The zebrafish dog-eared mutation disrupts eya1, a gene required for cell survival and differentiation in the inner ear and lateral line

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Abstract

To understand the molecular basis of sensory organ development and disease, we have cloned and characterized the zebrafish mutation dog-eared (\textit{dog}) that is defective in formation of the inner ear and lateral line sensory systems. The \textit{dog} locus encodes the \textit{eyes absent-1} (\textit{eya1}) gene and single point mutations were found in three independent \textit{dog} alleles, each prematurely truncating the expressed protein within the Eya domain. Moreover, morpholino-mediated knockdown of \textit{eya1} gene function phenocopies the \textit{dog-eared} mutation. In zebrafish, the \textit{eya1} gene is widely expressed in placode-derived sensory organs during embryogenesis but Eya1 function appears to be primarily required for survival of sensory hair cells in the developing ear and lateral line neuromasts. Increased levels of apoptosis occur in the migrating primordia of the posterior lateral line in \textit{dog} embryos and as well as in regions of the developing otocyst that are mainly fated to give rise to sensory cells of the cristae. Importantly, mutation of the \textit{EYA1} or \textit{EYA4} gene causes hereditary syndromic deafness in humans. Determination of \textit{eya} gene function during zebrafish organogenesis will facilitate understanding the molecular etiology of human vestibular and hearing disorders.

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Introduction

The inner ear is a complex sensory organ that serves to monitor environmental stimuli such as sound, motion, and gravity. Highly conserved among vertebrates, the inner ear is functionally and structurally divided into two subsystems: auditory (cochlea) and vestibular (membranous labyrinth and semicircular canals). Both the auditory and vestibular systems are sensitive to environmental, chemical, and genetic insults that can lead to hearing impairment or balance deficits. Current efforts to understand the molecular etiology of hearing-related disorders rely on animal models to identify developmental and regulatory mechanisms, including their component genes, necessary for normal morphogenesis and function of the inner ear.

The zebrafish (\textit{Danio rerio}) has become an increasingly important model system to understand the molecular genetic basis of vertebrate organogenesis, including the inner ear, and disease (Whitfield, 2002; Whitfield et al., 2002). Over 60 complementation groups affecting development or function of the zebrafish inner ear have been identified.
(Malicki et al., 1996; Whitfield et al., 1996), and many of the inner ear phenotypes resemble mutations affecting size, shape, or function of the mammalian vestibular system (Ahituv and Avraham, 2000; Hrabe de Angelis et al., 2000; Steel, 1995; Steel et al., 1997; Torres and Giraldez, 1998). An increasing number of these zebrafish mutations are being cloned and the affected genes identified found to be involved in hereditary deafness syndromes. These genes include myoVIIA, pax2a, foxl1, thbl1, and sox10 (Dutton et al., 2001; Ernest et al., 2000; Lee et al., 2003; Lun and Brand, 1998; Moens et al., 1998; Nissen et al., 2003; Piotrowski et al., 2003; Solomon et al., 2003). In this study, we report the identification of the dog-eared locus as encoding the zebrafish eya1 gene.

eya1 is an ortholog of the Drosophila gene eyes absent and is required for cell differentiation and survival in the fly eye (Bonini et al., 1993). In vertebrates, there are four known eya genes and mutations in EYA1 or EYA4 cause human hereditary deafness disorders (Abdelhak et al., 1997; Azuma et al., 2000; Kumar et al., 2000; Wayne et al., 2001). In humans, haploinsufficiency of the EYA1 locus is a cause of Branchio-Oto-Renal syndrome (BOR), a disorder characterized by craniofacial and kidney defects and sensorineural deafness (Abdelhak et al., 1997). Mice heterozygous for an Eya1 null allele display otic and renal defects similar to those in human BOR patients, while homozygous mutants develop with numerous anomalies, including outer, middle, and inner ear defects (Xu et al., 1999). The zebrafish provides an attractive model system to study genes implicated in inherited human disorders because phenotypic consequences of mutations are readily discerned and gene expression is easily manipulated.

In this study, we have analyzed the zebrafish dog-eared (dog) mutation that was identified in a large-scale ENU mutagenesis screen by abnormal morphology of the inner ear (Whitfield et al., 1996). The phenotype of all four dog alleles is recessive, fully penetrant, and variably expressed. dog-eared embryos are less responsive to vibrational stimuli, fail to maintain balance when swimming, and may circle when disturbed, a behavior characteristic of fish with vestibular defects (Nicolson et al., 1998). Initial characterization of the dog inner ear phenotype (Whitfield et al., 1996) revealed defects of both sensory and non-sensory tissue of the inner ear. Specifically, hair cells in the sensory cristae fail to differentiate, while those in the maculae are reduced in number. In addition, otoliths were found to be smaller. The dog mutation, thus, does not affect sensory hair cell formation per se but primarily affects regions of the otocyst containing the sensory patches (Whitfield et al., 1996).

Using a positional candidate cloning approach, we show that the dog-eared locus encodes the zebrafish eya1 gene, which is expressed during embryogenesis in cells of the anterior pituitary, olfactory, otic, and lateral line placodes, as well as in the somites, branchial arches, and pectoral fins (Sahly et al., 1999). Homozygous dog embryos have ectopic and increased levels of cell death in the developing otic vesicle and migrating primordium of the posterior lateral line. These results demonstrate that Eya1 is required for survival of cells in the otic vesicle, including those cells that will differentiate into crista hair cells.

Materials and methods

Zebrafish strains and maintenance

Adult zebrafish were maintained under standard conditions (Westerfield, 1993). Embryos were collected from pairwise matings of dog heterozygous adults and staged by hours post-fertilization (hpf) at approximately 28.5°C. Homozygous dog embryos were identified by abnormal ear morphology. For the dog<sup>neo60b</sup> allele, homozygous embryos were also identified by genotyping for the dog<sup>neo60b</sup>-specific RFLP marker described below.

Genetic mapping, PCR, and sequencing

A meiotic mapping cross was established by mating a fish heterozygous for the dog<sup>neo60b</sup> allele on a Tü background with a WIK fish (Knapik et al., 1996; Rauch et al., 1997). F<sub>1</sub> progeny, heterozygous for the dog<sup>neo60b</sup> locus, were identified by random pair mating and subsequently used to generate F<sub>2</sub> embryos that were sorted by phenotype. Genomic DNA was extracted from wild-type and dog F<sub>2</sub> embryos for genotyping by PCR (Rauch et al., 1997). Bulked segregant analysis (Talbot and Schier, 1999) using 50 dog<sup>neo60b</sup> embryos and 50 phenotypically wild-type siblings and a genome-wide panel of polymorphic CA repeat markers (Shimoda et al., 1999) placed the dog locus on LG24. Further mapping using 750 individual mutant embryos (1500 meiososes) positioned the dog locus between CA repeat markers z7823 and z9321. Candidate genes and ESTs located near the dog locus were positioned and linearly ordered relative to dog flanking markers by mapping to the LN54 radiation hybrid (RH) panel (Hukriede et al., 1999). The EST fe13c10, corresponding to the eya1 gene, was mapped onto the RH panel using two sets of primers that gave the same result; 5′UTR1-1GCTGG-GCCTTGAGTTTAC and 5′UTR1-1GCTGG-GCCTTGAGTTTAC.

RT-PCR of the zebrafish eya1 cDNA

Total RNA was isolated from 72 to 96 hpf embryos (using Trizol; Life Technologies, Gibco BRL) and used as a template to generate eya1 cDNA (Superscript Preamplification System For First Strand cDNA Synthesis, Life Technologies, Gibco BRL). Primers were designed from the published eya1 sequence (Sahly et al., 1999). The gene-
specific primer R2 (5'-CTATCAGAACGCAGACCGTCG-3') was used to prime reverse transcription, and primer pairs, F1 (5'-GCAAGGATTCTACCATATGTG-3') and R2, F1 and R3 (5'-CGTGCGGATTGGAGATAGTCG-3'), or F3 (5'-GGACTATCTCTCTCCACCAGCG-3') and R2 were used to generate full-length 5' or 3' portions of the eya1 cDNA, respectively. PCR products were purified by agarose gel electrophoresis and cloned using the pGEM-Teasy Vector System (Promega). Both cloned cDNAs and bulk cDNA PCR products were sequenced and yielded similar results.

To assess correct splicing of eya1 mRNA (Figs. 2G and H), total RNA was prepared from 24 and 48 hpf embryos, reverse transcribed using a polyT primer, and the eya1 exon 10/11 boundary PCR amplified using primers F3 (see above) and R4 (5'-GTTGCGAAGCGTGAATCGTCG-3').

Genomic sequence of the eya1 locus

An arrayed zebrafish BAC library (Incyte Genomics, Inc.) was screened by PCR using the eya1 primers 3'UTR1 (5'-AGTGGCACTGCGCCATATAAA-3') and 3'UTR2 (5'-AAAACGTCAACACTTTCAAGC-3'). Two BAC clones (54D17 and 71D17) were identified and directly sequenced with CEQ dye terminator (Beckman Coulter) and run on an ABI Prism 310 Genetic Analyzer to obtain sequence of (54D17 and 71D17) were identified and directly sequenced.
GATGATGACCTACTTCC-3’) or containing a 5 nucleotide mismatch (mutMO: S’-AAAATACATGTTACCC- TAGTTC-3’) were microinjected into the one-cell embryo. Initial titration experiments determined that microinjection of 20 ng of eya1MO was sufficient to generate specific embryonic defects without general toxicity. Microinjection of 20 ng mutMO had no effect on embryonic development.

Results

Identification and molecular characterization of the dog locus

The dog gene was mapped to linkage group 24 (Fig. 1A) by genome-wide linkage analysis of simple sequence length polymorphisms (SSLP) tested on a panel of mutant embryos (100 meioses). Higher resolution mapping using 750 individual mutant embryos (1500 meioses) demonstrated that SSLP markers z7823 and z9321 flank the mutant locus and define a 1.7-cM interval containing the dog gene. These and other closely linked SSLP markers were placed on the LN54 radiation hybrid panel (Hukriede et al., 1999) to identify candidate genes or ESTs located within the region defined by markers z7823 and z9321. One EST fc13c10, corresponding to the zebrafish eyes absent-1 (eya1) gene (Fig. 1A), was located within this 1.7-cM genomic interval. Because mutations of the EYA1 gene in humans (Abdelhak et al., 1997) and Eya1 gene in mouse (Xu et al., 1999) result in defects in inner ear development and function, we chose to investigate zebrafish eya1 further as the candidate gene for the dog-eared locus. The zebrafish eya1 ortholog has already been cloned and its expression in the otic vesicle, facial ganglia, and lateral line systems (Sahly et al., 1999) is also consistent with it being the gene affected in dog-eared mutants.

We cloned and sequenced eya1 cDNAs from both dog and phenotypically wild-type sibling embryos using PCR primers designed from the published eya1 sequence (Sahly et al., 1999). Sequence comparison of cDNA from phenotypically wild-type sibling embryos and the published eya1 cDNAs identified a single difference in the ORF sequence. The published zebrafish eya1 cDNA contains an extra 66-bp fragment located 124-bp downstream of the A of the ATG start codon and this inserted sequence is unique to the zebrafish eya1 cDNA reported (Sahly et al., 1999). In the multiple eya1 cDNA clones derived from either dog or wild-type siblings embryos, we never observed this 66-bp fragment. The reported 66-bp fragment is defined by GT and AG dinucleotides that are canonical splice donor and acceptor sequences, is present in the eya1 genomic DNA sequence, and likely represents a splice variant of the eya1 gene. We thus found the size of the predicted Eya1 isoform to be 587 amino acids.

Analysis of eya1 cDNA sequences from three independent alleles of dog revealed two classes of mutations, each predicted to truncate the expressed protein. cDNA derived from dog-eared allele tp85b had a single nucleotide C to T transition changing a codon for arginine (CGA) at position 420 to a termination codon (TGA; Fig. 1B). This mutation is predicted to truncate the Eya1 protein in the N-terminal half of the Eya domain (Fig. 1E).

Sequence analysis of eya1 cDNAs from the two other dog alleles tm90b and tc257e revealed similar but not identical 4-bp insertions located at the same position in the cDNA, just downstream of the glutamic acid codon at amino acid position 375 (Fig. 1D). Each 4-bp insertion is predicted to give rise to a frame-shift in the coding sequence, resulting in a truncated protein lacking most of the Eya domain. Insertions are not characteristic of the mutagen (ENU) used to generate the dog alleles, and the sequence of each insertion suggested the possibility of a mutated splice donor sequence in the adjacent intron. To identify adjacent intronic sequence, we isolated a genomic BAC clone containing this region of the eya1 gene and obtained genomic sequence adjacent to the 4-bp insertions. Sequence analysis revealed an exon–intron boundary adjacent to the 4-bp insertions. Further analysis of this sequence in each dog allele revealed a single point mutation in the dinucleotide splice donor sequence (Fig. 1B). Each mutation was located in one nucleotide of the conserved splice donor sequence for putative intron 10 (Fig. 1D). Sequence of the cloned eya1 cDNAs from each of these alleles indicates that a cryptic splice donor sequence located 4-bp downstream was utilized and appended four nucleotides to the 3’ end of putative exon 10 (Fig. 1D). (Exons and introns are numbered by analogy to human EYA1.)

The identified point mutation in the eya1/dog<sup>tm90b</sup> genomic DNA creates a novel MnlI RFLP that can be used to follow the tm90b allele in our map cross (Fig. 1C). Fourteen phenotypically mutant embryos that were recombinant for flanking SSLP markers z7823 or z9321 (data not shown) are all homozygous for the mutant allele of dog-eared.

To further confirm that disruption of the eya1 gene is responsible for the dog-eared phenotype, we tested whether a morpholino oligonucleotide (eya1MO; see Materials and methods) complementary to the exon10–intron10 splice sequences in the eya1 primary RNA could phenocopy the dog-eared mutation. Targeting of antisense morpholinos to primary RNA splice sequences is an effective approach to alter normal mRNA processing and “knockdown” gene function (Draper et al., 2001). Wild-type embryos microinjected with 20 ng eya1MO at the one-cell stage were assayed for the presence of correctly spliced eya1 mRNA at 24 and 48 hpf (Figs. 2G and H). eya1 mRNA correctly spliced at the exon10–exon11 junction is undetectable in eya1MO-injected embryos using PCR primers spanning this splice junction. In contrast, eya1 mRNA correctly spliced in this region is detected in embryos injected with buffer only or a control morpholino containing a 5 nucleotide mismatch to the eya1 exon10–intron10 splice junction (Figs. 2G and H).
Microinjection of eya1MO phenocopies the dog-eared mutation. Wild-type embryos microinjected with 20 ng of eya1MO have abnormal otic vesicle morphology (compare Fig. 2B with Fig. 7) characteristic of the dog-eared mutant embryos (see below). Additionally, eya1MO-injected embryos have ectopic cell death in the developing otic vesicle (acridine orange positive cells; Fig. 2D), and these cells are apoptotic (TUNEL positive; Fig. 2F). Each of these phenotypes is characteristic of the dog-eared mutant phenotype (see below). Together, genetic mapping, identification of point mutations in the eya1 gene, and phenocopy by knockdown of eya1 gene function demonstrate that the dog-eared locus encodes the zebrafish eyes absent-1 gene. Below we detail the phenotypic defects associated with eya1 mutations in the zebrafish embryo.

**Formation of the otic placode occurs normally in dog-eared embryos**

Expression of the eya1 gene in the zebrafish embryo has been described in detail (Sahly et al., 1999). We find that the defects in dog-eared embryos largely correspond to the spatial and temporal domains of eya1 expression. However, despite the expression of eya1 in the presumptive otic region at pre-placodal and placodal stages (Sahly et al., 1999), induction of the otic and lateral line placodes appears to occur relatively normally in dog-eared embryos, and at
increases (Figs. 3G and J), than wild-type siblings (Fig. 3A). As the phenotypic severity pooled embryos.

fragment amplified from correctly spliced mRNA. Each lane represents five eya1MO-injected, or mismatched morpholino (mutMO)-injected, or buffer-injected embryos at 24 hpf (G) and 48 hpf (H). Products of reactions of wild-type siblings have fused in the ear lumen, forming a characteristic cruciform appearance when viewed from the lateral side (Fig. 3B). In dog embryos of the same stage, the protrusions do not have a cruciform appearance but, rather, are disorganized and folded within the otic vesicle lumen (Figs. 3E, H, and K). By 96 hpf, the lumen of the phenotypically most severe dog otic vesicles is nearly filled by these epithelial projections; the canals themselves are misshapen with narrow lumens.

Sensory hair cells are reduced in all sensory patches of the dog-eared otic vesicle and lateral line

Initial phenotypic characterization of the dog phenotype revealed the absence of sensory hair cells in the three cristae (Whitfield et al., 1996). We documented this aspect of the inner ear phenotype more closely using confocal microscopy of FITC-phalloidin stains to detect the actin-rich stereocilia that are present on hair cells in both cristae and maculae in the otic vesicle. Fig. 4A shows the normal pattern of hair cells in a 5.5-day embryo. In agreement with previous results, we find that most phenotypically dog embryos lack crista hair cells as judged by the absence of phalloidin-stained stereocilia (Fig. 4B). Only in rare cases were we able to identify dog embryos with 2–3 hair cells in one of the three cristae (Fig. 4B arrowhead; higher magnification view in inset). This indicates that the dog gene is not required to make crista hair cells per se, but rather the gene product may be required for maintenance or survival of crista hair cells.

Although macular hair cells are present in dog otic vesicles, both the saccular and utricular maculae show an abnormal shape and overall reduced hair cell number in dog embryos. In the saccular macula, there may be additional hair cells in the anterior region of the patch, which is sometimes wider than normal, while there are fewer hair cells in the posterior part of the patch, which is sometimes separated from the anterior part by a hair cell-free region.
Cell number and patch morphology are also variable between and within alleles for the utricular macula; in the examples shown, about half the normal number of hair cells is present at d5.5 (Figs. 4K–N). Of 24 dog otic vesicles examined carefully for macular morphology, 8 showed a mild phenotype (Figs. 4D and H), 11 a moderate phenotype (Figs. 4E and I), and 5 a severe phenotype (Figs. 4F and J). Additionally, hair cells that are present in the utricular macula have short stereociliary bundles; those with longer bundles, abundant at the lateral edges of the wild-type macula, appear to be absent (Figs. 4K–N).

Expression of bmp4 mRNA is abnormal in dog otic vesicles

To investigate the earliest molecular effects of the dog mutation on sensory crista formation, we examined gene expression in presumptive crista cells. Previously, it has been shown that dog embryos have reduced or absent staining of mscx in three ventrolateral domains of the otic vesicle at 48 hpf (Whitfield et al., 1996). At this stage in otocyst development, domains of mscx expression correspond to thickened regions of the otic epithelium that will become the sensory cristae.

Earlier in otocyst development, both bmp4 and the related bmp2b gene are expressed at the anterior and posterior ends of the 24 hpf otic vesicle (Mowbray et al., 2001); these are regions likely to contain prospective crista hair cells (Haddon, 1997). In two dog alleles examined (tm90b and tp85b), expression of bmp2b at 24 hpf is present but abnormally weak (Mowbray, 2002), whereas bmp4 expression is reduced in one dog allele (tp85b) but normal in the other (tm90b). By 36 hpf, three ventrolateral domains of bmp4 mRNA expression are apparent in wild-type sibling otic vesicles (Fig. 5A; Mowbray et al., 2001), but this expression is variably reduced or absent in the otic vesicles of dog (tm90b and tp85b) mutant embryos (Mowbray, 2002). We found a variable reduction of bmp4 expression in the anterior and lateral cristae, and in some cases an increase in bmp4 expression in the posterior crista and in a dorsal region of the otocyst in dogtm90b embryos at this stage (Fig. 5C). By 55 hpf, ventrolateral expression of bmp4 mRNA is clearly associated with epithelial thickenings that will become the three cristae in wild-type embryos (Fig. 5B). In contrast, bmp4 mRNA expression is nearly extinguished in these domains in the mutants (Fig. 5D). By this stage, expression of brn3.1 mRNA, which normally marks nascent hair cells (Mowbray et al., 2001) and of eya1 itself, is greatly reduced or absent in the same three ventrolateral regions, but remains in the developing maculae (data not shown). Together, the progressive loss of specific gene expression in presumptive crista cells is consistent with the absence of crista hair cells later in development.
Although the bmp4 gene is considered to be expressed in presumptive crista cells, this has not been formally shown. To show that cells within the three ventrolateral domains of bmp4 expression give rise to the cristae, we fate mapped 10–15 cells in each ventrolateral region of bmp4 expression in the 36 hpf otic vesicle. Cells in the lateral half of the otic vesicle labeled by uncaging of fluorescein at this stage stay together as a recognizable group (Kozlowski and Weinberg, unpublished observations) and do not scatter to the extent seen in the frog (Kil and Collazo, 2001). This finding permits fate mapping of the 36 hpf otic vesicle.

Fig. 5E and F illustrate two examples of cells labeled by the photoactivation of caged fluorescein in the anterior (Fig. 5E) or posterior (Fig. 5F) ventrolateral domain of bmp4 expression (to illustrate the spatial relationship of uncaged cells to the axes of the otic vesicle more clearly, the focal plane shown in each panel is slightly more medial than the plane of uncaging). At 72 hpf, cells containing uncaged fluorescein were detected immuno-histochemically and their fates determined. Cells labeled in Fig. 5E gave rise to hair and supporting cells in the anterior crista (Fig. 5G) and portions of the semicircular canals, but not to more posterior regions of the otic vesicle. Cells labeled in the posterior region of the otic vesicle gave rise to cells in the posterior crista (Fig. 5H) and portions of the semicircular canals, but not to more anterior regions of the otic vesicle. In both cases, we intentionally labeled large regions that include both presumptive crista and semicircular canal cells. These data indicate that cells within the ventrolateral domains of bmp4 expression at 36 hpf give rise to at least the sensory cristae. Similar attempts to fate map presumptive crista cells in dog embryos consistently resulted in fewer than expected cells at 72 hpf (data not shown). This result suggests that the absence of crista hair cells in the mutants may not be due to changes in cell fate of presumptive crista cells, but rather that presumptive crista cells die or do not proliferate during otic vesicle morphogenesis.

Fig. 4. Sensory patch phenotype in the ears of dog-eared homozygous embryos; confocal images of embryos and larvae stained with FITC-phalloidin. (A) Dorsal view (anterior to top) of a dissected preparation of the two ears of a 5 dpf wild-type sibling larva showing five distinct sensory patches in each ear: the utricular and saccular maculae (um, sm), and the anterior, lateral, and posterior cristae (ac, lc, pc). Scale bar, 50 μm. (B) The ears of a dogtm9b homozygote. Hair cells of the cristae are not visible in the left ear; in the right ear, three hair cells are present in the region where the lateral crista would normally form (magnified in inset). (C–J) Abnormalities in the saccular macula (orientation shown in J: anterior to left, dorsal to top): (C) lateral view of wild type, 3 dpf; (D–F) examples of saccular maculae from dogtm9b homozygous mutants, 3 dpf; (G) wild type, 5 dpf; (H–J) examples of saccular maculae from dogtm9b homozygous mutants, 5 dpf. Scale bar, 20 μm. (K–N) Abnormalities in the utricular macula (orientation shown in N: anterior to left, medial to top): (K and M) dorsal views of utricular maculae from phenotypically wild-type siblings, 5.5 dpf; (L) dogtm9b homozygote, 5.5 dpf; (N) dogtm9b homozygote, 5.5 dpf. Brackets to the left and right of the wild-type maculae shown in K and M indicate the hair cells with long stereociliary bundles located at the lateral edges of the macula; this type of hair cell is mostly absent in the maculae of dog embryos (L and N). Scale bar, 20 μm.
Apoptosis is increased in the otic vesicles of dog-eared embryos

To understand the basis of decreased bmp4 gene expression and the absence of sensory cristae in dog-eared otic vesicles, we examined apoptosis-mediated cell death in the developing otic vesicles of dog embryos. During development of the zebrafish otic vesicle, there is very little, if any, cell death (Bever and Fekete, 1999; Cole and Ross, 2001). Consistent with published observations, we found very little cell death in wild-type siblings of dog-eared embryos at 28 hpf (Figs. 6A and C). In contrast, the otic vesicles of dog embryos had increased numbers of TUNEL-positive cells in the otic vesicle (Figs. 6B and D). TUNEL-positive cells were generally found in groups located primarily in a lateral domain of the otic vesicle.

Fig. 5. Otocyst expression of bmp4 mRNA in dogmt90b and fates of cells within ventrolateral domains. (A and B) Four domains of bmp4 mRNA expression are detected in wild-type siblings at 36 (A) and 55 hpf (B) (Mowbray et al., 2001). (C and D) In dogmt90b, bmp4 mRNA expression is variably reduced in two of the ventrolateral domains and in some embryos is increased in the posterior ventrolateral domain and in the dorsal domain at 36 hpf (C). At 55 hpf, bmp4 mRNA expression in dogmt90b is virtually absent in all three ventrolateral domains, but not in the dorsal domain (D). For each of the 55 hpf embryo panels (B and D), a line has been drawn at the margin of the otic vesicle to better indicate the position of bmp4-expressing cells. (E and F) Labeling of cells by uncaging of fluorescein within anterior (E) or posterior (F) ventrolateral domains of bmp4 expression. Focal planes shown are more medial than the uncaged cells to demonstrate more clearly the location of uncaged cells relative to anterior–posterior and dorsal–ventral axes of the otocyst. The uncaged cells are more lateral than the cells in the focal plane shown. (G) Anterior ventrolateral cells labeled in E are detected in the anterior crista (white arrow) and semicircular canal at 72 hpf. (H) Posterior ventrolateral cells labeled in F are detected in the posterior crista (white arrow) and semicircular canal at 72 hpf. Embryos in A–F are shown in lateral views with anterior to the left. Embryos in G and H are shown in dorsal views with anterior to the left and medial to the top.

Fig. 6. Increased apoptotic cell death in dogmt90b. Whole mount visualization of TUNEL-positive cells (blue) in wild type (A, C, E, and G) and dogmt90b mutant (B, D, F, and H) embryos. (A and B) Lateral views of wild-type sibling (A) and dogmt90b (B) embryos at 28 hpf. In mutant embryos, increased numbers of TUNEL-positive cells are seen in the region of the otocyst (arrow) and migrating primordium of the posterior lateral line (bracket). (C and D). Higher magnification view of otocysts in wild-type sibling (C) and dogmt90b (D) embryos. (E and F) Transverse sections through the otic vesicles of embryos shown in panels A and B. Note that TUNEL-positive cells are located within the lateral otocyst wall of dogmt90b embryo. (G and H) Higher magnification view of TUNEL-positive cells in the migrating primordium of the posterior lateral line in wild-type sibling (G) and dogmt90b (H) embryos shown in A and B, respectively. Panels E and F: dorsal to top and lateral to right. All other panels: lateral views with dorsal to top and anterior to left.
To be sure that these cells are within the otic epithelium and not located on the embryo surface, we cut transverse sections through dog and wild-type sibling embryos. Fig. 6F illustrates that TUNEL-positive cells lie within the lateral otic epithelium of dog embryos. Additionally, increased numbers of TUNEL-positive cells were found in the migrating primordium of the posterior lateral line (Fig. 6H). The results of TUNEL and acridine orange staining demonstrate that ectopic cell death occurs in the otic vesicle of dog embryos. Clearance of apoptotic cells is thought to be rapid in the zebrafish (Bever and Fekete, 1999; Cole and Ross, 2001) so that the presence of dying otic vesicle cells at several developmental stages indicates that cells are continually dying during otic vesicle morphogenesis, rather than simply accumulating over time.

Reduced cell numbers in the statoacoustic ganglion of dog embryos

To determine whether other structures derived from otic vesicle cells are affected in dog embryos, we examined the statoacoustic ganglion (SAg; VIIIth ganglion) during development. Cells giving rise to the neurons of the SAg are derived from the ventral floor of the otic vesicle (Haddon and Lewis, 1996). The sna2 gene is expressed in these cells as they delaminate from the otic vesicle at 24 hpf and sna2 expression is variably reduced in this region of dog embryos (Whitfield et al., 2002). Similarly, we find fewer cells expressing the homeobox gene tlxA (Andermann and Weinberg, 2001) in the presumptive SAg (double-headed arrow, panel B) embryos when compared to wild-type siblings (A). Numbers of tlxA-expressing cells in the trigeminal (line arrow) and anterior lateral line ganglia (open arrow) are comparable in wild-type siblings (A) and dog (B, D, and F) embryos. Axonal projections around the otocyst expressing the 3A10 epitope. In dog embryos (D), there are longer, but fewer, axonal projections in the VIIIth nerve (black arrow). (A and B) Ventral view, anterior to left. (C and D) Dorsal to top and anterior to left. Asterisks mark the otic vesicles.
headed arrow, Fig. 8B) but not the trigeminal or anterior lateral line ganglia (arrows, Fig. 8B) when compared to wild-type sibling embryos (Fig. 8A). At 96 hpf, axonal projections of the SAg can be detected by the neurofilament antibody 3A10 (Hatta, 1992) and there are fewer projections in dog embryos (Fig. 8D). Axonal projections of the Vth, VIIth, and Xth nerves appear to be unaffected in dog embryos (cf. Figs. 8C and D).

**Defects in other eya1-expressing tissues**

eya1 is also expressed in the lateral line, pituitary, olfactory placode, somites, pectoral fins, and branchial arch region of zebrafish embryos (Sahly et al., 1999). Initial characterization of the dog phenotype found that the terminal neuromasts of the posterior (trunk) lateral line were often absent in dog embryos (Whitfield et al., 1996). Examination of neuromasts of the anterior lateral line system by DASPEI and FITC-phalloidin staining reveals that neuromasts are consistently smaller (have fewer hair cells) than normal, with the opercular neuromast usually missing (Figs. 9E and F; J–M and data not shown). Expression of eya1 itself, which marks both the migrating posterior primordium and nascent neuromasts, reveals that migration of the posterior primordium in dog embryos lags behind that of phenotypically wild-type siblings (Figs. 9A–D); by 50 hpf, when the primordium has normally reached the tail tip, both the primordium and axons of the posterior lateral line nerve remain several somites behind (Figs. 9G–I). Although expression levels of eya1 are initially normal in dog embryos, by 7 dpf expression of eya1 in lateral line neuromasts is reduced (Figs. 9E and F); it is not clear whether this is due to reduced cell numbers in the lateral line, reduced expression levels of eya1, or both reduced

**Fig. 9. Lateral line phenotype in dog alleles.** (A) Expression of eya1 mRNA in posterior lateral line neuromasts (arrows) of a phenotypically normal sibling embryo at 60 hpf. Migration of the posterior lateral line primordium (arrowhead) is complete. (B) In the tail of a dog<sup>gspb</sup> mutant, eya1 is expressed in the nascent neuromasts (arrows), but the primordium has not reached the tail tip (arrowhead). (C and D) Higher magnification view of eya1 expression at the tip of the tail at 50 hpf. eya1 is strongly expressed in the primordium and neuromast (arrowheads) in the wild-type sibling embryo, but no eya1-expressing lateral line cells have reached the tail tip in the dog<sup>c257e</sup> mutant. (E and F) By 7 dpf, eya1 is still strongly expressed in all neuromasts in the wild-type sibling. Expression is weaker in the dog<sup>gspb</sup> mutant, and neuromasts of the opercular line (arrowhead) are missing. (The eyes have been removed for clarity.) (G, H, and I) Dissected preparations of tail skin from 54-hpf embryos stained as whole mounts with an antibody to acetylated tubulin showing the extent of the posterior lateral line nerve. In the dog<sup>c257e</sup> mutant, the nerve terminates before the tail tip. (J–M) Confocal images of live embryos stained with the vital dye DASPEI showing hair cells in the neuromasts of the anterior lateral lines (green dots). In all three dog alleles, the neuromasts have fewer hair cells than normal, and neuromasts of the opercular line (arrowhead) are missing. Note also the rounded shape of the jaw in all three dog alleles.
**eya1** expression and cell numbers. No obvious defects have been found in the somitic and pectoral fin musculature; the somites have a normal morphology, while the pectoral fins show a normal fiber number (P. Currie, personal communication). Similarly, the paired thymus forms in **dog**mutants; levels of expression of **rag1** (Willett et al., 1997) and **ikaros** (Willett et al., 2001) in and around the paired thymus are essentially normal by 6 dpf, although the more diffuse domain of **rag1** expression at 4 dpf indicates that there may be some morphological abnormalities (data not shown). By 5 dpf, homozygous **dog** mutants also have a slightly rounder jaw than normal (Whitfield et al., 1996; Fig. 9). All cartilaginous elements of the head skeleton are present, but muscles articulating the jaw are underdeveloped (Whitfield et al., 1996).

**Discussion**

The **dog** locus encodes the zebrafish **eya1** gene

Using a positional-candidate gene cloning strategy, we have shown that the **dog** locus encodes the zebrafish **eya1** gene. Single point mutations in three **dog** alleles were identified and each mutation is predicted to terminate the predicted protein prematurely within the first half of the Eya domain. Similarly located mutations in the Eya homology domain of the human EYA1 gene are responsible for BOR syndrome (Abdelhak et al., 1997; Azuma et al., 2000; Kumar et al., 1998). Recently, it has been shown that the Eya domain contains a protein phosphatase enzyme activity that is necessary for gene function (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). Mutations in each **dog** allele are predicted to remove carboxy-terminal amino acids (phosphatase Motifs II and III) necessary for this activity.

**eya1** cDNA sequences recovered from phenotypically wild-type embryos were not identical to the previously reported zebrafish **eya1** cDNA. The published sequence contains a 66-bp insertion at nucleotide position 123. Neither our **eya1** sequence nor numerous reported EST sequences contain this insertion. However, the cDNA-derived 66-bp fragment is flanked by consensus splice donor and acceptor sites and is present in genomic DNA containing the **eya1** gene, suggesting that this a splice variant. The published **eya1** sequence is derived from a 20–28 hpf cDNA library (Sahly et al., 1999) and our cDNAs were recovered from later developmental stages (72–96 hpf). The different sources of mRNA and splice variants recovered might reflect a temporal difference in expression of different **eya1** isoforms.

Role of **dog**/*eya1* in development of the otic vesicle

**eya1** mRNA transcripts are first observed at the tailbud stage (10 hpf) in a region of the anterior neural keel termed the preplacodal domain (Baker and Bronner-Fraser, 2001; Sahly et al., 1999; Whitfield et al., 2002). During development, this expression progressively restricts to the olfactory and otic placodes (Sahly et al., 1999). By 24 hpf, **eya1** is expressed in the otic vesicle and neuroblasts are delaminating from the ventral otic vesicle floor. During this stage, we can detect variably weaker expression of **bmp4** (this work) and **bmp2b** (Mowbray, 2002) in the anterior and posterior domains of the otic vesicle of **dog** embryos and reduced expression of **sna2** in the neuroblasts delaminating from the ventral otic vesicle (Whitfield et al., 2002). The expression of several genes (**bmp4, msxc, brn3.1**) is either not maintained or does not initiate normally in presumptive crista cells of mutant embryos.

Normally, there are few, if any, apoptotic cells in the otic vesicle at 24–28 hpf (Bever and Fekete, 1999; Cole and Ross, 2001). However, we detected increased apoptotic cell death in the otic vesicles of **dog** mutant embryos between 24 and 28 hpf, and this continues through 48 hpf (Fig. 6F). Consistent with the notion that these cells are lost through cell death, we find that ectopic apoptosis frequently occurs in the lateral otic vesicle wall, a portion of which is fated to give rise to the cristae (Figs. 5E–H; Kozlowski and Weinberg, unpublished observations). Moreover, fate mapping of cells specifically within the three ventrolateral domains of **bmp4** expression in **dog** embryos consistently resulted in fewer labeled cells present at 72 hpf when compared to similarly labeled wild-type sibling embryos (Kozlowski and Weinberg, unpublished observations).

However, ectopic cell death in the developing otic vesicle is not restricted to prospective crista cells in the lateral wall. Acridine orange staining of **dog** embryos and wild-type siblings at several times during development revealed that cell death can occur throughout the **dog** otic vesicle (Fig. 7). Ectopic cell death throughout the otic vesicle is the likely cause of the smaller otic vesicles observed in **dog** embryos during embryogenesis (Fig. 3). Because the clearance of apoptotic cells is thought to be rapid in zebrafish (Bever and Fekete, 1999; Cole and Ross, 2001), the large number of acridine orange- or TUNEL-positive cells observed during otic vesicle development is likely due to continued cell death and not simply an accumulation of dead cells. By 55 hpf, the expression of crista-specific genes is severely reduced or absent in **dog** embryos and crista sensory hair cell bundles are absent at 72 hpf, suggesting that they have failed to differentiate (Whitfield et al., 1996).

Role of **dog**/*eya1* in development of the statoacoustic ganglion and lateral line system

The **eya1** gene is expressed in cells delaminating from the ventral otic vesicle and the expression of **sna2** in these cells is variably reduced in **dog** embryos. Later in development, these cells express the homeobox gene **tlxA** and, like **sna2**, there are fewer **tlxA**-expressing cells in the forming SAg in **dog** embryos (Figs. 8A and B). Interestingly, **tlxA**
expression in cells of the trigeminal and anterior lateral line ganglion cells appears to be unaffected in dog embryos at the same stage (Figs. 8A and B). The reduced numbers of tlx4 cells in the developing SAg are more apparent later in development with fewer axonal projections in this ganglion at 96 hpf (Figs. 8C and D). The lack of crista hair cells and axonal projections in the SAg is likely the cause of vestibular defects seen in dog embryos.

Both the anterior and posterior lateral line systems are affected in dog/eya1 mutant embryos. Frequently, the terminal most neuromasts of the posterior lateral line are absent (Whitfield et al., 1996) and the opercular neuromast of the anterior lateral line system is absent (Fig. 9). We observed ectopic cell death in the migrating primordium of the posterior lateral line as it begins migration along the trunk (Figs. 6G,H and C,D). Although we did not determine which cells in the migrating primordium were undergoing apoptosis, it is possible that at least some of the neuromast progenitors (possibly stem cells) are affected. Too few progenitors could lead to the absence of the terminal neuromast rather than having a global effect on all neuromasts.

dog/eya1 as a model system to determine gene function in development and disease

Haploinsufficiency of the human eyes absent-1 gene is responsible for hereditary deafness as part of the Branchio-Oto-Renal syndrome (Abdelhak et al., 1997). Similarly, mice heterozygous for eya1 alleles have phenotypic defects reminiscent of the human BOR syndrome (Xu et al., 1999). Mice homozygous for eya1 alleles are embryonic lethal and have severe developmental defects in multiple organ systems, including the otic vesicle, kidney, thymus, thyroid, and skeleton (Xu et al., 1999, 2002). Similarly, dog embryos have an otic vesicle phenotype, with minor skeletal defects such as smaller jaw elements (Fig. 9). We have not identified any defects in the developing renal system in dog-eared embryos, but because teleosts do not form a metanephros, the major site of Eya1 expression in the mammalian kidney, and because the eya1 gene is not expressed in the developing zebrafish renal system (Sahly et al., 1999), this is not unexpected.

The zebrafish embryo should provide a useful system to study the roles of Eya1 further in development of the cristae, statoacoustic ganglia, and lateral line system. Our findings indicate that a primary consequence of loss of eya1 function in the zebrafish embryo is premature apoptosis in precursors to these structures. As apoptosis has also resulted from loss of eya gene function in Drosophila and mouse (Bonini et al., 1993; Xu et al., 1999), these findings may reflect a general mechanism of suppression of apoptosis by Eya proteins. Indeed, recent evidence indicates a role of Eya protein in regulating genes controlling precursor cell proliferation and survival during mammalian organogenesis (Li et al., 2003).

The specific morphological changes we observe in dog-eared embryos, in contrast to the widespread embryonic expression of the eya1 gene, are likely the consequence of functional redundancy between various Eya family members (to date, four EYA genes (EYA1-4) have been identified in vertebrates). Morpholino-mediated knockdown of Eya gene expression, singly or in combination, using wild-type and eya1/dog mutant embryos will be an important step to resolve both unique and redundant functions of eyes absent genes during vertebrate organogenesis.

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References


