Development of the semicircular canals and otolithic organs of the vertebrate inner ear

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Abbreviations

aGPCR	Adhesion class G-protein-coupled receptor
BMP	Bone Morphogenetic Protein
BPPV	Benign paroxysmal positional vertigo (the sensation of spinning when at rest, due to misplaced otoconia in the inner ear)
cAMP	Cyclic adenosine monophosphate
CS	Chondroitin sulphate
CSPG	Chondroitin sulphate proteoglycan
(μ)CT	(Micro) Computed Tomography
cryoEM	Cryogenic electron microscopy
DAPI	4′,6-diamidino-2-phenylindole (a DNA dye)
Dpp	Decapentaplegic
dpf	Days post fertilisation (zebrafish)
E	Embryonic day (chick, mouse)
ECM	Extracellular matrix
FGF	Fibroblast Growth Factor
GAG	Glycosaminoglycan
GFP	Green Fluorescent Protein
GlcNAc	N-acetylglucosamine
GRN	Gene regulatory network
HA	Hyaluronic acid (also known as hyaluronan)
HCR	Hybridisation chain reaction

HR	H^+-ATPase-rich (a type of ionocyte)
HSPG	Heparan sulphate proteoglycan
MAPK	Mitogen-activated protein kinase
MZ	Maternal-zygotic
RA	Retinoic acid
RFP	Red Fluorescent Protein
SHH	Sonic Hedgehog
V-ATPase	Vacuolar-type ATP-dependent proton pump
(a)VOR	(Angular) Vestibulo-Ocular Reflex (counter-movement of the eyes in response to vestibular stimulation)
WNT	Wingless/Int

Abstract

The vestibular apparatus of the inner ear functions to sense gravity and motion. Working together with the visual and proprioceptive systems via various muscular reflexes, it enables an organism to stabilise head position, gaze and body posture, both at rest and during movement. These functions are dependent on sensory hair cells that respond to mechanical stimulation, together with their associated non-sensory structures, including fluid-filled ducts and chambers, specialised extracellular matrices, and biomineralised crystalline deposits. The focus of this review is on the embryonic development of selected elements of the vestibular system: morphogenesis of the semicircular canal ducts, development of the ampullae and sensory cristae, and formation of the biomineralised otoliths and otoconia. Recent findings have identified new genetic players, dynamic cross-repressive gene regulatory networks, and morphogenetic mechanisms that act to shape the developing vestibular system. A final section of the review highlights approaches that link developmental genetic studies to an understanding of cell and tissue mechanics, vestibular-driven behaviour, evolution and human disease.

Key words

Inner ear, vestibular system, semicircular canal, extracellular matrix, otolith, otoconia, biomineralisation, development, mouse, chick, zebrafish

1 Introduction

The inner ear: an organ that captures the imagination, not only for its exquisite sensitivity to sound, gravity and motion, but also its intricate and complex morphology (Fig. 1). The ear has both auditory (hearing) and vestibular (balance) functions. In all jawed vertebrates, the vestibular components of the inner ear comprise the three semicircular canals, used to sense rotational movement (angular acceleration) of the head, and the otoconial or otolithic organs, which sense gravity and other linear accelerations. Vestibular sensory inputs, together with visual and proprioceptive inputs, feed into neuronal circuitry to control muscular reflexes that stabilise head position and gaze, and maintain a normal body position or postural equilibrium. In fishes, the otolithic organs are also important for hearing. Mechanosensitive sensory hair cells of the vestibular system function in the context of various non-sensory structures—the semicircular canal ducts and ampullae, specialised extracellular matrices and the biomineralised otoconia or otoliths—that are essential components of the functioning whole.

The focus of this review will be on the development of selected components of the vestibular system: the semicircular canal system, and the otoconia and otoliths. Our knowledge of how these structures develop in the embryo largely comes from a limited range of model systems (mouse, chick, frog and fish). Classical, hypothesis-driven genetic and pharmacological perturbation studies are now being complemented by information-rich, unbiased discovery approaches, such as single-cell transcriptomics, providing information on a whole-tissue or whole-organism scale. These approaches are both validating previous observations and revealing new and unexpected findings—and are uncovering molecular mechanisms in exquisite detail.

2 Development of the semicircular canal system

2.1 Anatomy of the semicircular canal system

The inner ear has three semicircular canal ducts, arranged roughly orthogonally to one another, providing sensitivity to movement in all directions in three-dimensional space (Fig. 1). Each canal duct is a curved tube of epithelium (part of the membranous labyrinth) ending in a rounded chamber, the ampulla, which houses sensory a saddle-shaped strip of sensory tissue containing hair cells, known as the crista (meaning 'crest'). The epithelial canal ducts are partly or wholly surrounded by bone, depending on the species. This protective casing is known as the bony labyrinth, visible in computed tomography (CT) scans and well-preserved in fossils, as it is located within the dense petrous pyramid of the temporal bone of the skull (Spoor & Zonneveld, 1998).

The fundamental arrangement of three orthogonally-oriented semicircular canals is conserved across all jawed vertebrates (Higuchi et al., 2019). However, the inner ear is a highly evolvable organ (Grunstra et al., 2024), with considerable variation in semicircular canal morphology between and within different taxa. In mammals, differences in semicircular canal shape often correlate with lifestyle (Spoor et al., 2007): long thin canals in animals that rely heavily on balancing skills for their locomotion, such as predators requiring high speed and manoeverability (Grohé et al., 2018); variable canal morphologies in the slow-moving three-toed sloth (Billet et al., 2012); and reduced canal size in aquatic mammals with limited neck movements, such as the cetaceans (Spoor et al., 2002), manatee and dugong (Grunstra et al., 2024). By contrast, phylogeny—rather than life habit—is a better predictor of semicircular canal shape in reptiles, with some exceptions (Latimer et al. (2023) and references within). The relative size of the inner ear can also vary: although inner ear size correlates with body mass in mammals and reptiles (Latimer et al., 2023), several species of deep-sea fishes have enormous semicircular canals relative to brain size (X. Deng et al. (2011, 2023) and references within).

2.2 Morphogenesis of the semicircular canal system

Almost all tissues and cell types in the membranous labyrinth derive from the otic vesicle or otocyst (Fig. 1). This placodally-derived structure is an epithelial monolayer surrounding a fluid-filled cavity, which forms on either side of the developing hindbrain (reviewed in (Groves & Fekete, 2012; Sai & Ladher, 2015; Singh & Groves, 2016)). Generation of the curved semicircular canal ducts from the otic vesicle requires a whole series of tissue gymnastics, involving the folding, fusion and perforation of epithelial sheets. In amniote vertebrates (birds and mammals), a first step is the formation of flattened pouches or diverticula of the otic vesicle (Fig. 1B,C). A single vertical pouch gives rise to both the anterior and posterior canals, and a separate lateral pouch forms the lateral (horizontal) canal. As development proceeds, the epithelial sides of each pouch move towards each other, meeting at their apical surfaces to form a transient epithelial bilayer known as the fusion plate (Figs. 1,2). Two fusion plates form from the vertical pouch, and a single fusion plate forms in the lateral pouch. Cells are subsequently cleared from each fused region, leaving behind the tubular pouch rim as the nascent canal duct (Bissonnette & Fekete, 1996; Fekete et al., 1997; Martin & Swanson, 1993; Morsli et al., 1998) (Fig. 2F). A central portion of the vertical pouch becomes the crus commune, a structure that links and supports the anterior and posterior canals. In anamniote vertebrates (frogs and fish), finger-like projections and bulges of epithelium—topologically equivalent to, but much smaller than, the amniote pouch walls—evaginate, fuse and perforate to form pillar-like structures that span the otic lumen (Hertwig,

1987; Waterman & Bell, 1984) (Figs. 1A,2B,C). These pillars subsequently widen, forming the inner walls of the canal ducts. In the fish ear, incomplete septa, most prominently the dorsolateral septum (Fig. 2A), divide the canal duct lumens from one another. In all vertebrates, these epithelial fusion and perforation events change the topology of the otic epithelium, converting the original simple vesicle into an interconnected series of tubes, whose lumens remain in communication with one another (Fig. 2C,F). For previous reviews of semicircular canal development, see (Alsina & Whitfield, 2017; D. K. Wu & Kelley, 2012); selected mechanistic updates are presented here.

2.3 Gene regulatory networks required for semicircular canal formation

2.3.1 Signalling pathways required for semicircular canal development

The gene regulatory networks (GRNs) that underlie specification and morphogenesis of the semicircular canals are initiated through signalling pathways that are used reiteratively in different developmental contexts. BMP, FGF, Hedgehog, WNT, Notch, Retinoic Acid, adhesion G-protein-coupled receptor (aGPCR) and purinergic signalling pathways are all implicated in development of the vestibular system (reviewed in (Alsina & Whitfield, 2017; R. Brown & Groves, 2020; Kim & Choi, 2022; Ohta & Schoenwolf, 2018)). Often, there is cross-talk between them, making the GRNs leading to semicircular canal formation complex and context-dependent. The roles of selected signalling pathways (BMP, FGF and WNT) are discussed below.

The developing inner ear is a strong site of expression of genes coding for Bone Morphogenetic Proteins (BMPs), together with their receptors, downstream transducers (Smads), and regulatory proteins (including Dan and Bmper); expression domains are conserved across mouse, chick, frog and zebrafish (Baldera et al., 2023; Baxendale et al., 2021; W. Chang et al., 2002, 1999; Dewulf et al., 1995; Gerlach-Bank et al., 2004; Morsli et al., 1998; Mowbray et al., 2001; Oh et al., 1996; Ohta et al., 2010; D. K. Wu & Oh, 1996; Yamanishi et al., 2007). Both BMP2 and BMP4 are critical for formation of the semicircular canal ducts. Treatment of the chick otocyst with the BMP antagonist Noggin results in semicircular canal truncations (W. Chang et al., 1999; Gerlach et al., 2000). In the mouse, inner-ear-specific conditional disruption of *Bmp2* or *Bmp4* results in a loss of all three canal ducts (W. Chang et al., 2008; C. H. Hwang et al., 2019); in zebrafish, a late loss of Bmp2b function, bypassing its early role in embryonic axial patterning, also results in loss of all three canal ducts (Hammond et al., 2009).

BMP signalling is required for cell thinning in the pouches that prefigure the canal ducts in the chick otocyst (Ohta et al., 2010), and mediates its effects in the developing vestibular system through both positive and negative regulation of gene expression. Transcriptional targets of BMP signalling in the developing chick ear include the transcription factor genes *DLX5* and *HMX3* in dorsal otic epithelium, activated through canonical SMAD and non-canonical protein kinase A pathways, respectively (Ohta et al., 2016a). BMP signalling in the dorsal otocyst opposes the action of Sonic Hedgehog (SHH), which represses expression of *HMX3* in ventral regions (Ohta et al., 2016b). BMP2 signalling also acts as a negative regulator of *Netrin1* (*Ntn1*) expression (C. H. Hwang et al., 2019), driving the reciprocal feedback loops that regulate cell behaviour at the fusion plate in the mouse (see below). In the developing cristae in the chick, *BMP4* expression is maintained by Notch signalling (Daudet et al., 2007), and acts to regulate the expression of target genes in both sensory and non-sensory zones (W. Chang et al., 2004). At later stages, BMP signalling continues to play a role in development of the semicircular canal system, mediating interactions between

the epithelium and surrounding mesenchyme, important for chondrogenesis of the otic capsule (W. Chang et al., 2002).

Fibroblast Growth Factor (FGF) signalling—from sources both extrinsic to the ear and within the otic epithelium—also plays critical roles at several stages of inner ear development. FGFs have an early and conserved role in otic placode induction; homozygous mutations in the human *FGF3* gene, for example, can result in Michel aplasia (the complete absence of inner ear structures) (reviewed in (Hutchings & Sela-Donenfeld, 2024; Riley, 2021; Sai & Ladher, 2015)). FGFs are also important for regional patterning of the otic vesicle and for otic neurogenesis (reviewed in (Riley, 2021)). In addition to these early roles in otic development, FGF signalling is required for formation of the semicircular canals. In the mouse, mutations in both *Fgf9* and *Fgf10* result in a loss of semicircular canal ducts (Ohuchi et al., 2005; Pauley et al., 2003; Pirvola et al., 2004; Urness et al., 2015). *Fgf10* is expressed in prosensory epithelium and later in the cristae, whereas genes coding for FGF receptors, together with FGF target genes including *Bmp2*, are expressed in the nonsensory epithelium of the prospective canal duct (W. Chang et al., 2004; Pirvola et al., 2000; Sánchez-Guardado et al., 2013). Together, these findings have led to a model where FGF signalling from the cristae regulates specification of the non-sensory semicircular canal duct epithelium by establishing a ‘canal genesis zone’ in the chick otocyst (W. Chang et al., 2004). This model continues to be supported by analysis of a number of mutant phenotypes, with a few exceptions (see discussion in (C. H. Hwang et al., 2009)).

To identify additional targets of FGF signalling in the mouse ear, Urness and colleagues used conditional expression of a soluble dominant-negative FGF receptor ectodomain (dnFGFR2b) to trap FGF ligands, followed by RNA sequencing and validation of selected candidate genes (Urness et al., 2018). This approach identified both expected and novel targets of FGF signalling in the developing ear. Interestingly, one up-regulated target after FGF inhibition was *Bmper*, orthologue of the Dpp (BMP) regulator gene *crossveinless-2* in *Drosophila* (Urness et al., 2018). In situ hybridisation analysis confirmed that FGF signalling normally restricts expression of *Bmper* to dorsomedial regions of the mouse otocyst (Urness et al., 2018), where it is also a known transcriptional target of DLX5 (Sajan et al., 2011) (see below). Although an inner ear phenotype is yet to be described in the mouse *Bmper* mutant, mutations in the zebrafish *bmper* gene result in truncations of the anterior and posterior semicircular canal ducts, with several lines of evidence indicating that Bmper acts to promote BMP signalling during semicircular canal morphogenesis (Baxendale et al., 2021).

Wingless/Int (WNT) signalling also plays important roles in semicircular canal development, where the main focus of study has been the mouse. WNT signalling from the hindbrain, mediated by WNT1 and WNT3a, is important for specifying dorsal regions of the murine otocyst, where target genes include *Dlx5/6* and *Gbx2* (Noda et al., 2012; Riccomagno et al., 2005). Conditional loss- and gain-of-function approaches, together with pharmacological manipulation of pathway activity, have established that WNT signalling is required to maintain *Dlx5* expression and preserve epithelial integrity in the pouch rim, repressing *Ntn1* expression and protecting rim cells from resorption at the fusion plate (Noda et al., 2012; Rakowiecki & Epstein, 2013) (see below). Later, however, WNT signalling is also active at the fusion plate itself (Rakowiecki & Epstein, 2013). Rapid progress is being made in elucidating the details of WNT transport mechanisms in a range of different model systems, including the roles played by signalling filopodia (cytonemes) and exosomes (reviewed in (Routledge & Scholpp, 2019)). It will be interesting to see how these apply to the developing

ear, where, for example, WNT signalling from the hindbrain to the otocyst necessitates the crossing of two basement membranes.

2.3.2 Transcription factors required for semicircular canal development

Many families of transcription factor genes, including *Dlx*, *Eya*, *Gata*, *Gbx*, *Hmx*, *Lmx*, *Maf*, *Msx*, *Otx*, *Prx*, *Tbx*, *Six*, *Sox* and *Zic*, have roles in semicircular canal duct development, acting upstream or downstream of, or in feedback loops with, the various signalling pathways detailed above (reviewed in (Alsina & Whitfield, 2017)). Some of these genes have very early roles in otic morphogenesis, with loss-of-function mutations affecting the entire ear, including development of all three semicircular canals; in others, mutations result in more specific defects, with just one or two canals affected. Here, I have selected gene families where recent progress has been made in identifying downstream targets and mechanisms of action of transcription factors in the developing ear: *Dlx, Hmx*, *Lmx,* and *Sox* genes.

The *Dlx* family of transcription factor genes—orthologues of *Distal-less* (*Dll*) in *Drosophila*—show conserved expression in the developing inner ear (S. T. Brown et al., 2005; Ekker et al., 1992; Robledo & Lufkin, 2006), where they act as readouts of both BMP and WNT signalling. As detailed above, *Dlx5* expression is a target of BMP signalling in the chick ear (Ohta et al., 2016a), and is maintained in the pouch rim (prospective canal duct) of the mouse ear by WNT signalling (Noda et al., 2012; Rakowiecki & Epstein, 2013). *Dlx5* expression is complementary to that of *Netrin1* in the canal duct (C. H. Hwang et al., 2019) (see also below). Mutation of *Dlx5*, *Dlx6* or both genes in the mouse results in a loss of *Bmp4* expression and semicircular canal defects (Acampora, Merlo, et al., 1999; Depew et al., 1999; Merlo et al., 2002; Robledo & Lufkin, 2006). Studies to identify transcriptional targets of DLX proteins in otic development in both the mouse (Sajan et al., 2011) and zebrafish (Ezhkova et al., 2023) have yielded a number of candidates. Of particular relevance for semicircular canal development is the BMP regulator gene *Bmper*, identified and validated as a direct transcriptional target of DLX5 in the mouse ear (Sajan et al., 2011) (see above). In turn, Bmper is also required for maintenance of *dlx5a* expression in the zebrafish ear, most likely through promotion of BMP signalling (Baxendale et al., 2021). Together, these results highlight the importance of finely tuned WNT-BMP-DLX feedback loops for semicircular canal morphogenesis.

Hmx family genes—orthologues of the *Drosophila* homeobox gene *Hmx*—have conserved patterns of expression in the developing ear, where they are known targets of BMP, FGF and SHH signalling, as described above (Feng & Xu, 2010; Hartwell et al., 2019; Herbrand et al., 1998; Ohta et al., 2016a, 2016b; Rinkwitz-Brandt et al., 1995; W. Wang et al., 1998). Mouse mutants in both *Hmx2* and *Hmx3* have severe semicircular canal defects (Hadrys et al., 1998; W. Wang et al., 1998, 2001, 2004). In the zebrafish, a role for *hmx* genes in semicircular canal development is less clear: although otic anterior patterning is disrupted following their knockdown or mutation, initial steps of semicircular canal morphogenesis appear to proceed normally (Feng & Xu, 2010; Hartwell et al., 2019). However, a double *hmx2;hmx3* mutant has not yet been studied in this species.

LIM-domain (LMX) transcription factors have important roles at several stages of formation of the semicircular canal ducts. Mutations in *Lmx1a* underlie the mouse *dreher* (‘turner’) mutant (Millonig et al., 2000); this and other allelic variants have a complete lack of semicircular canals (Steffes et al., 2012). In the zebrafish, mutations in *lmx1bb* cause semicircular canal and endolymphatic duct abnormalities (Obholzer et al., 2012; Swinburne

et al., 2018). Further analysis in this species has established that Lmx1bb is an upstream regulator of *versican* and genes leading to the production of hyaluronan, placing it upstream of a GRN required for ECM production and epithelial outgrowth (Mori et al., 2025) (Fig. 3; see below). In mouse and chick, LMX1 proteins antagonise Notch signalling to allow the correct segregation of sensory patches, including the cristae (Mann et al., 2017). LMX1a also plays key roles in regulating the formation and extent of the fusion plate, as discussed below.

SOX9 and SOX10 are members of the SOXE family of DNA-binding transcription factors. Consistent with their widespread and conserved expression in the otic vesicle, mutation of *SoxE* family genes affects the development of several different otic tissues, including sensory hair cells, semicircular canals and the endolymphatic duct (Dutton et al., 2009; Szeto et al., 2022). Transcriptional targets of SOX proteins in the mouse ear include the Type II Collagen gene *Col2a1* (Bell et al., 1997) (see also below) and the forkhead family transcription factor gene *Foxg1* (H. Yang et al., 2022). In the zebrafish, *sox10*$^{-/-}$ mutations result in severe and wide-ranging inner ear defects, including disrupted morphogenesis of the epithelial projections and pillars of the semicircular canal system (Dutton et al., 2009). These defects are preceded by dramatic patterning alterations: expanded otic expression domains of *bmp4*, *fgf8* and *pax5*, and a loss of expression of *connexin* genes (Dutton et al., 2009). Other genes down-regulated in the *sox10* zebrafish mutant or morphant ear include the aGPCR gene *adgrg6* (*gpr126*) (Geng et al., 2013) (see below) and the protein disulphide isomerase gene *agr2* (Tang et al., 2014). Both these molecular changes are likely to disrupt production of extracellular matrix, thus contributing to the semicircular canal defects in *sox10*$^{-/-}$ mutants.

An important role for SOXE factors in the developing inner ear is that of fluid regulation. In the mouse, SOX9 and SOX10 co-operate to repress expression of *Aqp3* (*Aquaporin3*) in the stria vascularis of the cochlea (Szeto et al., 2022). Aquaporin3 is a water channel implicated in endolymphatic hydrops and Menière's disease (Nevoux et al., 2015). Variable (and occasionally extreme) ear swelling has been reported in the zebrafish *sox10*$^{-/-}$ mutant (Dutton et al., 2009); although fish ears have no cochlea, and thus no direct equivalent of the stria vascularis, they still require regulation of endolymph production and volume. In the early zebrafish otic vesicle, the semicircular canal pillar epithelium is the site of expression for many of the genes required for endolymph production (Abbas & Whitfield, 2009). It will be interesting to test whether a de-repression of otic *aqp* genes contributes to the ear swelling seen in the zebrafish *sox10*$^{-/-}$ mutant ear.

2.4 Morphogenetic mechanisms in formation of the semicircular canals

2.4.1 Role of the extracellular matrix in semicircular canal morphogenesis

Activity of the various signalling pathways and transcription factors required for semicircular canal development result in new patterns of gene expression and cell behaviour that re-model the otic epithelium. A key consideration here is the role of the extracellular matrix (ECM), which has been studied most intensively in anamniote vertebrates (frogs and fish). In zebrafish, the ECM filling the acellular core of the epithelial projections that initiate semicircular canal formation is a complex hydrated mix of proteins, proteoglycans and glycosaminoglycans (GAGs) (Fig. 3). A major component is the GAG hyaluronan (hyaluronic acid, HA): localised synthesis and retention of this giant space-filling polymer has been proposed as a major contributor to the driving force for epithelial projection outgrowth (Haddon & Lewis, 1991). In support of this idea, mutation or knockdown of genes required

for HA production, or its experimentally-induced degradation, disrupt epithelial projection outgrowth and semicircular canal formation (Busch-Nentwich et al., 2004; Geng et al., 2013; Haddon & Lewis, 1991; Mori et al., 2025; Munjal et al., 2021; Neuhauss et al., 1996; Walsh & Stainier, 2001).

Sulphated proteoglycans, including chondroitin sulphate proteoglycans (CSPGs) and heparan sulphate proteoglycans (HSPGs), are also present in the mix. These molecules have a brush-like structure, with a core protein decorated by numerous unbranched GAG chains; the core binds to HA via link proteins (Haplns) to generate charged supramolecular complexes (reviewed in (Richter et al., 2018; Toole, 2001)). Transcripts coding for proteoglycan core proteins, including Versicans, show strong, specific and transient expression in the epithelial projections of the zebrafish ear during outgrowth, becoming rapidly down-regulated after epithelial fusion (Geng et al., 2013). Several glycosyltransferase genes, coding for enzymes that assemble GAG chains, are expressed in the developing zebrafish ear (Filipek-Górniok et al., 2013; Y. Li et al., 2010). Indeed, the very first indication of the sites of epithelial projection outgrowth—before any overt morphological change—is the appearance of discrete CS-positive foci (Jones et al., 2022). Mutations in *chondroitin synthase 1* (*chsy1*) (Y. Li et al., 2010), *versican* (Mori et al., 2025) and *exostosin 2* (*ext2*) (Jones et al., 2022) all disrupt epithelial projection outgrowth, indicating a requirement for both CSPGs and HSPGs in zebrafish semicircular canal morphogenesis (Fig. 3).

Epithelial projections in the zebrafish ear also express a whole host of genes coding for other ECM components, or proteins involved in ECM synthesis and processing. For example, genes coding for Type II Collagen are strongly expressed throughout the embryonic teleost ear (Matsumoto et al., 2012; Yan et al., 1995) (Fig. 3), and the protein accumulates in the evaginating epithelial projections when epithelial fusion is disrupted (Geng et al., 2013). Otic expression of *col2a1a* is beautifully recapitulated by a zebrafish transgenic GFP reporter driven by *col2a1a* regulatory elements (Dale & Topczewski, 2011). Mutations in the zebrafish gene *cog4*, which codes for a Golgi-localised protein required for ECM protein processing, result in reduced proteoglycan and Type II Collagen expression within the projections, and delayed projection outgrowth (Clément et al., 2019). Pharmacological disruption of Type II Collagen expression also correlates with epithelial fusion defects in the zebrafish (Cintrón-Rivera et al., 2023).

Surprisingly, given their rapid growth, cell divisions are very rare within the zebrafish epithelial projections during outgrowth (Munjal et al., 2021; Waterman & Bell, 1984). Instead, growth is thought to be driven by synthesis and hydrostatic swelling of the ECM core of the projection, and collective cell movement of the epithelial sheet (Haddon & Lewis, 1991; Mori et al., 2025; Munjal et al., 2021). In the mouse, *Fgf9*- and *Ntn1*-dependent cell proliferation in the surrounding mesenchyme is thought to push the epithelial sides of the pouches together (Pirvola et al., 2004; Salminen et al., 2000). A role for the ECM in remodelling of the semicircular canal pouches in the amniote ear has not been addressed to the same extent as in the fish, but it is likely to have similar contributions. *Col2a1*, for example, is a transcriptional target of SOX9 in the mouse ear (Bell et al., 1997) (see above), while Netrin1 (see below) is a laminin-like ECM protein. In further support of a role for the ECM in the mouse, some histological sections reveal an acellular space beneath the fusion plate, likely to be ECM-filled (Fig. 2E; see, for example, (Pirvola et al., 2004)).

2.4.2 Mechanisms of cell adhesion and cell clearance at the fusion plate

One of the most intriguing events in the developing ear is the formation and resolution of the fusion plate. As soon as it has formed, this structure is rapidly remodelled through cell death, perforation or resorption, leaving behind and sealing off a tubular rim of tissue, which forms the nascent canal duct. All three canals are formed in this way, across all jawed vertebrates. Expression of several key genes is conserved, yet surprising differences in mechanism are being uncovered, both between species and between the individual semicircular canals within species. Clearly, the embryo has found more than one way to solve the same morphogenetic problem.

A fusion plate is generated when opposing layers of the otic epithelium meet and adhere at their apical surfaces, forming a transient bilayer. In zebrafish, formation of the fusion plates for all three canals is critically dependent on the aGPCR Adgrg6 (also known as Gpr126): in homozygous *adgrg6*$^{-/-}$ mutants, fusion fails altogether (Diamantopoulou et al., 2019; Geng et al., 2013). Transcripts for *adgrg6* are expressed on both sides of the epithelial tissue (projection and bulge) that meet at the fusion plate, suggestive of a homotypic adhesion event. However, fusion can be partially rescued in hypomorphic *adgrg6*$^{-/-}$ mutants by the application of compounds that increase intracellular cyclic AMP (cAMP) levels, bypassing receptor signalling, indicating that Adgrg6 may not be the (or not the only) adhesion factor involved (Geng et al., 2013). In addition to its role in fusion, signalling through Adgrg6 is required for the transcriptional down-regulation of numerous ECM genes expressed in the outgrowing epithelial projections (Geng et al., 2013). Overall, therefore, Adgrg6 signalling appears to mediate the switch in cell behaviour from an outgrowth-like state to one of adhesion at the zebrafish fusion plate (Fig. 3). In mice, *Adgrg6* (*Gpr126*) is expressed in the otocyst (Patra et al., 2013), but an otic phenotype in mouse *Adgrg6* mutants has not yet been described.

Following its formation, cells are rapidly cleared from the fusion plate, although the mechanism of clearance differs between species. In the frog and chick, cells are removed through apoptosis (Bever & Fekete, 1999; Fekete et al., 1997; Haddon & Lewis, 1991; Lang et al., 2000). In the mouse, cell death is not thought to be so important; instead, cells are thought to intercalate to allow perforation of the plate (Martin & Swanson, 1993). However, mutation of *Apaf1*, leading to a lack of apoptosis in the otic epithelium, does disrupt semicircular canal formation in the mouse, particularly the anterior (superior) canal (Cecconi et al., 2004). In the zebrafish, the tiny fusion plate may consist of fewer than 10 cells, and cell death does not appear to be a major player in this species (Bever & Fekete, 1999; Waterman & Bell, 1984).

Whether or not they are destined to die, cells at the fusion plate lose some of their epithelial characteristics. Histological studies show that fusing cells in both mouse and zebrafish lack a basal lamina (Martin & Swanson, 1993; Waterman & Bell, 1984); in the chick, the basal lamina becomes disrupted at the fusion plate, but does not disappear entirely (Fekete et al., 1997). The exact sequence and timing of events leading to loss of the basal lamina are still obscure. Several studies describe a detachment of the epithelium from the underlying basal lamina prior to its breakdown (Hurd et al., 2012; Matilainen et al., 2007; Salminen et al., 2000; W. Wang et al., 2001). In the mouse, Netrin1 is required (Salminen et al., 2000)—but possibly not sufficient (C. H. Hwang et al., 2019)—for local disruption of the basal lamina (see discussion below). In the chick, the onset of apoptosis precedes breakdown of the basal lamina (Nishitani et al., 2017). Presumably, at some later developmental stage, a basement membrane is re-established beneath the remodelled epithelium forming the inner

walls of the canal ducts, although little is known about the timing of this process. In addition, the space generated by clearance of the fusion plate is invaded by mesenchyme; in the chick, this happens rapidly, within a day of the fusion event (Nishitani et al., 2017).

2.4.3 Cross-repressive gene networks acting at the fusion plate

Clearance of cells at the fusion plate must be tightly regulated: in the mouse, if too many cells are cleared, the pouch rim is depleted, and the semicircular canals are truncated, thin or absent; conversely, if fusion fails, the rim fails to be isolated and a canal duct cannot form. These observations suggest that a balance of both positive and negative regulators is required: this is borne out by the analysis of single and double mutant phenotypes and mis-expression experiments. These elegant approaches have identified several cross-repressive gene regulatory networks operating at the fusion plate, controlling both the timing and extent of epithelial fusion during semicircular canal morphogenesis (Fig. 4).

The Netrins, secreted molecules related to the Laminins, were originally identified as key molecules required for semicircular canal formation over twenty years ago, with a pro-fusogenic role in the mouse embryo. *Netrin1* (*Ntn1*) is normally expressed in the fusion plate (resorption domain) of the canal pouch, and is repressed in the pouch rim by DLX5, in response to both BMP and WNT signalling (C. H. Hwang et al., 2019; Rakowiecki & Epstein, 2013). In *Ntn1* loss-of-function mouse mutants, the basement membrane fails to break down, and epithelial fusion is blocked or delayed: the posterior and lateral (horizontal) canals fail to form altogether (Salminen et al., 2000) (Fig. 4B). Likewise, forced WNT/β-catenin signalling results in an expansion of *Dlx5* expression and a repression of *Ntn1*, blocking fusion plate formation (Rakowiecki & Epstein, 2013). An opposite phenotype is found in mutants for *Lrig* genes, which code for single-pass transmembrane proteins (Abraira et al., 2008; del Rio et al., 2013). In *Lrig3*$^{-/-}$ mutants, *Ntn1* expression is expanded in the lateral pouch, correlating with precocious breakdown of the basement membrane, ectopic epithelial fusion, and truncation of the lateral canal (Abraira et al., 2008) (Fig. 4B). Mutual antagonism between *Lrig3* and *Ntn1* was confirmed by rescue experiments in double mutant combinations, where lowering the dose of one factor could restore normal canal formation and vestibular behaviour in a homozygous mutant background for the other (Abraira et al., 2008).

An antagonistic relationship between *Ntn1* and *Bmp2* has also been uncovered in the mouse ear (C. H. Hwang et al., 2019). Here, inner-ear-specific conditional knockout of *Bmp2* results in an expansion of *Ntn1* expression into the pouch rim zone, reducing *Dlx5* and *Lmo4* expression and disrupting canal formation. Interestingly, however, there was no premature breakdown of the basal lamina in these mutants, as assessed by anti-Laminin staining (C. H. Hwang et al., 2019). Again, reciprocal inhibition between *Ntn1* and *Bmp2* was confirmed by double mutant combinations, showing that the phenotype in homozgyous *Ntn1* mutants can be partially rescued in a heterozygous background for *Bmp2*, and vice versa (C. H. Hwang et al., 2019).

Two *Netrin* genes are expressed at the chick fusion plate (Abraira et al., 2010), with *cNTN2* expressed prior to, during, and after fusion. *cNTN1* expression marks the fusion plate itself, but *cNTN2* expression extends beyond this, and remains expressed in the inner walls of the nascent semicircular canal duct (Nishitani et al., 2017). Despite these largely conserved expression patterns, over-expression experiments suggest that the exact role of Netrins at the fusion plate differs between chick and mouse (Nishitani et al., 2017). In the chick, over-expression of cNTN1-MYC disrupted canal formation by blocking or delaying apoptosis,

basement membrane breakdown, *DLX5* down-regulation, and epithelial fusion, with no effect on *LRIG3* expression. By contrast, similar over-expression of the chick gene in the mouse embryo generated partially penetrant and variably expressive phenotypes suggestive of ectopic fusion (Nishitani et al., 2017). Thus, while Netrin is pro-fusogenic in the mouse ear (based on both loss- and gain-of-function approaches), over-expression experiments suggest an anti-fusogenic role in the chick.

A further important cross-repressive interaction at the fusion plate is that between the transcription factor genes *Lmx1a* (see above) and *Lmo4*. Expression domains of these two genes in the semicircular canal pouches of the developing mouse ear are largely mutually exclusive, with *Lmo4* most strongly expressed in the pouch rim (prospective canal) and crista, and *Lmx1a* most strongly in the prospective fusion plate (M. Huang et al., 2008; Y. Huang et al., 2018) (Fig. 4A). Their functions have been explored in an allelic series of systemic mutants (Chizhikov et al., 2021; Koo et al., 2009; Nichols et al., 2008; Steffes et al., 2012) and in inner-ear-specific conditional knockouts (M. Deng et al., 2010; Y. Huang et al., 2018). Mutations in either gene result in a loss of all three canals. Variable rescue of the anterior canal in double mutant combinations suggests that transcriptional repression of *Lmo4* by LMX1a is important for resorption of the dorsal canal pouch and thus for formation of the anterior and posterior canals, whereas repression of *Lmx1a* by LMO4 is important for formation of the anterior canal rim and the three cristae (Y. Huang et al., 2018).

Taken together, the available data—at least in the mouse—suggest that mutually exclusive expression domains and antagonistic interactions between *Ntn1, Lrig3, Dlx5, Lmx1a* and *Lmo4*, regulated by BMP and WNT signalling, pattern the semicircular canal pouches and the nascent canal ducts (Fig. 4C). These key players and others distinguish the fusion plate resorption zone from the protected outer rim of the pouch. Within the fusion plate, changes in cell behaviour lead to cell detachment from and/or destruction of the basement membrane, cell thinning, and cell clearance. However, although required, *Ntn1* is not sufficient to direct cell clearance: its expression in the mouse persists in the inner wall of the nascent canal duct, indicating the presence of an intermediate zone between the fusion plate and pouch rim (Fig. 4A). It is also not yet clear whether NTN1 and LRIG3 act in a receptor-dependent or receptor-independent manner at the fusion plate; their receptors in this context have not yet been identified, despite consideration of several candidates (Abraira et al., 2010; Matilainen et al., 2007). In addition, the roles of other downstream effector molecules, such as enzymes involved in breakdown of the basal lamina, are yet to be explored.

2.5 Development of ampullae and cristae

At the base of each canal duct is the ampulla, a rounded chamber housing a thickened ridge of sensory tissue, known as the crista, which runs across the ampullary floor (Desai et al., 2005) (Fig. 5). Crista sensory hair cells project their stereocilia and kinocilia into the cupula, a sheet of extracellular matrix that can be deflected by relative movements of the ampulla and endolymph (Goodyear & Richardson, 2002). Some crista sensory hair cells have spectacularly long stereocilia and kinocilia in both zebrafish and mice (Forge et al., 2017; Stawicki et al., 2014). A non-sensory region, known as the cruciate eminence (mouse) or septum cruciatum (chick), bisects the sensory epithelium of the anterior and posterior cristae (Desai et al., 2005) (Fig. 5B,D). Hair cells in any one crista are all oriented in the same direction (Y. Wang et al., 2006); together, the arrangement of canals and cristae allows

sensitivity to turning movements (angular accelerations) of the head in three-dimensional space.

2.5.1 Morphogenesis of ampullae

Little is known about how the rounded ampullae are shaped during development. Although mutations in some genes, including *Lmo4*, result in a loss of both canal ducts and ampullae (M. Deng et al., 2010), mutations in others result in selective canal duct truncations, leaving ampullae intact (Abraira et al., 2008; Baxendale et al., 2021; Hammond et al., 2009; C. H. Hwang et al., 2019; Pauley et al., 2003). Conversely, mutations in the Notch pathway gene *Jag1*, or some alleles of *Sox2*, can result in the selective loss of ampullae, with canals relatively unaffected (Kiernan et al., 2001, 2005). Ampullae appear flattened in the mouse mutant for *Nr4a3* (formerly *Nor-1*), which codes for a transcription factor in the nuclear receptor class; the ampullary phenotype in the mutant is attributed to reduced cell proliferation (Ponnio et al., 2002).

Single-cell RNA sequencing of dissected ampullae from mouse pups at different developmental stages (Wilkerson et al., 2021) has revealed distinct spatial patterning of the non-sensory ampullary walls, corroborating earlier histological observations. A new finding is the presence of a 'rooftop' domain expressing *Wnt3, Crtac1* and *Sfrp5*, with a *Wnt3*-expressing stripe of small epithelial cells extending along the inner wall of the canal duct (Wilkerson et al., 2021) (Figs. 4A,5B). This domain also expresses *Nr4a3* (Ponnio et al., 2002), and is likely to relate to the earlier events of cell clearance at the fusion plate. In this context, it is interesting to note that *Wnt* genes, most notably *Wnt1* and *Wnt3A*, are expressed in the neural tube roof plate (Parr et al., 1993; Roelink & Nüsse, 1991), another tissue formed through epithelial fusion, where they play important roles in epithelial morphogenesis (Ventriglia & Kalcheim (2024) and references within). Shaping of the ampullae, including possible parallels between the rooftop domain and the neural tube roof plate, therefore remains an interesting area to explore further.

2.5.2 Specification of cristae and crista sensory hair cell types

BMP signalling is a key player in the development of all three cristae. Expression of *bmp4* in the developing cristae is conserved across mouse, chick and zebrafish (Morsli et al., 1998; Mowbray et al., 2001; D. K. Wu & Oh, 1996). In zebrafish, cristae stain for an antibody to P-Smad1/5/9 and express a transgenic BMP reporter, both indicating sites of active BMP signalling (Baxendale et al., 2021; Feng et al., 2024). In the mouse, BMP4 signalling promotes *Lmo4* expression throughout the crista (in both sensory and non-sensory regions) (W. Chang et al., 2008), where, as discussed above, it represses expression of *Lmx1a* (Y. Huang et al., 2018). By contrast, additional BMP target genes have more specific expression patterns, distinguishing the non-sensory zone (cruciate eminence), or marking sensory hair cells and supporting cells within the sensory zone (W. Chang et al., 2008).

Notch and FGF signalling also play a role in crista development, but here mutations in pathway genes sometimes affect just one or two of the developing cristae. For example, zebrafish mutations in *jag1b*, which codes for a Notch ligand, result in loss of the anterior and posterior cristae, but not the lateral crista (W. R. Ma & Zhang, 2015). Loss of the posterior crista in *jag1b*$^{-/-}$ mutants has been attributed to cell death and a loss of *fgf10a* expression, whereas loss of the anterior crista was attributed to an expansion of mitogen-activated protein kinase (MAPK) activation and ectopic cell flattening (W. R. Ma & Zhang, 2015). As discussed above, FGF signalling from the cristae is proposed to establish a 'canal genesis zone' in the chick otocyst (W. Chang et al., 2004).

Crista sensory hair cells can be subdivided into two main types, originally distinguished by morphology and synaptic connections (Desai et al., 2005). Type I and type II hair cells are present throughout the mammalian sensory epithelium (Lysakowski & Goldberg, 1997), in both central and peripheral regions, making four potential hair cell sub-types. However, recent transcriptomic profiling identifies two main groups of crista hair cells: *Oncomodulin* (*Ocm*)-positive striolar-like type I cells and *Annexin a4* (*Anxa4*)-positive extrastriolar-like type II cells, which largely correspond to central and peripheral hair cells, respectively, in the fish crista (Shi et al., 2023; Wilkerson et al., 2021). Expression of candidate genes of interest has been validated by in situ hybridisation, including the use of hybridisation chain reaction (HCR), a multiplex fluorescence technique. For example, expression of the calcium-binding protein gene *cabp5b* marks zebrafish crista hair cells with exquisite specificity (Fig. 5F), with expression of *cabp2b* and *cabp1b* distinguishing central and peripheral hair cells, respectively (Shi et al., 2023). Transcripts for *parvalbumin9* (*pvalb9*), the zebrafish orthologue of *Ocm*, are expressed in a subset of crista hair cells (Shi et al., 2023). Central, but not peripheral, hair cells express the voltage-gated sodium channel gene *scn5lab*, suggesting functional specialisation between different zebrafish crista hair cell types (Shi et al., 2023).

Supporting cells of the crista also show differences in gene expression between central and peripheral zones. In the mouse, central supporting cells are marked by the expression of *Cyp26b1*, whereas peripheral cells (including transitional epithelial cells) are marked by expression of *Aldh1a3* (Ono, Keller, et al., 2020; Wilkerson et al., 2021), a pattern that is conserved in the zebrafish (Pittlik et al., 2008). These genes code for enzymes important for degradation and production, respectively, of retinoic acid (RA): analysis of conditional mutant phenotypes confirms a requirement for the regulation of RA signalling in patterning the striolar/central zone of vestibular organs in the mouse, including the cristae (Ono, Keller, et al., 2020; Ono, Sandell, et al., 2020).

Developmental trajectory analysis, comparing single-cell transcriptomic datasets from different developmental stages in the mouse, hints at the differentiation of crista hair cells from immature supporting cells in the postnatal crista (Wilkerson et al., 2021). Similarly, in the zebrafish ear, damaged crista hair cells are gradually replenished by transdifferentation of supporting cells, with striking parallels to mechanisms of growth and regeneration in the mammalian vestibular system (Beaulieu et al., 2024) (see also (Jimenez et al., 2022)). These findings establish the zebrafish ear as a valuable model for studies of hair cell regeneration.

2.5.3 Development and composition of the cupula

The cupula is a specialised extracellular matrix that sits over the crista, forming a structure spanning the ampullary lumen, where it is deflected by movement of the canal duct relative to the inertial endolymph (Fig. 5A,B). The stereocilia and kinocilia of crista sensory hair cells are embedded within the cupula and therefore respond directly to its deflection. Although the cupula shares some components, such as Otogelin, with other specialised ECM structures in the ear (for example, the tectorial and otolithic membranes), it also has unique characteristics; the existence of a cupula-specific ECM component, ‘Cupulin’, was proposed in 2002 (Goodyear & Richardson, 2002). This protein has now been discovered in salmon, where immunostaining shows it strongly localised to the cupula (Dernedde et al., 2014); mRNA expression has also been documented in supporting cells of the crista in the three-day-old zebrafish embryo (Shi et al., 2023; C. H. Yang et al., 2011) (Fig. 5E,F) and in newborn mice (Vijayakumar et al., 2019). The Cupulin protein contains domains

641 characteristic of ECM proteins found in the zona pellucida, the clear jelly-like coat that
642 surrounds mammalian egg cells; its alternative name is ZPLD1 (zona pellucida-like domain-
643 containing protein 1).

644 There is still much to learn about the development of the semicircular canal system and its
645 constituent cell types. I have not had space to discuss the innervation of crista hair cells, or
646 the interactions between epithelium and mesenchyme that give rise to the protective casing
647 surrounding the delicate tissues of the membranous labyrinth. The next section of the
648 review deals with formation of the otoliths and otoconia; a final section returns to the
649 semicircular canal system to consider it in the context of the development of vestibular-
650 driven behaviours, evolution and disease.

3 Development of otoconia and otoliths

3.1 Form and function of otoconia and otoliths

Both amniotes and fishes sense gravity and linear acceleration through sensory hair cells that are stimulated by the movement or inertia of dense biomineralised structures. In fish, these are the otoliths ('ear stones'), single macroscopic entities that sit above sensory hair cells in the vestibular sensory patches or maculae. In mammals, collections of multiple small crystals, known as otoconia, perform a similar function (Figs. 1,6). The otoliths or otoconia are physically coupled to their cognate sensory patches by the otolithic membrane, a thick layered extracellular matrix consisting of both fibrous and gelatinous components (reviewed in (Schulz-Mirbach et al., 2019)). Together, the otolith, otolithic membrane, sensory epithelium and innervating axons form a functional unit, with the planar-polarised orientation patterns of the sensory hair cells giving each otolithic organ a particular range of directional sensitivity. In fishes, otoliths not only form a dense inertial mass for the detection of gravity and linear acceleration (Riley & Moorman, 2000), but are also critical for the reception of sound (reviewed in (Baeza-Loya & Raible, 2023; Schulz-Mirbach et al., 2019)). In otophysan (hearing specialist) fish, including the zebrafish, auditory sensitivity is enhanced by the Weberian ossicles, a series of accessory bones that link the swim bladder to the inner ear (Grande & Young (2004) and references within).

Fish otoliths have a huge diversity of form, both within an individual and between species. The three main classes of otolith are the rounded lapillus ('pebble'), slender sagitta ('arrow'), and elaborately crenellated asteriscus ('star'), situated in the utricle, saccule and lagena, respectively. Otoliths may also display species-specific features, including spurs (X. Deng et al., 2013). The composition, evolution and diversity of fish otoliths have been covered extensively elsewhere (Lundberg et al., 2015; Schulz-Mirbach et al., 2019). Here, I have selected studies that give insight into developmental genetic mechanisms of otolith formation, including the role of cilia in otolith formation in zebrafish, developmental requirements for the formation of different crystal polymorphs, and the importance of pH for otolith biomineralisation.

3.2 Developmental requirements for otolith formation

3.2.1 Generation of otolith precursor particles

Several gene products are required for the earliest steps of otolith formation in teleost fish, with mutations resulting in an 'empty ear' phenotype, lacking otoliths altogether (Fig. 6B,M). For example, normal formation or nucleation of otolith precursor particles is critically dependent on function of the enzyme Polyketide Synthase in zebrafish and medaka (Hojo et al., 2015; Lee et al., 2019; Thiessen et al., 2019). Although the polyketide products of this enzyme have yet to be identified, it has been proposed that they would be secreted into the lumen of the otic vesicle, where they would nucleate organic and inorganic components of the otolith (Hojo et al., 2015). Maternal-zygotic zebrafish mutants in the gene *mat1a*, coding for the serine protease Matriptase1a, have a very similar phenotype to those of *polyketide synthase 1* (*pks1*) mutants, with a swollen otic vesicle that lacks otoliths completely (J. Ma et al., 2021). Although a possible link between *mat1a* and *pks1* has yet to be explored, overexpression and rescue experiments indicate that the serine protease inhibitor Hai1 preserves epithelial integrity by antagonising Matriptase activity (Carney et al., 2007). Unsurprisingly, mutations in other genes that disrupt epithelial integrity also perturb the early stages of otolith formation in the zebrafish. These include the transcription factor gene *grhl2b* (Han et al., 2011), the epithelial cell adhesion molecule gene *epcam* (Slanchev et al.,

2009), and the claudin gene *cldnj* (Hardison et al., 2005). Grhl2b regulates expression of another claudin, *cldnb*, together with *epcam*, in the early otic vesicle (Han et al., 2011). Ionic composition and pH of the endolymph (see below) are also important for otolith formation (Ito et al., 2024; Jędrychowska et al., 2024).

3.2.2 The role of cilia in otolith formation

In the zebrafish embryo, the development of otoliths is intimately linked with the presence of cilia. Immunostaining for acetylated alpha-Tubulin reveals that the entire lumen of the zebrafish otic vesicle—like that of many epithelia—is ciliated (Riley et al., 1997). Different types of cilia are present in different locations in the otic epithelium: short primary cilia, present on most cells; long immotile kinocilia present on pairs of sensory hair cells at the anterior and posterior poles of the vesicle; and single motile cilia, found on a small number of cells surrounding the sensory hair cells (Stooke-Vaughan et al., 2012). Free-floating otolith precursor particles, initially distributed throughout the nascent otic vesicle lumen, are locally swept along by the vortical flow generated by motile cilia at the poles (Riley et al., 1997; D. Wu et al., 2011), and soon become firmly attached to the tips of the hair cell kinocilia, in a process known as otolith seeding or tethering. Additional particles coalesce, forming the otolith, a single structure associated with each pair of hair cells, which then becomes biomineralised.

Mutation or morpholino-mediated knockdown of any of the multitude of genes required for formation or motility of cilia in zebrafish results in mild (and sometimes temporary) defects in otolith number or formation (reviewed in (Whitfield, 2020); see also (Jin et al., 2022; Ka et al., 2023; Kuang et al., 2022; Leventea et al., 2021; Ott et al., 2023)) (Fig. 6D). For this reason, defects in otolith formation have become one of a standard checklist of indicators for ciliary dysfunction in the zebrafish embryo, alongside whole-body curvature, kidney cysts and left-right axial patterning defects.

Given the close association of otolith development with cilia, it is perhaps surprising to learn that zebrafish otoliths can still form and biomineralise even when cilia are missing. In the zebrafish maternal-zygotic *ift88*$^{-/-}$ mutant, which lacks cilia altogether, otolith precursor particles adhere directly to the apical surface of sensory hair cells, where they become biomineralised (Stooke-Vaughan et al., 2012). The otoliths do not form entirely normally in the absence of cilia or ciliary movement, however: not only do hair cell kinocilia provide the normal site for otolith nucleation, but ciliary motility helps to prevent the formation of supernumerary otoliths (Stooke-Vaughan et al., 2012).

The large glycoprotein Otogelin appears to be one of the factors responsible for the tethering of otolith precursor particles to the tips of hair cell kinocilia in the developing zebrafish ear. Otogelin is a component of the otolithic membrane, and antibody staining indicates that it may become concentrated near the kinociliary tips in wild-type zebrafish embryos (Thiessen et al., 2019), although this needs further validation. Otolith seeding on kinocilia is disrupted in the *otogelin* mutant, and formation of the utricular otolith is delayed (Bagnall & McLean, 2014; Goldblatt et al., 2023; Mo et al., 2010; Roberts et al., 2017; Stooke-Vaughan et al., 2015; Whitfield et al., 1996) (Fig. 6C; see also the section on behaviour below). In mammals, Otogelin is a constituent of all acellular membranes in the developing ear (Cohen-Salmon et al., 1997; El-Amraoui et al., 2001), and is essential for attachment of both the otoconial membrane and otoconia (Simmler et al., 2000).

3.2.3 Regulation of crystal growth by otolith matrix proteins

Fish otoliths are largely (>90%) composed of crystalline calcium carbonate, accounting for their stone-like structure. This inorganic material is deposited on an organic scaffold, making up less than 10% of the otolith mass, but with many different protein, glycoprotein, proteoglycan and polysaccharide components (Lundberg et al., 2015; Schulz-Mirbach et al., 2019) (Fig. 6). Correct selection of the crystalline calcium carbonate growth form or polymorph (aragonite, calcite or vaterite) in each otolith is controlled by several of the organic components, and the lack of individual proteins can result in dramatic alterations to otolith shape. The abnormal morphology of otoliths in the absence of particular proteins thus reveals how proteins can constrain the way in which inorganic crystals grow.

A striking example is the requirement for Starmaker, a secreted protein with no direct mammalian counterpart, in fish otolith growth. Morpholino-mediated knockdown of *starmaker* in zebrafish disrupts growth of the aragonitic polymorph of calcium carbonate, with some otoliths developing a star-like morphology, and the most extreme examples containing crystals of calcite (Söllner et al., 2003) (Fig. 6E,F). This initial observation has since triggered a whole series of biochemical and biophysical studies that are giving further insights into the mechanisms involved, with interesting implications for the design of biologically-inspired materials. Starmaker and Starmaker-like proteins are now understood to be intrinsically disordered acidic proteins that bind calcium ions (Kapłon et al., 2008; Różycka et al., 2014) (Fig. 6G,H). Additional studies in vitro have demonstrated the importance of phosphorylation of Starmaker, essential for its calcium-binding activity and ability to control growth of the crystal lattice (Wojtas et al., 2012, 2015). Phosphorylation of zebrafish Starmaker and carp Starmaker-like proteins has also been shown to be important for their function in vivo, for formation of both the aragonitic lapillus and the vateritic asteriscus (Kalka et al., 2019, 2024). Starmaker-like genes are also expressed in the ears of medaka (Bajoghli et al., 2009), a teleost fish distantly related to zebrafish and carp. Starmaker is not the only calcium-binding protein present in otoliths: other intrinsically disordered acidic proteins, for example Otolith Matrix Macromolecule-64 (OMM-64), regulate otolith growth in a similar way (Poznar et al., 2017, 2020).

3.2.4 The role of pH in otolith and otoconial biomineralisation

Just as acid rainwater dissolves limestone, so the generation and maintenance of biomineralised structures in living organisms is at risk if the pH is too low. This means that the milieu in which otoliths form must be kept sufficiently alkaline for the formation and maintenance of crystalline calcium carbonate. Otolith biomineralisation therefore requires a system to regulate pH and remove protons from the calcification front. It was thus an exciting (but nevertheless unsurprising) discovery that one of the key conserved proteins required for both otolith and otoconial formation, Otopetrin1 (Otop1, the ‘ear-stone protein’), functions as a proton-selective transmembrane ion channel to regulate cytoplasmic and extracellular pH (Tu et al., 2018).

Otop1 is conserved across a wide range of invertebrate and vertebrate organisms (Hurle et al., 2011; Tu et al., 2018), and is required both for the formation of otoliths in zebrafish (Hughes et al., 2004; Söllner et al., 2004) and otoconia in mammals (Hurle et al., 2003; Ornitz et al., 1998) (Fig. 6I–M). Although otoconia are usually missing altogether in the mouse *Otop1* (*tilted*) mutant ear, giant otoconia occasionally form (Ornitz et al., 1998). Elucidation of the structure of zebrafish Otop1 in lipid nanodiscs using cryogenic electron microscopy (cryoEM) indicates that the protein forms a homodimer, with three alternative models for the transmembrane conduction of protons (Saotome et al., 2019). In an

interesting cross-over with other senses, mammalian Otopetrin1 is also expressed in Type III taste receptor cells in the tongue, where it is thought to contribute to the detection of acid (sour taste) (Tu et al., 2018) and ammonium (Liang et al., 2023), both strong and normally aversive gustatory stimulants.

The importance of pH regulation for otolith formation is also underlined by the phenotype of zebrafish embryos in which embryo-wide acid-base homeostasis is disrupted. These include the *glial cells missing 2* (*gcm2*) mutant (Stawicki et al., 2014), and mutants or morphants for *atp6v1b1* (Ikeuchi et al., 2024): loss-of-function of either gene results in a systemic extracellular acidosis, and reduced or absent otoliths in the ear. The *gcm2* gene is required for specification of H^+-ATPase-rich (HR) ionocytes, which regulate whole-body pH via acid secretion (W. J. Chang et al., 2009; P. P. Hwang & Chou, 2013) (and references within). The *atp6v1b1* gene codes for the B1 subunit of a vacuolar-type ATP-dependent proton pump (V-ATPase), which is expressed in HR ionocytes and has a direct role in H^+ transport; mutations in the human *ATP6V1B1* gene result in distal renal tubular acidosis and sensorineural hearing loss (Karet et al., 1999). For the zebrafish mutants, it is not clear whether embryo acidification prevents otolith biomineralisation directly, or if there is also an indirect effect on other cellular processes, for example the regulation of calcium ions; see discussion in (Ikeuchi et al., 2024; Stawicki et al., 2014).

Unexpected parallels between evolutionarily distant organisms demonstrate just how fundamental the conserved molecular mechanisms of biomineralisation are. Orthologues of both *polyketide synthase* and *otopetrin*, for example, are also essential for biomineralisation in echinoderms. In the sea urchin pluteus larva, these genes are expressed exclusively in the skeletogenic primary mesenchymal cells that generate the exquisitely patterned calcitic larval skeleton (Beeble & Calestani, 2012; W. W. Chang et al., 2021; Hojo et al., 2015). Here, Otopetrin channels allow the removal of protons—generated by the intracellular chemistry of calcification—from the cytoplasm, making it more alkaline (W. W. Chang et al., 2021).

4 Future directions and applications

A greater understanding of the development of semicircular canals and otoliths has the potential for impact in surprisingly diverse areas. From many interesting studies, I have selected four broad areas where developmental studies are synergising with other disciplines to reveal new insights and understanding: cell and tissue mechanics, behaviour, evolution and human disease.

4.1 Cell and tissue mechanics of the developing inner ear

The burgeoning discipline of mechanobiology—where developmental biology meets soft-matter physics—is leading to studies of an increasingly interdisciplinary nature. These are making exciting progress in linking molecular and cellular developmental mechanisms to the mechanical forces, such as compression, tension and pressure, which together contribute to shaping the developing inner ear.

4.1.1 Physical properties of the extracellular matrix

The physical properties of the ECM core of the finger-like epithelial projections in the fish ear are still only partially understood. Waterman and Bell (Waterman & Bell, 1984) cite early work by Noorden, who noted that the epithelial projections in the fish ear ‘resisted pinching’, perhaps suggesting that the ECM within behaves like a solid gel. By contrast, Munjal and colleagues have modelled the ECM in the zebrafish ear as a viscoelastic fluid constrained by

tension in the overlying epithelium (Munjal et al., 2021). Filopodial extensions, termed cytocinches, form transient tensioned links between cells of the epithelial projections (Munjal et al., 2021) (Fig. 2B). Clearly, interplay between the mechanics of both the matrix and overlying tissue is important. Hapln-mediated HA-proteoglycan cross-linking has been proposed as a mechanism leading to local hydration and swelling of the matrix (Jones et al., 2022); here, there are interesting parallels with heart development (Derrick et al., 2021). Mori and colleagues have tested the contribution of the CSPG Versican to this process, concluding that the Versican protein core regulates HA density, and that charged CS groups enable hydration and swelling of the matrix (Mori et al., 2025). It will also be interesting to test, for example, whether there is any directional alignment of HA and fibrillar proteins such as collagens within the growing epithelial projections (Fig. 3), as has been suggested for the fish ampullary cupula (Silver et al., 1998).

4.1.2 Epithelial mechanics at the fusion plate: parallels with other fusion events

Beyond a loss of some epithelial characteristics, breakdown of the basement membrane, and the variable role of apoptosis, little is known about the cell behaviours at the fusion plate. By definition (unless they die), cells must drastically rearrange their apicobasal polarity and junctional contacts in order to perforate the plate and create the inner walls of the canal ducts. This merits further investigation: the importance of adherens junctions and cell packing in morphogenesis is apparent in many developing systems (reviewed in (Campàs et al., 2024)). It will also be important to explore parallels with similar morphogenetic rearrangements in other developing tissues, including the neural tube, palate, heart and eye. Some commonalities are already evident: LMX1a, for example, is critically required for formation of the mammalian roof plate, generated on fusion of the neural folds to form the neural tube (Millonig et al., 2000; Mishima et al., 2009), and Netrin1 has a conserved pro-fusogenic role in optic fissure closure in the vertebrate eye (Hardy et al., 2019; Richardson et al., 2019). Other studies are providing new candidates to test in the context of ear development. For example, recent work has established a role for the Rab11a recycling endosome pathway in epithelial fusion and perforation in the developing trachea and oesophagus, where it is required for the trafficking of planar cell polarity complexes, highlighting the dramatic reorganisation of the cell membrane that is required for these events (Edwards et al., 2024).

4.2 Integration of developmental understanding with studies of animal behaviour

Rapid progress is being made in the study of the onset and maturation of vestibular-driven reflexes, linking an understanding of development and morphology to neuronal circuitry and behaviour. This is especially true in the zebrafish model, where ingenious experimental approaches have been employed, including the use of tiltable microscopes for inner-ear or whole-brain calcium imaging of live fish in response to vestibular stimulation (Goldblatt et al., 2023; Hamling et al., 2022; Migault et al., 2018, 2024; Tanimoto et al., 2022), the use of magnetised otoliths to evoke vestibular behaviours in response to magnetic stimulation (Beiza-Canelo et al., 2023), and three-dimensional reconstructions of axonal trajectories from serial-section electron micrographs (Hildebrand et al., 2017; Liu et al., 2022). These approaches have been used, for example, to establish the developmental principles and organisational logic of the vestibulo-ocular reflex (VOR) and escape reflex circuits (Goldblatt et al., 2023; Liu et al., 2022). Neurophysiology of the zebrafish vestibular system has been covered comprehensively elsewhere (reviewed in (Baeza-Loya & Raible, 2023; Liu & Bagnall, 2023)); selected highlights relevant to developmental studies are presented here.

4.2.1 Onset of vestibular and auditory function in zebrafish

In zebrafish, the otolithic organs begin to function early, as demonstrated by the loss of balance and self-righting behaviour in embryos lacking otoliths. For example, *otogelin* mutants, which initially lack the gravity-sensing utricular otolith (Fig. 6C; see above), show a loss of various vestibular-driven reflex behaviours in the four- and five-day-old embryo (Bagnall & McLean, 2014; Goldblatt et al., 2023; Mo et al., 2010; Stooke-Vaughan et al., 2015). Moreover, fictive stimulation of the larval vestibular system (effected through movement of the otolith using optical traps) can elicit compensatory muscular responses (Favre-Bulle et al., 2017). Zebrafish *otogelin* mutants have proved ideal tools to explore the development of neuronal circuitry in the absence of sensory input (Bagnall & McLean, 2014; Leary et al., 2025; Roberts et al., 2017). Delayed formation of the utricular otolith in some *otogelin* mutant alleles, followed by the rapid restoration of reflex responses once the otolith appears, has demonstrated that vestibulospinal and vestibuloocular circuits—including the assembly of neuromuscular junctions—can develop unexpectedly robustly, despite early sensory deprivation (Leary et al., 2025; Roberts et al., 2017). Zebrafish *otogelin* mutants have also been used to demonstrate that gravity-sensing is essential for larval zebrafish to develop co-ordinated swimming behaviour (Ehrlich & Schoppik, 2019) and to navigate vertically through the water column (Zhu et al., 2024).

The timing of onset of semicircular canal function in fishes and amphibians remains a source of debate. Crista hair cells are already evident in the three-day-old zebrafish embryo (Haddon & Lewis, 1996) (Fig. 5A,C,E), but some authors have argued that the semicircular canal duct diameter is simply too small to allow response to angular accelerations until around one month of age (Beck et al., 2004; Lambert et al., 2008). Another study identified an angular vestibulo-ocular reflex (aVOR) in three-day-old embryos (Easter & Nicola, 1997), but this was later attributed to an optokinetic response (Beck et al., 2004). However, in addition to crista hair cells, the relevant neuronal circuitry is already in place in the five-day-old zebrafish embryo (Goldblatt et al., 2023). Moreover, lesion of anterior and posterior semicircular canal nerves resulted in measurable differences in vestibular response at this age (Goldblatt et al., 2023). Thus, although the utricular otolith is the predominant vestibular sensor in the zebrafish embryo and larva (Bianco et al., 2012; Mo et al., 2010; Riley & Moorman, 2000) (reviewed in (Bagnall & Schoppik, 2018; Liu & Bagnall, 2023)), the onset of semicircular canal function in this species may be earlier than previously thought.

In fishes, the otoliths also function to detect sound. Acoustic startle—the conserved escape response to a sudden auditory stimulus—is an early and robust behaviour in the zebrafish, underlain by a well-characterised neuronal circuit (Meserve et al. (2024) and references within). An afferent pathway from macular sensory hair cells to hindbrain Mauthner reticulospinal neurons is functional by the end of the first day of development, with auditory-evoked responses measurable from 40 hours post fertilisation (Tanimoto et al., 2009). Recent work has uncovered critical roles for the aGPCRs Celsr2 and Celsr3, together with their binding partner Fzd3a, in development and axon growth of the Mauthner cell (Meserve et al., 2024). Another zebrafish study has highlighted an essential role for the cytoplasmic protein Cyfip2 in establishing the threshold for acoustic startle: *cyfip2* (*triggerhappy*) mutants are hypersensitive to sound. The Cyfip2 protein acts on a range of targets to influence actin dynamics and protein translation, helping to set the correct excitatory/inhibitory balance for the auditory startle response (Deslauriers et al., 2024).

Studies on how the otoliths and inner ear accessory structures (the swim bladder and Weberian ossicles) contribute to fish hearing are revealing that bony fish live in a rich

auditory world. The tiny transparent teleost fish *Danionella*, for example—one of the smallest known vertebrates—has a highly conserved inner ear morphology, and uses sound for communication. This species has been used to resolve a long-standing debate on how such a miniature anatomy could enable the fish to localise sound direction. *Danionella* achieves this feat through separate detection and comparison of sound pressure and particle motion, a mechanism likely to be general for all otophysan ('hearing specialist') fish (Veith et al., 2024).

4.3 'Evo-devo': Evolutionary developmental biology of the inner ear

The complex anatomy of the semicircular canals lends itself well to morphometrics, using data from either magnetic resonance imaging (MRI) (Sauer et al., 2023) or micro-computed tomography (μCT) (Schulz-Mirbach et al., 2014). Although these approaches are most often applied to the bony labyrinth, staining protocols have also been developed to enhance contrast and allow reconstruction of soft tissue in μCT scans, allowing visualisation of the membranous labyrinth (David et al., 2016; Schulz-Mirbach et al., 2013; Veith et al., 2024). Conveniently, morphometrics of the bony labyrinth can be applied to both extant and fossil specimens. Geometric morphometric studies of semicircular canal shape are helping to sort out 'perplexing' fossils (Johanson et al., 2017), elucidate the origins of mammalian endothermy (Araújo et al., 2022), and to understand evolutionary selective pressures on morphology (Grunstra et al., 2024; Renaud et al., 2024). Morphometric studies of the bony labyrinth have also been used to infer phylogenetic relationships between extinct and living hominoids (great apes and humans) (Urciuoli et al., 2021), and to track the origin of human bipedalism in the fossil record (Spoor et al., 1994; Zhang et al., 2024).

The role of *Otx1* genes in development and evolution of the lateral (horizontal) semicircular canal has been a long-standing subject of interest and debate. In jawed vertebrates (mouse and zebrafish), *Otx1* is expressed in lateral and ventral otic epithelium, where it has a conserved role in formation of the lateral (horizontal) semicircular canal, ampulla and crista (Acampora et al., 1996; Acampora, Avantaggiato, et al., 1999; Hammond & Whitfield, 2006; Mazan et al., 2000; Morsli et al., 1999). Jawless fish, including lampreys and hagfish, have inner ears that lack a lateral horizontal canal (see references within Hammond & Whitfield (2006)). Initial studies in two species of lamprey (*Petromyzon marinus*, *Lampetra japonica*) failed to find otic expression of *Otx* genes (Tomsa & Langeland, 1999; Ueki et al., 1998), seemingly providing a neat explanation for the role of *Otx* genes in evolution of the horizontal canal in jawed vertebrates (Hammond & Whitfield, 2006). However, *Otx1* expression has now been described in the ventral otic vesicle of a third lamprey species (*Lethenteron camtschaticum*) and in the hagfish (*Eptatretus burgeri*) (Higuchi et al., 2019). These findings have led to a revised model for evolution of the lateral canal through the acquisition of a genetic programme downstream of Otx1, rather than acquisition of a new otic *Otx1* expression domain (Higuchi et al., 2019). Additional details remain to be elucidated; for example, the developmental origin and morphogenetic steps leading to formation of the ciliated chambers of the lamprey ear, which separate the anterior and posterior canals, are not yet fully understood. Moreover, careful 3D reconstructions of the adult inner ear in *P. marinus* have revealed its unexpected and quite unique anatomy, including the presence of two horizontal canal-like structures on the medial side of each ear (Maklad et al., 2014). The authors of this study suggest that sensitivity to movement in the horizontal plane has been achieved differently in lampreys and jawed vertebrates through parallel evolution (Maklad et al., 2014).

4.4 Relevance of developmental studies to human disease

Because visual and proprioceptive inputs help to compensate for any deficits in inner ear function, disorders of the vestibular system are a largely unappreciated clinical issue. However, an acute loss of vestibular function can be highly debilitating, and undetected chronic vestibular dysfunction may underlie several relatively common problems, including developmental delay in infancy (Janky et al., 2018; Kaga et al., 2008), idiopathic scoliosis (Lambert et al., 2009), and anxiety (Shefer et al., 2010). Dizziness is frequently reported in the elderly, and is a risk factor for falls (Colledge et al., 1994). Disorders of the vestibular system can have many causes (genetic or environmental); they may involve semicircular canal anomalies, otoconial degradation or displacement, hair cell loss, or synaptopathy, and frequently accompany syndromes involving deafness (see (Maudoux et al., 2022) for a review).

4.4.1 Human disease involving abnormalities of the semicircular canals

One well-studied example of a human disease involving semicircular canal anomalies is CHARGE syndrome, where the genetic cause—haploinsufficiency of the *CHD7* gene—has been identified. A common characteristic of this multi-system condition is reduced growth (hypoplasia) or even complete absence (aplasia) of the semicircular canals (reviewed in Choo et al. (2017)). New genetic and clinical variants of CHARGE syndrome continue to be uncovered (see, for example, (Boschann et al., 2023)). Mice carrying both systemic and inner-ear-specific heterozygous *Chd7* mutations recapitulate the semicircular canal defects seen in people (Adams et al., 2007; Bosman et al., 2005; Hurd et al., 2007, 2010, 2012). In addition to canal defects, there may be other craniofacial and inner ear defects, including a loss of innervation to the posterior crista (Adams et al., 2007; Hurd et al., 2007, 2011).

CHD7 codes for an ATP-dependent chromatin remodelling protein (see references within Choo et al. (2017)), and the mouse models are being used to identify its molecular interactions and transcriptional targets in the ear. A host of familiar genes are either down-regulated (*Sox2*, *Eya1, Pax2, Otx2, Fgf10*) or up-regulated (*Tbx1*) in ears lacking Chd7 function (Gao et al., 2024; Hurd et al., 2010), several of which are likely to contribute to the effects on semicircular canal morphogenesis. Lateral semicircular canal truncations in *Chd7*$^{Gt/+}$ heterozygous mice can be fully or partially rescued by citral, an inhibitor of RA synthesis, indicating interaction with the RA signalling pathway (Micucci et al., 2014). In a zebrafish *chd7*$^{-/-}$ model, there are abnormalities in the Weberian ossicles (Breuer et al., 2024). Recent work in chick and human has used epigenomic profiling to identify conserved enhancers regulating *Chd7* expression, including those driving expression in the otic placode (Williams et al., 2024).

While CHARGE syndrome remains the best characterised, other syndromes are known that involve semicircular canal abnormalities. Mutations in *SOX10*, for example, frequently result in semicircular canal malformations in the deafness-pigmentation disorder Waardenburg Syndrome Type IV (Elmaleh-Bergès et al., 2013; S. Li et al., 2022; Moldenæs et al., 2021; Song et al., 2016; Sznajer et al., 2008) (reviewed in (Pingault et al., 2022)). This is consistent with the widespread expression of *Sox10* in the otic epithelium (Watanabe et al., 2000), and corroborates analysis of the zebrafish model (Dutton et al., 2009), demonstrating that the inner ear defects in patients not only result from a loss of neural-crest-derived pigment cells affecting hearing function, but also reflect a conserved role for SOX10 in the otic epithelium. Interestingly, SOX10 and CHD7 interact as a complex and co-operate to

regulate enhancer activity in the central nervous system in mice (He et al., 2016), and so it is likely that they also synergise to regulate target gene expression in the inner ear.

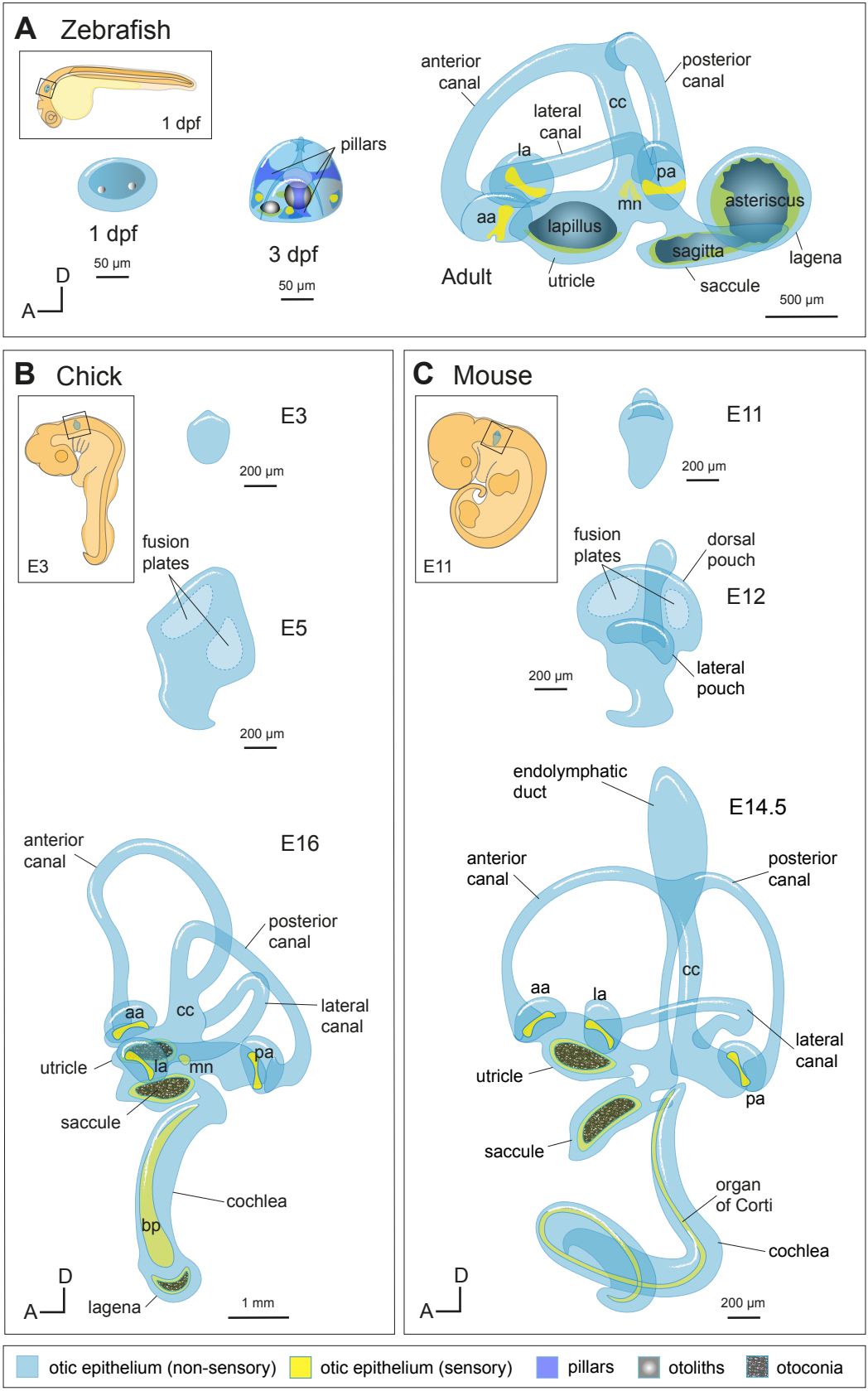
Developmental studies are likely to provide additional candidate genes for idiopathic disorders of the semicircular canal system. In addition, direct sequencing approaches are uncovering new genes with roles in semicircular canal development, which can then be modelled in animal or cellular systems to understand mechanism; see, for example, identification of the gene *ZSCAN10*, mutations in which affect development of the horizontal (lateral) canal (Laugwitz et al., 2024).

4.4.2 Human disease involving otoconial displacement

Demineralisation of otoconia or their displacement from the otolithic membrane can result in dizziness and vertigo (the disabling sensation of spinning). Otoconia or the otolithic membrane may degrade over time, a process that—like osteoporosis—can be hormone-dependent (L. Yang et al., 2018). Benign paroxysmal positional vertigo (BPPV) describes the symptoms experienced when otoconia become dislodged or form ectopically, ending up in the canal duct (canalithiasis) or attached to the cupula in the ampulla (cupulolithiasis) (Argaet et al., 2019). This can happen due to abnormalities in the otolithic membrane, head trauma, or even space flight (see references within Argaet et al. (2019); Dror et al. (2020)). For many BPPV patients, relief from symptoms can be provided by a sequence of controlled head movements designed to re-position the displaced otoconia back in the utricle (Argaet et al., 2019). Dror and colleagues have used the mouse *Slc26a4* (*Pendrin*) mutant as an animal model for BPPV (Dror et al., 2020). In *Slc26a4* mutant mice (which are also deaf), there are disruptions to endolymph pH and calcium concentration. As a consequence, otoconial formation is affected; giant otoconia form (similar to those occasionally found in *Otop1* mutants; Fig. 6K), and are sometimes found in the canal ducts and ampullae (Dror et al., 2020). This study supports the idea that certain genetic backgrounds can predispose individuals to BPPV, paving the way for improved management and treatment of this common disorder.

5 Conclusion

At first sight, the vestibular system may seem like a niche topic for research, lacking the immediate broad appeal of the well-studied auditory functions of the inner ear. However, the studies reviewed here demonstrate that vestibular research is generating exciting and novel findings of wide applicability and interest. These are truly at the cutting edge of interdisciplinary science, linking developmental biology to crystallography, soft-matter physics and neuroscience. Large-scale methodologies such as single-cell transcriptomics have identified new genetic players and cell types, revealing complexities in the vestibular system that are a match for those in the cochlea. Imaging and modelling tools are enabling us to probe and understand the physical forces that accompany and drive morphological change in the developing embryo, so well exemplified by the formation of the semicircular canal ducts. Analysis of otolith formation has highlighted general principles that underpin the chemistry of biomineralisation. Together, these studies are building a systems-level understanding of the vestibular system, from its earliest developmental origins in the embryo to its ancient function as a sensor for gravity and motion, with far-reaching impact for our understanding of fundamental processes in development, evolution, neuroscience and medicine.


A Zebrafish
1 dpf
1 dpf
50 µm
pillars
3 dpf
50 µm
D
A
anterior canal
posterior canal
lateral canal
cc
la
pa
mn
aa
lapillus
asteriscus
sagitta
lagena
Adult
utricle
saccule
500 µm
B Chick
E3
E3
200 µm
fusion plates
E5
200 µm
E16
anterior canal
posterior canal
lateral canal
aa
cc
pa
utricle
la
mn
saccule
cochlea
bp
lagena
1 mm
C Mouse
E11
E11
200 µm
fusion plates
dorsal pouch
E12
200 µm
lateral pouch
endolymphatic duct
E14.5
anterior canal
posterior canal
cc
aa
la
lateral canal
utricle
pa
saccule
organ of Corti
cochlea
200 µm
otic epithelium (non-sensory)
otic epithelium (sensory)
pillars
otoliths
otoconia

Figures

Figure 1. Comparative morphogenesis and anatomy of the inner ear in three widely used model vertebrates

A. Morphogenesis of the inner ear in the zebrafish
B. Morphogenesis of the inner ear in the chick
C. Morphogenesis of the inner ear in the mouse

Drawings are presented to allow comparison of the semicircular canal morphology between species; see scale bars for approximate sizes. Lateral views; dorsal (D) to the top, anterior (A) to the left. Insets (not to scale) show the position of the otic vesicle or otocyst (boxed) in the embryo at the earliest embryonic stage shown. Abbreviations: aa, anterior ampulla; bp, basilar papilla; la, lateral ampulla; lag, lagenar macula; pa, posterior ampulla; cc, crus commune; mn, macula neglecta; dpf (zebrafish), days post fertilisation, E (chick, mouse), embryonic day. Drawings are based on data from references cited in the text.

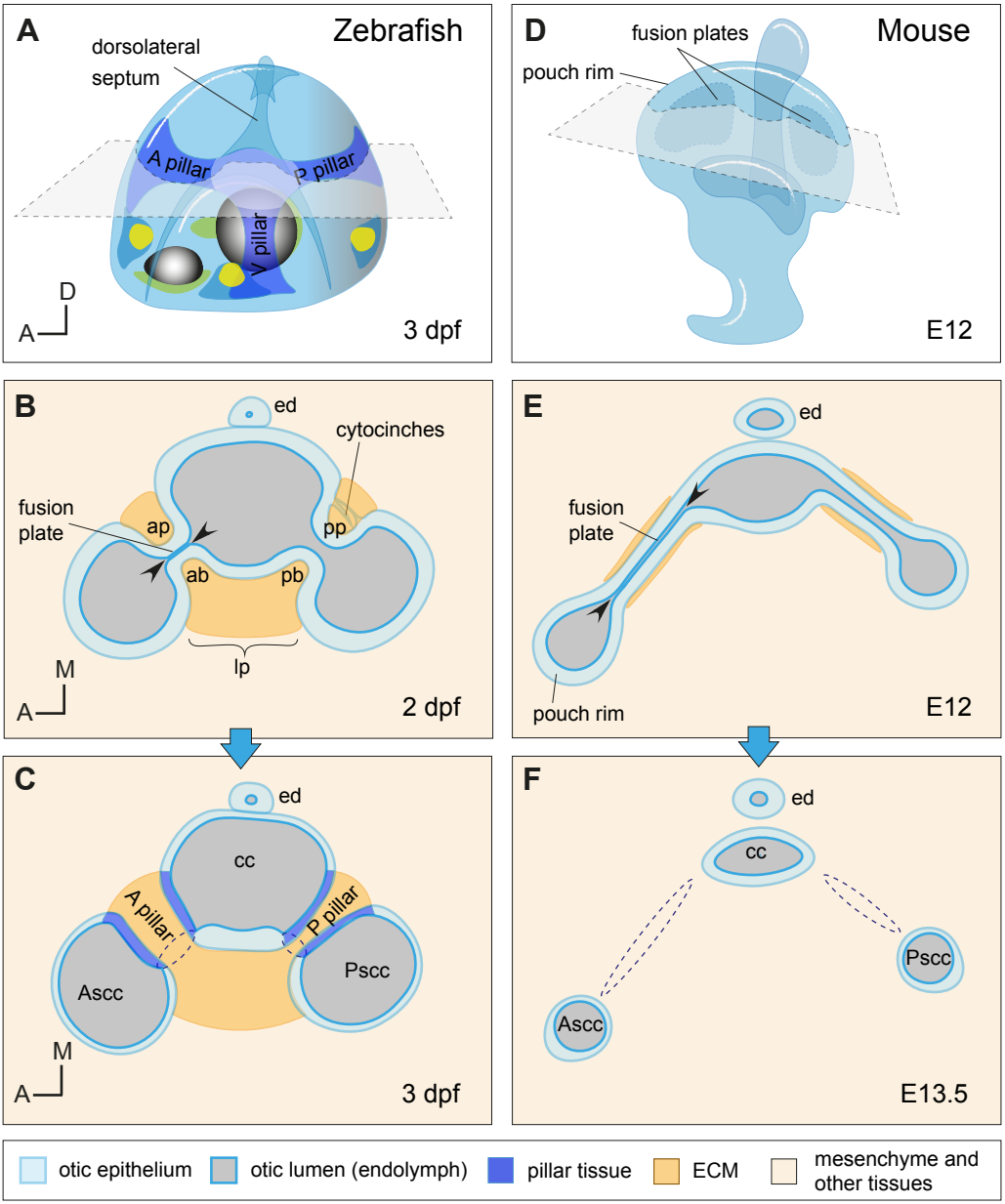

A
Zebrafish
dorsolateral
septum
A pillar
P pillar
V pillar
D
A
3 dpf
D
Mouse
fusion plates
pouch rim
E12
B
ed
cytocinches
fusion
plate
ap
pp
ab
pb
lp
M
A
2 dpf
E
ed
fusion
plate
pouch rim
E12
C
ed
cc
A pillar
P pillar
Ascc
Pscc
M
A
3 dpf
F
ed
cc
Pscc
Ascc
E13.5
otic epithelium
otic lumen (endolymph)
pillar tissue
ECM
mesenchyme and
other tissues

Figure 2. Comparison of fusion plate formation and perforation in the zebrafish and mouse

A–C. Fusion plate formation and perforation in the zebrafish inner ear. **A.** Drawing of a zebrafish ear at 3 dpf (lateral view) showing the approximate position of the transverse section (grey plane) through the forming anterior and posterior canals in the panels below. The positions of the anterior (A), posterior (P), and ventral (V) pillars, together with the dorsolateral septum, are shown. Together, these structures have the appearance of a cross. **B.** Schematic diagram depicting a transverse section through the ear during fusion plate formation (2 dpf). Anterior and posterior epithelial projections (ap, pp) meet anterior and posterior bulges (ab, pb) from a lateral projection (lp). The acellular cores of each projection and bulge are filled with extracellular matrix (ECM, orange). The anterior projection-bulge pair has made contact to form a bilayered fusion plate (arrowheads), consisting of about 8–10 cells; the posterior pair has not yet made contact. Cytocinches link the basal sides of cells on either side of a projection before fusion. **C.** After fusion plate perforation (3 dpf). Lumens of the anterior and posterior semicircular canal ducts (Ascc, Pscc), together with the crus commune (cc), are now resolved. ECM-filled tissue pillars now span the otic lumen, but are not yet invaded by mesenchyme. Dotted lines indicate the cleared regions. The canal walls continue to thin at this time. Note how the orthogonal arrangement of the semicircular canals is set by the positions of the growing projections and pillars.

D–F. Fusion plate formation and perforation in the mouse inner ear. **D.** Drawing of a mouse ear at E12 showing the approximate position of the section through the forming anterior and posterior canals in the panels below. The positions of the fusion plates and pouch rim for the dorsal pouch (giving rise to the anterior and posterior semicircular canals) are shown. **E.** Formation of the fusion plate (arrowheads) for the anterior semicircular canal. (A cell-free area beneath the fusion plate is likely to be ECM-filled (orange), but this is not well described in the literature.) Compare to B. **F.** After fusion plate perforation (E13.5), showing resolution of the anterior and posterior semicircular canal duct lumens and the crus commune. Compare to C. Dotted lines indicate the cleared regions: mesenchyme soon invades to fill these spaces.

Orientations: A, anterior; D, dorsal; M, medial. Diagrams are based on data from references cited in the text.

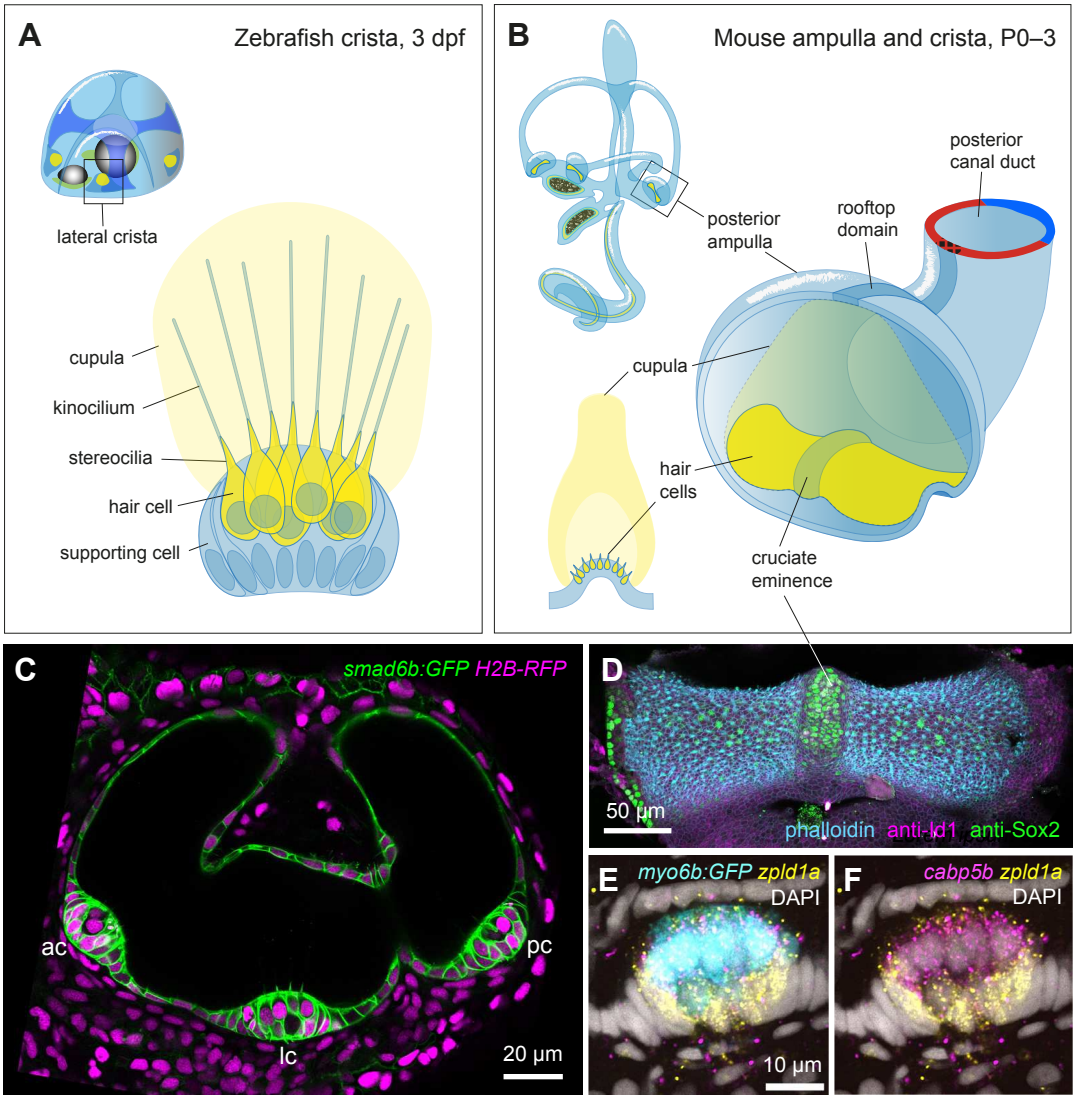

A
Zebrafish crista, 3 dpf
lateral crista
cupula
kinocilium
stereocilia
hair cell
supporting cell
B
Mouse ampulla and crista, P0–3
posterior canal duct
posterior ampulla
rooftop domain
cupula
hair cells
cruciate eminence
C
smad6b:GFP H2B-RFP
ac
pc
lc
20 µm
D
50 µm
phalloidin anti-ld1 anti-Sox2
E
myo6b:GFP zpld1a
DAPI
10 µm
F
cabp5b zpld1a
DAPI

Figure 3. Regulation of ECM production during early steps of semicircular canal formation in the zebrafish ear

Model for assembly of the ECM that contributes to driving epithelial projection and bulge outgrowth in the developing zebrafish ear. 1. Events initiated by key transcription factors lead to the localised production of ECM components, which together form a hydrated ECM complex that assembles beneath the basal side of cells in the nascent epithelial projections and bulges. The possible anisotropic alignment of HA and collagen fibres within the matrix is hypothesised, but has not been formally demonstrated. 2. When cells adhere at the fusion plate, Adgrg6 signalling pathway activity results in the rapid transcriptional down-regulation of various ECM genes, together with changes in cell behaviour. The ECM itself persists in the pillars beyond the fusion plate stage. The diagram is based on references cited in the text. The dotted line is inferred from data in the mouse. Only selected players are shown; for an overview of the complexities of GAG biosynthesis in the zebrafish, see Habicher et al. (2022).

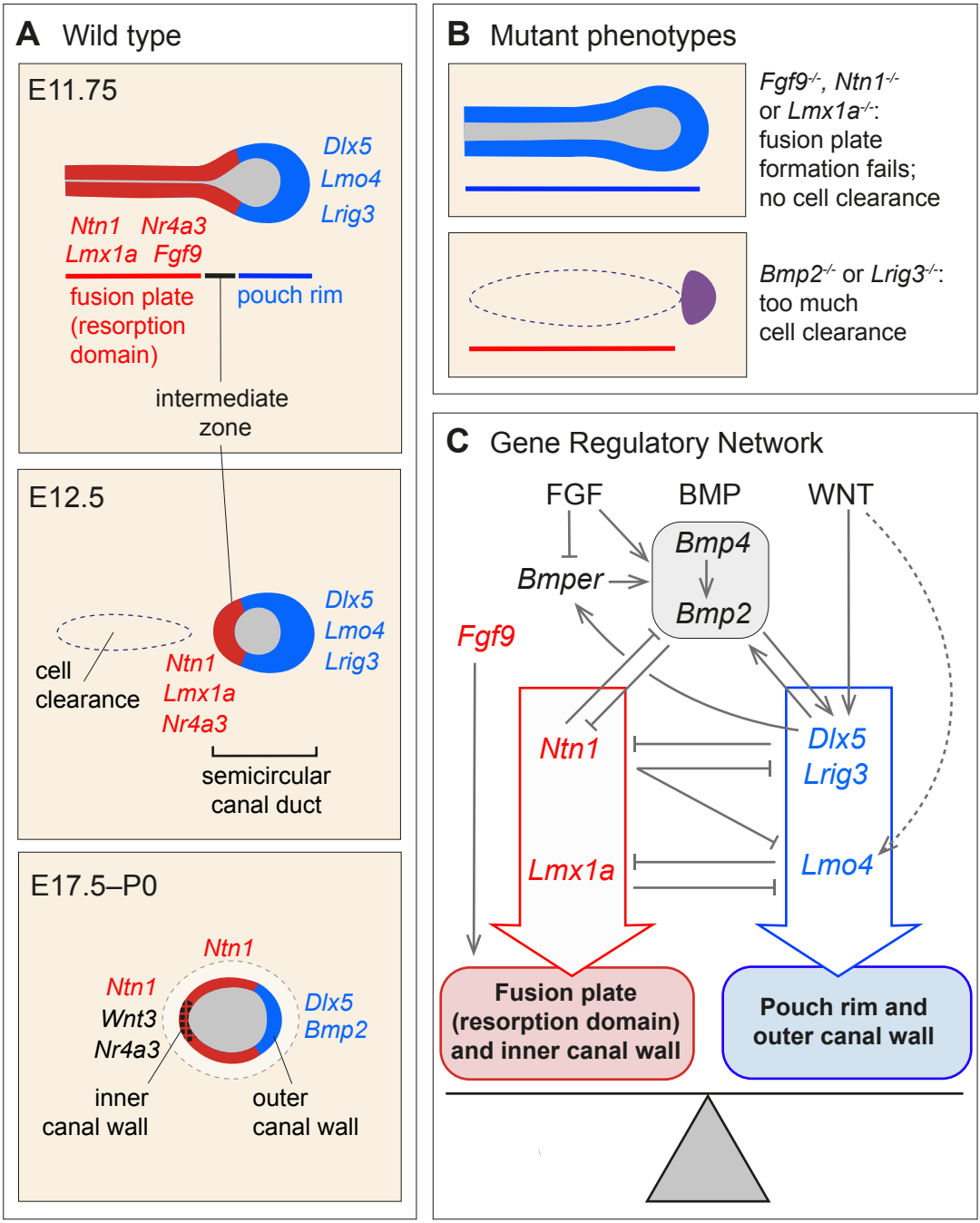

A Wild type
E11.75
Dlx5
Lmo4
Lrig3
Ntn1 Nr4a3
Lmx1a Fgf9
fusion plate
(resorption
domain)
pouch rim
intermediate
zone
E12.5
Dlx5
Lmo4
Lrig3
cell
clearance
Ntn1
Lmx1a
Nr4a3
semicircular
canal duct
E17.5–P0
Ntn1
Ntn1
Wnt3
Nr4a3
Dlx5
Bmp2
inner
canal wall
outer
canal wall
B Mutant phenotypes
Fgf9-/-, Ntn1-/-
or Lmx1a-/-:
fusion plate
formation fails;
no cell clearance
Bmp2-/- or Lrig3-/-:
too much
cell clearance
C Gene Regulatory Network
FGF
BMP
WNT
Bmp4
Bmper
Bmp2
Fgf9
Ntn1
Dlx5
Lrig3
Lmx1a
Lmo4
Fusion plate
(resorption domain)
and inner canal wall
Pouch rim and
outer canal wall

Figure 4. Cross-repressive gene regulatory interactions at the fusion plate in the mouse

A. Sequence of events leading to formation and patterning of a semicircular canal duct in the wild-type mouse. Selected genes expressed in the fusion plate and the canal rim are shown. An intermediate zone, which expresses *Ntn1* (red), but does not fuse, separates the fusion plate from the pouch rim, and becomes the inner wall of the canal duct. After fusion (E17.5), expression of *Ntn1* (red) persists in the inner and lateral canal walls, and expression of *Dlx5* and *Bmp2* (blue) becomes restricted to the outer canal wall. By postnatal day 0 (P0), *Wnt3* and *Nr4a3* (hatched shading) are expressed in a subset of the *Ntn1*-expressing domain on the inner wall of the canal duct, contiguous with the rooftop domain of the ampulla.

B. Selected mutant phenotypes characterised by failure of fusion (top), or excessive fusion and cell clearance (bottom). Both phenotypes result in a failure to define the semicircular canal ducts.

C. Model for the cross-repressive regulatory relationships between selected genes in the mouse semicircular canal pouch. Arrows indicate promotion (or inhibition) of either transcription or activity of the factors shown. (Not all the genes listed code for transcription factors.) The scales highlight the delicate balance between the alternative output cell fates, which can be tipped in either direction through genetic or pharmacological disruption of the GRN. See text for details.

Diagrams are based on data from references cited in the text.

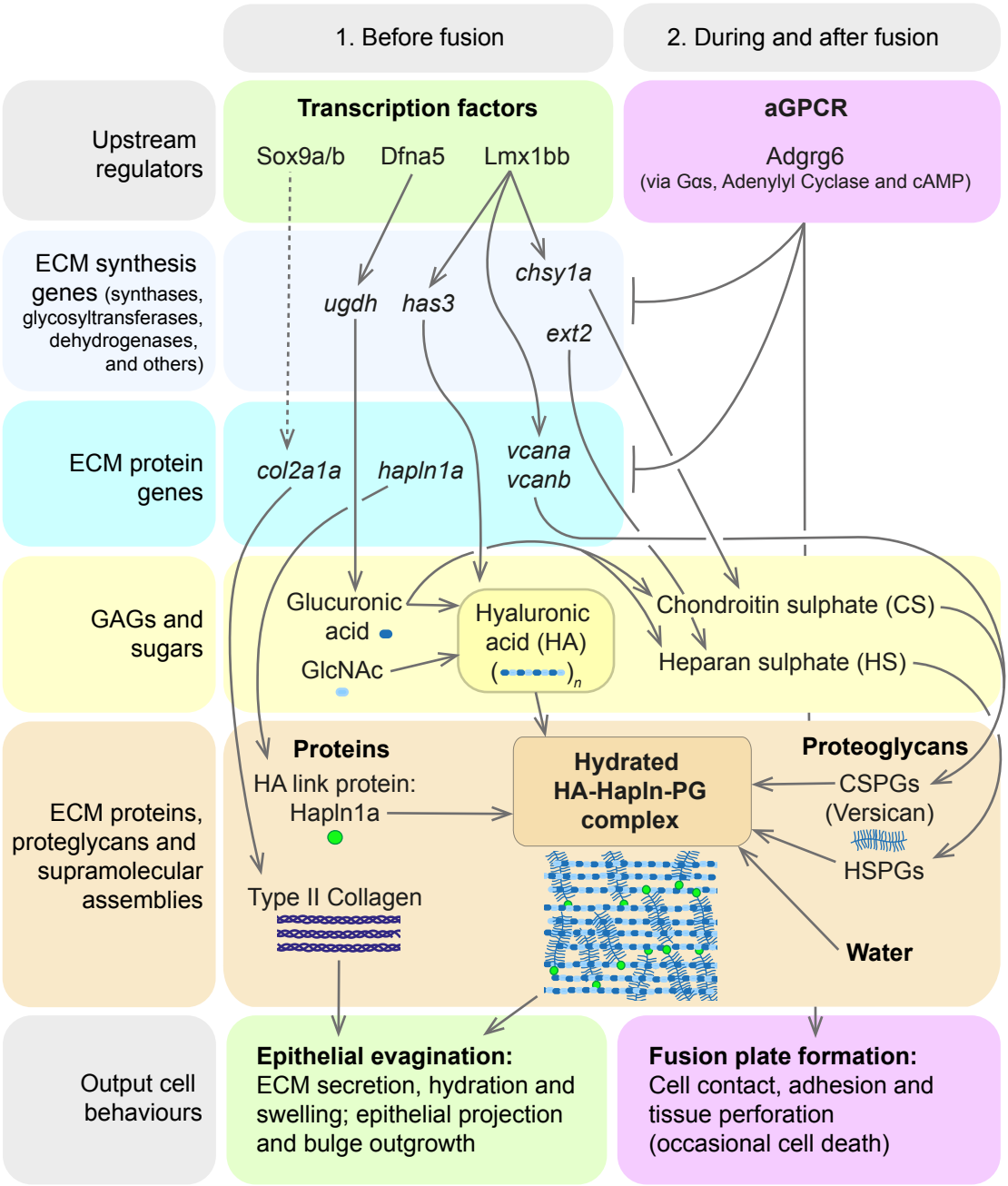

1. Before fusion
2. During and after fusion
Upstream regulators
Transcription factors
Sox9a/b
Dfna5
Lmx1bb
aGPCR
Adgrg6
(via Gαs, Adenylyl Cyclase and cAMP)
ECM synthesis genes (synthases, glycosyltransferases, dehydrogenases, and others)
ugdh
has3
chsy1a
ext2
ECM protein genes
col2a1a
hapln1a
vcana
vcanb
GAGs and sugars
Glucuronic acid
GlcNAc
Hyaluronic acid (HA)
Chondroitin sulphate (CS)
Heparan sulphate (HS)
ECM proteins, proteglycans and supramolecular assemblies
Proteins
HA link protein:
Hapln1a
Type II Collagen
Hydrated HA-Hapln-PG complex
Proteoglycans
CSPGs
(Versican)
HSPGs
Water
Output cell behaviours
Epithelial evagination:
ECM secretion, hydration and swelling; epithelial projection and bulge outgrowth
Fusion plate formation:
Cell contact, adhesion and tissue perforation
(occasional cell death)

Figure 5. Development of ampullae and cristae in the zebrafish and mouse

A. Schematic drawing of the lateral crista in a 3-day-old zebrafish inner ear. Hair cells (yellow) project their stereocilia and long, straight kinocilia into the overlying cupula (pale yellow).
B. Schematic drawings of the posterior ampulla and crista in the postnatal (P0–3) mouse inner ear. Note the zone of *Nr4a3*- and *Wnt3*-expressing cells defining a 'rooftop domain' of the ampulla, which continues along the inner curvature of the canal duct. Colour coding of the section through the duct corresponds to Fig. 4A.
C. Airyscan confocal section through a 3-day-old zebrafish ear, showing the anterior, lateral and posterior cristae (ac, lc, pc). The transgenic marker *smad6b:GFP* (green) marks otic epithelial cell membranes. Cell nuclei are stained with RFP (magenta). Dark spaces are the fluid-filled lumens of the nascent semicircular canal ducts. Reproduced from Baldera et al. (2023).
D. A crista from a postnatal day 0 (P0) mouse inner ear, stained with phalloidin (cyan) to show the actin-rich stereociliary bundles on sensory hair cells. An antibody to Sox2 (green) labels supporting cells, including those in the cruciate eminence, and type II hair cells. Reproduced from Wilkerson et al. (2021).
E, F. Sensory hair cells in a 3-day-old zebrafish lateral crista are marked by expression of the pan-hair-cell transgenic marker *myo6b:GFP* (cyan), and the crista-hair-cell-specific expression of *cabp5b* (HCR; magenta). Supporting cells express *zpld1a* (HCR; yellow), which codes for Cupulin. Nuclei (grey) are stained with DAPI. Reproduced from Shi et al. (2023).

Diagrams are based on data from references cited in the text. Note: innervation of the cristae is not illustrated.

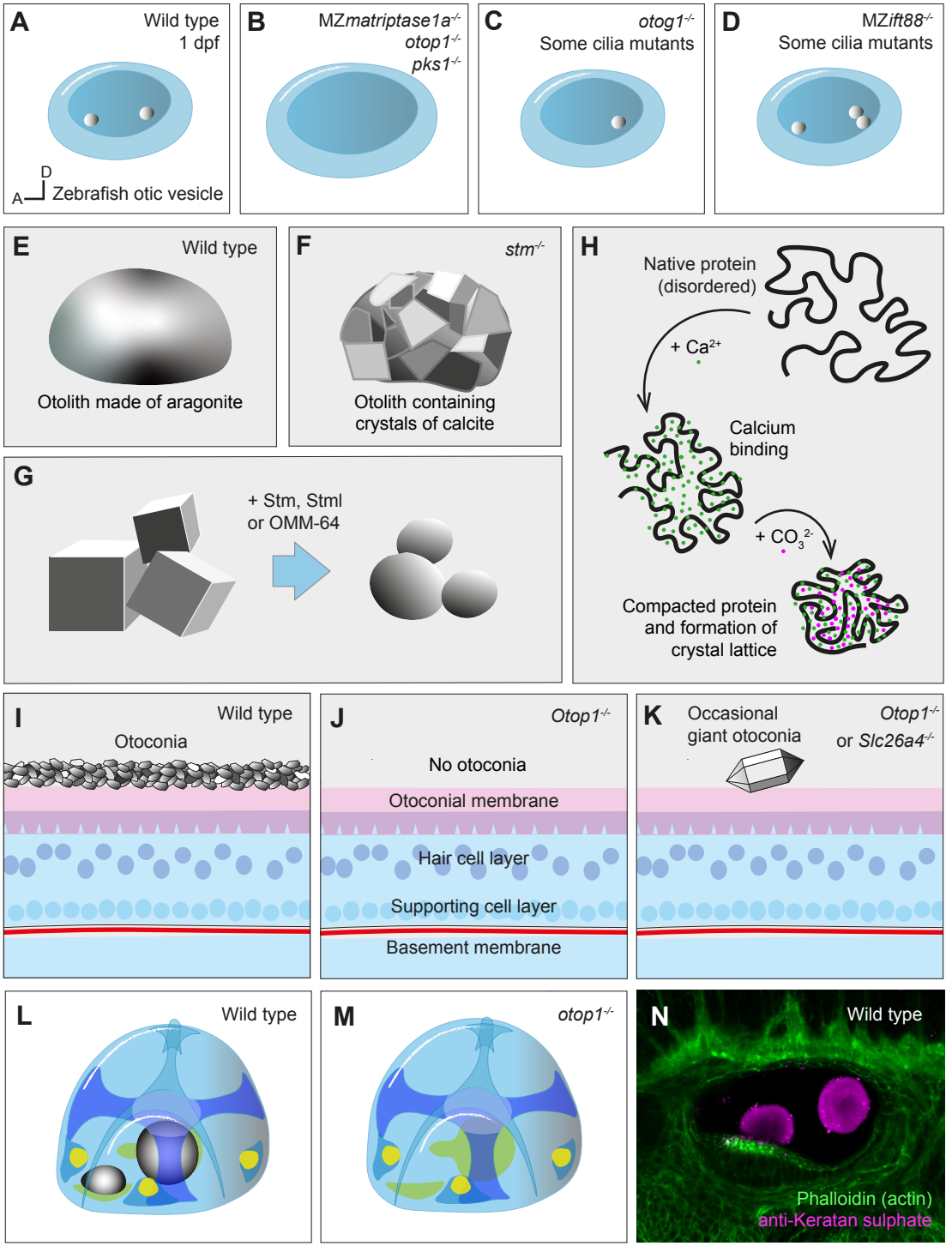

A
Wild type
1 dpf
D
A
Zebrafish otic vesicle
B
MZmatriptase1a-/-
otop1-/-
pks1-/-
C
otog1-/-
Some cilia mutants
D
MZift88-/-
Some cilia mutants
E
Wild type
Otolith made of aragonite
F
stm-/-
Otolith containing
crystals of calcite
G
+ Stm, Stml
or OMM-64
H
Native protein
(disordered)
+ Ca2+
Calcium
binding
+ CO3 2-
Compacted protein
and formation of
crystal lattice
I
Wild type
Otoconia
J
Otop1-/-
No otoconia
Otoconial membrane
Hair cell layer
Supporting cell layer
Basement membrane
K
Occasional
giant otoconia
Otop1-/-
or Slc26a4-/-
L
Wild type
M
otop1-/-
N
Wild type
Phalloidin (actin)
anti-Keratan sulphate

Figure 6. Development of otoliths and otoconia in the zebrafish and mouse

A–D. Counting stones: Disruption of otolith formation in the one-day-old zebrafish otic vesicle. **A.** Two otoliths (precursors of the lapillus in the utricle and the sagitta in the saccule) are present in the wild-type otic vesicle, and are already becoming mineralised at this stage. **B.** Absence of otoliths and presence of ear swelling in mutants for *matriptase1a*, *otopetrin1* (*otop1*) or *polyketide synthase 1* (*pks1*). **C.** Delayed formation of the utricular (anterior) otolith in the *otogelin1* (*otog1*) mutant, used in many behavioural studies. **D.** Supernumerary and mis-positioned otoliths in a mutant lacking cilia (MZ*ift88*$^{-/-}$). Diagrams are based on data from references cited in the text.

E–H. Smoothing it over: Intrinsically disordered calcium-binding proteins select calcium carbonate crystal polymorph growth in vivo and in vitro. **E,F.** Zebrafish otoliths (normally aragonitic) grow as calcite in the *starmaker* (*stm*$^{-/-}$) morphant or mutant ear. After (Pachoensuk et al., 2021; Söllner et al., 2003). **G.** Modulation of calcium carbonate polymorph growth in vitro by addition of otolith matrix proteins. After (Kalka et al., 2024; Poznar et al., 2020; Różycka et al., 2014, 2019; Wojtas et al., 2012). **H.** Model for binding of calcium and carbonate ions by Starmaker and OMM-64. After (Poznar et al., 2020; Wojtas et al., 2015).

I–M. Acid test: The conserved role of Otopetrin1, a proton channel, in otoconial and otolith development. **I,J.** Lack of saccular otoconia (or occasional giant otoconia) in the mouse *Otop1* (*tilted*) mutant. Giant otoconia are also seen in the *Slc26a4* (*Pendrin*) mutant. After (Dror et al., 2020; Ornitz et al., 1998). Organisation of the sensory epithelium and otolithic membrane is unaffected. **L,M.** Lack of utricular and saccular otoliths in the zebrafish *otop1*$^{-/-}$ (*backstroke*) mutant. After (Hughes et al., 2004; Söllner et al., 2004; Whitfield et al., 1996). Apart from the lack of otoliths and a mild swelling, morphology of the ear otherwise appears normal.

N. Sugar-coated: Otoliths in the two-day-old wild-type zebrafish otic vesicle are positive for the polysaccharide keratan sulphate (magenta). Phalloidin staining (green) marks the actin-rich stereociliary bundles of sensory hair cells beneath the utricular (anterior) otolith. Reproduced from Jones et al. (2022).

Orientations: A, anterior; D, dorsal (applies to A–F, L–N).

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