The Role of *Zic* Genes During Inner Ear Development in the Mouse

by

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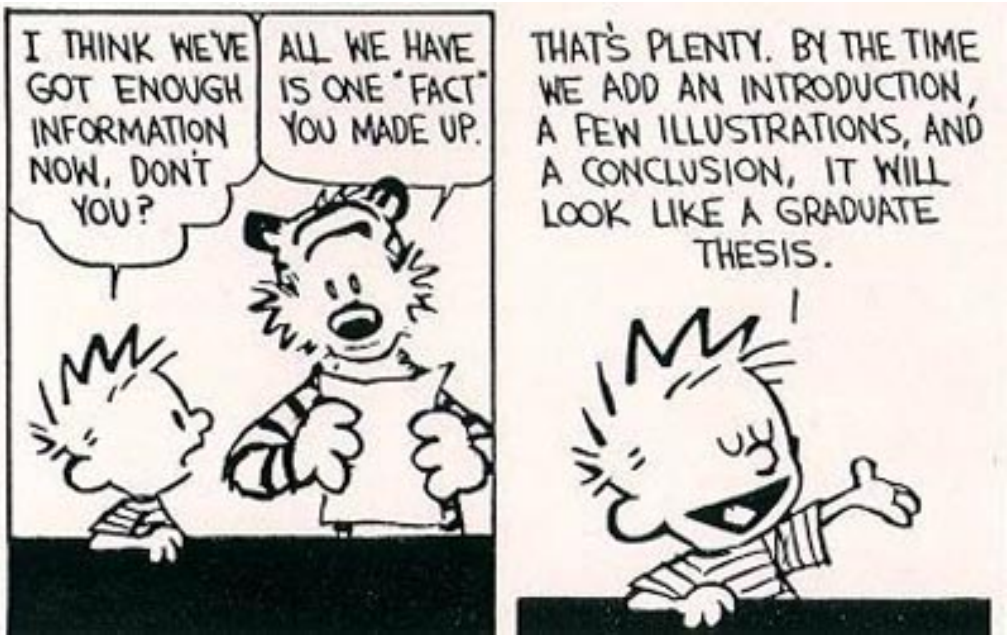

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

- Isaac Asimov

DEDICATION

To my wife Linnea, whose constant encouragement and unwavering belief in me guided me through all of the difficult times throughout graduate school, and to my daughter Elin, may my graduate school experience demonstrate to you that no matter what it is you want to achieve in life, drive, perseverance, and hard work will always be rewarded.

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LIST OF ABBREVIATIONS

A	ampulla
AA	ampulla of the anterior semicircular canal
ABR	auditory brainstem response
A-P	anterior-posterior
ApoE	apolipoprotein E
ASC	anterior semicircular canal
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BOR	branchio-oto-renal
C2H2	$Cysteine_2$-$Histidine_2$ (C_2H_2)
Ca^{2+}	calcium
CC	common crus
CD	cochlear duct
CI	cochlear implant
CN	cochlear nerve
CO	cochlea
COOH	carboxy terminal
CVG	cochleo-vestibular ganglion
CWCH2	Cysteine-Tryptophan-Cysteine-$Histidine_2$
Dm	*Drosophila melanogaster*

Dr	*Danio rerio*
DRG	dorsal root gangion
D-V	dorsal-ventral
E	embryonic day
ED	endolymphatic duct
ES	endolymphatic sac
EUCOMM	European Conditional Mouse Mutagenesis Program
FBS	fetal bovine serum
FGF	fibroblast growth factor
GC	glial cell
Gg	*Gallus gallus*
GL	ganglion
HC	hair cell(s)
HH	Hamburger-Hamilton
Hs	*Homo sapiens*
HV	head vein
I-mfa	inhibitor of MyoD family protein
IHC	inner hair cell
iPSC	induced pluripotent stem cell
kd	knock down (*Zic2* allele)
kHz	kilohertz
KOMP	Knockout Mouse Project
Ku	Kumba (*Zic2* allele)

LA	ampulla of the lateral semicircular canal
LAG	lagena
LM	lagena macula
LSC	lateral semicircular canal
mESC	mouse embryonic stem cell(s)
M-L	medial-lateral
miRNA	micro RNA
Mm	*Mus musculus*
N	neuron
NH2	amino terminal
NLS	nuclear localization signal
NT	neural tube
OC	organ of Corti
OE	otic epithelium
OHC	outer hair cell
OTC	otocyst
PA	ampulla of the posterior semicircular canal
PAP	papilla
PFA	paraformaldehyde
PBS	phosphate buffered saline
PPA	posterior placodal area
PSC	posterior semicircular canal
r3	rhombomere 3

r5	rhombomere 5
r6	rhombomere 6
RA	retinoic acid
RCAS	replication competent avian splice (vector)
S or SAC	saccule
SAG	statoacoustic ganglion
SC	supporting cell(s)
SCC	semicircular canal
SGN	spiral ganglion neuron(s)
SHH	sonic hedgehog
siRNA	small interfering RNA
SNHL	sensorineural hearing loss
TGF-β	transforming growth factor β
U	utricle
UTR	untranslated region
VN	vestibular nerve
WNT	wingless/integrated
Xl	*Xenopus laevis*
Zic	Zinc finger of the cerebellum (gene)
ZIC	Zinc finger of the cerebellum (protein)
ZOC	*Zic*-odd-paired conserved domain
ZF	zinc finger
ZF-NC	zinc finger nucleocapsid domain

ABSTRACT

The *Zic* (zinc finger of the cerebellum) family of transcription factors is involved in many developmental processes. *Zic* gene dysfunction causes developmental defects in mouse and man, including neural tube and skeletal defects and abnormal left-right axis formation. We focused on the role of *Zic* genes in inner ear development and found that *Zic1-5* are expressed in the hindbrain adjacent to the ear and in the periotic mesenchyme (POM) in unique but partially overlapping patterns. Although mice lacking both *Zic1* and *Zic4* have normal ears, partial (*Zic2*$^{kd/kd}$) or complete (*Zic2*$^{Ku/Ku}$) loss of *Zic2* results in severely malformed inner ears. Ears from the *Zic2*$^{kd/kd}$ mice were normal in size but had malformations of the cochlear duct and semicircular canals. In the *Zic2*$^{Ku/Ku}$ mice, ears were misoriented and considerably smaller, and the spatial relationship between the ear and hindbrain was altered. We could not assess whether canal formation was affected in the inner ears from the *Zic2*$^{Ku/Ku}$ mice, as embryonic lethality prevented us from looking at embryos older than embryonic day 12.5 (E12.5) when canal development is initiated. Despite the altered orientation relative to one another, SHH and WNT signaling from the hindbrain to the ear were relatively unaffected in the mutants we analyzed, though we could not conclude the same about BMP signaling due to inconsistent data and low numbers of replicates. Otocyst patterning appeared to be unaffected in the *Zic2*$^{Ku/Ku}$ mutants, as the regionalized expression of *Pax2*, *Lfng*, *Dlx5*, *Gbx2*,

Tbx1 in the otocyst were relatively unaffected in the *Zic2*$^{Ku/Ku}$ mutants we analyzed. Outside of the otic epithelium, there appeared to be an increase in the number of *Tbx1*$^{+}$ cells in the POM of the *Zic2*$^{Ku/Ku}$ mice, but we cannot conclude whether this was due to an increase in proliferation, a decrease in apoptosis, or a shift in the localization of POM cells. Based on these data, it is clear that *Zic2* has an important function during inner ear development. This function may include regulation of cell proliferation in the otic epithelium or POM, or regulation of otic capsule formation, but further experiments are required to test these hypotheses.

Chapter 1

Introduction

1.1. The Inner Ear—A Complex Organ That Develops From The Integration Of Signals From Multiple Pathways

The inner ear has been the focus of developmental studies for almost 100 years, and molecular mechanisms underlying the development of this key sensory system are beginning to emerge (reviewed in Barald and Kelley 2004; Kelley 2006; Ladher, O'Neill et al. 2010; Groves and Fekete 2012). Development of the inner ear is tightly controlled by BMP, WNT, SHH, and FGF signaling, in addition to many other signaling molecules that provide crosstalk between the pathways. These signals emanate from the endoderm and mesoderm to initially specify the region where the ear will develop (reviewed in Barald and Kelley 2004; Kelley 2006; Ladher, O'Neill et al. 2010; Groves and Fekete 2012). After the broad otic domain is specified, complex molecular- and extracellular matrix-based cues from the hindbrain, periotic mesenchyme, and migrating neural crest cells pattern the otic domain as inner ear specification begins (reviewed in Barald and Kelley 2004; Kelley 2006; Ladher, O'Neill et al. 2010; Groves and Fekete 2012). In addition to having the correct signals expressed in the correct regions of the inner ear, the timing of signaling events is also critical for inner ear development. Correct timing of these signaling events is required for the proper development of

inner ear structures and the sensory cells contained within, as well as for the delamination of neuroblasts and their subsequent development and maturation (neurogenesis, synaptogenesis) into the neurons that will innervate these structures (Lee, Liu et al. 2006; Appler and Goodrich 2011; Okano, Xuan et al. 2011; Cadot, Frenz et al. 2012; Kopecky, Jahan et al. 2013). How cells in the neural tube, periotic mesenchyme, and the otic epithelium integrate the signals they receive from multiple pathways (FGF, SHH, WNT, BMP) and use this information to then activate or repress transcription of key downstream genes is a key question that remains to be answered.

1.2. Inner Ear Development in Chick and Mouse

Formation of the otocyst

The inner ear begins as a simple, flat region of head ectoderm adjacent to rhombomeres 5 and 6 (r5, r6) of the hindbrain (Figure 1-1). *Fgf8* from the endoderm induces the expression of *Fgf3* and *Fgf19* (chick) or *Fgf3* and *Fgf10* (mouse) in the overlying mesoderm (Mahmood, Kiefer et al. 1995; Vendrell, Carnicero et al. 2000; Wright and Mansour 2003; Ladher, Wright et al. 2005; Zelarayan, Vendrell et al. 2007; Freter, Muta et al. 2008; Dominguez-Frutos, Vendrell et al. 2009). These mesodermal signals then induce the expression of *Pax2* in the overlying non-neural ectoderm in a broad region called the posterior placodal area, or PPA (Groves and Bronner-Fraser 2000; Ohyama and Groves 2004). Further refinement of the PPA into the otic placode, epidermis, and epibranchial placode is regulated by WNT signaling from the neural tube

(Ohyama, Mohamed et al. 2006). The region of the PPA receiving the highest levels of WNT signal is specified as otic placode and the region receiving the lowest level of WNT signal becomes the epibranchial placode (Ohyama, Mohamed et al. 2006). The otic placode domain is further specified and restricted through WNT-induced upregulation of NOTCH pathway components combined with upregulation of FGF inhibitors, including *sprouty2* (chick) and *Dusp6/7/9* (mouse) in the otic placode (Chambers and Mason 2000; Jayasena, Ohyama et al. 2008; Urness, Li et al. 2008).

After the otic placode has been specified, the cells begin to change shape and the placode gradually invaginates to form the otic cup (Figure 1-1; reviewed in Torres and Giraldez 1998). The edges of the otic cup move towards each other until the otic cup has closed to form the otocyst, a hollow sphere of cells underneath the overlying ectoderm (Figure 1-1; reviewed in Torres and Giraldez 1998).

Patterning the otocyst

The early otocyst (~E9.5 in mouse, ~HH stage 17-18 in chick) already shows asymmetric gene expression, as *Neurogenin-1* (*Ngn1*), a marker of vestibular and auditory neuroblasts, is expressed in the anteroventral region of the mouse otocyst (Raft, Nowotschin et al. 2004). As development proceeds, the ear is patterned along its three axes: dorsal-ventral (D-V), anterior-posterior (A-P), and medial-lateral (M-L). It was hypothesized that the r5-r6 boundary is involved in A-P axis specification, as it is located halfway along the A-P axis of the otocyst

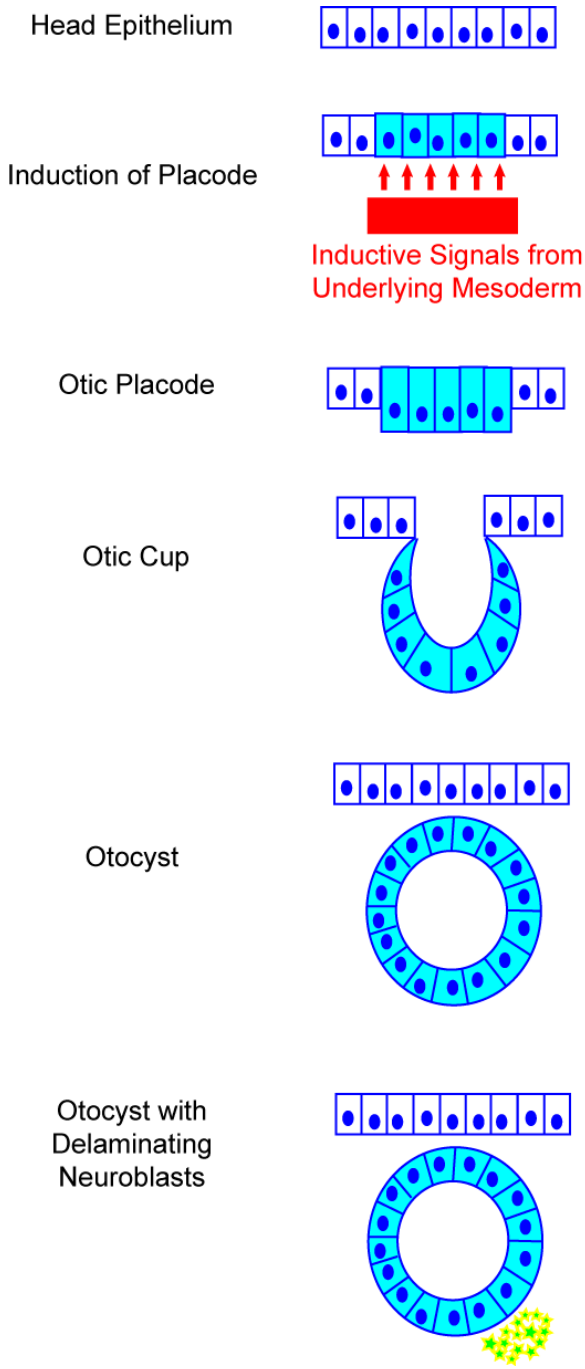

Figure 1-1. Overview of Early Stages of Inner Ear Development in Mammals and Birds. Epithelial cells in the head region (white) receive inductive signals from the underlying mesoderm (red) and become specified as the otic placode (blue cells). A series of morphological changes transform the flat placode first into the otic cup, and then the otocyst that is separate from, and lies underneath, the epithelium. Later, neuroblasts (yellow/green) delaminate from the anteroventral surface of the otic epithelium and migrate away to form the cochleovestibular ganglion (CVG) that innervates the ear.

(Brigande, Kiernan et al. 2000; Bok, Chang et al. 2007). Recent work demonstrated that retinoic acid (RA) signaling confers A-P axis identity to the otocyst (Bok, Raft et al. 2011). *Tbx1*, a T-box family transcription factor, is expressed in the posterior half of the otocyst and responsible for the posterior expression of the orthodenticle homeobox transcription factors *Otx1* and *Otx2* and the homeobox transcription factor *Goosecoid*, and the anterior restriction of expression of genes such as *Ngn1*, the basic helix-loop-helix (bHLH) transcription factor *NeuroD1*, *Lunatic Fringe* (*Lfng*) and *Fgf3*, a member of the fibroblast growth factor family (Vitelli, Viola et al. 2003; Raft, Nowotschin et al. 2004; Arnold, Braunstein et al. 2006). In addition, *Tbx1* was also found to be a direct downstream target of RA signaling (Bok, Raft et al. 2011). In the chick inner ear, implantation of RA-soaked beads into the middle of the otocyst led to a preferential loss of anterior structures, such as the semicircular canals (SCC), which further argues for a role of RA signaling in A-P axis specification (Thompson, Gerlach-Bank et al. 2003).

It is still not clear exactly what signals or tissues specify the M-L axis of the inner ear. The M-L axis forms only after the otic cup has closed to form the otocyst, but it is possible that signals present in the otic epithelium or external tissues (hindbrain, periotic mesenchyme) may pre-pattern this axis. WNTs and SHH from the hindbrain activate expression of the transcription factors *Gbx2* (*Gastrulation Brain Homeobox 2*) and *Pax2* (*Paired Box Gene 2*), two genes expressed in the medial wall of the otocyst (Riccomagno, Martinu et al. 2002; Riccomagno, Takada et al. 2005).

Patterning of the inner ear along its D-V axis is accomplished by SHH signaling from the notochord and floor plate of the ventral neural tube and WNT signals from the dorsal neural tube (Riccomagno, Martinu et al. 2002; Riccomagno, Takada et al. 2005; Bok, Chang et al. 2007; Bok, Dolson et al. 2007; Whitfield and Hammond 2007; Brown and Epstein 2011). BMPs are also expressed in the dorsal neural tube, but their exact role in D-V axis specification has yet to be determined (Groves and Fekete 2012). The interplay of the WNT and SHH signals results in tightly regulated, regionalized expression of genes within the otic epithelium (Riccomagno, Martinu et al. 2002; Riccomagno, Takada et al. 2005; Brown and Epstein 2011). The combinatorial expression of specific genes within the otic epithelium along the D-V axis of the developing inner ear further subdivides the ear into regions where the future endolymphatic duct/sac (ED/ES), SCC, saccule/utricle, and cochlea will emerge. As development of the inner ear progresses, these regions undergo morphological changes, such that the ear lengthens along the D-V axis and narrows along the M-L axis, eventually resulting in the three-dimensional shape of the mature inner ear (Bok, Chang et al. 2007; Chatterjee, Kraus et al. 2010; Groves and Fekete 2012).

Sensory cell specification

In addition to specifying the locations of the different inner ear structures, combinatorial gene expression in the otocyst also pre-patterns the epithelium into prosensory and non-sensory domains. These genes include *Bone Morphogenetic Protein 4* (*Bmp4*), a ligand involved in Transforming Growth

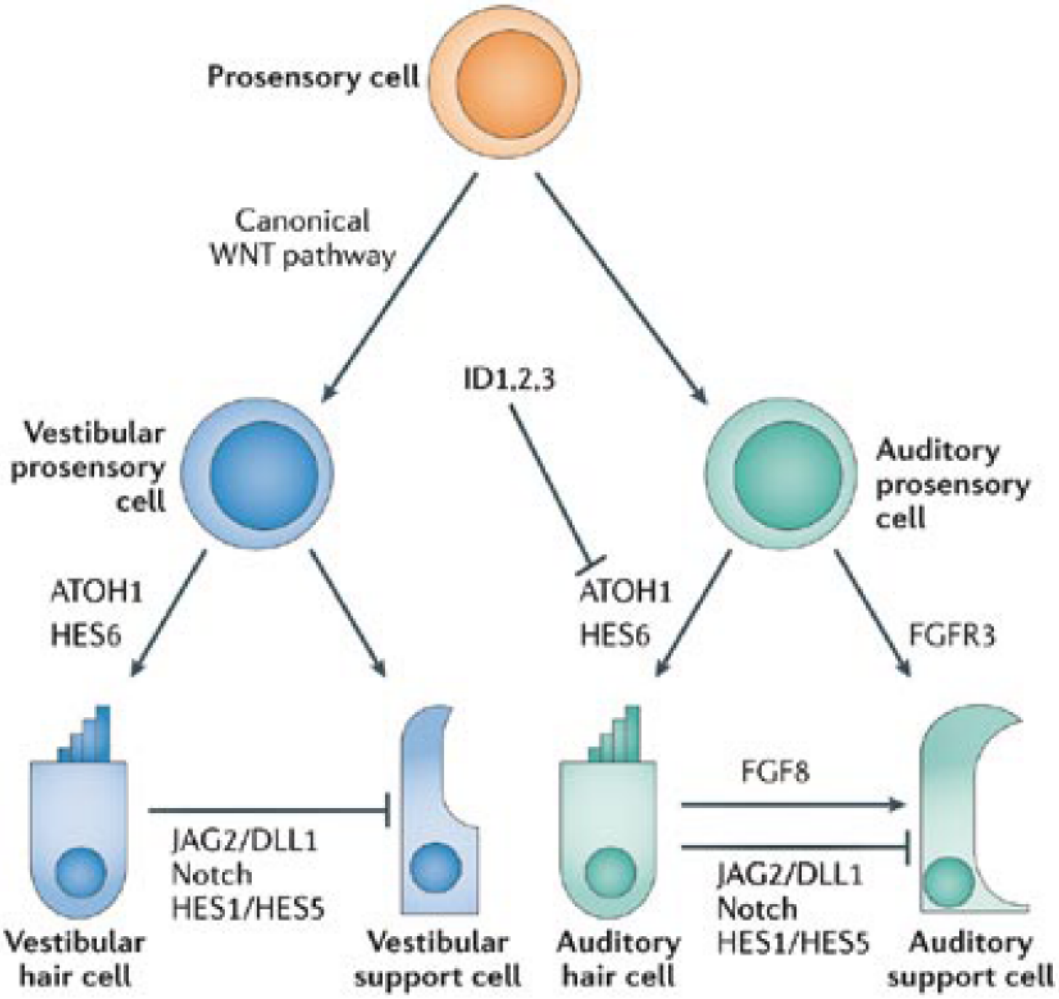

Figure 1-2. Signaling Events Involved in Sensory Cell Specification. Prosensory cells (orange) are specified in different patches in the developing inner ear. Some of these early prosensory cell patches are exposed to WNT signals and the cells within are further committed to a vestibular fate (blue), while other patches do not receive WNT signals and their cells are committed to an auditory fate (green). Prosensory cells expressing *Atoh1* differentiate into auditory and vestibular hair cells (HC), while other prosensory cells receive different signals and differentiate into supporting cells (SC). The specification of HC is tightly controlled through NOTCH-mediated lateral inhibition, ensuring both proper numbers and spacing of HC within a sensory patch. (Image modified from Kelley 2006).

Factor Beta (TGF-β) signaling, the transcription factor *Islet1*, and *Jag1* and *Lfng*, two members of the NOTCH signaling pathway. These genes (*Bmp4*, *Islet1*, *Jag1*, *Lfng*) are among the genes that specify the prosensory domains in the otocyst (Kelley 2006). Once the inner ear has begun to adopt its morphologically distinct structures around embryonic day (E) 12.5 in mouse and E5-E6 in chick, the sensory patches (6 in mammals, 7 in birds) begin to emerge from the prosensory domains that were originally specified in the otocyst. *Atoh1* expression promotes differentiation of hair cells within the sensory patches, and lateral inhibition via NOTCH signaling serves to limit the number of hair cells within a sensory patch (Figure 1-2; Kelley 2006).

1.3. Inner Ear Structure

Vertebrate inner ears share many of the same structures, as the inner ears of birds, mammals, amphibians, and fish all contain semicircular canals, a utricle, and a saccule (Figure 1-3). However, there are a few structures that are only found in some species, such as the cochlea (mouse and chick) and the papilla (frog; Figure 1-3). The dorsal structures of the mature inner ear comprise the vestibular system, and include the saccule, utricle, and three semicircular canals (SCC) that are oriented at ninety-degree angles relative to one another. An additional dorsal structure not found in fish is the endolymphatic sac/endolymphatic duct (ES/ED). A single ventral tubed structure forms the auditory component of the inner ear in both birds (the basilar papilla) and mammals (the cochlea). Fish and amphibians lack a cochlea—instead, the

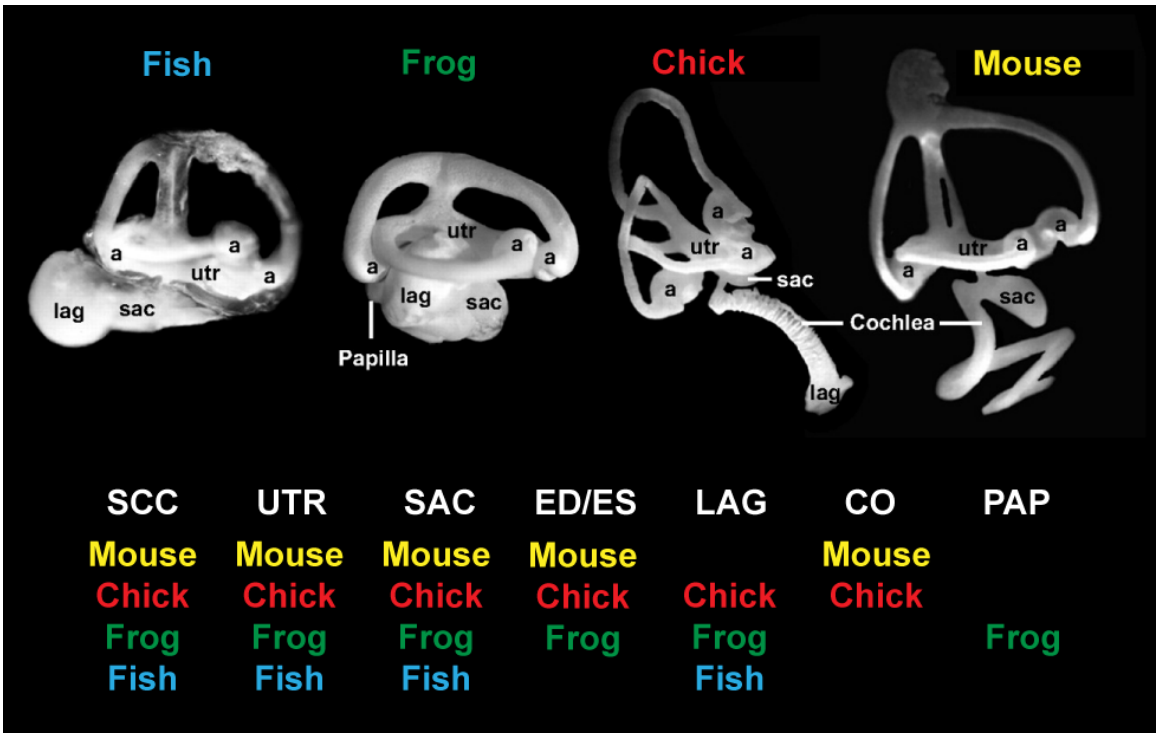

Figure 1-3. Comparison of Vertebrate Inner Ears. (*Top row*) Inner ears from zebrafish, frog, chick, and mouse were paint-filled to illustrate the complex three-dimensional structures present. ***(Bottom row)*** The table shows the different structures found within the inner ears of the vertebrates from the top row. Abbreviations: a, ampulla; lag, lagena; sac, saccule; utr, utricle; scc, semicircular canals; co, cochlea; pap, papilla; ed/es, endolymphatic duct/sac. (Image in top row modified from Groves and Fekete 2012).

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saccule and lagena (fish) and the papilla (amphibians) have evolved to function as the auditory components of the inner ear (Popper and Fay 1993; Riley and Phillips 2003; Groves and Fekete 2012). As in fish, birds and amphibians have a ventrally-located lagena.

Within each of the inner ear structures, there are specialized regions of the epithelium known as sensory patches (Figure 1-4). These sensory patches have specific names according to their location within the inner ear (from this point forward only the inner ears from birds or mammals will be discussed).

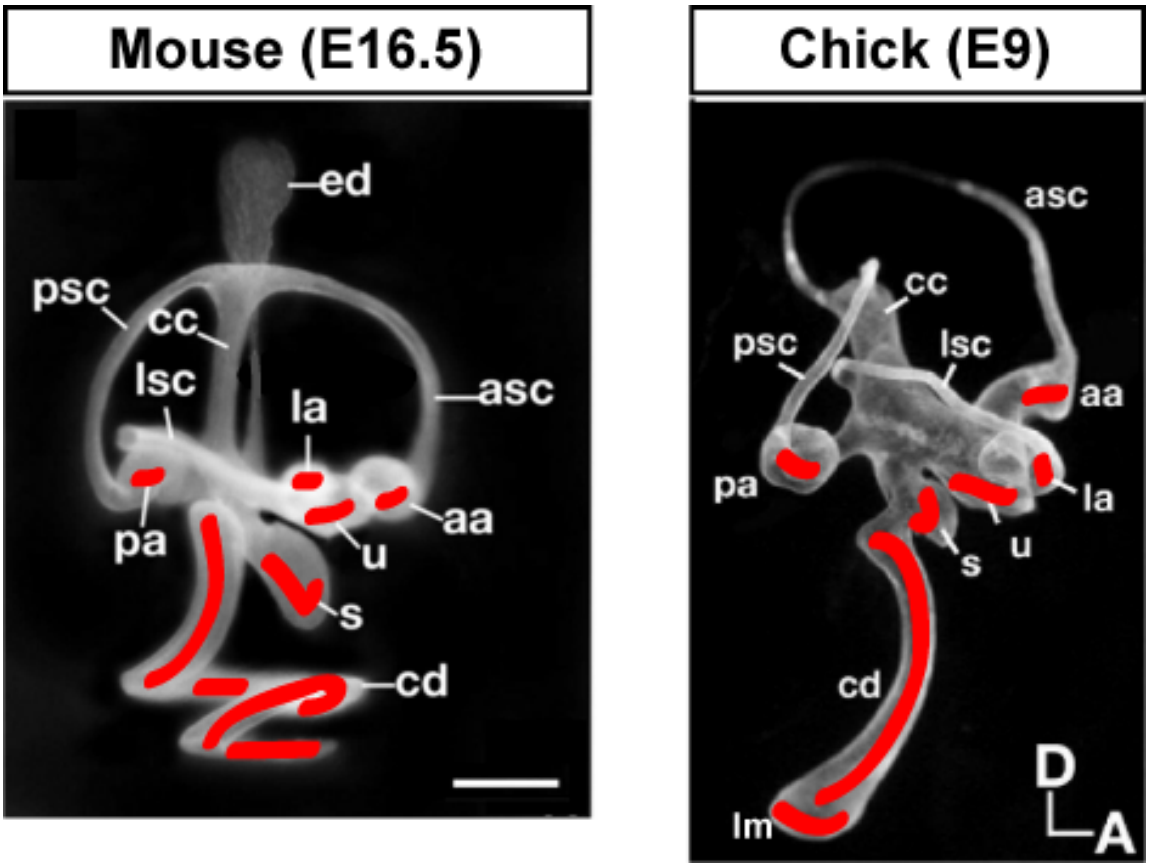

Figure 1-4. Sensory Patches Are Located Within Distinct Structures of the Inner Ear. Photographs of paint-filled inner ears from an E16.5 mouse embryo (*left*) and an E9 chick embryo (*right*) with their sensory patches (shown in red). Sensory patches located in the semicircular canals are called cristae ampullares, those in the utricle, saccule, and lagena are called maculae, while those in the cochlea are the organ of Corti (mouse) or basilar papilla (chick). Abbreviations: asc, anterior semicircular canal; lsc, lateral semicircular canal psc, posterior semicircular canal; aa, ampulla of the anterior semicircular canal; la, ampulla of the lateral semicircular canal; pa, ampulla of the posterior semicircular canal; ed, endolymphatic duct; cc, common crus; lm, lagena macula; s, saccule; u, utricle; cd, cochlear duct; d, dorsal; a, anterior. (Image modified from Bok, Brunet et al. 2007).

Sensory patches are found in the crista amupllaris of the lateral, posterior, and anterior SCC, saccular macula, utricular macula, lagenar macula (birds), organ of Corti (OC; mammals), and basilar papilla (birds).

Contained within the sensory patches are mechanosensory hair cells (HC) and nonsensory supporting cells (SC) that provide trophic support for the HC (the function of these SC at the cellular level will be discussed in more detail below) (the function of these SC at the cellular level will be discussed in more detail below; Kelley 2006). Depending on the organism, the HC within a sensory patch can be exquisitely ordered, such as the 3 by 1 array of HC (3 outer/1 inner) that forms a continuous ribbon of 4 rows of HC that spirals within the mammalian cochlea, or more variable, such as the HC in the basilar papilla of birds, where the number of HC that span the width of the basilar papilla varies along its length (Barald and Kelley 2004; Daudet and Lewis 2005; Kelley 2006). Further, HC can be arranged in circular or rectangular arrays, such as in the mammalian cristae ampullares and saccular/utricular maculae, respectively (Kelley 2006). Bipolar spiral ganglion neurons (SGN), a subset of neurons of the cochleovestibular ganglion (CVG), innervate the HC, and Schwann cells associate with those bipolar neurons (Safieddine, El-Amraoui et al. 2012). Together, these four cell types (HC, SC, SGN, and Schwann cells) form the functional unit of the inner ear (Alsina, Giraldez et al. 2009). This depicted functional unit (Figure 1-5) is a highly simplified version, as the situation is more complicated in the cochlea of mammals where HC have evolved to have different functions and where there are different types of neurons that innervate the HC (Fritzsch, Pan et al. 2013).

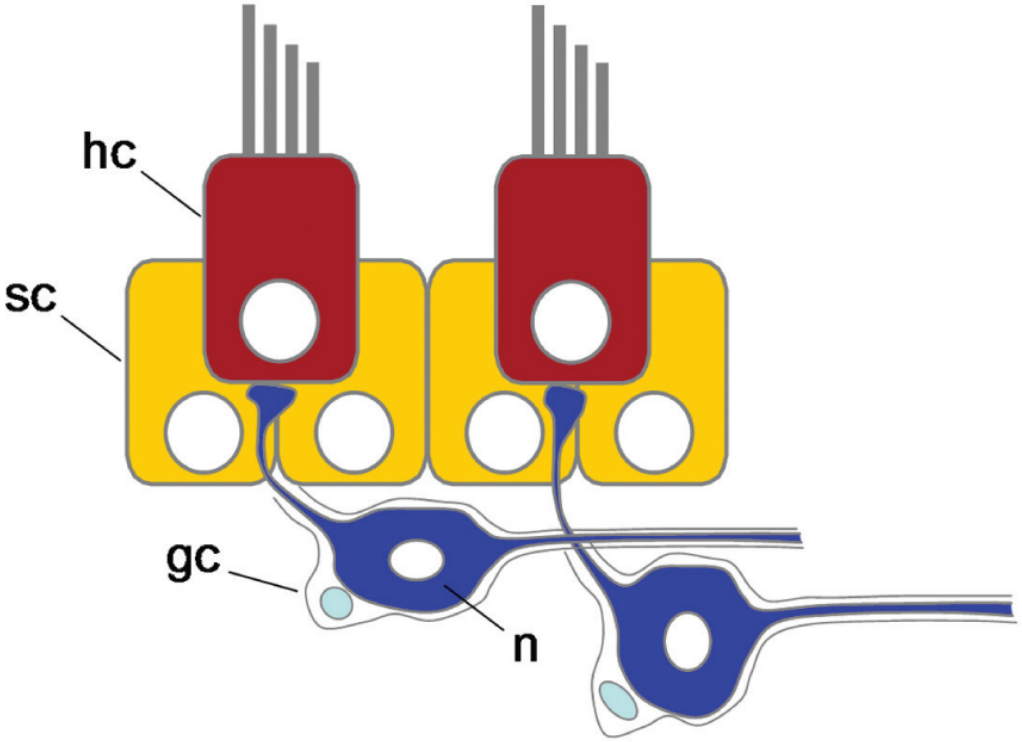

Figure 1-5. The Functional Unit of the Inner Ear. In this simplified diagram, hair cells (red) are intimately associated with supporting cells (yellow) that nourish and support the hair cells. A single cochleovestibular neuron (blue) innervates the hair cell. Schwann cells (white) associate with and support the spiral ganglion neurons. Abbreviations: hc, hair cell; sc, supporting cell; n, neuron; gc, glial cell. (Image modified from Alsina, Giraldez et al. 2009).

Mammals have two types of HC, inner (IHC) and outer (OHC) whose functions differ from one another (Fritzsch, Pan et al. 2013). IHC act as receivers, converting mechanical stimulation into an action potential in the neurons that innervate them, while OHC act as amplifiers that amplify the incoming sound waves (discussed in more detail below; Fritzsch, Pan et al. 2013). In addition to different functional characteristics, IHC and OHC are innervated by two different types of neurons (Rubel and Fritzsch 2002). Type I neurons are myelinated and create afferent (to the brain) synapses with IHC, while Type II neurons are

unmyelinated and create efferent (from the brain) synapses with both OHC and the dendrites of Type I neurons (Rubel and Fritzsch 2002). Further, multiple Type I neurons may innervate a single IHC; in contrast, a single Type II neuron may innervate multiple OHC (Rubel and Fritzsch 2002).

1.4. Inner Ear Function

The functions of the mammalian inner ear are divided between the vestibular and auditory systems. In the vestibular system, linear acceleration is detected by the saccule (up/down) and utricule (forward/backward; Figure 1-6A, 1-6B). To accomplish this, their sensory patches, the maculae, are oriented perpendicular to one another. Within the maculae, the sensory HC are covered by tiny otoconia (calcium-based “ear rocks”) that stimulate the HC when the head moves, bending the stereocilia and causing the HC to become slightly polarized (Buttner-Ennever 1999). When the head moves, the otoconia move, causing the stereocilia to bend more or less depending on the direction of movement. This change in the bending of the stereocilia causes the HC to depolarize or hyperpolarize, depending on the direction of bending (Buttner-Ennever 1999). The resulting changes in HC polarization are converted to electrical impulses that are transmitted by the vestibular nerve (VN), a subset of neurons of the CVG to the brain where inputs from both ears are coordinated and interpreted (Buttner-Ennever 1999).

The three semicircular canals (SCC), oriented at ninety-degree angles relative to one another, detect angular momentum and form the other component

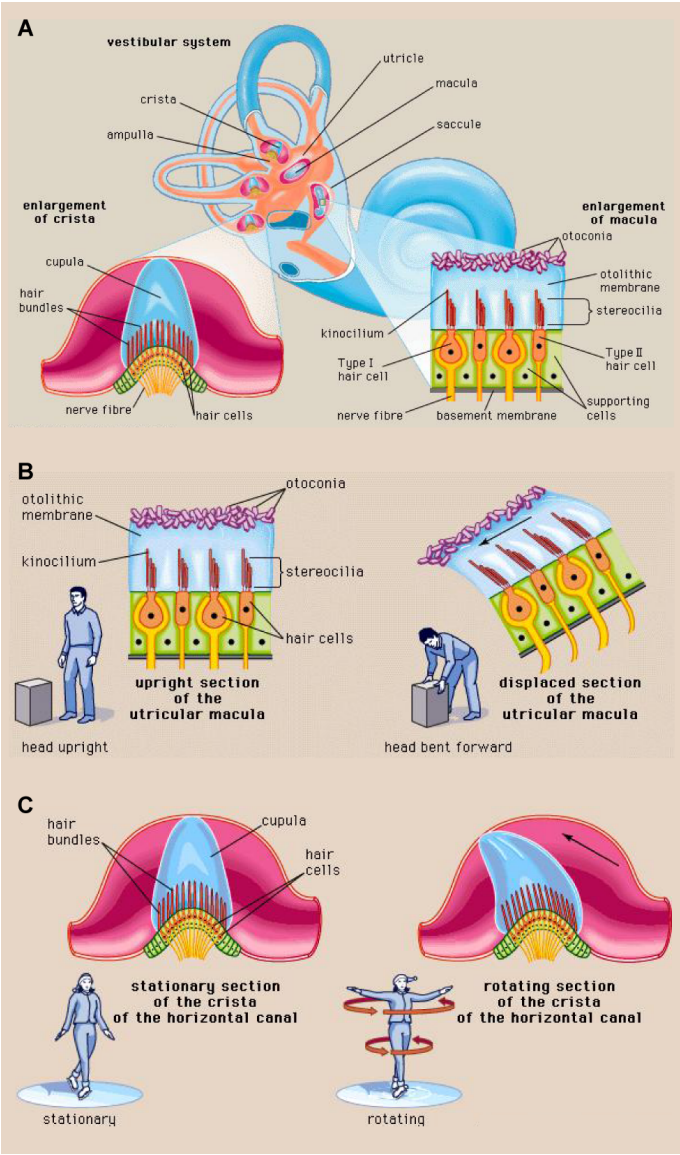

Figure 1-6. Organization and Function of the Vestibular Organs in Mammals. (A) Location of the crista (semicircular canals, SCC) and macula (saccule, utricle) within the vestibular system of the inner ear. **(B)** Hair cells (HC) within the utricular macula are in contact with the otolithic membrane. Otoconia sit on top of the otolithic membrane and push down slightly on the sterocilia when the head is upright in normal gravity (*left*). When the head is bent forward (*right*), the otoconia push on the stereocilia, causing the innervating neuron to fire and transmit positional information to the brain. The saccular macula functions in a similar way. **(C)** HC within the crista ampullaris of the horizontal (lateral) SCC have their stereocilia embedded in a gel-like material, the cupula, that resists movement (*left*). When the head rotates in the horizontal plane (*right*), the cupula is displaced and the stereocilia bend, causing the innervating neuron to fire and transmit positional information to the brain. The anterior and posterior SCC function in a similar way. For a more detailed description of how the maculae and cristae ampullares function, see main text. Abbreviations: HC, hair cell; SCC, semicircular canal. (Image modified from EBO).

of the vestibular system (Figure 1-6A, 1-6C). The lateral semicircular canal (LSC) detects rotation of the head around a vertical axis (the neck), the anterior semicircular canal (ASC) detects rotation of the head around an anterior-posterior axis, and the posterior semicircular canal (PSC) detects rotation of the head in the sagittal plane (Angelaki and Cullen 2008). Within each SCC, a thickened region of epithelium called the ampulla contains the sensory hair cells within a smaller region known as the crista ampullaris (Kelley 2006). The stereocilia of the hair cells are embedded in a dome of gelatinous material, the cupula that overlies the HC and protrudes into the endolymph that fills the SCC (Angelaki and Cullen 2008). As the head turns or rotates in a “normal” gravitational field (e.g. 1g), the canal moves while the endolymph “wave” lags behind. This difference in movement pushes or pulls on the cupula, which in turn pushes or pulls on the stereocilia and depolarizes or hyperpolarizes the hair cells (Angelaki and Cullen 2008). The VN then relay this information to the brain where information from the three SCC from both the left and right ears are coordinated and interpreted (Angelaki and Cullen 2008).

The auditory component of the inner ear consists of a single, ventrally-located structure, the cochlea (mouse) or basilar papilla (bird) that projects dorsally and spirals (in the case of the mouse) or curves (in the case of birds) away from the vestibular organs (Figure 1-4; Fritzsch, Pan et al. 2013). The mammalian organ of Corti (OC) follows the spiral of the cochlea and contains highly organized rows of inner (IHC) and outer (OHC) hair cells that are unique to mammals (Figure 1-7A; Fritzsch, Pan et al. 2013). The basal side of hair cells

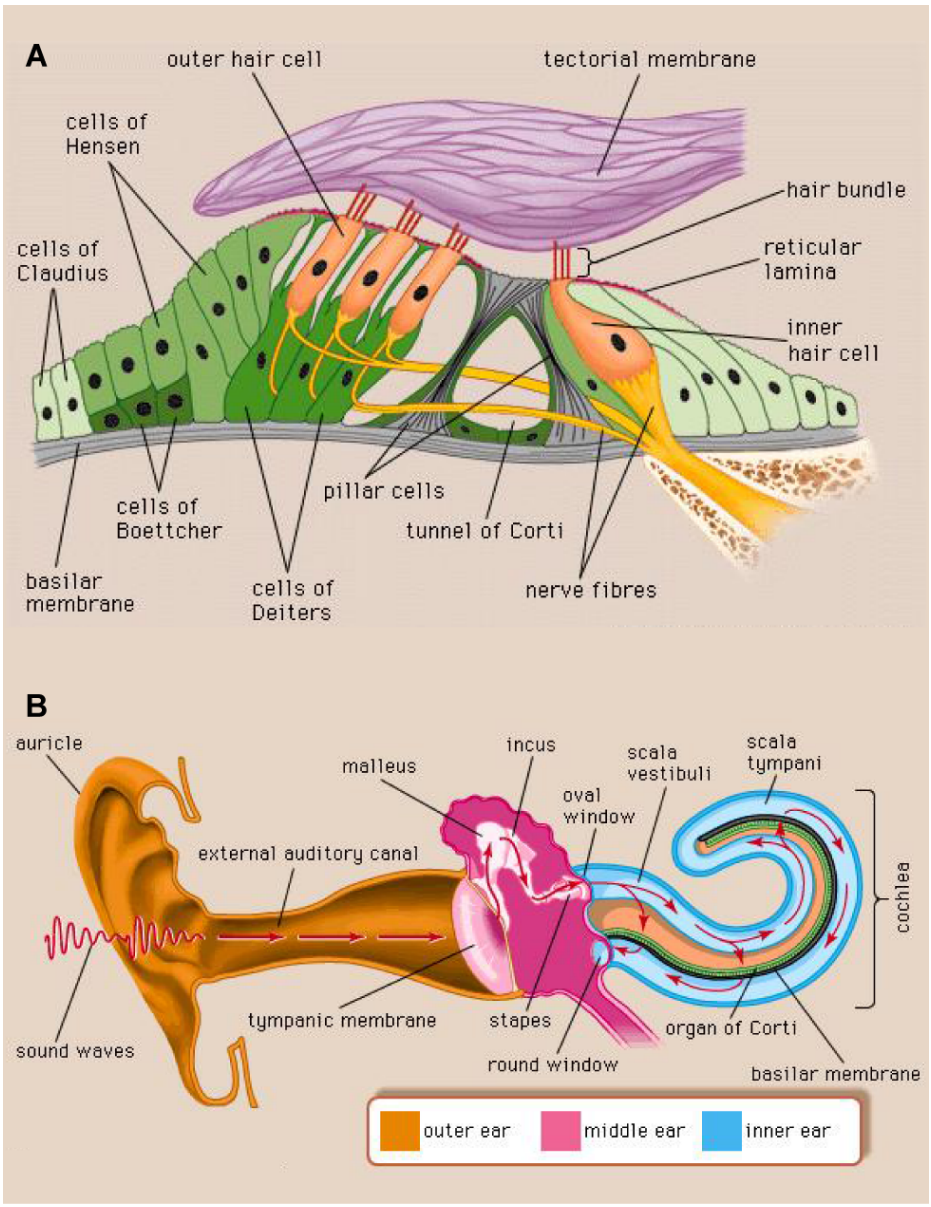

Figure 1-7. Organization and Function of the Auditory Organ of the Mammalian Inner Ear. (A) Diagram showing a cross-section through the organ of Corti, the sensory patch found in the cochlea of mammals. Hair cells (HC) are arranged into rows of inner (IHC) and outer (OHC) HC that contact underlying supporting cells (basal surface) and the tectorial membrane (apical surface). Sound waves cause the basilar membrane to move, in turn causing the HC to move. Stereocilia of the HC bend as a result of movement of the tectorial membrane causing depolarization/polarization of the HC and the firing of Type I neurons. **(B)** How sound travels through the ear. Sound waves enter the outer ear (orange), are converted into mechanical waves in the middle ear (magenta), and are sent through the inner ear (blue) as pressure waves. For a more detailed description of how auditory HC function and how sound travels through the ear, see main text. (Image modified from EBO).

contacts the underlying SC, while on their apical sides, their stereocilia contact the tectorial membrane, another gelatinous structure (Fritzsch, Pan et al. 2013). Sound waves that enter the outer external ear through the pinna and external ear canal impinge on the eardrum, which in turn vibrates the three bones of the middle ear (Figure 1-7B). These bones convert sound energy moving through air into mechanical energy that is then transferred to the fluid endolymph within the cochlea, resulting in pressure waves throughout the endolymph (Volandri, Di Puccio et al. 2011). The movement of the endolymph presses against the basilar membrane, causing the hair cells to move up and down. The stereocilia are then moved into or away from the tectorial membrane, causing them to bend and the HC to depolarize or hyperpolarize depending on the direction of stereocilia bending (Volandri, Di Puccio et al. 2011). When the HC depolarize, a receptor potential is created. In IHC, the receptor potential causes voltage-gated calcium channels to open and an influx of calcium ions (Ca^{2+}) into the IHC, resulting in the release of neurotransmitters that then trigger action potentials in the Type I nerves that innervate the IHC (Safieddine, El-Amraoui et al. 2012). The Type I neurons of the cochlear nerve (CN) convey the information to the auditory processing centers in the brain where the electrical impulses are decoded and interpreted as sound. The receptor potential in OHC causes the OHC to lengthen and shorten at a frequency matching that of the incoming soundwave, resulting in the amplification of the soundwave (Safieddine, El-Amraoui et al. 2012). Type II neurons of the CN modulate the response of the HC to the pressure waves in the endolymph by relaying signals from the brain to the OHC and the afferent Type I

neurons that synapse with the IHC (Rubel and Fritzsch 2002). Inhibitory signals from the brain to the OHC dampen the shortening/lengthening of the OHC, limiting their ability to amplify the sound waves. These same inhibitory signals from the brain to the dendrites of the afferent Type I neurons dampen the signals to the brain from the IHC by increasing the action potential required for the neuron to fire (Rubel and Fritzsch 2002). In a similar way, excitatory signals from the brain increase the sensitivity of the IHC by lowering the action potential of the Type I neurons while increasing the extent, but not the frequency, of OHC shortening and lengthening. This feedback loop allows for amplification of quiet sounds and protects the HC from damage by limiting their response to very loud sounds (Rubel and Fritzsch 2002). In addition to conveying information about the intensity of sounds, the cochlea is also sensitive to the frequency of sound waves. The positioning of a given HC within the OC along the spiral of the cochlea correlates with the frequency of sound that can best be detected by that HC, creating a tonotopic map where high frequencies are detected by HC at the base of the cochlea and low frequencies are detected by HC at the apex of the cochlea (Scheich 1991).

1.5. Hearing Loss

There are three main types of hearing loss—conductive hearing loss (problems with the middle ear, ear canal, or ear drum), sensorineural hearing loss (SNHL; problems with the inner ear, the vestibulocochlear nerve, or auditory processing centers of the brain), and mixed hearing loss, which is a combination of

conductive and SNHL (Hu and Ulfendahl 2013). SNHL accounts for the vast majority of hearing loss, so “hearing loss” from this point forward will refer to SNHL (Masindova, Varga et al. 2012).

The etiology of hearing loss can be either inherited or acquired. Most inherited hearing loss is non-syndromic, meaning it is not associated with other symptoms. Within this category, mutations in connexin 26 are the most common genetic causes of non-syndromic hearing loss (Masindova, Varga et al. 2012). The other cases of inherited hearing loss are syndromic, and are associated with a variety of other symptoms. Though there are many types of syndromic hearing loss, the most common are associated with Usher syndrome (also affects sight), Waardenburg syndrome (also presents with craniofacial abnormalities and abnormal pigmentation of the hair and irises), Pendred syndrome (also affects the thyroid), Stickler syndrome (also presents with skeletal defects), Alport syndrome (also affects the kidneys), and branchio-oto-renal (BOR) syndrome (Karaman and Aliagaoglu 2006; Choi, Muskett et al. 2011; Friedman, Schultz et al. 2011; Kimberling, Borsa et al. 2011; Acke, Dhooge et al. 2012; Bonnet and El-Amraoui 2012; Masindova, Varga et al. 2012). In the United States, approximately 2-3 children out of every 1000 are born deaf or hard-of-hearing (NIDCD 2013). Acquired hearing loss, affecting 36 million adults in the United States alone, can result from traumatic injuries, such as repeated or prolonged exposure to loud noise or exposure to ototoxic drugs (which are frequently those used for chemotherapy, such as cisplatin, or aminoglycoside antibiotics, which have often been prescribed for inner ear infections) or as a result of aging

(Schacht, Talaska et al. 2012; NIDCD 2013). Aging is a significant factor associated with hearing loss, as 47% of those age 75 or older have some form of hearing loss, compared to only 18% of those aged 45-64 (NIDCD 2013).

In the aging population, most hearing loss results from the gradual loss of spiral ganglion neurons (SGN) over time (Barald and Kelley 2004). Other acquired hearing loss usually results from the loss of sensory hair cells (HC) within the inner ear and/or from loss of SGN (Barald and Kelley 2004). Hearing loss in certain subtypes of one of the congenital syndromes, Usher syndrome, also results from a variety of defects in the HC (Friedman, Schultz et al. 2011; Bonnet and El-Amraoui 2012).

1.6. Therapies for Hearing Loss

Hearing loss results from many different causes, among them the total or partial loss of hair cells and/or SGN, either as a result of inherited genetic disorders (e.g. Usher syndrome, connexin 26 mutations), trauma, or aging. Currently the only known “cure” for various forms of profound deafness is the cochlear implant (CI). Cochlear implants require that at least some of the SGN remain, and fitting the implant damages or destroys the remaining HC in the cochlea. Therefore, cochlear implants are only valid treatments in a subset of patients with hearing loss. Finding ways to preserve the remaining SGN in the aged inner ear will improve the efficacy of cochlear implants, but additional approaches that would preserve both SGN and the HC, a situation not requiring a cochlear implant, would be ideal. Brain stem implants, another therapy currently being researched,

would bypass the ear altogether (Cervera-Paz and Manrique 2007; Kuchta 2007; Schwartz, Otto et al. 2008; Colletti, Shannon et al. 2009; Lim, Lenarz et al. 2009; Sennaroglu and Ziyal 2012).

The ideal solution would be to replace the lost or damaged cells with new HC and SGN. Mammals do not have the ability to spontaneously regenerate HC or neurons in response to injury, except for a limited capacity to regenerate HC in the vestibular system (Warchol, Lambert et al. 1993; Cotanche and Lee 1994; Rubel, Dew et al. 1995; Walsh, Hackney et al. 2000; Kawamoto, Izumikawa et al. 2009; Lin, Golub et al. 2011). In contrast, damage to the inner ear in birds sets in motion a wave of regeneration, replacing damaged HC with new ones (Rubel, Oesterle et al. 1991; Stone and Cotanche 1992; Oesterle, Tsue et al. 1993; Cotanche and Lee 1994; Cotanche, Lee et al. 1994; Tsue, Oesterle et al. 1994; Oesterle and Rubel 1996; Rubel and Stone 1996; Stone, Leano et al. 1996; Stone, Oesterle et al. 1998). Initially, asymmetric division of supporting cells was thought to be the only mechanism for regenerating HC, but later studies showed that new HC could be derived directly from SC through a trans-differentiation mechanism (Figure 1-8; reviewed in Stone and Cotanche 2007). Thus, for this strategy to be feasible in mammals, a mechanism for inducing regeneration would need to be created.

Several plausible alternative approaches exist. For example, it would be attractive to discover a mechanism to selectively engineer hair cells and neurons from progenitor cells that remain within the adult inner ear (Chen, Jongkamonwiwat et al. 2012). Another approach would be to “engineer” HC

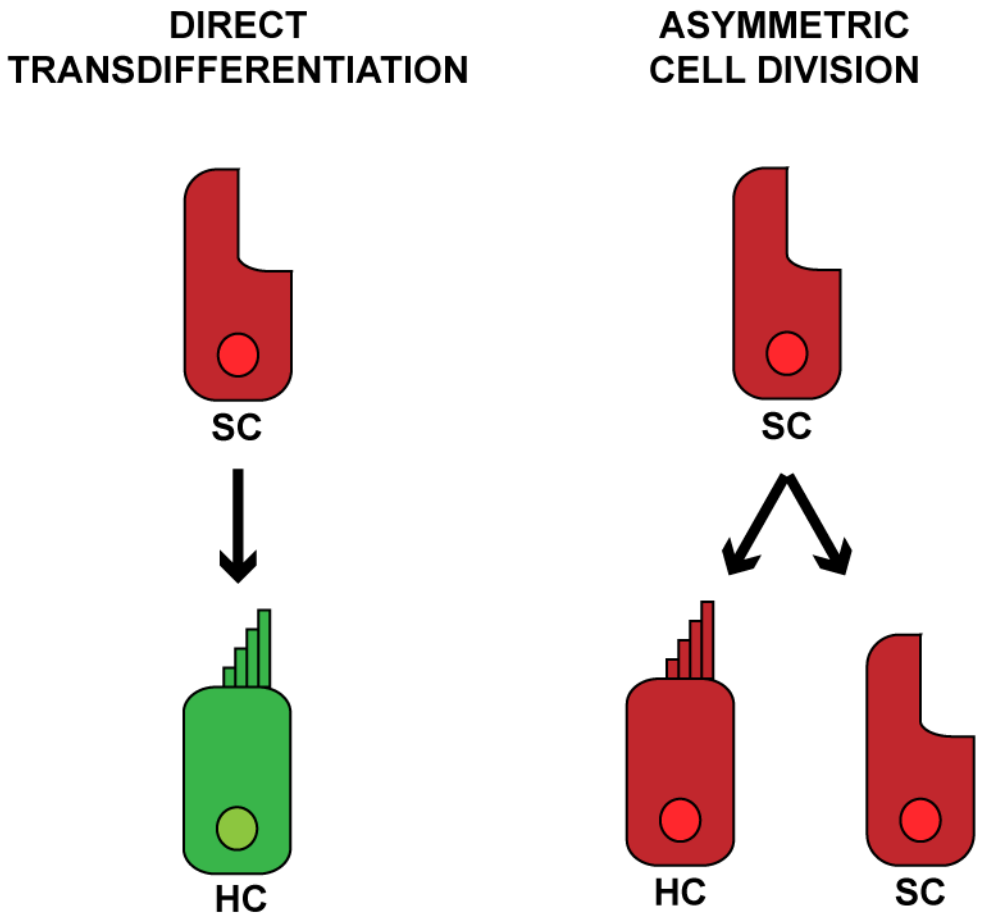

Figure 1-8. Mechanisms of Hair Cell Regeneration. In regenerating sensory epithelia, HC can be produced from SC in two different ways: SC can trans-differentiate directly into HC (*left*) or SC can asymmetrically divide, forming both a new HC and a new SC (*right*). Abbreviations: HC, hair cell; SC, supporting cell.

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and/or SGN from embryonic stem cells or isolated inner ear precursor cells and then implant these cells into the damaged inner ear. These strategies could be applied not only to treating hearing loss, but also to treating balance disorders, as the loss of HC within the vestibular organs is the cause of many balance disorders (Hawkins, Bashiardes et al. 2007).

So far, limited progress has been made on these fronts. Most successful efforts are limited to regenerating vestibular hair cells in mammals, as stimulating

SGN to regenerate *in vivo* has not been possible to date. One of the most promising strategies has been to overexpress the basic helix-loop-helix (bHLH) transcription factor *Atoh1/Math1* in cells within the damaged inner ear. *Atoh1/Math1* is required for the formation of HC in the inner ear during development, as the loss of *Math1* expression in *Math1* null mice (*Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$) resulted in the complete loss of hair cells in both the auditory and vestibular organs of the inner ear (Bermingham et al., 1999). Limited numbers of auditory hair cells have been produced both *in vitro*and *in vivo*. In cultures of postnatal rat cochleae, extra hair cells developed after overexpression of *Math1* (Zheng and Gao 2000). Another group showed that adenoviral vector delivery of *Atoh1* to nonsensory cells in the inner ears of deafened guinea pigs resulted in the regeneration of hair cells that were morphologically and functionally similar to the endogenous HC (Izumikawa, Minoda et al. 2005). A more recent study showed that treatment of the noise-damaged cochleae of guinea pigs with a viral vector encoding *Atoh1* not only led to regeneration of hair cells, but also repair of stereocilia bundles in damaged hair cells (Yang, Chen et al. 2012). Just as in the earlier study (Izumikawa, Minoda et al. 2005), hearing function was partially restored by the repaired/regenerated HC as demonstrated by improved auditory brainstem response (ABR) thresholds (Yang, Chen et al. 2012).

Similar results have been reported in the vestibular system. Cultured maculae from the utricle and saccule of mice were treated with aminoglycoside antibiotics to damage all of the hair cells before being treated with viral vectors encoding *Atoh1* (Staecker, Praetorius et al. 2007). After forced expression of

Atoh1, new hair cell-like cells were identified in the cultures based on expression of hair cell markers (Staecker, Praetorius et al. 2007). These results were repeated *in vivo* in mice that had undergone the same treatments (treatment with aminoglycoside antibiotics to damage all of the hair cells, then treated with viral vectors encoding *Atoh1*), and in addition to regenerating hair cells, the balance problems associated with the loss of hair cells in the utricle and saccule were eliminated (Staecker, Praetorius et al. 2007). Other labs have replicated these *in vivo* results both in mouse (Baker, Brough et al. 2009; Schlecker, Praetorius et al. 2011) and in rat (Xu, Huang et al. 2012).

Previous research (discussed above) demonstrated that mammalian vestibular hair cells have some regenerative potential and that forced expression of the bHLH transcription factor *Atoh1/Math1* could induce the repair of damaged hair cells or regeneration of new hair cells. Recent studies have found a connection between these two events and have shown that inhibition of NOTCH signaling improves the regenerative potential of vestibular hair cells. Lin *et al.* (2011) discovered that supporting cells in cultured mouse utricles began to express *Atoh1* three days after treatment with neomycin to induce HC death (Lin, Golub et al. 2011). The supporting cells could be induced to become more “hair cell-like” through inhibition of the NOTCH signaling pathway, either through addition of the gamma-secretase inhibitor DAPT to the cultures or by reducing *Hes5* levels via small-interfering RNA (siRNA) delivery to the inner ear of deafened mice (Lin, Golub et al. 2011; Jung, Avenarius et al. 2013).

The other strategy to replace hair cells and SGN in the inner ear is to transplant some type of stem cells into the site of hair cell or neuron loss and differentiate the cells *in situ*. Hair cells and neurons have been successfully produced by *in vitro* differentiation of embryonic stem cells (Li, Roblin et al. 2003; Rivolta, Li et al. 2006; Reyes, O'Shea et al. 2008). Numerous difficulties exist when moving from the *in vitro* experiments to *in vivo* tests, however, including whether the injected cells can survive and then "home" to the correct site within the tissue. In the case of the inner ear, this would mean getting cells to the sensory patches and the CVG. Previously, neural stem cells were injected into the scala media/modiolus of the cochlea of a deafened guinea pig and triggered to differentiate, resulting in a few exogenous SGN-like neurons being detected in the correct location (Reyes, O'Shea et al. 2008). In adult rats, dorsal root ganglion (DRG) neurons and mouse embryonic stem cells (mESC) were injected into the modiolus at the site where the rat's SGN had been severed, but the long-term (more than 3 weeks post-injection) survival of both cell types was very low (Hu, Ulfendahl et al. 2004). Similarly low survival of transplanted cells was observed when mouse neural stem cells were injected into the inner ear of deafened guinea pigs (Hu, Wei et al. 2005). Finding ways to increase the number of correctly-targeted replacement cells, as well as demonstrating that these cells are able to integrate functionally will be key points to address in the development of stem cell-based therapies for hearing loss. One recent study has demonstrated that injection of neural stem cells in the lateral wall of the cochlea results in stem cells migrating to Rosenthal's canal (where SGN cell bodies are

located) and the differentiation of some of these cells into neurons as evidenced by the expression of TUJ1 (Zhang, He et al. 2013). Another study had more promising results, as mESC injected into the modiolous of deafened gerbils were able to engraft and differentitate into neurons (Chen, Jongkamonwiwat et al. 2012). Excitingly, these neurons were able to restore some function to the inner ears, as ABR measurements recovered at all frequencies tested, with statistically-significantly recoveries observed at 22, 26, and 30kHz (Chen, Jongkamonwiwat et al. 2012).

Despite the successes in generating functional hair cells and neurons *in vivo*, none of the damaged inner ear structures (vestibular organs, cochlea, CVG) completely regenerate all of the cells present prior to injury. In addition, many of these cells are immature or incomplete versions of the cells they are meant to replace. This could reflect differences in the environment (cellular, extracellular matrix, etc.) during development compared to the environment in the adult inner ear. Stem-cell based therapies provide another method for replacing damaged inner ear structures, but there are still many issues to address before these can be viable options. The basic mechanisms of inner ear development, including cell fate specification, must be better characterized.

1.7. Linking *Zic* Genes with Inner Ear Development

The inner ear lies adjacent to the neural tube, and alterations in specification and patterning of the neural tube are known to affect inner ear development. Signaling from the hindbrain, especially rhombomeres 5 and 6 (r5, r6), is critical

for proper patterning and development of the inner ear, as well as development of the CVG that innervates the ear (Bok, Bronner-Fraser et al. 2005; Kil, Streit et al. 2005; Bok, Brunet et al. 2007; Choo 2007; Vazquez-Echeverria, Dominguez-Frutos et al. 2008; Liang, Bok et al. 2010).

Neural tube closure defects, including exencephaly, in or near the hindbrain, are seen in *Zic2*$^{kd/kd}$ (Nagai, Aruga et al. 2000), *Zic3*^{Bn} (Klootwijk, Franke et al. 2000) and *Zic5*$^{-/-}$ (Inoue, Hatayama et al. 2004) mutant mice. In *Zic2*$^{Ku/Ku}$ mutants, rhombomeres 3 and 5 (r3, r5) are smaller than those in wild type embryos, and ectopic *follistatin* expression is detected in these rhombomeres (Elms, Siggers et al. 2003). In addition to effects on hindbrain development, mutations in *Zic* genes affect signaling from the hindbrain to the inner ear. Expression of *Wnt3a*, a signaling molecule from the hindbrain important for inner ear development (Riccomagno, Takada et al. 2005), is delayed in the dorsal hindbrain of both *Zic2*$^{kd/kd}$ (Nagai, Aruga et al. 2000) and *Zic5*$^{-/-}$ (Inoue, Hatayama et al. 2004) mutant mice.

In addition to their projected role(s) in the inner ear, *Zic* genes regulate the expression of key genes involved in the development of HC and neurons of the CVG. In the chick neural tube, over-expression of *Zic1* inhibited the expression of *Cath1*, the chicken homologue of *Math1/Atoh1* (Ebert, Timmer et al. 2003). *Atoh1/Math1* is required for HC development in the inner ears of rodents, as *Math1* null mice (*Math1*$^{\beta\text{-gal}/\beta\text{-gal}}$) lack HC in both the auditory and vestibular organs of the inner ear (Bermingham, Hassan et al. 1999). Similar to *Zic1*, *Zic2* negatively regulates a bHLH transcription factor, *neurogenin1* (Brewster, Lee et

al. 1998) that is required for the formation of the CVG (Ma, Chen et al. 1998). In *Xenopus* embryos, injection of *Zic2* mRNA led to a complete loss of *neurogenin1* (*Ngn1*) expression in the neural plate. Conversely, injection of *Ngn1* mRNA led to an increase in neural induction in the neural plate and ectopic neurons in the surrounding ectoderm, which were drastically reduced when equal amounts of *Zic2* and *Ngn1* mRNA were co-injected (Brewster et al. 1998).

The involvement of *Zic* genes in the development of tissues that affect inner ear development and the regulation of key inner ear development genes in other tissues makes them attractive candidates for further study as regulators of inner ear morphogenesis.

To date, limited studies have examined *Zic* gene expression in the context of inner ear development. One early study identified *Zic2* as one of the first genes up-regulated in the regenerating sensory epithelium of the chicken inner ear after noise exposure (Gong, Hegeman et al. 1996). Published data conflicts regarding the location of *Zic* gene expression during inner ear development (Warner, Hutson et al. 2003; McMahon and Merzdorf 2010). Early studies reported expression of *Zic1* and *Zic2* in the neural crest, the periotic mesenchyme, and the hindbrain adjacent to the developing inner ear, while *Zic3* expression was only detected in the periotic mesenchyme (Warner, Hutson et al. 2003). Of the three *Zic* genes, only *Zic1* expression was detected in the otic epithelium (Warner, Hutson et al. 2003). In contrast, a later analysis found a more restricted *Zic* expression pattern in the developing chick ear (McMahon and Merzdorf 2010). In that study, expression of the *Zic* genes (*Zic1-4*) was not

detected in the otic epithelium, and only *Zic2* expression was detected in the periotic mesenchyme (McMahon and Merzdorf 2010). *Zic1-3* expression was found in the dorsal neural tube in the hindbrain, but *Zic4* expression was not detected in the hindbrain at any of the stages examined, up to HH stage 19 (McMahon and Merzdorf 2010). Until recently, limited *Zic* expression patterns had been described in the mouse, and none of these examined *Zic* gene expression in the developing inner ear (reviewed in Merzdorf 2007; Ali, Bellchambers et al. 2012).

A recent study from our laboratory reconciled the conflicting *Zic* expression patterns in the chick inner ear and expanded the expression patterns to include chick *Zic4* and *Zic1-5* in mouse (Chervenak, Hakim et al. 2013). None of the *Zic* genes (*Zic1-4* in chick, *Zic1-5* in mouse) was expressed in the otic epithelium at any stage of inner ear development studied; instead, expression was found in the dorsal neural tube and in the periotic mesenchyme (Chervenak, Hakim et al. 2013). Each *Zic* gene had a unique spatiotemporal expression pattern, though regions of overlapping expression did exist (Chervenak, Hakim et al. 2013). Interestingly, the expression pattern of equivalent *Zic* genes (e.g., chick and mouse *Zic1*) was similar (Chervenak, Hakim et al. 2013). Based on their spatiotemporal expression patterns during inner ear development in both mouse and chick, the *Zic* genes may regulate WNT or BMP signaling from the dorsal neural tube, function as intermediates in mesenchymal-epithelial signaling, or have a novel ear-specific function.

1.8. Biological Functions of *Zic* Genes

Based on the results from *in vitro* studies and limited *in vivo* studies, ZIC proteins are thought to function in two different ways: as transcription factors that directly bind to DNA or as transcriptional co-factors that interact with other transcription factors that themselves bind to DNA (Figure 1-9).

Although a number of studies have investigated *Zic* gene function, the exact *in vivo* role these genes play still remains elusive. In Xenopus, *zic1* activates WNT/β-catenin signaling (Merzdorf and Sive 2006); conversely *zic2* and *zic3* inhibit WNT/β-catenin signaling (Pourebrahim, Houtmeyers et al. 2011; Fujimi, Hatayama et al. 2012). In zebrafish, *zic1* positively regulates expression of *shh* and *nodal* in the forebrain (Maurus and Harris 2009). Similarly, *zic2a* modulates Hedgehog-mediated gene expression in the forebrain (Sanek, Taylor et al. 2009). At shield stage, *zic2a* is expressed in the zebrafish organizer and at the blastoderm margin, and later demarcates a population of proliferating neuroectoderm in the presumptive forebrain.

The *Zic* genes are involved in a multitude of developmental processes. *Zic* gene function is well-studied during neural development in the control of neural patterning, generation of neural crest, cerebellar development, and formation of the neural tube (Aruga 2004; Merzdorf 2007). In addition to these more well-known roles, *Zic* genes also are involved in determining left-right symmetry (Herman and El-Hodiri 2002), function during somite myogenesis (Pan, Gustafsson et al. 2011), and play a role in retinal development (Watabe, Baba et al. 2011). *Zic* genes are also expressed and function in adult tissues, including

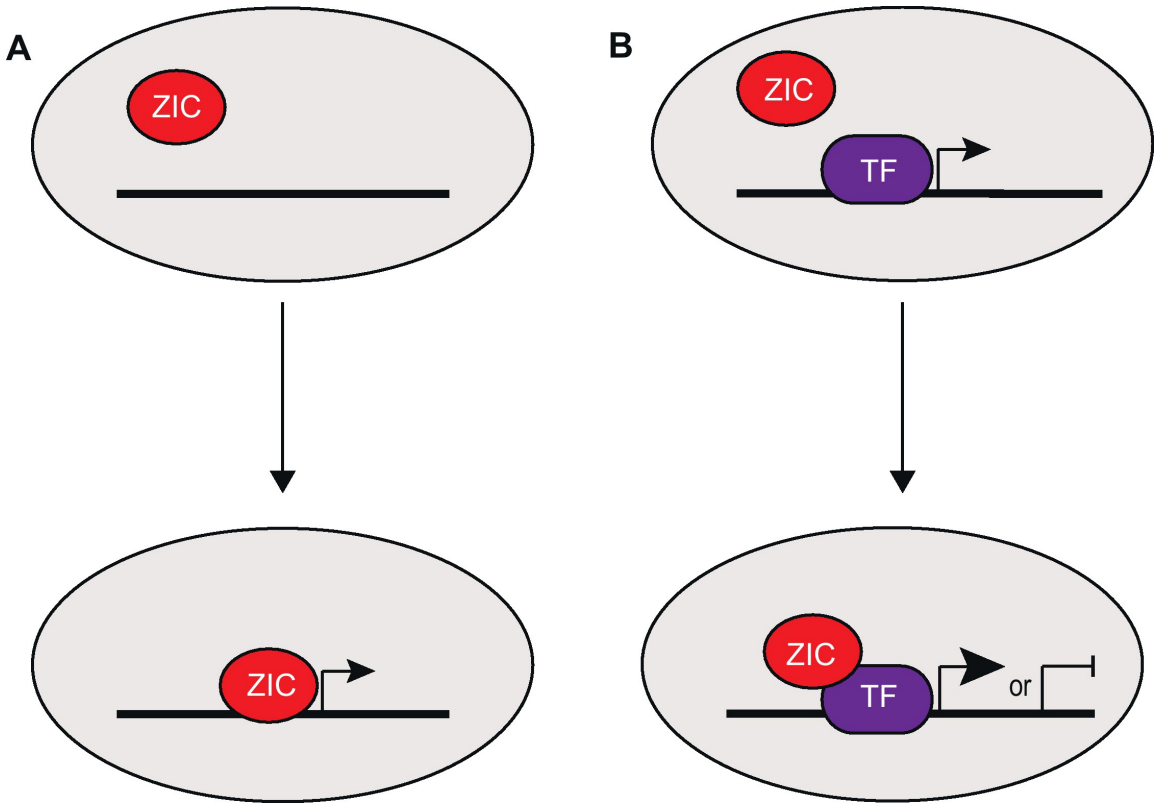

Figure 1-9. Dual Function of ZIC Proteins. (A) As transcription factors, ZIC proteins translocate to the nucleus and directly bind to DNA target sequences, activating transcription. (B) As co-factors, ZIC proteins translocate to the nucleus where they bind to other transcription factors that directly bind to DNA target sequences. In this role, ZIC proteins can either promote or repress transcription. (Image from Ali, Bellchambers et al. 2012).

Zic2 in the brain of adult mice (Brown and Brown 2009) and *Zic1* in the osteocytes of bone in adult humans (Kalogeropoulos, Varanasi et al. 2010). In a recent study, addition of *Zic3* to the Yamanaka factors *Oct4*, *Sox2*, and *Klf4* enhanced the generation of induced pluripotent stem cells (iPSC), identifying *Zic3* as a pluripotency reprogramming factor (Declercq, Sheshadri et al. 2013).

Although the various *Zic* genes have similar functions, mutations in each of the *Zic* genes result in different phenotypes (Table 1-1). Loss of *Zic1* in mice results in abnormalities in axial skeleton formation and cerebellar ataxia (Aruga,

Minowa et al. 1998; Aruga, Mizugishi et al. 1999; Blank, Grinberg et al. 2011). Mice homozygous for a hypomorphic allele of *Zic2* (*Zic2*$^{kd/kd}$) or a loss-of-function allele (*Zic2*$^{Ku/Ku}$) develop severe neural tube defects, including spina bifida and holoprosencephaly (Nagai, Aruga et al. 2000; Elms, Siggers et al. 2003; Warr, Powles-Glover et al. 2008). Importantly, *Zic2* mutations have been identified in human patients with holoprosencephaly (Brown, Warburton et al. 1998). Mutation or deletion of *Zic3* in mice leads to exencephaly, abnormalities in left-right asymmetry, and heart defects (Klootwijk, Franke et al. 2000; Purandare, Ware et al. 2002). Notably, similar effects are seen in humans with deletions or mutations in *Zic3* (Gebbia, Ferrero et al. 1997; Ware, Peng et al. 2004). Loss of *Zic4* in mice has only mild effects, but the combined loss of both *Zic1* and *Zic4* results in

Table 1-1. Selected Phenotypes Resulting from Mutations in *Zic* Genes

Gene	Organism	Phenotype	Reference
Zic1	mouse	skeletal abnormalities, cerebellar ataxia	(Aruga, Minowa et al. 1998; Aruga, Mizugishi et al. 1999; Blank, Grinberg et al. 2011)
Zic2	mouse	neural tube defects (spina bifida, holoprosencephaly)	(Nagai, Aruga et al. 2000; Elms, Siggers et al. 2003; Warr, Powles-Glover et al. 2008)
Zic2	human	neural tube defects (holoprosencephaly)	(Brown, Warburton et al. 1998).
Zic3	mouse	exencephaly, heart defects, left-right asymmetry defects	(Klootwijk, Franke et al. 2000; Purandare, Ware et al. 2002)
Zic3	human	exencephaly, heart defects, left-right asymmetry defects	Gebbia, Ferrero et al. 1997; Ware, Peng et al. 2004)
Zic4	mouse	mild (smaller in size, no other changes in viability or behavior)	(Blank, Grinberg et al. 2011)
Zic1/Zic4	mouse	cerebellar defects (smaller, abnormal foliation patterns, abnormal righting reflexes, ataxia)	(Grinberg, Northrup et al. 2004; Blank, Grinberg et al. 2011)
Zic1/Zic4	human	Dandy-Walker malformation (cerebellar defects)	(Grinberg, Northrup et al. 2004; Blank, Grinberg et al. 2011)
Zic5	mouse	neural tube closure defects, defects in neural crest development, craniofacial abnormalities	(Inoue, Hatayama et al. 2004; Furushima, Murata et al. 2005)

severe cerebellar defects that mimic the Dandy Walker malformation seen in human patients heterozygous for mutations in both *Zic1* and *Zic4*, including smaller cerebella with abnormal foliation patterns, abnormal righting reflexes, and ataxia (Grinberg, Northrup et al. 2004; Blank, Grinberg et al. 2011). Mice lacking *Zic5* exhibit defects in neural tube closure and neural crest development, leading to craniofacial abnormalities (Inoue, Hatayama et al. 2004; Furushima, Murata et al. 2005). Thus, correct expression and function of the *Zic* genes is critical for proper development of the neural tube, among other tissues.

1.9. Phylogenetic Distribution of the *Zic* Genes

The *Zic* (zinc finger of the cerebellum) genes comprise a family of transcription factors found in both vertebrates and invertebrates (reviewed in Grinberg and Millen 2005; Merzdorf 2007; Ali, Bellchambers et al. 2012; Houtmeyers, Souopgui et al. 2013) that are related to the *Drosophila Zic* homologue, *odd-paired* (*opa*) (Nusslein-Volhard and Wieschaus 1980; Benedyk, Mullen et al. 1994). To date, *Zic* gene homologs have only been identified in metazoans and not in organisms from any of the other kingdoms (Aruga, Kamiya et al. 2006). *Zic* gene homologs have been identified in organisms throughout the metazoan kingdom, from the basal metazoans of the phylum *Cnidaria* to the higher metazoans of the phylum *Chordata* (Table 1-2; Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010). The number of *Zic* genes varies widely among different phyla, and can even vary within phyla. A single *Zic* gene has been identified in molluscs, arthropods, and echinoderms, while cnidarians have as

Table 1-2. Metazoan *Zic* Genes

Phylum	Organism	# of *Zic* genes	Reference
Porifera	*Amphimedon queenslandica*	0	(Layden, Meyer et al. 2010)
	Ephydatia muelleri	0	(Layden, Meyer et al. 2010)
Ctenophora	*Mnemiopsis leidyi*	0	(Layden, Meyer et al. 2010)
Placozoa	*Trichoplax adhaerens*	1	(Layden, Meyer et al. 2010)
Cnidaria	*Scolionema suvaense*	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Hydra vulgaris	1	(Aruga, Kamiya et al. 2006)
	Hydra magnipapillata	1	(Layden, Meyer et al. 2010)
	Nematostella vectensis	5	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
Acoelomorpha	*Convolutribola longifissura*	1	(Layden, Meyer et al. 2010)
Platyhelminthes	*Dugesia japonica*	2	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Schmidtea mediterranea	2	(Aruga, Kamiya et al. 2006)
	Schistosoma mansoni	1	(Aruga, Kamiya et al. 2006)
Dicyemida	*Dicyema acuticephalum*	2	(Layden, Meyer et al. 2010)
Annelida	*Tubifex tubifex*	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Capitella teleta	1	(Layden, Meyer et al. 2010)
Mollusca	*Loligo bleekeri*	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Octopus ocellatus	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Corbicula sp.	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Spisula solidissima	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
Nematoda	*Caenorhabditis elegans*	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
Arthropoda	*Drosophila melanogaster*	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Anopheles gambiae	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Pandinus imperator	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Artemia franciscana	1	(Aruga, Kamiya et al. 2006)
Echinodermata	*Strongylocentrotus purpuratus*	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Asterina pectinifera	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
Chordata	*Ciona intestinalis*	2	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Ciona savignyi	2	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Halocynthia roretzi	2	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Branchiostoma floridae	1	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Xenopus sp.	6	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Danio rerio	8	(Layden, Meyer et al. 2010)
	Gallus gallus	4	(Layden, Meyer et al. 2010)
	Mus musculus	5	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)
	Homo sapiens	5	(Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010)

few as one (*Hydra vulgaris*, *Hydra magnipapillata, Scolionema suvaense*) or as many as five (*Nematostella vectensis*) *Zic* genes (Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010). Among vertebrate species, humans and mice have five *Zic* genes (*Zic1-5*), while other species have more (zebrafish, 8 *Zic* genes; frog, 6 *Zic* genes) or less (chicken, 4 *Zic* genes).

1.10. Structural Features of ZIC Proteins

Structurally, ZIC proteins are characterized by a zinc finger (ZF) region that contains five tandem C_2H_2 (C2H2) ZF domains that are closely related to the ZF domain of the GLI/GLIS/NKL families (Aruga 2004; Merzdorf 2007; Ali, Bellchambers et al. 2012). The amino acid sequence of ZF2 through 5 are almost identical among ZIC1-5 for a given species, while the sequence outside of this region can be highly variable (Aruga 2004; Grinberg and Millen 2005). Later studies found that the similarity among ZIC proteins also held true for ZIC proteins from different species. When the amino acid sequences of 45 ZIC proteins from 25 species were compared, the sequence similarity between *Zic* genes from any two species was greater than 75% (Aruga, Kamiya et al. 2006). The area of highest conservation among these 45 *Zic* genes was the region encompassing ZF2-5 where 60 of 104 amino acids were absolutely conserved, with many of these conserved residues existing as clusters (Aruga, Kamiya et al. 2006). Further, although there were 44 amino acids that differed among the *Zic* genes, many of these were conservative amino acid changes (Aruga, Kamiya et al. 2006). Upon closer examination of the highly conserved ZF region, Aruga et

al. found that ZF1 was more divergent than ZF2-5, with a wide, species-specific variation in the number of amino acids separating the two cysteines of the C2H2 domain of ZF1 and that vertebrate ZIC proteins have additional variation in the spacing between the cysteine residues in ZF5: ZIC1-3 has 2 amino acids (CxxC) and ZIC4-5 has 4 amino acids (CxxxxC) (Aruga, Kamiya et al. 2006).

Outside of the ZF region, 2 additional evolutionarily conserved sequences, the zinc finger nucleocapsid (ZF-NC) domain and the Zic-Opa conserved (ZOC) domain, were identified (Figure 1-10; Aruga, Kamiya et al. 2006). The ZF-NC domain is located just outside of the ZF region on its N-terminal side and is present in all *Zic* genes identified to date, either as the fully conserved sequence or as a cluster of minimally conserved amino acids hypothesized to be remnants

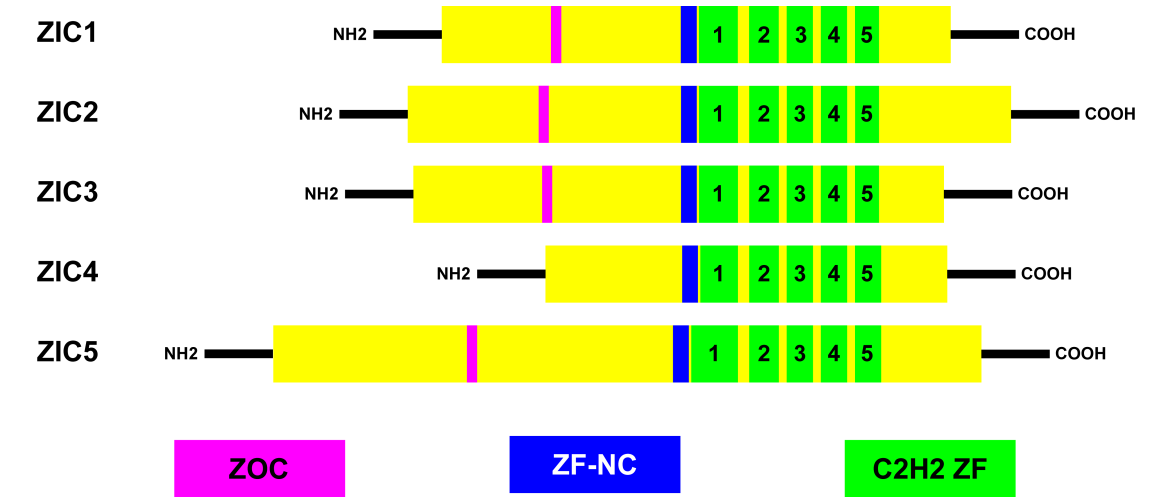

Figure 1-10. Structure of the Murine ZIC Proteins. All ZIC proteins contain a ZF domain consisting of 5 tandemly arrayed C2H2 ZF (green) as well as a ZF-NC domain (blue). ZIC1-3 and ZIC5 contain an additional feature, the ZOC domain (magenta). Numbers in the ZF domain refer to the number of the C2H2 ZF. Abbreviations: ZF, zinc finger; ZOC, *Zic-Opa* conserved domain; ZF-NC, zinc finger nucleocapsid domain; C2H2 ZF, C_2H_2 zinc finger; NH2, amino terminal; COOH, carboxy terminal.

of the original ZF-NC (Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010). Closer to the N-terminal end of the protein is the ZOC domain. The ZOC domain was originally identified as a region conserved between the *odd-paired* (*opa*) gene in Drosophila and *Zic1-3* in mice, but has now been identified in some, but not all, *Zic* genes from a variety of species (Aruga, Nagai et al. 1996; Mizugishi, Hatayama et al. 2004; Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010). Interestingly, there are now two slightly different consensus sequences described for the ZOC: (S/T)RDFLxxxR (Aruga, Nagai et al. 1996; Mizugishi, Hatayama et al. 2004) and RDFL-(1-2aa)-RR (Layden, Meyer et al. 2010). The former was proposed based on a limited, biased sequence alignment between mouse ZIC1-3 and *Drosophila* OPA and the latter was based on an unbiased alignment of the N-terminal regions from more than 30 ZIC proteins from at least 20 different species (Aruga, Nagai et al. 1996; Mizugishi, Hatayama et al. 2004; Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010). The more recent consensus sequence described for the ZOC domain includes all of the previous ZOC-containing *Zic* genes and now includes previously excluded *Zic* genes and taxa, such as *ZicA*, *C*, *D*, and *E* (*N. vectensis*; Cnidarians), *Ref2* (*C. elegans*; Nematodes), *ZicA* (*D. japonica*; Platyhelminthes), *amphiZic* (*B. floridae*; Urochordates), and *Zic4-5* (*Xenopus*, mouse; Vertebrates) (Layden, Meyer et al. 2010).

In addition to the C2H2 ZF, ZF-NC, and ZOC domains, the ZIC proteins also have regions of low complexity located outside of the ZF region. These include poly-alanine, poly-histidine, poly-proline, and poly-serine/glycine tracts on

the N-terminal side of the ZF region and poly-alanine and poly-serine/glycine tracts on the C-terminal side of the ZF region. The exact function of these regions is unknown, but in some patients with holoprosenchphaly, expansions of the C-terminal alanine tract of ZIC2 are observed (Brown, Odent et al. 2001). Similarly, one patient with heterotaxy had an expansion of the N-terminal alanine tract of ZIC3 (Wessels, Kuchinka et al. 2010). Modeling of the ZIC2 mutation *in vitro* demonstrated that expansion of the alanine tract reduced the ability of ZIC2 to bind DNA despite having no effects on the zinc finger region (Brown, Paraso et al. 2005).

1.11. Functions of ZIC Protein Structural Domains

As described previously, ZIC proteins contain a zinc finger domain composed of 5 tandemly-arranged C2H2-type zinc fingers and a ZF-NC domain, as well as ZOC domain that is found in some, but not all, ZIC proteins (Figure 1-10). To date, the ZF and ZOC domains have been shown to directly bind DNA, participate in protein-protein interactions, and to function in regulating the cellular localization of ZIC proteins (described in detail below); however, the function of the ZF-NC is unknown (Aruga, Kamiya et al. 2006; Houtmeyers, Souopgui et al. 2013).

DNA binding

As transcription factors, one of the primary ways the ZIC proteins function is through direct binding to DNA. When the first *Zic* gene was cloned (originally

known as *zic*, now *Zic1*), gel shift assays showed that ZIC protein bound to the human GLI3 binding sequence and that this binding depended on the ZF domain (Aruga, Yokota et al. 1994). Since then, other ZIC proteins have been shown to directly bind to DNA via their ZF domains *in vitro*: ZIC1 and ZIC2 to the *ApoE* promoter (Salero, Perez-Sen et al. 2001), ZIC1 to a novel conserved site within the *Math1* enhancer (Ebert, Timmer et al. 2003), and ZIC3 to the *Nanog* promoter (Lim, Hong et al. 2010). The ZOC domain also is important for binding to DNA and activating transcription. In experiments utilizing N-terminal and C-terminal deletions of ZIC2 *in vitro*, the expression of reporter genes was reduced to similar levels in ZIC2 lacking either the ZOC domain or ZF3-5 (Mizugishi, Hatayama et al. 2004). Interestingly, when a different assay was used, the ZOC domain behaved as a transcriptional repressor (Mizugishi, Hatayama et al. 2004). Other direct interactions between ZIC proteins and DNA have been shown, although the exact region of the ZIC protein required for DNA binding was not determined. ZIC2 binds to and activates the alpha-CaM kinase II promoter (Sakurada, Mima et al. 2005) and binds to the activator region within the promoter of the human *dopamine receptor 1a* gene, repressing its Sp1-induced activation (Yang, Hwang et al. 2000).

Two ZIC proteins, ZIC2 and ZIC3, have unique features that are believed to be involved in DNA binding, though the exact mechanisms have yet to be determined. ZIC2 has a C-terminal poly-alanine tract that when expanded, interrupts DNA binding (Brown, Paraso et al. 2005). The first and second zinc fingers of ZIC3 have a tryptophan residue between the two cysteines, forming a

CWCH2 motif (Hatayama and Aruga 2010). The exact function of the CWCH2 domain is not known, but it has been hypothesized that it may indirectly affect DNA binding by altering the position of the protein relative to DNA or by participating in protein-protein interactions (Hatayama and Aruga 2010).

Protein-protein interactions

ZIC proteins can also affect transcription indirectly through protein-protein interactions with other transcription factors. *In vitro*, ZIC2 inhibits WNT/β-catenin signaling through a direct interaction between its zinc finger and the high mobility group box of TCF4 (Pourebrahim, Houtmeyers et al. 2011). In addition, ZIC proteins interact with GLI transcription factors to either suppress or enhance GLI-mediated transactivation (Koyabu, Nakata et al. 2001; Mizugishi, Aruga et al. 2001; Pan, Gustafsson et al. 2011). Zinc fingers 3-5 (ZF3-5) of both GLI3 and ZIC1 are critical for these interactions—GLI3 lacking ZF3-5 cannot bind to ZIC1-3 and ZIC1 lacking ZF3-5 cannot bind to GLI1-3 (Koyabu, Nakata et al. 2001). The ZOC domain also participates in protein-protein interactions. Binding of I-mfa to ZIC1-3 via the ZOC domain inhibits the transcriptional activity of ZIC1-3 *in vitro* (Mizugishi, Hatayama et al. 2004).

Subcellular localization of ZIC proteins

In vitro, ZIC proteins are found in both the nucleus and the cytoplasm (Bedard, Purnell et al. 2007). Since ZIC proteins act as transcriptional regulators (directly binding to DNA or indirectly through protein-protein interactions with other

transcription factors), they must translocate from the cytoplasm into the nucleus to function. Unlike many other proteins that translocate to the nucleus, ZIC proteins do not contain a traditional nuclear localization signal (NLS). Independent results from two labs characterized the ZF region of ZIC3 as having an NLS spread out across the ZF region, and alterations in the amino acid sequence within this region resulted in the accumulation of ZIC3 in the cytoplasm (Bedard, Purnell et al. 2007; Hatayama, Tomizawa et al. 2008). Interactions between I-mfa and ZIC1-3 via the ZOC domain also result in cytoplasmic accumulation of ZIC1-3, but this is not due to an effect on the NLS, as the ZOC domain is more than 100 amino acids N-terminal to the NLS-containing ZF region (Mizugishi, Hatayama et al. 2004).

1.12. Genomic Organization and Evolution of the *Zic* Genes

Sequence analysis of 45 *Zic* genes revealed the presence of 5 different intron-exon boundaries within the ZF region (Aruga, Kamiya et al. 2006). One of these intron-exon boundaries (designated “A”) is located between ZF3 and ZF4 and was conserved across most *Zic* genes, except for the seven cnidarian *Zics* (Aruga, Kamiya et al. 2006). Within the vertebrate *Zics*, one other conserved intron-exon boundary (designated “B”) was observed, while the other metazoan *Zics* contained from 0 (*Hyzic* from *Hydra vulgaris*; *ZicA-E* from *Nematostella vectensis*) up to 4 (*Ref-2* from *C. elegans*) different intron-exon boundaries within each *Zic* gene (Aruga, Kamiya et al. 2006).

In vertebrates, the *Zic* genes have an unusual arrangement within the

Table 1-3. Location of *Zic* Genes in Representative Vertebrates

	Chromosome		
Organism	***Zic1* / *Zic4***	***Zic2* / *Zic5***	***Zic3* / *Zic6***
Human	3	13	X[a]
Mouse	9	14	X[a]
Chick	9	1[b]	4[a]
Zebrafish	24	9[c]	14

[a] no *Zic6* exists; [b] existence of *Zic5* has not been confirmed; [c] *zic2a*

genome. *Zic1*/*Zic4* and *Zic2*/*Zic5* are located in tandem on the same chromosome, flanking an intervening bidirectional promoter, while *Zic3* is located by itself on another chromosome (Table 1-3, Figure 1-11). In addition to *Zic1-5*, zebrafish have a *Zic6* gene, which is located on the same chromosome as *Zic3* in the exact same tandem arrangement as observed for *Zic1*/*Zic4* and *Zic2*/*Zic5*. Outside of vertebrates, the only other *Zic* genes to cluster together on the same chromosome are the *ZicB*/*ZicC*/*ZicD*/*ZicE* genes from the Cnidarian, *Nematostella vectensis*. Unlike in vertebrates, however, these genes are not transcribed in opposite directions from the same promoter but rather are oriented such that transcription of *ZicB*, *ZicC*, and *ZicD* proceeds in the same direction and transcription of *ZicD* and *ZicE* oppose one another.

The *Zic* gene family is hypothesized to have evolved from a single ancestral *Zic* gene that shares a common ancestor with the *Gli*/*Glis*/*Nkl* gene family (Aruga, Kamiya et al. 2006; Layden, Meyer et al. 2010). This hypothesis is based on the various structural features (ZF-NC, ZOC, ZF) present in some, or all, of the *Zic* genes, as well as the presence of the different intron-exon boundaries within the zinc finger region (Aruga, Kamiya et al. 2006). The

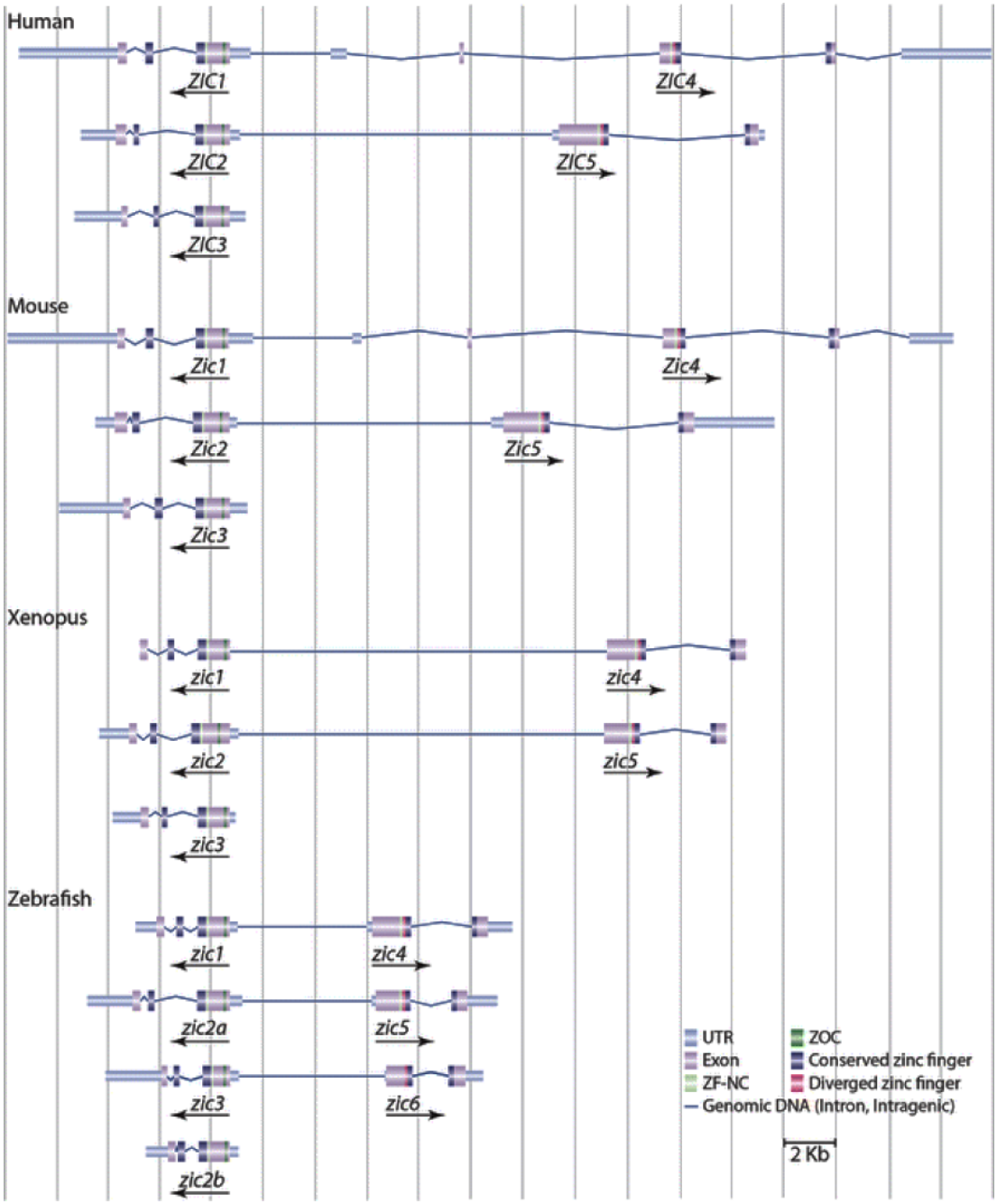

Figure 1-11. Chromosomal Location of *Zic* Genes in Representative Vertebrates. *Zic* genes from human, mouse, frog (*Xenopus*), and zebrafish are located as pairs of genes on the same chromosome that are transcribed in opposite directions relative to one another. In human, mouse, and frog, *Zic3* exists as a lone gene, presumably due to the loss of its paired *Zic* gene during evolution. Similarly, *zic2b* in zebrafish exists as a lone gene. Abbreviations: ZOC, *Zic*-*Opa* conserved domain; ZF-NC, zinc finger nucleocapsid domain; UTR, untranslated region. (Image from Houtmeyers, Souopgui et al. 2013).

ancestral *Zic* gene likely did not contain any introns within the ZF region, but as the genes evolved, they acquired various combinations of these introns (Figure 1-12). Additionally, duplication of the evolving *Zic* gene in certain species led to the presence of multiple *Zic* genes, such as in *N. vectensis*, *D. japonica*, *S. mediterranea*, *C. intestinalis*, *C. savignyi*, and *H. roretzi*. The vertebrate *Zic* genes present a very interesting case of gene duplication followed by subsequent evolution of individual *Zic* genes coupled with gene loss (Figure 1-13). After gaining the "A" intron-exon boundary, it is hypothesized that this gene, containing the ZF, ZF-NC, and ZOC domains, was duplicated on the same chromosome and then one of the copies of the gene acquired the "B" intron-exon boundary. Then, this cluster was duplicated twice more, resulting in 8 *Zic* genes (4 with the "A" intron-exon boundary and 4 with both the "A" and "B" intron-exon boundaries in the ZF region). Over time, loss of one (zebrafish), two (*Xenopus*), or three (human, mouse) *Zic* genes resulted in the current numbers of *Zic* genes observed for each species. Although only *Zic1-4* have been identified in the chicken, it is likely that as the chicken genome is further annotated, *Zic5* will be found on the same chromosome as *Zic2*, mirroring the arrangement seen for the mammalian *Zic* genes (McMahon and Merzdorf 2010). In addition, changes in the sequence over time led to the loss or partial loss of the ZOC domain, the creation of the low complexity regions characterized by poly-alanine, -histidine, -proline, and -serine/glycine tracts, the divergence of ZF1, and the variation in ZF3 (*Zic3*) and ZF5 (*Zic1-3* compared to *Zic4-5*). The hypotheses proposed by Aruga et al. for the evolution of the metazoan and vertebrate *Zic* genes based on

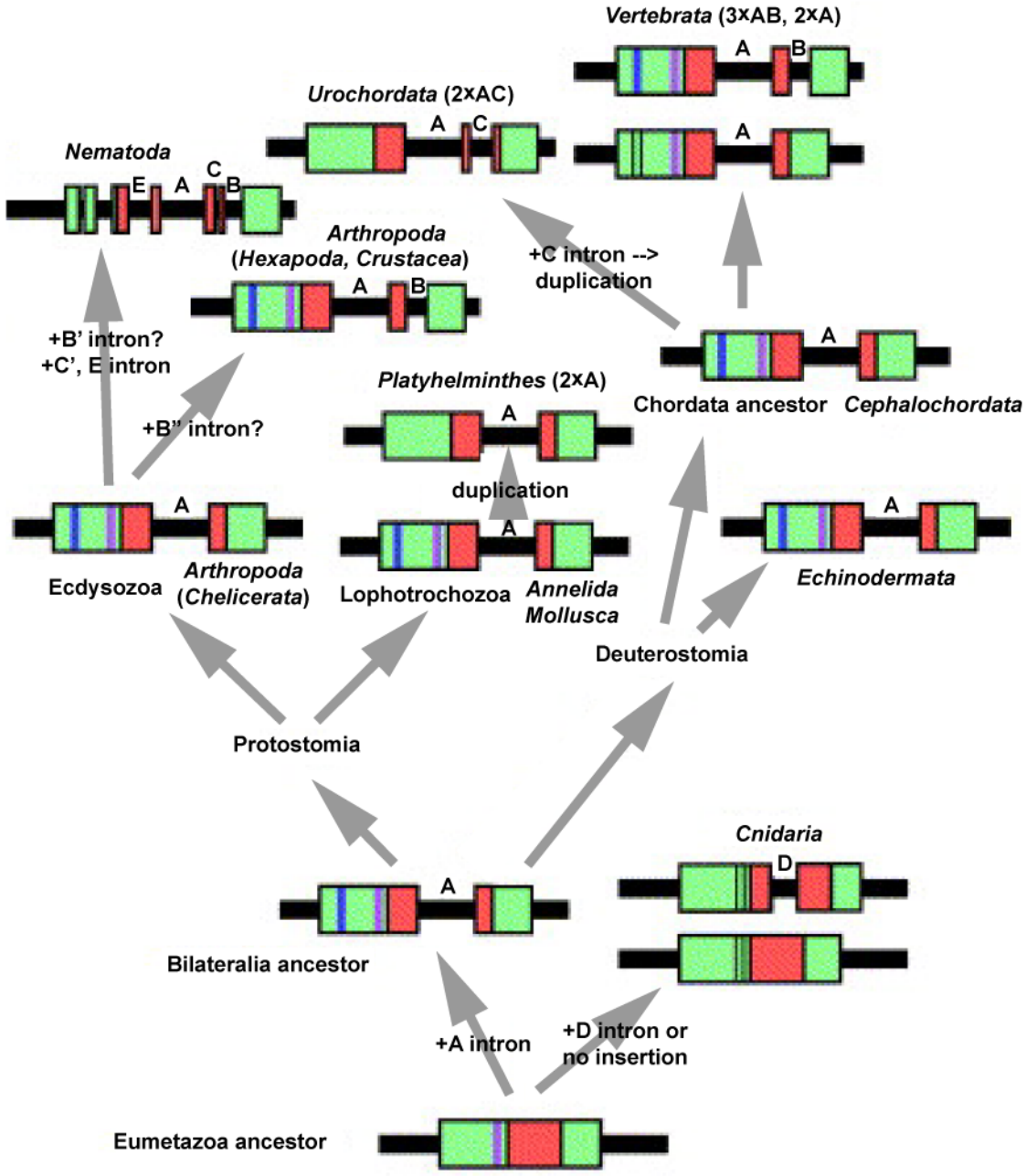

Figure 1-12. Hypothesized Evolution of Metazoan *Zic* Genes. A single ancestral *Zic* gene containing the C2H2 ZF domain (red) and the ZF-NC domain (purple) initially gained the ZOC domain (blue) and an intron (designated by A, B, C, D, or E) in the Bilaterian ancestor. This *Zic* gene evolved further, gaining other introns and modifying (designated by stippled blue or purple rectangle) or losing the ZOC and ZF-NC domains, and underwent occasional gene duplications, resulting in the diversity of *Zic* genes observed in metazoans. Abbreviations: ZF, zinc finger; ZOC, *Zic-Opa* conserved domain; ZF-NC, zinc finger nucleocapsid domain; C2H2 ZF, C_2H_2 zinc finger. (Image modified from Aruga, Kamiya et al. 2006).

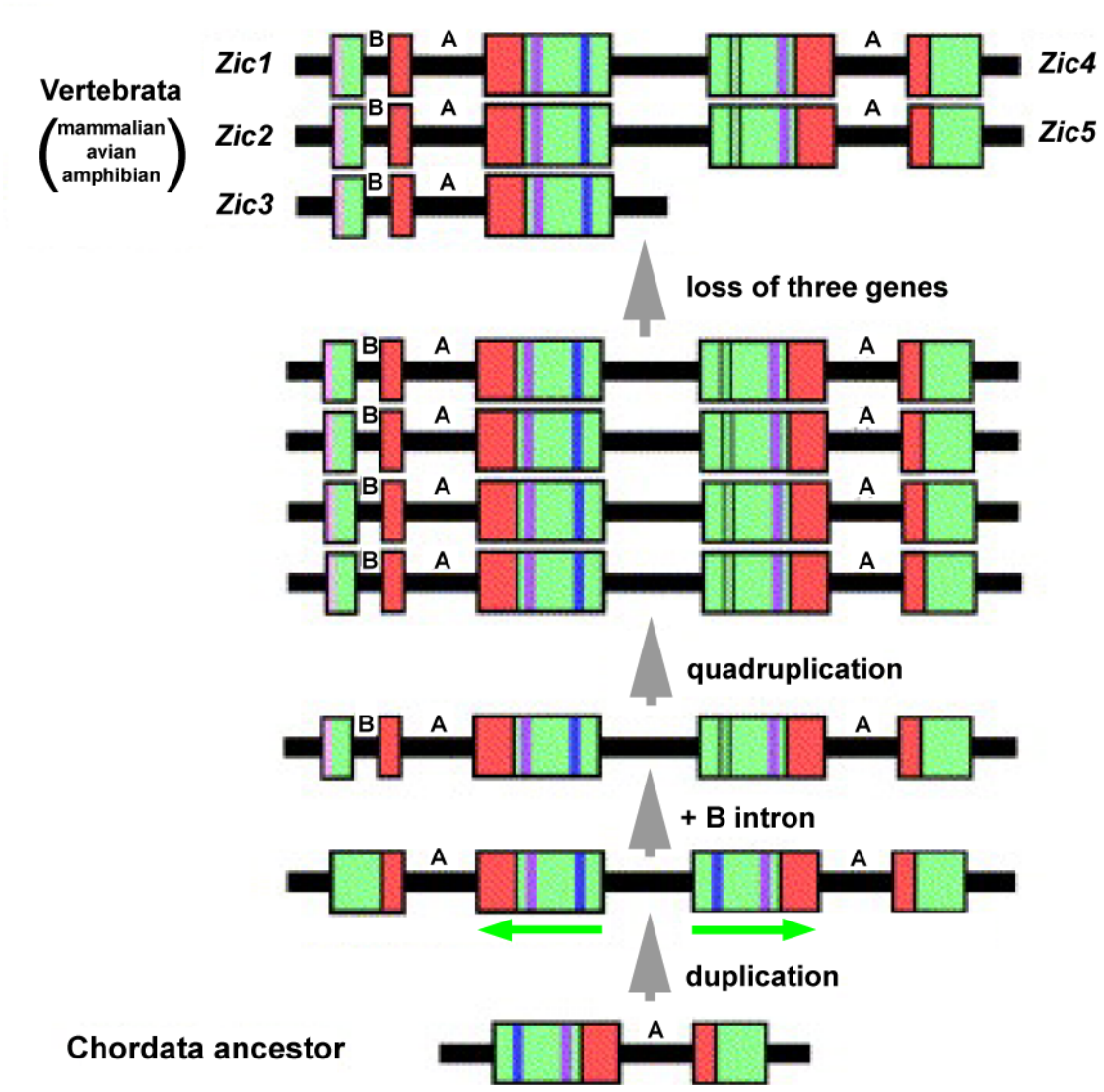

Figure 1-13. Hypothesized Evolution of Vertebrate *Zic* Genes. A single *Zic* gene descended from the ancestral *Zic* gene contained the C2H2 ZF, ZOC, and ZF-NC domains, as well as one intron (designated "A"). Subsequent gene duplications and the introduction of a second intron (designated "B") resulted in pairs of *Zic* genes on four different chromosomes. Over time, some of these genes were lost and the remaining *Zic* genes evolved, resulting in the current numbers of vertebrate *Zic* genes (five in mouse/human, six in frog, seven in fish). Green arrows (after duplication event) show direction of transcription of the two genes. Abbreviations: ZF, zinc finger; ZOC, *Zic*-*Opa* conserved domain; ZF-NC, zinc finger nucleocapsid domain; C2H2 ZF, C_2H_2 zinc finger. (Image modified from Aruga, Kamiya et al. 2006).

intron-exon boundaries is plausible (Aruga, Kamiya et al. 2006); however, with the recent reclassification of the consensus ZOC sequence leading to the inclusion of more *Zic* genes into the ZOC-containing group (including the discovery of the ZOC domain in the Cnidarian *Zic* genes), the emergence of the ZOC domain must be placed before the *Bilateria*/*Cnidaria* divergence (Layden, Meyer et al. 2010).

1.13. Investigating the Role of *Zic* Genes in Inner Ear Development

Previous studies in chick embryos reported that expression of *Zic2* was upregulated in the regenerating sensory epithelium of the inner ear (Gong, Hegeman et al. 1996). During development, *Zic1* expression was detected at early stages in the otic epithelium and at later stages in the sensory patches of the inner ear (Warner, Hutson et al. 2003). However, a more careful analysis of the expression patterns of the *Zic* genes (*Zic1-4*) during development of the inner ear in chick found that none of the *Zic* genes are expressed in the otic epithelium between HH stages 13 and 32, but rather are expressed to varying degrees in the periotic mesenchyme and dorsal neural tube adjacent to the developing inner ear (Chervenak, Hakim et al. 2013). In the developing mouse inner ear, for which *Zic* expression data was previously unavailable, a similar expression pattern for the *Zic* genes (*Zic1-5*) was reported (Chervenak, Hakim et al. 2013).

In both mouse and chick, *Zic2* expression completely surrounds the developing inner ear, indicating that *Zic2* may be a key factor in signaling between the mesenchyme and the otic epithelium (Chervenak, Hakim et al.

2013). We investigated changes in ear development in two different *Zic2* mouse mutants, the *Zic2*^{kd} mouse (hypomorphic allele; Nagai, Aruga et al. 2000) and the *Zic2*^{Kumba} mouse (complete loss-of-function allele; Elms, Siggers et al. 2003). Paint-fills of the inner ears were used to assess morphological changes in embryonic inner ears from both the *Zic2*^{kd} and *Zic2*^{Kumba} mice. To investigate the function of *Zic2* during inner ear development, *in situ* hybridization and immunofluorescence experiments were performed with sections through the ears of *Zic2*^{Kumba} embryos to examine changes in the activation of key signaling pathways (FGF, SHH, WNT, BMP) involved in inner ear development, as well as to look for changes in the expression of ear development genes downstream of these pathways.

Our studies of *Zic* genes during inner ear development have expanded to include *Zic1* and *Zic4*, as we have recently received compound *Zic1*/*Zic4* heterozygous and mutant embryos (*Zic1*$^{+/-}$;*Zic4*$^{+/-}$, *Zic1*$^{-/-}$;*Zic4*$^{-/-}$), as well as wild type controls (*Zic1*$^{+/+}$;*Zic4*$^{+/+}$) from our collaborator, Dr. Kathy Millen (University of Washington). The expression patterns of *Zic1* and *Zic4* in the region of the developing inner ear in mouse have a very high degree of overlap, especially at later stages of development (Chervenak, Hakim et al. 2013). These *Zic1*/*Zic4* mice have enabled us to study the effects of loss of both *Zic1* and *Zic4* on inner ear development, and allow us to begin to address the possibility of redundancy among the *Zic* genes. Our initial experiments with these mice have been to paint-fill the inner ears to look for changes in inner ear morphology.

Inner ear morphology is severely affected in both *Zic2*$^{kd/kd}$ and *Zic2*$^{Ku/Ku}$ mice, but apparently unaffected in *Zic1*$^{-/-}$;*Zic4*$^{-/-}$ mice. Otocyst patterning in the *Zic2*$^{Ku/Ku}$ mice is relatively unchanged, but the inner ears are smaller and misoriented compared to ears from control mice. It is clear that *Zic2* has a role in inner ear development in mice, but what that exact role is remains to be determined. Further, despite the inner ears from the *Zic1*$^{-/-}$;*Zic4*$^{-/-}$ mice appearing to be relatively unaffected, the other *Zic* genes are expressed at the right time and in the right place to be involved in inner ear development. A comprehensive analysis of inner ears from single and compound *Zic* mutants is needed to fully understand the roles that the *Zic* genes play during inner ear development.

Chapter 2

Spatiotemporal Expression of *Zic* Genes During Vertebrate Inner Ear Development.

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2.1. Abstract

Background: Inner ear development involves signaling from surrounding tissues, including the adjacent hindbrain, periotic mesenchyme and notochord. These signals include SHH, FGFs, BMPs and WNTs from the hindbrain and SHH from the notochord. *Zic* genes, which are expressed in the dorsal neural tube and act during neural development, have been implicated as effectors of these pathways. This report examines whether *Zic* genes' involvement in inner ear development is a tenable hypothesis based on their expression patterns. Results: In the developing inner ear of both the chick and mouse, all of the *Zic* genes were expressed in the dorsal neural tube and variably in the periotic mesenchyme, but expression of the *Zic* genes in the otic epithelium was not found. The onset of expression differed among the *Zic* genes; within any given region surrounding the otic epithelium, multiple *Zic* genes were expressed in the same place at the same time. Conclusions: *Zic* gene expression in the region of the developing inner ear is similar between mouse and chick. *Zic* expression domains overlap

with sites of WNT and SHH signaling during otocyst patterning, suggesting a role for *Zic* genes in modulating signaling from these pathways.

2.2. Introduction

The *Zic* (zinc finger of the cerebellum) genes comprise a family of transcription factors found in both vertebrates and invertebrates (reviewed in Ali, Bellchambers et al. 2012) that are related to the *Drosophila Zic* homologue, *odd-paired* (*opa*) (Nusslein-Volhard and Wieschaus 1980; Benedyk, Mullen et al. 1994). Among vertebrate species, humans and mice have five *Zic* genes (*Zic1-5*); other species have more (zebrafish, 8 *Zic* genes; frog, 6 *Zic* genes) or fewer (chicken, 4 *Zic* genes) *Zic* genes. Structurally, ZIC proteins are characterized by a zinc finger region that contains five tandem C_2H_2 zinc finger domains (Aruga 2004; Merzdorf 2007; Ali, Bellchambers et al. 2012). The amino acid sequence of zinc fingers 2 though 5 is almost identical among *Zic1-5* for a given species, while the sequence outside of this region can be highly variable (Aruga 2004; Grinberg and Millen 2005).

The *Zic* genes are involved in a multitude of developmental processes. *Zic* gene function has predominantly been studied during neural development, where they have roles in controlling neural patterning, in generation of neural crest, in cerebellar development, and in the formation of the neural tube (Aruga 2004; Merzdorf 2007). However, *Zic* genes also function in somite myogenesis (Pan, Gustafsson et al. 2011), left-right asymmetry (Herman and El-Hodiri 2002), and retinal development. Although there have been a number of studies focused on