Hoxa2 Selectively Enhances Meis Binding to Change a Branchial Arch Ground State

Highlights
- Meis provides a ground state that is common to all the branchial arches
- Hoxa2 recognizes Meis prebound sites in the second arch that contain Hox motifs
- Hoxa2 enhances Meis binding, which coincides with active enhancers, at these sites
- Hoxa2 modulates the ground-state binding of Meis to instruct second arch identity

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In Brief
Hox transcription factors instruct specific morphologies in the branchial arches, a series of segments in the developing vertebrate head. Amin et al. found that Hoxa2 operates as a tissue-specific cofactor, enhancing binding of the homeodomain transcription factor Meis to specific sites that provide the second arch with its anatomical identity.
Hoxa2 Selectively Enhances Meis Binding to Change a Branchial Arch Ground State

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SUMMARY

Hox transcription factors (TFs) are essential for vertebrate development, but how these evolutionary conserved proteins function in vivo remains unclear. Because Hox proteins have notoriously low binding specificity, they are believed to bind with cofactors, mainly homeodomain TFs Pbx and Meis, to select their specific targets. We mapped binding of Meis, Pbx, and Hoxa2 in the branchial arches, a series of segments in the developing vertebrate head. Meis occupancy is largely similar in Hox-positive and -negative arches. Hoxa2, which specifies second arch (IIBA) identity, recognizes a subset of Meis pre-bound sites that contain Hox motifs. Importantly, at these sites Meis binding is strongly increased. This enhanced Meis binding coincides with active enhancers, which are linked to genes highly expressed in the IIBA and regulated by Hoxa2. These findings show that Hoxa2 operates as a tissue-specific cofactor, enhancing Meis binding to specific sites that provide the IIBA with its anatomical identity.

INTRODUCTION

The body plan of vertebrates involves the formation of similar repetitive segments, which subsequently diversify to give rise to different body parts. A key discovery has been that Hox genes endow the initially identical segments with their distinct identities (McGinnis et al., 1984a, 1984b; Scott and Weiner, 1984).

Branchial arches are a useful model of segmental specification. This transient, metameric series of tissue bands appears in the head region of vertebrate embryos. Cranial neural crest (CNC) cells, emerging from areas of the hindbrain that express different Hox genes, colonize the branchial arches (Le Douarin and Kalcheim, 1999). All branchial arches share a ground-patterning program that is fully executed in the Hox-free first branchial arch. Hox proteins reprogram the execution of this first arch-like program in the subsequent arches. Hoxa2, a member of the Hox paralog group 2, patterns the second arch skeleton. In embryos that lack Hoxa2 function, the second branchial arch (IIBA) forms mirror image copies of first arch skeletal derivatives (Gendron-Maguire et al., 1993; Rijli et al., 1993). In addition, simultaneous inactivation of all HoxA cluster genes in the CNC leads to partial replacement of second, third, and fourth arch skeletal derivatives with multiple first arch-like structures (Minoux et al., 2009). Thus, Hox proteins appear to instruct arch-specific morphologies by overriding a ground-patterning program. Accordingly, in IIBA territory, Hoxa2 represses the expression of genes that are expressed in the anterior first branchial arch (IBA) (Bobola et al., 2003; Geisen et al., 2008; Kirilenko et al., 2011; Kutejova et al., 2005, 2008).

Intensive work in the past years has revealed that Hox genes are present in nearly all animals, and the principles of Hox gene organization and function are highly conserved throughout the animal kingdom (Carroll, 1995; Krumlauf, 1994; Lemons and McGinnis, 2006; Noordermeer and Duboule, 2013; Pearson et al., 2005; Trainor and Krumlauf, 2001). Despite this enormous progress, the logic of segment specification remains difficult to grasp.

As DNA-binding transcription factors, Hox display notoriously low binding specificity in vitro, yet they accomplish the task of selecting specific target genes to control segment morphology (Berger et al., 2008; Mann et al., 2009; Noyes et al., 2008). Hox proteins bind cooperatively with three amino acid loop extension (TALE) homeodomain transcription factors Pbx and Meis (Mann et al., 2009). Because complex formation improves the affinity and sequence selectivity of Hox proteins, the prevailing view has been that Pbx and Meis act as ancillary cofactors and assist Hox proteins in identifying their specific targets in the genome (Mann et al., 2009; Moens and Selleri, 2006). The main limitation of this view is that it is largely based on molecular and biochemical analyses in vitro. Insight into the functional interactions of Hox and TALE cofactors in their native environment in the embryo remains limited, in large part because these interactions have yet to be mapped on a genome-wide scale.
To understand how Hox operate in vivo, we mapped Meis, Pbx, and Hoxa2 binding in the native area of competence of Hoxa2, the IIBA. Through this analysis we uncovered a synergistic interaction between Hoxa2 and Meis TFs. Collectively, our findings show that Meis TFs provide a ground state that is common to all segments (arches). Hoxa2 recognizes a subset of Meis prelabeled sites, which contain Hox recognition motifs. By selectively binding to and enhancing a subset of Meis binding, Hoxa2 modifies the branchial arch ground state, established by Meis, to instruct IIBA-specific morphologies. This specific synergy between Hoxa2 and Meis is further reinforced by a positive-feedback loop, which locks IIBA cells in a state of high Hoxa2 and Meis levels. Our results highlight the importance of genomic profiling TFs in their native, tissue-specific expression domains to understand the mechanisms governing segment-specific identity.

**RESULTS**

**Hoxa2 Activates Meis Genes**

Hoxa2 controls the correct development of the IIBA (Gendron-Maguire et al., 1993; Rijli et al., 1993), most likely by binding to and regulating the expression of target genes. By analyzing Hoxa2 chromatin immunoprecipitation sequencing (ChIP-seq) (Donaldson et al., 2012), we observed frequent binding of Hoxa2 to the genomic regions that contain Meis1 and Meis2 (13 and 15 Hoxa2-bound regions were associated with Meis1 and Meis2, respectively; the average Hoxa2-bound regions per gene = 2.7). We extracted chromatin from the IIBA of E11.5 embryos and confirmed that Hoxa2 binds to Meis1 and Meis2 (Figures 1A and 1B). At the same embryonic stage (E11.5), whole-mount in situ hybridization (ISH) revealed high expression of Meis1 and Meis2 in the main Hoxa2 domain of expression, the IIBA, and in the posterior margin of the IIBA. We found that both transcripts were downregulated, and the proximal domains of Meis1 and Meis2 expression were absent in the IIBA of Hoxa2 mutant embryos (Figures 1C–1F, arrows). The binding of Hoxa2 to Meis1 and Meis2 and their downregulation in Hoxa2 loss-of-function embryos strongly suggest that Hoxa2 is directly upstream of Meis1 and Meis2 in vivo.

**Meis Transcription Factors Control the Formation of the Visceral Skeleton**

The above results suggest that Meis1/2 TFs are part of the gene regulatory network controlled by Hoxa2 to instruct the IIBA fate. Meis1 mutant mice display neither abnormalities in CNC derivatives nor Hox-related phenotypes (Azcoitia et al., 2005; Hisa et al., 2004), possibly due to the redundant functions of Meis2 (Meis3 is not expressed in the branchial arches). Therefore, because Meis1 and 2 are highly conserved in vertebrates (Longobardi et al., 2014), we used a zebrafish embryo model to systematically deplete Meis proteins. After injecting one-to-two-cell-stage embryos with morpholino oligonucleotides targeting translation of Meis transcripts expressed in the IIBA (meis1, meis2a, meis3, and meis4.1a) (Thisse and Thisse, 2005), we observed an almost complete absence of the visceral, neural crest-derived skeleton, including the skeleton derived from the Hoxa2-positive domain (Figures 2A and 2B; 14/14 embryos affected). Abnormalities in the skeletal derivatives of the branchial arches also were observed in embryos injected with a dominant-negative construct, which interfered with Meis nuclear entry (data not shown). Our findings are consistent with
Figure 2. Meis TFs Are Required to Form the Branchial Arch-Derived Skeleton

(A and B) Ventral view of zebrafish larval (6 days postfertilization) control (mismatched morpholino, MM) (A) and Meis-morpholino-injected embryos (B) head skeleton. The IIBA-derived skeleton (ceratohyal) is labeled by ch.

(C) Craniofacial region of a E11.5 mouse embryo hybridized with Meis1 antisense probe; IBA (gray) and IIBA (red) are outlined. Overlap of Meis summit regions (200 nt, overlap at least 1 nt) in the IIBA (red), with Meis summit regions in the IBA (dark gray) and Pbx summit regions in the IIBA (yellow).

(D) Distance of Meis peaks relative to Pbx peaks. Meis peaks (IIBA) are binned according to the distance to the nearest Pbx peak and labeled according to FE (high FE, dark red bars; low FE, dark blue bars).

(E) Top overrepresented functional categories associated to common Meis/Pbx-bound regions in the branchial arches. The length of the bars corresponds to the binomial raw (uncorrected) p values (x axis values). Cb, ceratobranchials; ch, ceratohyals; e, ethmoid plate; m, Meckel cartilage; and pq, palatoquadrate. See also Figure S2.
the identification of Meis1 and Meis2 as primarily involved in the formation of the viscerocranium in zebrafish (Melvin et al., 2013), and mirror the inactivation of Prep1.1, another TALE family member (Deflorian et al., 2004). Collectively, these observations indicate that Meis genes are essential for the development of the entire branchial arch-derived skeleton in zebrafish.

To identify the molecular mechanisms controlled by Meis1/2 in the branchial arches, we mapped Meis genomic occupancy in both Hox-negative (IBA) and Hox-positive (IIBA) arches in mouse. Meis bound many regions (>60,000 regions) in both Hox-positive (II) and -negative (I) branchial arches (Tables S1 and S2), which were widely distributed across the genome (Figures S2A and S2B). Importantly, we found that a large fraction of Meis binding (>30,000 Meis-bound regions) were common to both Hox-positive (II) and -negative (I) branchial arches, and largely overlapped Pbx binding in the IIBA (Figure 2C; Table S3). These binding overlaps are close in size to the binding overlaps expected across biological replicates of genome-wide-binding experiments (>50%) (Bardet et al., 2012). Indeed, the majority of Meis binding, including the highest Meis peaks, occurred within 1 kb of Pbx binding; unrelated binding to Pbx mainly consisted of low-enrichment binding (Figure 2D). According to gene ontology terms analysis, Meis and Pbx combinatorial binding in the branchial arches mapped close to genes involved in skeletal development (Figure 2E, red arrows), stem cell maintenance and differentiation, and the IBA- and IIBA-specific process middle ear morphogenesis (Figure 2E, red arrows).

In sum, Meis TFs occupy a large pool of common regions in the branchial arches and control the formation of the entire visceral skeleton, which derives from both the Hox-negative and Hox-positive branchial arches. Collectively, these observations suggest that Meis TFs have a widespread regulatory role in the CNC.

Meis Regulates Hoxa2 in the IIBA

The differential expression of Hox genes along the anteroposterior axis of the embryo is imposed by their relative positions in the chromosome and is crucial for Hox patterning activities (Duboule and Dole, 1989; Graham et al., 1989). In the branchial arches, members of the paralog group 2 (Hoxa2 and Hoxb2) are expressed in arch II, and paralogs of group 3 are expressed in arch III, while the first arch does not express any Hox genes (Hunt et al., 1991). We observed strong Meis binding at the HoxA cluster in IIBA-chromatin, peaking at the Hox2 promoter and stretching to the neighboring Hoxa1/Hoxa3 genes (Figure 3A). In the HoxB cluster, Meis binding was restricted to the Hoxa2 paralog Hoxb2 (albeit with a lower peak) (Figure 3A). In contrast, we did not detect binding of Meis to the HoxA or HoxB clusters in the adjacent anterior IBA, where Hox genes are not expressed (Figure 3A). In sum, Meis binding appears to specifically mark actively transcribed areas of the Hox clusters. Confirming these results, we found that the Hox2 proximal promoter was specifically enriched in IIBA chromatin after immunoprecipitation with Meis antibodies, but not when chromatin was extracted from the adjacent, Hox-negative IBA (Figure 3B). The Meis binding located upstream of the Hoxa2 gene (including the highest Meis peak overlapping the Hoxa2 proximal promoter) was contained in a 4.0 kb fragment sufficient to drive gene expression in the hindbrain rhombomere 3 (r3) and r5 and in the CNC migrating from r4 into the IIBA (Nonchev et al., 1996). In a cotransfection assay, Hoxa2 proximal promoter showed the strongest transactivation when Hoxa2 and Meis1 were used together (Figure 3C). The Hoxa2 proximal promoter was extremely conserved across vertebrates (Figure 3D), and, as in the mouse, the zebrafish hoxa2b proximal promoter was highly enriched in chromatin immunoprecipitated with Meis3 antibodies (Figure 3E).

Having established that Meis binding to the Hoxa2 proximal promoter is conserved from mouse to zebrafish, we turned to the zebrafish embryo model to examine the effects of Meis knockdown on Hoxa2 expression. After injecting one-to-two-cell-stage embryos with morpholino oligonucleotides targeting translation of meis transcripts expressed in the branchial arches, we observed a downregulation of hoxa2b expression in the hindbrain and IIBA of all embryos (39/39 affected). These embryos still expressed ddx2, which labeled the developing branchial arches (Figure 3F). In sum, our results indicate that Meis TFs control Hoxa2 expression and that this mechanism is conserved in vertebrates. Together with the converse activation of Meis1/2 by Hoxa2, this mechanism defines a positive-feedback loop that maintains and amplifies Hoxa2 expression in the IIBA. It also secures high levels of Hoxa2 and Meis transcripts in the same cells of the IIBA.

Hoxa2 Largely Binds Close to TALE Proteins

The prevailing view is that TALE homeodomain proteins act as ancillary cofactors for Hox; however, these interactions have yet to be mapped in vivo on a genome-wide scale. For this reason, and to fully understand the role of Meis TFs in Hoxa2-positive domain, we investigated whether Hoxa2 occupied common cis-regulatory modules with Pbx and Meis in the IIBA.

We found that the majority of Hoxa2 binding clustered within 1 kb of Meis binding. The highest Hoxa2 peaks occurred within a closer (200 nt) distance from Meis binding, and only a small fraction of Hoxa2-binding events was apparently unrelated to Meis binding (Figure 4A). A similar distribution was observed with Pbx (Figure S3A). Hoxa2 combinatorial binding largely involved the three factors (Figure 4B). Chromatin accessibility is a major determinant of TFs binding (Biggin, 2011). By mapping the binding of Foxc1 (an unrelated, nonhomeodomain TF) in E11.5 IIBAs, we observed that the binding overlap of Hoxa2 and Foxc1 was significantly lower than that of Hoxa2 and Meis (Figures S3B–S3D). Similarly, Meis bound at a significantly higher frequency with Pbx than with Foxc1 (Figure S3E), indicating that the extensive binding overlap of Hox and TALE proteins is determined by other factors in addition to the chromatin structure.

Functional annotation of Hoxa2/Meis/Pbx-shared regions identified enrichment of genes in overlapping functional categories with the entire Hoxa2 ChIP-seq (Figure 4C), while the genomic regions enrichment of annotations tool (GREAT) analysis of Hoxa2 unique binding sites did not detect association with any functional processes (not shown). Regions occupied by Hoxa2, Meis, and Pbx displayed a higher sequence conservation compared to the entire Hoxa2 ChIP-seq (Figure 4D). Collectively, these observations indicate that Hoxa2 largely binds in combination with Meis and Pbx in the IIBA, and suggest that combinatorial binding underlies Hoxa2 function.
Hox proteins form dimers with either a Pbx protein or a Meis protein, as well as trimers with one Pbx protein and one Meis protein (reviewed in Mann et al., 2009). Because complex formation improves the affinity and sequence selectivity of Hox proteins, we asked if Hoxa2/TALE complexes occupy different genomic regions relative to TALE proteins alone. Noticeably, the large majority (67%) of the regions occupied by Hoxa2 with Meis and Pbx in the IIBA overlapped with regions bound by Meis in the IBA, where Hoxa2 is absent (Figure 4E), suggesting that Hoxa2 binds Meis-prelabeled sites. Thus, binding of Meis, possibly with Pbx, provides an accessible chromatin platform for Hoxa2 to bind.

**Hoxa2 Enhances Meis Binding in the IIBA**

Next, we investigated whether the presence of Hoxa2 affects Meis binding to chromatin. As Hoxa2 does not alter the spatial occupancy of Meis TFs in the IIBA, we focused on the binding signal of Meis peaks in Hoxa2-positive (IIBA) and
Figure 4. Combinatorial Binding of Hoxa2, Meis, and Pbx

(A) Distance of Hoxa2 peaks relative to Meis peaks. Hoxa2 peaks are binned according to distance to the nearest Meis peak and labeled according to FE (high FE, dark red bars; low FE, dark blue bars).

(B) Binding overlap of Hoxa2 with Meis and Pbx in the IIBA (200 nt summit regions, overlap at least 1 nt). Only the overlapping portion of the larger Pbx and Meis data sets has been included in the figure.

(C) Functional categories identified by GREAT analysis of whole Hoxa2 ChIP-seq data set (blue bars) and Hoxa2/Meis/Pbx-shared regions (red bars). The length of the bars corresponds to the binomial raw (uncorrected) p values (x axis values).

(D) Average sequence conservation (vertebrates) of Hoxa2 binding (entire Hoxa2 ChIP-seq, continuous line) and Hoxa2 combinatorial binding with Meis and Pbx (dashed line), centered on the summit of the peaks.

(E) Overlap of Hoxa2/Meis/Pbx combinatorial binding in the IIBA (blue circle) with Meis binding in the IBA (dark gray) and Meis binding in the IIBA (red). See also Figure S3.
Hoxa2-negative (IIBA) branchial arches. Meis peaks with a higher binding signal in the IIBA (measured by fold enrichment [FE]) were preferentially located close (200 nt) to Hoxa2 binding (Figure 5A), suggesting that the presence of Hoxa2 enhances Meis occupancy on chromatin. Next, we examined the entire distribution of Meis binding in the IIBA relative to Hoxa2 binding. Whereas the vast majority of Meis binding was apparently unrelated to Hoxa2 (Figure S4A), we observed that high Meis peaks (FE > 40) preferentially occurred close to Hoxa2 binding, with a marked tendency for top Meis peaks (FE > 60) to occur close to a Hoxa2-binding event (Figure 5B). In contrast, we found that high Pbx and high Foxc1 peaks were equally distributed close to and far from Hoxa2 (Figures S4B–S4D), and occurred at a significantly lower frequency close to a Hoxa2-binding event than high Meis peaks (Figure S4E). In line with these observations, we found that Meis binding at a subset of regions bound by Hoxa2 in the IIBA (Donaldson et al., 2012) was markedly increased in the IIBA compared to the IBA, while Pbx binding was only modestly affected (Figure S4F). In summary, these observations suggest that Hoxa2 enhances Meis binding to chromatin. Reciprocally, the highest Hoxa2 peaks occurred within 200 nt of Meis binding (Figure 4A), suggesting that Hoxa2 and Meis proximity reinforces their binding to chromatin. We name this effect, which may result from cooperativity, Hoxa2 and Meis synergistic binding.

We selected a set of high-confidence, synergistic binding events to test if Hoxa2 affects binding of Meis (Figure 5C). First, we confirmed that Meis binding is increased at these regions in IIBA cells infected with pMY-Hoxa2-HA (orange) by ChIP qPCR. Asterisks indicate Hoxa2/Meis synergistic binding regions; Fbn1 and Adamts8 are control regions; and Ith4 is a negative control region. Values represent the average of duplicate samples and error bars indicate the SEM.
the IIBA (Figure 5D). Upon expressing Hoxa2 in Hox-negative IBA cells (Anderson et al., 2013; Kirilenko et al., 2011), we observed increased levels of Meis binding, and high levels of Hoxa2 binding, at these high-confidence regions. In contrast, Meis binding levels remained unaffected at regions that were not bound by Hoxa2 (Figure 5E). In this system, the Hoxa2/Meis positive-feedback loop was inactive: Meis did not bind to the Hox clusters (Figure 5A), and Hox genes were not transcribed (Hoxa2 was ectopically expressed using a heterologous promoter). As a result, Meis transcript levels were only modestly not bound by Hoxa2 (Figure 5E). In this system, the Hoxa2/Meis binding levels remained unaffected at regions that were observed increased levels of Meis binding, and high levels of Hoxa2 binding, at these high-confidence regions. In contrast, Meis binding levels remained unaffected at regions that were not bound by Hoxa2 (Figure 5E), while Meis did not bind to the Hox clusters (Figure 5A), and Hox genes were not transcribed (Hoxa2 was ectopically expressed using a heterologous promoter). As a result, Meis transcript levels were only modestly increased by Hoxa2 in this system (no change in Meis; 1.3-fold change in Meis2) (Anderson et al., 2013) and were unlikely to account for the increase in Meis-binding levels. We therefore conclude that Hoxa2 binding specifically enhances binding of Meis.

Finally, we asked whether enhancement of Meis binding in the IIBA reflects biologically meaningful differences in the branchial arches. We focused on the top 1% of Meis peaks for analysis (containing peaks with FE > 60), as only high-FE Meis-bound regions showed a skewed distribution relative to Hoxa2 distance (Figure 5B). Functional annotation of arch-specific high-confidence Meis binding showed an association with different biological processes in the IBA and IIBA (Figure 5F). Top Meis-binding events in the IIBA (n = 630) mapped close to genes that negatively regulate transcription/gene expression. These terms were exclusively enriched within the fraction of Meis peaks that overlap Hoxa2 binding (n = 347), and not with top Meis peaks unrelated to Hoxa2 in the IBA (n = 283) (Figure 5A) or top Meis peaks in the IBA (cutoff p < 1 × 10^-3). GREAT showed that top Meis binding in both branchial arches was associated with genes involved in skeletal development, consistent with the control of skeletal development by Meis TFs. Association with middle ear morphogenesis was also detected, which is consistent with the middle ear forming from both IBA and IIBA. We observed the same differential association using the top 1% of Meis ChIP-seq replicates in the IBA and IIBA (data not shown). In sum, the enhanced Meis binding in the IIBA, caused by Hoxa2, appears to target specific biological processes in the IIBA.

Synergistic Binding of Hoxa2 and Meis Is Sequence Specific

We interrogated DNA sequence motifs to identify the mechanisms underlying synergistic binding of Hoxa2 and Meis in the IIBA. We found TGAACAG, which corresponds to the canonical Meis recognition motif, as the most highly overrepresented motif in the entire Meis ChIP-seq and in highly enriched Meis peaks unrelated to Hoxa2 binding (FE > 70, closest Hoxa2-binding event > 10 kb; n = 78) (Figure 6A). In contrast, scanning Meis summit regions corresponding to synergistic binding (Figure 5C; Table S4) identified the reverse complement of GATNNAT (Figure 6A), an almost perfect match of the Hox-Pbx recognition motif (TGATNNAT). In agreement with de novo motif discovery, Meis summit regions corresponding to synergistic binding with Hoxa2 displayed a significantly higher occurrence of GATNNAT (76%; 16/21) (Figure 6B), and also a high occurrence of the single Hox motif TAAT (present in 19/21 sequences with an average 3 TAAT/peak). The occurrence of the Meis recognition motif TGAACAG was similar to the entire Meis ChIP-seq, but most Meis/Hoxa2 synergistic binding regions contained a partial Meis consensus, TGACA (86%; 18/21). Collectively, these observations suggest that the presence of closely arranged recognition motifs is important to bring Hoxa2 and Meis together. Indeed, we observed a high occurrence of GATNNAT in the entire set of Meis summits overlapping Hoxa2 binding (48%; 2,457/5,115). Further supporting this conclusion, TGAACAG was identified as the most recurrent flanking motif to TGATNNAT in Hoxa2 ChIP-seq (Donaldson et al., 2012). Moreover, Hoxa2 summit regions (200 nt) enriched in Meis and Hox recognition motifs (≥1 GATNNAT; ≥1 TGACAD; ≥3 TAAT; average sequence conservation ≥ 40%; n = 450) showed association with the gene ontology (GO) terms negative regulation of transcription and negative regulation of gene expression (p value = 1 × 10^-15), which also were enriched in the fraction of top Meis binding in the IIBA. In sum, these observations indicate that the assembly of Hox-Meis complexes is sequence based.

Cooperation of Meis and Hoxa2 at Hoxa2-Regulated Genes

Finally, we examined whether the selective enhancement of Meis binding in the IIBA underlies Hoxa2 function. As a direct readout of Hoxa2 activity, we used the changes in gene expression detected in the IIBA in the absence of Hoxa2 (Donaldson et al., 2012). We observed a strong positive correlation between Hoxa2/Meis synergistic binding and genes activated by Hoxa2. Only a small fraction of Hoxa2-binding events (8% of Hoxa2 ChIP-seq) was associated with genes regulated by Hoxa2, while up to 48% of Meis/Hoxa2 synergistic binding (Figure 5C; Table S4) was associated with genes dysregulated in Hoxa2 mutants (Figure 6C; Figure S5A). In all cases, genes displayed a decrease in expression in the Hoxa2 mutant, suggesting that Meis/Hoxa2 synergistic binding defines enhancer regions. The majority of Meis/Hoxa2 synergistic binding was located far away (>50 kb) from genes (Figure S5B), and all the regions tested displayed high enrichment of the histone mark H3K27Ac, which maps active enhancers (Figure 6D). Further suggesting that Meis/Hoxa2 synergistic binding labels IIBA long-range enhancers, insertions of a reporter gene in topologically associated domains (Chen et al., 2013; Dixon et al., 2012), which contain Hoxa2/Meis synergistic binding, highlighted the presence of regulatory domains of IIBA-specific expression (Figure 6E; Figure S5C). These domains of expression correspond to the expression of distant but associated Hoxa2-regulated genes (Figure 6E). These observations strongly suggest that Hoxa2 and Meis bind cooperatively to activate transcription. Supporting this conclusion, Meis has a positive effect on Hox-dependent transcription (Cho et al., 2009).

We therefore focused on the genes linked to synergistic binding of Hoxa2 and Meis (Table S4). Meis2 and Wnt5a were highly expressed in the IBA and downregulated in Hoxa2 mutant embryos (Figures 1C–1F; Donaldson et al., 2012). Wnt5a is required for the formation of the pinna, a IIBA-specific derivative (Donaldson et al., 2012; Minoux et al., 2013; Yamaguchi et al., 1999). Interestingly, a few of these genes were included in the GO category negative regulation of transcription/gene expression, which is specifically associated with high Meis peaks in the IBA (Zfp703, Zfp503, and Wnt5a). Zfp703 and Zfp503 encode for two related zinc-finger proteins that act as transcriptional repressors. In zebrafish, they are required for the formation of r4
Figure 6. Synergistic Binding Is Sequence Based and Associated with Hoxa2-Activated Genes

(A) Sequence logo of the top motifs identified using de novo motif discovery.

(B) Distribution of motifs in Meis peaks (entire ChIP-seq), top Meis peaks far from Hoxa2, and Meis peaks corresponding to Hoxa2-Meis synergistic binding (200 nt summit regions). Red, yellow, and blue columns represent the occurrence of TGACAG, GATNNAT, and TAAT, respectively. The occurrence of GATNNAT is significantly higher in synergistic binding events relative to Meis ChIP-seq (p values shown on the tops of columns).

(C) Percentage of Hoxa2-bound regions associated to a Hoxa2-regulated gene in the entire Hoxa2 ChIP-seq (Hoxa2_ALL), Hoxa2/Meis synergistic binding, top 1% Hoxa2 peaks (TOP_Hoxa2_ONLY), and top 1% Meis peaks (TOP_Meis_ONLY). For each category, the corresponding number of regions is indicated on the x axis.

(D) High enrichment of the histone mark H3K27Ac on Hoxa2/Meis synergistic binding regions in IIBA chromatin, relative to a negative control region using ChIP qPCR. Data are presented as the average of two independent experiments in duplicate and error bars indicate the SEM.

(E) Integration of a lacZ reporter gene in genomic regions containing Hoxa2/Meis synergistic binding events. The expression of the reporter (top) and the expression of Hoxa2-regulated genes associated to the integration sites (bottom) are shown.

(F) ChIP-seq tracks corresponding to the genomic region containing Zfp703. Meis binding in the IIBA overlapping a Hoxa2 peak (enclosed by the red rectangle) is enhanced relative to Meis binding in the IBA. Black arrow shows similar binding of Meis in the IBA and IIBA in regions not bound by Hoxa2.

(G–J) Whole-mount ISH on E11.5 wild-type (G and I) and Hoxa2 mutant (H and J) embryos, using Zfp703 (G and H) and Zfp503 (I and J) probes. Both Zfp703 and Zfp503 are specifically downregulated in the IIBA (black arrows) of Hoxa2 mutant embryos. See also Figure S5.
and the expression of r4-specific genes, including hoxa2 (Nakamura et al., 2004, 2008; Runko and Sagerström, 2003, 2004). A survey of Zfp703 and Zfp503 genomic regions revealed a high density of Hoxa2- and Meis-binding events, and a higher Meis-binding signal in the IIBA in locations that were cooccupied with Hoxa2 (Figure 6F). We examined the expression of Zfp703 and Zfp503 and found that both genes were highly expressed in the IIBA (Figures 6G and 6I, arrows) and specifically downregulated in the IIBA of Hoxa2 mutant embryos (Figures 6G–6J). In addition, consistent with their transcription being directly regulated in the IIBA of Hoxa2 (Figure 6F). We examined the expression of both genes and found that both genes were highly expressed in the IIBA (Figures 6G and 6I, arrows) and specifically downregulated in the IIBA of Hoxa2 mutant embryos (Figures 6G–6J).

**DISCUSSION**

It is clear that Hox genes specify the identities of embryonic segments, yet how they do it is still poorly understood. Our results show that Hoxa2 selectively enhances a ground-state binding of Meis in the branchial arches to instruct the IIBA-specific identity. Reinforcing Meis binding at selected enhancers can generate large phenotypic differences, because these enhancers regulate transcriptional repressors.

These findings change our interpretation of Hox/TALE cooperative binding. Rather than simply increasing DNA-binding specificity, binding with TALE enables Hox to modify the function of TALE and change a branchial arch ground state established by TALE to generate specific anatomical identities.

**TALE Proteins Recruit Hoxa2 to Chromatin**

Our results provide a molecular explanation for the recruitment of Hoxa2 to chromatin. Hoxa2 occupancy largely overlaps with Meis binding in the branchial arches, indicating that Meis creates an accessible platform that is recognized by Hoxa2. While chromatin accessibility is a main determinant of TF binding (Biggin, 2011), chromatin structure and indirect cooperativity (increase in the occupancy of a TF caused by other proteins’ binding and partial displacement of the nucleosome from the DNA) (Polach and Widom, 1996) do not entirely explain the extensive overlap of Hoxa2 and TALE.

Because Hoxa2 can interact with Meis (Williams et al., 2005), it is highly likely that Hoxa2 is recruited to chromatin by direct interaction with prebound Meis, and possibly Pbx. Collectively, the observations that Pbx acts as a pioneer factor and that it largely binds with Meis in the branchial arches and in the entire embryo (Penkov et al., 2013) suggest that Pbx could be the first determinant for Meis binding. The similar distribution of Hoxa2 peaks relative to Meis and to Pbx binding (Figure 4A; Figure S3) likely reflects the recruitment of Hoxa2 by both Meis and Pbx, or alternatively a requirement of prebound Pbx for Meis binding. Dissecting the sequence of the binding events responsible for loading Meis/Pbx complexes onto chromatin, and the precise temporal order of such events, will require additional experiments. Supporting an active role for Pbx, Pbx1-null mutant embryos display IBA defects (Selleri et al., 2001); additionally, Pbx is required for Hox and Meis binding to DNA in vitro (Longobardi et al., 2014) and is ubiquitously expressed (Yokoyama et al., 2009). Within the accessible platform provided by TALE proteins, Hoxa2 selects a subset of Meis/Pbx-prelabeled regions that contain a Hox recognition motif.

**Hoxa2 and Meis Cooperative Binding**

While Meis and Pbx similarly promote Hoxa2 binding, Hoxa2 exerts a reciprocal effect on Meis, but not Pbx. Enhanced Meis binding is observed close to Hoxa2 in the IIBA, at sites highly enriched in Meis and Hox recognition motifs. Importantly, the addition of Hoxa2 to Hox-negative cells reinforces Meis binding at selected sites. Collectively, these observations suggest that Hoxa2 and Meis bound to adjacent sites on DNA could reinforce each other’s occupancy by direct protein-protein interactions (Spitz and Furlong, 2012; Williams et al., 2005), although indirect mechanisms (e.g., the requirement of additional proteins that bridge Hoxa2 and Meis) cannot be excluded at this stage.

A range of possibilities likely contributes to higher Meis binding levels. Because TFs exchange rapidly between the DNA-bound and -unbound states, higher Meis binding levels could reflect a longer residence time at the same genomic location in the presence of Hoxa2 (e.g., Meis-binding events are stabilized by interactions with Hoxa2). In addition, the synergy between Hoxa2 and Meis is reinforced by genetic interaction. A positive-feedback loop expands Meis expression domain to Hoxa2-positive cells in the IIBA, resulting in more cells displaying Meis binding at the same sites in the IIBA relative to the IBA. In addition, the resulting higher levels of Meis1/2 and Hoxa2 in IIBA cells could allow formation of a stable Hoxa2/Meis complex on sites that may not have optimal Meis-binding sites per se.

Reciprocal Meis/Hox activation appears to be a broadly used, possibly general mechanism: XMeis3 activates Hox genes in Xenopus (In der Rieden et al., 2011) and anterior Hox and Meis2 are concomitantly induced by retinoic acid (Colberg-Poley et al., 1985; Oulad-Abdelghani et al., 1997).

**Hoxa2 Specifies IIBA Identity by Reinforcing a Ground-State Binding of Meis**

Branchial arches develop following a first arch ground program, which is modified in Hox-positive segments (arches) to shape arch-specific morphologies (Minoux et al., 2009). The widespread and largely similar occupancy of Meis TFs in Hox-negative and Hox-positive branchial arches, and the observation that Meis TFs are essential for development of the entire branchial arch-derived skeleton (including the Hoxa2-positive IIBA), implicate Meis TFs in establishing the first arch ground state. We observed that the presence of Hoxa2 mainly induces quantitative, rather than qualitative, changes in Meis binding in the branchial arches (although the existence of a limited number of functional qualitative changes in Meis binding cannot be completely ruled out). By selectively reinforcing Meis binding, Hoxa2 appears to modulate the transcriptional program controlled by Meis in the branchial arches toward IIBA-specific transcription (Figure 7). Translating these genomic findings into
a genetic network is the requisite next step, and will require defining the functional contribution of individual nodes to the network. Indeed, Meis has a positive effect on Hox-dependent transcription (Choe et al., 2009, 2014), which is partly exerted by interfering with histone deacetylase recruitment by Pbx. In agreement, our results suggest that enhancement of Meis binding may turn poised enhancers into active ones.

Likewise, more posterior arches (third and fourth arches) share a common lack of binding specificity, Hox proteins display multiple, paralog-specific Hox/TALE interaction modes (Hudy et al., 2012). These paralog-specific Hox/TALE interactions could affect the ground-state binding of Meis in different ways, thus creating the basis for instructing diverse, branchial arch-specific identities by Hox proteins from different paralog groups.

Our work shows that, molecularly, Hoxa2 does not entirely reprogram the epigenomic landscapes provided by Meis Tfs in the branchial arches. Rather, Hoxa2 truly acts as a tissue-specific cofactor to specify the identity of the second arch, where it modulates the ground-state program established by Meis proteins. Our results provide a radically new framework to understand how Hox transcription factors control development in vertebrates.

**EXPERIMENTAL PROCEDURES**

**Animal Experiments**

Hoxa2 mutant mice were described previously (Gendron-Maguire et al., 1993; Rijli et al., 1993). CD1 mice were time-mated to isolate branchial arches from E11.5 embryos. Mouse experiments were carried out under ASPA 1986. Wild-type zebrafish were raised in the University of Massachusetts Medical Center Zebrafish Facility. In situ hybridization in mouse and zebrafish were carried out as described previously (Kanzler et al., 1996; Zannino and Appel, 2009).

**Transfections**

HEK293T cells were transfected using Fugene 6 (Promega), with a total of 1 μg DNA, containing 550 ng pGL3-Hoxa2 promoter (Hoxa2 promoter from −220 to +1 cloned in pGL3 [Promega]) and 150 ng of each Hoxa2, Meis1, and Pbx1a in pcDNA3 expression vector or pcDNA3 control (Life Technologies). Cells were harvested 24 hr after transfection for luciferase reporter assays (Promega). IBAs were dissociated into single cells and infected using supernatants from Ecotropic-Phoenix packaging cells, transfected with pMYS-IRES-GFP (Cell Biolabs) or pMYS-Hoxa2-IRES-GFP (Anderson et al., 2013). The infection efficiency, evaluated by fluorescence-activated cell sorting, was 70%. Cells were cultured for 72 hr and their chromatin extracted for chromatin immunoprecipitation (ChIP).

**ChIP Assays and ChIP-Seq**

ChIP-seq experiments have been deposited in ArrayExpress (Pbx IBA, E-MTAB-1633; Meis IIBA, E-MTAB-1632; Meis IBA, E-MTAB-1631; and Foxc1 IBA, E-MTAB-2696). ChIP-seq and ChIP assays were performed as described previously (Donaldson et al., 2012; Amin and Bobola, 2014) using the following antibodies: Meis1/2 (Santa Cruz Biotechnology sc-10599X), pan-Pbx (Santa Cruz Biotechnology, sc-25411X), H3K27ac (Abcam, ab4729), Hoxa2 (Kutejova et al., 2008), Foxc1 (Abcam, ab5079), and rabbit or goat immunoglobulin G (IgG) controls. Approximately 70 IBA pairs and 100 IIBA pairs were processed for each of the ChIP-seq experiments. Enrichment of IP material was validated by SYBR green quantitative PCR (qPCR) and percentage input was calculated for at least two duplicate samples. Primer sequences are listed in Table S5. ChIP was performed on zebrafish whole embryos (24 hours postfertilization) using an antibody that crossreacts with Meis1, Meis2, and Meis3 (Choe et al., 2009).

**Bioinformatics Analysis**

For ChIP-seq analysis, 50 bp sequences from Meis IBA ChIP, Meis IIBA ChIP, Pbx IBA ChIP, Foxc1 ChIP-seq, and matched-input DNA controls were used. The Meis samples were run using two biological duplicates for the ChIP and matched-input DNA controls. Sequence reads were mapped to the NCBI37 (mm9/July 2007) release of the entire mouse (Mus musculus) genome using BFAST 0.7.0a (Homer et al., 2009a, 2009b). The mapped reads were converted into BED format for downstream analysis. Peak calling was performed using MACS version 1.4.2 (Zhang et al., 2008; http://liulab.dfci.harvard.edu/MACS/), using the matched-input DNA reads as a control. For peak calling, the “nomodel” parameter was used and the mean fragment size set at 200 bp. The threshold p value was set to p < 1 × 10⁻⁸. Binding regions with false discovery rate < 10% were selected (Meis IBA = 6,047, Meis IBA-rpt = 64,406, Meis IIBA = 17,676, Meis IIBA-rpt = 62,627, Pbx IBA = 59,341, and Foxc1 IBA = 30,834). The first replicate of Meis IBA and Meis IIBA underperformed, but 85.4% and 84.5% of the called regions were contained within the second replicates. In view of this, the second replicate was used in downstream analyses. The location of binding regions, defined by their summit coordinates relative to RefSeq gene structure, was determined using GALAXY (Goecks et al., 2010). Motif discovery and the scan of genome coordinates and the generation of the conservation profile used GALAXY (Goecks et al., 2010). Motif discovery and the scanning of known motifs in 200 bp summit regions and background sequences were performed previously (Donaldson et al., 2012). The analysis of gene annotation enrichment was performed using GREAT version 2.0.2 (McLean et al., 2010; http://bejerano.stanford.edu/great/) using the “basal plus
Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.12.024.

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Developmental Cell
Supplemental Information

Hoxa2 Selectively Enhances Meis Binding
to Change a Branchial Arch Ground State

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Supplemental Figures

Figure S1

E9.5  10.5  11.5

Meis1

A  B  C

Meis2

D  E  F
Figure S3

(A) Number of Hox peaks over distance to closest Pbx peak.

(B) Number of Hox peaks over distance to closest Foxc1 peak.

(C) Table showing counts for different categories:

|        | Hoxa2 | Meis  | Pbx   | Foxc1 |
|--------|-------|-------|-------|-------|
| Hoxa2  | 10031 | 5116  | 4588  | 1852  |
| Meis   | 62627 | 31603 | 6015  |       |
| Pbx    | 59341 | 7083  |       |       |
| Foxc1  | 30384 |       |       |       |

(D) Bar graph showing Hoxa2-Meis and Hoxa2-Foxc1 ratios.

(E) Bar graph showing Meis-Pbx and Meis-Foxc1 ratios.
Supplemental Figures legends

Figure S1 (related to Figure 1). Expression of *Meis1* and *Meis2* in the craniofacial region. Whole-mount ISH on wild-type E9.5 (A, D), E10.5 (B, E) and late E11.5 (C, F) mouse embryos, using *Meis1* (A-C) and *Meis2* (D-F) probes. The IIBA (arrows) is one of the main domains of expression of *Meis1* and *Meis2*. Arrowheads indicate the first arch.

Figure S2 (related to Figure 2). Genome-wide occupancy of Meis TFs in the IIBA. Distribution of Meis summit regions relative to Reference Sequence (RefSeq) gene structures. The pie charts indicate the proportion of reads for each gene structure; the corresponding numeric values are included. B. Distribution of Meis summits relative to the absolute distance to a transcription start site (TSS).

Figure S3 (related to Figure 4). Combinatorial binding of Hoxa2, Meis, Pbx and Foxc1 A. Distance of Hoxa2 peaks (IIBA) relative to Pbx peaks. Hoxa2 peaks are binned according to distance to the nearest Pbx peak and labeled according to fold enrichment (FE; high FE is represented by dark red bars and low FE is represented by dark blue bars). B. Distance of Hoxa2 peaks relative to Foxc1 peaks. Hoxa2 peaks are binned according to distance to a nearest Foxc1 peak and labeled according to FE, as above. C. Total number of peaks for Hoxa2, Meis, Pbx and Foxc1 followed by pairwise combinations (number of overlapping 200nt summits). D, E. Significance of pairwise overlaps The co-binding frequency of Hoxa2/Meis is significantly higher than Hoxa2/Foxc1 (D). The co-binding frequency of Meis/Pbx is significantly higher than Meis/Foxc1 (E). Using a binomial test, similar results were observed using the entire ChIP-seq (blue bars) and peaks with FE>50 (orange bars). Asteriks indicate a P value <1E-8.
Figure S4 (related to Figure 5). Hoxa2 enhances Meis binding. A. Distance of Meis peaks (IIBA) relative to Hoxa2 peaks (non cropped). Meis peaks are binned according to distance to the nearest Hoxa2 peak and labeled according to fold enrichment (FE; high FE is represented by dark red bars and low FE is represented by dark blue bars). B, C. Distance of Pbx peaks relative to Hox peaks, cropped (B) and uncropped (C). Pbx peaks are binned according to distance to a nearest Hoxa2 peak and labeled according to FE, as above. D. Distance of Foxc1 peaks relative to Hox peaks (cropped). Foxc1 peaks are binned according to distance to a nearest Hoxa2 peak and labeled according to FE, as above. E. Distribution of Meis, Pbx and Foxc1 peaks relative to Hoxa2 binding. The fraction of Meis (red), Pbx (yellow) and Foxc1 (green) peaks close (1kb) to a Hoxa2 peak are plotted with error bars showing 95% confidence region. High Meis peaks (FE≥40) occur at significantly higher frequencies close to Hoxa2 (1kb) than Pbx or Foxc1 peaks. F. Meis and Pbx occupancy in the IBA (grey panel) and IIBA (red panel) by ChIP-qPCR. Fzd1, Fzd4, Sfrp2 and Axin2 are Hoxa2-bound regions; the IIBA/IBA average enrichment ratios at these regions were 10 and 2.41 for Meis and Pbx, respectively. Regions bound by Meis alone (i.e., Fbn1, Adamts8, closest Hoxa2 binding >10 kb) showed comparable enrichments when chromatin was extracted from the IBA or the IIBA. Art3 is a negative control (unbound region). Asterisks indicate regions associated with Hoxa2-regulated genes. Data are presented as average of two independent experiments in duplicate and error bars represent the standard error of the mean. G. Functional categories associated with the top Meis peaks in the IIBA that overlap (red) or do not overlap (green) a Hoxa2 peak (200nt peak summit regions). Meis peaks overlapping Hoxa2 binding display enrichment in the GO term ‘negative regulation of transcription’ (red arrows), similar to the entire set of top Meis peaks in the IIBA. The length of the bars corresponds to the binomial raw (uncorrected) P-values (x-axis values).
**Figure S5 (Related To Figure 6). Hoxa2 and Meis synergistic binding. A.**

Association of Hoxa2/Meis synergistic binding regions with Hoxa2-regulated genes. Increasing the number of ‘synergistic binding events’ (by relaxing the FE threshold) results in lower, but still significant associations with Hoxa2-regulated genes, relative to the entire Hoxa2 ChIP-seq (dashed horizontal line at 8%). Top1% (x-axis) indicates the set of top 1% Meis peaks that overlap top1% Hoxa2 peaks; top 2% indicates the set of top 2% Meis peaks that overlap top 2% Hoxa2 peaks, etc. The number of overlapping binding events are: top1%: n = 34; top 2%: n = 70; top 3%: n = 109; top 4%: n = 155; top 5%: n = 209. B. Distribution of Hoxa2/Meis synergistic binding regions relative to the transcription start site (TSS). C. Transposon insertion in the genomic regions containing Zfp503. The insertion is located downstream of Zfp503 (red triangle).

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**Supplemental Tables and Legends**

**Table S1 (related to Figure 2).** Meis ChIP-seq dataset in the IIBA

**Table S2 (related to Figure 2).** Meis ChIP-seq dataset in the IBA

**Table S3 (related to Figure 2).** Pbx ChIP-seq dataset in the IIBA

**Table S4 (related to Figure 5). Hoxa2-Meis synergistic binding regions.** List of high confidence Hoxa2-Meis cooperative binding, their associated genes and occurrence of Hox and Meis motifs. Genes dysregulated in *Hoxa2* mutant embryos are in bold.
Table S5 (related to Experimental procedure). List of primers used in ChIP-qPCR

| Gene          | Forwards primer       | Reverse primer       |
|---------------|-----------------------|----------------------|
| Meis1 (1) enhancer | AGATGCCCAGAGAAAGCAA  | GGGGTGTGCATAGAAGGAAA |
| Meis1 (2) transcript | GCCAATCTTAGCTCCATT   | TCACTCCGCCATTCTAAACA |
| Meis2 (1) enhancer | GGGGCGATGCTGTTTTATT  | GGGTGACAGGAATGATGGAT |
| Meis2 (2) promoter | TCCCCGCACACATTTGTTA | AAATTTGGCCTCGTTCTCCT |
| Pou6f2 (Donaldson et al. 2012) | CCTGGATTTGACTCGGAAAG | CAGCTGTAACCCGACATTATGA |
| Itih4 neg (Donaldson et al. 2012) | GAGTCTGCTTTGCTTGAAACC | AAGATTTGGGCACTTTTTGGA |
| Hoxa2         | TGAGCCTTCTTCTGACT    | GGGACCAGCTCTTTTAAAA  |
| Fzd1 (Donaldson et al. 2012) | GCCAGAAAATGGTTTGCATAA | AGAGGCTGTGGAATTTCTGA |
| Fzd4 (Donaldson et al. 2012) | CTCTCCTGGCTCTGCTGAAAG | CAAGGACTCCCATTTACCTCA |
| Sfrp2 (Donaldson et al. 2012) | TCACCATGACAGGTGGAAAA | CGGAGCTGAAACAGAAGCTC |
| Axin2 (Donaldson et al. 2012) | TGCTAAAAGCCGGAAGTA  | TCGCAGTCCCTTTGAACCTC |
| Art3 neg (Donaldson et al. 2012) | GCCAAATTTAGCCAGATCCA | ACGGGGCAACAATAGCTTT |
| Fbn1          | CTTACACGCTTGTCAATT   | TCCCTTTCCCAAGTCTCCAGT |
| Adamts8       | CAGCCTCCACTTAGCAGAATT | GGCAACATTCTGCGACTTTT |


### Supplemental Experimental procedures

**ISH probes**

Probes used for *in situ* hybridization were *Meis1*, *Zfp503* (gifts from Dorothea Schulte and Carol Wicking), *Meis2* (targeting Meis2 3' UTR), *Zfp703* (Slorach et al., 2011) for mouse and *dlx2a* (Akimenko et al., 1994) and *hoxa2b* (Prince and Lumsden, 1994; Prince et al., 1998) for zebrafish.

**Antisense morpholino oligonucleotides injections**

Antisense morpholino (MO) oligonucleotides were designed to the translation start sites of *meis1* (5'- TATCTTCGTACCTCTGCGCCATCGC-3'), *meis2a* (5'- CTCATCGTACCTTTGCGCCATCAGC-3'), *meis3* (5'- AACTCCTCATACTTCTTGTGAGCCCATGC-3'), and *meis4.1a* (5'- AGATCCTCGTACCGTTGCGCCATGA-3'), with a corresponding 5bp mismatch MO designed to *meis4.1a* (5'- AGtTgCTCcTACCcTTcCGCCATGA-3') (Gene Tools, LLC).

A combination of four antisense morpholinos (MOs) was injected, each with a concentration of 0.7 mM (final combined concentration of 2.8 mM) in 1 nl total volume. A corresponding 2.8 mM mismatch control MO was also used.