to retinoic acid receptor elements (RAREs) of target genes involved in cellular processes such as proliferation, differentiation, and apoptosis, restricting their transcription by recruiting nuclear receptor co-repressors (NCORs) in the absence of RA (Chatzi, Brade, & Duester, 2011; J. D. Chen & Evans, 1995; De Genaro, Simón, Rotstein, & Politi, 2013; Hu, Gao, Liao, Tang, & Lu, 2013; Janesick et al., 2014; Kurokawa et al., 1995; Molotkova, Molotkov, & Duester, 2007; Rajaii, Bitzer, Xu, &

Sockanathan, 2008; Ribes, Wang, Dollé, & Niederreither, 2006; Weston, Blumberg, & Underhill, 2003). Once RA binds RAR-RXR, a conformational change in the heterodimer promotes the exchange of co-repressors for nuclear receptor co-activators (NCOAs; also known as SRCs), which specifically interact with RA-liganded RAR, the key regulatory component of RAR-RXR (Kashyap & Gudas, 2010; McInerney et al., 1998; Rochel et al., 2011). In zebrafish, there are four *rar* genes (*rarαa, rarαb, rarγa,* and *rarγb*) and six *rxr* genes (*rxrαa, rxrαb, rxrβa, rxrβb, rxrγa,* and *rxrγb*), each of which has a distinct spatiotemporal expression pattern during embryogenesis (Hale et al., 2006; Tallafuss et al., 2006; Waxman & Yelon, 2007). While RXRs can be involved in cellular processes outside RA-mediated transcriptional regulation by forming heterodimeric complexes with other nuclear receptors (including vitamin D receptors, liver x receptors, thyroid-hormone receptors, and peroxisome proliferator-activated receptors), RARs function primarily in the RA signaling pathway (Umesono & Evans, 1989; Willy & Mangelsdorf, 1998). During otic development, all four *rar* genes are expressed within the inner ear, imparting another layer of spatiotemporal regulation of the RA signaling pathway (Maier & Whitfield, 2014).

1.4 Summary and Outline of Research Performed

Our sensory organs provide us with the ability to perceive mechanical, chemical, or thermal stimuli and transduce this information to the brain for interpretation (Vosshall & Carandini, 2009). The stimuli that vertebrate inner ear is specialized to detect include sound and motion, although more research has been invested over the years in understanding how its auditory apparatus, the cochlea, detects sound. The detection of motion relies on the vestibular apparatuses of the inner ear. These include the semi-circular canals, which detect rotational accelerations of the head, and the maculae, sensory patches overlain by small calcium concretions that detect linear accelerations

and gravity (Hain & Helminski, 2007; Mescher, 2010; Purves et al., 2001). Congenital disorders of the inner ear can result in delayed motor development, chronic dizziness, or balance issues from birth, although symptoms can also appear later in life, such as bouts of vertigo, the illusionary spinning or tilting of oneself or the environment (Gurr & Moffat, 2001; C.-M. Li et al., 2016). To understand how dysgenesis of the inner ear contributes to vestibular dysfunction and to develop better treatments for people suffering from this dysfunction, further research needs to be conducted into the development of the vestibular apparatuses of the inner ear.

Much of what we already know about inner ear development pertains to the earliest stages of its developmental programme. This includes the specification of the pre-placodal region along the anterior edge of the neural plate where an ectodermal thickening known as the otic placode will be induced (Arima et al., 2005; Janesick et al., 2012; Litsiou et al., 2005; Hakim Morsli, Choo, Ryan, Johnson, & Wu, 1998; Shiotsugu et al., 2004). This placode is then invaginated in amniotes or cavitated in fish to form the otic vesicle (otocyst), which will be subjected to various signaling cues to pattern the sensory, non-sensory, or neuronal cell types that will form the vestibular apparatuses of the inner ear (Kaufman, 1992; Maroon et al., 2002; G. Schlosser & Northcutt, 2000). One signaling molecule that has already been implicated in early inner ear development is the vitamin A derivative retinoic acid (RA), which binds nuclear receptors upstream target genes to promote their transcription (Kashyap & Gudas, 2010; McInerney et al., 1998; Rochel et al., 2011). Severe embryological malformations of the inner ear arise with either elevated doses of vitamin A or inadequate nutrition during pregnancy; some of these defects include small or missing otic vesicles and epithelial atrophy (Chole & Quick, 1976; Kil et al., 2005; Löhle, 1985; Maden, Gale, Kostetskii, & Zile, 1996; Wackym et al., 1987; J. C. White, Highland, Kaiser, & Clagett-Dame, 2000). Animal models of mutations in the metabolizing enzymes for RA recapitulate these

phenotypes, and exogenous treatment with RA leads to the agenesis or dysgenesis of the maculae and semi-circular canals (Burk & Willhite, 1992; Frenz, Liu, Galinovic-Schwartz, & Van De Water, 1996; Granström, 1990; Jarvis, Johnston, & Sulik, 1990; Karen Niederreither et al., 1999; R. Romand et al., 2002). These malformations are reflected in human infants exposed to retinoids, resulting in the Mondini-Alexander defect, the symptoms of which include an enlarged saccule or utricle, and Michel aplasia, characterized by the agenesis of the inner ear or a rudimentary cavity void of vestibular or auditory structures (Berger, 2014; Lammer et al., 1985).

Research concerning the role RA plays in early inner development reveals that it is involved with several processes, including: (1) regulating the posterior expression of pre-placodal markers; (2) regulating the expression of negative regulators of neurogenesis in the otic vesicle; (3) promoting the development of sensory cells within the neuro-sensory-competent domain of the otic vesicle; and (4) allowing the maturation of the otic neuroblasts that give rise to the afferent neurons of the vestibulocochlear cranial ganglion (Arima et al., 2005; Bok et al., 2011; Janesick et al., 2012; Maier & Whitfield, 2014; Shiotsugu et al., 2004). Given the severity of inner ear malformations involving later structures of the inner ear and the expression of RA metabolizing genes throughout otic development, we hypothesized that this pathway also plays a late role in otic development, beyond the earlier patterning stages and coinciding with the morphogenesis of the canals. However, though research modeling mutations in the metabolizing enzymes of the RA pathway in non-human vertebrates result in the agenesis or dysgenesis of late otic structures, it is difficult to discern if RA plays an independent role at these stages or if earlier phenotypes generated by interfering with this pathway are contributing to or masking those that would appear later.

Therefore, in Chapter 3, we tested our hypothesis through the pharmacological treatment of zebrafish embryos with an RA agonist or antagonist beyond the point at which RA-responsive otic markers have been studied in the literature. Our work identified novel targets of the RA pathway expressed in the protrusions of the semi-circular canals that are involved with the extracellular matrix. We also demonstrate that RA impacts the levels of other signaling pathways involved in semi-circular canal morphogenesis, though unlike studies that investigate the role these independent pathways play in this process, canal morphogenesis is complete, if aberrant. We also find that late treatments with excess RA significantly impact the overall shape of the developing ear. Additionally, we demonstrate that ablating RA late in otic development results in a loss or reduction in the size of the otoliths, implicating a significant role for RA in otolith maintenance, at least in teleosts (Ono, Keller, et al., 2020; Ono, Sandell, Trainor, & Wu, 2020). In conclusion, our work shows that the RA signaling pathway has a unique and previously undescribed late phase of activity in otic development.

During our investigation of a late role for RA in otic development, one of the problems we encountered was the lack of a fast or reliable way to observe inner ear structures in zebrafish. Therefore, we sough to identify transgenic lines that could be utilized to visualize either the whole inner ear or key features of the inner ear during otic development, particularly at later stages. To do so, we performed a screen at the National Institute of Genetics, Japan, to identify zebrafish gene or enhancer trap lines expressing an engineered yeast Gal4 in various structures of the developing ear. In Chapter 4, we provide an analysis of the expression profiles of six lines that could facilitate research at later stages of otic development, either by enabling the visualization of distinct inner ear structures with the help of visual reporters, like GFP, or the manipulation of target gene expression.

1.5 Tables

Table 1.5. 1. Genes associated with vestibular dysfunction in human syndromes or disease phenotypes, as well as their phenotypes in animal disease models. OMIM reference numbers have been included where available.

Human gene name	Possible human behavioral or anatomical phenotypes	Animal model gene name and disease phenotypes	Citations
CADHERIN 23; CDH23 (605516)	- Delayed motor skills associated and vestibular dysfunction - Associated with Usher syndrome, type 1D (601067) and typed 1D/F digenic (601067); Deafness, autosomal recessive 12 (601386); and Pituitary adenoma 5, multiple types (617540)	- *Cdh23* (mouse): hyperactivity, head-tossing, and circling behavior; disorganized stereociliary bundles - *cdh23* (zebrafish): balance defects and circling swimming behavior; disorganized stereociliary bundles	- (Bolz et al., 2001) - (Di Palma et al., 2001) - (Holme & Steel, 2002) - (Smith, Lee, et al., 1992) - (Söllner et al., 2004) - (T. Wada et al., 2001) - (Q. Y. Zheng et al., 2004)
CHLORIDE INTRACELLULAR CHANNEL 5; CLIC5 (607293)	- Vestibular dysfunction later in life - Associated with Deafness, autosomal recessive 103 (616042)	- *Clic5* (mouse): impaired balance; reduced number and increased degeneration of hair cells; abnormal or missing stereocilia	- (Gagnon et al., 2006) - (Seco et al., 2015)
CHROMODOMAIN HELICASE DNA-BINDING PROTEIN 7; CHD7 (608892)	- Semi-circular canal hypoplasia or agenesis - Vestibulocochlear nerve hypoplasia - Associated with CHARGE syndrome (214800) and Hypogonadotropic hypogonadism 5 with or without anosmia (612370)	- *Chd7* (mouse): head-shaking and circling; truncation or size-reduction of the posterior and lateral semi-circular canals; improper fusion within the central pouch prior to canal formation	- (E. A. Bosman et al., 2005) - (Jongmans et al., 2008) - (Van de Laar et al., 2007) - (Vissers et al., 2004)
COAGULATION FACTOR C HOMOLOGY; COCH (603196)	- Variable vestibular dysfunction - Caused by mucopolysaccharide depositions within the inner ear, resulting in	- *Coch* (mouse): vestibular dysfunction (as determined through analysis of vestibular-evoked potential)	- (S. M. Jones et al., 2011) - (Khetarpal, Schuknecht, Gacek, & Holmes, 1991) - (Manolis et al., 1996) - (Robertson et al., 2006)

	the degeneration of dendritic fibers - Associated with Deafness, autosomal recessive 110 (618094) and autosomal dominant 9 (601369)		
ELONGATION FACTOR Tu GTP-BINDING DOMAIN-CONTAINING 2; EFTUD2 (603892)	- Agenesis of lateral canals - Associated with Mandibulofacial dysostosis, Guin-Almedia type (MFDGA) (610536)		- (Gordon et al., 2012)
EPHRIN RECEPTOR EPHA2; EPHA2 (176946)	- Enlarged vestibular aqueduct -Associated with Pendred syndrome (274600) and Cataract 6, multiple types (116600)		- (M. Li et al., 2020)
EPHRIN RECEPTOR EPHB2; EPHB2 (600997)	- Enlarged vestibular aqueduct - Associated with Pendred syndrome (274600); Bleeding disorder, platelet-type, 22 (618462); and prostate cancer/brain cancer susceptibility, somatic (603688)	- *Ephb2* (mouse): abnormal otoconia; absence of the endolymphatic duct; dysplasia of endolymphatic fluid space (particularly of the semicircular canals); mis-localized endolymphatic sac ion-transport cells	- (Cowan, Yokoyama, Bianchi, Henkemeyer, & Fritzsch, 2000) - (M. Li et al., 2020) - (Raft et al., 2014)
EPITHELIAL SPLICING REGULATORY PROTEIN 1; ESRP1 (612959)	- Hypoplasia of the lateral semi-circular canal (deficiency restricted to the central bony island) - Associated with Deafness, autosomal recessive 109 (618013)	- *Esrp1* (mouse): common crus and lateral semi-circular canal dysgenesis; low incidence of central cyst without vestibular outgrowths	- (Rohacek et al., 2017)
EYA TRANSCRIPTION COACTIVATOR AND PHOSPHATASE 1; EYA1 (601653)	- Enlarged vestibule; enlarged vestibular aqueduct; lateral semi-circular canal dysplasia - Associated with Otofaciocervical	- *Eya1* (mouse): inner ear agenesis; truncated endolymphatic duct, truncated anterior or posterior ampullae	- (Ceruti et al., 2002) - (Kozlowski, Whitfield, Hukriede, Lam, & Weinberg, 2005) - (Namba, Abe, Shinkawa,

	syndrome (166780); Anterior segment anomalies with or without cataract (602588); Branchio-otic syndrome 1 (602588); and Branchio-oto-renal syndrome 1 with or without cataracts (113650)	and canals; absent semi-circular canals; small or missing maculae; reduced number of hair cells - *eya1* (zebrafish): imbalanced and circling swimming behavior; reduced response to auditory/vibrational stimuli; small otoliths, absent hair cells in cristae; reduced number of hair cells in maculae	Kimberling, & Usami, 2001) - (Song et al., 2013) - (T.T. Whitfield et al., 1996) - (P.-X. Xu et al., 1999) - (Dan Zou et al., 2008)
FIBROBLAST GROWTH FACTOR 3; FGF3 (164950)	- Delayed gross motor skills in infancy due to balance impairment - Inner ear agenesis (Michel aplasia) or hypoplasia (cystic vestibulum) - Absence of the vestibulocochlear nerve - Associated with Deafness, congenital with inner ear agenesis, microtia, and microdontia (610706)	- *Fgf3* (mouse): inner ear agenesis - *fgf3* (zebrafish): inner ear agenesis	- (Alsmadi et al., 2009) - (Sensi et al., 2011) - (Tekin et al., 2007) - (Tekin et al., 2008) - (Wright & Mansour, 2003)
FIBROBLAST GROWTH FACTOR RECEPTOR 2; FGFR2 (176943)	- Semi-circular canal hypoplasia - Associated with Apert syndrome (101200); Antley-Bixler Syndrome without genital anomalies or disordered steroidogenesis (207410); Beare-Stevenson cutis gyrata syndrome (123790); Bent bone dysplasia syndrome (614592); Craniofacial-skeletal-dermatologic dysplasia (101600);	- *Fgfr-2* (mouse; IIIb isoform): disrupted morphogenesis: dysgenesis of membranous labyrinth, resulting in cystic cavities; rudimentary vestibulocochlear ganglion and sensory patches	- (W.-J. Park et al., 1995) - (Quintero-Rivera et al., 2006) - (T. J. Wang et al., 2002)

	Craniosynostosis, nonspecific (N.A.); Crouzon syndrome (123500); Gastric cancer, somatic (613659); Jackson-Weiss syndrome (123150); LADD syndrome (149730); Pfeiffer syndrome (101600); Saethre-Chotzen syndrome (101400); Scaphocephaly and Axenfeld-Rieger anomaly (N.A.); and Scaphocephaly, maxillary retrusion, and mental retardation (609579)		
GAP JUNCTION PROTEIN, BETA-2; GJB2 (121011)	- Vertigo - Associated with Bart-Pumphrey syndrome (149200); Deafness, autosomal dominant 3A (601544) and autosomal recessive 1A (220290); Hystrix-like ichthyosis with deafness (602540); Keratitis-ichthyosis-deafness syndrome (148210); Karetoderma, palmoplantar, with deafness (148350); and Vohwinkel syndrome (124500)	- *cx30.3* (zebrafish): reduced ear size	- (Chang-Chien et al., 2014) - (Dodson et al., 2011)
GAP JUNCTION PROTEIN, BETA-6; GJB6 (604418)	- Vertigo - Associated with Deafness, autosomal dominant 3B (612643), autosomal recessive 1B (612645), and digenic GJB2/GJB6 (220290); also associated with Ectodermal dysplasia 2, Clouston type (129500)		- (Dodson et al., 2011)

H6 FAMILY HOMEOBOX 2; HMX2 (600647)	- Unsteady, wide-based gait, and delayed walking in children - Mondini malformations: enlarged vestibule and endolymphatic duct (N.A)	- *Hmx2* (mouse): failed enlargement of the pars superior and semi-circular canal aplasia - *hmx2* (zebrafish): larval circling, looping, and spiraling swimming behaviour; mono-otolith (fused otoliths) situated over saccular macula; reduced number of utricular hair cells	- (Feng & Xu, 2010) - (Miller et al., 2009) - (W. Wang, Chan, Baron, Van De Water, & Lufkin, 2001) - (W. Wang, Grimmer, Van De Water, & Lufkin, 2004)
H6 FAMILY HOMEOBOX 3; HMX3 (613380)	- Unsteady, wide-based gait, and delayed walking - Mondini malformations: enlarged vestibule and endolymphatic duct (N.A.)	- *Hmx3* (mouse): loss of hair cells; absence of lateral semi-circular duct crista - *hmx3* (zebrafish): larval circling, looping, and spiraling swimming behaviour; mono-otolith (fused otoliths) situated over saccular macula; reduced number of utricular hair cells	- (Feng & Xu, 2010) - (Miller et al., 2009) - (W. Wang et al., 2004)
HOMEOBOX A1; HOXA1 (142955)	- Semi-circular canal aplasia; common cavity - Associated with Athabaskin brainstem dysgenesis syndrome (601536) and Bosley-Salih-Alorainy syndrome (601536)	-*Hoxa1* (mouse): variable malformations, including dilated membranous labyrinth, incomplete periotic capsule ventromedially, and missing semi-circular canals	- (Lufkin, Dierich, LeMeur, Mark, & Chambon, 1991) - (Pasqualetti, Neun, Davenne, & Rijli, 2001) - (Tischfield et al., 2005)
HOMEOBOX A2; HOXA2 (604685)	- Inner ear agenesis - Associated with Microtia, hearing impairment, and cleft palate (AR) (612290) and Microtia with or without hearing impairment (AD) (612290)		- (Alasti et al., 2008)

JAGGED 1; JAG1 (601920)	- Vestibular pathology, as characterized with disequilibrium when walking - Hypoplasia of the posterior canal and aplasia of the anterior canal - Associated with Deafness, congenital heart defects, and embryotoxon (617992); Alagille syndrome 1 (118450); and Tetralogy of Fallot (187500)	- *Jag1* (mouse): heading-shaking/weaving behaviour and poor negative geotaxis (vertical climbing ability); missing ampullae	- (Le Caignec et al., 2002) - (Tsai et al., 2001)
LIM HOMEOBOX TRANSCRIPTION FACTOR 1, ALPHA; LMX1A (600298)	- Vertigo - Associated with Deafness, autosomal dominant 7 (601412)	- *Lmx1a* (mouse): impaired righting reflex, circling behaviour, hyperactivity, and ataxic gate	- (Bergstrom, Gagnon, & Eicher, 1999) - (Millonig, Millen, & Hatten, 2000) - (Wesdorp et al., 2018)
LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 2; LRP2 (600073)	- Malformation of the semi-circular canal and vestibule - Associated with Donnai-Barrow syndrome (222448)		- (S. Kantarci et al., 2007)
LYSINE-SPECIFIC METHYLTRANSFERASE 2D; KMT2D (602113)	- Malformations associated with Mondini dysplasia: variable aplasia, hypoplasia, or dysplasia of the semi-circular canals and an enlarged endolymphatic sac and duct - Associated with Kabuki syndrome 1 (147920)	- *Kmt2d* (mouse): difficulty maintaining balance and gait abnormalities	- (Igawa, Nishizawa, Sugihara, & Inuyama, 2000) - (Yamamoto et al., 2019)
MYOSIN, HEAVY CHAIN 9, NONMUSCLE; MYH9 (160775)	- Scheibe dysplasia: cochleosaccular degeneration (CSD) - Associated with Deafness, autosomal dominant 17 (603622) and Macrothrombocytopenia		- (Lalwani et al., 1997) - (Lalwani et al., 1999)

	and granulocyte inclusions with or without nephritis or sensorineural hearing loss (155100)		
MYOSIN VIIA; MYO7A (276903)	- Vestibular dysfunction - Associated with Usher syndrome type 1B (276900) and Deafness, autosomal dominant 11 (601317) and autosomal recessive 2 (600060)	- *Myo7a* (mouse): splayed/disorganized stereociliary bundles; hyperactivity, head-shaking, and circling behaviors - *myo7aa* (zebrafish): splayed/disorganized stereociliary bundles; reduced response to auditory/vibrational stimuli and looping or corkscrewing swimming patterns	- (Ernest et al., 2000) - (Gibson et al., 1995) - (Holme & Steel, 2002) - (Kristi Nguyen, Amanda L. Hall, & Jennifer M. Jones, 2015) - (Weil et al., 1996)
OTOGELIN; OTOG (604487)	- Delayed motor development; persistent vestibular hyporeflexia -Associated with Deafness, autosomal recessive 18B (614945)	- *Otog* (mouse): Abnormal head and body posture, abnormal landing pattern (commonly land on back), and circular underwater swimming behaviour; detached otoconia and ampullary cupula	- (Schraders et al., 2012) - (M.-C. Simmler et al., 2000)
PAIRED BOX 2; PAX2 (167409)	- Enlarged chamber of the inner ear - Associated with Papillorenal ('Renal-coloboma') syndrome (120330) and Glomerulosclerosis, focal segmental, 7 (616002)	- *Pax2* (mouse): enlarged central vestibule; absent utricle, saccule, and ampullae	- (Eccles & Schimmenti, 1999) - (Favor et al., 1996) - (Sanyanusin, McNoe, Sullivan, Weaver, & Eccles, 1995)
POTASSIUM CHANNEL, VOLTAGE-GATED, KQT-LIKE SUBUNIT SUBFAMILY, MEMBER 1; KCNQ1 (607542)	- Gross motor developmental delay and impaired balance - Associated with Jervell and Lange-Nielsen syndrome (220400); Long QT syndrome 1 (195200); Short QT	- *Kcnq1* (mouse): head bobbing, intermittent bidirectional circling, and trouble landing on feet/righting themselves; reduced endolymph; shrunken	- (Casimiro et al., 2001) - (Winbo & Rydberg, 2015)

	syndrome 2 (609621); and Atrial fibrillation, familial, 3 (607554)	or collapsed vestibular epithelia; hair cell degeneration	
POU DOMAIN, CLASS 3, TRANSCRIPTION FACTOR 4; POU3F4 (300039)	- Semi-circular canal dehiscence - Reduced vestibular response; mild vestibular hyperactivity (nystagmus) - Associated with Deafness, X-linked 2 (304400)	- *Pou4f3* (mouse) impaired balance; absent hair cells in cristae and maculae; loss of vestibular ganglia	- (Cremers & Huygen, 1984) - (Crovetto, Whyte, Sarasola, Rodriguez, & García-Barcina, 2012) - (Erkman et al., 1996) - (Shine & Watson, 1967) - (Xiang et al., 1997)
PROTOCADHERIN 15; PCDH15	- Delayed motor skills associated and vestibular dysfunction - Associated with Associated with Usher syndrome, type 1F (602083) and typed 1D/F digenic (601067), and Deafness, autosomal recessive 23 (609533)	- *Pcdh15* (mouse): impaired balance; shorter, distorted stereociliary bundles and degeneration of neuroepithelia	- (Z. M. Ahmed et al., 2003) - (Alagramam et al., 2001) - (Raphael et al., 2001) - (Smith, Lee, et al., 1992) - (Q. Y. Zheng et al., 2004)
RIBOSOMAL PROTEIN S26; RPS26 (603701)	- Anomalous vestibule and semi-circular canals - Associated with Diamond-Blackfin anemia 10 (613309)		- (McFarren, Jayabose, Ozkaynak, Tugal, & Sandoval, 2007)
SEMAPHORIN 3E; SEMA3E (608166)	- Semi-circular canal agenesis - Associated with CHARGE syndrome (214800)	- *Sema3a* (mouse): defects in the afferent protrusions of the vestibulocochlear ganglion	- (Katayama, Imai, Suto, & Yoshida, 2013) - (D. M. Martin, Sheldon, & Gorski, 2001)
SIX HOMEOBOX 1; SIX1 (601205)	- Widened semicircular canals; enlarged vestibular aqueduct; Mondini dysplasia - Associated with Branchio-otic syndrome 3 (608389) and Deafness, autosomal dominant 23 (605192)	- *Six1*(mouse): head-bobbing and absence of righting reflexes; inner ear agenesis; truncated semi-circular canals; reduced number of utricular hair cells; missing hair cells in ampullae	- (Ceruti et al., 2002) - (Erika A Bosman, Quint, Fuchs, de Angelis, & Steel, 2009) - (X. Li et al., 2003) - (Ruf et al., 2004)
SMALL NUCLEAR RIBONUCLEOPROTEIN POLYPEPTIDES B	- Agenesis of the inner ear - Associated with Cerebrocostomandibular		- (Hennekam & Goldschmeding, 1998)

AND B1; SNRPB (182282)	syndrome (CCMS) (117650)		
SOLUTE CARRIER FAMILY 12, MEMBER 2; SLC12A2 (600840)	- Delayed motor skills associated with vestibular dysfunction; inability to walk - Associated with Deafness, autosomal dominant 78 (619081), Delpire-McNeill syndrome (619083), and Kilquist syndrome (619080)	- *Slc12a2* (mouse): head bobbing, bidirectional circling, and difficulties with locomotion; collapse of vestibular compartments and epithelia	- (Delpire et al., 1999) - (Dixon et al., 1999) - (Flagella et al., 1999) - (McNeill et al., 2020) - (Mutai et al., 2020)
SOLUTE CARRIER FAMILY 26, MEMBER 4; SLC26A4 (605646)	- Enlarged vestibular aqueduct - Vertigo - Associated with Pendred syndrome (274600) and Deafness, autosomal recessive 4, with enlarged vestibular aqueduct (600791)	- *Pds* (mouse): head tilting and bobbing, circling, unsteady gait, and abnormal reaching response; endolymphatic dilation; abnormal otolith mineralization	- (Dror et al., 2010) - (Lorraine A Everett et al., 2001) - (Stinckens et al., 2001) - (Tsukamoto et al., 2003)
SORTING NEXIN 10; SNX10 (614780)	- Sclerosis of the semi-circular canals - Associated with autosomal recessive osteopetrosis 8 (OPTB8) (615085)		- (Aker et al., 2012)
SRY-BOX 10; SOX10 (602229)	- Semi-circular canal hypoplasia or agenesis - Enlarged central vestibule - Atresia of the vestibulocochlear nerve - Associated with Waardenburg syndrome, type 2E, with or without neurologic involvement (611584); Waardenburg syndrome, type 4C (613266); and PCWH syndrome (609136)		- (Elmaleh-Bergès et al., 2013) - (Sznajer et al., 2008)
TRANSCRIPTION FACTOR AP2-ALPHA; TFAP2A (107580)	- Enlarged vestibule and vestibular aqueduct	- *Ap-2* (mouse): dysmorphogenesis of the inner ear	- (H. Kantarci, Edlund, Groves, & Riley, 2015)

	- Associated with Barnchiooculofacial syndrome (113620)	- *tfap2a* (zebrafish): inhibition of neurogenesis and reduced neural maturation	- (Schorle, Meier, Buchert, Jaenisch, & Mitchell, 1996) - (Tekin, Sırmacı, Yüksel-Konuk, Fitoz, & Sennaroğlu, 2009)
USH1 PROTEIN NETWORK COMPONENT HARMONIN; USH1C (605242)	- Delayed motor skills associated with vestibular dysfunction; difficulty walking - Associated with Usher syndrome, type 1C (276904), and Deafness, autosomal recessive 18A (602092)	- *Ush1c* (mouse): splayed/disorganized stereociliary bundles; reduced response to auditory/vibrational stimuli, hyperactivity, head-tossing, and circling behavior - *ush1c* (zebrafish): reduced number of and disorganized stereociliary bundles; circling swimming behavior and reduced or abnormal evasive swimming behavior	- (J. Lentz, Pan, Ng, Deininger, & Keats, 2007) - (J. B. Phillips et al., 2011) - (Smith, Pelias, et al., 1992)

1.6. Figures

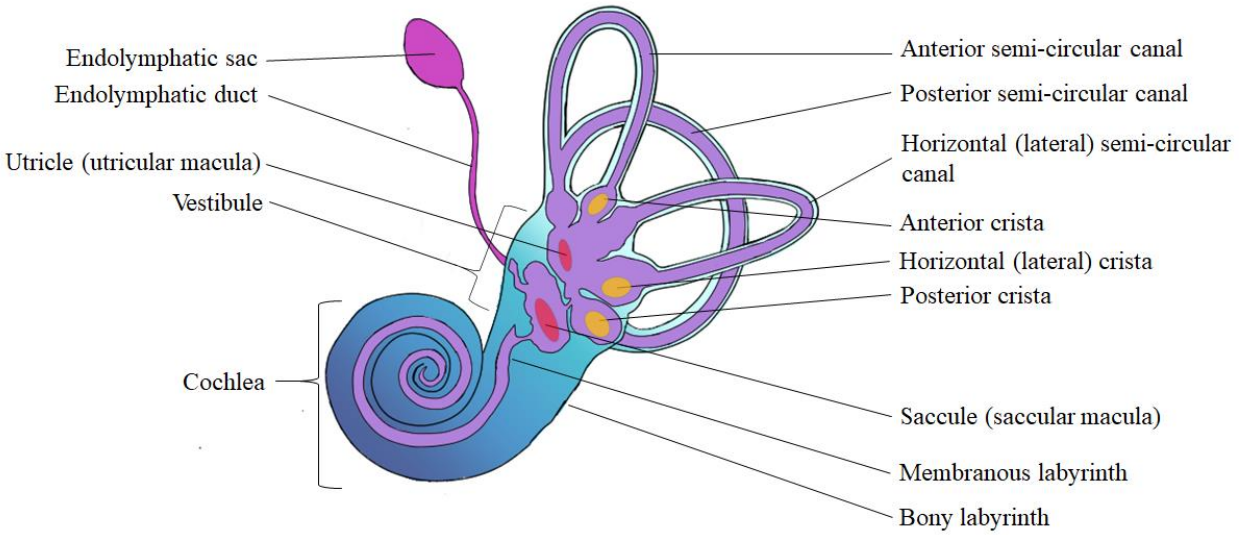

Figure 1.6. 1. Schematic illustration of the adult human inner ear, with an emphasis on the vestibular apparatuses (not to scale). Illustrated by Emily Mackowetzky.

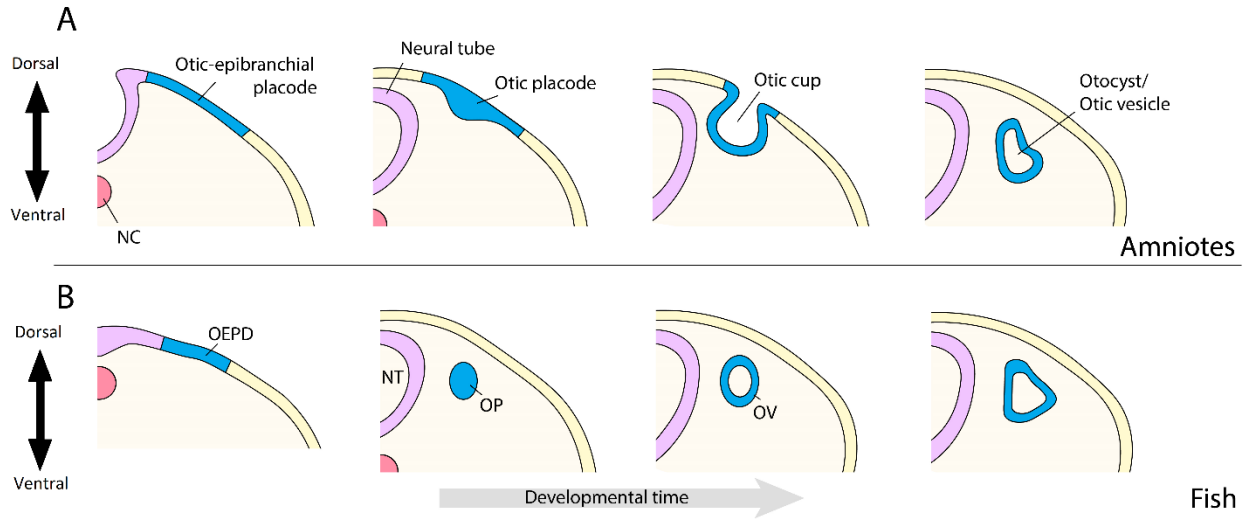

Figure 1.6. 2. Transverse images portraying early inner ear development. The inner ear is formed within the pre-placodal region of the ectoderm that is first segregated into the otic/epibranchial precursor domain. A portion of this domain will then give rise to the otic placode, a thickening of the endoderm that invaginates in amniotes (A) or cavitates in fish (B) to form the otocyst/otic vesicle. This vesicle will be subjected to signaling cues that confer various otic identities on the vesicle and shape it into a labyrinth structure that houses both vestibular and auditory apparatuses. Illustrated by Kevin Yoon.

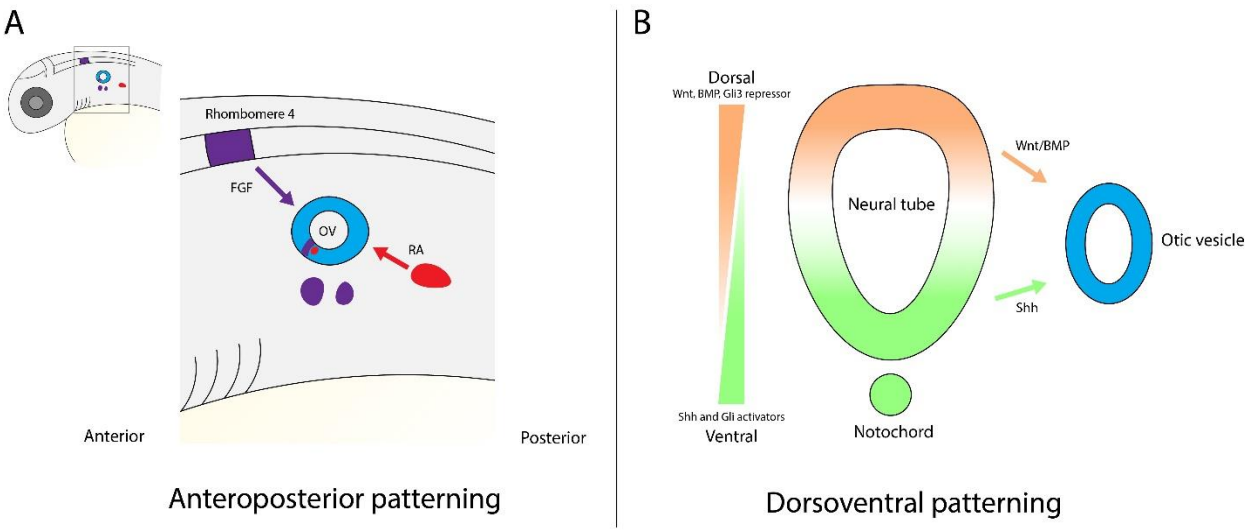

Figure 1.6. 3. Axial patterning regulators of the inner ear. (A) The Fibroblast growth Factor (FGF) and Retinoic Acid (RA) signaling pathways are responsible for conferring anteroposterior identities during early inner ear development. Initially, *fgf3* from the hindbrain confers anterior identities within the placode while RA, metabolized by *aldh1a3* in the head mesenchyme posteroventral to the placode, promotes posterior identities. Once the otic vesicle forms, it continues to be subjected to FGF and RA signaling, particularly within the neuro-sensory domain of the vesicle; at this time, FGFs are also expressed ventral to the ear. (B) Dorsoventral patterning of the ear is regulated by the Wnt, Bone Morphogenetic Protein (BMP), and Sonic Hedgehog (SHH) signaling pathways. SHH, secreted from the floor plate of the hindbrain and the notochord, is mediated by opposing gradients of the Gli3 repressor within the dorsal epithelium of the otic vesicle and Gli2/3 activators within the ventral domain. Wnt signaling is driven by the secretion of ligands from the dorsal hindbrain, whereas BMP signaling is driven by the secretion of ligands from the dorsal hindbrain and within the otic epithelium. Both Wnt and BMP have been implicated in restricting SHH signaling to the ventromedial region of the ear; ultimately, SHH acts as a ventralizing agent within the ear while WNT and BMP act as dorsalizing agents. Illustrated by Kevin Yoon.

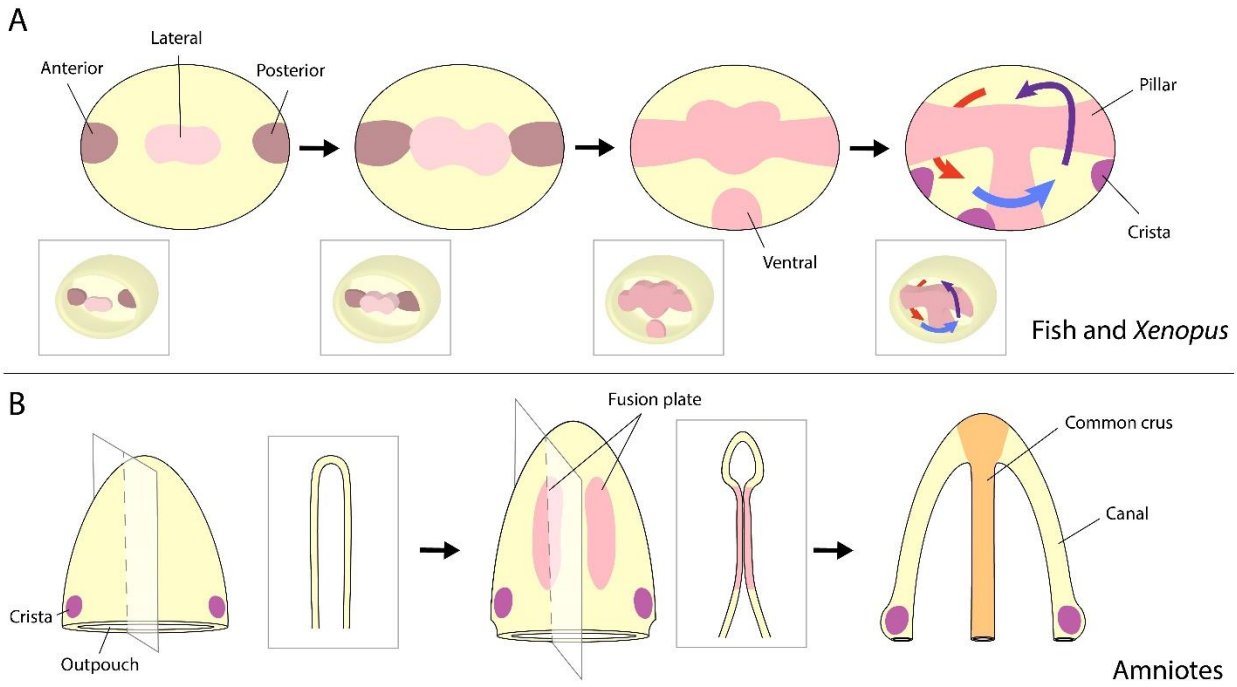

Figure 1.6. 4. Semi-circular canal morphogenesis. The cells that give rise to the canals originate in a lateral region adjacent to the presumptive cristae. However, there are variations between how these canals form in anamniotes, such as zebrafish and *Xenopus*, and amniotes, such as mouse and chick. (A) Canal formation in zebrafish and *Xenopus* begins with an outgrowth of protrusions that bulge inward into the otic vesicle. When these protrusions fuse, they become the pillars around which the canals are formed. (B) In amniotes, the canals are sculpted from two out-pouches, one ventral (shown above) and one horizontal. Fusion plates are formed by opposing epithelium that extend toward one another. Following fusion, these plates are resorbed through apoptosis. In the above example, this process forms the anterior and posterior canals, with the common crux situated between them. Illustrated by Kevin Yoon.

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Chapter 2: Materials and Methods

2.1 Ethics statement

All zebrafish embryonic and adult strains were cared for according to the guidelines laid out by the Canadian Council for Animal Care (CCAC). This study was approved by the University of Alberta's Animal Care and Use Committee (ACUC) - Biosciences (Protocol 427).

2.2 Animal care and fish lines

The database for the transgenic fish generously supplied to us by the Kawakami lab (National Institute of Genetics, Japan) can be accessed here: https://ztrap.nig.ac.jp/ztrap/. These lines were developed by the Kawakami lab using the *Tol2*-transposon-mediated gene and enhancer trap methods previously described (Asakawa & Kawakami, 2009; Asakawa et al., 2008; Kawakami et al., 2016; Takeuchi et al., 2015). The six lines selected during our shelf screen (using the Kawakami lab nomenclature) were *nkhspgff75a*, *nkhspgffdmc12a, nkgsaigff266c*, *nkgsaizgffd262a*, *nkgsaizgffm789a*, and *nkgsaizgffm1225a*. The transgenic lines *nkhspgff75a* and *nkhspgffdmc12a* are enhancer trap constructs with Gal4FF (Also known as 'GFF'), a fusion protein of the Gal4 DNA binding domain and a tandem repeat of the VP16 transactivation domain (2xPADALDDFDLDML), under the control of a *hsp70l* promotor. *nkgsaigff266c*, *nkgsaizgffd262a*, *nkgsaizgffm789a*, and *nkgsaizgffm1225a* are gene trap constructs that contain the *gata6* splice acceptor, GFF, and the internal ribosomal entry site of the encephalomyocarditis virus. The transposon integration sites for *nkgsaigff266c*, *nkgsaizgffd262a*, *nkgsaizgffm789a*, *nkhspgff75a*, and *nkhspgffdmc12a* have already been cloned and determined via inverse PCR by the Kawakami laboratory (Table 4.4.1). Visualization of GFF expression was achieved by outcrossing these strains to transgenic lines containing the *green fluorescent protein* (*gfp*) gene downstream a UAS recognition sequence, *Tg(UAS:gfp)*, from the Zebrafish International Resource

Center (ZIRC; Catalog ID: ZL624). *nkhspgffdmc12a* was used for the visualization of inner ear structures at 72 hours post fertilization (hpf).

Expression of canonical Wnt signaling was analyzed with the *Tg(top:gfp)* canonical Wnt reporter zebrafish line supplied to us by the Dorsky lab (Dorsky, Sheldahl, & Moon, 2002). Expression of Shh signaling was analyzed with the *Tg(ptc2:kaede)* reporter line from the Huang lab (Huang, Xiong, Megason, & Schier, 2012). Wildtype AB and TL fish were utilized for all other experiments.

All Embryos were grown in embryo media (EM) at 28.5ºC and staged according to standard morphological criteria (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Westerfield, 1995). A 20X stock solution of EM can be made from the following: 17.5g NaCl, 0.75 KCl, 2.9g CaCl-2H_2O, 0.41g KH_2PO_4 anhydrous, 4.9g $MgSO_4$-7H_2O, and MilliQ water up to 1 litre; when using a 1X working solution, adjust pH to 7.2-7.4 with $NaHCO_3$. Prior to mRNA extraction, mRNA in situ hybridization analysis, or immunohistochemical analysis, embryos or larvae were manually dechorionated using Dumont No. 5 forceps.

2.3 Pharmacological treatments

To activate retinoic acid (RA) signaling, embryos were exposed to a 5 nM solution of all-*trans*-RA (Sigma, St. Louis, USA). Inhibition of RA synthesis by aldehyde dehydrogenase enzymes was accomplished with 1 µM or 5 µM solutions of Diethylaminobenzaldehyde (DEAB; Sigma, St. Louis, USA) (Maves & Kimmel, 2005). All compounds were dissolved in Dimethyl sulfoxide (DMSO; Sigma, St. Louis, USA), which was used as a solvent control. Working concentrations were diluted with EM. All embryos were treated at 28 hpf onward and grown in a

darkened incubator at 28.5ºC. Treatments were refreshed every 24 hours. Larvae undergoing behavioral analysis were washed with EM prior to testing.

2.4 Behavioral testing

For the behavioral assay, 5 days post fertilization (dpf) larva were individually recorded in a 24 well Corning Costar cell culture plate (Sigma, St. Louis, USA) for 10 minutes between 10.00 to 15.00h using a CCTV camera (WV-CL930; Panasonic) and the Ethovision XT10 software by Noldus (Wageningen, Netherlands). Three larvae from the control group and each treatment group were tested per plate, with a 20-minute acclimatization period prior to recording. Each 18mm diameter well was filled with 2ml EM. The sample arrangement for each plate was randomized and blinded prior to quantification of circling phenotype.

For this experiment, circling phenotype was defined as any continuous and complete 360º revolution in the horizontal plane that did not exceed 4.5mm in diameter (1/4 the diameter of a well). Left- and right-handed circles were counted individually. To account for variations in total distance travelled between samples, circling phenotype was quantified by the total number of circles (left and right) observed per 100cm travelled.

2.5 Template mRNA extraction

Template mRNA was extracted from 50-100 embryos with the RNAqueous-PCR kit (Ambion, Austin, USA). The embryos were then added to a tube containing 350µl of Lysis/Binding solution and homogenized by vortex-based agitation. After 350µl was added to the tube, embryos were again vortexed for 30 seconds (sec). This combined solution was transferred

to a filter column in a collection tube, centrifuged for 1 min at full speed, and the flow-through was discarded. 700µl of Wash Solution #1 was added to the column. The column in the collection tube was again centrifuged for 1 min at full speed, and the flow-through was discarded. Next, 500µl of Wash Solution #2/3 was added to the column in the collection tube, both of which were centrifuged 1 min at full speed, and the flow-through was discarded. The previous step was repeated once. After the column was transferred to a fresh collection tube with a lid, 40µl of pre-heated 70ºC Elution Solution was carefully pipetted onto the filter cartridge within the column. The column and collection tube were then centrifuged 30 sec at full speed. The column was then discarded. At this point, the tube could be stored at -80ºC prior to RNA purification.

2.6 Template mRNA purification

To remove DNA from the eluted RNA, 19µl diethylpyrocarbonate-treated water, 10µl 10X DNase I Buffer (Thermo Scientific, Waltham, USA), and 1µl DNase I (Thermo Scientific, Waltham, USA) was added to the tube and incubated for 30 min at 37ºC. RNA was then purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany): 350µl Buffer RLT + 1% β-mercaptoethanol was added to the tube, which was then vortexed for 5 sec. 250µl 100% ethanol was then added to the solution, which was mixed by pipetting. This solution was transferred to a column inside a collection tube and centrifuged for 15 sec at 10,000 revolutions per min (rpm). The flow-through was discarded. The column was transferred to a new collection tube, at which point 500µl of Buffer RPE was added to the column. The column and collection tube were centrifuged for 15 sec at 10,000 rpm, and the flow-through was discarded. 500µl of Buffer RPE was again added to the column, but this time the column and collection tube were centrifuged for 2 min at 10,000 rpm. The column was then transferred to a new collection tube and centrifuged

for 1 min at full speed to remove the dead volume. The column was again transferred to a new collection tube. 10ul diethylpyrocarbonate (DEPC)-treated water was carefully pipetted onto the filter cartridge, and the column and collection tube were centrifuged 1 min at 10,000 rpm. The quality and quantity of this eluted, purified RNA was assessed by spectrophotometry. RNA then was stored at -80ºC.

2.7 Probe synthesis and purification for whole mount mRNA in situ hybridization

Targeted sequences for probe synthesis were either transcribed from linearized plasmid DNA containing the probe template and a T3 promoter sequence (Table 2.1) or PCR-amplified with the Superscript III One-Step Reverse Transcriptase (RT)-PCR kit with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, USA) to incorporate a T7 promoter sequence (Table 2.2).

To generate template DNA from a plasmid, the plasmid was linearized by digesting it with the appropriate restriction endonuclease (RE) and then purified following electrophoresis on a 1% agarose gel (made with 1X Tris-EDTA buffer). After running the DNA sample on a gel, a band of the desired length was excised with a clean razor blade and placed in a 1.7mL tube. The DNA was then extracted with the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany): 300µl of QG buffer was added to the tube, which was incubated in a water bath at 50ºC for 10 min, with periodic vertexing, until the gel melted. 100µl of 100% Isopropanol was mixed into the solution by pipetting, which was then transferred to a column in a collection tube. The column and collection tube were centrifuged for 1 min at full speed, and the flow-through was discarded. 500µl QG buffer was added to the column, which was again centrifuged for 1 min at full speed. The flow-through was discarded. 750µl PE buffer was added to the filter cartridge of the column and incubated at RT for 5 min. The column was centrifuged in the collection tube for 1 min at full speed and then

transferred to a new 1.7mL tube with a lid. 30-35µl of Elution Buffer was carefully pipetted onto the filter cartridge, and the column was left to stand for 1 min at RT. The column and collection tube were then centrifuged for 1 min at max speed. The eluted DNA could then be stored at -20ºC.

To generate template DNA from mRNA with the Superscript III One-Step Reverse Transcriptase (RT)-PCR kit (Invitrogen, Carlsbad, USA), each reaction was set up as follows: 12.5µl of 2x Reaction mix, 8.5µl of DEPC-treated water, 1µl of 5µM forward primer, 1µl of 5µM reverse primer, 1µl of purified RNA template, and 1µl of Superscript III platinum Taq. The PCR cycle conditions were as follows: (1) 54ºC for 30 min (cDNA synthesis); (2) 94ºC for 2 min (denaturation); (3) 30 cycles of 94ºC for 15 sec (denaturation), 55-65ºC for 30 sec (primer annealing), and 68ºC for 1 min/kb (extension); and (4) 68ºC for 5 min (final extension). The final products were analyzed by gel electrophoresis and could be stored at -20ºC.

RNA probe synthesis was then accomplished with the PCR DIG Probe Synthesis Kit from Roche (Penzberg, Germany) (Thisse & Thisse, 2008). 200-400ng of template DNA was incubated in a tube with the following: 2 µl of 10X transcription buffer (Roche), 2µl of dioxigenin (DIG) RNA labeling mix (Roche, Penzberg, Germany), 1µl RNA polymerase (T7 or T3; Roche, Penzberg, Germany), 1µl RNaseOUT (Life Technologies, Carlsbad, USA), and DEPC-treated water (up to 20µl total volume in the tube). This solution was incubated at 37ºC for 1 hour. Another 1µl of the appropriate RNA polymerase was added, and the solution was again incubated at 37ºC for 1 hour. To remove the template DNA, 1µl of TURBO DNase (Life Technologies, Carlsbad, USA) was added to the solution, which was then incubated at 37ºC for 5 min. The reaction was stopped by adding 2µl of 0.2M EDTA (pH 8) in DEPC-treated water.

Probes were purified using a SigmaSpin™ Sequencing Reaction Clean-Up kit (Sigma, St. Louis, USA): an empty column was placed in a collection tube and centrifuged for 2 min at 2,500

rpm. The base of the column was then removed, and the column was centrifuged in the collection tube for 2 min at 2,500 rpm. The column was then placed in a new collection tube with a lid, and the probe synthesis solution was pipetted into the column. The column and collection tube were centrifuged for 4 min at 2,500 rpm. 0.5µl RNase OUT (Life Technologies, Carlsbad, USA) and 25µl DEPC-treated water were added to the collection tube to prevent degradation by RNAses. The probe was stored at -80ºC. However, working 1:200 probe dilutions in DEPC-treated water could be stored at -20ºC.

2.8 Whole mount mRNA in situ hybridization

Embryos were fixed in 4% paraformaldehyde (PFA) for 4 hours at RT or overnight at 4ºC with gentle agitation and then dechorionated and bleached (3% H_2O_2/0.5%KOH; 15 minutes for 48 hpf embryos and 20-25 minutes for 72 hpf larvae) to remove melanin pigments. Embryos were then washed 3x 5 minutes in phosphate buffered saline (PBS) and permeabilized with 10µg/ml Proteinase K (Sigma, St. Louis, USA) for 30 minutes (Thisse & Thisse, 2008). Embryos were re-fixed in PFA for 20 minutes, followed by 5x 5-minute washes in phosphate buffered saline with Tween-20 (PBST). Pre-hybridization was carried out at 65ºC for approximately 2 hours, and hybridization was carried out using a 1:200 probe dilution at 65ºC overnight. Embryos were then washed at 65 ºC under the following conditions: 5 minutes in 66% hybridization mix + 33% 2x saline sodium citrate (SSC); 5 minutes in 33% hybridization mix + 66% 2x SSC; 5 minutes in 2x SSC; 1x 20 minutes in 0.2x SSC+0.1% Tween-20; and 2x 20 minutes in 0.1x SSC, 0.1% Tween-20. Subsequently, embryos were washed at RT: 5 minutes in 66% 0.2x SSC + 33% PBST; 5 minutes in 33% 0.2x SSC + 66% PBST; and 5 minutes in PBST.

Embryos were blocked with a 2% sheep serum, 2mg/ml bovine serum albumin (BSA) solution for 1 hour and then incubated with a 1:500 dilution of primary antibody (sheep anti-DIG-AP Fab fragments; Roche) for 2 hours. 5x 15-minute PBST washes and 4x 5-minute coloration buffer (100mM Tris, pH 9.5; 50nM $MgCl_2$;100mM NaCl; and 0.1% Tween-20) washes were performed prior to visualization with a nitro-blue tetrazolium (NBT, 45:1000 dilution; Roche, Penzberg, Germany)/5-bromo-4-chloro-3-indolyl phosphate (BCIP, 35:1000 dilution; Roche, Penzberg, Germany) solution. Following coloration, embryos were washed for 15 minutes in PBST (pH 5.5) and either stored overnight at 4 ºC with methanol + 0.1% Tween-20 to prevent excess coloration or immediately mounted for imaging.

2.9 Immunohistochemistry: Active Caspase

Embryos were fixed in 4% PFA for 2 hours at RT with gentle agitation. To remove melanin pigments, they were then dechorionated and bleached (3% H_2O_2/0.5%KOH; 15 minutes for 48 hpf embryos and 20-25 minutes for 72 hpf larvae). Following 3x 20-minute washes with PBST, embryos were washed 1x in MilliQ + 0.1% Tween-20 for 5 minutes and then permeabilized in ice-cold (-20ºC) acetone for 7 minutes. Embryos were again washed in MilliQ + 0.1% Tween-20 for 5 minutes prior to being blocked for 30-90 minutes in PBSDTT (PBST + 1% DMSO + 0.1% Triton X-100) + 5% goat serum (GS). They were then incubated in a solution of Anti-Active Caspase 3 (BD Biosciences, Franklin Lakes, USA) diluted 1:400 in block for 2 hours at RT or overnight at 4ºC with gentle agitation. After being rinsed 2x in PBSDTT, embryos were washed 2x for 20 minutes in PBSDTT and then incubated for 2 hours at RT or overnight at 4ºC with gentle agitation in a solution of Goat anti-Rabbit IgG Alexa Flour 488 (Invitrogen, Carlsbad, USA) and TO-PRO-

3 (Invitrogen, Carlsbad, USA), each diluted 1:1000 in block. Finally, embryos were rinsed 2x with PBSDTT and washed 4x for 15 minutes in PBSDTT.

2.10 Immunohistochemistry: Phospho-histone H3

Embryos were fixed in 4% PFA for 2 hours at RT with gentle agitation, then dechorionated and bleached (3% H_2O_2/0.5%KOH; 15 minutes for 48 hpf embryos and 20-25 minutes for 72 hpf larvae) to remove melanin pigments. They were then washed 3x 5 minutes in PBS, permeabilized with -20ºC acetone for 30 minutes, and washed again 3x 5 minutes in PBST prior to antigen retrieval. This was achieved by incubating embryos in Tris-HCL, pH 9, first for 5 minutes at RT and then 15 minutes at 70ºC (Inoue & Wittbrodt, 2011). Subsequently, embryos were blocked in PBS + 3% BSA + 1% GS + 0.5% Triton X-100 for 1 hour at RT. Embryos were then incubated for 2 hours at RT in a solution of Anti-phospho-histone H3 (Abcam, Cambridge, UK) diluted 1:1000 in block. Following 5x 15-minute washes in PBST, embryos were incubated for 2 hours at RT in a solution of Goat anti-Rabbit IgG Alexa Fluor 488 (Invitrogen, Carlsbad, USA) and TO-PRO-3 (Invitrogen, Carlsbad, USA) each diluted 1:1000 in block. Finally, embryos were washed 5x for 15 minutes in PBST.

2.11 DiI injections into the inner ear

The fabricated micropipette needles used for these injections were prepared by heating and pulling borosilicate glass capillary tubes (Sutter Instruments Inc., O.D.: 1.2mm, I.D.: 0.94mm, 10 cm length) in a micropipette puller device (Sutter Instruments Inc., Flaming/Brown P-87) and stored in a clean petri dish, mounted on a small piece of adhesive tape. 5 dpf larvae were fixed in

4% PFA for 2 hours at RT with gentle agitation and then dechorionated and bleached (3% H_2O_2/0.5%KOH) to remove melanin pigments. Larvae were washed 3x 5 minutes in PBST and then 5x 5 minutes in PBS prior to being mounted in 1% Ultrapure Low-Melting Point Agarose (Invitrogen, Carlsbad, USA) on a microscope slide. Then a 2mg/ml solution of DiI (Thermo Scientific, Waltham, USA), dissolved in DMSO (Sigma, St. Louis, USA), was backfilled into a prepared micropipette needle and mounted on a pressure-pulsed micro injector apparatus. Once the tip of the needle was clipped with forceps, approximately 1-2nl DiI was directly injected into the ear.

2.12 Mounting and imaging embryos

Following mRNA in situ hybridization, inner ear injections, or any immunohistochemical techniques, embryos or larvae were washed 5x for 5 minutes in PBS and mounted in 1% Ultrapure Low-Melting Point Agarose (Invitrogen, Carlsbad, USA). Following *in situ* hybridization, embryos or larvae were photographed with an Olympus stereoscope (Shinjuku City, Tokyo, Japan) and a QImaging micropublisher camera (Surrey, Canada). For the observation of otolith phenotypes, embryos or larvae were also photographed with an Olympus stereoscope (Shinjuku City, Tokyo, Japan) and a QImaging micropublisher camera (Surrey, Canada). All other embryos or larvae were photographed with a Zeiss LSM510 confocal microscope (Carl Zeiss AG, Jena, Germany) and Zeiss Zen imaging software (Carl Zeiss AG, Jena, Germany).

Only live images of *nkgsaigff266c*, *nkgsaizgffd262a*, *nkgsaizgffm789a*, *nkgsaizgffm1225a*, *nkhspgff75a*, and *nkhspgffdmc12a* were used in this publication. As such, zebrafish embryos and larvae were transferred to a petri dish of EM containing approximately 100 mg L^{-1} tricaine methanesulfonate (MS222; Syndel, Nanaimo, Canada) (Rombough, 2007). Once anesthetized, as

determined by a lack of response to direct mechanical stimulation (i.e., a gentle prod to the head with Dumont No. 5 forceps), embryos and larvae were photographed with an Olympus stereoscope (Surrey, Canada) and Zeiss Zen imaging software (Carl Zeiss AG, Jena, Germany). Following imaging, embryos and larvae were immediately transferred to a dish of fresh EM in a 28.5ºC air incubator to revitalize them.

2.13 Statistical analyses

For the behavioral assay, experimental data was analyzed using a one-way ANOVA with a post-hoc Dunn's test for pairwise comparisons between each group. For inner ear measurements made with the Imaris (Bitplane, Belfast, UK) software, data was analyzed using a one-way ANOVA with a post-hoc Holm-Sidak test for pairwise comparisons between each group. All data is reported as mean ± SD.

2.14 Tables

Table 2.14. 1. Plasmid based probes for *in situ* hybridization.

Gene	Vector	Linearize	Promotor
aldh1a3	pSPORT	EcoRI	T3
neurod1	pCR4-TOPO	NotI	T3

Table 2.14. 2. PCR based probes for *in situ* hybridization.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Promotor
bmp4	ATGTGGATTTCAGCGACG	AAGTAATACGACTCACTATAGGGTTG AGACTGATCTGGCTC	T7
hapln1a	ATTGCTCTGTTTTCTGTGGC	AAGTAATACGACTCACTATAGGGGGA AGTTAAGGTTGTACCGC	T7
has3	CACACAGAGCAGCACCATCT	AAGTAATACGACTCACTATAGGGGGA TTGAGCCAACGCAGGTATC	T7
stm	TTGAGCATGGCACAGATG	AAGTAATACGACTCACTATAGGGAAC TCCAGATCCTCATGC	T7
ugdh	GACGTACGGTATGGGCAAAG	AAGTAATACGACTCACTATAGGGTTG ATTTCCAGCAATGGTCA	T7
vcana	AGGATTTCCTGCATGAGC	AAGTAATACGACTCACTATAGGGATG AATGGTATGCTGGC	T7

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Chapter 3: A Late Role for Retinoic Acid Signaling in Inner Ear Morphogenesis and Otolith Maintenance

The equipment and software utilized in the behavioral assay was provided by Dr. Keith Tierney, with Danielle Philibert providing the necessary training. Hayley Todesco and Jainil Doshi contributed to this chapter by editing and confirming the larval traces generated by Noldus in the behavioral assay. The shelf screen from which *nkhspdmc12a* was selected for our work was performed with the help of the Kawakami laboratory at the National Institute of Genetics (Mishima, Japan).

3.1 Introduction

Sensory organs provide an organism with the ability to perceive thermal, mechanical, or chemical stimuli, either internally or in the environment, and transduce this information to the brain for interpretation and response determination (Vosshall & Carandini, 2009). Altogether, the sensory organs improve the survivability of a species by aiding in navigation, predator avoidance, and the collection of food and other resources (Dominy, Ross, & Smith, 2004). One of these organs, the vertebrate inner ear, detects motion and stabilizes the head and body during movement, (Ekdale, 2016). The inner ear is comprised of multiple components including the semi-circular canals, three orthogonally-oriented ducts that each terminate in a sac that houses a sensory epithelium sheathed by a gelatinous membrane known as cupula (Mescher, 2010). During angular accelerations of the head, the cupula presses up against the potassium-rich endolymph fluid contained within the ear, deflecting the stereocilia of the hair cells embedded within it. In addition to the semicircular canals, the inner ear contains the maculae, the two sensory epithelia housed within the central vestibule adjacent to the canals (Hain & Helminski, 2007; Purves et al., 2001). They are similarly overlain by a gelatinous mass, although this macular membrane is embedded with small calcium crystals known as otoliths or otoconia (Hain & Helminski, 2007; Purves et al., 2001). This heavy otolithic membrane shifts according to the direction of gravity or linear accelerations, the saccular along the vertical plane and the utricular along the horizontal, although the utricle alone is considered essential to vestibular function in fish while the saccule plays a more essential role in detecting auditory stimuli (Hain & Helminski, 2007; Riley & Moorman, 2000; Sisneros & Rogers, 2016). Together, these vestibular apparatuses detect a wide array of motion, though there is an incomplete understanding of the development programme behind these vestibular structures.

Inner ear development begins with the specification of the pre-placodal region along the anterior border of the neural plate and the subsequent induction of the otic placode, an ectodermal thickening that will give rise to the inner ear cell types (Arima et al., 2005; Janesick, Shiotsugu, Taketani, & Blumberg, 2012; Litsiou, Hanson, & Streit, 2005; Morsli, Choo, Ryan, Johnson, & Wu, 1998; Shiotsugu et al., 2004). This is followed by the cavitation of the otic placode in fish or its invagination in amniotes to form the otic vesicle/otocyst (Kaufman, 1992; Maroon et al., 2002; Schlosser & Northcutt, 2000). The anteroventral portion of this vesicle gives rise to the neuro-sensory competent domain, from which we see the delamination of the neuroblasts that will form the vestibulocochlear nerve, as well as the development of sensory hair cells and their support cells (Ahmed, Xu, & Xu, 2012; Chen, Johnson, Zoghbi, & Segil, 2002; Kiernan et al., 2005; N. Pan et al., 2011; W. Pan et al., 2013; Petrovic et al., 2014; Xu et al., 1999; Zheng et al., 2003; Zou et al., 2008). This domain will then separate into the sensory epithelia of the ear, the maculae and the cristae (Hwang, Simeone, Lai, & Wu, 2009; Nichols et al., 2008; Pauley et al., 2003). The otoliths (or otoconia) tether and grow upon the maculae, while the inner ear relies upon inductive signals originating from the cristae to form the semi-circular canals (Chang, Brigande, Fekete, & Wu, 2004; Chang et al., 2008; Hammond et al., 2009; Mansour, Goddard, & Capecchi, 1993; Omata et al., 2007; Pauley et al., 2003; Riley, Zhu, Janetopoulos, & Aufderheide, 1997; Tanimoto, Ota, Inoue, & Oda, 2011). There are, however, variations in how different species form these canals. In anamniotes like *Xenopus* and zebrafish, protrusions of the otic epithelium bulge inward and then fuse together, forming the pillars of the canals (Paterson, 1949; Robert Earle Waterman & Danny H Bell, 1984). In amniotes like chick and mice, canal morphogenesis instead begins with the formation of a vertical and horizontal out-pouch, within which opposing epithelia extend toward one another to form fusion plates (Bissonnette & Fekete, 1996; Fekete, 1999; Kopecky, Johnson,

Schmitz, Santi, & Fritzsch, 2012). Upon fusion, these plates are resorbed through apoptosis, with the canal ducts forming along the rim.

During otic development, the otic vesicle will be subjected to various signaling cues that help to pattern the non-sensory, sensory, or neuronal cell types that give rise to the vestibular structures of the inner ear. One such signaling molecule is retinoic acid (RA), a derivative of vitamin A (all-*trans* retinol) that controls gene expression in developing organs by binding nuclear receptors upstream target genes and generally promoting their transcription (Ghyselinck & Duester, 2019). Otic regulation of RA synthesis is undertaken by a number of enzymes expressed either within or nearby the developing ear. During prenatal development, all-*trans* retinol is first metabolized into all-*trans* retinal by the retinol dehydrogenase RDH10 (Duester, 2000; Farjo et al., 2011; Metzler & Sandell, 2016; Penzes & Napoli, 1999; Sandell, Lynn, Inman, McDowell, & Trainor, 2012; Sandell et al., 2007). It is then further metabolized into RA primarily by retinaldehyde dehydrogenases (RALDHs, also known as aldehyde dehydrogenases or ALDHs) (Niederreither, Subbarayan, Dollé, & Chambon, 1999; Niederreither, Vermot, Fraulob, Chambon, & Dollé, 2002). Studies in mice have demonstrated that there are three RALDHs responsible for RA metabolism in mammals, Raldh1, Raldh2, and Raldh3 (Niederreither et al., 1999; Niederreither et al., 2002). *Raldh2* expression in the somites contributes RA to regulate target genes expressed in both the hindbrain and the early otocyst, followed shortly by the expression of all three RALDHs within the developing otic epithelium, although there are variations in the number of RALDHs and their expression patterns in other species (Gale, Zile, & Maden, 1999; Niederreither, Vermot, Schuhbaur, Chambon, & Dollé, 2000; Raymond Romand, Dollé, & Hashino, 2006; Raymond Romand et al., 2004). For example, zebrafish have only two ALDH genes, *aldh1a2* and *aldh1a3* (Cañestro, Postlethwait, Gonzàlez-Duarte, & Albalat, 2006; Silke

Pittlik, Domingues, Meyer, & Begemann, 2008; Xi, Yue, & Yang, 2015). *aldh1a2* is expressed in the paraxial mesoderm at 12 hours post fertilization (hpf) and both the developing somites and the head mesenchyme posteroventral to the otic placode by 15 hpf, although its expression adjacent to the developing ear only persists until 19 hpf (Begemann, Schilling, Rauch, Geisler, & Ingham, 2001; Grandel et al., 2002; Maier & Whitfield, 2014; S. Pittlik & Begemann, 2012). At this time, *aldh1a3* is expressed within the anteroventral region of the otic vesicle (Maier & Whitfield, 2014; Silke Pittlik et al., 2008). By 48 hpf, *aldh1a3* expression expands to the cristae of the semi-circular canals, the utricular (anterior) macule, and a non-sensory region of the otic epithelium known as the endolymphatic duct (an apparatus responsible for regulating inner ear pressure), being the only aldehyde dehydrogenase to provide the inner ear with RA during late otic development.

Both inadequate nutrition and high doses of vitamin A during pregnancy have been linked to severe embryological malformations of the inner ear. For example, animal models of a complete vitamin A deficiency have no otic vesicle, whereas a reduction in vitamin A results in a wide range of phenotypes including: small otic vesicles that often fail to close and sink beneath the ectoderm; hypertrophy of the otic capsule; epithelial atrophy; missing or reduced otoliths/otoconia; and an absence of the endolymphatic duct (Chole & Quick, 1976; Kil et al., 2005; Löhle, 1985; Maden, Gale, Kostetskii, & Zile, 1996; Raymond Romand et al., 2013; Wackym et al., 1987; White, Highland, Kaiser, & Clagett-Dame, 2000). Treatment with excess retinoids leads to malformations of the semi-circular canals, maculae, vestibulocochlear nerve, and cochlea (an apparatus that houses the auditory machinery of the ear) or, in the most severe cases, the complete absence of any identifiable inner ear structures (Burk & Willhite, 1992; Frenz, Liu, Galinovic-Schwartz, & Van De Water, 1996; Granström, 1990; Jarvis, Johnston, & Sulik, 1990). These abnormalities are also seen in human patients, where an exposure to retinoids during pregnancy can result in either

the Mondini-Alexander defect, which includes a malformed or missing cochlea, an enlarged saccule and utricle, and missing neurons, or Michel aplasia, which is a missing or rudimentary inner ear void of discernible structures (Berger, 2014; Lammer et al., 1985).

Research in animal models utilizing mutants in the enzymes required for RA metabolism or early exogenous RA treatment have identified multiple roles for this signaling pathway during early otic development. They demonstrate that RA regulates the posterior expression of pre-placodal markers and subsequently promotes the expression of negative regulators of neurogenesis within the otic vesicle (Arima et al., 2005; Bok et al., 2011; Janesick et al., 2012; Maier & Whitfield, 2014; Shiotsugu et al., 2004). It also permits the development of sensory cells within the neuro-sensory-competent domain of the otic vesicle and allows for the maturation of the otic neuroblasts, which will eventually give rise to the afferent neurons of the vestibulocochlear (VIII) cranial ganglion (Maier & Whitfield, 2014). However, though the severity of inner ear malformations that occur with misregulated RA during early embryogenesis is well documented, study of late inner ear development is complicated by the fact that the organization of early otic structures is essential for the induction of later structures. To address this problem, we used the pharmacological treatment of the RA signaling antagonist diethylaminobenzaldehyde (DEAB) or exogenous all-trans RA to perturb the activity of this pathway during later stages of otic development in zebrafish (Bang, Sewell, & Malicki, 2001; Bever & Fekete, 2002; C. Haddon & Lewis, 1996; R. E. Waterman & D. H. Bell, 1984; T. Whitfield, 2000). In this work, embryos were treated with DEAB or excess RA from 28 hpf onward, which falls after the point that already well established RA-responsive otic markers have been studied during early stages of inner ear development (Arima et al., 2005; Bok et al., 2011; Janesick et al., 2012; Maier & Whitfield, 2014; Shiotsugu et al., 2004). Zebrafish with RA signaling perturbations display the circling behavioral

phenotype typical of animal models with inner ear malformations, demonstrating that altering late RA levels impacts inner ear function (Raymond Romand et al., 2013; T. T. Whitfield et al., 1996). Furthermore, we observe anatomical changes within the developing zebrafish inner ear, implicating RA as an important player in its morphogenesis. This is supported by changes in the expression of novel RA-responsive genes involved with the extracellular matrix or otolith formation within developing vestibular structures. Altogether, this work suggests that the RA signaling pathway plays an important role in late otic development.

3.2 Results

3.2.1 Altering Retinoic Acid levels during later stages of inner ear development disrupts vestibular function

Animals models of inner ear malformations exhibit circling behavior (Alagramam et al., 1999; Bloom & Hultcrantz, 1994; Kitamura, Yagi, Yoshikawa, Ochidubo, & Kato, 1991; Lv et al., 2010; Rogers et al., 1999; Raymond Romand et al., 2013; T. T. Whitfield et al., 1996). Additionally, either an RA deficiency or a lack of RA degradation prior to inner ear development will result in vestibular dysfunction (Ono, Sandell, Trainor, & Wu, 2020; Raymond Romand et al., 2013). As late otic synthesis of RA is regulated by *aldh1a3* in the utricular macula, the endolymphatic duct, and the cristae, it is likely that RA participates in the development of these vestibular structures (Supplemental Figure 3.4.1A). However, because the induction and organization of early inner ear cell types is essential for the subsequent induction of later vestibular structures, we wanted to determine a timeframe for RA agonist or antagonist treatment that would not affect the expression of RA-responsive target genes already identified in the literature. To determine an ideal timeframe, we observed the expression of a known downstream otic target of RA signaling, *neurogenic differentiation 1* (*neurod1*), which is required for the maturation of neuroblasts in the statoacoustic ganglion (Supplemental Figure 3.4.1B) (Maier & Whitfield, 2014). While early perturbations of RA signaling, such as at 20 hpf, result in changes in *neurod1* expression, treatment at 28 hpf does not. This suggests that treating embryos from 28 hpf point onward would allow for the discovery of novel behavioral, morphological, or genetic responses to changes in RA signaling.

To determine the impact of perturbations to RA signaling on the vestibular function of zebrafish within this timeframe, embryos were treated from 28 hpf onward with either exogenous

RA (5nM), to simulate increased RA signaling, or diethylaminobenzaldehyde (DEAB), which blocks RA signaling by inhibiting the activity of the *aldh1* family of RA metabolizing genes (Chute et al., 2006). While a dose of 5μM DEAB is classically used to ablate RA-signaling in zebrafish embryos, these fish display swelling around the heart, which could affect their swimming behavior. Therefore, a separate group of embryos were treated with 1μM DEAB. For this experiment, circling phenotype was defined as any continuous and complete 360º revolution on the horizontal plane that did not exceed 4.5mm in diameter (1/4 diameter of a well; also, one length of the zebrafish larva) at 5dpf, which is when zebrafish larvae are capable of mature swimming (Hernandez, Galitan, Cameron, Goodwin, & Ramakrishnan, 2018; Nicolson et al., 1998). When including all larvae (moving and stationary), the 1μM DEAB and 5nM RA treatment groups exhibited a higher rate of circling (Figure 3.4.1B; 1μM DEAB: $Q = 2.491$, $p = 0.038$; 5μM DEAB: $Q = 0.588$, $p = 1.000$; 5nM RA: $Q = 2.550$, $p = 0.032$). With the omittance of larvae that remained stationary during the 10-minute recording period, all treatment groups exhibited a higher rate of circling per 100cm travelled in comparison to the DMSO control (Figure 3.4.1C: 1μM DEAB: $Q = 4.634$, $p < 0.001$; 5μM DEAB: $Q = 2.975$, $p = 0.009$; 5nM RA: $Q = 4.241$, $p < 0.001$). There was no bias found in left- or right- handed turns during this behavioral assay (Supplemental Figure 3.4.2). As these results are consistent with vestibular dysfunction, this suggests that altering RA levels during late otic development is detrimental to vestibular function.

3.2.2 Perturbations of RA signaling during later stages of otic development impact inner ear dimensions and result in malformations of the semi-circular canals

Either the ablation of RA signaling with DEAB or exogenous RA treatment at 40% epiboly (5 hpf) in zebrafish has been shown to impact the size of the inner ear and disrupt protrusion

formation of the semi-circular canals by 50 hpf (Hans & Westerfield, 2007). Early DEAB treatment results in small otic vesicles and impaired protrusion formation, whereas exogenous RA treatment results in the formation of additional protrusions, although how this ultimately affects the shape of the pillars or the inner ear as a whole is unclear. To determine if changes in the size or shape of the inner ear continue to be made with altered RA signaling late in otic development, we decided to perform a screen at the National Institute of Genetics (Mishima, Japan) to identify zebrafish transgenic lines that could facilitate the visualization of otic tissues. This enhancer and gene trap database contains transgenic lines expressing a zebrafish optimized form of the yeast Gal4 (GFF) generated by the Kawakami laboratory in various embryonic tissues. One of these enhancer trap lines, *nkhspdmc12a*, expresses GFF throughout the otic epithelium at 3dpf (Supplemental Figure 3.4.3). Therefore, crossing these lines with *Tg(UAS:gfp)* enables the visualization of the entire inner ear. 2D confocal images were then taken of the resulting larvae at 3dpf and reconstructed into 3D models of the inner ear using the imaging software Imaris. 3 dpf was chosen for our initial analysis as this is the time at which the protrusions of the inner ear should have fused to form the pillars of the semi-circular canals, allowing us to determine if this process is disrupted with perturbations to RA signaling. We chose a second time point of 5 dpf to confirm our analysis, as this is the stage at which vestibular dysfunction was observed in our behavioral assay. However, because GFF expression throughout the otic epithelium in the *nkhspgffdmc12a* transgenic line does not extend to 5 dpf, visualization of inner ear structures was instead achieved at this stage by injecting the fluorescent lipophilic dye DiI into the ear.

Analysis of the surface area (μm^2) and volume (μm^3) of the inner ear reveals only a statistically significant increase in surface area in larvae with ablated RA signaling at 3 dpf (Figure 3.4.2A; 3 dpf RA: $t = 0.0117$, $p = 0.991$), with there being no significant changes in volume

between the different treatment groups at either stage (Figure 3.4.2B). However, there were other dimensional alterations observed between the treatment groups. While there were no significant differences in "length" along the anteroposterior (AP) axis (Figure 3.4.2C), there was a significant decrease in the "height" of the inner ear along the dorsoventral (DV) axis in exogenous RA treated larvae at both 3 dpf and 5 dpf (Figure 3.4.2D; 3 dpf RA: $t = 2.458$, $p = 0.035$; 5 dpf RA: $t = 3.550$, $p = 0.003$). Additionally, with respect to the ratio of the height along the DV axis to the length along the AP axis, there was significant increase in embryos with DEAB treatment at 3 dpf and a significant decrease in exogenous RA treated larvae at both stages (Figure 3.4.2E; 3 dpf DEAB: $t = 2.328$, $p = 0.024$; 3 dpf RA: $t = 3.386$, $p = 0.003$; 5 dpf RA: $t = 3.452$, $p = 0.004$). To summarize, these results demonstrate that perturbations to RA signaling impact the dimensions of the inner ear at 3dpf, although at 5 dpf only significantly with exogenous RA treatment.

A morphological analysis of the inner ear also revealed irregularities in pillar formation (Figure 3.4.2F; Figure 3.4.3). Both the DEAB and RA treated groups demonstrated a higher occurrence of irregularities at both stages (Figure 3.4.2F; Figure 3.4.3; 3dpf DMSO = 6.25%; 5 dpf DMSO = 0%; 3 dpf DEAB = 31.58%; 5 dpf DEAB = 30%; 3 dpf RA = 52.94%, 5 dpf RA = 50%). In the DEAB treated larvae, these irregularities are characterized as an abnormal branching pattern at the base of the pillars, typically the ventral or the posterior, which could be due to the formation of extra protrusions over the course of canal morphogenesis (Figure 3.4.3B). In the RA treated larvae, there were often multiple such branches observed at the base of the anterior, posterior, or ventral pillars (Figure 3.4.3C). This data suggests that alterations to RA signaling during later stages of inner ear development impact the morphology of the semi-circular canals.

Canal morphogenesis can be impacted by cellular processes such as proliferation, apoptosis, and migration (Hynes, 2009; Monboisse, Oudart, Ramont, Brassart-Pasco, & Maquart,

2014; Saupe et al., 2013; Sethi et al., 1999; Su, Shi, Stolow, & Shi, 1997). Both apoptosis and cell proliferation have been identified as key cellular processes that drive the formation and subsequent sculpting of the canals in vertebrates, although apoptosis has not been observed as an essential process in canal morphogenesis in zebrafish (Bissonnette & Fekete, 1996; Deng, Pan, Xie, & Gan, 2010; Fekete, 1999; Kopecky et al., 2012; Noda et al., 2012; Paterson, 1949; Salminen, Meyer, Bober, & Gruss, 2000; Robert Earle Waterman & Danny H Bell, 1984). Therefore, we wanted to determine if the changes in RA signaling could impact either of these processes. However, we found that there was no observable change in apoptosis in or around the ear in response to either pharmacological treatment (Supplemental Figure 3.4.4). Additionally, there were no observable differences in cell proliferation, either within or around the ear (Supplemental Figure 3.4.5). There were also no observable differences in cell proliferation later in the ear at 3 dpf (Supplemental Figure 3.4.6). These results suggest that RA signaling is either directly or indirectly regulating canal morphogenesis through cellular processes other than apoptosis and cell proliferation.

3.2.3 Perturbations of RA signaling during later stages of inner ear development disrupt the expression of extracellular matrix genes

As a key signaling molecule that controls gene expression in developing organs, we wanted to identify RA-responsive genes during later stages of inner ear development (Ghyselinck & Duester, 2019). Due to our observed malformations of the semi-circular canal pillars, we decided to analyze otic markers known to be expressed within the pillars themselves or the protrusions that give rise to them. In doing so, we uncovered four novel targets of RA signaling (Figure 3.4.4). Each of these genes are implicated in reshaping the extracellular matrix (ECM) and are expressed either within the protrusions that bulge from the otic vesicle and fuse together to generate the

pillars of the semi-circular canals (Figure 3.4.4A) or within the head mesenchyme adjacent to the ear. The first target we uncovered was *versican a* (*vcana*), which encodes a chondroitin sulphate proteoglycan core protein considered to be an anti-adhesion molecule (Figure 3.4.4B-C″)(Ricciardelli, Sakko, Ween, Russell, & Horsfall, 2009). From 48-72 hpf, ablating RA signaling with DEAB treatment reduces the intensity of its expression at the inner ear protrusions (Figure 3.4.4B, C), whereas increasing RA signaling promotes its ectopic expression in the ventral protrusion (Figure 3.4.4B″, C″). The second target we identified was *hyaluronan and proteoglycan link protein 1a* (*hapln1a*), which encodes a cartilage oligomeric matrix protein essential for cartilage and bone development that stabilizes proteoglycans and hyaluronic acid in the ECM (Figure 3.4.4D-E″)(Watanabe & Yamada, 1999). While *hapln1a* expression at 48 hpf is only altered with exogenous RA treatment, displaying a marked reduction of the area of expression within the anterior, lateral, and posterior protrusions (Figure 3.4.4D″), at 72 hpf it additionally demonstrates an expansion in the expression area within and around the ventral aspect of the ear upon DEAB treatment (Figure 3.4.4E). The third gene we found to be RA-responsive was *UDP-glucose 6-dehydrogenase* (*ugdh*), which encodes a cytosolic enzyme that participates in the glycosylation of ECM components, such as heparan sulfate, hyaluronan, and chondroitin (Figure 3.4.4F-G″)(Alhamoudi et al., 2020). At 48 hpf, reduced RA signaling results in ectopic *ugdh* expression between the anterior and posterior protrusions (Figure 3.4.4F), while increasing RA represses expression in the head mesenchyme ventral to the ear (Figure 3.4.4F″). At 72 hpf, there is an increase in the area of expression with low levels of RA (Figure 3.4.4G) and a loss of expression within the ear with high levels of RA (Figure 3.4.4G″). The final target we uncovered was *hyaluronan synthase 3* (*has3*), a membrane-associated enzyme responsible for synthesizing hyaluronan (Figure 3.4.4H-I″)(Weigel, Hascall, & Tammi, 1997). At 48 hpf, the only notable

difference in its expression is an unusual 'hooked' pattern with reduced RA signaling (Figure 3.4.4H), demonstrating expression presumably where the ventral protrusion originates as opposed to within the posterior protrusion. At 72 hpf, low levels of RA increase its expression within the ear (Figure 3.4.4I) and high levels of RA decreases its expression within the ear and around the ventral aspect of the ear (Figure 3.4.4I″). To summarize, these results demonstrate that RA signaling regulates the expression of genes encoding ECM proteins or modifying enzymes during later stages of inner ear development.

3.2.4 Perturbations of RA signaling during later stages of inner ear development disrupt the activity of other signaling pathways

RA has already been demonstrated to interact with multiple signaling pathways to regulate early otic development (Hans & Westerfield, 2007; Maier & Whitfield, 2014; Radosevic, Robert-Moreno, Coolen, Bally-Cuif, & Alsina, 2011; Thompson, Gerlach-Bank, Barald, & Koenig, 2003). As such, we wanted to determine if RA signaling continues to regulate or participate within a network of other signaling pathways during late otic development. To identify other signaling pathways that might respond to perturbations in RA signaling, we performed an *in situ* hybridization analysis of *bone morphogenetic protein 4* (*bmp4*), a critical signaling molecule of the Bone Morphogenetic (BMP) pathway (Figure 3.4.5A-A″), or monitored the expression of reporter zebrafish lines, such as *Tg(top:gpf)* for the canonical WNT pathway (Figure 3.4.5B-B″) or *Tg(ptc2:kaede)* for the Sonic Hedgehog (SHH) pathway (Figure 3.4.5C-C″). Changes in the level of BMP signaling within the ear were observed, such that ablating RA signaling with DEAB resulted in a decrease in the intensity of *bmp4* expression (Figure 3.4.5A) and increasing RA signaling with exogenous RA resulted in an increase in the intensity of *bmp4* expression (Figure

3.4.5A″). We also observed a moderate decrease in the intensity of WNT signaling with lower levels of RA (Figure 3.4.5B). In contrast to this, the intensity of SHH signaling was moderately reduced with higher levels of RA (Figure 3.4.5C″). These results suggest that changes to RA signaling late in otic development are capable of altering the activity of other signaling pathways.

3.2.5 Late ablation of RA signaling results in a loss or reduction in the size of otoliths late in otic development

Studies in mice have demonstrated that a knockout of *Raldh3* (the homolog of *aldh1a3*) without RA supplementation at the onset of otoconia (otolith) formation results in fused saccular and utricular maculae and a complete loss or reduction in the number of otoconia (Raymond Romand et al., 2013). In zebrafish, the ablation of RA signaling with DEAB at 40% epiboly, well before the onset of otolith formation at 18 hpf, also results in fewer otoliths (Hans & Westerfield, 2007; Riley et al., 1997; Tanimoto et al., 2011). Alternatively, early treatment with exogenous RA results in the formation of three otoliths (Hans & Westerfield, 2007). Therefore, we wanted to determine if perturbations to RA signaling late in otic development would have an effect on otolith size or number. As seen at 3 dpf, otolith formation commences normally, with the appearance of both saccular and utricular otoliths of appropriate sizes between treatment groups (Figure 3.4.6A-B). However, observation at 5 dpf reveals that late DEAB treatment results in a loss of the utricular otolith and a smaller saccular otolith (Figure 3.4.6D). Meanwhile, exogenous RA treatment does not result in a change in otolith number or size (Figure 3.4.6F). While few RA target genes of otolith formation or maintenance in zebrafish are known, we discovered that the expression of *starmaker* (*stm*), which regulates otolith nucleation and composition, is responsive to perturbations in RA signaling (Figure 3.4.6G-L) (Kalka et al., 2019; Raymond Romand et al., 2013; Söllner et

al., 2003). Late DEAB treatment results in a reduction in the location and intensity of *stm* expression (Figure 3.4.6G, J), while late RA treatment results in an increase in the intensity of *stm* expression (Figure 3.4.6I, L). Together, these results indicate that perturbations to RA signaling disrupt otolith maintenance late in otic development.

3.3 Discussion

Previous work into the Retinoic Acid (RA) signaling pathway has demonstrated that it is essential at multiple stages during early otic development. For example, studies in *Xenopus* have shown that RA signaling plays an essential role in specifying the pre-placodal region (PPR) within the ectoderm where the otic placode originates, which RA accomplishes by restricting the posterior domain of *fgf8* and inducing the posterior expression of the PPR genes *tbx1* and *ripply3* (Arima et al., 2005; Janesick et al., 2012; Shiotsugu et al., 2004). Subsequently, studies in zebrafish and chick demonstrated that RA participates in anteroposterior patterning of the otic placode by promoting the posterior expression of *tbx1*, a negative regulator of neurogenesis (Bok et al., 2011; Maier & Whitfield, 2014; Radosevic, 2011; Radosevic et al., 2011; Steven Raft, Nowotschin, Liao, & Morrow, 2004). Once the placode invaginates or cavitates into the otic vesicle, RA continues to maintain anteroposterior patterning of the ear and regulates the development of sensory hair cells (Maier & Whitfield, 2014). Studies in zebrafish demonstrate that RA accomplishes this by restricting the posterior expression of *otxb1*, a transcription factor that negatively regulates neurogenesis while promoting the development of structural cells, thereby allowing the development of sensory cells. Concurrently, it continues to promote *tbx1* expression, permitting the maturation of otic neuroblasts.

Together, these studies describe multiple phases for RA in the earliest stages of otic development, which would help to explain why disruptions to RA signaling via the modification of maternal vitamin A intake or mutations in key components of this pathway lead to a range of inner ear malformations (Chole & Quick, 1976; Kil et al., 2005; Löhle, 1985; Maden et al., 1996; Niederreither et al., 1999; R. Romand et al., 2002; Raymond Romand et al., 2013; Wackym et al., 1987; White et al., 2000). However, the early disruption of a signaling pathway can produce robust

phenotypes in a tissue that obscure those that might occur later. For example, early disruptions in RA signaling can result in missing or reduced otic vesicles, which would presumably affect the later morphology of the inner ear, such as the formation of the semi-circular canals; additionally, as the sensory epithelia of the inner ear participate in both the formation of the otoliths and canals, RA's early role in sensory development could indirectly affect these processes (Maier & Whitfield, 2014; S. Raft et al., 2007; Satoh & Fekete, 2005). Therefore, while previous work helps to explain how early disruptions in RA signaling can impact the development of later vestibular structures, by treating embryos with exogenous RA or the RA antagonist diethylaminobenzaldehyde (DEAB) beyond the timeframe that early RA-responsive targets have been studied in zebrafish, we have identified a much later phase of RA activity in the developing ear.

Our initial evidence toward another, late phrase for RA signaling in otic development first includes the disruption of vestibular function, both with a reduction or increase in RA signaling. While pharmacological treatments to whole embryos could impact the development of non-otic tissues that participate in vestibular function, the anatomical abnormalities of the inner ear observed in our work imply that this dysfunction could be attributed, at least in part, directly to perturbations in otic development. Because the precise toroidal dimensions of the semi-circular canals are essential for the endolymph fluid dynamics of the inner ear, presumably the peculiar branching formation at the base of the canal pillars observed with both a reduction or increase in RA signaling in our work, as well as the change in axial otic dimensions observed with exogenous RA treatment, might interfere with these dynamics (Kassemi, Deserranno, & Oas, 2005; Michaels, 1987; Spoor et al., 2007). However, it is questionable whether the zebrafish semi-circular canal system is capable of distinguishing angular accelerations during the timeframe analyzed in our work (Beck, Gilland, Tank, & Baker, 2004). This is largely assumed to be due to the small size of

the larval ear, in which endolymph movement would be severely constrained (Beck et al., 2004; Hullar, 2006; Muller, 1999; Rabbitt, Damiano, & Grant, 2004). In fact, canal activity is not readily detected until approximately the 1-month mark of development, at which point the inner ear has undergone significant growth and the further morphological refinement of the canals, resulting in more recognizably independent ducts (Beck et al., 2004; Bever & Fekete, 2002). Therefore, while it is unlikely that the irregular canal morphology of our larvae affects vestibular function at 5 dpf, it would be interesting to see if these malformations are sustained up to the 1-month mark and could impact the functionality of the canal system at that stage. Regardless, while previous work in zebrafish demonstrate that disruptions in RA signaling can impair the formation of the protrusions that fuse together to form the pillars of the semi-circular canals, and studies in chick demonstrate that exogenous RA can altogether inhibit the development of the canals, the complete formation of the pillars in our work suggests that from 28 hpf onward RA enters a new phase of regulating canal morphogenesis (Hans & Westerfield, 2007; Thompson et al., 2003). To summarize, our work indicates that RA participates in initial canal formation prior to 28 hpf, after which point it appears to be involved with the further sculpting of the canals.

Due to the observed abnormalities in the structure of the pillars, we wanted to confirm these morphological changes by analyzing otic markers known to be expressed within the pillars or their initial protrusions. In zebrafish and *Xenopus*, glycosaminoglycan hyaluronan production and the proper regulation of extracellular matrix (ECM) core proteins or modifying enzymes have been implicated in proper canal morphogenesis (Busch-Nentwich, Söllner, Roehl, & Nicolson, 2004; C. M. Haddon & Lewis, 1991; Maier & Whitfield, 2014; Paterson, 1949; Robert Earle Waterman & Danny H Bell, 1984). In analyzing such markers, we uncovered four novel RA-responsive targets in otic development, the ECM proteins *vcana* and *hapln1a* and the ECM

modifying enzymes *ugdh* and *has3*. However, the misregulation of these genes in *gpr126* mutants coincides with the failed fusion of the canal pillars (Geng et al., 2013). In contrast to this, pillar formation in our work is complete, if abnormal. Of course, while each of these genes is upregulated in *gpr126* mutants, it is only with ablated RA signaling that we see an increase in *hapln1a*, *ugdh*, and *has3* expression, with *vcana* expression alternatively decreased, and while there is a clear disruption in the expression of ECM genes with perturbations to RA signaling, this disruption is not as robust in *gpr126* mutants. Because functional studies have yet to be conducted on any of these ECM genes in relation to otic development, it is difficult to decipher how the individual changes in expression of each gene could affect canal morphogenesis, although this would be an intriguing avenue for future research. Presently, we can only conclude that RA regulates the expression of novel target genes involved with the ECM that may or may not participate in sculpting of the canals during this late phase of otic development.

Because evidence that RA interacts with additional pathways during early otic development is already well documented, we wanted to know if RA is interacting with other pathways during this late phase of activity. For example, studies in chick demonstrate that early RA signaling directly downregulates *Bmp4* within the ear to inhibit canal formation, and work in zebrafish shows that SHH and RA regulate otic markers such as *tbx1* in an opposing manner (Radosevic et al., 2011; Thompson et al., 2003). RA has also been shown to directly inhibit Gli activity in some non-otic tissues, such as within neural and mesodermal progenitors, while promoting the expression of *shh* in others, such as the prechordal plate and limb buds, implicating various tissue-specific roles for RA in regulating SHH signaling (Goyette et al., 2000; Halilagic, Zile, & Studer, 2003; Power, Lancman, & Smith, 1999; Stratford, Horton, & Maden, 1996). Canonical WNT signaling is another pathway that RA is known to interact with; while RA

typically suppresses this pathway in non-otic tissues, studies in zebrafish show that RA promotes canonical *wnt8b* expression in the hindbrain to increase the competence of ectodermal cells to respond to otic induction signals (Bonney, Dennison, Wendlandt, & Siegenthaler, 2018; Hans & Westerfield, 2007; Osei-Sarfo & Gudas, 2014; Roa, Bloemen, Carels, Wagener, & Von den Hoff, 2019). Our work demonstrates that RA continues to participate within a network of various signaling pathways during this late phase of activity, where it maintains a moderately inhibitive role in regulating SHH and promotes canonical WNT activity within the developing ear. Additionally, RA conversely regulates *bmp4* expression in a positive manner at this stage, further supporting the notion that RA plays a unique role in late otic development. Determining how RA interacts with each of these pathways to regulate gene expression during this late phase of activity is another possibility for further research.

While the aberrant canal formation observed in our work already suggests a new phase for RA activity in otic development, the observed otolith phenotypes in this study demonstrate another late role for RA signaling. In our work, otolith formation initiates normally, regardless of pharmacological treatment, generating utricular and saccular otoliths of appropriate size and position within the ear. However, by 5 dpf, DEAB treated larvae are missing their utricular otoliths and display saccular otoliths that are reduced in size. What this demonstrates is that maintenance of the otoliths requires an appropriate amount of RA over the course of otic development. This could explain the behavioral abnormalities observed in our DEAB treated larvae at 5dpf, as the otoliths, particularly the utricular, is critical for vestibular function and survival at this stage (Beck et al., 2004; Riley & Moorman, 2000). A more in-depth histological and chemical analysis of the otoliths of RA treated larvae is required to definitively ascertain if they are unaltered in thickness or composition or if the development or maintenance of the underlying macular hair cells are

alternatively affected, though given that RA treated larvae also display vestibular dysfunction at 5 dpf, it is likely that they also suffer from otolith or macular abnormalities to some degree.

When considering potential RA-responsive genes that could participate in otolith maintenance at this stage, our work has demonstrated that *stm* is a candidate as it has already been implicated in promoting the nucleation of the otoliths and the preferential formation of aragonite within the developing otoliths (Kalka et al., 2019; Söllner et al., 2003). However, *stm* has not been implicated in otoconial formation in mammals and, overall, many variations exist in otolith composition, morphology, and crystalline structure between fish and other vertebrates (Campana, 1999; Campana & Neilson, 1985; Carlstrom, 1963; Mann, Parker, Ross, Skarnulis, & Williams, 1983; Ross & Pote, 1984; Salamat, Ross, & Peacor, 1980; Steyger & Wiederhold, 1995; Zhao, Yang, Yamoah, & Lundberg, 2007). Therefore, it is not clear how applicable this finding is in understanding otoconial maintenance in other species. Even so, future research could look into uncovering other RA-responsive genes potentially involved in otolith maintenance to help identify protective factors against otoconial degradation.

In conclusion, while previous work has identified crucial roles for RA signaling in early otic development, the temporal analysis undertaken in our work has identified a previously undescribed late phase of RA activity. By using late pharmacological treatments to perturb RA signaling, we have revealed that RA signaling acts during this later phase to sculpt the canals and regulate maintenance of the otoliths.

3.4 Figures

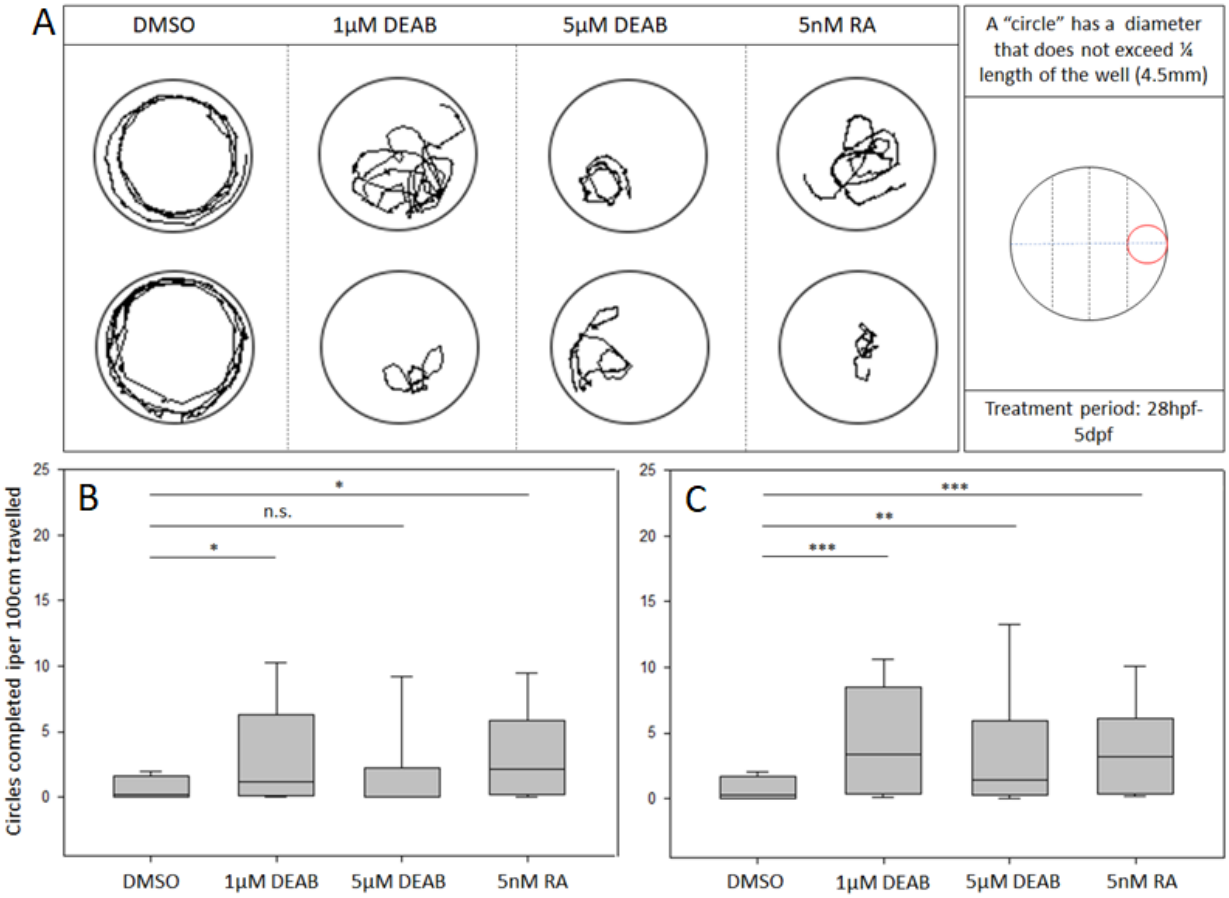


Figure 3.4. 1. Circling behavioral phenotype assay following pharmacological perturbations of the Retinoic Acid signaling pathway in zebrafish larvae. RA signaling was ablated from 28 hpf onward with diethylaminobenzaldehyde (DEAB; 5µM) or increased with exogenous RA (5nM); dimethyl sulfoxide (DMSO) was used as a solvent control. Larvae were recorded for a total of 10 minutes using a CCTV camera (WV-CL930) and the Ethovision XT10 software by Noldus (Wageningen, Netherlands). Three larvae from the control group and each treatment group were tested per 12-well plate, with a 20-minute acclimatization period prior to recording. The sample arrangement for each plate was randomized and blinded prior to quantification of circling phenotype. Circling phenotype was defined as any continuous and complete 360◦ revolution in the horizontal plane that did not exceed 4.5mm in diameter (1/4 the diameter of a well), which is the

approximate length of a zebrafish larva at this stage. To account for variations in total distance travelled between samples, circling phenotype was quantified by the total number of circles (left and right) observed per 100cm travelled. 50-second representative swimming traces of two 5 dpf zebrafish larvae for the control group and each treatment group (A). Total number of circles completed per 100cm, including larvae that did not move within the 10-minute recording period (B). Total number of circles completed per 100cm, omitting larvae that did not move within the 10-minute recording period, (C). Experimental data was analyzed using a one-way ANOVA with a post-hoc Dunn's test for pairwise comparisons between each group.

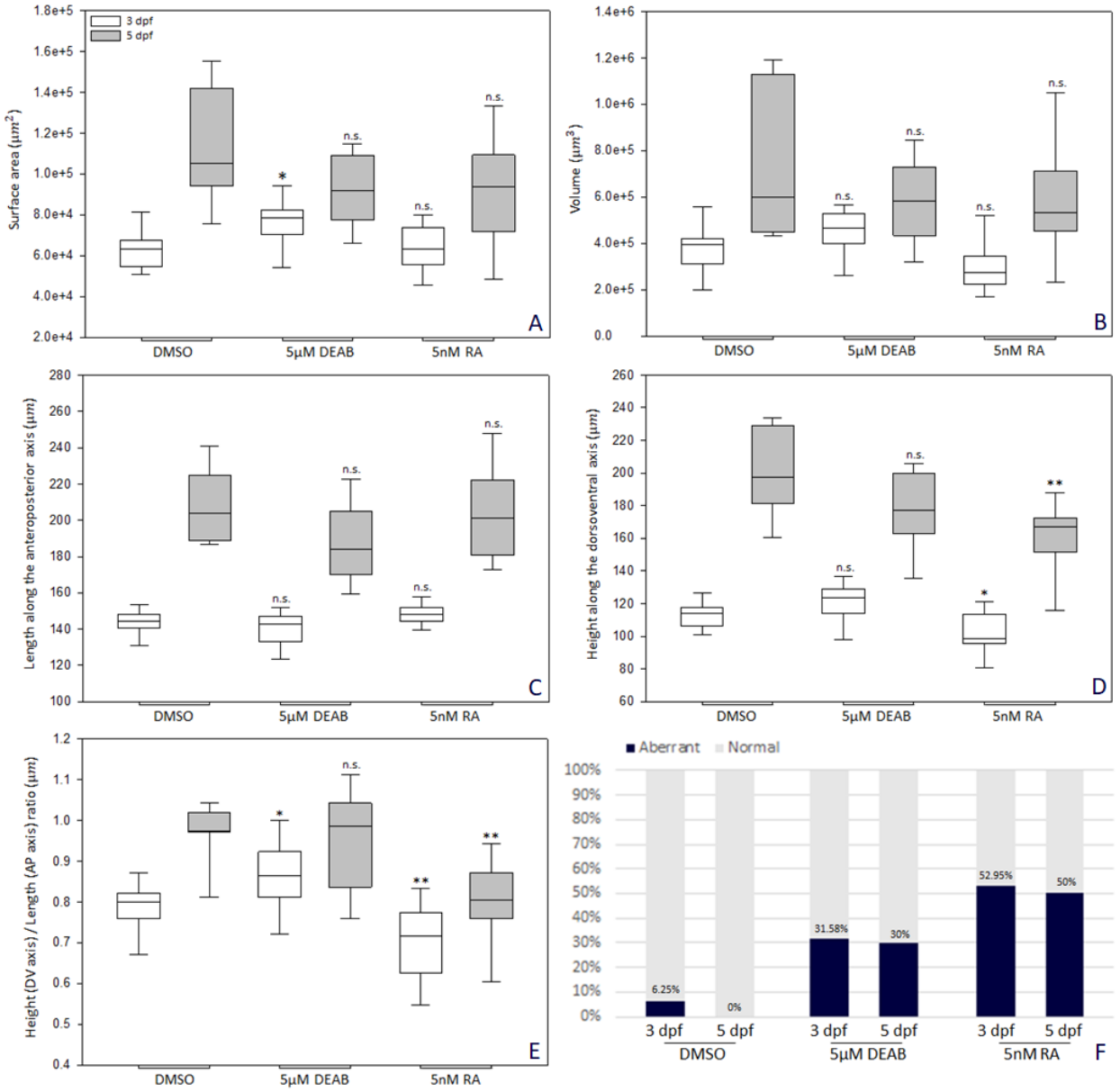


Figure 3.4. 2. Dimensional alterations and the occurrence of morphological irregularities in response to pharmacological perturbations of the Retinoic Acid signaling pathway in zebrafish larvae at 3 and 5 dpf. RA signaling was ablated 28 hpf onward with diethylaminobenzaldehyde (DEAB; 5μM) or increased with exogenous RA (5nM); dimethyl sulfoxide (DMSO) was used as a solvent control. 3D images of these larvae were generated and measured with the Imaris (Bitplane) software. The surface area (μm^2) of the inner ear (A). The volume (μm^3) of the inner ear (B). The "length" along the anteroposterior/AP axis of the inner ear

(C). The “height” of the inner ear along the dorsoventral/DV axis (D). The ratio of the height of DV axis to the length of the AP axis (E). Percentage of larvae demonstrating pillar irregularities, described as a branching pattern at the base of the pillars (F). Experimental data was analyzed using a one-way ANOVA with a post-hoc Holm-Sidak test for pairwise comparisons between each group. Sample size for each group: 3dpf DMSO, n = 16, 5dpf DMSO, n = 7; 3dpf DEAB, n = 19; 5 dpf DEAB, n = 10; 3 dpf RA, n= 17; 5 dpf RA, n = 12.

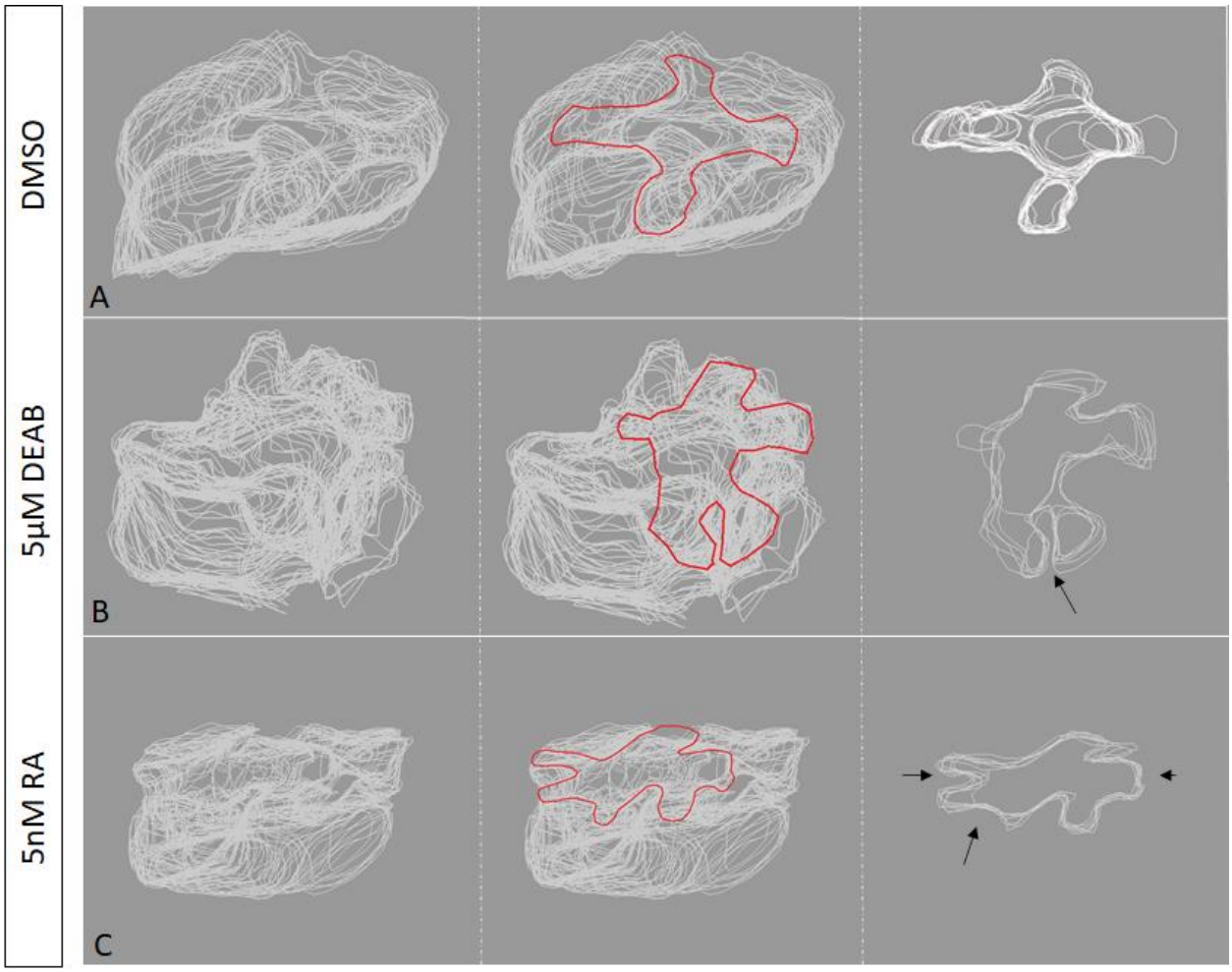


Figure 3.4. 3. Inner ear traces representing the morphological irregularities that occur in response to pharmacological perturbations of the Retinoic Acid signaling pathway in zebrafish larvae at 3 and 5 dpf. RA signaling was ablated from 28 hpf onward with diethylaminobenzaldehyde (DEAB; 5µM) or increased with exogenous RA (5nM); dimethyl sulfoxide (DMSO) was used as a solvent control. These lateral images are situated with the anterior to the left. A stack of confocal images was taken with the Zeiss Zen imaging software and the lumenal space was traced (in white) with the Imaris (Bitplane) software to generate a 3D image of the inner ear. The pillars were traced in subset of the slides from each 3D image (in red) to highlight the abnormal branching at their base (black arrows), where apparent. Inner ear traces representing the DMSO control group (A), the DEAB treated group (B), and the RA treated group (C).

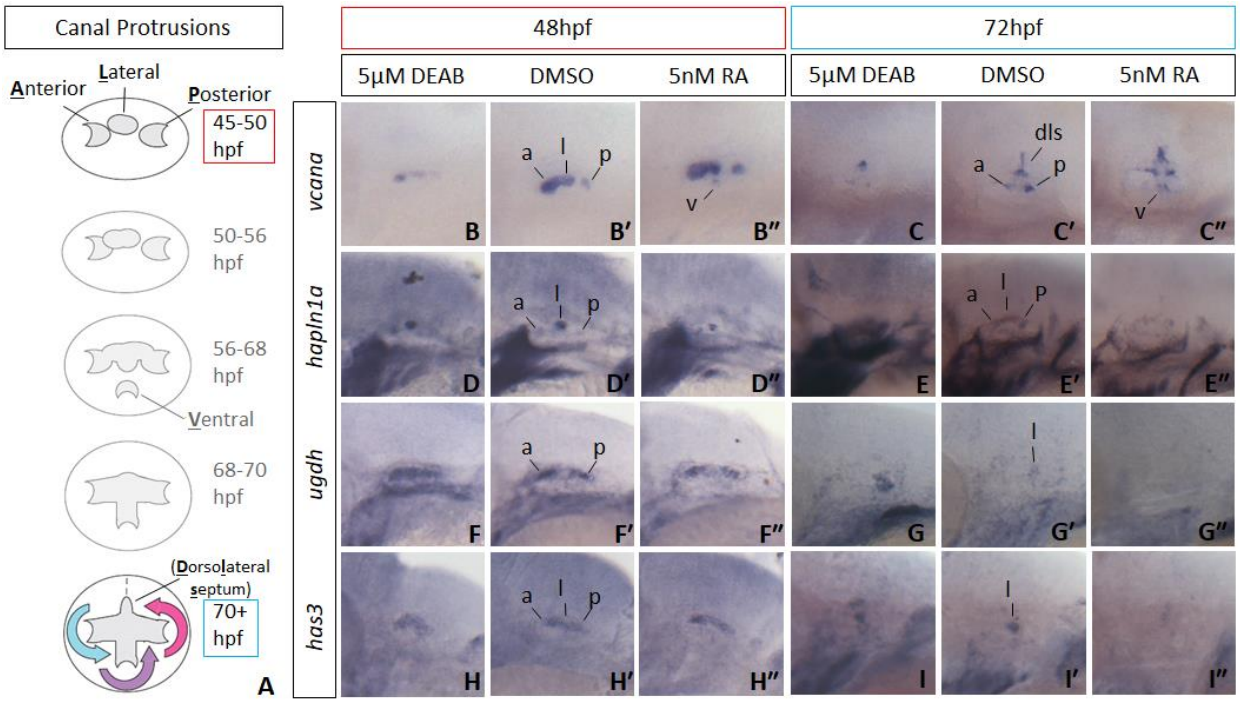


Figure 3.4. 4. *In situ* hybridization analysis of the expression of the extracellular matrix proteins or regulating enzyme *vcana*, *hapln1a*, *ugdh*, and *has3* following pharmacological perturbations of the Retinoic Acid signaling pathway in zebrafish embryos. RA signaling was ablated from 28 hpf onward with diethylaminobenzaldehyde (DEAB; 5µM) or increased with exogenous RA (5nM); dimethyl sulfoxide (DMSO) was used as a solvent control. The anterior direction is to the left in these lateral stereoscopic images. Starting at approximately 45-48 hpf, a series of protrusions bulge into the otic vesicle and fuse together, forming the three pillars around which the semi-circular canals are formed (A). *vcana* expression within the inner ear at 48 hpf (B-B″) and 72 hpf (C-C″). *hapln1a* expression within the inner ear and the head mesenchyme ventral to the otic vesicle at 48 hpf (D-D″) and 72 hpf (E-E″). *ugdh* expression within the inner ear and the head mesenchyme ventral to the otic vesicle at 48 hpf (F-F″) and 72 hpf (G-G″). *has3* expression within the inner ear at 48hpf (H-H″) and 72hpf (I-I″). Abbreviations: a, anterior

protrusion; dls, dorsolateral septum; l, lateral protrusion; p, posterior protrusion; v, ventral protrusion.

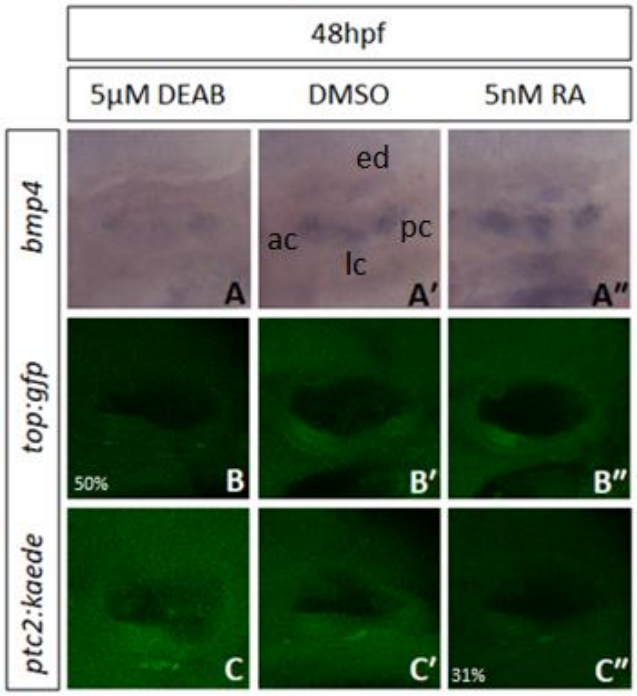


Figure 3.4. 5. Otic expression of BMP, WNT, and SHH signaling in and around the otic vesicle at the onset of semi-circular development at 48 hpf following pharmacological perturbations of the Retinoic Acid signaling pathway in zebrafish embryos. RA signaling was ablated from 28 hpf onward with DEAB (5µM) or increased with exogenous RA (5nM); DMSO was used as a solvent control. These lateral images are situated with the anterior to the left. Stereoscopic images of the *in situ* hybridization analysis of the critical BMP signaling molecule, *bmp4* (A-A″). Confocal images of the *Tg(top:gpf)* zebrafish reporter line for the WNT signaling pathway (B-B″). Confocal images of the *Tg(ptc2:kaede)* zebrafish reporter line for the SHH signaling pathway (C- C″). Percentages represent the number of embryos with the described observation. Abbreviations: ac, anterior crista; ed, endolymphatic duct; lc, lateral crista; pc, posterior crista.

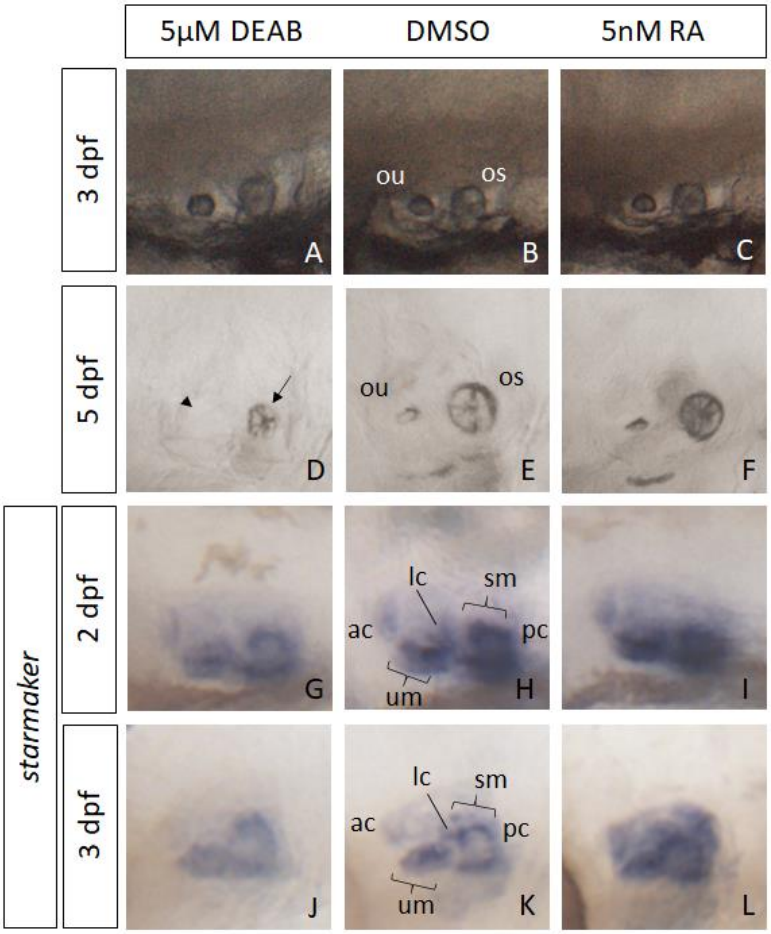
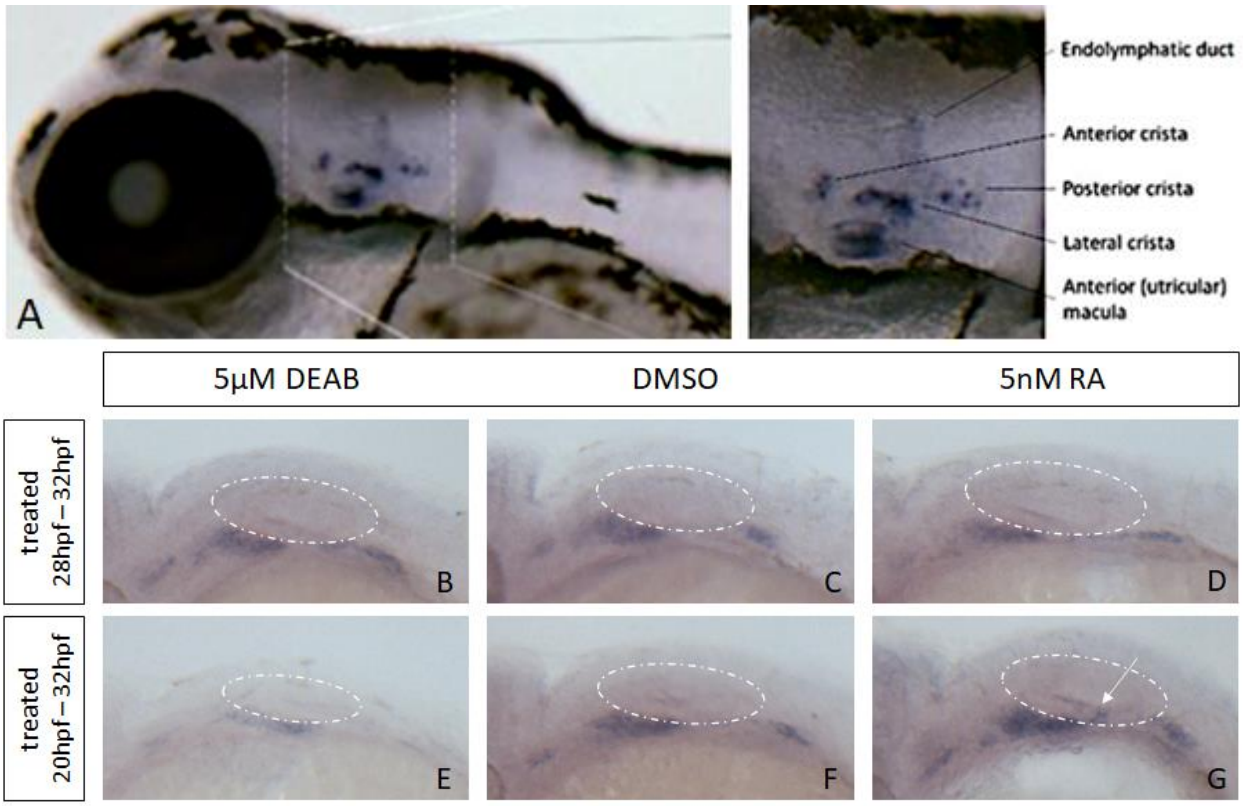
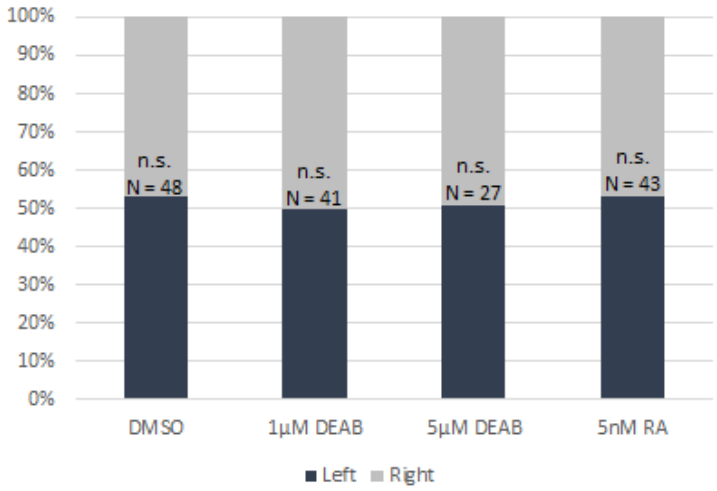


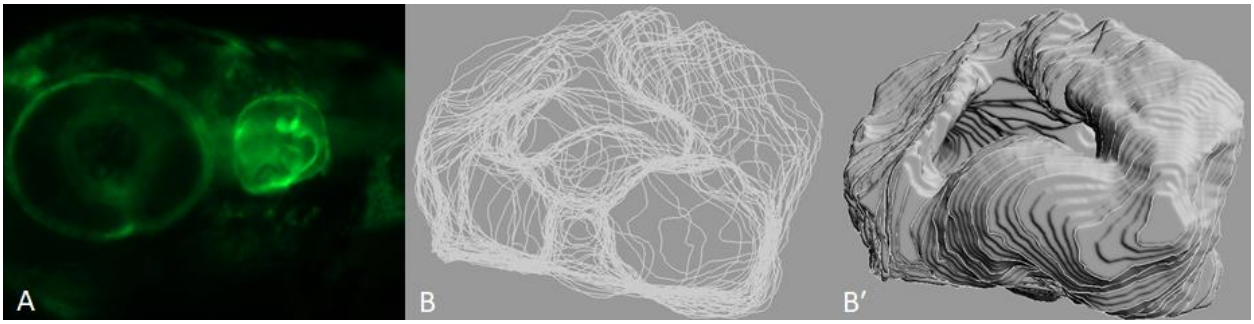
Figure 3.4. 6. Changes in otolith size and number in response to pharmacological perturbations of the Retinoic Acid signaling pathway in zebrafish larvae. RA signaling was ablated from 28 hpf with diethylaminobenzaldehyde (DEAB; 5µM) or increased with exogenous RA (5nM); dimethyl sulfoxide (DMSO) was used as a solvent control. These lateral stereoscopic images are situated with the anterior to the left. The loss of an otolith is indicated with an arrowhead; a reduction in the size of an otolith is indicated with an arrow. Inner ear images of unbleached larvae at 3dpf (A-C). Inner ear images of bleached larvae at 5 dpf (D-F). *In situ* hybridization analysis of *stm* expression at 48 hpf (2 dpf) (G-I) and 72 hpf (3 dpf) (J-L). Abbreviations: ac, anterior crista; lc, lateral crista; pc, posterior crista; ou, otolith (utricular); os, otolith (saccular); sm, saccular macula; um, utricular macula



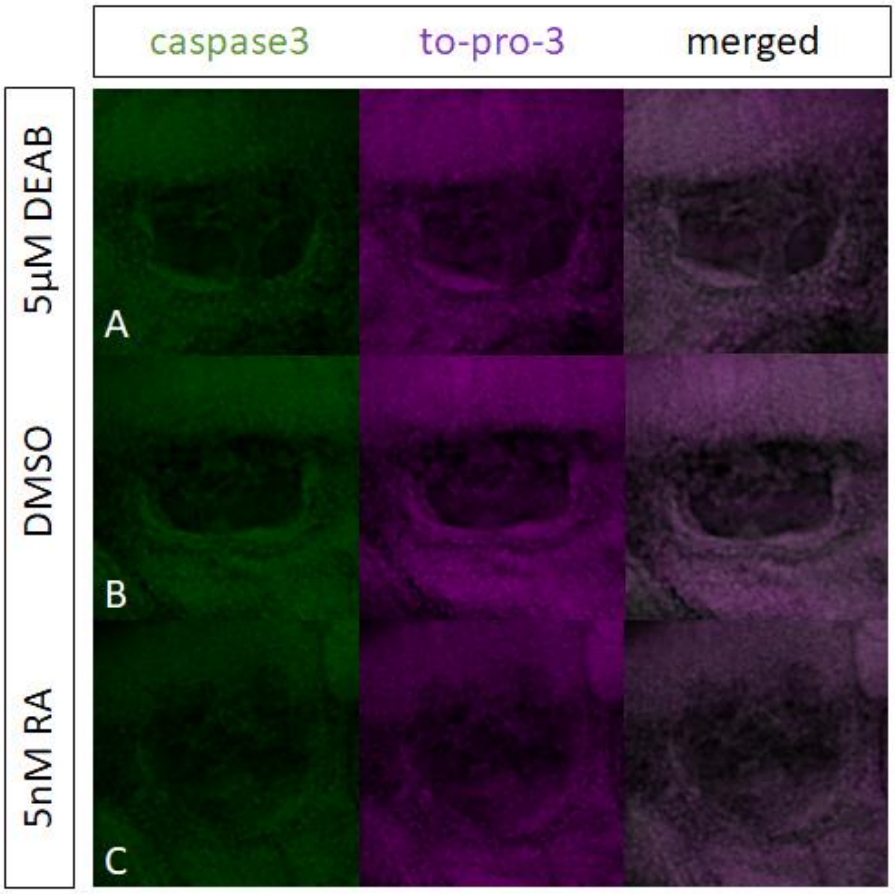
Supplemental Figure 3.4. 1. *In situ* hybridization analysis of *aldh1a3* and *neurod1* otic expression in zebrafish embryos. RA signaling was ablated with diethylaminobenzaldehyde (DEAB; 5µM) or increased with exogenous RA (5nM); dimethyl sulfoxide (DMSO) was used as a solvent control. These stereoscopic images are situated with the anterior to the left. The white oval traces represent the position of the otic vesicle; a white arrow is used to indicate any expansion within the otic vesicle. Retinoic Acid is synthesized within the developing inner ear from 48-96 hpf by *aldh1a3*, which is expressed in the anterior (utricular) macule, the three cristae of the semi-circular canals, and the endolymphatic duct (A). Otic *neurod1* expression following perturbations to RA signaling from 28 hpf onwards (B-D) or 20 hpf onwards (E-G).



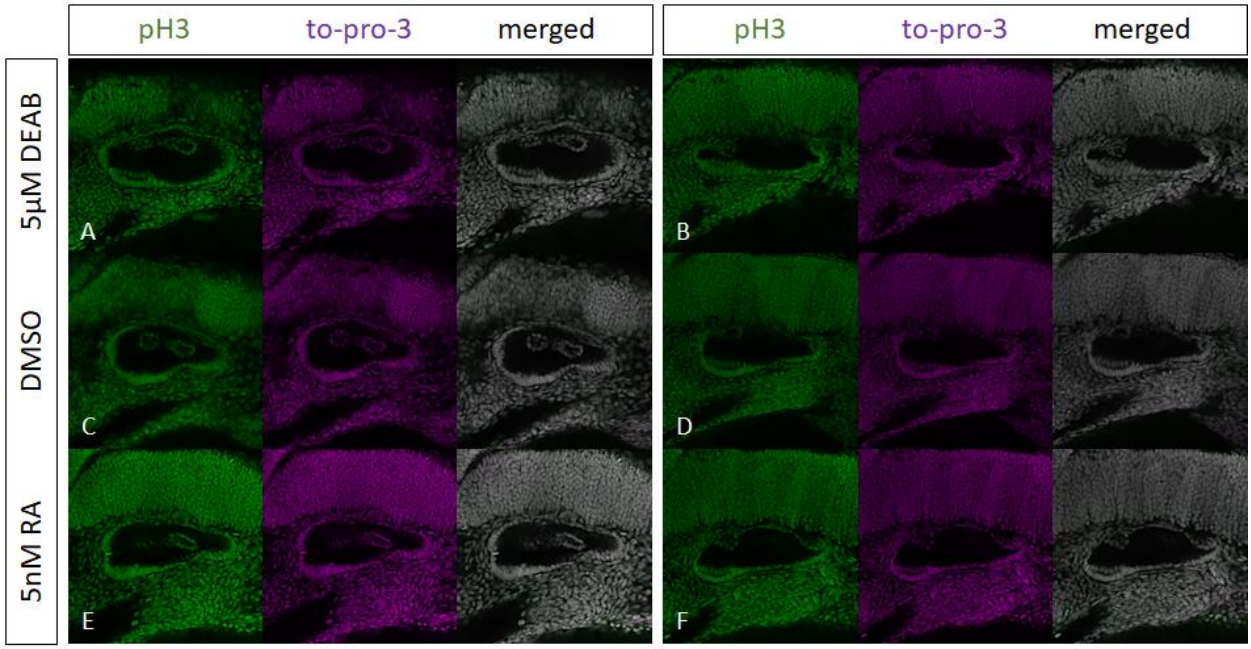
Supplemental Figure 3.4. 2. Percentage of left- and right- handed circles completed in response to pharmacological perturbations of the Retinoic Acid signaling pathway in zebrafish larvae. Larvae were recorded for a total of 10 minutes using a CCTV camera (WV-CL930) and the Ethovision XT10 software by Noldus (Wageningen, Netherlands). Three larvae from the control group and each treatment group were tested per 12-well plate, with a 20-minute acclimatization period prior to recording. The sample arrangement for each plate was randomized and blinded prior to quantification of circling phenotype. Circling phenotype was defined as any continuous and complete 360◦ revolution in the horizontal plane that did not exceed 4.5mm in diameter (1/4 the diameter of a well). RA signaling was ablated with DEAB (1 µM or 5µM) or increased with exogenous RA (5nM); DMSO was used as a solvent control. The percentage of left handed turns for each group are as follows: DMSO = 52.9%; 1 µM DEAB = 49.8%; 5µM DEAB = 51.0%; 5nM RA = 53.2%. N represents the total number of larvae included in the count; larvae that did not move within the recording period were omitted. Abbreviations: DEAB, diethylaminobenzaldehyde; DMSO, dimethyl sulfoxide (DMSO); RA, retinoic acid.



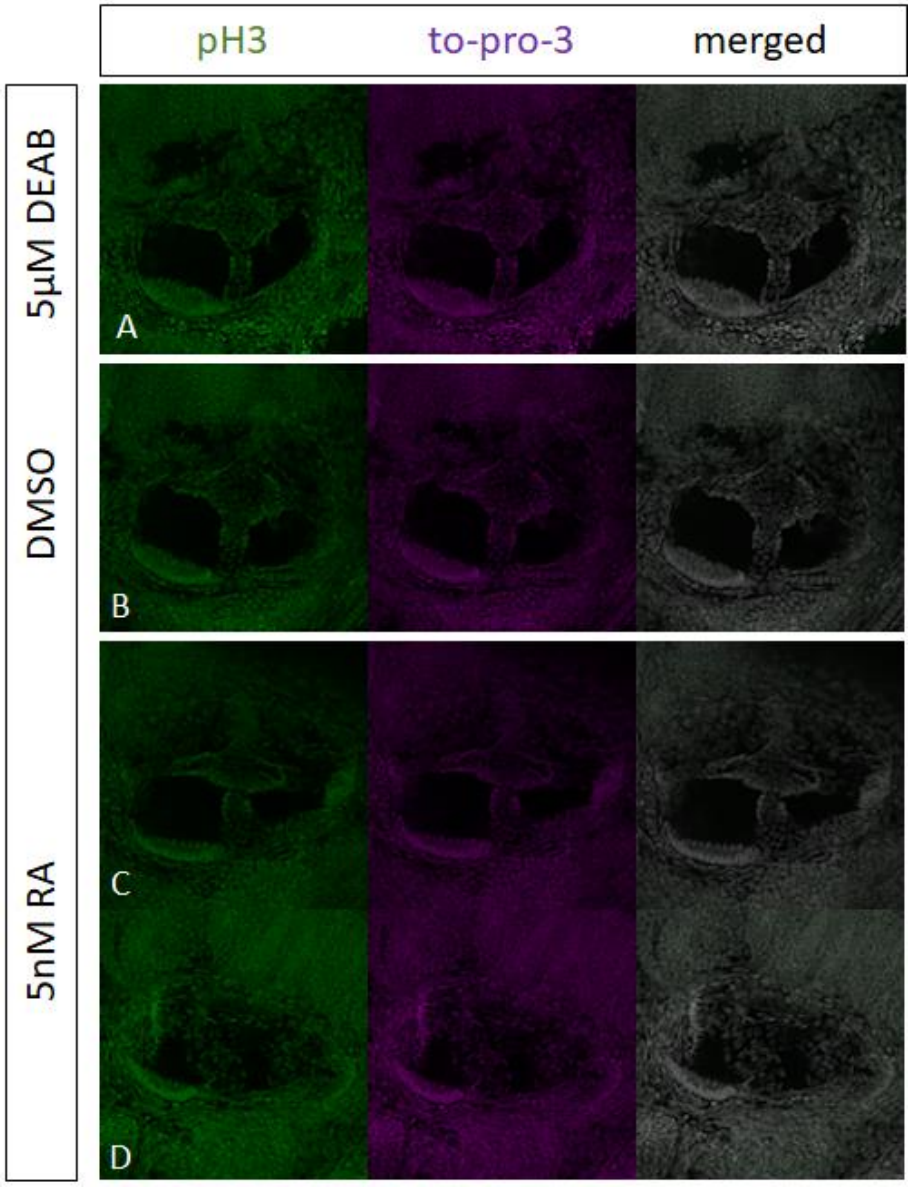
Supplemental Figure 3.4. 3. Otic expression of the enhancer trap *nk12aEt* (insertion line 'hspGFFDMC12A') generated by the Kawakami Lab (NIG, Japan). A stereoscopic lateral image of the inner ear at 3 dpf. Traces of a stack of confocal images taken of an inner ear generated with the Imaris (Bitplane) software (B). A 3D reconstruction of these traces for measurements of the inner ear with the Imaris (Bitplane) software (B′).



Supplemental Figure 3.4. 4. Immunohistochemistry analysis of apoptosis using an activated caspase 3 antibody following perturbations of the Retinoic acid signaling pathway in zebrafish larvae (3 dpf). RA signaling was ablated with diethylaminobenzaldehyde (DEAB; 5µM) or increased with exogenous RA (5nM) from 28 hpf onward; dimethyl sulfoxide (DMSO) was used as a solvent control. These confocal images are situated with the anterior to the left. Individual and merged images of an antibody against active caspase3 (green) or the nuclear counterstain To-pro-3 (magenta) in the DEAB treated (A), DMSO control (B), or exogenous RA treated (C) groups.



Supplemental Figure 3.4. 5. Immunohistochemistry analysis of cell proliferation using a phospho-histone H3 (Ser10) antibody following perturbations of the Retinoic acid signaling pathway in zebrafish larvae (2 dpf). RA signaling was ablated with diethylaminobenzaldehyde (DEAB; 5µM) or increased with exogenous RA (5nM) from 28 hpf onward; dimethyl sulfoxide (DMSO) was used as a solvent control. These confocal images are situated with the anterior to the left. Individual and merged images of an antibody against phospho-histone H3 (Ser 10) (green) or the nuclear counterstain To-pro-3 (magenta) in the DEAB treated group either mid-way, mediolaterally, through the otic vesicle (A) or more medially (B). Staining for phospho-histone H3 in the DMSO control group either mid-way, mediolaterally, through the otic vesicle (C) or more medially (D). Staining for phospho-histone H3 in RA treated group either mid-way, mediolaterally, through the otic vesicle (E) or more medially (F).



Supplemental Figure 3.4. 6. Immunohistochemistry analysis of cell proliferation using a phospho-histone H3 (Ser10) antibody following perturbations of the Retinoic acid signaling pathway in zebrafish larvae (3 dpf). RA signaling was ablated with diethylaminobenzaldehyde (DEAB; 5µM) or increased with exogenous RA (5nM) from 28 hpf onward; dimethyl sulfoxide (DMSO) was used as a solvent control. These confocal images are situated with the anterior to the left. Individual and merged images of an antibody against phospho-histone H3 (Ser 10) (green) or

the nuclear counterstain To-pro-3 (magenta) taken midway, mediolaterally, in the DEAB treated (A), DMSO control (B), or RA treated (C-D) groups.

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Chapter 4: Utilizing the Gal4:UAS system to visualize vestibular features of the vertebrate inner ear

4.1 Introduction

The inner ear is a fluid-filled organ that is essential for the perception of motion and gravity. It contains two specialized apparatuses that enable the detection of different accelerations of the head, both of which appear across vertebrates (Abbas & Whitfield, 2010; Ekdale, 2016). Angular accelerations are detected by the semi-circular canals, three orthogonally-oriented ducts that each terminate in a sac housing a sensory epithelium known as the crista ampullaris (Mescher, 2010). The hair cells of this epithelium are sheathed in a gelatinous mass called cupula that is deflected by the fluid in the ear during rotational motions of the head. Linear accelerations and gravity are instead detected by the maculae, which are two sensory epithelia housed in the central vestibule of the inner ear adjacent to the semi-circular canals (Hain & Helminski, 2007; Purves et al., 2001). These epithelia are overlain by an otolithic membrane, a gelatinous mass imbedded with small calcium crystallites called otoliths or otoconia (Hain & Helminski, 2007; Purves et al., 2001). The macula that senses accelerations along the horizontal plane is known as the utricle, whereas the macula that senses accelerations along the vertical plane is called the saccule, although in fish the utricle is shown to be the only macula critical to vestibular function, with the saccule playing more of a role in detecting auditory stimuli (Hain & Helminski, 2007; Riley & Moorman, 2000; Sisneros & Rogers, 2016).

Some headway has been made over the years in understanding the primary morphological steps behind the developmental programme of the inner ear and its vestibular apparatuses. For example, we know that it begins with an ectodermal thickening along the anterior border of the neural plate that will give rise to the otic placode, the source of the various inner ear cell types. This placode then cavitates in fish or invaginates in amniotes to form a hollow organ known as the otic vesicle (Kaufman, 1992; Kwon, Bhat, Sweet, Cornell, & Riley, 2010; Maroon et al., 2002;

McCarroll et al., 2012; Ohyama, Mohamed, Taketo, Dufort, & Groves, 2006; Schlosser & Northcutt, 2000; Steventon, Mayor, & Streit, 2012). The anteroventral portion of this vesicle gives rise to the neuro-sensory competent domain, the region from which the neuroblasts of the vestibulocochlear nerve delaminate and where sensory hair cells and their support cells originate (Ahmed, Xu, & Xu, 2012; Chen, Johnson, Zoghbi, & Segil, 2002; A. E. Kiernan et al., 2005; N. Pan et al., 2011; W. Pan et al., 2013; Petrovic et al., 2014; Xu et al., 1999; Zheng et al., 2003; Zou et al., 2008). As otic development progresses, this domain separates into the various sensory epithelia of the inner ear, the maculae and the cristae (Hwang, Simeone, Lai, & Wu, 2009; Nichols et al., 2008; Pauley et al., 2003). It is upon the maculae that the otoliths or otoconia then tether and grow, and it is with the aid of signaling cues from the cristae that the formation of semi-circular canals is then induced, although canal morphogenesis varies between species (Chang, Brigande, Fekete, & Wu, 2004; Chang et al., 2008; Hammond et al., 2009; Mansour, Goddard, & Capecchi, 1993; Omata et al., 2007; Pauley et al., 2003; Riley, Zhu, Janetopoulos, & Aufderheide, 1997; Tanimoto, Ota, Inoue, & Oda, 2011). In zebrafish and *Xenopus*, protrusions of the otic epithelium protrude into the vesicle and fuse together to generate the pillars of the canals (Paterson, 1949; Robert Earle Waterman & Danny H Bell, 1984). In amniotes, canal morphogenesis begins with the formation of two out-pouches, one vertical and one horizontal (J. P. Bissonnette & D. M. Fekete, 1996; Fekete, 1999; Kopecky, Johnson, Schmitz, Santi, & Fritzsch, 2012). Opposing epithelial within each out-pouch extend toward one another to form fusion plates, which are resorbed through apoptosis to create the canal ducts along the rim, the anterior and posterior canals from the vertical out-pouch and the lateral canal from the horizontal.

Although the fundamental steps of otic development have been identified, there is still a gap in our knowledge concerning all the genetic factors involved in these morphological processes,

a field of research that is hindered by the lack of a fast and reliable way to measure the inner ear or visualize key otic structures. Current methods include the generation of 2D images taken by a camera affixed to a microscope, usually with the aid of more specialized microscope techniques like differential interference contrast microscopy to capture the finer details of specific inner ear cell types or features (Hans & Westerfield, 2007; Ryan D Hartwell et al., 2019; Kawashima et al., 2011; Whitfield, Riley, Chiang, & Phillips, 2002). The observation of key inner ear features can also be accomplished by the *in situ* hybridization analysis of genes known to be expressed in specific tissues (Geng et al., 2013; Hans & Westerfield, 2007). However, this method requires that the target transcripts be of a high enough copy number to allow for detection and relies on the unaltered expression pattern of these genes under the conditions studied, requiring previous knowledge of their regulation (Gall & Pardue, 1969; Harrison, Conkie, Paul, & Jones, 1973; Jones & Robertson, 1970). Additionally, both techniques do not allow for the generation of a 3D model of the ear, making them ill-suited for visualizing and measuring the inner ear as a whole.

Other methods exist that allow for modelling of the entire inner ear. These include the reconstruction of inner ear images captured with computed tomography, magnetic resonance imaging, or in histological sections, although these are time-consuming and expensive techniques (Agarwal, Singh, Ghuman, Sharma, & Lahiri, 2014; Jun et al., 2005; Kendi, Arikan, & Koc, 2005; Rau, Würfel, Lenarz, & Majdani, 2013). A more inexpensive method is the paint-fill technique, whereby an opaque marker, such as white gloss paint, can be injected directly into the inner ear with a needle pulled from a capillary tube (John P Bissonnette & Donna M Fekete, 1996; Amy E. Kiernan, 2006; Martin & Swanson, 1993; Miller Bever, Jean, & Fekete, 2003). However, this method also suffers from its share of drawbacks. Injections into the ear at later stages of inner ear development can be hindered by the formation of cartilage or bone around the ear, and these

injection sites are prone to leakage if the needle is too large (Amy E. Kiernan, 2006). Furthermore, injecting the inner ear with an opaque substance can inadvertently obscure internal otic features, such as with zebrafish, whose semi-circular canals form from the inward growth of protrusions toward a central point within otic vesicle (R. E. Waterman & D. H. Bell, 1984).

An inexpensive and elegant solution to this problem would be the use of fluorescent markers that label either the entire otic epithelium or select features of the inner ear, of which 2D confocal images could be reconstructed into 3D images and measured by modeling software, such as Imaris, Amira, or Fiji (Bloch et al., 2019; Ethell & Cameron, 2014; Goetz et al., 2014; Packard et al., 2017). However, an obstacle to this solution is the development of genetic tools that drive visual reporters in unique spatial patterns within the inner ear. Of course, several methods already exist for the *in vivo* observation and spatiotemporal manipulation of gene expression in vertebrate and invertebrate models. One of the most well known is the GAL4-UAS system, which uses a yeast transcription activator protein (Gal4)—which, alone, is lacking the necessary regulatory elements for its own transcription—and its targeted upstream activator sequence (UAS) to drive the expression of a target gene, such as a fluorescent reporter (Brand & Perrimon, 1993; Duffy, 2002; Kakidani & Ptashne, 1988; Webster, Jin, Green, Hollis, & Chambon, 1988). Therefore, utilizing the Tol2 transposon system, which uses a transposase to facilitate the random insertion of foreign DNA into the genome, gene traps, where the insertion is made within a gene locus, and enhancer traps, where the insertion is made within the region of an enhancer's influence, can be generated to drive Gal4 expression in a temporal and tissue specific manner, as determined by the endogenous spatiotemporal regulatory elements or behaviors of the targeted gene or enhancer (Koga, Iida, Hori, Shimada, & Shima, 2006; Stuart, McMurray, & Westerfield, 1988). In doing so, crossing these lines with those possessing a target gene downstream UAS will confer that same

spatiotemporal specificity on its expression in the resulting progeny. This precise mechanism has already been used by the Kawakami laboratory at the National Institute of Genetics (NIG) in Mishima, Japan, to generate a gene and enhancer trap database of zebrafish transgenic lines expressing 'GFF,' a fusion protein of the Gal4 DNA binding domain and a tandem repeat of the VP16 transactivation domain that is more strongly expressed in zebrafish (Asakawa & Kawakami, 2009; Asakawa et al., 2008; Kawakami et al., 2016). They have so far generated many lines that express GFF in various embryonic tissues, including the developing inner ear.

In this paper, we describe the otic expression profile of six zebrafish transgenic lines, four gene traps and two enhancer traps, analyzed during a screen of the NIG database. These lines express GFF in distinct otic structures, such as the cristae, the maculae, the semi-circular canals, and broadly throughout the otic epithelium. Together, these lines enable the visualization of key structures and can aid future research involving the manipulation of target gene expression in a spatial manner within the developing inner ear.

4.2 Results

4.2.1 Shelf screen analysis

The database at the NIG (accessible here: https://ztrap.nig.ac.jp/ztrap/) currently hosts 130 zebrafish transgenic lines that display GFF expression in otic tissue. In order to identify what we would consider to be the most beneficial strains for visualizing otic development, we adhered to the following criteria during our screen. First, we wanted to isolate strains that demonstrated otic expression of GFF from 24 hpf until 5 days post fertilization (5 dpf). This timeframe was chosen because it overlaps many morphological processes in the developing zebrafish ear, such as the separation of the sensory epithelia, otolith formation, and morphogenesis of the semi-circular canals. 5 dpf is also the stage at which the swim bladder has developed and zebrafish larva are capable of mature swimming, coinciding with the observation of behaviours indicative of vestibular dysfunction, such as circling, looping, and rolling (Hernandez, Galitan, Cameron, Goodwin, & Ramakrishnan, 2018; Nicolson et al., 1998). Second, we selected for strains that strongly expressed GFF in distinct otic structures or cell types relevant to vestibular function, such as within the sensory epithelia, like the cristae or the maculae, or the epithelia involved in canal morphogenesis, such as the protrusions that fuse to form the pillars of canals. This was to facilitate the observation of malformations in any of these tissues that might correlate with vestibular dysfunction. Additionally, we wanted to identify strains that alternatively demonstrated strong GFF expression throughout the otic epithelium, which would allow for modeling of the entire inner ear. This would enable the observation of gross malformations in the size or shape of the inner ear and allow for more precise computational measurements of the inner ear, such as with respect to its volume, lumenal surface area, and axial dimensions.

Through our shelf screen, we identified four gene traps (*nkgsaigff266c*, *nkgsaizgffd262a*, *nkgsaizgffm789a*, and *nkgsaizgffm1225a*) and two enhancer traps (*nkhspgff75a* and *nkhspgffdmc12a*) of interest that fulfilled our criteria. We describe their unique otic expression patterns in further detail below.

4.2.2 nkgsaigff266c

Through inverse PCR, the Kawakami laboratory at the NIG has already identified this transgenic line as a gene trap of *UDP-glucose 6-dehydrogenase* (*ugdh*) (Table 4.4.1). The enzyme encoded by this gene converts UDP-glucose to UDP-glucuronic acid, an obligate precursor for the biosynthesis of glycosaminoglycans, such as hyaluronan, heparan sulfate, and chondroitin sulfate (Casale & Crane, 2019; Egger, Chaikuad, Kavanagh, Oppermann, & Nidetzky, 2011; Egger et al., 2012). Previous work in zebrafish has shown that a loss of *ugdh* expression within the protrusions of the semi-circular canals coincides with a reduction of hyaluronan production, resulting in malformed protrusions that often fail to fuse (Busch-Nentwich, Söllner, Roehl, & Nicolson, 2004; Geng et al., 2013). Outside the inner ear, *ugdh* expression is observed from approximately 30 to 48 hpf in the fin bud, pectoral fin, pharyngeal arches, mandibular arch skeleton, muscle precursor adaxial cells, and heart tube (Busch-Nentwich et al., 2004; Walsh & Stainier, 2001).

In *nkgsaigff266c*, GFF expression in the developing ear reflects what is seen in the literature from 3-5 dpf (Table 4.4.1; Figure 4.5.1)(Busch-Nentwich et al., 2004; Geng et al., 2013). At both 3 dpf and 4 dpf, GFF is evident at the leading edge of the anterior, posterior, and ventral protrusions (Figure 4.5.1A-B′). At 5 dpf, expression is also evident within the ventral otic epithelium (Figure 4.5.1C-C′). However, from 3-5 dpf, there is additional expression in what appears to be the anterior lateral line ganglia (Figure 4.5.1A- C′). Because the *in situ* hybridization

analysis of gene expression requires a target transcript be of a high enough copy number to allow for detection, *ugdh* expression in the anterior lateral line ganglia might have been missed in previous research.

4.2.3 nkgsaizgffd262a

nkgsaizgffd262a has already been identified by the NIG as a gene trap of *otogelin* (*otog*) (Table 4.4.1). The protein encoded by this gene is a glycoprotein expressed in the hair cells and support cells of the sensory epithelia, as well as their acellular membranes (Cohen-Salmon, El-Amraoui, Leibovici, & Petit, 1997; Stooke-Vaughan, Obholzer, Baxendale, Megason, & Whitfield, 2015). Previous work has identified it as an essential component in the adherence of otolith precursor cells to the tether hair cells of the presumptive maculae, a crucial step in otolith seeding (Riley et al., 1997; Stooke-Vaughan et al., 2015). The *in situ* hybridization analysis of *otog* expression localizes it only to the developing inner ear, placing it in the presumptive maculae at the anterior and posterior poles of the otic vesicle from approximately 10 to 24 hpf (Schwarzer, Spieß, Brand, & Hans, 2017; Stooke-Vaughan et al., 2015). From 24 hpf to 4 dpf, the literature also describes the expression of *otog* in all three cristae and both maculae (Stooke-Vaughan et al., 2015).

The expression of GFF in *nkgsaizgffd262a* closely resembles *otog* expression in the literature (Table 4.4.1; Figure 4.5.2)(Schwarzer et al., 2017; Stooke-Vaughan et al., 2015). At 24 hpf, GFF is faintly expressed at the anterior and posterior poles of the otic vesicle (Figure 4.5.2A). At 48 hpf, this expression is localized to the anterior, posterior, and lateral crista primordia and the utricular macula (Figure 4.5.2B-B′). At 3, 4, and 5 dpf, expression persists in the anterior, posterior, and lateral crista and utricular macula and is also now evident at the dorsal edge of the saccule

(Figure 4.5.2C-E′). In extending our observations to 5 dpf, beyond which *otog* expression is analyzed in the literature, we also note novel *otog* expression within the eye, heart, and musculature of the torso.

4.2.4 nkgsaizgffm789a

nkgsaizgffm789a has been determined by the NIG to be a gene trap of *carbonic anhydrase* (*cahz*) (Table 4.4.1). Zebrafish *cahz* is homologous to the carbonic anhydrases found in other species, most closely resembling *CA2* in humans (Ito et al., 2013; Peterson, Tu, & Linser, 1997). *cahz* belongs to a family of zinc metalloenzymes responsible for the reversible interconversion between CO_2 and HCO_3^-, a crucial process in maintaining an alkaline endolymph pH, which directly contributes to proper otoconial/otolith composition (Maren, 1984; Peterson et al., 1997; Silverman & Lindskog, 1988). Previous work has found that *cahz* is faintly expressed in numerous cell types and tissues from the 1-cell stage up until 5 dpf in zebrafish, but it is most strongly expressed in the yolk, brain, retina, blood, pancreas, and spinal cord (Gilmour, Thomas, Esbaugh, & Perry, 2009; Greenhough et al., 2018; Kudoh et al., 2001; Li et al., 2015; Lin et al., 2008; Rauch et al., 2003).

With our analysis of *nkgsaizgffm789a*, we provide a more detailed description of *cahz*, particularly within the inner ear. From 24-48 hpf, GFF is broadly expressed throughout the otic epithelium, as well as the yolk (Table 4.4.1; Figure 4.5.3A-B). At 48 hpf, it is also evident outside the ear in the eye, brain, and spinal cord, where it persists until at least 5 dpf. By 3 dpf, this widespread expression is weaker, although it is more noticeable in some structures, such as the semi-circular canals (Figure 4.5.3C-D). At this time, it is also more strongly expressed in the anterior, posterior, and lateral cristae, as well the utricle. Widespread expression in the otic

epithelium continues to decrease at 4-5 dpf, although it is still strongly expressed in the cristae and the utricle (Figure 4.5.3E-G). By 4 dpf, expression outside the ear is also evident in the pancreas.

4.2.5 nkgsaizgffm1225a

While the insertion locus of *nkgsaizgffm1225a* has yet to be determined, GFF expression is evident in sensory and non-sensory otic tissues from 3-5 dpf (Table 4.4.1; Figure 4.5.4). At 3 dpf, GFF is expressed in the semi-circular canals, localized to the leading edge of the protrusions, where they fuse, most strongly between the lateral protrusion and the anterior and posterior protrusions (Figure 4.5.4A). Expression in the semi-circular canals continues into 4 dpf, although it is now additionally evident in what appears to be the anterior crista and possibly an anterior portion of the utricle, as well as within the otic epithelium near or at the dorsolateral septum, the partition between the anterior and posterior canals (Figure 4.5.4B). At 5dpf, GFF expression is strong in the utricle and faint within the anterior, posterior, and lateral cristae (Figure 4.5.4C-D). Additionally, expression is still evident either near or at the dorsolateral septum (Figure 4.5.4C). From 3-5 dpf, GFF expression is also weakly evident outside the ear in the eye, gut, brain, and spinal cord.

4.2.6 nkhspgff75a

The insertion for the *nkhspgff75a* enhancer trap line has been found on Chromosome 20, region 9,009,953-9,010,133 (Table 4.4.1). At 24 hpf, GFP expression is evident within the anteroventral and posterior otic vesicle, as well as in the skin and heart (Figure 4.5.5A). At 48 hpf, it is expressed in the anterior, posterior, and lateral crista primordia and the utricle (Figure 4.5.5B).

It is also still evident in the heart and skin. From 3-5 dpf, expression continues in the three cristae and the utricle, and it is weakly expressed in the saccule (Figure 4.5.5C-G′). From 3-4 dpf, it is also evident outside the ear in the heart, neuromasts of the lateral line system, retina, and musculature of the torso, although this expression pattern of GFF does not extend to 5 dpf.

4.2.7 nkhspgffdmc12a

The insertion for the *nkhspgffdmc12a* enhancer trap line has been found on Chromosome 12: 43,435,503-43,435,993 (Table 4.4.1). At 24 hpf, GFF expression is evident throughout the otic vesicle (Figure 4.5.6A). From 48 hpf to 5 dpf, GFF continues to be expressed throughout the otic epithelium, although it is significantly weaker at 5 dpf (Figure 4.5.6B-G). From 24 hpf to 5 dpf, it is also expressed strongly throughout the eye and weakly throughout the rest of the body.

4.3 Discussion

Though headway has been made in our understanding of the primary morphological steps behind the developmental programme of the inner ear, this field of research suffers from the lack of a fast and reliable way to measure the inner ear or visualize key otic structures, especially those responsible for vestibular function. Currently, the visualization of the inner ear is usually carried out with microscope techniques that generate only 2D images or the reconstruction of a 3D model of the inner ear with methods that are often laborious and time-consuming, such as magnetic resonance imaging, computed tomography, or the paint-fill technique (Agarwal et al., 2014; John P Bissonnette & Donna M Fekete, 1996; Ryan D. Hartwell et al., 2019; Jun et al., 2005; Kawashima et al., 2011; Kendi et al., 2005; Amy E. Kiernan, 2006; Martin & Swanson, 1993; Miller Bever et al., 2003; Rau et al., 2013; Whitfield et al., 2002). A possible solution to this problem is the utilization of a system that could drive target gene expression, such as a visual reporter, either throughout the otic epithelium or in distinct otic features. This would then enable more detailed measurements of the inner ear when used in conjunction with remodeling software or allow for the live time-lapse analysis of morphogenetic processes involving only specific otic features.

The Gal4/UAS system is a powerful tool for driving the expression of transgenes in a spatial and temporal manner during embryogenesis in invertebrate and vertebrate models. By utilizing the Tol2 transposon system, several gene and enhancer trap lines driving an optimized form of Gal4 (GFF) in a time and tissue specific manner have been made available by the Kawakami Laboratory at the National Institute of Genetics in Japan (accessible here: https://ztrap.nig.ac.jp/ztrap/). Each of the transgenic zebrafish selected in our screen of this database can be used for the targeted expression of fluorescent reporters to observe the

morphogenesis of inner ear structures during later stages of development. For example, both *nkgsaigff266c* and *nkgsaizgffm1225a* express GFF in the protrusions of the semi-circular canals. These transgenic lines would therefore be useful in visualizing the shape and number of these protrusions, as well as performing a time-lapse of the fusion of these protrusions as they give rise to the pillars of the canals. Additionally, because these lines are not limited to driving the tissue specific expression of visual reporters, they can also be used to drive the overexpression of genes normally transcribed in the protrusions or constructs that interfere with their transcription, translation, or activity (Chavous, Jackson, & O'Connor, 2001; Elefant & Palter, 1999; Hara et al., 2017; Scheer & Campos-Ortega, 1999). This would allow, for example, the targeted manipulation of genes localized in or around the protrusions, such as *hapln1a*, *versicana*, *versicanb*, *col2a1a,* or *has3*, which have not yet been investigated regarding their independent roles in semi-circular canal morphogenesis (Busch-Nentwich et al., 2004; Geng et al., 2013; Lee et al., 2015; Sarmah et al., 2010; van der Velden, Wang, Querol Cano, & Haramis, 2013).

There are additional avenues of research into semi-circular canal morphogenesis and other otic developmental processes made possible by the transgenic lines described in this paper. For example, *nkgsaizgffd262a*, *nkgsaizgffm789a*, *nkgsaizgffm1225a*, and *nkhspgff75a* express GFF in the cristae of the semi-circular canals and the utricle, with the addition of the saccule in *nkgsaizgffd262a* and *nkhspgff75a*. These sensory epithelia originate from one large sensory domain present early in otic development; in fact, the separation of this domain into the various sensory epithelia precedes many important morphological processes in the development of the inner ear. For example, given their role in inducing the formation of the canal ducts, the improper separation, size, or positioning of the cristae is likely to affect canal morphogenesis, such as what is seen in studies investigating the improper separation of cristae and lack of canals in *Fgf10*$^{-/-}$

chicks (Chang et al., 2004; Chang et al., 2008; Hammond et al., 2009; Omata et al., 2007). Not only do these transgenic lines allow for the observation of malformations involving cristae separation, size, or position under certain conditions, but they can also be used for the genetic manipulation of suspected inducers of canal morphogenesis and their potential downstream targets within the cristae.

Of course, these lines are not limited to research involving canal morphogenesis. Given the macular expression of GFF in *nkgsaizgffd262a*, *nkgsaizgffm789a*, *nkgsaizgffm1225a,* and *nkhspgff75a*, these lines can therefore facilitate research in the generation or tethering of the otoliths, the otolithic membranes, or the cupulae. For example, *nkgsaizgffd262a* represents a line carrying an insertion in *otogelin*, a gene that is responsible for tethering the cupulae and otolithic membranes to their underlying sensory epithelia (Stooke-Vaughan et al., 2015). GFF is therefore expressed in the same cells that express *otogelin*, such as hair cells, tether cells (precocious hair cells upon which the otolith precursor particles form), and the surrounding epithelial cells. Potential candidate genes involved with the generation, tethering, or maintenance of the cupulae, otolithic membranes, or otoliths can therefore be investigated by *nkgsaizgffd262a*, as well as *nkgsaizgffm789a*, *nkgsaizgffm1225a,* and *nkhspgff75a*. Furthermore, these transgenics can also be used to further research into the regenerative abilities of teleost hair cells, which have been studied in great detail in the maculae (Lombarte, Yan, Popper, Chang, & Platt, 1993; Smith, Coffin, Miller, & Popper, 2006; Sun, Lin, & Smith, 2011). Mammalian hair cells typically do not regenerate following trauma caused by ototoxic agents or noise, and the loss of inner ear hair cells is a common cause of peripheral vestibular disorders (Desmadryl, Dechesne, & Raymond, 1992; Lysakowski, 1999; Nadol Jr, 1993; Stone, Oesterle, & Rubel, 1998). These lines can therefore facilitate the investigation of candidate genes potentially involved with hair cell regeneration.

Finally, some of these transgenic lines can be used to measure the volume, lumenal surface area, or dimensions of the inner ear, as its size and shape can have a direct effect on its internal fluid dynamics and, by extension, vestibular function (Kassemi, Deserranno, & Oas, 2005; Michaels, 1987; Spoor et al., 2007). *nkgsaizgffm789a* demonstrates strong GFF expression throughout the inner ear from 24 hpf to at least 3 dpf, enough so that the protrusions and pillars of the semi-circular canals are easily visible. *nkhspgffdmc12a* likewise demonstrates strong expression throughout the otic epithelium, at least until 4 dpf. 2D confocal images of either of these lines can be reconstructed into 3D images and measured by software such as Imaris, Fiji, or Amira, which have already been used to model the brain, heart, and vasculature in zebrafish (Bloch et al., 2019; Ethell & Cameron, 2014; Goetz et al., 2014; Packard et al., 2017).

In conclusion, the transgenic lines described here demonstrate the expression of GFF in key vestibular structures over the course of inner ear development, from at least 24 hpf until 5 dpf. Therefore, they provide an opportunity to visualize these structures or manipulate the expression of target genes in a spatial manner, particularly at later stages of development, facilitating future research of several areas within the field of otic development.

4.4 Tables

Table 4.4. 1. Summary of insertion locus and otic expression patterns (24 hpf – 5 dpf) for Gal4 (GFF) driver transgenic zebrafish generated by the Kawakami laboratory at the National Institute of Genetics (NIG), Japan.

Line names	Transgenic type	Locus (gene)	Otic expression pattern
nkgsaigff266c	Gene trap	Chromosome 1: 23,294,023-23,293,351 (*ugdh*)	▪ Ventral otic epithelium ▪ Semi-circular canals (protrusions)
nkgsaizgffd262a,	Gene trap	Chromosome 7: 39,536,010-39,536,409 (*otog*)	▪ Anterior and posterior otic epithelium ▪ Anterior, posterior, lateral crista primordia ▪ Anterior, posterior, lateral cristae ▪ Utricular and saccular otoliths/maculae
nkgsaizgffm789a	Gene trap	Chromosome 2: 29,195,642-29,196,354 (*cahz*)	▪ Throughout otic epithelium ▪ Anterior, posterior, lateral cristae ▪ Utricle
nkgsaizgffm1225a	Gene trap	Undetermined	▪ Semi-circular canals (protrusions, particularly where the anterior and posterior fuse with the lateral) ▪ Anterior, posterior, lateral cristae ▪ Utricle ▪ Otic epithelium near or at the dorsolateral septum
nkhspgff75a	Enhancer trap	Chromosome 20: 9,009,953-9,010,133	▪ Anterior and posterior otic epithelium ▪ Anterior, posterior, lateral crista primordium ▪ Anterior, posterior, lateral cristae, ▪ Utricle ▪ Possibly saccule

nkhspgffdmc12a	Enhancer trap	Chromosome 12: 43,435,503-43,435,993	▪ Throughout otic epithelium

4.5 Figures

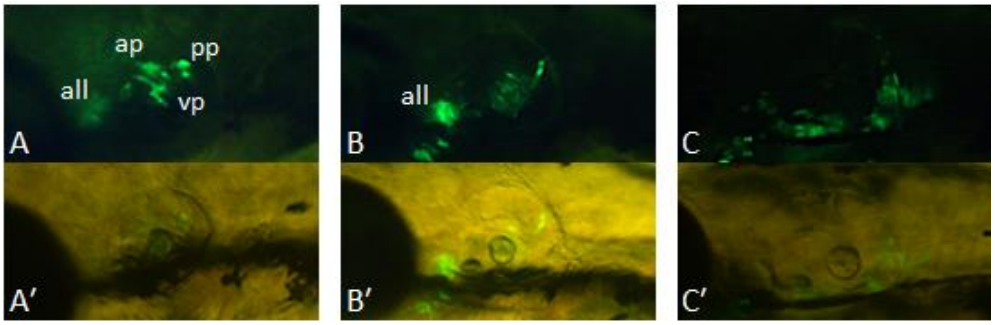

Figure 4.5. 1. Otic expression of the gene trap line *nkgsaigff266c*, which represents an insertion in *ugdh*. The anterior direction is to the left in these lateral images. Images A, B, and C are visualized solely with fluorescent microscopy; images A′, B′, and C′ are additionally back lit with a halogen lightbulb to aid visualization of GFF expression in relation to overall otic anatomy. Expression is evident at 3 dpf (A-A′) and 4 dpf (B-B′) in the pillars of the semi-circular canals at the leading end of each of the (anterior, lateral, posterior, and ventral) protrusions, where they fuse; however, unlike previous descriptions of *ugdh*, expression also appears in the anterior lateral line ganglia. At 5dpf, expression is evident in the ventral otic epithelium (C-C′). Abbreviations: all, anterior lateral line ganglia; ap, anterior protrusion/fusion point; pp, posterior protrusion/fusion point; vp, ventral protrusion/fusion point.

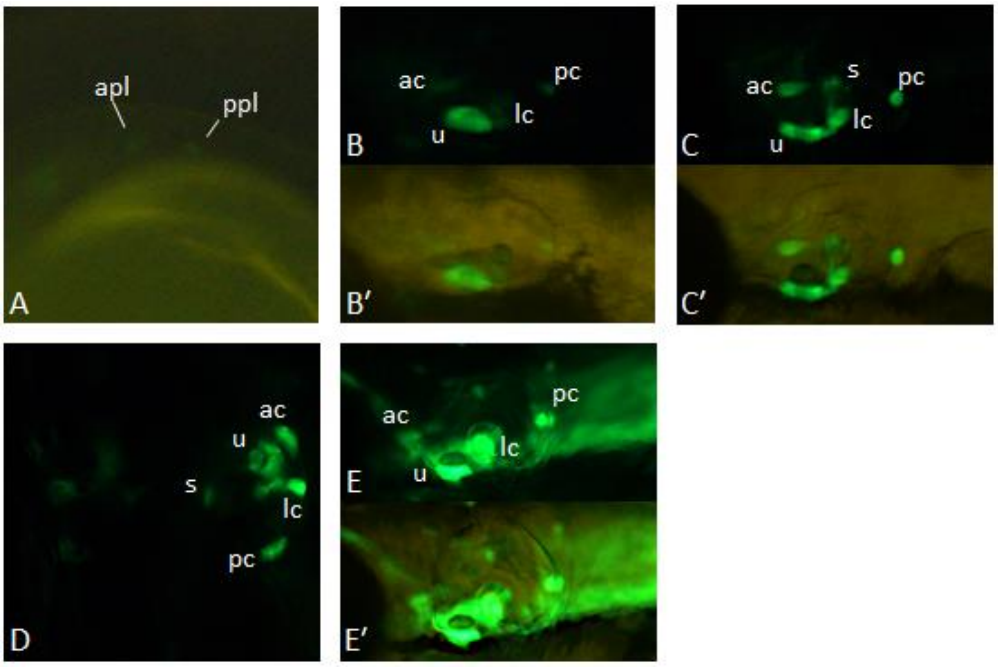

Figure 4.5. 2. Otic expression of the gene trap line *nkgsaizgffd262a*, which represents an insertion in *otog*. The anterior direction is to the left in these lateral images (upward in dorsal images, where specified). Images A, B, C, D, and E are visualized solely with fluorescent microscopy; images B′, C′, and D′ are additionally back lit with a halogen lightbulb to aid visualization of GFF expression in relation to overall otic anatomy. At 24 hpf, expression is evident at the anterior and posterior poles of the otic vesicle (A). At 48 hpf, expression is localized to the anterior, posterior, and lateral crista primordia and the utricular macula (B-B′). At 3 dpf (C-C′), 4 dpf (D, dorsal view), and 5 dpf (E-E′), expression persists in the anterior, posterior, and lateral crista and utricular macula, as well as the dorsal edge of the saccule. Abbreviations: ac, anterior crista; apl, anterior pole; lc, lateral crista; pc, posterior crista; ppl, posterior pole; s, saccule; u, utricle.

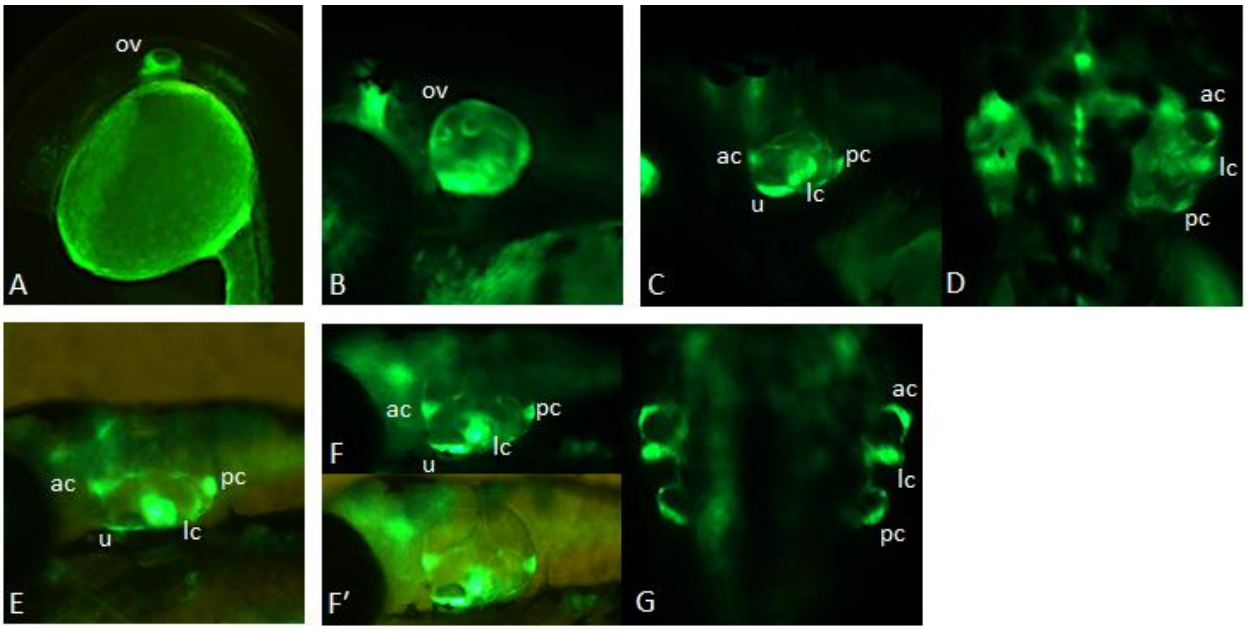

Figure 4.5. 3. Otic expression of the gene trap line *nkgsaizgffm789a*, which represents an insertion in *cahz*. The anterior direction is to the left in lateral images (upward in dorsal images, where specified). Images A, B, C, D, E, F, and G are visualized solely with fluorescent microscopy; image F′ is additionally back lit with a halogen lightbulb to aid in visualizing GFF expression in relation to overall otic anatomy. At 24 hpf (A) and 48 hpf (B) there is broad expression throughout the otic vesicle/epithelium. At 3dpf, expression is still evident throughout the ear, but it is weaker in some areas, most notably in the dorsal region of the ear, and stronger in others, such as the cristae and utricle (C lateral view; D, dorsal view). Broad expression continues to decrease at 4 dpf (E) and 5 dpf (F-F′, lateral view; G, dorsal view), but remains strong in the cristae and utricle. Abbreviations: ac, anterior crista; lc, lateral crista; ov, otic vesicle; pc, posterior crista; u, utricle.

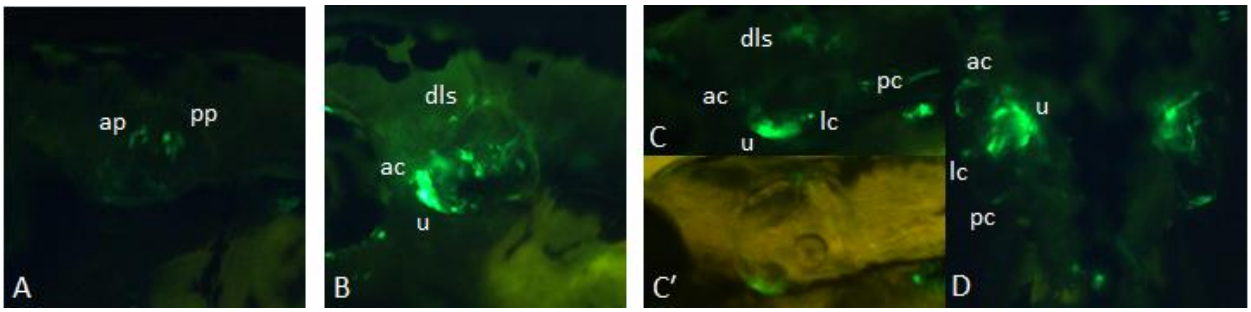

Figure 4.5. 4. Otic expression of the gene trap line *nkgsaizgffm1225a*. The anterior direction is to the left in lateral images (upward in dorsal images, where specified). Images A, B, C, and D are visualized solely with fluorescent microscopy; image C′ is additionally back lit with a halogen lightbulb to aid visualization of GFF expression in relation to overall otic anatomy. Expression is evident at 3 dpf in the pillars of the semi-circular canals where the anterior and posterior fuse with the lateral (A). Expression is still evident at 4 dpf in the pillars of the canal and is additionally in the anterior crista, utricle, and epithelium at or near the dorsolateral septum (B). At 5dpf, expression is evident in the cristae, the utricle, and at or near the dorsolateral septum (C-C′, lateral view; D, dorsal view). Abbreviations: ac, anterior crista; ap, anterior protrusion/fusion point; dls, dorsolateral septum; lc, lateral crista; pc, posterior crista pp, posterior protrusion/fusion point; u, utricle.

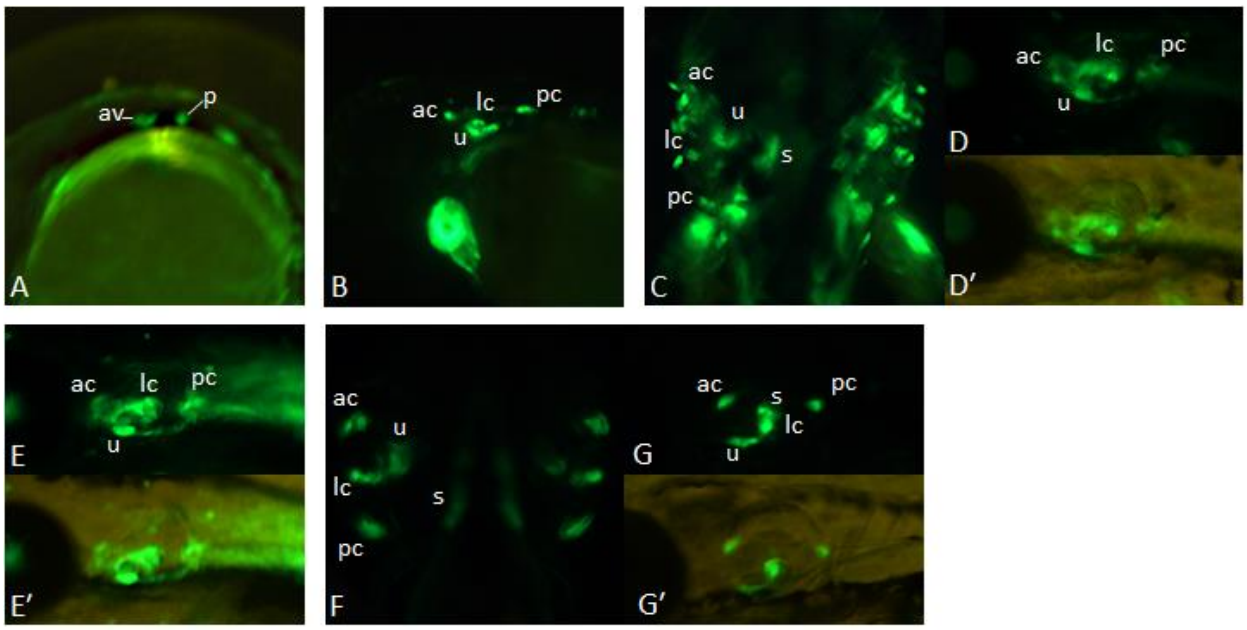

Figure 4.5. 5. Otic expression of the enhancer trap line *nkhspgff75a*. The anterior direction is to the left in lateral images (upward in dorsal images, where specified). Images A, B, C, D, E, F, and G are visualized solely with fluorescent microscopy; images D′, E′, F′, and G′ are additionally back lit with a halogen lightbulb to aid visualization of GFF expression in relation to overall otic anatomy. At 24 hpf, expression is evident within the anteroventral and posterior otic vesicle (A). At 48 hpf, expression is evident in the crista primordia and the utricle (B). From 3 dpf (C, lateral; D-D′, dorsal), 4 dpf (E-E′), and 5 dpf (F, lateral; G-G′) expression is evident in the cristae, the utricle, and weakly in the saccule (Figure C-G′). Abbreviations: av, anteroventral otic vesicle; ac, anterior crista; lc, lateral crista; p, posterior otic vesicle; pc, posterior crista; s, saccule; u, utricle.

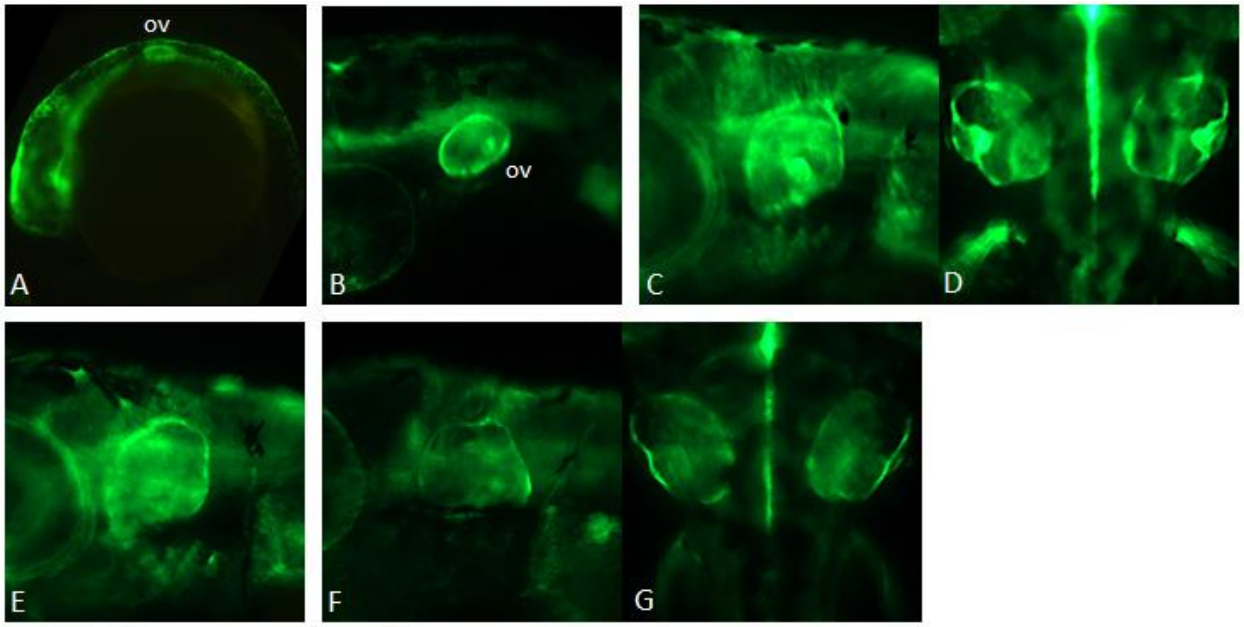

Figure 4.5. 6. Otic expression of the enhancer trap line *nkhspgffdmc12a*. The anterior direction is to the left in lateral images (upward in dorsal images, where specified). At 24 hpf, expression is evident throughout the otic vesicle (A). A 48 hpf (B), 3 dpf (C, lateral; D, dorsal), and 4 dpf (E), expression is throughout the ear. At 5 dpf, this broad expression is still evident but weaker (F, lateral view; G, dorsal view). Abbreviations: ov, otic vesicle

4.6 References

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Chapter 5: Conclusions and Future Directions

Previous research has identified several causative factors contributing to the agenesis or dysgenesis of inner ear structures, such as those required for vestibular function. However, a large body of this work has relied on research involving disruptions to the expression or activity of genes implicated in the earliest stages of otic development, including those involved with the metabolism of Retinoic Acid (RA), a crucial signaling molecule in embryogenesis (Karen Niederreither, Subbarayan, Dollé, & Chambon, 1999; Ono, Keller, et al., 2020; Ono, Sandell, Trainor, & Wu, 2020). To investigate the role RA plays in later stages of otic development in the absence of earlier disruptions to this signaling pathway, we observed for changes in inner ear morphogenesis following late exposure to an RA agonist or antagonist in zebrafish, which share similar anatomy and function of their vestibular apparatuses to other vertebrates (Abbas & Whitfield, 2010; Baxendale & Whitfield, 2014). By utilizing this method, we identified a late role for RA signaling in canal morphogenesis and otolith maintenance. To aid future research in the analysis of later stages of inner ear development, we also performed a shelf screen at the National Institute of Genetics in Japan to analyze zebrafish gene or enhancer trap lines expressing a zebrafish-optimized form of yeast Gal4 in various tissues of the developing ear. We then identified six transgenic lines that could be used to visualize distinct vestibular features of the inner ear or manipulate target gene expression in these structures.

5.1. Retinoic acid signaling in later stages of inner ear development

Many studies approach research into congenital disorders with the intention of determining the key disrupted function of the gene(s) presumed to be responsible for a given disease phenotype. However, while a fraction of genes can appear to control a single characteristic or biological process, more complicated genetic phenomena can impede research into determining the various

roles that a gene might play in the development of a disease phenotype. For example, a pleiotropic gene can affect two or more seemingly unrelated traits, either because the product it encodes is utilized in different ways or because it encodes multiple gene products (He & Zhang, 2006; Plate, 1910). Epistasis can further confound these studies given the many ways in which the phenotype generated by mutations in one gene can depend in a complicated way on the phenotype generated by others (Bateson, 1902). Both of these phenomena touch upon the difficulty in determining how many genes are implicated in a particular phenotype, which genes are involved, and how their gene products might interact to generate that phenotype. This process is further complicated by the spatiotemporal variations in the expression of a gene, which can confound our understanding of its function between different tissues or developmental stages.

However, these complications are not always taken into consideration when determining the full array of biological functions that a gene product can perform, either alone or in participation with others. For example, while lethal mutations or mutations that result in the initial agenesis or dysgenesis of certain tissues are useful in identifying essential genes in embryogenesis, they can prevent the analysis of gene function at later stages of development. A more detailed example is *sine oculis* (*so*), a member of the SIX family of transcription factors required for eye development discovered during an enhancer trap screen in Drosophila (Cheyette et al., 1994). Mutations in *so* demonstrate an increase in cell death within the eye primordia and result in eyeless flies, preventing the identification of other potential functions of this gene at later stages of eye development. It was only through the later use of a conditional gene knockout generated with the Flp-FRT site-directed recombination system in differentiated cells that the postmitoic roles for *so* could be ascertained, such as its requirement in cone cell development and interommatidial bristle differentiation (Jin et al., 2016). Studies such as these emphasize the need for utilizing tools to

analyze the full array of a gene's function, particularly at later stages of a tissue's development, in the absence of any earlier defects generated by that gene's dysfunction.

Of course, this difficulty in determining late phenotypes in embryogenesis is not restricted to single genes. A few key signaling pathways act repeatedly in different tissues and at different developmental stages to control the expression of a large suite of genes. The benefit to an organism in utilizing cell signaling pathways during embryogenesis is that they independently often target the same subset of genes responsible for the same biological process in different tissues across development, ensuring that these gene are expressed together precisely when and where they need to be. A disadvantage to this is that the misregulated expression of a few of these target genes could result in embryonic death or produce early and robust phenotypes in a tissue that obscure those that might appear later. Therefore, disturbances to a signaling pathway can have multiple downstream effects, making it difficult to elucidate the many transcriptional targets of a signaling pathway over the course of a tissue's development and hindering the identification of which targets are the true causative factors behind certain disease phenotypes.

This problem applies to the Retinoic Acid (RA) signaling pathway, which utilizes RA, a derivative of vitamin A, to control gene expression (Ghyselinck & Duester, 2019). The discovery of this pathway began in the late 1950s, where it was observed that administering large doses of vitamin A to mammals during pregnancy had widespread teratogenic effects on their offspring (Cohlan, 1953; Giroud & Martinet, 1959). These initial experiments demonstrated that an overexposure to retinoids resulted in a lower birth rate and malformations of the head, such as a cleft palate, an enlargement of the tongue, an extrusion of the brain, and gross defects of the eye, already implicating it in the development of various embryonic tissues (Cohlan, 1953). Since then, further research has uncovered the more precise biological processes that the RA signaling

pathway is involved in, such as proliferation, apoptosis, and differentiation, and determined that it partakes in the development of several additional tissues (Clagett-Dame & DeLuca, 2002; Clagett-Dame & Knutson, 2011; Collop et al., 2006; Diez del Corral et al., 2003; Encinas, Iglesias, Llecha, & Comella, 1999; Falasca, Favale, Gualandi, Maietta, & Devirgiliis, 1998; Glover, Renaud, & Rijli, 2006; Jimenez-Lara, Clarke, Altucci, & Gronemeyer, 2004; Kelley, Turner, & Reh, 1994; Kholodenko et al., 2007; Lin et al., 2010; Lupo et al., 2011; Maden, Gale, Kostetskii, & Zile, 1996; K. Niederreither et al., 2001; Oström et al., 2008; van Noesel & Versteeg, 2004; Wolf, 2008; Zhang, Satyamoorthy, Herlyn, & Rosdahl, 2003). This includes the vertebrate inner ear, where previous work demonstrates that an overexposure to retinoids can lead to inner ear defects ranging from an otic vesicle/otocyst void of identifiable vestibular structures to more singular malformations of the semi-circular canals, maculae, and vestibulocochlear nerve (Burk & Willhite, 1992; Frenz, Liu, Galinovic-Schwartz, & Van De Water, 1996; Granström, 1990; Jarvis, Johnston, & Sulik, 1990). Animal models of vitamin A deficiency also present a range of vestibular defects, from no otic vesicle to misplaced or enlarged otic vesicles, epithelial atrophy, and missing or reduced otoliths (Chole & Quick, 1976; Kil et al., 2005; Romand et al., 2013; White, Highland, Kaiser, & Clagett-Dame, 2000). However, given the number of otic phenotypes generated by disruptions to the RA pathway, it is not sufficient to assume that these are merely the downstream consequences of earlier perturbations to the developmental programme of the inner ear.

For example, pharmacological treatments in zebrafish at 40% epiboly, near the end of the Blastula period, demonstrate that a reduction in RA signaling results in smaller otic vesicles, smaller and fewer otoliths, and reduced or ‘impaired’ protrusions of the semi-circular canals, whereas an overexposure to RA results in an enlarged vesicle and a greater number of both otoliths and protrusions (Hans & Westerfield, 2007). It might therefore be assumed that canal

malformations and protrusion defects can be partially attributed to the size of the vesicle, given that an early role for RA is determining the number of cells that are competent to adopt an otic fate. At least with respect to smaller otic vesicles, a reduction in the size of the otic epithelium might not allow for a large enough dedicated region for the development of these protrusions or the epithelium responsible for separating them, resulting in fewer protrusions overall. Additionally, canal malformations might be attributed to the fact that RA signaling restricts *otx1b* expression in the otic vesicle, a transcription factor that has been implicated in the evolution of the third (lateral) semi-circular canal (Hammond & Whitfield, 2006; Maier & Whitfield, 2014). However, because *otx1b* is expected to promote the formation of a third canal, it is therefore curious that an early reduction in RA signaling in zebrafish results in fewer protrusions, while treatment with excess RA instead increases the number of protrusions (Hans & Westerfield, 2007). Considering it was only recently discovered that there is expression of *OtxA* within the ventral domain of the otic vesicle in lamprey, which lack a lateral canal, this then suggests that the OTX family of transcription factors are not the sole factors responsible for the development of additional canals (Higuchi et al., 2019). This has left a gap in the literature for understanding the precise mechanism by which RA signaling is involved in canal morphogenesis.

The search for RA-responsive factors that impact semi-circular canal morphogenesis is therefore aided by our work. We determined that embryos treated with RA agonists or antagonists from 28 hpf onward continue to demonstrate malformations of the canals, indicating that the dysgenesis of these vestibular structures observed with either a vitamin A deficiency or an overexposure to retinoids are not only due to early disruptions to this pathway during otic development. These phenotypes include both the peculiar branching formation observed with a reduction or increase in RA signaling, as well as a change in the axial ratio observed with excess

RA exposure. Our work also identified RA-responsive genes known to be involved in remodeling of the extracellular matrix that could be contributing to our observed canal phenotypes, *hapln1a*, *has3*, *ugdh*, and *vcana* (Cañibano-Hernández et al., 2019; Gesteira et al., 2017; Goodwin, Ahmed, & Xia, 2019; Takabe et al., 2015; Teoh, Ogrodzinski, & Lunt, 2020). As these genes have yet to be investigated for their individual roles in canal morphogenesis, a possible avenue of future research is the observation for canal phenotypes in the independent mutant models of these genes or following their overexpression.

However, the link between the canal phenotypes observed in our work and late perturbations to RA signaling represent only one example of a novel role for RA signaling in later stages of otic development. Previous work demonstrates that early disruptions of RA signaling also result in otolith defects, those being a reduction in the size and number of otoliths with ablated RA signaling and an increase in the number of otoliths with increased RA signaling (Hans & Westerfield, 2007; Romand et al., 2013). There are many reasons why these phenotypes may occur. For example, previous research has found that RA promotes sensory fates in the anteroventral sensory domain of the otic vesicle, which gives rise to the support cells and sensory hair cells that comprise the sensory epithelia of the inner ear, including the maculae (Maier & Whitfield, 2014; Raft et al., 2007; Satoh & Fekete, 2005). It is upon the kinocilia of the first hair cells that appear in the presumptive utricular and saccular maculae that otolith precursor particles tether and growth, a process that begins at approximately 18 hpf in zebrafish (Riley, Zhu, Janetopoulos, & Aufderheide, 1997; Tanimoto, Ota, Inoue, & Oda, 2011). Seeding particles continue to coalesce with these precursor particles, greatly increasing the size of the otoliths until 24 hpf, at which point their growth rate decreases by approximately 90% (Riley et al., 1997). Because disruptions to RA signaling could impact the timing of hair cell development and number

of hair cells that appear in the sensory domain, this could interfere with the initial tethering and formation of the otoliths. Additionally, early disruptions to RA signaling have demonstrated that a reduction in RA results in the failed separation between the utricle and the saccule, implying that the reduced number of otoliths could be attributed to there being only one fused maculae upon which an otolith can form (Ono, Sandell, et al., 2020; Romand et al., 2013). The loss of otoconia with RA deficiency could also be attributed to the reduced expression of *Otopetrin1*, an RA-responsive gene responsible for calcium regulation during otolith mineralization (Kim et al., 2010; Romand et al., 2013). However, these collective works implicitly suggest that these otolith defects are due to early disruptions in RA signaling.

Our work demonstrates that otolith defects can also be a result of later disruptions to RA signaling during otic development, particularly the reduction in otolith size and number observed with vitamin A deficiency. Through our work, we have shown that treating embryos with RA agonists or antagonists from 28 hpf onward bypasses both the initial formation of the otoliths and the period of their highest growth rate, thereby allowing for the identification of RA-responsive factors that are involved in otolith maintenance. We determined that one potentially contributing factor in this process is *starmaker* (*stm*), which is already known to be involved with otolith nucleation and regulation of otolith composition (Kalka et al., 2019; Söllner et al., 2003). Curiously, it is difficult to discern if *stm* expression is responsive to early perturbations in RA signaling, at least until 28 hpf, as early research into this pathway's role in otic patterning used *stm* expression to mark the initial size of the otic vesicle (Hans & Westerfield, 2007). Unlike previous work, we observe a noticeable decrease in the intensity of *stm* expression rather than merely a reduction in the 'area' of expression, a phenotype that is presumably the indirect effect of a

reduction in inner ear size, implying that *stm* might be uniquely RA-responsive at later stages of otic development.

In conclusion, our work emphasizes the importance of studying the role genetic factors, such as the RA signaling pathway, play at later stages of development without disrupting their expression or activity at earlier stages. However, because the independent analysis of known otic markers via *in situ* hybridization does not reveal the full scope of biological processes that RA signaling regulates, a more prudent search for late RA-responsive transcriptional targets could be achieved through the RNA-sequence (RNA-seq) analysis of dissected ears comparing wild type larvae to those treated with either an RA agonist or antagonist. Additionally, while we found no changes in apoptosis or cell proliferation over the course of canal morphogenesis, other biological processes, such as cell adhesion, migration, survival, or differentiation, should be investigated between treatment groups to better determine how changes in RA signaling generate the canal malformations observed in our work.

5.2. The utilization of transgenic lines in the visualization of vestibular structures

One of the difficulties in studying otic development is the lack of a reliable way to visualize and measure inner ear structures. While robust phenotypes that disrupt the size or shape of the whole inner ear or key internal structures can be observed under a stereoscope or by more specialized techniques, like differential interference contrast microscopy, often only 2D images can be generated of these features (Hans & Westerfield, 2007; Whitfield, Riley, Chiang, & Phillips, 2002). Paint-filling the inner ear is a popular technique for generating 3D models of the lumenal space, but this is a laborious technique that can require multiple injection sites into the ear, which are prone to leakage, and does not allow for the greatest resolution of small inner ear features

(Bissonnette & Fekete, 1996; Kiernan, 2006; Martin & Swanson, 1993; Miller Bever, Jean, & Fekete, 2003). Finally, while the *in situ* hybridization analysis of genes known to be expressed in specific otic structures can allow for the observation of key structures or cell types, this method is also laborious and time-consuming, and it requires that the target transcripts be of a high enough copy number to allow for detection (Gall & Pardue, 1969; Harrison, Conkie, Paul, & Jones, 1973; Jones & Robertson, 1970). Furthermore, this method relies on the unaltered expression pattern of these genes under the conditions studied, requiring previous knowledge of their regulation. Therefore, there is a need for the development of genetic tools that would allow for a more easy and precise way to visualize inner ear structures.

Currently, a popular method for the *in vivo* observation of gene expression or the morphogenesis of select tissues in vertebrate and invertebrate models is the Gal4-UAS system, which uses a yeast transcription activator protein (Gal4) and its targeted upstream activator sequence (UAS) to drive the expression of a target gene, such as a visual reporter like GFP (Brand & Perrimon, 1993; Duffy, 2002; Kakidani & Ptashne, 1988; Webster, Jin, Green, Hollis, & Chambon, 1988). The Kawakami laboratory at the National Institute of Genetics (Japan) has utilized the Tol2 transposon system to generate various gene and enhancer trap lines in zebrafish that drive Gal4 expression in a temporal and tissue specific manner. Therefore, we performed a screen at their institute to identify transgenic lines with distinct otic Gal4 expression patterns, with the intent of describing strains that could be used by zebrafish researchers to visualize key inner ear structures, particularly at later stages of development.

During this screen, we were particularly interested in identifying strains with expression during later stages of otic development, particularly from 24 hpf to 5 dpf, to facilitate the observation of the key vestibular apparatuses that form and are refined during this timeframe. One

of the enhancer traps selected in this screen, *nkhspgffdmc12a*, demonstrated strong expression throughout the otic epithelium, especially in the protrusions of the semi-circular canals and the fused pillars. In Chapter 3, we demonstrated that by using imaging software like Imaris, reliable 3D reconstructions of the lumenal space of the inner ear could be made from confocal images of this transgenic line, at least at 3 dpf. We also identified transgenic lines, such as the gene traps *nkgsaigff266c* and *nkgsaizgffm1225a*, that demonstrate expression at the protrusions of the semi-circular canals, allowing for the more precise analysis of protrusion formation and fusion. *nkgsaizgffm1225a*, as well as *nkgsaizgffd262a*, *nkgsaizgffm789a*, and *nkhspgff75a*, demonstrate expression in the sensory epithelia of the inner ear, promoting their use in visualizing the separation of these epithelia over the course of otic development and their resulting size and position. Their macular expression could also facilitate research concerning the formation of the otoliths or otolithic membranes.

While our initial intent was to identify and describe strains that could allow for a more simplified method of visualizing key inner ear structures, these strains can also be used for the targeted manipulation of genes localized in the described tissues. These transgenic lines therefore lend themselves toward studies that require the more refined spatial regulation of target genes.

5.3. References

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