Zebrafish foxi1 mediates otic placode formation and jaw development

Keely S. Solomon1,*, Tetsuhiro Kudoh2,*, Igor B. Dawid2 and Andreas Fritz1,§

1Department of Biology, Emory University, Atlanta, GA 30322, USA
2Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA
*These authors contributed equally to this work
1Present address: Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK
§Author for correspondence (e-mail: afrtiz@biology.emory.edu)

SUMMARY

The otic placode is a transient embryonic structure that gives rise to the inner ear. Although inductive signals for otic placode formation have been characterized, less is known about the molecules that respond to these signals within otic primordia. Here, we identify a mutation in zebrafish, hearsay, which disrupts the initiation of placode formation. We show that hearsay disrupts foxi1, a forkhead domain-containing gene, which is expressed in otic precursor cells before placodes become visible; foxi1 appears to be the earliest marker known for the otic anlage. We provide evidence that foxi1 regulates expression of pax8, indicating a very early role for this gene in placode formation. In addition, foxi1 is expressed in the developing branchial arches, and jaw formation is disrupted in hearsay mutant embryos.

Key words: Foxi1, Forkhead, Zebrafish, Otic Placode, Ear, Jaw

INTRODUCTION

The vertebrate inner ear develops from the otic placode, a thickening of embryonic ectoderm that becomes morphologically visible around mid-somitogenesis in most vertebrate species (Baker and Bronner-Fraser, 2001; Torres and Giraldez, 1998). The otic placode is a transient structure, giving rise to the otic vesicle, which then proliferates and differentiates to form specialized cell types necessary for hearing and balance. Transplantation experiments in chick (Waddington, 1937) and amphibians (Jacobson, 1936; Yntema, 1933; Yntema, 1950) have shown that competence to form an otic placode is initially broadly distributed throughout the ectoderm that lies laterally to the neural plate. This region of competence becomes increasingly restricted as an ongoing response to multiple, overlapping signals emanating from surrounding tissues (Jacobson, 1963). Analyses of mutants that disrupt development of the hindbrain (Chisaka et al., 1992; Cordes and Barsh, 1994; Epstein et al., 1991; Lufkin et al., 1991; Moens et al., 1998), paraxial cephalic mesoderm and prechordal plate (Mendonsa and Riley, 1999) have demonstrated an inductive, partially redundant role for these tissues in the process of otic placode formation.

Recent work in chick and zebrafish has identified several of the signaling molecules that function in the earliest inductive steps. Chick FGF19 and FGF3 are expressed adjacent to the otic anlagen in the paraxial cephalic mesoderm and later in the hindbrain, and FGF3 is additionally expressed in the otic placode and vesicle (Ladher et al., 2000; Mahmood et al., 1995). FGF19 induces Wnt-8c expression in the neural tissue, and together these two signals can induce expression of otic markers (Ladher et al., 2000). In addition, misexpression of FGF3 induces ectopic otic vesicle formation (Vendrell et al., 2000), and depletion of this molecule in explants of otic precursor tissue results in failure to form otic vesicles (Represa et al., 1991). During gastrulation, zebrafish fgf3 and fgf8 are expressed in mesendoderm and the presumptive hindbrain (Phillips et al., 2001). Loss of function of these two partially redundant genes leads to a severe reduction in otic vesicle size and a loss of pax8 expression in the otic anlagen (Phillips et al., 2001); pax8 is the earliest known marker of otic precursor cells in both fish and mammals (Pfeffer et al., 1998). Thus, these and other experiments (Kozlowski et al., 1997; Woo and Fraser, 1997) show that induction of the otic placode begins as early as mid-gastrulation and that Fgf-signaling molecules provide some of the earliest inductive signals in this process.

Less is known about early acting genes that function within the otic precursor cells to respond to inductive signals and elicit placode formation. No otic defect has been reported for a knockout of mouse Pax8 (Mansouri et al., 1998), and otic function of zebrafish pax8 has not been characterized. Zebrafish dlx3b and dlx4b (formerly dlx3 and dlx7, respectively), two members of the Distal-less family of transcription factors, are expressed shortly after the onset of pax8 expression in presumptive otic tissue (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997). Embryos homozygous for a deletion of these genes lack otic placodes, and morpholino-mediated knockdown of one or both genes demonstrates their partially redundant function in otic placode development (Solomon and Fritz, 2002). In mouse, targeted disruption of the Drosophila eyes absent homologue Eya1
leads to a severe defect, as development arrests at the otic vesicle stage and the otic vesicle regresses by apoptosis; however, early placode induction occurs normally in these embryos (Xu et al., 1999). We have identified a mutation in zebrafish, *hearsay* (*hsy*), which disrupts the *foxi1* gene and results in a reduction or loss of the otic placode and vesicle. Foxi1 is a member of the forkhead family of winged-helix transcription factors that share a highly conserved, 110 amino acid DNA-binding domain (Kauffman and Knochel, 1996). Founding forkhead family members, *Drosophila* Forkhead (*Weigel et al., 1989*) and mammalian HNF-3α (*Lai et al., 1990*), have been shown to function in the development of terminal structures in the embryo and in regulation of liver gene expression, respectively. Currently, more than 100 forkhead genes have been identified in species ranging from yeast to humans (see the Winged Helix Proteins site: http://www.biology.pomona.edu/fox.html). These genes have been implicated in a broad range of biological functions, including early patterning and morphogenesis (*Pogoda et al., 2000*), cell specification (*Miller et al., 1993*), gene regulation in differentiated tissues (*Clevidence et al., 1994*), cell proliferation (*Dou et al., 1999*) and tumorigenesis (*Kops and Burgering, 1999*). So far, few forkhead genes have been implicated specifically in otic development. Of the more than a dozen forkhead genes identified in zebrafish (*Biggs and Cavenee, 2001*; *Boggetti et al., 2000*; *Dirksen and Jamrich, 1995*; *Odenthal and Nusslein-Volhard, 1998*; *Strahle et al., 1993*; *Topczewska et al., 2001*), only one, *foxi1* (formerly *zBF-1*), is expressed in the otic vesicle (*Toresson et al., 1998*), and no otic defect is reported in mice that are homozygous null for the *foxi1* gene (Xuan et al., 1994). In mouse, *Foxf2* is expressed in the otic vesicle, as well as other tissues (*Aitola et al., 2000*), but no functional analysis has been reported for this gene. *Foxi1* is expressed in the otic vesicle, and mice homozygous for a targeted inactivation of this gene have severely malformed inner ears and exhibit both cochlear and vestibular dysfunction (Hulander et al., 1998).

Here we report the functional characterization of the *hsy/foxi1* gene in zebrafish. Embryos homozygous for the *hsy* mutation display a severe reduction or loss of the otic placode, providing the first example of a single gene mutation that can lead to a complete loss of otic placodes. In addition, *hsy* mutant embryos also exhibit a malformation of the jaw. Expression analysis of *foxi1* suggests a direct role for this gene in the development of the otic placode and branchial arch derivatives. We demonstrate that otic expression of *pax8* is absent in *hsy* mutants, and that misexpression of *foxi1* can induce ectopic *pax8* expression. Together, these data suggest that *foxi1* function is required within otic precursor cells for the earliest stages of otic placode induction.

**Materials and Methods**

**Strains**

Zebrasfish (*Danio rerio*) were raised and maintained according to standard procedure (Westerfield, 2000). The *hearsay* mutation was identified through random intercrosses among a partially inbred line of store-bought fish. Embryos were raised at 28.5°C and staged as described (Kimmel et al., 1995).

**Cloning and construction of expression plasmid**

A partial zebrafish *foxi1* clone was identified in an expression pattern screen using high-throughput in situ hybridization (clone 3015) (Kudoh et al., 2001). The 5’ end of *foxi1* was subsequently generated by 5’-RACE using the SMART RACE Kit (Clontech) with a gene-specific primer (5’-cgctgtcgctgacgacctgct-3’), and sequenced. Full-length cDNA was then amplified and subcloned into the expression vector pCS2+.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed as described (Thissie and Thissie, 1998), and double labeling as outlined (Itoh et al., 2002; Jowett, 2001). Embryos were prepared for sectioning as described (Westerfield, 2000), and 5 μm plastic sections or 15 μm frozen sections were generated. The zebrafish *dlx3b* (*Ekker et al., 1992*), *dlx4b* (*Ellies et al., 1997*), *dlx5a* (*Akimenko et al., 1994*), *dlx2a* (*Akimenko et al., 1994*), *pax8* (*Pfeffer et al., 1998*), *pax2a* (*Krauss et al., 1991*), *krox20* (*Oxtohy and Jowett, 1993*), *crestin* (*Luo et al., 2001; Rubinstein et al., 2000*) and *otx2* (Li et al., 1994) probes were previously described.

**Morpholino injections**

Morpholino antisense oligonucleotides (Gene Tools, LLC, Corvallis, OR) were as follows (complementary bases to the predicted start codon are indicated in italics): *foxi1*: 5’-TAATCCGCTCTCCCTCAGAGAAACATC-3’. Gene Tools, LLC standard control oligo (http://www.gene-tools.com/).

**Alcian Green staining**

Embryos were fixed overnight in 4% paraformaldehyde, followed by several washes in PBT (phosphate-buffered solution +0.1% Tween 20) the next day. The embryos were then placed in 30% hydrogen peroxide for approximately 20 minutes to bleach pigment cells, washed in PBT several more times, then stained in 0.37% HCl/70% ethanol solution/0.1% Alcian Green for 1.5 hours. This was followed by several brief washes in 1%HCl/70% ethanol. The tissues were cleared in 50% glycerol/0.5% KOH and the cartilages were photographed.

**Genotyping**

DNA was prepared after in situ hybridization from embryos sorted by phenotype, and a 607 bp PCR fragment was amplified with the following primers: F, 5’-CTGCCAGCCGGCTTTACTTTTCTTGT-3’; R, 5’-CGCCACGGCGTATTCACTTCTGT-3’. The amplification products were digested with TaqI, which only cuts the mutant sequence, and analyzed on a 1.5% agarose gel.

**RESULTS**

**Isolation and morphological characterization of *hearsay***

The *hearsay* mutation was identified in a screen for mutations affecting early ear development in zebrafish embryos. At 24 hours post-fertilization (24 hpf), wild-type embryos display easily discernable otic vesicles that have cavitated to form lumens, each containing two otoliths (Fig. 1A). The vesicles are larger but retain a similar structure at two days post-fertilization (2d) (Fig. 1I). In contrast, homozygous *hsy* embryos display small or no otic placodes and vesicles (Fig. 1B). This defect is visible by the 14-somite stage and persists through the remainder of the embryogenesis and larval stages. The *hearsay* phenotype is variable in that some mutants lack otic vesicles entirely (Fig. 1F), whereas others develop a small lumen with no or one otolith (Fig. 1K,L). A fraction of embryos
Fig. 1. *hearsay* mutant embryos display defects in otic and jaw development. All panels show lateral views of live embryos with anterior to the left. (A) Wild-type and (B) *hsy* embryos at 24 hpf. (C-D) Twenty-seven hpf wild-type embryos injected with (C) control morpholino and (D) *foxi1* morpholino. (E-H) Six-day-old embryos: (E) wild type, (F) *hsy*, (G) wild type injected with control morpholino and (H) wild type injected with *foxi1* morpholino. (I-L) Otic vesicles in (I) wild-type and (J-L) *hsy* mutant embryos at 2 days. j, jaw; ot, otolith; ov, otic vesicle.

have two small lumens that each contains a single otolith (Fig. 1J), a phenotype reminiscent of *quadro* mutants (Malicki et al., 1996), although no complementation tests between the two mutations have been performed. In addition to this otic defect, *hsy* mutants exhibit a defect in jaw development that becomes apparent by four days post-fertilization (4d). In 6d wild-type embryos, most of the cartilaginous elements of the jaw have begun to develop and the mouth appears to protrude from the anterior-most region of the head (Fig. 1E), whereas *hsy* mutant jaws are much smaller than in wild type and the protruding mouth is not visible (Fig. 1F). The *hsy* phenotype is recessive and fully penetrant; approximately 25% of embryos from a cross between *hsy* carriers exhibit some degree of otic and jaw defects, whereas the remaining 75% appear normal. The *hsy* mutation is lethal; mutant larvae fail to inflate the swimbladder and die at around 7-8d.

The *hearsay* mutation disrupts the *foxi1* gene

To identify the gene disrupted by the *hsy* mutation, DNA was prepared from individual mutant and wild-type embryos and tested for linkage to polymorphic microsatellite markers (http://zebrafish.mgh.harvard.edu) (Shimoda et al., 1999). The mutation was localized to LG12, between markers Z14982 (two recombinants/265 chromosomes tested) and Z8344 (two recombinants/253 chromosomes tested, data not shown).

One candidate gene in this region, 3015, was identified as part of a screen for expression patterns of embryonic cDNAs (Kudoh et al., 2001). Clone 3015 was localized by radiation hybrid (RH) mapping (LN54 panel) (Hukriede et al., 2001; Hukriede et al., 1999) on LG12 in the vicinity of the *hsy* map position; we will provide evidence below that the *hsy* locus encodes the 3015 sequence. A BLAST search of the NCBI database with the 3015 sequence identified it as encoding a forkhead domain-containing protein. This sequence was classified in the Fox I class and consequently named *foxi1* (see the Winged Helix Proteins site; http://www.biology.pomona.edu/fox.html) (Fig. 2A). The zebrafish FoxI1 protein shares 52% identity with *Xenopus* FoxIIc and 40% with human FOXI1; the forkhead domains are 95% and 94% identical, respectively.

To test whether *foxi1* is disrupted by the *hsy* mutation, we amplified and sequenced fragments representing the gene from individual haploid mutant and wild-type embryos. Mutant embryo DNA contained a two base-pair deletion close to the amino-terminus of the protein, creating a shift in the reading frame in codon 41 (Fig. 2B). The shifted sequence continues for an additional 117 amino acids with no similarity to known proteins. Thus, the mutation eliminates most of the wild-type FoxI1 protein including the forkhead domain, and most likely represents a null mutation. No additional differences were found between mutant and wild-type sequences. The two base-pair deletion in *hsy* creates a TaqI restriction site, and we used this polymorphism to test for linkage of the mutant phenotype to this sequence change. Genomic DNA was isolated from individual embryos generated from crosses of *hsy* heterozygous parents, amplified and tested for the TaqI polymorphism: no recombinants between the *hsy* mutation and the TaqI polymorphism were found in more than 450 meioses tested. Conversely, no homozygotes for the TaqI polymorphism were found in 700 wild-type siblings. Thus, the *hsy* mutation is tightly linked to the two-base deletion, providing direct genetic evidence for the conclusion that *hsy* disrupts *foxi1*.

To test whether inhibition of *foxi1* translation could phenocopy the *hsy* mutation, we injected wild-type embryos with a morpholino antisense oligonucleotide (Nasevicius and Ekker, 2000) against *foxi1* mRNA. Injection of this morpholino produced a phenotype almost identical to that of *hsy*; otic vesicles of injected embryos were very small or absent (Fig. 1D), and jaws were reduced in size (Fig. 1H). These injected embryos displayed variability in severity of the otic defect, similar to the variability seen in the mutants. None of these phenotypes were observed in embryos injected with a control morpholino (Fig. 1C,G). This observation confirms the conclusion, based on our linkage and sequencing data, that the *hsy* phenotype is caused by disruption of the *foxi1* gene.

*foxi1* expression

The expression profile of *foxi1* is shown in Fig. 3. At 50% epiboly, the earliest stage examined, *foxi1* is expressed in a portion of the ectoderm (Fig. 3A), and this domain of expression becomes broader and stronger by 75% epiboly (Fig. 3B). To determine whether *foxi1* is expressed in neural or non-
neural ectoderm, double in situ labeling was performed with otx2, a marker of neural ectoderm (Li et al., 1994). foxi1 expression does not overlap with otx2, indicating that it is expressed in non-neural ectoderm (Fig. 3C).

By the beginning of somitogenesis, foxi1 expression has become confined to two domains that lie laterally to the neural plate in the dorsal ectoderm (Fig. 3D-F), whereas expression is no longer detectable in ventral ectoderm. Based on double in situ labeling with otx2, the anterior-most region of foxi1 expression lies just posterior to the midbrain-hindbrain boundary (Fig. 3D,F). At the three-somite stage, the two domains of foxi1 expression have become more compact, but are still located in approximately the same position lateral to the hindbrain (Fig. 3G,H). A transverse section of an embryo at this stage shows that the more lateral region of foxi1 expression is confined to the upper, ectodermal layer of cells, whereas more medially, ectodermal and mesendodermal expression can be seen (Fig. 3I).

To determine whether foxi1 is expressed in otic precursor cells, double labeling was performed with both pax8 and dlx3b at the one-somite stage. The foxi1 expression domain overlaps pax8 otic expression at this stage, sharing approximately the same posterior and medial boundaries, but extending further than pax8 in the anterior and lateral directions (Fig. 3J,K). foxi1 expression also overlaps with dlx3b at the one-somite stage, although dlx3b expression is more extensive along the a-p axis. Within the region of overlap, foxi1 and dlx3b share the same medial border, but foxi1 extends further laterally (Fig. 3L). These results indicate that foxi1 is expressed in otic precursor cells at late gastrula and early somitogenesis stages. At mid- and late-somitogenesis stages, foxi1 expression is no longer detectable in the otic placode and vesicle, respectively (Fig. 4A-C and data not shown).

At the 18-somite stage, foxi1 is expressed in throughout the pharyngeal arches (Fig. 4A,B), and expression in this region remains detectable at 28 hpf (Fig. 4C), the latest stage examined. Sections through the pharyngeal arches of 28 hpf embryos reveal that foxi1 expression appears to extend into the pouches that separate the individual arches (Fig. 4D,E). dlx2a (formerly dlx2) is expressed in cranial neural crest cells that migrate to reside within the pharyngeal arch primordia by 16 hpf, and expression in the arches persists through the beginning of the second day of development (Akimenko et al., 1994). Sections through 26 hpf embryos double-labeled for expression of dlx2a (red) and foxi1 (purple) show that most of the dlx2a-expressing presumptive neural crest cells do not express foxi1 (Fig. 4F,G). Rather, foxi1 expression partially surrounds these cells, consistent with the position of the pouches and/or clefts that separate the neural crest-containing arches. It is unclear whether any of the foxi1-expressing cells also express dlx2a, as the purple

Fig. 2. The hsy phenotype is caused by a disruption of foxi1. (A) Nucleotide sequence and predicted ORF of zebrafish foxi1 (GenBank Accession Number, AY204207). (B) Schematic of Foxi1 protein in wild type and hsy. A two-base deletion produces a frameshift in codon 41 and results in a truncated protein of 158 amino acids.
substrate masks the expression of the red. Thus, foxi1 is expressed predominantly in cells other than neural crest in the pharyngeal arches.

**foxi1 function in otic placode specification**

To study the timing of foxi1 function in otic development, we examined the expression of several early otic markers in hsy mutant embryos. pax8 expression is detectable in the presumptive otic domain of wild-type embryos as early as 90% epiboly, whereas pax2a is expressed in otic cell precursors beginning in early somitogenesis, several hours before otic placodes become visible (Krauss et al., 1991; Pfeffer et al., 1998). We found that both pax8 and pax2a expression is completely absent in the otic domain of approximately one quarter of three- to five-somite stage embryos from a cross between hsy heterozygotes. At this stage, dlx3b is expressed in a continuous ectodermal stripe around the edge of the neural plate, and expression is upregulated in the otic and olfactory primordia (Akimenko et al., 1994; Ekker et al., 1992). We found that one-quarter of the embryos from this cross lack this upregulation in the otic region (Fig. 5E,F). To confirm that these embryos were mutants, we sorted them based on this phenotype and individually genotyped them for the hsy mutation (Fig. 5G). One-hundred per cent (9/9) of the embryos that lacked dlx3b otic upregulation were homozygous for the hsy mutation, and 100% (21/21) of those that had been judged as having normal expression were heterozygous or homozygous for the wild-type allele.

**foxi1 induces ectopic pax8 expression**

To learn more about the function of foxi1, we injected an expression construct containing the full-length coding sequence under the control of the cytomegalovirus (CMV) promoter into one-cell stage wild-type embryos. pax8 expression was examined in injected embryos fixed at three- to five-somite stage. We found, in addition to the normal domains of otic and pronephric expression, pax8 was also expressed in many ectopic locations in the injected embryos (Fig. 6A). Double in situ labeling with foxi1 reveals that the ectopic domains of pax8 expression coincide with ectopic foxi1 expression (Fig. 6A,B).
This co-localization of ectopic expression was observed in 11/16 embryos injected with the foxi1 expression construct.

Ectopic expression of dlx3b, dlx5a (formerly dlx4), and dlx4b, but not pax2a, was also observed in these embryos (data not shown).

Expression of late otic markers in hearsay mutants

We examined the effect of the hsy mutation on later stages of otic development by in situ hybridization with dlx3b, dlx4b, and dlx5a. These three genes are expressed in several developing cranial structures during mid-late somitogenesis, including the otic vesicle and the visceral arches (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997). At 16-somite stage, dlx4b and dlx3b are expressed in the olfactory placode and the otic vesicle in wild-type embryos (Fig. 7A; dlx3b not shown), and this otic expression was reduced or absent in all hsy mutants examined (Fig. 7B; dlx3b not shown). In 24 hpf wild-type embryos, dlx3b, dlx5a and dlx4b are expressed in the olfactory placode, visceral arches and otic vesicle, and dlx5a is additionally expressed in several structures of the forebrain (Fig. 7C; dlx4b not shown). hsy mutants display a variable reduction or absence of otic staining for all of these genes at this stage (Fig. 7D,F,G; dlx4b not shown). Although all mutant embryos examined had reduced otic expression of these genes, the degree of reduction was variable, ranging from no otic expression to a reduction in size of the otic expression domain. This observation is in concordance with the variability in the otic phenotype observed by morphological inspection, as described above. In addition, we observe a loss of expression in the branchial arches in some of the mutant embryos, although this phenotype is not as penetrant as the reduction in otic expression (Fig. 7D for dlx3b, F and G for dlx5a; dlx4b not shown).

Effects of hearsay in jaw formation

Alcian Green staining reveals that all of the major cartilaginous elements of the jaw are present in hsy mutants at 5 d (Fig. 8A,B). However, these elements are spaced more compactly in the a-p dimension, and this compression presumably results in the reduced jaw phenotype observed in the mutant.

Fig. 5. Markers of early placode induction are absent in hsy. (A-F) In situ hybridizations with three- to five-somite stage embryos: dorsal views, anterior towards the left. (A,C,E) wild-type and (B,D,F) hsy embryos. (A,B) pax8 expression, (C,D) pax2a expression and (E,F) dlx3b expression. (G) TaqI genotyping for dlx3b in situ. Migration position for hsy mutant and wild-type fragments are indicated at the left of the gel. mhb, midbrain-hindbrain boundary; ot, otic primordia; pn, pronephros.

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Expression of late otic markers in hearsay mutants

We examined the effect of the hsy mutation on later stages of otic development by in situ hybridization with dlx3b, dlx4b and dlx5a. These three genes are expressed in several developing cranial structures during mid-late somitogenesis, including the otic vesicle and the visceral arches (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997). At 16-somite stage, dlx4b and dlx3b are expressed in the olfactory placode and the otic vesicle in wild-type embryos (Fig. 7A; dlx3b not shown), and this otic expression was reduced or absent in all hsy mutants examined (Fig. 7B; dlx3b not shown). In 24 hpf wild-type embryos, dlx3b, dlx5a and dlx4b are expressed in the olfactory placode, visceral arches and otic vesicle, and dlx5a is additionally expressed in several structures of the forebrain (Fig. 7C; dlx4b not shown). hsy mutants display a variable reduction or absence of otic staining for all of these genes at this stage (Fig. 7D,F,G; dlx4b not shown). Although all mutant embryos examined had reduced otic expression of these genes, the degree of reduction was variable, ranging from no otic expression to a reduction in size of the otic expression domain. This observation is in concordance with the variability in the otic phenotype observed by morphological inspection, as described above. In addition, we observe a loss of expression in the branchial arches in some of the mutant embryos, although this phenotype is not as penetrant as the reduction in otic expression (Fig. 7D for dlx3b, F and G for dlx5a; dlx4b not shown).
Specifically, the first or mandibular arch derivatives, which include the mandibular and palatoquadrate elements, appear normal in hsy mutants. The second or hyoid arch derivatives appear to be differentially affected: whereas the basihyal element seems to form normally, the ceratohyal (ch) element is often in a more perpendicular position with respect to the a-p axis. In addition, the ch element is shorter and thicker than in wild-type larvae, and this thickening may in part be caused by a fusion with the first gill arch. The posterior or gill arch elements are derived from the third through seventh pharyngeal arches. Like the ch, the gill arches of hsy mutants often lie in a more perpendicular orientation with respect to the a-p axis, and they are shorter and more closely spaced.

The cartilage elements of the jaw are largely derived from cranial neural crest cells that migrate into the area of the branchial arches. foxi1 is expressed in the region of the branchial arches (Fig. 4), providing a basis for its potential function in the formation of this tissue. In addition to the jaw, cranial neural crest contributes to pigmentation, the developing heart, the enteric nervous system (ENS) and the sensory portion of cranial ganglia. We have not been able to detect any defects in pigmentation or heart morphology, and anti-Hu staining revealed no ENS defects (data not shown). Cranial ganglia were not examined. To address the possible effect of the hsy mutation on the migration and differentiation of neural crest within the branchial arches, we examined expression of crestin, a marker for neural crest cells (Luo et al., 2001; Rubinstein et al., 2000). All of the major domains of crestin expression were present in hsy mutants, including the mandibular, hyoid and vagal cranial crest streams (Fig. 8C,D). However, double in situ labeling with krox20, which marks rhombomeres 3 and 5 (r3 and r5), reveals that the hyoid and vagal crestin domains are more closely spaced in mutant embryos (Fig. 8C,D). In particular, the vagal crest domain extends past the anterior boundary of the krox20 r5 stripe in hsy mutants, ending in a region adjacent to r4 (Fig. 8D), whereas in wild-type embryos, the anterior boundary of this expression domain lies laterally to the middle of the krox20 r5 stripe (Fig. 8C). This observation may provide an explanation for the fusion of the ch element (hyoid crest derivative) and the third gill arch (vagal crest derivative) seen in hsy mutant larvae.

dlx2a expression in the cranial neural crest also appears to be affected in hsy mutant embryos at the 18-somite stage (Akimenko et al., 1994; Ellies et al., 1997) (Fig. 8E,F). First, similar to crestin, the three domains of expression in the cranial crest are more closely spaced. Second, we consistently observe an increase in intensity of dlx2a staining in the mutants. Likewise, the expression of foxi1 itself is more intense in hsy mutant embryos (Fig. 8G,H). This conclusion was supported by genotype analysis. All embryos derived from a cross between hsy heterozygotes were hybridized in situ with dlx2a or foxi1, sorted by ‘increased’ or ‘normal’ expression of the examined marker, and genotyped for the hsy mutation using the TaqI restriction polymorphism (see Fig. 2B). For foxi1 expression at the 18-somite stage, genotyping revealed that 100% (11/11) of increased expression embryos were homozygous mutant, and 100% (32/32) of normal expression embryos were heterozygous or homozygous wild type (Fig. 8I). For dlx2a at the 18-somite stage, 86% (12/14) of increased expression embryos were mutant and 100% (32/32) of normal expression embryos were heterozygous or wild type. Using 24 hpf embryos we found that, for foxi1, 81% (13/16) of increased embryos were mutant and 91% (51/56) of normal embryos were wild type, and for dlx2a, 100% (19/19) increased embryos were mutant and 100% (33/33) of normal embryos were wild type. Thus, increased expression of dlx2a and foxi1 in the branchial arches is a consequence of the hsy mutation. No increase in expression of foxi1 was detectable during gastrulation or early somitogenesis (data not shown). This increased expression of foxi1 in hsy mutant embryos is suggestive of a function for foxi1 as a negative transcriptional regulator. However, it remains unclear whether this

![Fig. 8. Analysis of jaw and neural crest in hsy. (A,C,E,G) Wild-type and (B,D,F,H) hsy mutant embryos. (A,B) Alcian Green staining of cartilaginous jaw elements at 5 days. Ventral views, anterior towards the left. Cartilages: bh, basihyal; ch, ceratohyal; m, mandibular; pq, palatoquadrate; 3-7, gill cartilages derived from branchial arches P3-P7; m and pq are P1 derivatives; ch and bh are derived from P2. ch+3 indicates unilateral fusion of ceratohyal with gill arch 3 in mutant. (C-H) Expression analysis at the 18-somite stage: dorsal views, anterior towards the top. (C,D) Double labeling with crestin (dark purple) and krox20 (red). (E,F) dlx2a expression. (G,H) foxi1 expression. (I) TaqI restriction polymorphism genotyping data for foxi1 in situ hybridization at the 18-somite stage, sorted as either ‘upregulated’ or ‘normal expression’. m, mandibular neural crest (nc) stream; h, hyoid nc; v, vagal nc; 3 and 5, krox20 expression in rhombomeres 3 and 5.
autoregulation is direct or indirect and whether the increased expression of foxi1 or dlx2a is caused by an increase in the number of cells expressing these genes or elevated expression levels.

**DISCUSSION**

**The role of foxi1 in otic placode formation**

Although several inductive signals for otic placode formation have been characterized, including chick FGF2 and FGF8 (Adamska et al., 2001), FGF3 (Mahmood et al., 1995; Vendrell et al., 2000), FGF19 and Wnt8c (Ladher et al., 2000), and zebrafish fgf3 and fgf8 (Phillips et al., 2001), little is known about the genes that respond to these signals within the pre-placodal tissue and initiate the program of otic development. One gene that might participate in this inductive response is pax8, which, previous to this study, was the earliest gene reported to be expressed in otic precursor tissue (Pfeffer et al., 1998). However, no otic defects were observed in mice homozygous for a targeted inactivation of Pax8 (Mansouri et al., 1998), and no mutations in this gene have been described in zebrafish. dlx3b and dlx4b are also expressed in the otic primordia, shortly after the onset of pax8 expression, and are additionally expressed in the olfactory primordia (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997). Embryos homozygous for b380, a deletion for both of these genes, fail to form otic and olfactory placodes, revealing an early role for dlx3b and dlx4b in placode induction (Solomon and Fritz, 2002). Although pax2a, a slightly later otic marker than pax8, fails to be expressed in the otic precursor domain of these embryos, the transient expression of pax8 in otic primordia is normal, indicating that early otic characteristics are induced in b380 mutant embryos.

In this work we have identified a mutation, hearsay, which leads to a severe reduction or loss of the otic placode in zebrafish. Further, we show that the hsy phenotype is caused by a disruption of foxi1, which was identified in a screen for genes that are differentially expressed during zebrafish embryogenesis (Kudoh et al., 2001). Double in situ labeling with known otic markers shows that foxi1 is expressed very early in otic precursor cells, as early or earlier than pax8. Loss of pax8, dlx3b and pax2a otic expression in hsy mutant embryos demonstrates a defect in the initiation of otic placode induction and suggests that foxi1 is the earliest-acting gene characterized thus far within the responding otic precursor domain.

Forkhead genes are known to function as transcriptional regulators (for a review, see Kaufmann and Knochel, 1996). Thus, it is possible that foxi1 regulates the transcription of genes necessary for development of the otic placode in response to signals from surrounding tissues. However, we have shown that foxi1 is also expressed in some mesendodermal cells underlying the presumptive otic anlagen of the ectodermal layer. Therefore, it is also possible that foxi1 functions in a non-cell-autonomous manner to regulate otic development. In support of the first view we have demonstrated that misexpression of foxi1 can induce ectopic pax8 expression within the same cells. This observation, together with the loss of pax8 otic expression in hsy, suggests a simple model in which foxi1 would activate transcription of pax8. However, although it appears that foxi1 is necessary, it is not sufficient for pax8 otic expression, because not every cell that expresses foxi1 also expresses pax8 (Fig. 3). We also find that pax8 is not expressed in every location where foxi1 is ectopically expressed (Fig. 6). Therefore it appears that expression of pax8 requires the function of regulatory factors in addition to foxi1.

**Variability of the hearsay phenotype: multiple steps for otic placode induction**

The loss of pax8 expression observed in hsy embryos is a robust phenotype; expression of this gene was always absent in the presumptive otic domains of hsy mutant embryos. However, many hsy embryos develop an otic vesicle, albeit a small and malformed one. Thus, it appears that there is no absolute requirement for foxi1 or pax8 to form at least some aspects of an otic vesicle, nor is ectopic expression of these two genes sufficient to produce one.

Why this variability in otic placode and vesicle formation? One possibility is that another foxi1-like gene is present in the zebrafish genome. It has been suggested that ray-finned fish have undergone an additional round of genome duplication after diverging from the tetrapod lineage, and many gene families (Hox, Dlx, Msx, Eng) contain additional genes in the zebrafish genome (Postlethwait et al., 1998). If a second foxi1 gene exists, it may be able to partially overcome the loss of the foxi1/hsy gene.

Another possibility arises from observations that embryonic induction of the otic placode occurs in multiple, overlapping steps (Baker and Bronner-Fraser, 2001; Torres and Giraldez, 1998). Recently, Groves and Bronner-Fraser have elegantly combined transplantation experiments with molecular analysis of otic placode induction (Groves and Bronner-Fraser, 2000). In chick, Pax-2 is expressed in the presumptive otic placode at the four- to five-somite stage, followed by Sox-3 at the six-somite stage, BMP-7 at the seven-somite stage, and Notch at the nine- to ten-somite stage, a few hours before otic placodes become morphologically visible. When anterior, non-otic epiblast was grafted to the presumptive otic region of progressively older (11- to 21-somite stage) hosts, the grafted tissue formed an epithelial (otic) vesicle and often expressed Sox-3 and BMP-7. However, older hosts rarely induced Pax-2 expression, the earliest otic marker used, in the graft. Similarly, we observe later otic expression of markers such as dlx3b, dlx5a and dlx4b in the subset of hsy embryos that form a small otic vesicle, indicating that foxi1 and/or pax8 are not absolutely required for the expression of these genes. It has also been shown that signals for otic placode induction emanate from multiple tissues. For example, in zebrafish, heterotopic transplants of mesendoderm from early gastrula embryos can induce ectopic otic vesicles (Woo and Fraser, 1997). Mendonsa and Riley have used one eyed pinhead (oep) mutant and notail (ntl)/oep double mutant zebrafish embryos to show that genetic removal of mesendodermal cells leads to a severe delay in otic placode induction (Mendonsa and Riley, 1999). This delay is concomitant with a loss of pax8 induction in the otic primordia (Phillips et al., 2001). Despite these defects, otic vesicles form, although they are variably smaller than wild type and lack one or both otoliths. Together, these observations demonstrate that otic vesicle formation can be uncoupled from the expression of early marker genes, and that a later source of otic vesicle-inducing signals can partially compensate for the loss of early
mesendodermal signaling. Thus, foxi1, like pax8, might only be transiently required in early otic primordia, and continued inductive influences can partially and variably bypass this requirement.

**Induction of the otic placode**

In zebrafish, fgf3 and fgf8 are expressed in both early mesendoderm and in the developing hindbrain during the stage when otic placode induction occurs, but not within the otic precursor tissues. These two genes play a redundant role in otic induction; inactivation of both fgf3 and fgf8 at the same time blocks otic placode induction and expression of pax8 in the otic primordia (Phillips et al., 2001). Interestingly, some of these embryos still form a small otic vesicle, reminiscent of hsy.

Evidence from zebrafish and chick suggests that there are other factors in addition to fgf3/8 involved in otic placode induction. In chick, FGFR9 is co-expressed with FGFR3 in paraxial cephalic mesoderm around the time of otic induction (Ladher et al., 2000), and transplants of mesoderm expressing FGFR3 to anterior ectoderm can induce expression of otic markers. Furthermore, FGFR9 requires the synergistic action of Wnt8c, which is induced by FGFR9 in overlying neural tissue. Although fgf9 has not been reported in zebrafish, fgfβ is expressed early in gastrulation in cephalic mesoderm, subjacent to the presumptive otic primordia (Phillips et al., 2001). Genetic removal of mesendodermal cells in oep mutant embryos (Schier et al., 1997) abolishes this fgf3 domain and leads to a delay in otic placode induction (Mendonsa and Riley, 1999), but inactivation of fgf3 in wild-type embryos does not produce a similar delay (Phillips et al., 2001). This suggests that mesendodermal signals in addition to fgf3 are disrupted in oep.

**foxi1 and jaw formation**

In addition to otic abnormalities, hsy embryos also display a defect in jaw formation. Although all the cartilaginous elements of the jaw are present, the spacing is more compact, and this compression presumably leads to a reduction in size of the jaw. The cartilaginous elements arise from the branchial arches, which are formed through complex interactions between endoderm, mesoderm and neural crest cells that migrate to the arches from the hindbrain. In zebrafish, several mutations have been identified that lead to defects in inner ear and jaw formation (Malicki et al., 1996; Piotrowski et al., 1996; Whitfield et al., 1996), but this association is not well understood. Both defects may be the consequence of inappropriate hindbrain patterning, an example of which is seen in the valentino mutant, which disrupts a bZip transcription factor expressed in the hindbrain (Moens et al., 1998). However, morphological analysis and expression of hindbrain markers krox20 (Fig. 8) and fgf3/8 (not shown) show no indication of hindbrain defects in hsy, and cranial crest derivatives outside the jaw appear unaffected.

In an alternative model, the otic placode may provide positional cues for neural crest cell migration (Malicki et al., 1996). In particular, the preotic hyoid neural crest and the postotic vagal crest migrate in close apposition to the otic placode (Schilling and Kimmel, 1994), and misguided migration could be the result of otic placode defects. In support of this model, we find that the hyoid and postotic neural crest domains are positioned more closely to each other in hsy mutant embryos (Fig. 7C-F). However, we have previously shown that loss of dlx3b and dlx4b function blocks otic placode formation but has no detectable effect on the formation of the jaws (Solomon and Fritz, 2002).

In a third model, foxi1 may play a direct role in the development of the branchial arches. We have shown that foxi1 is expressed within the branchial arches, apparently within the pouches that separate the individual arches. It is possible that foxi1 may regulate the expression of factors that control neural crest migration within the ectoderm. Another possibility is that foxi1 may be required for the proper development of the pouches and that loss of foxi1 function in hsy leads to a failure of the neural crest populations within the individual arches to become fully separated. In support of this, we have shown that expression domains of neural crest markers dlx2a and crestin of the otic vesicle, reminiscent of the hsy.

In the light of these results, it appears more probable that foxi1 is required for branchial arch formation. In this context, hsy is reminiscent of the van gogh (vge) mutation, which disrupts the segmentation of the pharyngeal pouches, leading to malformation of the jaw, and displays an inner ear defect, but does not affect hindbrain patterning (Piotrowski and Nusslein-Volhard, 2000; Whitfield et al., 1996).

**Foxi I class genes**

Foxi I class genes have been described in humans (Larsson et al., 1995; Pierrou et al., 1994), mouse (Halander et al., 1998; Overdier et al., 1997), rat (Clevidence et al., 1993) and Xenopus (Lef et al., 1994; Lef et al., 1996). However, it is unclear whether zebrafish foxi1 is orthologous to any one of these genes. The Xenopus FoxI1c (Lef et al., 1996), FoxIIa and FoxIb genes (Lef et al., 1994) share the highest degree of sequence conservation with the zebrafish gene. The expression pattern of the two Xenopus pseudoallelic variants FoxI1a/b (XFD-2, XFD-2) does not suggest functional similarity to zebrafish foxi1. Of the three Xenopus FoxI1 genes, FoxI1c (XFD-10) is most similar to foxi1 in sequence. However, Xenopus FoxI1c was reported to be expressed in the neuroectoderm and somites but not in the otic placode, unlike the pattern for foxi1 we report (Lef et al., 1996). A very recent report provides a more detailed description of Xenopus FoxI1c (Pohl et al., 2002), which suggests that this gene is expressed in preplacodal tissue and the branchial arches, similar to our observations for foxi1. Thus, it now appears probable that Xenopus FoxI1c represents the ortholog of zebrafish foxi1.

Functional analysis for a foxi paralogue has only been reported in mouse (Halander et al., 1998). Mice homozygous for a targeted disruption of the Fkh10 locus (Foxi1 – Mouse Genome Informatics) exhibit circling behavior, poor swimming ability and abnormal reaching response as well as profound hearing impairment, demonstrating vestibular and cochlear dysfunction. In addition, these mice display malformations of the inner ear in that a large, irregular cavity replaces the cochlea and vestibulum. At 9.5 days post-coitus (dpc), Fkh10 is expressed exclusively in the otic vesicle, the stage when the otic vesicle first forms by invagination. Mouse Fkh10 is also expressed in the epithelium of renal distal convoluted tubules at 16.0 dpc (Overdier et al., 1997), however
no renal dysfunction has been observed in mutant mice (Hulander et al., 1998), and we have not examined expression of zebrafish foxi1 at larval or adult stages. These observations suggest that Foxi1 genes in fish and mammals play a role in the development of the inner ear, but clear discrepancies exist between the mouse and fish Foxi1 genes. Most notably, zebrafish foxi1 is expressed early in the otic primordia, but is no longer detectable in the otic placodes or otic vesicles, whereas mouse Fkh10 is expressed at a later stage in the otic vesicle. In addition, Fkh10 mutant mice do not show any craniofacial abnormalities, and, unlike the fish foxi1 gene, the mouse gene is not expressed in the branchial arches at the otic vesicle stage. As branchial arch expression is shared by the related zebrafish foxi1 and Xenopus FoxI1c genes, it is possible that as yet unexamined members of this subfamily may show expression in the arches during murine development.

Individuals affected by Treacher Collins Syndrome (TCS; MIM #154500) display a phenotype that includes inner ear defects and craniofacial malformations reminiscent of the hsy phenotype. In most cases, this syndrome is inherited in an autosomal dominant fashion, and the pathogenic mutation has been shown to lie within the TCOF1 gene, which maps to 5q32 (The Treacher Collins Syndrome Collaborative Group, 1996; Edwards et al., 1997; Gladwin et al., 1996; Wise et al., 1997). However, individuals forming a subset of TCS patients appear to inherit the syndrome in a recessive fashion and do not carry any obvious defects in the TCOF1 gene, even though haplotype analysis suggests that the genetic defect is linked closely to the TCOF1 locus (Edwards et al., 1997). The human FOXI1 (FKHL10) gene, which encodes a protein with 40% sequence identity to zebrafish Foxi1, has been localized to chromosome 5q34 (Larsson et al., 1995). Northern analysis with 16 adult and five fetal tissues demonstrated kidney-specific expression, but no otic tissues were analyzed (Pierrou et al., 1994). Based on our data, the nearby FOXI2 gene is an intriguing candidate for this recessively inherited syndrome, and this should be easily testable.

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