**moz regulates Hox expression and pharyngeal segmental identity in zebrafish**

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Summary

In vertebrate embryos, streams of cranial neural crest (CNC) cells migrate to form segmental pharyngeal arches and differentiate into segment-specific parts of the facial skeleton. To identify genes involved in specifying segmental identity in the vertebrate head, we screened for mutations affecting cartilage patterning in the zebrafish larval pharynx. We present the positional cloning and initial activity for Moz in regulating Hox expression and embryonic role and a crucial histone acetyltransferase genetic evidence that *moz* contributes to the *bapx1* function in wild types causes loss of the jaw joint. Reduction of *bapx1* function in both first and second arch joints, providing functional gene expression changes in postmigratory CNC prefiguring the homeotic phenotype in *moz* mutants. Early second arch ventral expression of *goosecoid* (gsc) in *moz* mutants and in animals injected with hox2-MOs shifts from lateral to medial, mirroring the first arch pattern. *bapx1*, which is normally expressed in first arch postmigratory CNC prefiguring the jaw joint, is ectopically expressed in second arch CNC of *moz* mutants and hox2-MO injected animals. Reduction of *bapx1* function in wild types causes loss of the jaw joint. Reduction of *bapx1* function in *moz* mutants causes loss of both first and second arch joints, providing functional evidence that *bapx1* contributes to the *moz*-deficient homeotic pattern. Together, our results reveal an essential embryonic role and a crucial histone acetyltransferase activity for Moz in regulating Hox expression and segmental identity, and provide two early targets, *bapx1* and *gsc*, of *moz* and hox2 signaling in the second pharyngeal arch.

Key words: *moz*, Hox, *hoxa2*, Zebrafish, Cranial neural crest, Bapx1, Goosecoid, Homeosis, Pharynx

Introduction

The jaw forms embryonically as hinged dorsal and ventral cartilages in the first (mandibular) pharyngeal arch. The jaw cartilages are classically considered segmentally homologous with the jaw-supporting cartilages in the second (hyoid) pharyngeal arch and the gill-supporting (branchial) cartilages in more posterior pharyngeal arches (Kimmel et al., 2001b). First arch cranial neural crest (CNC), which forms the jaw, lacks Hox gene expression. Second and more posterior arch CNC displays a nested pattern of Hox gene expression (Hunt et al., 1991). Hox2 genes act as selector genes for second arch segmental identity: mutation of *Hoxa2* in the mouse or reduction of *hoxa2b* and *hoxb2a* function in zebrafish results in homeotic transformation of second pharyngeal arch skeletal elements into first arch identity (Gendron-Maguire et al., 1993; Rijli et al., 1993; Hunter and Prince, 2002) (C. T. Miller, PhD Thesis, University of Oregon, 2001). Downregulation of *Hoxa2* expression by ectopic sources of FGF8 results in a similar transformation of the second arch-derived skeletal elements in the chick (Trainor et al., 2002). Forcing ectopic expression of *Hoxa2* in the first arch of *Xenopus* or chicks, or *hoxa2b* and *hoxb2a* in zebrafish, results in the converse phenotype, where the jaw segment adopts a second arch morphology (Grammatopoulos et al., 2000; Pasqualetti et al., 2000; Hunter and Prince, 2002). Other Hox genes are proposed to specify segmental identity throughout the pharyngeal arches (Hunt et al., 1991). In support of this, ectopic expression of *Hoxa2* can divert the *Xenopus* third pharyngeal arch (the first...
gill-bearing or branchial arch) towards hyoid fate (Pasqualetti et al., 2000). Furthermore, third arch CNC in valentinino (val) mutant zebrafish inappropriately expresses hoxb2a, which is normally restricted to second arch CNC, perhaps contributing to a mild transformation of the val mutant third arch cartilages to a second arch pattern (Moens et al., 1998; Kimmel et al., 2001a).

Although it is still not clear why Hox2 dysfunction results in homeotic transformations, analyses in the mouse, chick and Xenopus have begun to unravel the Hox2-responsive genetic circuitry. A subtractive screen in mice discovered that Pitx1 is ectopically expressed in Hoxa2 mutant second arch primordia, and inactivating Pitx1 in a Hoxa2 mutant partially rescues the homeosis (Bobola et al., 2003). Expression analyses in mice reveal that at late stages Hoxa2 represses expression of the chondrogenic factor Sox9 and the osteogenic factor Runx2 (Cbfa1) (Kanzerl et al., 1998). Two other known Hoxa2 target genes are the homeobox genes bapx1 and goosecoid (gsc). In Xenopus, ectopic expression of Hoxa2 in the first arch represses expression of Bapx1 (Pasqualetti et al., 2000). In chicks, ectopic expression of Hoxa2 induces Gsc expression (Grammatopolous et al., 2000), and in zebrafish early gsc expression is reported to be downregulated in hox2-MO injected animals (Hunter and Prince, 2002). Both Gsc and bapx1 are essential for craniofacial development, although reducing function of either gene does not result in homeosis (Rivera-Perez et al., 1995; Yamada et al., 1995; Miller et al., 2003).

Hox gene expression is maintained by trithorax group (trxG) activity, which involves chromatin remodeling, including histone acetylation (Simon and Tamkun, 1998). In humans, mutations in trxG members cause leukemia (Look, 1997; Ernst et al., 2002). The MYST family histone acetyltransferase MOZ (monocytic leukemia zinc finger protein; MYST3 – Human Gene Nomenclature Database) is mutated in human leukemias (Borrow et al., 1996). Human MOZ is a large protein of 2004 amino acids and biochemical analyses reveal MOZ to possess both histone acetyltransferase (HAT) and transcriptional activation activity (Champagne et al., 2001; Kitabayashi et al., 2001a). Targets of either of these activities in vivo are unknown and the function of MOZ during embryonic development has not been reported.

Previous screens in zebrafish have identified a large number of mutations causing craniofacial defects (Schilling et al., 1996a; Piotrowski et al., 1996; Neuhaus et al., 1996). The cloning of several of these mutations [endothelin1 (edn1 or sucker) (Miller et al., 2000); tbxl (van gogh) (Piotrowski et al., 2003); tf2ap2a (lockjaw) (Knight et al., 2003)] reveals remarkable conservation in the genetic control of vertebrate craniofacial development, as each of these molecules is also required for patterning the mammalian pharyngeal arches (Kurihara et al., 1994; Jerome and Papaioannou, 2001; Schorle et al., 1996; Zhang et al., 1996). To identify genes required for segmental identity in the pharyngeal arches, we directly screened for mutations affecting cartilage patterning in zebrafish.

We present the molecular identification and phenotypic characterization of a zebrafish homeotic mutant discovered in this screen. Fine mapping, positional cloning, sequencing and morpholino phenocopy experiments reveal this homeotic locus to encode a zebrafish ortholog of the human oncogene MOZ, a MYST family HAT. Severely reduced hox2 expression in moz mutant zebrafish contributes to a mirror-image duplication of jaw cartilages in place of second arch cartilages. moz is also more broadly required for maintenance of most hox1-4 expression domains, probably resulting in the homeotic transformation of the third and fourth arch gill support cartilages. In the hindbrain, moz is required for maintenance, but not initiation, of Hox gene expression, and moz mutants display aberrant facial motoneuron migration. Inhibition of histone deacetylase activity with Trichostatin A rescues Hox maintenance defects and homeotic cartilage transformations in moz mutants, indicating that HAT activity is essential for moz function. Pharyngeal musculature appears transformed late but not early in moz mutants. We find little evidence for patterning defects in arch epithelia of moz mutants. However, striking gene expression changes in moz mutant postmigratory hyoid CNC are apparent. Expression of bapx1, which is normally restricted to the jaw joint (Miller et al., 2003) is robustly duplicated in second arch CNC of moz mutants. Although reduction of bapx1 function in wild-type embryos results in absence of the jaw joint (Miller et al., 2003), reduction of bapx1 function results in absence of both the first and second arch joints in moz mutants. Expression of gsc is profoundly reorganized in the moz mutant second arch, with lateral CNC expression shifting to medial, mirroring the wild-type first arch pattern. Together our results reveal that a zebrafish ortholog of the human oncogene MOZ regulates Hox gene expression and segmental identity in the vertebrate pharynx.

Materials and methods

Fish maintenance and Alcian screen

Fish were raised and staged as described (Westerfield, 1995; Kimmel et al., 1995). For the head cartilage screen, ENU-mutagenized F2 gynogenetic diploid clutches were generated by EP treatment (Streisinger et al., 1981). Mutagenesis was postmeiotic (Riley and Grunwald, 1995) for b719 and premeiotic (Solnica-Krezel et al., 1994) for b999. Survivors at day four were fixed, stained with Alcian Green, and bleached to remove pigmentation as described (Miller et al., 2003). Clutches were screened for cartilage morphology under a Zeiss STEMI SR dissecting microscope at 50× magnification. The recessive larval lethal mutations b719 and b999 were outcrossed to the AB strain. All detailed phenotypic analyses were carried out with the b719 allele.

Mapping and positional cloning

Initial mapping was performed with mozb719 on an outbred wik background. Fine mapping was performed with mozb719 crossed onto the Islet1-GFP background (Higashijima et al., 2000), which was found to be highly polymorphic relative to AB within the z6371-z7351 interval. In these fish, primers 1 and 2 (Table 1) were used to amplify the microsatellite z6371. Primers and enzymes were used to reveal co-dominant polymorphisms in the 5′ and 3′ UTRs, respectively, of f332e05 (3+4, MnlI) and fc15g12 (5+6, XmnI). All size polymorphisms were resolved on 1-4% agarose gels using standard techniques. The 3′ end of mk1671 was not present on PAC74G4. The SP6 end of PAC 14P16 begins with the ninth nucleotide of the fc15g12 ORF. PAC ends were sequenced and the following primers and enzymes used to reveal codominant polymorphisms: 4T (T7 end of PAC 4O19, 7+4, BclI); 14T (T7 end of PAC 14P16, 9+10, ScfI); 114T (T7 end of PAC 11E16, 11+12, DraI). Accession numbers are: AY600370 (moz cDNA) and CL525848-CL525855 (PAC end sequences).

Phenotypic analyses

In situ hybridization was performed using standard techniques with
Table 1. Primer and morpholino oligo (MO) sequences

<table>
<thead>
<tr>
<th>Primer/MO</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>1</td>
<td>GCCTGGCATTTAGAAGCG TTCC</td>
</tr>
<tr>
<td>2</td>
<td>GAGAGCCGACCTGCTAGGG</td>
</tr>
<tr>
<td>3</td>
<td>GCTACTCCTGGCCTCTAGAA</td>
</tr>
<tr>
<td>4</td>
<td>CTGCTGAGGGGAGCGAGTC</td>
</tr>
<tr>
<td>5</td>
<td>CATGATATATATTGTCTGAT</td>
</tr>
<tr>
<td>6</td>
<td>CGATGCTGTTTTAGTCTAGT</td>
</tr>
<tr>
<td>7</td>
<td>GAGTGCTGGTTCTGCAAG</td>
</tr>
<tr>
<td>8</td>
<td>ACCCTTTGAAGAGATTGTTTG</td>
</tr>
<tr>
<td>9</td>
<td>GGCTGCTGGCTGCTTCAAAG</td>
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<tr>
<td>10</td>
<td>AAAAAATCATAAGCTTCCAC</td>
</tr>
<tr>
<td>11</td>
<td>CCAATCTACATGCTTACC</td>
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<tr>
<td>12</td>
<td>ACTACCAAGTACGAAAAAAC</td>
</tr>
<tr>
<td>13</td>
<td>GGGAGAGGAGGAAAGAGGGTT</td>
</tr>
<tr>
<td>14</td>
<td>TGCAAAAGGAAAGGATGTCCTCAGACCT</td>
</tr>
</tbody>
</table>

See Materials and methods for description and number of primers. ATG translation start site is underlined for moz-MO1, both hox-MOs and bapx1-MO. For the splice-blocking moz-MOs (MO2 and 3), the intron complementary sequence is in lower case. Although the hoxa2b-MO sequence is identical to that reported in Hunter and Prince (Hunter and Prince, 1993), the hoxb2a-MO sequence we injected was three bases longer than the sequence they report.

Regulation of Hox activity

Development of wild-type embryos (L.M., unpublished). Embryos in their chorions were incubated in 0.1 M TSA, 0.003% DMSO beginning at 15 hours postfertilization and were maintained in this treatment until fixation for in situ hybridization or Alcian staining. Control sibling embryos were incubated in 0.003% DMSO.

BODIPY labeling

Vital imaging with the fluorescent dye BODIPY-ceramide was performed as described (Yan et al., 2002). Briefly, clutches were soaked in dye continually from late gastrulation onwards. Although animals continue to develop normally in this dye, it does slightly retard development, as does keeping the fish at room temperature, which we did while viewing repeatedly under a Zeiss LSM confocal microscope. Therefore stages given are the corresponding stages at 28°C, based on the head-trunk angle and other morphological criteria (Kimmel et al., 1995). A total of 31 fish were examined, eight mutants and 23 wild-type siblings. One side of the head was imaged from the outer surface to the midline with optical sections 3 μm apart.

Results

A new screen reveals a homeotic locus

Although large scale zebrafish screens revealed over 100 mutations affecting craniofacial development (Schilling et al., 1996a; Piotrowski et al., 1996; Neuhauss et al., 1996), no mutants with clear homeotic pharyngeal arch phenotypes were found. Such phenotypes were possibly to be expected based on homeotic mutant phenotypes in the mouse (Rijli et al., 1993; Gendron-Macguire et al., 1993; Selleri et al., 2001). We reasoned that homeotic phenotypes in fish might not result in a severe overall morphological phenotype and that directly screening pharyngeal cartilage shapes might reveal homeotic loci. We designed a screen in which F2 ENU-mutagenized gynogenetic clutches were grown up to 4 days, and surviving larvae were fixed, stained with Alcian Green to visualize cartilage, and then bleached to remove pigmentation. This enabled rapid scoring of head cartilage shapes under a dissecting microscope. This screen revealed two non-complementing alleles, b719 and b999, of a homeotic locus. We initially named the b719 locus binmandibular, for the apparent homeotic duplication of the first arch (see below).

Positional cloning reveals the homeotic locus to encode moz

We mapped b719 using bulk segregant analysis and subsequent fine mapping to an ~4 cM interval between z7351 and z6371 on LG5 (Fig. 1A, data not shown). Two zebrafish ESTs, fc15g12 and fc32e05, mapped to this region (http://wwwmap.tuebingen.mpg.de; http://134.174.23.167/zonrhmapper/Maps.htm) were found to closely flank the b719 locus, by 0.11 cM and 0.05 cM, respectively (Fig. 1A). Two PACs for each of these two ESTs were isolated by PCR from DNA pools of an arrayed zebrafish PAC library (Amemiya and Zon, 1999). Mapping polymorphisms derived from the PAC ends revealed that ends of two of the PACs had crossed the recombinants (Fig. 1A). Sequencing the T7 end of PAC4019 revealed an exon highly homologous to the human histone acetyltransferase MOZ, positioning moz as within the non-recombinant interval (Fig. 1A). By aligning vertebrate Moz sequences and using degenerate PCR, the rest of the predicted zebrafish moz ORF was isolated and is predicted to encode a 2246 amino acid protein. The first five exons were found to
reside on PAC 114E16, whereas exons 8-16 were contained on PAC4O19. Zebrafish Moz is highly conserved with human MOZ (Fig. 1B), a MYST family histone acetyltransferase (HAT, Fig. 1). In addition to activity of its HAT domain, human MOZ contains a transcriptional activation (TA) domain at its C terminus (Champagne et al., 2001; Kitabayashi et al., 2001a). A 1 bp deletion at position 2590 of 6738 in zebrafish moz b719 mutants results in an early frameshift, predicted to produce a truncated protein lacking this TA domain (Fig. 1A,B). A C-to-T nonsense mutation at position 4977 of 6738 in moz b999 mutants is predicted to also truncate the TA domain (Fig. 1A,B).

Expression of zebrafish moz at 28-48 hours postfertilization (hpf) appears ubiquitous in the head but expression is undetectable in the trunk and tail (Fig. 1C-E). The diffuse posterior boundary of moz expression during this time frame roughly coincides with the boundary between the hindbrain and spinal cord (Fig. 1C-E). Analyzing moz expression at 24, 28, 36 and 48 hpf in clutches of embryos from moz b719 heterozygotes yielded three clear classes of animals based on moz expression: strong, intermediate and faint. PCR genotyping revealed the strong class to be homozygous wild types, the intermediate class to be heterozygous for the moz b719 mutation, and the faint class to be moz b719 homozygous mutants. The reduction of moz expression in both classes of moz mutants appeared to globally affect moz expression levels in all cranial tissues (data not shown).

Fig. 1. Mutations in a zebrafish moz. (A) Positional cloning of the gene disrupted by the b719 mutation. The LG5 genomic region is schematized at the top, with informative genetic markers shown. 4T, 14T and 114T are polymorphisms in the T7 end of PACs 4O19, 14P16 and 114E16, respectively. Position of four PACs are shown below the genomic region, with the moz region expanded underneath. moz spans the non-recombinant interval, with exons on both non-recombinant PAC ends. Lesions are schematized in purple and shown in the chromatograms on the left side of this panel: b719 deletes one bp (cytosine) in exon 14 of moz. A C-to-T missense mutation in b999 introduces an early stop codon in the 16th exon. Positions of morpholino oligos (MOs) are shown in green (see Tables 1 and 2). (B) Schematic of protein domains of human, zebrafish wild type, b719 mutant and b999 mutant. Amino acid domains (Kitabayashi et al., 2001a): H, H15 nuclear localization; PHD, PHD fingers; basic; MYST HAT, MYST family histone acetyltransferase; acidic; serine rich; glutamate rich; methionine rich. The percent identity between human and wild-type zebrafish is listed for each region beneath human MOZ. The C-terminal transactivation domain of human MOZ (Champagne et al., 2001; Kitabayashi et al., 2001a) is labeled. The gray domain in b719 mutant is frame-shifted prior to translation stop. (C-E) Embryonic expression of moz in wild types at 28 hpf (C,D) and 48 hpf (E). Lateral views of head (C), head/trunk interface (D) and whole larva (E). At both stages, moz expression appears ubiquitous in cranial tissues, and has a diffuse posterior border of expression near the boundary (arrowhead in D,E) of the hindbrain and spinal cord. Expression at these stages is not detected in the trunk and tail. Scale bars: 50 μm.
Reducing moz function results in pharyngeal cartilage homeosis

In moz mutants, second arch cartilages adopt a mirror-image first arch pattern, forming an ectopic jaw (Fig. 2A,B). A novel large opening to the pharynx is present on either side of the head, resembling an ectopic mouth (Fig. 2B, but see below). Especially ventrally, this homeotic phenotype resembles the phenotype seen upon reducing function of both hoxa2b and hoxb2a (C. T. Miller, PhD Thesis, University of Oregon, 2001) (Hunter and Prince, 2002). Flat-mounting dissected cartilages from moz mutants reveals that the second arch cartilages adopt shapes characteristic of first arch cartilages (Fig. 2D,E). In the moz mutant second arch, the hyomandibular region of the dorsal second arch cartilage that normally articulates with the otic capsule is missing (Fig. 2D,E; Table 2), presenting a more complete homeotic transformation than observed in the earlier work (C. T. Miller, PhD Thesis, University of Oregon, 2001) (Hunter and Prince, 2002). The moz mutant dorsal second arch cartilage, in the more ventral position of the thin symplectic cartilage (Kimmel et al., 1998) is thicker than its wild-type counterpart, resembling the wild-type first arch dorsal (upper) jaw cartilage, the palatoquadrate. The moz mutant ventral second arch cartilage is shorter, thinner, contains fewer rows of chondrocytes, and forms a knob on its lateral end, resembling the wild-type first arch ventral (lower) jaw cartilage, Meckel’s (Fig. 2; and see below). Furthermore, in the first two arches of moz mutants, the dorsal cartilages fuse to one another and the ventral cartilages fuse to one another. By

Table 2. Morpholino oligo phenocopy of the moz mutant phenotype

<table>
<thead>
<tr>
<th></th>
<th>b719/b719</th>
<th>b999/b999</th>
<th>moz-MO1</th>
<th>moz-MO2</th>
<th>moz-MO3</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5 ng</td>
<td>15 ng</td>
<td>5 ng</td>
<td>15 ng</td>
<td>5 ng</td>
</tr>
<tr>
<td>n</td>
<td>86</td>
<td>23</td>
<td>41</td>
<td>89</td>
<td>161</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% with phenotype (n)</th>
<th>Moz-MO1</th>
<th>Moz-MO2</th>
<th>Moz-MO3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal deletion</td>
<td>81 (70.0)</td>
<td>22 (5.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Dorsal shape change</td>
<td>100 (86.0)</td>
<td>100 (23.0)</td>
<td>52 (21.5)</td>
</tr>
<tr>
<td>Ventral inversion</td>
<td>99 (85.5)</td>
<td>54 (12.5)</td>
<td>12 (5.0)</td>
</tr>
<tr>
<td>Ventral shape change</td>
<td>108 (60.0)</td>
<td>72 (16.5)</td>
<td>35 (14.5)</td>
</tr>
<tr>
<td>Ventral fusion</td>
<td>98 (84.5)</td>
<td>33 (7.5)</td>
<td>15 (6.0)</td>
</tr>
<tr>
<td>Dorsal fusion</td>
<td>99 (85.5)</td>
<td>44 (10.0)</td>
<td>18 (7.5)</td>
</tr>
</tbody>
</table>

Percentage of animals with each phenotype is listed. Of 75 uninjected wild-type control animals, none had any of these six phenotypes (data not shown). The position of each MO is shown in Fig. 1A. For each class, animals were scored on both right and left sides, with each side counting as one-half of an animal. ‘Dorsal deletion’ includes animals in which no Alcian-positive cartilage articulated with the neurocranium, which in all cases included deletion of the square dorsal half (HM) of HS. ‘Dorsal shape change’ class contains all phenotypes with mispatterned HSs, thus includes the ‘HM deletion’ class. Other mispatterned HSs include absent foramen, shortening of SY, or other shape changes of HM and/or SY, all of which make HS more simply triangular, resembling its first arch counterpart. ‘Ventral inversion’ contains animals in which CH, the ventral second arch cartilage, which normally forms a ~45° angle between the lateral side of CH and the midline, makes over a 90° angle between the posterior side of CH and the midline, and branchial cartilage was not deleted. ‘Ventral shape change’ contains animals in which the second arch ventral cartilage forms an enlarged process on its lateral end (see Fig. 2). In all cases, this second arch ventral cartilage was also smaller and thinner than the wild-type condition, but the presence of the distinctive knob was the criterion for inclusion in this class. ‘Ventral fusion’ and ‘dorsal fusion’ contain animals in which the first and second arch ventral and dorsal cartilages were visibly connected with Alcian-positive cartilage. All scoring was made on a high-power dissecting microscope in depigmented and cleared specimens. CH, ceratohyal; HM, hyomandibula; HS, hyosymplectic; SY, symplectic.
contrast, dorsal/ventral fusions within either arch are only rarely seen (see below).

Cartilages in more posterior pharyngeal arches of moz mutants are also mispatterned. The third arch ventral cartilage in moz mutants appears slightly shorter and thicker and also has a distinctive knob on its lateral end (Fig. 2G,H), resembling the retroarticual process of Meckel’s cartilage and thus suggesting a mild transformation of arch three to arch one fate. A similar phenotype is seen in the moz mutant fourth arch (data not shown).

To confirm that these homeotic phenotypes in moz mutants are due to reduction of Moz function, we injected moz morpholino antisense oligonucleotides (MOs). We have previously shown that MO injections can efficiently phenocopy severe phenotypes of larval head skeletal mutants, as well as reveal hypomorphic phenotypes at lower doses (Miller and Kimmel, 2001). Animals injected with any of three moz MOs display dose-dependent homeotic phenotypes seen in moz mutants (Fig. 2C,F,I; Table 2), strongly supporting our conclusion that reduction of moz function causes the b719 and b999 homeotic phenotypes.

Injection of lower doses of each morpholino, as well as analyses of the slightly variable b719 and hypomorphic b999 phenotypes (Table 2), show that the several homeotic phenotypes described above are separable, and that homeosis is not an all-or-nothing phenomenon. Some mutant animals display shape changes of the dorsal hyoid cartilage (the HM cartilage) without having deletions of HM (Table 2), showing moz controls at least two processes, positioning and shaping, of dorsal second arch cartilage formation. Likewise, some mutant animals display homeotic shape changes of the ventral hyoid cartilage without displaying the inversion (Table 2), similarly arguing that these two processes are separable. Interestingly, shape changes are more frequently seen than fusions, regardless of MO or dose (Table 2). The dorsal deletion of the hyomandibular portion of the hyosymplectic was the least penetrant for both mutant alleles and with both doses of all three MOs (Table 2). Thus, sensitivity to reduction of moz function ranges from high for shape changes to intermediate for fusions to low for dorsal deletions.

**moz is required for most Hox group 1-4 expression domains**

As anterior transformation of arch two to one resembles the mouse Hoxa2 and zebrafish hoxa2b/hoxb2a loss-of-function phenotypes (Gendron-Maguire et al., 1993; Rijli et al., 1993; Hunter and Prince, 2002) (C. T. Miller, PhD Thesis, University of Oregon, 2001), we asked if moz functions upstream of hox2 genes. Although zebrafish have at least seven Hox clusters, only two hox2 genes are retained, hoxa2b and hoxb2a (Amores et al., 1998). Expression of both hoxa2b and hoxb2a is broadly and severely downregulated in the moz mutant second arch primordia by 33 hpf (Fig. 3A-D, see below). Like the second pharyngeal arch expression and despite the separable regulation of pharyngeal arch and CNS Hox gene expression domains (Prince and Lumsden, 1994; Macdonachie et al., 1999), hindbrain expression of hoxa2b is also severely reduced in moz mutants at 33 hpf (Fig. 3E,F). All rhombomeres that express hoxa2b (rhombomeres 2-5 or r2-r5) appear to do so at a lower level in moz mutants, although r2 appears to be the most strongly affected. Expression of hoxa2b is strikingly reduced in medial r2 and lateral r4 (Fig. 3E,F). To determine if hoxa2b expression was simply delayed in moz mutants, we assayed a later time point, 48 hpf. Even with overdeveloped in situ hybridization and similar to expression at 33 hpf, arch expression of hoxa2b is undetectable (Fig. 3G,H), and hindbrain expression is still drastically reduced (data not shown).

To determine if moz is required for the initiation of expression of the Hox genes it regulates, we examined hoxa2b expression at 11 hpf, soon after it initiates embryonic expression (Prince et al., 1998). Initiation of hoxa2b expression in the hindbrain in moz mutants appears unaffected (Fig. 3J,L). However, initiation of hoxa2b in second arch CNC around 12-14 somites occurs but is substantially reduced in moz mutants (data not shown). Thus, moz regulates hoxa2b expression in distinct manners in the hindbrain and CNC.

We next asked if moz is required for expression of other Hox genes. hoxa1a expression is normally not present in a typical Hox domain spanning one or multiple segments but instead is in clusters of cells in the ventral forebrain and midbrain and in scattered cells in the anterior hindbrain (McClelntock et al., 2001; McClintock et al., 2003). In contrast to the moz requirement for later expression of hoxa2b and hoxb2a, hoxa1a expression is not appreciably affected in moz mutants (Fig. 3K,L).

For the hoxb3a cluster, expression of group 1-4 genes are affected in a graded fashion in moz mutants, with hoxb1a being the most severely affected and hoxb4a the most mildly affected. Severe Hox expression defects in moz mutants are also present in the embryonic hindbrain. At 36 hpf, the r4-restricted hindbrain expression of hoxb1a is nearly abolished in moz mutants (Fig. 3M,N). In addition to the missing second arch domain (see above), hindbrain expression of hoxb2a is reduced in moz mutants (Fig. 3O,P). hoxb3a expression is reduced in the hindbrain and in the third to fifth pharyngeal arch primordia in moz mutants (Fig. 3Q,R), perhaps contributing to the third arch homeotic phenotype presented above. Expression of hoxb4a is mildly reduced in both the hindbrain and pharyngeal arches four through six of moz mutants (Fig. 3S,T). Expression of the other hox3-4 genes is similarly affected as their respective hoxb3a and hoxb4a paralogs (data not shown). Initiation of Hox genes b1a-b4a, like hoxa2b, appears unaffected in the hindbrain of moz mutants. Hence, similar to trithorax group (trxG) genes (Simon and Tamkun, 2002; Ernst et al., 2002), moz appears to be required for the maintenance, but not initiation, of expression of particular Hox genes in the hindbrain.

However, hox5-6 gene expression appears unaffected in moz mutants (Fig. 3U,V; data not shown). Thus, moz expression, present throughout the embryonic head (see Fig. 1C-E), is specifically required for most hox1-4 expression domains in the hindbrain and pharyngeal arches.

**Inhibition of histone deacetylase activity partially rescues the moz mutant phenotype**

Because human MOZ has been shown to have histone deacetyltransferase (HAT) activity (Champagne et al., 2001), and because trxG factors that maintain Hox gene expression are associated with HAT activity (Petruk et al., 2001; Milne et al., 2002), we wondered whether the inability of moz mutants to maintain Hox gene expression was due to hypoacetylation. The histone deacetylase inhibitor trichostatin A (TSA) has been
shown to rescue defects caused by trxG mutations in *Drosophila* (Sollars et al., 2003) and human cells (Milne et al., 2002). We therefore asked whether TSA treatment could rescue the *moz* mutant phenotype. *moz* mutant embryos that are incubated in 0.1 M TSA starting at about 15 hpf show striking rescue of arch cartilage homeosis (Table 3; Fig. 4A-D) and rescue of Hox gene expression (Fig. 4E-H).

TSA-treatment partially rescues *hoxa2b* expression in the hindbrain and second arch CNC of *moz* mutants (*n*=12/12) compared with DMSO-treated *moz* mutant controls (*n*=6/6; Fig. 4E-H). TSA-treated *moz* mutants (*n*=5/5) have increased, but not wild-type levels, of r4 expression of *hoxb1a* compared with DMSO-treated *moz* mutant controls (*n*=8/8) (not shown). TSA-treated *moz* mutants have weakly rescued expression of *hoxb2a* in hyoid CNC (*n*=6/7) compared with DMSO-treated *moz* mutant controls (*n*=5/5, data not shown). Therefore, even though neither of our *moz* mutant alleles directly affect the Moz HA T domain, these results suggest that the *moz* homeotic phenotype is at least partially dependent on the function of Moz HA T activity.

**moz** mutants display late hindbrain neuronal phenotypes

Given the broad expression defects of group 1-4 Hox genes, we analyzed hindbrain neuronal development in *moz* mutants. Facial motoneurons differentiate in r4 and begin to migrate posteriorly towards r5-6 around 15 hpf (Chandrasekhar et al., 1997; Maves et al., 2002). At 48 hpf, some mutants display
mispositioned facial motoneurons (Fig. 5A,B), resembling hoxb1 loss-of-function phenotypes seen in zebrafish and mice (McClintock et al., 2002; Studer et al., 1996; Goddard et al., 1996; Gavalas et al., 2003). In contrast to Mll/Trithorax mutant mice, which also fail to maintain Hox expression (Yu et al., 1998), cranial ganglia appear to innervate each pharyngeal arch (Fig. 5C,D). The early reticulospinal neurons, some of which are born by 10 hpf (Mendelson, 1986) and show anterior transformations upon reduced function of hoxb1 (McClintock et al., 2002), display no detectable alterations in moz mutants (data not shown). Thus, early segmentation and neuronal specification of the moz mutant hindbrain occurs relatively normally, while later hindbrain phenotypes in moz mutants are consistent with a defect in maintenance, but not initiation, of Hox gene expression.

Late patterning defects in moz mutant arch mesoderm
Segmental identity in the vertebrate head periphery involves complex crosstalk between CNC and head mesoderm. In the mouse, Hox gene expression in CNC requires unknown factors emanating from cranial mesoderm (Trainor and Krumlauf, 2000). Conversely, in the chick, transplanting (presumably Hoxa2-negative) first arch CNC into the second arch results in second arch muscles non-autonomously adopting a first arch pattern of beak muscles (Noden, 1983a; Trainor et al., 2002). In zebrafish, mosaic analyses have revealed that CNC patterns arch mesoderm (Schilling et al., 1996b; Knight et al., 2003). Additionally, zebrafish injected with hox2-MOs and Hoxa2 mutant mice display altered head musculature (Rijli et al., 1993; Barrow and Capecchi, 1999; Hunter and Prince, 2002). Thus, we expected head musculature to be affected in moz mutants but wondered at what stage segmental identity defects in the head mesoderm occur.

Pharyngeal arch muscles are derived from paraxial mesoderm, which initially occupies central locations (arch ‘cores’) in the pharyngeal arch, ensheathed by postmigratory CNC (reviewed by Kimmel et al., 2001b). Each arch mesodermal core subdivides into a discrete pattern of identified myogenic cores. The first and second arches display different sequences of mesodermal core subdivision. Although at intermediate stages in fish the first arch contains three myogenic cores [constrictor dorsalis (CD), adductor mandibulae (AM) and intermandibularis (IM)], the second arch contains only two [constrictor hyoideus dorsalis and ventralis (CHD and CHV)]. These myogenic cores subsequently subdivide into primordia for individual muscles (Edgeworth, 1935) (reviewed by Kimmel et al., 2001b). This early difference in the wild-type arch one and two intermediate

Table 3. Histone deacetylase inhibitor trichostatin A (TSA) rescues moz mutant homeosis and hox gene expression defects

<table>
<thead>
<tr>
<th>Genotype and treatment</th>
<th>b719+/b719+ +DMSO</th>
<th>b719−/b719− +DMSO</th>
<th>b719+/b719+ +TSA</th>
<th>b719−/b719− +TSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>26</td>
<td>24</td>
<td>39</td>
</tr>
<tr>
<td>% with phenotype (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal deletion</td>
<td>0 (0.0)</td>
<td>69 (18.0)</td>
<td>0 (0.0)</td>
<td>22 (8.5)</td>
</tr>
<tr>
<td>Dorsal shape change</td>
<td>0 (0.0)</td>
<td>100 (26.0)</td>
<td>46 (11.0)</td>
<td>76 (29.5)</td>
</tr>
<tr>
<td>Ventral inversion</td>
<td>0 (0.0)</td>
<td>95 (25.0)</td>
<td>0 (0.0)</td>
<td>8 (3.0)</td>
</tr>
<tr>
<td>Ventral shape change</td>
<td>0 (0.0)</td>
<td>52 (13.5)</td>
<td>0 (0.0)</td>
<td>6 (2.5)</td>
</tr>
<tr>
<td>Ventral fusion</td>
<td>0 (0.0)</td>
<td>60 (15.5)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Dorsal fusion</td>
<td>0 (0.0)</td>
<td>39 (10.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

All animals were PCR-genotyped. See Table 2 legend for scoring method and explanation of phenotypes and abbreviations. The HS disorganization in TSA-treated embryos, while disorganized relative to the untreated wild-type pattern, lacked shape changes characteristic of moz mutants.
myogenic core pattern precludes assigning segmental homology to subsequently-forming dorsal and intermediate muscles. However, this intermediate pattern serves as a segmental character distinguishing the first two arches. Thus, we wondered whether this aspect of segmental identity was transformed in moz mutants.

Eng2 expression marks the dorsal first arch myogenic condensation, constrictor dorsalis (Hatta et al., 1990; Ekker et al., 1992) (reviewed by Kimmel et al., 2001b). Expression of eng2 in moz mutants at 28 hpf is not seen homeotically duplicated in the second arch (Fig. 6A,B). myod expression marks all pharyngeal arch myogenic condensations (Schilling and Kimmel, 1997). We examined myod expression in moz mutants at 44 hpf, a stage soon after myod expression labels the first and second arch myogenic condensations (Fig. 6C,D). The arrangement of myod-expressing cores in moz mutants at this stage appears grossly indistinguishable from the wild-type pattern (Fig. 6C,D). Slightly later in development at 54 hpf, subtle defects are observed in moz mutant myod-expressing myogenic condensations. Ectopic patches of myod expression are seen in the intermediate second arch of moz mutants (Fig. 6E,F).

Despite the lack of dramatic early muscle phenotypes, the larval musculature at 4 days is radically transformed in moz mutants (Fig. 6G,H). In wild types, a large jaw-closing adductor mandibulae muscle connects the dorsal and ventral cartilages in the first arch, whereas no prominent muscles connect the dorsal and ventral cartilages in the second arch (Fig. 6G). In moz mutants, the first arch muscle pattern appears unaffected, except for late subdivision and patterning of CD, which normally inserts upon the HM cartilage. In most (61%, 14/23) moz mutants, a muscle of variable size in the second arch connects the dorsal and ventral cartilages (Fig. 6H). These late muscle phenotypes in moz mutants resemble a slightly stronger version of those reported for hox2-MO injected zebrafish (Hunter and Prince, 2002). In moz mutants in which a muscle did not connect the dorsal and ventral hyoid cartilages, hyoid musculature was variably disorganized. Without specific markers for individual hyoid muscles, we were unable to assign identity to these. The third arch ventral muscle (transversus ventralis) (Schilling and Kimmel, 1997) was slightly enlarged
in \textit{moz} mutants, suggesting, like the cartilage phenotype, a mild anterior homeotic transformation. Together these results indicate that \textit{moz} mutants display late, but not early, anterior homeotic transformations of second and third arch musculature.

**Defects in pharyngeal epithelia are not detected in \textit{moz} mutants**

Pharyngeal endoderm is required for many aspects of CNC patterning (Piotrowski and Nüsslein-Volhard, 2000; Piotrowski et al., 2003; Couly et al., 2002) and chondrification of CNC requires contact with pharyngeal endoderm (Epperlein, 1974). Given that the hyomandibular (HM) region of the dorsal second arch cartilage almost never chondrifies in \textit{moz} mutants (Table 2), we wondered whether missing, mispositioned or mis-specified pharyngeal pouches contribute to the \textit{moz} mutant phenotype.

We directly assayed developing pharyngeal pouch morphology and specification by following the expression of the FGF target gene \textit{pea3} (Roehl and Nüsslein-Volhard, 2001) at 24, 34 and 54 hpf. Each pharyngeal pouch, which separates the arch primordia, consists of an epithelial bilayer with an AP polarity: expression of \textit{pea3} and the secreted ligand \textit{edn1} are both expressed in posterior, but not anterior, pharyngeal endodermal epithelia (Fig. 7A,B) (Miller et al., 2000). The first pharyngeal pouch, which abuts dorsal second arch CNC, is present in \textit{moz} mutants and appears similarly patterned as its wild-type counterpart at all three stages examined (Fig. 7A,B; data not shown). The same was true for more posterior pharyngeal pouches (Fig. 7A,B; data not shown). We wondered if the mirror-image duplication of the first arch pattern in the \textit{moz} mutant second arch could be due to defects or possibly even reversals in pharyngeal pouch polarity. However, expression of \textit{pea3} (Fig. 7A,B) and \textit{edn1} (data not shown) in \textit{moz} mutants revealed no defects in pouch polarity.

Surrounding ectodermal epithelia also contribute to CNC patterning (Tyler and Hall, 1977). \textit{shh} is specifically expressed in a thin stripe of second arch surface ectoderm at the posterior margin of the second arch resembling an identified \textit{shh} expression domain in chicks and mice (posterior ectodermal margin of the second arch resembling an identified...). Given the second arch specificity of this pattern and the defect in second arch identity in \textit{moz} mutants, we wondered whether \textit{moz} mutants might lack this expression domain of \textit{shh}. Instead, expression of \textit{shh} in \textit{moz} mutants is still present in the PEM of the second arch, although expression is perhaps slightly reduced ventrally (Fig. 7C,D). Thus, this epithelial aspect of hyoid segmental identity is retained in \textit{moz} mutants.

Between the inverted ectopic jaw and the enlarged third arch in \textit{moz} mutants, large bilateral openings in the pharynx resemble ectopic mouths (see Fig. 2B). To determine if gene expression data support this interpretation, we examined expression of a stomadeal marker, \textit{pitx2c} (Essner et al., 2000; Schweickert et al., 2001), in \textit{moz} mutants. \textit{pitx2c} expression in wild-type embryos at 54 hpf strongly labels the mouth, presumably the ectodermal derivatives of the stomodeum (Fig. 7E,F). Expression of \textit{pitx2c} in \textit{moz} mutants was not detected ectopically in these enlarged pharyngeal openings, providing no evidence for stomadeal identity.

In chicks, the thymus forms largely from the third and fourth endodermal pharyngeal pouches, which attract blood-borne lymphocyte precursors (LeDouarin and Jotereau, 1975). In mice, \textit{hox3} genes regulate thymus formation (Manley and Capecchi, 1995; Manley and Capecchi, 1998). In zebrafish, \textit{ragl} expression in lymphocytes marks the early thymus (Willett et al., 1997). In \textit{phox4(lzr)} mutants, which have reduced \textit{hox3} expression, the thymus fails to form as assayed by \textit{ragl} expression (Popperl et al., 2000). Thus, we similarly asked whether \textit{moz} mutants form a thymus by examining \textit{ragl} expression at 4 days. \textit{ragl} expression in the thymus is present in \textit{moz} mutants, although reduced (Fig. 7G,H). Taken together, these results reveal relatively minor defects in arch epithelial tissues in \textit{moz} mutants and are consistent with the idea that many aspects of the arch environment are set up independent of the CNC (Veitch et al., 1999; Gavalas et al., 2001).

**Early CNC generation appears normal in \textit{moz} mutants but mispositioned and misshapen condensations form**

Finding evidence suggesting that early patterning of non-CNC
arch tissues is normal in moz mutants, we next analyzed the CNC. In mice, Hox genes not only control segmental identity, but also control the generation of CNC (Gavalas et al., 2001). To determine whether the broad Hox expression defects result in a defect in CNC generation, we examined early pharyngeal arch primordia in living embryos with the fluorescent dye BODIPY ceramide. This vital labeling offers nice histological resolution of all major differentiated cell types (Kimmel et al., 2001b; Yan et al., 2002). Examining arch primordia in labeled sections confirm our in situ results that no gross changes are revealed in wild-type mutants (data not shown). In moz mutants, no dorsal second arch hyomandibular condensation is seen and cells appear as lose mesenchyme in this region (arrow in F). A dorsal mutant condensation does form, in the position of the wild-type symplectic condensation. The ventral mutant condensation is mispositioned anteriorly (arrowhead), and is abutting the first arch ventral condensation. The condensation pattern (E,F) largely prefigures the resultant larval cartilage pattern (G,H; compare with Fig. 2D,E). This figure shows two time points of four different animals: A and C are the same animal, as are B and D, E and G, and F and H. Arches are numbered in A-D.

Ref. 8. Hyoid CNC is generated in moz mutants but forms mispositioned and misshapen condensations. Confocal micrographs of lateral views of the anterior pharyngeal arches in live wild-type (A,C,E,G) and moz mutant (B,D,F,H) embryos (A-F) and larvae (G-H) stained with the vital fluorescent dye BODIPY-ceramide at 28 hpf, 34 hpf, 48 hpf and 3.5 day stages. Images have been inverted; the dye fills interstitial spaces, so the inverted images show cells labeled in white and interstitial space in black. (A,B) Early CNC appears morphologically indistinguishable from wild type in moz mutants. Both first and second arches are filled with a cylinder of postmigratory CNC (Miller et al., 2000; Kimmel et al., 2001b), with no obvious defect in hyoid or branchial CNC in mutants. Other arch tissues also fail to show obvious morphological defects (C,D). At a slightly later stage the second pharyngeal arch in moz mutants appears slightly hypoplastic (D), but is still filled with CNC. (E,F) A day later, condensations have formed in the wild type (E), including in the second arch a dorsal hyomandibular condensation (arrow, compare with Fig. 2D) and a ventral ceratohyal condensation (arrowhead). In moz mutants, no dorsal second arch hyomandibular condensation is seen and cells appear as lose mesenchyme in this region (arrow in F). A dorsal mutant condensation does form, in the position of the wild-type symplectic condensation. The ventral mutant condensation is mispositioned anteriorly (arrowhead), and is abutting the first arch ventral condensation. The condensation pattern (E,F) largely prefigures the resultant larval cartilage pattern (G,H; compare with Fig. 2D,E). This figure shows two time points of four different animals: A and C are the same animal, as are B and D, E and G, and F and H. Arches are numbered in A-D.

 moz and hox2 genes repress early second arch expression of bapx1, which is required for aspects of moz-mediated homeosis

Reduced hox2 expression can at least in part account for the anterior transformation homeotic skeletal and muscular phenotypes observed in the second arch of moz mutants. To investigate the molecular consequences of hox2 downregulation in the early second arch primordium, we analyzed embryonic expression of a known hox2 target gene, bapx1 (Pasqualetti et al., 2000), in moz mutants and in embryos injected with morpholinos to reduce function of hoxa2b and hoxb2a.

In embryos and larvae, bapx1 is expressed in a patch of intermediate first arch, but not second arch, mesenchyme (Fig. 9A,D) (Miller et al., 2003). In moz mutants at 33 hpf, first arch bapx1 expression is present while an ectopic bapx1 domain is seen in the second arch (Fig. 9B,E), providing molecular confirmation of an anterior transformation in second arch CNC of moz mutants. Ectopic second arch bapx1 expression is also observed in embryos injected with hoxa2b and hoxb2a morpholinos (95%, 37/39; Fig. 9C), demonstrating that hox2...
The first two pharyngeal arches are numbered. e, eye. Scale bars: and second arch joints (asterisks in K). Reduction of mutants also can rescue the ventral arch one and two fusions.

Ectopic dysfunction is sufficient to result in these homeotic molecular changes. Ectopic bapx1 expression is present in moz mutants and hox2-MO injected animals when bapx1 expression first initiates arch expression, around 30 hpf (data not shown). This molecular homeosis is stable, as bapx1 expression is maintained in the second arch at 54 hpf (Fig. 9F,G) and 4 days (Fig. 9H,I).

We next asked if this ectopic bapx1 expression domain is functional in moz mutants. Reducing bapx1 function in a wild-type background specifically eliminates the jaw joint (asterisk in J), while reducing function of moz mutants causes specific loss of the jaw joint (asterisk in K). Reducing bapx1 function in moz mutant second arch mesenchyme of bapx1-MO co-injected animals (C). (F-I) Ectopic bapx1 expression (arrowheads) is maintained in second arch mesenchyme of moz mutants. (J,K) Reduction of bapx1 function in wild type (J) and moz mutant (K). Reducing bapx1 function in wild type causes specific loss of the jaw joint (asterisk in J), while reducing function of bapx1 in moz mutants causes loss of both first and second arch joints (asterisks in K). Reduction of bapx1 function in moz mutants also can rescue the ventral arch one and two fusions.

The first two pharyngeal arches are numbered. e, eye. Scale bars: 50 μm.

results in loss of joints in both arch one and two (Fig. 9K; Table 4). Thus, the moz mutant second arch requires bapx1 function for formation of the dorsal/ventral joint, providing functional genetic evidence that the moz mutant second arch phenotype is homeotic.

moz and hox2 genes regulate the mediolateral pattern of goosecoid expression in the second arch

We extended our analyses of early CNC patterning in moz mutants by studying expression of a second known hox2 target gene, goosecoid (gsc) (Grammatopoulos et al., 2000; Pasqualetti et al., 2000; Hunter and Prince, 2002). Misexpression of hox2 in the chick and Xenopus induces gsc expression (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). In zebrafish, hoxa2b and hoxb2a were reported to positively regulate early second arch gsc expression, although only lateral views were reported (Hunter and Prince, 2002).

Viewing early embryonic gsc expression from a ventral aspect reveals gsc expression to consist of a thin medial crescent in the ventral first arch and a broad lateral crescent in the ventral second arch (Fig. 10A). Examination of gsc expression in moz mutants from this ventral aspect reveals a startling patterning change. In moz mutants, ventral second arch expression of gsc appears as a thin medial crescent, mirroring the first arch pattern (Fig. 10B). Like the bapx1 expression change, this shifting of gsc expression is also observed in embryos injected with hoxa2b and hoxb2a morpholinos (92%, 23/25; Fig. 10C), demonstrating that hox2 dysfunction is also sufficient to result in this homeotic molecular change of gsc expression. At 41 hpf, dorsal arch two expression of gsc is reduced (Fig. 10D,F). However, lateral views do not reveal maintenance of the patterning change that ventral views do: the shifting of lateral gsc expression to the medial second arch (Fig. 10E,G). The pattern at 41 hpf is slightly different than the 33 hpf pattern (Fig. 10H), suggesting gsc expression is either dynamic, and/or that movements of gsc-expressing cells occur. Together our results suggest moz not only controls maintenance of an early pattern, but also specification of subsequent dynamic changes in patterning in the second arch CNC well before differentiation begins.

Discussion

An essential embryonic role for the oncogene moz

In humans, translocation breakpoints within MOZ result in leukemia (Borrow et al., 1996; Aguiar et al., 1997; Carapeti et
The anterior medial arch. Relevant pharyngeal arches are numbered.

Fig. 10. Mirror-image duplication of gsc expression in the moz

Mutations and morpholino phenotypes are all hypomorphic until deletion alleles are found. Although we have no evidence of a fish-specific duplication of moz, vertebrates do have a closely related gene, Morf (monocytic leukemia zinc finger protein related factor; Myst4 – Mouse Genome Informatics), which is also mutated in human leukemias (Champagne et al., 1999; Panagopoulos et al., 2001). An embryonic function has been reported for Morf (named Querkopf) in mice (Thomas et al., 2000). An insertion in the 5′UTR of mouse MORF causes skull and forebrain defects, but hindbrain or homeotic pharyngeal arch defects were not reported (Thomas et al., 2000). Whether MORF and MOZ have overlapping functions is unknown.

Biochemical analyses of human MOZ have also revealed multiple MOZ-interacting partners. MOZ physically interacts with RUNX1 (AML1) and RUNX2 (AML3 or CBFA1) (Kitabayashi et al., 2001b; Pelletier et al., 2002). The RUNX2 interaction is particularly interesting, as this is the osteogenic gene shown in mice to be repressed by Hoxa2 (Kanzler et al., 1998). Thus, in our analyses of segmental identity in the pharyngeal arches of moz mutants, we examined pharyngeal bones expecting to see ectopic bone formation. However, the hyoid bone pattern (Kimmel et al., 2003) was undetectable and duplicated mandibular bones were not seen in moz mutants (C.M., unpublished). Although we did not examine bone patterning in hox2-MO injected fish, one possibility is that MOZ functionally interacts with RUNX2 during pharyngeal development in zebrafish.

Whether mammalian MOZ regulates Hox gene expression, as we predict, awaits generation of moz mutant mice. The regulation of Hoxa2 by the transcription factor AP2 is conserved from mammals to fish (Maconochie et al., 1999; Knight et al., 2003). However, no reduction in AP2 expression was observed in moz mutants at 28 hpf (C.M., unpublished results). Thus, the
regulation of hox2 genes by moz appears to act through another mechanism, possibly by directly transactivating Hox genes.

**moz regulates segmental identity of pharyngeal cartilages**

Our results extend the understanding that Hox genes specify segmental identity in the vertebrate pharynx, as the transformed pharyngeal segments in moz mutants correlate with reduced Hox expression in pharyngeal arch primordia. A mirror-image duplicated jaw replaces the hyoid cartilages in moz mutants, resembling the phenotype seen in animals injected with hoxa2b and hoxb2a morpholinos (C. T. Miller, PhD Thesis, University of Oregon, 2001) (Hunter and Prince, 2002). However, the moz mutant transformation is more complete, as dorsal transformations are more severe and dorsal fusions more common than in hox2-MO injected animals. It is likely that morpholinos cause incomplete loss-of-function at later developmental timepoints when the injected morpholino is significantly diluted. Alternatively, moz might regulate other genes that are also expressed in hyoid CNC and contribute to segmental identity.

Despite this stronger phenotype, the homeotic transformation in the hyoid arch of moz mutants is still not complete in that the pterygoid process of the palatoquadrate (PTP) is not seen duplicated. Perhaps in moz mutants, as has been proposed for mouse Hoxa2 mutants, only certain axial levels of CNC are transformed, i.e. perhaps PTP is derived from midbrain crest whose derivatives are not seen duplicated in the second arch of Hoxa2 mutants (Köntges and Lumsden, 1996). de Beer (de Beer, 1937) proposed that PTP was a premandibular element.

The pharyngeal arches are more sensitive to partial reduction of Hoxa2 function in the mouse (Ohnemus et al., 2001). The partial transformations observed in these hypomorphomic mouse mutants led these authors to propose that homeosis was not an ‘all-or-nothing’ phenomenon, as the second arch did not act as a developmental unit as a whole. Our results, in which a hypomorphic allele and low-level injections of Moz-MOs also separate particular homeotic phenotypes from others, strongly support this conclusion.

Experiments in Xenopus with an inducible Hoxa2 construct revealed that the time of Hoxa2 overexpression affected the resultant phenotype: overexpressing Hoxa2 early during CNC migration resulted in ‘segmentation’ phenotypes, where arch derivatives were fused, while overexpressing late in postmigratory CNC resulted in ‘homeotic’ phenotypes, where cartilage shapes were altered (Pasqualetti et al., 2000). As interarch fusions could also be interpreted as homeosis (i.e. loss of individual arch identity), this distinction is debatable. However, we note that the moz mutant phenotype contains more frequent shape changes (‘homeotic’) than fusions (‘segmentation’), both dorsally and ventrally for both mutant alleles and for all three morpholinos at two different doses each. pbx4(lzr) mutants might display the converse phenotype, i.e. segmentation appears more affected than homeosis. Interarch fusions seen in moz mutants resemble the pbx4(lzr) mutant phenotype, although fusions are more severe in pbx4(lzr) mutants (Pöpperl et al., 2000). The more severe phenotype of pbx4(lzr) mutants might reflect differences in the set of affected target genes and/or temporal differences of target gene regulation (e.g. initiation versus maintenance).

**moz mutants also present with mild anterior homeotic transformations of pharyngeal arches three and four (branchial or gill-bearing arches one and two).** Arch three and four cartilages in moz mutants are slightly thicker, and typically contain an enlarged process on their lateral end, resembling the retroarticular process of Meckel’s (the lower jaw) cartilage. Especially in the third arch of moz mutants at 5 days, an ectopic dorsal cartilage is also frequently seen. These transformations in the moz mutant anterior branchial arches are not seen in hox2-MO injected animals (Hunter and Prince, 2002). These phenotypes probably result from additional Hox genes (e.g. Hox3 and Hox4 genes) that moz regulates (see above). Once genetic alleles of these zebrafish Hox genes are isolated, their function in specifying pharyngeal segmental identity can be assessed.

This third arch cartilage phenotype in moz mutants somewhat resembles the phenotype of valentino (val) mutants (Moens et al., 1998) (reviewed by Kimmel et al., 2001), which was interpreted to be an ectopic interhyal cartilage based on ectopic hoxb2a expression in the third arch of val mutants. As neither hoxa2 nor hoxb2 is expressed in the moz mutant third arch, we propose that the moz mutant third arch has adopted mandibular fate. We did not detect ectopic bapx1 expression in third or fourth arch CNC of moz mutants. However, given the subtle nature of the skeletal change, the causative gene expression changes would probably be subtle as well.

**Moz is required for Hox maintenance and behaves like a trithorax group factor**

Consistent with the homeotic pharyngeal arch phenotype, we observe defects in hoxl-4 gene expression in moz mutants. Three pieces of evidence suggest that Moz functions similarly to trithorax (trxG) factors in regulating maintenance of Hox gene expression, as we discuss below.

First, we do not detect changes in initiation of Hox gene expression in the hindbrain of moz mutants. By later stages, graded reduction of most hoxl-4 expression domains in the CNS domains is apparent. Defects in Hox gene maintenance, but not initiation, are hallmarks of trx mutants in Drosophila (Breen and Harte, 1993) and mouse (Yu et al., 1998). Loss of Hox group 1 or 2 gene function in zebrafish or in mice can cause severe homeotic neuronal transformations in the hindbrain and motor axon pathfinding defects (McClintock et al., 2002; Cooper et al., 2003; Studer et al., 1996; Gavalas et al., 1997; Gavalas et al., 1998; Gavalas et al., 2003; Rossel and Capecci, 1999; Gendron-Maguire et al., 1993; Rijli et al., 1993). Supporting a role for moz in maintenance of Hox expression in the hindbrain, we find that early neuronal specification in the hindbrain and axonal trajectories in the head periphery are approximately normal in moz mutants. The only consistent neuronal defect we are able to detect in moz mutants is the disruption of facial motor neuron migration. This phenotype may be consistent with the defect in maintenance of hoxb1a expression in moz mutants, as loss of hoxb1a in zebrafish or Hoxb1 in mice causes a similar defect (McClintock et al., 2002; Studer et al., 1996). In the mouse Mll (trx) mutant, cranial ganglia are condensed and fail to innervate the pharyngeal arches (Yu et al., 1998), but more specific neuronal defects have not been reported and MLL mutant zebrafish have not been described.

Second, Moz has a HAT domain, which for human MOZ has
been demonstrated to have HAT activity (Champagne et al., 2001), and HAT activity has been associated with trxG factors (Petruk et al., 2001; Milne et al., 2002). Furthermore, HAT activity is required for Moz function, as treatment with a histone deacetylase inhibitor rescues many aspects of the moz mutant phenotype.

Third, we find that moz mutant homeosis and Hox maintenance defects are rescued by TSA, and TSA has been shown to rescue defects caused by trxG mutations in Drosophila (Sollars et al., 2003) and human cells (Milne et al., 2002). Why does inhibition of histone deacetylase activity rescue a putative decrease of histone acetylation? It seems likely that the transcriptional on or off state of Hox genes is maintained through a balance of chromatin modification activities, including histone acetylation by trxG factors and histone deacetylation by Polycomb group (PcG) factors (Milne et al., 2002) (reviewed by Francis and Kingston, 2001; Simon and Tamkun, 2002). In support of this idea, trxG and PcG factors are antagonistic for proper Hox expression. For example, homeotic axial transformations and Hox expression defects of Mll-deficient mice and Bml-deficient mice are rescued when function of both genes is removed (Hanson et al., 1999; Yu et al., 1995; Yu et al., 1998). We do not yet know whether Moz directly acetylates histones associated with Hox regulatory regions. Western analyses show no detectable decrease of acetylated histone H4 levels in moz mutants compared with wild-type siblings (L.M., unpublished). We might expect to see rhombomere- or arch-specific defects in acetylated histone H4 levels at specific Hox genes, but at the present time this is very difficult to test.

Taken together, these findings implicate Moz as a trxG factor. trxG genes have been genetically defined as suppressors of PcG mutant phenotypes (reviewed by Kennison, 1995). Further studies demonstrating genetic interactions between moz and PcG genes would provide firm support for moz as a trxG gene.

One interesting aspect of Hox regulation that has emerged from our analysis of moz mutants is that in general, there appears to be a gradient effect of Moz activity within a Hox complex. We find that the hox1-4 requirement for Moz activity ranges from strong for group 1 to weak for group 4, while group 5 and 6 Hox genes show no Moz requirement. We see this gradient effect on similar paralogs, where they have segmental domains in hindbrain and CNC, but we do not see a moz requirement for hox10a expression, even though moz appears to be expressed ubiquitously throughout the head. Our findings suggest that Moz activity plays a global role in Hox locus regulation, possibly through HAT activity. MYST family HAT activity has been shown to have a chromosomal gradient of transcription control in yeast (Kimura et al., 2002). Whether a Moz-mediated gradient of histone acetylation exists across group 1-5 genes in Hox clusters remains to be determined.

moz is required for late but not early patterning of head musculature

Our data suggest that at times when severe hox2, bapx1 and gsc expression defects are present in postmigratory hyoid CNC of moz mutants, head mesodermal patterning appears unaffected. eng2, with eng1 the only segmentally restricted head mesodermal marker that we know of (Ekker et al., 1992; Hatta et al., 1990), appears appropriately confined to the first arch dorsal muscle core (constrictor dorsalis) of moz mutants. Likewise, the early myod expression pattern, which labels all proposed arch myogenic cores (see Kimmel et al., 2001b), appears normal in moz mutants. The absence of eng2 duplication or myod pattern disruption could be due to residual hox2 activity in moz mutants. Alternatively, moz and hox2 genes could play no role in restricting eng expression to the first arch or setting up the pattern of myod-expressing myogenic cores. The homeotic late muscle pattern seen in moz mutants perhaps results from transformed CNC-derived connective tissue, which has been shown to pattern paraxial-mesodermally derived and somitic-derived myocytes (Noden, 1983a; Noden, 1983b; Noden, 1986).

Dramatic changes in CNC expression of bapx1 and gsc prefigure the moz mutant phenotype

In stark contrast to the apparently normal early patterning of moz mutant mesoderm, endoderm and surface ectoderm, expression of two known hox2 target genes, bapx1 and goosecoid (gsc), is radically perturbed in postmigratory CNC of moz mutant second arch primordia.

Within postmigratory CNC, bapx1 expression is confined to a patch of intermediate first arch mesenchyme which appears to prefigure the jaw joint (Miller et al., 2003). We previously identified edn1 and hand2 (dHAND) as positive and negative regulators, respectively, of bapx1 expression. bapx1 expression spreads ventrally in hand2 mutants (Miller et al., 2003). We report that moz and hox2 genes also contribute to positioning bapx1 to the jaw joint, although these genes prevent bapx1 from being expressed in an intermediate domain of the hyoid arch. Thus, bapx1 integrates positional information from both the DV (edn1, hand2) and AP axes (moz, hox2) to achieve its jaw-joint-restricted expression. Furthermore, as an aspect of the moz mutant homeotic pattern (the jaw joint) requires bapx1, these results identify bapx1 as a crucial downstream effector contributing to the homeotic transformation.

Microarray comparisons of gene expression in the second arches of wild-type and Hoxa2 mutant mice revealed Ptx1 to be upregulated in the Hoxa2 mutant second pharyngeal arch primordial (Bobola et al., 2003), similar to what we report here for bapx1. These authors report finding no confirmed gene that is downregulated in Hoxa2 mutant second arches, and suggest that Hoxa2-mediated segmental identity in the second arch might largely involve repression of the first arch program. Although microarray analyses promise to provide a global view of overall changes in gene expression in Hoxa2 mutant arches, our demonstration of spatially shifted gsc expression highlights the need to also analyze potential spatial reorganization of affected genes.

In the mouse, gsc expression is spatially restricted within first and second arch CNC (Gaunt et al., 1993). Although gsc is required for specific aspects of mouse craniofacial development, defects in first, but not second, arch derivatives were reported (Rivera-Perez et al., 1995; Yamada et al., 1995). In both fruitflies and vertebrates, gsc functions as a transcriptional repressor (Danilov et al., 1998; Ferreiro et al., 1998; Mailhos et al., 1998; Latinkic and Smith, 1999; Yao and Kessler, 2001), although precedent exists for gsc positively regulating target genes (frzb) (Yasuuo and Lemaire, 2001). The identity of gsc target genes and the nature of their regulation in the pharyngeal arches remains to be determined.
gsc are expressed in strikingly complementary patterns in the first two arches (compare Fig. 9A-C with Fig. 10A-C), suggesting one might repress expression of the other. Our previous report that in hand2 mutant zebrafish ventral first arch expression of gsc is lost, while bapx1 expression expands ectopically into this domain (Miller et al., 2003), is consistent with gsc repressing bapx1 arch expression.

The inverted gsc expression domain in the early second arch primordia of moz mutants suggests reorganization of the fate map at an early prechondrogenic stage has occurred. This model is consistent with our finding that moz mutant prechondrogenic condensations are mispositioned and the primordia of mutants suggests reorganization of the fate domain partially phenocopied the Sox9 Hoxa2 mutant (Kanzler et al., 1998). This latter study additionally showed the finding that in the mouse Hoxa2 mutant, chondrogenesis is induced in different regions of the arch than in wild types (Kanzler et al., 1998). This latter study additionally showed that transgenically driving Sox9 expression in the Hoxa2 domain partially phenocopied the Hoxa2 mutant phenotype. Furthermore, transgenically driving Hoxa2 with an Msx2 promoter resulted in loss of cranial bones, suggesting Hoxa2 represses both cartilage and bone formation (Kanzler et al., 1998). Our bapx1 and gsc expression data suggests that moz and hox2 affect patterning within the second arch primordia long before cartilage or bone differentiation occurs.

Perhaps gsc expression labels chondrogenic cells and these cells shift medial in the early CNC cylinder. Alternatively, gsc expression could label non-chondrogenic cells. Although the first model is more consistent with the demonstrated cell-autonomous function of gsc in mice (Rivera-Perez et al., 1999), the latter is more consistent with the moz mutant phenotype, in which the lateral end of the duplicated lower jaw cartilage fuses laterally with the lower jaw near the jaw joint. In mice, although gsc was found to be cell autonomous, gsc-null cells in the presence of wild-type cells could contribute to the condensation of the tympanic bone, a bone that never forms in gsc mutants (Rivera-Perez et al., 1995; Rivera-Perez et al., 1999; Yamada et al., 1995). However, these gsc-null cells were not maintained (Rivera-Perez et al., 1999). Exogenous gsc can induce neighboring cells to form a secondary axis, suggesting in some contexts, Gsc can have non cell-autonomous functions (Cho et al., 1991; Niehrs et al., 1993).

A central mystery remaining is why the duplication in moz or hox2-deficient animals is mirror image. At the time the gsc expression defect appears, hand2 expression appears to mark all hyoid postmigratory CNC. Thus, the spatially complex gsc defect in hox2-injected animals is hard to reconcile with a model in which hox2 genes simply positively regulate gsc. The gsc expression defect is also hard to reconcile with a model in which hox2 genes modify responsiveness of second arch CNC to a single cue emanating from the arch 1/2 boundary (Rijli et al., 1993). We propose a modified version of the model of Rijli et al., in which hox2 modifies the responsiveness of hyoid CNC to multiple environmental signals. The mediolateral inversion of gsc could be explained if hox2 genes conferred responsiveness of second arch CNC to a lateral surface ectodermal signal cue to activate gsc while repressing responsiveness to a medial endodermal cue that normally repressed gsc expression. Continued forward genetic screens in zebrafish could reveal components of these putative signaling pathways that underlie segmental identity in the pharyngeal arches.

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Regulation of Hox activity


