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oto is a homeotic locus with a role in anteroposterior development that is partially redundant with *Lim1*

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SUMMARY

Genetic control of mammalian head development involves mechanisms that are shared with trunk development as well as mechanisms that are independent. For example, mutations in the *nodal* gene disrupt axis formation and head development while mutations in the *Otx2* or *Lim1* genes block head development without disrupting development of the trunk. We show here that the *oto* mutation on mouse chromosome 1 defines a locus with a critical role in anterior development. The *oto* mutation disrupts development of the telencephalic and optic vesicles, the pharyngeal endoderm and the first branchial arch. Also, *oto* embryos have dose-dependent, posterior homeotic transformations throughout the axial skeleton. To further dissect the role of the *oto* locus in head development, we crossed mice carrying *oto* and *Lim1* mutations. Interactions between the two mutations indicate that the role of *oto* in the regulation of head development is partially redundant with that of *Lim1*. The phenotype of *oto* embryos points to an early and critical role for *oto* in the development of forebrain subregions. Transformations of the vertebrae in *oto* embryos reveal a *Lim1*-independent role in the establishment of positional information in the trunk.

Key words: Telencephalon, Pharynx, Jaw, Homeotic, *Lim1*, Mouse, Human

INTRODUCTION

The complexity of the adult forebrain arises by elaboration of basic themes laid out during early development. By mid-gestation three major subdivisions, the telencephalon, the optic vesicles and the diencephalon, are easily recognized in the mouse embryo and as many as six subdivisions have been proposed based upon the analysis of gene expression patterns (Rubenstein et al., 1994). How and when these subdivisions are defined, and to what extent each is independent of the others, remain important questions. Fate mapping studies in the chick have shown that the subdivisions of the forebrain can be traced back to the neural plate, suggesting that the mechanisms that subdivide the forebrain begin operating as early as gastrulation (Couley and Le Douarin, 1988; Rubenstein et al., 1998).

Induction and patterning of the vertebrate body axis is controlled by signals emanating from the gastrula organizer (for review see Harland and Gerhart, 1997; Tam and Behringer, 1997). Studies in amphibians have shown for example that organizer function can be subdivided into distinct head and trunk organizer activities. Careful assessment of gene expression patterns in the dorsal blastopore lip of the early gastrula *Xenopus* embryo reveals molecularly distinct territories (Vodicka and Gerhart, 1995; Zoltewicz and Gerhart, 1997). An anterior domain, expressing *Gsc*, is fated to form prechordal mesendoderm (the prechordal plate or PcP) and can induce the expression of forebrain markers in animal cap explants, whereas a posterior domain, expressing *Xnot*, is fated to form notochord and somites and induces the expression of both anterior and posterior markers. The small number of cells that the organizer contains and its dynamic nature make it difficult to more accurately dissect out those aspects of organizer function that are directly related to the development of the forebrain using embryological techniques.

Genetic evidence for a distinct head organizer has been provided by the phenotype of the *Lim1* (*Lhx1*) knockout mouse (Shawlot and Behringer, 1995). The *Lim1* gene encodes a transcription factor with both LIM domains and a homeodomain. *Lim1* is expressed in the node and primitive streak of gastrulating mouse embryos. In homozygous *Lim1* mutant embryos, none of the structures anterior to rhombomere 3 develop, while the trunk develops normally. The node is not present initially in mutant embryos but can subsequently be found in the tail region as gastrulation proceeds. These defects are consistent with the concept of a head organizer that has come from amphibian studies. Chimera analysis has shown that *Lim1* expression is required in the organizer-derived anterior mesendoderm, consistent with the classical notion of the head organizer. *Lim1* function is also required in the anterior visceral endoderm (AVE) (Shawlot et al., 1999), a

requirement that is consistent with other recent insights into the regulation of mammalian anterior development.

The first obvious sign of anteroposterior (AP) pattern in the mouse embryo is provided by the formation of the primitive streak. Molecular studies have shown that AP pattern in the visceral endoderm, in the form of restricted expression of genes such as *Lim1* and *Hex*, precedes discernible pattern in the overlying epiblast by at least 12 hours (reviewed in Beddington and Robertson, 1998, 1999). More recently a specific role for the AVE of the pregastrula mouse embryo in patterning the anterior of the embryo has been shown by both embryological and genetic methods. Embryological evidence comes from the demonstration that surgical removal of the AVE reduces or extinguishes expression of forebrain markers in the mouse embryo. Similarly, transplantation of the rabbit AVE shows that it has the ability to induce the expression of an anterior neural marker in the epiblast of a recipient chick embryo (Knoetgen et al., 1999). Genetic evidence comes from the characterization of mutations in genes that are expressed in the visceral endoderm. Chimera experiments using mutant embryos or ES cell lines show that *Lim1*, *Otx2*, *nodal* and *Hnf3β* expression in the AVE is required for proper development of both the forebrain and midbrain. It is not clear though, how the AVE functions to regulate forebrain development. Study of zebrafish embryos indicates that differential competence of the epiblast determines whether anterior or posterior neurectoderm is produced in response to neural inducers (Koshida et al., 1998). It may be that the AVE acts to regulate the competence of the epiblast to respond to patterning signals from the axial mesendoderm or it may have neural inducing capabilities on its own as indicated by heterospecific transplantation experiments. Gene expression patterns within the AVE indicate significant AP pattern within this structure that could produce significant AP pattern in the overlying epiblast during neural induction (Beddington and Robertson, 1998, 1999).

The *oto* (otocephaly) mutation was identified in a screen for lethal mutations on chromosome 1 (Juriloff et al., 1985). Preliminary characterization of the mutant phenotype using scanning electron microscopy revealed defects in the development of the telencephalon and the lower jaw. Examination of progressively younger embryos revealed a deficiency of the anterior midline of the neural plate that could be detected as early as 8 days of gestation. The severity of the phenotype is variable, at least partly as a result of the mutation being carried on a mixed strain background.

We have characterized the developmental defects in *oto* embryos in greater detail and have uncovered novel and fundamental aspects of the phenotype. *oto* embryos have dramatic defects in the development of the anterior neural tube, the first branchial arch and the foregut. The pattern of the defects and their onset is consistent with an early defect in anterior development. To explore this idea further we crossed *oto* carriers to mice carrying a null mutation in the *Lim1* gene. Interactions between *oto* and *Lim1* mutations were seen with all of the genotype combinations. Thus our results reveal redundancy between *oto* and *Lim1* functions in the regulation of anterior development. The indication of a role for *oto* in regulating the anterior axis led us to re-examine the development of the rest of the AP axis more carefully. Analysis of the skeleton revealed posterior homeotic transformations along the length of the vertebral column. The transformations are dose-dependent but are not affected by the *Lim1* mutation indicating that *oto* regulates positional values in the trunk by a mechanism independent of *Lim1*.

MATERIALS AND METHODS

Mapping and generation of *oto* embryos

The method for distinguishing *oto* carriers from noncarriers utilized of a set of PCR-based Simple Sequence Length Polymorphism (SSLP) markers generated by the Whitehead Institute Center for Genome Research (Dietrich et al., 1996). Genomic DNA was prepared from tail tips by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. PCR was performed and markers were visualized on 3.5% (2.5 NuSieve, FMC; 1% low EEO, Fisher) agarose.

Mice

The *oto* mutation was obtained as a single male carrier on a mixed strain background by recovery from the frozen embryo stocks at the Jackson Laboratory, Bar Harbor Maine. The $Pax3^{Sp\text{-}r}$ deletion mutation was obtained as frozen embryos from the stocks of the Medical Research Council, Harwell, UK. Transfer of $Pax3^{Sp\text{-}r}$ embryos to pseudopregnant females was kindly carried out by Cheryl Bock of the Transgenic Facility of the Duke Comprehensive Cancer Center.

Null mutations in the *Lim1* and *Gsc* genes ($Lim1^{ko}$ and Gsc^{ko}) were obtained from Richard Behringer as heterozygous males on a C57BL/6J background. *oto* carrying females were mated to males heterozygous for knockout mutations to produce *oto/+;* $Lim1^{ko}$/+ or Gsc^{ko}/+, compound heterozygotes. Animals carrying the $Lim1^{ko}$ allele were identified by PCR, using primers complementary to the PGK promoter and neomycin sequences unique to the $Lim1^{ko}$ allele. A set of male compound heterozygotes were mated to *oto* carrying females or compound heterozygote females in timed pregnancies.

In situ hybridization and histology

Whole-mount in situ hybrization was performed as described (Henrique et al., 1995; Hentges et al., 1999). For double labels, digoxigenin and fluorescein-labeled RNA probes were synthesized in vitro using standard techniques. After visualization with Magenta Phos substrate (Biosynth AG), the first alkaline phosphatase-coupled antibody was inactivated by incubating embryos in 0.1 M glycine-HCl pH 2.2, 0.1% Tween-20 for 1 hour. The stain was then bleached with 5% hydrogen peroxide in maleic acid buffer for 1-2 hours at room temperature until the color turned pink. The second label was also visualized with Magenta Phos but was not bleached to yield a blue-purple second color. Probe plasmids were kind gifts of: *Fgf8* (Gail Martin), *dHand* and *eHand* (Deepak Srivistava), *Pax6* (Peter Gruss), *Nkx2.1*, *Otx2* and *Emx2* (John Rubenstein), *Lim1* (Richard Behringer, Heiner Westphal and Brigid Hogan), *Nkx2.5* (Richard Harvey), *Shh* (Andy McMahon). Embryos to be examined histologically were dehydrated, embedded in paraffin wax, and cut into 10 μm sections. Embryo images were captured via computer using a Sony 3CCD color video camera mounted on a Leica Wild M420 stereomicroscope.

Double staining of fetal skeletons

Cartilage and bone were visualized in skeletons from 18.5 dpc or neonate animals by staining with Alcian blue and Alizarin red S, respectively (Peters, 1977). Following the procedure, specimens were stored in 50:50 glycerol:ethanol.

RESULTS

Localization of the *oto* gene

The *oto* mutation was induced by X-irradiation and mapped within a pre-existing inversion of mouse chromosome 1

(Juriloff et al., 1985). We obtained a single male carrying the *oto* mutation from stocks of frozen embryos at the Jackson Laboratory where the mutation had been maintained on a mixed strain background. Since previous studies had indicated that penetrance of the *oto* phenotype was dependent upon strain background, we have stabilized the background by crossing to C57BL/6J. The crosses to C57BL/6J allowed us to confirm the tight linkage between the inversion on chromosome 1 and the *oto* mutation. Characterization of embryonic phenotypes has been done using mice back-crossed for 1-3 generations.

To allow the mutation to be more accurately localized using meiotic crossover events, we transferred the *oto* mutation to a non-inverted chromosome. This was accomplished by identifying rare double recombination events using SSLP markers that distinguish the *oto* carrying chromosomal segment of DBA/2J origin from a non-inverted C57Bl/6J chromosome. Animals carrying recombinant chromosomes were mated to *oto* carriers to determine whether or not the recombinant chromosome carried the *oto* mutation. A series of meiotic crossover events allowed us to define an interval, between D1mit79 and D1mit134, that contains the *oto* locus (Fig. 1). This interval is just distal to *Pax3* in a region of synteny with human chromosome 2q35-36. To provide further information about the location of the *oto* mutation, we also took advantage of the Sp^r deletion allele of *Pax3* (Doolittle et al., 1996; Epstein et al., 1991). $Pax3^{Sp\text{-}r}$ is a deletion of about 10 cM that confers the dominant belly spot phenotype characteristic of *Pax3* mutations and recessive, preimplantation lethality. We mapped the distal breakpoint of the $Pax3^{Sp\text{-}r}$ deletion to a location within the *oto* interval, between D1mit79 and D1mit134. We used two criteria to determine whether *oto* was uncovered by the deletion: we examined the vertebrae of $Pax3^{Sp\text{-}r}$ heterozygotes for dominant homeotic transformations, described in detail below, that are characteristic of *oto*, and we examined compound, *oto/+;* $Pax3^{Sp\text{-}r/+}$ heterozygotes for both anterior defects and homeotic transformations. Both of these assays indicated that *oto* is not uncovered by the $Pax3^{Sp\text{-}r}$ deletion. The combination of meiotic and deficiency mapping places *oto* between D1mit134 and the distal endpoint of $Pax3^{Sp\text{-}r}$ (Fig. 1).

oto embryos have anterior defects

Crosses between *oto* carriers produced embryos and pups with obvious anterior defects (Fig. 2). The externally visible defects were variable but were limited to specific structures: the jaws, the eyes and the outer ears. By late gestation, the jaws were absent or dramatically reduced in size (Fig. 2A-D). The eyes approached the midline, fused to form a cyclopic eye, were reduced in size or absent. More extensive forebrain defects were suggested by the obvious reduction in the size of the heads of affected pups. Defects are obvious in embryos at mid-gestation (Fig. 2E,F) and as early as 8-8.5 dpc (Fig. 2G,H) indicating that the defects are due to the disruption of anterior development at an early stage of development.

The importance of genetic background for the penetrance of the *oto* phenotype was suggested in a previous report from outcrosses to several different inbred strains (Juriloff et al., 1985). In our hands, the penetrance stabilized at about 50% after crossing for 3-4 generations onto the C57BL/6J background, suggesting that some of the phenotypic variability results from threshold or stochastic effects (McAdams and Arkin, 1999). We examined both moderately and severely affected embryos and found a consistent pattern of developmental defects as described in more detail below.

Forebrain defects in *oto* mice

To characterize the forebrain defects in more detail, we examined embryos from mid-gestation and earlier stages. At 9.5 dpc, forebrain phenotypes in *oto* embryos ranged from apparently normal to severely affected. To better understand the defects, we categorized embryos as mildly, moderately or severely affected and used in situ hybridization and markers relevant to each set of defects.

Mildly affected embryos have subtle defects in the anterior midline of the telencephalon (Fig. 3A,B). The commissural plate (CP) is a structure at the anterior midline of the telencephalon that expresses Fgf8, a signaling molecule with important roles in a number of developmental processes including an early role in regulating telencephalic development (Shimamura and Rubenstein, 1997). Although a role for *Fgf8* expression in the CP has not been clearly defined, it is a useful marker for this structure. The expression of *Fgf8* was reduced in mildly affected embryos, although expression at other sites such as the mid-hindbrain junction remained at a normal level (Fig. 3C,D).

Moderately affected embryos have telencephalic vesicles that are obviously reduced in size (Fig. 3G,H). The appearance of the residual telencephalon suggested that the ventral telencephalon was more affected by the mutation than the dorsal telencephalon. To test this idea, we examined the expression of *Nkx2.1* as a marker of the ventral telencephalon and *Emx2* and *Pax6* as markers of the dorsal forebrain. Expression of *Nkx2.1* is normally found in two domains in the forebrain (Lazzaro et al., 1991; Price, 1993; Shimamura et al., 1995), in the ventral region of both the anterior diencephalon and the telencephalon (Fig. 3E,F). Expression of *Nkx2.1* was reduced or absent in the *oto* telencephalon (Fig. 3G-J). In the wild-type, *Emx2* is expressed in the dorsal telencephalon and *Pax6* is expressed in both the dorsal diencephalon and telencephalon (Boncinelli et al., 1993; Puschel et al., 1992; Simeone et al., 1992a,b; Walther and Gruss, 1991). In moderate mutants in which the telencephalic expression of *Nkx2.1* expression was lost, expression of both *Emx2* (not shown) and *Pax6* (Fig. 3K,L) was retained. Staining with a probe for *Otx2* indicated that the remaining tissue was properly specified as anterior neurectoderm (Fig. 3M,N).

Severely affected embryos have telencephalic and optic vesicles that are absent or reduced to a single, small vesicle (Figs 2F, 3I). Diencephalic tissue is present in severely affected embryos but it is not apparent from simple inspection whether the diencephalon is anteriorly truncated. To examine this issue, we looked at the expression of *Nkx2.1* and *Shh* as ventral markers. The persistence of *Nkx2.1* expression in severely affected mutants indicates that most of the anterior diencephalon is present. However, the anterior midline of the diencephalon, the preoptic area and pituitary region are apparently reduced or absent in severely affected mutants (Fig. 3J) indicating that the posterior boundary of *oto*'s effects is within the anterior diencephalon. Wild-type expression of *Shh* is also ventrally restricted but extends throughout the neuraxis (Fig. 3M). The expression pattern of *Shh* in severely affected

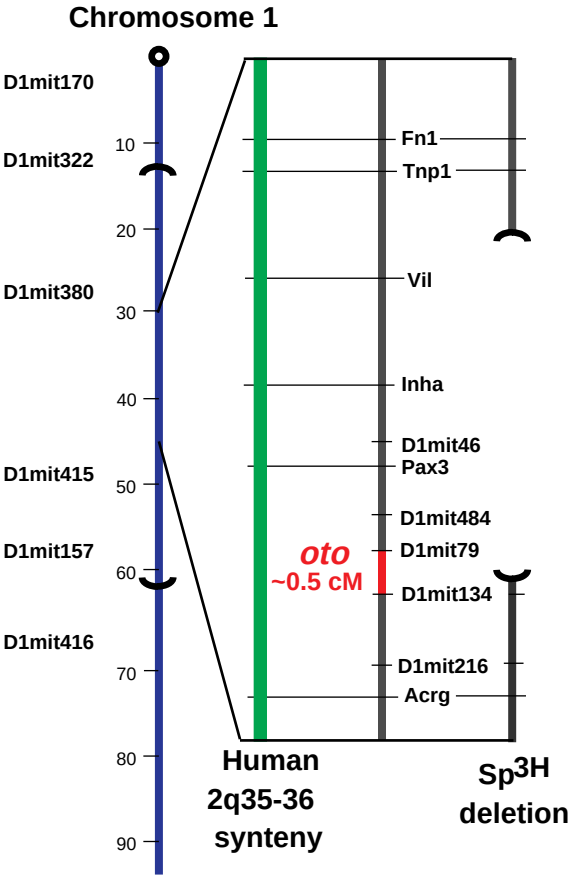

Fig. 1. Mapping of the *oto* gene to mouse chromosome 1. The *oto* mutation has been mapped meiotically to mouse chromosome 1 between D1mit79 and D1mit134, in a region of synteny with human chromosome 2q35-36. The location of genes that have been mapped in both human and mouse are shown. The approximate end-points of the inverted segment upon which *oto* was induced are indicated by brackets. The $Pax3^{Sp\text{-}r}$ deletion shown on the right has a proximal endpoint between Tnp1 and Vil and a distal endpoint between D1mit79 and D1mit134.

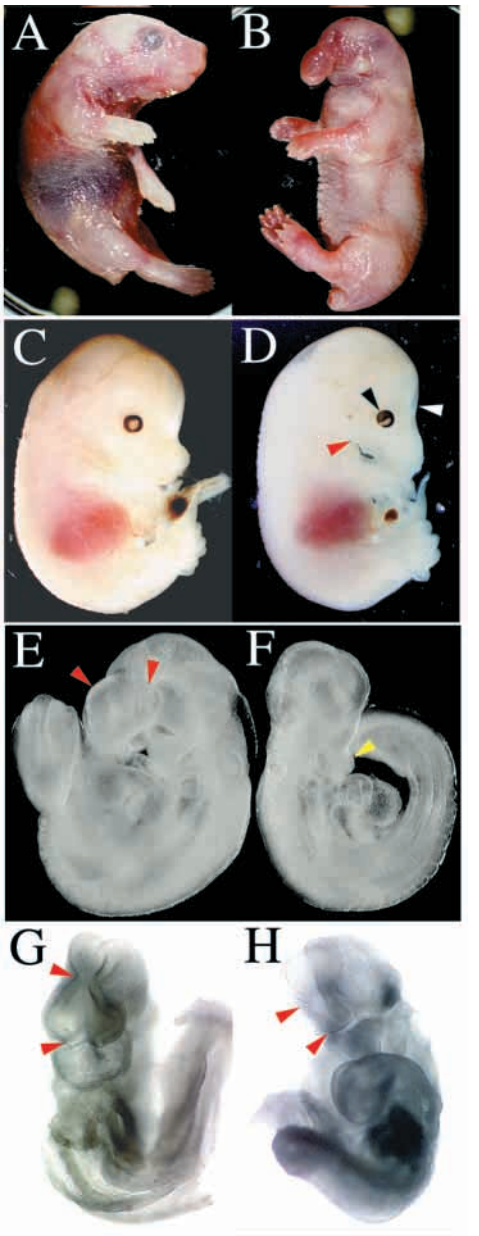

Fig. 2. *oto* homozygotes have anterior defects. Wild-type (A) and *oto* (B) newborn pups. The *oto* pup is lacking a lower jaw and eyes. The head is reduced in size and terminates in a proboscis. The tails of both pups were removed for genotyping. A~14 dpc wild-type (C) pup is shown in comparison to an *oto* littermate (D). The *oto* pup is lacking a lower jaw (red arrowhead) and has eye defects (black arrowhead) and a reduced forehead (white arrowhead). In contrast to its wild-type littermate (E), the telencephalic and optic vesicles (indicated by red arrowheads in E) are completely absent in an *oto* embryo (F) at 9.5 dpc. The first branchial arch is reduced to a single midline structure (yellow arrowhead). At 8.5 dpc (10 somites) the size of the anterior forebrain (between red arrowheads) is reduced in *oto* embryos (H) relative to wild-type (G).

mutants (Fig. 3N) supports the conclusion that the posterior as well as the majority of the anterior diencephalon are intact.

Defects in the anterior neurectoderm can be seen in some *oto* embryos as early as the 2- to 3-somite stage (Fig. 4). The anterior margin of the neurectoderm (the neural ridge) normally has an inward deflection at the midline that is not present in severely affected *oto* embryos and the width of the neurectoderm is obviously narrowed, suggesting that the anterior midline is not properly specified (Fig. 4). To examine the defects in these embryos in more detail, we examined embryos from 0-8 somites for the expression of *Hesx1* (also known as *Rpx1*) and *Otx2*. Both genes play important roles in regulating the development of the forebrain at early stages and both have dynamic patterns of expression with early expression in the AVE followed by later expression in the PcP and in the anterior neurectoderm of wild-type embryos. At 4-6 somites, *Hesx1* expression is restricted to the anteriormost portions of the neurectoderm and the adjoining ectoderm whereas *Otx2* expression is found throughout the presumptive forebrain and midbrain region.

The expression of *Hesx1* appeared normal in presomitic embryos (data not shown). By 2-3 somites, however, defects in the pattern of *Hesx1* expression in the forebrain neurectoderm became apparent (Fig. 4A,B). Embryos lacked expression of *Hesx1* at the anterior midline and lateral edges of the normal

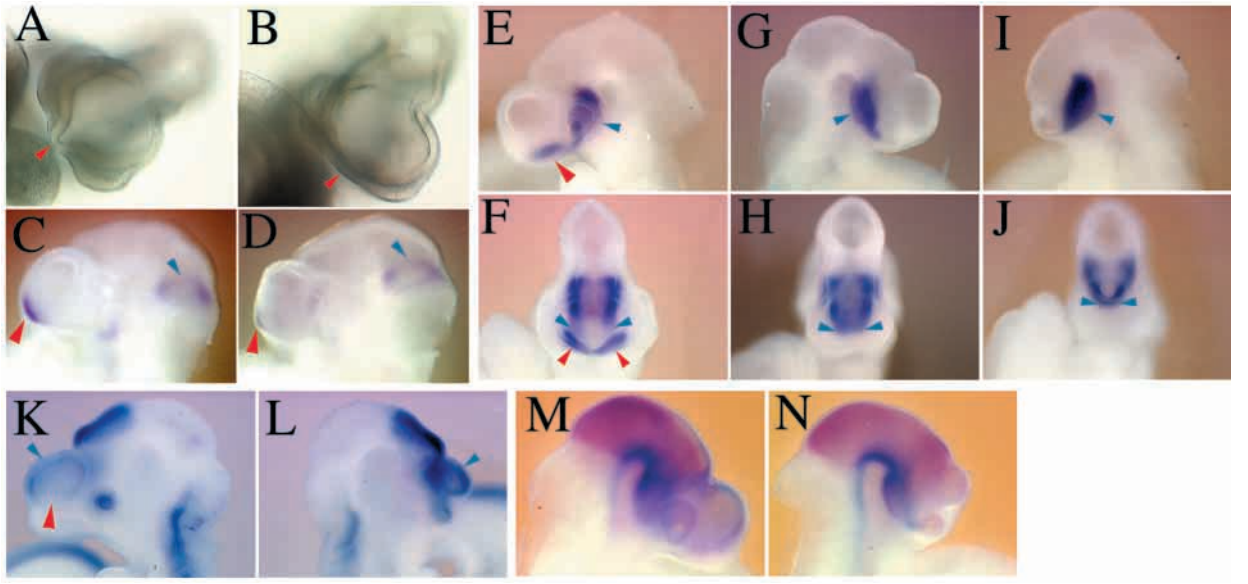

Fig. 3. Forebrain defects in *oto* embryos at 9.5 dpc. Mildly affected embryos (wild-type in A,C and *oto* in B,D) have defects in the midline of the telencephalic vesicles, indicated by red arrowheads. *Fgf8* is expressed in the central nervous system at two sites in wild-type embryos (C), the commissural plate (red arrowhead) and the isthmus (blue arrowhead). Expression is reduced in the commissural plate of *oto* embryos (D). *Nkx2.1* is expressed in two domains in wild-type embryos (E,F), the ventral diencephalon (blue arrowheads) and the ventral telencephalon (red arrowheads). The expression pattern of *Nkx2.1* in moderately (G,H) and severely (I,J) affected embryos reveals loss of ventral telencephalon. The narrowing of the diencephalic expression domain at the anterior (blue arrowheads in F,H,J) is the result of loss of the preoptic region. *Pax6* expression in wild-type embryos (K) is found in the dorsal diencephalon and dorsal telencephalon (blue arrowhead) but is absent in the ventral telencephalon (red arrowhead). In the reduced telencephalic vesicle of an *oto* embryo (blue arrowhead in F), *pax6* is expressed throughout the vesicle. Double-label in situ hybridization shows the expression of *Otx2* (in magenta) and *Shh* (in purple) in wild-type (M) and *oto* (N) embryos.

expression domain while expression in the medial portion of the neural plate remained. This aberrant expression pattern was maintained as development proceeded (Fig. 4C,D). The expression of *Otx2* was not obviously affected by the *oto* mutation (Fig. 4E,F).

Defects in development of the first branchial arch and pharynx

Mildly affected *oto* embryos at late-gestation have a reduced lower jaw in which distal midline structures are preferentially disrupted (Fig. 5A,B). A possible explanation for this phenotype is that distal fates in the first branchial arch are lost leading to the subsequent loss of distal and midline structures of the mandible. *oto* embryos at 9.5-10.5 dpc characteristically exhibit abnormal fusion of the distal tips of the first branchial arch, consistent with this interpretation. We investigated this possibility more directly by staining the first arch of mid-gestation embryos with markers for distal (*dHAND* and *eHAND*) and proximal (*Fgf8*) fates. Expression of the *dHAND* and *eHAND* genes in the first arch is restricted to the distal tips at 9.5-10.5 dpc (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995), while *Fgf8* is restricted to the anterior and proximal region of the arch (Crossley and Martin, 1995). In *oto* embryos expression of both *dHAND* (Fig. 4C,D) and *eHAND* (not shown) is reduced or absent while *Fgf8* is expressed in a continous stripe across the anterior edge of the arch (not shown). This is consistent with the idea that a loss of distal fates, and a resultant fusion of proximal elements, in the first arch may be responsible for defective jaw development.

The lack of an externally apparent oral cavity in severely affected *oto* embryos at midgestation suggested that development of the oral ectoderm was impaired. To examine this further, we sectioned embryos. In a wild-type embryo at 9.5 dpc, the oral ectoderm extends posteriorly under the ventral neurectoderm to the infundibulum (Fig. 5E). Here the oral

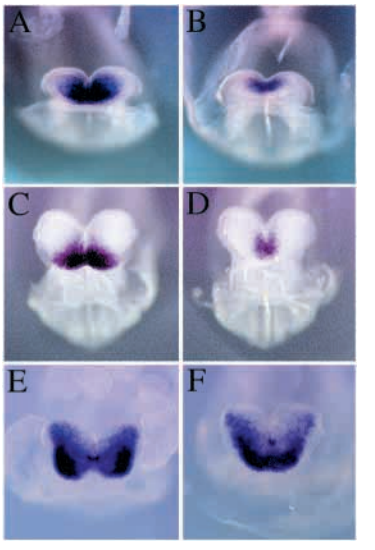

Fig. 4. Forebrain defects in *oto* embryos at 2-8 somites. Frontal views of wild-type (A,C,E) and homozygous *oto* (B,D,F) embryos. *Hesx1* expression (A-D) is diminished in the lateral and anterior parts of its normal expression domain in the anterior neural plate but maintained in the medial portion. At 2-3 somites (A,B), a relatively subtle deficit in the amount of neurectoderm at the anterior midline is accompanied by an obvious diminution of *Hesx1* expression. The deficit in anterior neurectoderm is more apparent by 4-6 somites (C,D), when the anterior neural plate is obviously narrowed and the aberrant pattern of *Hesx1* expression is maintained. The expression of *Otx2* (shown at 6-8 somites in E,F) in *oto* embryos, on the contrary, appears to be unaffected by the *oto* mutation.

ectoderm forms a specialized structure, Rathke's pouch, which will form the anterior pituitary. Just beyond Rathke's pouch the oral ectoderm meets the foregut endoderm to form the oropharynx. *oto* embryos lack an oropharynx (Fig. 5F). This could result from a failure in either the formation of the oral ectoderm, the foregut endoderm or both. In *oto* embryos, defects in both lineages are apparent. The absence of Rathke's pouch indicates defects in the oral ectoderm (Fig. 5F). The truncation of the foregut (Fig. 5F) and the failure of the eustachian tubes to form from the first pharyngeal pouch in some embryos (not shown) indicates that the development of the foregut endoderm is also affected.

Redundancy of *oto* and *Lim1* function in anterior development

The set of anterior defects in *oto* embryos and their early onset suggests that *oto* may be acting during gastrulation. To assess this possibility, we crossed *oto* carriers with animals carrying characterized null mutations in *goosecoid* (*Gsc*) or *Lim1*. A role for *Gsc* in regulating axis formation during gastrulation has been suggested from studies in *Xenopus* (Cho et al., 1991). $Gsc^{-/-}$ mice do not have a gastrulation phenotype, perhaps because of compensation by closely related genes (Rivera-Perez et al., 1995; Yamada et al., 1995). A function for *Lim1* in the regulation of anterior development is clear from the headless phenotype in mice (Shawlot and Behringer, 1995).

Compound heterozygotes were generated for each mutation and then intercrossed or back-crossed to *oto* carriers. Pilot crosses with each of the mutations indicated that *oto/oto*; $Lim1^{ko/+}$ embryos had a novel phenotype as described in more detail below. The Gsc^{ko} mutation did not show an obvious interaction with *oto* and these studies were not pursued further.

Lim1 has a dynamic pattern of expression in the early embryo. *Lim1* is initially expressed in the AVE at 6.5 dpc and then subsequently in the primitive streak, the migrating mesodermal wings, the node and the prechordal mesendoderm. The phenotype of a null mutation indicates that *Lim1* is required for development of the neural tube anterior to rhombomere 3 in the hindbrain. Chimeric embryos and in vitro tissue recombination experiments indicate that *Lim1* expression is required in both the AVE and the prechordal plate (PcP) for normal development although the exact role that *Lim1* plays at each site has not been determined (Rhinn et al., 1998 and Richard Behringer, personal communication).

Heterozygosity for either *Lim1* or *oto* alone did not have phenotypic consequences. Compound heterozygosity did however have developmental effects. 15% of the doubly heterozygous (*oto*/+; $Lim1^{ko}$/+) mice had anterior defects. Affected embryos showed reductions of the telencephalon at 8.5-9.5 dpc and some showed the lateral bias seen in *oto* homozygotes (Fig. 6A,B,E,F).

We also observed clear effects of removing one copy of *Lim1* on *oto* homozygotes at 9.5 dpc. While only ~50% of *oto* homozygotes had obvious anterior defects at 9.5 dpc, 75% of the *oto/oto;* $Lim1^{ko}$/+ embryos had anterior defects. The defects ranged from a phenotype that appeared to be the same as the *oto* phenotype (not shown) to phenotypes that were obviously more severe (Fig. 6G-J). The more severely affected embryos had deletions of the anterior CNS that were more extensive than are found in *oto* homozygotes. To determine the extent of the deletions, we examine embryos representing both phenotypic classes for expression of *Nkx2.1*. As described previously, the posterior limit of *Nkx2.1* expression in the forebrain is in the mid-diencephalon, at the the prechordal-notochordal boundary.

In the most severely affected *oto/oto;* $Lim1^{ko/+}$ embryos, the entire *Nkx2.1* domain is deleted (not shown). Some, slightly less affected embryos retained a small portion of the rostral diencephalon as a residual *Nkx2.1*-expressing domain (Fig. 6I,J). Some of the less affected embryos, those that outwardly appeared identical to moderately affected *oto* embryos, nonetheless had a novel phenotype (Fig. 6G,H). The expression of *Nkx2.1* is reduced in these embryos compared to *oto* homozygotes indicating that development of the ventral diencephalon is affected when one copy of *Lim1* is removed from *oto/oto* embryos.

oto regulates positional values in the trunk by a *Lim1*-independent mechanism

In carrying out the *Lim1* interaction studies, we also observed effects of *oto* heterozygosity on the *Lim1* homozygous phenotype. The *Lim1* knockout characteristically causes truncation of the CNS anterior to rhombomere 3 without affecting trunk development. We obtained $Lim1^{ko}/Lim1^{ko}$*; oto/*+ embryos at 9-9.5 dpc that had variable, but consistently more severe, anterior truncations than those typically seen in $Lim1^{ko}$ homozygotes alone (Fig. 7B-D). The phenotype varied from embryos with a well-developed body axis to those without. We stained embryos that had an apparent body axis with a marker for heart tissue, *Nkx2.5*. This revealed the presence of cardiac tissue with severe morphogenetic defects at the anterior end of the embryo indicating truncation of the body axis posterior to rhombomere 3. Staining of embryos with *Hoxb1*, a marker for rhombomere 4 confirmed this analysis (not shown). Somewhat surprisingly we also saw morphogenetic defects in the hearts of $Lim1^{ko}$/+ (not shown) and $Lim1^{ko}/Lim1^{ko}$ embryos (Fig. 7B). Defects were seen in 13% of $Lim1^{ko}$ heterozygotes indicating a previously undescribed role for *Lim1* in the development of the heart.

The variable phenotype of $Lim1^{ko}/Lim1^{ko}$*; oto/*+ embryos precluded a detailed characterization, but since the defects in some doubly homozygous embryos involved regions of the trunk, they raised the possibility that *oto* plays a role, not only in anterior development, but also in regulating trunk development. To test this hypothesis more directly, we examined the vertebrae of late gestation *oto* heterozygotes and homozygotes. The structure of vertebral elements varies along the AP axis, providing a sensitive indicator of AP patterning in the trunk. Alcian blue and alizarin red staining of skeletons revealed a dose-dependent effect of *oto* on trunk development. All homozygotes and more than half of heterozygotes show posterior transformations of the axial skeleton (Fig. 8). The transformations are most apparent at the junctions between the cervical, thoracic, lumbar and sacral regions apparently because the morphology of the vertebrae changes dramatically at these points. For example, the seventh cervical (C7) vertebrae of *oto* homozygotes commonly carried a rib, a feature characteristic of the thoracic vertebrae (Fig. 8B,C). Posterior transformations of the first thoracic rib (Fig. 8D,E) and thoracic, lumbar and sacral vertebrae (Fig. 8F,G) indicate that the posterior shifts in identity occur all along the trunk.

Since *Lim1* heterozygosity has clear effects on the *oto*

anterior phenotype, we determined whether the axial skeleton phenotype was also affected by *Lim1* heterozygosity. In contrast to the anterior phenotype, we saw no effect of the *Lim1*^{ko} heterozygosity on the establishment of positional values in the trunk (data not shown).

DISCUSSION

The role of *oto* in the establishment of forebrain subregions

The regions of the forebrain that are deleted in the most severe *oto* homozygotes at 9.5 dpc, the telencephalic and optic vesicles, arise from precursor regions that are adjacent in the anterior neural plate (Rubenstein et al., 1998). *oto* embryos have forebrain defects as early as 2-3 somites (Fig. 4A,B) indicating that the wild-type *oto* gene functions before or during the neural plate stage to regulate development of the anteriormost portions of the CNS. It is not possible to determine the site of action of the *oto* gene product from the phenotype alone but it is interesting to note the very similar forebrain phenotype of *Hesx1*/*Rpx1* mutants (Dattani et al., 1998). The *Hesx1* gene on chromosome 14 encodes a homeobox-containing transcription factor that is first expressed in the AVE (Hermesz et al., 1996; Thomas and Beddington, 1996). Expression is subsequently found in the PcP and in the anterior neurectoderm that is fated to form the ventral portion of the anterior forebrain. Later in development expression becomes restricted to the anterior pituitary. The telencephalic and optic vesicles fail to develop in *Hesx1*$^{-/-}$ embryos – a pattern of defects that is similar to that seen in *oto*.

One explanation for the similarity in the *oto* and *Hesx1*$^{ko/ko}$ phenotypes is that *oto* is an upstream regulator of *Hesx1* expression. Supporting this hypothesis is the observation that severely affected *oto* embryos lack expression of *Hesx1* in the anterior midline and lateral portions of the neural plate (Fig. 4 A-D). Since *oto* homozygotes do not appear to have defects in *Hesx1* expresion at earlier stages, this would suggest that the similarity in the *oto* and *Hesx1*$^{ko/ko}$ phenotypes results from a critical role for *Hesx1* expression in the anterior neurectoderm and not in the AVE or PcP.

An alternative explanation for the similarity in the *oto* and *Hesx1*$^{ko/ko}$ phenotypes is that *oto* acts downstream of or in parallel to *Hesx1* in regulating development of the anterior neurectoderm. The anterior neurectoderm that fails to express *Hesx1* in *oto* homozygotes is fated to form the forebrain structures that are lost in *oto* homozygotes. This observation, together with the fact that expression of *Hesx1* in the visceral endoderm and the PcP is not obviously disrupted (data not shown), could mean that the loss of *Hesx1* expression is the result of forebrain defects rather than a cause. In this view, the loss of *Hesx1* expression is an early indicator of forebrain defects, i.e., the midline of the anterior neurectoderm has not been properly induced or specified. This explanation does not preclude an important role for *Hesx1* expression in the anterior neurectoderm; however, it differs significantly from the view that the *oto* phenotype results from a role for *oto* as as a direct regulator of the expression of *Hesx1*. It suggests instead that *oto* functions downstream of, or in parallel to, *Hesx1* in regulating development of the anterior neural plate. Similarly, the apparently normal expression of *Otx2* in the anterior neurectoderm also suggests that *oto* acts downstream of, or in parallel to, the action of *Otx2* at this site.

Interaction with *Lim1*^{ko} suggests roles for *oto* in the organizer and prechordal plate

Interaction between *oto* and *Lim1* mutations produces progressively greater defects in anterior development as embryos lose wild-type copies of the *oto* and *Lim1* genes. Forebrain defects in *oto* homozygotes range from loss of only the ventral midline of the telencephalon in mild mutants, to loss of the entire telencephalon, eyes and preoptic region in severe mutants (Fig. 3). In *oto/oto*; *Lim1*^{ko}/+ embryos, defects extend to the anterior diencephalon (Fig. 6). In *Lim1*$^{ko/ko}$ embryos, all structures anterior to rhombomere 3 are deleted. Finally, in *Lim1*$^{ko/ko}$; *oto*/+ embryos, defects range from loss of all head structures anterior to the heart, to loss of all but the ventral-derived allantois (Fig. 7). These phenotypes are similar to those resulting from surgical depletion of the *Xenopus* organizer (Stewart and Gerhart, 1990). When more and more organizer cells are removed from the *Xenopus* late blastula, progressively less dorsal and anterior axial structures are formed in the resulting embryo. Removal of the whole organizer results in an embryo composed of only ventral cell types. Mutating both *oto* and *Lim1* in the mouse has a similar phenotypic consequence to surgical depletion of the organizer in *Xenopus*, suggesting partially redundant roles for *oto* and *Lim1* in mouse organizer function.

Lim1 expression is required in both the AVE and the PcP for normal forebrain development (Shawlot et al., 1999). Removal of one copy of *Lim1* from *oto* homozygotes causes a loss of the entire *Nkx2.1* expression in the ventral forebrain of the most severely affected embryos, a phenotype that is not seen with either *oto* homozygotes or *Lim1* heterozygotes. Because expression of *Nkx2.1* in the ventral diencephalon is initiated by signals from the PcP (Shimamura and Rubenstein, 1997), this phenotype is likely to be the result of reduction of *Lim1* function in the PcP. The mechanisms that produce genetic interactions vary from direct physical contact between two gene products to indirect contributions to a common endpoint, so the requirement for *oto* could be in the overlying neurectoderm rather than in the PcP. *oto* is required for the development of the PcP-derived anterior foregut endoderm, supporting the hypothesis that *oto* is required for normal PcP formation and function. Thus the defects seen in the diencephalon of *oto/oto; Lim1*$^{ko/+}$ embryos are likely the result of synergistic effects on PcP function.

We observed effects of the *Lim1*^{ko} allele on heart morphogenesis in the mouse. In *Lim*^{ko}*/Lim1*^{ko}*; oto*/+ embryos, the heart consists of an arc of cells expressing the cardiac marker *Nkx2.5*. We also found that 13% of *Lim1*$^{ko/+}$ embryos had reduced or absent left ventricles (data not shown), and that *Lim1*^{ko} homozygotes developed with defects in the morphology of both ventricles. Prechordal endoderm is thought to be a source of heart-inducing activity (Jacobson and Sater, 1988). Thus these heart defects may result from a deficit in prechordal plate function caused by mutations in the *Lim1* and *oto* genes.

The role of *oto* in trunk development

Mutations in a diverse group of genes cause homeotic transformations of the vertebrae. Anterior transformations are

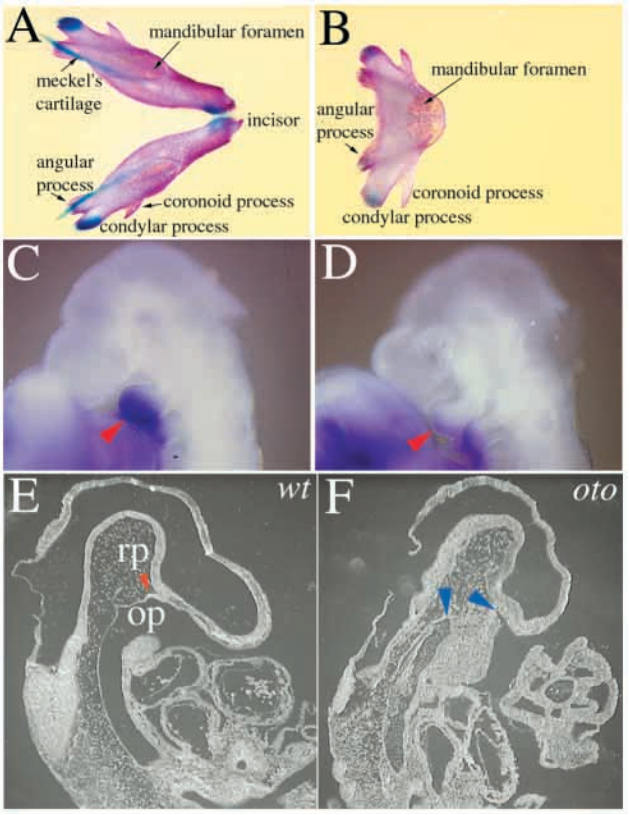

Fig. 5. Defects in the development of the mandible, oral ectoderm and foregut of *oto* embryos. A dorsal view of the lower jaw of a wild-type 18.5 dpc embryo (A) stained with alizarin red (bone) and alcian blue (cartilage) shows characteristic features along the proximal-to-distal axis such as the angular process and incisors respectively. The jaw of a mildly affected *oto* littermate (B) shows distal truncations. Meckel's cartilage was present in the mutant embryo but was lost during dissection of the jaw after staining. In *oto* embryos at 9.5 dpc (wild-type in C and *oto* in D), the expression of dHAND in the distal tip of the first branchial arch (red arrowheads) is reduced. The defective oropharynx (op) of severely affected embryos is shown in dark-field views of sagittal section (wild-type in E and *oto* in F). Rathke's pouch (rp), indicated with a red arrowhead in E is absent in the *oto* embryo. The foregut endoderm normally fuses with the oral ectoderm to form a seamless tube (E). The foregut is truncated in the *oto* embryo and the oral cavity does not form (blue arrowheads in F).

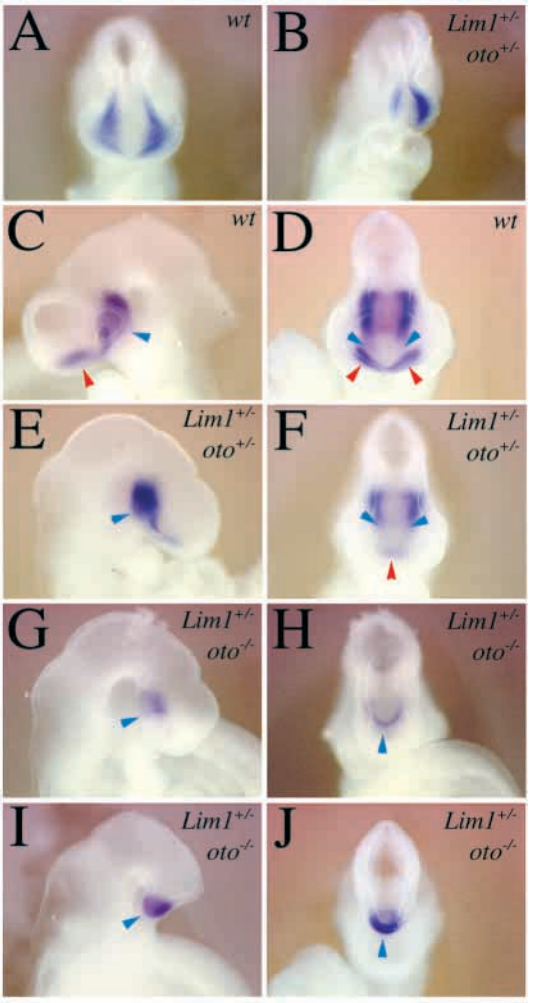

Fig. 6. Effects of Lim1 heterozygosity on the *oto* phenotype. Compound heterozygotes sometimes have defects in anterior forebrain development that are indistinguishable from those of *oto* homozygotes. A dorsal view of *Emx2* expression in the telencephalon of wild-type (A) and *oto/+; Lim1^{ko}/+* (B) embryos at 8.5 dpc shows a reduction of the telencephalic region, with a right-sided bias identical to defects that are seen in *oto* homozygotes. The blue arrowheads in C-J indicate diencephalic expression and the red arrowheads indicate telencephalic expression of *Nkx2.1* in embryos at 9.5 dpc. A wild-type embryo (C,D) has both domains. Some *oto*/+; *Lim1^{ko}*/+ embryos have a novel phenotype in the form of an anterior reduction in staining for *Nkx2.1* in the ventral telencephalon (F). *oto*/*oto*; *Lim1^{ko}*/+ exhibit an obviously novel phenotype – hypoplasia of the anterior-ventral diencephalon (G-J).

caused by loss-of-function mutations in Hox genes or in loci that regulate the AP pattern of Hox gene expression during early development. For example, mutations in genes encoding BMP/TGFβ family members or their receptors (Gdf11 and ActRIIB), an FGF and a retinoic acid receptor (FGFR1 and RARγ2) and the caudal related transcription factors Cdx1 and Cdx2 all cause anterior transformations of vertebral identity.

Posterior transformations on the contrary are less commonly seen and loss-of-function mutations causing a uniform pattern of posteriorward transformations are characteristic of mutations in mammalian Polycomb-Group (PcG) genes (reviewed in Gould, 1997; Santamaria, 1998; Schumacher and Magnuson, 1997). Loss-of-function mutations in PcG genes cause ectopic expression of PcG target genes. In the case of the Hox genes, this means an anterior spread of Hox gene expression domains and a posteriorization of the trunk.

PcG genes were first recognized in *Drosophila* and cloning of *Drosophila* PcG genes led to the conclusion that they encode a structurally diverse group of genes that are involved in establishing and maintaining stable and transcriptionally repressive chromatin structures. Mammalian homologues have been identified for 9 of the 15 PcG genes that are known in *Drosophila*. Mutations in mouse PcG homologues produce posterior transformations of the axial skeleton, consistent with a conserved role in the repressive regulation of Hox genes.

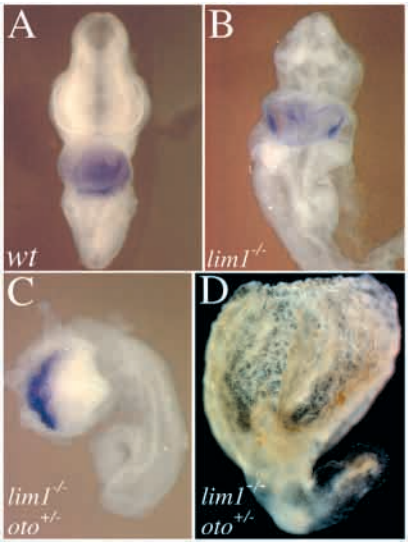

Fig. 7. Effects of the *oto* mutation on the *Lim1* phenotype at 9.5 dpc. Staining of a wild-type (A), a *Lim1*^{ko} homozygous (B) and a *Lim1*$^{ko/ko}$; *oto*/+ embryo (C) with *Nkx2.5*, a marker of the cardiac mesoderm. The cardiac tissue stained by *Nkx2.5* in C is at the extreme anterior end of the embryo. The compound mutant embryo in D has posterior mesoderm in the form of an allantois but otherwise lacks evidence of an embryonic body axis.

None of the mammalian PcG homologues are known to map to the *oto* locus. Nonetheless, many mammalian PcG genes are probably still unidentified. Based on genetic screens in *Drosophila*, the total number of PcG genes is estimated to be as high as 30-40 making it a near certainty that additional mammalian PcG genes exist. More than 100 loci are likely to be under the control of PcG repression in the fly so independent actions of *oto* on head and trunk development are not surprising if *oto* is a PcG gene.

oto and human holoprosencephalies

Holoprosencephaly is the term used to describe defects in the formation of the midline of the forebrain and associated facial structures in humans (Roessler and Muenke, 1998). The severity of the defects ranges from complete absence of the forebrain to the presence of a single upper incisor. Four loci have been defined based on the non-random association of chromosomal abnormalities in affected individuals: 21q22.3, 2p21, 7q36 and 18p. A mild autosomal dominant familial form has been recognized and has been mapped to chromosome 7q36. Most cases are sporadic, i.e., they have no obvious genetic lesion. Maternal alcoholism or diabetes are found in association with sporadic cases in a pattern that may indicate an interaction between environmental and genetic factors. In most cases, the lower jaw is not affected but sporadic cases do occur with head defects that are indistinguishable from the phenotype of the *oto* mouse described here (Hersh et al., 1989).

Holoprosencephaly is usually fatal, often prenatally, and even in families with multiple affected offspring it is difficult

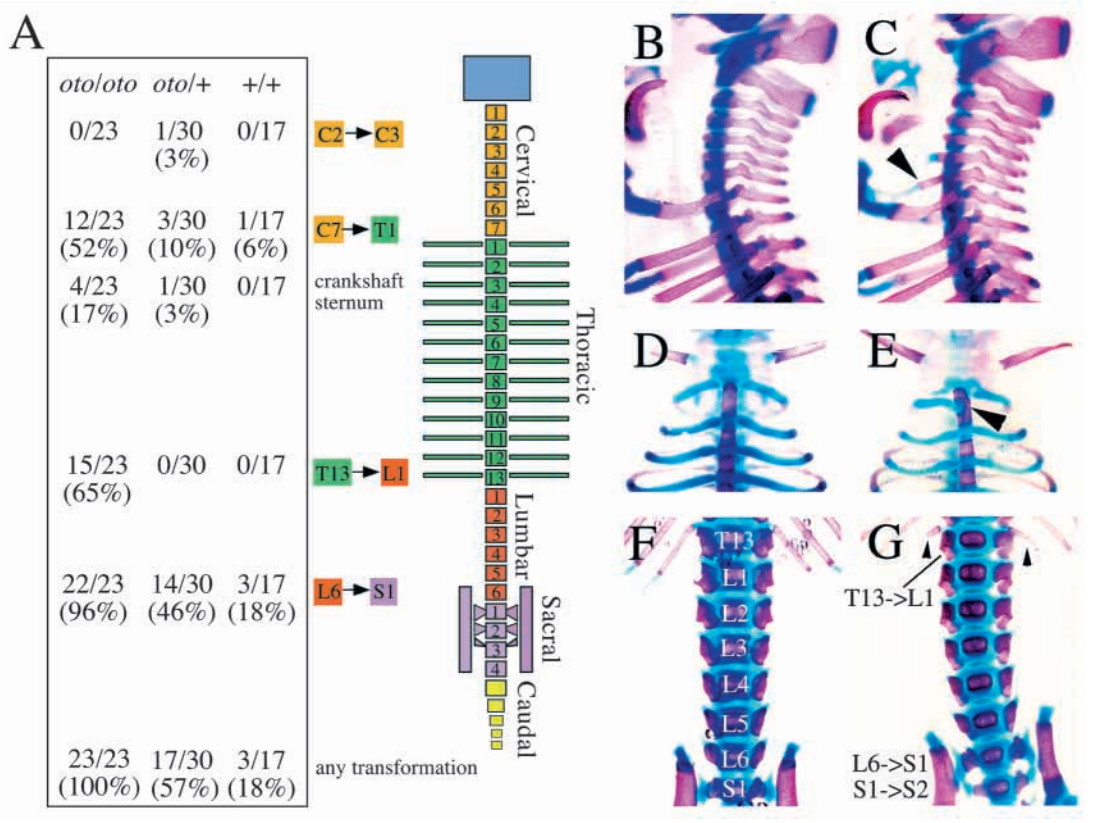

Fig. 8. The *oto* mutation causes homeotic transformations of the vertebrae in a dose-dependent fashion. The frequency, type and axial position of transformed vertebrae are shown in A. Cleared and stained skeletons from 18.5 dpc wild-type (B,D,F) and *oto*/*oto* (C,E,G) embryos. (B,C) Ventral is to the left and (D-G) ventral is toward the viewer. An ectopic rib on the 7th cervical vertebra of an *oto* embryo (arrowhead in C) indicates transformation towards a thoracic fate. The crankshaft sternum (Bel et al., 1998; Theiler, 1989) (arrowhead in E) results from transformation of the first thoracic rib towards a 2nd thoracic fate on the right but not the left side of the embryo. Reduction of the ribs on the thirteenth thoracic (T13) vertebrae (arrowheads in G) result from transformation towards a lumbar (L1-6) fate. The transformation of the sixth lumbar (L6) vertebrae towards a first sacral (S1) can also be seen.

or impossible to study the heritability of the disorder. Family studies have shown that the 7q36 locus corresponds to the *Shh* and the 2p21 locus to the *Six3* gene (Roessler et al., 1996; Wallis et al., 1999). Other loci have been identified by screening collections of sporadic cases for mutations in candidate loci (Brown et al., 1998; Dattani et al., 1998). No familial forms of holoprosencephaly have been linked to the 2q35 region on the long arm of chromsome 2 where the human *oto* locus is predicted to reside. Sporadic cases are much more common than familial cases though and it is likely that mutations in the human homologue of the *oto* locus occur in some fraction of these cases. Identification of human mutations and evaluation of the interaction between *oto* and environmental causes awaits molecular identification of the *oto* gene.

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