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Paralog group 1 *hox* genes regulate rhombomere 5/6 expression of *vhnf1*, a repressor of rostral hindbrain fates, in a *meis*-dependent manner

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Abstract

The vertebrate hindbrain is segmented into an array of rhombomeres (r), but it remains to be fully understood how segmentation is achieved. Here we report that reducing *meis* function transforms the caudal hindbrain to an r4-like fate, and we exploit this experimental state to explore how r4 versus r5–r6 segments are set aside. We demonstrate that r4 transformation of the caudal hindbrain is mediated by paralog group 1 (PG1) *hox* genes and can be repressed by *vhnf1*, a gene expressed in r5–r6. We further find that *vhnf1* expression is regulated by PG1 *hox* genes in a *meis*-dependent manner. This implies that PG1 *hox* genes not only induce r4 fates throughout the caudal hindbrain, but also induce expression of *vhnf1*, which then represses r4 fates in the future r5–r6. Our results further indicate that r4 transformation of the caudal hindbrain occurs at intermediate levels of *meis* function, while extensive removal of *meis* function produces a hindbrain completely devoid of segments, suggesting that different *hox*-dependent processes may have distinct *meis* requirements. Notably, reductions in the function of another Hox cofactor, *pbx*, have not been reported to transform the caudal hindbrain, suggesting that Meis and Pbx proteins may also function differently in their roles as Hox cofactors.

Keywords: Hindbrain; Rhombomere; Segmentation; *Hox*; *Meis*; *Pbx*; *Vhnf1*; Homeodomain

Introduction

The embryonic hindbrain is transiently divided into a series of segments, termed rhombomeres, during early development. Rhombomeres share a basic underlying developmental program, but individual rhombomeres display unique variations on this program. Accordingly, reticulospinal interneurons form in several rhombomeres, but display rhombomere-specific features such that, for instance, Mauthner neurons in rhombomere 4 (r4) have different morphology and axonal projections than Ro3 neurons in r3. Similarly, branchiomotor (BM) neurons also differentiate in several rhombomeres, but display features specific to individual rhombomeres.

Formation of r4 versus r5–r6 provides a paradigm for how the hindbrain primordium becomes subdivided into rhombomeres with unique fates. Paralog group 1 (PG1) *hox* genes (*Hoxa1* and *Hoxb1* in mouse as well as *hoxb1a* and *hoxb1b* in zebrafish) control formation of r4 (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Gavalas et al., 1998; Lufkin et al., 1991; Mark et al., 1993; McClintock et al., 2002; Rossel and Capecchi, 1999; Studer et al., 1998), at least in part by regulating expression of downstream genes such as *hoxb2* (Maconochie et al., 1997), and several other genes (*krox20*, *kreisler*/*valentino*, *vhnf1*, and PG3 *hox* genes) are required for development of r5–r6 (Frohman et al., 1993; Gaufo et al., 2003; McKay et al., 1994; Moens et al., 1996; Schneider-Maunoury et al., 1993; Sun and Hopkins, 2001; Swiatek and Gridley, 1993). To generate adjacent rhombomeres with distinct fates, the actions of each of these genes must be restricted to the appropriate domain within the hindbrain primordium. Although this appears to involve cross regulation between genes expressed in presumptive r4 and r5–r6, the nature of these interactions remains unclear. In particular, mutations in *vhnf1* (Sun and

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Hopkins, 2001; Wiellette and Sive, 2003) or PG3 *hox* genes (Gaufo et al., 2003) cause a caudalward expansion of *hoxb1* expression, suggesting that r5–r6 genes negatively regulate r4 fates. On the other hand, disruption of PG1 *hox* function disrupts not only r4, but also r5 (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Gavalas et al., 1998; Lufkin et al., 1991; Mark et al., 1993; McClintock et al., 2002; Rossel and Capecchi, 1999; Studer et al., 1998), indicating a requirement for PG1 *hox* genes in r5–r6 development. The fact that PG1 *hox* genes may be required for r5–r6 development in spite of their expression being repressed by r5–r6-specific genes suggests that we do not fully understand the cross regulation of genes acting in presumptive r4 and r5–r6. Further, while recent findings suggest that PG1 *hox* genes acting in r4 may indirectly regulate development of r5–r6 (Maves et al., 2002; Walshe et al., 2002), the fact that PG1 *hox* genes are transiently expressed in r5–r6 (Alexandre et al., 1996; Frohman et al., 1990; Murphy and Hill, 1991; Prince et al., 1998; Sundin and Eichele, 1990) is consistent with these genes playing a direct role also within r5–r6.

Although PG1 Hox proteins appear required for the formation of r4 as well as r5–r6, these (and other) Hox proteins do not function as monomers. Instead, they require cofactors of the Pbx and Meis/Prep homeodomain families (reviewed in Mann and Affolter, 1998). Accordingly, disruption of *pbx* function in zebrafish completely blocks hindbrain segmentation (Waskiewicz et al., 2002), consistent with all *hox* function being lost in the absence of *pbx* function. In contrast, disruption of *meis*/*prep* function does not completely block hindbrain segmentation, but gene expression and neuronal differentiation proceed in several rhombomeres (Choe et al., 2002; Deflorian et al., 2004; Dibner et al., 2001; Waskiewicz et al., 2001). We have previously used a dominant-negative construct (ΔCPbx4) to interfere with *meis* function (Choe et al., 2002), and we have now explored its effect on hindbrain development in more detail. We find that reducing *meis* function leads the caudal hindbrain to take on an r4-like fate characterized by ectopic Mauthner neurons and ectopic *hoxb1a* expression. This effect is similar to what is observed upon disrupting r5–r6 gene function, and we have exploited this experimental condition to further examine cross regulation between r4 and r5–r6 genes. We demonstrate that this caudal r4-like fate is induced by PG1, but not PG2 or PG3, *hox* genes and that it is repressed by *vhnf1*. Strikingly, *vhnf1* expression in r5–r6 is regulated by PG1 *hox* genes in a *meis*-dependent manner. This clarifies the relationship between r4 and r5–r6 genes by indicating that PG1 *hox* genes induce r4 fates throughout the caudal hindbrain, but that they also induce *vhnf1*, which subsequently represses r4 fates in the future r5–r6 domain. We next reasoned that the ΔCPbx4 construct may not completely remove *meis* function and that transformation of the caudal hindbrain may occur at an intermediate level of *meis* function. Accordingly, co-injection of ΔCPbx4 with a second dominant-negative construct produces a hindbrain completely devoid of segments. The resulting unsegmented structure does not correspond to any one mature rhombomere, but is similar to a hindbrain 'ground state' observed upon disrupting *pbx* function (Waskiewicz et al., 2002). We conclude that extensive loss of *meis* or *pbx* function completely blocks hindbrain segmentation, consistent with these genes acting together as Hox cofactors. Notably, while incomplete disruption of *meis* function transforms the caudal hindbrain to an r4 fate, incomplete disruption of *pbx* function does not appear to have such an effect (Pöpperl et al., 2000; Waskiewicz et al., 2002), suggesting that Meis and Pbx may play distinct roles as Hox cofactors.

Materials and methods

DNA constructs

Constructs for the in vitro synthesis of mRNA were generated by cloning genes into 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 *Eco*RI 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 one- to two-cell stage. For morpholino injections, 2-mM stocks of anti-Hoxb1a and anti-Hoxb1b MOs were combined, diluted to 1–4 mg/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 (Perkin-Elmer Life Sciences, Inc.) to visualize the primary reticulospinal interneurons.

Results

Reducing meis function leads to a rostral transformation of the caudal hindbrain

We have previously utilized a dominant-negative construct that blocks nuclear translocation of Meis proteins (ΔCPbx4) to explore the role of Meis proteins in hindbrain development (Choe et al., 2002). We demonstrated that interfering with *meis* function disrupts segment-specific gene expression in r3, r4, and r5, but has little or no effect in more rostral (r1 and r2) or caudal (r6 and r7) rhombomeres. However, in the course of these experiments, we also noticed two incidences of what appeared to be ectopic gene expression. First, ΔCPbx4 not only disrupted *hoxb1a* expression in r4, but also induced ectopic *hoxb1a* expression. This expression was seen in r5 and further caudally, but was never seen rostral to r4 (Fig. 1B and (Choe et al., 2002)). Second, ΔCPbx4 not only disrupted high-level *ephA4* expression in r3 and r5, but also induced low-level *ephA4* expression rostral to r5 (Fig. 1D and (Choe et al., 2002)). These effects of the ΔCPbx4 construct appear restricted to *hoxb1a* and *ephA4* since we did not observe ectopic expression of other genes in the hindbrain (e.g., *krox20*, *val*, *hoxb3*, *hoxa2*; (Choe et al., 2002) and data not shown).

To explore whether this ectopic gene expression affects development of the hindbrain, we next examined differentiation of reticulospinal neurons that show rhombomere-specific morphologies and axonal trajectories (Metcalfe et al., 1986). Using the anti-neurofilament antibody RMO44 (Pleasure et al., 1989) to detect a broad complement of reticulospinal interneurons, we found that rostral reticulospinal neurons (r2 and r3) were only variably detected in ΔCPbx4-injected embryos (Fig. 1F), consistent with the abnormal gene expression in r2–r3 of these embryos. More strikingly, caudal reticulospinal neurons (r4–r7) that normally display unique morphologies appeared to have become homogeneous in ΔCPbx4-injected embryos. This was seen most clearly the case of r7, where reticulospinal neurons had large round cell bodies and 'T'-shaped axonal projections in control embryos (arrow in Fig. 1E). In ΔCPbx4-injected embryos, cells with T-interneuron morphology were often lacking in r7. Instead, neurons with elongated cell bodies and axons that project contralaterally were observed at the level of r7 (arrow in Fig. 1F). Indeed, the majority of reticulospinal neurons detected in the hindbrain of ΔCPbx4-injected embryos had 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, which specifically detects Mauthner neurons at early stages of development

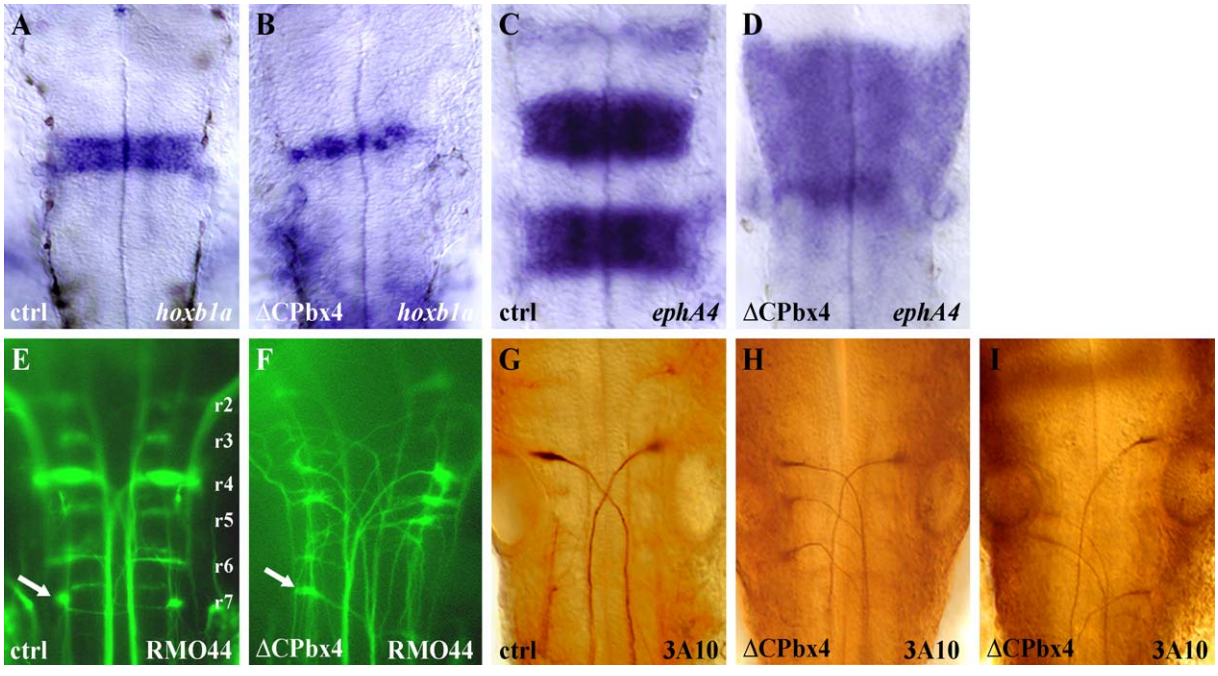

Fig. 1. Expression of ΔCPbx4 reveals an r4-like state in the caudal hindbrain. (A–D) ΔCPbx4 induces ectopic *hoxb1a* and *ephA4* expression. One- to two-cell stage embryos were injected with 150 pg of *ΔCPbx4* mRNA (B and D) or *lacZ* mRNA (A and C), raised to 24 hpf, and analyzed for expression of *hoxb1a* (A and B) or *ephA4* (C and D) by in situ hybridization. (E–I) ΔCPbx4 induces ectopic Mauthner neurons in the caudal hindbrain. Embryos were injected as in (A–D), raised to 28 hpf (G–I) or 48 hpf (E and F), and stained with RMO44 (E and F) or 3A10 (G–I) antibody. Arrows in E and F indicate reticulospinal neurons in r7 (note that the arrowed neuron in F is different from the one in E in its shape and axonal projection). All panels are dorsal views of the hindbrain with anterior to the top.

(Hatta, 1992). We found that while control-injected embryos displayed a single pair of 3A10-positive Mauthner neurons in r4 (Fig. 1G), ΔCPbx4-injected embryos contained multiple 3A10-positive neurons (Figs. 1H and I). These caudal Mauthner neurons were observed in r5, r6, and r7, as well as occasionally caudal to r7, but never rostral to r4. The frequency of this phenotype varied between experiments (likely depending on the level of residual Meis function; see below) such that 5–30% of ΔCPbx4-injected embryos showed caudal Mauthner neurons in a particular experiment (n = 20 experiments, >1000 embryos). We conclude that interfering with Meis function leads to a rostral transformation of the caudal hindbrain to an r4-like fate.

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

Specification of r4 fates is mediated by the paralog group 1 (PG1) *hox* genes *hoxb1a* and *hoxb1b* in zebrafish (McClintock et al., 2002). Since *hoxb1a* was expressed in the caudal hindbrain of ΔCPbx4-injected embryos (Fig. 1B), it was possible that PG1 *hox* genes were 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 *ΔCPbx4* mRNA and assayed formation of Mauthner neurons in the caudal hindbrain (Fig. 2). Expressing PG1 *hox* genes alone never induced ectopic caudal Mauthner neurons (not shown), but we found that co-expressing *hoxb1b* or *hoxb1a* with ΔCPbx4 increased the frequency of embryos with caudal Mauthner neurons by about 4- to 5-fold on average, with as many as 48% of embryos showing ectopic caudal Mauthner neurons in some experiments. In contrast, formation of ectopic Mauthner neurons was not significantly enhanced by co-expressing ΔCPbx4 with *hoxa2*, *hoxb2*, or *hoxa3* (Fig. 2), 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 r4 transformation of the caudal hindbrain. The use of MOs to simultaneously knockdown Hoxb1a and Hoxb1b (PG1MOs) induces bilateral loss of r4 Mauthner neurons in 40% of embryos (McClintock et al., 2002). We found that knockdown of Hoxb1a and Hoxb1b reduced the number of ΔCPbx4-injected embryos displaying caudal Mauthner neurons to a similar extent (45%; Fig. 2). We conclude that endogenous *hoxb1a* and *hoxb1b* are required for induction of an r4-like fate in the caudal hindbrain of ΔCPbx4-injected embryos.

vhnf1 represses rostral hindbrain fates

Based on our results, we hypothesized that the ΔCPbx4 construct interferes with a factor that normally represses r4 fates in the caudal hindbrain. *vhnf1*, one of the earliest

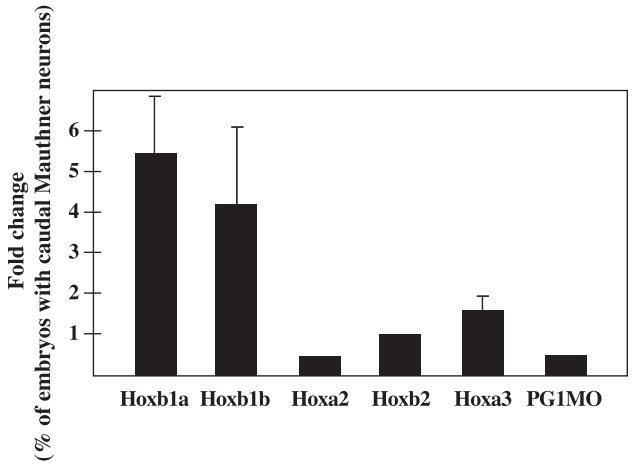

Fig. 2. PG1, but not PG2 or PG3, *hox* genes mediate transformation of the caudal hindbrain to an r4 fate. One to two-cell stage embryos were injected with *ΔCPbx4* mRNA together with the indicated *hox* mRNA, or together with PG1MOs, raised to 28 hpf, stained with 3A10 antibody, and scored for the presence of ectopic caudal Mauthner neurons. The data are presented as fold-change in the frequency of embryos showing caudal Mauthner neurons relative to control injections of ΔCPbx4 with *lacZ* mRNA or control MOs. The average change is shown for each experimental condition and standard deviations are indicated except for *hoxa2*, *hoxb2*, and PG1MO injections, which were only performed twice. The data for individual experiments were as follows (presented as percent experimental/percent control embryos showing caudal Mauthner neurons): *hoxb1a* (Experiment 1: 23.1/3.7 = 6.2-fold; Experiment 2: 35.1/3.2 = 11-fold; Experiment 3: 13.4/3.1 = 4.3-fold; Experiment 4: 13.5/10.2 = 1.3-fold; Experiment 5: 18.9/5.4 = 3.5-fold), *hoxb1b* (Experiment 1: 20.5/4.8 = 4.3-fold; Experiment 2: 47.8/23.7 = 2-fold; Experiment 3: 4.4/10.0 = 0.4-fold; Experiment 4: 41.2/3.6 = 11.4-fold; Experiment 5: 11.9/5.5 = 2.2-fold), *hoxa2* (Experiment 1: 6.2/10.0 = 0.6-fold; Experiment 2: 1.3/4.4 = 0.3-fold), *hoxb2* (Experiment 1: 7.6/10.0 = 0.8-fold; Experiment 2: 5.0/4.4 = 1.1-fold), *hoxa3* (Experiment 1: 10.1/5.6 = 1.8-fold; Experiment 2: 17.8/8.1 = 2.2-fold; Experiment 3: 31.3/33.8 = 0.9-fold), and PG1MO (Experiment 1: 13.2/26.1 = 0.5-fold; Experiment 2: 8.8/15.2 = 0.6-fold). Between 50 and 100 embryos were scored for each experiment.

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. Furthermore, misexpression of *vhnf1* represses gene expression in r4 (Sun and Hopkins, 2001; Wiellette and Sive, 2003). To directly test if *vhnf1* is capable of repressing Mauthner neuron differentiation, we misexpressed *vhnf1* in wild-type embryos. We found that embryos injected with *vhnf1* mRNA lacked one or both Mauthner neurons in r4 (Fig. 3B; 40% affected, n = 3 experiments, 160 embryos) and an additional 10–15% showed mispositioning or abnormal axonal projection of one Mauthner neuron (Fig. 3C shows a Mauthner axon projecting laterally before turning to the midline). However, it also appeared that reticulospinal neurons rostral to r4 were lost (Figs. 3B and C), although detection of neurons in this region was less robust than in r4. To further explore the possibility that *vhnf1* represses rostral fates in addition to r4, we next examined differentiation of branchiomotor (BM) neurons of the cranial nerves using an *islet1-GFP* transgenic line that

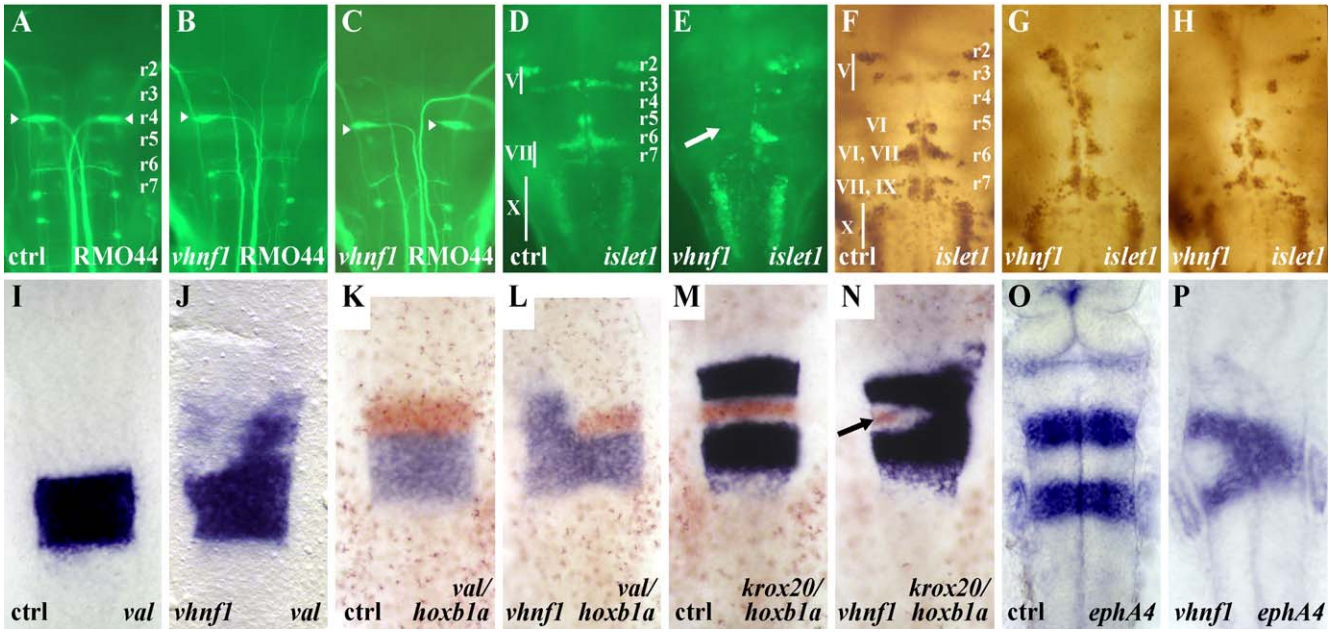

Fig. 3. Misexpression of *vhnf1* represses r4 and anterior fates. (A–H) *vhnf1* disrupts neuronal differentiation. One to two-cell stage embryos were injected with 25 pg of *vhnf1* (B, C, E, G, and H) or 25 pg of *lacZ* mRNA (A, D, and F), raised to 48 hpf, and processed by immunohistochemistry using 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. White arrow in E indicates loss of nVII neurons on the left side. (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 (I–N) or 24 hpf (O and 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 *hoxb1a*-expressing region. All panels are dorsal views with anterior to the top.

drives GFP expression in BM neurons (Higashijima et al., 2000). We found that nVII neurons, which are born in r4 and migrate caudally into r6–r7, were lost in 51% (43/85) of embryos injected with *vhnf1* (white arrow in Fig. 3E), consistent with *vhnf1* repressing r4 fates. However, we also observed an effect on r2–r3, where nV neurons were missing in 26% of *vhnf1*-injected embryos (22/85; left hand side of embryo in Fig. 3E), demonstrating that *vhnf1* also represses r2–r3 fates. Notably, when *islet1* expression was 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 observed in the *islet1*-GFP line), we found that Islet1 expression was never completely lost in r5–r6–r7 (Figs. 3G and H) although nV neurons in r2–r3 were again lost in 25% of embryos (15/63; left-hand side of embryo in Fig. 3H). 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. These results demonstrate that *vhnf1* misexpression represses differentiation of BM neurons in r2–r4 while BM neurons in r5–r7 (where *vhnf1* is normally expressed) are unaffected.

This effect on neuronal differentiation correlated well with the effect of *vhnf1* on gene expression. In particular, gene expression in r5–r6 was unaffected in *vhnf1*-injected embryos and instead r5–r6-specific expression of *valentino, hoxa3*, and *krox20* expanded rostrally in 90–95% of these embryos (Figs. 3I–N and data not shown). This expansion extended rostral to r4, at least into r2–r3 (Figs. 3J and L) and occasionally as far rostrally as the midbrain (approximately 14%; $n = 2$ experiments and 142 embryos, data not shown). Concomitantly, *hoxb1a* expression in r4 (Figs. 3K–N; 95% affected), *hoxa2* expression in r2–r3 (approximately 30% affected; not shown), and *ephA4* expression in r1 (Figs. 3O and P; approximately 17% affected) were reduced in *vhnf1*-injected embryos. Our results not only confirm that *vhnf1* misexpression represses gene expression in r4, but also demonstrate that *vhnf1* can repress gene expression and neuronal differentiation in rhombomeres rostral to r4. Taken together, these findings make *vhnf1* a likely candidate for a caudal gene whose function is blocked in ΔCPbx4-injected embryos.

The ΔCPbx4 construct interferes with vhnf1 expression, but not vhnf1 function

We next examined how *vhnf1* activity might be disrupted by the ΔCPbx4 construct. We reasoned that ΔCPbx4 might interfere either with *vhnf1* expression or *vhnf1* function. Notably, the ΔCPbx4 construct disrupts expression of several r5–r6-specific genes (*krox20, valentino*, and *hoxb3*; (Choe et al., 2002)), suggesting that ΔCPbx4 might also block expression of *vhnf1*. To test this possibility, we examined *vhnf1* expression in ΔCPbx4-injected embryos. We found that *vhnf1* expression is partially lost in 48% (41/86) of ΔCPbx4-expressing embryos (Fig. 4B), demonstra-

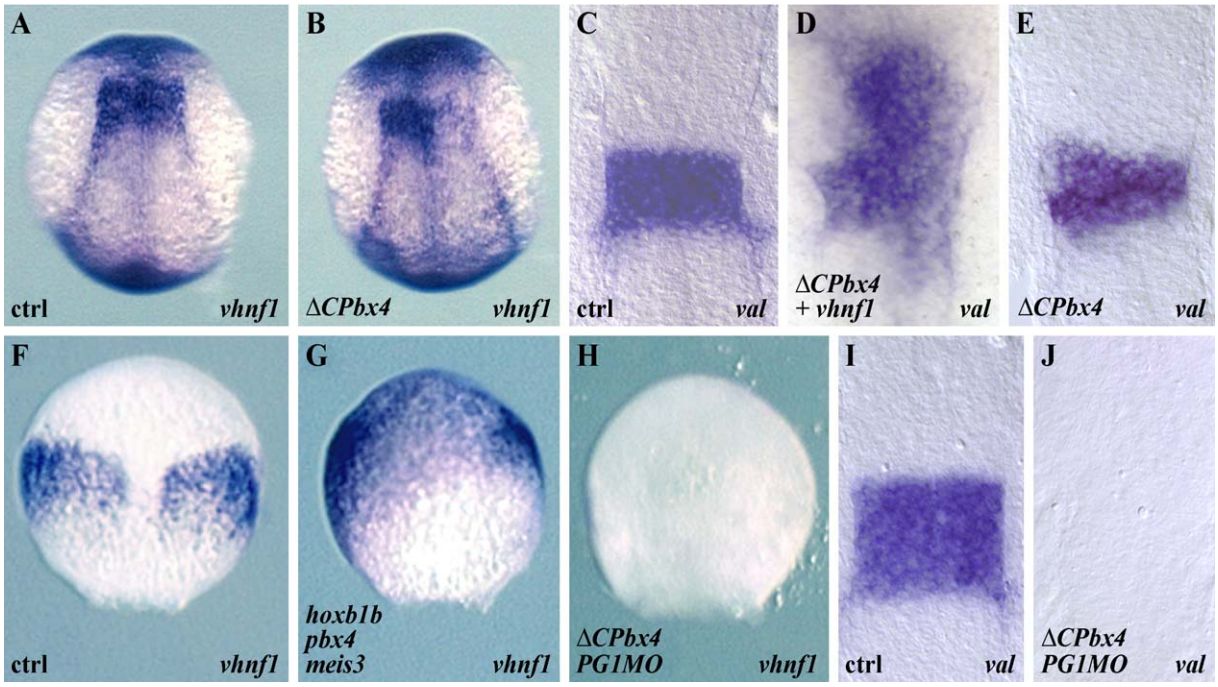

Fig. 4. PG1 *hox* genes regulate *vhnf1* expression in r5–r6. (A and B) *vhnf1* expression is dependent on *meis* function. One to two-cell stage embryos were injected with 300 pg of *lacZ* (A) or *ΔCPbx4* (B) mRNA, raised to 11 hpf, and analyzed by in situ hybridization for *vhnf1* expression. (C–E) *vhnf1* function is largely independent of *meis* function. One to two-cell stage embryos were injected with 300 pg of *lacZ* mRNA (C), 300 pg of *ΔCPbx4* mRNA (E), or 300 pg of *ΔCPbx4* + 25 pg of *vhnf1* mRNA (D), raised to 14 hpf, and analyzed by in situ hybridization for *valentino* expression. (F–J) PG1 *hox* genes are necessary and sufficient to induce r5–r6 gene expression. One- to two-cell stage embryos were injected with 300 pg of *lacZ* mRNA (F and I), 166 pg each of *hoxb1b*, *pbx4*, and *meis3* mRNA (G), or 300 pg of *ΔCPbx4* + *PG1MO* (H and J), raised to 10 hpf (F–H) or 14 hpf (I and J), and analyzed by in situ hybridization for *vhnf1* (F–H) or *val* (I and J) expression. All panels are dorsal views with anterior to the top.

ting 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 *ΔCPbx4* mRNA reduced r5 gene expression (*valentino*, *hoxb3*, and *krox20*; Fig. 4E shows effect on *valentino* expression, see also Choe et al., 2002) in 68% of embryos (112/164), we did not observe any repression of r5 gene expression in embryos injected with both *vhnf1* mRNA and *ΔCPbx4* mRNA (Fig. 4D). Instead, we found that 61% of embryos (220/358) injected with both *vhnf1* and *ΔCPbx4* mRNA showed a rostral expansion of r5 gene expression (Fig. 4D), similar to the phenotype observed when only *vhnf1* mRNA is injected (see Fig. 3). 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*, but not with *vhnf1* function.

Lastly, if ΔCPbx4 induces caudal Mauthner neurons by blocking *vhnf1* expression, re-establishing *vhnf1* expression in ΔCPbx4-expressing embryos should prevent the formation of such caudal Mauthner neurons. We found this to be the case as *vhnf1* repressed the formation of caudal Mauthner neurons by approximately 6-fold in ΔCPbx4-expressing embryos. Specifically, only 3.7% of embryos co-injected with *ΔCPbx4* and *vhnf1* mRNA (7/187 embryos, $n = 4$ experiments) displayed ectopic caudal Mauthner neurons while 23.5% of control embryos (injected with *ΔCPbx4* and *βgal* mRNA; 38/162 embryos, $n = 3$ experiments) displayed such ectopic neurons. In summary, these results suggest that *vhnf1* normally represses r4 fates in the caudal hindbrain and that *vhnf1* expression is *meis*-dependent while *vhnf1* function is largely *meis*-independent.

PG1 hox genes are necessary and sufficient to induce vhnf1 expression in r5–r6

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 found that this leads to ectopic *vhnf1* expression in the rostral embryo in 55% of embryos (54/98; Fig. 4G). In contrast, co-injecting *hoxb1b* and *pbx4* mRNA without *meis3* mRNA did 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 *vhnf1* expression in r5–r6. Previous work using MOs to disrupt PG1 function reported a very mild hindbrain phenotype (McClintock et al., 2002). In particular, r4 was reduced in size and Mauthner neurons were lost in r4, but r5 and r6 gene expression was not lost (McClintock et al., 2002). This is likely due to the anti-PG1 MOs not completely removing PG1 *hox* function (Waskiewicz et al., 2002). We therefore co-injected anti-PG1 MOs with the ΔCPbx4 construct to simultaneously interfere with PG1 *hox* and *meis* function. Although ΔCPbx4 never completely eliminated gene expression in r5 and r6 (*krox20*, *valentino*, *hoxb3*; Fig. 4E and (Choe et al., 2002)), co-injection of PG1 MOs and ΔCPbx4 completely eliminated *valentino* expression in 29% of embryos (Fig. 4J). Similarly, while ΔCPbx4 partially blocked *vhnf1* expression in 48% of injected embryos, only in 3% did this effect encompass more than half of the expression domain. In contrast, 17% of embryos co-injected with anti-PG1 MOs and ΔCPbx4 showed loss of *vhnf1* expression in more than half of the expression domain and many of these embryos lacked *vhnf1* expression altogether (Fig. 4H). We conclude that PG1 Hox proteins and their cofactors are necessary and sufficient to induce expression of *vhnf1* in r5–r6.

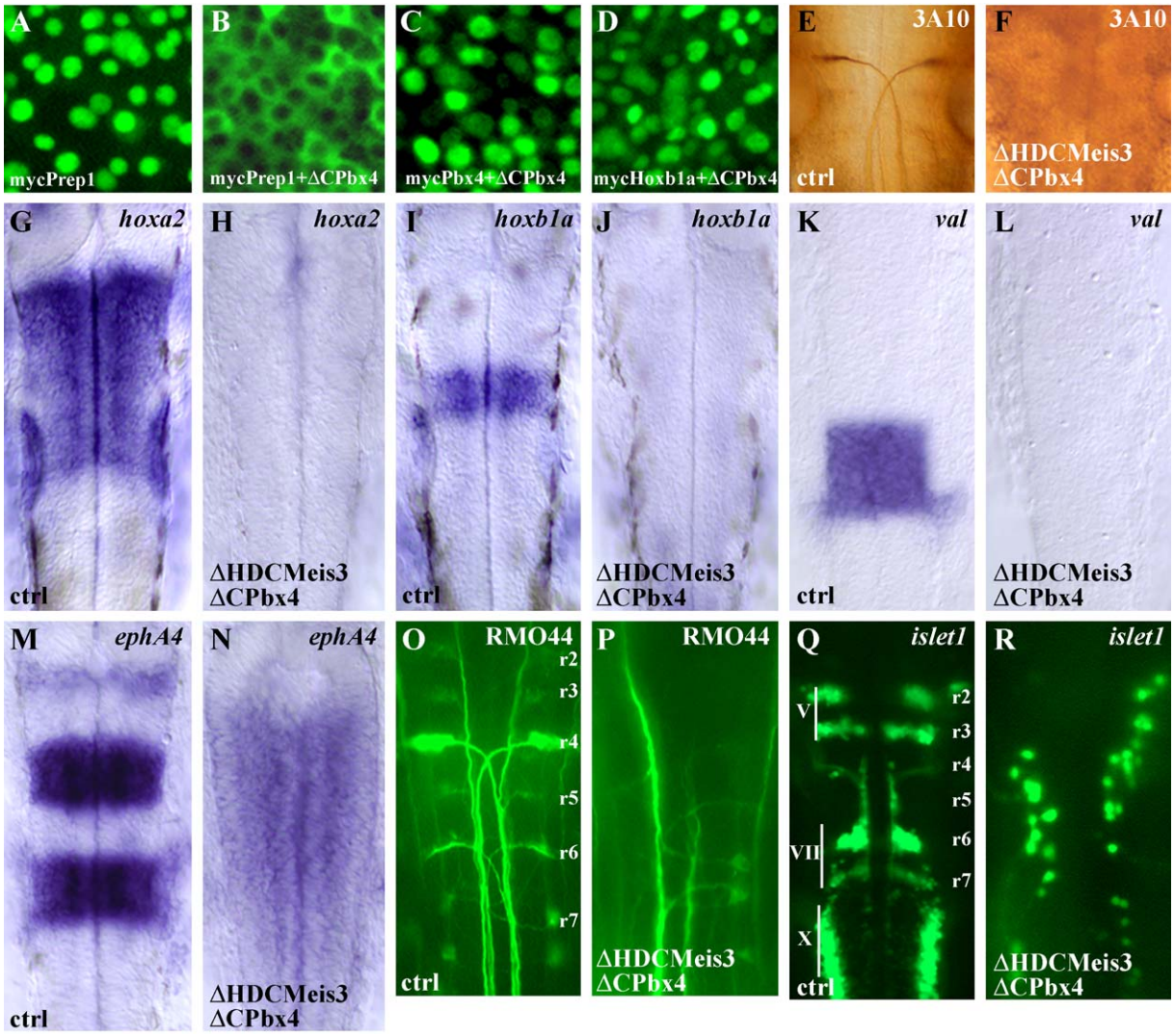

Fig. 5. Co-expression of ΔCPbx4 and ΔHDCMeis3 completely disrupts hindbrain gene expression and neuronal differentiation. (A–D) ΔCPbx4 interferes with the nuclear localization of Prep1, but not Pbx4 or Hoxb1a. Embryos were injected with 300 pg of the mRNAs indicated at the bottom of each panel, raised to 12 hpf, and stained with anti-Myc antibody. (E and F) Co-expression of ΔCPbx4 and ΔHDCMeis3 does not induce caudal Mauthner neurons. One- to two-cell stage embryos were injected with 250 pg each of *ΔCPbx4* and *ΔHDCMeis3* mRNA (F) or 500 pg of *lacZ* mRNA (E), raised to 28 hpf, and stained with 3A10 antibody. (G–N) Co-expression of ΔCPbx4 and ΔHDCMeis3 completely abrogates gene expression in the hindbrain. Embryos were injected with the indicated mRNA as in E and F, raised to 14 hpf (K and L) or 24 hpf (G–J, M, and N), and analyzed by in situ hybridization for genes indicated at the top right of each panel. (O–R) Co-expression of ΔCPbx4 and ΔHDCMeis3 severely disrupts neuronal differentiation. The indicated mRNA was injected as in E and F, embryos were raised to 48 hpf and stained with RMO44 antibody (O and P). In Q and R, an *islet1-GFP* transgenic line was instead used to visualize branchiomotor neurons. E–R are dorsal views of the hindbrain with anterior to the top.

Co-expression of two dominant negative meis constructs abolishes hindbrain segmentation

Although both Meis and Pbx proteins function as Hox cofactors, using the ΔCPbx4 construct to interfere with *meis* function gives a less severe phenotype than removal of all *pbx* function, which completely blocks segmentation of the hindbrain (Waskiewicz et al., 2002). This finding suggests that the ΔCPbx4 construct might be unable to eliminate all *meis* function in vivo. Notably, this scenario would also explain the variable transformation of the caudal hindbrain (Fig. 1), which might take place only in a narrow range of residual *meis* activity.

This model suggests that more extensive removal of *meis* function might give a more severe phenotype. In particular, transformation of the caudal hindbrain may not take place and the phenotype may be more similar to the effect of completely eliminating *pbx* function. To test this possibility, we attempted to reduce *meis* function further. We first considered that ΔCPbx4 might not affect all Meis family proteins. In particular, we have demonstrated that the ΔCPbx4 construct blocks nuclear translocation of Meis proteins (Choe et al., 2002), but it has been suggested that translocation of Prep1, a more divergent member of the Meis family, might not be blocked by ΔCPbx4 (Deflorian et al., 2004). However, we find that Prep1 is also sequestered in the cytoplasm by ΔCPbx4 (Figs. 5A and B), suggesting that ΔCPbx4 affects all Meis family members similarly.

We next considered that ΔCPbx4 might be quantitatively unable to block all endogenous *meis* activity. 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 the C-terminus (and therefore lacks the homeodomain required for DNA binding) reportedly interferes with endogenous *meis* function (Waskiewicz et al., 2001), and we generated an analogous form of Meis3 (ΔHDCMeis3). Although we did not observe any reproducible developmental defects when ΔHDCMeis3 was expressed by itself (not shown; (Choe et al., 2002)), co-expression of ΔHDCMeis3 with ΔCPbx4 blocked segmentation of the hindbrain without inducing ectopic caudal Mauthner neurons (Fig. 5F). In particular, while embryos injected with ΔCPbx4 never showed loss of *hoxa2* expression in r2 (99% have normal r2 expression; Choe et al., 2002), embryos co-injected with ΔCPbx4 and ΔHDCMeis3 exhibited a partial (79%) or complete (8%) loss of *hoxa2* expression in r2 (Fig. 5H). Furthermore, only approximately 10% of ΔCPbx4-injected embryos showed 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 increased to approximately 40%, and the phenotype was more severe as high-level *ephA4* expression was completely lost in r3 and r5, and the low-level *ephA4* expression was detected throughout the hindbrain rather than just rostrally (Fig. 5N). Lastly, *hoxb1a* expression in r4 (Fig. 5J) and *valentino* expression in r5–r6 (Fig. 5L) were affected more strongly in embryos co-injected with ΔCPbx4 and ΔHDCMeis3, although the difference was less marked since these genes are partially affected by ΔCPbx4 alone (Choe et al., 2002). A similar effect was also seen at the level of neuronal differentiation, as reticulospinal neurons were largely absent from embryos co-injected with ΔCPbx4 and ΔHDCMeis3 (Fig. 5P) and the number of *islet1*-positive cells in the hindbrain of such embryos was drastically reduced (Fig. 5R). Notably, the remaining *islet1*-positive cells were not segmentally organized, but were arranged in a continuous array that extended into the caudal region normally occupied by nX neurons. Since this phenotype is more severe than that generated by ΔCPbx4 alone, we conclude that ΔCPbx4 only partially blocks *meis* function and that this transforms the caudal hindbrain to an r4 fate, while more extensive removal of *meis* function completely blocks segmentation of the hindbrain. This result also demonstrates that *meis* function is required for hindbrain segmentation and, since this phenotype is very similar to that observed upon extensive disruption of *pbx* function (Waskiewicz et al., 2002), is consistent with Pbx and Meis acting together as Hox cofactors during hindbrain segmentation.

Discussion

Here we report that a construct that blocks *meis* function (ΔCPbx4) transforms the caudal hindbrain to an r4-like fate, and we exploit this experimental state to explore hindbrain segmentation and the role of Meis proteins in this process. We demonstrate that this transformation requires PG1 *hox* function, consistent with the known role for PG1 *hox* genes in promoting r4 fates. However, we also find that PG1 *hox* genes regulate expression of *vhnf1*, a repressor of rostral hindbrain fates, in r5–r6. We propose that PG1 *hox* genes not only specify a broad r4-like domain in the caudal hindbrain, but also induce expression of *vhnf1*, which then restricts this domain to the future r4. While ΔCPbx4 transforms the caudal hindbrain to an r4-like fate, combining ΔCPbx4 with a second dominant-negative construct eliminates segmentation of the hindbrain altogether, leaving in its stead an apparently uniform and undifferentiated structure. We propose that this represents a more severe phenotype due to more complete disruption of *meis* function. Notably, this phenotype is similar to that resulting from extensive removal of *pbx* function, consistent with Meis and Pbx proteins acting together as Hox cofactors during hindbrain segmentation.

A model for patterning of the caudal hindbrain

Based on our results, we present a model for patterning of the caudal hindbrain (Fig. 6A). We propose that a broad

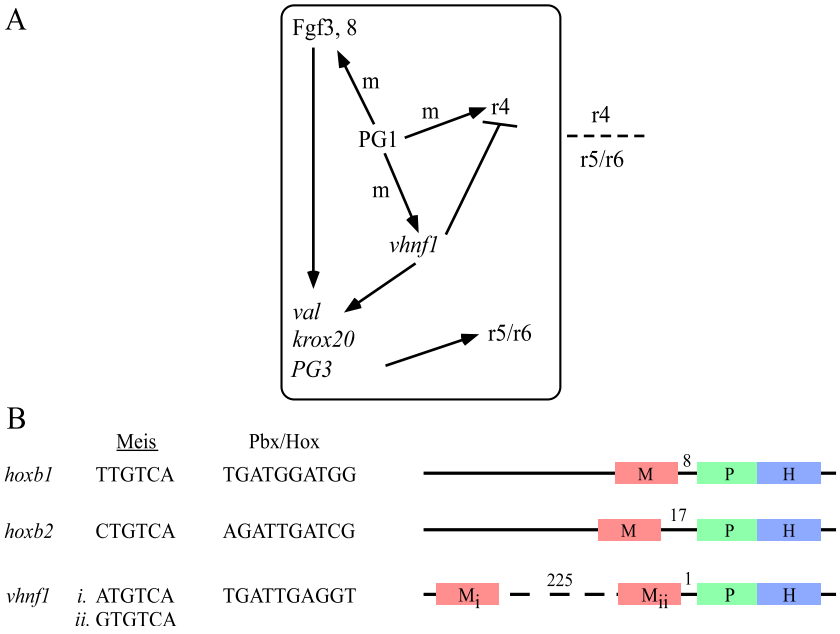

Fig. 6. (A) Proposed model for the role of PG1 *hox* genes in development of the caudal hindbrain. PG1 *hox* genes induce r4 fates throughout the caudal hindbrain and also induce *vhnf1* expression in the future r5–r6. *vhnf1* represses r4 fates and also promotes r5–r6 fates by cooperating with Fgf signals from r4 to induce *val*, *krox20*, and PG3 *hox* gene expression. m indicates steps that require Meis activity. See text for further details. (B) A putative PG1 Hox-regulated element is present in the *vhnf1* promoter. The left hand side shows a sequence comparison of Meis and Pbx/Hox binding sites found in the *vhnf1* promoter to those found in the *hoxb1* and *hoxb2* promoters. The right hand diagram shows the arrangement of Meis (M), Pbx (P), and Hox (H) elements in the three promoters. Numbers above the black line indicate spacing between the elements (number of nucleotides). Note that there are two Meis sites adjacent to the Pbx/Hox site in the *vhnf1* promoter.

domain of the hindbrain primordium is specified to an r4 fate by the onset of PG1 *hox* gene expression caudal to the presumptive r3–r4 boundary. Accordingly, PG1 *hox* genes are transiently expressed in the caudal hindbrain primordium of zebrafish, mouse, and 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 further postulate that PG1 *hox* genes induce expression of *vhnf1*, which represses r4 fates in the future r5–r6 (directly or via its downstream targets). *vhnf1* also cooperates with Fgf3 and Fgf8 secreted from r4 to promote r5–r6 fates (Wiellette and Sive, 2003) by inducing *krox20*, *valentino*, and PG3 *hox* gene expression. Among these, *valentino* is of particular importance since it has been shown to divide the r5–r6 proto-segment into definitive r5 and r6 (Moens et al., 1996).

Of the known zebrafish *meis* family members, *prep1* is expressed throughout the hindbrain (Choe et al., 2002; Deflorian et al., 2004; Waskiewicz et al., 2001) and, although their patterns vary somewhat during embryogenesis, *meis1*, *2*, and *3* are expressed in the caudal hindbrain with anterior limits in r2 (*meis1* and *2*) or in r4 (*meis3*) (Sagerström et al., 2001; Waskiewicz et al., 2001; Zerucha and Prince, 2001). Since the dominant-negative constructs used in this study appear active against each of these genes (Fig. 5; (Choe et al., 2002; Waskiewicz et al., 2001)), we cannot conclude whether one specific or a combination of several *meis* family members is required for PG1 *hox* function in the hindbrain.

Disruption of vhnf1 function may also explain other conditions that expand r4 fates

We propose that reducing *meis* function promotes transformation of the caudal hindbrain by blocking *vhnf1* expression. Several other experimental conditions have been shown to similarly transform the hindbrain to an r4-like fate, and we hypothesize that these may also be explained by disruption of *vhnf1* expression or function. In particular, ectopic expression of a dominant-negative retinoic acid receptor-β (RARβ) construct induces ectopic Mauthner neurons in the caudal hindbrain of *Xenopus* embryos (van der Wees et al., 1998), 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 retinoic acid (RA) synthesizing enzyme RALDH2 leads to expression of *krox20* and *hoxb1* in the caudal hindbrain (Niederreither et al., 2000). Since we find that *vhnf1* expression is RA-dependent (not shown), disrupting RA signaling by these different methods might also expand r4 fates by blocking *vhnf1* expression. Other cases of r4 expansion may be indirectly attributable to disruption of *vhnf1* function, since they interfere with genes downstream of *vhnf1*. For instance, mutations in PG3 *hox* genes result in ectopic *Hoxb1* expression and ectopic nVII BM neurons in r6 (Gaufo et al., 2003). However, interference with the *vhnf1* pathway unlikely explains all cases of r4 expansion. In

particular, *valentino* has the ability to repress r4 fates (Giudicelli et al., 2003), but it unlikely acts downstream of *vhnf1* in this capacity (Wiellette and Sive, 2003), suggesting that several r5–r6 genes may independently repress r4 fates.

PG1 hox genes may act within r5–r6 to regulate vhnf1 expression

Disruption of PG1 *hox* genes in mouse (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Gavalas et al., 1998; Lufkin et al., 1991; Mark et al., 1993; Rossel and Capecchi, 1999; Studer et al., 1998) and zebrafish (McClintock et al., 2002) affects both r4 and r5. PG1 *hox* genes control a regulatory cascade in r4 (*Hoxa1* regulates *Hoxb1* that regulates *Hoxa2*; (Maconochie et al., 1997; Pöpperl et al., 1995)), but the role of PG1 *hox* genes in r5 remains unclear. Recent work demonstrated that PG1 *hox* genes induce expression of *fgf3* and *fgf8* in r4 (Waskiewicz et al., 2002), and that Fgf produced in r4 is required for the formation of r5–r6 (Maves et al., 2002; Walshe et al., 2002), leading to the hypothesis that PG1 *hox* genes regulate r5–r6 development indirectly by regulating Fgf production in r4. However, several aspects of our results suggest that PG1 *hox* genes may regulate *vhnf1* expression directly within the future r5–r6. First, the phenotypes we observe are distinct from the reported effect of disrupting Fgf3 and Fgf8 function. In particular, loss of 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 we observe transformation of r5–r6 to an r4 fate. 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 (n = 147 embryos, not shown; also see (Wiellette and Sive, 2003)).

Consistent with PG1 *hox* genes activating *vhnf1* expression directly, we found that the *vhnf1* promoter contains at least one element with binding sites for Meis, Pbx, and Hox proteins (Fig. 6B) similar to the ones observed in the murine *hoxb1* and *hoxb2* promoters (Ferretti et al., 2000; Jacobs et al., 1999), both of which are regulated by PG1 *hox* genes, as well as in the zebrafish *hoxb1a* promoter (McClintock et al., 2002). We note that the Meis site is located closer to the Pbx/Hox site in the *vhnf1* element than in the *hoxb1* or *hoxb2* elements. However, experiments have revealed considerable flexibility in the distance and orientation of Meis sites relative to Pbx/Hox sites (Jacobs et al., 1999), suggesting that this is unlikely to affect function of the element. A second difference is that an additional Meis site is found at a further distance from the Pbx/Hox site in the *vhnf1* element. We also note that the *vhnf1* promoter contains several Hnf1 binding sites, suggesting that *vhnf1* may maintain its own expression via an autoregulatory loop. This finding potentially explains how *vhnf1* expression persists in r5–r6 once PG1 *hox* expression disappears. It is not clear how *vhnf1* expression becomes restricted to the future r5–r6 without expanding into the future r4, but it is likely that other factors prevent *vhnf1* expression in the future r4. For instance, we have recently characterized a family of zinc-finger proteins (*nlz1* and *nlz2*) that appears required in r4 to repress transcription of non-r4 genes (Runko and Sagerstrom, 2003, 2004). Furthermore, expression of *nlz* in the hindbrain requires RA and Fgf signaling, and both factors can induce *nlz* expression in the absence of protein synthesis (Roy and Sagerstrom, 2004), consistent with *nlz* being a direct target of RA and Fgf signaling during early hindbrain development.

Differential requirements for Hox cofactors during hindbrain development

There appear to be differences in the extent to which various steps of hindbrain development require *meis* or *pbx* function. First, extensive elimination of *meis* function (by combining two dominant-negative constructs) disrupts both induction of caudal r4 fates and *vhnf1* expression, demonstrating that both of these events are *meis*-dependent. However, partial reduction in *meis* function (using the ΔCPbx4 construct) disrupts *vhnf1* expression in r5–r6 without repressing r4 fates in this region, suggesting that *vhnf1* expression is more dependent on *meis* function than induction of r4 fates. Further characterization of the *vhnf1* promoter will reveal if such differences in *meis* dependence may be encoded in the PG1 response elements. For instance, the presence of two Meis binding sites near the Pbx/Hox site in the *vhnf1* element may indicate that *vhnf1* expression is more highly dependent on *meis* function.

Second, partial removal of *meis* function reveals an r4-like state in the caudal hindbrain, but partial removal of *pbx* function does not (Pöpperl et al., 2000; Waskiewicz et al., 2002). 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). The 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 *hox* function during hindbrain development, Meis proteins may play a more modulatory role.

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