Cdx1 refines positional identity of the vertebrate hindbrain by directly repressing Mafb expression

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SUMMARY
An interplay of transcription factors interprets signalling pathways to define anteroposterior positions along the vertebrate axis. In the hindbrain, these transcription factors prompt the position-appropriate appearance of seven to eight segmental structures, known as rhombomeres (r1-r8). The evolutionarily conserved Cdx caudal-type homeodomain transcription factors help specify the vertebrate trunk and tail, but have not been shown to directly regulate hindbrain patterning genes. Mafb (Kreisler, Krml1, valentino), a basic domain leucine zipper transcription factor, is required for development of r5 and r6 and is the first gene to show restricted expression within these two segments. The homeodomain protein vHnf1 (Hnf1b) directly activates Mafb expression in its normal r5 and r6 domain, even in the absence of Cdx1. Our findings identify Mafb as one of the earliest direct targets of Cdx1 and show that Cdx1 plays a direct role in early hindbrain patterning. Thus, just as Cdx2 and Cdx4 govern the trunk-to-tail transition, Cdx1 may regulate the hindbrain-to-spinal cord transition.

KEY WORDS: Caudal related proteins (Cdx), Mafb (Kreisler, valentino), Axial patterning, Developmental gene regulation, Hindbrain patterning, Rhombomere

INTRODUCTION
Early anteroposterior (AP) patterning of vertebrates occurs through the interplay of transcription factors that interpret fibroblast growth factor (Fgfs), cysteine-rich secreted Wnt glycoprotein and retinoic acid (RA) signalling pathways. These transcription factors prompt the position-appropriate appearance of segmental and, thereby, segmentally derived structures: the rhombomeres in the hindbrain, which give rise to specific cranial motor nerves, and the somites, from which vertebra in the trunk and tail are derived. Hindbrain segmentation has been a particularly useful tool for understanding early AP neural patterning. The developing hindbrain is segmented early in embryogenesis along its AP axis into seven to eight lineage-restricted compartments, the rhombomeres (Lumsden, 2004; Lumsden and Krumlauf, 1996; Schneider-Maunoury et al., 1996; Schneider-Maunoury et al., 1998; Tumpel et al., 2009). The first seven segments (r1-r7) form from the midbrain/hindbrain boundary to the first somite pair, and pseudo-rhombomere eight (r8) forms next to somites two to five (Lumsden, 2004; Schneider-Maunoury et al., 1998). Many gene expression patterns, including those of Hox genes, the most studied segmentation genes, respect rhombomere boundaries and are limited to specific subsets of rhombomeres (Lumsden, 2004; Lumsden and Krumlauf, 1996; Schneider-Maunoury et al., 1998; Tumpel et al., 2009). Their physical compartmentalization and defined rhombomere-specific gene expression patterns facilitate identification of regulatory regions and, thereby, molecules required for establishment, maintenance and refinement of the positional identity of individual rhombomeres. This provides an exceptional model for the study of vertebrate AP patterning.

RA, Fgfs and Wnts have all been implicated in AP patterning of the brain (Diez del Corral and Storey, 2004; Nordstrom et al., 2006). Many studies have identified RA, a product of vitamin A (retinol) metabolism, as the dominant posteriorizing factor in hindbrain development (reviewed in Glover et al., 2006; McCaffery et al., 2003). It is thought that the RA gradient is set up by a posterior source and an anterior sink. The source of RA is provided by retinaldehyde dehydrogenase 2 (Raldh2), which synthesizes RA in the presomitic posterior mesoderm that flanks the future r7, r8 and spinal cord. The sink is provided by RA-catabolizing enzymes, cytochrome P450s (Cyp26s), expressed in the floor plate region of the forebrain and midbrain (Dupe and Lumsden, 2001; Niederreither et al., 2000; Wendling et al., 2001). RA can act directly to induce transcription of some segmentation genes via RA receptors (RARs) and the retinoid X receptors (RXRs), which are ligand-dependent transcription factors that bind as homo- or heterodimers to specific sequences, known as RA response elements (RAREs). RAREs have been found to govern the hindbrain-specific expression of some Hox genes, such as Hoxa1, Hoxb1, Hoxb4 and...
Hoxd4 (Frasch et al., 1995; Gould et al., 1998; Langston and Gudas, 1992; Marshall et al., 1994; Moroni et al., 1993; Nolette et al., 2003; Popperl and Featherstone, 1993; Studer et al., 1998; Whiting et al., 1991). These, along with many other studies performed in the chick, quail, mouse, fish and frog have identified RA as a posteriorizing factor in vertebrate hindbrain patterning.

In response to the RA gradient, initial segmentation genes are expressed in subsets of prospective rhombomeres and induce a molecular cascade that promotes further refinement of region-specific expression patterns and segment identity (Lumsden and Krumlauf, 1996; Schneider-Maunoury et al., 1998). One of the earliest genes to be expressed segmentally in the posterior hindbrain is Mafb [previously known as kreisler, Krm1, valentino (Val)], a basic leucine zipper transcription factor (Cordes and Barsh, 1994). Mafb is expressed in the future rhombomere five and six (r5-r6) domain and is required for formation of these rhombomeres (Cordes and Barsh, 1994; Frohman et al., 1993). The original X-ray-induced Mafb mouse mutant, kreisler (kr), is a near-perfect chromosomal inversion that abolishes rhombomere-specific Mafb expression (Cordes and Barsh, 1994). In kr/kr mutants and in mouse embryos homozygous for the severely hypomorphic or null allele krmut, the r5-r6 domain does not develop properly, Hox genes expressed in the hindbrain are misregulated, and morphological segmentation in the posterior hindbrain (r4-r7) is lost (Cordes and Barsh, 1994; Frohman et al., 1993; McKay et al., 1994; Sadl et al., 2003). Mafb has been shown to directly activate rhombomere-specific expression of Hoxa3 and Hoxb3 (Manzanares et al., 1999; Manzanares et al., 1997; Yau et al., 2002), but whether it directly regulates other Hox genes is unknown.

Expression of Mafb is regulated positively and negatively by RA (Dupe and Lumsden, 2001; Gavalas and Krumlauf, 2000; Grapin-Botton et al., 1998; Hernandez et al., 2004). Mafb activation in the hindbrain depends on RA. For example, application of anRARα antagonist at the 0-somite stage, eliminates Mafb expression in mouse and chick embryos. In mouse embryos that are Raldh2−/− or RARα−/−:RARγ−/−, Mafb expression is lost completely (Niederreither et al., 2000; Wendling et al., 2001). RA levels also control the position of the posterior boundary of Mafb expression. So, reducing RA signalling in the posterior hindbrain and neural tube allows Mafb expression to expand further posteriorly beyond the r6/r7 boundary. For instance, in Raldh2 mutant zebrafish, RA signalling is decreased (Grandel et al., 2002), and embryos show posterior expansion of Mafb (val) (Grandel et al., 2002; Maves and Kimmel, 2005). These results suggest that Mafb induction by RA is concentration dependent: higher RA levels in the spinal cord and r7-r8 suppress it, whereas lower ones activate it in r5-r6.

RA-dependent activation of Mafb depends, at least in part, on the homeodomain protein, variant hepatic nuclear factor 1 (vHNF1/HNF1B) (Aragon et al., 2005; Hernandez et al., 2004; Kim et al., 2005; Lecaudey et al., 2004; Maves and Kimmel, 2005; Wiellette and Sive, 2003). At the 0-somite stage, Hnf1b expression, which is induced by RA, extends from the future r4/r5 boundary posteriorly into the posterior hindbrain and spinal cord of mouse, chick and zebrafish embryos (Aragon et al., 2005; Kim et al., 2005; Lecaudey et al., 2004; Maves and Kimmel, 2005). In the mouse, identification of the r5-r6 specific S5 enhancer from Mafb illustrated that vHNF1 is essential, but not sufficient, for driving r5-r6 expression in 0- to 10-somite-stage embryos. In vHNF1−/− zebrafish, Mafb(val) is not expressed (Sun and Hopkins, 2001). Treatment of zebrafish with anRAR panagonist leads to loss of vHNF1 and Mafb(val) expression, but Mafb(val) expression can be restored through the addition of exogenous Hnf1b (Hernandez et al., 2004). Thus, vHNF1 directly regulates RA-dependent, r5-r6-specific Mafb activation. However, although the anterior expression limits of Hnf1b and Mafb coincide at the r4/r5 boundary, Hnf1b expression extends more posteriorly in the neural tube than Mafb and, therefore, does not establish the posterior boundary of Mafb expression (Kim et al., 2005). The initial posterior boundary of Mafb expression could be established by two simple mechanisms: (1) An unknown activator, expressed in r5 and r6, but not posteriorly of the r6/r7 boundary, collaborates with vHNF1 to activate Mafb in r5-r6; or (2) a repressor with an anterior expression limit at the r5/r7 boundary inhibits Mafb expression in the posterior neural tube (Kim et al., 2005). Because the earliest genes expressed in the hindbrain have well-defined anterior expression boundaries and show diffuse expression posteriorly (Cordes, 2001; Deschamps et al., 1999; Hunt and Krumlauf, 1992) and Mafb is the earliest gene confined to r5-r6, we hypothesized that a repressive mechanism might be likely. Given the absence of RARE elements in the S5 enhancer, such a repressive mechanism might depend directly on an RA-inducible transcription factor(s).

The RA-responsive causal-type homeodomain transcription factors, which have conserved roles in posterior axial patterning of animals, are candidate Mafb repressors (Young and Deschamps, 2009). In the early Drosophila embryo, Caudal protein is present in a posterior to anterior gradient and initiates expression of posterior gap and pair rule genes that establish embryonic segmentation and induce Hox gene expression (Hader et al., 1998; La Rosee et al., 1997; Ochoa-Espinosa et al., 2005; Rivera-Pomar et al., 1995). In the mouse, there are three Cdx genes, Cdx1, Cdx2 and Cdx4, that are expressed in a stepwise manner along the AP axis of the embryo, with Cdx1 being the most anterior, followed by Cdx2, then by Cdx4 (Beck et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993). Although their anterior limits are staggered, they are all expressed into the posterior of the embryo, essentially creating a posterior-to-anterior gradient of Cdx protein (Gaunt et al., 2003; Gaunt et al., 2005). This nested pattern of expression is highly conserved across vertebrates (Charite et al., 1998; Deschamps et al., 1999; Gaunt et al., 2005; Houle et al., 2000; Lohnes, 2003; Savory et al., 2009a). Cdx genes are regulated by signalling molecules involved in AP patterning, including RA, Fgf5 and Wnts (Gaunt et al., 2005; Keenan et al., 2006; Lohnes, 2003; Pilon et al., 2006). In the mouse, RA directly regulates Cdx1 through an upstream RARE (Houle et al., 2000). Once Cdx1 expression is initiated, Wnt3a, the expression of which overlaps with that of Cdx1, acts to maintain Cdx1 expression through two response elements for the lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF), the nuclear interpreters of canonical Wnt signalling (Ikeda and Takada, 2001; Prinos et al., 2001). Cdx1 binds as a heterodimer with LEF1 at the LREs to maintain its own expression (Beland et al., 2004). Loss of the RARE and the LREs strongly reduces Cdx1 expression (Pilon et al., 2007), indicating that RA and Wnt3a signalling act synergistically to regulate Cdx1 expression (Beland et al., 2004; Lohnes, 2003; Pilon et al., 2007; Prinos et al., 2001). Experiments in Xenopus and chick indicate that Cdx1 is also induced by Fgf5 (Bel-Vialar et al., 2002; Keenan et al., 2006). Thus, Cdx1 responds to and integrates these signalling pathways during AP patterning (Young and Deschamps, 2009).

Cdx proteins specify the vertebrate trunk and tail by activating posteriorly expressed Hox genes. For example, loss of Cdx genes causes a posterior shift in Hox gene expression and anterior transformations of vertebra in Cdx1−/− and Cdx1−/−, Cdx2−/− mouse embryos (Allan et al., 2001; Subramanian et al., 1995; van den Akker et al., 2002). The homeotic transformations seen in Cdx1−/−
Cdx2+/− mice are more severe than either of the single mutants, indicating an overlapping role in vertebral patterning (van den Akker et al., 2002). Conversely, ectopic or overexpression of Cdxs expands trunk regions at the expense of the posterior hindbrain in Xenopus, zebrafish and mice (Epstein et al., 1997; Gaunt et al., 2008; Isaacs et al., 1998; Shimizu et al., 2006; Skromne et al., 2007; Young et al., 2009). For example, ectopic expression of Cdx1a or Cdx4 in the zebrafish eliminates expression of posterior hindbrain specific markers, including MafB (val) and the MafB target Hoxb3 (Shimizu et al., 2006; Skromne et al., 2007). These and other observations suggest that Cdx proteins might activate trunk-specific Hox genes and may also repress MafB in the hindbrain. Of course, such repression of hindbrain character could be a secondary consequence of misexpressing trunk-specific Hox genes.

Cdx proteins might transduce AP positional information to Hox and other segmentation genes in several ways. First, in the mouse, Cdx proteins have been shown to directly regulate some trunk-specific Hox genes, including Hoxa5 (Tabaries et al., 2005), Hoxa7 (Gaunt, 2001), Hoxb7 (Charite et al., 1998; Subramanian et al., 1995), Hoxc8 (Shashikant et al., 1995) and Hoxc9 (Papenbrock et al., 1998). Alternatively, Cdxs might regulate another layer of transcription factor(s), such as vertebrate equivalents of the ‘Gap’ genes in the fly, which then govern Hox gene expression directly (Rivera-Pomar and Jackle, 1996). Finally, Cdxs can affect Hox gene expression indirectly by regulating RA and Wnt3a signalling. For example, in Cdx2−/−; Cdx4−/− mouse embryos, the RA-degrading enzyme Cyp26a1 is downregulated, RA localization is shifted posteriorly and Wnt3a expression is also decreased (Young et al., 2009). So, Cdx proteins might affect MafB expression by altering RA signalling.

Based on the role of Cdx1 in segmental skeletal development, its expression at the appropriate region of the hindbrain, its proposed role as a transducer of RA signalling, and given that MafB expression is highly sensitive to positive and negative regulation via RA, we hypothesized that Cdx1 might repress MafB, thereby establishing the posterior boundary of MafB expression in the hindbrain. Here we show that Cdx1 is present at the right time and place to act as a posterior repressor of MafB; that Cdx1-binding sites are present in a regulatory element required to repress reporter gene expression in the posterior hindbrain and spinal cord. MafB expression is shifted posteriorly in Cdx1−/− embryos. Chromatin immunoprecipitation (ChIP) experiments demonstrate that in embryos at embryonic day 8.0-8.5 (E8.0-E8.5) Cdx1 binds within the Cdx1-binding site containing S5 enhancer. Together, these data reveal that Cdx1 acts as an early posterior repressor of MafB and, thus, plays a direct role in refining posterior hindbrain identity.

**MATERIALS AND METHODS**

**Mice**

Transgenic mice were generated by pronuclear injection into fertilized oocytes from superovulated mice from the FVB/NJ strain. The 240 bp containing the predicted Cdx1 sites (ASS-LacZ) was removed from the S5 regulatory element by digestion with XbaI and partial digestion with BglII and, after filling in, was cloned into the Norl site of the hsp88LacZ construct. The S5Hox mutation was introduced using Quickchange Site directed mutagenesis (Stratagene, La Jolla, CA, USA) using the oligonucleotides: 5′-GTATCCCTCCCCATATAGTTC-AGGCCTAGGACCTCATAACGTGATTTGTC-3′ and 5′-GCAAATA-TGATTGGAATGCTAGGCTCTGATAACTTTATGATTGGGGAG-3′-TAC-3′ to generate the S5Hoxmu-LacZ reporter. For microinjection, linear digestion followed by DNA electrophoresis separated inserts from vector sequences, and inserts were purified using GeneClean beads (Bio101, Vista, CA). LacZ expression was analyzed in embryos from two independent transgenic lines. Embryos were stained for LacZ as previously described (Whiting et al., 1991). Transgenic lines were maintained by crossing with CD1 mice.

Cdx1−/− mice, first described by Subramanian et al. (Subramanian et al., 1995), were a generous gift from D. Lohnes (University of Ottawa) and P. Gruss, and were maintained by intercrossing. Cdx2−/− embryos were generated by tetraploid aggregation of Cdx2−/− embryonic stem cells (Chawengsaksophak et al., 2004; Nagy et al., 2010) and harvested at E8.0-8.5. All animal husbandry and embryo harvesting were performed according to Canadian Council on Animal Care guidelines and approved by the Samuel Lunenfeld Research Institute animal care committee.

**Immunofluorescence and RNA in situ hybridization analyses**

RNA in situ hybridization and LacZ staining were performed as described (Kim et al., 2005). Images were captured with QImaging MicroPublisher 3.3 RTV.

Immunofluorescence was performed on 10 mm frozen sections prepared as previously described (Sadl et al., 2003). Imaging was performed on Nikon Eclipse 80i fluorescence microscope with Nikon DS-Ri1 Camera.

**Electrophoretic mobility shift assays**

Mouse Cdx1 was in vitro transcribed and translated from the Cdx1-prECMV plasmid, provided by J. Rossant, using the TNT reticulocyte lysate system (Promega). DNA-binding reactions using in vitro transcribed and translated Cdx1 protein and radio-labelled S5-S5A, S5-1SB or Cdx1-55C double-stranded oligonucleotides were performed and resolved by gel electrophoresis as described by Kim et al. (Kim et al., 2005). The forward strand oligonucleotide sequences used in these experiments were: 5′-CAATCATAGAAGTTATTATATTTATGC-3′, Cdx1-S5A-Mut1: 5′-CAATCATAGAAGTTATTATATTTATGC-3′, Cdx1-S5A-Mut2: 5′-CAATCATAGAAGTTATTATATTTATGC-3′, Cdx1-S5A-Mut1+2: 5′-CAATCATAGAAGTTATTATATTTATGC-3′, Cdx1-S5B-Mut1: 5′-GAC- CTTGAGGCGGTATATTTATGC-3′, Cdx1-S5B-Mut2: 5′-GAC- CTTGAGGCGGTATATTTATGC-3′, Cdx1-S5B-Mut1+2: 5′-GAC- CTTGAGGCGGTATATTTATGC-3′.

**ChIP on embryos**

ChIP was performed on sets of ~150 embryos at the 0- to 10-somite stage that had been fixed in 4% paraformaldehyde and stored in 100% methanol according to a protocol provided by D. Lohnes and previously described by Pilon et al. (Pilon et al., 2006). To verify IP of Cdx1 protein, western blots were performed on a tenth of each IP using standard protocols. Remaining samples were eluted from agarose beads; formaldehyde cross-links were reversed. Samples were purified using Qiagen Quick PCR Purification Kit. Real-time PCR was performed on the Applied Biosystems 7900HT Real time PCR system. Samples were prepared using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with a total volume of 20 μl and were run for a total of 40 cycles with an annealing temperature of 60°C. Each experiment was performed a minimum of twice from the embryo stage and PCRs were performed in triplicate. ChIP assays were analyzed by a two-way ANOVA using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA). ChIP values with IgG and with Cdx1 antibody were entered as percent of input for two separate IP reactions. The ANOVA test compared each group with every other group followed by an ANOVA test compared each group with every other group followed by a Bonferroni post-hoc test. Primers used were: Cdx1-autoF 5′-GAGGAGCTGGAAGCCCAACAGCT-3′, Cdx1-autoR 5′-GCGGTTTCATTTGAC-3′, Cdx1-autoF 5′-GAGGAGCTGGAAGCCCAACAGCT-3′, S5-2kF 5′-CCACGAGAGGCTCATCACA-3′, S5-2kR 5′-AGAGGATTCCATTCTTCAGATGAGGCTCATCACA-3′, 5′-S5R 5′-CATTCTCACCTGAGAGGCTCATCACA-3′, S5-2kF 5′-ATCTAGAAGGAGGCAGCTCATCACA-3′, krprF 5′-ATCG- CTAATTTAAGGGCCAGCCGCT-3′, krprR 5′-TAGAAGCCCAAAGAAGTCCGAGAG-3′.
RESULTS
A Cdx-binding-site-containing region restricts posterior S5-LacZ reporter expression

To address the molecular mechanisms that act to exclude Mafb expression from the neural tube posterior of the r6/r7 boundary in the hindbrain, we analyzed the early-acting, rhombomere-specific S5 enhancer from the Mafb gene, which directs expression of a LacZ reporter in r5-r6 in 0- to 10-somite-stage embryos (Fig. 1A,B) (Kim et al., 2005). Upon dissection of this regulatory region, we found that removing a 240 bp sequence from S5 (ΔS5-LacZ) resulted in expansion of LacZ expression past the r6/r7 boundary into the posterior neural tube in 0- to 10-somite-stage embryos from two out of two transgenic lines (Fig. 1C,D). These observations suggest that binding site(s) for a repressor may reside within this region.

Analysis of the transcription-factor-binding sites within the deleted segment identified multiple candidate Cdx-binding sites and one candidate Hox/Pbx-complex-binding site, referred to as SSHox (Fig. 1F). Hox proteins achieve high-affinity DNA binding by forming heterodimers with the Pbx family of transcription factors (Chan et al., 1997; Monica et al., 1991). Such Hox/Pbx complexes have been shown to directly govern some rhombomere-specific genes (Gould et al., 1997; Popperl et al., 1995; Saleh et al., 2000). Hoxb4 and Hoxd4 genes show expression up to the r6/r7 boundary in E8.5 embryos (Breder et al., 2003; Chan et al., 1997; Folberg et al., 1999; Gould et al., 1998; Morrison et al., 1997; Zhang et al., 2000; Zhang et al., 1997), when the posterior boundary of Mafb expression is first established, and thus, might be candidate Mafb repressors. To test possible involvement of the SSHox site in Mafb repression, we introduced mutations, which had been shown to eliminate Hox/Pbx binding in other studies, to generate the SS-Hox-mut reporter (Di Rocco et al., 1997; Gould et al., 1997; Kutejova et al., 2005; Serpente et al., 2005). In mouse embryos, from three out of three lines carrying the SS-Hox-mut transgene, LacZ expression was still restricted to r5 and r6 (Fig. 1E). A few speculles of LacZ expression were observed posterior of the r6/r7 boundary, but an equivalent amount of staining was also seen in the S5-LacZ reporter (Fig. 1B) and in embryos carrying an enhancerless hspa8-LacZ reporter (data not shown). Thus, Hox/Pbx complexes do not play a significant role in directly establishing the posterior boundary of Mafb expression via the SS-Hox site. Of course, these findings do not exclude the possibility that Hox proteins could influence Mafb repression by unconventional sites.

Because the 240 bp deleted region contains multiple consensus Cdx-binding sites (Margalit et al., 1993) (Fig. 1F,G) and such arrays of Cdx-binding sites have been shown to regulate Hox gene expression within the trunk (Tabaries et al., 2005; Gaunt, 2001; Charite et al., 1998; Subramanian et al., 1995; Shashidhar et al., 1995; Papenbrock et al., 1998), we tested whether any of these could bind Cdx proteins in vitro in electrophoretic mobility shift assays (EMSA). In vitro Cdx proteins are thought to preferentially bind as homo- or heterodimers to two closely juxtaposed Cdx sites. Hence we refer to pairs of juxtaposed Cdx sites as homodimer sites. Out of four predicted Cdx homodimer binding sites, three homodimer sites, which we named Cdx1-SSA, Cdx1-SSB and Cdx1-SSC, bind in vitro transcribed-translated Cdx1 protein and did so specifically. Cdx1 binding could be competed away by addition of a double-stranded oligonucleotide containing an established Cdx1-binding site from the Sif1 gene (Suh et al., 1994) or the Cdx1-SSA, Cdx1-SSB or Cdx1-SSC sites themselves (Fig. 2A-C: lanes 3-6). Mutating a single Cdx site within each homodimer binding site did not abrogate its ability to compete for Cdx1 binding (Fig. 2A-C: lanes 7-10). However, simultaneously mutating both single binding sites in each of the Cdx1-SSA, Cdx1-SSB or Cdx1-SSC oligonucleotides eliminated the ability of these oligonucleotides to effectively compete for Cdx1 binding (Fig. 2A-C: lanes 11, 12). Competition with oligonucleotides containing a mutated SIF1 site, that had previously been shown to no longer bind Cdxs, had no effect on Cdx1 binding to any of the S5-Cdx sites (data not shown). These three Cdx homodimer sites were also bound specifically by Cdx2 and Cdx4 in EMSA assays and testing overlapping fragments spanning the 240 bp region demonstrated that Cdx binding localized only to the 70 bp containing these three Cdx homodimer sites (data not shown). Thus, Cdx proteins bind specifically to all three S5-Cdx homodimer sites in vitro and these sites may be involved in restricting Mafb expression.

The anterior boundary of Cdx1 and posterior boundary of Mafb expression coincide

In order for Cdx1 to establish the posterior boundary of Mafb expression Cdx1 protein must be present in the hindbrain at the r6/r7 boundary and more posteriorly early in embryogenesis. Previous reports determined that Cdx1 is present in the posterior hindbrain from the 1- to 10-somite stages (E8.0-E8.75) (Houle et al., 2000; Meyer and Gruss, 1993), a period that coincides with the presence of Hnf1b expression and Mafb induction (Kim et al., 2005).
Expression is maintained throughout the posterior neural tube from the appearance of the first somite to the 35-somite stage (E8.0-E10.5), but the anterior boundary of expression regresses into the spinal cord during development (Meyer and Gruss, 1993). Cdx2 and Cdx4 expression does not extend into the hindbrain (Beck et al., 1995; Gamer and Wright, 1993). To localize the Cdx1 expression domain relative to the Mafb-expressing r5-r6 region, we performed immunofluorescence experiments with Cdx1- and Mafb-specific antibodies on serial sections of embryos at the 0- to 12-somite stages (E8.0–E8.5). In these experiments we found that the anterior boundary of Cdx1 expression coincided with the posterior boundary of Mafb expression at 0- to 8-somite stages (E8.0–E8.5) (Fig. 3A-D). At the 10-somite stage (E8.75), there was a gap between the posterior boundary of Mafb and the anterior boundary of Cdx1 expression (data not shown). By the 12-somite stage, Cdx1 expression had receded from the hindbrain and anterior spinal cord altogether (Fig. 3E,F). These findings are in line with other analyses of Cdx1 expression (Meyer and Gruss, 1993; Gaunt et al., 2003; Gaunt et al., 2005; Houle et al., 2000; Lohnes, 2003; Béland et al., 2004; Pilon et al., 2007). Moreover, analyses of E9.5 embryos doubly transgenic for the Z/AP reporter, in which LacZ expression is not expressed in the hindbrain, but is expressed early in a domain that extends from the tailbud to the anterior spinal cord. Cdx2 embryos die at preimplantation because of placental defects, which can be rescued in embryos generated by tetraploid aggregation. When we examined Mafb expression in Cdx2–/– embryos by examining Cdx2–/– embryos, expansion of Mafb expression into the posterior neural tube was observed (Fig. 4). However, this expansion was seen only in 0- to 8-somite stage (E8.0–E8.5) embryos (Fig. 4A-D), after which, by the 10-somite stage, ectopic posterior Mafb expression disappeared and the normal r5-r6 expression pattern was regained (Fig. 4E,F). This expansion was most pronounced at four somites (E8.0), and thereafter expression began to regress towards the anterior. Expansion of Maf protein expression was also seen in Cdx1–/– embryos at the four-somite stage (E8.0) (Fig. 4G,H). We also examined whether lowering overall Cdx dosage in the posterior neural tube might affect Mafb expression by examining Cdx2–/– embryos. Cdx2 is not expressed in the hindbrain, but is expressed in a domain that extends from the tailbud to the anterior spinal cord. Cdx2 embryos die at preimplantation because of placental defects, which can be rescued in embryos generated by tetraploid aggregation. When we examined Mafb expression in Cdx2–/– embryos generated by tetraploid aggregation, Mafb was expressed essentially normally in r5 and r6 in 0- to 18-somite stage embryos (see Fig. S1 in the supplementary material; data not shown). So, Cdx2 on its own is not required for repressing Mafb in the posterior neural tube. Thus, Cdx1 directly or indirectly establishes the initial posterior boundary of Mafb expression and a Cdx1-independent mechanism acts to maintain r5-r6-specific Mafb expression in r5-r6 after the 10-somite stage.

**Cdx1 binds at the S5 enhancer in vivo**

To test whether Cdx1 might bind within the S5 regulatory element in embryos, we performed ChIP analyses with an anti-Cdx1 antibody on 0- to 12-somite-stage embryos (E8.0–E8.5). The embryo ChIP samples were analyzed with quantitative PCR (qPCR) using primers within and flanking the S5 Cdx-binding sites as well as primers at the cdx1 auto-regulatory region and the Mafb promoter as positive and negative controls, respectively. Cdx1

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**Fig. 2. Cdx1 protein binds to three predicted Cdx homodimer-binding sites from the 240 bp deleted S5 sequence in vitro.** (A-C) EMSA using in vitro transcribed-translated Cdx1 protein detect binding to oligonucleotides containing each of three predicted Cdx1 homodimer-binding sites: (A) Cdx1-SSA, (B) Cdx1-SSB and (C) Cdx1-SSC. Adding 10- or 100-fold molar excess of the SIF1 oligonucleotide containing the known Cdx1-binding sites from SIF1 gene (lanes 3,4) abolished binding, as did competition with 10- or 100-fold excess of Cdx1-SSA (A, lanes 5,6), Cdx1-SSB (B, lanes 5,6) and Cdx1-SSC (C, lanes 5,6). Oligonucleotides containing mutations within in only one half binding site still effectively competed for binding (lanes 7-10). However, oligonucleotides, in which both Cdx1 half binding sites were mutated, could no longer compete for Cdx1 binding (lanes 11,12). (D) Sequences of the predicted normal and mutated Cdx1-SSA, Cdx1-SSB and Cdx1-SSC binding sites are shown.
bound at its auto-regulatory region as expected ($P<0.001$), but not at the $Mafb$ promoter, or $2\text{ kb}$ upstream or downstream of the S5 $Cdx1$-binding sites (Fig. 5). $Cdx1$ binding was detected within the S5 enhancer in embryos confirming an in vivo interaction ($P<0.001$) (Fig. 5). This is the first evidence for in vivo binding of a transcription factor to the endogenous S5 rhombomere-specific $Mafb$ enhancer and is consistent with a direct role of $Cdx1$ in regulating early $Mafb$ expression. All of these data point towards a cell-autonomous role for $Cdx1$ in repressing $Mafb$ expression in the posterior hindbrain and spinal cord and, thereby, preventing these from assuming more anterior r5-r6 like identity.

**DISCUSSION**

Here we have uncovered a role for $Cdx1$ in the direct repression of the r5-r6 specific hindbrain segmentation gene $Mafb$. Ectopic or overexpression of $Cdx$ genes in *Xenopus* mouse and zebrafish eliminates expression of posterior hindbrain patterning genes, including $Mafb$ in some cases (Epstein et al., 1997; Gaunt et al., 2008; Isaacs et al., 1998; Shimizu et al., 2006; Skromne et al., 2007; Young et al., 2009). Conversely, reducing $Cdx$ gene dosage leads to expansion of the posterior hindbrain and $Mafb$ expression into the posterior neural tube. However, these effects on $Mafb$ expression could have been mediated indirectly by $Cdx$ target genes or by effects of $Cdx$ dosage on overall Wnt and RA signalling. Notably, transplantation experiments in zebrafish showed that simultaneous reduction of both $Cdx1a$ and $Cdx4$ leads to cell-autonomous $Mafb$ derepression in the posterior neural tube and, thus, suggested that either $Cdx$s or $Cdx$ targets might normally repress $Mafb$ in the posterior neural tube (Skromne et al., 2007). All past observations are consistent with our discovery of direct $Mafb$ repression by $Cdx1$. Thus, $Mafb$ is the earliest AP patterning gene known to be directly regulated by $Cdx1$ – aside from $Cdx1$ autoregulation. $Cdx1$ and $Cdx2$ are known to interpret the RA signal to Hox genes expressed along the spinal cord (Allan et al., 2001; Gaunt et al., 2008; Tabaries et al., 2005; van den Akker et al., 2002). Similarly, $Cdx1$ appears to be responsible, at least in part, for the early RA-dependent repression of $Mafb$ expression posterior of the r6/r7 boundary.

A redundancy in the actions of $Cdx1$ and $Cdx2$ could explain the differences in expansion observed for $\Delta S5$-$LacZ$ in wild-type embryos when compared with endogenous $Mafb$ expression in $Cdx1^{+/+}$ embryos. Although there is obvious posterior expansion of $Mafb$ expression in $Cdx1^{+/+}$ embryos, it is not as dramatic as that observed for the $\Delta S5$-$LacZ$ reporter in which all $Cdx$ sites were deleted. A simple explanation is that, when all $Cdx$ binding is eliminated, the $LacZ$ reporter is free to be expressed throughout the posterior neural tube. By contrast, when only binding of $Cdx1$ is eliminated, $Mafb$ expansion is limited to the posterior neural tube and anterior spinal cord, where $Cdx1$ is the only $Cdx$ gene expressed. In the posterior neural tube, levels of $Cdx2$ and $Cdx4$...
Cdx1 directly regulates hindbrain patterning

In transgenic mice, where Cdx1, Cdx2 and Cdx4 may all collaborate to repress MafB in the posterior neural tube, and the three Cdx homodimer sites present in S5 may require only low levels of Cdx; for example in the hindbrain only Cdx1 is required to mediate repression. Although our findings are all in line with a pivotal repressive role for Cdx1 in the posterior hindbrain and possibly Cdx1, Cdx2 and Cdx4 in the spinal cord, we cannot exclude the possibility that other sites within the 240 bp region and/or additional regulators other than Cdxs may contribute to the posterior repression of MafB. Nonetheless, our data do ascertain direct involvement of Cdx1 in establishing the early r6/r7 boundary of MafB expression.

Although MafB is now the earliest known axial Cdx target, it is not the only segmentation gene repressed by Cdxs. Previously, Cdxs have been implicated in defining the posterior boundary of Hoxa5 expression in the spinal cord (Tabaries et al., 2005). Hoxa5 expression extends from the caudal end of the posterior myelencephalon (the r8/spinal cord boundary) in the neural tube to vertebra 3 in the preaxial skeleton. In the mouse, two Cdx-binding sites in a Hoxa5 enhancer, essential for mesodermal expression in cervical and upper thoracic regions of E12.5 embryos, were required for establishing the posterior boundary of LacZ reporter gene expression in transgenic mice. In contrast to our findings, in vivo data did not corroborate a possible repressive role for Cdxs in defining the Hoxa5 expression domain, as endogenous Hoxa5 expression was not affected in Cdx1−/− or Cdx2−/− embryos. Because Cdx1, Cdx2 and Cdx4 are co-expressed posterior of the normal Hoxa5 expression domain, redundancy among Cdxs may have obscured any effects of reducing Cdx dosage in these experiments. In the case of MafB, the in vivo binding of Cdx1 detected by ChIP analyses and the posterior expansion of MafB expression in Cdx1−/− embryos strongly support Cdx1 involvement in direct MafB repression.

Cdxs may play a more global role in establishing posterior expression boundaries of segmentation genes. Previously, HoxB genes were placed into two groups based on their reciprocal sensitivity to RA or Fgf during early axial patterning (Bel-Vialar et

Fig. 5. Cdx1 binds at the S5 enhancer in vivo. (A) Representative qPCR results for ChIP experiments performed with an anti-Cdx1 antibody on 0- to 12-somite-stage embryos detect Cdx1 binding at the Cdx1 autoregulatory region (Cdx1 auto) of the Cdx1 gene, as a positive control, and at the Cdx1-site-containing region of the S5 enhancer (P<0.001). No Cdx1 binding can be detected in regions 2 kb 5' (S5-2kb) or 2 kb 3' (S5+2kb) of the Cdx1 sites within S5 or at the MafB promoter (MafB prom). qPCR products are expressed as percent of input (sonicated embryo DNA before IP) and compared with background levels (+IgG) using a two-way Anova and post-hoc t-test. Error bars indicate the standard error of the mean (s.e.m.). (B) A schematic diagram shows the relative locations of the primer pairs used in ChIP-qPCR analyses.

suffice to suppress endogenous MafB in Cdx1−/− animals. Although Cdx1, Cdx2 and Cdx4 differ in their expression patterns and some structural details, at least in the case of Cdx1 and Cdx2 they have been shown to act redundantly in vertebral patterning (Savory et al., 2009b; van den Akker et al., 2002). In transgenic mice, where Cdx1 was replaced by Cdx2 at the endogenous locus, Cdx2 could effectively replace Cdx1 function (Savory et al., 2009b). Aside from redundancy among Cdx genes, the number of Cdx sites within the S5 enhancer are likely to make it exquisitely responsive to even low levels of Cdx. Thus, Cdx1, Cdx2 and Cdx4 may all collaborate to repress MafB in the posterior neural tube, and the three Cdx homodimer sites present in S5 may require only low levels of Cdx; for example in the hindbrain only Cdx1 is required to mediate repression. Although our findings are all in line with a pivotal repressive role for Cdx1 in the posterior hindbrain and possibly Cdx1, Cdx2 and Cdx4 in the spinal cord, we cannot exclude the possibility that other sites within the 240 bp region and/or additional regulators other than Cdxs may contribute to the posterior repression of MafB. Nonetheless, our data do ascertain direct involvement of Cdx1 in establishing the early r6/r7 boundary of MafB expression.

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Fig. 6. Two-step regulation of rhombomere-specific MafB expression. (A) During the 0- to 8-somite stages, an RA gradient establishes vHNF1 expression (green) up to the r4/r5 boundary and Cdx1 (orange) expression to the r6/r7 boundary. MafB is expressed in r5-r6 and Hoxb4 in r7-r8 in Cdx1+ve embryos. In Cdx1−/− embryos, MafB expression extends from the r4/r5 boundary posteriorly into the future spinal cord and Hoxb4 expression is shifted posteriorly. (B) During the ~10- to 20-somite stages, Hnf1b and Cdx1 are absent and a mechanism that depends on MafB autoregulation and probably an unidentified Fgf-responsive activator maintains MafB expression in r5-r6 in both Cdx1+ve and Cdx1−/− embryos. Hoxb4 expression, which is autoregulated, but not known to depend on Fgf signalling at these stages, remains shifted posteriorly in Cdx1−/− embryos (van den Acker et al., 2002). (C) Schematic outline of action of MafB regulators on the S5 enhancer and later-acting, unidentified rhombomere-specific regulatory elements.
al., 2002). Exogenous Fgf expands the expression domains of the Cdx genes and 5' members from the HoxB complex (Hoxb6-Hoxb9), which are normally trunk specific, anteriorly in the neural tube up to the level of the otic vesicle. Conversely, the more anteriorly expressed 3' Hoxb genes (Hoxb1 and Hoxb3-Hoxb5) are sensitive to RA, but not Fgf, treatment at these stages. These analyses focused on RA and Fgf sensitivity of Hox gene activation and anterior expression boundary establishment. Extrapolation from our findings suggests that FgfS and RA via Cdxs may also selectively repress specific Hox genes and establish posterior expression boundaries. Given the observations of direct Cdx-dependent repression of Hoxa3 and Mafb, perhaps the repressive effects of Cdx proteins may be specific to ‘anterior’ segmentation genes. Such repressive influences could either be masked or enhanced by subsequent regulatory mechanisms, e.g. inter-Hox gene crossregulation or autoregulatory loops. Thus, as with Mafb and Hoxa5, such mechanisms might only become apparent in enhancer dissection experiments that reveal transient regulatory events and developmental stages.

Our findings indicate that Cdx1-dependent repression is only temporary, suggesting that a later-acting mechanism stabilizes Mafb expression in r5 and r6 after the 10-somite stage. Initial rhombomere-specific Mafb expression occurs through the S5 enhancer, which is active from the 0- to 10-somite stages. The activities of Cdx1 and Hnf1b in Mafb regulation are limited to the early induction period, because these molecules regress towards the posterior during embryonic development and are absent from the hindbrain by the 10-somite stage (Fig. 6) (Houle et al., 2000; Kim et al., 2005; Meyer and Gruss, 1993), long before Mafb expression is extinguished (Cordes and Barsh, 1994; Frohman et al., 1993; Theil et al., 2002). Regulatory elements governing Mafb expression between the 10- and 20-somite stages have not yet been described. However, post-initiation Mafb(val) expression depends on functional Mafb(val) protein in both mice and zebrafish, indicative of an autoregulatory loop (Hernandez et al., 2004; Huang et al., 2000; Moens et al., 1998; Sadl et al., 2003). For some Hox genes, such as Hoxb4 (Gould et al., 1998), an RA-responsive early enhancer establishes rhombomere-specific expression and, subsequently, an autoregulatory enhancer helps maintain expression. Consistent with this model, in Cdx1-/- mice, Hoxb4 expression is shifted posteriorly throughout the 0- to 20-somite stages (van den Acker et al., 2002) (Fig. 6). However, the Maf autoregulatory loop is unique in that it appears to depend not only on Maf but also on the presence of a regionally restrictive signal, possibly an Fgf (Marin and Charnay, 2000; Maves et al., 2002; Walshe et al., 2002; Wielliste and Sive, 2003). This regionally restricted signal might affect either Maf itself by perhaps a post-translational modification, such as phosphorylation, or may act via inducing another transcriptional activator. In any case, it is probably the regionally dependent signal that promotes the post-initiation retraction of Mafb expression into its normal r5-r6 domain in Cdx1-/- mice.

The molecular mechanism by which Cdx1 acts to repress Mafb in the same regions in which it activates trunk-specific Hox genes, is unknown. It seems probable that Cdx actions depend on the sequence-specific context of the Cdx-binding sites within the S5 enhancer. In addition to its roles in embryonic patterning, Cdx2 plays a role in tumorigenicity in a subset of human colorectal cancer cell lines (Chawengsaksophak et al., 1997). In an established model for human colon cancer, the human colonic adenocarcinoma cell line (LOVO), Cdx2 directly represses the insulin-like growth-factor-binding protein 3 gene (Chun et al., 2007). Here the existence of unknown repressive interactors(s) has been invoked as a possible explanation and provides a plausible mechanism for Cdx1-dependent repression of Mafb as well. Core promoter composition might further influence the choice between Cdx-dependent activation and repression. In flies, Caudal preferentially activates promoters containing a downstream core promoter element (DPE) (Juven-Gershon et al., 2008). The promoters of Drosophila Hox genes are TATA-less, except for those from the abdominal-B ( Abd-B) and Ultrabithorax ( Ubx) genes, and can be activated by Caudal in preference to TATA-containing promoters. Whereas generally the focus in developmental gene expression has been on enhancers, there has been precedent, in studies on mouse Hoxb2 and Hoxb4 regulation, for promoter selectivity among Hox gene enhancers (Gould et al., 1997). The functional composition of vertebrate Hox and Mafb gene promoters have not been examined. Although Cdxs have not been shown to block the use of specific promoters, it is, theoretically, conceivable that core promoter composition in combination with Cdx interactors acting via specific enhancers might influence the choice between Cdx-responsive activation or repression.

In conclusion, we have shown for the first time that Cdx1 promotes not just trunk specification, but plays a role in defining the r7-r8 domain of the hindbrain by directly repressing Mafb expression. Thus, the role of Cdx1 within the hindbrain is an active but transient one. Our observations suggest that other transient effects of Cdxs may have been missed. Do such transient perturbations subtly alter neuronal fate assignments or distribution? Many important neuronal subtypes and neural circuits are established during hindbrain patterning. Thus, it is not difficult to imagine that subtle gene expression perturbations even when apparently corrected later on – may have consequences later in life. Thus, further analyses of Mafb and hindbrain regulation will continue to offer scientific and possibly clinically relevant insights.

Acknowledgements

We thank D. Lohnes for providing the anti-Cdx1 antibody and Cdx1-/- mice, originally generated by Subramanian et al. (Subramanian et al., 1995). We are grateful to M. Genterstein, S. Tondat and the Samuel Lunenfeld transgenic core facility for generating transgenic mice and embryos using tetraploid aggregation. This research was funded by CIHR grants MOP 14312 and MOP 97966 to S.P.C.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/doi/10.1242/dev.058727/-/DC1

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