REFINING POSITIONAL IDENTITY IN THE VERTEBRATE HINDBRAIN

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ABSTRACT

The vertebrate hindbrain is divided early in embryogenesis along its anterior-posterior axis into eight segments known as rhombomeres. This provides an excellent model for studying early segmentation and region-specific transcriptional domains. MafB, a basic domain leucine zipper transcription factor, is the first gene known to be expressed in the presumptive rhombomere 5 and 6 domain (r5-r6). MafB expression is directly activated by the homeodomain protein vHnf1. vHnf1 and MafB share an anterior expression limit at the r4/r5 boundary but vHnf1 expression extends beyond the posterior limit of MafB and, therefore, cannot establish the posterior expression boundary of MafB. Through the use of in situ hybridization, immunofluorescence, and chromatin immunoprecipitation analyses, I have determined that the caudal-related homeodomain protein Cdx1 establishes the posterior boundary of MafB by directly inhibiting expression beyond the r6/r7 boundary. My results indicate that MafB is one of the earliest direct targets of Cdx1.
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# TABLE OF CONTENTS

ABSTRACT ........................................................................................................... ii

ACKNOWLEDGEMENTS .......................................................... iii

TABLE OF CONTENTS ................................................................. iv

LIST OF FIGURES ............................................................... vii

CHAPTER 1 – INTRODUCTION ................................................................. 1

1.1 Hindbrain patterning................................................................. 1

1.1.1 The retinoic acid morphogen gradient............................. 2

1.1.2 Hox genes................................................................. 4

1.1.3 Even and odd type rhombomeres................................. 5

1.2 MafB: a hindbrain segmentation gene.............................. 6

1.2.1 Retinoic acid and MafB expression................................. 9

1.2.2 Induction of MafB by vHnf1.......................................... 10

1.2.3 FGFs and MafB expression....................................... 10

1.2.4 MafB in the mouse.................................................... 11

1.3 A potential role for Cdx proteins in hindbrain patterning...... 13

1.3.1 cdx1 expression.......................................................... 18

1.4 This thesis................................................................. 19
CHAPTER 2 – MATERIALS AND METHODS .................................................. 21
  2.1 Embryo harvesting ........................................................................... 21
  2.2 MafB RNA in situ hybridization .................................................... 21
      2.2.1 Embryo powder ..................................................................... 21
      2.2.2 MafB RNA in situ probe preparation ..................................... 22
      2.2.3 In situ hybridization ............................................................... 22
  2.3 Immunofluorescence ................................................................. 24
  2.4 LacZ staining ............................................................................. 25
  2.5 Chromatin immunoprecipitation assay ........................................ 26
      2.5.1 Chromatin extraction from F9 embryonic carcinoma cells..... 26
      2.5.2 Chromatin extraction from embryos at 8-8.5dpc ............... 27
      2.5.3 Chromatin immunoprecipitation ......................................... 28
      2.5.4 Quantitative PCR ................................................................. 29
  2.6 Time lapse Western ................................................................. 29

CHAPTER 3 – RESULTS - Establishment of the posterior boundary of MafB expression in the embryonic hindbrain ................................................................. 30
  3.1 Introduction .................................................................................. 30
  3.2 Functional Cdx binding sites reside within the S5 enhancer .......... 31
  3.3 Posterior expansion of MafB expression in cdx1-/— embryos ....... 33
  3.4 The anterior boundary of Cdx1 lies at the posterior boundary of MafB .... 34
  3.5 Cdx1 binds at the S5 enhancer in vivo .......................................... 37
CHAPTER 4 – DISCUSSION ................................................................. 41

4.1 Cdx1 establishes the posterior expression boundary of MafB .......... 41

4.2 A potential MafB activator ...................................................... 43

4.3 MafB maintenance ................................................................. 44

4.4 The Cdx-Hox code: a conserved mechanism of axial patterning ....... 46

REFERENCES .................................................................................... 51
LIST OF FIGURES

Figure 1.1 Rhombomere segmentation and hindbrain development ............ 3
Figure 1.2 The MafB rhombomeric null kr/kr..................................... 8
Figure 1.3 Two models for establishing early rhombomere-specific
MafB expression.............................................................. 14
Figure 1.4 Hoxb4 hindbrain expression is altered in the absence
of MafB and cdx1............................................................ 17
Figure 3.1 Cdx binding sites, but not the Hox/Pbx binding site, are necessary
to establish r5-r6 specific expression..................................... 32
Figure 3.2 MafB expression in wild type and cdx1/- embryos ................. 35
Figure 3.3 Posterior expansion of MafB protein in cdx1/- embryos.......... 36
Figure 3.4 Cdx1 is present at the posterior limits of MafB....................... 38
Figure 3.5 Cdx1 binds at the S5 enhancer in vivo............................... 40
Figure 4.1 Initiation of MafB expression........................................ 42
Figure 4.2 Induction and Maintenance of MafB expression.................... 47
CHAPTER 1
INTRODUCTION

Segmentation is an evolutionarily conserved developmental process that is used repeatedly during vertebrate embryonic development. This strategy underlies the construction and diversification of many body regions including the central nervous system. Segmentation occurs through the organization of cells along an axis into metameric structures whereupon each segment adopts a specific developmental program. Given that segmentation has a recurring role in axial patterning, gaining insight into the molecular mechanisms that govern segment formation will lead to a greater understanding of embryonic development as a whole [1-4].

1.1 Hindbrain patterning

In vertebrates the central nervous system develops from a dorsal strip of epithelial cells at the gastrula stage which later forms the neural tube by folding along its anterior-posterior (A/P) axis and, later still, becoming subdivided along this axis to form the presumptive forebrain, midbrain, hindbrain and spinal cord [5, 6]. The developing vertebrate hindbrain, which will grow to form the cerebellum, the pons and the medulla in the adult brain, is segmented early in embryogenesis along its A/P axis into 7 to 8 lineage-restricted compartments known as rhombomeres [5, 7, 8]. 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 2 through 5 [7, 8]. The morphological rhombomeric bulges first appear upon neural tube closure.
and rhombomeric segmentation is complete prior to neurogenesis [7, 8]. Rhombomeres represent true embryonic compartments as they share the same cell lineage, morphology, and genetic identity. The cells within each rhombomere follow a common developmental program that coordinates formation of adult structures such as the cranial motor nerves (Fig 1.1). Many gene expression patterns, including those of Hox genes, the most extensively studied segmentation genes, respect rhombomere boundaries and are limited to specific subsets of rhombomeres (Fig 1.1) [5, 7-9]. The physical compartmentalization and defined rhombomere-specific gene expression patterns facilitate identification of regulatory regions and molecules required for the establishment, maintenance, and refinement of the transcriptional state of individual rhombomeres. This provides a premier model for the study of developmental vertebrate segmentation [9, 10].

1.1.1 The retinoic acid morphogen gradient

A/P patterning in the vertebrate hindbrain is induced by a posteriorizing morphogen gradient. While retinoic acid (RA), fibroblast growth factors (FGFs) and Wnts have all been implicated in A/P patterning of the brain, the precise role of the FGFs and Wnts in hindbrain segmentation is still unclear. In contrast, multiple studies have identified RA, a product of vitamin A (retinol) metabolism, as the dominant posteriorizing factor in hindbrain development. 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 pre-somitic
Figure 1.1 Rhombomere segmentation and hindbrain development

A schematic representation depicting the developing hindbrain at embryonic day 9 including rhombomere segments (numbered 1 through 8) and associated cranial motor nerves (V to XII). The retinoic acid (RA) gradient is depicted above and the rhombomeric expression patterns of the segmentation genes $MafB$ and $Krox20$, and the $Hox$ genes are depicted below.
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 [8, 11, 12]. RA acts to induce transcription of segmentation genes via RA receptors (RARs) and the retinoid X receptors (RXRs), which are ligand-dependent transcription factors that bind as homodimers or heterodimers to specific cis-acting elements termed RA response elements (RAREs). RAREs have been found for many of the \textit{Hox} genes expressed in the hindbrain [7, 9, 11, 13]. The dependence of hindbrain development on RA signalling was first demonstrated in quail embryos from mothers deprived of vitamin A in which the posterior hindbrain (r4-r7) fails to develop [11]. A similar effect is seen in zebrafish and chick embryos when an RAR pan-agonist is added to abolish RA signalling [14], in mouse double knock-outs for two RAR subtypes (RAR\textalpha and RAR\textgamma)[15] and in \textit{Raldh}2 mutant mouse embryos [16]. These, along with many other studies performed in the chick, mouse, fish and frog have identified RA as the posteriorizing factor in vertebrate hindbrain patterning [15-17].

\textbf{1.1.2 \textit{Hox} genes}

In response to the RA morphogen gradient initial segmentation genes, such as \textit{MafB} and \textit{Krox20}, are expressed in discrete regions along the hindbrain which induce a molecular cascade, including activation of the \textit{Hox} genes, resulting in region-specific expression patterns and segment identity (Fig 1.1) [5, 7]. Vertebrate \textit{Hox} genes, homologs of the \textit{Drosophila} HOM-C genes, are organized into four linkage
groups (a-d) thought to have arisen through duplication of a common ancestral complex. The genes on each cluster are further classified into 13 paralogous classes according to sequence similarity [5, 7, 9]. The 3’-to-5’ organization of the paralogs within each cluster is conserved and the location of a particular gene within a cluster will determine when and where it is expressed with the 3’-most genes being expressed first and most anteriorly [5, 7]. Classes 1-4 of the a, b and d linkage groups are expressed in the developing vertebrate hindbrain. Typically Hox genes of the same class have overlapping expression patterns but their level of expression in particular rhombomeres may differ [5, 9]. The complement of Hox genes expressed by a particular rhombomere and the sequential timing of expression of these Hox genes helps determine rhombomeric identity [7, 9, 18].

1.1.3 Even and odd type rhombomeres

In addition to the identity procured through transcriptional specificity, segmentation appears to be aided further by inter-rhombomeric interactions, through which, rhombomeres are assigned an even or odd type identity. When cells from an even-numbered and an odd-numbered rhombomere are mixed they segregate via differential adhesion while cells isolated from two evenly or two odd-numbered rhombomeres mix freely [7, 9]. This mutual repulsion is thought to arise, at least in part, through alternate expression of the Eph family of receptor tyrosine kinases in odd rhombomeres and their ligands, the ephrins, in the even-numbered rhombomeres. EphA4 expression, for example, is directly induced in rhombomeres 3 and 5 by Krox20 [7, 8]. This additional layer of regulation is crucial to the maintenance of
rhombomeric boundaries and segment identity required for the development of adult structures [7, 8]. The organization of the branchiomotor neurons, for instance is dependent on the even/odd identity of the rhombomere from which they emerge. Branchiomotor neurons of even-numbered rhombomeres (r2, r4 and r6) send out lateral axonal projections to an exit point within the rhombomere of origin whereas those originating from odd-numbered rhombomeres (r3, r5 and r7) send out lateral axonal projections that then turn and project rostrally to join the exit point within the neighbouring anterior even-numbered rhombomere. Neuronal differentiation also occurs first in even-numbered rhombomeres and is delayed in odd-numbered rhombomeres [7, 9] and neural crest cells are derived from even rhombomeres [2, 9]. Completion of hindbrain segmentation ensures proper development and organization of hindbrain derived adult structures [5, 7, 9]. For example, early segmentation is crucial to the formation of neuronal networks that form the rhythm centres that control respiration, prevent apnoea, and allow the body to respond to hypoxia and hyperoxia [19].

1.2 MafB: a hindbrain segmentation gene

Prior to morphological rhombomere development a few master regulatory genes are expressed in specific A/P locations along the neural tube and govern the expression of Hox genes. One of the first genes to be expressed segmentally in the posterior hindbrain is MafB (also known as Kreisler, Krml1, and in zebrafish as Valentino), a basic leucine zipper transcription factor [1, 8, 10]. MafB, is the first gene known to be expressed in a segmental fashion in the future rhombomere 5 and 6
(r5-r6) domain prior to the appearance of rhombomeric boundaries [1, 2, 8] and directly activates expression of the early-acting segmentation genes \textit{Hoxa3} and \textit{Hoxb3} [20-22]. The original X-ray induced \textit{MafB} mouse mutation, \textit{kr}, is a near perfect chromosomal inversion which abolishes rhombomere specific \textit{MafB} expression [1, 2]. In \textit{kr}/\textit{kr} mutants, and in mouse embryos homozygous for the severely hypomorphic or null allele \textit{kr\textsuperscript{nu}}, the r5-r6 domain does not develop properly, \textit{Hox} genes expressed in the hindbrain are misregulated, and there is a loss of morphological segmentation in the posterior hindbrain (r4-r7) (Fig 1.2) [1, 2, 23, 24]. In \textit{kr}/\textit{kr} mice, adult structures derived from and induced by r5-r6 are also affected: cranial motor nerve VII is reduced and VI is absent, and the otic vesicle, which normally forms the inner ear, fails to develop properly leading to deafness and balance deficits in adult mice and the characteristic circling behaviour for which this mutant was named (kreisler is German for circler) [1, 2, 23]. The phenotypes of these mutants reveals \textit{MafB} as a true segmentation gene, as, in its absence, its rhombomeric territory fails to form [1, 7, 23]. Although it is clear that the \textit{Hox} genes play a definitive role in determining regional specification, their organization into linkage groups and the overlapping roles of members of the same class make it more difficult to dissect out the function of any individual \textit{Hox} gene. \textit{MafB}, in contrast, is a single, non-redundant, and independently regulated gene. This, along with its early expression and clear role as a segmentation gene, make \textit{MafB} an ideal candidate for the study of early hindbrain gene regulation [1, 2, 9].
Figure 1.2 The rhombomeric MafB null kr/kr

A schematic of the developing hindbrain illustrating expression patterns of MafB and the Hox genes in normal (WT) and MafB rhombomere null (kr/kr) embryonic hindbrains. In the absence of MafB rhombomeres 5 and 6 (r5-r6) and the otic vesicle (OV) fail to develop properly and Hox gene expression is highly disrupted in the presumptive r5-r6 region.
1.2.1 Retinoic acid and MafB expression

Expression of MafB is highly dependent on RA [14, 17, 25]. In mouse embryos that are Raldh2-/- or RARα-/-;RARγ-/-, MafB expression is completely lost [15, 16]. The induction of MafB by RA is both concentration and time dependent. MafB is usually induced at a specific A/P location in the hindbrain but reduction in RA signalling produces a permissive environment for MafB expression further into the posterior hindbrain. For instance, in Raldh2 mutant zebrafish where RA signalling is attenuated but not fully blocked [26], embryos show posterior expansion of MafB (Val) [12, 26]. Posterior expansion of MafB is also seen when an RAR pan-agonist is added to mouse or chick embryos at the 8-to-12 somite stages after MafB expression has begun [15, 17]. In addition, increasing the concentration of the agonist at this stage leads to increased posterior expansion of MafB expression in mouse embryos [15]. These results clearly indicate that induction of MafB by RA is concentration dependent. In contrast to the expansion seen at the 8-to-12 somite stage, application of the RAR pan-agonist at the same concentrations at the 0-somite stage, eliminates MafB expression in both mouse and chick embryos. This demonstrates that at the time of induction MafB expression is highly dependent on RA signalling. These data hint at a two step regulatory process: first, in early segmentation, RA is necessary to induce MafB expression, but, slightly later, RA is necessary to restrict MafB expression to r5-r6 by directly or indirectly inhibiting posterior MafB expression [15, 17].
1.2.2 Induction of MafB by vHnf1

Induction of MafB by RA occurs, in part, through the homeodomain protein, variant hepatic nuclear factor 1 (vHnf1) [6, 10, 12, 14, 27, 28]. vHnf1 expression is induced by RA at the 0-somite stage in the presumptive hindbrain of mouse, chick and zebrafish embryos from the future r4/r5 boundary into the posterior hindbrain [6, 10, 12, 28]. As mentioned, in the zebrafish Raldh2 mutant RA signalling is reduced but not lost. Addition of an RA agonist to these embryos results in the absence of both vHnf1 and MafB(Val) [12, 14]. Normal expression of vHnf1 and MafB(Val) in these embryos can be restored by addition of RA [12]. Similarly, treatment of zebrafish with an RAR pan-agonist leads to loss of vHnf1 and MafB(Val) expression, but MafB(Val) expression can be restored through the addition of exogenous vHnf1 [14]. This indicates that loss of vHnf1 due to the absence of RA is linked to the loss of MafB(Val) expression. In further support, vHnf1 mutant zebrafish embryos show no MafB(Val) expression [12, 27], and, in chick embryos exogenous vHnf1 is sufficient to induce MafB expression [6]. Our lab has determined that in mice MafB expression is directly regulated by vHnf1. The rhombomere specific MafB enhancer contains a functional vHnf1 site that is necessary but not sufficient to induce rhombomere specific MafB expression [10]. Therefore, although it is clear that vHnf1 is required for MafB induction, it does not act alone [10].

1.2.3 FGFs and MafB expression

Mounting evidence suggests that FGFs act in concert with vHnf1 to regulate MafB expression [27, 29, 30]. In chick embryos adding an inhibitor of FGF activity
results in loss of \textit{MafB} expression [6, 27, 31] and exogenous \textit{FGFs} can induce \textit{MafB} but the specific \textit{FGFs} involved in \textit{MafB} regulation have not been identified [31]. In zebrafish, \textit{FGF3} and \textit{FGF8} expressed in r4 have been implicated in \textit{MafB(Val)} induction. The addition of double morpholinos for \textit{FGF3} and \textit{FGF8} results in the absence of \textit{MafB(Val)} induction [29, 30] and overexpression of \textit{vHnf1} and \textit{FGF3} in zebrafish leads to upregulation of \textit{MafB(Val)}[29, 30]. In the mouse, \textit{FGF3} mutants show abnormal inner ear development as is seen in \textit{MafB} mutants [32-35]. However, \textit{MafB} expression is not altered in the absence of \textit{FGF3} [32, 36]. In addition, ectopic expression of \textit{MafB} in mouse embryos induces \textit{FGF3} [37], and \textit{FGF3} expression is nearly abolished in the absence of \textit{MafB} expression indicating that in mice, \textit{FGF3} is downstream of \textit{MafB} [2, 33, 38]. In accordance with this, \textit{FGF3} expression in mice is not restricted to r4 but has an expression pattern that mimics that of \textit{MafB}. At early somitic stages there is broad low level expression of \textit{FGF3} in the presumptive hindbrain [38, 39] that becomes upregulated in r5-r6 by the 6-somite stage [38, 40, 41] where it is maintained until the 20-somite stage [2, 16, 32, 37, 38, 42-44]. These dissimilar expression patterns and differences in the hierarchy of gene expression indicate that the particular \textit{FGF} molecules involved in \textit{MafB} induction are variable among vertebrates.

\subsection*{1.2.4 \textit{MafB} in the mouse}

Although the segment specific \textit{MafB} expression pattern, and its requirement for the proper formation of the posterior hindbrain, is conserved across vertebrates, perhaps different species have adopted slightly variant methods to achieve this
expression. As all model systems are used as a means for discovering the mechanisms underlying human development, the use of a mammalian model, such as the mouse, is more appropriate in this case as it is more likely to recapitulate human hindbrain segmentation than a non mammalian model. In addition to the physiological and genetic similarities to humans, the mouse is an ideal model system because of the ability to alter its genome. The mouse can be used as a powerful genetic tool through the creation of transgenic mice or production of knock-in, knock-out and conditional null mice for specific genes at their endogenous locus through homologous recombination. These tools provide a means to examine gene regulation and function in vivo. In fact, the S5 rhombomere specific MafB enhancer was discovered through the use of transgenic mice and further investigations into direct regulators of MafB expression will be facilitated by this knowledge. For these reasons I have used the mouse as a model system.

In mice rhombomere-specific MafB expression is first seen in the future r5-r6 domain at the 0-somite stage or embryonic day 8 (E8.0), and then in both r5 and r6 once rhombomeric boundaries have formed (E8.25 to E8.5). It is down-regulated in r5 by the 14-somite stage (E8.75 to E9.0), and cannot be detected in r6 past the 22-somite stage (E9.75) [10]. In an effort to understand the mechanisms that establish such precise anterior and posterior boundaries of gene expression, our lab previously identified the S5 enhancer, which initiates expression of MafB in the future r5-r6 domain. This has allowed us to identify molecules that act directly upstream of MafB to establish its segment-specific expression. Using the S5 enhancer and analyses in transgenic mice, the lab determined that vHnf1 is necessary but not sufficient to
induce $MafB$ expression. While the anterior expression limit of $vHnf1$ and $MafB$ coincide at the r4/r5 boundary, $vHnf1$ expression is observed far more posteriorly in the neural tube than that of $MafB$ and therefore does not establish the posterior boundary of $MafB$ expression [10]. This observation can be explained by two simple models that are not mutually exclusive: (1) $vHnf1$ induces expression of $MafB$ along with an unknown activator that is restrictively expressed in rhombomeres 5 and 6; and/or (2) $vHnf1$ and an unknown activator regulate the anterior expression boundary of $MafB$ while a separate repressor restricts the posterior boundary of expression [10] (Fig 1.3). Because the earliest genes expressed in the hindbrain have sharp, well-defined anterior expression boundaries and more diffuse expression at their posterior limits [9, 45, 46], and from the results described in more detail below, I propose the second model plays a significant role in the establishment of segmental $MafB$ expression.

1.3 A potential role for Cdx proteins in hindbrain patterning

The caudal related homeobox ($cdx$) genes are a group of transcription factors which are vertebrate homologues of the *Drosophila caudal* gene [47]. In the mouse, there are three $cdx$ genes, $cdx1$, $cdx2$ and $cdx4$, that are expressed in a stepwise manner along the A/P axis of the developing mouse embryo with $cdx1$ being the most anterior, followed by $cdx2$, then by $cdx4$. While 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 [46-51]. The Cdx proteins act to transduce A/P positional information to the *Hox* genes and directly regulate *Hoxa7* and *Hoxb8*.
Figure 1.3 Two models for establishing early rhombomere-specific $MafB$ expression

A. vHnf1 along with an unknown activator which is restrictively expressed in rhombomeres 5 & 6 act together to induce $MafB$ (depicted in red). B. vHnf1 and an unknown activator establish the anterior boundary while a repressor establishes the posterior boundary of $MafB$ expression.
Not surprisingly, the \textit{cdx} genes are regulated by signalling molecules involved in A/P patterning including RA, FGFs and Wnts \cite{48, 49, 54, 55}. During development, as posteriorizing signals regress into the posterior, the gradient of Cdx proteins regresses as well \cite{48, 56}.

In mice, \textit{cdx1} and \textit{cdx2} have clearly defined roles in A/P patterning of the vertebrae. \textit{cdx1-/-} and \textit{cdx2+/-} mice show anterior homeotic transformations of the vertebra. In \textit{cdx1-/-} mice these transformations occur from cervical vertebra 1 (C1) through thoracic vertebra 8 (T8) and, in \textit{cdx2+/-} mice, C6 through T8 are affected. The more anterior transformations in \textit{cdx1-/-} mice are expected due to the more anterior expression of \textit{cdx1} compared to that of \textit{cdx2} \cite{53, 57, 58}. The homeotic transformations seen in \textit{cdx1-/-;cdx2+/-} mice are more severe than either of the single mutants indicating an overlapping role in vertebral patterning \cite{57}. \textit{cdx1-/-} and \textit{cdx1-/-;cdx2+/-} embryos also show a posterior shift in \textit{Hox} gene expression \cite{53, 57, 58}. Overexpression of \textit{cdx1}, \textit{cdx2} or \textit{cdx4} in mouse embryos results in posterior vertebral homeotic transformations and an anterior shift in \textit{Hox} gene expression \cite{59}. The addition of exogenous RA also results in more anterior expression of \textit{Hox} genes as well as posterior vertebral homeotic transformations \cite{51, 60, 61}. Together these phenotypes suggest that loss of \textit{cdx1} and \textit{cdx2} may reduce RA signalling resulting in aberrant \textit{Hox} gene expression and misspecification of vertebral identity. Although the role of the \textit{cdx} genes in hindbrain development has not been explored, their graded expression in the neural tube, and their activity as transducers of RA signalling in vertebral patterning suggest the Cdx proteins may play a similar role in A/P hindbrain patterning.
In mouse embryos, *Hoxb4* is normally expressed in the posterior neural tube with an anterior limit at the r6/r7 boundary. In *kr/kr* mutants, where rhombomere specific *MafB* expression is lost, *Hoxb4* extends more anteriorly into the presumptive r5-r6 domain [2, 23, 24, 62]. This observation suggests that *Hoxb4* is a direct or indirect *MafB* target gene. In contrast to this anterior shift, the rostral expression limit of *Hoxb4* is pushed significantly more posteriorly in *cdx1-/-* and *cdx1-/-;cdx2+/-* embryos [48], and thus, Cdx1 and Cdx2 proteins may be involved in *Hoxb4* and/or *MafB* expression. These opposing phenotypes suggest that perhaps Cdx1 and Cdx2 restrict the posterior expression limit of *MafB* which, in turn, regulates the anterior expression boundary of *Hoxb4* (Fig 1.4). Of course, none of these observations exclude the possibility that *Hoxb4* itself represses *MafB*; however, detectable *Hoxb4* expression in the hindbrain has first been reported a few somite stages later than that of *MafB* [24, 62]. Based on these observations, we hypothesized that Cdx1 and Cdx2 are potential regulators of posterior *MafB* expression. In addition, ectopic expression of *MafB(Val)* is observed in the posterior neural tube of zebrafish embryos in cells that are deficient for *cdx1a* and *cdx4*, but not in wild-type cells, implicating the Cdx proteins as repressors of *MafB*. Unfortunately, *cdx2* expression appears in the embryonic mouse hindbrain at the 6-somite stage (E8.25) [49], clearly too late to restrict early *MafB* expression. This, combined with the more anterior expression of *cdx1*, makes Cdx1 the most likely repressor candidate. Therefore, I am investigating Cdx1 as a potential posterior repressor of *MafB*.
Figure 1.4 Hoxb4 hindbrain expression is altered in the absence of MafB and cdx1

Schematic representation of expression patterns of MafB (red) and Hoxb4 (blue) in the hindbrains of normal (A), MafB rhombomere null (B), and cdx1 null (C) embryos. Normally expression of these two genes meets at the r6/r7 boundary (A), but in the absence of MafB, the anterior boundary of Hoxb4 shifts rostrally (B). In the absence of cdx1, Hoxb4 expression is pushed posteriorly and it is hypothesized that this is due to a posterior expansion of MafB expression (C).
1.3.1 *cdx1* expression

*cdx1* expression is first observed in the presumptive hindbrain of mouse embryos in the primitive streak and then the neural plate ectoderm (E7.0-E7.5). At the head fold stage (E7.75), the anterior boundary of expression is found at the level of the preotic sulcus, the region of *MafB* expression, with expression extending throughout the posterior presumptive hindbrain. 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 [63]. *cdx1* is present in the posterior hindbrain from the 1 to 10-somite stages (E8.0-E8.75) [47, 63], a period that coincides with the presence of *vHnf1* expression and *MafB* induction [10].

Like *MafB*, *cdx1* expression is reliant on RA [47]. Upregulation of *cdx1* is seen with the addition of exogenous RA to mouse embryos from the 0-to-20 somite stages (E7.5-E9.5) and *cdx1* expression is significantly reduced in *RARα-/-;RARγ-/-*, embryos [47]. If *cdx1* acts to repress *MafB* at its posterior limit, loss of Cdx1 in embryos treated with RA agonists may account for the posterior expansion of *MafB* observed in these embryos. RA directly regulates *cdx1* through an upstream RARE [47]. Mutation of this RARE nearly eliminates *cdx1* expression. However, *cdx1* can still be activated by exogenous RA in these embryos suggesting an additional RARE or an indirect RA-dependent mechanism that has yet to be identified [64]. The anterior boundary of *cdx1* expression is maintained by COUP-TF1 binding to the *cdx1* RARE, which actively blocks RA signalling [65]. Once *cdx1* expression is
initiated, Wnt3a, whose expression overlaps with that of cdx1 from the early head fold stage, is required to maintain expression [66, 67]. Wnt3a signalling preserves cdx1 expression through two upstream LEF/T-cell factor (TCF) response elements (LREs) [66]. Maintenance of cdx1 is also dependent on autoregulation. Interestingly, Cdx1 binds as a heterodimer with LEF1 at the LREs to self-maintain [68]. When both the RARE and the LREs are mutated, cdx1 expression is completely lost [69], indicating that RA and Wnt3a signalling act synergistically to regulate cdx1 expression [49, 66, 68, 69]. Preliminary experiments in Xenopus and chick indicate that cdx1 is also induced by FGFs [55, 70], making cdx1 an attractive subject in the study of regional specification as it is responsive to all three major signalling molecules involved in A/P patterning.

1.4 This thesis

Based on its role in segmental skeletal development, its expression at the appropriate region of the hindbrain, its proposed role as a transducer of the RA gradient at the transcriptional level, and given that MafB expression is highly sensitive to RA, I hypothesized that Cdx1 acts to repress MafB, thereby establishing the posterior boundary of MafB expression in the hindbrain.

This thesis will address this hypothesis through three main goals. **Specific aim 1:** To confirm that Cdx1 is present at the right time and place to act as a posterior repressor of MafB. In other words, is Cdx1 localized at the posterior boundary of MafB expression at the time of MafB induction? To answer this question, immunofluorescence experiments using Cdx1 and MafB specific antibodies were
performed on embryos from the 4-to-12 somite stages to observe the localization of Cdx1 relative to MafB. **Specific aim 2:** To demonstrate that Cdx1 is necessary to establish the posterior boundary of *MafB* expression. To address this aim, *in situ* and immunofluorescence analyses were performed on *cdx1*−/− embryos to examine *MafB* expression and MafB protein localization in the absence of Cdx1. **Specific aim 3:** To determine if Cdx1 acts directly to repress MafB expression. Chromatin immunoprecipitation experiments were used to investigate an *in vivo* interaction between Cdx1 and the rhombomere specific *MafB* enhancer. Together, these specific aims will reveal if Cdx1 acts as a posterior repressor of *MafB*.
CHAPTER 2
MATERIALS AND METHODS

2.1 Embryo harvesting

Pregnant mice were euthanised at 8 to 11.5 days post coitus (dpc), their uteri removed and placed into ice-cold phosphate-buffered saline (PBS) (7mM Na₂HPO₄, 3mM NaH₂PO₄, 0.13M NaCl). Embryos were dissected out of the uteri and all extra-embryonic tissue removed. Once dissected, embryos were fixed in ice cold 4% paraformaldehyde (PFA) in PBS for 2 hours (8-9dpc) to overnight (9-11.5dpc) at 4°C. After fixation, embryos were passed through three 5 minute washes in PBS with 0.1% tween-20 (PBST). Embryos were then taken through a dehydration series by washing twice for 10 minutes in each of the following: 25%, 50%, 75% and 100% methanol in PBST. Dehydrated embryos were stored at -20°C in 100% methanol until needed. All of the above solutions were treated with Diethyl pyrocarbonate (DEPC).

2.2 MafB in situ hybridization

2.2.1 Embryo powder

One or two litters of 10.5-11.5 dpc embryos were harvested from naturally mated mice. Dissected embryos were homogenized in a minimal amount of ice-cold PBS using a glass homogenizer. The homogenate was poured into four volumes of ice-cold acetone, mixed and incubated on ice for 30 minutes. Samples were
centrifuged for 10 minutes at 10,000 x g, supernatant was removed, followed by a wash with fresh ice-cold acetone. The pellet was placed on a glass slide and chopped into a fine powder, allowed to air dry, and stored in an air-tight tube at 4°C.

2.2.2 MafB RNA in situ probe preparation

4ck template plasmid was linearized by digestion with NcoI (New England Biolabs) (NcoI cuts the plasmid twice, complete digestion produces two fragments of 3.5kb and 1kb). Linearized template was treated with proteinase K (Invitrogen) for 30 minutes at 37°C. The template was purified by performing two phenol/chloroform extractions followed by one chloroform extraction and then an ethanol precipitation. RNA MafB probe was synthesized using T7 RNA polymerase (Invitrogen, Cat. #18033-019) and digoxigenin (DIG) labelling mix (Roche, Cat. #11277073910) in the following reaction for 2 hours at 37°C: 1x transcription buffer, 0.01M Dithiothreitol (DTT), 1 ug/ul linearized template, 1 ul RNase OUT (Invitrogen, Cat. #10777-019), 2 ul T7 RNA polymerase, 4 ul DIG labelling mix and sterile water to a total volume of 40 ul. After synthesis, DNA was removed with RNase free DNaseI (Invitrogen) by incubating for 30 minutes at 37°C. The RNA probe was purified by ethanol precipitation and stored at -80°C until use.

2.2.3 In situ hybridization

Day 1: Embryos were rehydrated through a methanol gradient by washing for 10 minutes twice each in 75%, 50%, 25% methanol in PBST and then washed for 5
minutes three times in PBST. Embryos were bleached in 6% hydrogen peroxide in PBST for 1 hour and then washed three times for 5 minutes in PBST. Embryos were treated with 5 ug/ml proteinase K (Invitrogen) for 30 seconds (this step was omitted for 0-to-2 somite embryos). Proteinase K reaction was stopped with two 5 minute washes in 2 mg/ml glycine (Sigma Aldrich) in PBST followed by three 5 minute washes in PBST. Embryos were then transferred to prehybridization buffer (50% formamide, 5x SSC pH4.5, 50 ug/ml yeast tRNA, 1% SDS, 50 ug/ml heparin, 0.1% CHAPS, 5 mM EDTA) and incubated at 70°C for 1 hour with occasional swirling. Finally embryos were transferred to hybridization buffer (prehybridization buffer plus 1 ug/ml DIG-labelled MafB RNA probe) and incubated with rocking at 70°C overnight. Probe was pre-cleared in hybridization buffer with three 10.5dpc embryos incubated at 70°C with agitation for 3 hours. All solutions used on day 1 are treated with DEPC.

Day 2: Embryos were washed three times for 30 minutes at 70°C in solution 1 (50% formamide, 5x SSC pH 4.5, 1% SDS, 0.1% CHAPS) pre-warmed to 70°C followed by three 30 minute washes at 65°C in solution 3 (50% formamide, 2x SSC pH4.5, 0.1% CHAPS) pre-warmed to 65°C. Next, embryos were washed three times for 5 minutes in Tris-buffered saline with Tween-20 (TBST) (140 mM NaCl, 30 mM KCl, 25 mM TrisHCl pH7.4, 1% Tween-20) at room temperature then pre-blocked by incubating at 4°C with rocking for 1.5 hours in 10% heat-inactivated sheep serum (high pure, Sigma) in TBST (10% serum was heat inactivated by incubating for 30 minutes at 70°C). Embryos were then transferred to 1% heat inactivated sheep serum
in TBST containing 0.5 ul/ml pre-adsorbed α-DIG antibody (Roche) and incubated with rocking overnight at 4°C. α-DIG antibody was pre-adsorbed by incubating in 1% heat inactivated sheep serum in TBST with 6 mg/ml embryo powder at 4°C for 1 hour. All solutions used on day 2 are treated with DEPC.

Day 3: Embryos were washed three times for 5 minutes then seven times for 45 minutes in TBST with rocking at room temperature. Embryos were then washed three times for 10 minutes each in alkaline phosphatase buffer (NTMT) (100 mM NaCl, 100 mM TrisHCl pH9.5, 50 mM MgCl2, 0.1% Tween-20, 2 mM Levamisole) and then transferred to 20 ul/ml NBT/BCIP (Roche) in NTMT and allowed to develop in the dark for 3 to 4 hours. This reaction was stopped by 5 minute washes, once in NTMT and then three times in PBST-1mM EDTA. Embryos were post-fixed in 4% PFA / 0.1% gluteraldehyde for 1 hour at 4°C, washed for 5 minutes three times in PBST and stored in PBST at 4°C. Images were captured with QImaging MicroPublisher 3.3 RTV.

2.3 Immunofluorescence

Embryos were harvested as outlined above but were not dehydrated following fixation. Once embryos were fixed, they were washed three times for 5 minutes each in PBST and then incubated over night at 4°C in 30% sucrose in PBST. The following day embryos were incubated in a 50:50 solution of 30% sucrose/PBST: optimal cutting temperature (OCT) compound (Sakura Finetek) for 3 hours at room temperature in moulding trays, then transferred to new trays containing fresh OCT
three times for 1 hour incubations. Embryos were transferred once more, oriented for ideal sectioning and frozen on a bed of dry ice. Frozen samples were wrapped in foil and stored at -80°C until sectioning. Embedded embryos were sectioned into 10 um cryosections onto superfrost plus slides (Thermo Scientific) and stored at -80°C.

Slides with 10 um cryosectioned embryos were allowed to come to room temperature and then rinsed in PBS* (PBS* = high salt PBS; 0.3M NaCl,) three times for 10 minutes. Samples were then blocked in 10% normal donkey serum (NDS) in PBS*T for 2.5 hours at room temperature in a humidified chamber. The block was removed and primary antibody (Rabbit αCdx1, provided by Dr. David Lohnes; Rabbit αMafB, purified in the Cordes lab) was added at 1:1000 in 1%NDS/PBS*T and incubated over night at 4°C. Slides were washed twice for 5 minutes and then three times for 10 minutes in PBS*. Slides were returned to the humidified container and incubated with secondary antibody, Cy3-Donkey αRabbit (Jackson Immunoresearch Laboratories) at 1:250 in 1%NDS/PBS*T for 2 hours at room temperature in the dark. Slides were then washed three times for 5 minutes each and six times for 10 minutes each with PBS* while remaining protected from the light. Following washes, one drop of VECTASHEILD with DAPI (Vector Laboratories) was placed on each slide followed by a cover slip. Imaging was performed on Nikon Eclipse 80i fluorescence microscope with Nikon DS-Ri1 Camera.

2.4 LacZ staining

LacZ staining performed as previously described [10].
2.5 Chromatin immunoprecipitation assay

(adapted from Fernandez et al., 2001 [71], and protocol provided by Dr. Lohnes)

2.5.1 Chromatin extraction from F9 embryonic carcinoma cells

For each sample 2x10^7 cells (3x100mm plates at ~80 to 90% confluency) were collected. Culture dishes were coated with 0.1% gelatin and contained 10 ml of growth media each. Plates were seeded at 48 hours prior to fixation, given fresh media 24 hours after seeding and then treated with 1 uM of all-trans retinoic acid (Sigma-Aldrich) or ethanol carrier 12 hours prior to fixation. To each plate, containing 10ml of growth media, 313 ul of 32% formaldehyde solution was added (final concentration of 1%) swirled to mix and incubated at room temperature for 10 minutes. To quench any remaining formaldehyde 1ml 1.25 M Glycine was added (0.125M final concentration) swirled to mix and incubated at room temperature for 5 min. Dishes were placed on ice and culture media was aspirated to remove as much liquid as possible. Cells were washed twice with 10ml ice cold PBS. 1ml PBS was added to plates and cells were scraped into microfuge tubes. Cells were spun at down at 200 x g for 5 min at 4°C, supernatant removed and incubated on ice for 15 minutes in 1ml of Triton buffer (10mM Tris-HCl pH 8.0, 10mM EDTA, 0.5mM EGTA, 0.25% Triton X-100) then spun down again, supernatant removed and incubated on ice for 15 minutes in 1ml of NaCl buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Samples were spun down once more and cells from three replicate plates were combined in 1ml RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% Deoxycholate)
containing protease inhibitors (one Roche “mini complete” protease inhibitor tablet per 7 ml of buffer) and incubated on ice for 30 minutes. Samples were sonicated with four 10-second bursts with the sonicator tuned at 25 and set to 30%, spun down and supernatants collected. Input DNA was collected at this stage. The remainder of the samples were stored as bulk chromatin extracts split into four 250 ul samples with individual volumes increased to 1 ml with RIPA Buffer and stored at -80°C.

2.5.2 Chromatin extraction from embryos at 8-8.5dpc (0-12 somites)

Embryos were rehydrated by going through the MeOH series outlined above in reverse then washing three times for 5 minutes each in PBST (150 embryos per sample). After rehydration, chromatin was cross-linked with 1% formaldehyde in PBS for 15 minutes at room temperature. Formaldehyde was quenched by adding glycine to a final concentration of 0.125 M. Samples were centrifuged (2000rpm 5 min) and pellets washed in ice-cold PBS, then resuspended in 1 ml RIPA Buffer containing protease inhibitors. Tissues were disrupted with a syringe with no needle, to break up embryos into smaller pieces, then with a fine gauge 26 3/8 needle and incubated on ice for 1 hour. Chromatin samples were then sonicated with four 10 second bursts (sonicator tuned at 25 and set to 30%). Sonicate was spun down to remove cell debris and supernatants were collected. Input DNA was collected at this stage (20 ul each of each chromatin sample, diluted to 100 ul in RIPA buffer and stored at -80°C). Samples were split in two and volumes increased to 1ml with RIPA buffer containing protease inhibitors and stored at -80°C.
2.5.3 Chromatin immunoprecipitation

Day 1: Samples were pre-cleared by adding 2 ug sheared herring sperm DNA, 20 ul rabbit serum and 50 ul A/G Agarose (Santa Cruz Biotech, cat # sc-2003) to each sample and incubating for 4 hours on a rotator at 4°C. Samples were centrifuged at 4500rpm for 2 minutes and supernatant collected, 5 ul Cdx1 antibody was added to experimental samples and none to controls, and samples were incubated on a rotator at 4°C over night.

Day 2: 50 ul A/G Agarose plus 2 ug sheared herring sperm DNA were added to each sample followed by a 4 hour incubation with rotation at 4°C. Agarose beads containing sample were collected by centrifugation (2 minutes at 4500rpm) and washed sequentially in 1ml each of the following: TSE I (20 mM Tris-HCl pH8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), TSE II (20 mM Tris-HCl pH8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), Buffer III (10 mM Tris-HCl pH8.0, 1 mM EDTA, 1% Deoxycholate, 0.25 M LiCl, 1% NP-40), TE pH8.0 (10 mM Tris-HCl pH8.0, 1 mM EDTA). Each wash was incubated on a rotator for 10 minutes at 4°C. To verify immunoprecipitation of Cdx1 protein a subset of ChIP samples were prepared for Western analysis by adding 10 ul Western lysis buffer, 10 ul 4X SDS loading buffer and heated to 95°C for 10 minutes to reverse all cross-linking. A Western blot was performed on the IP using standard protocols. Remaining samples were eluted from agarose beads by adding 100 ul of freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃) and incubating, along with
input samples, at 65°C overnight to reverse formaldehyde cross-links. Samples were purified using Qiagen QIAquick PCR Purification Kit per manufacturer’s instructions.

### 2.5.4 Quantitative PCR

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 ul and were run for a total of 40 cycles with an annealing temperature of 60°C.

### 2.6 Time lapse western

Plates were seeded at 48 hours prior to sample collection, given fresh media 24 hours after seeding and then treated with 1 uM of all-trans retinoic acid (Sigma-Aldrich) or ethanol carrier at 2, 6, 12, and 24 hours prior to protein collection. To collect protein samples, media was removed, cells were washed once with room temperature PBS and then resuspended in 500 ul of RIPA buffer with protease inhibitors (one Roche “mini complete” protease inhibitor tablet per 7 ml of buffer) Cells were disrupted with a 21 gauge needle and transferred to an ependorf tube and incubated on ice for 30 minutes. Samples were stored at -80°C until needed. Western blot was performed using standard protocols and primary antibodies at the following concentrations: rabbit αCdx1 1/1000, mouse αtubulin 1/2500.
CHAPTER 3
RESULTS

Material included in this chapter is currently in preparation for submission:

Establishment of the posterior boundary of MafB expression in the embryonic hindbrain

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Melissa Biemann & Dr. Sabine Cordes

3.1 Introduction

Most genes expressed early in hindbrain development show clearly defined anterior boundaries, but more diffuse expression at the posterior. In contrast, MafB, one of the earliest genes to be expressed in the hindbrain, shows crisp boundaries of expression at both its anterior and posterior expression limits [9, 45, 46]. Here I show that the caudal related homeodomain protein Cdx1, which is only present transiently in the hindbrain, acts to repress MafB in the posterior hindbrain at the time of MafB induction. This is the first investigation into the role of cdx genes in hindbrain patterning. Both cdx1 and cdx2 are known to interpret the RA signal to Hox genes
expressed along the spinal cord, thus providing an indirect means of responding to the RA gradient [57-59, 72]. As RAREs have not been identified for all of the hox genes expressed in the hindbrain [13, 15], one can imagine that the Cdx proteins could play the same role in hindbrain segmentation. The induction of segmentation genes is the first step in the molecular cascade that eventually leads to segment-specific transcription and developmental programs [2, 7, 8]. Therefore, deciphering the role of cdx1 in regulation of the early acting segmentation gene MafB will lead to a greater understanding of the molecular mechanisms underlying hindbrain segmentation.

3.2 Functional Cdx binding sites reside within the S5 enhancer

Previously, our lab identified an early-acting, rhombomere-specific enhancer, S5, which initiates MafB expression in the future r5-r6 domain. The 2kb S5 enhancer directs expression of a LacZ reporter in r5-r6 in 0-to-10 somite stage embryos [10]. However, removal of a 240bp region from S5 (ΔS5-LacZ) results in dramatic expansion of LacZ expression past the r6/r7 boundary into the posterior neural tube in 2 out of 2 transgenic lines (Fig 3.1). Posterior expansion of lacZ reporter expression indicates that binding sites for a potential repressor may reside within this region. Analysis of the transcription factor binding sites within the deleted segment, and previous analyses of Hoxb4 gene expression in normal and mutant animals, suggested two possible candidate repressors of MafB in the posterior neural tube: Hoxb4 and the Cdx family of homeodomain proteins.
Figure 3.1 Cdx binding sites, but not the Hox/Pbx binding site, are necessary to establish r5-r6 specific expression

A. LacZ reporter driven by the S5 enhancer in r5-r6.  B. LacZ reporter driven by the S5 enhancer with a mutated Hox/Pbx site (mutated to TCAGGCCTAGG) C. LacZ reporter driven by the S5 enhancer with a 240bp deleted region showing dramatic posterior expansion of the LacZ reporter.  D. 240bp region deleted from the S5 enhancer in C with Cdx sites indicated in red and the Hox/Pbx site highlighted in yellow. (LacZ staining done by Melissa Biemann)
Hoxb4 often binds as a heterodimer with Pbx homeobox proteins to activate gene expression [73, 74]. A consensus Hox/Pbx site (Fig 3.1D) through which Hoxb4 could potentially act was found in the deleted region. When this site was mutated (ΔHox/Pbx-LacZ), a few cells posterior to the r5/r6 were positive for the LacZ reporter but there was no significant expansion of the normal r5-r6 domain (Fig 3.1B). Therefore, Hoxb4 is not the dominant repressor of posterior *MafB* expression. In addition, *Hoxb4* expression is not detected in the hindbrain at the time of *MafB* induction so it cannot establish the initial posterior boundary.

Hindbrain specific *cdx1* expression occurs at the right developmental stage to restrict posterior *MafB* expression and the 240bp deleted region contains multiple consensus Cdx binding sites (NTTTANN based on the SIFI site of sucrase-isomaltase [76]) (Fig 3.1D). A similar distribution of Cdx binding sites was found within the *Hoxa7* regulatory element, which is directly regulated by Cdx proteins [52]. The functionality of the Cdx binding sites within the 240bp deletion fragment was confirmed for Cdx1 *in vitro*, using electrophoretic mobility shift assays (Tomomi Kaneko unpublished data).

### 3.3 Posterior expansion of *MafB* expression in *cdx1-/-* embryos

If the posterior extension of LacZ staining seen in the ΔS5-LacZ reporter were due to the loss of Cdx1 binding sites then, in the absence of Cdx1 protein, a similar posterior expansion of *MafB* expression should be observed. To test this hypothesis, I performed *in situ* hybridization analyses on *cdx1-/-* embryos from the 0-to-22
somite stages (E7.5-E9.5). In cdx1-/− embryos expansion of MafB expression into the posterior neural tube was observed. However, this expansion was only seen for a brief developmental period, after which, the normal r5-r6 expression pattern was regained. Posterior expansion was present from the 0-to-8 somite stages (E8.0-8.5). This expansion was most pronounced at 4-somites (E8.0) and, thereafter, expression began to regress towards the anterior. By 10-somites (E8.75) MafB expression appeared almost normal and was for the most part restricted to the r5-r6 domain (Fig 3.2). Expansion of MafB protein was also seen in cdx1-/− embryos at the 4-somite stage (E8.0) (Fig 3.3). These data suggest that Cdx1 is necessary to establish the initial posterior boundary of MafB expression and indicate that after r5-r6 specific expression is established a separate mechanism acts to maintain it.

3.4 The anterior boundary of Cdx1 lies at the posterior boundary of MafB

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. To investigate the rhombomeric boundaries of Cdx1 protein in comparison to the MafB r5-r6 domain, I performed immunofluorescence experiments with Cdx1 and MafB specific antibodies on serial sections of embryos at the 4-to-12 somite stages (E8.0-8.5). These experiments revealed that the anterior boundary of Cdx1 protein was present at the posterior boundary of MafB protein from the 4-to-8 somite stages (E8.0-8.5). At the 10-somite stage (E8.75), there was a significant gap between the posterior boundary of MafB
Figure 3.2 MafB expression in wild type and cdx1/- embryos.

Whole-mount in situ hybridizations showing normal (A,C,E) MafB expression restricted to rhombomeres 5 and 6. At the 4 (A,B) and 6 (C,D) somite stage there is expansion of MafB expression into the posterior neural tube in cdx1/- embryos (B,D) with posterior limits indicated by arrows. At the 10 somite stage (E,F) MafB expression in cdx1/- embryos (F) is restricted to rhombomeres 5 and 6 and is comparable to normal expression (E).
Figure 3.3 Posterior expansion of MafB protein in cdx1-/– embryos

Immunofluorescence with a MafB specific antibody (A,B) in 4 somite stage embryos demonstrating the normal (WT) pattern of MafB protein localization (A) in rhombomeres 5 and 6 (r5-r6). Lines demark the boundaries of these rhombomeres. In cdx1-null embryos cells positive for MafB staining appear beyond the posterior boundary of r6 (B). DAPI staining for the same sections appear below (C&D).
and the anterior boundary of Cdx1 and, by the 12-somite stage (E9.0), Cdx1 staining was absent from anterior sections of the embryo that were positive for MafB staining (Fig 3.4). The presence of Cdx1 at the r6/r7 boundary at early somite stages further implicates Cdx1 as a repressor of MafB in the posterior hindbrain at the time of induction. These results support the findings of the in situ analysis, that Cdx1 is necessary to establish the posterior boundary of MafB expression, as Cdx1 protein is present at the posterior boundary of MafB at the same stages that posterior expansion of MafB expression was seen in cdx1-/- embryos. Therefore, from 0-to-8 somites (E8.0-8.5) Cdx1 is present at the posterior boarder of MafB where it is required to restrict MafB expression to the r5-r6 domain.

3.5 Cdx1 binds at the S5 enhancer in vivo

Although we have demonstrated an in vitro interaction between Cdx1 and the S5 regulatory element, to confirm that Cdx1 is acting directly to repress MafB expression it is necessary to demonstrate an in vivo interaction. As a preliminary investigation, I conducted chromatin immunoprecipitation (ChIP) analyses on F9 embryonic carcinoma cells. Increased Cdx1 protein has been observed in F9 cells differentiated by addition of RA [47, 63]. Inducing F9 cells with RA could therefore increase the probability of capturing a Cdx1-S5 interaction. A time-course experiment was performed to determine the optimal duration of RA exposure to increase Cdx1 protein levels (Fig 3.5 A). At 0 hours there was no detectable Cdx1 protein, whereas, at 12 hours, there was a peak in Cdx1 protein levels. Therefore, cells with no RA exposure (no Cdx1) were used as a negative control and compared
Figure 3.4 Cdx1 is present at the posterior limits of MafB

Immunofluorescence on sequential sections of embryos using MafB (A,C,E) and Cdx1 (B,D,F) specific antibodies. The typical restrictive r5-r6 localization of MafB protein is seen at the 4 (A), 8 (C) and 12 (E) somite stages. Cdx1 protein is localized at the caudal limit of MafB at the 4 (B) and 8 (D) somite stages. By the 12 somite stage, no Cdx1 staining is seen in the hindbrain (F).
to cells that were induced with 12 hours of RA exposure (high Cdx1). ChIP samples were analyzed with primers within the S5 enhancer flanking the region enriched for Cdx binding sites, primers specific to the \textit{cdx1} auto-regulatory region (as a positive control), and primers at the \textit{MafB} promoter (as a negative control). When ChIP samples were analyzed by standard PCR, Cdx1 binding was present at the \textit{cdx1} auto-regulatory region but not at the \textit{MafB} promoter, as expected. Cdx1 binding was clearly evident at the S5 enhancer and this binding was RA dependent (Fig 3.5B). This experiment confirms \textit{in vivo} binding of Cdx1 at the S5 enhancer.

Once binding was confirmed in F9 cells, I next performed ChIP on embryos to demonstrate a true \textit{in vivo} interaction. In order to capture a Cdx1-S5 interaction at the developmental stage of interest ChIP was performed on embryos staged from 0-to-12 somites (E8-8.5). The embryo ChIP samples were analyzed with qPCR using the same primers as for the F9 ChIP (at the S5 Cdx binding sites, flanking the \textit{cdx1} auto-regulatory region and at the \textit{MafB} promoter) as well as two additional primer sets (at 2kb upstream and at 2kb downstream of the S5 enhancer). Cdx1 bound at its auto-regulatory region as expected (p<0.001). There was no binding at the \textit{MafB} promoter, or 2kb upstream or downstream of the S5 enhancer. Cdx1 binding was detected at the S5 enhancer in embryos confirming an \textit{in vivo} interaction (p<0.001) (Fig 3.5C). This is the first evidence for \textit{in vivo} binding of a transcription factor to the endogenous S5 rhombomere-specific \textit{MafB} enhancer.
Figure 3.5 Cdx1 binds at the S5 enhancer in vivo

A. Time course western of Cdx1 protein on retinoic acid (RA) induced F9 embryonic carcinoma cells. Cdx1 protein concentration is highest after 12 hours of RA induction. B. PCR amplification of ChIP on RA induced (12 hours) and non-induced cells. Input is sonicated DNA prior to IP. Cdx1 binds at its auto-regulatory element and at the S5 enhancer in a RA dependent manner and there is no detectible binding at the MafB promoter. C. Representative quantitative PCR results for one of two replicate embryo ChIP experiements The Cdx1 auto primers flank the Cdx1 autoregulatory region and act as a positive control. qPCR products are expressed as percent of input (sonicated embryo DNA prior to IP) and compared to background levels (no antibody) using a 2 way anova and post hoc t-test. Cdx1 binds at its autoregulatory region as expected and also binds at the S5 enhancer (*p<0.001).
CHAPTER 4

DISCUSSION

4.1 Cdx1 establishes the posterior expression boundary of MafB

Initiation of MafB expression in the mouse embryo is accomplished through the rhombomere specific enhancer S5. This induction is RA dependent and requires vHnf1 (Fig 4.1). However, because vHnf1 is expressed more posteriorly than the r6/r7 boundary it cannot establish the posterior boundary of MafB. This thesis has investigated Cdx1 as a potential posterior repressor of MafB. Using immunofluorescence experiments, I have confirmed that Cdx1 is at the right place and time to act as a MafB repressor in the posterior neural tube: the posterior boundary of MafB protein and the anterior boundary of Cdx1 protein coincide until the 8-somite stage (E8.5). I have also demonstrated that, in the absence of Cdx1, there is expansion of MafB RNA and protein beyond the normal posterior boundary from 0-to-8 somites (E8.0-8.5), further implicating Cdx1 in the establishment of the posterior expression boundary of MafB. To demonstrate that Cdx1 acts directly to inhibit MafB, I performed ChIP experiments on 0-to-12 somite (E8.0-8.75) embryos and demonstrated that Cdx1 binds in vivo at the endogenous S5 enhancer. Together these results confirm Cdx1 as a direct repressor of MafB expression in the posterior hindbrain from the 0-to-8 somite stages (E8-8.5) (Fig 4.1). This is the first demonstration that the Cdx family of homeodomain proteins is directly involved in A/P hindbrain patterning.
Figure 4.1 Initiation of *MafB* expression

A. At the 0 somite stage vHnf1 and an unknown activator induce *MafB* expression in r5-r6 while Cdx1 represses expression from the r6/r7 boundary and more posteriorly. B. By the 10 somite stage Cdx1, vHnf1 and perhaps the unknown activator have receded into the posterior neural tube in response to the regressing retinoic acid gradient.
4.2 A potential MafB activator

With a repressor identified, restriction of MafB to the r5/r6 domain could, in theory, be accomplished via induction by vHnf1 in r5-r6 and repression by Cdx1 from r6/r7 and more posteriorly. However, vHnf1 alone is not sufficient to induce MafB expression, indicating that a second activator is required. Evidence from the chick and zebrafish has implicated FGF signalling in MafB activation. In zebrafish, FGF3 works through Mitogen-activated Protein Kinase (MAPK) to activate MafB(Val) [14]. FGF-dependent ETS transcription factors, Pea3 and Erm, which are activated downstream of MAPK, are absent in double FGF morpholino-treated (FGF3 & FGF8) zebrafish embryos in which MafB(Val) expression is also extinguished [29]. Pea3 and erm are expressed in zebrafish from the mid/hindbrain boundary to the r6/r7 boundary as of the 1-somite stage [29, 77]. However, in mouse embryos the expression of Pea3 is limited to the posterior at the time of MafB induction [78], eliminating it as a potential activator. Expression of erm, in contrast, has been reported in the posterior hindbrain of E9.5 mouse embryos but earlier expression as well as the specific rhombomeric expression pattern remains to be explored [79]. The localization of erm at the appropriate level of the hindbrain, and the absence of detectable levels in double-morpholino-treated zebrafish embryos make this FGF dependent transcription factor a likely candidate for the translation of the FGF signal to MafB(Val) induction. In addition, functional ETS binding sites have been found in the r5-specific Hoxb3 enhancer and these sites are required for proper expression, indicating that an ETS transcription factor is present in this region of the hindbrain [21, 22, 80]. To determine if Erm, or another ETS transcription
factor, is acting as an activator, the first step would be to examine the S5 enhancer for conserved ETS binding sites. If such sites are found, the next step would be to examine the expression patterns of erm, and perhaps other ETS family members, with in situ analysis in 0-to-4 somite embryos (E8.0). If appropriate expression patterns are observed, then ChIP in embryos could be performed to demonstrate direct binding of the ETS transcription factor at the S5 enhancer. Alternatively, if ChIP-grade antibodies are unavailable for the protein in question, a series of transgenic LacZ reporters could be used to demonstrate necessity of ETS transcription factors for MafB induction. LacZ driven by the normal S5 enhancer, and the S5 enhancer with the vHnf1 site mutated would serve as controls. If an ETS transcription factor is necessary for MafB induction then diminished LacZ staining would be expected when driven by the S5 enhancer with the ETS binding site(s) mutated. If vHnf1, Cdx1 and the ETS transcription factor are sufficient to induce MafB rhombomere specific expression, then a series of Cdx, ETS and vHnf1 binding sites should be sufficient to drive the LacZ reporter in an r5-r6 specific manner.

4.3 Maintenance of MafB expression

While initial rhombomere specific expression of MafB occurs through the S5 enhancer, this enhancer is only active from 0-to-10 somite stages, indicating that a separate mechanism must be responsible for the maintenance of MafB expression. In further support of a separate maintenance program, posterior expansion of MafB in cdx1-/- embryos is seen within a restricted developmental stage (0-to-8 somites, E8.0-8.5), after which expression becomes properly restricted to the r5-r6 domain. The
activities of Cdx1 as well as vHnf1 in \textit{MafB} regulation are limited to this early induction period because these molecules regress towards the posterior during embryonic development and both are absent from the hindbrain by the 10-somite stage (Fig 4.1) [10, 47, 63], long before \textit{MafB} expression is extinguished [1, 2, 37].

Proper \textit{MafB(Val)} expression is dependent on functional \textit{MafB(Val)} protein, in both zebrafish and mice, indicative of an auto-regulatory loop [14, 24, 81, 82]. However, this auto-regulatory loop must be dependent on a regionally restrictive signal because \textit{MafB} expression is not maintained posterior to the r6/r7 boundary in \textit{cdx1-/-} embryos. Preliminary experiments in the lab have determined that the \textit{MafB} auto-regulatory region lies within a 2kb region directly upstream of the promoter. ChIP experiments using primers spanning this region and a \textit{MafB} specific antibody could serve to locate the auto-regulatory segment. Once the region of MafB binding has been identified, this region could be screened for MafB binding sites and binding sites for potential activators to determine how MafB acts to maintain its own expression. The minimal auto-regulatory unit could also be deduced through the use of the transgenic LacZ reporter system.

Chromatin remodelling may also contribute to the maintenance of \textit{MafB} expression. Recently our lab identified and functionally confirmed a Polycomb response element (PRE) for \textit{MafB} (PRE-kr) [83]. PREs are cis-acting DNA regulatory sequences through which Polycomb group (PcG) proteins act to maintain the silent or repressed state, and the trithorax group (trxG) proteins act to preserve the active state [84, 85]. Our data suggest that PRE-kr acts during very brief developmental window and its activity is dependent on RA [83]. As mentioned
above, the S5 enhancer directs LacZ expression in r5 and r6 [10]; however, addition of the PRE-kr upstream of the S5 enhancer results in a complete loss of rhombomere-specific LacZ expression. Interestingly, r5-r6 specific LacZ expression was regained through the addition of an evolutionarily conserved RARE upstream of the PRE-kr. Therefore, in its ground state PRE-kr acts as a silencer, and can be reset in an RA-dependent manner [83]. While this series of experiments outlined the role of PRE at the time of MafB induction, it is reasonable to infer that PRE-kr is also actively involved in the maintenance of the MafB transcriptional state.

Combining these data with the aforementioned knowledge, a model of MafB induction and maintenance can be outlined. At the time of induction PRE-kr grants access to MafB in an RA dependent manner allowing vHnf1, an FGF dependent cofactor and Cdx1 to bind at the S5 enhancer to establish r5-r6 specific expression. After expression is established, MafB maintains its own expression through autoregulation, and PcG and trxG complexes bind at PRE-kr to help in this maintenance through chromatin remodelling (Fig 4.2).

4.4 The Cdx-Hox code: a conserved mechanism of axial patterning

The caudal-type transcription factors have an evolutionarily conserved role in axis patterning and segmentation. In Drosophila, caudal mRNA is maternally provided and its protein is present in a posterior-to-anterior gradient. Drosophila Caudal initiates expression of posterior gap and pair rule genes that establish embryonic segmentation and induce Hox gene expression thus determining segment identity [86-89]. Although the genetic cascades have diverged, the general role of the
Figure 4.2 Induction and Maintenance of *MafB* expression

A. At the time of induction, in response to the RA gradient TrxG proteins bind at the PRE and promote chromatin remodelling granting regulatory molecules access to the S5 enhancer. vHnf1 and an FGF dependent activator induce *MafB* in r5-r6 and Cdx1 represses expression beyond the r6/r7 boundary. B. *MafB* maintenance is achieved through autoregulation in r5-r6 and the TrxG and PcG proteins act through the PRE to aid in this maintenance.
caudal genes in posterior segmentation has been conserved. The vertebrate caudal genes also form a posterior-to-anterior protein gradient via the nested expression patterns of \textit{cdx1}, \textit{cdx2} and \textit{cdx4}. This nested pattern of expression is highly conserved across vertebrates and has been observed in amphibians, mammals, fish and birds [46-51]. Interestingly, rather than acting through intermediate transcription factors, the vertebrate Cdx proteins activate some \textit{Hox} genes directly [50, 52, 53]. In fact, close examination of the \textit{Hoxb} group in the chick neural tube has determined that the 3’ genes (1, 3-5) are responsive to RA and the 5’ genes (6-9) are responsive to FGF, and that this differential signalling is dependent on Cdx activity, further implicating the Cdx proteins as transducers of positional information [70]. Recent work in zebrafish has also implicated Cdx proteins in A/P neural tube patterning. Double-morpholino-treated embryos for \textit{cdx1a} and \textit{cdx4} exhibited a mirror-image axis-duplication phenotype of the anterior spinal cord and hindbrain. In these double-morpholino-treated embryos, the 5’ \textit{Hox} genes were not expressed in the posterior neural tube, indicating that the Cdx proteins are crucial for posterior \textit{Hox} gene expression, repression of the hindbrain fate in the posterior neural tube and the normal patterning of the spinal cord [90, 91]. The role of Cdx proteins in A/P segmentation therefore includes not only axial skeletal patterning, but the neural tube as well.

If positional information is dependent on the gradient of Cdx proteins, one would expect their effect to be dose-dependent. Evidence for a dosage effect comes from experiments where multiple copies of the \textit{Hoxa7} enhancer, containing Cdx binding sites, were placed upstream of a LacZ reporter. Multimerization of this
enhancer resulted in an anterior shift of the LacZ reporter when compared to one copy. This anterior shift was significantly reduced when LacZ was driven by multiple enhancers with the majority of the Cdx sites mutated indicating that the Cdx sites are necessary for the anterior expansion to occur [92]. Cdx1 and Cdx2 are known to act cooperatively in vertebral patterning [51, 57] and a combined role could be imagined in the hindbrain as well. A redundancy in the actions of Cdx1 and Cdx2 could explain the differences in expansion that I observed for ΔS5-LacZ when compared to MafB expression in cdx1/- embryos. While there is obvious posterior expansion of MafB expression in cdx1/- embryos, it is not as dramatic as that observed for the ΔS5-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 into the posterior neural tube. In contrast, when only binding of Cdx1 is eliminated, expansion is limited to the anterior boundary of Cdx2 expression, or a region where Cdx2 protein concentration is high enough to compensate for the absence of Cdx1. Although these two genes differ in their expression patterns they have been shown to act redundantly in vertebral patterning [51, 57]. In transgenic mice where cdx1 was replaced by cdx2 at the endogenous locus, so that it was driven by the cdx1 regulatory elements, Cdx2 can effectively replace Cdx1 function. Although these mice are essentially null for cdx1, they display no skeletal abnormalities due to the presence of cdx2 in the normal cdx1 expression domain [51]. In order to demonstrate a dosage effect in MafB repression, in situ hybridizations in cdx1+/− and cdx1−/-; cdx2+/- embryos could be performed. Vertebral homeotic
transformations seen in $cdx1^{+/-}$ mice are less frequent and severe than those observed in $cdx1^{-/-}$ embryos [58], therefore, either a less dramatic $MafB$ expansion or reduction in the frequency of expansion may be observed in $cdx1^{+/-}$ embryos when compared to $cdx1^{-/-}$ embryos. In $cdx1^{-/-}; cdx2^{+/-}$ embryos, a more dramatic posterior expansion would be expected than that observed in $cdx1^{-/-}$ embryos based on the more severe anterior vertebral homeotic transformations seen in these compound mutant mice [51, 57].

The discovery that Cdx1 is actively involved in hindbrain segmentation is a logical extension of the conserved role of the Cdx proteins in axis patterning. In addition to the aforementioned role of the Cdx proteins in axial skeletal patterning [51, 57, 58], and preliminary evidence of a role in neural tube patterning [90, 91], the Cdx proteins are also involved in A/P patterning of the gut and digestive system development [93, 94]. Therefore, in vertebrates, the Cdx protein family has a recurring role in A/P patterning and segmentation. Future examinations of the role of the Cdx proteins in neural tube patterning and neural development are likely to reveal new Cdx targets and provide further insight into Cdx proteins as transducers of positional signals.
References


