Transient activation of *Meox1* is an early component of the gene regulatory network downstream of Hoxa2.

Pavel Kirilenko[1], Guiyuan He[1], Baljinder Mankoo[2], Moises Mallo[3], Richard Jones[4, 5] and Nicoletta Bobola[1,*]

(1) School of Dentistry, Faculty of Medical and Human Sciences, University of Manchester, Manchester, UK.

(2) Randall Division of Cell and Molecular Biophysics, King's College London, UK.

(3) Instituto Gulbenkian de Ciência, Oeiras, Portugal.

(4) Genetic Medicine, Manchester Academic Health Science Centre, Central Manchester University Hospitals NHS Foundation Trust, Manchester, UK.

(5) Present address: Department of Biology, University of York, York, UK.

Running title: Hoxa2 activates *Meox1* expression.

Keywords: Meox1, Hoxa2, homeodomain, development, mouse

[*]**Words Count:** Material and Methods: 344; Introduction, Results and Discussion: 3679

[*] Author for correspondence at:

AV Hill Building

The University of Manchester

Manchester M13 9PT

United Kingdom

Phone: (+44) 161 3060642

E-mail: Nicoletta.Bobola@manchester.ac.uk

Abstract

Hox genes encode transcription factors that regulate morphogenesis in all animals with bilateral symmetry. Although Hox genes have been extensively studied, their molecular function is not clear in vertebrates, and only a limited number of genes regulated by Hox transcription factors have been identified. *Hoxa2* is required for correct development of the second branchial arch, its major domain of expression. We now show that *Meox1* is genetically downstream from *Hoxa2* and is a direct target. *Meox1* expression is downregulated in the second arch of *Hoxa2* mouse mutant embryos. In chromatin immunoprecipitation (ChIP), Hoxa2 binds to *Meox1* proximal promoter. Two highly conserved binding sites contained in this sequence are required for Hoxa2-dependent activation of the *Meox1* promoter. Remarkably, in the absence of Meox1 and its close homolog Meox2, the second branchial arch develops abnormally and two of the three the skeletal elements patterned by Hoxa2 are malformed. Finally, we show that Meox1 can specifically bind the DNA sequences recognized by Hoxa2 on its functional target genes. These results provide new insight into the Hoxa2 regulatory network that controls branchial arch identity.

Introduction

In vertebrates, development of the face and neck starts with formation of the frontonasal mass and the branchial arches. The branchial arches are transient, repetitive structures in which cells of the cranial neural crest (CNC) and mesoderm are encapsulated by epithelia. Upon differentiation, each of the branchial arches contributes to head and neck specific skeletal elements, their associated muscles, blood supply and nerves. Morphogenesis of the branchial arches depends on a number of transcription factors and signaling molecules. Transcription factors of the Dlx and Hox families have a most prominent role, the first by regulating proximo-distal patterning within each branchial arch (8), the latter by controlling branchial arch identity (3, 11, 17, 25).

The second branchial arch (IIBA) contributes to the outer and middle ear and to part of the neck. Development of the IIBA is controlled by the transcription factor Hoxa2. In Hoxa2 mutant embryos, IIBA-skeletal derivatives are replaced by typical first branchial arch (IBA) skeletal elements in mirror image configuration (3, 11, 25). Hoxa2 belongs to the large family of Hox transcription factors, whose members specify the body axis of bilaterian organism, whether a segment of the embryo will form head, thorax or abdomen (7). The identification of Hox downstream effectors in vertebrates is complicated by the high redundancy of Hox genes, represented by multiple paralogs in vertebrate genomes. Although Hox genes have been the subject of extensive genetic analysis, few target genes have been identified (22, 29). The IIBA is one of the few embryonic districts in which inactivation of a single Hox gene has an unambiguous effect in mouse, and an effective model system to define Hox molecular function in vertebrate embryogenesis.

How does Hoxa2 specify IIBA identity? Hoxa2 control of IIBA development appears to involve the repression of few transcription factors. At E10.0, before overt differentiation has begun in the IIBA, Hoxa2 negatively regulates transcription of *Ptx1* and *Lhx6*; their characterization has suggested that *Hoxa2* patterns the IIBA by changing the competence of the CNC to respond to skeletogenic signals (5). At the same developmental stage, Hoxa2 directly represses *Six2* expression, and this partially mediates Hoxa2 control over the IGF system (13, 14). Genetic experiments to dissect the role of the genes downstream of Hoxa2 have shown that correcting the expression levels of *Six2* and *Ptx1* ameliorates the *Hoxa2* mutant phenotype (5, 13, 14), indicating that repression of *Six2* and *Ptx1* is indeed required for IIBA-specific morphogenesis. However the limited extent of the rescue by inactivation of individual targets and its incomplete penetrance, suggest the existence of strong redundancies among these genes in the morphogenesis of the IIBA.

A number of observations indicates that Hoxa2 controls IIBA identity by blocking a first arch default fate. *Ptx1, Lhx6* and *Six2*, and the few additional genes identified as

regulated by Hoxa2 (6, 28) are normally expressed in the IBA, but not in the IIBA, suggesting that Hoxa2 functions as a transcriptional repressor to prevent the expression of IBA- specific genes in the IIBA. Moreover, the IIBA gives rise to duplicates of IBA-skeletal elements in the absence of Hoxa2. Is IIBA identity simply achieved by blocking a default IBA state or is there more to it? The finding that Hoxa2 is functionally relevant at E9.5 (Santagati et al., 2005), which is earlier than the stages at which expression of most identified Hoxa2 targets become apparent in *Hoxa2* mutant IIBAs, indicates that the gene regulatory network (GRN) controlled by Hoxa2 in the IIBA must include additional target genes expressed at earlier developmental stages. Their identification is essential to clarify the structure and organization of the GRN that governs IIBA identity downstream of Hoxa2.

Here we show that the earliest event controlled by Hoxa2 in the IIBA is the activation of the gene encoding the transcription factor Meox1. The *Meox1* homeobox gene is strongly expressed in the somites during embryogenesis, and controls formation and differentiation of the somites and their derivatives (16). We find that, in the developing IIBA, Hoxa2 is associated to a highly conserved region of *Meox1* chromatin. We further show that the interaction of Hoxa2 with *Meox1* promoter is sequence-specific and required for *Meox1* activation. Our results show that *Meox1* is genetically downstream of Hoxa2 and is a direct target. In addition, we find that Meox1 can specifically bind the DNA sequences recognized by Hoxa2 on its functional target genes, suggesting these transcription factors may share the control of downstream targets in the IIBA. Finally, the analysis of *Meox1*; *Meox2* combined mutants reveals that IIBA derivatives develop abnormally in the absence of *Meox* genes. These findings link two co-expressed transcription factors in a novel molecular pathway in mouse embryonic development. In addition, they uncover a previously unidentified role for *Meox* genes in the morphogenesis of the branchial arches.

Materials and methods

Mutant and transgenic animals and embryos

Hoxa2, *Meox1*, *Meox2* null mice are described in Gendron-Maguire et al., 1993; Mankoo et al., 1999; Mankoo et al., 2003; Skuntz et al. 2009. *Meox2*$^{+}$/$^{tm1(lacZ)Mnko}$ contains a ires-nls-*lacZ* knocked into *Meox2* first exon. Animals experiments were carried out under ASPA 1986.

Molecular and phenotypic analyses

Whole mount and section in situ hybridization was performed as described (Kanzler et al., 1998), using *Hoxa2* (Mallo, 1997), *Crabp1* (a gift from Rudolph Grosschedl) and *Meox1* probe, amplified from IIBA cDNA using primers *Meox1*F 5'-CACAGGAGCAGGACCGAGAGG -3'; *Meox1*R 5'-CCGGAACACGCAGGATAGGTCC-3'. Skeletal phenotypes were analyzed by alcian blue/alizarin red staining (Mallo and Brändlin, 1997).

RT-PCR

RT-PCR was performed as described (Kutejova et al., 2005), using RNA extracted from 10^6 cells dissociated from IBA and IIBA of E10.5 embryos, using 1% trypsin, 0.1% EDTA, filtered through a cell strainer (BD), and cultured in DMEM 10%FCS for 3 days. cDNA was subjected to 24 cycles of amplification using the following primers: *Lhx6F* 5'-GGAGATCTACTGCAAGATGGACTAC-3; *Lhx6R* 5'-CCGTCATGTCCGCTAGCTTCTG-3; *Pitx1F* 5'-AATCGTCCGACGCTGATCTGCC-3; *Pitx1R* 5'-CCTTGCACAGGTCCAACTGCTG-3'; *Six2* and *Hoxa2* primers are described in Kutejova et al. 2008.

Chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay (EMSA)

ChIP was performed according to Kutejova et al. 2008. *Meox1* mouse cDNA was amplified from E10.0 IIBA cDNA and cloned into pcDNA3 (Invitrogen). pcDNA3-*Meox1-HA* contains a HA tag before the stop codon; pcDNA3-*Hoxa2* construct has been described (Kutejova et al., 2005). EMSA was performed as described (Kutejova

et al., 2005). *Meox1* promoter and its mutant versions were labeled using γ^{32}P-dATP. For the supershift experiment, 40 ng of anti-HA antibodies (rat monoclonal 3F10, Roche) were added to the reaction.

Cell transfection

IBA mandibular components were isolated from 10 mouse embryos collected at E10.5. Cells were dissociated and resuspended in DMEM 10%FCS. 500 ng total DNA (250ng *Meox1-lacZ* or mut*Meox1-lacZ* construct and 250 ng pCDNA3-*Hoxa2* or pCDNA3) and 3 ul Fugene (Roche) were added to identical aliquots of the resuspended cells. Each aliquot was plated onto a well of a 24-well plate. After 24 hr incubation at 37ºC, cells were fixed and stained for B-galactosidase activity.

Results

Hoxa2 regulates *Meox1* expression in the IIBA

To identify early targets of Hoxa2, we compared the expression profiles of wild-type and *Hoxa2* mutant IIBAs at E9.5 in mouse, when migration of the *Hoxa2*-positive CNC into the IIBA has just been completed (15, 24). The gene encoding the transcription factor Meox1 was among the few genes differentially expressed, and its signal was decreased two-fold in the absence of Hoxa2.

Meox1 signal was not detected in the *Hoxa2*-positive CNC prior or during migration to the IIBA (not shown). *Meox1* signal was first evident in the branchial area at E9.0, and predominantly in the IIBA (Fig. 1A). At E9.5 strong expression of *Meox1* was detected in the proximal area of the IIBA, and expression was excluded from the distal domain of the arch; strong expression was also found in the third arch (IIIBA) (Fig. 1B). Around E10.0 only a small area of the IIBA and the IIIBA remained positive for *Meox1* (Fig. 1C) and after E10.5 no *Meox1* signal could be detected in the IIBA (not shown). At all the stages examined, *Meox1* expression was excluded from the IBA.

To confirm that Hoxa2 regulates *Meox1* expression, we performed ISH on E9.5 *Hoxa2* mutant embryos. *Meox1* expression was specifically lost in the IIBA of *Hoxa2* mutant embryos (Fig. 1E).

At E9.5, when migration of *Hoxa2*-positive cells is completed, *Hoxa2* was expressed in most of the IIBA (Fig. 1F), while *Meox1* was transcribed only in the proximal area of the IIBA (Fig. 1D). *Meox1* is expressed in a subpopulation of the *Hoxa2*-positive, *Crabp1*-positive cranial neural crest of the IIBA (Fig. 1G-I).

These results indicate that *Hoxa2* positively controls *Meox1* expression in the IIBA. They also show that the temporal and spatial expression of *Meox1* is more restricted as compared to the one of *Hoxa2*. Therefore Hoxa2 appears to be necessary, but not sufficient to activate *Meox1* expression.

Hoxa2 binds a conserved sequence within *Meox1* proximal promoter

Hoxa2 contains a DNA binding domain, which interacts in a sequence-specific fashion with the chromatin of target genes to control their transcription. Association of Hoxa2 to *Meox1* chromatin in vivo is therefore an essential requisite for *Meox1* direct regulation by Hoxa2. Loss of *Hoxa2* function generates an identical IIBA phenotype in mouse, frog and fish (2, 3, 11, 12, 25), suggesting that Hoxa2 responsive elements should be conserved across vertebrates. The CORG database (9) identified *Meox1* proximal promoter (corresponding to positions -236 to -33 in mouse) as perfectly conserved from human to fish (Fig. 2A). IIBA-extracted, Hoxa2-immunoprecipitated chromatin showed a substantial enrichment for the most proximal *Meox1* promoter region, while no enrichment was detected for an unrelated, control promoter (Fig. 2B). These results demonstrate that at E10.0, when *Hoxa2* is strongly expressed (Fig. 2C) and still required for activation of *Meox1* transcription in the IIBA, Hoxa2 is bound to *Meox1* chromatin in vivo.

Binding of Hoxa2 to its in vivo target *Six2* is mediated by two conserved TAAT consensus sites (13). The *Meox1* promoter region enriched in ChIP assays contains

an ATTA motif, embedded in a perfectly conserved stretch of flanking nucleotides, and a second one located on the opposite strand (Fig. 2A). Incubation of the conserved *Meox1* proximal promoter with in vitro translated Hoxa2 in electrophoretic mobility shift assay (EMSA) resulted in the formation of a retarded complex, which was supershifted by the addition of the anti-Hoxa2 antibody (Fig. 2D). In contrast, incubation of the probe in the presence of unprogrammed reticulocytes did not result in any retarded complex, nor did addition of the antibody have any effect (Fig. 2D). Mutating both TAAT consensus completely abolished binding of Hoxa2 to the probe (Fig. 2D), while nucleotide substitutions introduced in each of the single TAAT sites partially reduced Hoxa2 binding (Fig. 2D). Finally, Hoxa2 binding to the probe was competed by increasing molar concentration of wild-type oligonucleotides reproducing the TAAT motif and flanking nucleotides, but not mutant oligonucleotides (Fig. 2E). The oligonucleotide reproducing binding site 1 (BS1) was more efficient in competing Hoxa2 binding to the probe, indicating a higher affinity of Hoxa2 for binding site 1 (Fig. 2E).

We used IBA-derived cells to investigate if binding of Hoxa2 to *Meox1* promoter may control gene transcription. Experimental evidence indicates that IBA and IIBA cells share a similar ground state, which is modified in the IIBA by the presence of Hoxa2 (3, 11, 21, 25); IBA cells appear therefore capable to provide an environment equivalent to IIBA cells (without Hoxa2). When isolated from E10.0 branchial arches and grown in monolayers, these cells maintain the molecular identity of the area of origin, as estimated by expression of Hoxa2 and few of its target genes in IBA- and IIBA-derived cells after three days in culture (Fig 3A, B).

Cells dissociated from E10.0 IBAs were transfected with a 4.6kb*Meox1-lacZ* construct, containing the *Meox1* promoter (-4642 to + 22) fused to a *lacZ* reporter gene. *Meox1* promoter activity, assessed by counting the number of cells that turned blue after transfection, was very low in these cells (less than 20 blue cells were counted in each individual experiment). A significant increase in the number of cells

expressing *lacZ* (> 200, i.e. 10 fold increase) was observed when *Hoxa2* was co-transfected together with 4.6kb*Meox1-lacZ* (Fig. 3C). Introducing the nucleotide substitutions shown to abolish Hoxa2 binding (4.6kbmut*Meox1-lacZ*) (Fig. 2D) did not affect the basal activity of the promoter. However, unlike its wild-type version, addition of *Hoxa2* failed to activate the reporter gene (Fig. 3C). The lack of effect described above shows that expression of Hoxa2 in these cells did not affect cell number; this observation was also confirmed by using an unrelated promoter (not shown).

In conclusion, these results show that Hoxa2 interacts with the proximal region of the *Meox1* promoter in vivo and that this interaction, which is sequence-specific and mediated by two TAAT, is required for *Meox1* activation.

Meox homeodomain transcription factors control formation of the IIBA-specific cartilages

We analyzed *Meox1* mutant mice to understand whether *Meox1* activation contributes to IIBA morphogenesis. Skeletal analysis of *Meox1* mutant mice showed no evident phenotype in CNC derivatives of the IIBA, but when the *Meox1* mutation was combined with mutation in the homolog *Meox2* (also not showing a IIBA phenotype), two of the three elements that are controlled by Hoxa2 were abnormal. As sporadically observed in *Hoxa2* heterozygous (20), the styloid process was split in two fragments (Fig. 4B). Its distal part failed to extend distally and formed close to IBA cartilages, resembling an intermediate situation towards the *Hoxa2* mutant phenotype, in which the IIBA cartilages are formed much closer to their IBA mirror-image counterparts with respect to the wild-type (Figs. 4A-C). The lesser horn of the hyoid bone also developed abnormally (not shown). In addition, similar to the *Hoxa2* mutants, whose basioccipital bone has an abnormal shape, *Meox1* single mutants showed defects in this structure. These abnormalities were enhanced in $Meox1^{-/-}$; $Meox2^{-/-}$ mutants, resulting in a hypoplastic basioccipital bone (Fig. 4E).

The above data indicate that Meox1 functions redundantly with its homolog Meox2 during the development of the IIBA, consistent with the experimental evidence that Meox1 and Meox2 act redundantly to control somite development (Mankoo et al., 2003). *Meox2* expression in the IIBA is not equivalent to *Meox1* and appears later in the development of the IIBA; although we could detect *Meox2* transcripts in the developing somites by ISH, we were unable to detect *Meox2* expression in the branchial arches at stages earlier than E12.5. However *LacZ* staining of *Meox2*$^{+/tm1(lacZ)Mnko}$ embryos indicated that the *Meox2* allele is expressed in the posterior-distal domain of the IIBA already at E10.75 (Fig. 5).

Meox1 binds Hoxa2 target sequences

The identification of the genes regulated by Meox1 in the IIBA is complicated by the apparent redundant function of *Meox1* and *Meox2*. Meox1 regulates *Bapx1* expression in developing somites (26) and *Bapx1* is under Hoxa2 regulation in the IIBA (28), suggesting that Meox1 may mediate Hoxa2 regulation of *Bapx1* in the IIBA.

Meox1 binding site on its functional target *Bapx1* (Rodrigo et al., 2004) is very similar to the binding sites recognized by Hoxa2 on its direct targets, *Six2* (13) and *Meox1* itself. High-resolution analysis of sequence preferences attributes identical DNA-binding specificities to Meox1 and Hoxa2 (4). We ran a blast search (1) using Meox1 homeodomain and identified Hoxa2 homeodomain as a hit, with 68% residues identity and up to 75% positives residues. These observations predict that Meox1 and Hoxa2 may bind the same sequences in vitro. Hoxa2 directly regulates transcription of *Six2* gene by binding two closely spaced sites on *Six2* proximal promoter (13). In band-shift assays, *Six2* proximal promoter interacted similarly with Meox1 and Hoxa2-programmed reticulocytes (Fig. 6A). Double stranded oligonucleotides, reproducing the Hoxa2 binding sites identified in the *Six2* gene, specifically competed Meox1 and Hoxa2 binding to *Six2* promoter, and both Hoxa2

and Meox1 showed a higher affinity for binding site 1 (BS1). Conversely, double stranded oligonucleotides containing mutations in the TAAT core did not disturb complexes formation (Fig. 6A). Meox1, like Hoxa2, binds *Meox1* promoter: incubation of a probe containing *Meox1* proximal promoter gave rise to a strong complex in the presence of Meox1-HA programmed-reticulocytes. The specificity of the complex was confirmed by the addition of an anti-HA antibody (Fig. 6B).

The finding that Meox1 and Hoxa2 bind the same sequences in vitro suggests that these transcription factors could, at least in part, share the control of downstream targets in the IIBA.

Discussion

Activation of Meox1 by Hoxa2

Hoxa2 controls IIBA identity. Diverse experimental evidence indicates that Hoxa2 controls the production of IIBA structures by blocking a first arch default fate. First, morphogenesis in the IIBA follows patterns typical of the proximal region of the IBA in the absence of Hoxa2 (3, 11, 25). Second, Hoxa2 appears to act as a repressor and mainly negatively regulates the expression of IBA-specific developmental regulators in the IIBA (5, 6, 13, 28).

Here we identified a new downstream target of Hoxa2, the transcription factor Meox1. The addition of Meox1 to Hoxa2 GRN uncovers a novel aspect of Hoxa2 activity in the IIBA, which does not directly function to prevent the execution of the molecular program that imposes a first arch fate. Unlike all the previously identified Hoxa2 target genes, *Meox1* expression is never detected in the IBA. Activation of *Meox1* by Hoxa2 is mediated by direct interaction of Hoxa2 with a phylogenetically conserved region in the *Meox1* promoter. *Meox1* expression is detected as early as E9.0, when *Hoxa2*-positive cells settle in the IIBA. *Meox1* is transiently expressed and by the time the other known Hoxa2 targets appear in the Hoxa2 mutant IIBA (i.e. around E10.5), its expression has ceased.

Few similarities can be found between the regulation of *Meox1* and another bona fide Hoxa2-regulated promoter in the IIBA, *Six2* (13, 14). Hoxa2 binding sites are closely spaced and located in the proximity of the transcription start site in both *Six2* and *Meox1* promoters. Also in both cases the activity of Hoxa2 appears to require additional factors, namely Pax and Eya to regulate *Six2* (31). Spatial and temporal expression of *Meox1* in the IIBA is more restricted than the expression of *Hoxa2* (15, 24), indicating that Hoxa2 is necessary, but it is not sufficient to activate *Meox1* expression. However, Hoxa2 activates *Meox1* and represses *Six2* transcription; Hoxa2 repressor activity has been mapped to the protein region N-terminal to the homeodomain (27, 31). The molecular basis of this regulatory switch, also common to other Hox proteins (23, 27), is unknown. The molecular composition of the IIBAs at different developmental stages (the temporal dynamics of *Meox1* activation and *Six2* repression are clearly different, almost complementary) or the presence of additional cis-regulatory modules in *Six2* and *Meox1* promoters, able to recruit specific co-activators or co-repressors, may affect Hoxa2 function.

The Hoxa2 GRN: a highly redundant network?

Hoxa2 controls morphogenesis of the IIBA, and defines the shape and position of IIBA cartilages. Hoxa2 regulates *Meox1* expression and Meox1 and Meox2 control morphogenesis of IIBA skeletal elements. Taken together, it is highly likely that Meox1 partially mediates Hoxa2 function in the IIBA. The finding that *Meox1* null mutants do not display a IIBA phenotype, but *Meox1; Meox2* null mutants have defects in the skeletal elements that are controlled by Hoxa2, suggests that Meox1 function in the IIBA can be compensated by Meox2. This is similar to what observed in the developing somites, where *Meox2* and *Meox1* act redundantly (16). However, differently from the somites, *Meox1* and *Meox2* spatio-temporal expressions in the IIBA are almost complementary. *Meox2* is expressed in distal areas of the IIBA, opposite to the antero-proximal expression of *Meox1*. Expression profiling of wild-type and mutant IIBAs, collected from E10.0 to E11.5, indicates that in the absence

of Hoxa2 *Meox1* transcript levels are downregulated at all stages examined, while changes in *Meox2* expression are only detected at the latest stage examined (E11.5), suggesting they are likely to be indirect effects of Hoxa2 absence (N. Bobola, unpublished results). The combined *Meox1*; *Meox2* null phenotype in the IIBA indicates that Meox1 and Meox2 can compensate for each other's loss. Their differences in expression, however, raise questions about the effective capacity of Meox2 to compensate the loss of Meox1 in the cells where *Meox1* is normally expressed. Could other factors, in addition to Meox2, compensate for Meox1 absence?

One of the most intriguing findings resulting from the analysis of Hoxa2 activity during branchial arch development is that, while *Hoxa2* loss of function generates strong phenotypes, the network downstream of Hoxa2 appears highly resistant to perturbations. Correcting *Six2, Ptx1* and *Gbx2* upregulation in *Hoxa2* mutant embryos has mild or no effect on the development of the IIBA (5, 14) (M. Carapuco and M. Mallo, unpublished results). Similarly we show here that *Meox1*-null mice do not have a phenotype, while *Meox1*; *Meox2* null mutants have IIBA defects. The simplest interpretation is that we observe mild defects because the genes analyzed do not include the crucial Hoxa2 functional targets, which still need to be identified. An alternative possibility, which takes into account that two of the four genes analyzed (*Meox1* and *Six2*) are bona fide direct targets of Hoxa2, is that the GRN downstream of Hoxa2 is highly robust and is able to cope with modifications in the activity of its members. Indeed, the variability observed in the rescue of the phenotype in *Hoxa2; Six2*-null mutants (5, 14), indicates a high degree of redundancy, with other genes able to compensate *Six2* function. Although we currently lack systematic evidence to substantiate this hypothesis, it is interesting to speculate further. Hoxa2 GRN robustness may derive from recruiting genes with considerable redundant roles, e.g. genes that belong to families, and whose members are also present in the IIBA (*Meox1* and *Meox2*; *Six2*, *Six1* and *Six4*).

It is often found that changing the level of a transcription factor alters the expression level of a small subset of its predicted target genes; one of the possible explanations for this finding, other than a lack of function, is that related family members might bind to the same sites and have the same function (10). The structure of the Hoxa2 transcriptional network, where most of the Hoxa2 downstream targets identified so far encode for homeodomain transcription factors (5, 13, 14), may also contribute to the functional stability of the network. Homeodomain proteins regulate transcription of their target genes by binding to specific nucleotide sequences. A survey of the binding preferences of Hoxa2 targets, according to the interactive prediction tool developed in Noyes et al. (19) and to high-throughput binding site selection (4), shows that Meox1, Lhx6 (5), Ptx1 (5), Msx1 (28), Gbx2 (6) and Hoxa2 itself interact with very similar, if not identical nucleotides sequences (Fig. 7). We showed in this paper that Meox1 specifically interacts with the sequences recognized by Hoxa2, and its binding abilities are comparable to the ones of Hoxa2.

Recent genome- wide profiling of site-specific transcription factors has discovered that transcription factors bind thousands of binding sites in the genome (10), pointing at functional redundancy as a built-in safeguard for maintaining accurate regulation of the genome. The observed enrichment in transcription factors with similar binding affinities could provide a quantitative backup to the Hoxa2 GRN function. For instance, elimination of one GRN member could allow a higher level of binding of another GRN member. Homeodomain proteins recognize short sequences that are widespread throughout the genome, and it is believed that only a small percentage of all occurrences of a motif are actually bound by these proteins (18). A global map of the binding sites of Hoxa2 GRN members in vivo could discover potential overlaps on target promoters. These data would provide a molecular basis for the network redundancy and explain the recurring finding that loss of function of Hoxa2 targets has only a very partial, if no effect on the phenotype.

The developing somites represent the main domain of *Meox1* expression, and the

area of the embryo most affected in the absence of Meox1 (16). Hox genes are expressed in somites and control morphogenesis of the axial skeleton (30). These observations raise the intriguing possibility that *Meox1* might be a target of other Hox proteins, in addition to Hoxa2. The use of common target genes to control diverse developmental processes has been documented only for *Six2* (13, 14, 31), but it could represent a more widespread aspect of Hox function in vertebrates.

Acknowledgments

The authors thank members of the Piper-Hanley and the Hentges labs for help with sectioning, and Rudolf Grosschedl for the *Crabp1* probe. This work was supported by BBSRC grant BB/E017355/1 to N.B. The Bobola group is supported by the Manchester Academic Health Science Centre and the Manchester NIHR Biomedical Research Centre. M.M. was supported by grants PTDC/BIA-BCM/71619/2006 and by Centro de Biologia do Desenvolvimento POCTI-ISFL-4-664.

References

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-402.
2. **Baltzinger, M., M. Ori, M. Pasqualetti, I. Nardi, and F. M. Rijli.** 2005. Hoxa2 knockdown in Xenopus results in hyoid to mandibular homeosis. Dev Dyn **234:**858-67.
3. **Barrow, J. R., and M. R. Capecchi.** 1999. Compensatory defects associated with mutations in Hoxa1 restore normal palatogenesis to Hoxa2 mutants. Development **126:**5011-26.

4. **Berger, M. F., G. Badis, A. R. Gehrke, S. Talukder, A. A. Philippakis, L. Pena-Castillo, T. M. Alleyne, S. Mnaimneh, O. B. Botvinnik, E. T. Chan, F. Khalid, W. Zhang, D. Newburger, S. A. Jaeger, Q. D. Morris, M. L. Bulyk, and T. R. Hughes.** 2008. Variation in homeodomain DNA binding revealed by high-resolution analysis of sequence preferences. Cell **133:**1266-76.

5. **Bobola, N., M. Carapuco, S. Ohnemus, B. Kanzler, A. Leibbrandt, A. Neubuser, J. Drouin, and M. Mallo.** 2003. Mesenchymal patterning by Hoxa2 requires blocking Fgf-dependent activation of Ptx1. Development **130:**3403-14.

6. **Carapuco, M., A. Novoa, N. Bobola, and M. Mallo.** 2005. Hox genes specify vertebral types in the presomitic mesoderm. Genes Dev **19:**2116-21.

7. **Carroll, S. B.** 1995. Homeotic genes and the evolution of arthropods and chordates. Nature **376:**479-485.

8. **Depew, M. J., T. Lufkin, and J. L. Rubenstein.** 2002. Specification of jaw subdivisions by Dlx genes. Science **298:**381-5.

9. **Dieterich, C., H. Wang, K. Rateitschak, H. Luz, and M. Vingron.** 2003. CORG: a database for COmparative Regulatory Genomics. Nucleic Acids Res **31:**55-7.

10. **Farnham, P. J.** 2009. Insights from genomic profiling of transcription factors. Nat Rev Genet **10:**605-16.

11. **Gendron-Maguire, M., M. Mallo, M. Zhang, and T. Gridley.** 1993. Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. Cell **75:**1317-31.

12. **Hunter, M. P., and V. E. Prince.** 2002. Zebrafish hox paralogue group 2 genes function redundantly as selector genes to pattern the second pharyngeal arch. Dev Biol **247:**367-89.

13. **Kutejova, E., B. Engist, M. Mallo, B. Kanzler, and N. Bobola.** 2005. Hoxa2 downregulates Six2 in the neural crest-derived mesenchyme. Development **132:**469-78.

14. **Kutejova, E., B. Engist, M. Self, G. Oliver, P. Kirilenko, and N. Bobola.** 2008. Six2 functions redundantly immediately downstream of Hoxa2. Development **135:**1463-70.

15. **Mallo, M.** 1997. Retinoic acid disturbs mouse middle ear development in a stage-dependent fashion. Dev Biol **184:**175-86.

16. **Mankoo, B. S., S. Skuntz, I. Harrigan, E. Grigorieva, A. Candia, C. V. Wright, H. Arnheiter, and V. Pachnis.** 2003. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. Development **130:**4655-64.

17. **Manley, N. R., and M. R. Capecchi.** 1997. Hox group 3 paralogous genes act synergistically in the formation of somitic and neural crest-derived structures. Dev Biol **192:**274-88.

18. **Moens, C. B., and L. Selleri.** 2006. Hox cofactors in vertebrate development. Dev Biol **291:**193-206.

19. **Noyes, M. B., R. G. Christensen, A. Wakabayashi, G. D. Stormo, M. H. Brodsky, and S. A. Wolfe.** 2008. Analysis of homeodomain specificities allows the family-wide prediction of preferred recognition sites. Cell **133:**1277-89.

20. **Ohnemus, S., N. Bobola, B. Kanzler, and M. Mallo.** 2001. Different levels of Hoxa2 are required for particular developmental processes. Mech Dev **108:**135-47.

21. **Pasqualetti, M., M. Ori, I. Nardi, and F. M. Rijli.** 2000. Ectopic Hoxa2 induction after neural crest migration results in homeosis of jaw elements in Xenopus. Development **127:**5367-78.

22. **Pearson, J. C., D. Lemons, and W. McGinnis.** 2005. Modulating Hox gene functions during animal body patterning. Nat Rev Genet **6:**893-904.

23. **Pinsonneault, J., B. Florence, H. Vaessin, and W. McGinnis.** 1997. A model for extradenticle function as a switch that changes HOX proteins from repressors to activators. EMBO J **16:**2032-42.

24. **Prince, V., and A. Lumsden.** 1994. Hoxa-2 expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. Development **120:**911-23.

25. **Rijli, F. M., M. Mark, S. Lakkaraju, A. Dierich, P. Dolle, and P. Chambon.** 1993. A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene. Cell **75:**1333-49.

26. **Rodrigo, I., P. Bovolenta, B. S. Mankoo, and K. Imai.** 2004. Meox homeodomain proteins are required for Bapx1 expression in the sclerotome and activate its transcription by direct binding to its promoter. Mol Cell Biol **24:**2757-66.

27. **Saleh, M., I. Rambaldi, X. J. Yang, and M. S. Featherstone.** 2000. Cell signaling switches HOX-PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases. Mol Cell Biol **20:**8623-33.

28. **Santagati, F., M. Minoux, S. Y. Ren, and F. M. Rijli.** 2005. Temporal requirement of Hoxa2 in cranial neural crest skeletal morphogenesis. Development **132:**4927-36.

29. **Svingen, T. a. T., K.F. .** 2006. Hox transcription factors and their elusive mammalian gene targets. . Heredity **97:**88-96

30. **Wellik, D. M.** 2007. Hox patterning of the vertebrate axial skeleton. Dev Dyn **236:**2454-63.

31. **Yallowitz, A. R., K. Q. Gong, I. T. Swinehart, L. T. Nelson, and D. M. Wellik.** 2009. Non-homeodomain regions of Hox proteins mediate activation versus repression of Six2 via a single enhancer site in vivo. Dev Biol **335:**156-65.

Figure legends

Fig. 1. *Meox1* expression in the branchial arches.

Whole-mount and section ISH on wild-type (A, B, C, D, F, G, H, I) and *Hoxa2* mutant (E) embryos, using *Meox1* (A, B, C, D, E, H), *Hoxa2* (F, G), and *Crabp1* (I) probes. *Meox1* expression is first detected at E9.0 in the IIBA (A). At E9.5 *Meox1*-positive cells occupy most of the proximal area of the IIBA (B), they are still detected at E10.0 (C) and no longer visible after E10.5 (not shown). *Meox1* is expressed at E9.5 in both II and IIIBA (D). *Meox1* expression is specifically lost in the IIBA of *Hoxa2* mutant (E, arrowhead). F, *Hoxa2*-positive cells are detected in the entire IIBA and in rhombomere 4 (asterisk). The dotted line delimitates the proximal, *Meox1*-positive area of the IIBA. G, H, I. Adjacent parasagittal sections of E9.5 embryo. *Hoxa2* (G) and *Crabp1* (I) expressions demarcate cranial neural crest cells. *Meox1* (H) is transcribed in a subpopulation of the cranial neural crest, anterior to the second arch artery (asterisk). In A, B, C, D, E, arrowheads and arrows point at IIBA and IIIBA, respectively; I= first branchial arch.

Fig. 2. Hoxa2 binds the *Meox1* proximal promoter. A, Clustal alignment of *Meox1* proximal promoter sequences from different vertebrate species. Numbers indicate nucleotides position relatively to +1 (transcriptional start site) in mouse. Light red highlights Hoxa2 binding sites. The chromatin sequence amplified in ChIP is enclosed in a red rectangle. B, The conserved *Meox1* proximal promoter (red rectangle in A) is enriched in ChIP assays performed on E10.0 IIBAs in the presence of Hoxa2 antibody (a-a2= Hoxa2 antibody; IgG= non-specific antibody; EB= elution buffer. The input was diluted 1:300 prior to amplification). Specific enrichment is also

detected for Hoxa2 direct target *Six2*, while no enrichment is observed for *Intein*, a control, unrelated promoter. ChIP was performed on three independent pools of samples. PCRs were performed in duplicate on each pool. Results shown are from a representative set. C, ISH on E10.5 embryo shows strong *Hoxa2* expression in the IIBA (surrounded by red dots). D, Labeled *Meox1* proximal promoter (red rectangle in A), incubated in the presence of Hoxa2-programmed reticulocytes, gives rise to a retarded complex (arrowhead), supershifted by the addition of anti-Hoxa2 antibody (arrow). Nucleotide substitutions in single Hoxa2 binding sites (mBS1 or mBS2, changes are shown in A) do not abolish complex formation, while no complex formation is observed when the probe contains nucleotide substitutions in both binding sites (mBS1+ 2). E, The formation of the complexes (arrowhead) is competed by the addition of cold double-stranded oligonucleotides containing Hoxa2 binding sites (wtBS1, wtBS2), but not of oligonucleotides with the mutated site (mBS1, mBS2). Cold oligonucleotides were added at 200 (3,4,8,9) and 400 folds (5,6,10,11) molar excess.

Fig. 3. Hoxa2 activates the *Meox1* promoter. A, Craniofacial area of E10.5 mouse embryo showing first (I) and second (II) arch (red and blue, respectively). B, Semi-quantitative RT-PCR on RNA extracted from duplicates of IBA- and IIBA-derived mesenchymal cells cultured for three days (red and blue cells on top, respectively). The expression of IBA-specific genes (*Six2, Ptx1, Lhx6*) is maintained in IBA-derived cultures. IIBA-derived cultures do not express these genes. Hoxa2 expression is still detected in IIBA-, and is absent from IBA-derived cell cultures. C,
The number of ß-galactosidase-stained IBA cells is significantly increased when 4.6kb-*Meox1-lacZ* is co-transfected with Hoxa2 (a $P < 0.001$ was measured in three independent experiments). Hoxa2 fails to activate *LacZ* expression driven by the same promoter containing point mutation in BS1 and BS2 (4.6-mut*Meox1*; mutations as shown in Fig. 2A). Empty bars show the basal activity of *lacZ* constructs; black

bars show *lacZ* constructs activity in the presence of Hoxa2. The results shown are the average of three independent experiments, each performed in duplicate.

Fig. 4. Middle ear skeletal phenotype of *Meox1*$^{-/-}$*; Meox2*$^{-/-}$ mouse mutants. Skeletal phenotype of wild-type (A, D), *Meox1*$^{-/-}$*; Meox2*$^{-/-}$ (B, E) and *Hoxa2*$^{-/-}$ (C, F) E18.5 fetuses. A-C, Dissected otic capsules. In the absence of Hoxa2 stapes (s), styloid process (st) (highlighted in green in A) are replaced by mirror image copies of IBA skeletal elements (highlighted in green in C); wild-type IBA cartilages, incus (i) and malleus (m) are highlighted in orange in A, B, C. In *Meox1*$^{-/-}$*; Meox2*$^{-/-}$ mutants, the styloid process is truncated and is formed much closer to IBA skeletal derivatives with respect to the wild-type (compare double arrow in A and B; dissection of six otic capsules revealed an identical phenotype). D-F, Ventral view of the posterior cranial base, showing an hypomorphic basioccipital bone (bo) in *Meox1*$^{-/-}$*; Meox2*$^{-/-}$ and *Hoxa2*$^{-/-}$ mutants compared to wild-type.

Fig 5. *Meox2* expression in the branchial arches. Whole mount *LacZ* staining of E10.75 *Meox2*$^{+/tm1(lacZ)Mnko}$ embryos reveals *lacZ*-positive cells in the posterior region of the IIBA (arrow).

Fig 6. Meox1 and Hoxa2 display very similar binding activities in vitro. A, Labeled mouse *Six2* promoter (nucleotide -181 to -48), incubated in the presence of Meox1- or Hoxa2- programmed reticulocytes, gives rise to a retarded complex (arrowhead and arrow, respectively). Both complexes are similarly competed by the addition of cold double-stranded oligonucleotides containing the Hoxa2 binding sites identified on *Six2* promoter (wtS1, wtS2), but not by oligonucleotides containing mutated Hoxa2 binding sites (mS1, mS2). Cold oligonucleotides (sequences are shown) were added at 200 and 400 fold molar excess. B, Labeled *Meox1* promoter (nucleotide -235 to -102), incubated in the presence of Meox1-HA- or Hoxa2- programmed reticulocytes, gives rise to retarded complexes (arrows), supershifted by the addition of specific antibodies (arrowheads).

Fig 7. Members of the Hoxa2 GRN display similar binding preferences. The sequence logo indicates the DNA recognition sequence determined by the prediction tool at http://ural.wustl.edu/flyhd. The same recognition sequences were independently identified by high-throughput binding site selection (4).

Figure 1

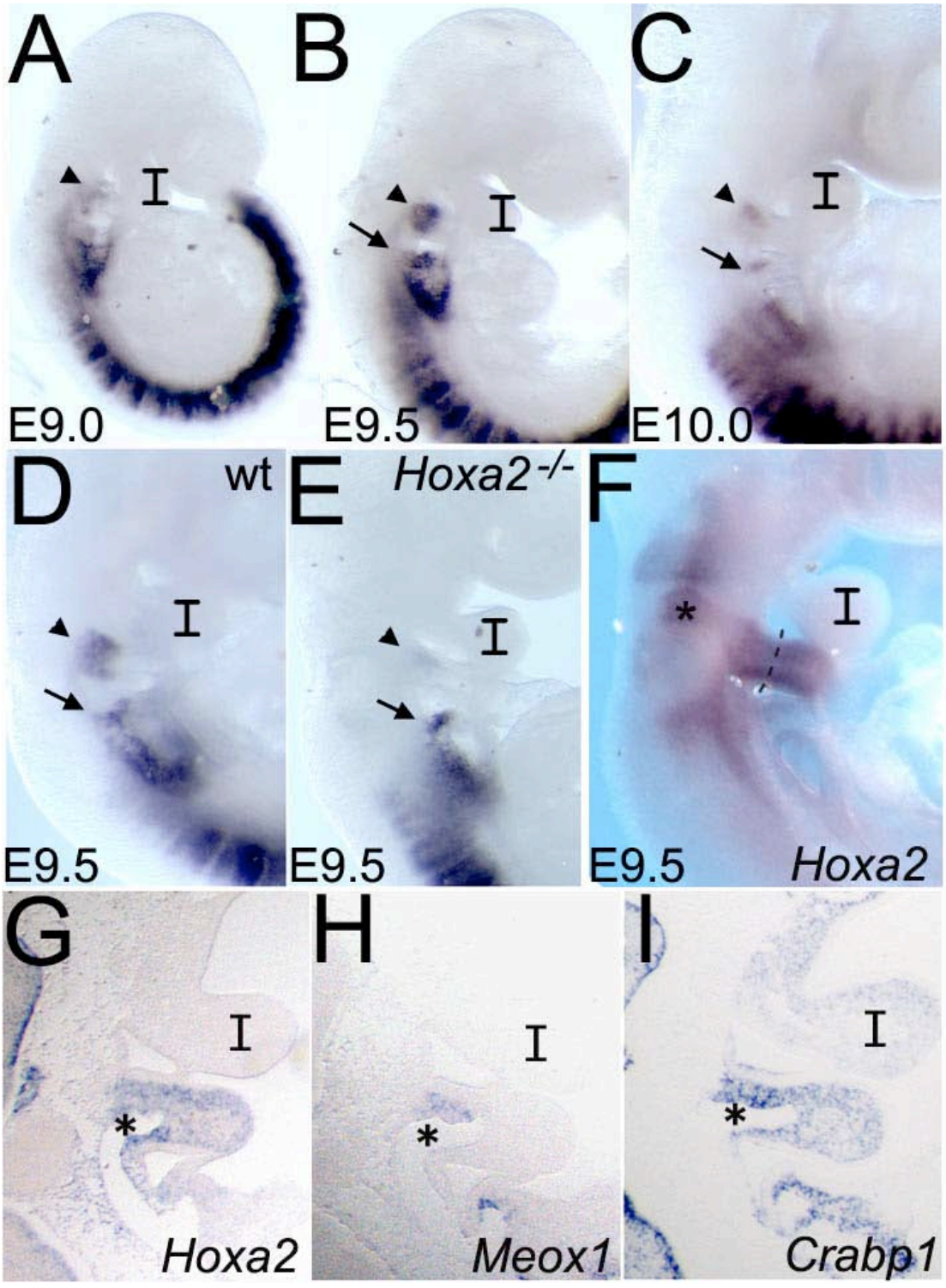
A
B
C
E9.0
E9.5
E10.0
D
wt
E
Hoxa2 -/-
F
E9.5
E9.5
E9.5
Hoxa2
G
H
I
Hoxa2
Meox1
Crabp1

Figure 2

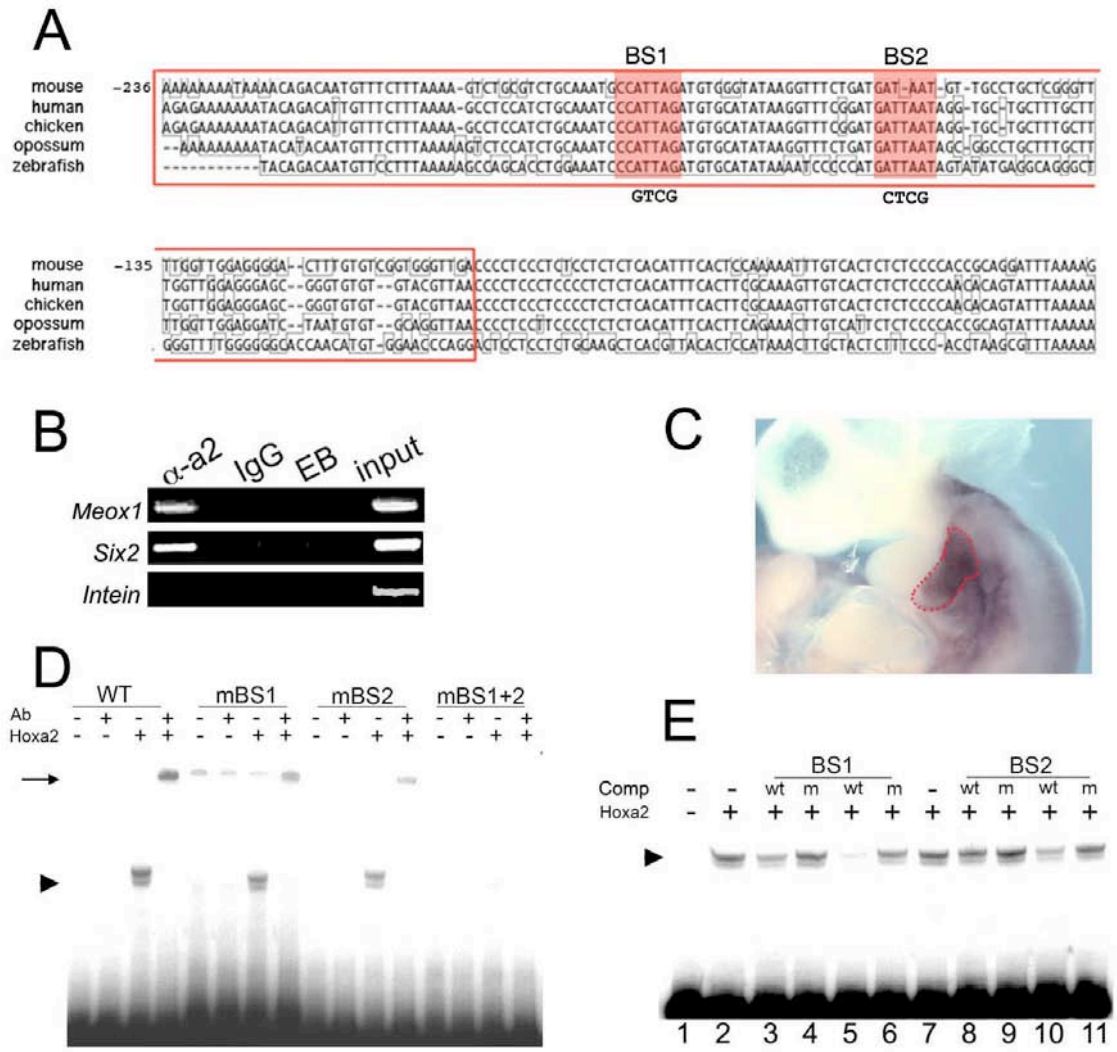
A
BS1
BS2
mouse
human
chicken
opossum
zebrafish
GTCG
CTCG
B
α-a2
IgG
EB
input
Meox1
Six2
Intein
C
D
WT
mBS1
mBS2
mBS1+2
Ab
Hoxa2
E
BS1
BS2
Comp
Hoxa2
wt
m
1 2 3 4 5 6 7 8 9 10 11

Figure 3

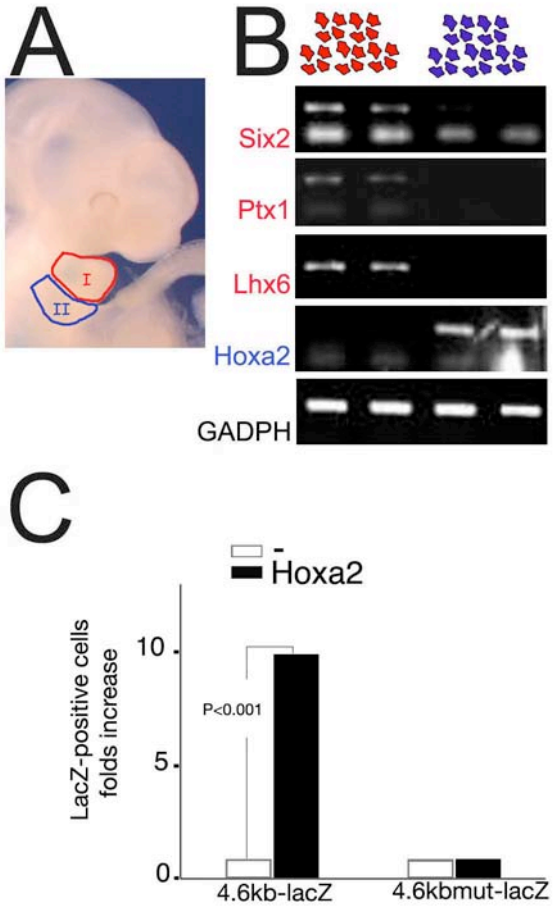
A
B
I
II
Six2
Ptx1
Lhx6
Hoxa2
GADPH
C
-
Hoxa2
LacZ-positive cells
folds increase
10
5
0
P<0.001
4.6kb-lacZ
4.6kbmut-lacZ

Figure 4

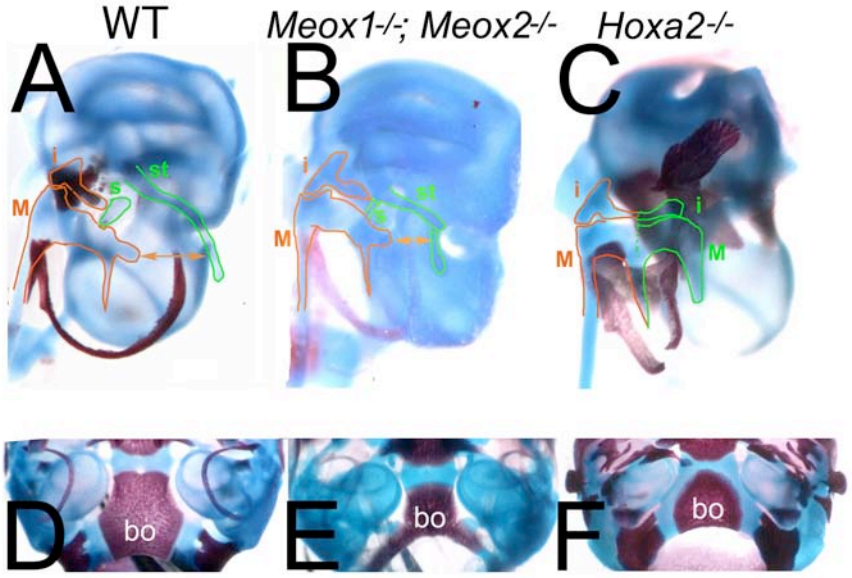

Figure 5

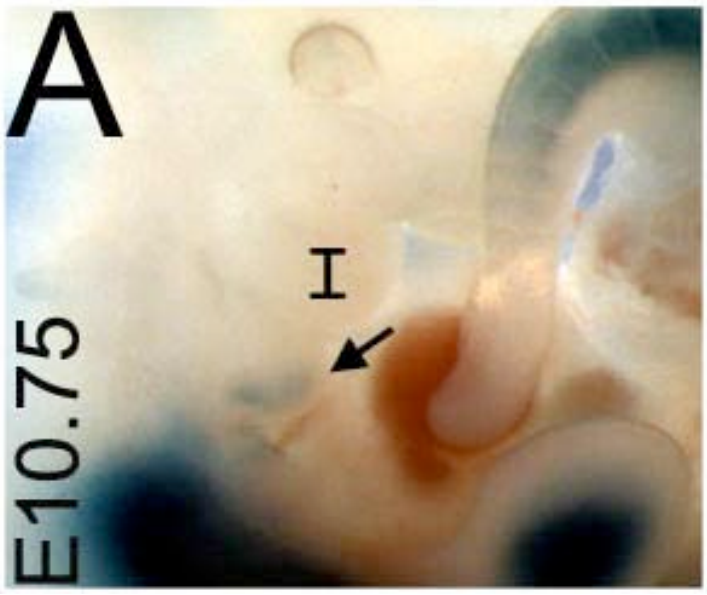

Figure 6

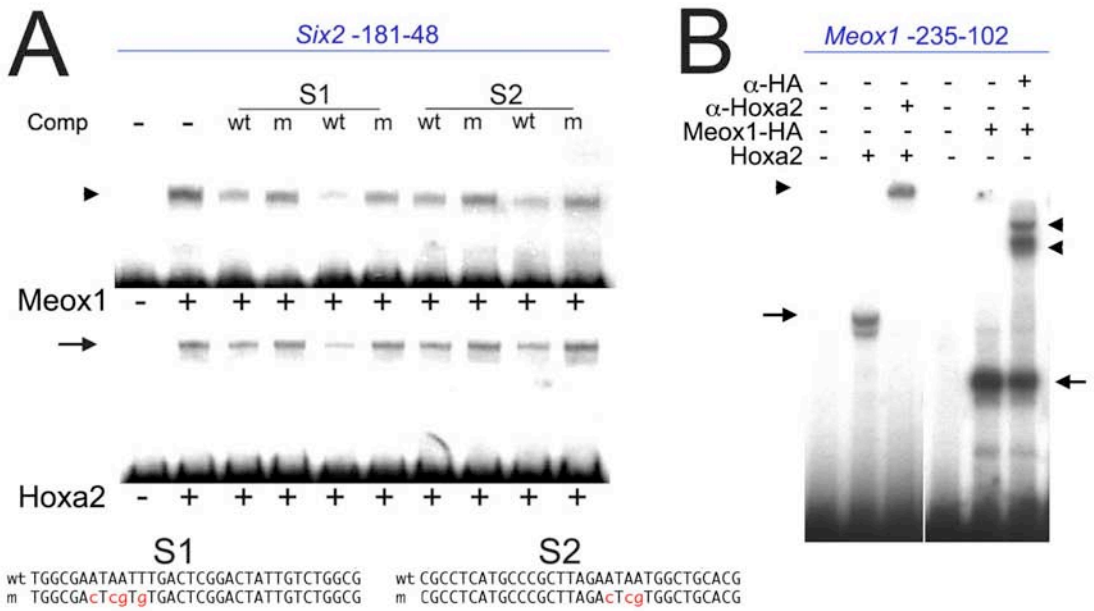
A
Six2 -181-48
S1
S2
Comp - - wt m wt m wt m wt m
Meox1 - + + + + + + + + +
Hoxa2 - + + + + + + + + +
S1
S2
wt TGGCGAATAATTTGACTCGGACTATTGTCTGGCG
m TGGCGAcTcgTgTGACTCGGACTATTGTCTGGCG
wt CGCCTCATGCCCGCTTAGAATAATGGCTGCACG
m CGCCTCATGCCCGCTTAGAcTcgTGGCTGCACG
B
Meox1 -235-102
α-HA - - - - - +
α-Hoxa2 - - + - - -
Meox1-HA - - - - + +
Hoxa2 - + + - - -

Figure 7