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Cilia in the developing zebrafish ear

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Abstract
The inner ear, which mediates the senses of hearing and balance, derives from a simple ectodermal vesicle in the vertebrate embryo. In the zebrafish, the otic placode and vesicle express a whole suite of genes required for ciliogenesis and ciliary motility. Every cell of the otic epithelium is ciliated at early stages; at least three different ciliary subtypes can be distinguished on the basis of length, motility, genetic requirements and function. In the early otic vesicle, most cilia are short and immotile. Long, immotile kinocilia on the first sensory hair cells tether the otoliths, biomineralised aggregates of calcium carbonate and protein. Small numbers of motile cilia at the poles of the otic vesicle contribute to the accuracy of otolith tethering, but neither the presence of cilia nor ciliary motility are absolutely required for this process. Instead, otolith tethering is dependent on the presence of hair cells and function of the glycoprotein Otogelin. Otic cilia or ciliary proteins also mediate sensitivity to ototoxins and co-ordinate responses to extracellular signals. Other studies are beginning to unravel the role of ciliary proteins in cellular compartments other than the kinocilium, where they are important for the integrity and survival of the sensory hair cell.

Introduction

The cilia of vertebrate sensory organs are extraordinarily diverse in morphology and function, forming an integral part of cellular receptors for light, odorants, sound, gravity and motion. The zebrafish, *Danio rerio*, is a widely-used vertebrate model system for development and disease, with particular advantages for combining imaging in the live animal with genetic or pharmacological manipulation of protein function. Cilia in the zebrafish retina, nose[1], inner ear and lateral line have all been the subject of intense study; in this review, I focus on cilia in the inner ear, with occasional reference to the lateral line. From the earliest stages of its development, the inner ear is a major site of expression for genes coding for ciliary proteins. Analysis of the different ciliary types in the early otic vesicle has revealed some fascinating biology linking both immotile kinocilia and motile-cilia-driven fluid flow with formation of the otoliths. In the lateral line, a closely related sensory system to the inner ear, kinocilia of the sensory hair cells in superficial neuromasts are uniquely accessible for study. Both the ear and lateral line in zebrafish form important models for our understanding of hearing, balance and deafness in humans.

Three distinct types of cilia in the zebrafish otic vesicle

There are at least three distinct types of cilia in the zebrafish ear at early stages. From the onset of hollowing of the otic placode to form a vesicle at 18 hours post fertilisation (hpf), most cells of the otic epithelium bear a single immotile cilium on their apical (luminal) side (Stooke-Vaughan et al., 2012). Each sensory hair cell also bears a single cilium, the kinocilium, located on one side of the stereociliary bundle, an apical collection of microvillar-like projections that respond to mechanical deflection. In the early otic vesicle (20–25 somite stage), hair cells (tether cells) form in two pairs, one at each pole of the otic vesicle (Haddon and Lewis, 1996; Riley et al., 1997), and thus initially there are only four kinocilia amongst the 200 or so cilia in total in each ear (Fig. 1A,B). Despite the prefix *kino*-, indicating movement, zebrafish hair cell kinocilia are not thought to generate active motility. However, motile cilia (distinct from the kinocilia and the immotile cilia) are also found in the ear, with most concentrated at the poles in close proximity to the hair cells (Stooke-Vaughan et al., 2012; Wu et al., 2011; Yu et al., 2011). Although some reports have suggested otherwise, motile cilia only appear to make up a small fraction of the total number of cilia in the ear, with the vast majority (92–98%) being immotile (Stooke-Vaughan et al., 2012).

[1] For a review of motile cilia in the olfactory system, see **Ringers C, Olstad E and Jurisch-Yaksi N** (2019). The role of motile cilia in the development and physiology of the nervous system. *Phil. Trans. Roy. Soc. B, this issue*.

Ultrastructure of cilia in the zebrafish ear

Transmission electron microscopy of kinocilia from zebrafish inner ear hair cells reveals a 9+2 axoneme (9 doublet microtubules surrounding a central pair of single microtubules) (Leventea et al., 2016), as described for the supernumerary ear kinocilia in homozygous *mib*^{ta52b} mutants (Yu et al., 2011). Although based on very few examples in zebrafish, these studies corroborate findings for utricular and lateral line kinocilia in another teleost fish, the burbot (*Lota lota*) (Flock, 1964; Flock and Duvall, 1965; Flock and Wersäll, 1962). Fish sensory hair cell kinocilia thus make up a class of cilium (9+2, but immotile) often ignored in overviews of ciliary ultrastructure. Hair cell kinocilia are thought to have outer dynein arms and radial spokes, but to lack inner dynein arms and nexin links, as summarised by McHenry and van Netten (McHenry and van Netten, 2007), although this has not been shown definitively for the zebrafish. It will be exciting to see whether the relatively new technique of cryo-electron tomography, already so successful in revealing spectacular ultrastructural detail in the motile 9+2 flagellum of the unicellular alga *Chlamydomonas* (Bui et al., 2011; Nicastro, 2009), and of the actin filaments in mammalian vestibular hair cell stereocilia (Metlagel et al., 2019; Sazzed et al., 2018), can be applied to zebrafish hair cell kinocilia.

Kinocilia of amphibian vestibular hair cells also have the 9+2 pattern (for example, those of the marsh frog *Pelophylax ridibundus* (Nagel et al., 1991)). However, kinocilia of mammalian (rat and guinea-pig) vestibular hair cells have been described to have a 9+0 axoneme, with electron-dense material (but no clear microtubules) at the centre (Hamilton, 1969; Kikuchi et al., 1989). In amniote auditory hair cells, there are further variations: the transient kinocilia of immature hair cells in cochleae of the chick and mouse, for example, have a 9+0 arrangement near the base, but a pattern of eight doublet microtubules surrounding one central doublet (presumed to be one of the nine outer doublets displaced inward) further up the ciliary shaft (Honda et al., 2018; Sobkowicz et al., 1995). The axonemal configuration of the short immotile cilia and the motile cilia present at early stages in the zebrafish otic vesicle have not yet been described. Given the similarity of the immotile short cilia to primary cilia, and the rotational movement of the motile cilia (see below), it might be expected that both types have a 9+0 axoneme.

Ciliary motility and cilia-driven fluid flow in the zebrafish ear

Motile cilia in the zebrafish ear move in a rotational fashion, with an estimated beat frequency of 28–44 Hz, depending on stage (and possibly other factors such as strain and temperature). Beating cilia can be imaged using high-speed video microscopy at 300 frames per second and above, and are found to be concentrated at the anterior and posterior poles of the early otic vesicle, close to, but distinct from, the immotile tether kinocilia (Stooke-

Vaughan et al., 2012; Wu et al., 2011; Yu et al., 2011). In the early otic vesicle, fluid flow driven by ciliary movement can be inferred from observations of the free otolith precursor particles within the vesicle lumen, which move in vortices near the anterior and posterior poles (Riley et al., 1997). The flow has been mapped using Particle Image Velocimetry, by collecting otolith precursor particles in an optical trap and releasing them into the flow near motile cilia (Wu et al., 2011). These measurements, together with a physical model simulating cilia-driven flow in a spherical cavity, indicated that the flow field near the tether kinocilia (where most motile cilia are concentrated) is advective, whereas the flow field further from the otolith is dominated by diffusion (Wu et al., 2011).

In contrast to the motile cilia in the ear, the kinocilia of zebrafish ear and lateral line hair cells are immotile and rigid in the living animal (McHenry and van Netten, 2007; Stooke-Vaughan et al., 2012). This is beautifully illustrated by live imaging of the *Tg(pou4f3:gfp)* line, which expresses a membrane-tethered GFP specifically in hair cells, revealing the long straight kinocilium, bright stereociliary bundle and hair cell soma of crista hair cells (Stawicki et al., 2014) (Fig. 1H). Tether cell kinocilia at the poles of the early otic vesicle show some passive displacement due to the beating of nearby motile cilia, but do not display active movement (Stooke-Vaughan et al., 2012).

Expression of genes required for ciliary motility in the zebrafish ear

The transcription factor Foxj1 is required for the expression of a suite of genes coding for proteins expressed in motile cilia (Hellman et al., 2010; Yu et al., 2011; Yu et al., 2008). There are two *foxj1* genes in the zebrafish genome: the ear is a major site of expression for *foxj1b*, but not *foxj1a* (Hellman et al., 2010; Tian et al., 2009; Yu et al., 2008). *foxj1b* is initially expressed throughout the otic placode at 10 hours post fertilisation (hpf), some eight hours before a lumen forms in the vesicle and motile cilia are apparent. In the otic vesicle, *foxj1b* expression becomes restricted to the poles at 22 hpf, apparently marking the first hair cells (the tether cells) developing there (Yu et al., 2011). (Expression in hair cells is surprising, as the kinocilia, as discussed above, are immotile.) Otic expression of *foxj1b* is dependent on Wnt signalling: it is strongly down-regulated after heat shock-induced mis-expression of the Wnt inhibitor Dkk (Caron et al., 2012).

In a screen for genes regulated by Foxj1 transcription factors, over 600 differentially expressed genes were uncovered in zebrafish embryos overexpressing *foxj1a* (Choksi et al., 2014). In this study, the authors assayed for otolith abnormalities (see below) as one of five tissue-specific phenotypic criteria to indicate a successful disruption of motile cilia. Of 50 randomly-selected genes tested for function by morpholino-mediated knockdown, two genes

appeared to be required for the function of motile cilia in all tissues except the otic vesicle, whereas knockdown of another two (*arhgef18b* and *ect2l*, coding for guanine nucleotide exchange factors) gave defects that were specific to the ear and Kupffer's vesicle (Choksi et al., 2014). These results highlight potential commonalities between the motile cilia in the ear and those in Kupffer's vesicle, both of which have rotational movement.

As expected for tissues expressing *foxj1b*, the zebrafish otic placode and vesicle are prominent sites of expression for many genes known to be required for ciliogenesis and ciliary motility (see (Stooke-Vaughan et al., 2012) and references within). These include genes coding for dyneins and dynein axonemal assembly factors (DNAAFs) (Hjeij et al., 2014; Jaffe et al., 2016; Jerber et al., 2014; Sullivan-Brown et al., 2008; van Rooijen et al., 2008; Yamaguchi et al., 2018; Yu et al., 2008). A comprehensive expression analysis of dynein axonemal heavy chain (*dnah*) genes in the zebrafish revealed several expressed in the ear, including one (*dnah9l*) that was ear-specific (Yamaguchi et al., 2018). The tissue specificity of the *dnah* expression patterns appears to correlate with the type of ciliary movement present (oscillatory or rotational): notably, the subset of *dnah* genes expressed in the ear largely seems to overlap with that expressed in the floorplate and Kupffer's vesicle, again highlighting tissues with rotational ciliary movement (Yamaguchi et al., 2018). All four zebrafish PIH (Protein interacting with Hsp90) genes are also expressed in the otic placode or vesicle, some very strongly (Yamaguchi et al., 2018). PIH gene products are DNAAFs implicated in the pre-assembly of dynein arms prior to their transport into cilia. Transcripts for components of the radial spokes of the axoneme (leucine-rich repeat-containing protein 23, *lrrc23*) and radial spoke head proteins (*rsph9* and *rsph4a*) are also expressed in the zebrafish otic placode during somite stages (Han et al., 2018; Sedykh et al., 2016).

The role of zebrafish otic cilia in otolith formation

Many organisms, both invertebrate and vertebrate, sense gravity and other linear acceleration through an inertial mass coupled to a mechanosensitive sensory cell type, which is often ciliated. In ctenophores (comb jellies), for example, lithocytes—entire cells whose cytoplasm harbours a membrane-bound concretion—are actively transported from the base to the tip of sensory cilia in a truly astonishing example of ciliary surface transport (Noda and Tamm, 2014) (and references within). In teleost fish, the inertial mass is the otolith, a dense crystalline aggregate of calcium carbonate and protein that sits over hair cells in the sensory maculae, embedded within an extracellular matrix, the otolithic membrane (for a recent review, see (Schulz-Mirbach et al., 2019)). Otoliths grow by daily incremental deposition of otolithic material: sectioning through an otolith thus reveals rings that can be used to

determine the age and life history of a fish (Campana and Neilson, 1985), just as in the trunk of a tree.

Both kinocilia and motile cilia are implicated in the earliest stages of otolith formation in the zebrafish, as summarised in Figure 2. Otolith precursor particles are initially found distributed throughout the otic vesicle lumen, but adhere specifically to the tips of kinocilia on the first hair cells to differentiate in the ear (named tether cells for this reason) (Riley et al., 1997; Stooke-Vaughan et al., 2012; Yu et al., 2011). The composition of the particles has not been fully described, but they are likely to contain Cadherin11 (Clendenon et al., 2009), Otoconin90 (Petko et al., 2009; Thiessen et al., 2019) and Starmaker (Söllner et al., 2003; Söllner et al., 2004). At the kinociliary tip, the bound otolith precursor particles serve as a nucleation site for deposition of further layers of protein and mineral (calcium carbonate), and thus the tether kinocilia become partially embedded within the structure of the otolith. Efficient production or nucleation of the precursor particles, in both zebrafish and medaka, appears to require the activity of the enzyme polyketide synthase (Hojo et al., 2015; Lee et al., 2019; Thiessen et al., 2019). Growing zebrafish otoliths incorporate a number of proteins, including Otolith Matrix Protein-1, Sparc, Starmaker and others (Kalka et al., 2019; Kang et al., 2008; Murayama et al., 2005; Rotllant et al., 2008). Exciting progress is being made in elucidating the mechanism of otolith biomineralisation, which is dependent on activity of the calcium-binding protein Starmaker (Kalka et al., 2019; Söllner et al., 2003; Wojtas et al., 2015) and the conserved proton-selective ion channel Otopetrin 1 (Hughes et al., 2004; Saotome et al., 2019; Söllner et al., 2004; Tu et al., 2018).

A defect in otolith formation is a signature phenotype of almost all zebrafish ciliary mutants and morphants described to date, including those with a loss of ciliary motility (Gao et al., 2010; Han et al., 2018; Jin et al., 2014; Jurisch-Yaksi et al., 2013; Neugebauer et al., 2009; Panizzi et al., 2007; Pathak et al., 2011; Stooke-Vaughan et al., 2012; Wilkinson et al., 2009; Yu et al., 2011). This observation led to the idea that ciliary motility must somehow be critical for otolith formation. As discussed above, ciliary flow can readily be demonstrated in the zebrafish ear, and various interpretations have been proposed for its function: that it agitates and distributes the otolith precursor particles, preventing their premature aggregation (Riley et al., 1997; Wu et al., 2011; Yu et al., 2011), that it creates an advection zone to draw free precursor particles towards the nucleating otolith (Wu et al., 2011), or that it contributes to otolith shape (Wu et al., 2011). Nevertheless, the otolith phenotype of ciliary mutants is often mild, variable and incompletely penetrant: a typical defect is the presence of three otoliths rather than two, which may resolve over time to give the normal pattern.

A breakthrough in understanding came from the unexpected observation that homozygous maternal-zygotic (MZ) mutants for an intraflagellar transport (IFT) gene, *ift88*, which lack cilia altogether (Huang and Schier, 2009), have a similarly mild otolith phenotype (Stooke-Vaughan et al., 2012) (Fig. 1E,F). On close inspection, the otoliths in MZ*ift88*^{tz288b} mutants formed close to the surface of the sensory epithelium, but still nucleated on time at the poles of the ear, in close proximity to the tether cells, which are present but lack kinocilia in this mutant. This suggested that both the immotile tether kinocilia and ciliary motility are, in fact, dispensable for otolith formation. Notably, premature aggregation of otolith precursor particles was not observed in either MZ*ift88*^{tz288b} mutants (lacking cilia altogether) or in the *lrrc50* (*dnaaf1*) mutant, which lacks ciliary motility (Fig. 1C,D) (Stooke-Vaughan et al., 2012; Sullivan-Brown et al., 2008; van Rooijen et al., 2008), and so the proposition that ciliary flow acts to prevent premature aggregation of otolith precursor particles seems unlikely.

A much more striking otolith phenotype—quite distinct from that of the ciliary or biomineralisation mutants—results from the lack of tether cells in the zebrafish ear. Injection of an *atoh1b* morpholino into wild-type embryos, which prevents the formation of tether cells, but does not compromise ciliogenesis or ciliary motility, disrupts the early stages of otolith formation completely: otolith precursor particles remain distributed throughout the ear. Here, they become larger than normal and appear birefringent, demonstrating that the process of otolith biomineralisation does not require attachment to kinocilia. Eventually, the crystalline particles coalesce to form a single untethered otolith (Stooke-Vaughan et al., 2012). This observation strongly suggested that it is the presence of tether cells, rather than cilia or cilia motility per se, that is essential for otolith development, implicating the function of a hair cell-specific factor or factors in the initial tethering step. Such a factor, perhaps produced by the tether cells, might become transported to the kinociliary tip.

In a quest to identify the otolith precursor-binding factor, we and others turned to mutants that phenocopied the *atoh1b* morphant phenotype. Isolated in forward mutagenesis screens on the basis of their phenotype—a single otolith, rather than two, in each ear—there were several promising candidates, initially given the charming names of *einstein* (German: one stone) (Whitfield et al., 1996), *rock solo* (Roberts et al., 2017) and *monolith* (Riley and Grunwald, 1996). Both the *einstein* (Stooke-Vaughan et al., 2015) and *rock solo* (Roberts et al., 2017) mutants were found to disrupt the gene *otogelin*, which codes for a large glycoprotein. Otogelin remains the best candidate to date for an otolith precursor-binding factor. Although expression of *otogelin* mRNA is not restricted to hair cells (Stooke-Vaughan et al., 2015), staining with an anti-Otogelin antibody suggests that the protein itself becomes concentrated at the distal half of the tether kinocilia (Thiessen et al., 2019). How Otogelin

gets there is not currently understood. It is possible that one or more adhesion proteins, trafficked to the kinociliary tip, help to localise it there. In this context, it is interesting to note that the integrin Itga8 and protocadherin Pcdh15 localise in a complex at the kinociliary tip of zebrafish lateral line hair cells (Goodman and Zallocchi, 2017). In chick auditory hair cells, transport of Pcdh15 to the kinociliary tip is dependent on the IFT machinery and FGFR1 activity (Honda et al., 2018). Zebrafish lateral line and amniote auditory kinocilia, of course, do not bind otoliths, but it is possible that a tether cell-specific factor contributes to the localisation of both Otogelin protein and otolith precursor particles at the tips of tether kinocilia via a similar mechanism.

Note that ear and otolith morphology vary enormously between different teleosts, and it is not known whether otoliths are nucleated in the same way in other species. Otolith formation was reported to be normal in the medaka mutant *kintoun*, which lacks ciliary motility due to mutation in a gene coding for a dynein arm pre-assembly factor (Hojo et al., 2015). As in zebrafish, this argues against an essential requirement for ciliary motility in otolith formation in this species. In the zebrafish, after the initial nucleation step, it is unlikely that cilia play a further role in otolith growth. Additional hair cell kinocilia do not all contact the otolith directly as the tether kinocilia do (Fig. 1G), and total otolith growth is unaffected by focal laser ablation of motile cilia after otolith nucleation (Wu et al., 2011). As the sensory patch and otolith develop further, a layer of extracellular matrix, the otolithic membrane, is deposited on the apical surface of the sensory patch, and it is this structure that now tethers to otolith to its cognate sensory epithelium (Stooke-Vaughan et al., 2015). Several protein components of the otolith and otolithic membrane in zebrafish are conserved with those of otoconia and the otoconial membrane, respectively, in vestibular end organs of the mammalian inner ear (reviewed in (Lundberg et al., 2014)). Here, multiple small otoconia are present rather than the large individual otoliths found in bony fish. A role for cilia in the initial formation of mammalian otoconia has not been described, to my knowledge.

Length control of cilia in the zebrafish ear and lateral line

The length of cilia in the zebrafish ear is both dynamic and sensitive to perturbation. All cilia in the ear are initially of similar length at the very earliest otic vesicle stages (19 somites), but rapidly become differentiated from one another (Stooke-Vaughan et al., 2012). By the 21-somite stage, cilia at the poles of the ear (tether cell kinocilia and motile cilia) are longer than other cilia throughout the vesicle lumen. Within a few hours, the motile cilia have regressed in length, and by 24 hpf are about half the length of the kinocilia (Stooke-Vaughan et al., 2012). Kinocilia of different sensory hair cell types can also be distinguished on the basis of length. At 24–25 hpf, the tether kinocilia in the presumptive sensory maculae are about 8 μm

long, with the distal 3 µm embedded in the otolith (Stooke-Vaughan et al., 2012). Kinocilia of the crista hair cells are the longest, projecting up to 30 µm into the lumen of the ear at 4 days post fertilisation (dpf) (Imtiaz et al., 2018; Stawicki et al., 2014) (Fig. 1H). Kinocilia on lateral line hair cells are 14–20 µm long in the zebrafish larva, occupying about half the length of the gelatinous cupula that covers them (Imtiaz et al., 2018; Kindt et al., 2012; McHenry and van Netten, 2007). In adult teleost fish, kinociliary length can also vary in different regions of the sensory maculae, as shown for the blue gourami *Trichogaster trichopterus* (Popper and Hoxter, 1981).

The length of a cilium depends on a balance between kinesin-driven anterograde (IFT-B) and dynein-driven retrograde (IFT-A) transport along the axoneme. Mutations in genes coding for proteins involved in ciliogenesis or IFT can thus disrupt cilia length, resulting in either shorter or longer cilia than normal. Disruption of *foxj1b* results in shorter motile cilia and kinocilia in the ear, whereas transgenic mis-expression results in the development of long motile cilia throughout the otic vesicle (Yu et al., 2011). Ubiquitous transgenic expression of a GFP-tagged murine Arl13b protein in the zebrafish, whilst labelling most cilia very effectively (Borovina et al., 2010), also increases their length, and is reported to disrupt both ciliary motility and otolith formation in the ear (Lu et al., 2015). Mutation of *arl13b* results in shorter lateral line kinocilia; cilia in the ear were not examined (Stawicki et al., 2016). Cilia in all tissues, including the ear, are missing in maternal-zygotic mutants for the IFT-B gene *ift88* (Huang and Schier, 2009) (see above) and the ciliogenesis gene *ta3* (Ben et al., 2011), while disruption of another IFT-B gene, *ift46* (Lee et al., 2015), or the IFT-A gene *ift122* (Boubakri et al., 2016) both result in fewer or abnormal cilia in many tissues, including the ear and lateral line.

Different ciliary types in the zebrafish ear also have different genetic requirements for some gene products. Mutation of the *abcc4* gene, which codes for a transporter involved in prostaglandin signalling, appears to disrupt short cilia, but not tether kinocilia, in the early otic vesicle (Jin et al., 2014). The long kinocilia of the cristae seem particularly resistant to genetic perturbation of kinesin subunit genes, which may indicate the existence of additional crista-specific IFT-B components. Crista cilia were unaffected by mutation of *kif3b*, which codes for a heterotrimeric kinesin II subunit, whereas cilia in maculae were lost altogether (Zhao et al., 2012). Short crista kinocilia were also able to develop in a strong loss-of-function *kif3a* mutant, which otherwise eliminates cilia in maculae of the ear and in several other tissues, including the lateral line (Pooranachandran and Malicki, 2016). Cilia length in the zebrafish ear is also governed by the activity of Crumbs genes (Hazime and Malicki, 2017; Omori and Malicki, 2006). mRNA for *crb3a* is strongly and almost exclusively

expressed in the ear; the Crb3a protein localises to the base of hair cell kinocilia at the otic vesicle stage, and to the basal body of kinocilia in adult hair cells. Morpholino-mediated knockdown of *crb3a* results in shorter kinocilia in maculae (Omori and Malicki, 2006), but interestingly, in loss-of-function mutants for *crb3a* or *crb2b*, cilia in the cristae get longer (Hazime and Malicki, 2017).

The role of kinocilia and ciliary proteins in hair cell planar polarity, stereociliary bundle assembly and mechanosensitivity

The eccentric position of the kinocilium on the hair cell apical surface defines the directional sensitivity of the cell: deflection of the stereociliary bundle in the direction of the kinocilium depolarises the cell, whereas deflection in the opposite direction hyperpolarises the cell (Kindt et al., 2012) (and references within). This can be demonstrated in the zebrafish larva by imaging of lateral-line hair cells labelled with the calcium indicator GCaMP6s after deflection of kinocilia in the neuromast cupula with a glass fibre or fluid jet (Kindt et al., 2012; Lukasz and Kindt, 2018). The kinociliary axoneme is arranged in a polar fashion with respect to the hair bundle, with the first doublet microtubule (number 1) located on the side facing the stereociliary bundle (Flock and Duvall, 1965).

In mammalian auditory hair cells, the kinocilium plays a role in orientation of the hair cell stereociliary bundle, together with planar cell polarity genes (Jones et al., 2008) (and references within). However, there is currently no strong evidence to suggest a role for kinocilia in establishing hair bundle polarity in zebrafish hair cells, which are thought to be more closely related to mammalian vestibular hair cells. Normal stereocilia polarity was observed in both ear and lateral line hair cells in the zebrafish *ift88*^{tz288b} mutant, which has missing or stunted kinocilia (Kindt et al., 2012; Stooke-Vaughan, 2013). This contrasts with the obvious disruption of planar cell polarity in mammalian auditory hair cells in the murine *Ift88* mutant, where mis-oriented and even circular stereociliary bundles were observed in the organ of Corti (Jones et al., 2008). Although hair bundle polarity is not affected in zebrafish zygotic *ift88*^{tz288b} mutants, hair bundle morphology is abnormal, and fewer hair cells form stereocilia (Blanco-Sánchez et al., 2014; Stooke-Vaughan, 2013). Analysis of individual mutant phenotypes and assays for protein colocalisation revealed that the Ift88 protein helps to stabilise a complex of the Usher Syndrome 1 (USH1) proteins Cdh23, Harmonin and MyoVIIa in the endoplasmic reticulum (ER), ensuring their correct trafficking for assembly of the stereociliary bundle. Disruption of any one component of the complex can result in ER stress and eventual hair cell death (Blanco-Sánchez et al., 2014). Thus, Ift88 and possibly other ciliary proteins have additional roles in protein or vesicular traffic within hair cells.

Kinocilia of immature zebrafish lateral line hair cells also have an early role in mediating hair cell mechanosensitivity, a property restricted to the stereociliary bundle in mature hair cells (Kindt et al., 2012). Small but robust responses were measured in hair cells with morphologically immature stereociliary bundles, but with an opposite polarity to responses found in mature hair cells. By using the *ift88*^{tz288b} mutant, in which all cilia are absent or stunted, the authors were able to show that these early responses were mediated by kinocilia. The loss of responses in mutants for the cadherin genes *cdh23* and *pcdh15*, together with pharmacological disruption, demonstrated that the early polarity-reversed responses were dependent on the integrity of kinocilial links between the kinocilium and adjacent stereocilia (Kindt et al., 2012).

The role of kinocilia and ciliary proteins in sensitivity to aminoglycoside ototoxicity

Aminoglycoside antibiotics are potent ototoxins, killing sensory hair cells in both zebrafish and mammals (Harris et al., 2003) (and references within). As a result, off-target damage to the auditory and vestibular systems can be a considerable clinical problem in patients treated with aminoglycosides for serious acute or chronic bacterial infections. Work in the zebrafish has revealed that cilia and ciliary proteins appear to play a role in the uptake and/or toxicity of these compounds. Mutations in anterograde IFT-B, retrograde IFT-A and ciliary transition zone genes in the zebrafish all confer protection against neomycin ototoxicity (Owens et al., 2008; Stawicki et al., 2016; Stawicki et al., 2019). In most IFT mutants, where ciliary morphology and hair cell mechanotransduction are compromised, neomycin uptake into the cell was disrupted (Stawicki et al., 2016; Stawicki et al., 2019). However, mutations in transition zone genes (*cc2d2a, mks1, cep290*)—where ciliogenesis, mechanotransduction and neomycin uptake were unaffected—still conferred partial protection, suggesting that transition zone proteins may modulate trafficking or metabolism of neomycin once it has entered the hair cell (Stawicki et al., 2016). These studies are important for our understanding of aminoglycoside uptake and toxicity in hair cells, with implications for the search and design of new otoprotective compounds or alternative antibiotic agents.

Role of cilia in signalling in the zebrafish ear

Cilia are well known to act as cellular antennae for the reception of various extracellular signals (reviewed in (Nachury and Mick, 2019)). As the zebrafish ear is a prominent site of expression for many ciliary genes, it is perhaps surprising that few studies to date have addressed the contribution of cilia to cell signalling in this tissue. In zebrafish, the complete loss of cilia in MZ*ift88*^{tz288b} mutants, and depletion of cilia in *igu* (*dzip1*) mutants, reduces levels but expands the reach of Hedgehog pathway activity, rather than eliminating it (Huang and Schier, 2009). This finding corroborates preliminary observations of the later ear

phenotype of MZ*ift88* mutants, which have morphological abnormalities that resemble those of *igu* (*dzip1*) mutants (Stooke-Vaughan, 2013), but are not a close phenocopy of the mirror-symmetric double-anterior ears generated by a strong loss of Hh signalling (Hartwell et al., 2019) (and references within). Signalling via cilia or cilia-driven flow therefore does not appear to play a major role in otic axial patterning in the otic vesicle. This contrasts with the similarly-sized Kupffer's vesicle, where ciliary-driven flow is essential for left-right patterning of the embryo (Sampaio et al., 2014) (and references within). However, given the mix of motile and non-motile ciliary types in both the early otic vesicle and Kupffer's vesicle (Sampaio et al., 2014), their shared dependence on Wnt signalling (Caron et al., 2012), and the overlap in their ciliary transcriptome profile (Choksi et al., 2014; Yamaguchi et al., 2018), this remains an interesting area to explore further.

The active phosphorylated form of type II calcium/calmodulin kinase CaMK-II is expressed in the zebrafish ear in hair cell kinocilia, most strongly at their base, where it may localise to the ciliary transition zone and/or stereociliary bundle (Rothschild et al., 2013). Interestingly, disruption of CaMK-II activity—either pharmacologically, or via morpholino-mediated knockdown of *camk2g1*—results in a disruption of Delta-Notch signalling, and the development of supernumerary tether cells. Morphants and treated embryos had otolith defects that resemble those of *mib1*^{ta52b} (loss of Notch signalling) mutants at 24 hpf, where multiple tether kinocilia make contact with otolithic material (Rothschild et al., 2013; Stooke-Vaughan et al., 2012). At later stages (30–72 hpf), *camk2g1* morphants or treated embryos showed a phenotype characteristic of ciliary mutants, with ectopic fused or third otoliths in the ear (Rothschild et al., 2013). Thus, signalling via calcium, CaMK-II, Delta and cilia appear to be closely linked in the zebrafish ear.

Conclusion

Our understanding of ciliary structure and function has come a long way in the last 50 years, although many of the stunning electron micrographs of kinocilia from the 1960s (Flock and Duvall, 1965; Flock and Wersäll, 1962; Hamilton, 1969) are still among the clearest in the literature, and repay careful attention. There remains enormous interest in cilia, not least for their important link to rare genetic disease: mutations in the human orthologues of many of the genes discussed here result in ciliopathies that affect quality of life and are challenging to diagnose and treat. Sensorineural deafness is a feature of several of these syndromes, underlining the need to understand cilia function in the inner ear. Here, the zebrafish holds promise as a model system, partly because the otoliths are a highly visible proxy readout of ciliary integrity. As technologies to achieve tissue-specific disruption of zebrafish gene function improve (see, for example, (Kirchgeorg et al., 2018; Savage et al., 2019; Yamaguchi

et al., 2019)), new ways of working to analyse ciliary function in the ear will become possible, where—unlike in the brain or kidney—loss of function should not compromise adult viability. Cilia in the zebrafish ear are also interesting in their own right; key among the problems remaining to be solved is an understanding of the unique protein or lipid composition at the tip of the tether kinocilium that distinguishes it from other cilia, allowing otolith precursor particles to bind there so precisely and robustly. Comparison of the rotational motile cilia in the otic vesicle to those in Kupffer's vesicle or the floorplate may also lead to general insights into mechanisms of cilia-driven flow and its consequences for developmental patterning.

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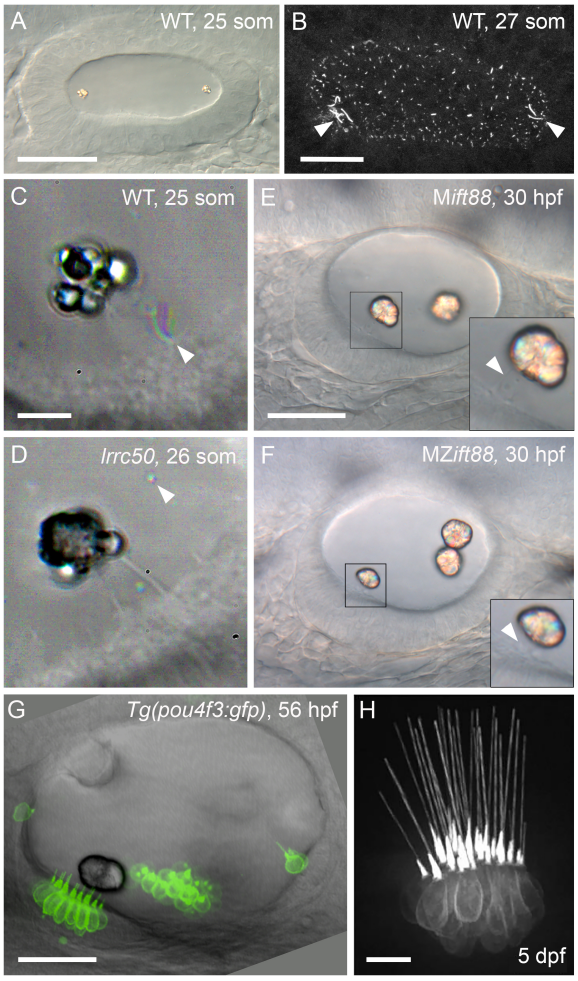

Figure 1. Cilia and otolith formation in the zebrafish ear.

A. Differential interference contrast (DIC) image of the wild-type otic vesicle at the 25-somite stage, showing the two birefringent otoliths. Scale bar, 40 µm.
B. Lumen of the otic vesicle at the 27-somite stage, stained with an anti-acetylated tubulin antibody. The arrowheads mark longer tether kinocilia and motile cilia at the anterior and posterior poles of the otic lumen. Scale bar, 20 µm.
C. Time-to-colour merged image of the nascent otolith in a phenotypically wild-type embryo at the 25-somite stage (sibling of the embryo in D). The colour (arrowhead) indicates a motile cilium near the otolith. Scale bar, 10 µm (also applies to D).
D. Time-to-colour merged image of the nascent otolith in a homozygous *lrrc50* (*dnaaf1*) mutant. The otolith has adhered to the tips of two kinocilia (clearly visible in this image), despite the lack of ciliary motility. The arrowhead marks an otolith precursor particle.
E. Phenotypically wild-type embryo (sibling of the embryo in F) lacking maternal *ift88* contribution but with normal zygotic *ift88* function at 30 hpf. Two otoliths are present, attached to kinocilia (arrowhead, inset). Scale bar, 30 µm (also applies to F).
F. Typical otolith phenotype for a ciliary mutant, in this case an embryo lacking both maternal and zygotic *ift88* function. Three otoliths are present, but still localise to the anterior and posterior poles of the ear. The anterior otolith sits directly on the hair cell stereociliary bundles (arrowhead, inset); all cilia are absent.
G. Transgenic zebrafish otic vesicle at 56 hpf expressing GFP in hair cells. Note that the kinocilia of hair cells in the utricular macula do not all contact the anterior otolith (bottom left).
H. Image of a crista in the same transgenic line at 5 dpf. Note the long straight kinocilia. Scale bar, 10 µm.

A, B, E, F and G are lateral views with anterior to the left. Abbreviations: dpf, days post fertilisation; hpf, hours post fertilisation; M, maternal; MZ, maternal-zygotic; som, somite stage; WT, phenotypically wild type. A, C, D and E are reproduced from (Stooke-Vaughan, 2013); B and F are reproduced from (Stooke-Vaughan et al., 2012); G is reproduced from (Maier et al., 2014); H is reproduced from (Stawicki et al., 2014).

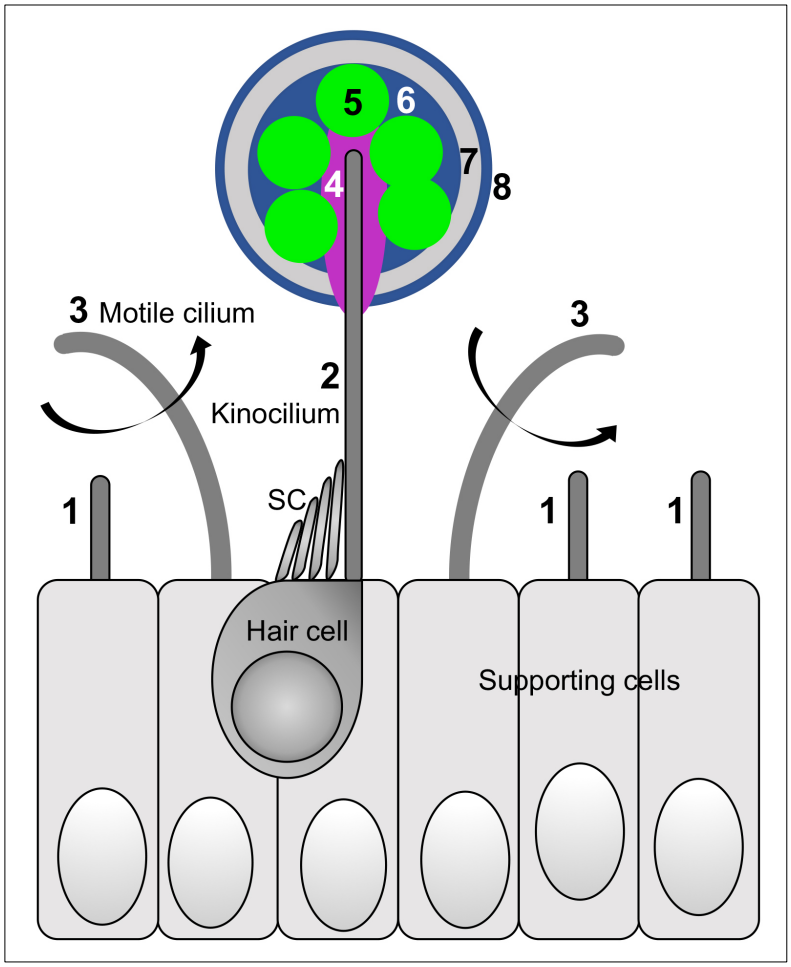

Figure 2. Schematic diagram summarising the main early steps in zebrafish otolith formation discussed in this review.

A single sensory hair cell is shown for simplicity, but tether cells normally form in pairs (see Fig. 1D). Not to scale. SC, stereocilia. For details and supporting references, see the text.

Key:

1. Short, immotile cilia, present on most otic epithelial cells
2. Tether kinocilium, present on each of the first sensory hair cells to develop in the ear
3. Motile cilia; these contribute to the accuracy of otolith precursor particle tethering
4. Extracellular proteins, possibly bound to the kinociliary tip, required for otolith precursor particle tethering: Otogelin (and others?)
5. Otolith precursor particles, thought to contain Cadherin11, Otoconin90 and Starmaker
6. Otolith proteins incorporated after initial nucleation, including Otolith Matrix Protein-1, Sparc, Starmaker and others
7. Biomineralisation: deposition of crystalline calcium carbonate, requiring the activity of Starmaker and Otopetrin 1
8. Further layers added daily throughout life

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