

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

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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).

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

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

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

1 regulated by *Hoxa2* (6, 28) are normally expressed in the IBA, but not in the IIBA,
2 suggesting that *Hoxa2* functions as a transcriptional repressor to prevent the
3 expression of IBA- specific genes in the IIBA. Moreover, the IIBA gives rise to
4 duplicates of IBA-skeletal elements in the absence of *Hoxa2*. Is IIBA identity simply
5 achieved by blocking a default IBA state or is there more to it? The finding that
6 *Hoxa2* is functionally relevant at E9.5 (Santagati et al., 2005), which is earlier than
7 the stages at which expression of most identified *Hoxa2* targets become apparent in
8 *Hoxa2* mutant IIBAs, indicates that the gene regulatory network (GRN) controlled by
9 *Hoxa2* in the IIBA must include additional target genes expressed at earlier
10 developmental stages. Their identification is essential to clarify the structure and
11 organization of the GRN that governs IIBA identity downstream of *Hoxa2*.
12 Here we show that the earliest event controlled by *Hoxa2* in the IIBA is the activation
13 of the gene encoding the transcription factor *Meox1*. The *Meox1* homeobox gene is
14 strongly expressed in the somites during embryogenesis, and controls formation and
15 differentiation of the somites and their derivatives (16). We find that, in the
16 developing IIBA, *Hoxa2* is associated to a highly conserved region of *Meox1*
17 chromatin. We further show that the interaction of *Hoxa2* with *Meox1* promoter is
18 sequence-specific and required for *Meox1* activation. Our results show that *Meox1* is
19 genetically downstream of *Hoxa2* and is a direct target. In addition, we find that
20 *Meox1* can specifically bind the DNA sequences recognized by *Hoxa2* on its
21 functional target genes, suggesting these transcription factors may share the control
22 of downstream targets in the IIBA. Finally, the analysis of *Meox1*; *Meox2* combined
23 mutants reveals that IIBA derivatives develop abnormally in the absence of *Meox*
24 genes. These findings link two co-expressed transcription factors in a novel
25 molecular pathway in mouse embryonic development. In addition, they uncover a
26 previously unidentified role for *Meox* genes in the morphogenesis of the branchial
27 arches.

28

1 **Materials and methods**

2 **Mutant and transgenic animals and embryos**

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

7 **Molecular and phenotypic analyses**

8 Whole mount and section in situ hybridization was performed as described (Kanzler
9 et al., 1998), using *Hoxa2* (Mallo, 1997), *Crabp1* (a gift from Rudolph Grosschedl)
10 and *Meox1* probe, amplified from IIBA cDNA using primers *Meox1F* 5'-
11 CACAGGAGCAGGACCGAGAGG -3'; *Meox1R* 5'-
12 CCGGAACACGCAGGATAGGTCC-3'. Skeletal phenotypes were analyzed by alcian
13 blue/alizarin red staining (Mallo and Brändlin, 1997).

14 **RT-PCR**

15 RT-PCR was performed as described (Kutejova et al., 2005), using RNA extracted
16 from 10⁶ cells dissociated from IBA and IIBA of E10.5 embryos, using 1% trypsin,
17 0.1% EDTA, filtered through a cell strainer (BD), and cultured in DMEM 10%FCS for
18 3 days. cDNA was subjected to 24 cycles of amplification using the following primers:
19 *Lhx6F* 5'-GGAGATCTACTGCAAGATGGACTAC-3; *Lhx6R* 5'-
20 CCGTCATGTCCGCTAGCTTCTG-3; *Pitx1F* 5'-AATCGTCCGACGCTGATCTGCC-3;
21 *Pitx1R* 5'-CCTTGACAGGTCCAAGTCTG-3'; *Six2* and *Hoxa2* primers are
22 described in Kutejova et al. 2008.

23 **Chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay**
24 **(EMSA)**

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

et al., 2005). *Meox1* promoter and its mutant versions were labeled using $\gamma^{32}\text{P}$ -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 μl 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 37°C, 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.

1 To confirm that *Hoxa2* regulates *Meox1* expression, we performed ISH on E9.5
2 *Hoxa2* mutant embryos. *Meox1* expression was specifically lost in the IIBA of *Hoxa2*
3 mutant embryos (Fig. 1E).
4 At E9.5, when migration of *Hoxa2*-positive cells is completed, *Hoxa2* was expressed
5 in most of the IIBA (Fig. 1F), while *Meox1* was transcribed only in the proximal area
6 of the IIBA (Fig. 1D). *Meox1* is expressed in a subpopulation of the *Hoxa2*-positive,
7 *Crabp1*-positive cranial neural crest of the IIBA (Fig. 1G-I).
8 These results indicate that *Hoxa2* positively controls *Meox1* expression in the IIBA.
9 They also show that the temporal and spatial expression of *Meox1* is more restricted
10 as compared to the one of *Hoxa2*. Therefore *Hoxa2* appears to be necessary, but not
11 sufficient to activate *Meox1* expression.

12

13 ***Hoxa2* binds a conserved sequence within *Meox1* proximal promoter**

14 *Hoxa2* contains a DNA binding domain, which interacts in a sequence-specific
15 fashion with the chromatin of target genes to control their transcription. Association of
16 *Hoxa2* to *Meox1* chromatin in vivo is therefore an essential requisite for *Meox1* direct
17 regulation by *Hoxa2*. Loss of *Hoxa2* function generates an identical IIBA phenotype
18 in mouse, frog and fish (2, 3, 11, 12, 25), suggesting that *Hoxa2* responsive elements
19 should be conserved across vertebrates. The CORG database (9) identified *Meox1*
20 proximal promoter (corresponding to positions -236 to -33 in mouse) as perfectly
21 conserved from human to fish (Fig. 2A). IIBA-extracted, *Hoxa2*-immunoprecipitated
22 chromatin showed a substantial enrichment for the most proximal *Meox1* promoter
23 region, while no enrichment was detected for an unrelated, control promoter (Fig.
24 2B). These results demonstrate that at E10.0, when *Hoxa2* is strongly expressed
25 (Fig. 2C) and still required for activation of *Meox1* transcription in the IIBA, *Hoxa2* is
26 bound to *Meox1* chromatin in vivo.
27 Binding of *Hoxa2* to its in vivo target *Six2* is mediated by two conserved TAAT
28 consensus sites (13). The *Meox1* promoter region enriched in ChIP assays contains

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

16 We used IBA-derived cells to investigate if binding of Hoxa2 to *Meox1* promoter may
17 control gene transcription. Experimental evidence indicates that IBA and IIBA cells
18 share a similar ground state, which is modified in the IIBA by the presence of Hoxa2
19 (3, 11, 21, 25); IBA cells appear therefore capable to provide an environment
20 equivalent to IIBA cells (without Hoxa2). When isolated from E10.0 branchial arches
21 and grown in monolayers, these cells maintain the molecular identity of the area of
22 origin, as estimated by expression of Hoxa2 and few of its target genes in IBA- and
23 IIBA-derived cells after three days in culture (Fig 3A, B).

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

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

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

12

13 **Meox homeodomain transcription factors control formation of the IIBA-specific** 14 **cartilages**

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

1 The above data indicate that Meox1 functions redundantly with its homolog Meox2
2 during the development of the IIBA, consistent with the experimental evidence that
3 Meox1 and Meox2 act redundantly to control somite development (Mankoo et al.,
4 2003). *Meox2* expression in the IIBA is not equivalent to *Meox1* and appears later in
5 the development of the IIBA; although we could detect *Meox2* transcripts in the
6 developing somites by ISH, we were unable to detect *Meox2* expression in the
7 branchial arches at stages earlier than E12.5. However *LacZ* staining of
8 *Meox2*^{+tm1(lacZ)Mnko} embryos indicated that the *Meox2* allele is expressed in the
9 posterior-distal domain of the IIBA already at E10.75 (Fig. 5).

10

11 **Meox1 binds Hoxa2 target sequences**

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

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

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

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

11 Discussion

12 Activation of Meox1 by Hoxa2

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

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

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

16 **The *Hoxa2* GRN: a highly redundant network?**

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

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

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

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

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

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

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

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6 **Figure legends**

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

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

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

1 detected for *Hoxa2* direct target *Six2*, while no enrichment is observed for *Intein*, a
 2 control, unrelated promoter. ChIP was performed on three independent pools of
 3 samples. PCRs were performed in duplicate on each pool. Results shown are from a
 4 representative set. C, ISH on E10.5 embryo shows strong *Hoxa2* expression in the
 5 IIBA (surrounded by red dots). D, Labeled *Meox1* proximal promoter (red rectangle in
 6 A), incubated in the presence of *Hoxa2*-programmed reticulocytes, gives rise to a
 7 retarded complex (arrowhead), supershifted by the addition of anti-*Hoxa2* antibody
 8 (arrow). Nucleotide substitutions in single *Hoxa2* binding sites (mBS1 or mBS2,
 9 changes are shown in A) do not abolish complex formation, while no complex
 10 formation is observed when the probe contains nucleotide substitutions in both
 11 binding sites (mBS1+ 2). E, The formation of the complexes (arrowhead) is
 12 competed by the addition of cold double-stranded oligonucleotides containing *Hoxa2*
 13 binding sites (wtBS1, wtBS2), but not of oligonucleotides with the mutated site
 14 (mBS1, mBS2). Cold oligonucleotides were added at 200 (3,4,8,9) and 400 folds
 15 (5,6,10,11) molar excess.

16 **Fig. 3.** *Hoxa2* activates the *Meox1* promoter. A, Craniofacial area of E10.5 mouse
 17 embryo showing first (I) and second (II) arch (red and blue, respectively). B, Semi-
 18 quantitative RT-PCR on RNA extracted from duplicates of IBA- and IIBA-derived
 19 mesenchymal cells cultured for three days (red and blue cells on top, respectively).
 20 The expression of IBA-specific genes (*Six2*, *Ptx1*, *Lhx6*) is maintained in IBA-derived
 21 cultures. IIBA-derived cultures do not express these genes. *Hoxa2* expression is still
 22 detected in IIBA-, and is absent from IBA-derived cell cultures. C,
 23 The number of β -galactosidase-stained IBA cells is significantly increased when
 24 4.6kb-*Meox1-lacZ* is co-transfected with *Hoxa2* (a $P < 0.001$ was measured in three
 25 independent experiments). *Hoxa2* fails to activate *LacZ* expression driven by the
 26 same promoter containing point mutation in BS1 and BS2 (4.6-mut*Meox1*; mutations
 27 as shown in Fig. 2A). Empty bars show the basal activity of *lacZ* constructs; black

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

3 **Fig. 4.** Middle ear skeletal phenotype of *Meox1*^{-/-}; *Meox2*^{-/-} mouse mutants.

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

14 **Fig 5.** *Meox2* expression in the branchial arches.

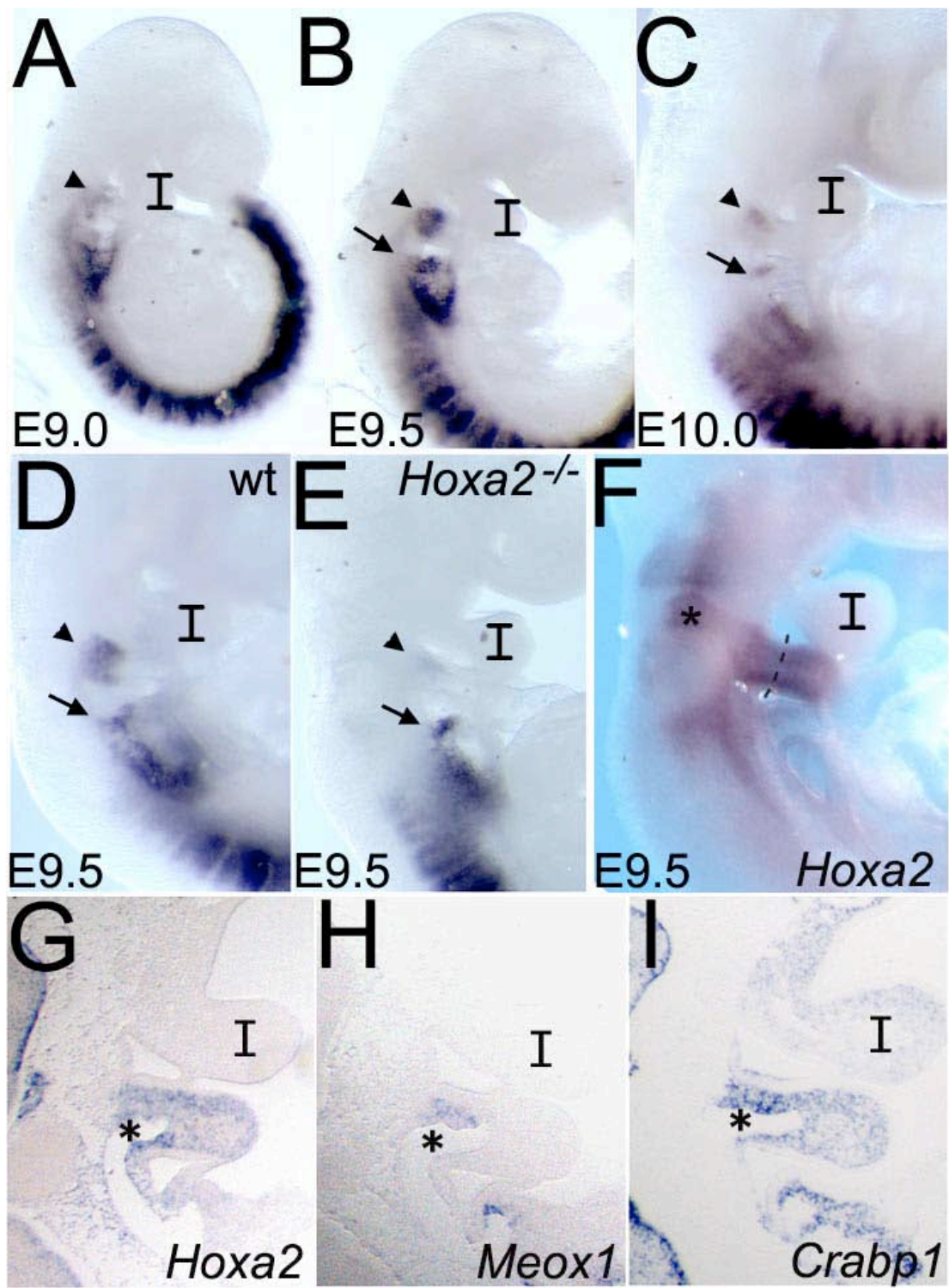
15 Whole mount *LacZ* staining of E10.75 *Meox2*^{+/tm1(lacZ)Mnko} embryos reveals *lacZ*-
16 positive cells in the posterior region of the IIBA (arrow).

17 **Fig 6.** *Meox1* and *Hoxa2* display very similar binding activities in vitro. A, Labeled
18 mouse *Six2* promoter (nucleotide -181 to -48), incubated in the presence of *Meox1*-
19 or *Hoxa2*- programmed reticulocytes, gives rise to a retarded complex (arrowhead
20 and arrow, respectively). Both complexes are similarly competed by the addition of
21 cold double-stranded oligonucleotides containing the *Hoxa2* binding sites identified
22 on *Six2* promoter (wtS1, wtS2), but not by oligonucleotides containing mutated
23 *Hoxa2* binding sites (mS1, mS2). Cold oligonucleotides (sequences are shown) were
24 added at 200 and 400 fold molar excess. B, Labeled *Meox1* promoter (nucleotide -
25 235 to -102), incubated in the presence of *Meox1*-HA- or *Hoxa2*- programmed
26 reticulocytes, gives rise to retarded complexes (arrows), supershifted by the addition
27 of specific antibodies (arrowheads).

1 **Fig 7.** Members of the Hoxa2 GRN display similar binding preferences. The
2 sequence logo indicates the DNA recognition sequence determined by the prediction
3 tool at <http://ural.wustl.edu/flyhd>. The same recognition sequences were
4 independently identified by high-throughput binding site selection (4).
5

1 Figure 1

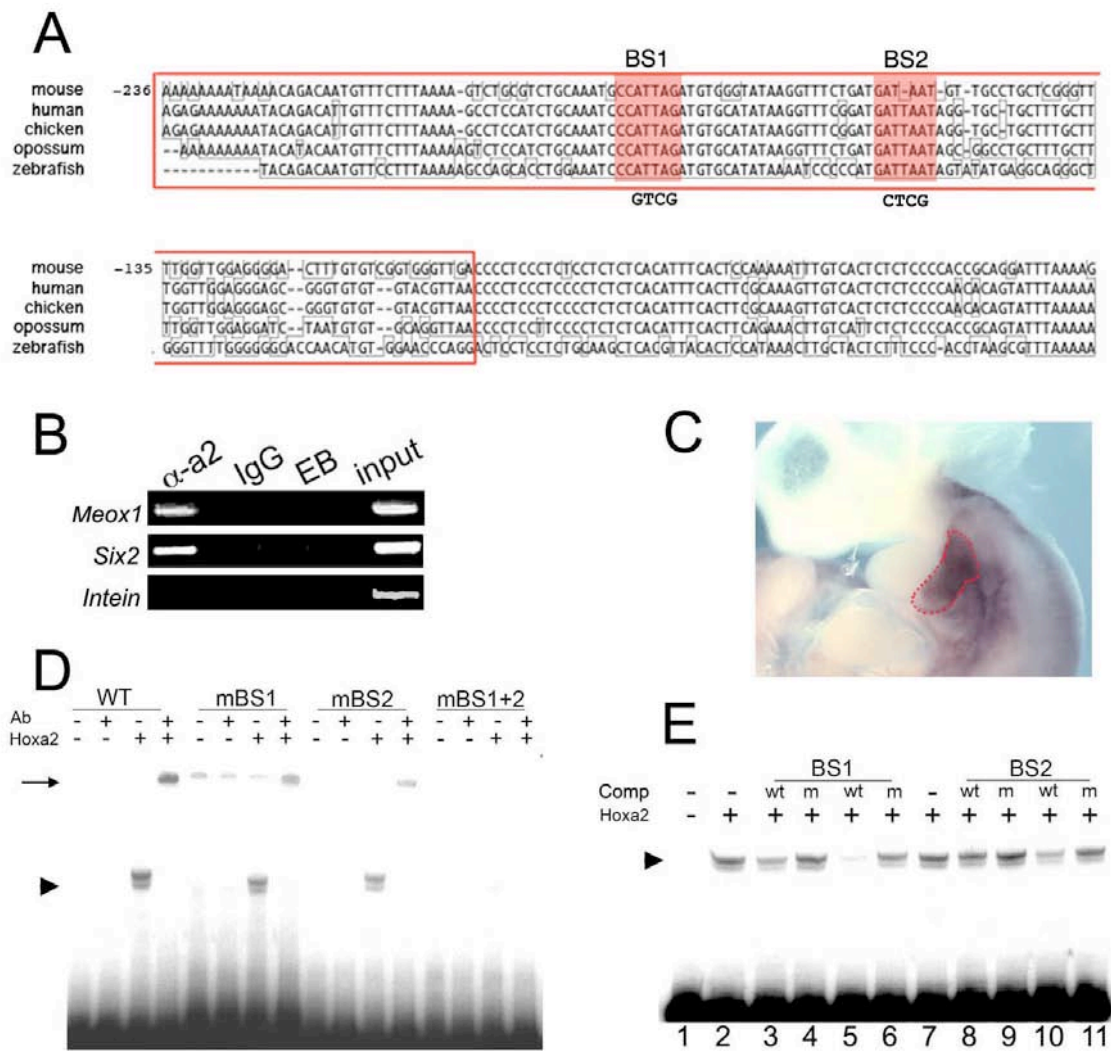
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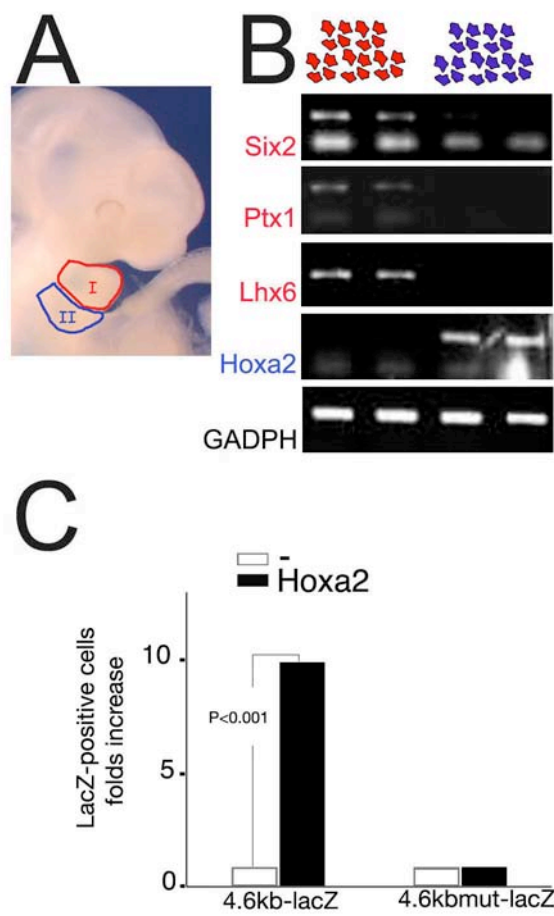
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1 **Figure 2**



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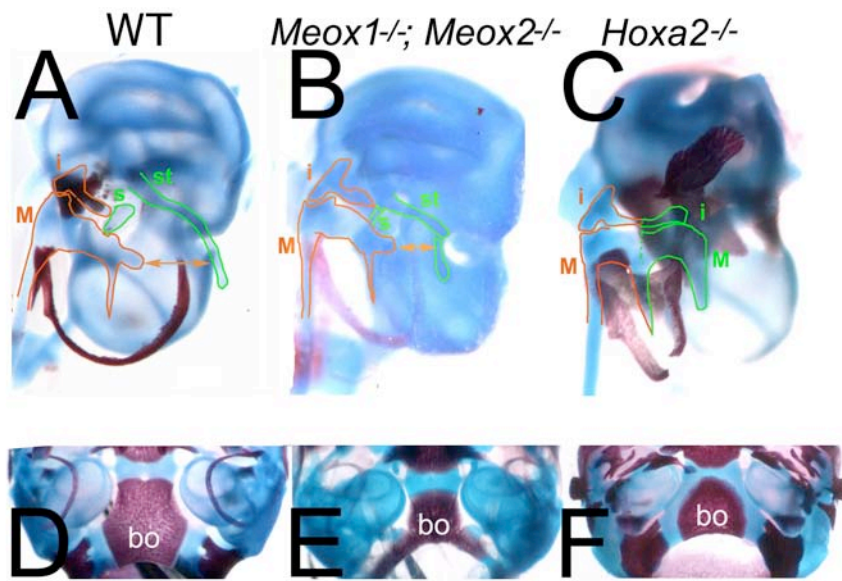
1 **Figure 3**



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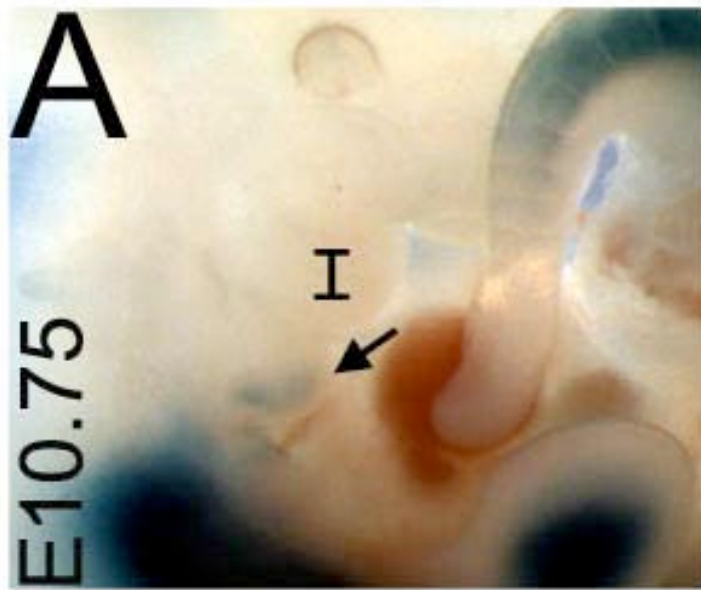
1 **Figure 4**



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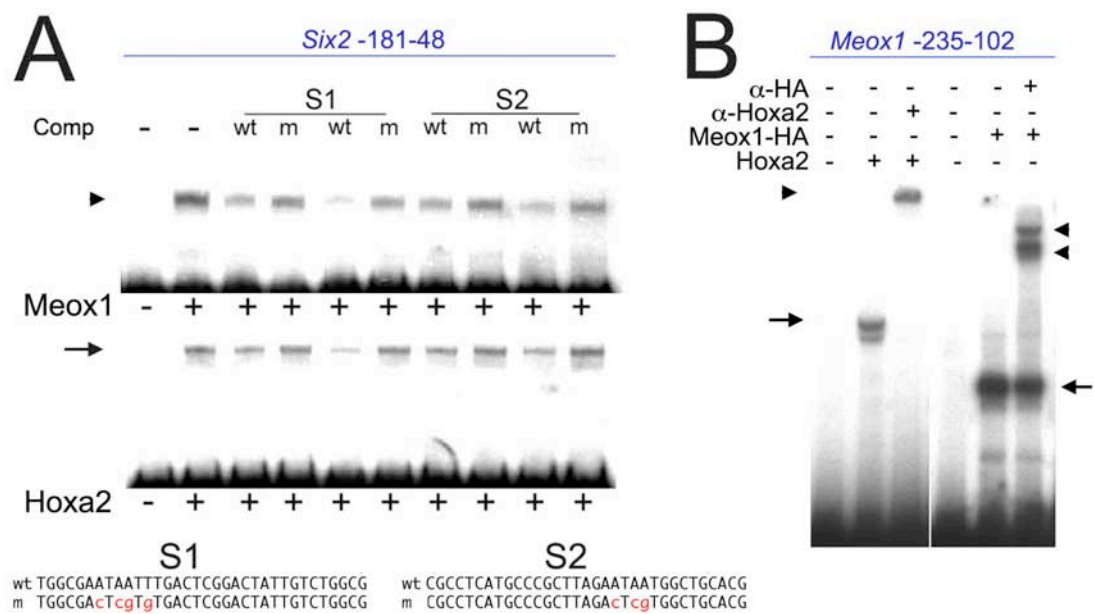
1 **Figure 5**



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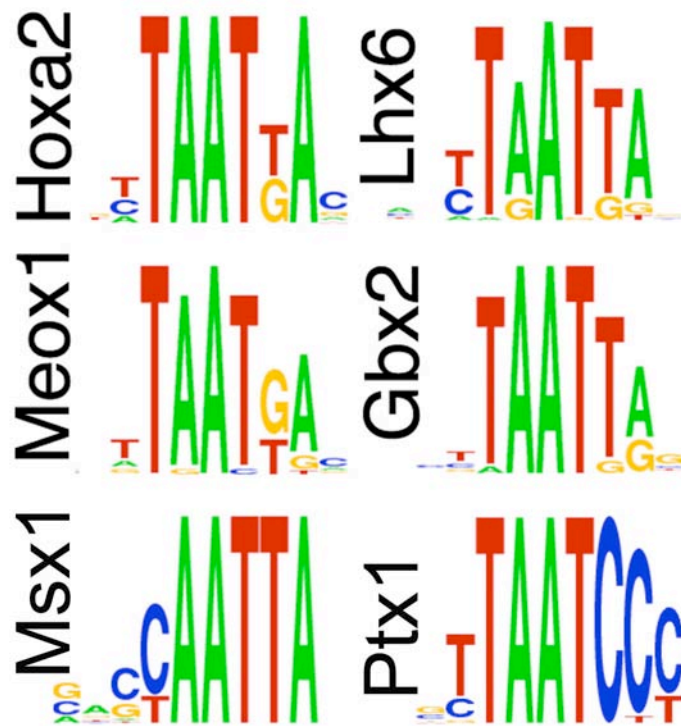
1 **Figure 6**



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1 Figure 7



2