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Essential Roles of the Meis Family Proteins During Segmentation of the Zebrafish Hindbrain : a Dissertation

Seong-Kyu Choe
University of Massachusetts

ESSENTIAL ROLES OF THE MEIS FAMILY PROTEINS DURING SEGMENTATION OF THE ZEBRAFISH HINDBRAIN

A Dissertation Presented

By

SEONG-KYU CHOE

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Approved as to the style and content by

Dr. Kendall L. Knight, Chair of Committee

Dr. Kai Lin, Member of Committee

Dr. Nicholas R. Rhind, Member of Committee

Dr. Nathan D. Lawson, Member of Committee

Dr. Rolf O. Karlstrom, Member of Commttee

Dr. Charles G. Sagerstrom, Dissertation Advisor

Dr. Anthony Carruthers, Dean of Graduate School of Biomedical Sciences

Department of Biochemistry and Molecular Pharmacology

December 11, 2003

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To my family

ABSTRACT

Hindbrain patterning requires many factors involved in early segmentation and later segment identity of the specific domains of the hindbrain. Hox proteins and their cofactors are of great importance during segmentation of the hindbrain, because segmentation and/or segment identity are lost when any of them are lost. Previously, we have reported that Meis proteins synergize with Pbx, another Hox cofactor, and Hox proteins expressed in the hindbrain. To further investigate Meis function during hindbrain development, we utilized a Meis dominant-negative molecule, ΔCPbx4, and expressed it in zebrafish embryos. We find that ΔCPbx4 affects gene expression and neuronal differentiation especially in r3 through r5. Further, we combined ΔCPbx4 with another Meis dominant-negative molecule (ΔHDCMeis) to disrupt Meis function more extensively. Under these conditions, we find that the entire hindbrain loses gene expression as well as its complement of neuronal differentiation. This phenotype is strikingly similar to that of loss of Pbx function, suggesting that Meis proteins act in the same pathway as Pbx. Therefore, Meis family proteins are indispensable for the entire hindbrain segmentation. In addition to the milder effect on hindbrain patterning, we also found upon expressing ΔCPbx4 that the caudal hindbrain transforms to r4-like fates, supported by expression of r4-specific marker gene (*hoxb1a*) and specification of r4-specifc Mauthner neurons in the domain. This phenotype is not reported upon loss of Pbx function, suggesting that Meis proteins may play a more modulatory role, while Pbx is

absolutely required during hindbrain development. Through several in vivo assays, we find that this r4 transformation is induced by Hox PG1 proteins and that *vhnf1* represses r4 fates in the caudal hindbrain to further specify caudal fates in this region. Based on these results, we propose a model by which hindbrain patterning is achieved. Initially, un-segmented hindbrain is segmented into two domains wherein the caudal domain displays an r4 fate. This caudal r4 fate is then repressed by vhnf1 function which restricts the r4 fate to the presumptive r4 domain and specifies r5 and r6 by inducing its downstream genes such as *valentino* and *hox PG3*. Taken together, we conclude that Meis family proteins are essentially involved in function of Hox complexes to specify distinct rhombomeres during segmentation of the zebrafish hindbrain.

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INTRODUCTION

The vertebrate body plan is achieved through the establishment of both anteroposterior (head to tail) and dorsoventral (back to front) axes during early embryonic development. Within the axes, different germ layers are specified and different organs are derived. Studies have been focused for decades on hindbrain patterning since it deals with many aspects of developmental processes, for instances, how pattern formation occurs, how cell specification/differentiation occurs and how morphogenesis occurs. As a sophisticated developmental program driving the genesis of the hindbrain requires many players, I will here discuss known regulators and their proposed action governing hindbrain segmentation and describe my thesis research aims driven from the basics using the zebrafish as a model.

ZEBRAFISH AS A MODEL ORGANISM

Developmental biology focuses on many biological processes including gene regulation, signaling pathways, cell proliferation, germ cell biology, organogenesis, body patterning, tissue regeneration, cell motility and cell guidance using many biological tools such as cell biology, molecular biology, chemical biology, biochemistry, genetics and so on. To understand such broad topics, many model organisms have been studied and each model gives a specific advantage over others in a particular field of study. For instance, *Xenopus* has been used for experimental manipulations to reveal embryonic patterning in early days because of the large size of its eggs (1 to 2 mm in diameter) and ease of availability of eggs. In spite of these advantages, *Xenopus* can not be used for genetic studies due to its long generation time and pseudo-tetraploidy. *Drosophila*, on the other hand, has been used for genetic studies due to the ease of genetic manipulation and a plethora of available mutants, although it does not provide any of vertebrate developmental processes. Recently, the zebrafish has drawn much attention because of its short life cycle, its genetic accessibility and the transparency of its embryos providing easy visualization of developmental events. These advantages have led to successful forward genetics screenings using zebrafish to isolate genes responsible for many developmental processes covering virtually from head to tail (Grosshans et al., 1994; Haffter and Nusslein-Volhard, 1996; Jiang et al., 1996). Recently, with increasing evidence of conserved developmental mechanisms between zebrafish and humans, the zebrafish has become a model to study human

diseases such as cancer (Amatruda et al., 2002; Stern and Zon, 2003). With a promising advancement in gene knock-out technique, studies using zebrafish as a model will provide a great deal of understanding in general biology.

DEVELOPMENTAL STAGES OF THE ZEBRAFISH

To explain how the hindbrain develops from a single cell embryo, I will discuss general aspects of zebrafish development in this section with a particular focus on hindbrain development (zebrafish developmental staging is based on Kimmel et al., 1995). This description will help define terms and processes that will be encountered in this thesis.

Early cell divisions and fate decisions

A single newly fertilized zebrafish embryo starts its cleavage by exhibiting cytoplasmic movements that deliver yolky nutrients to the single cell sitting on top of the vegetal yolk (0 h; Figure 1A). After the first cell division, cells divide rapidly about every 15 minutes (Figure 1B and C). The early cell divisions occur with an incomplete cytokinesis, called meroblastic division and cell cycles are propelled by maternal gene products. At the 512-cell stage (Figure 1D), cells begin to exhibit longer cell cycles, some of which include an interphase, indicating the midblastula transition (MBT). The MBT marks the starting point of zygotic gene expression and cell motility. This cell motility leads to epiboly, through which cells on top of the yolk spread out to cover the yolk. The yolk syncytial layer (YSL), comprised of deep blastomeres that lie on the

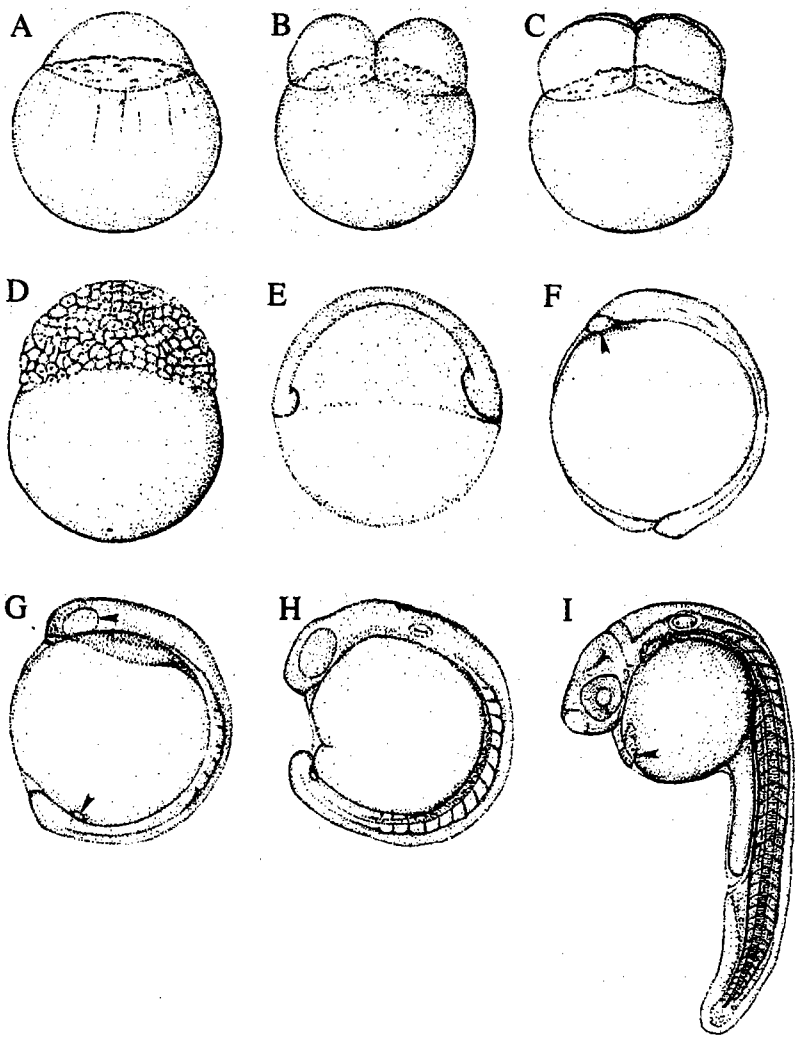

Figure 1. A zebrafish embryo at selected stages. (A) 1-cell stage, (B) 2-cell stage, (C) 4-cell stage, (D) 512-cell stage (2.75 h; onset of the MBT), (E) shield stage (6 h; onset of gastrulation), (F) bud stage (10h), (G) 6-somite stage (12 h); upper arrow head indicates eye primordium, (H) 14-somite stage (16 h), and (I) prim-5 (25 h); hatching gland is indicated by an arrow head on the yolk. Reproduced from Kimmel et al., 1995.

yolk cell, act as a motor for the epiboly process. As epiboly continues, cell movements featuring involution, convergence and extension also occur to produce three primary germ layers as well as the embryonic axis (5.25 h; 50% epiboly). The three germ layers are the epiblast-derived ectoderm (future skin and nervous system), the hypoblast-derived mesoderm (future muscle and internal organs) and the endoderm (future gut and associated organs). The formation of the germ layers begins with involution of the cells in the future dorsal side of the embryo, which also defines the onset of gastrulation. Morphologically, a thickened marginal region called the germ ring is then apparent all around the blastoderm rim at this stage (5.7 h). By 6 h, the embryonic shield is formed through convergence movements of cells towards the dorsal side of the embryo (Figure 1E). The embryonic shield is most easily visible from an animal pole view and determines the dorsoventral axis. Developmentally, the embryonic shield corresponds to Spemann's organizer in the frogs (Harland and Gerhart, 1997; Spemann and Mangold, 1924) and Hensen's node in the chick (Boettger et al, 2001; Joubin and Stern, 1999) because of its ability to induce a secondary axis when transplanted (Saude et al., 2000). Simultaneously with the convergence movements that accumulate cells at the embryonic shield, the extension process occurs to locate cells further anteriorly. Fate mapping analyses have been performed to link early positional information to later cell fates (Woo and Fraser, 1995; Figure 2). Cells at the anterior pole will develop into head structure (nose and eye) and cells near the embryonic shield give rise to the notochord. Therefore, one can easily determine both the anteroposterior and the dorsoventral axes

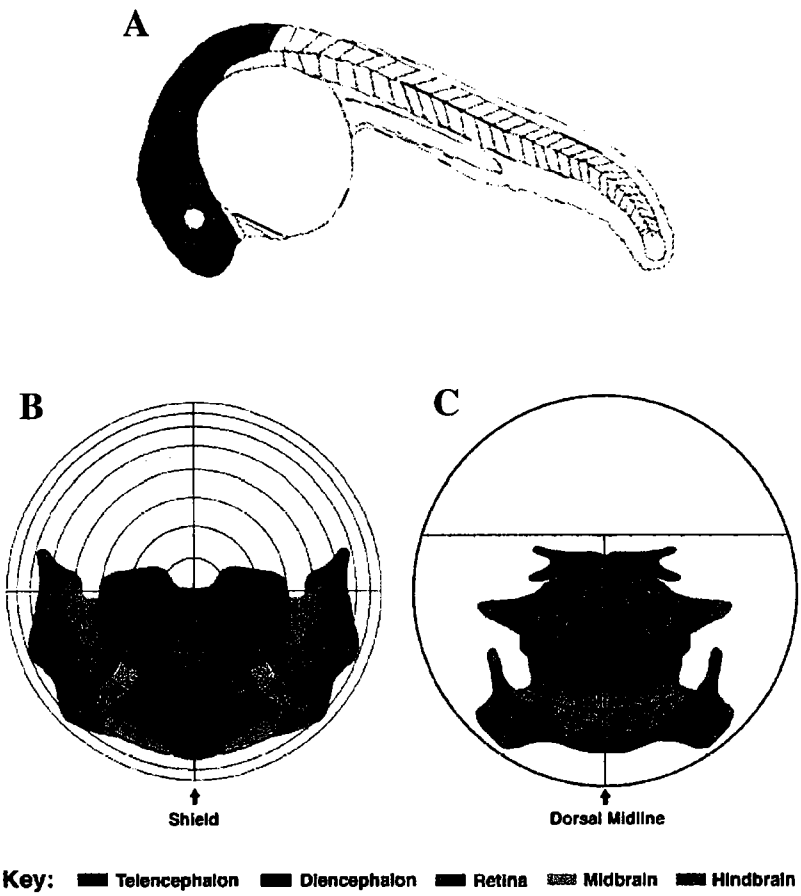

Figure 2. Zebrafish brain fate maps. (A) An embryo at 24 h is shown with colored head region. Each color represents a specific brain region as indicated in the key box at the bottom of the figure. (B and C) Fate map at 6 h and 10 h, respectively. Note that at 6 h, progenitors are spreaded more laterally than cells at 10 h. Reproduced from Woo and Fraser, 1995.

at early gastrulation stage. During the gastulation period, hindbrain fates as well as other fates are pre-determined. For instance, genes known to be critical for the formation of the hindbrain are expressed as early as 70% epiboly stage (e.g. *hoxb1b*; (Prince et al., 1998) and neuronal precursors that will consist of the hindbrain neuronal structure are believed to form at this stage (e.g. precursors for Mauthner neurons (McClintock et al., 2002).

Morphogenesis and differentiation into specialized tissues

By 10 h (tail bud stage; Figure 1F), the yolk is completely covered by the blastoderm and from this stage an enormous morphological differentiation occurs. One such event is the formation of somites that appear in the trunk and tail. Somites are formed sequentially with anterior one first and are used as the staging index (Figure 1G and H). These somites contribute to the body muscles, vertebral cartilages and dermis. Another prominent event is the formation of the neural plate from which the central nervous system is derived. The neural plate then forms the neural keel structure which in turn rounds up in a cylindrical shape into the neural tube. The brain rudiment is formed at segmentation stages with about ten distinct swellings (neuromeres) in the anterior region of the neural tube. The first three correspond to the telencephalon (forebrain), the diencephalon (forebrain) and the mesencephalon (midbrain). The remaining seven are called rhombomeres and make up the hindbrain (Figure 3A). Along with the emergence of the neuromeres, neural crest cells begin to migrate from the dorsolateral position of the neural tube to give rise to structures such as peripheral

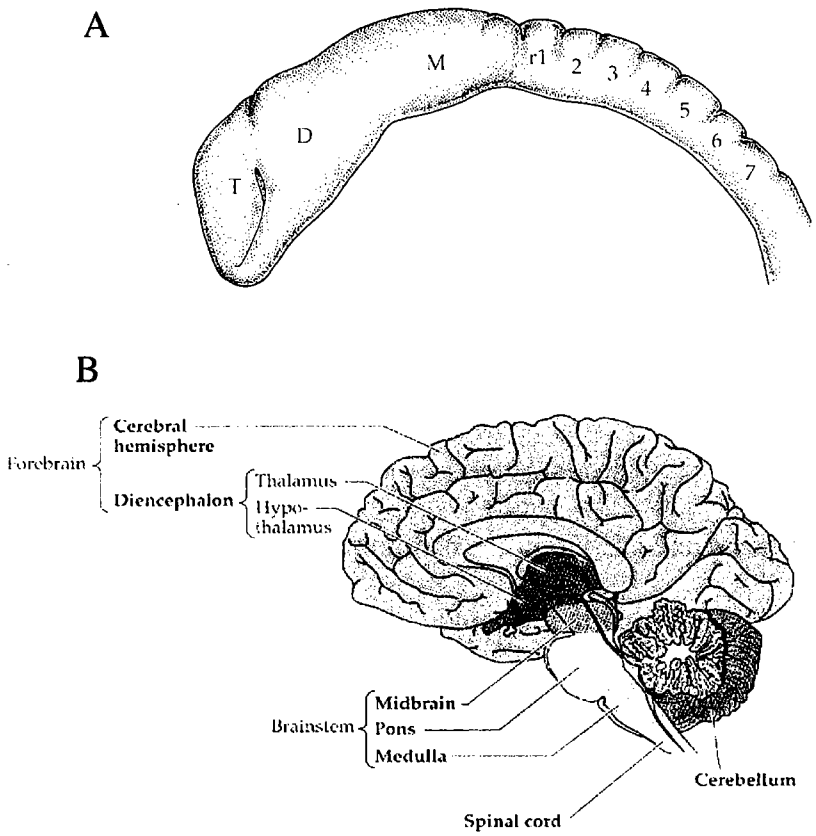

Figure 3. A developing zebrafish brain rudiment and adult mammalian brain anatomy.
(A) At 18 h (18-somite stage), a zebrafish embryo develops about ten neuromeres; T (telencephalon), D (diencephalon), M (mesencephalon) and r1-7 (rhombomeres). (B) Adult brain anatomy. Structures derived from the hindbrain are pons, medulla and cerebellum. Reproduced from Kimmel et al., 1995 (A) and from Kandel et al., Principles of Neural Science.

neurons, ganglia and cartilage. Moreover, other morphological processes such as formation of the eyes and otic vesicles are also apparent during segmentation stages. Neuronal differentiation, one of the central features during the hindbrain development, is also first observed at the early segmentation period; primary neurons that connect the hindbrain with the spinal cord are formed with their large cell bodies and long axonal projections. Sensory and motor neurons are also formed in the central area of the neuromeres.

Formation of organs and end of the embryonic development

By 24 h after fertilization, the zebrafish embryo looks like a vertebrate organism (Figure 1I). It now contains a differentiated notochord, a segmented hindbrain with advanced general brain morphology, a complete set of somites, a more complete nervous system and a circulatory system. An embryo at this stage responds to a stimulus. Heart formation, pigmentation, fin generation, protruding mouth and jaw structure are apparent as development proceeds.

HINDBRAIN DEVELOPMENT

Patterning of the hindbrain requires segmentation of the posterior brain region to provide positional identity along the anteroposterior axis of the neural tube. Differential gene expression throughout the hindbrain ensures normal segmentation of the hindbrain, which eventually develops into adult structures, such as cerebellum, medulla oblongata and pons to perform important daily function including coordinated motor

responses, autonomic regulation and bridging information shared between the cerebral hemisphere and the cerebellum, respectively (Figure 3B). Several key players will further be discussed in the following sections. The developing hindbrain is comprised of 7 segments, each known as a rhombomere (r) (Lumsden, 1990; Vaage, 1969). Distinct types of cranial nerves innervating the face and neck are specified from the rhombomeres and neural crest cells differentiating to a variety of cell types such as peripheral neurons, skeleton and pigment cells are derived from the dorsal neural tube (Guthrie, 1995). In addition, pharyngeal arches are also derived from the neural crest cells to constitute major skeletal structure in this region (Ghislain et al., 2003; Hunt et al., 1998; Hunt et al., 1991; Krumlauf et al., 1993; Noden, 1991).

Induction of neural fates from dorsal ectoderm

Neural fate are induced in the dorsal ectoderm during gastrulation. Initial induction of dorsal fate is thought to depend on signals from the vegetal most portion of yolk at late blastula stage of the embryo. Although the determinant has not yet been identified, it is thought that the determinant translocates to the future dorsal side of the embryo and that this translocation stabilizes and translocates β-catenin to the nucleus of the dorsal YSL. Once the nuclear β-catenin activates genes on the dorsal side of the embryo, these genes antagonize the activity of the bone morphogenetic protein family members, such as BMP2 and BMP4, in dorsal ectoderm. These β-catenin induced genes include *noggin*, *chodin* and *follistatin* and are important to derive the dorsal fate as well as the neural fate, since mutants defective in each function have been shown to generate

a ventralized phenotype and fail to form a neural tube (Hammerschmidt et al., 1996; Schulte-Merker et al., 1997; Bauer et al., 1998; Furthauer et al., 1999). Further, mutation in either *bmp7* (*snailhouse*) or *bmp2* (*swirl*) results in a dorsalized embryo with expanded neural ectoderm (Dick et al., 2000; Kishimoto et al., 1997). Therefore, the dorsoventral axis is established through the concerted actions of BMP proteins and BMP-antagonizing proteins, and a neural fate is specified from a portion of the dorsal ectoderm.

Factors that posteriorize the anterior neural tube

During the gastrulation stage, the anteroposterior axis is established as a part of the body plan. Secreted factors such as retinoic acid (RA), fibroblast growth factor (FGF), and Wnt ligands are known as the posteriorizing factors that induce the anterior neuroectoderm to more posterior fates (Doniach, 1995; Kolm et al., 1997; McGrew et al., 1997). Evidence that supports RA as an endogenous signal in this process comes from studies on quail embryos deficient in dietary vitamin A, whose hindbrain is abnormally patterned (Gale et al., 1999; Maden, 1995; Maden et al., 1996; Maden et al., 1997). Gain-of-function and loss-of-function studies have further demonstrated RA action in hindbrain patterning (Blumberg et al., 1997; Dupe et al., 1999; Durston et al., 1989; Kolm et al., 1997; Marshall et al., 1992; van der Wees et al., 1998). Studies on FGF and Wnt ligands were similarly performed and suggested that these secreted molecules also function in the formation of posterior neural structures (Erter et al., 2001; Fekany-Lee et al., 2000; Griffin et al., 1995; Kim et al., 2000; Lekven et al.,

2001; Pownall et al., 1998; Pownall et al., 1996). Recently, a study dealing with combinatorial effects and distinct roles of these signaling molecules in inducing posterior fates of the neural tube was completed (Kudoh et al., 2002). In this study, both FGFs and Wnts initially suppressed anterior genes in an RA-independent manner, after which RA activation was necessary and sufficient to induce posterior genes (Figure 4). Therefore, distinct roles for these factors in neural patterning have begun to be elucidated.

Neuronal specification and differentiation in the zebrafish hindbrain

Neuronal organization of the zebrafish hindbrain correlates with its morphological segmentation into rhombomeres. The hindbrain contains cranial nerves from the trigeminal (Vth) to the hypoglossal (XIIth) nerves. These nerves contain either motor or sensory neurons, or both types of neurons, and performing a variety of activities including facial expression, eye movement, hearing, balancing, taste and so on. The cranial nerves are functionally homologous to the spinal nerves of the spinal cord, except that the cranial nerves tend to perform a specific function while the spinal nerves provide all sensory and motor function to a specific segment. For instances, the facial nerve (VIIth), which is specified in r4 and r5 and further differentiates in r6 and r7 after migration, provides sensory input from the skin, motor output to muscles of the face and taste; the trigeminal nerve (Vth), on the other hand, which is specified in r2 and r3, senses light touch and controls jaw movement. The cranial nerves in the hindbrain also innervate neighboring pharyngeal arches to control jaw as well as gill

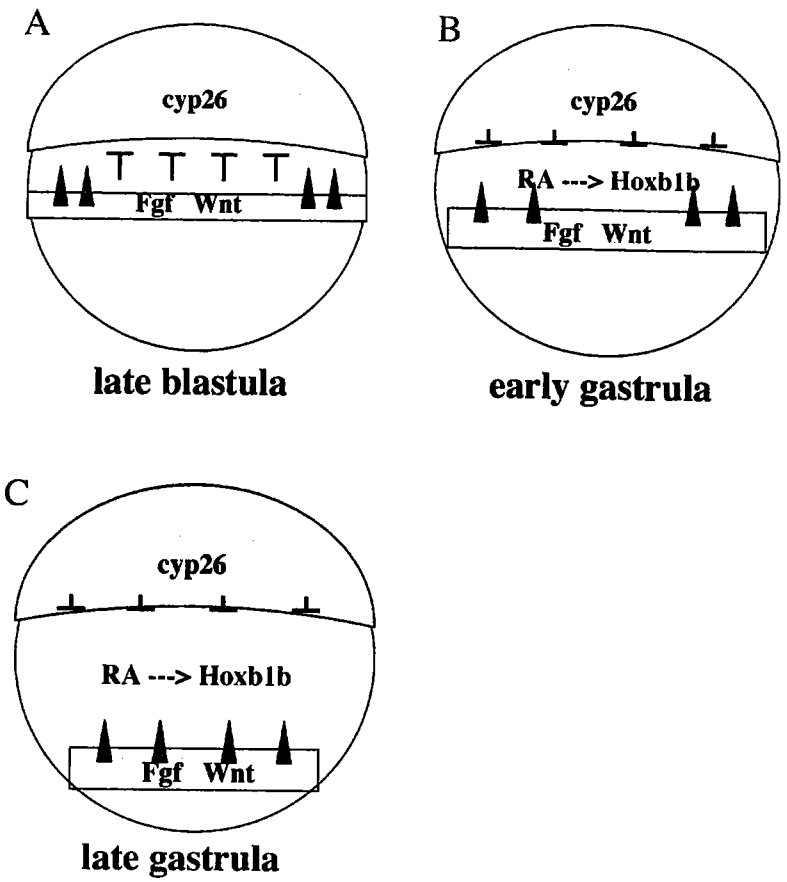

Figure 4. Summary of interactions between Fgf, Wnt and RA during early development. (A) At the late blastula stage, Fgf and Wnt signals (shown in red triangles) from the blastoderm margin induce posterior neural fates by blocking the expression of cyp26. (B) As the gastrulation begins, an area between cyp26 and Fgf and Wnt domains becomes widened and accumulates RA which induces posterior gene expression such as hoxb1b. (C) As the gastrulation proceeds, the domain expands and gives rise to the posterior neuroectoderm. Reproduced from Kudoh et al., 2002.

structures. Therefore, the organization of the cranial nerves is a representation of the hindbrain segmentation (Figure 5A). Another neuronal type in the hindbrain, called reticulospinal interneurons, is also derived in a segment-restricted manner. These neurons are responsible for coordinated reflexes and simple stereotyped movements and are the principal pathways for voluntary movements of organs in the head and body of most vertebrates. In zebrafish, these neurons are located bilaterally along the anteroposterior axis and in the center of the rhombomeres. Various types of the neurons are named after their position through the axis; there are rostral neurons (Ro), middle region neurons (Mi) and caudal neurons (Ca). Among the reticulospinal interneurons, the most prominent ones are the Mauthner neurons that are specified and differentiate only in r4 (Figure 5B). As the specification and differentiation of the hindbrain-derived neurons reflect the intrinsic fate of specific rhombomeres, experiments dealing with fate changes utilize these specific characteristics as markers of rhombomere identity.

Segmentation of the hindbrain

Hindbrain segmentation is first visible at the 5-somite stage and r4 seems to be the first rhombomere to form (Maves et al., 2002). These observations suggest that the formation of rhombomeres is sequentially achieved; the first rhombomere, r4, acts as a signaling center from which adjacent rhombomeres are further specified (Maves et al., 2002). Moreover, it was shown that the r4-restricted expression of FGF3 and FGF8 is required to give rise to r5/6 segmentation. Specifically, by using both the *acerebellar* mutant fish line that lacks FGF8 and morpholino antisense oligos against FGF3 for

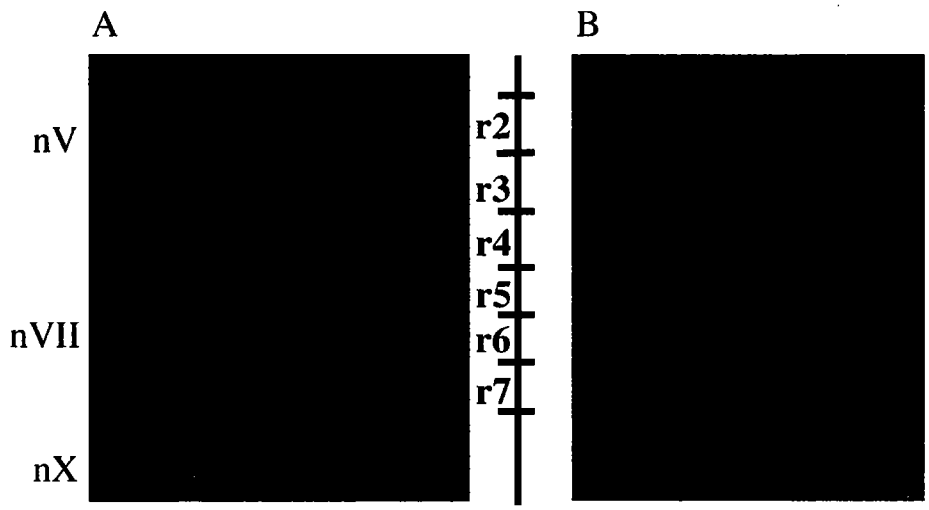

Figure 5. Hindbrain-derived neurons.
(A) The branchiomotor neurons are detected through a *GFP-islet1* trangenic line. nV neurons are specified in r2/3 and nVII neurons appear primarily in r6/7 following their original specification in r4/5. nX neurons are shown in the caudal hindbrain. (B) The reticulospinal interneurons are also segmentally restricted. The most prominent, r4-derived Mauthner neurons are shown as a pair projecting their axons contralaterally.

knocking down FGF3 transcripts, they generated embryos lacking r5 and r6 segments (Maves et al., 2002). Very recently, a zebrafish mutant that lacks vHnh1 function was found to lose r5 and r6 of the hindbrain (Wiellette and Sive, 2003). vHnf1 is believed to be an upstream regulator of *valentino* (*val*), which is required for proper r5/r6 segmentation. In this study, Fgf3 and Fgf8 were shown to synergize with vHnf1 to effect r5 and r6 segmentation by inducing *val* expression, suggesting an indispensable role for Fgf3 and Fgf8 in r4 to induce r5 and r6 fates (for a summary model, see Figure 6). Further evidence that supports the sequential development of the hindbrain rhombomeres comes from interference with RA signaling. Double mutant mice lacking both RA receptor (RAR) α and RARγ display an expansion of *Hoxb1* expression into the caudal hindbrain and application of RA antagonists generates an enlarged region of r4 identity (Dupe et al., 1997; Dupe and Lumsden, 2001; Wendling et al., 2001). Moreover, a dominant-negative RAR-β construct induces ectopic caudal Mauthner neurons in *Xenopus* (van der Wees et al., 1998). Taken together, it is very likely that the hindbrain segmentation is achieved through several distinct steps by which the hindbrain primordium is first divided into broad domains which are further divided into more defined rhombomeres. Although many genes have been identified and studied to ultimately resolve how the hindbrain segmentation is achieved, there are many gaps between the individual phenomena observed. Identification of more genes that act early enough to distinguish events between the various segmentation steps is warranted to further understand the hindbrain segmentation.

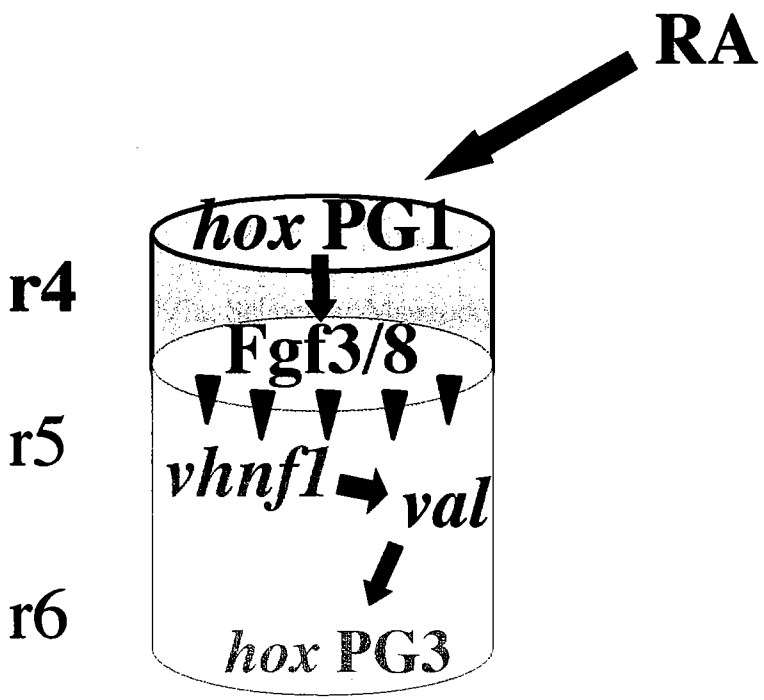

Figure 6. A simplified diagram showing current view of the R4-signaling center and the segmentation of r5 and r6. RA-induced *hox* PG1 induces Fgf3/8 in r4, which acts as a signaling center to further specify caudal rhombomeres (r5/6). Fgf3/8 work together with *vhnf1* in order to activate downstream target genes, especially *val* expression. Based on Maves et al., 2002 and wiellette and sive, 2003.

Key regulators required for segmentation

Under the regulation of posteriorizing factors, segmentation genes such as *hoxb1b*, *krox20* and *valentino* (the zebrafish homolog of mouse *Kreisler*) are thought to be activated to further specify hindbrain rhombomeres. *Krox20* encodes a zinc-finger transcription factor and is expressed only in r3 and r5 (Wilkinson et al., 1989). Phenotypes from targeted *Krox20* inactivation show loss of these rhombomeres, suggesting indispensable role of *krox20* in the formation/maintenance of r3/5 (Schneider-Maunoury et al., 1997a; Schneider-Maunoury et al., 1997b; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). *Valentino* was identified in a mutagenic screen for genes responsible for brain regionalization (Moens et al., 1996). In *val* mutants, r5/6 are formed as a mis-specified domain, termed rX, and thus *val* is required for r5/r6 specification (Moens et al., 1996). These segmentation genes are known to activate downstream *hox* target genes within their expression domain and the *hox* gene expression is essential for the segmental identity along the anteroposterior axis (Manzanares et al., 2001; Manzanares et al., 1999; Manzanares et al., 1997; Manzanares et al., 2002; Nonchev et al., 1996; Sham et al., 1993).

Recently, a zebrafish *vhnf1* mutant was discovered and found to have a hindbrain patterning defects (Sun and Hopkins, 2001). Mutations in *vHnf1* were originally implicated in the development of human disease such as MODY5 (maturity-onset diabetes of the young, type V) and familial GCKD (glomerulocystic kidney disease) suggesting that *vHnf1* has an important function in development of the pancreas and the kidney (Bingham et al., 2001; Horikawa et al., 1997; Lindner et al.,

1999; Nishigori et al., 1998). In the developing zebrafish hindbrain, *vhnf1* is transiently expressed and this expression disappears by the 8-somite stage (Sun and Hopkins, 2001). Zebrafish embryos bearing *vhnf1* mutation show expanded r4 marker gene expression while ectopic expression of *vhnf1* gives rise to expansion of r5/6 marker gene expression, suggesting an indispensable role of vhnf1 in segmentation of the caudal hindbrain region, especially r5 and r6 (Sun and Hopkins, 2001). Further, study of *vhnf1* function in relation to FGF3 and FGF8 expression in r4 revealed that vHnf1, FGF3 and FGF8 synergize to specify r5/6 by inducing downstream genes, in particular *val* (Wiellette and Sive, 2003).

Hox genes

In vertebrates, there are 4-7 different chromosomal *Hox* complexes depending on the organism (Krumlauf, 1992; McGinnis and Krumlauf, 1992). The close relationship between expression and function of the *Hox* complexes and their physical order in the genome is referred to as 'colinearity' (Duboule and Dolle, 1989; Graham et al., 1989). *Hox* genes encode homeodomain-containing transcription factors and their expression provides a 'hox code' which delivers positional identity along the anteroposterior axis (Krumlauf, 1994; McGinnis and Krumlauf, 1992). The ability of Hox genes to specify positional value was originally found in studies of the fruit fly *Drosophila* where homeotic transformations were induced by disruptions of the *hox* gene expression (Gehring, 1967; Schneuwly et al., 1987; Struhl, 1981). In the developing hindbrain, there are 4 different paralog groups (PG) of *hox* genes expressed

in overlapping domains (Figure 7; Lumsden and Krumlauf, 1996). *Hox* gene expression in the hindbrain is believed to be induced by the action of RA because upstream regulatory elements of many *Hox* genes include retinoic acid response elements (Dupe et al., 1997) and because both disruption of RA signaling and application of exogenous RA affect *hox* gene expression (Alexandre et al., 1996; Kolm and Sive, 1995; Marshall et al., 1992). The initial expression of *hoxb1b*, equivalent to mouse *Hoxa1* (McClintock et al., 2002), in the presumptive caudal hindbrain of zebrafish (Vlachakis et al., 2000) induces expression of *hoxb1a*, equivalent to mouse *Hoxb1* (McClintock et al., 2002), in a similar region (Gavalas et al., 1998; Popperl et al., 1995; Rossel and Capecchi, 1999; Studer et al., 1998). Expression of more downstream *hox* genes such as *hoxb2* is then induced by *hoxb1a* in r4 (Maconochie et al., 1997). In addition to this regulation, *hox* genes from PG2 and PG3 are also regulated by *krox20* and *valentino* in r3 and r5/6. The overlapping *hox* gene expression in a given rhombomere together with expression of other segment identity genes provides a positional identity distinct from that of neighboring rhombomeres. Experiments demonstrating critical *hox* gene regulation on hindbrain patterning come largely from genetic studies. When disrupted, *hox* PG1 either alters r4 fate (for *hoxb1a*) or generates loss of r4/5 (for *hoxb1b*) (Carpenter et al., 1993; Dolle et al., 1993; Mark et al., 1993; McClintock et al., 2002; Studer et al., 1996). On the other hand, disruption of *hox* PG2 and PG3 genes causes much milder defects revealing redundant Hox function among the paralogs (Davenne et al., 1999; Gavalas et al., 1997; Greer et al., 2000; Hunter and Prince, 2002; Manley and Capecchi, 1997).

Mouse *Hox* gene expression **Zebrafish *hox* gene expression**

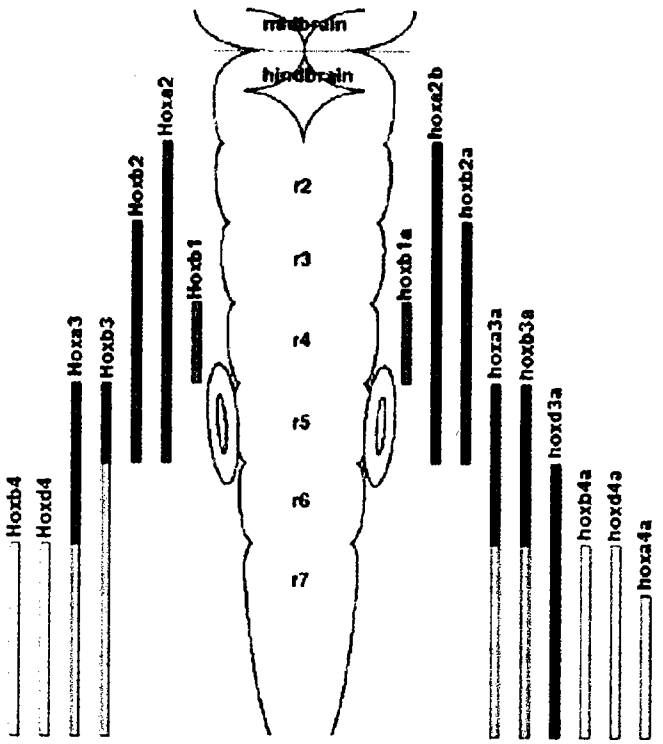

Figure 7. Expression of *Hox* PG1-4 genes in mouse and zebrafish during hindbrain development. Each paralog group is shown in the same color. Although the zebrafish has more *hox* genes than the mouse expressed in the hindbrain, a similar complement of *Hox* genes that cover the same rhombomeres are expressed. Reproduced from Moens and Prince, 2002.

Therefore, understanding Hox function as well as their targets is crucial to understanding the segmentation processes during hindbrain development.

Hox Cofactors

Hox proteins cannot function as monomers since they have display poor affinity and/or selectivity for DNA target sequences. Identification of TALE (Three Amino acid Loop Extension) homeodomain Hox cofactors of the Pbx (Pre-B cell leukemia; *Drosophila* Extradenticle, Exd) and Meis/Prep (Myeloid Ecotropic viral-Integration Site; *Drosophila* Homothorax, Hth) families has facilitated understanding how Hox proteins function *in vivo* (Mann and Affolter, 1998; Ryoo et al., 1999). It has been shown that dimers and trimers between Hox proteins and their cofactors can be formed (Chang et al., 1997; Ferretti et al., 2000; Knoepfler et al., 1997; Shen et al., 1999) and the formation of such complexes is critical for the function of Hox proteins *in vivo* (Vlachakis et al., 2001) . Specifically, dominant-negative forms of Hth in *Drosophila* induced many developmental defects including inactivation of direct Hox target gene (Jaw et al., 2000; Ryoo et al., 1999). Furthermore, several Hox target promoters have been shown to include Meis, Pbx and Hox binding sites (Ferretti et al., 2000; Jacobs et al., 1999; Popperl et al., 1995; Ryoo et al., 1999).

Pbx family proteins consist of at least 4 members in vertebrates, Pbx1 through Pbx4. Initial identification of Pbx as a Hox cofactor came from studies in *Drosophila* where mutations in the *Drosophila* ortholog of Pbx, Exd, generates a phenotype similar to loss of *hox* gene function. Further efforts have demonstrated that Exd interacts with

Drosophila Hox proteins and enhances their DNA binding specificity. Biochemical studies have shown that Pbx interacts with Hox PG 1-10 proteins *in vitro* (Shen et al., 1997b) and studies in *Drosophila* as well as in mice suggested Pbx as an important Hox partners *in vivo* (Mann and Affolter, 1998; Ryoo et al., 1999; Maconochie et al., 1997; Manzanares et al., 2000; Popperl et al., 1995). Therefore, Pbx functions as a Hox cofactor to enhances Hox affinity for its target by binding cooperatively to target DNA sequences as a Pbx/Hox dimer (Knoepfler and Kamps, 1995). The presence of bipartite Hox/Pbx binding sites in the regulatory regions of *Hox* genes further supports such a role (Ferretti et al., 2000; Jacobs et al., 1999; Ryoo et al., 1999). In the developing hindbrain, a zebrafish *lazarus* mutant, which lacks zygotic Pbx4 function, shows a defect in the segmentation of r3 and r4, demonstrating a crucial Pbx requirement for the segmentation of the hindbrain (Popperl et al., 2000). Furthermore, phenotypes generated from the simultaneous loss of Pbx2 and Pbx4, called the'hindbrain ground-state' in which no distinct rhombomeric fate is observed, is evidence that Pbx function is indispensable for segmentation of the entire hindbrain (Waskiewicz et al., 2002).

The Meis family is comprised of Meis1 through Meis3 and the more distantly related Prep1 and Prep2 in vertebrates. As is the case of Pbx, an indication that Meis is a Hox cofactor came from studies in *Drosophila* where loss of Hth function disrupts the embryonic patterning and resembles the complete loss of Exd function (Rieckhof et al., 1997). Biochemically, Meis has been shown to interact with Hox PG 9-13 *in vitro* (Shen et al., 1997a) and also with Pbx in solution as well as on enhancer sequences of DNA. However, the formation of dimeric and trimeric complexes among Hox, Pbx and Meis

suggests that Meis-containing complexes may have different *in vivo* roles from Hox/Pbx dimers (Chang et al., 1997; Ferretti et al., 2000; Jacobs et al., 1999; Knoepfler et al., 1997; Ryoo et al., 1999; Shen et al., 1999). Therefore, it is likely that Meis is not required for all Hox function (Ferretti et al., 2000) or that it performs a more modulatory role in the complex (Moens and Prince, 2002). A clue to Meis function during hindbrain development comes from its expression pattern. Several reports in zebrafish indicate that *meis* is dynamically expressed during early development and this expression overlaps with early-acting *hox* genes as well as *hox* genes belonging to other palarog groups (Figure 8; Sagerstrom et al., 2001; Vlachakis et al., 2000; Waskiewicz et al., 2001). In the hindbrain, Meis can not interact with Hox proteins from PG1-4 directly (Chang et al., 1997; Knoepfler et al., 1997; Shen et al., 1999). Instead, it may bind Pbx to exert its role on Hox function during hindbrain development. Gain-of-function studies in zebrafish have demonstrated that Meis promotes caudal hindbrain fates synergistically with Pbx4 and Hoxb1b and that the Pbx-interacting domains of Meis3 and Hoxb1b are absolutely required for this effect (Vlachakis et al., 2001). Additional evidence for Meis function in hindbrain development comes from a *Xenopus* study where *Xmeis3* misexpression in *Xenopus* embryos leads to mispatterning of the anteroposterior axis indicating the Meis involvement in axis patterning (Salzberg et al., 1999). Taken together, these results suggest that Meis proteins have important roles in hindbrain development by participating in Hox-containing complexes.

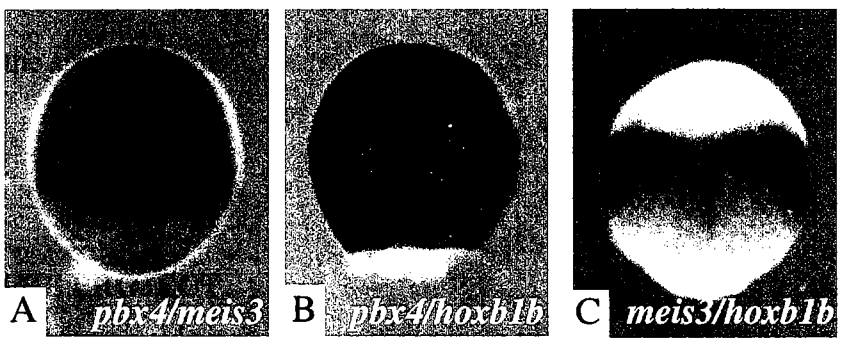

Figure 8. *meis3* expression overlaps with *hoxb1b* and *pbx4* expression at late gastrula stage. (A-C) double in situ hybridization analyses reveal that *meis3*, *pbx4* and *hoxb1b* are all expressed in the presumptive hindbrain. *pbx4* is shown in red while *hoxb1b* and *meis3* are shown in purple. Reproduced from Vlachakis et al., 2000.

Although earlier studies suggested that Meis genes are involved in hindbrain development, a critical investigation will be Meis loss-of-function studies. Further, direct *in vivo* evidence that supports the relationship between Hox and Meis proteins during the hindbrain segmentation is still lacking. To evaluate the function of Meis and link Meis to the function of the Hox-containing complex, I performed the experiments described in the specific aims.

GOAL OF THE PROJECT

Study the role of *meis* in conjunction with *hox* and *pbx* during hindbrain development in vertebrates

SPECIFIC AIMS

A. Determine the role of *meis* during hindbrain development

1. Define functional Meis domains required for hindbrain development
2. Determine if Meis is required for normal hindbrain segmentation by using Meis dominant-negative constructs

B. Determine if *meis* synergizes with *hox* genes during hindbrain patterning

1. Determine if *meis* genetically interacts with *hox* genes
2. Determine Meis-dependent Hox targets during normal hindbrain segmentation

CHAPTER I

MEIS FAMILY PROTEINS ARE REQUIRED FOR HINDBRAIN DEVELOPMENT IN THE ZEBRAFISH

Seong-Kyu Choe^, Nikolaos Vlachakis^ and Charles G. Sagerström*
Department of Biochemistry and Molecular Pharmacology, and Program in Neuroscience, University of Massachusetts Medical School, Worcester, MA

^These authors contributed equally to this work
*To whom correspondence should be addressed at:
Department of Biochemistry and Molecular Pharmacology
University of Massachusetts Medical School
55 Lake Ave N.
Worcester MA 01655
Phone: (508) 856 8006
Fax: (508) 856 8007
Email: charles.sagerstrom@umassmed.edu

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Key words: Hox, Pbx, Meis, Prep, hindbrain, homeodomain, rhombomere, segmentation, zebrafish

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SUMMARY

Meis homeodomain proteins function as Hox-cofactors by binding Pbx and Hox proteins to form multimeric complexes that control transcription of genes involved in development and differentiation. It is not known what role Meis proteins play in these complexes, nor is it clear which Hox functions require Meis proteins in vivo. Here we demonstrate that a divergent Meis family member, Prep1, acts as a Hox co-factor in zebrafish. This suggests that all Meis family members have at least one shared function and that this function must be carried out by a conserved domain. We proceed to demonstrate that the Meinox domain, an N-terminal conserved domain shown to mediate Pbx binding, is sufficient to provide Meis activity to a Pbx/Hox complex. We find that this activity is separable from Pbx binding and resides within the M1 subdomain. This finding also presents a rational strategy for interfering with Meis activity in vivo. We accomplish this by expressing the Pbx4/Lzr N-terminus, which we demonstrate sequesters Meis proteins in the cytoplasm away from the nuclear transcription complexes. Sequestering Meis proteins in the cytoplasm leads to extensive loss of rhombomere (r) 3 and r4 -specific gene expression, as well as defective rhombomere boundary formation in this region. These changes in gene expression correlate with impaired neuronal differentiation in r3 and r4, e.g. the loss of r3-specific nV branchiomotor neurons and r4-specific Mauthner neurons. We conclude that Meis

family proteins are essential for the specification of rhombomere 3 and 4 of the hindbrain.

INTRODUCTION

Hox proteins are transcriptional regulators that function to specify cell fate during early embryonic development and organogenesis (reviewed in Krumlauf, 1994). However, Hox protein monomers display poor specificity and affinity for enhancer sequences, suggesting that they do not act in isolation. Recently, two families of Hox cofactors, Pbx and Meis, belonging to the TALE (Three Amino acid Loop Extension) homeodomain superfamily, were identified (reviewed in Mann and Affolter, 1998). In vitro analyses indicate that Meis and Pbx function by forming multimeric complexes with Hox proteins. In particular, Pbx binds to Hox proteins from paralog group 1-10 (Shen et al., 1997b) and Meis binds to Hox proteins from paralog group 9-13 (Shen et al., 1997a). Meis and Pbx also interact, via the Meinox domain (particularly the M1 and M2 subdomains) in Meis and the PBC-A and -B domains in Pbx (reviewed in Mann and Affolter, 1998), to permit the formation of Meis/Pbx/Hox trimers (Berthelsen et al., 1998a; Jacobs et al., 1999; Ryoo et al., 1999; Shen et al., 1999; Vlachakis et al., 2000). The formation of multimeric complexes improves the affinity and specificity of Hox proteins for particular DNA sequences, potentially explaining the need for Pbx and Meis cofactors (reviewed in Mann and Affolter, 1998). However, since Hox proteins are transcription factors it seems likely that Meis and Pbx might also contribute functions that regulate the transcriptional activity of the complexes. Indeed, Hox proteins contain activation domains (Di Rocco et al., 1997; Rambaldi et al., 1994; Vigano et al., 1998) that may interact with the coactivator CREB-binding protein

(CBP)/p300 (a histone acetyl transferase; Chariot et al., 1999; Saleh et al., 2000) and Pbx proteins reportedly interact with corepressors such as the histone deacetylases (HDACs) as well as N-CoR/SMRT (Asahara et al., 1999; Saleh et al., 2000). Although no transcription regulatory functions have been demonstrated for Meis proteins, the Meis homeodomain is not required for all Meis functions (e.g. Berthelsen et al., 1998a; Vlachakis et al., 2001), suggesting that Meis may also have roles beyond merely enhancing the affinity and specificity of Hox binding to DNA.

An in vivo role for Hox cofactors was first demonstrated by analyzing mutations in the *Drosophila homothorax* (*hth*, the Meis ortholog; Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997) and *extradenticle* (*exd*, the Pbx ortholog; Rauskolb et al., 1993) genes. Mutations in either gene lead to posterior transformations of embryonic segments, without affecting *hox* gene expression, demonstrating that both Exd and Hth are required for Hox protein function during fly development. Loss-of-function analyses in vertebrates have also revealed a requirement for *pbx* genes in segmentation processes during development. This is seen particularly clearly in the segmented hindbrain where disruption of the *pbx4* gene in the zebrafish *lazarus* mutant (Pöpperl et al., 2000) leads to abnormal segmentation. The *lazarus* phenotype is similar to that observed upon targeted deletion of *hox* genes from paralog groups 1 and 2 in the mouse (e.g. Davenne et al., 1999; Gendron-Maguire et al., 1993; Goddard et al., 1996; Lufkin et al., 1991; Rijli et al., 1993; Studer et al., 1996), consistent with a role for Pbx proteins in regulating Hox function in the vertebrate hindbrain. In contrast, although several *meis* genes are expressed in the developing hindbrain (Sagerström et al., 2001; Salzberg et

al., 1999; Zerucha and Prince, 2001), no loss-of-function analyses have been reported for *meis* genes to date. Instead, support for *meis* genes acting in hindbrain development come from ectopic expression analyses demonstrating that Meis proteins posteriorize the rostral CNS in *Xenopus* (Salzberg et al., 1999) and cooperate with Pbx and Hox proteins to promote hindbrain fates in zebrafish (Vlachakis et al., 2001). Since vertebrates have several closely related, and perhaps functionally redundant, *meis* genes, loss-of-function analyses for *meis* may best be performed by using dominant negative constructs that interfere with all Meis family members. A basis for dominant negative strategies presents itself by the fact that Meis proteins act as part of larger complexes. These complexes are likely the functional units in vivo, as evidenced by dimers and trimers being detected by co-immunopreciptation from cell extracts (Chang et al., 1997; Ferretti et al., 2000; Knoepfler et al., 1997; Shen et al., 1999), Meis sites being found adjacent to Pbx and Hox sites in several Hox-dependent promoters (Ferretti et al., 2000; Jacobs et al., 1999; Ryoo et al., 1999) and the Pbx interaction domain of Meis being required for Meis function in vivo (Vlachakis et al., 2001). Thus, expressing a Meis protein that retains its ability to bind Pbx, but lacks other essential functions, might interfere with endogenous Meis activity. However, attempts at accomplishing this by introducing point mutations into the homeodomain (thereby preventing DNA binding) of zebrafish Meis3 and *Drosophila* Hth (Ryoo et al., 1999; Vlachakis et al., 2001) did not generate a dominant negative protein. Similarly, expressing the Meinox domain of *Xenopus* Meis3 in vivo did not have a dominant negative effect (Salzberg et

al., 1999), while expressing the Meinox domain of Hth only partially interfered with Hox function in *Drosophila* embryos (Ryoo et al., 1999).

Here we first demonstrate that highly divergent members of the Meis family display the same activity in promoting hindbrain fates, suggesting that conserved regions within Meis family members carry out this function. We proceed to define this essential region and find that it resides within the Meinox domain, a region previously implicated in Pbx binding. The activity of this region, M1, is independent of Pbx binding, suggesting that Meis proteins contribute a distinct activity to the complex. The M1 region does not encode a known motif and we hypothesize that it may interact with an auxiliary protein. This data predicts that in order to inhibit Meis function, the M1 domain must be removed from the Hox-cofactor complex and we took advantage of the fact that nuclear localization of zebrafish Meis proteins is mediated by Pbx proteins (Vlachakis et al., 2001). We find that expressing the Pbx4/Lzr N-terminus in zebrafish embryos sequesters Meis proteins in the cytoplasm, thereby keeping them out of transcription complexes in the nucleus. Embryos without nuclear Meis displayed severe defects in hindbrain development. In particular, gene expression specific to rhombomere (r) 3 and r4 was largely lost and rhombomere boundaries do not form properly in this region. Neuronal differentiation in this region was also affected, e.g. nV branchiomotor neurons in r3 and Mauthner neurons in r4 were lost. Our results suggest that the Meis Meinox domain contributes an activity in addition to Pbx binding and demonstrate that Meis proteins are required for proper specification of r3 and r4 during hindbrain development.

MATERIALS AND METHODS

Constructs

All genes used were derived from zebrafish and all constructs were verified by sequencing. *meis3, hoxb1b* and *pbx4* expression vectors were described previously (Vlachakis et al., 2001; Vlachakis et al., 2000). All Meis and Prep1 constructs were engineered to contain a MYC-epitope tag. A *prep1* cDNA was obtained as an EST database clone from Incyte Genomics. The *prep1* ORF was amplified by PCR using primers 5'-CCGACCGCTCGAGTTAGTCGCTGACGTCTAAACCCAGACCGGG-3' and 5'-CCCGCCGGAATTCATGATGGCTGCCCAGTCTGTGTCC-3' and subcloned via EcoRI/XhoI sites in the primers into *pCS2+MT*. In ΔNMeis3, the N-terminal 37aa of the *meis3* ORF were deleted. Primers 5'-GCGAATTCAGTGCCTGACTCTCTGAAACAC-3' and 5'-GCTCTAGATTATCAGTGGGCATGTATGTC-3' amplified the domain of the *meis3* ORF C-terminal to aa37, which was subcloned via EcoRI and XbaI sites in the primers into the *pCS2+MT* vector. In ΔCMeis3, the C-terminal 93aa of the *meis3* ORF were deleted. Primers 5'-CGGAATTCCATGGATAAGAGGTATGA-3' and 5' GCTCTAGATTCATGAGCGATTTGTTTGGTCAAT-3' amplified the N-terminal 322aa domain of the *meis3* ORF, which was subcloned via EcoRI and XbaI sites in the primers into the *pCS2+MT* vector. In ΔNCMeis3, both the N-terminal 37aa and the C-terminal 93aa of *meis3* ORF were deleted. Primers 5'-GCGAATTCAGTGCCTGACTCTCTGAAACAC-3' and 5'-

GCTCTAGATTCATGAGCGATTTGTTTGGTCAAT-3' amplified an aa38-aa322 domain of *meis3* ORF, which was subcloned via EcoRI and XbaI sites in the primers into the *pCS2+MT* vector. In ΔHDCMeis3 the C-terminal 191aa of the *meis3* ORF were deleted by digesting *pCS2+Meis3* with PstI/XmaI, inserting oligonucleotide 5'-GATGATAATAGGCGGCCGC-3' and then moving an EcoRI/NsiI fragment into the *pCS2+MT* vector. In ΔNXCMeis3 the N-terminal 37aa, the C-terminal 93aa as well as an internal domain, aa145-aa253, were deleted. Primers 5'-CCACTAGTAACCTTTTCTAGTTCTAATAG-3' and 5'-GGACTAGTAACAACAAGAAAAGAGGAATC-3' amplified *pCS2+MTΔNCMeis3*, which was then digested with SpeI (site in the primers) and re-ligated. For ΔIMeis3 the M1 domain was amplified by primers 5'-CGGAATTCCATGGATAAGAGGTATGA-3' and 5'-CGGCTCGAGGGAGTCTCGTGGTGAGCAAGT-3' and digested with EcoRI/XhoI. The region C-terminal to the I domain was amplified by primers 5'-CGGCTCGAGCTGGATAATCTGATCCAG-3' and 5'-GCTCTAGATTATCAGTGGGCATGTATGTC-3' and digested with XhoI/XbaI. The two fragments were then cloned into *pCS2+MT* digested with EcoRI/XbaI. For C->IMeis3 the C-terminal 56 aa of Prep1 (lacking any known activity) was amplified with primers 5'- CGGCTCGAGGACGGCTTCCAGGCGCTTTCTTCA-3' and 5'-CCGCTCGAGGTCGCTGACGTCTAAACCCAGACC-3' and cloned into the XhoI site of ΔIMeis3. In M1IM2Meis3 the N-terminal 37aa, and aa143-415 were deleted by digesting *pCS2+MTΔNXCMeis3* with SpeI/XbaI and religating. In BMNPbx4 the N-terminal 171aa of $BM^{M1/2}$Meis3 were fused in frame with the C-terminal aa230-344 of

the Pbx4 ORF. PCR primers 5'-GGTCTAGACCAGACGTAAGAGACGCAAC-3' and 5'-GGTCTAGATCATAGCCTGCCGTCAGGTGT-3' amplified aa230-344 of the Pbx4 ORF, which was subcloned into *pCS2+MT* (*pCS2+MTΔpbx4*) via XbaI sites in the primers. PCR primers 5'-CGGGATCCCCCGGGATGGCTCCAAAGAAGAAGCGTAAGGTAAATC-3' and 5'- GCTCTAGAGTCTTCCAGCACCAAATCAGTGGG-3' amplified aa1-171 of $BM^{M1/2}$Meis3, which was subcloned into *pCS2+MTΔpbx4* via BamHI/XbaI sites in the primers. For IPbx4 the I domain was amplified by primers 5'-GCTCTAGATTCTGGATTTGATGAAAATATGG-3' and 5'-CGGCTCGAGGAACTTGCCACTTGC-3' and cloned via XhoI/XbaI sites together with a XbaI/NotI fragment from BMNPbx4 into the *pCS2+MT* vector cut with XhoI/NotI. For BM1IPbx4 a BM1+I fragment was amplified with primers 5'-CGGCTCGAGGTGCCTGACTCTCTGAAACAC-3' and 5'-GCTCTAGATTCTGGATTTGATGAAAATATGG-3' and cloned via XhoI/XbaI sites in the primers into IPbx4 cut with XhoI/XbaI. For ΔCPbx4 the N-terminus of Pbx4 was amplified with primers 5'-GGAATTCTATGGATGATCAGACCCGAATGCTG-3' and 5'-GGGCTCGAGTCATTCGTGCCATTCGATTTTCTGAGCTTCGAAGATGCTGTTCAGGCCGGACATGTCGAGGAAGCGGGAGCG-3'digested with EcoRI/XhoI and cloned into *pCS2+* (for ΔCPbx4) or *pCS2+MT* (for MycΔCPbx4) digested with EcoRI/XhoI. This also introduces a biotin tag at the ΔCPbx4 C-terminus.

RNA injections, Western blots, immunoprecipitations, in situ hybridization and immunostaining was performed as described previously (Vlachakis et al., 2001).

RESULTS

Divergent Meis family members share the ability to promote hindbrain fates

We have previously demonstrated that Meis3 cooperates with Hoxb1b and Pbx4 to induce hindbrain fates ectopically in the zebrafish (Vlachakis et al., 2001). To better understand the role of Meis proteins in this process we isolated the Meis family member Prep1 from zebrafish and compared it to Meis3. Analyses in mouse and human have demonstrated that *prep1*, while clearly part of the Meis family, represents the most divergent family member identified to date both in terms of its sequence and its expression pattern (Berthelsen et al., 1998a; Berthelsen et al., 1998b; Ferretti et al., 1999).

A search of the zebrafish EST database revealed several ESTs with sequence homology to murine Prep1. One of these, fc13f10, was obtained and sequenced. Sequence analysis revealed that zebrafish Prep1 has a similar domain structure to other Meis proteins (Fig. 1a; Prep1 accession # AY052752). Prep1 is most similar to Meis3 in the homeodomain (71% identical at the amino acid level) and in the M1 and M2 domains (55% and 86% identical, respectively) that have been implicated in Pbx binding (Knoepfler et al., 1997). Other regions of Prep1; the N-terminus, the region between the M1 and M2 domains, the C-terminus and the region between the M2 domain and the homeodomain, were less than 26% identical. The fc13f10 Prep1 EST has been mapped to between 52.2 and 52.3 cM from the top of LG9 by the zebrafish mapping consortium.

prep1 transcripts are present in zebrafish embryos from the earliest stage analyzed (1 hpf; Fig. 1b), suggesting that they are maternally deposited. *prep1* mRNA is detectable throughout the embryo, with highest levels at the germ ring during early gastrula stages (6 hpf; Fig. 1d) and dorsally and posteriorly at late gastrula stages (9 hpf; Fig. 1e). During segmentation stages (13 hpf; Fig. 1f and 25hpf; Fig. 1h) *prep1* expression is detected throughout the embryo at low levels. This expression pattern is distinct from other *meis* genes which show very restricted expression (e.g. to the eyes, finbuds, hindbrain/spinal cord and somites; Sagerström et al., 2001; Zerucha and Prince, 2001). Indeed, the expression pattern of *prep1* at gastrula and segmentation stages is more similar to that of *pbx4/lzr* (Pöpperl et al., 2000; Vlachakis et al., 2000). A *prep1* sense probe used as a control did not hybridize to embryos at any stage tested (Fig. 1c, g, i).

Our sequence comparison (Fig. 1a) revealed that the M1 and M2 domains, which have been implicated in binding to Pbx, are well conserved between Meis3 and Prep1, suggesting that Prep1 may interact with Pbx proteins in a manner similar to Meis3. To determine if Prep1 interacts with Pbx4/Lzr, the most prevalent Pbx protein during early zebrafish development (Pöpperl et al., 2000), we used an in vitro co-immunoprecipitation assay. Pbx4/Lzr was expressed alone or together with MYCMeis3 or MYCPrep1 and precipitated with anti-MYC antibody. We find that both MYCMeis3 (Fig. 1j, lane 2) and MYCPrep1 (lane 4) interact with Pbx4/Lzr. The anti-MYC antibody did not cross-react with Pbx4/Lzr (lane 6). We have previously demonstrated that zebrafish Meis3 depends on Pbx proteins for its nuclear localization (Vlachakis et

al., 2001), and that this requires an intact Meinox motif in Meis3, consistent with Meis3 interacting with Pbx proteins to access the nucleus in vivo. To determine if Prep1 behaves the same way, we tested its subcellular localization in the presence or absence of co-expressed Pbx4/Lzr. We find that at 5 hpf MYCPrep1 is primarily cytoplasmic in the absence of Pbx4/Lzr (Fig. 1k), but localizes to the nucleus when Pbx4/Lzr is co-expressed (Fig. 1l).

We have previously demonstrated that while Hoxb1b can interact with Pbx4/Lzr to induce ectopic expression of *hoxb1a* in rhombomere 2 of the hindbrain, co-expression of Meis3 with Pbx4/Lzr and Hoxb1b leads to ectopic expression of both *hoxb1a* and *hoxb2* in a broad domain, resulting in transformation of the rostral CNS to a hindbrain fate (Vlachakis et al., 2001). To determine if Prep1 can function to induce hindbrain fates in a manner similar to Meis3, we co-expressed Prep1 with Pbx4/Lzr and Hoxb1b in developing zebrafish embryos and scored for ectopic expression of the *hoxb1a* and *hoxb2* hindbrain genes. Western blot analysis demonstrated that MYCMeis3 and MYCPrep1 were expressed at similar levels (Fig. 1p). Expression of MYCPrep1 or MYCMeis3 by themselves had no effect on *hoxb1a* or *hoxb2* expression (not shown). In contrast, expressing MYCMeis3 or MYCPrep1 together with Pbx4/Lzr and Hoxb1b resulted in massive ectopic expression of both *hoxb1a* (not shown) and *hoxb2* (Fig. 1m-o) anterior to their normal expression domains, leading to anterior truncations. Since Prep1 represents the most divergent Meis family member known, these results suggest that all known members of the zebrafish Meis family, despite

differences in sequence and expression pattern, share the ability to promote hindbrain fates.

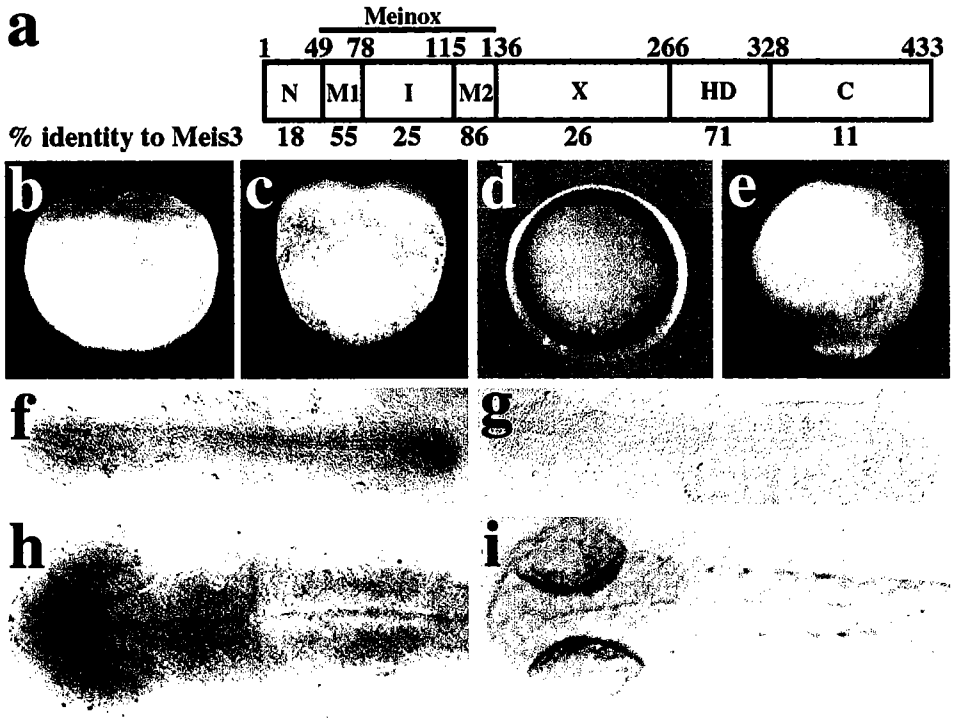

Figure 1A. Prep1 retains functions similar to Meis3.
a. Schematic representation of the Prep1 protein. Letters indicate the name of individual domains, the Meinox domain includes the M1, I and M2 domains. Numbers on top represent amino acid positions in Prep1 and numbers on the bottom indicate percent identity of each domain between Prep1 and Meis3. b-i. Expression pattern of prep1 during zebrafish embryogenesis. An antisense (b, d, e, f, h) or sense (c, g, i) probe for prep1 was hybridized to zebrafish embryos at the 2-cell stage (1 hpf; b, c), early gastrula (6 hpf; d), late gastrula (9 hpf; e), early segmentation (13 hpf; f, g) and late segmentation (25 hpf; h, i). b, c are lateral views with animal pole to the top, d is an animal pole view, e is a lateral view with dorsal to the right and anterior to the top, f-i are dorsal views with anterior to the left.

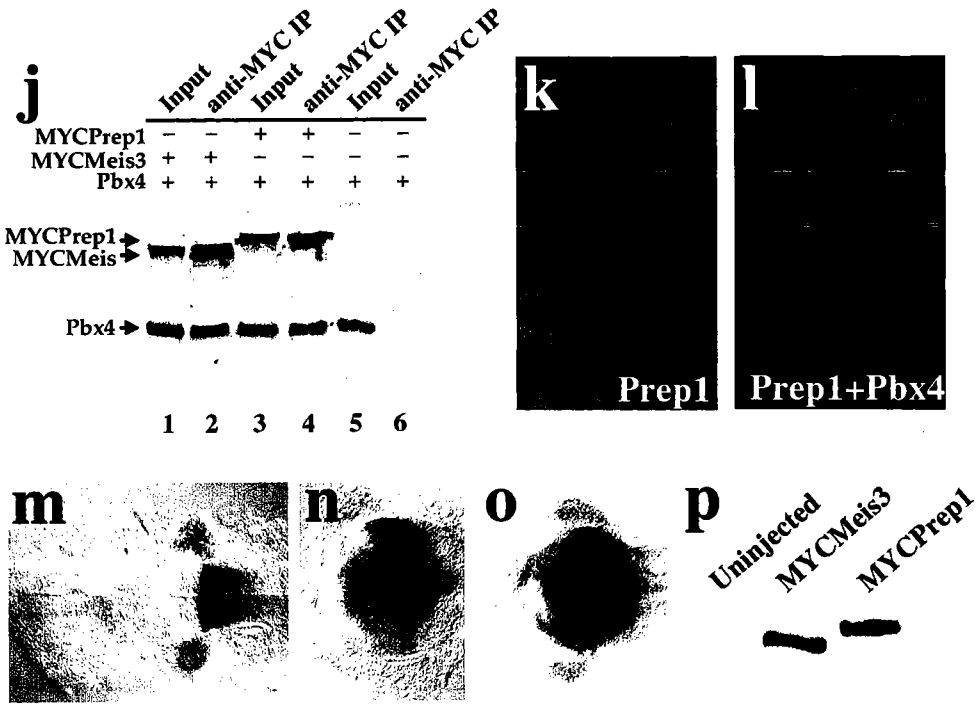

Figure 1B. Prep1 retains functions similar to Meis3.
j. Prep1 binds to Pbx4/Lzr in vitro. Pbx4/Lzr was in vitro transcribed in the presence of 35S-Methionine together with MycMeis3 (lanes 1, 2), MycPrep1 (lanes 3, 4) or by itself (lanes 5, 6), immunoprecipitated with anti-Myc antibody, resolve on a 10% SDS-PAGE gel and exposed to film. k, l Prep1 is brought to the nucleus by Pbx4/Lzr. 1-2 cell stage embryos were injected with 300pg *MycPrep1* mRNA by itself (k) or together with 300pg *pbx4/lzr* mRNA (l), raised to 5 hpf and stained with anti-Myc antibody. m-o. Prep1 induces hindbrain fates similarly to Meis3. 1-2 cell stage embryos were injected with 500pg *lacZ* RNA (m), *meis3+ pbx4+hoxb1b* mRNA (n; 165pg each), or *prep1+pbx4+hoxb1b* mRNA (o; 165pg each), raised to 25 hpf and analyzed for hoxb2 expression by in situ hybridization. All three embryos are dorsal views with anterior to the left. p. MycMeis3 and MycPrep1 are expressed at similar levels. 1-2 cell stage embryos were injected with 300pg *MycMeis3* mRNA or *MycPrep1* mRNA, raised to 5 hpf, lysed, resolved on a 10% SDS-PAGE gel, Western blotted and probed with anti-Myc antibody.

The Meinox domain is sufficient to mediate the activity of Meis family proteins

Since Prep1 and Meis3 can both promote hindbrain fates, the sequences responsible for this activity must be shared between the two proteins. Meis3 and Prep1 demonstrate highest sequence identity in the Meinox domain (consisting of the M1, I and M2 regions) and in the homeodomain. While this is consistent with Meis proteins mediating their in vivo effects solely by binding Pbx and DNA, thereby perhaps stabilizing Pbx/Hox complexes, it remains possible that other domains in Meis proteins are essential for function, or that the Meinox and homeodomain have activities in addition to Pbx and DNA binding. To determine which domains are necessary for Meis protein function we generated a series of Meis3 deletion constructs (Fig. 2A) and tested whether they could promote hindbrain fates upon co-expression with *pbx4/lzr* and *hoxb1b* in zebrafish embryos.

All constructs shown in Fig. 2A are expressed at comparable levels in vivo as determined by Western blotting of lysates from injected embryos (Fig. 3a, lanes 2-10). In order to determine whether the deletion constructs can still interact with Pbx, we tested if they localized to the nucleus following co-expression with Pbx4/Lzr. All constructs shown in Fig. 2A translocated to the nucleus in the presence of Pbx4/Lzr, except for ΔNXCMeis3 (Fig. 3 d, e) and ΔIMeis3 (Fig. 3 f, g), both of which remained at least partly cytoplasmic. We conclude that while most constructs interact well with Pbx4/Lzr, ΔNXCMeis3 and ΔIMeis3 do so inefficiently or not at all. We do not think that the Pbx interaction domain was removed in the ΔNXCMeis3 or ΔIMeis3 constructs, rather that the Pbx binding motif (i.e. the Meinox domain) was interfered

with indirectly. This is supported by the observation that removing the HD from ΔNXCMeis3 (to generate M1IM2Meis3) and inserting an unrelated sequence in place of the I domain of ΔIMeis3 (to generate C->IMeis3) restored Pbx-dependent nuclear localization (Fig. 3 h-k).

When expressed alone in zebrafish embryos, none of the constructs in Fig. 2A lead to ectopic expression of *hoxb1a* and *hoxb2*, nor do they affect endogenous gene expression in the hindbrain, demonstrating that they do not have a dominant negative effect (not shown). When co-expressed with Pbx4/Lzr and Hoxb1b each of the constructs generated phenotypes quantitatively and qualitatively similar to those seen when wild-type Meis3 is co-expressed with Pbx4/Lzr and Hoxb1b. In particular, they promote ectopic *hoxb1a* and *hoxb2* expression as well as anterior truncations (Fig. 3, compare p, t with o, s; Table 1). However, the ΔNXCMeis3 and ΔIMeis3 constructs were less effective and rarely displayed the type of anterior truncations indicative of the rostral CNS being transformed to a hindbrain fate (Table 1). This result is likely due to reduced Pbx binding by these constructs (see above), rather than to the homeodomain or I domain being required for function. Indeed, the M1IM2Meis3 (with the HD deleted) and C->IMeis3 (with the I domain replaced) constructs, which bind Pbx4/Lzr well, retain high activity (Fig 3 p, t; Table 1). We conclude that the Meinox domain is sufficient to provide Meis activity in this ectopic expression system. Since we find that the sequence of the I region is irrelevant for Meis activity, we also suggest that the I region serves primarily to space the M1 and M2 domains properly, and that the sequences essential for Meis activity reside within the M1 and/or M2 domains.

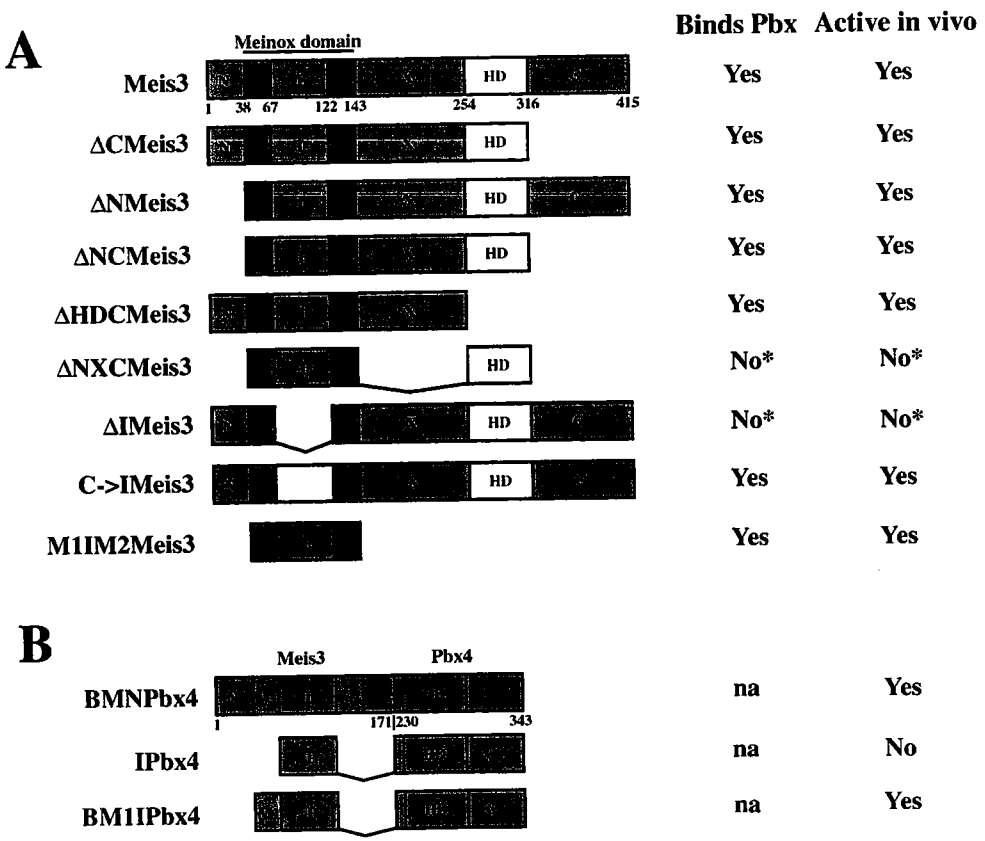

Figure 2. Schematic representation of Meis constructs.
Meis3 deletion constructs (A) and fusions with Pbx4/Lzr (B) are shown schematically to the left. Columns to the right indicate whether each protein binds Pbx4/Lzr and displays activity in vivo. Asterisks indicate two constructs that have drastically reduced Pbx-binding and in vivo activity, but retain some function (see text for details). na, not applicable since the fusion constructs were designed not to require Pbx binding (see text for details). Meis3 is colored blue, except for the homeodomain (HD; white) and M1 and M2 (red). Yellow indicates sequences from the Prep1 C-terminus that were inserted in place of the I domain in the C->I Meis3 construct. The M1 and M2 domains in several fusion constructs (B) were mutated to abolish Pbx binding (purple). These domains are referred to as BM1 and BM2 in the text. Pbx4/Lzr is colored green.

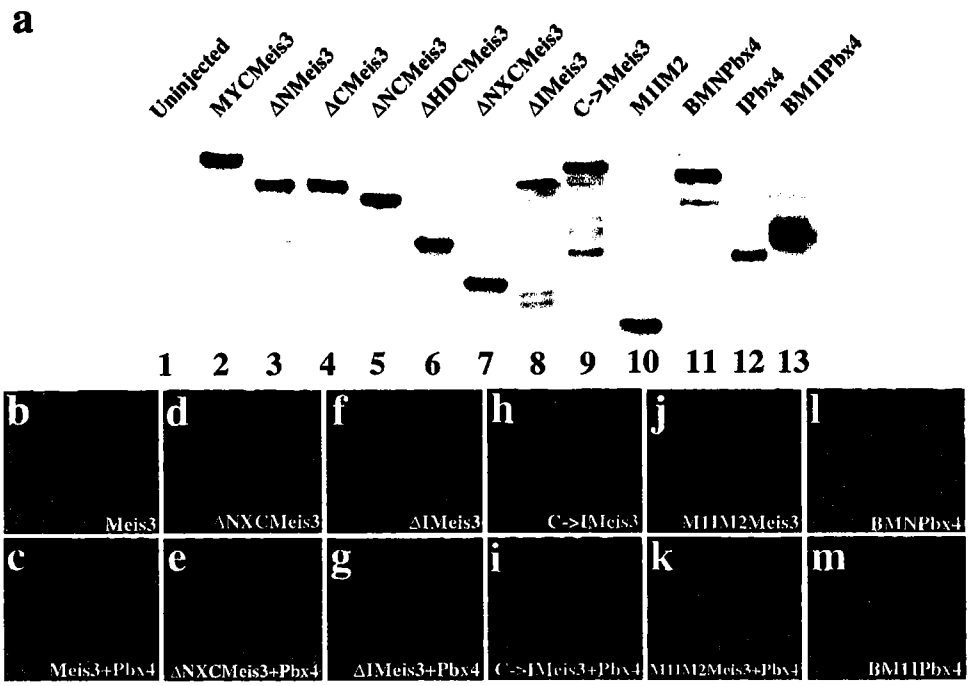

Figure 3A. The M1 domain is sufficient to confer Meis activity.
a. All constructs used are expressed at comparable levels in embryos. 1-2 cell stage embryos were injected with 300pg of each mRNA encoding Myc-tagged constructs as indicated at the top of each lane. Embryos were raised to 5 hpf, lysed, resolved on a 10% SDS-PAGE gel, Western blotted and probed with anti-Myc antibody.
b-m. Analysis of Pbx4/Lzr-mediated nuclear localization of Meis constructs. 1-2 cell stage embryos were injected with 300pg of each mRNA as indicated at the bottom right of each panel, raised to 5 hpf and stained with anti-Myc antibody. All Meis constructs were Myc-tagged while Pbx4/Lzr was untagged.

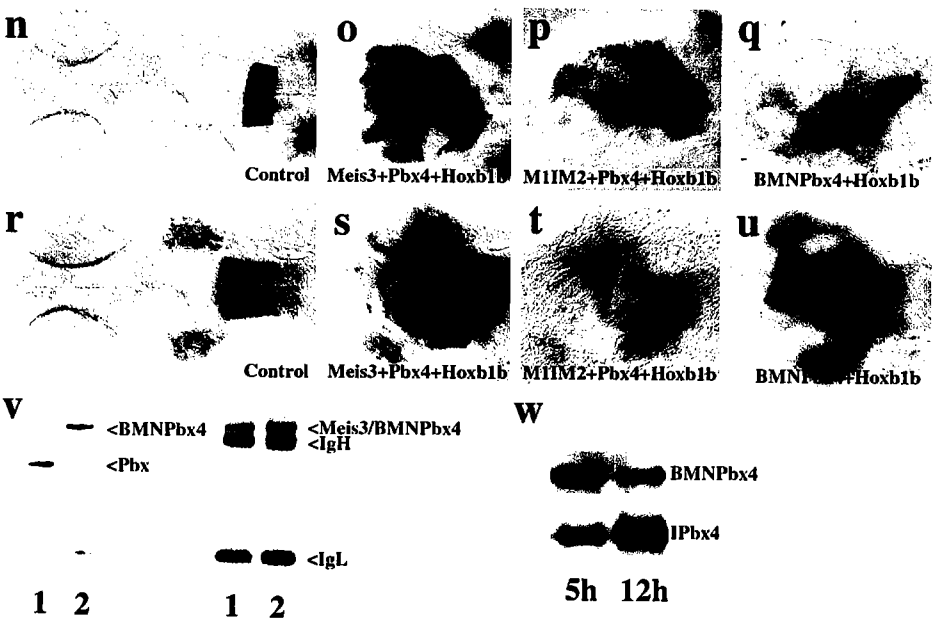

Figure 3B. The M1 domain is sufficient to confer Meis acivity.
n-u. Analysis of in vivo activity of Meis constructs. 1-2 cell stage embryos were injected with 500pg lacZ RNA (control) or 165pg of each mRNA as indicated in the lower right corner of each panel, raised to 25 hpf and analyzed for expression of hoxb1a (n-q) or hoxb2 (r-u) by in situ hybridization. All embryos are dorsal views with anterior to the left.
v. Meis3-Pbx4 fusion constructs do not bind endogenous Pbx. 1-2 cell stage embryos were injected with 300pg MycMeis3 (lane 1) or MycBMNPbx4 (lane 2) and raised to 10 hpf. Embryos were lysed, immunoprecipitated with anti-Myc, resolved on a 10% SDS-PAGE gel, Western blotted and probed with anti-Pbx4 antiserum (left panel), or anti-Myc antiserum (right panel). Note that the BMNPbx4 fusion protein in lane 2 of the left hand panel is detected by the anti-Pbx4 antiserum. MycMeis3 and BMNPbx4 are the same size. IgH = antibody heavy chain. IgL = antibody light chain. w. Meis3-Pbx4 fusion proteins remain stable at 12 hpf. 1-2 cell stage embryos were injected with 300pg MycBMNPbx4 or MycIPbx4 mRNA and harvested at 5hpf or 12 hpf. Embryos were lysed, 3 embryo equivalents were resolved on a 10% SDS-PAGE gel, Western blotted and probed with anti-Myc antiserum.

Table1: Activity of Meis deletion and fusion constructs.

Injected RNA[a]	Outcome			Probe
	Unaffected	Ectopic staining[b]	Ectopic staining/ truncated axis[c]	
pbx4+hoxb1b	37% (18/49)	63% (31/49)	0% (0/49)	*hoxb1a*
	93% (40/43)	7% (3/43)	0% (0/43)	*hoxb2*
meis3+pbx4+hoxb1b	7% (4/61)	49% (30/61)	44% (27/61)	*hoxb1a*
	3% (2/57)	46% (26/57)	51% (29/57)	*hoxb2*
ΔNMeis3+pbx4+hoxb1b	10% (8/80)	40% (32/80)	50% (40/80)	*hoxb1a*
	12% (11/93)	34% (32/93)	54% (50/93)	*hoxb2*
ΔCMeis3+pbx4+hoxb1b	7% (6/85)	62% (53/85)	31% (26/85)	*hoxb1a*
	20% (22/110)	49% (54/110)	31% (34/110)	*hoxb2*
ΔNCMeis3+pbx4+hoxb1b	15% (34/228)	63% (143/228)	22% (51/228)	*hoxb1a*
	16% (31/192)	48% (92/192)	36% (69/192)	*hoxb2*
ΔHDCMeis3+pbx4+hoxb1b	25% (36/141)	42% (59/141)	33% (46/141)	*hoxb1a*
	34% (40/117)	37% (43/117)	29% (34/117)	*hoxb2*
ΔNXCMeis3+pbx4+hoxb1b	40% (46/116)	58% (68/116)	2% (2/116)	*hoxb1a*
	77% (63/82)	23% (19/82)	0% (0/82)	*hoxb2*
ΔIMeis3+pbx4+hoxb1b	24% (13/54)	74% (40/54)	2% (1/54)	*hoxb1a*
	69% (37/54)	31% (17/54)	0% (0/54)	*hoxb2*
C->IMeis3+pbx4+hoxb1b	40% (33/83)	29% (24/83)	31% (26/83)	*hoxb1a*
	52% (33/64)	27% (17/64)	22% (14/64)	*hoxb2*
M1IM2Meis3+pbx4+hoxb1b	21% (37/175)	58% (101/175)	21% (37/175)	*hoxb1a*
	51% (61/119)	34% (40/119)	15% (18/119)	*hoxb2*
BMNPbx4+hoxb1b	27% (48/179)	60% (108/179)	13% (23/179)	*hoxb1a*
	55% (96/176)	38% (67/176)	7% (13/176)	*hoxb2*
BM1IPbx4+hoxb1b	38% (31/82)	39% (32/82)	23% (19/82)	*hoxb1a*
	40% (27/68)	25% (17/68)	35% (24/68)	*hoxb2*
IPbx4+hoxb1b	100% (194/194)	0% (0/194)	0% (0/194)	*hoxb1a*
	99% (202/203)	0% (0/203)	1% (1/203)	*hoxb2*

a. 1-2 cell stage embryos were injected with the indicated mRNAs, fixed at 25hpf and analyzed by in situ hybridization for *hoxb1a* and *hoxb2* expression.

b. Embryos showing normal morphology but ectopic gene expression. Note that *pbx4+hoxb1b* induces ectopic expression of *hoxb1a* in r2, but not elsewhere, and has a minimal effect on *hoxb2* expression or embryo morphology.

c. Embryos with anterior truncations and ectopic gene expression

The Meinox domain contributes a function in addition to Pbx binding

Our results demonstrate that the Meinox domain is sufficient to confer Meis activity to Pbx/Hox complexes, but it is unclear exactly what function is provided by this domain. Since Meis proteins utilize the Meinox domain to bind Pbx, it is possible that the function provided by the M1IM2Meis3 construct is simply Pbx binding, perhaps because it thereby stabilizes the Pbx/Hox complex.

To test this possibility, we set out to determine if a Meinox domain lacking the ability to bind Pbx still retains activity. To carry out this experiment it became necessary to devise a means for the Meinox domain to participate in Pbx/Hox complexes without being able to interact with Pbx (Fig. 2B). To this end we replaced the N-terminus of Pbx4/Lzr (containing the PBC-A and -B domains required for Meis binding) with the Meis N-terminus (containing the Meinox domain). This eliminates the normal interaction between the Meinox domain and Pbx4/Lzr, but since the chimaeric protein retains the Hox interaction motif in Pbx4/Lzr, it still ensures that the Meinox domain is part of the Pbx/Hox transcription complex bound to DNA. Notably, since this construct lacks the PBC-A and B domains it can not bind endogenous Meis proteins. To also eliminate the ability of this construct to bind endogenous Pbx proteins, we used a Meinox domain that contains multiple amino acid substitutions in the M1 (aa 64-67 KCEL->NNSQ) and M2 (L141->A; E142->A) motifs. We have previously demonstrated that this mutated Meinox domain can not bind to Pbx4/Lzr in vivo (Vlachakis et al., 2001) and we confirmed that the resulting fusion protein, BMNPbx4, does not bind endogenous Pbx by performing co-immunoprecipitations on lysates from

embryos expressing BMNPbx4 (Fig. 3v). To ensure that BMNPbx4 localizes to the nucleus we also introduced a nuclear localization signal (NLS) at its N-terminus.

BMNPbx4 is expressed at similar levels to Meis3 following microinjection (Fig. 3a, compare lanes 2 and 11) and localizes to the nucleus (Fig. 3l), as expected. Expression of BMNPbx4 alone resulted in embryos with normal expression of *hoxb1a* and *hoxb2* (not shown), while co-injection with Hoxb1b resulted in embryos exhibiting ectopic *hoxb1a* (Fig. 3q) and *hoxb2* (Fig. 3u). This phenotype was qualitatively and quantitatively similar to that of expressing the Meinox domain together with Pbx4/Lzr and Hoxb1b (Fig. 3p, t; Table 1). This result indicates that the BMNPbx4 chimaera now contains the combined activities of Pbx4/Lzr and Meis3.

Additional constructs were generated to better delineate the region of the Meis3 N-terminus required for this activity. We first generated a construct containing only the I domain fused to Pbx4/Lzr. This construct (IPbx4; Fig. 2B) is expressed at the same level as Meis3 following injection (Fig. 3a, lane 12) and localizes to the nucleus (not shown). IPbx4 lacks in vivo activity (Table 1), confirming that the I domain is not required for function and also demonstrating that simply fusing sequences to the Pbx4/Lzr C-terminus is not sufficient for activity. We then added the M1 domain (containing the same amino acid substitutions as in BMNPbx4) onto the IPbx4 construct to generate BM1IPbx4 (Fig. 2B). This construct is expressed at the same level as other constructs (Fig. 3, lane 13) and localizes to the nucleus (Fig. 3m). BM1IPbx4 has no effect when expressed by itself (not shown), but leads to ectopic *hoxb1a* and *hoxb2* as well as anterior truncation similar to those seen with the BMNPbx4 construct, when co-

expressed with Hoxb1b (Table 1). Based on the data from the deletion analysis and the chimaeric constructs we conclude that the Meinox domain has a function in addition to Pbx binding and that the M1 domain is sufficient for this function, at least in our ectopic expression system. We do not think that the M1 domain acts by stabilizing the fusion protein, since a fusion protein lacking the M1 domain (IPbx4) does not appear less stable over time in vivo than one which retains the M1 domain (BMNPbx4; Fig. 3w). Instead we speculate that the M1 domain may serve as a binding site for an auxiliary protein.

Expression of the Pbx4/Lzr N-terminus sequesters Meis proteins in the cytoplasm. Our finding that the M1 domain is sufficient for Meis activity provides a rationale for a dominant negative strategy. In particular, it might not be sufficient to eliminate the DNA binding capacity of Meis to generate a dominant negative construct, since such a construct will retain the M1 domain. Instead we set out to devise a strategy where the M1 domain is kept out of Pbx/Hox complexes. Specifically, since the M1 domain is also involved in Pbx binding we hypothesized that expressing a construct that sequesters Meis proteins away from Pbx/Hox complexes might act in a dominant negative fashion. To test this possibility we generated a construct expressing only the N-terminus of Pbx4/Lzr, containing the PBC-A and –B domains required for binding to Meis, but lacking the motifs required for binding Hox proteins and for nuclear localization (Fig. 4a). We observed that this construct (MycΔCPbx4) was

cytoplasmically located at 12 hpf following expression in zebrafish embryos (Fig. 4b). In contrast, injected MycMeis3 is found exclusively in the nucleus at this stage of development (Fig. 4c), likely as a result of nuclear transport by endogenous Pbx which has become highly expressed by this stage (Vlachakis et al., 2001). Strikingly, when ΔCPbx4 is co-expressed with MycMeis3, MycMeis3 is found primarily in the cytoplasm (Fig. 4d). These data are consistent with ΔCPbx4 competing with endogenous Pbx proteins for binding to Meis3 in the cytoplasm and subsequently retaining Meis3 in the cytoplasm. This result raises the possibility that ΔCPbx4 might act in a dominant negative fashion by keeping Meis proteins out of nuclear Pbx/Hox complexes.

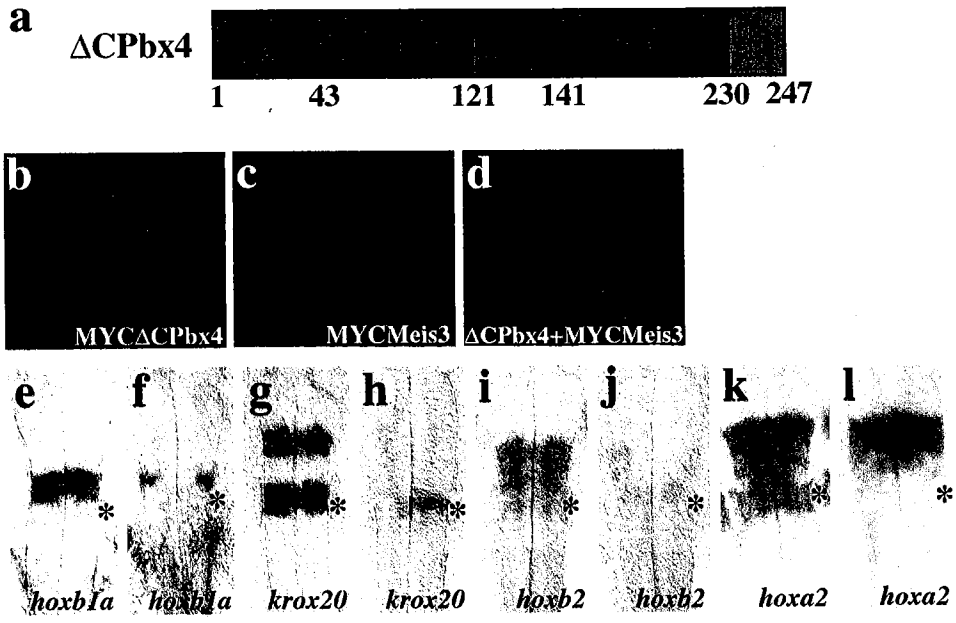

Figure 4A. Loss of Meis function disrupts hindbrain development. a. Schematic representation of the ΔCPbx4 construct with amino acid positions indicated at the bottom. The red boxes indicate the PBC-A and –B domains. The blue domain represents a biotin tag introduced at the C-terminus. b-d. ΔCPbx4 sequesters Meis3 in the cytoplasm. 1-2 cell stage embryos were injected with 300pg of MycΔCPbx4 (b) MycMeis3 (c) or ΔCPbx4 +MycMeis3 (d), raised to 12 hpf and stained with anti-Myc antibody. e-l. ΔCPbx4 affects gene expression in the hindbrain. 1-2 cell stage embryos were injected with 300pg of ΔCPbx4 mRNA (f, h, j, l) or lacZ mRNA (e, g, i, k), raised to 24 hpf and analyzed by in situ hybridization for the genes indicated at the bottom of each panel. Black asterisks indicate the level of the otic vesicle on the right side of each embryo.

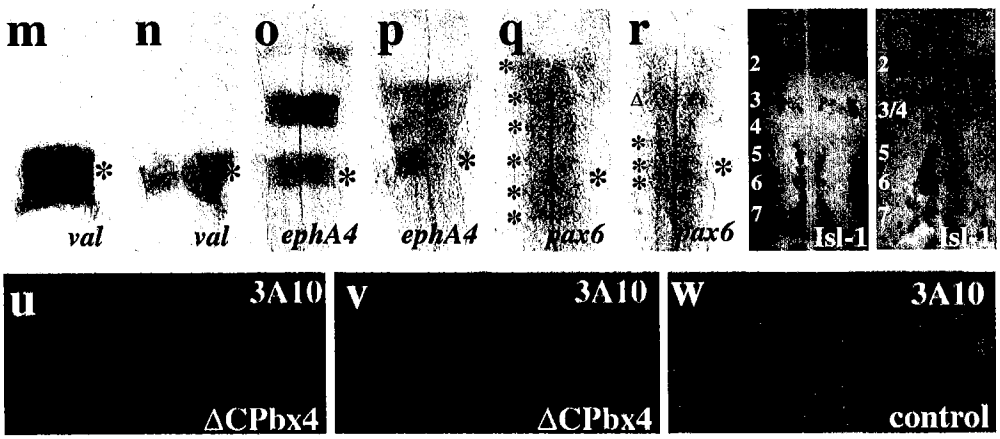

Figure 4B. Loss of Meis function disrupts hindbrain development. m-r. ΔCPbx4 affects gene expression in the hindbrain. 1-2 cell stage embryos were injected with 300pg of ΔCPbx4 mRNA (n, p, r,) or lacZ mRNA (m, o, q,), raised to 14 hpf (m, n) or 24 hpf (o-r) and analyzed by in situ hybridization for the genes indicated at the bottom of each panel. Black asterisks indicate the level of the otic vesicle on the right side of each embryo. Black asterisks on left side in q and r indicate rhombomere boundaries. Black triangle in r indicates region of strong pax6 expression. s-w. ΔCPbx4 affects neuronal differentiation. 1-2 cell stage embryos were injected with 300pg of ΔCPbx4 mRNA (s, u, v) or lacZ mRNA (t, w), raised to 48 hpf (s, t) or 28 hpf (u-w) and stained with anti-islet (s, t) or 3A10 (u-w) antibody. Black asterisks indicate the otic vesicle and rhombomeres are numbered on the left.

Table 2. Effect of ΔCPbx4 on hindbrain gene expression[a].

Gene	Effect on gene expression		
	Normal	**Partial[b]**	**Absent[c]**
ephA4			
r3	28% (30/106)	56% (59/106)	16% (17/106)
r5	78% (83/106)	21% (22/106)	1% (1/106)
hoxa2			
r2	99% (106/107)	1% (1/107)	0% (0/107)
r3-r5	28% (30/107)	66% (71/107)	6% (6/107)
krox20			
r3	19% (17/88)	39% (34/88)	42% (37/88)
r5	49% (43/88)	49% (43/88)	2% (2/88)
hoxb2			
r3-r5	5% (4/78)	78% (61/78)	17% (13/78)
hoxb1a			
r4	7% (4/60)	80% (48/60)	13% (8/60)
hoxb3			
r5-r6	33% (26/78)	67% (52/78)	0% (0/78)
valentino			
r5-r6	26% (49/192)	74% (143/192)	0% (0/192)

a. 300 pg of *ΔCPbx4* mRNA was injected at the 1-2 cell stage, embryos were harvested at 24 hpf (except for *valentino* and *hoxb3*, which were harvested at 14 hpf) and assayed by in situ hybridization for the expression of the indicated gene. For genes expressed in more than one non-adjacent rhombomere, the rhombomeres are scored separately. For genes expressed in more than one adjacent rhombomere, the rhombomeres are scored together because of the difficulty in unequivocally assigning rhombomere boundaries, except for *hoxa2* where the anteriormost domain (r2) was clearly regulated differently. A comparable number of embryos injected with 300pg control mRNA (*lacZ*) and assayed for expression of each gene showed >98% normal staining.

b. Partial gene expression is defined as loss of gene expression within a portion of a rhombomere

c. Absence of gene expression indicates that no expression was detectable within a rhombomere

Meis function is required for proper formation of r3 and r4 during hindbrain development

To test if cytoplasmic retention of endogenous Meis proteins results in developmental defects we expressed ΔCPbx4 in developing zebrafish embryos. Since Meis3 acts together with Pbx4/Lzr and Hoxb1b to promote r4 fates when expressed ectopically (Vlachakis et al., 2001), we first tested whether ΔCPbx4 interfered with endogenous gene expression in r4. We found that *hoxb1a* (Fig. 4f) expression was reduced or absent in 93% of *ΔCPbx4* injected embryos (Table 2), consistent with a role for Meis proteins in regulating gene expression in r4, while embryos injected with an equivalent amount of *lacZ* RNA (Fig. 4e) were unaffected. *hoxb1a* expression was affected in 83% (72/87; not shown) of *ΔCPbx4* injected embryos already at the end of gastrulation, suggesting that Meis proteins are required for *hoxb1a* expression soon after its onset. This is consistent with reports that expression of murine *hoxb1* (the ortholog of zebrafish *hoxb1a*) is dependent on Hox activity (Pöpperl et al., 1995). In contrast, expression of *hoxb1b*, which precedes *hoxb1a* expression and is the earliest *hox* gene expressed in zebrafish, was unaffected by ΔCPbx4 (not shown), indicating that expression of *hoxb1b* is independent of Meis function. ΔCPbx4 also interfered with gene expression in r3 at a frequency similar to r4, as illustrated by *krox20*, which was affected in r3 in 81% of *ΔCPbx4* injected embryos (Fig. 4g, h; Table 2). Other genes whose expression domains include r3 and r4 were also affected. For instance, *hoxb2* expression was affected in r3 and r4 in 95% (Fig. 4i, j; Table 2) and *hoxa2* expression was affected in r3-r5 in 72% (Fig. 4k, l; Table 2) of *ΔCPbx4* injected embryos. Other rhombomeres appear to be less

affected. In particular, although *hoxa2* expression (Fig. 4k, l; Table 2) is affected in r3-r5, it is largely normal in r2 of *ΔCPbx4* injected embryos. In addition, although *krox20* and *ephA4* expression (Fig. 4g, h, o, p; Table 2) is strongly affected in r3 (42% and 16% lack expression, respectively) these genes are less affected in r5 (only 1-2% lack expression). Furthermore, *hoxb3* and *valentino* expression is only mildly affected in r5 and r6 and no *ΔCPbx4* injected embryos lacked expression of these genes (Fig. 4m, n; Table 2). Analysis of gene expression outside the hindbrain demonstrated that the fore and midbrain (*otx2*), midbrain-hindbrain boundary (*pax2.1*) and somites (*MyoD, hoxb3*) were essentially normal (not shown). We conclude that r3 and r4 do not develop properly in the presence of ΔCPbx4, consistent with the formation of these rhombomeres requiring Meis proteins.

We next analyzed expression of *pax6*, which is present throughout the hindbrain, but also outlines rhombomere boundaries (black asterisks on left in Fig. 4q). *pax6* expression reveals six boundaries in control embryos (Fig. 4q), but in most ΔCPbx4 injected embryos only three boundaries are observed (black asterisks on left in Fig. 4r). Using the otic vesicle as a landmark (black asterisk on right) we conclude that these boundaries correspond to r4/r5, r5/r6 and r6/r7. We sometimes also observe a strongly staining region in the rostral hindbrain (black triangle in Fig. 4r) of ΔCPbx4 injected embryos. This domain may correspond to the r2/r3 boundary, in agreement with r2 retaining normal *hoxa2* expression. Thus, boundary formation in the rostral hindbrain is affected. We also observe that *ephA4* expression is occasionally (~10% of affected embryos) found at low levels throughout the hindbrain of ΔCPbx4 injected

embryos (compare Fig. 4p and o). This expression level is similar to that normally seen in r1 and may indicate that r1-specific gene expression expands caudally when rhombomere formation is interrupted, although this remains speculative in the absence of r1-restricted markers.

To further explore the effect of ΔCPbx4 on r3 and r4 development we analyzed neuronal differentiation in this region. Both the primary reticulospinal neurons and the branchiomotor neurons display a segment specific distribution in the hindbrain, permitting us to characterize the effect of ΔCPbx4 on neuronal differentiation in individual rhombomeres. We find that 73% (30/41) of ΔCPbx4 injected embryos lack one or both r4-specific Mauthner neurons (Fig. 4u-w). Using an anti-Islet1 antibody we also observe an effect on branchiomotor neurons in 70% (21/30) ΔCPbx4 injected embryos. This effect is strongest in r3, as most embryos lack nV branchiomotor neurons on at least one side of the midline in r3 (Fig. 4s, t). Since there are only a few islet-1 positive cells in r4 it is difficult to determine if it is affected, although this region occasionally seems to be reduced in size, in agreement with the observed loss of r4 Mauthner neurons. nVII neurons in r6 and r7 are also affected, although less severely, perhaps as a result of these neurons originating in r4 before migrating to r6 and r7 (Chandrasekhar et al., 1997). In contrast, nV neurons in r2 are largely unaffected. These results are consistent with the observed effect of ΔCPbx4 on gene expression and suggest that specification of r3 and r4 is particularly dependent on Meis function.

To confirm that this phenotype is specific we attempted to rescue *ΔCPbx4* injected embryos by co-expressing *pbx4/lzr* mRNA. We expect Pbx4/Lzr to compete

with ΔCPbx4 for Meis binding in the cytoplasm and bring Meis proteins to the nucleus where they can interact with Hox proteins and activate transcription. We find that expressing *pbx4/lzr* mRNA along with *ΔCPbx4* mRNA rescues *hoxb1a* expression to virtually normal levels in all embryos (43/43). We attribute this high frequency of rescue to ΔCPbx4 not entering the nucleus. Thus, once Meis proteins have entered the nucleus together with Pbx4/Lzr, they are inaccessible to the ΔCPbx4 dominant negative protein. We also used the *BMNPbx4* construct to rescue *ΔCPbx4* injected embryos. Since BMNPbx4 does not interact with Pbx, it should not be affected by the ΔCPbx4 dominant negative construct. Furthermore, since it contains the M1 domain it should be able to rescue Meis activity in ΔCPbx4 expressing embryos. We find that expression of *BMNPbx4* together with *ΔCPbx4* restores *hoxb1a* expression in all embryos (30/30), but that the rescued expression is less complete than following rescue with *pbx4/lzr*. We attribute this difference to BMNPbx4 being less active than wild type Meis3 in vivo (Table 1). This result further demonstrates that the effect of ΔCPbx4 is due to it interfering with endogenous Meis activity.

DISCUSSION

Meis family proteins have been implicated as Hox cofactors (reviewed in Mann and Affolter, 1998), but a requirement for Meis proteins during vertebrate embryonic development has not been established, primarily because of the lack of an appropriate loss-of-function approach. Here we first demonstrate that two divergent members of the Meis family display similar activities in vivo. We then demonstrate that the M1 domain is sufficient for this function. The M1 domain resides within the Meinox domain, in close proximity to the Pbx interaction domain, but this activity is independent of Pbx binding. We then use the Pbx4/Lzr N-terminus, containing the Meis interaction domain, to sequester Meis family proteins in the cytoplasm, thereby preventing them from acting in transcriptional complexes in the nucleus. We find that sequestering Meis proteins in the cytoplasm leads to developmental defects in the hindbrain. In particular, gene expression, boundary formation and neuronal differentiation is disrupted in r3 and r4. Our results are consistent with Meis family proteins being required for development of the hindbrain, particularly rhombomere 3 and 4.

What role do Meis proteins play in the multimeric transcription complexes?

Several reports have demonstrated that Meis, Pbx and Hox proteins can interact to form trimeric complexes (Berthelsen et al., 1998a; Ferretti et al., 2000; Jacobs et al., 1999; Ryoo et al., 1999; Shen et al., 1999; Vlachakis et al., 2000) and that Hox and Meis need to interact with Pbx to function in vivo (Vlachakis et al., 2001). Although

these data suggest that Meis/Pbx/Hox complexes exist in vivo, it remains unclear what role each protein plays within the complex. Possible roles for Hox and Pbx proteins derive from their interaction with transcriptional coactivators (Chariot et al., 1999; Saleh et al., 2000) and corepressors (Asahara et al., 1999; Saleh et al., 2000). The absence of such demonstrated interactions for Meis proteins has led to the suggestion that they serve to stabilize Pbx/Hox complexes by binding both to DNA and to Pbx. In possible disagreement with this hypothesis, it has been found that while Meis proteins require an intact Pbx interaction domain, they do not require an intact homeodomain to synergize with Pbx and Hox proteins (e.g. Berthelsen et al., 1998a; Vlachakis et al., 2001), although this has only been analyzed during conditions of Meis overexpression. In this report we identify a domain essential for function near the Pbx interaction motif of Meis3. By mutating residues required for Pbx binding and transferring the domain from Meis3 onto Pbx4/Lzr we demonstrate that this activity is retained even when Pbx binding is abolished. We interpret our results to mean that Meis proteins contribute an activity to the multimeric complexes in addition to stabilization. Since this domain does not contain any known motifs we hypothesize that it serves as a binding site for an auxiliary protein required for transcription activity.

Furthermore, if Meis proteins serve only to stabilize Pbx/Hox complexes it should be possible to generate a dominant negative form of Meis by disrupting DNA binding while retaining Pbx binding. We did not observe reproducible dominant negative phenotypes using such constructs (Vlachakis et al., 2001; N. V. and C. G. S. unpublished) and while a similar construct does not have an effect in *Xenopus* embryos

(Salzberg et al., 1999), expressing a homeodomain-less Hth construct in *Drosophila* has a mild dominant negative effect on Hox-dependent functions (Ryoo et al, 1999). Our identification of a required domain adjacent to the Pbx interaction domain explains these results since constructs lacking the homeodomain will retain the M1 domain and will not be strongly dominant negative. Our results instead support the idea that to interfere with Meis function, this essential domain must be kept out of the multimeric complexes.

For what Hox-dependent processes are Meis proteins required?

Our experiments reveal a role for Meis proteins in development of the hindbrain, particularly r3 and r4. Notably, this region of the hindbrain expresses *hox* genes only from paralog group 1 and 2, and the phenotype we observe is similar to that of mice lacking paralog group 1 and 2 *hox* genes (Barrow and Capecchi, 1996; Davenne et al., 1999; Studer et al., 1996). Since expression of paralog group 1 and 2 *hox* genes is controlled by Hox proteins acting in an auto and cross regulatory fashion, we suggest that Meis proteins are essential cofactors for Hox proteins in this capacity. Although both murine *hoxb1* and *hoxb2* have Meis binding sites adjacent to Hox and Pbx binding sites in their enhancers (Ferretti et al., 2000; Jacobs et al., 1999), the Meis site in the *hoxb1* enhancer is not essential for expression (Ferretti et al., 2000). These data may indicate that while Meis proteins are required for both *hoxb1* and *hoxb2* expression, binding to the Meis site is dispensable for *hoxb1* expression.

Our results also indicate that *hoxb1a* and *hoxb2* expression is dependent on Meis, while *hoxb1b* expression is not. This finding correlates with the fact that *hoxb1b* (the zebrafish counterpart to murine *hoxA1*) is the earliest *hox* gene expressed in zebrafish. Since there are no other Hox proteins present to regulate initial *hoxb1b* expression, it is possible that its expression is regulated by a Hox-independent mechanism, and that Meis proteins are therefore not required. Once *hoxb1b* is expressed it may then act with *meis* and *pbx* to cross regulate the transcription of later expressed *hox* genes. Indeed, we have shown that co-expression of *hoxb1b* with *meis3* and *pbx4/lzr* is sufficient to induce ectopic *hoxb1a* and *hoxb2* expression in zebrafish (Vlachakis et al,. 2001) and murine *hoxA1* likely directly regulates the expression of *hoxB1* (the murine counterpart to zebrafish *hoxb1a*; Pöpperl et al., 1995).

Meis proteins may also be required for the proper formation of other structures. For instance, although r2 retains *hoxa2* expression in *ΔCPbx4* injected embryos, it occasionally also expresses ectopic *ephA4* and there may be similar subtle effects on more caudal rhombomeres, as well as on regions outside the hindbrain. Furthermore, since our dominant negative approach relies on the ΔCPbx4 construct binding to Meis, any Meis functions that are independent of Pbx binding would not be detected in our experiments.

The phenotype we observe as a result of interfering with Meis activity is also qualitatively similar to that of the *lazarus* mutant (which carries a mutation in the *pbx4* gene; Pöpperl et al., 2000). Particularly, in both cases gene expression is affected primarily in r3 and r4 and less in r1, r2 or r5-r7. This suggests that Pbx and Meis

function in the same pathway during hindbrain development. This is consistent with work in *Drosophila*, where the phenotypes of *hth* and *exd* mutants are largely indistinguishable (Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997) and the genes are thought to act in the same pathway. An explanation for Meis and Pbx acting in the same pathway in the hindbrain likely comes from Meis proteins not interacting directly with Hox proteins expressed in the hindbrain (primarily paralog group 1-4), while Pbx proteins do. Therefore, Meis proteins can only act as Hox cofactors in the hindbrain by binding to Pbx. Our finding that Meis and Pbx loss-of-function give similar hindbrain phenotypes are therefore consistent with all hindbrain Hox functions that require Pbx also requiring Meis. However, while the *meis* loss-of-function and *lazarus* phenotypes are qualitatively similar, they differ quantitatively. Surprisingly, we observe both a higher frequency and a more severe effect on hindbrain gene expression in the absence of Meis function than reported for the *lazarus* mutant. We speculate that this is unlikely due to Pbx-independent effects of Meis proteins on Hox function, but may instead stem from the presence of maternal *pbx4/lzr* transcript as well as additional *pbx* genes expressed in the *lazarus* mutant (Pöpperl et al., 2000). If this is correct, complete removal of Pbx activity might be required to conclusively define the relative roles of Pbx and Meis in regulating Hox function.

FOOTNOTE

While this work was under review two other manuscripts reporting Meis loss of function phenotypes appeared in press: Dibner et al., Development 128, 3415-3426; Waskiewicz et al., Development 128:4139-4151.

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CHAPTER II

PARALOG GROUP 1 *HOX* GENES REGULATE EXPRESSION OF *VHNF1*, A REPRESSOR OF RHOMBOMERE 4-FATES, IN RHOMBOMERE 5 AND 6

Seong-Kyu Choe and Charles G. Sagerström*

Department of Biochemistry and Molecular Pharmacology, and Program in Neuroscience, University of Massachusetts Medical School, Worcester,MA

*To whom correspondence should be addressed:

Department of Biochemistry and Molecular Pharmacology
364 Plantation Street/LRB 822
Worcester, MA 01605
Phone: (506) 856-8006
Fax: (508) 856-8007
Email: charles.sagerstrom@umassmed.edu

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SUMMARY

The vertebrate hindbrain is segmented into an array of rhombomeres whose fates represent unique variations on a basic developmental program. Hox proteins, acting together with cofactors from the Meis and Pbx families, are thought to specify the fate of individual rhombomeres. Here we report that disrupting Meis function blocks hindbrain segmentation completely, similar to the effect of disrupting Pbx function. However, incomplete disruption of Meis function reveals a developmental state where the entire caudal hindbrain takes on a rhombomere 4 (r4)-like fate, suggesting that a Meis-dependent factor is normally repressing r4-fates in the caudal hindbrain. We identify this repressor as *vhnf1* and demonstrate that *vhnf1* expression is regulated by paralog group 1 (PG1) *hox* genes in a Meis-dependent manner while *vhnf1* function is Meis-independent. Although PG1 *hox* genes have been implicated in the formation of r4 and r5/r6, it has been unclear what role they play in r5/r6. Our results indicate that PG1 *hox* genes induce r4 fates throughout the caudal hindbrain and that they also induce a repressor of r4-fates in r5/r6. Notably, incomplete disruption of Pbx function has not been reported to induce r4 fates in the caudal hindbrain. Thus, our results also suggest that there are different requirements for Meis and Pbx in hindbrain development.

Running Title: Requirement for PG1 *hox* genes within r5/r6

INTRODUCTION

The embryonic hindbrain, which gives rise to the adult brainstem and cerebellum, is transiently divided into a series of segments, termed rhombomeres, during early development. These rhombomeres share a basic underlying developmental program and individual rhombomeres display unique variations on this program. Accordingly, reticulospinal interneurons form in most rhombomeres, but display rhombomere-specific features such that, for instance, Mauthner neurons differentiating in rhombomere 4 (r4) have different morphology and axonal projections than do Ro3 neurons differentiating in r3. Similarly, branchiomotor (BM) neurons of the cranial nerves differentiate in several rhombomeres, but display rhombomere-specific features such that, for instance, the BM neurons of the Vth (trigeminal) nerve differentiate in r2 and r3 while BM neurons of the VIIth (facial) nerve differentiate in r4 and r5.

The formation of individual rhombomeres appears to depend on rhombomere-specific genes, or on unique combinations of more broadly expressed genes. This process is perhaps best understood for r4, r5 and r6. Specifically, a regulatory cascade has been proposed for r4, wherein *Hoxa1* [functionally equivalent to zebrafish *hoxb1b* (McClintock et al., 2002)] regulates the expression of *Hoxb1* [(Gavalas et al., 1998; Pöpperl et al., 1995; Rossel and Capecchi, 1999; Studer et al., 1998); functionally equivalent to zebrafish *hoxb1a* (McClintock et al., 2002)], which in turn regulates expression of *hoxb2* (Maconochie et al., 1997). At least three genes, *krox20* (Wilkinson et al., 1989), *kreisler/valentino* (Cordes and Barsh, 1994; Moens et al.,

1998) and *vhnf1* (Sun and Hopkins, 2001) are expressed in r5 and/or r6 and are required for development of these rhombomeres (Frohman et al., 1993; McKay et al., 1994; Moens et al., 1996; Schneider-Maunoury et al., 1993; Sun and Hopkins, 2001; Swiatek and Gridley, 1993). In particular, *vhnf1* is thought to activate *valentino* expression in r5/r6, which in turn regulates *krox20* and *hoxb3* expression in r5 and *hoxa3* expression in r5 and r6 (Manzanares et al., 1999; Manzanares et al., 1997; Prince et al., 1998; Sun and Hopkins, 2001; Theil et al., 2002). There also appears to be cross-regulation between genes promoting r4 versus r5/r6 fates. In particular, disruption of *Hoxa1* function disrupts not only r4, but also r5 (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Lufkin et al., 1991; Mark et al., 1993), and this effect is more pronounced when *Hoxb1* is simultaneously disrupted (Gavalas et al., 1998; McClintock et al., 2002; Rossel and Capecchi, 1999; Studer et al., 1998). It has recently been demonstrated that Fgf3 and 8 produced in r4 is required for development of r5/r6 (Maves et al., 2002; Walshe et al., 2002), suggesting that paralog group 1 (PG1) *hox* genes affect r5/r6 indirectly. In contrast to the positive regulation of r5/r6 fates by r4 genes, r5/r6 genes appear to negatively regulate r4-fates, since mutations in *vhnf1* (Sun and Hopkins, 2001; Wiellette and Sive, 2003) or paralog group 3 *hox* genes (Gaufo et al., 2003) permits a caudalward expansion of r4 fates. At present it is not clear how r4 and r5/r6 genes cross-regulate each other's function to subdivide the hindbrain into rhombomere segments.

Hox proteins play an important role in hindbrain development by regulating transcription, but they do not function as monomers. Instead they require homeodomain

proteins of the Pbx and Meis families as cofactors (reviewed in (Mann and Affolter, 1998)). To understand the role of Hox proteins in hindbrain segmentation, it is therefore important to understand the function of these cofactors. Disruption of Pbx function in zebrafish completely blocks hindbrain segmentation (Waskiewicz et al., 2002), consistent with Pbx proteins acting as Hox cofactors in hindbrain development. However, Pbx proteins are also required for the nuclear localization of Meis proteins (Vlachakis et al., 2001), suggesting that defects observed upon blocking Pbx function may not be due to the disruption of activities intrinsic to Pbx proteins, but may at least in part stem from interference with Meis function. Indeed, disruption of Meis function reveals effects on hindbrain segmentation that represent a subset of the defects observed upon blocking Pbx function (Choe et al., 2002; Dibner et al., 2001; Waskiewicz et al., 2001).

Here we further explore the role of Meis proteins as Hox cofactors during hindbrain development. We find that extensive removal of Meis function leads to a hindbrain completely devoid of segments. The resulting unsegmented structure displays a generic 'pre-hindbrain' fate that does not correspond to any one mature rhombomere, but is similar to the hindbrain 'ground state' observed upon disrupting Pbx function (Waskiewicz et al., 2002). Surprisingly, we find that partial removal of Meis function leads to the caudal hindbrain taking on an r4-like fate characterized by ectopic Mauthner neurons and ectopic *hoxb1a* expression. We demonstrate that this r4-like fate is induced by PG1 *hox* genes and can be repressed by at least one gene expressed in the caudal hindbrain (*vhnf1*). We further demonstrate that *vhnf1*

expression is regulated by PG1 *hox* genes in a Meis-dependent manner, while *vhnf1* function is Meis-independent. Our results therefore suggest that PG1 *hox* genes transiently induce r4 fates throughout the caudal hindbrain and that they also induce *vhnf1*, which subsequently acts as a repressor of r4-fates to subdivide the caudal hindbrain. Notably, incomplete disruption of Pbx function has not been reported to induce r4 fates in the caudal hindbrain. Thus, our results also indicate that, while both Meis and Pbx are required for hindbrain segmentation, they may have distinct roles.

MATERIALS AND METHODS

DNA Constructs

Constructs for the in vitro synthesis of mRNA were generated by cloning genes into the pCS2+ or pCS2+MT vectors and were verified by sequencing. The Hoxb1a, Hoxa2, Hoxb2 and Hoxa3 constructs contain HA epitope tags (as previously reported for HAHoxb1b (Vlachakis et al., 2001)) and were cloned into pCS2+. MycPbx4 was generated by transferring Pbx4 into pCS2+MT. A Myc-tagged form of Hoxb1a was generated by inserting six Myc tags into the EcoRI site of HAHoxb1a. The MycPrep1, MycΔCPbx4, ΔCPbx4, MycΔHDCMeis3, FlagPbx4 and MycMeis3 expression vectors were described previously (Choe et al., 2002; Vlachakis et al., 2001).

Microinjections

mRNAs for injections were synthesized *in vitro* using the Ambion SP6 mMessage mMachine kit. mRNAs were diluted in nuclease-free water including 0.25-0.5% phenol red to the concentrations indicated in the figure legends. All microinjections were done at the 1- to 2-cell stage. For morpholino injections 2mM stocks of anti-Hoxb1a and anti-Hoxb1b MOs were combined, diluted to 1-4mg/ml of each MO and injected as described (McClintock et al., 2002).

In situ hybridization and Immunohistochemistry

In situ hybridizations were described previously (Vlachakis et al., 2001). Whole mount immunohistochemistry with 3A10 (Hatta, 1992) or anti-Islet (39.4D5: (Korzh et al., 1993)) antibody and protein localization with anti-Myc (clone 9E10) antibody were performed as previously described (Vlachakis et al., 2001). Immunostaining with RMO44 (Zymed laboratories) antibody was performed using goat anti-mouse secondary antibodies conjugated to horseradish peroxidase and then incubating with FITC-conjugated tyramide (PerkinElmer Life Sciences, Inc) to visualize the primary reticulospinal interneurons.

RESULTS

Extensive removal of Meis activity completely abolishes hindbrain segmentation.

We have previously utilized a dominant negative construct (ΔCPbx4) to explore the role of Meis proteins in hindbrain development (Choe et al., 2002). We reported that interfering with Meis function leads to loss of segment-specific gene expression in r3, r4 and r5, but has little or no effect in more rostral (r1, r2) or caudal (r6, r7) rhombomeres (Choe et al., 2002). Meis proteins are thought to act as Hox cofactors during hindbrain development, but they require Pbx proteins both to enter the nucleus and to interact with Hox proteins present in the hindbrain. It is therefore notable that the ΔCPbx4 phenotype is less severe than the phenotype resulting from extensive removal of Pbx function (Waskiewicz et al., 2002) and more similar to the effect of partially removing Pbx function (Pöpperl et al., 2000; Waskiewicz et al., 2002). This suggests that Meis is either not required for all Pbx/Hox functions in the hindbrain, or that the ΔCPbx4 construct is unable to eliminate all Meis function in vivo.

The dominant negative effect of the ΔCPbx4 construct stems from it blocking nuclear localization of Meis proteins and we initially demonstrated that ΔCPbx4 sequesters Meis3 in the cytoplasm (Choe et al., 2002). Although additional Meis-family members are expressed in the zebrafish hindbrain (Choe et al., 2002; Sagerström et al., 2001; Waskiewicz et al., 2001; Zerucha and Prince, 2001), we have not found any qualitative differences in their sensitivity to ΔCPbx4. For instance, Prep1 is efficiently sequestered in the cytoplasm by ΔCPbx4 (Fig. 1A, B), while nuclear localization of

Pbx4 and Hoxb1a is unaffected (Fig. 1C, D). Since Prep1 is the most divergent Meis family member identified to date, this finding is consistent with all Meis family members being affected by ΔCPbx4.

We next considered that ΔCPbx4 might be quantitatively unable to sequester all endogenous Meis proteins. To address this possibility we combined ΔCPbx4 with a second dominant negative construct that acts by a different mechanism. In particular, a form of Meis1.1 that lacks its C-terminus (and therefore lacks the homeodomain required for DNA binding) reportedly interferes with endogenous Meis function (Waskiewicz et al., 2001). We have generated an analogous form of Meis3 (ΔHDCMeis3) and co-expressed it with ΔCPbx4 to test if this further reduces endogenous Meis function (Fig. 1). We do not observe any developmental defects when ΔHDCMeis3 is expressed by itself (not shown; (Choe et al., 2002)), but the combination of ΔCPbx4 and ΔHDCMeis3 gives a more severe phenotype than ΔCPbx4 alone. In particular, while embryos injected with ΔCPbx4 never show loss of *hoxa2* expression in r2 (99% have normal r2 expression (Choe et al., 2002)), embryos co-injected with ΔCPbx4 and ΔHDCMeis3 exhibit a partial (79%) or complete (8%) loss of *hoxa2* expression in r2 (Fig. 1F). We also observe a more severe effect on *ephA4* expression. Specifically, ~10% of ΔCPbx4-injected embryos show low-level *ephA4* expression in the rostral hindbrain, concomitant with partial loss of high-level *ephA4* expression in r3 and r5 (Choe et al., 2002). In embryos co-injected with ΔCPbx4 and ΔHDCMeis3, the frequency of embryos with such diffuse low-level *ephA4* expression increases to ~40% and the phenotype is more severe. In particular, high-level *ephA4*

expression is completely lost in r3 and r5 and the low-level *ephA4* expression is detected throughout the hindbrain rather than just rostrally (Fig. 1L). Lastly, *hoxb1a* expression in r4 (Fig. 1H) and *val* expression in r5/r6 (Fig. 1J) is affected more strongly in embryos co-injected with ΔCPbx4 and ΔHDCMeis3, although the difference is less marked since these genes are partially affected by ΔCPbx4 alone (Choe et al., 2002).

We next examined differentiation of reticulospinal neurons, which show rhombomere-specific morphologies and axonal trajectories (Metcalfe et al., 1986). Using the 3A10 antibody, which specifically detects Mauthner neurons at early stages of development (Hatta, 1992), we find that Mauthner neurons do not form in r4 of embryos co-injected with ΔCPbx4 and ΔHDCMeis3 (Fig. 1N). Furthermore, using the anti-neurofilament antibody RMO44 (Pleasure et al., 1989) to detect the entire complement of reticulospinal interneurons, we find that most of these neurons are absent from embryos co-injected with ΔCPbx4 and ΔHDCMeis3 (Fig. 1P). We also examined the segmental differentiation of branchiomotor (BM) neurons of the cranial nerves, using an *islet1-GFP* transgenic line that drives GFP expression in BM neurons (Higashijima et al., 2000) (Fig. 1Q, R). We find that the number of *islet1*-positive cells in the hindbrain of embryos co-injected with ΔCPbx4 and ΔHDCMeis3 is drastically reduced (Fig. 1R). Further, the remaining *islet1*-positive cells are not segmentally organized, but are arranged in a continuous array along the rostrocaudal axis of the hindbrain. This arrangement extends into the caudal region normally occupied by nX neurons, which are also reduced in number. We conclude that extensive removal of

endogenous Meis function completely blocks segmentation of the hindbrain, similar to the effect of extensively disrupting Pbx function (Waskiewicz et al. 2002).

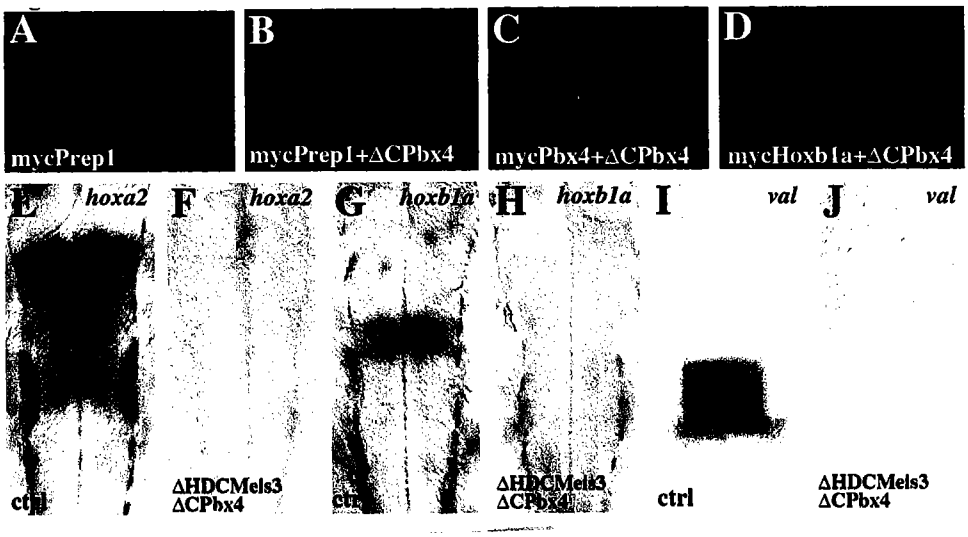

Figure 1A. Co-expression of ΔCPbx4 and ΔHDCMeis3 completely disrupts hindbrain gene expression and neuronal differentiation. (A-D) ΔCPbx4 interferes with the nuclear localization of Prep1, but not Pbx4 or Hoxb1a. Embryos were injected with 300 pg of the mRNAs indicated at the bottom of each panel, raised to 12 hpf and stained with anti-Myc antibody. (E-J) Co-expression of ΔCPbx4 and ΔHDCMeis3 completely abrogates gene expression in the hindbrain. Embryos were injected with 250 pg of each Δ*CPbx4* and Δ*HDCMeis3* mRNA (F, H, J) or 500 pg of *lacZ* mRNA (E, G, I), raised to 14 hpf (I, J) or 24 hpf (E-H) and analyzed by in situ hybridization for genes indicated at the top right of each panel.

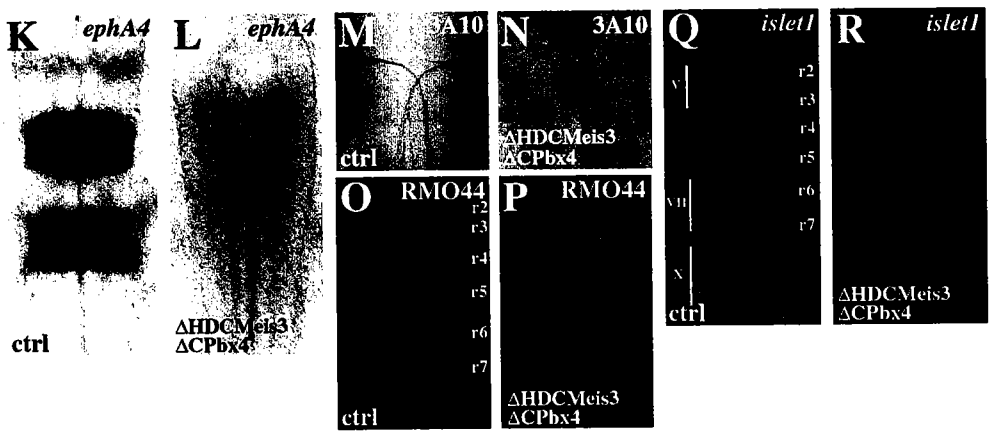

Figure 1B. Co-expression of ΔCPbx4 and ΔHDCMeis3 completely disrupts hindbrain gene expression and neuronal differentiation.
(K-L) Co-expression of ΔCPbx4 and ΔHDCMeis3 completely abrogates gene expression in the hindbrain. Embryos were injected with 250 pg of each Δ*CPbx4* and Δ*HDCMeis3* mRNA (L) or 500 pg of *lacZ* mRNA (K), raised to 24 hpf and analyzed by in situ hybridization for genes indicated at the top right of each panel. (M-R) Co-expression of ΔCPbx4 and ΔHDCMeis3 severely disrupts neuronal differentiation. mRNA was injected as in E-L and embryos were raised to 28 hpf (M, N) or 48 hpf (O-R) and stained with 3A10 (M, N) or RMO44 (O, P) antibody. In Q and R, an *islet1-GFP* transgenic line was instead used to visualize branchiomotor neurons. All panels are dorsal views with anterior to the top.

Partial removal of Meis activity leads to anterior transformation of the caudal hindbrain.

In the course of these experiments we noted that the phenotype induced by the ΔCPbx4 construct alone was not simply a milder version of that induced by co-expressing ΔCPbx4 together with ΔHDCMeis3, but appeared qualitatively distinct. In particular, while RMO44 staining revealed that rostral reticulospinal neurons (r2 and r3) are only variably detected in ΔCPbx4-injected embryos, similar to the effect seen upon co-expression of ΔCPbx4 and ΔHDCMeis3, the caudal reticulospinal neurons (r4-r7) appear to have become homogenized (Fig. 2A,B). This is seen most clearly in the case of r7 interneurons that have large round cell bodies and 'T'-shaped axonal projections in control embryos (arrow in Fig. 2A). In ΔCPbx4-injected embryos, cells with T-interneuron morphology are often lacking in r7. Instead, neurons with elongated cell bodies and axons that project contralaterally are observed at the level of r7 (arrow in Fig. 2B). Indeed, the majority of reticulospinal neurons detected in the hindbrain of ΔCPbx4-injected embryos have elongated cell bodies and contralateral projections. These features are characteristic of reticulospinal neurons in r2, r4 and r6, but the Mauthner neurons in r4 are the most prominent neurons of this type. To determine if caudal reticulospinal neurons in ΔCPbx4-injected embryos take on an r4 Mauthner neuron fate, we made use of the 3A10 antibody. We find that, while control-injected embryos display a single pair of 3A10 positive Mauthner neurons in r4 (Fig. 2C), ΔCPbx4-injected embryos contain multiple 3A10 positive neurons (Fig. 2D, E). The frequency of ΔCPbx4-injected embryos showing caudal Mauthner neurons is about 10%

on average (n= more than 20 experiments and >1,000 embryos), but varies between experiments and in some cases as many as 30% of embryos show this phenotype. The supernumerary Mauthner neurons appear to largely respect the segmental spacing of rhombomeres, although they are not always centered within the rhombomere, and are observed in r5, r6 and r7, as well as occasionally caudal to r7, but never rostral to r4. We conclude that a partial reduction in Meis function leads to an anterior transformation of caudal reticulospinal neurons to an r4-fate.

This conclusion is supported by our examination of branchiomotor (BM) neuron differentiation in ΔCPbx4-injected embryos using the *islet1-GFP* transgenic line (Fig. 2F, G). In wild type and control-injected embryos (Fig. 2F) nV (trigeminal) cell bodies form as a major rostral cluster in r2 (nVa) and a smaller caudal cluster in r3 (nVp), nVII (facial) cell bodies form as a cluster in r4 that migrates to r6/r7 by 36hpf and nX (vagal) cell bodies form as an extended cluster caudal to r7. We find that BM neuron differentiation in r2 and r3 is affected in ΔCPbx4-injected embryos, in agreement with the observed effect on reticulospinal neurons in this region. In particular, nVa neurons reside more medially than in control embryos, suggesting that they do not undergo their normal lateral migration within r2, and nVp neurons in r3 are largely lost (Fig. 2G). Since *islet1* positive neurons do not form in r5 and r6 of *islet1-GFP* fish, an anterior transformation of this domain cannot be directly observed. However, we find that nVII BM neurons remain in r4, suggesting that they cannot migrate through r5/6, consistent with formation of r5/6 being disrupted in ΔCPbx4-injected embryos (Fig. 2G). Lastly, we find that nX neurons caudal to r7 are unaffected in ΔCPbx4-injected embryos.

We next examined gene expression in ΔCPbx4-injected embryos. As we reported previously (Choe et al., 2002), ΔCPbx4 disrupts *hoxb1a* expression in r4, but we also observe ectopic *hoxb1a* expression in the caudal hindbrain (Fig. 2I). This ectopic expression is strongest caudally and extends at least as far rostrally as r5. In contrast, co-expressing ΔHDCMeis3 and ΔCPbx4 disrupts *hoxb1a* expression in r4, but never induces ectopic *hoxb1a* expression (Fig. 1H). In addition, ΔCPbx4 disrupts high-level *ephA4* expression in r3 and r5 and induces low-level *ephA4* expression, as we reported previously (Choe et al., 2002), but this low-level expression does not appear to expand caudal to r5 (Fig. 2K). This is in contrast to embryos co-expressing ΔCPbx4 and ΔHDCMeis3, where low-level *ephA4* expression is found throughout the hindbrain (Fig. 1L). These effects of the ΔCPbx4 construct appear restricted to *hoxb1a* and *ephA4* since we do not observe ectopic expression of other genes in the hindbrain (e.g. *krox20, val, hoxb3, hoxa2*; (Choe et al., 2002) and data not shown). Taken together, our results demonstrate that a partial reduction in endogenous Meis function reveals an intrinsic capacity of the caudal hindbrain to take on an r4 fate. Since this phenotype is observed in 10-30% of embryos expressing ΔCPbx4 alone, but not in embryos co-expressing ΔCPbx4 and ΔHDCMeis3, it is likely induced only in a relatively narrow range of Meis activity.

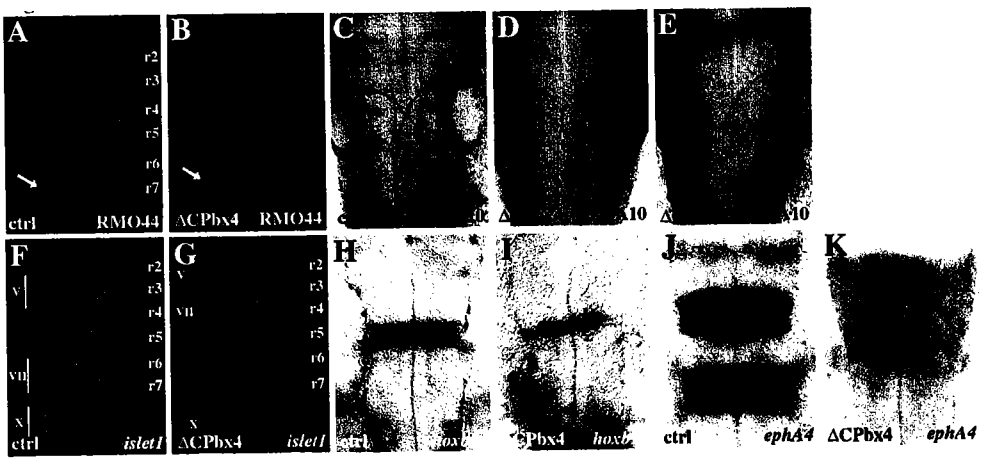

Figure 2. Expression of ΔCPbx4 reveals a transient r4-like state in the caudal hindbrain. (A-E) ΔCPbx4 induces ectopic Mauthner neurons in the caudal hindbrain. 1 to 2-cell stage embryos were injected with 150 pg of Δ*CPbx4* mRNA (B, D, E) or *lacZ* mRNA (A, C), raised to 28 hpf (C-E) or 48 hpf (A, B) and stained with RMO44 (A, B) or 3A10 (C-E) antibody. Arrows in A and B indicate reticulospinal neurons in r7 (Note that the arrowed neuron in B is different from the one in A in its shape and axonal projection). (F, G) ΔCPbx4 impairs differentiation of branchiomotor neurons. islet1-GFP embryos were injected as in A-E, raised to 48 hpf, fixed and flat-mounted to visualize GFP-positive branchiomotor neurons. (H-K) ΔCPbx4 induces ectopic *hoxb1a* and *ephA4* expression. Embryos were injected as in A-E, raised to 24 hpf and analyzed for expression of *hoxb1a* (H, I) or *ephA4* (J, K) by in situ hybridization.

Transformation of the caudal hindbrain to an r4-fate is mediated by PG1 hox genes.

The specification of r4 fates is mediated by paralog group 1 (PG1) *hox* genes (*hoxb1a* and *hoxb1b* in zebrafish, (McClintock et al., 2002)). Since *hoxb1a* is expressed in the caudal hindbrain of ΔCPbx4-injected embryos (Fig. 2I), it is possible that PG1 *hox* genes are responsible for inducing r4 fates in the caudal hindbrain of these embryos. To test this possibility we co-injected *hoxb1b* or *hoxb1a* mRNA together with Δ*CPbx4* mRNA and assayed formation of Mauthner neurons in the caudal hindbrain (Fig. 3). We find that co-expressing *hoxb1b* or *hoxb1a* with ΔCPbx4 increases the frequency of embryos with caudal Mauthner neurons by about 5-fold on average (n=5 experiments, 661 embryos for *hoxb1b* and n=5 experiments, 342 embryos for *hoxb1a*), with as many as 48% of embryos showing ectopic caudal Mauthner neurons in some experiments. In contrast, formation of ectopic Mauthner neurons is not enhanced by co-expressing ΔCPbx4 with *hoxa2* (n=2 experiments, 155 embryos), *hoxb2* (n=2 experiments; 139 embryos) or *hoxa3* (n=3 experiments; 158 embryos), demonstrating that this effect is specific to *hoxb1a* and *hoxb1b*.

We next used morpholino antisense oligos (MOs) specific to *hoxb1a* and *hoxb1b* mRNAs (McClintock et al., 2002) to test if Hoxb1a and Hoxb1b are required for induction of the caudal r4-fate. The use of MOs to simultaneously knock-down Hoxb1a and Hoxb1b, but not either protein alone, induces complete loss of r4 Mauthner neurons in 40% of embryos (McClintock et al., 2002). We find that simultaneous knock-down of Hoxb1a and Hoxb1b reduces the number of ΔCPbx4-injected embryos with caudal Mauthner neurons to a similar extent (40-50%; n=2 experiments, 176 embryos), while

control MOs do not have an effect (n=2 experiments, 187 embryos). We conclude that endogenous *hoxb1a* and *hoxb1b* are required for induction of an r4-like fate in the caudal hindbrain of ΔCPbx4-injected embryos.

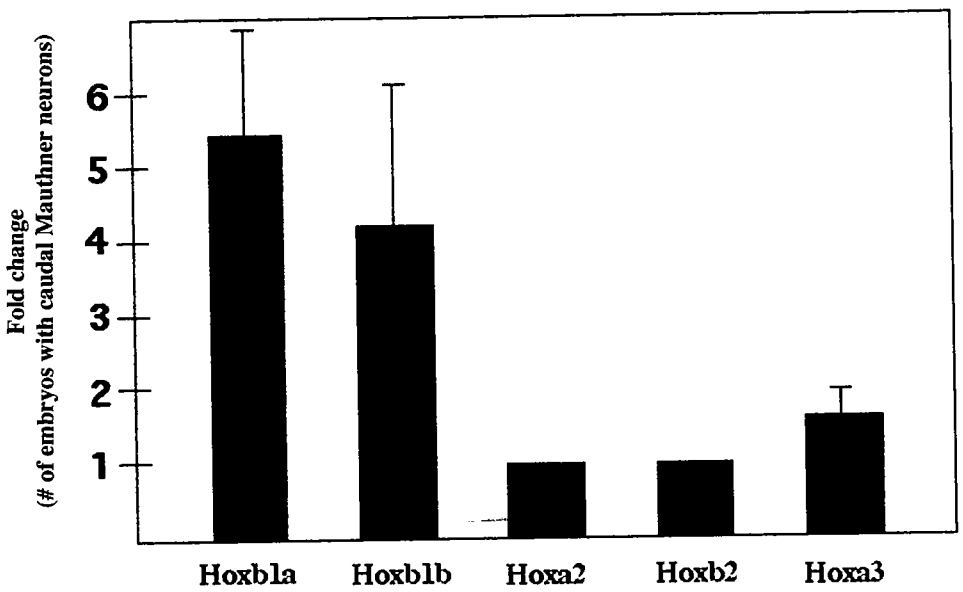

Figure 3. PG1, but not PG2 or PG3, hox genes enhance transformation of the caudal hindbrain to an r4 fate. 1 to 2-cell stage embryos were injected with 200 pg of ΔCPbx4 mRNA together with 400pg of hox mRNA as indicated at the bottom of each bar, raised to 28 hpf, stained with 3A10 antibody and scored for the presence of ectopic caudal Mauthner neurons. The data is presented as fold-increases in the number of embryos showing caudal Mauthner neurons following injection of 400pg hox mRNA relative to control injections with 400 pg lacZ mRNA.

vhnf1 represses r4-specific neuronal differentiation.

Our results suggest that the ΔCPbx4 construct interferes with a factor that normally represses r4 fates in the caudal hindbrain. *vhnf1*, one of the earliest expressed genes in r5/r6, is a strong candidate for this role. In particular, zebrafish embryos mutant for *vhnf1* display a loss of r5/r6 fates, as well as a caudalward expansion of r4 fates, and misexpression of *vhnf1* represses gene expression in r4 (Sun and Hopkins, 2001; Wiellette and Sive, 2003). To determine whether *vhnf1* also affects neuronal differentiation in r4, we misexpressed *vhnf1* in developing zebrafish embryos by mRNA microinjection. We find that 40% of embryos injected with *vhnf1* mRNA lack one or both Mauthner neurons in r4 (Fig. 4B; n=3 experiments, 160 embryos). An additional 10-15% of *vhnf1*-injected embryos show mispositioning and/or abnormal axonal projection of one Mauthner neuron (Fig. 4C shows a Mauthner axon projecting laterally before turning to the midline). Mispositioned Mauthner neurons are most often found in or near r4, but are occasionally found at a distance from r4. Although detection of reticulospinal neurons in r2/r3 is less robust than in r4, it appears that neurons in this region are also lost (Fig. 4B, C). To further assess these effects we examined differentiation of BM neurons in *islet1-GFP* transgenic embryos injected with *vhnf1*. We find that nVII neurons, which are born in r4 and migrate caudally into r6/r7, are lost in 51% (43/85) of embryos, consistent with r4 being affected by *vhnf1* misexpression (Fig. 4E). We observe a lesser effect on r2/r3, where nV neurons are missing in 26% of *vhnf1*-injected embryos (22/85). In contrast, when *islet1* expression is analyzed by anti-Islet1 immunohistochemistry (which detects motor neurons of the VIth and IXth cranial

nerves in addition to the Vth, VIIth and Xth nerves), we find that Islet1 expression is never completely lost in r5/r6/r7 (Fig. 4G, H) although nV neurons in r2/r3 are again lost in 25% of embryos (15/63; left-hand side of embryo in Fig. 4H). We conclude that although nVII neurons fail to migrate into r6/r7 of *vhnf1*-injected embryos, nVI and nIX neurons still develop in r5-r7. This suggests that differentiation of r4 is affected while differentiation of r5-r7 occurs normally. In addition, we observe an effect in r2/r3 of *vhnf1*-injected embryos, suggesting that *vhnf1* can affect differentiation also of more rostral rhombomeres.

This effect on neuronal differentiation correlates well with the effect of *vhnf1* on gene expression. In particular, r5/r6-specific expression of *valentino, hoxa3* and *krox20* expands rostrally in 90-95% of *vhnf1*-injected embryos (Fig. 4I-N and data not shown). This expansion extends rostral to r4, at least into r2/3 (Fig. 4J, L) and occasionally as far rostrally as the midbrain (~14%; n=2 experiments and 142 embryos, data not shown). Concomitantly, *hoxb1a* expression in r4 (Fig. 4K-N; 95% affected), *hoxa2* expression in r2/3 (~30% affected; not shown) and *ephA4* expression in r1 (Fig. 4O, P; ~17% affected) is reduced in *vhnf1*-injected embryos. We conclude that *vhnf1* has the ability to repress r4 fates as well as more rostral fates.

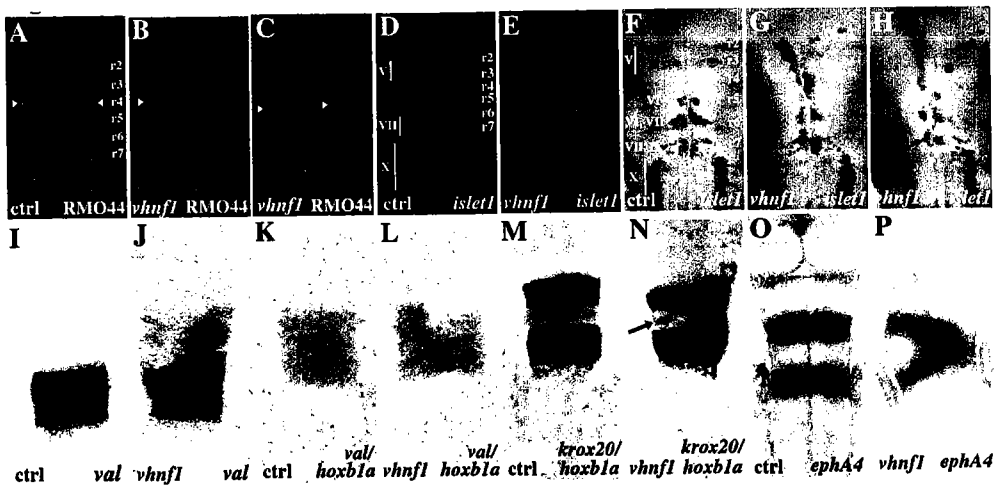

Figure 4. Misexpression of vhnf1 represses r4 and anterior fates. (A-H) vhnf1 disrupts neuronal differentiation. 1 to 2-cell stage embryos were injected with 25 pg of vhnf1 (B, C, E, G, H) or 25 pg of lacZ mRNA (A, D, F), raised to 48 hpf and processed by immunohistochemistry using RMO44 (A-C) or anti-Islet (F-H) antibody. In D and E, the islet1-GFP transgenic line was used to detect branchiomotor neurons. White arrowheads in A-C point to Mauthner neurons in r4. (I-P) vhnf1 expands r5/r6 gene expression and represses hoxb1a expression in r4. Embryos were injected as in A-H, raised to 14 hpf (IN) or 24 hpf (O, P) and analyzed by in situ hybridization for expression of the genes indicated at bottom right of each panel. In K-N double in situ hybridizations were performed with hoxb1a expression in red. Arrow in N indicates a small hoxb1aexpressing region. All panels are dorsal views with anterior to the top.

A Meis-dependent step upstream of vhnf1 is required to repress r4 fates in the caudal hindbrain.

While *vhnf1* has the ability to repress r4 fates, it is not clear how *vhnf1* function might be disrupted by the ΔCPbx4 construct. We reasoned that ΔCPbx4 might either interfere with *vhnf1* expression or with its function. Notably, the ΔCPbx4 construct disrupts expression of a number of r5/6-specific genes (*krox20, valentino* and *hoxb3*; (Choe et al., 2002)), suggesting that ΔCPbx4 might block expression also of *vhnf1*. To test this possibility we examined *vhnf1* expression in ΔCPbx4-injected embryos. We find that *vhnf1* expression is partially lost in 48% (41/86) of ΔCPbx4-expressing embryos (Fig. 5B), demonstrating that ΔCPbx4 acts upstream of *vhnf1* to block its expression.

We next reasoned that if ΔCPbx4 transforms the caudal hindbrain to an r4 fate by blocking *vhnf1* expression, re-establishing *vhnf1* expression in r5 and r6 of ΔCPbx4-expressing embryos should restore normal gene expression to this domain. Indeed, while injecting Δ*CPbx4* mRNA represses r5 gene expression (*valentino, hoxb3* and *krox20*; Fig. 5E shows effect on *valentino* expression, see also (Choe et al., 2002)), in 68% of embryos (112/164) we do not observe any repression of r5 gene expression in embryos injected with both *vhnf1* mRNA and Δ*CPbx4* mRNA (Fig. 5D). Instead, we find that 61% (220/358) of embryos injected with both *vhnf1* and Δ*CPbx4* mRNA show a rostral expansion of r5 gene expression into r4 (Fig. 5D), similar to the phenotype observed when only *vhnf1* mRNA is injected (see Fig. 4). Thus, *vhnf1* functions in the

presence of ΔCPbx4, both to restore r5/r6 gene expression and to repress r4 fates, consistent with ΔCPbx4 interfering with a Meis-sensitive step upstream of *vhnf1*.

PG1 Hox proteins are necessary and sufficient to induce vhnf1 expression in r5/6.

We next explored the nature of the Meis-sensitive step regulating *vhnf1* expression. Early-acting *hox* genes are likely candidates to regulate this step, particularly since Meis proteins have known roles as Hox cofactors. In fact, we have previously demonstrated that ectopic expression of *hoxb1b* together with the *meis3* and *pbx4* cofactors induces ectopic expression of *valentino* in the rostral embryo (Vlachakis et al., 2001). To test whether PG1 *hox* genes induce *vhnf1* expression, we co-injected *hoxb1b, pbx4* and *meis3* mRNA. We find that this leads to ectopic *vhnf1* expression in the rostral embryo in 55% of embryos (54/98; Fig. 5G). In contrast, co-injecting *hoxb1b* and *pbx4* mRNA without *meis3* mRNA does not induce ectopic *vhnf1* expression (94 embryos analyzed; not shown). We conclude that Hoxb1b is capable of inducing *vhnf1* expression and that it requires Meis cofactors for this purpose.

We next examined whether PG1 *hox* genes are required for gene expression in r5 and r6. Previous workers using MOs to disrupt PG1 function reported a very mild hindbrain phenotype (McClintock et al., 2002). In particular, r4 is reduced in size and Mauthner neurons are lost in r4, but r5 and r6 gene expression is not lost (McClintock et al., 2002). We reasoned that the anti-PG1 MOs might not completely remove PG1 Hox function and co-injected anti-PG1 MOs with the ΔCPbx4 construct to simultaneously interfere with PG1 Hox and Meis function. Although ΔCPbx4 never completely

eliminates gene expression in r5 and r6 (*krox20, valentino, hoxb3*; Fig. 5E and (Choe et al., 2002)), co-injection of PG1 MOs and ΔCPbx4 completely eliminates *valentino* expression in 29% of embryos (Fig. 5J). Similarly, while ΔCPbx4 partially blocks *vhnf1* expression in 48% of injected embryos, only in 3% does this effect encompass more than half of the expression domain. In contrast, 17% of embryos co-injected with anti-PG1 MOs and ΔCPbx4 show loss of *vhnf1* expression in more than half of the expression domain and many of these embryos lack *vhnf1* expression altogether (Fig. 5H). We conclude that PG1 proteins and their cofactors are necessary and sufficient to induce expression of several genes, including *vhnf1*, in r5/r6.

PG1 hox genes interact genetically with meis genes to pattern the entire hindbrain.

As expected, our analysis of embryos co-injected with ΔCPbx4 and anti-PG1 MOs also revealed an effect in r4. In particular, *hoxb1a* expression which is never lost in response to anti-PG1 MOs (McClintock et al., 2002) and only rarely lost in response to ΔCPbx4 (13%; (Choe et al., 2002)), is completely lost in 53% and severely affected in 47% of embryos co-injected with anti-PG1 MOs and ΔCPbx4 (Fig. 6B). However, we also observe an effect on gene expression in the rostral hindbrain. Specifically, we find that *hoxa2* expression, which is unaffected by injection of anti-PG1 MOs (McClintock et al., 2002) and affected in r3-r5, but not in r2, by ΔCPbx4 (Choe et al., 2002), is affected in r2 in 58% (and completely lost in 9%) of embryos co-injected with PG1 MOs and ΔCPbx4 (Fig. 6F). In addition, ectopic expression of *ephA4*, which is never seen in anti-PG1 MO injected embryos and observed in only 10% of ΔCPbx4-

injected embryos (Choe et al., 2002)), is observed in 54% of embryos co-injected with anti-PG1 MOs and ΔCPbx4 (Fig. 6D) and this ectopic *ephA4* staining extends further caudally than is seen in embryos injected with ΔCPbx4 alone.

To further examine this widespread effect of simultaneously reducing Meis and PG1 Hox function, we next examined neuronal differentiation. We find that, using concentrations where one or both Mauthner neurons are lost in 15-30% of embryos injected with anti-PG1 MOs alone or ΔCPbx4 alone, co-injecting anti-PG1 MOs with ΔCPbx4 leads to loss of one or both Mauthner neurons in r4 of >90% of embryos (Fig. 6J; n=3 experiments and 271 embryos). Using the RMO44 antibody to detect all reticulospinal neurons in these embryos, we observe a near-complete loss of reticulospinal neurons not only in r4, but also rostrally and caudally (Fig. 6H). We also examined differentiation of branchiomotor neurons using anti-islet immunostaining (Fig. 6K, L). We find that, while anti-PG1 MOs alone or ΔCPbx4 alone affect primarily BM neurons differentiating in r4 (Choe et al., 2002; McClintock et al., 2002), combining anti-PG1 MOs and ΔCPbx4 drastically reduces the number of Islet1-positive cells throughout the hindbrain (Fig. 6L). Further, the remaining Islet1-positive cells are not segmentally organized, but are scattered throughout the hindbrain. As seen for reticulospinal neurons, BM neurons are affected in the rostral as well as the caudal hindbrain, although nX neurons found caudal to r7 are affected less severely and nIII/nIV neurons in the midbrain are unaffected. We conclude that simultaneous reduction in Meis and PG1 Hox function disrupts development of the entire hindbrain. Since PG1 *hox* genes are not expressed in the rostral hindbrain, this effect must be

mediated indirectly. We also note that this phenotype is indistinguishable from that observed upon extensive disruption of Meis function (Fig. 1), consistent with Meis and PG1 Hox proteins cooperating to regulate hindbrain development.

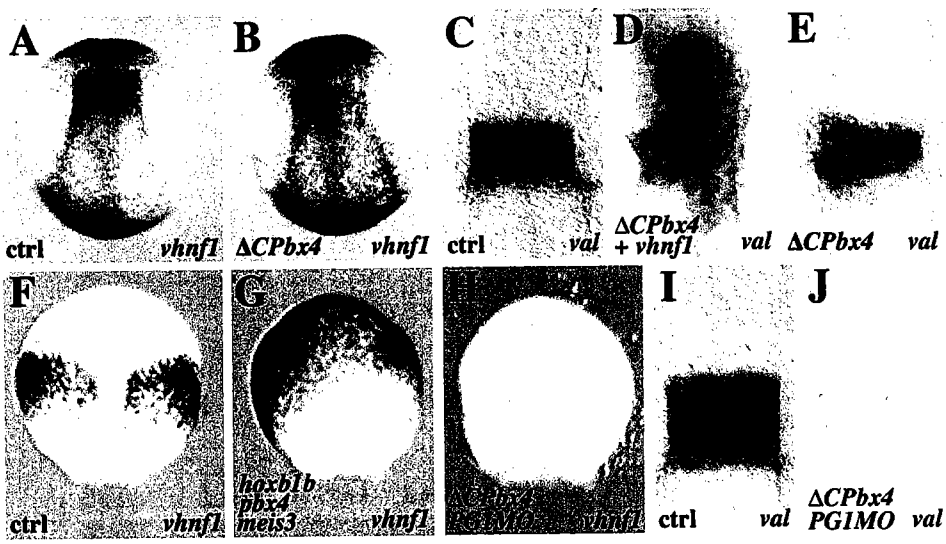

Figure 5. PG1 *hox* genes regulate *vhnf1* expression in r5/r6.
(A, B) *vhnf1* expression is dependent on Meis function. 1 to 2-cell stage embryos were injected with 300pg of *lacZ* (A) or Δ*CPbx4* (B) mRNA, raised to 11 hpf and analyzed by in situ hybridization for vhnf1 expression. (C-E) vhnf1 function is largely independent of Meis function. 1 to 2- cell stage embryos were injected with 300pg of *lacZ* mRNA (C), 300pg of Δ*CPbx4* mRNA (E) or 300pg of Δ*CPbx4* + 25pg of *vhnf1* mRNA (D), raised to 14 hpf and analyzed by in situ hybridization for val expression. (F-J) PG1 hox genes are necessary and sufficient to induce r5/r6 gene expression. 1-2 cell stage embryos were injected with 300pg of *lacZ* mRNA (F, I), 166pg each of *hoxb1b, pbx4* and *meis3* mRNA (G) or 300pg of Δ*CPbx4*+ PG1MO (H, J), raised to 10 hpf (F-H) or 14 hpf (I, J) and analyzed by in situ hybridization for *vhnf1* (F-H) or *val* (I, J) expression. All panels are dorsal views with anterior to the top.

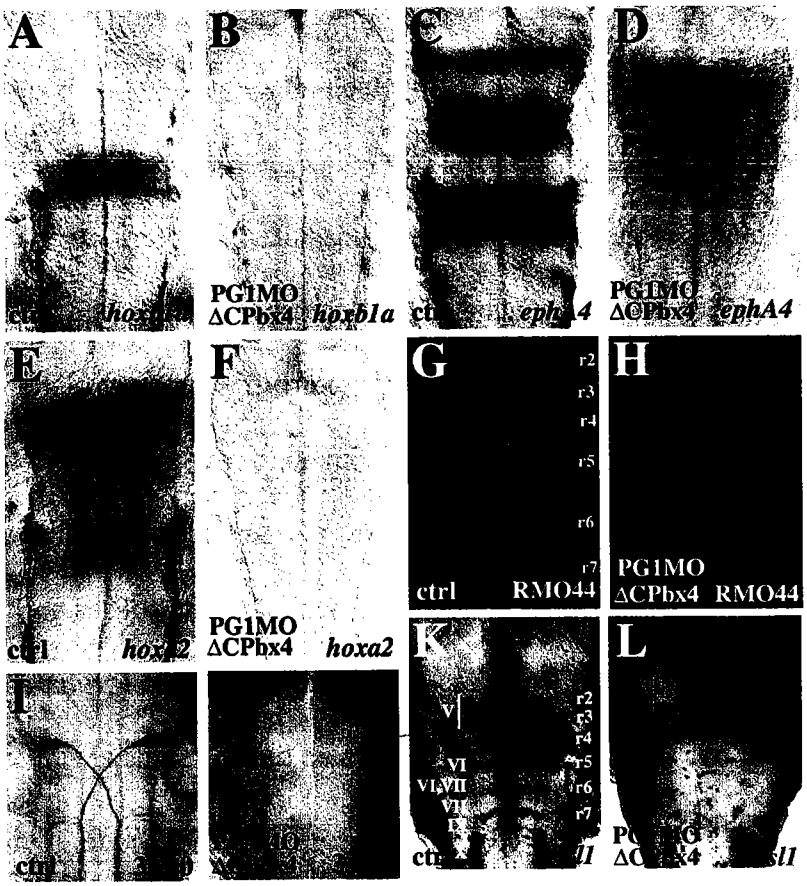

Figure 6. Simultaneous reduction in PG1 Hox and Meis function completely abolishes hindbrain patterning. (A-F) Hindbrain gene expression is completely impaired by the combined application of anti-PG1 MO and ΔCPbx4. 1 to 2-cell stage embryos were coinjected with control MO and *lacZ* mRNA (A, C, E) or anti-PG1MO (see Methods section) and 300pg Δ*CPbx4* mRNA (B, D, F), raised to 24 hpf and analyzed by in situ hybridization for expression of genes indicated at bottom right of each panel. (G-L) The combined action of anti-PG1 MO and ΔCPbx4 completely disrupts hindbrain neuronal differentiation. Embryos were injected as in A-F, raised to 28 hpf (I, J) or 48 hpf (G, H, K, L) and stained with 3A10 (I, J), RMO44 (G, H) or anti-Islet (K, L) antibody. All panels are dorsal views with anterior to the top.

DISCUSSION

In this report we first examine the function of Meis proteins as Hox cofactors in hindbrain development. By combining two different constructs that interfere with Meis function we demonstrate that extensive removal of Meis activity blocks development of the hindbrain. Specifically, segment-specific gene expression and neuronal differentiation is completely disrupted in the hindbrain, leaving in its stead a uniform structure. Further, simultaneous removal of *meis* and PG1 *hox* function reveals a strong genetic interaction between *meis* genes and PG1 *hox* genes, consistent with Meis proteins acting as Hox cofactors during hindbrain development. We then note that incomplete removal of Meis function produces an r4-like fate in the caudal hindbrain. This r4-like fate is characterized by ectopic Mauthner neurons and is induced by PG1 *hox* genes. Lastly, we demonstrate that *vhnf1* represses r4-fates in the caudal hindbrain and that *vhnf1* expression is regulated by PG1 *hox* genes. Our results indicate that PG1 *hox* genes not only induce a broad caudal r4 domain, but also induce expression of *vhnf1*, which then acts to repress caudal r4 fates and promotes subdivision the caudal hindbrain.

PG1 hox genes act within r5/r6 to regulate vhnf1 expression

A role for PG1 *hox* genes in the development of the caudal hindbrain was first indicated in embryos with targeted deletions of the *Hoxa1* gene (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Lufkin et al., 1991; Mark et al., 1993). In

particular, these embryos had marked reduction not only in r4, but also in r5. This effect becomes more pronounced when *Hoxa1* and *Hoxb1* are simultaneously deleted (Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998), leading to the loss of both r4 and r5. Simultaneous disruption of zebrafish *hoxb1b* and *hoxb1a* using morpholino antisense oligonucleotides also affects both r4 and r5 (McClintock et al., 2002), but the effect is significantly milder than in the mouse. While the role for PG1 *hox* genes in regulating formation of r4 has become clear (*Hoxa1* regulates *Hoxb1* which regulates *Hoxa2* etc; (Maconochie et al., 1997; Pöpperl et al., 1995)), it has remained unclear what role PG1 *hox* genes play in r5. It has been shown recently that PG1 *hox* genes induce expression of *fgf3* and *fgf8* in r4 (Waskiewicz et al., 2002), and that this Fgf-signal is required for formation of r5/r6 (Maves et al., 2002; Walshe et al., 2002). These findings have been taken as indications that PG1 *hox* genes play an indirect role in regulating r5/r6 development by regulating Fgf production in r4.

Here we demonstrate that PG1 *hox* genes regulate *vhnf1* expression and we propose that they do so not by producing Fgfs in r4, but instead by acting within r5/r6 to induce *vhnf1* expression. This is supported by several observations. First, if our experiments affect r5/r6 development by blocking PG1-mediated Fgf production in r4, we should observe phenotypes similar to those generated by disrupting Fgf3 and Fgf8 function, but we do not. In particular, reduced Fgf function leads to loss of r5/r6 (observed as a juxtaposition of r7 T-interneurons immediately caudal to r4 Mauthner neurons; (Maves et al., 2002)) while in our experiments the caudal hindbrain is either transformed to an r4 fate (by ΔCPbx4; observed as transformation of caudal

reticulospinal neurons to a Mauthner neuron fate) or lacks a discernible fate (by co-expressing ΔCPbx4 with ΔHDCMeis3 or PG1 MOs; observed as a loss of all caudal reticulospinal neurons). Second, if *vhnf1* expression is regulated by Fgf-signals from r4, *vhnf1* expression should be lost in embryos with reduced Fgf3 and Fgf8 function, but it is not. Indeed, *vhnf1* expression appears completely independent of Fgf3 and Fgf8 signaling (#'s not shown and (Wiellette and Sive, 2003)). This is in contrast to expression of *krox20, val* and PG3 *hox* genes, which is lost upon disruption of Fgf3 and Fgf8 (Maves et al., 2002; Walshe et al., 2002). Thus, our results help explain why PG1 genes are required for r5/r6 development by demonstrating that PG1 *hox* genes act within r5/r6 to activate *vhnf1* expression.

PG1 hox genes promote r4-fates and induce a repressor of r4-fates

Our results suggest that PG1 *hox* genes induce r4-fates in a broad caudal domain during hindbrain development. Several other experimental conditions have been shown to similarly transform the hindbrain to an r4-like fate. For instance, ectopic expression of a dominant negative retinoic acid receptor-β construct (dnRARβ) induces ectopic Mauthner neurons in the caudal hindbrain of *Xenopus* embryos (van der Wees et al., 1998), although a dnRARα construct appears to instead block development of the caudal hindbrain (Blumberg et al., 1997; Kolm et al., 1997). Furthermore, RARα/RARγ double mutant mice or application of RAR antagonists promotes expansion of *hoxb1* expression into the caudal hindbrain (Dupe and Lumsden, 2001;

Wendling et al., 2001) and disruption of the RA-synthesizing enzyme RALDH2 leads to expression of *krox20* and *hoxb1* in the caudal hindbrain (Niederreither et al., 2000). Similarly, mutations in *vhnf1* (Wiellette and Sive, 2003) or PG3 *hox* genes (Gaufo et al., 2003) promote an expansion of r4 fates into the caudal hindbrain. Thus, there is little doubt that the caudal hindbrain is transiently specified to an r4 fate and there appear to be factors responsible for the subsequent subdivision of this broad r4 domain into r4-r7.

vhnf1 has been shown to repress r4-specific gene expression in mis-expression experiments (Wiellette and Sive, 2003), making it a candidate to act as a repressor of r4 fates in the caudal embryo in vivo. We therefore tested if *vhnf1* might be the repressor affected in our experiments and found that *vhnf1* expression is Meis-dependent. Disruption of *vhnf1* expression might therefore explain why blocking Meis function promotes caudal r4 fates. This may also explain the expansion of caudal r4 fates under some other experimental conditions. In particular, disrupting retinoic acid (RA) signaling promotes caudal r4 fates (see above) and we find that *vhnf1* expression is RA-dependent (not shown), although we cannot distinguish whether RA acts directly to activate *vhnf1* expression or indirectly by first activating PG1 *hox* expression.

It is surprising that PG1 *hox* genes both promote r4 fates and induce a repressor of r4 fates. In particular, this raises the question of how *vhnf1* activity becomes restricted to the future r5/r6 and does not repress r4 fates in the future r4. The most likely explanation is that other factors are responsible for protecting the future r4 from the effect of *vhnf1*. For instance, we have recently identified a zinc-finger protein that

appears to be required in r4 to repress transcription of non-r4 genes (Runko and Sagerstrom, 2003). Regulation of PG1 gene expression also appears to be different in r4, where an autoregulatory loop is established (Pöpperl et al., 1995), than in the rest of the caudal hindbrain.

A model for the role of PG1 hox genes in patterning the caudal hindbrain

We consider the early hindbrain primordium to be a uniform structure that is specified to a generic 'pre-segmentation' fate, as revealed by extensive removal of Meis function (this study) or Pbx function (Waskiewicz et al., 2002). We postulate that the caudal hindbrain primordium is next specified to an r4 fate by the onset of PG1 *hox* gene expression caudal to the r3/r4 boundary. Consistent with the presence of a transient r4-like state during normal development, PG1 *hox* genes are transiently expressed in the hindbrain primordium of zebrafish, mouse and the chick (Alexandre et al., 1996; Frohman et al., 1990; Murphy and Hill, 1991; Prince et al., 1998; Sagerström et al., 2001; Sundin and Eichele, 1990). We propose that PG1 *hox* genes induce expression of *vhnf1*, which represses r4 fates (Figure 7). *vhnf1* also cooperates with Fgf3 and Fgf8 produced in r4 to promote r5/r6 fates by inducing *krox20, val* and PG3 *hox* gene expression. It is possible that genes downstream of *vhnf1* (e.g. *krox20, valentino,* PG3 and PG4 *hox* genes) share the ability to repress r4-fates, as indicated by recent reports (Gaufo et al., 2003; Giudicelli et al., 2003).

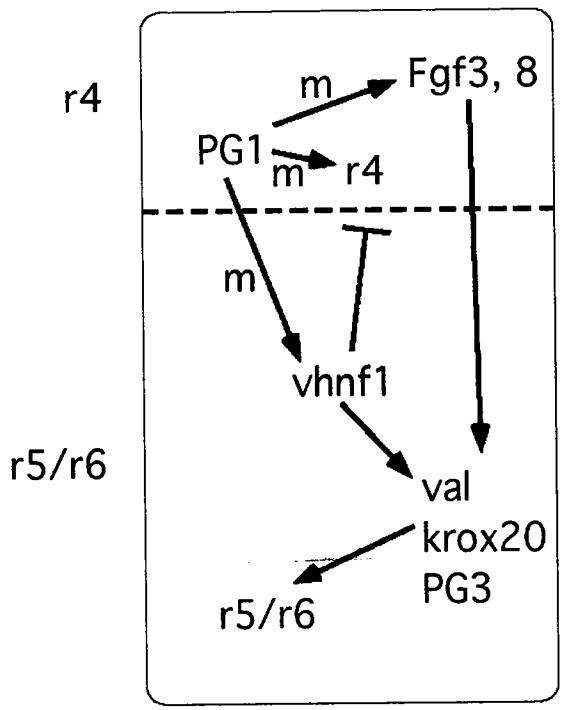

Figure 7. Proposed model for role of PG1 *hox* genes in development of the caudal hindbrain. PG1 *hox* genes induce r4 fates throughout the caudal hindbrain and also induce *vhnf1* expression in the future r5/r6. *vhnf1* represses r4 fates and also promotes r5/r6 fates by cooperating with Fgf signals from r4 to induce *val*, *krox20* and PG3 *hox* gene expression. m indicates steps that require Meis activity. See text for further details.

Different requirements for Meis and Pbx cofactors in hindbrain development.

There appear to be differences in the extent to which different steps require a given cofactors, as well as in the relative importance of each cofactor. First, our results suggest that although there are several Meis-dependent steps in hindbrain development, these are differentially sensitive to reduced Meis function. In particular, partial reduction in Meis function (using the ΔCPbx4 construct), does not block induction of caudal r4 fates, but it disrupts *vhnf1* expression sufficiently that caudal r4 fates are not repressed. This suggests that induction of *vhnf1* expression and induction of r4 fates require different levels of Meis function. The role of Meis proteins as Hox cofactors likely involves both stabilization of Hox binding to DNA and the recruitment of additional cofactors. Differences in Meis-dependence might therefore be explained by differences in either of these roles. For instance, if the DNA binding site required for the promotion of r4 fates is a closer match to the PG1 consensus than the binding site required to induce *vhnf1* expression, Meis proteins would be less important for the induction of r4 fates.

Second, while extensive removal of Meis function leads to a phenotype very similar to that observed upon extensive disruption of Pbx function (Waskiewicz et al., 2002), less complete interference with Pbx or Meis function reveals differences. In particular, partial removal of Meis function reveals an r4-like state in the caudal hindbrain, but partial removal of Pbx function does not induce such a fate. Specifically, two different *pbx* genes (*pbx2* and *pbx4*; (Pöpperl et al., 2000; Vlachakis et al., 2000)) are expressed in the hindbrain and several levels of Pbx function have been tested by

removing *pbx4* alone (eliminating only zygotic or both maternal and zygotic *pbx4* in the *lazarus* mutant; (Pöpperl et al., 2000)), *pbx2* alone (using anti-*pbx2* MOs; (Waskiewicz et al., 2002)) or both together (Waskiewicz et al., 2002), but ectopic *hoxb1a* expression or ectopic Mauthner neuron differentiation was not reported. This correlates with other differences between Meis and Pbx proteins. In particular, Pbx proteins bind directly to Hox proteins expressed in the hindbrain (PG1-4) and bind DNA sites immediately adjacent to the Hox site in many Hox-dependent enhancers (reviewed in (Mann and Affolter, 1998)). This Pbx site is absolutely required for Hox proteins to drive expression from these enhancers. In contrast, Meis proteins do not bind directly to Hox proteins expressed in the hindbrain, but instead associate with such Hox proteins indirectly, via Pbx. Further, Meis sites are found at a variable distance from the Pbx/Hox sites and the Meis binding site is required for expression from some, but not all, Hox-dependent enhancers (Ferretti et al., 2000; Jacobs et al., 1999). This suggests that while both Meis and Pbx are required for hindbrain development, Meis proteins may play a more modulatory role. Accordingly, several studies have demonstrated that Meis proteins function even when their DNA-binding homeodomain is mutated (Berthelsen et al., 1998; Vlachakis et al., 2001) and we have defined an N-terminal domain in Meis that is sufficient to confer Meis function (Choe et al., 2002).

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CHAPTER III

CONCLUSION

Both Meis and Pbx family members are known to interact with Hox proteins and stabilize Hox binding to DNA sequences. It has been shown that Meis can not interact directly with Hox proteins expressed in the hindbrain but must interact via Pbx for their nuclear localization in zebrafish (Vlachakis et al., 2001). While a role for Pbx has conclusively been shown in zebrafish hindbrain development (Waskiewicz et al., 2002), roles for Meis have not been clearly demonstrated for normal hindbrain development. Our experiments have begun to investigate the role of Meis during hindbrain development using an ectopic expression assay (Vlachakis et al., 2001). Co-expression of *hoxb1b, pbx4* and *meis3* gives rise to ectopic expression of *hoxb1a* and *hoxb2* in the anterior domain and causes severe anterior truncations, while expression of either *pbx4* and *meis3* or *hoxb1b* and *pbx4* does not show such phenotypes, suggesting that Meis proteins may function together with Pbx/Hox complexes.

To better understand the role of Meis *in vivo*, we designed a Meis dominant negative molecule (ΔCPbx4) to interfere with nuclear localization of Meis family members and found severe defects in gene expression as well as neuronal differentiation demarking r3 through r5 of the hindbrain upon expression of ΔCPbx4 (Choe et al.,

2002). This result suggests that Meis proteins are required for proper formation of r3 through r5 during normal hindbrain development. However, the phenotype we observed is not as severe as that observed upon extensive removal of Pbx activity (Waskiewicz et al., 2002), but instead are more similar to that of partially removing Pbx function (Popperl et al., 2000). This raised the question of how Meis proteins function during hindbrain development: either Meis is only required for some Pbx/Hox functions or ΔCPbx4 does not interfere with all Meis function *in vivo*. To test if ΔCPbx4 is unable to remove all Meis activity, we combined two different Meis dominant-negative molecules to see if this further reduces endogenous Meis activity. In particular, a Meis construct lacking the homeodomain-containing C-terminus (ΔHDCMeis) has been reported to interfere with Meis function during hindbrain development (Waskiewicz et al., 2001). We found that a more severe phenotype is generated upon co-expression of the two Meis dominant-negative molecules (see Chapter II). Specifically, embryos co-injected with ΔCPbx4 and ΔHDCMeis3 occasionally lose gene expression from r2 to r6 in hindbrain while embryos injected with ΔCPbx4 alone never show complete loss of gene expression in r2 and r6. Consistent with this result, neuronal specification and differentiation is also severely affected. Most segmentally specified and differentiated reticulospinal interneurons and branchiomotor neurons are largely lost with expression of ΔCPbx4 and ΔHDCMeis3. Instead, the entire hindbrain displays weak *ephA4* expression similar to r1-like expression of normal *ephA4*. These phenotypes are strikingly similar to that upon extensive removal of Pbx function (Waskiewicz et al.,

2002), suggesting that Meis proteins function in the same pathway as Pbx proteins to regulate hindbrain development.

While expression of ΔCPbx4 gives rise to partial defects in hindbrain segmentation, we also observe an anterior transformation of the caudal hindbrain when Meis activity is reduced (see Chapter II). Specifically, the caudal hindbrain from r5 through r7 is transformed to r4-like fate, evidenced by *hoxb1a* expression and by formation of Mauthner neurons in this region. Such phenotypes have never been reported upon removal of Pbx activity (Waskiewicz et al., 2002), suggesting functional difference between Meis and Pbx as Hox cofactors. We found that the anterior transformation of the caudal hindbrain upon partial removal of Meis function is enhanced by overexpression of Hox PG1 proteins and that *vhnf1* represses r4 fates in the caudal hindbrain. Further, we have shown that expression of *vhnf1* depends on the function of Hox PG1 and Meis proteins. These results suggest that *hox* PG1 has a role in the formation of r5/6 as well as r4 and Meis proteins are also required for this process. Based on these results, we propose a model by which segmentation of the caudal hindbrain is achieved (described in detail below; Figure 1).

Although our experiments have clearly demonstrated a role for Meis proteins during hindbrain development, they also raised several questions: First, what makes Meis proteins different from Pbx? We have shown that Meis and Pbx act together to induce hindbrain fates (Vlachakis et al., 2001; Choe et al., 2002), but partial removal of Meis only induces r4-like fate in the caudal hindbrain (Choe et al., 2002; Popperl et al., 2000). Second, how do different Hox targets require different Meis activity? We find

that ectopic Mauthner neurons are still specified in the caudal hindbrain in ΔCPbx4-injected embryos whereas *vhnf1* expression in the same domain is not induced (Choe et al., 2002), suggesting that functions of Hox PG1 proteins show different Meis dependence. Lastly, what are the targets of Meis? Consistent with the role of Meis as a Hox cofactor, we find that expression of *hox* genes is lost upon extensive removal of Meis function. Further, we also find that loss of Meis function gives rise to complete absence of segmentation of the hindbrain (Chapter II). This phenotype, however, is not in agreement with such a restricted role of Meis. Since expression of *hox* genes is restricted in r2 through r7 of the hindbrain, it suggests that Meis proteins may be involved in regulation of all segmentation genes responsible for patterning of the entire hindbrain. Supporting this idea, we have found that Hox PG1 and Meis proteins are required and sufficient for expression of *vhnf1*, an upstream regulator of at least one caudal segmentation gene, *val*. However, we do not know a target of Meis proteins for segmentation of the rostral hindbrain. These questions will be discussed below in the sections following a proposed model.

A model for segmentation of the caudal hindbrain: progressive segmentation of the caudal rhombomeres and roles for factors involved in hindbrain development

Many experiments addressing specific rhombomere formation have discovered segmentation genes such as *krox20, val* (a zebrafish counterpart of *kreisler* in mouse), *vhnf1* and *hoxb1b* (a zebrafish counterpart of *Hoxa1* in mammals). Upon loss of either gene, specific rhombomeres are lost; loss of *krox20* removes r3, loss of either *vhnf1* or

val removes r5/6 and loss of *Hoxa1* removes r4/5. While the mechanism that links their actions throughout the hindbrain is still elusive, our observations upon interfering with the function of Meis have begun to reveal how early hindbrain fates are determined and which factors are involved in the process.

From Pre-segment hindbrain to two-segment hindbrain

Although several lines of evidence indicate that segmentation genes such as *hoxb1b, krox20* and *val* are essential for individual rhombomere formation, questions regarding hindbrain segmentation have not been directly addressed yet. First, what is the initial hindbrain fate? Does it correspond to a particular rhombomere? While it is likely that factors that caudalize the neural tube specify hindbrain fates as well as more caudally located spinal cord, it is not still clear what the initial hindbrain looks like. Recent observations upon loss of both Pbx2 and Pbx4 provide clues to this question (Waskiewicz et al., 2002). Specifically, complete loss of Pbx2/4 function display a phenotype where the hindbrain primordium is transformed to an r1 fate, suggesting r1 as a hindbrain 'ground-state' identity (Waskiewicz et al., 2002). Similarly, we observe that simultaneous reduction in both Hox PG1 and Meis function results in a pre-segmented hindbrain structure which partially expresses r1 marker gene, *ephA4*, but otherwise does not correspond to any of rhombomere fates (see Chapter II). These experiments suggest that the initial hindbrain fate may be r1-like. Second, how is segmentation achieved? Is it simultaneous or sequential? That is, are seven rhombomeres segmented at the same time and kept throughout the development, or are

these rhombomeres specified as broad domains at a time before distinct and final rhombomere fate is determined? In relation to this question, evidence that supports sequential specification of hindbrain rhombomeres comes from observations where r4 expands progressively in response to gradual loss of RA signaling, suggesting that hindbrain may be segmented into more broad domains before further specification of each rhombomere (Dupe and Lumsden, 2001; Wendling et al., 2001). A recent report demonstrating r4 as the first visible rhombomere directly supports this sequential model for formation of rhombomeres. Further, our observations also support the sequential model. In particular, partial loss of Meis function results in an anterior transformation of the caudal hindbrain (r5-r7) to r4 fates; the expansion of the r4 marker gene expression, *hoxb1a*, and the formation of the Mauthner neurons (differentiated only in r4) in the caudal domain of the hindbrain (see Chapter II). While the caudal domain is transformed to r4 upon partial loss of Meis function, the anterior region may still take on r1-like, because it shows weak *ephA4* expression only seen in r1 during normal hindbrain development. These results suggest that the hindbrain structure is segmented into 2 distinct domains where the anterior domain takes on r1-like fate while the caudal domain is r4-like. Taken together, these observations indicate that an initial r1-like hindbrain is segmented into two-segment structure in which the caudal hindbrain takes on r4 fates before being specified into particular rhombomeres (Figure 1).

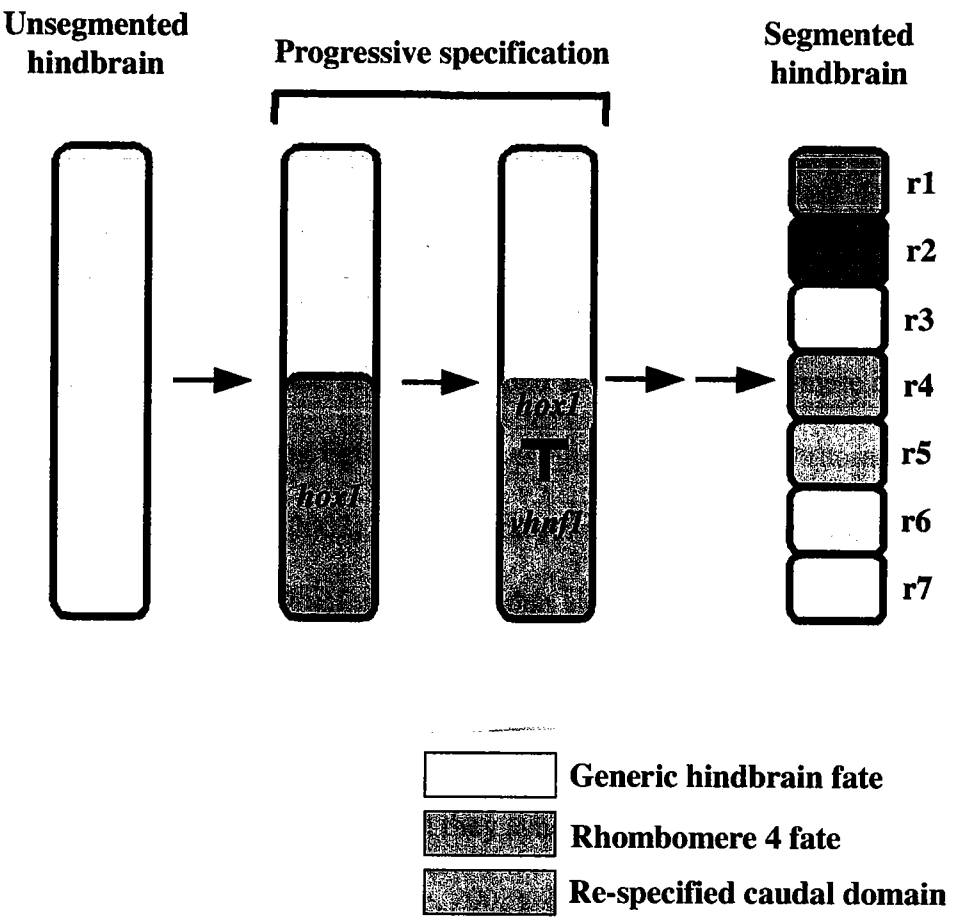

Figure 1. Proposed model for specification of the caudal hindbrain. The hindbrain primordium is initially unsegmented. Subsequently, the entire hindbrain takes on an r4 fate. This r4 fate becomes restricted to the future r4 by genes acting further caudally. For instance, *vhnf1* represses r4 fates and promotes r5/6 fates. See text for further details.

hox PG1 genes are required for formation of r5/6 as well as r4

hox PG1 genes are expressed during gastrulation and are responsible for induction of many critical genes for hindbrain segmentation including other paralog group of *hox* genes. Experiments have demonstrated that loss of *hox* PG1 expression results in loss of r4 segment as well as more caudal r5 segment. This suggests that *hox* PG1 function is required for the formation of r4 and r5. Supporting this idea, a recent observation has demonstrated that r4 is formed as the first rhombomere and acts as a signaling center by inducing Fgf3/8 to further specify more caudal rhombomeres such as r5 and r6 (Maves et al., 2002). Further, the *vhnf1* mutant displays loss of r5/6 marker gene expression, especially *val*, suggesting that *vhnf1* may act as an upstream regulator of genes required for r5/6 segmentation including *val* and possibly other genes in this region (Sun and Hopkins, 2001). A direct connection between Fgf3/8 and vHnf1 has come from a study demonstrating that they synergize to induce r5/6 fate by inducing r5/6 marker genes (Wiellette and Sive, 2003). Taken together, it is plausible that *hox* PG1 involvement in the formation of r5/6 may be achieved indirectly through activating Fgf3/8 in the r4 and the Fgf3/8 act together with vHnf1 to induce genes responsible for the r5/6 segmentation.

In addition to such a role for *hox* PG1, a direct role of *hox* PG1 during the formation of r5/6 may be through activating *vhnf1* expression. We have previously demonstrated that co-expression of *hoxb1b*, *pbx4* and *meis3* results in posterior transformation of the embryo with ectopic expression of hindbrain segmentation genes such as *hoxb1a*, *krox20* and *val* (Vlachakis et al., 2001). Similarly, we observed ectopic

vhnf1 expression upon co-expression of *hoxb1a, pbx4* and *meis3* (see Chapter II). Furthermore, we found that the *vhnf1* expression is partially affected with expression of ΔCPbx4 and that the *vhnf1* expression is completely lost when both *hox* PG1 and Meis function are interfered (see Chapter II). These experiments indicate that *hox* PG1 genes along with their cofactors are necessary and sufficient to drive *vhnf1* expression *in vivo* and thus required for specification of r5.

vhnf1 and/or its downstream targets further specify r5/6

Once *vhnf1* expression commences, *vhnf1* seems to repress the caudal r4 fate. The exact mechanism how *vhnf1* represses r4 fate in the caudal domain is not known, but we observe that ectopic *vhnf1* can repress *hoxb1a* expression in r4 and that it represses the specification of Mauthner neurons in r4 as well (Sun and Hopkins, 2001; see Chapter II). Further, we also observe that re-established *vhnf1* expression represses the formation of ectopic Mauthner neurons in the caudal hindbrain in the presence of ΔCPbx4 (S.-K. C. and C.G.S., unpublished result), suggesting that *vhnf1* may repress the caudal r4 fate during normal hindbrain development.

Although *vhnf1* is known to be an upstream regulator of *val* and other r5/6 marker genes, *vhnf1* is expressed transiently during early development (Sun and Hopkins, 2001). This raises the question of how r5/6 is specified. Is *vhnf1* only required for *val* expression, which in turn acts as the executor responsible for r5/6 segmentation? Or is it required for other processes? In the *vhnf1* mutant, *val* expression is lost and presumably *hox* PG3 expression as well. Instead, *hoxb1a* expression expands caudally

into the domain of normal *val* expression (Sun and Hopkins, 2001). Moreover, ectopic *val* expression in the *vhnf1* mutant partially rescues *vhnf1* loss of function phenotype (Wiellette and Sive, 2003). Therefore, it is likely that *vhnf1* functions to induce *val* expression and this expression may be sufficient for specification of r5/6.

What about *hox* PG3 genes then? As is the case with *val*, *hox* PG3 genes may repress caudal r4 fates induced by *hox* PG1 genes during normal hindbrain development. Such a role for Hox PG3 proteins is strongly supported by a recent experiment where homozygous *Hox* PG3 mutants display ectopic expression of *Hoxb1* in the caudal hindbrain and this ectopic *Hoxb1* expression results in the differentiation of r4-like facial neuron in the same domain (Gaufo et al., 2003). This result suggests that Hox PG3 may normally function to repress *Hoxb1* expression during hindbrain development. A role for *hox* PG3 genes in mediating the repressive effect of *vhnf1*, however, will require direct analysis of *hox* PG3 function *in vivo*. Taken together, the initial pre-segmented hindbrain is segmented into two-segment hindbrain with an r4-fate caudal region and the caudal r4 fate of the two-segment hindbrain is repressed by the *vhnf1* function, which cooperating with Fgf3 and Fgf8 induces *val* expression which in turn induces expression of *hox* PG3 and other downstream genes in r5/6 (Figure 1).

What makes Meis proteins different from Pbx?

Previously, we found that the Pbx-interacting domain of Meis family proteins, called the Meinox domain and consisting of M (Meinox) 1, I (intervening sequence) and

M2, is indispensable for Meis function in our ectopic expression assays (Vlachakis et al., 2001). Likewise, domain analysis of Meis indicates that the Meinox domain is sufficient to mediate the activity of Meis family proteins in a similar ectopic expression assay (Choe et al., 2002). These results may suggest that a role of Meis merely stabilizes Pbx/Hox complexes by binding Pbx and DNA. However, several pieces of evidences counteract this suggestion. First, we find that the Meinox domain confers a function in addition to Pbx binding in our ectopic expression assay (Choe et al., 2002). In particular, a mutated Meinox domain (which can not bind Pbx) fused to the C-terminus of Pbx (containing the Hox-interaction domain and homeodomain) is still functional when co-expressed with *hoxb1b*, suggesting that Meis may function by recruiting some other factor to the Pbx/Hox complex. Consistent with this idea, we found that Meis proteins interact with one of coactivators, CBP (CREB-binding proteins) (S.-K. C and C.G.S. unpublished result). To better understand how Meis proteins contribute to Pbx/Hox complexes, it may require identification/characterization of Meis-interacting molecules. Second, partial removal of Meis activity gives rise to anterior transformation of the caudal hindbrain, which is not seen with partial loss of Pbx activity (see Chapter 2). Loss of Pbx function studies have been performed in a step-wise manner where two *pbx* genes expressed in hindbrain are sequentially removed. Either eliminating zygotic *pbx4* (the *lazarus* mutant) or both maternal and zygotic *pbx4* (Popperl et al., 2000), removing *pbx2* using morpholino (Waskiewicz et al., 2002) or complete removal of both *pbx2* and *pbx4* (Waskiewicz et al., 2002) does not generate anterior transformation, suggesting that Meis proteins may have a

modulatory role in the complex where Pbx proteins are absolutely required for all Hox functions while Meis is not. This idea is supported by our observations that Pbx does not require Meis proteins to enter the nucleus while Meis does require Pbx for nuclear translocation, and that expression of the C-terminus of Pbx which lacks the Meis-interaction domain does not generate any hindbrain defects (unpublished result). Lastly, disrupting DNA binding of Meis while retaining Pbx binding does not give any defect (unpublished result). This result contradicts our prediction that as it would act as a Meis dominant-negative molecule if Meis stabilizes simply Pbx/Hox complex. Taken together, these observation suggest that Meis proteins may contribute an activity to the Pbx/Hox complex in addition to stabilization.

How do different Hox targets require different Meis activity?

While Meis proteins act as Hox cofactors, it is possible that different Hox proteins may require different Meis activities to mediate their function *in vivo*. Several lines of evidence support this hypothesis: First, we observed that expression of ΔCPbx4 affects *vhnf1* expression, but not *hoxb1a* expression, in the caudal hindbrain (see Chapter II). As we have demonstrated that both *hoxb1a* and *vhnf1* expression require Hox PG1 function (see Chapter II), the observation suggests different functions of Hox PG1 proteins may require different Meis activity. Second, expression of different *hox* genes is differently affected by the same amount of ΔCPbx4 (Choe et al., 2002). As illustrated in the Introduction, expression of *hox* genes is regulated by auto- and cross-

regulation and we have shown that *hoxb1a* and *hoxb2* expression in r4 is more severely affected than *hoxa2* or *hoxa3/b3* expression in other domains upon partial loss of Meis function, suggesting that earlier *hox* regulation is more dependent on Meis function (Choe et al., 2002). Lastly, upstream regulatory regions of *hox* genes such as *hoxb1a* and *hoxb2* contain Meis binding sequence, but Meis binding is not always required to mediate Hox function (Ferretti et al., 2000; Vlachakis et al., 2001; Jacobs et al., 1999). These results strongly suggest that different Hox targets display different Meis requirement, which may be achieved by several mechanisms. One way to accomplish this is that Pbx/Hox complexes possess differential binding affinity to Meis family members. Thus, expression of each Meis family member may be correlated with a Pbx/Hox complex in the same domain and different Meis family members may possess preferential binding to specific Hox targets. Alternatively, upstream regulatory elements may have different degrees of similarity to the Meis binding consensus sequence. Thus, an enhancer element close to the consensus may confer higher affinity to Meis proteins than others more divergent from the consensus. Ultimately, resolution of these questions will require sequence analysis of upstream regulatory elements of Hox targets and biochemical assays measuring binding affinities between different Meis family members and Pbx/Hox complexes.

What are the targets of Meis?

We find that partial removal of Meis activity using ΔCPbx4 primarily interferes with r3-5 segmentation and sometimes induces anterior transformation of the caudal

hindbrain (Choe et al., 2002). This phenotype shows a striking correlation with phenotype of mice lacking *Hox* PG1 and 2 (Barrow and Capecchi, 1996; Davenne et al., 1999; Studer et al., 1996) and with the phenotype of RAR interference in chick (Dupe and Lumsden, 2001), respectively. Since it is known that *hox* PG1 and 2 genes are auto- and cross-regulated, these results suggest that Meis proteins are essentially required for this Hox function. The conclusion that Meis family proteins act as Hox cofactors is further demonstrated by our findings with more extensive removal of Meis activity using two different Meis dominant-negative constructs, which completely abolish segmentation of the entire hindbrain (see Chapter II). Given that expression of Hox PG1-4 proteins are essential for both segmentation and segmental identity in hindbrain development, Meis proteins are indispensable for expression of *hox* genes in the hindbrain. Further, simultaneous partial removal of Meis and Hox PG1 function gives a phenotype identical to that observed upon extensive removal of Meis activity, suggesting a strong genetic interaction between *meis* and *hox* PG1 (Chapter II).

While Meis proteins are required for expression of *hox* genes expressed in hindbrain, they are also involved in expression of other genes, such as *vhnf1*, *Fgf3/8*, and *krox20* (see Chapter II and Waskiewicz et al., 2002). Considering that these genes belong to segmentation genes required for initial patterning of rhombomeres, Meis proteins must have an additional function as upstream regulators of these genes to ensure early segmentation events during hindbrain development. Therefore, Meis proteins clearly regulate early events during segmentation of the entire hindbrain, although it is not clear how Meis proteins are involved in segmentation of the rostal

hindbrain. Taken together, these results indicate that Meis proteins function as essential Hox cofactors and as upstream regulators of segmentation genes to pattern the entire hindbrain during development.

Future directions

Although our experiments have demonstrated essential roles of Meis proteins during hindbrain development, a complete picture of hindbrain development may require more experiments addressing the following questions. First, it is possible that each Meis family member possesses a distinct role in hindbrain development, even if our experiments have revealed that each Meis family member can similarly promote hindbrain fates in ectopic expression assay (Choe et al., 2002; Vlachakis et al., 2001), While it is not easy to investigate such a distinct role of each isoform because an application of single morpholino against Meis3 does not give any defect (N.V. unpublished result) and the most divergent member of Meis, Prep, is expressed throughout the embryo (Choe et al., 2002), a combinatorial application of morpholinos against Meis family members would facilitate to delineate functional difference among Meis family members. Second, how do Meis proteins contribute Pbx/Hox complexes? As discussed earlier, Meis proteins may recruit some other factor to Pbx/Hox complexes. We have found using GST pull-down assays that Meis proteins can bind CBP and vHnf1 (unpublished results), suggesting that Meis proteins may interact with some unknown factor which contributes its function to the Pbx/Hox complex. To

identify such a molecule, it may require a screening by which Meis-interacting molecules could be found. Third, how is r4 fate in the caudal hindbrain repressed? Is it repressed directly by *vhnf1* or by downstream effectors, such as *val* or PG3 *hox* genes? Since the *vhnf1* mutants are available, it might worth trying to rescue the phenotype generated by loss of *vhnf1* function by re-establishing Val or Hox PG3 proteins. Clearly, there are some cases that support this idea; First, *val* can partially rescue loss of *vhnf1* function (Wiellette and Sive, 2003); Second, loss of *hox* PG3 genes induces ectopic *Hoxb1* expression in r5/6 and induces facial neurons in the caudal domain (Gaufo et al., 2003). Therefore, it is plausible that the function of *vhnf1* is relayed by its downstream effectors, such as *val* and *hox* PG3 genes. Taken together, it certainly requires other experiments to elucidate the mechanism involving the function of Meis proteins and to completely envision segmentation process during hindbrain development.

APPENDIX I

HOXB1B AND MEIS3 REQUIRE AN INTACT PBX-INTERACTION DOMAIN FOR THEIR FUNCTION IN VIVO

Meis3 and Hoxb1b can not interact with each other, but both bind Pbx4 (Vlachakis et al., 2000), raising the possibility that they interact with Pbx4 in vivo. Adjacent Pbx and Hox binding site are present in the murine HoxB1 enhancer and both sites are required for HoxB1 expression in a transgenic model (Pöpperl et al., 1995), suggesting that Pbx and Hox proteins might interact to induce murine HoxB1. Thus, to induce ectopic hoxb1a in zebrafish, Hoxb1b might interact with an endogenous Pbx protein. The most likely candidate is Pbx4, which is expressed broadly in the zebrafish embryo (Vlachakis et al., 2000) and is the predominant Pbx protein at this stage (Popperl et al., 2000). Meis3, on the other hand, can not interact with Hox proteins expressed in hindbrain, but binds Pbx4 in solution as well as on DNA (Vlachakis et al., 2000), suggesting that Meis3 must participate in the Pbx/Hox complex by binding Pbx4. Consistent with Meis binding to Pbx/Hox complexes, Meis binding site as well as Pbx/Hox binding site are found in several enhancer elements, such as murine HoxB1 and HoxB2. To test if Hoxb1b and Meis3 interact with Pbx4 in vivo, we generated

several constructs that are deficient in Pbx-binding (see Methods). The mutants of Hoxb1b and Meis3 were first tested by in vitro binding assays and then used for in vivo activity assays (Vlachakis et al., 2001).

Hoxb1b requires an intact Pbx-interaction domain for its activity in vivo

To test if Hoxb1b interacts with Pbx4 in vivo, we generated BMHoxb1b, a mutant form that is unable to bind Pbx4 (compare lanes 2 and 5 in Fig. 2A), by introducing a single amino acid substitution (W186->F) into the pentapeptide of Hoxb1b (see Methods). Analogous mutations abolish Pbx binding of other Hox proteins without altering their DNA binding (e.g. Knoepfler and Kamps, 1995; Rambaldi et al., 1994). BMHoxb1b is expressed at levels comparable to wild type Hoxb1b following microinjection, as assayed by Western blotting (compare lanes 2 and 3 in Fig. 2B). Expression of Hoxb1b resulted in ectopic expression of hoxb1a, while BMHoxb1b expression led to essentially normal embryos (Vlachakis et al., 2001). This is consistent with the idea that Hoxb1b requires an intact Pbx-interaction domain, suggesting that Hoxb1b and Pbx4 interact to activate Hoxb1b target gene expression in vivo.

Meis3 also requires an intact Pbx-interaction domain for its function in vivo

We next generated forms of Meis3 with reduced Pbx4 binding activity (BMMeis3 mutants, see Methods) by mutating two Meis N-terminal domains (M1 and M2) thought to mediate Pbx binding (reviewed in Mann and Affolter, 1998). Since Meis-Pbx binding is not completely characterized and mutating M1 or M2 alone may

not eliminate all Pbx-binding (Jaw et al., 2000; Knoepfler et al., 1997), we generated several constructs based on a previous report (Knoepfler et al., 1997; see Figure 1). BM^{M2}Meis3 carries two amino acid substitutions in M2, BM^{wM2}Meis3 has 5 amino acid substitutions in M2, $BM^{M1/2}$Meis3 has the same substitution as BM^{M2}Meis3 plus a four amino acid substitution in M1 and BM^{N}Meis3 has had its N-terminus replaced by a protein interaction domain from the unrelated FRAP protein. None of these proteins bind Pbx4 in vitro (Fig. 2C), but all still binds DNA (Fig. 2D). However, expression of BM^{M2}Meis3 mutant together with Pbx4 and Hoxb1b does show an activity (even though less active than wild-type Hoxb1b) while other BMMeis3 mutants are essentially inactive (see Vlachakis et al., 2001). This suggests that BM^{M2}Meis3 retains the ability to bind Pbx in vivo although we can not detect this by co-immunoprecipitation in vitro. Taken together, our results indicate that both Hoxb1b and Meis3 require intact Pbx-interaction domains to mediate their activities in vivo.

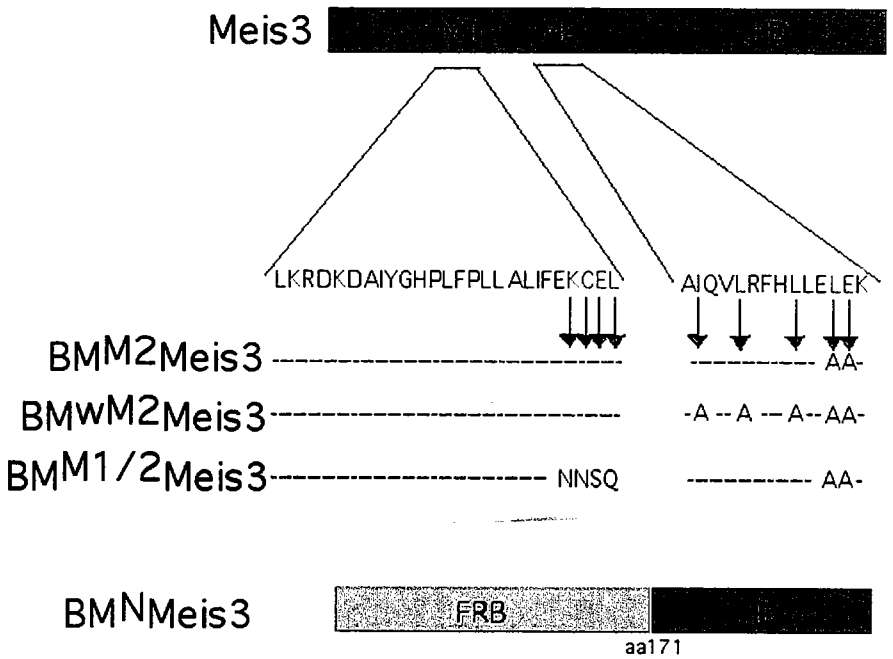

Figure 1. A schematic diagram showing Meis3 mutant constructs. Since Meis-Pbx binding is not fully characterized, we generated several constructs based on a previous report (Knoepfler et al., 1997). Amino acids in red are replaced by others as shown above. M1 and M2 consist of the Meinox domain. HD indicates the homeodomain.

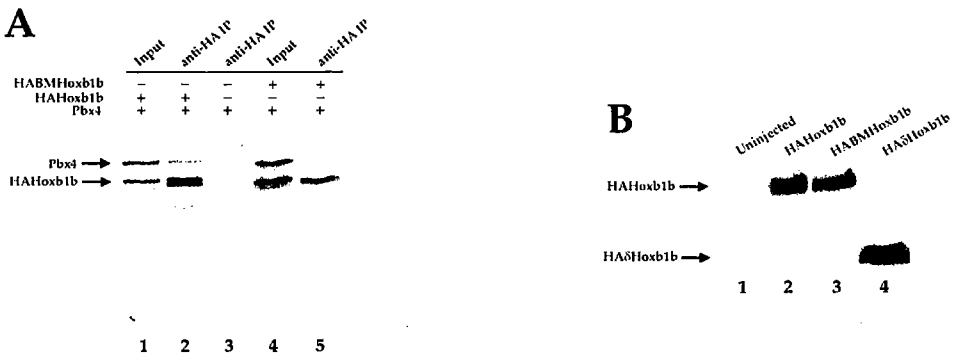

Figure 2A. Expression and Pbx4-interaction of Hoxb1b.
(A) Pbx4 was expressed alone (lane 3) or together with HAHoxb1b (lanes 1 and 2), or HABMHoxb1b (lanes 4 and 5) in vitro in the presence of ^{35}S-Methionine and either analyzed directly (input; lanes 1 and 4) or first immunoprecipitated with anti-HA antibody (lanes 2, 3, 5). All immunoprecipitations were performed in the presence of an oligonucleotide containing a Pbx/Hox binding site (P/H). (B) Western blot analysis (10 embryos/lane) of uninjected (lane 1), *HAhoxb1b* (lane 2), *HAbmhoxb1b* (lane 3), or *HAδhoxb1b* (lane 4) injected embryos, probed with anti-HA.

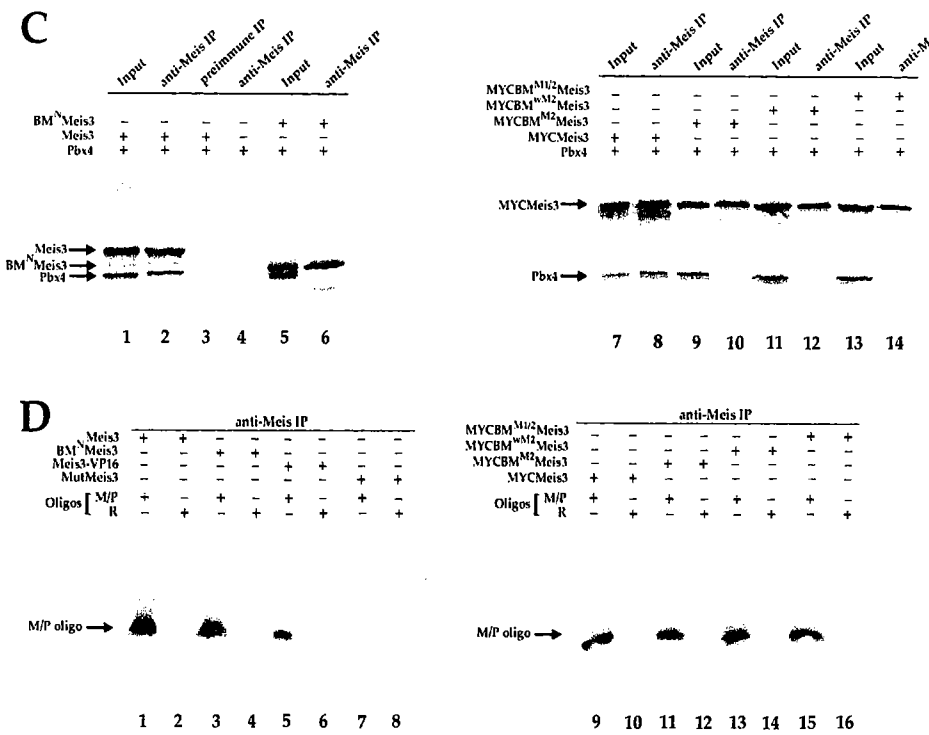

Figure 2B. Pbx4-interaction and DNA-binding of Meis3.
(C) Pbx4 was expressed alone (lane 4) or together with Meis3 (lanes 1-3), BM^{N}Meis3 (lanes 5 and 6), MYCMeis3 (lanes 7 and 8), $MYCBM^{M2}$Meis3 (lanes 9 and 10), $MYCBM^{wM2}$Meis3 (lanes 11 and 12) or $MYCBM^{M1/2}$Meis3 (lanes 13 and 14) in vitro in the presence of ^{35}S-Methionine and either analyzed directly (input; lanes 1, 5, 7, 9, 11, 13) or first immunoprecipitated with anti-Meis antisera (lanes 2, 4, 6, 8, 10, 12, 14) or with preimmune sera (lane 3). Immunoprecipitations were performed in the presence of an oligonucleotide containing a Meis/Pbx binding site (M/P). (D) Meis3 (lanes 1 and 2), BM^{N}Meis3 (lanes 3 and 4), MutMeis3 (lanes 7 and 8), MYCMeis3 (lanes 9 and 10), $MYCBM^{M2}$Meis3 (lanes 11 and 12), $MYCBM^{wM2}$Meis3 (lanes 13 and 14) or $MYCBM^{M1/2}$Meis3 (lanes 15 and 16) were expressed in vitro and incubated with ^{32}P-labeled oligonucleotide containing a Meis/Pbx binding site (M/P; lanes 1, 3, 5, 7, 9, 11, 13, 15) or a random sequence (R; lanes 2, 4, 6, 8, 10, 12, 14, 16). The samples were immunoprecipitated with anti-Meis antisera, resolved on a 5% acrylamide gel, and exposed to detect the presence of labeled oligonucleotides.

MATERIALS AND METHODS

Cloning

All genes used were derived from zebrafish, all expression constructs were in the pCS2+ vector and all constructs were verified by sequencing. Meis3, Pbx4, HA-Hoxb1b and MutMeis3 (carries two point mutations in the homeodomain, Q44->E and N51->A) have been described (Vlachakis et al., 2000, Sagerström et al, 2001). In BM^{N}Meis3 the N-terminal 171 aa were replaced with the FRB (FKBP 12-Rapamycin Binding) domain from FRAP (FKBP 12-Rapamycin Associated Protein) (Chen et al., 1995). All point mutations were generated with the QuikChange kit from Stratagene: BMHoxb1b (has a substitution in the pentapeptide FDWMK, W186->F) was generated using primer 5'-GGGGGATTCCTCTTGACTTTCATAAAGTCAAAGGTTGGCGC-3', BM^{M2}Meis3 (has two substitutions in the M2 motif, L141->A and E142->A) using primer 5'-CGGTTTCATCTATTAGAAGCAGCAAAGGTTCATGACCTCTGTGATAATTTCTGCC-3', BM^{wM2}Meis3 (has five substitutions in the M2 motif, I131->A, L134->A, L138->A, L141->A and E142->A) using primer 5'-CTGATGATCCAGGCCGCTCAAGTTGCACGGTTTCATGCATTAGAAGCAGC-3' with BM^{M2}Meis3 as a template and $BM^{M1/2}$Meis3 (has four substitutions in the M1 motif, aa 64-67 KCEL->NNSQ and two substitutions in the M2 motif, L141->A and E142->A) using primer 5'-GGCTCTGGTATTTGAAAACAATTCACAGCCACTTGCTCACC-3' with

BM^{M2}Meis3 as a template. NLS $BM^{M1/2}$Meis3 was generated by cloning oligonucleotide 5'- GATCCCCCGGGATGGCTCCAAAGAAGAAGCGTAAGGTAAA-3' into BamHI/ClaI digested pCS2+MT $BM^{M1/2}$Meis3.

Immunoprecipitations and western blots

immunoprecipitations have been described (Vlachakis et al., 2000). Rabbit polyclonal anti-Pbx4 antiserum was raised to a peptide containing the 13 C-terminal residues of Pbx4 and used at 1:1000 for Western blots.

The work in this section has appeared in a separate publication;

Vlackakis, N., Choe, S.-K. and Sagerstrom, C. G. (2001). Meis3 synergize with Pbx4 and Hoxb1b in promoting hindbrain fates in the zebrafish. *Development* **128,** 1299-1312.

APPENDIX II

HOXB1B AND HOXB1A SIMILARLY REQUIRE MEIS ACTIVITY IN VIVO

We have previously reported that Hoxb1b synergizes with Meis3 and Pbx4 to promote hindbrain fate in vivo and that Hoxb1b require Meis function to induce ectopic *hoxb1a* expression in r2 and more anterior regions (Vlachakis et al., 2001). Further, we have shown that expression of several *hox* genes in the hindbrain is affected by expressing Meis dominant-negative molecule (ΔCPbx4) (Choe et al., 2002). These results suggest that Hox proteins expressed in the hindbrain require Meis function to mediate their activities in vivo. However, a recent report has demonstrated a difference in activities between Hoxb1b and Hoxb1a, since expression of *hoxb1a* generates a more severe phenotype than expression of *hoxb1b* (McClintock et al., 2001). This observation suggests that there may be a differential requirement for their cofactors between Hox PG1 proteins. To test if activities of Hox PG1 proteins, Hoxb1b and Hoxb1a, similarly depend on Meis function, we utilized an ectopic expression assay (described in Vlachakis et al., 2001) and Meis dominant-negative approach (described in Chapter 2).

PG1 Hox proteins induce a truncation phenotype when co-expressed with Pbx4 and Meis3

Previously, we have demonstrated that expression of Hoxb1b together with Pbx4 and Meis3 results in ectopic expression of caudal hindbrain genes anteriorly (Vlachakis et al., 2001). To examine if this activity is shared between PG1 Hox proteins, we generated myc-tagged Hoxb1a and co-expressed along with Pbx4 and Meis3. We found that both Hoxb1b and Hoxb1a proteins are expressed at similar level 12 hours post expression (Fig. 1A). Further, we found that similar expression of either Hoxb1b or Hoxb1a together with Pbx4 and Meis3 gives rise to an anterior truncation as well as ectopic expression of k*rox20* anteriorly (Fig. 1C, D). This suggests that PG1 Hox proteins share their ability to promote hindbrain fates when ectopically co-expressed with *pbx4* and *meis3*.

Both PG1 Hox proteins show similar Meis dependence, but Hoxb1a can induce a more severe phenotype

To further test if PG1 Hox proteins require Meis function in vivo, we co-expressed each Hox protein along with ΔCPbx4 and examined if each Hox-alone phenotype is sustained in the presence of ΔCPbx4. When Hoxb1b alone is expressed, it results in ectopic *hoxb1a* expression in r2 (49 embryos/170 total; Fig. 2; Vlachakis et al., 2001; McClintock et al., 2001) and in midbrain (96 embryos/170 total), whereas Hoxb1a alone induces a more severe phenotype with an anterior truncation (37 embryos/155 total) and a phenotype similar to that upon Hoxb1b alone (128

embryos/155 total) as well (Fig. 2). This suggests that Hoxb1a can induce ectopic *hoxb1a* (endogenous) more anteriorly up to the forebrain, and that the resulting phenotype is a severe version of that upon Hoxb1b expression. However, the frequency of induction of these phenotypes by Hox PG1 proteins is significantly reduced in the presence of ΔCPbx4 (12 embryos/64 total show a partial r2 ectopic *hoxb1a* expression in Hoxb1b+ΔCPbx4 and 15 embryos/65 total show an r2 ectopic *hoxb1a* expression in Hoxb1a+ΔCPbx4; Fig. 2), suggesting that both Hoxb1b and Hoxb1a similarly require Meis function to induce the phenotype we observed.

The N-terminus of Hoxb1a possesses higher activity than that of Hoxb1b

We observed that Hoxb1a alone generates an anterior truncation phenotype seen upon expressing Hoxb1b together with Pbx4 and Meis3 and a more severe phenotype than that seen upon expressing Hoxb1b alone. This suggests that there may be a difference between Hoxb1b and Hoxb1a induction of their downstream targets. To locate the domain of Hoxb1a that distinguishes it from Hoxb1b, we generated chimeric proteins between Hoxb1a and Hoxb1b (Fig. 3A). For Hoxb1bb1a chimeric protein, the N-terminus of Hoxb1b (from amino acid #1 to #177; contains the trans-activation domain) is fused to the C-terminus of Hoxb1a (from amino acid # 193 to #316; contains Pbx-interaction domain and homeodomain). For Hoxb1ab1b, the N-terminus of Hoxb1a (from amino acid # 1 to # 192) is fused to the C-terminus of Hoxb1b (from amino acid # 178 to # 307). When expressed in the zebrafish embryos, the proteins are expressed at similar level (see Fig. 1A). In contrast to their expression level, the phenotypes

generated by expressing each chimera alone are very different. In particular, expressing Hoxb1bb1a results in a phenotype similar to that of Hoxb1b expression (28 embryos/50 total show an r2 ectopic and 18 embryos display *hoxb1a* expression in the midbrain) while expressing Hoxb1ab1b generates severe truncation with higher frequency than expressing Hoxb1a (38 embryos/46 total show an anterior truncation phenotype and 8 embryos/46 total display an r2 ectopic *hoxb1a* expression; Fig. 3B). This suggests that there is a difference between the N-termini of Hox PG1 proteins in mediating their activities in vivo. Since N-termini of Hox PG1 proteins contain the trans-activation domain, transcriptional activation by Hoxb1a may be more efficient than that by Hoxb1b.

Difference between Hox PG1 proteins may not be located in their binding ability to Pbx4, CBP or HDAC3

Our observation that Hoxb1a can induce a more severe phenotype than Hoxb1b prompted us to test if they have different abilities to bind their cofactors. We performed GST pull-down assays and found that Hoxb1b and Hoxb1a possess similar binding affinities to their binding partners, such as Pbx4 and CBP. Further, we also find that Hox PG1 proteins also interact with one of co-repressor family, HDAC3, which has not been reported yet. However, we are unable to delineate different binding abilities between Hoxb1b and Hoxb1a (Figure 4). These results suggest that there might be no significant differences between Hoxb1b and Hoxb1a in their interactions with known cofactors. However, we can not rule out the possibility that the difference between

Hoxb1b and Hoxb1a stems from a differential ability to interact with their binding partners, since we have previously shown that interaction undetectable by a biochemical assay may happen and be important in living cells (see Appendix I and Vlachakis et al., 2001). Furthermore, we have not tested their binding abilities in complexes.

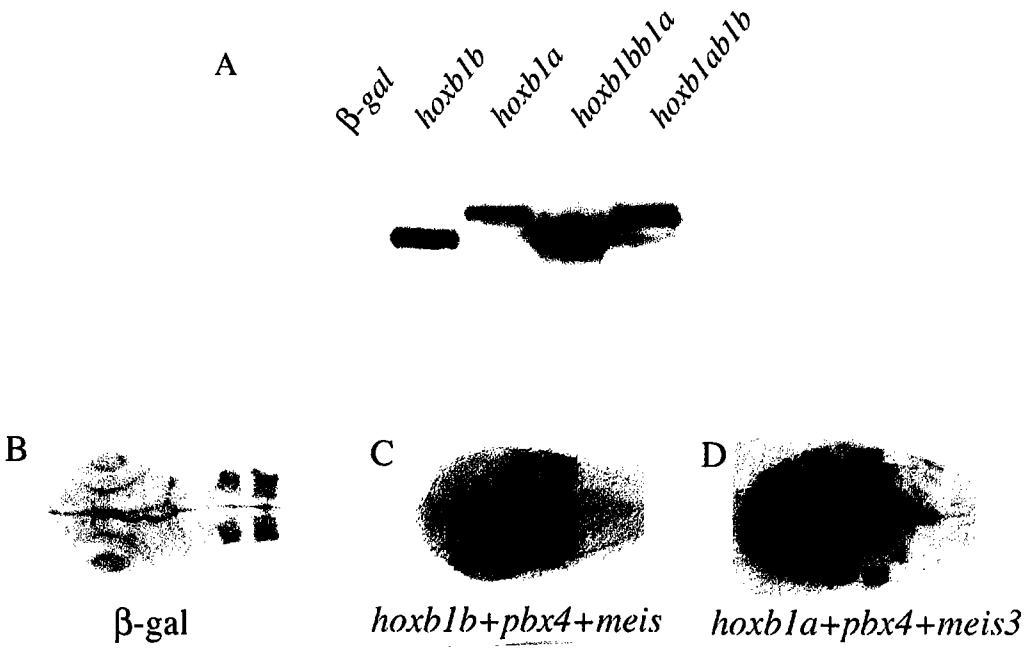

Figure 1. Hox PG1 proteins synergize with Pbx4 and Meis3 to promote hindbrain fates. (A) All constructs used are expressed at comparable levels in embryos. One cell stage embryos were injected with 150 pg of each mRNA encoding MYC-tagged constructs as indicated at the top of each lane. Embryos were raised to 12 hpf, lysed, resolved on a 10% SDS-PAGE gel, western blotted and probed with anti-MYC antibody. (B-D) Both Hoxb1b and Hoxb1a induce a similar phenotype with an anterior truncation when expressed with Pbx4 and Meis3. One- to two-cell stage embryos were injected with 500 pg of β-gal (B), 166 pg of each *hoxb1b+pbx4+meis3* mRNA (C) or 166 pg of each *hoxb1a+pbx4+meis3* mRNA (D), raised to 24 hpf and analyzed by in situ hybridization for *krox20* expression.

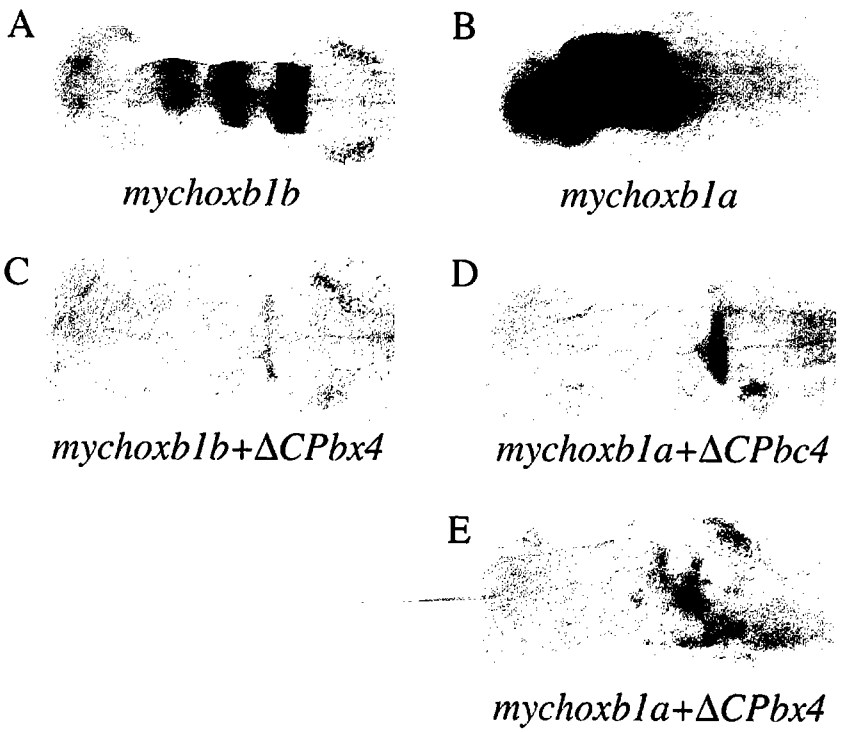

Figure 2. Hox PG1 proteins show similar Meis requirement. (A-E) One or two-cell stage embryos were injected with *mRNA* indicated at the bottom of each panel, raised to 24 hpf and analyzed by in situ hybridization for endogenous *hoxb1a* expression.

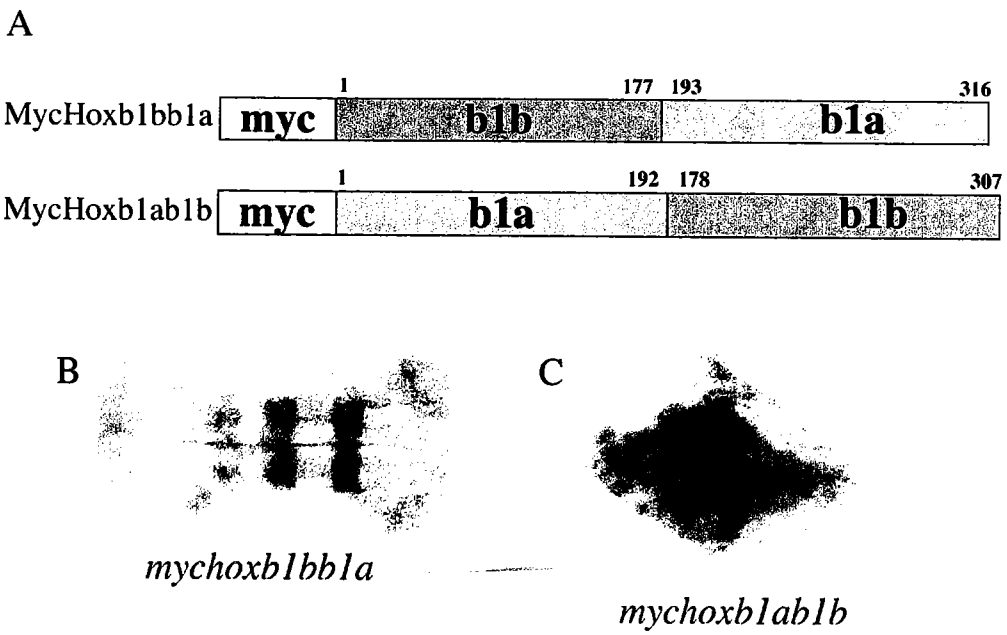

Figure 3. The N-terminus of Hoxb1a may have higher activity than that of Hoxb1b. (A) Constructs of MycHoxb1bb1a and MycHoxb1ab1b with amino acid positions indicated at the top of each diagram. myc indicates MYC-tag at the N-terminus. (B, C) One- to two-cell stage embryos were injected with *mychoxb1bb1a* (B) or *mychoxb1ab1a* (C), raised to 24 hpf and analyzed by in situ hybridization for endogenous *hoxb1a* expression.

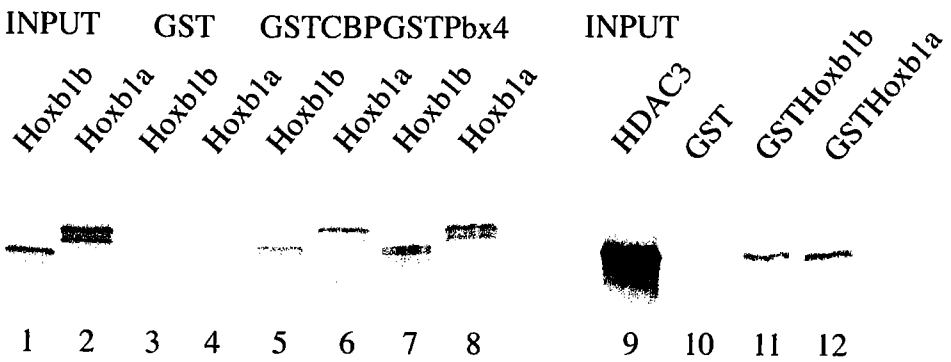

Figure 4. Hox PG1 proteins may not have different binding affinities to their cofactors. ^{35}S-labelled Hoxb1b (3, 5, 7) or Hoxb1a (4, 6, 8) proteins were incubated in the presence of either GST (3, 4), GSTCBP (5. 6) or GSTPbx4 (7, 8) to allow their interactions. INPUT lanes 1 and 2 show the position of in vitro translated, ^{35}S-labelled Hoxb1b or Hoxb1a (lane 1-8). ^{35}S-labelled HDAC3 proteins were incubated in the presence of either GST (10), GSTHoxb1b (11) or GSTHoxb1a (12).

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