ABSTRACT  Recent years have seen a renaissance of investigation into the mechanisms of inner ear development. Genetic analysis of zebrafish has contributed significantly to this endeavour, with several dramatic advances reported over the past year or two. Here, we review the major findings from recent work in zebrafish. Several cellular and molecular mechanisms have been elucidated, including the signaling pathways controlling induction of the otic placode, morphogenesis and patterning of the otic vesicle, and elaboration of functional attributes of inner ear.

INTRODUCTION  One of the greatest advantages of studying inner ear development in the zebrafish is the optical clarity of its embryo, enabling direct observation of internal anatomic structures. This facilitates analysis of normal developmental processes and makes it much easier to detect and interpret changes caused by mutations or experimental manipulations. Mutations are efficiently induced in zebrafish by using a variety of mutagens, including ethyl-nitrosourea (ENU), gamma rays, and viral insertion, each of which has its own characteristic advantages (Detrich et al., 1999). Several mutagenesis screens have provided a rich assortment of randomly induced mutations affecting inner ear development. Several of these mutant loci have been cloned (Table 1), and analysis of their phenotypes has uncovered detailed mechanisms of otic development and physiology.

It has also proven feasible to identify mutations in previously cloned genes by screening for ENU-induced point mutations that fail to complement defined deletions that remove genes of interest (Appel et al., 1999). It is not possible currently to perform directed gene-targeting analogous to that used in the mouse, but several alternative methods are available for conducting “reverse genetics,” including the use of antisense morpholino (MO) oligonucleotides (Nasevicius and Ekker, 2000). These are short, stable sequences designed to hybridize to specific mRNAs at or near the translation initiation codon, thereby blocking translation of the corresponding protein.

In this review, we aim to give a comprehensive coverage of the development of the zebrafish ear, highlighting the roles of genes identified by mutational studies and those with specific patterns of expression in the ear. These recent data complement detailed descriptions of the development and structure of the wild-type zebrafish ear (Waterman and Bell, 1984; Platt, 1993; Haddon and Lewis, 1996; Whitfield, 2000; Bang et al., 2001; Bever and Fekete, 2002), which are summarized briefly below. Stages are given according to Kimmel et al. (1995) or as hours post fertilisation (hpf) or days post fertilisation (dpf) at 28.5°C. Note that unless otherwise stated, all the zebrafish mutations described here are recessive; therefore, descriptions of mutant phenotypes refer to embryos homozygous for the mutation of interest.

INTRODUCTION TO ZEBRAFISH EAR ANATOMY  The ear develops from an ectodermal thickening, the otic placode, visible on either side of the hindbrain from mid-somite stages. In the zebrafish, this placode cavi-
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Gene and zebrafish cloning reference</th>
<th>Type of protein</th>
<th>Ear phenotype in zebrafish homozygous mutant</th>
<th>Murine model (knockout/mutation)</th>
<th>Human disease or syndrome</th>
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<tr>
<td>colourless (cls)</td>
<td>sox10 (Dutton et al., 2001b)</td>
<td>HMG box transcription factor</td>
<td>Small ear, abnormal sensory patches, rudimentary semicircular canal formation (Whitfield et al., 1996; Dutton et al., 2001a)</td>
<td>Dominant megacolon (Herbart et al., 1998; Southard-Smith et al., 1998)</td>
<td>Usher syndrome type IB (and some forms of nonsyndromic deafness) (Weil et al., 1995)</td>
</tr>
<tr>
<td>dlAdx2</td>
<td>deltaA (Appel et al., 1999)</td>
<td>DSL family Notch ligand</td>
<td>Neurogenic (excess hair cells) (Riley et al., 1999)</td>
<td>Eya1bw (Johnson et al., 1999); reviewed in Libby and Steel (2000)</td>
<td>Branchio-Oto-Renal syndrome (Abdelhak et al., 1997)</td>
</tr>
<tr>
<td>dog-eared (dog)</td>
<td>eya1 (Kozlowski et al., in preparation)</td>
<td>Transcription cofactor</td>
<td>Loss of cristae; abnormal maculae, semicircular canal system and neurogenesis; cell death (Whitfield et al., 1996; Kozlowski et al., in preparation)</td>
<td>Pax2lneu (Favor et al., 1996); targeted disruption (Torres et al., 1996)</td>
<td>Renal-coloboma syndrome (Sanyanusin et al., 1995)</td>
</tr>
<tr>
<td>jekyll (jek)</td>
<td>ugdh1 (Walsh and Stainier, 2001)</td>
<td>UDP-glucose dehydrogenase</td>
<td>Failure of epithelial projections to form the semicircular canal system (Neuhauss et al., 1996)</td>
<td>shaker1 (Gibson et al., 1995); reviewed in Libby and Steel (2000)</td>
<td>Usher syndrome type IB (and some forms of nonsyndromic deafness) (Weil et al., 1995)</td>
</tr>
<tr>
<td>mariner (mar)</td>
<td>myoVIIA (Ernest et al., 2000)</td>
<td>Unconventional myosin</td>
<td>Deaf; splayed stereociliary bundles (Nicolson et al., 1998)</td>
<td>Not described</td>
<td>Renal-coloboma syndrome (Sanyanusin et al., 1995)</td>
</tr>
<tr>
<td>no isthmus (noi)</td>
<td>pax2.1 (Lun and Brand, 1998)</td>
<td>Paired/homeodomain</td>
<td>Weakly neurogenic (excess hair cells) (Riley et al., 1999)</td>
<td>Not described</td>
<td>Usher syndrome type IB (and some forms of nonsyndromic deafness) (Weil et al., 1995)</td>
</tr>
<tr>
<td>neckless (nls)</td>
<td>raldh2 (Begemann et al., 2001)</td>
<td>Dehydrogenase retinoic acid pathway</td>
<td>Not described</td>
<td>Not described</td>
<td>Renal-coloboma syndrome (Sanyanusin et al., 1995)</td>
</tr>
<tr>
<td>spiel ohne grenzen (spg)</td>
<td>pou2 (Belting et al., 2001; S. Burgess, personal communication)</td>
<td>POU-domain transcription factor</td>
<td>Small otic vesicle (BBR, unpublished data)</td>
<td>Not described</td>
<td>Usher syndrome type ID (Bolz et al., 2001)</td>
</tr>
<tr>
<td>valentino (val)</td>
<td>krml1 (Moens et al., 1998)</td>
<td>b-zip MAF transcription factor</td>
<td>Small otic vesicle (BBR, unpublished data); later cystic (Moens et al., 1998)</td>
<td>kreisler (Cordes and Barsh, 1994)</td>
<td>Usher syndrome type ID (Bolz et al., 2001)</td>
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tates to form a hollow ball of epithelium, the otic ves-
icle, from which all structures of the membranous lab-
yrinth and the neurons of the statoacoustic (VIIIth)
ganglion arise. The main components of the larval ze-
brafish ear are illustrated in Figure 1. Five sensory
patches of epithelium, two maculae and three cristae,
develop in the embryonic and larval ear. These are thickened regions of epithelium consisting of arrays of sensory hair cells and supporting cells. The two maculae are the first sensory patches to develop. Each is overlain by an otolith, a crystalline deposit of calcium carbonate and protein; the two otoliths are easily visible within the ear lumen under a dissecting microscope. The rostral sensory patch will occupy the utricle of the adult ear and is thought to have a primarily vestibular role. The second macula lies in a caudalmedial position, will occupy the saccule, and has a primarily auditory role. Two further maculae, the lagena and macula neglecta, arise during larval stages. The three cristae, sensory patches for each of the three semicircular canals, differentiate slightly later than the utricular and saccular maculae. The canals themselves are formed by projections of epithelium that fuse in the centre of the ear to generate pillars of tissue, forming the hubs of the developing canals.

EARLY EAR DEVELOPMENT
Origin of Ear Placodal Ectoderm

Fate mapping studies have revealed the early origins of the zebrafish ear at the 50% epiboly/shield stage (late blastula/early gastrula; Kozlowski et al., 1997). The ears arise from groups of cells on the ventral side of the embryo, one on either side of the ventral midline, located between 45 degrees and 65 degrees from the margin (Fig. 2). At this stage, the fate map is not precise and the presumptive ear territory overlaps with those contributing to other tissues, including some of placodal origin (lens, olfactory epithelium, lateral line, and cranial ganglia). This finding indicates that cells may shift positions at later stages and that placodal cells are not specified by the shield stage. In general, however, a clear axial organisation is apparent, with the animal-vegetal position within the labelled region corresponding to the future rostrocaudal position of its derivatives. The map also indicates that tissue fated to form ear is originally not in direct contact with prospective ear inducing tissues (mesendoderm and hindbrain), but is brought into contact with these during gastrulation.

Placode Induction

The ear placode becomes morphologically distinguishable with differential interference contrast (DIC) optics at 13.5–14 hpf (9–10 somites; Kimmel et al., 1995). The developing placode shows high levels of intracellular calcium at this time (Créton et al., 1998). The presumptive ear field, however, can be visualized at much earlier stages by virtue of its expression of several marker genes (see below). Mutants with early ear phenotypes are being used to dissect the signals that induce the formation of otic tissue. No single gene mutants lacking a placode altogether have yet been isolated, suggesting that there may be no one “master” gene responsible for placode induction. Instead, combinations of factors appear to be involved, and members of the fibroblast growth factor (FGF) family of signaling molecules are currently the best candidates (see below).

Molecular markers of placode induction. By late gastrulation (90% epiboly onward), gene expression patterns corroborate the fate map data, marking a “preplacodal ectoderm field” around the edges of the rostral neural plate (Baker and Bronner-Fraser, 2001). Genes including dlx3, dlx7, eya1, and six4.1 are expressed here (Akimenko et al., 1994; Ellies et al., 1997;
Sahly et al., 1999; Kobayashi et al., 2000; Fig. 4), marking precursor cells not only of the ear, but also of several other cranial placodes. During the early somite stages, expression of genes in the preplacodal domain becomes refined and begins to mark individual placodes of thickened ectoderm.

The earliest ear-specific markers include members of the pax2/5/8 family of transcription factor genes (Pfeffer et al., 1998). Indeed, all known members of this family are expressed in the developing otic placode or preplacode. The first pre-otic marker, pax8, is induced in the otic primordium during the latter half of gastrulation, between 8.5 and 9 hpf (85% epiboly). Subsequently, pax2.1 is induced in the preplacode at around 11 hpf, and pax2.2 is induced at 13 hpf just before the onset of placode morphogenesis. Expression of pax8 gradually diminishes after the placode forms and can no longer be detected in the otic vesicle after 19 hpf (20 somites). pax5 is first induced in the otic placode by 17 hpf (16 somites) and is later confined to the developing utricular macula. Expression of pax2.1 becomes localized to ventromedial cells in the otic vesicle and is eventually restricted to sensory hair cells in both the maculae and cristae (Riley et al., 1999). pax2.2 is expressed in a similar pattern, albeit at lower levels. The dynamic and complex expression patterns of these genes have made them useful as markers of several definitive stages of pre-otic and otic development. Functions of pax genes, and other markers in the ear, are described in later sections.

Several other markers of the otic placode are known (Whitfield, 2000; Baker and Bronner-Fraser, 2001). Of particular interest are the claudin genes cldna and cldnb, members of the tetraspanin family of membrane proteins; cldna is the only completely ear-specific marker so far described, being expressed exclusively in the otic placode, preplacode, and vesicle from the end of gastrulation until at least 2 dpf (Kollmar et al., 2001). Expression of scyba, which codes for a CXC-type chemokine, is also strongly expressed in the otic placodes from 12 hpf (Long et al., 2000). Transcripts for the ETS-domain transcription factors etsn and pea3, and the FGF antagonist sprouty4, mark the otic placodes from early stages and appear to be direct transcriptional targets of FGF-mediated induction (Brown et al., 1998; Münchberg et al., 1999; Fürthauer et al., 2001; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001; see the Role of FGF Signaling section). Expression of these genes overlaps with that of pax2.1 (Raible and Brand, 2001). Competence and candidate inducing tissues: mesendoderm and hindbrain. For decades, investigators have attempted to elucidate the tissue interactions required for otic placode induction. Experimental embryologic studies in amphibian and avian embryos have shown that otic induction is a prolonged process involving interactions with a variety of tissues (Baker and Bronner-Fraser, 2001). The hindbrain, in particular, is thought to be a potent source of otic inducing factors. Subjacent mesoderm has also been implicated, although much less is known about which specific tissue is involved. Transplantation studies in zebrafish also support an inductive role for the hindbrain, as well as the germling, the site of mesoderm induction and involution. Specifically, grafting prospective hindbrain tissue to the ventral side of a shield stage embryo (near the germling) results in the induction of ectopic otic vesicles, whereas grafting to the animal pole (prospective forebrain) does not (Woo and Fraser, 1998). In contrast, transplanting nonaxial germling to the animal pole induces an ectopic hindbrain as well as accompanying otic placodes in host tissue (Woo and Fraser, 1997). These results suggest that competence to form otic placodes is widespread in the ectoderm at this stage, and that signals from nonaxial germling (mesendoderm) can strongly potentiate hindbrain-derived signals.

Although the above studies do not identify which mesodermal tissues participate in otic induction, the availability of numerous zebrafish mutations with tissue-specific defects has proven useful in this regard (Mendonsa and Riley, 1999). For example, although the notochord has been considered a strong candidate for an otic inducing tissue, mutations in no tail (ntl) or floating head (flh), which totally ablate chordamesoderm, do not detectably alter otic induction or subsequent otic development. In contrast, loss of zygotic One-eyed pinhead (Oep) function, which ablates prechordal mesoderm and all endoderm, leads to a modest delay of 30–60 min in induction of pre-otic markers. A much longer delay of 2–3 hr is observed in ntl/oep double mutants, which lack virtually all cephalic mesendoderm. The delay is specific for otic induction because several midbrain and hindbrain markers are expressed with normal timing (Mendonsa and Riley, 1999). Similar results are obtained by injection of oep-MO into wild-type embryos, which depletes both maternal and zygotic Oep function and ablates nearly all mesendoderm (Nasevicius and Ekker, 2000; Phillips et al., 2001). In such embryos, induction of pax8 is delayed by several hours, despite normal timing of gene expression in the hindbrain. These results suggest that prechordal and paraxial cephalic mesendoderm, although not essential for otic induction, are likely to play important roles in this process, a conclusion further supported by recent studies of FGF signaling (Ladher et al., 2000; Phillips et al., 2001). In the next sections, we discuss possible candidate signaling molecules for the inductive signals. Role of FGF signaling. At least two members of the FGF family of peptide ligands, Fgf3 and Fgf8, are strong candidates for otic inducing factors. Both genes are expressed in the primordium of rhombomere 4 (r4) by 8 hpf (75–80% epiboly; Phillips et al., 2001). In addition, fgf3 is expressed in prechordal and paraxial cephalic mesendoderm during the latter half of gastrulation. Thus, these genes are expressed at the right time and place to influence induction of pax8 in the otic anlagen by 9 hpf. The function of Fgf8 is disrupted by the acerebellar (ace) mutation, which was initially
identified in a large scale mutagenesis screen by virtue of morphologic defects in brain development (Brand et al., 1996; Reifers et al., 1998). Analysis of inner ear development shows that the ace mutation dramatically reduces the number of pre-otic cells expressing pax8 and pax2.1 and results in formation of small, abnormally patterned otic vesicles (Brand et al., 1996; Whitfield et al., 1996; Phillips et al., 2001). Mutant alleles of fgf3 have not yet been identified, but injecting wild-type blastulae with an fgf3-specific morpholino oligomer (fgf3-MO) reduces otic induction to nearly the same extent as does the ace mutation. Moreover, injecting fgf3-MO into ace embryos (to disrupt both fgf8 and fgf3) results in the near or total ablation of otic tissue (Phillips et al., 2001). Similar results are obtained by coinjecting fgf3-MO and fgf8-MO (Raible and Brand, 2001). These data indicate that Fgf3 and Fgf8 provide partially redundant functions required for induction of the otic placode.

Further evidence for redundant roles for Fgf3 and Fgf8 comes from analysis of expression of genes thought to be direct transcriptional targets of both signaling pathways. erm and pea3, which encode ETS domain transcription factors, are expressed in and around embryonic tissues that express fgf3 and/or fgf8 (Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001). Ectopic sources of either Fgf3 or Fgf8 are sufficient to induce erm and pea3 in surrounding cells. In contrast, disrupting either fgf3 or fgf8 strongly reduces expression of erm and pea3; disrupting both fgf3 and fgf8 abolishes their expression (Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001). Another FGF-induced gene, sprouty4 (spry4), is expressed in similar domains and is similarly dependent on Fgf3 and Fgf8 (Furthauer et al., 2001). Spry4 acts as an antagonist of FGF signaling and presumably serves to limit the duration or intensity of signaling. Over-expression of spry4 leads to a phenotype strongly resembling embryos deficient for both Fgf3 and Fgf8. Although otic vesicles are produced, they are extremely small. These studies provide a molecular link between Fgf3 and Fgf8 signal transduction and otic placode induction.

Fgf3 has been shown to play an important role in otic development in other vertebrate species. Misexpression of Fgf3 in the chick is sufficient to induce ectopic otic vesicles (Vendrell et al., 2000). Depletion of Fgf3 in cultured explants of chick periotic ectoderm specifically blocks formation of otic vesicles (Represa et al., 1991). A later role has been proposed in the mouse because targeted disruption of Fgf3 causes malformation of the otic vesicle but does not block otic induction (Mansour et al., 1993). However, redundant factors could partially compensate for loss of Fgf3 in the mouse, just as in zebrafish.

The activity of additional signals from mesendodermal sources is highlighted by the effects of mesendoderm ablation in Oep-depleted embryos (Phillips et al., 2001). In such embryos, fgf3 and fgf8 are expressed on time in the r4 anlage, yet otic induction is delayed by several hours. Depletion of Fgf3 alone does not cause such a delay, suggesting that mesendoderm provides additional factors that are critical for timely otic induction.

A clear example of an additional signal involved in chick otic induction is Fgf19. The expression pattern of Fgf19 is strikingly similar to that of Fgf3 (Mahmood et al., 1995; Ladher et al., 2000). Both genes are initially expressed in paraxial mesendoderm beneath the prospective otic ectoderm during gastrulation and are later expressed in the hindbrain and pharyngeal endoderm. In explant cultures, application of Fgf19 can induce only a few early otic markers in head ectoderm. However, application of Fgf19 plus the hindbrain factor Wnt8c can induce a full complement of otic markers. In addition, periotic mesoderm (presumably expressing Fgf3 and Fgf19) can induce a wide range of otic markers in head ectoderm. Thus Fgf19 interacts synergistically with a variety of other factors expressed in periotic tissues to induce otic differentiation (Ladher et al., 2000).

Whether similar interactions occur in zebrafish is not yet known. No homologs of Fgf19 have been described in zebrafish, but there are at least three gene products homologous to Wnt8. One wnt8 locus encodes a bicistronic mRNA with two complete open reading frames. One of these (ORF2) is expressed in r5 and r6 in the developing hindbrain by 75% epiboly (Lekven et al., 2001) and could contribute to placode induction. A second locus, wnt8b, is also expressed in r5 (and r1 and r3) by 16 hpf (Kelly et al., 1995). Although this is too late to play a role in placode induction, Wnt8b could influence later patterning of the otic placode or vesicle. Future investigation of the functions of these genes will provide a useful comparison with the chick. It will also be important to establish the relative roles of, and relationships between, the various signaling molecules implicated in otic induction.

**Indirect disruption of FGF signaling.** Mutations that alter hindbrain patterning can impair FGF signaling from this tissue, leading secondarily to inner ear defects. This mechanism appears to be the case in spiel ohne grenzen (spg) mutants, which have lesions in the POU-domain transcription factor gene pou2 (Belting et al., 2001; S. Burgess, personal communication). In spg mutants, the prosencephalon, mid-hindbrain boundary, hindbrain, and inner ear all show severe patterning defects. The inner ear phenotype of spg mutants strongly resembles that of ace (fgf8) mutants: The otic vesicle is small and often contains only a single (utricular) macula and otolith (BBR, unpublished data). Pou2 is coexpressed with fgf8 in the prospective hindbrain adjacent to the developing ear (Takeda et al., 1994; Hauptmann and Gerster, 1995), and the level of fgf8 expression is reduced in spg mutants (Belting et al., 2001; S. Burgess, personal communication). Thus the ear defects in spg (pou2) mutants are likely to result from a deficiency of FGF signaling from the hindbrain.

Another mutation that indirectly perturbs inner ear
development is valentino (val). val encodes a MaF bZIP transcription factor homologous to the mouse kreasler (kr/Krml1) gene (Moens et al., 1998). In mouse kr mutants, an otic placode is induced, but the ear later becomes severely cystic and malformed, with near or total loss of the endolympathic duct (Deol, 1964, and references therein). These defects are attributed to a deficiency of Fgf3 signaling from rhombomeres 4, 5, and 6 (McKay et al., 1996). In the fish, val/krml1 is primarily expressed in the primordia of rhombomeres 5 and 6 and is required for early differentiation of these hindbrain segments. val mutants fail to form a distinct r5 and r6, and instead produce a single rhombomere, termed rX, which has some early attributes of both r5 and r6 but fails to achieve the full regional identity of either. This confused segment identity disrupts normal interaction with the developing inner ear and results in formation of small otic vesicles. Vesicles may later become enlarged and cystic, sometimes with no distinct semicircular canal compartments (Fig. 3). There are concomitant behavioural (circling) defects, characteristic of vestibular dysfunction (Moens et al., 1998). The wild-type zebrafish ear has a small endolympathic duct (see Development of the Endolympathic Duct section), and the similarity of the val phenotype to that in the kr mouse suggests that this structure may also be disrupted in val mutants. Despite these similarities, a key difference between zebrafish and mouse is that fgf3 is not expressed in the r5–r6 region of the hindbrain in zebrafish. The possibility remains, however, that the val mutation nonautonomously reduces FGF signaling from r4, or, alternatively, that another fgf gene normally expressed in r5 and r6 is not properly expressed in mutant embryos. Thus, FGF signaling from the hindbrain may be involved not only in placode induction in the fish, but also in later patterning of the vesicle.

Retinoic acid and ear induction. In the mouse, retinoic acid (RA) signaling also appears to play a role in otic induction. In mice carrying a targeted disruption of the Raldh2 gene, which codes for an enzyme essential for RA synthesis, there are numerous developmental defects, including a severely reduced and abnormally patterned otic vesicle (Niederreither et al., 1999, 2000; see also the Vesicle Formation and the Specification of Axes section). Note, however, that hindbrain patterning in these mutants is severely disrupted; Fgf3 expression in the hindbrain is much weaker than normal, which could explain the inner ear defects (Niederreither et al., 1999).

A mutation in the zebrafish raldh2 gene, neckless (nls), has been described (Begemann et al., 2001). In general, the phenotype of this mutant—including pectoral fin, hindbrain, branchial arch, and head mesoderm defects—is less severe than in the mouse knockout, suggesting that the nls mutant may be a hypomorph, or that other similar genes in zebrafish compensate for loss of Raldh2 function. Hindbrain rhombomeres in the ear region, for example, express appropriate markers (including val/krml1 in r5 and r6), although these territories are slightly enlarged; in the mouse Raldh2 knockout, by contrast, expression of kr/Krml1 in the hindbrain is undetectable (Niederreither et al., 2000). The ear phenotype in nls has not been characterized in detail; otic vesicles do form, however. During gastrulation, raldh2 is expressed in the involuting paraxial mesendoderm and, at later stages, in head mesoderm abutting the ear; thus, these tissues are candidates for a source of RA that may influence development of the vesicle (Begemann et al., 2001). As in the mouse, however, any ear defects may be a secondary consequence of defective fgf3 expression. RA treatment in the fish can induce an expanded domain of fgf3 expression and give rise to ectopic pax8-expressing otic placodes, whereas a morpholino directed against fgf3 can block the effects of exogenous RA (Phillips et al., 2001). These results provide additional evidence that RA is required for normal fgf3 expression, which in turn mediates induction of the placode.

Analysis of the van gogh (vgo) mutation supports a role for pharyngeal endoderm, and possibly RA signaling, in regulation of otic development. In vgo mutants, pharyngeal arches and derivatives are fused and disorganized. Analysis of cell migration patterns and several molecular markers suggests that the primary defect lies in differentiation of pharyngeal endoderm, whereas patterning defects in neural crest and mesodermal derivatives are secondary (Piotrowski and Nüsslein-Volhard, 2000). The otic vesicle is small and fails to form any aspect of the semicircular canals or cristae. Two otoliths form, but by 4 dpf, these appear to lie over a single sensory patch, probably consisting of closely juxtaposed utricular and saccular elements. Several markers show abnormal expression; otx1 expression is missing at 24 hpf and crista-specific expression of mscC and bmp4 is absent at 48 hpf (Whitfield et al., 1996; Piotrowski and Nüsslein-Volhard, 2000). pax2.1, however, is expressed at normal levels, although in a smaller domain, in the early vesicle (TTW, unpublished data). The affected gene has not yet been identified, but it possibly perturbs RA signaling based on similarities with mouse mutants with defects in RA signaling.
Role for \textit{sox} Genes

The \textit{colourless (cls)} gene is so named because mutants fail to form almost all pigmented cells other than those in the pigmented retina. Dutton and colleagues (Dutton et al., 2001a,b) have shown that \textit{cls} disrupts the \textit{sox10} gene, which codes for a transcription factor expressed in migratory and premigratory neural crest cells, as well as in the developing inner ear. Analysis of mutant embryos shows that neural crest cells form normally, but all neural crest cells die before or soon after beginning migration, with the exception of ectomesenchymal derivatives (Kelsh and Eisen, 2000). \textit{cls} mutants also show defects in patterning of the otic vesicle, with sensory and morphogenetic defects (Whitfield et al., 1996). As in \textit{ugo} and \textit{ace} mutants, the early otic vesicle is small, suggesting that there may be a defect in otic induction or early development. However, it is still unknown whether this results directly from a cell autonomous defect in the inner ear or a secondary consequence of loss of neural crest-derived signals. The \textit{sox10} gene, however, is strongly expressed in the otic epithelium at otic vesicle stages (Dutton et al., 2001b), suggesting that a direct role for Sox10 in the ear is likely.

In humans, the disruption of \textit{SOX10} function leads to Waardenburg-Shah syndrome, characterized by sensorineural deafness, pigmentation defects and Hirschsprung’s disease (a deficit of enteric ganglia). Here, the deafness phenotype is usually attributed to a loss of melanocytes in the stria vascularis of the cochlea, a tissue that maintains the ionic composition of the endolymph (reviewed in Price and Fisher, 2001). Because zebrafish do not have a cochlea, or a direct counterpart of the stria, it is uncertain to what extent ear defects in \textit{cls} mutants will mimic those in the human.

Other \textit{sox} gene family members may also play roles in the development of the zebrafish ear; \textit{sox9a}, \textit{9b}, \textit{11a}, and \textit{11b} are also all expressed in the otic vesicle (De Martino et al., 2000; Chiang et al., 2001). \textit{Sox3} is expressed in all sensory placodes in the medaka (\textit{Oryzias latipes}), and ectopic \textit{Sox3} expression in this species can lead to the formation of ectopic otic vesicle-like structures, which express \textit{Pax2} and \textit{Eya1} (Köster et al., 2000).

Summary: Ear Induction

Recent genetic studies support classic experimental embryologic studies showing that the hindbrain and subjacent mesoderm emit otic inducing factors. In zebrafish, \textit{Fgf3} and \textit{Fgf8} appear to be the primary factors involved in otic induction. Wnt8 is another candidate, based on its early expression in the hindbrain, as well as the proposed role of Wnt8c in chick. The role of Wnt8 in zebrafish otic induction has yet to be determined. Genes that regulate hindbrain patterning, such as \textit{pou2} and \textit{val/krm1}, indirectly affect otic development by controlling signaling from that tissue. Similarly, RA signaling affects otic development by influencing expression of otic inducing factors in the hindbrain, although a more direct role cannot be excluded (see below). Finally, several transcription factors for the otic placode.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{signaling_interactions.pdf}
\caption{Signaling interactions influencing otic induction. Schematic diagrams of embryos at the shield stage (animal pole view), late epiboly/bud stage (view of rostral neural plate) and 10 somite stage (dorsal view of the developing head). Otic competence is initially widely distributed throughout the ectoderm. The fate map, however, already shows a restricted area that will become otic tissue (see also Fig. 2). Signals that specify the dorsoventral and rostrocaudal axes of the embryo eventually restrict the area of competence during the course of gastrulation. The nature of the response to axial signals, as it pertains to otic competence, is unknown. Several axial specifiers, including \textit{fgf3}, \textit{fgf8}, and \textit{wnt8}, are later expressed in tissues surrounding the prospective otic ectoderm and appear to play a direct role in otic induction during the latter half of gastrulation. The response to otic induction involves expression of a variety of transcription factors, many of which are expressed well before overt morphogenesis of the otic placode. By the time the otic placode forms at 10 somites, constituent cells express a diverse array of transcription factors, signaling molecules, and cell adhesion molecules. In addition, \textit{fgf3}, \textit{wnt8}, and \textit{wnt8b} continue to be expressed in the hindbrain and may help pattern the otic placode.}
\end{figure}
factors are expressed in the otic primordium and presumably mediate otic induction or regulate early placode development (Fig. 4).

**Future Questions**

Prospective otic cells presumably experience an array of earlier signals that make them competent to receive and respond appropriately to otic inducing factors. How otic competence is regulated is currently unknown, but signals that specify the dorsoventral and rostrocaudal axes of the gastrula appear to be essential. In severely dorsIALIZED mutants lacking *bmp2b* (swirl) or *smad5* (somitabun), the rostral neural plate expands to enwrap the embryo fully, and no otic tissue is specified. In more moderately dorsIALIZED mutants (*bmp7/snailhouse*), otic induction occurs but is displaced to the ventral side of the embryo by the enlarged neural plate (Mullins et al., 1996). Analysis of *dlx3* expression in such mutants shows that BMP signaling plays a general role in otic development by establishing the preplacodal domain at the neural-nonnerve interface (Nguyen et al., 1998). Thus, signals that specify positional identity of the ectoderm can be viewed as early determinants of otic competence. From this perspective, it is noteworthy that putative otic inducers Wnt8 and Fgf8 also play early roles in axial specification (Fürthauer et al., 1997; Lekven et al., 2001). The graded expression of *fgf3* during early gastrulation, and the effects of misexpression of *fgf3*, suggest that it, too, regulates axial development (Phillips et al., 2001; Raible and Brand, 2001). These genes may initially help define the region of otic competence before overtly inducing otic differentiation. The rostrocaudal determinant RA could also influence otic competence in this way.

Other than transcriptional activation of early otic markers, such as *pax8* and *pax2.1*, the nature of the response to otic induction is unknown. The number of preplacodal cells expressing *pax2.1* undergoes a three- to fourfold increase between 11 hpf and 14 hpf (B.B. Riley, unpublished data). This rapid expansion could involve cell division and/or additional recruitment of cells around the principle inductive sources. Despite the increase in cell number, the planar area of the preplacodal domain contracts during this period, reflecting cell shape changes and convergence of cells within the field; note that the zebrafish olfactory placode forms by cell convergence, rather than localized cell proliferation (Whitlock and Westerfield, 2000). Thus, otic development is highly dynamic even before the placode forms. Furthermore, the sequential activation of multiple members of the *pax2/5/8* gene family suggests that they may be involved in a regulatory cascade. Indeed, *pax2.1* is required for induction of *pax5* in the inner ear (Pfeffer et al., 1998). Similarly, it is possible that *pax8* is required to induce *pax2.1* and *pax2.2* in the preplacode, and these in turn could play a role in down-regulating *pax8* in the nascent otic vesicle. A combination of lineage tracing and analysis of the effects of various mutations and morpholino oligomers will be required to address these fundamental issues.

**PATTERNING OF SENSORY EPITHELIUM**

**Development of Sensory Patches**

Sensory patches are thickened regions of epithelium containing two major cell types, hair cells and supporting cells. In a mature patch, hair cells and supporting cells are generally regularly interspersed such that hair cells are surrounded and isolated from one another by the supporting cells. Separate thickenings for the maculae do not appear until after hair cells begin to differentiate. Instead, the whole of the ventral floor of the ear initially becomes thicker than the dorsal roof of the vesicle. The first hair cells of the utricular and saccular maculae appear at the rostral and caudal ends, respectively, of this macula communis (Haddon and Lewis, 1996).

**Early Markers of Sensory Patches**

Several genes mark the positions of the sensory patches in the otic placode well before the differentiation of hair cells. Neurogenic genes of the Delta/Serrate/Lag2 (DSL) family of Notch ligands (*deltaA, B, C, D, and serrateB*) are expressed in two small groups of cells at the rostromedial and caudomedial ends of the otic placode, as early as the 10 somite (14 hpf) stage, 4 hr before the placode cavitates to form the otic vesicle (Haddon et al., 1998a; Smithers et al., 2000; Fig. 5A). At the onset of vesicle formation, these two *delta*-expressing sites fate map to the utricular and saccular maculae, respectively (Haddon et al., 1998a; Fig. 6). Expression of *delta* genes eventually becomes restricted to emerging hair cells. Subsequently, expression of *deltaA, C*, and *D* is down-regulated as hair cells mature, but is intermittently re-expressed within the sensory patches as new hair cells form (Haddon et al., 1998a; Smithers et al., 2000). By contrast, expression of *serrateB*, and to a lesser extent *deltaB*, persists in mature hair cells (Haddon et al., 1998a). By 24 hpf, the macular sensory epithelium is clearly pseudostratified, with nuclei of the two cell types arranged in distinct layers. Sensory hair cells sit apically within the epithelium, where they are identified by their expression of *brn3.1* (a gene coding for a POU-domain transcription factor; Sampaio and Stuart, 1996; Mowbray et al., 2001). Although *pax2.1* initially marks all cells in the otic placode, it becomes restricted to cells in the ventromedial portion of the otic vesicle and up-regulates in hair cells by 24 hr (Fig. 5C). Later, *pax2.1* expression is restricted to hair cells alone (Riley et al., 1999). At this time, the first maturing hair cells begin to express *myoVIIA*, a component of stereociliary bundles (Ernest et al., 2000, and see the “Circler” Mutants and Hair Cell Function section; Fig. 5B). Supporting cells have basally positioned nuclei, but span the full thickness of the epithelium. No specific zebrafish supporting cell markers have yet been identified, but ongoing mutant studies may soon provide such markers (see the Supporting Cell Function section).
Markers of early crista development include members of the bone morphogenetic protein (BMP) family of signaling molecules. In all the major model vertebrate organisms (mouse, chick, *Xenopus*, and zebrafish), BMP4 mRNA expression at the rostral and caudal ends of the otic vesicle figures the developing rostral (superior) and caudal cristae (Hemmati-Brivanlou and Thomsen, 1995; Wu and Oh, 1996; Morsli et al., 1998; Mowbray et al., 2001; Fig. 5D). In the fish, a third ventral domain of *bmp2b* and *bmp4* expression appears at 36 hpf, marking the appearance of the lateral crista. Epithelial thickenings corresponding to all three cristae are visible at 48 hpf, and expression of *bmp2b* and *bmp4* continues to mark their development (Mowbray et al., 2001). The homeobox genes *msxC* and *msxD* also become expressed in the cristae at this time (Ekker et al., 1992), possibly as targets of BMP function. Thickening for the rostral and caudal cristae appear to arise de novo, whereas the thickening for the developing lateral crista is initially contiguous with that of the utricular macula (Mowbray et al., 2001). By 60 hpf, however, when differentiated hair cells begin to appear in these regions, all three cristae are distinct thickenings of epithelium. Genes coding for receptors, mediators, and antagonists of BMP signaling are also expressed in the zebrafish ear, in patterns that do not always correspond to those of the known BMP genes (Mowbray et al., 2001). It is, therefore, unclear at present exactly where the BMPs are acting in the zebrafish ear.

**Specification of Hair Cells and Supporting Cells in Sensory Patches**

At first glance, it appears that the first hair cells form no sooner than 24 hpf (30 somites), when their actin-rich stereociliary bundles can first be detected by phalloidin staining (Haddon and Lewis, 1996). However, examination of other aspects of hair cell morphology indicates that nascent hair cells have already formed by the time the vesicle cavitates at 18 hpf. Hair cell kinocilia, which can be observed in live specimens and stain positively for acetylated tubulin, serve to...
tether otoliths in place at the onset of vesicle formation. These early hair cells are atypical in several regards and are referred to as tether cells to distinguish them from the more typical hair cells that form later (Riley et al., 1997). Despite the presence of kinocilia, the cell bodies of tether cells do not take on the morphology of mature hair cells until 22 hpf (25-26 somites). In addition, tether cells always form in pairs at both the rostral and caudal ends of the otic vesicle, in contrast to the variability observed in the number of later hair cells. The stereotyped and early differentiation of tether cells in otolith development is discussed in a recent study (Artavanis-Tsakonas et al., 1999). If Notch signaling is disrupted, lateral inhibition fails, and all cells of an equivalence group are predicted to adopt the primary (hair) and secondary (supporting) cell fates in a regular and precisely spaced pattern (reviewed by Lewis, 1998; Artavanis-Tsakonas et al., 1999). If Notch signaling is disrupted, lateral inhibition fails, and all cells of an equivalence group are predicted to adopt the primary fate.

**Delta-Notch signaling: lateral inhibition and asymmetric cell division.** In zebrafish, as in other vertebrates, hair cells and supporting cells are thought to arise from a common equivalence group in response to Delta-Notch signaling. DSL family transmembrane proteins signal to Notch family transmembrane receptors on adjacent cells in a juxtacrine manner (Bosenberg and Massagué, 1993; Fagotto and Gumbiner, 1996). Activation of Notch by a DSL ligand results in a down-regulation of ligand expression in the receiving cell, and prevents it from acquiring a default or “primary” fate. This process, often referred to as lateral inhibition, leads to the adoption of primary (hair) and secondary (supporting) cell fates in a regular and precisely spaced pattern (reviewed by Lewis, 1998; Artavanis-Tsakonas et al., 1999). If Notch signaling is disrupted, lateral inhibition fails, and all cells of an equivalence group are predicted to adopt the primary fate.

Genetic studies in zebrafish strongly support the lateral inhibition model. Mutations that disrupt Delta-Notch interactions result in production of excess hair cells at the expense of supporting cells. Two such mutations have been described so far in zebrafish: One affects the mind bomb (mib) gene, the identity of which has not been reported, whereas the other affects the deltaA (dIA) gene (Jiang et al., 1996; Schier et al., 1996; Appel et al., 1999; Table 1). The mib mutation appears to block activation of all Notch homologs and results in severe specification defects in the inner ear and lateral line (Haddon et al., 1998a; Riley et al., 1999; Itoh and Chitnis, 2001). Analysis of genetic mosaics confirms...
that mib functions cell-autonomously (Fig. 7). mib mutants typically produce a 10-fold excess of hair cells and few, if any, supporting cells in developing maculae. The excess hair cells are produced prematurely, in a rapid wave, reaching a maximum at approximately 36 hpf (Fig. 8). In contrast, crista hair cells never develop in the mib ear, possibly because the supply of hair cell precursors has been exhausted by the first early wave of macular hair cell production. Subsequent otic development is severely perturbed; the huge sensory patches are extruded from the ear, and the vesicle remains small, with only rudimentary attempts at semicircular canal formation (Malicki et al., 1996; Haddon et al., 1998a).

A missense mutant allele of deltaA, \(dla^{dx2}\), encodes a dominant-negative protein that appears to impair the functions of wild-type Delta proteins, even those encoded by other delta genes that are normally coexpressed with \(dla\). Accordingly, the neurogenic defects caused by \(dla^{dx2}\) are considerably more severe than those caused by a deletion that removes the \(dla\) locus (Appel et al., 1999; Riley et al., 1999). However, \(dla^{dx2}\) is generally less severe than mib. Homozygous \(dla^{dx2}\) mutants typically produce a fivefold excess of hair cells in the maculae and, unlike mib mutants, \(dla^{dx2}\) mutants are usually able to form cristae. As in the maculae, hair cells are overproduced in the cristae of \(dla^{dx2}\) mutants.

Analysis of these neurogenic mutants highlights several aspects of hair cell patterning that are still poorly understood. For example, the mib phenotype may simply reflect the normal size of a hair cell-competent equivalence group, in which all but a very few cells are normally prevented from prematurely differentiating by means of the early phase of mutual Delta-Notch signaling between precursor cells (Haddon et al., 1999). However, such a large increase in hair cell number in the mutant may indicate that, in addition to the lateral inhibition defect, the equivalence group itself is greatly expanded. This finding is consistent with studies in Drosophila showing that Notch activity is required to limit the size of proneural clusters (Rusconi and Corbin, 1999). Cell lineage tracing, and analysis of expression of proneural genes that define the early equivalence group (see the Transcription Factors Required for Hair Cell Specification section below) will help to test these models.

Another issue raised by mib and \(dla^{dx2}\) stems from the observation that, although hair cells differentiate precipitously in these mutants, they do not form simultaneously. Rather, they are produced in a wave that propagates from the macular center outward, just as in the wild-type. Little is known about the events that initiate hair cell differentiation within equivalence groups, but they probably do not involve stochastic variation in Delta-Notch activity. The site of initiation is not random, as would be predicted by a stochastic mechanism. In addition, cells at the centre of an equivalence group, being surrounded on all sides by Delta-expressing neighbours, should experience more inhibitory signaling than cells at the periphery (Chitnis, 1995). Additional factor(s) must operate to overcome this geometrically imposed bias. During development of the eye, chordotonal organs, and sensory bristles in Drosophila, this function is mediated by the EGF receptor (EGFR) pathway, which preferentially antagonizes Notch activity in cells located at the centre of an equivalence group (Spencer et al., 1998; zur Lage and Jarman, 1999; Culi et al., 2001). This mechanism allows central cells to adopt the primary cell fate and initiate the lateral inhibition process. In addition, these organ systems show progressive recruitment of new sensory cells, a function that also requires EGFR. In the eye, for example, newly differentiating photoreceptors express Hedgehog, Decapentaplegic, and Vein (an EGFR ligand). These factors cooperate to induce a self-propagating wave of differentiation that spatially organizes Delta-Notch interactions and leads to the regular pattern of ommatidia in the compound eye. At present, there are no functional studies indicating how the orderly progression of hair cell differentiation is regulated in the vertebrate ear. However, several secreted factors are expressed in developing maculae and cristae, including FGF and BMP, that could mediate initiation of hair cell differentiation, wave progression, or both. Indeed, FGF and BMP stimulate signal trans-
duction pathways homologous to those of *Drosophila* EGFR and Dpp, respectively, and might, therefore, show similar interactions with the Delta-Notch pathway.

There are several other aspects of Delta-Notch signaling in the ear that need to be resolved. First, lateral inhibition may not be the only mechanism used to specify hair cell and support cell fates. In the fly, factors such as Numb, fringe, and Wg serve to bias or modulate Notch signaling (Artavanis-Tsakonas et al., 1999) and homologues of these proteins are expressed in the developing ear. Numb protein—a Notch antagonist—is initially expressed throughout a sensory patch and later becomes localized to hair cells in the chick ear (Eddison et al., 2000), whereas Prox1, a homolog of the Numb-associated protein Prospero, is expressed in the zebrafish ear at relevant stages (Glasgow and Tomarev, 1998). Both proteins are associated with asymmetric cell division, which acts in concert with lateral inhibition to diversify cell fates in the nervous system (Campos-Ortega, 1997; Jan and Jan, 1998). In the chick ear, elaborations to the lateral inhibition model postulate that *Serrate1* is up-regulated in supporting cells in response to Notch signaling (a process termed lateral induction; Lewis, 1998; Eddison et al., 2000). In this model, nascent hair cells are immune to inhibition from *Serrate1* due to their expression of Numb, whereas supporting cells, which contact one another, retain high Notch activation by means of *Serrate1* and are, thus, inhibited from adopting a hair cell fate. It is not known whether supporting cells in zebrafish also express Notch ligands, although a homologue of *Serrate1* may exist (Haddon et al., 1998b). In mammals, the *Serrate1* homolog *Jagged1* is expressed in supporting cells of the ear, but mouse mutants in *Jagged1* indicate that this gene may also have an earlier role in sensory patch specification (Kiernan et al., 2001; Tsai et al., 2001, and references therein).

Note that in hair cells, persistent expression of Notch ligands (such as zebrafish *serrateB*) after differentiation may stabilize initial fate choices (Haddon et al., 1998a), whereas cell rearrangements (Goodyear and Richardson, 1997) and cell death (Fekete et al., 1997) may fine tune the initial pattern produced by lateral inhibition and asymmetric cell division.

**Transcription factors required for hair cell specification.** During development of most sensory organs, equivalence groups are initially marked by expression of proneural genes related to *achaete-scute* and *atalon* genes of *Drosophila*. Proneural genes encode bHLH transcription factors that give all cells in the equivalence group the potential to adopt the primary (e.g., neural) cell fate. However, these proteins also induce expression of *Delta* genes, which inhibit proneural gene expression and delay differentiation. This negative feedback is relieved only in a subset of cells in which Notch activity is depressed or antagonized, allowing continuation of differentiation and initiation of lateral inhibition. In rodents, *Math1* (a murine *atalon* homolog) is required for hair cell specification: targeted disruption of murine *Math1* ablates all hair cells (Bermingham et al., 1999), whereas over-expression of *Math1* in the rat ear is sufficient to induce the development of extra cochlear hair cells (Zheng and Gao, 2000). In zebrafish, the equivalent gene, *zath1*, is also expressed in the developing ear in association with developing maculae (Fig. 8C, and A.B. Chitnis, personal communication). This expression domain is greatly expanded in *mib* mutants (Fig. 8D), but it is not yet clear whether finding this reflects expansion of the hair cell equivalence group and/or early onset of hair cell differentiation by all cells within the equivalence group. In addition to its expression in the ear, *zath1* is also associated with hair cell formation in the developing caudal lateral line primordium. Here, *zath1* expression initially marks all cells in each equivalence group and subsequently becomes restricted to hair cells in nascent neuromasts (Itoh and Chitnis, 2001).

Regulation of *delta* genes in the fish ear is also influenced by the Pax2.1 protein (Riley et al., 1999). Expression of *pax2.1* is down-regulated in the majority of cells in the otic vesicle between 24 and 30 hpf, whereas expression is maintained in mature hair cells. As the maculae grow, *pax2.1* is induced in newly specified hair cell precursors around the periphery and is then maintained after their terminal differentiation. *pax2.1* is also expressed in hair cells in the cristae. Surprisingly, the only overt change in otic development in *no isthmus* (*noi*) mutants, which lack Pax2.1 function, is that they produce roughly twice the normal number of hair cells (Fig. 9). This defect appears to result from faulty regulation of *delta* gene expression. Specifically, the *noi* mutation reduces and/or abbreviates expression of several *delta* genes during early hair cell differentiation. This process would be expected to weaken lateral inhibition of hair cell specification, an interpretation supported by genetic interactions between *noi* and mutant alleles of *dlA*. It is not yet known whether Pax2.1 regulates *delta* gene expression directly at the transcriptional level or indirectly by perturbing the general course of early hair cell differentiation.

Duplication and divergence of *pax* genes probably accounts for the mild effects of the *noi* mutation. In *noi* mutants, *pax5* is not expressed in the developing inner ear, but *pax2.2* continues to be expressed in the domain normally shared by *pax2.1* (*Pfeffer* et al., 1998). Mutant alleles of *pax2.2* have not yet been identified. However, injection of *pax2.2-MO* into wild-type embryos impairs formation of hair cells that normally form after the tether cells. Later hair cells normally begin to accumulate by 24 hpf, but in *Pax2.2*-depleted embryos they do not begin to accumulate until after 30–36 hpf. Injection of *pax2.2-MO* into *noi* mutants also impairs formation of later hair cells, with no further loss of otic structures (Fig. 9). Thus, *pax2.1* and *pax2.2* may have evolved to regulate distinct aspects of early hair cell differentiation, with *pax2.2* being indispensable for their initial specification and *pax2.1* reg-
ululating the attendant process of lateral inhibition. It is not yet known whether the block to later hair cell formation is eventually overcome by another as yet unidentified gene or instead reflects “leakiness” or gradual depletion of pax2.2-MO. Notably, tether cells still form in all embryos lacking function in both pax2.1 and pax2.2. The early formation of tether cells raises the possibility that pax8 is sufficient for their specification. Designing a morpholino to test pax8 function is not yet possible because the 5’ terminus of pax8 mRNA has not yet been sequenced (Pfeffer et al., 1998).

In the mouse, the loss of Pax2 function in the ear is more severe: targeted disruption of the locus reveals that Pax2 is necessary for development of the cochlea, its associated ganglion and sensory patch, the organ of Corti (Favor et al., 1996; Torres et al., 1996; note, however, that there is a direct counterpart of the cochlea in the teleost ear). In humans, PAX2 mutations result in Renal-Coloboma syndrome, symptoms of which may include high-frequency hearing loss (Schimenti et al., 1997; Eccles and Schimenti, 1999).

A Pax-Eya-Six-Dach Pathway in the Ear

Pax genes may also function in other genetic networks in the ear. In the Drosophila eye disc, the pax family proteins Eyeless (Ey) and Twin of eyeless (To) function in a network together with the nuclear proteins Eyes Absent (Eya), Sine Oculis (So), and Dachshund (Dach), characterized by transcriptional feedback loops and protein-protein interactions. Together, these proteins perform multiple functions, including initiation and progression of the morphogenetic furrow, an apical depression that sweeps across the eye disc, and neuronal differentiation behind it (Chen et al., 1997; Pignoni et al., 1997). Homologues of all four gene families (Pax, Eya, Six, and Dach, respectively), often with several representatives of each, exist in vertebrates. Particular combinations of these are thought to be involved in different developmental processes; for example, Pax3, Eya2, Six1, and Dach2 contribute to muscle patterning in the developing somite (Heanue et al., 1999).

The eya1 gene, an eyes absent homologue, is disrupted in the zebrafish dog-eared (dog) mutant (Kozłowski et al., manuscript in preparation; Table 1), and the most obvious defects in homozygous embryos involve the ear. Although eya1 is a preplacodal marker (Sahly et al., 1999), the earliest defects seen in the ear are at otic vesicle stages. These defects include an increase in cell death, and abnormal neurogenesis (Kozłowski et al., manuscript in preparation; see the Neurogenesis and Formation of the Statoacoustic Ganglion section). The sensory patch phenotype is striking. There is an almost total lack of crista hair cell differentiation and failure to maintain expression of cristaspecific markers. Hair cells in the maculae differentiate, but these patches are smaller than normal and may be abnormally patterned. The ear itself is small and misshapen. Semicircular canal projections form and fuse in the centre of the ear but are disorganized and swollen. Defects are also seen in the lateral line system (Whitfield et al., 1996; Kozłowski et al., manuscript in preparation). In the human, EYA1 mutations result in the autosomal dominant disorder Branchio-Oto-Renal (BOR) syndrome, of which a common feature is sensorineural deafness (Abdelhak et al., 1997).

The expression pattern of eya1 mRNA has been described in detail in the zebrafish (Sahly et al., 1999). At
the end of gastrulation (bud stage; 10 hpf), it is expressed in the horseshoe-shaped preplacodal domain surrounding the rostral edge of the neural plate, encompassing presumptive ear, lateral line, olfactory, and hypophyseal placodal tissue (see Fig. 4). By 13 hpf, there is a clear concentration of expression in the developing otic placode. By 20 hpf, initial uniform expression in the placode becomes restricted to the ventral half of the otic vesicle. Expression continues in the ear throughout larval stages, becoming localized to sensory epithelium.

In the zebrafish ear, it is likely that Eya1 acts in a transcriptional network, as in the fly eye. As mentioned earlier, several pax family members (although not pax6 itself) are expressed strongly in the ear, together with at least one othereya gene, eya4 (J. Ungos and D. Raible, personal communication). Homologues of six2, 3, and 4 are known in zebrafish (Kobayashi et al., 1998; 1998a,b; Kobayashi et al., 2000; Kobayashi et al., 2001). Of these, three six4 genes are expressed in the ear; the expression of six4.1 closely mimics that of eya1 (Kobayashi et al., 2000). Three dachshund homologues, dachA, B, and C, are all expressed in the zebrafish ear, in largely nonoverlapping patterns (Hammond et al., 2002). Interestingly, these homologues mark very different domains of epithelium. dachA is expressed primarily in nonsensory regions, marking the dorsal part of the ear epithelium and the semicircular canal projections. dachB marks all sensory patches in the ear and the lateral line neuromasts. In the maculae, at 50 hpf, expression is restricted to the hair cell layer. dachC marks a ventral domain of nonsensory epithelium abutting the utricular macula.

The existence of a Pax-Six-Eya-Dach network appears to be a conserved and common feature of the development of several different placodally derived tissues (Baker and Bronner-Fraser, 2001). In the zebrafish ear, Eya1 appears to be required not in the specification of a single cell type but for overall patterning and cell survival in the otic epithelium. Transcriptional interactions between differenteya, pax, six, and dach genes in the ear have not yet been established. Given that several members of each gene family are expressed in the zebrafish ear, there is potential for complex combinatorial control. The network(s) may also interact or cooperate with BMP-mediated signals; in the fly eye disc, the BMP homologue Decapentaplegic (Dpp) is an integral member of the feedback loops controlling expression of network members (Chen et al., 1999; Curtiss and Modzik, 2000). bmp4 expression is lost in the developing cristae in dog (Kozlowski et al., manuscript in preparation), but it is not known whether this finding reflects a failure of maintenance of expression by Eya1 or whether bmp4-expressing cells in the dog ear simply die.

**Hair Cell Polarity Patterns**

Differentiated hair cells are characterized by their apical cytoskeletal specialisations—the stereociliary bundle and kinocilium. The relative position of these structures gives each hair cell a distinct polarity (and directional sensitivity) in the plane of the epithelium. Each sensory patch has its own characteristic pattern of hair cell planar polarities (Fig. 10). The utricular macula lies on the rostral ventral floor of the vesicle, and by 5 dpf has approximately 80 hair cells. Hair cells within it display a range of stereociliary bundle heights (2–5 μm), with the greatest being toward the lateral edge of the patch. Actin and tubulin stains show that hair cell polarities fan out from a medial pole, with an outer rim of hair cells all polarized in the opposite direction (Haddon et al., 1999; Whitfield, 2000). This pattern matches that of the otic and striolar regions of the adult utricle (Platt, 1993).

By 5 dpf, the saccular macula is positioned on the medial wall of the vesicle, immediately underneath the hindbrain. It has a caudal rounded region, where hair cell polarities point away from a dorsoventral midline, and a slim rostral projection, where hair cells are arranged in an antiparallel pattern about the same axis (Haddon et al., 1999). This is the “standard” four-quadrant pattern for a teleost sacculus, but the adult sacculus has been described to have a “vertical” pattern, lacking the antiparallel region (Platt, 1993).

Hair cells in the cristae are visible by phalloidin staining at 60 hpf. Stereociliary bundles (>5 μm long) and kinocilia (>20 μm) are longer than those of the macular hair cells. From 60 hpf onward, crista kinocilia are readily evident in live specimens with DIC optics under the compound microscope (Nicolson et al., 1998). By 5 dpf, each crista has approximately 20 hair cells (Haddon and Lewis, 1996). These, too, have a unique polarity pattern, which corresponds to the adult arrangement; all hair cells within a single crista point in the same direction (Whitfield, 2000).

**Summary: Sensory Patterning**

It is clear from the above sections that much of our current understanding of sensory patch specification and patterning has been driven by analogy to comparable processes in the fly. The striking similarities in form, function, and development between sensory hair cells in vertebrates and sensory bristles inDrosophila have often been noted (Parks et al., 1997; Adam et al., 1998; Kavaler et al., 1999; Riley et al., 1999), and these similarities include conserved roles for proneural and neurogenic pathways in hair cell specification and differentiation. Likewise, other genetic pathways, such as the Pax-Six-Eya-Dach network, discovered and elucidated in the fly eye, are now known to be used in the specification of vertebrate sense organs. Conserved roles may extend to later stages of sensory patch specification: it has been suggested that vertebrate homologs of genes with roles in generating planar (tissue) polarity in the fly may function in establishing hair bundle morphogenesis and polarity in the ear (Eaton, 1997; Kollmar, 1999).

Detailed comparative studies, however, reveal differences between vertebrate and invertebrate mechanisms. In the fly bristle, shaft cells and socket cells...
(analogous to hair cells and supporting cells, respectively) emerge from a common equivalence group in response to Delta-Notch interactions. Proneural genes \textit{achaete} and \textit{scute}, and \textit{Drosophila Pax2}, which appears to act as a downstream effector of proneural gene function, initially mark all cells in the equivalence group and are later required for differentiation of shaft cells. In the vetebrate ear, members of both gene families are likely to be required for hair cell specification, but it is not clear whether they act in parallel or form a regulatory hierarchy. \textit{pax} genes are expressed first during otic development, and may be required for placa
d induction. At later stages, however, \textit{pax} gene expression is limited to hair cells, whereas proneural gene expression marks precursors of both hair cells and supporting cells before specifying hair cell fate.

Also inspired by studies in \textit{Drosophila}, Fekete and colleagues have proposed a compartment boundary model for the specification of sensory organs and nonsensory structures in the chick ear (Fekete, 1996; Kiernan et al., 1997; Brigande et al., 2000). In this model, asymmetric domains of transcription factor expression define compartments in the ear; signals across compartment boundaries specify the positions of differ-
ent ear structures. The authors propose that morphologically elongated sensory organs (the chick basilar papilla and vestibular maculae) may arise at the juncture of just two expression domains, whereas specification of more compact organs (crista) might require the juncture of three expression domains (Brigande et al., 2000).

To what extent do the current data support such a model in the zebrafish ear? The positions of the maculae appear to be specified very early, possibly before any asymmetries in gene expression domains within the otic placode have been described (see the Vesicle Formation and the Specification of Axes section). Moreover, the first macular hair cells, although they arise within a larger thickened domain, appear only in discrete patches within this domain (see Fig. 5A, for example). Early on, therefore, the zebrafish data do not fit the chick model well, although differences may partly reflect the difference in size between the otic vesicles of the two species. By 24 hpf, however, clear asymmetric domains of gene expression, often with sharp boundaries, appear (Fig. 5B–D; see the Vesicle Formation and the Specification of Axes section). These domains may serve to delimit domains of sensory competence, or define boundaries between sensory and nonsensory epithelium. Eya1 may have a role here, as its expression becomes progressively restricted to sensory epithelium, and possibly correlates with sensory competence. Expression of BMP family members, however, does not seem to define sensory competent regions, because no single bmp gene appears to mark all sensory patches, unlike bmp4 in the chick ear (Wu and Oh, 1996; Mowbray et al., 2001).

One outstanding question in sensory patch specification concerns lineage relationships in the ear. Fate mapping of the early (18–19 hpf) zebrafish otic vesicle by single cell injection reveals that single precursor cells can divide to yield both a supporting cell and a hair cell (Haddon et al., 1998a; Fig. 6). However, although hair cells and supporting cells can clearly share a common precursor, it is unclear whether they also share a fixed lineage relationship with neurons in the ear, analogous to the shaft, socket, and neural cells in a Drosophila sensory bristle. If they did, it might be expected that mutants that disrupted Delta-Notch signaling would generate otic neurons at the expense of hair cells, which is clearly not the case, at least in mind bomb mutants (see the Discussion section in Haddon et al., 1998a). Note that hair cells and neurons do not share a fixed lineage in the developing zebrafish caudal lateral line (Gompel et al., 2001), and no evidence has been found for such a relationship in the chick ear (Lang and Fekete, 2001). In this respect, therefore, development of the vertebrate ear sensory patch and Drosophila sensory bristle appear to differ.

**OTIC MORPHOGENESIS**

**Vesicle Formation and the Specification of Axes**

Formation of the otic vesicle from the placode in the zebrafish does not involve invagination as in higher vertebrates. Instead, the placode sinks beneath the surface ectoderm and forms a vesicle by cavitation. By the 14 somite stage (16 hpf), placodal cells are already showing signs of apicobasal polarisation; staining with fluorescein-phalloidin reveals that actin is concentrated at their future apical surfaces in the centre of the placode. At approximately the 18 somite stage (18 hpf), a lumen appears in the placode, first as a narrow slit. As cavitation proceeds, organisation of the placodal cells into a polarized epithelium becomes more evident; actin remains concentrated at the apical (luminal) surfaces of the cells, whereas the nuclei are found basally (Haddon and Lewis, 1996).

Over the next few days, the ear increases in size. Cell division rates in the epithelium, however, appear to be relatively low (Haddon, 1997), as do levels of cell death in the early ear (Bever and Fekete, 1999; see also the Cell Death in the Zebrafish Ear section). Some cells also leave the epithelium to form the statoacoustic ganglion. Growth of the ear during these stages, therefore, may be primarily due to an increase in fluid volume in the lumen, rather than a vast increase in epithelial cell number. The dorsal part of the epithelium thins during this time, whereas the ventral part thickens, marking the site of future sensory patches.

Little is known about the definition of axes in the zebrafish ear. Patterns of gene expression at placode stages are typically throughout the placode, or symmetrical about an axis. Expression of pax5 in the rostral part of the placode marks the first known asymmetry about the rostrocaudal axis at the 17 somite stage (Pfeffer et al., 1998). The early rostral and caudal patches of delta expression, whereas symmetrical about the rostrocaudal axis, are restricted to the medial side of the otic placode by 10 somites (14 hpf; Haddon et al., 1998a; Fig. 5A). By 24 hpf, however, there are clear asymmetries in the expression patterns of several genes along all three axes: rostrocaudal (e.g., continued expression of pax5 in the rostroventral part of the placode); dorsoventral (e.g., restriction of eya1 expression to the ventral half of the placode and dix3 expression to the dorsal half); and mediolateral (e.g., restriction of pax2.1 expression to the medial side of the placode and the appearance of bmp2b expression on the lateral side; Fig. 5C,D). It is likely that external signals, possibly including continuing signals from the hindbrain, influence this early symmetry-breaking. Rotation of the ear rudiment, otic placode, or vesicle in chick and amphibian embryos has established that the rostrocaudal axis is determined before the dorsoventral axis in the ear (Wu et al., 1998, and references therein). Similar experiments have not been performed in the zebrafish.

In the mouse, retinoic acid signaling may be important for defining the mediolateral axis of the ear. The reduced otic vesicle of mice carrying a targeted disruption of the Raldh2 gene (Table 1) appears to be lateralized: expression of Pax2, a medial marker, is lost, whereas that of Nkx5.1, a lateral marker, is found throughout the otic epithelium (Niederreither et al.,
2000). Note, however, that this may be a secondary effect of abnormal otic induction by the hindbrain (see the Retinoic Acid and Ear Induction section). It is not known whether there are mediolateral defects in the ears of neckless mutants, which carry mutations in the zebrafish raldh2 gene (Table 1; the Retinoic Acid and Ear Induction section), but it will be interesting to see whether pax2.1 and nkx5.1 are expressed normally in the nls ear.

**Neurogenesis and Formation of the Statoacoustic Ganglion**

If the otic vesicle is observed with DIC optics from the 26 somite stage (22 hpf) onward, the basal lamina is seen to be indistinct in an anteroventral region. Individual cells here show blebbing activity, and some—perhaps a few hundred in total—delaminate from the epithelium. Delamination can be visualized with time-lapse video recording and is also evident in semi-thin resin sections (Haddon and Lewis, 1996). Delaminating cells are neuroblasts undergoing an epithelial to mesenchymal transition to leave the otic vesicle. They do not travel far, but accumulate immediately beneath the rostral half of the otic vesicle, where, after further divisions, they differentiate into neurons of the statoacoustic (VIIIth) ganglion (SAg). Both sna2 and nkx5.1 appear to mark cells during and after this process, being expressed both in a ventral region of the epithelium and in the newly delaminated neuroblasts beneath (Thisse et al., 1995; Adamska et al., 2000; Fig. 11). Delamination ceases by 42 hpf, and the ganglion begins to compact, extending under each sensory macula of the developing ear. SAg neurons will send projections back to the ear’s sensory patches. Neuroanatomy of the VIIIth nerve has been briefly described with antibody staining for later stages (Raible and Kruse, 2000).

Both Fgf8 and Eya1 function are required for the correct formation of the SAg. In ace (fgf8) mutants, nkx5.1 expression is present at reduced levels in the ventroorostral half of the small otic vesicle, but very few expressing cells are seen beneath the ear (Adamska et al., 2000; Fig. 11). In dog-eared (eya1) embryos, which carry a mutation at the eya1 locus (Table 1; the Pax-Eya-Six-Dach Pathway in the Ear section; Fig. 11), sna2 expression appears to be reduced in the ventral part of the ear, with fewer cells expressing sna2 beneath it. This early reduction of gene expression correlates with later reduced expression of neural markers, suggesting that neuron number in the SAg is decreased in dog embryos (Kozlowski et al., manuscript in preparation).

Selection of otic neural cell fate, as in the central nervous system, also depends on the activity of the neurogenic genes; a roughly twofold increase in statoacoustic ganglion neuron number is seen in the mind bomb mutant, in which neurogenic gene is dysregulated (Haddon et al., 1998a). As with hair cells, high levels of delta expression are presumed to correlate with the selection of a neuronal fate, whereas high levels of Notch activity in a cell repress acquisition of this fate.

**Development of Otoliths**

The adult zebrafish ear contains three otoliths, one associated with each of the maculae in the utricle, saccule, and lagena. In the embryo, the utricular and saccular otoliths are formed initially, at the rostral and caudal ends of the cavity, respectively, although occasionally three or four small otoliths may be observed,
which later resolve into two. Otoliths initially grow rapidly by a process termed otolith seeding, which involves localized accretion of otolith precursor particles (Riley et al., 1997). These seeding particles are able to move freely throughout the lumen. Manipulation of seeding particles with laser tweezers shows that they are both dense and highly adhesive. Nevertheless, they are inhibited from prematurely agglutinating by the action of beating cilia, which are present on the apical surfaces of most cells in the vesicle. This constant agitation helps to distribute seeding particles to the ends of the vesicle where they bind to the stationary kinocilia of tether cells, thereby localizing accretion of seeding particles over the developing maculae. This initial seeding process, which lays the foundation for future otolith growth, occurs only during a critical period from 18 to 24 hpf. Soon after 24 hpf, the supply of visible seeding particles becomes depleted, beating cilia are resorbed, and otolith growth slows dramatically (Riley et al., 1997).

By the third day of development, the two otoliths already have distinct and characteristic sizes and shapes. The larger, rounded otolith (precursor of the sagitta of the saccus in the adult) lies in a caudal, medial position, whereas the smaller, ellipsoid rostral otolith (precursor of the lapillus of the utricle) occupies a more lateral position over the utricular macula. These characteristic shapes are a prelude to the individual and complex sculpting of the otoliths in the adult ear (Platt, 1993). The third otolith of the adult ear (the lagena’s asteriscus) starts to form at approximately day 9 (Riley and Moorman, 2000).

Several mutations have been identified that alter otolith formation (Malicki et al., 1996; Riley and Grunwald, 1996; Whitfield et al., 1996; Riley et al., 1997), one of which, monolith (mnl), has been described in detail. Embryos carrying the mnl mutation usually produce only a single otolith per vesicle due to a defect in otolith seeding (Riley et al., 1997). Although most aspects of inner ear development appear normal, mnl embryos show a 4-hr delay in the ability of tether cell kinocilia to bind seeding particles. This defect is more pronounced in utricular tether cells such that, once seeding begins, saccular tether cells acquire most or all seeding particles before the close of the critical period at 24 hpf. Without a foundation of seeding particles, utricular tether cells are unable to initiate or support subsequent otolith morphogenesis. The deficiency in seeding utricular otoliths is not absolute, however, as approximately 20% of mutant vesicles succeed in producing small utricular otoliths. Moreover, briefly immobilizing mutant embryos in agarose with the rostral end of the vesicle oriented downward concentrates the dense seeding particles at that end and permits formation of utricular otoliths in all embryos. In contrast, formation of utricular otoliths is totally blocked by immobilizing mutant embryos in the opposite orientation. After the close of the critical period, seeding particles are depleted and gravity no longer affects otolith morphogenesis (Riley et al., 1997).

Development of Semicircular Canals

The semicircular canal system has two main components; the canals themselves, which are bounded by nonsensory epithelium, and their associated sensory patches, the cristae (discussed in the Patterning of Sensory Epithelium section). Semicircular canal formation involves a complex reshaping of the ear epithelium, and has been described in detail in the zebrafish by Waterman and Bell (1984). No initial flattened pouch of presumptive semicircular canal tissue is seen, in contrast to the amniote ear (Fekete et al., 1997). Instead, the first sign of canal formation is the appearance of finger-like protrusions of epithelium (equivalent to the opposing walls of the pouches in amniote ears), which push inward toward the centre of the otic vesicle. There are four such projections, from the rostral, caudal, ventral, and lateral walls of the vesicle. The lateral projection later forms three bulges, pointing rostrally, caudally, and ventrally; these eventually contact the rostral, caudal, and ventral projections, respectively, forming fusion plates at the points of contact. Rearrangement of the epithelium here generates a continuous tube or pillar; cell death does not seem to be prevalent at the fusion plate (Waterman and Bell, 1984). The pillar can then be thought of as the hub of the developing semicircular canal, the canal itself being the fluid-filled toroidal space surrounding each hub. A dorsolateral septum later grows inward to separate the chambers of the anterior and posterior canals (Haddon and Lewis, 1996).

Genes expressed in the developing semicircular canal system. Few genes are known to mark the semicircular canal system specifically. dachA marks the anterior and posterior semicircular canal projections at 48 hpf, and remains in the fused epithelium in the centre of the ear at 72 hpf (Hammond et al., 2002). As described above, strong expression of several bmp genes is found in the cristae of the fish ear, and weak expression is also seen in the developing nonsensory semicircular canal epithelium (Mowbray et al., 2001). In the chick, treatment of the developing ear with noggin protein, a BMP antagonist, causes specific defects in semicircular canal development (Chang et al., 1999; Gerlach et al., 2000).

Gene knockout studies in the mouse have shown that transcription factors of the Dlx, Otx, Prx, and Nkx/Hmx families are essential for the normal development of semicircular canals in this species (Acampora et al., 1996, 1999; Hadrys et al., 1998; ten Berge et al., 1998; Mazan et al., 2000; reviewed in Fekete, 1999; Brigande et al., 2000). In the zebrafish, representatives of otx and nks families are expressed in the ear, but a functional role in semicircular canal development has not yet been demonstrated. otx1 and otx3, related to the Drosophila gene orthodenticle, are expressed in a discrete ventral region of the otic epithelium from mid-somite stages (Li et al., 1994; Mercier et al., 1995). nks5.1, as discussed in the Neurogenesis and Formation of the Statoacoustic Ganglion
Interestingly, this image suggests that cristae development has proceeded normally (arrowhead, posterior crista). C: Mutants at the tp219e locus have swollen ears. Here epithelial projections grow but fail to fuse and form pillars across the enlarged lumen of the ear (arrows). Swelling occurs in the mediolateral dimension, not visible in this image. Panel B reproduced, with permission, courtesy of Neuhauss et al. (1996). Scale bar = 50 μm.

Development of the Endolymphatic Duct

Although an endolymphatic duct has not been described in the embryonic or adult zebrafish ear, it is identifiable in larval ears in sectioned material, and occasionally in paint-filled specimens, as a narrow tube, running along the medial surface of the common crus (Bever and Fekete, 2002). In the embryo, dorsal domains of both bmp4 and dacha expression appear to mark the primordium of the duct as it begins to extend, between 2 and 3 dpf (Whitfield, 2000; Mowbray et al., 2001; Hammond et al., in press; C. Mowbray, K. Hammond, and TTW, unpublished data). The duct remains small, however, and its function in the zebrafish ear is unknown.

Cell Death in the Zebrafish Ear

Programmed cell death (apoptosis) is known to play a role in the morphogenesis of semicircular canals in the chick, where it removes cells at the large fusion plates of the semicircular canal pouches (Fekete et al., 1997). In zebrafish, the fusion plates are 5 to 10 times smaller than in the chick, and no increase in apoptosis has been observed during their remodelling, as assayed by transmission electron microscopy (Waterman and Bell, 1984), or by TUNEL staining just after fusion has taken place (Bever and Fekete, 1999). Occasional apoptotic cells are seen in zebrafish pillars later, at 4–5 dpf, suggesting that apoptosis may play a role in later remodelling of semicircular canal pillars (Bever and Fekete, 1999). Positions of the fusion plates are still visible by their concentrations of actin long after fusion has taken place (at 24 dpf; C. Mowbray, unpublished data), suggesting that remodelling takes some time after fusion.

Birds and mammals also show a ventromedial “hot spot” of apoptosis in the ear, located at the boundary between the pars superior and pars inferior (Lang et al., 2000). This boundary is not well-defined in zebrafish; even by 5 dpf, the saccular macula remains dorsal to the utricular macula, and there is no evidence
of a narrowing of the connection between the presumptive utricular and saccular chambers. A narrow utricle-ulosaccular canal is evident by 17 dpf, however (Bever and Fekete, 2002), but a focus of apoptosis associated with its formation has not been found, although it is possible that it has been missed (Bever and Fekete, 1999).

A low but persistent level of cell death is seen within or at the edges of all developing sensory organs in the zebrafish ear (Bever and Fekete, 1999). Several possible functions for this are proposed by the authors, including refinement of the hair cell/supporting cell pattern, elimination of inappropriately innervated hair cells, or generation of pathways for ingrowing nerve fibres. Note that in zebrafish lateral line neuromasts, there appears to be a rapid and constant turnover of hair cells, where dying cells at the centre of the neuromast are replaced by mantle cell proliferation at the edge (Williams and Holder, 2000, and see the Growth and Regeneration of Sensory Epithelia section below).

Haddon et al. (1999) describe numerous TUNEL-labelled cells in the region of the developing statoacoustic ganglion in otherwise normally developing siblings of mind bomb mutants at 36 hpf, but it is possible and even likely that some of these represented false positives, as no concomitant changes in nuclear morphology were seen. Numerous false positives were also reported in the study by Bever and Fekete (1999), but a variable and low level of bona fide cell death seen in the developing statoacoustic ganglion at later stages (3–12 dpf) may represent the elimination of neurons that have failed to connect appropriately with their targets (Bever and Fekete, 1999).

**Summary: Morphogenesis of the Otic Vesicle**

Although it remains a useful model of vertebrate otic morphogenesis, the developing zebrafish ear differs from that of an amniote in several regards: the ear forms by cavitation, not invagination; the endolymphatic duct is not so prominent (it is a key landmark in the chick inner ear, for example); there is no outpouching of the semicircular canal primordia; a specialized auditory organ is absent; and cell death is not a major player in otic morphogenesis. Many genes show similar, but not identical, expression patterns to those in the ears of other vertebrate species. In several mutant lines, it is difficult to ascertain whether a gene functions only at early stages (placode induction) or whether there is a continuing role in otic morphogenesis, as early phenotypes may mask later ones. On the basis of expression patterns, some genes, such as eya1, may have roles at multiple stages of ear development. Approaches to perturb gene function conditionally will be particularly useful in tackling these problems (see the Future Perspectives section).

Our understanding of both semicircular canal and otolith formation is hampered by the paucity of markers for nonsensory epithelium and supporting cells. The collection of mutants affecting these components, however, is rich, and cloning of these will afford insights into mechanisms of semicircular canal development, supporting cell function, and otolith biomineralisation. Further analysis of these mutants, for example by cell transplantation, can be used to assess the autonomy of these mutations, and the relative roles of surrounding tissues in shaping the vertebrate ear.

**DEVELOPMENT OF FUNCTIONAL ATTRIBUTES OF THE INNER EAR**

The first neurons to differentiate in the developing ear ganglion do so soon after the first neuroblasts have arrived there, at 24–30 hpf, and may be visualized in fixed specimens with a variety of antibody probes (Haddon and Lewis, 1996). By 72 hpf, these cells have made projections that contact hair cells in the sensory patches, and by observing the swimming behaviour of larvae, it can be inferred that these connections soon become functional. At 96 hpf, most larvae have an inflated swim bladder and make active swimming movements in three dimensions. Whereas younger larvae will rest on their sides, approximately half of 3-day-old and all 4-day-old fry consistently rest and swim dorsal side up under normal lighting conditions (Riley and Moorman, 2000). This behavior is due to an active vestibular righting reflex and not to passive inflation of the swim bladder, because anaesthetised fish roll over. In addition to the righting reflex, zebrafish have a well-defined startle reflex to auditory and lateral line stimulation that also becomes functional at approximately 96 hpf. This reflex can be observed by filming larval responses to high-frequency clicks, tapping, or other vibrational stimuli, which elicit a “tail-flip” escape response (Eaton and Farley, 1973; Kimmel et al., 1974; Eaton et al., 1977). Another vestibular behaviour, the vestibulo-oculomotor reflex (VOR), can be measured by the degree of counter-rotation of the eyes in response to tilting larvae in micropipettes (Moorman et al., 1999; Riley and Moorman, 2000). Thus by 96 hr of age, zebrafish larvae show clear signs of vestibular, auditory, and lateral line function, and display the appropriate muscular reflexes after the stimulation of these systems. Several mutations have been described that perturb the development or maintenance of these functions.

**Sensory Functions**

**“Circler” mutants and hair cell function.** The “circler” mutants show deficits in equilibrium and coordination due to defects in both the lateral line and inner ear (Nicolson et al., 1998). The eight identified mutant loci appear to disrupt distinct aspects of hair cell structure and function. Mutations in mariner and sputnik cause morphologic defects in hair cell ciliary bundles, and the skylab mutation causes hair cell degeneration. The stronger alleles of mariner and sputnik show an absence of externally measured (microphonic) potentials after mechanical stimulation of lateral line hair cells. Three other mutants, gemini, mercury, and


orbiter, show no overt defects in hair cell morphology but exhibit reduced or absent microphonic potentials. Finally, astronaut and cosmonaut mutants are morphologically normal and hair cells in the lateral line show near normal microphonic potentials. Loss of behavioural responses in the latter mutants presumably involves defects in later aspects of signal transduction, such as synaptic transmission.

Additional evidence for impaired hair cell function in circler mutants is the observation that most loci are associated with marked impairment of apical endocytosis (Seiler and Nicolson, 1999). Hair cell mechanosensory function is normally associated with rapid Ca++-dependent endocytosis of luminal material. The function of this vesicular transport is not clear but may play a role in maintenance or adaptation of the sensory system. The circler mutants mariner, sputnik, orbiter, mercury, and skylab all show reduced endocytosis, which also protects them from the cytotoxic effects of streptomycin. It is not clear whether defective endocytosis is a direct consequence of these mutations, but it could contribute to impairment of hair cell function.

Mutations in mariner disrupt the zebrafish homolog of myosin VIIa (Ernest et al., 2000; Table 1). This gene codes for an unconventional myosin, defects in which are associated with Usher syndrome type 1B and some forms of nonsyndromic deafness in humans. Myosin VIIa is also disrupted at the shaker1 locus of the mouse, and a series of 10 alleles have been characterized; homozygous mutants show both vestibular defects and hearing loss. The protein is hair cell-specific within the ear (as in the fish), but details of subcellular protein distribution are species-specific. In the mouse, Myosin VIIa appears to function to maintain the structural integrity of the stereociliary bundle (reviewed in Libby and Steel, 2000).

The sputnik (spu) gene codes for a cadherin-related protein and is homologous to the CDH23 gene in humans, mutations in which cause Usher syndrome type 1D and DFNB12 (C. Söllner, G.-J. Rauch, R. Geisler, S. Schuster, and T. Nicolson, unpublished data; Bolz et al., 2001; Table 1). In the equivalent mouse mutant, waltzer, the protein encoded by Cdhl23 has been called otocadherin and is suggested to play a role as a stereociliary cross-linker, essential for correct hair bundle morphology (Di Palma et al., 2001; Wilson et al., 2001). In the fish, spu mRNA is expressed in the ear, together with the eye and brain. Morpholinos directed against the transcript phenocopy the hair cell bundle defects of the mutant, which are similar to those seen in the mouse, with splayed stereociliary bundles (C. Söllner, G.-J. Rauch, R. Geisler, S. Schuster, and T. Nicolson, unpublished data).

Stimulus dependence of vestibular function. Normal development of vestibular function in zebrafish larvae is stimulus-dependent (Moorman et al., 1999). When embryos/larvae are developed for the first 72–96 hr under conditions that simulate microgravity, they subsequently display persistent deficits in vestibular function. Illuminating such animals from below (but not from above) induces erratic or upside-down swimming behaviour, an indication that zebrafish rely on visual cues to compensate for vestibular deficits. In addition, these animals show a dramatic reduction in the ability to sense changes in their orientation in the gravity field.

Analysis of mnl mutants has also revealed previously undocumented aspects of vestibular function (Riley and Moorman, 2000). It is possible to manipulate the distribution of otoliths in mnl embryos by briefly immobilizing them in agarose at various angles. All mnl embryos immobilized in a head-down orientation form utricular otoliths bilaterally, and approximately half form saccular otoliths. Such embryos show normal vestibular function and survive as well as wild-type controls. Surprisingly, saccular otoliths are dispensable for these functions. In contrast, immobilizing mnl embryos in a head-up orientation causes bilateral loss of utricular otoliths in all embryos. Embryos lacking utricular otoliths show almost no detectable vestibular function and invariably die during larval development. These data support a longstanding (but not fully tested) hypothesis that vestibular function is primarily limited to the utricle, whereas the saccule and lagena are primarily responsible for audition (Fay and Popper, 1999; Popper and Fay, 1999). An absolute requirement for vestibular function during embryogenesis could explain why the structure of the vestibular endorgans has been so highly conserved throughout the vertebrate lineage.

Supporting Cell Function

Compared with hair cells, very little is known about the development and function of supporting cells. As mentioned above, one difficulty in studying supporting cells is that there are few reliable markers available in the species that are commonly studied, and none have yet been described in zebrafish. Supporting cells are readily recognizable by morphologic criteria in mature sensory epithelia, but this observation offers few clues about detailed mechanisms of their differentiation, physiology, or function. Despite these limitations, several mutant studies have suggested several roles of supporting cells.

Regulation of tether cells and otolith seeding. The otolith seeding defect in mnl mutants appears to result from a defect in a supporting cell function that indirectly alters the binding properties of tether cells. Previous studies of mutant-wild type chimeras showed that utricular otoliths are produced in nearly all mutant embryos harboring wild-type supporting cells, but that wild-type hair cells, or wild-type cells outside the utricle, are not sufficient to rescue their mutant hosts (Riley and Gruenwald, 1991). More recent studies have shown that the presence of wild-type supporting cells dramatically improves the ability of mutant tether cells to bind otolith seeding particles (B.B. Riley, unpublished data). In mutant control embryos, tether cells never begin to bind seeding particles before 26 somites (22 hpf). Moreover, in the fraction of cases
(18 ± 8%) in which utricular otoliths do form, tether cells rarely begin to bind seeding particles before 28 somites (23 hpf). In chimeras, however, mutant tether cells that develop in close proximity to wild-type supporting cells invariably begin to bind seeding particles between 22 and 24 somites (20–21 hpf). Even a single wild-type supporting cell is sufficient to rescue the mutant in this way. The frequency of rescue falls off with distance, such that wild-type cells lying six or more cell diameters away from the nearest tether cell have no effect on otolith seeding. These data suggest that wild-type supporting cells provide factor(s) in trans that act over a short distance to stimulate otolith seeding. Considering the rapid dispersive motion of seeding particles, it seems unlikely that supporting cells modify the local population of seeding particles. Instead, the supporting cell factor probably affects some aspect of tether cell function.

Consistent with this interpretation is the observation that wild-type tether cells do not facilitate rescue of mutant hosts (Riley and Grunwald, 1996). Specifically, wild-type cells that differentiate as tether cells in mnl hosts also show a delay in otolith seeding. Thus, the mnl gene seems critical for supporting cell function but does not appear to directly contribute to tether cell function.

The mnl gene has not yet been identified, but it probably does not encode the putative supporting cell factor responsible for mutant rescue. The mnl mutation is dominant, indicating that a 1:1 ratio of wild-type:mutant gene product (or a half-dose of wild-type protein) is not sufficient for normal gene function. It is, therefore, doubtful that a single wild-type supporting cell could restore function to an otherwise mutant macula if the mnl gene product were a secreted factor. Instead, mnl probably encodes an autonomous function in supporting cells that is required for synthesis or secretion of the rescuing factor. Thus, cloning the mnl gene promises to shed light on fundamental aspects of supporting cell biology and could also provide a valuable supporting cell marker.

Analysis of mind bomb mutants provides further evidence for the proposed role of supporting cells in tether cell function. In these mutants, maculae in the ear have a gross deficiency of supporting cells (see the Delta-Notch signaling: Lateral Inhibition and Asymmetric Cell Division section; Fig. 8). Otoliths in mib are small but, nevertheless, appear normal in structure by transmission electron microscopy. This indicates that, although supporting cells may normally generate some otolith material, other cells are also able to do so (Haddon et al., 1999). However, mib mutants typically show a 4-hr delay in otolith seeding, just as in mnl mutants. Disruption of otolith seeding is greatly enhanced in mib; mnl double mutants. In some cases, this finding reflects gross deficiency of visible seeding particles. In over half of double mutants, however, seeding particles are plentiful but nevertheless fail to bind to any of the abundant hair cell kinocilia, and instead gradually aggregate to form untethered otolith-like masses (Fig. 13). The basis for this phenotypic enhancement could be that mib and mnl are both "leaky" mutations; mnl permitting residual supporting cell activity, whereas mib may retain a few functional supporting cells. In the double mutant, the compromised function of these few remaining cells may explain the more severe phenotype.

**Maintenance of epithelial integrity and cell survival.** Analysis of mib mutants has suggested additional roles for supporting cells. First, maculae of mib embryos fail to form a basal lamina and appear to bind only weakly to surrounding nonsensory cells. The maculae are eventually extruded from the otic vesicle (Haddon et al., 1998a, 1999). In mib mosaics, wild-type cells that initially develop within mutant maculae are often displaced to the periphery by 30 hpf (Fig. 7). This finding suggests that supporting cells maintain epithelial integrity by generating appropriate extracellular matrix and cell adhesion molecules. Second, TUNEL assays appear to reveal a greatly elevated rate of apoptosis in mib mutants, and it is likely that the dying cells include the hair cells extruded from the ear epi-
ovarian epithelium. This finding suggests that interactions with supporting cells are required for hair cell survival. In summary, mib mutants display a range of defects in epithelial integrity, hair cell survival, and otolith seeding, all of which appear to result from loss of extracellular structural or regulatory factors normally supplied by supporting cells. Hair cells themselves in mib, before their extrusion from the epithelium, look surprisingly normal, even at the ultrastructural level; supporting cells do not, therefore, seem to be required for correct hair cell differentiation or morphology (Haddon et al., 1999).

**Growth and regeneration of sensory epithelia.**

In adult endorgans, supporting cells provide a stem cell-like function, whereby their progeny give rise to both hair cells and new supporting cells. Such a role has not yet been demonstrated during development in the inner ear. However, patterns of cell turnover in neuromasts of the larval lateral line suggest that central hair cells, which frequently undergo apoptosis, are constantly being renewed by induced division of peripheral supporting cells (Williams and Holder, 2000). In contrast, apoptosis is quite rare in the developing inner ear, suggesting that cell populations are more stable in otic sensory epithelia (Bever and Fekete, 1999). Nevertheless, maculae and cristae continue to grow for the life of the fish, and it is likely that division of peripheral supporting cells is largely responsible for this expansion.

Supporting cells are also likely to play a role in regeneration of damaged hair cells, as seen in other species (Baird et al., 2000; Stone and Rubel, 2000). Supporting cells not only emit factors required for hair cell survival, they apparently receive feedback from hair cells to monitor hair cell status. Such feedback is possibly mediated by persistent expression of Delta in mature hair cells. Hair cell death then disrupts the feedback signal and triggers changes in one or more adjacent supporting cells, leading to a regenerative response. In all vertebrates (although not in the mammalian cochlea), hair cell regeneration can occur by either of two mechanisms (Baird et al., 2000; Stone and Rubel, 2000): Supporting cells can directly trans-differentiate into hair cells with no intervening cell division or can undergo asymmetric cell division to generate hair cells and new supporting cells. Supporting cells can also divide symmetrically to yield two new supporting cells. A balance between these processes is required to restore depleted populations and to adjust the ratio of supporting cells to hair cells.

The molecular events controlling hair cell regeneration are still poorly characterized. For example, it is not known whether genes required for initial development are re-deployed during regeneration. However, patterns of Delta gene expression in the chick (Stone and Rubel, 1999) and pax2.1 expression in the zebrafish (BBR, unpublished data) suggest that this might be the case.

**Summary**

The first clear manifestation of inner ear function occurs at 3 dpf, when larvae begin to maintain their balance and show coordinated swimming movements. Vestibular dysfunction leads to obvious defects in motor coordination and balance. Many mutants with such behavioural defects, including most of the circler mutants, have detectable changes in hair cell structure or function. Two circler loci, mariner and sputnik, encode proteins required for structural integrity of stereocilia. In some cases, vestibular mutants may have defects in supporting cell function. This finding appears to be the case in mnl mutants, whose tether cells fail to bind otolith seeding particles but can be rescued by the activities of wild-type supporting cells. Supporting cell defects could also underlie hair cell dysfunction in some circler mutants. Supporting cells appear to maintain overall epithelial integrity and promote hair cell development and survival. Moreover, supporting cells remain plastic in their capacity to divide and transdifferentiate, properties required to orchestrate hair cell regeneration.

**Future Questions**

From the perspective of human health, an important mission of inner ear research is to uncover detailed mechanisms of hair cell regeneration. Systematic studies in wild-type and mutant zebrafish could identify many of the genes required for support cell function and hair cell regeneration. This identification will be of general value in understanding regulation of the mature sensory epithelium but could also lead to insights into human hearing loss. In mammalian embryos, supporting cells in the cochlea can trans-differentiate into hair cells but typically do not renew cell populations by reentry into the cell division cycle (Kelley et al., 1995). Moreover, little or no hair cell regeneration occurs within the mammalian cochlea postembryonically, although regeneration apparently does occur in the utricle (Forge et al., 1993; reviewed by Staeker and Van De Water, 1998). By understanding how hair cell regeneration is normally initiated in other sensory epithelia, it may be possible to stimulate this process in the mammalian cochlea.

One of the greatest challenges to inner ear research is to understand how the organization of sensory patches in the ear and neural pathways in the brain facilitate vestibular and auditory functions. Hearing in mammals is mediated almost exclusively by the Organ of Corti in the cochlea, whereas mammalian vestibular function is shared by the maculae and cristae. Fishes possess no structure homologous to the cochlear apparatus. Instead, maculae appear to be regionally specialized, with audition occurring primarily in the sacculus and lagena and vestibular function primarily in the utricle. How macular specialisation is achieved is unknown, but it probably reflects distinct patterns of neural integration in the brain. In the cichlid fish *Astropterus ocellatis* (oscar), the afferent neurons of the
various sensory patches project axons to different regions in the hindbrain and midbrain (Meredith and Butler, 1983). Similarly, recent studies in zebrafish show that neurons from saccular and utricular maculae terminate at distinct sites in the brain (S. Moorman, personal communication).

The correlation between neural projection patterns and macular specialisation raises two questions: First, how do appropriate groups of neurons come to innervate the various sensory patches within the inner ear? In principle, each macula could emit specific guidance cues that attract axons from appropriate neurons. Specific macular guidance cues have not yet been identified, although general guidance cues have been identified in the mouse that are required for efferent neurons in the hindbrain to project axons toward the contralateral inner ear (Cowen et al., 2000). Second, how are neurons organised into higher order structures within the brain, and how does this facilitate processing of vestibular and auditory information? Several zebrafish mutants have been identified by virtue of defective locomotory behaviour, and some of these could have defects in neural circuitry associated with vestibular function. For example, the circler mutants cosmonaut and astronaut (see the “Circler” Mutants and Hair Cell Function section), which have no detectable inner ear defects, may well prove to have defects in neural architecture. Another locomotory mutant, space cadet, has subtle defects in formation of specific commissures in the hindbrain necessary for motor coordination (Lorent et al., 2001). These commissural defects cause misregulation of the Mauthner neuron and thereby may indirectly affect vestibular processing. The Mauthner cell is a reticulospinal neuron that receives input from an array of sensory systems, including the inner ear and lateral line, and subsequently coordinates motor output. Further analysis of such mutants promises to elucidate detailed facets of neural patterning on which the vestibular system relies. Some of these mutants could also prove to have problems with audition.

**POSTEMBRYONIC AND LARVAL DEVELOPMENT**

Although functional at 5 dpf, the zebrafish ear will undergo further morphogenetic change and growth to acquire its adult form. This process has been beautifully illustrated by Bever and Fekete (2002) by using the paint-filling method; examples from a stage series are shown in Figure 14. The pars inferior, in particular, matures during the larval period. Two new sensory patches, the lagenar macula and the macula neglecta, arise, and the saccule and lagena become separate and distinct chambers.

**Saccule, Lagena, and Transverse Canal**

From 8 to 12 dpf, the single outpocketing containing the saccular macula can now be considered as a “sacculolagenar” pouch, as it contains the rudiments of the lagena as well as the saccule. By 15 dpf, the two chambers are separate, but remain in communication with one another by means of the sacculolagenar foramen. The saccule also separates from the utricle, and by 22 dpf, the utriculosaccular foramen is only a mere 10 μm wide. A small evagination of the dorsomedial aspect of each saccule wall at 17 dpf is the first sign of the genesis of the transverse canal, which connects the saccules at the midline by 20 dpf (Bever and Fekete, 2002). This structure is prominent in the adult ear, where its caudal end is enclosed within, but is a distinct compartment from, the perilymph-filled space of the sinus impar (Bang et al., 2001). The lateral saccule wall, conversely, invaginates and thickens to form a saccular ridge, the function of which is unknown (see the Discussion section in Bever and Fekete, 2002).

**Further Development of Sensory Patches**

The lagener macula is evident as an undifferentiated thickening of epithelium, contiguous with the saccular macula, lying on the medial wall of the undivided sacculolagenar pouch. Hair cell differentiation begins at 15 dpf (Bever and Fekete, 2002), but despite this late onset, the lagener macula will eventually contain more hair cells than the saccular macula (Platt, 1977; Bang et al., 2001). The macula neglecta, which has no otolith, is located in the adult ear near the utriculosaccular canal; it is visible at 17–20 dpf and consists of two small patches of hair cells of opposing orientation (Platt, 1977; Bang et al., 2001). These patches may arise from the saccular macula (see the Discussion section in Bever and Fekete, 2002). During larval stages, all three maculae and the cristaæ continue to grow (Bang et al., 2001). The utricular macula develops a long thin extension, the lacinia, whereas the whole saccular macula becomes very elongated. Hair cell densities and polarity patterns have been mapped for each sensory patch in the adult ear (Platt, 1977; Bang et al., 2001). Hair cells probably continue to be produced into adulthood, as in the goldfish (Lanford et al., 1996).

**Otoliths**

A third otolith, the lagena’s asteriscus, appears at 9–17 dpf, depending on strain (Riley and Moorman, 2000; Bever and Fekete, 2002). All three otoliths continue to grow during larval stages, and by 30 dpf resemble their adult form (Bever and Fekete, 2002). The sagittæ of the saccules, initially rounded, becomes thin and elongated, tapering caudally; the lapillus of the utricle remains ovoid, whereas the asteriscus in the adult is round and flat, with fluted edges (Platt, 1993).

**Semicircular Canals**

Although the three canals and common crus have formed by 3 dpf, they must grow to acquire their distinctive form in the adult. The ampullæ, swellings at the bases of the canals that house the cristaæ, become evident at 17 dpf (Bever and Fekete, 2002; Fig. 14).

**Chondrogenesis and Development of Perilymphatic Spaces**

Development of the cartilaginous capsule surrounding the ear has not been well-studied in the zebrafish.
The ear becomes partly encased by cartilage by 4 dpf (Haddon and Lewis, 1996; for anatomy of the otic capsule, and a description of mutations affecting chondrogenesis and craniofacial development, see Neuhauss et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996).

The anatomy of the main perilymphatic compartment of the adult zebrafish ear, the sinus impar, has been described by Bang et al. (2001).

**Weberian Ossicles and the Swim Bladder**

Otophysan fish use a series of transformed vertebrae, the Weberian ossicles, to enhance their hearing (reviewed in Popper and Fay, 1999). These form a chain of four bones, the tripus, intercalarium, scaphium, and claustrum, which couple the inner ear to the swim bladder, and are thought to amplify vibrational stimulation of the ear. Bang et al. (2001) have described their anatomy in the adult zebrafish. The swim bladder becomes inflated by 120 hpf (Kimmel et al., 1995).

**FUTURE PERSPECTIVES**

Inner ear research has undergone rapid growth on many fronts. The field has made significant progress in answering some very old questions, and has begun to frame entirely new questions based on a fundamental new understanding. The rich assortment of genes and mutants identified in zebrafish has been seminal, yet in many ways the task of clarifying the functions and interrelationships of these genes is just beginning. It is also certain that many more genes with important...
functions in the inner ear are yet to be identified. Because basic mechanisms of ear development are conserved in all vertebrates, comparative studies will continue to be productive and many of the findings in zebrafish will be of broad relevance.

Many of the key questions facing the field of inner ear research have been outlined in preceding sections. Here we briefly consider how zebrafish genetics may contribute to answering these questions and discuss the broader significance of these findings.

A Continuing Role for Genetics

The zebrafish offers a wide range of genetic techniques which, combined with the relative ease of phenotypic analysis, will figure prominently in future studies. As more and more regulatory genes are identified, it will be increasingly important to analyze double and even triple mutants (relatively straightforward in zebrafish) to untangle the complex networks and pathways regulating the processes in question. Simultaneous use of morpholinos further expands the scope of experiments that might be contemplated. Mutagenesis screens in sensitized backgrounds (carrying known mutations) will be useful for identifying additional interacting loci. Screens can also be designed to identify temperature sensitive alleles of previously identified mutations, and behavioural screens will be an efficient way to identify additional loci with roles in hearing or balance, behaviours that can be readily assayed during larval development. Conditional (and reversible) disruption of key signaling pathways, such as FGF, Delta-Notch, and BMP, will permit detailed analysis of their stage-specific functions.

The use of caged nucleic acid constructs, which can be photoactivated to give temporal and spatial control of gene expression, may be useful here (Ando et al., 2001). A rapid and potentially powerful way of achieving conditional disruption of protein function is by the application of small molecules; because of its external accessibility for observation and study. As the embryo is optically clear, direct observation of the inner ear, at cellular resolution, is possible in the live embryo. For these reasons, several methods of phenotypic analysis, such as cell transplantation or fate mapping, are easily performed in the fish embryo, but would be difficult to apply to a murine model.

Zebrafish as Models for Human Deafness

With the cloning of genes disrupted in a variety of mutant lines, it is now becoming clear that the zebrafish can be considered as a powerful model organism in the understanding of human disease (Dooley and Zon, 2000). At least five forms of syndromic or nonsyndromic deafness are now known to have a zebrafish counterpart (Table 1). In the human, these conditions are important clinical conditions. Congenital deafness affects up to one in every thousand children at birth or in early (prelingual) childhood (Petit, 1996). Usher syndrome type IB is the most common cause of deaf-blindness in developed countries, whereas Branchio-Oto-Renal syndrome accounts for at least 2% of profoundly deaf children (Fraser et al., 1980; Online Mendelian Inheritance in Man, 2001). At present, for most of the cloned zebrafish genes outlined in Table 1, there also happen to be corresponding mouse mutations or knockout lines. Although the mouse provides a closer anatomic model for the human ear, the zebrafish offers unique advantages for the analysis of the developmental genetic mechanisms underlying human disease. Its main asset is that, unlike in the mouse, all embryologic stages are accessible for observation and study. As the embryo is optically clear, direct observation of the inner ear, at cellular resolution, is possible in the live embryo. For these reasons, several methods of phenotypic analysis, such as cell transplantation or fate mapping, are easily performed in the fish embryo, but would be difficult to apply to a murine model.
One difference between the fish models and the human diseases they model is that zebrafish mutations are generally less severe. Indeed, fish mutations are usually recessive, whereas the equivalent human disorders are often dominant conditions, the result of haploinsufficiency in the heterozygous state (e.g., Waardenburg-Shah syndrome and Branchio-Oto-Renal syndrome; Table 1). Homozygosity for the human disease genes (as well as knockouts of mouse homologs) usually results in much more severe phenotypes. The difference in phenotypic severity probably reflects duplication of many regions of the fish genome relative to mammals (Postlethwait et al., 1998). That is, zebrafish mutations are often less severe than their mammalian counterparts because ancestral functions are typically subdivided amongst two or more homologs. This subdivision offers a great advantage in specifying specific details of gene function. Whereas the severity of mammalian mutations often masks secondary or late-acting functions of individual genes, the splitting of functions between different fish homologs permits multiple functions to be studied independently. For this reason, zebrafish should provide a useful means of facilitating functional genomic studies of human sequences, and will continue to contribute to an understanding of how our own inner ears develop and function.

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