Deletion of a conserved regulatory element required for Hmx1 expression in craniofacial mesenchyme in the dumbo rat: a newly identified cause of congenital ear malformation

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SUMMARY

Hmx1 is a homeodomain transcription factor expressed in the developing eye, peripheral ganglia, and branchial arches of avian and mammalian embryos. Recent studies have identified a loss-of-function allele at the HMX1 locus as the causative mutation in the oculo-auricular syndrome (OAS) in humans, characterized by ear and eye malformations. The mouse dumbo (dmbo) mutation, with similar effects on ear and eye development, also results from a loss-of-function mutation in the Hmx1 gene. A recessive dmbo mutation causing ear malformation in rats has been mapped to the chromosomal region containing the Hmx1 gene, but the nature of the causative allele is unknown. Here we show that dumbo rats and mice exhibit similar neonatal ear and eye phenotypes. In midgestation embryos, dumbo rats show a specific loss of Hmx1 expression in neural-crest-derived craniofacial mesenchyme (CM), whereas Hmx1 is expressed normally in retinal progenitors, sensory ganglia and in CM, which is derived from mesoderm. High-throughput resequencing of 1 Mb of rat chromosome 14 from dmbo/dmbo rats, encompassing the Hmx1 locus, reveals numerous divergences from the rat genomic reference sequence, but no coding changes in Hmx1. Fine genetic mapping narrows the dmbo critical region to an interval of ~410 kb immediately downstream of the Hmx1 transcription unit. Further sequence analysis of this region reveals a 5777-bp deletion located ~80 kb downstream in dmbo/dmbo rats that is not apparent in 137 other rat strains. The dmbo deletion region contains a highly conserved domain of ~500 bp, which is a candidate distal enhancer and which exhibits a similar relationship to Hmx genes in all vertebrate species for which data are available. We conclude that the rat dumbo phenotype is likely to result from loss of function of an ultraconserved enhancer specifically regulating Hmx1 expression in neural-crest-derived CM. Dysregulation of Hmx1 expression is thus a candidate mechanism for congenital ear malformation, most cases of which remain unexplained.

INTRODUCTION

Hmx1 is a homeodomain transcription factor expressed in the developing eye, peripheral ganglia and branchial arches of avian and mammalian embryos (Wang et al., 2000; Yoshiura et al., 1998). Relatively little is known about the function of Hmx1, but recently an Hmx1 allele has been identified as the causative genetic defect in a human disorder, oculo-auricular syndrome (OAS) (Schorderet et al., 2008). Patients with OAS exhibit malformations of the outer ear (pinna) and defects of the eye, including microphthalmia, cataract, coloboma and retinal dystrophy (Schorderet et al., 2008; Vaclavik et al., 2011).

Animal models of OAS include the dumbo (dmbo) and misplaced ears (mpe) mutations in mice (Munroe et al., 2009). Mouse dmbo, mouse mpe and the known human Hmx1 allele causing OAS are all coding mutations that affect the Hmx1 DNA-binding homeodomain, and thus are predicted to result in loss of function. In addition to malformed ears, dumbo mice exhibit eye malformations, although less severe than those observed in the OAS patients identified to date. In both mouse and man, hearing is spared. In rats, the dumbo (dmbo) trait identified in animals kept by amateur ‘fancy rat’ breeders has also been mapped to a 5 Mb region encompassing the Hmx1 locus (Kuramoto et al., 2010), but the nature of the causative mutation is unknown.

Recent work in mice has also revealed a role for Hmx1 in the development of sensory neurons. Hmx1 is widely expressed in the sensory peripheral nervous system, including a subset of neurons in the trigeminal ganglion, geniculate ganglion, superior ganglion of the IX-X ganglion complex and dorsal root ganglia (Wang et al., 2000; Yoshiura et al., 1998). In the caudal cranial ganglia, Hmx1 is confined to somatosensory neurons and is not expressed in the distal viscerosensory component of these ganglia. Despite the wide expression of Hmx1 in the sensory system, only the geniculate

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**TRANSLATIONAL IMPACT**

**Clinical issue**
The oculo-auricular syndrome (OAS) is a rare human craniofacial disorder that involves multiple anomalies of the eyes (retinal dys trophy, microphthalmia, chor ioretinal colobomas and cataract) and ears (lobe malformation and simplification of ear morphology). OAS in humans has been linked to a deletion in the gene Hmx1, a transcription factor expressed in the developing eye, peripheral nervous system and branchial arches of birds and mammals. However, the function of this gene and the nature of the disrupted embryological processes that result in these anomalies are not known. Moreover, the genetic and non-genetic causes of much more prevalent congenital anomalies of the ear, with and without associated eye malformations, remain largely unknown. Eye and ear anomalies have profound effects on the lives of the affected individuals. The study of animal models that advance our understanding of these conditions, and enable specific diagnosis and ultimately treatment of patients, are an invaluable resource.

**Results**
In prior work, the dumbo phenotype, named for its characteristic ear malformation, has been linked to the Hmx1 gene in rats and mice. Our results show that neonatal dumbo rats have a similar phenotype to that observed in dumbo mice, with characteristic ventral displacement of the ear and a moderate degree of microphthalmia. Dumbo rats show a specific loss of Hmx1 expression in neural-crest-derived craniofacial mesenchyme, particularly the maxillary component and most of the mandibular component of the first branchial arch, and the distal part of the second branchial arch. Expression of Hmx1 in the early developing retina and peripheral nervous system is intact. Unlike the known OAS Hmx1 allele in humans and the dumbo mutation in mice (both of which prevent translation of the Hmx1 homeodomain), sequence analysis of the chromosomal region that encompasses the rat Hmx1 locus reveals no coding changes in the Hmx1 open reading frame. Instead, dumbo rats exhibit a novel 5777 bp deletion 79 kb downstream from Hmx1, which includes an ‘ultraconserved’ region of ~500 bp that is likely to be crucial for Hmx1 expression in the branchial arches.

**Implications and future directions**
This detailed genetic analysis of the dumbo rat suggests that the causative mutation is deletion of a conserved enhancer specifically affecting Hmx1 expression in neural-crest-derived mesenchyme, and future studies will focus on unraveling the nature of this regulatory allele. In future studies of individuals with ear malformations, exon sequencing alone will not be sufficient to exclude the Hmx1 locus; regulatory alleles of Hmx1 must also be considered. These results also suggest that Hmx1 lies in the middle of a poorly understood pathway for development of the pinna. Thus, genes both upstream and downstream of Hmx1, as well as the Hmx1 locus itself, are candidates for the site of the causative allele in studies of individuals with syndromic or isolated eye and ear anomalies.

somatosensory neurons appear to require it for neurogenesis (Quina et al., 2012), whereas early development of the trigeminal and dorsal root ganglia appear normal in dumbo mice.

In the present study, we show that dumbo rats and mice exhibit similar ear malformations and extent of microphthalmia. Hmx1 protein expression in dumbo rat embryos shows no change in the early developing eye and sensory ganglia compared with controls, but dumbo embryos exhibit specific loss of Hmx1 expression in the mesenchymal components of branchial arches 1 and 2 (BA1 and BA2), which give rise to the pinna. Fate-mapping of Hmx1-expressing craniofacial mesenchyme (CM) cells in transgenic mouse shows that this region of decreased Hmx1 expression in the dumbo rat corresponds to CM that is derived from neural crest, but not from cranial mesoderm. To better understand the genetic basis of this specific loss of Hmx1 expression, we resequenced 1 Mb of rat chromosome 14 encompassing the Hmx1 locus, revealing numerous divergences from the rat genomic reference sequence, but no coding changes in Hmx1. Single nucleotide polymorphisms within this span allowed the dumbo critical region to be narrowed to the interval of ~410 kb immediately downstream of the Hmx1 transcription unit. Within this region, we identified a 5777-bp deletion that encompasses a ~500-bp region conserved across all vertebrate species. Together, these results suggest that the rat dumbo mutation is a cis-acting regulatory allele of an ultraconserved enhancer that is necessary for Hmx1 expression in neural-crest-derived CM. Dysregulation of Hmx1 expression is thus a potential causative mechanism in many patients with congenital ear malformations, a common abnormality for which the etiology is largely unknown.

**RESULTS**
Dumbo rats exhibit congenital malformations of the pinna similar to dumbo mice, and modest reduction in ocular size at birth

In order to compare the phenotypes of dumbo mice and rats, we first examined the morphology of the ear and eye in newborn dumbo animals and controls. The characteristic ventral displacement of the ear in dumbo rats and mice, which gives the mutation its name, has been previously described in adults (Kuramoto et al., 2010; Munroe et al., 2009). To define an endpoint for developmental studies, we examined the morphology of the external ear in neonatal dumbo rats and mice at postnatal day 2 (P2) and P1, respectively. At P2, dumbo rats exhibited marked ventral displacement of the pinna compared with heterozygous controls (Fig. 1A,B). P1 Hmx1<sup>dm/dm</sup> mice also exhibited ventral displacement and, in addition, the top of the pinna was rotated posteriorly by 33° relative to controls (Fig. 1C,D).

To determine the extent of congenital microphthalmia in dumbo rats and mice we measured the neonatal eye. The average diameter of the newborn dumbo rat eye was modestly decreased, to 86% that of heterozygous controls (Fig. 1E). However, the eye was of normal size at E14 (data not shown), indicating that the ocular size difference must be generated during the period of extensive eye growth in late gestation. Prior characterization of the Hmx1<sup>dm/dm</sup> mouse identified a microphthalmia phenotype in adults but did not quantitatively assess the reduction in eye size (Munroe et al., 2009). In the present study, comparison of P1 dumbo mouse (Hmx1<sup>dm/dm</sup>) to littermate controls (Hmx1<sup>dm/+</sup> and Hmx1<sup>+/+</sup>) revealed that the mean diameter and length of the orbit in Hmx1<sup>dm/dm</sup> mice was reduced to 91% that of controls, corresponding to approximately 75% of normal ocular volume (Fig. 1F–H). We then undertook detailed developmental studies to examine the cellular expression of Hmx1 in the developing CM and eye in order to better understand these deformities.

**Expression of Hmx1 in the mouse craniofacial mesenchyme**
In developing mice, Hmx1 mRNA can be detected in BA2 at E10.5, and Hmx1 protein in the mesenchyme of proximal BA1 at E11.5 (Quina et al., 2012; Wang et al., 2000; Yoshiura et al., 1998). Malformation of the pinna is evident by E14.5 in Hmx1<sup>dm/dm</sup> embryos (data not shown). Thus the crucial events leading to the dumbo ear phenotype occur in the E10.5-E14.5 developmental interval in mice. To better understand the expression of Hmx1 at
In the E11.5 mouse embryo, Hmx1 was expressed in three regions of the CM. Region 1 consisted of the part of proximal BA1 overlying the trigeminal ganglion (Fig. 2A-D). Region 2 comprised the caudal half of distal BA2 (Fig. 2D-F), and a small region of the caudal or distal tip of the mandibular component of BA1 (Fig. 2F,G). Region 3 consisted of an extensive region of posterior mesenchyme, caudal to the head vein and branchial arches, ending at the top of the limb bud (Fig. 2A-H). By contrast, extensive regions of CM, particularly the maxillary component and most of the mandibular component of BA1 and the proximal part of BA2, do not express Hmx1. Based on past studies of the embryological origin of the external ear, only Region 2 CM in the distal part of BA1 and BA2 is likely to contribute directly to the pinna.

Expression of Hmx1 in the dumbo rat embryo
In order to understand the expression of Hmx1 in the dumbo rat embryo, and the basis of the rat dumbo phenotype, we examined Hmx1 expression in E13 rat embryos (equivalent to the E11.5 mouse described above) and in E14 rat embryos (equivalent to an E12.5 mouse). Dumbo rats and embryos were generated by interbreeding KFRS4/Kyo parental rats. Age-matched controls were generated from crosses of the PVG/seac strain (see Methods).

Examination of BA1 in E13 dumbo rat embryos and controls revealed loss of Hmx1 expression in the proximal part of BA1, overlying the trigeminal ganglion, defined as Region 1 (Fig. 3B-I). The thickness of the mesenchyme overlying the trigeminal ganglion, measured as the distance between the trigeminal ganglion itself and the surface ectoderm, was not altered, indicating that there was loss of Hmx1 expression in this domain rather than loss of the CM cells as such. Expression of Hmx1 was also diminished in the region of branchial arches 3 and 4, overlying the superior ganglion (Fig. 3F-G). Remarkably, expression of Hmx1 was not affected in the sensory neurons residing at the same axial levels, including the mandibular lobe of the trigeminal ganglion (Fig. 3H,I), the somatosensory neurons of the geniculate ganglion or the Hmx1-expressing neurons in the statoacoustic ganglion (Fig. 3J,K). The Hmx1 signal in the retina and the characteristic nasotemporal gradient of retinal expression were also unchanged in dumbo embryos compared with controls (Fig. 4B,C).

Similar loss of Hmx1 expression was seen in the ventral branchial arches of E13 dumbo rat embryos, designated as Region 2. Hmx1 was expressed in the caudal part of distal BA2 in control embryos, whereas dumbo embryos revealed a complete loss of Hmx1 expression in this region (Fig. 4D-G). By contrast, expression was unchanged in the posterior mesenchyme designated as Region 3 (Fig. 4D,E). The cervical dorsal root ganglia (DRG) of dumbo embryos also maintained normal Hmx1 expression (Fig. 4H,I), as did the cranial sensory ganglia.

In E14 rat embryos (equivalent to an E12.5 mouse), Hmx1 was prominently expressed in the lateral facial mesenchyme, including a region of expression posterior to the eye, and dorsal to the branchial arches, which was not evident at E13 (Fig. 5). Double-labeling experiments with Hmx1 and Ap2a demonstrated that Hmx1 is expressed in the mesenchyme of this region, but not in the surface ectoderm (supplementary material Fig. S1). To better define the Hmx1 expression differences in dumbo embryos, serial sections were examined throughout this dorsal domain, and the extent of Hmx1 expression was measured using semi-quantitative immunofluorescence (Fig. 5A-C). The overall size of the area of expression was reduced moderately (44%) in the most dorsal region of expression, at the level of the eye and lateral to the hypothalamus (Fig. 5B,C, level 0), and severely (74-92%) in the ventral region of expression, lateral to the mandibular lobe of the
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Fig. 2. Hmx1 expression in developing mouse craniofacial mesenchyme. (A-H) Serial sections of a wild-type E11.5 mouse embryo showing the expression domains of Hmx1 in the CM. Successively more ventral planes of section show distribution of Hmx1-expressing cells in the CM. Arrows in F and G indicate the region of expression in ventral BA1. (I) Planes of section in A-H. Shaded areas indicate regions of Hmx1 expression: 1, part of proximal BA1 overlying the trigeminal ganglion; 2, the caudal half of distal BA2 and a small region of the caudal/distal tip of the mandibular component of BA1; 3, an extensive region of posterior mesenchyme, caudal to the head vein and branchial arches, ending at the top of the limb bud. BA1, branchial arch 1; BA2, branchial arch 2; BA3, branchial arch 3; C1, brachial (pharyngeal) cleft (groove); C2, brachial cleft 2; Di, heart; D1, diencephalon; DRG, dorsal root ganglion; EAM, external auditory meatus; FL, forelimb; H1, hindbrain; GG, geniculate ganglion; Inf X, inferior ganglion of the IX-X ganglion complex (nodose/petrosal ganglion); Mn, mandibular process (of BA1); Mx, maxillary process (of BA1); mtTG, mandibular lobe trigeminal ganglion; mxTG, maxillary lobe, trigeminal ganglion; OV, otic vesicle; P, pons; RP, Rathke’s pouch; SAG, statoacoustic ganglion; SC, spinal cord; SG, superior ganglion; V, head vein. Scale bar: 200 μm.

Fig. 3. Loss of Hmx1 expression in proximal BA1 in the E13 Dumbo rat. E13 rat embryos, developmentally equivalent to E11.5 mice, were examined using immunofluorescence for Hmx1 and for Brn3a, which identifies somatosensory neurons. (A) Plane of section in subsequent views. (B-D) Control (B,D) and dumbo (C,E) embryos showing loss of expression of Hmx1 in the dorsalmost part of BA1 in the mutant. The CM overlying the TG is present (dashed line in E), but fails to express Hmx1. Expression of Hmx1 is unaffected in the mtTG and SAG. (F-K) Control (F,H,J) and dumbo (G,I,K) embryos showing loss of Hmx1 expression in BA1 in dumbo embryos. Some loss of Hmx1 expression is also noted in posterior mesenchyme overlying the head vein in the mutant (arrowheads, F). CM, craniofacial mesenchyme; Di, diencephalon; GG, geniculate ganglion; GGv, geniculate ganglion, viscerosensory component; GGv, geniculate ganglion, viscerosensory component; Hb, hindbrain; mtTG, mandibular lobe, trigeminal ganglion; mxTG, maxillary lobe, trigeminal ganglion; OV, otic vesicle; SAG, statoacoustic ganglion; SG, superior ganglion; V, head vein. Scale bar: 200 μm.

The craniofacial mesenchyme, which gives rise to the bones and cartilage of the head, is known to originate from both the cranial neural crest and mesoderm, depending on location (Le Douarin et al., 1993). Our observation that Hmx1 expression in dumbo rat embryos is lost only in specific regions of CM led us to hypothesize that the effect of the rat dmbo mutation might differ according to the embryological origin of the affected CM cells. In mice, tissue derived from neural crest in the cranium and branchial arches can be identified by embryological fate mapping, using a Wnt1-Cre
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driver and a LoxP-mediated reporter (Danielian et al., 1998). In mid-gestation embryos, a clear boundary can be shown between neural-crest-derived frontonasal structures and mesoderm-derived parietal structures (Jiang et al., 2002). Because no such approach is available for rats, we chose to determine the embryological origin of the Hmx1-expressing CM in a transgenic mouse model.

To assess the embryological origin of the Hmx1-expressing cells in the CM, we crossed mice carrying Wnt1-Cre with a reporter strain, Ai6, which conditionally expresses the fluorescent reporter ZS-green from a modified Rosa26 locus (Madisen et al., 2010). In E12.5 Wnt1-Cre/Ai6 mouse embryos, expression of the induced marker gene revealed an occult boundary between the neural-crest-derived CM in frontonasal and branchial arch regions and the mesoderm-derived CM in the parietal region (Fig. 6), which was consistent with prior studies (Jiang et al., 2002; Yoshida et al., 2008).

Hmx1-expressing CM cells were found in both the neural crest and mesoderm compartments, but the embryological origin of the Hmx1 cells differed markedly according to axial level. In the most dorsal and rostral domain of expression, at the level of the diencephalon (Fig. 6B), Hmx1-expressing CM cells were derived entirely from mesoderm. At the level of the trigeminal and geniculate ganglia and the future pons, an increasing proportion of the Hmx1-expressing CM cells resided in the neural-crest-
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Fig. 6. Hmx1 is expressed in CM of neural crest and non-crest origin. A Wnt1-Cre transgenic line was interbred with the reporter strain Ai6, expressing the fluorescent protein ZEG from a modified Rosa26 locus. Mouse embryos were analyzed at E12.5. (A) Whole-mount E12.5 embryo showing planes of section for levels analyzed in subsequent views. The ganglia and projections of the sensory peripheral nervous system are marked by expression of a LacZ transgene targeted to the Bm3α locus. (B-F) Progressively more ventral and caudal sections showing expression of Hmx1 in crest-derived and mesoderm-derived CM. The endogenous fluorescence of the ZEG reporter appears in green. Dashed lines demarcate the border of the cranial neural-crest-derived tissue. BA1-4, branchial arch 1-4; D1, diencephalon; GG, geniculate ganglion; Hb, hindbrain; inf IX/X, inferior part of IX-X ganglion complex (nodose and petrosal ganglia); mnTG, mandibular lobe, trigeminal ganglion; mxTG, maxillary lobe, trigeminal ganglion; Ret, retina; SAG, statoacoustic ganglion; SG, superior ganglion (of the IX-X ganglion complex).

derived compartment in progressively more ventral and caudal sections (Fig. 6C–E). Caudal to the otic region, in CM overlying the superior ganglion and future medulla, Hmx1 was again expressed predominantly in the mesoderm-derived CM (Fig. 6F).

Although it is not possible to directly fate-map the neural crest and parietal mesoderm in rat embryos, the loss of Hmx1 expression in the dumbo rat appears to be largely confined to the neural crest compartment, with persistence of Hmx1 expression in the mesoderm-derived CM (compare Fig. 5, L5 with Fig. 6D and Fig. 5, L7 with Fig. 6E). However, the Hmx1 expression is not affected in the trigeminal or dorsal root ganglia, which are also largely neural-crest-derived. Thus, it appears that the dumbo rat phenotype results from loss of Hmx1 expression specifically within the neural-crest-derived CM, suggesting a tissue-specific defect in the regulation of Hmx1 expression.

Sequence of the rat dumbo critical region reveals deletion of a unique distal conserved region.

Tissue-specific loss of Hmx1 expression in neural-crest-derived CM could in principle result from the loss of a trans-acting regulatory factor governing expression in this domain, or from the disruption of a cis-acting neural-crest- or CM-specific regulatory element at the Hmx1 locus. However, prior mapping of the rat dumbo allele to a critical region of ~5 Mb encompassing the Hmx1 locus suggested a cis-acting mechanism (Kuramoto et al., 2010).

In an effort to determine the nature of the dumbo mutation, we performed resequencing of a 1.0-Mb region of the dmbo/dmbo rat strain KFRS4, spanning the interval from 80.5 to 81.5 Mb of chromosome 14, which includes the core of the dumbo critical region (see Methods). Comparison of this region with the Brown Norway (BN) reference sequence revealed 31 single nucleotide polymorphisms (SNPs) residing in the coding sequences of nine of the ten annotated genes in this interval (supplementary material Table S1), as well as numerous SNPs in the intergenic regions. Eight of the SNPs resulted in conservative amino acid changes, and 23 were synonymous. The single SNP identified within the Hmx1 coding sequence was silent (L251L).

The identification of additional SNPs distinguishing KFRS4 from the BN reference sequence allowed further refinement of the genetic map of the dumbo critical region. To fine-map the dumbo locus, we assessed the progeny of a (BN/SsNslc × KFRS4/Kyo)F1 × KFRS4/Kyo backcross. Transmission or non-transmission of the dumbo allele was determined by the appearance of the pinna at 3 weeks of age. A total of 614 backcross progeny were examined, of which 322 expressed the dumbo ear phenotype and 292 had normal ears. Recombination between the chromosomal markers D14Rat10 and D14Rat57 was observed in 31 of the backcross progeny, and these animals were used for fine mapping (Fig. 7). By genotyping of recombinant progeny with Ablim2_SNPN(H42Y) and Hmx1_SNP(L251L), the minimal dumbo critical region was refined to a 411.5-kb segment between 80.58 and 81.0 Mb of Chr14 (Fig. 7A). The haplotypes of animals used to define the critical region appear in supplementary material Fig. S2.

Only two mis-sense mutations were localized to this refined dumbo critical region (supplementary material Table S1), Cpz_S23P and Acox3_N287H. However, these SNPs are not specific to the KFRS4/Kyo strain. Cpz_S23P has been identified as a homozygous SNP (rs64860291) between the BN reference strain and the Sprague Dawley (SD) strain, which does not have dumbo ears. Acox3_N287H has been identified by our group as a homozygous mutation in the NER rat strain (data not shown), an inbred line derived from Crj:Wistar rats, which does not exhibit dumbo ears.

Although resequencing provided ~99% coverage of the dumbo critical region, we wished to determine whether small discontinuities detected in the sequence data represented genomic rearrangements. To search for deletions or insertions, we used long PCR to amplify segments of DNA from dumbo and control rats spanning the 120-kb intergenic region from the 3’ end of the Hmx1 gene to the 3’ end of the Cpz gene. One primer set spanning the region 80-90 kb downstream of the Hmx1 gene yielded a ~6 kb shorter product in dumbo samples than in controls, and a mixed product in heterozygotes (supplementary material Table S2). Sequencing of the PCR product from this region revealed a 5777-bp deletion in KFRS4/Kyo genomic sequence relative to the reference strain (Fig. 7B). The deletion was not detected in a panel of 137 other rat strains, strongly suggesting that it is the causative allele for the rat dumbo phenotype. To further verify the relationship between this deletion and the dumbo phenotype, we amplified the breakpoint region in DNA samples obtained from

Sequence of the rat dumbo critical region reveals deletion of a unique distal conserved region.
four dumbo rats with diverse genetic backgrounds, obtained from an enthusiast breeder in California. Each of these animals exhibited the same 5777-bp deletion \(3/H11032\) to the \(Hmx1\) transcribed region observed in \(KFRS4/\text{Kyo}\) (supplementary material Fig. S3), and no coding changes in the \(Hmx1\) exonic sequence.

Because the 5.8-kb \(dmbo\) deletion lies in a presumed intergenic region, we used BLAST and VISTA genomic search tools to determine whether the deletion region contained sequence elements conserved across species. A BLAST search revealed a high degree of similarity between the rat \(dmbo\) deletion region and sequences in the \(Hmx1\)-\(Cpz\) intergenic region in the mouse (Chr:5) and human (Chr:4) genomes. The BLAST search did not reveal significant homology to identified transcripts or expressed sequence tags in any species, confirming the intergenic nature of the deletion region and suggesting instead a regulatory function for the conserved sequence.

Because of the limited annotation of the rat genome, we used the homologous region of the mouse genome to generate VISTA alignments (Dubchak et al., 2009) of conserved sequences in the \(dmbo\) deletion region across multiple species. VISTA analysis revealed a highly conserved region of \(~500\) bp within the deletion region that is present in all mammalian species (Fig. 8A). MultiZ alignment revealed that a core sequence is conserved across vertebrate classes (Fig. 8B). This \(Hmx\) distal conserved region (DCR) occurs consistently in a syntenic chromosomal region including the \(Hmx\) locus or loci and the \(Cpz\) locus in all species for which complete data are available. In chicken and zebrafish, two closely related \(Hmx\)-class genes are present in tandem in this region. Although the genomic distance between the \(Hmx\) transcribed region and the DCR varies from 45 kb (zebrafish) to 326 kb (opossum), no intervening identified transcripts occur between the \(Hmx\) loci and the DCR in any species. We conclude that the \(Hmx\) DCR is a strong candidate for an ancient distal enhancer that regulates \(Hmx\) expression in the branchial arch CM.

**DISCUSSION**

The dumbo mouse and rat strains were named for their most obvious physical characteristic, displaced and malformed ears, prior...
to any knowledge of the genetic mechanism underlying these malformations. The dumbo mouse phenotype was identified in a mutagenesis screen (Wilson et al., 2005), whereas the dumbo rat mutation arose spontaneously among 'fancy' rats kept by enthusiastic breeders, probably in the western United States, and has become a popular pet strain on the basis of its novel appearance. Given the large number of genes that are likely to play a role in the morphogenesis of the pinna, it is remarkable that both the mouse and rat dumbo strains converged on the same gene locus. The appearance of the mouse and rat dumbo ears are quite similar, but at the cellular level the phenotypes of the mouse and rat mutants reveal other striking differences. In recent work, we have shown at the cellular level the phenotypes of the mouse and rat mutants.

The distinctive nature of the Hmx1 mutant phenotypes, resulting either from the loss of protein function or defective regulation, is underscored by comparison with the effects of other genes that regulate morphogenesis of the pinna. One example is Hoxa2, which affects ear development through specification of segmental identity in the branchial arches, a classic homeotic function. BA2 development is particularly influenced by the loss of Hoxa2, which results in transformation of BA2-derived structures into BA1 phenotypes and absence of the pinna (Santagati et al., 2005). Although Hmx1 is a homeodomain protein, its late expression in a restricted set of neural and mesenchymal cell types across all cranial segments excludes a Hox-like role in the assignment of the segmental identity. Consistent with this, Hmx1 mutants do not show the extensive changes in multiple structures derived from BA2, such as the inner ear and brainstem, that are observed in Hoxa2 mutants. In chick embryos, mis-expression of Hoxa2 in BA1 can induce ectopic expression of the chick Hmx1-class factor SOHo1, suggesting that Hmx1 might lie downstream of this Hox gene (Grammatopoulos et al., 2000). However, this cannot reflect an exclusive requirement
for Hoxa2 because the normal boundaries of Hmx1 expression extend beyond the Hoxa2 expression domain.

An Hmx gene is also present in the Drosophila genome [H6-like-homeobox, FlyBase ID FBgn0264005 (Wang et al., 2000)]. The regulatory function of the Drosophila Hmx protein overlaps that of mouse Hmx2/3, because Drosophila Hmx can partially rescue the hypotalamic and inner ear phenotypes observed in Hmx2/3 double mutant mouse embryos (Wang et al., 2004). The ‘non-homeotic homeobox’ role of the Hmx family is underscored by the fact that although insertional mutants of Drosophila Hmx are known (http://flybase.org/reports/FBgn0264005.html), they apparently do not exhibit homeotic transformations and there are no published reports of Hmx function in flies.

Mutations affecting development of the external ear might also result from defective neural crest development, a mechanism exemplified by the role of Tcof1/Treacle in Treacher Collins Syndrome (TCS), an autosomal dominant disorder that frequently includes hypoplasia of the facial bones, cleft palate and middle ear defects in addition to malformation of the pinna (Dixon et al., 2007). Treacle is expressed throughout the dorsal neural tube and early neural crest. Mice haplo-insufficient for Tcof1/Treacle in Treacher Collins Syndrome (Tcs) and include hypoplasia of the facial bones, cleft palate and middle ear defects (Dixon et al., 2007). Mice were crossed to C57BL/6N (Charles River) for two to five additional generations prior to the experiments described. Experiments were performed with littermate controls. Genotyping of the Hmx1dmbo and wild-type Hmx1 (Hmx1+) alleles was performed by real-time PCR using oligonucleotide primers having 3' termini at the point mutation that characterizes the dmbo mutation, as previously described (Quina et al., 2012).

The founder rat expressing the dumbo ear phenotype [SRRO4, (Kuramoto et al., 2010)] was obtained from a commercial breeder in the United States and crossed with the PVG/seac strain of Black Hooded rats to generate the strain KFRS4, which is homozygous for the recessive alleles dumbo (dmbo) and the coat color allele head spot (hs). In prior work, the dmbo locus was mapped to the 5.7-Mb region defined by D14Rat10 and D14Rat57 (Kuramoto et al., 2010).

To further refine the dmbo locus, we produced 614 (BN/SsNSlc x KFRS4/Kyo)F1 x KFRS4/Kyo backcross progeny. The BN/SsNSlc rat strain was purchased from Japan SLC (Hamamatsu, Japan). Genotypes of the dmbo locus were determined by the appearance of the outer ear (dumbo malformation or wild-type) at 3 weeks of age. Animals with recombination events between D14Rat10 and D14Rat57 were used for the fine mapping of the dmbo critical region.

Newborn dumbo rats and E13 and E14 dumbo embryos were generated by interbreeding KFRS4/Kyo parental rats. For Hmx1 expression studies, age-matched wild-type controls were generated from PVG/seac rats. For measurements of ocular diameter, littermate controls were used. Mouse embryos were staged according to a published method (Theiler, 1972) and the corresponding rat embryo stages were identified according to Witschi (Altman and Katz, 1962).

**METHODS**

**Animals and genotyping**

Mice bearing the dmbo allele (Hmx1^dmbo), strain B6.C3Fe-Hmx1^dmbo/Rw/JcsKjn, were obtained from Jackson Laboratories (Stock #008677). The Hmx1^dmbo allele was originally the result of ENU mutagenesis on a C57BL/6 genetic background (Wilson et al., 2005). Mice were crossed to C57BL/6N (Charles River) for two to five additional generations prior to the experiments described. Experiments were performed with littermate controls. Genotyping of the Hmx1^dmbo and wild-type Hmx1 (Hmx1+) alleles was performed by real-time PCR using oligonucleotide primers having 3' termini at the point mutation that characterizes the dmbo mutation, as previously described (Quina et al., 2012).

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**Resequencing of the rat dmbo critical region**

Regions for targeted resequencing spanned from 80.5 to 81.5 Mb of the rat chromosome 14 and contained the dmbo critical region. The 1.0-Mb target region of KFRS4/Kyo DNA was enriched using the SureSelect oligonucleotide hybridization solution-based capture technique (Agilent). The enriched fragment library was then subjected to PCR amplification, followed by sequencing on an Illumina GAIIx platform. Coverage of 40x or greater was obtained for 98.86% of the target region. The reads generated were mapped to the rat genome reference sequence (Baylor HGSC v3.4/rn4) using Burrows-Wheeler alignment (Li and Durbin, 2009). The known variants between KFRS4 and the rat genome reference sequence were identified using the Ensembl, and the Ensembl database. Further resequencing of the dmbo critical region was performed using long PCR using PrimeSTAR GXL DNA polymerase (Takara, Otsu, Japan). VISTA alignment of the dmbo deletion region was performed using the
Lawrence Berkeley National Laboratory and US Department of Energy Joint Genome Institute VISTA browser http://genome.lbl.gov/vista/index.shtml (Dubchak et al., 2009). Identification of conserved sequences across mammalian and vertebrate species was performed using MultiZ alignment using the UCSC genome browser http://genome.ucsc.edu/ (Kent et al., 2002).

Immunofluorescence
Embryos for immunofluorescence were fixed in 4% paraformaldehyde in phosphate-buffered saline, equilibrated in 15% then 30% sucrose, embedded in OCT medium and cryo-sectioned at 14-20 μm, depending on stage. Antiserum to Hmx1 was prepared in rabbits to a glutathione-S-transferase (GST)/Hmx1 fusion protein containing amino acids 2-188 of the Hmx1 protein, excluding the conserved homeodomain, as previously described (Quina et al., 2012). The Hmx1 antiseraum shows no reactivity to CM in Hmx1<sup>sm/dm</sup> mice. Polyclonal rabbit and guinea pig antiserum against Brrn3a have also been previously described (Quina et al., 2005). Mouse monoclonal anti-Ap2α antibody (3B5) was obtained from the Developmental Studies Hybridoma Bank. Alexa-Fluor-conjugated species-specific secondary antibodies were obtained from Life Technologies (Grand Island, NY).

For semiquantitative immunofluorescence measurement of Hmx1 expression, serial sections of dumbo and control rat embryos were processed, immunostained and imaged in parallel, with the same imaging parameters and no post-processing. Areas of interest in the CM were defined manually, and the area, total fluorescence signal and mean fluorescence signal were calculated using ImageJ.

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COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
L.Q., E.E.T. and T.S. conceived and designed the experiments. L.Q. and T.K. performed the experiments. L.Q., E.E.T. and T.C.C. analyzed the data. L.Q., E.E.T., T.K. and T.C.C. wrote the paper.

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