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Pbx proteins cooperate with Engrailed to pattern the midbrain-hindbrain and diencephalic-mesencephalic boundaries

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Abstract

Pbx proteins are a family of TALE-class transcription factors that are well characterized as Hox co-factors acting to impart segmental identity to the hindbrain rhombomeres. However, no role for Pbx in establishing more anterior neural compartments has been demonstrated. Studies done in *Drosophila* show that Engrailed requires Exd (Pbx orthologue) for its biological activity. Here, we present evidence that zebrafish Pbx proteins cooperate with Engrailed to compartmentalize the midbrain by regulating the maintenance of the midbrain-hindbrain boundary (MHB) and the diencephalic-mesencephalic boundary (DMB). Embryos lacking Pbx function correctly initiate midbrain patterning, but fail to maintain *eng2a*, *pax2a*, *fgf8*, *gbx2,* and *wnt1* expression at the MHB. Formation of the DMB is also defective as shown by a caudal expansion of diencephalic *epha4a* and *pax6a* expression into midbrain territory. These phenotypes are similar to the phenotype of an Engrailed loss-of-function embryo, supporting the hypothesis that Pbx and Engrailed act together on a common genetic pathway. Consistent with this model, we demonstrate that zebrafish Engrailed and Pbx interact in vitro, and that this interaction is required for both the *eng2a* overexpression phenotype and Engrailed's role in patterning the MHB. Our data support a novel model of midbrain development in which Pbx and Engrailed proteins cooperatively pattern the mesencephalic region of the neural tube.

Keywords

zebrafish; Engrailed; Pbx; midbrain; midbrain-hindbrain boundary; diencephalic-mesencephalic boundary; hexapeptide; lineage restriction; neural patterning

Introduction

Over the course of vertebrate development, the neural plate is progressively subdivided into functionally specialized, lineage restricted compartments (Kiecker and Lumsden, 2005).

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Tissue compartmentalization is important to specify cell position, identity and function during vertebrate patterning. The seven rhombomeres of the hindbrain were the first observed lineage-restricted compartments in the vertebrate nervous system (Fraser et al., 1990; von Baer, 1828). Hindbrain segmentation has since been shown to occur downstream of Hox proteins and their DNA binding co-factors Pbx and Meis. Lineage-restriction has also been observed at the diencephalic-mesencephalic boundary (DMB) and the midbrain-hindbrain boundary (MHB), which enclose the midbrain at its rostral and caudal ends respectively. In this regard, the vertebrate neural tube is an excellent system in which to study the formation and maintenance of lineage-restricted boundaries.

The Pbx (pre-B-cell leukemia transcription factor) family of TALE class homeodomain transcription factors are best characterized as heterodimeric partners for Hox proteins (Mann and Chan, 1996; Moens and Selleri, 2006). Pbx proteins are hypothesized to reveal intrinsic DNA-binding specificity within the Hox proteins, as well as to coordinately bind an adjacent Pbx recognition site in the promoter of target genes (Chan et al., 1996; Knoepfler et al., 1996; Mann and Chan, 1996). As such, Pbx-Hox complexes often have a much higher DNA binding specificity and affinity than either Pbx or Hox alone. A zebrafish mutant in the *pbx4* gene (*lazarus* or *lzr*) was identified in a genetic screen for embryos that fail to properly express the rhombomere 3 (r3) and r5-specific transcription factor *egr2b (krox20)* (Popperl et al., 2000). Two partially redundant zebrafish *pbx* genes, *pbx2* and *pbx4*, are expressed during early embryogenesis at a time when the hindbrain is being patterned. These two Pbx proteins cooperate with Hox proteins to drive expression of early hindbrain patterning genes such as *fgf3*, *fgf8*, *hoxb1a*, and *vhnf1* (Hernandez et al., 2004; Maves et al., 2002; Popperl et al., 1995; Walshe et al., 2002; Waskiewicz et al., 2002). In the absence of Pbx2 and Pbx4 proteins, the region of hindbrain normally fated to give rise to r2-r6 is deprogrammed to adopt the default groundstate identity of r1, a segment that lacks expression of any *hox* gene (Waskiewicz et al., 2002). As such, the hindbrain region of Pbx-less embryos mimics the loss of all hindbrain *hox* gene function, demonstrating the importance of Pbx proteins in tissue compartmentalization during vertebrate hindbrain development. However, although Pbx genes are expressed ubiquitously throughout the developing zebrafish nervous system, no role for Pbx proteins in the formation or patterning of either forebrain or midbrain has been described.

Within the Hox proteins themselves, a motif called the hexapeptide is required for cooperative DNA binding with Pbx (Chang et al., 1995; Neuteboom et al., 1995). This evolutionarily conserved consensus motif, located just N-terminal of the Hox homeodomain, consists of the residues YQWPM. The hexapeptide motif, particularly the tryptophan residue, binds within a hydrophobic pocket formed by the extended loop between helix 1 and 2 in the Pbx homeodomain (LaRonde-LeBlanc and Wolberger, 2003; Piper et al., 1999). The mechanism of the homeodomain-hexapeptide interaction is conserved in fly Exd and Hox proteins as well (Passner et al., 1999), illustrating the importance of Pbx-Hox interactions during development.

Other hexapeptide-containing transcription factors have been found to bind Pbx proteins (In der Rieden et al., 2004). Amongst these Pbx-interacting proteins is the homeodomain transcription factor Engrailed (abbreviated Eng or En). In Engrailed proteins, a hexapeptide motif (WPAWVY) is located just upstream of the EH2 (Eng Homology-2) domain. The hexapeptide, along with the EH2 and EH3 domains, is required for the Pbx - Eng interaction (Peltenburg and Murre, 1996). Within the Engrailed hexapeptide itself, the two tryptophan residues are of particular importance in mediating cooperative binding between Pbx and Eng. Additionally, the three amino acid extension of the Pbx homeodomain is also required for the Pbx–Eng interaction (Peltenburg and Murre, 1997). All domains necessary for the Pbx-Eng interaction are conserved in flies and vertebrates, pointing to the importance of this interaction for metazoan development.

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Engrailed was originally identified in *Drosophila* as a factor required for the maintenance of cellular compartments during fly development (Hidalgo, 1996). In *Drosophila*, a genetic interaction between *engrailed* and the *pbx* orthologue *extradenticle* (*exd)* has been established based on the similarity in phenotypes between maternal, zygotic *exd* mutants and those of *en* mutant flies (Alexandre and Vincent, 2003; Kobayashi et al., 2003; Peifer and Wieschaus, 1990). Biochemical evidence suggests that the Pbx / Exd family of TALE-class homeodomain proteins can directly bind Engrailed in vitro and in vivo (Kobayashi et al., 2003; Peltenburg and Murre, 1996; Serrano and Maschat, 1998; van Dijk and Murre, 1994; van Dijk et al., 1995). Experimentally, Engrailed's role as a transcriptional regulator has been shown to require the presence of functional Exd and Homothorax (Hth; vertebrate Meis) proteins (Alexandre and Vincent, 2003; Kobayashi et al., 2003; Rieckhof et al., 1997). A trimeric complex of En, Exd, and Hth can cooperatively bind DNA and either activate or repress transcription of target genes (Alexandre and Vincent, 2003; Kobayashi et al., 2003). En expression is autoregulatory and is not maintained in maternal, zygotic *exd* mutants, suggesting that *en* requires *exd* to positively regulate its own expression (Peifer and Wieschaus, 1990). These studies have established a genetic and biochemical pathway involving Engrailed and TALE-class transcription factors. However, vertebrate developmental pathways involving a Pbx-Eng interaction have not been investigated.

In vertebrates, the best-described role for Engrailed is in patterning the mesencephalic region of the developing neural tube, especially the midbrain-hindbrain boundary (MHB). Formed at the interface between anterior (*otx2*-expressing) and posterior (*gbx2*-expressing) neural tissue, the isthmic organizer (IsO) at the MHB has been identified as an important source of signals required for specification of the mesencephalon and the rostral metencephalon, as well as formation and maintenance of the DMB and MHB (Alvarado-Mallart et al., 1990; Raible and Brand, 2004; Wurst and Bally-Cuif, 2001). Fgf8 is likely the main IsO signaling molecule as ectopic Fgf8 protein can mimic the organizer activity of the MHB (Crossley et al., 1996; Martinez et al., 1999) Although the interface of *otx2* and *gbx2* expression correlates with the position of the MHB, it is unclear how gene expression at the MHB organizer is initiated. In mice, expression of MHB markers can be initiated in the absence of *otx2* and *gbx2* function (Li and Joyner, 2001). This suggests that other factors are involved in MHB establishment, such as Wnt8 signals originating from the lateral mesendodermal cells (Rhinn et al., 2005), and transcriptional regulation by *pou5f1* (*spg)* and *sp5* (*bts1*) (Burgess et al., 2002; Tallafuss et al., 2001). Although MHB initiation is not well understood, it is clear that following establishment there is considerable transcriptional interdependence amongst the MHB patterning factors. Maintenance appears to involve a complicated cross-regulatory loop involving the secreted factors Wnt1 and Fgfs 8, 17, and 18, as well as transcriptional regulators including the Pax2/5/8 family, Irx1b, Irx7, Lmx1b.1, Lmx1b.2, and Engrailed proteins (Brand et al., 1996; Itoh et al., 2002; McMahon and Bradley, 1990; McMahon et al., 1992; O'Hara et al., 2005; Reifers et al., 1998). Functional perturbations in any of these genes can lead to a depletion of all other MHB markers and a loss of tectal and cerebellar structures.

Besides being a primary player in the cross-regulatory loop that maintains the isthmic organizer, Engrailed also performs more specialized functions in midbrain development. Specifically, Engrailed is required to position the caudal extent of the forebrain by maintaining the DMB and to polarize gene expression in the optic tectum (Araki and Nakamura, 1999; Liu and Joyner, 2001; Logan et al., 1996; Scholpp and Brand, 2001; Scholpp et al., 2003). Additionally, Engrailed can act as a cell-cell signaling molecule to guide retinal ganglion cell axons via a novel secretory mechanism (Brunet et al., 2005; Maizel et al., 1999). In mouse and zebrafish embryos lacking Engrailed function, expression of the forebrain markers *pax6a* and *epha4a* are expanded caudally, implying Eng proteins are required to maintain the integrity of the DMB. Conversely, ectopic overexpression of Engrailed can repress *pax6* expression in the forebrain and cause a rostral expansion of midbrain identity (Araki and Nakamura, 1999;

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Scholpp and Brand, 2001; Scholpp et al., 2003). Furthermore, Araki and Nakamura present evidence in chick that the repression of *pax6* by ectopic En-2 occurs prior to the induction of *pax2*, *pax5* and *fgf8*, suggesting that the foremost function of Engrailed is to maintain the DMB. Taken together, these studies highlight the importance of Engrailed protein function in the formation, patterning and maintenance of the vertebrate midbrain.

Here we present evidence that zebrafish Pbx proteins are important regulators of MHB and DMB formation by acting as biochemical partners with Engrailed proteins. Zebrafish embryos that lack Pbx2 and Pbx4 function initiate MHB development normally, but progressively lose *eng2a*, *pax2a, fgf8, gbx2, and wnt1* expression as well as the corresponding midbrain-derived structures. Likewise, we show that in the absence of Pbx function, the forebrain domain of *pax6a* expression is caudally expanded, suggesting that Pbx proteins are required to maintain the integrity of the DMB. We also show in vitro that zebrafish Pbx4 interacts biochemically with the zebrafish Eng2a protein and that this physical interaction is required for the biological activity of *eng2a* overexpression in vivo. Based on these results, we favor a model where Eng requires Pbx as a co-factor in the midbrain to properly pattern the MHB and DMB.

Materials and Methods

Whole mount in situ hybridization and mRNA injection

Examination of gene expression by whole-mount in situ hybridization was carried out essentially as described (Prince et al., 1998). For two-color in situs, we also labeled probes with fluorescein-UTP and detected alkaline phosphatase with either Fast-Red (Sigma) or Iodonitrotetrazolium-violet (Sigma). Embryos were manually deyolked, and photographed using a Zeiss Axioskop, Axioplan, or Axio Imager Z1 compound microscope and digital camera (SPOT RT, Retiga Exi, or Axiocam HR). Images were assembled in Photoshop (Adobe).

To make *eng2a* mRNA, a full-length *eng2a* ORF was cloned into pCS3+MT. *eng2a* mRNA was synthesized as a N-terminal 6X myc-tagged mRNA using the mMessage mMachine kit (Ambion). Following synthesis, mRNA was purified using four consecutive YM-30 microcon columns. mRNA concentration was estimated by spectrophotometry, and RNA was diluted to appropriate concentration in DEPC-treated H_2O. For *eng2a* mRNA injections, 25 pg was the minimal quantity required to cause profound defects in eye formation and repress *pax6a* expression. The *eng2a,2b*MO and *pbx2,4*MO rescue experiments used 100 pg of mRNA.

Zebrafish strains, genotyping, and morpholinos

The *pbx4* allele b557 (also known as *lazarus* or *lzr*) mutation was identified by aberrations in the expression pattern of *egr2b (krox20)* as described previously (Popperl et al., 2000). We used the following Pbx translation-blocking morpholinos in this paper:

Pbx2MO1: CCGTTGCCTGTGATGGGCTGCTGCG

Pbx2MO2: GCTGCAACATCCTGAGCACTACATT

Pbx4MO1: AATACTTTTGAGCCGAATCTCTCCG

Pbx4MO2: CGCCGCAAACCAATGAAAGCGTGTT

Pbx-depleted embryos were created using three methods. In the first, zebrafish embryos lacking maternal and zygotic *pbx4* gene function was accomplished by germ line transplantation as described previously (Waskiewicz et al., 2002). We generated Pbx-depleted embryos by injecting *pbx2*MO1 into mz*pbx4* one-cell stage embryos. In the second, we injected *pbx2*MO1 and *pbx4*MO1 into one-cell stage *lzr* (*pbx*$4^{-/-}$) embryos. In the third method, all four *pbx* morpholinos were injected into one-cell stage AB embryos. The phenotypes produced from

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all three methods were indistinguishable from one another. We used the morphological features of a small ear and malformed midbrain to identify live Pbx-depleted embryos at 24 hpf. By in situ analysis, we assayed for the absence of *egr2b* (*krox20*) expression in the hindbrain to identify Pbx-depleted embryos.

The *no isthmus* (*noi* / *pax2a*$^{-/-}$) b539 strain of fish was acquired from the Zebrafish International Resource Center. To create Engrailed-null embryos, we used *eng2a* (*eng2*) (CGCTCTGCTCATTCTCATCCATGCT) and *eng2b* (*eng3*) (CTATGATCATTTTCTTCCATAGTGA) morpholinos, as described previously (Scholpp and Brand, 2001).

Mobility shift assays and western immunoblotting

Electrophoretic mobility shift assays were performed using the EMSA core kit (Promega) and precast EMSA gels (Invitrogen) according to manufacturers recommendations. *eng2a* and *pbx4* open reading frames were subcloned into pCS3MT and pCS2MT, respectively and their sequences confirmed. Protein was synthesized using a coupled in vitro transcription and translation system (SP6 Wheat germ lysate TnT, Promega). Point mutations in Eng2a were created using the Quickchange site directed mutagenesis procedure according to manufacture's recommendations (Stratagene). For the Eng-Pbx cooperative binding experiments, the following oligonucleotide was synthesized and labeled using T4 polynucleotide kinase and ^{32}[P]-ATP: 5'-GTCAATTAAATGATCAATCAATTTCG-3'.

To assay for the in vivo translation efficiency of the various *eng2a* mRNAs, we injected one-cell embryos with 100pg of each mRNA construct, and extracted protein from 70% epiboly embryos essentially as previously described (Link et al., 2006). Western immunoblots were performed using precast 4–12% gradient gels (Invitrogen), followed by blotting to PVDF membranes (Millipore). Western detection for the myc-Eng fusion proteins were done using a 1:5000 dilution of anti-Myc 1° antibody (9E10, Covance), a 1:7500 dilution of sheep anti-mouse IgG HRP (Amersham), followed by chemiluminescent detection with Pierce Supersignal West Pico Chemiluminescent Substrate.

Results

Pbx proteins are required for the proper formation of the midbrain and for maintenance of gene expression at the MHB

Given the established genetic and biochemical interactions between Engrailed and Exd proteins in flies, we wanted to see if Pbx proteins cooperated with Engrailed to pattern the vertebrate midbrain. As a first step, we examined midbrain morphology in live wild type, *lazarus* (*lzr* / *pbx4*$^{-/-}$), and Pbx-depleted embryos at 24 hours post-fertilization (hpf). In wild type embryos, the characteristic isthmic constriction has formed at the MHB with the tectum and the cerebellum located rostrally and caudally to the MHB respectively. (Fig.1A, A′). In *lzr* embryos, the isthmus is poorly formed and the size of tectum is diminished (Fig.1 B, B′; n=15). To further reduce Pbx function, we injected *lzr* embryos with both *pbx2* and *pbx4* morpholinos (*lzr;pbx2,4*MO). In all Pbx-depleted embryos examined, the isthmic constriction is almost completely absent and the tectum is further reduced (Fig. 1C, C′; n=10). The phenotype of the Pbx-depleted embryos is similar to that of *eng2a* morphants, although not as severe as *eng2a/2b* double morphants or *noi* (*no isthmus / pax2a*$^{-/-}$) embryos (Fig.1 D, D′) (Brand et al., 1996; Scholpp and Brand, 2001). These results suggest that vertebrate midbrain development requires Pbx proteins in a dose dependent fashion, and that *pbx* genes may act on the same genetic or biochemical MHB patterning pathway as the *engrailed* family of genes.

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The MHB promotes separation between midbrain and hindbrain identities by restricting cell movements between the mesencephalon and metencephalon (Langenberg and Brand, 2005). MHB development consists of two early phases: initiation and maintenance. The genes involved in MHB maintenance are initiated largely independently of one another, and later become transcriptionally interdependent (Raible and Brand, 2004). To determine if Pbx proteins are required to initiate MHB gene expression, we compared the expression of *eng2a, pax2a, fgf8, gbx2*, and *wnt1* in wild type and Pbx-depleted embryos at 11 hpf (3 somite stage; Fig. 2A–J). Pbx-depleted embryos were identified by the absence of *egr2b* (*krox20*) expression in rhombomeres 3 and 5 of the presumptive hindbrain. *eng2a* and *pax2a* are expressed broadly across the MHB region, *fgf8* and *gbx2* are expressed in the posterior half of the MHB, while *wnt1* is expressed in the anterior region of the mesencephalon. We can detect no difference in the level or pattern of MHB gene expression between wild type and Pbx-depleted embryos at this stage (n=30). These data suggest that Pbx-proteins are not involved in the specification or positioning of the MHB and the immediately adjacent regions. Therefore, the loss of tectal and isthmic structures observed at 24 hpf may be due to a subsequent failure to maintain MHB gene expression.

In 18 hpf wild type embryos, a cross-regulatory loop between *eng2a, eng2b, pax2a*, *fgf8*, and *wnt1* maintains gene expression at the MHB and shapes the morphology of the isthmic constriction. We tested whether Pbx function is required to maintain the MHB by examining the expression of MHB marker genes in wild type, *lzr* and Pbx-depleted embryos (Fig. 3A–O). We used *eng2a* to mark the mesencephalon, MHB and metencephalon. In wild type embryos, *eng2a* is expressed in a wedge shape centered about the MHB (Fig. 3A). This domain is diminished slightly in *lzr* embryos (Fig. 3B). In Pbx-depleted embryos, this wedge-shaped domain of expression is greatly reduced and expression anterior to the MHB (the presumptive tectum) is absent (Fig. 3C). A similar loss of ventral expression is observed for *pax2a* expression at the MHB (Fig. 3D–F). We also performed in situs for *fgf8* and *gbx2* to examine the effects of Pbx depletion on the rostral metencephalon. *fgf8* expression is not changed in *lzr* mutants as compared to wild type (Fig. 3G, H). However, in Pbx-depleted embryos, the ventral domain is expanded caudally while medial expression is absent (Fig. 3I). Similar results were recorded for *gbx2* expression, although it appears to be more sensitive to Pbx-depletion (Fig. 3J–L). In wild type embryos, *wnt1* is expressed in the caudal mesencephalon and dorsal midbrain (Fig. 3M). In *lzr* and Pbx-depleted embryos, *wnt1* ventral expression is progressively lost, while the dorsal domain is unchanged. To summarize, in all cases where *egr2b* expression was completely or nearly absent in hindbrain rhombomeres 3 and 5, we observed a general decrease in the level of MHB marker expression and loss of medio-ventral gene expression at the MHB (n>300). Although less severe, the perturbation of MHB gene expression in Pbx-depleted embryos is similar to a *pax2a*, *fgf8* or *eng2a/2b* loss-of-function (Lun and Brand, 1998; Reifers et al., 1998; Scholpp and Brand, 2001). This comprehensive decrease in MHB gene expression supports the hypothesis that Pbx proteins act within the same regulatory pathway as *eng* to maintain the MHB.

The diencephalic-mesencephalic boundary is compromised in Pbx-depleted embryos

The diencephalic-mesencephalic boundary (DMB) is a lineage-restricted boundary that maintains separation between forebrain and midbrain identities. A loss of *eng*, *pax2a* or *fgf8* expression at the MHB has been shown to cause a caudal expansion of the forebrain at the expense of midbrain territory (Araki and Nakamura, 1999; Liu and Joyner, 2001; Scholpp and Brand, 2001; Scholpp and Brand, 2003; Scholpp et al., 2003). To determine the effect of Pbx-depletion on the DMB, we analyzed *epha4a*, *pax6a*, and *fgf8* expression by in situ hybridization on wild type and Pbx-depleted embryos (Fig. 4). In 16.5 hpf and 18 hpf Pbx-depleted embryos, forebrain-specific expression of *epha4a* (Fig. 4A, B) and *pax6a* (Fig. 4C, D) extends beyond its normal posterior limit while the distance between the DMB and MHB is reduced (all Pbx-

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null embryos affected, n>100). To quantify this, we compared the rostro-caudal extent of the *pax6a* domain and found it to be expanded by an average of 15% in Pbx-depleted embryos (P<0.025; n=5). Similarly, the distance between the MHB (*fgf8* expression) and the caudal limit of *pax6a* expression is reduced by 44% in Pbx-depleted embryos (P<0.01; n=5). Zebrafish embryos depleted of both Eng2a and Eng2b also exhibit a caudal expansion of forebrain markers *pax6a* and *epha4a* and a loss of midbrain territory. This demonstrates a strong similarity between the DMB defects in Pbx-depleted embryos and those lacking Engrailed function, implying that *pbx* and *eng* genes may function on a common genetic pathway in vertebrates.

To more closely examine the integrity of the DMB in Pbx-depleted embryos, we analyzed the expression of *pax6a* and *eng2a* in 16.5 hpf embryos (Fig. 4E-H′). In wild type embryos, cells expressing forebrain (*pax6a*) and midbrain (*eng2a*) markers exist as separate populations (arrows in Fig. 4E, G). In 16.5 hpf Pbx-depleted embryos, the integrity of the DMB has been compromised, as indicated by the region of overlap between forebrain and midbrain cells due to a caudal expansion of *pax6a* and a rostral expansion of *eng2a* (brackets in Fig. 4F, F′, H, H ′) Analysis of *eng2a* expression using fluorescent visualization of Fast Red-labeled *eng2a* probe details this anterior-ward expansion of the midbrain domain (Fig. 4E′-H′). These results demonstrate that Pbx proteins are required to maintain separate populations of forebrain and midbrain cells, a critical element of DMB formation and positioning.

Given the overlap between forebrain and midbrain genetic markers at 16.5 hpf, we examined 20 hpf and 28 hpf embryos to determine if cells were able to subsequently reorganize into proper domains. In 20 hpf Pbx-depleted embryos, the overlap between *pax6a* and *eng2a* expressing cells observed earlier at 16.5 hpf has diminished (Fig. 4J). *pax6a*-expressing cells from the diencephalon and hindbrain have encroached into the former mesencephalon and *eng2a* expression has retreated to small dorsal and ventral domains (compare brackets in Fig. 4I, J). At 28 hpf, we used *pax6a* as a marker for forebrain identity and included *isl1* to label interneurons of the posterior commissure, located just anterior to the DMB. In wild type embryos, the forebrain and hindbrain expression domains of *pax6a* are separated by the midbrain (Fig. 4K). However, like the 20 hpf Pbx-depleted embryos, these two domains of *pax6a* expression are nearly fused in 28 hpf Pbx-depleted embryos, and the caudal limit of forebrain *pax6a* has an obvious bulge midway along the dorsal-ventral axis (Fig. 4J, L). Additionally, the number and caudal position of the posterior commissure cell bodies are markedly expanded (brackets in Fig. 4I–L), whereas the neuronal cell population at the epiphysis is mostly unaffected (marked with an asterisk). This suggests, that the dorsal pretectal area (marked by the posterior commissure) expands posteriorly in Pbx-depleted embryos, whereas the more anteriorly positioned epithalamus (epiphysis) is less affected.

These data show that Pbx function is required to maintain the distinction between the forebrain and midbrain. In Pbx-depleted embryos, MHB development is initiated correctly, but expression of MHB patterning genes is not maintained. There is a transient period during which *pax6a* and *eng2a* expressing cells can share the same region of the midbrain, but eventually the mesencephalic region adopts a forebrain fate. It has been demonstrated in *noi* and *eng2a/2b*MO embryos that the midbrain adopts a forebrain fate (Scholpp and Brand, 2003; Scholpp et al., 2003). Other studies have demonstrated that a loss of MHB gene expression leads to a decrease in cell proliferation and / or an increase in cell death in the mesencephalon (Brand et al., 1996; Chi et al., 2003; Jaszai et al., 2003), and this may account for the eventual replacement of the mesencephalon with forebrain cells in Pbx-depleted embryos. In wild type embryos, Engrailed proteins are believed to be the principle factor that prevents the rostral midbrain from adopting a forebrain identity. From an early stage, *eng2a* is expressed immediately adjacent to the forebrain *pax6a* domain, thus placing it in an excellent position to repress diencephalic gene expression (Scholpp et al., 2003). Furthermore, in chick, ectopic Engrailed can repress

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pax6a expression in the forebrain, and does so before leading to the activation of other MHB genes (Araki and Nakamura, 1999). Therefore, together with the established genetic and biochemical interactions between fly Engrailed and Exd / Pbx proteins, the failure to maintain the MHB and DMB in Pbx-depleted embryos strongly suggests that zebrafish Pbx and Engrailed proteins cooperate to pattern the midbrain region of the neural tube.

Eng protein activity is dependent on the presence of Pbx proteins

Our finding that Pbx-depleted embryos resemble a loss of Engrailed function suggests a biochemical dependence of Eng function on Pbx proteins. To test this hypothesis directly, we determined whether Eng function is dependent on the presence of Pbx. We used an Eng overexpression assay in which we injected *eng2a* mRNA into single-cell zebrafish embryos and examined the resulting change in forebrain *pax6a* expression (Fig. 5) (Araki and Nakamura, 1999;Scholpp et al., 2003). Injection of low doses of *eng2a* mRNA caused strong reduction of *pax6a* expression, with only a vestigial stripe of *pax6a* typically remaining at the anterior-most region of the injected embryo (67.7%, n=341; Fig. 5A, B). The *eng2a*-dependent repression of *pax6a* in the forebrain was accompanied by a marked shortening of the forebrain region and a loss of eye formation. To determine whether the biological activity of ectopic Eng2a is dependent on the presence of Pbx4 protein, we also injected *eng2a* into maternal, zygotic *lzr* (mz*lzr*) mutant embryos. We chose to use mz*lzr* embryos to avoid the difficulty in scoring expression domains caused by the expansion of *pax6a* that is seen in Pbx-less embryos. The loss of maternal and zygotic Pbx4 potently attenuated the biological activity of injected *eng2a* mRNA. mz*lzr*, *eng2a*-injected embryos possessed both eyes, and near-normal levels of *pax6a* expression (strongly reduced in only 1.3% of injected embryos, n=75; Fig. 5C, D). The presence of Pbx2 protein in these embryos may account for some of the residual *pax6a* repressing activity of ectopic Eng2a. These results show that the ability of overexpressed Eng2a to repress *pax6a* expression is largely dependent upon the presence of Pbx4 protein. These data suggest that Eng and Pbx proteins act together to repress diencephalic fate in the vertebrate midbrain.

A biochemical interaction between Pbx and Eng is required for Eng function

A biochemical interaction between both vertebrate and *Drosophila* Eng and Pbx/Exd proteins has been documented previously (Peltenburg and Murre, 1996; van Dijk et al., 1995). To confirm that the zebrafish proteins possess similar biochemical properties, we performed EMSA using in vitro translated zebrafish Eng2a (fused to a 6X myc epitope) and Pbx4 (Fig. 6). We assayed for cooperative binding by mixing proteins together with a ^{32}P-labeled oligonucleotide that binds both Eng2a and Pbx4 (van Dijk et al., 1995). Whereas Eng2a has the ability to bind the oligo in the absence of Pbx4, we find that zebrafish Pbx4 will bind the oligo only in the presence of Eng2a proteins (compare lanes 1, 2 and 7 in Fig. 6). To examine which residues are required for an interaction between zebrafish Pbx and Eng, we mutated the orthologous residues to those which are required for mouse Pbx-Eng interactions, tryptophan residues 145 and 148 within the hexapeptide motif of Eng2a. We found that mutation of either tryptophan (W145K, W145S, or W148K) completely eliminated cooperative DNA binding with Pbx4 protein, implying that these mutated Eng proteins cannot bind effectively to Pbx (Fig. 6 lanes 8–11). The ability of Eng2a to bind the oligo was not affected by mutation of either tryptophan residue. However, we observed a general decrease in the ability of Eng2a with nonfunctional hexapeptide motifs to bind the oligo in the presence of Pbx4 (Fig. 6 lanes 8–11). We expect that this is a result of a residual in vitro interaction between Pbx4 and Eng2a that leaves both proteins in a conformation that is unfavorable for binding DNA. This incomplete interaction may involve the EH2 and EH3 domains of Eng that have previously been shown to be required for the Eng-Pbx interaction (Peltenburg and Murre, 1996). Our EMSA results show that there is an evolutionarily conserved biochemical interaction between

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zebrafish Eng2a and Pbx4 mediated by the tryptophan residues of the hexapeptide domain, agreeing with previous work performed on the orthologous murine and *Drosophila* proteins.

According to our experiments with Pbx-depleted embryos, reduction of *pax6a* expression by overexpressed Eng2a requires the presence of Pbx proteins (Fig. 5). To directly test whether ectopically expressed Eng2a must have the ability to bind Pbx proteins in order to reduce *pax6a* expression, we injected one-cell wild type embryos with the same mRNAs used in our gel shift assays. We then assayed for *pax6a* expression to compare the biological activity of wild type Eng2a with that of the hexapeptide mutants which cannot bind directly to Pbx proteins. We find that all of the Eng2a hexapeptide mutants have dramatically lowered biological activity. Whereas 76.2% (n=126) of *eng2a* WT injected embryos show reduced expression of *pax6a* (Fig. 7B, G), only 6.6% of embryos injected with *eng2a*-W148K and 17.2% of embryos injected with *eng2*a-W148S show any observable reduction of *pax6a* (Fig. 7C, F, G). Mutation of both tryptophan residues together (WWKK) was similar to mutation of the W148 alone (7.9% showing reduced expression; Fig. 7E, G). Mutation of the other conserved Eng2a tryptophan residue alone (W145) leads to a subtly smaller attenuation of biological activity (24% showing reduced *pax6a*; Fig. 7D, G). All *eng2a* mRNA constructs were expressed as full-length proteins and translated at similar efficiencies (Fig. 7H), showing that the point mutations introduced into the *eng2a* coding region did not affect the translation or stability of the protein product. Both wild type and WWKK forms of Eng were also correctly localized to the nucleus (data not shown). Taken together, these results agree with our in vitro gel-shift assays and show that the repression of *pax6a* expression by Eng2a in vivo requires the ability to directly bind Pbx4.

To establish whether Eng and Pbx proteins cooperate to pattern the MHB, we examined the ability of wild type and tryptophan-mutant forms of *eng2a* mRNA to rescue the MHB defects of *eng2a,2b* morphants and *pbx2,4* morphants (Fig. 8). First, we examined the effects of overexpressing the WT and WWKK forms of *eng2a* mRNA on the MHB by assaying for *pax2a* expression. As shown previously, the ectopic expression of wild type *eng2a* causes a loss of eye formation. In 65% embryos that exhibit this phenotype, we also observe a slight expansion of *pax2a* expression at the MHB (n=37) (Fig. 8B). On the other hand, overexpression of *eng2a* WWKK does not lead to an expansion of the MHB. Furthermore, in 47% of these embryos (n=73) we observe a decrease in *pax2a* expression, suggesting the tryptophan-mutant forms of Eng2a can act as a dominant negative (Fig. 8C). We speculate that Eng2a WWKK can bind to promoter sites normally occupied by Eng-Pbx heterodimers and prevent the normal regulation of target genes, thereby causing the dominant negative effect. Similar results were observed for both WT and WWKK forms of Eng2a when we assayed for *fgf8* and *wnt1* expression (data not shown). These results suggest that Eng2a requires an intact hexapeptide motif in order to properly regulate MHB development. To further test this, we attempted to rescue *eng2a* and *eng2b* double morphants (*eng2a,2b*MO) with wild type and WWKK forms of *eng2a* mRNA. At the dose of morpholino we used (8ng of each MO), knockdown of both Eng2a and Eng2b lead to a dramatic decrease in *pax2a* expression at the MHB (Fig. 8D). Injection of wild type *eng2a* RNA resulted in near normal levels of *pax2a* expression in 67% of *eng2a,2b*MO embryos (n=15) (Fig. 8E). *eng2a* WWKK was unable to rescue the MHB phenotype of *eng2a,2b*MO embryos (100%, n=20) (Fig. 8F). To see if *eng2a* RNA is able to rescue the MHB phenotype of Pbx-depleted embryos, we injected *pbx2* and *pbx4* double morphant embryos with either WT or WWKK forms of *eng2a* mRNA. The loss of *pax2a* expression at the MHB in Pbx-depleted embryos (Fig. 8G) cannot be fully rescued by injection of WT *eng2a* mRNA (n=14), though we do observe a partial rescue in 36% of embryos (Fig. 8H). We attribute this partial rescue to incomplete knockdown of Pbx proteins by morpholino treatment in some embryos, since the degree of rescue correlates with the amount of *egr2b* remaining in the hindbrain of Pbx-depleted embryos (data not shown). Injection of Pbx-depleted embryos with *eng2a* WWKK cannot rescue *pax2a* expression at the MHB (100%,

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n=26) (Fig. 8I). Taken together, the dominant negative effect of *eng2a* WWKK mRNA at the MHB, its inability to rescue *eng2a,2b* morphants, and the inability of WT *eng2a* mRNA to rescue Pbx-depleted embryos all suggest that Engrailed function at the MHB requires a biochemical interaction with Pbx proteins.

Discussion

In this paper, we present evidence that zebrafish Pbx2 and Pbx4 proteins act as biochemical partners with Eng proteins to pattern the mesencephalic territory of the developing vertebrate neural tube. This new role as a midbrain patterning factor expands upon the previously reported role of zebrafish Pbx2 and Pbx4 as Hox co-factors in patterning the hindbrain. We show that the expression of MHB markers is initiated, but not maintained in Pbx-depleted embryos, suggesting that Pbx participates in the cross-regulatory loop that maintains MHB gene expression. Furthermore, diencephalic markers *pax6a* and *epha4a* are expanded caudally and that there is an anomalous overlap between *pax6a* and *eng2a* expressing cells at the DMB in Pbx-depleted embryos. We used an Eng2a overexpression assay to demonstrate that the *pax6a* repressing activity of Eng2a depends largely upon the presence of Pbx4 protein. Lastly, we show that zebrafish Pbx4 and Eng2a interact biochemically in vitro via Engrailed's hexapeptide motif, and that this biochemical interaction is required for Engrailed's role in regulating MHB development. Taken together, these data suggest a model whereby Pbx and Eng proteins cooperate biochemically to pattern the developing vertebrate midbrain.

The interaction between Eng and Pbx/Exd is conserved in vertebrates

Drosophila Engrailed is an important factor in establishing and maintaining cellular compartments during development (Hidalgo, 1996). Engrailed fulfills this role in part through its biochemical interaction with Exd, the fly orthologue of Pbx (Alexandre and Vincent, 2003; Kobayashi et al., 2003; Peifer and Wieschaus, 1990). Engrailed's role in forming lineage restricted compartments is highly conserved in vertebrates, as evidenced by the Engrailed loss-of-function phenotype in the mesencephalon (Scholpp and Brand, 2001; Wurst et al., 1994). In this paper, we show that the ability of Engrailed to pattern the MHB and DMB is dependent on its interaction with Pbx proteins. Thus, the partnership between Engrailed and Pbx/Exd, their mechanism of biochemical interaction, and their role in the transcriptional regulation of boundary formation are all conserved between flies and vertebrates.

Pbx proteins act outside of the hindbrain to pattern the zebrafish embryo

Pbx proteins are well characterized as Hox co-factors that function to compartmentalize the vertebrate hindbrain. However, previous research has demonstrated that Pbx proteins do function outside of the hindbrain, sometimes in a Hox-independent fashion. *Pbx*-deficient mice have defects in Hox-dependent processes such as organogenesis (Manley et al., 2004; Schnabel et al., 2003), hematopoiesis (DiMartino et al., 2001), limb formation (Capellini et al., 2006), and skeletal and cartilage formation (Selleri et al., 2001). Other studies in zebrafish and mouse have demonstrated Hox-independent functions for Pbx proteins as cofactors for MyoD in muscle cells (Berkes et al., 2004), Pdx1/Ipf1 in pancreatic development (Dutta et al., 2001; Kim et al., 2002; Peers et al., 1995), and for the metaHox protein Rnx/Hox11L2/Tlx-3 to control development of the medullary respiratory control mechanisms (Rhee et al., 2004). Our finding that Pbx cooperates with Eng proteins to pattern the midbrain adds to this growing body of evidence that vertebrate Pbx proteins are involved in a myriad of developmental processes in multiple tissue types, and that some functions of Pbx proteins are Hox-independent.

Our results also suggest that Pbx proteins are key regulators of compartmental boundaries. In the absence of Pbx function, the primary division of the neuroectoderm into presumptive fore-, mid-, and hindbrain regions still occurs normally. Subsequent to this, these boundaries are

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reinforced and maintained while the tissues are secondarily subdivided. Our results show that, in the absence of Pbx function, the midbrain region is initiated correctly, but that secondary maintenance of the MHB and DMB is compromised. This result is consistent with the role already described for Pbx in the hindbrain. A Pbx (Waskiewicz et al., 2002), Meis (Choe and Sagerstrom, 2004) or Pknox (Deflorian et al., 2004) loss-of-function prevents the rhombomere boundaries from ever forming, but does not prevent the initial specification of the hindbrain region. Thus, it appears that a general role for Pbx proteins during vertebrate development is to act as transcriptional co-factors throughout the midbrain and hindbrain in the formation and maintenance of lineage-restricted boundaries. Whether or not Pbx participates in lineage restriction between compartments within the forebrain has not been investigated.

The requirement for zebrafish Pbx proteins in regulating midbrain gene expression

A loss of *pax2a* or *engrailed* function in the vertebrate midbrain is characterized by a reduction of the tectum and cerebellum, and a failure to maintain the morphological and genetic characteristics of the isthmic organizer at the MHB. In fish, it is possible to study a partial loss of *engrailed* function by using morpholinos against one of the two *eng* paralogues, *eng2a* (*eng2*) or *eng2b* (*eng3*) (Scholpp and Brand, 2001). Eng2b knockdown has very limited phenotypic effects, but the loss of *eng2a* function leads to a morphological phenotype that is intermediate between wild type and *eng2a/2b*MO or *noi* embryos. The ventral region of the MHB is especially sensitive to Eng2a knockdown, as the ventral domains of *pax2a* and *eng2b* expression are lost. Similar DV patterning defects can be observed in weak alleles of *pax2a* (Lun and Brand, 1998). We observe similar defects in Pbx-depleted embryos. The medio-ventral domain of MHB gene expression preferentially lost (Fig. 3), and the isthmic constriction is diminished, but not completely eliminated (Fig. 1). This result shows that a Pbx loss-of-function is not equivalent to a complete Engrailed loss-of-function, suggesting that some activities of Engrailed are Pbx-independent. The idea that Engrailed can act independently of Pbx is also supported by the observation that Engrailed proteins with mutated hexapeptide domains still possess some ability to repress *pax6a* expression in zebrafish embryos (Fig. 7). These data suggest either of two possibilities: that Engrailed can act independently of Pbx, or that the Pbx-Engrailed interaction in vivo is not solely dependent on a functional hexapeptide domain in Engrailed. Although either theory is possible, it has been demonstrated in flies that En requires Exd for activation of some targets, but not the repression of others (Alexandre and Vincent, 2003; Kobayashi et al., 2003; Serrano and Maschat, 1998). Therefore, it is likely that some functions of Eng are Pbx-independent.

Downstream effects of Pbx depletion on midbrain structures and function

Besides patterning the DMB and MHB regions of the neural tube, Engrailed function is also required to establish spatial polarity in the optic tectum. A rostrocaudal gradient of Engrailed expression in the tectum is necessary for correct topographic targeting of the retinal ganglion cell axons (Friedman and O’Leary, 1996; Itasaki et al., 1991; Itasaki and Nakamura, 1996; Logan et al., 1996; Nakamura and Sugiyama, 2004). Engrailed likely exerts that effect by regulating the gradient expression of Ephs and Ephrins in the tectum (Logan et al., 1996). The Eph family of RTKs and their Ephrin ligands are essential components in establishing tectal polarity, mediating axon guidance and forming the retinotectal topographic map (Drescher et al., 1997). Our analysis of Pbx-depleted embryos shows that the rostrocaudal gradient of *eng2a* is abolished by 21 hpf, suggesting that Pbx-depletion may cause tectal patterning defects later in development. We found that the normal patterns of *epha4a* (Fig. 1A–D) and *efna2* (French et al., in preparation) gene expression in the presumptive tectum are disrupted in Pbx-depleted embryos. Both *epha4a* and *efna2* have been implicated in retinal ganglion axon guidance (Marin et al., 2001; Pfeiffenberger et al., 2005; Walkenhorst et al., 2000). This result implies that, together with defects in the DMB, Pbx-depleted embryos may also exhibit abnormalities in tectal patterning and retinal ganglion cell axon projection defects.

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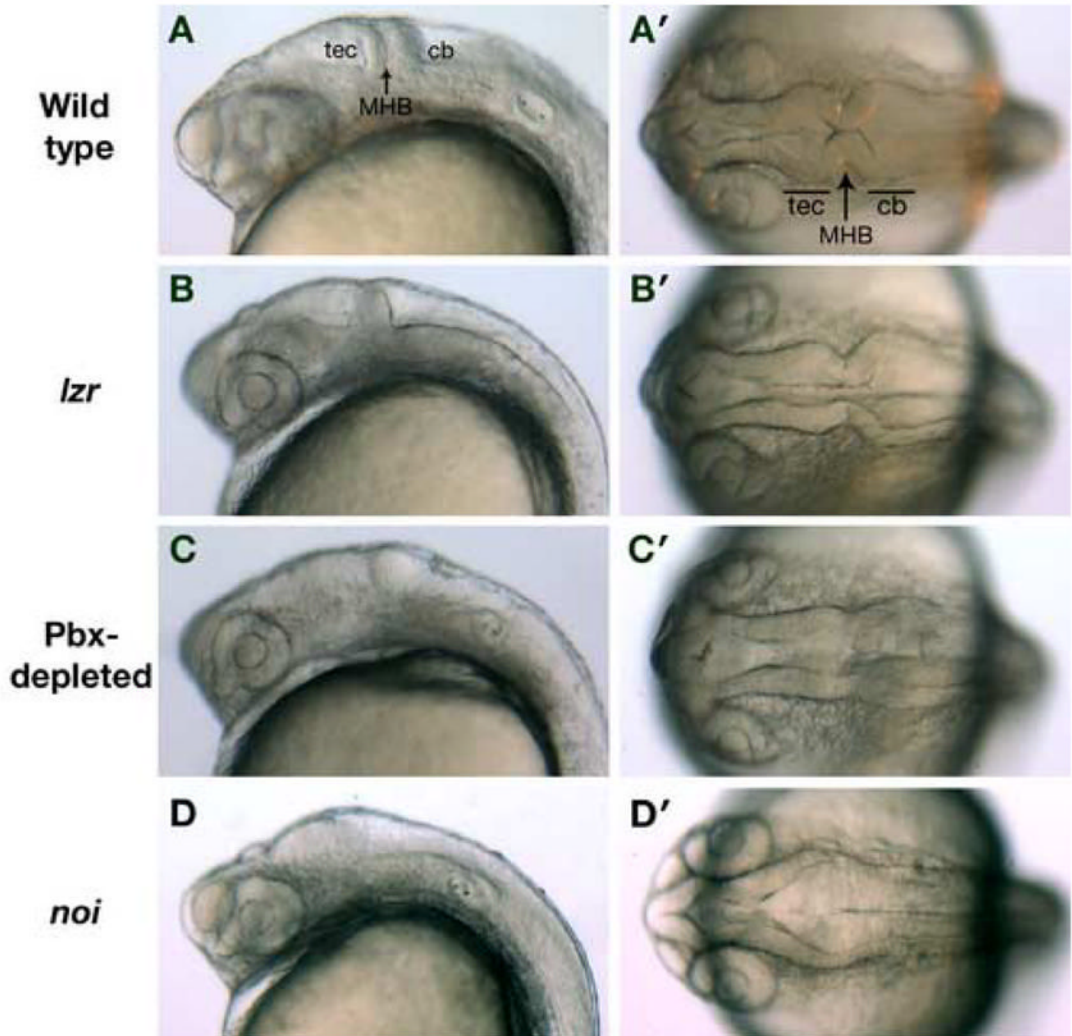

Fig. 1.

The morphology of the MHB, tectum and cerebellum is defective in Pbx-depleted embryos at 24 hpf. (A, A′) Wild type embryos at 24 hpf possess a well formed tectum (tec) and cerebellum (cb) separated by the isthmic constriction at the MHB. (B, B′) *lzr* (*pbx4*$^{-/-}$) embryos have a normal cerebellum, but the size of the tectum is diminished and the isthmus is not as well formed. (C, C′) In Pbx-depleted embryos, the isthmic constriction at the MHB is indistinct, and neither the tectum nor cerebellum has formed properly. (D, D′) By way of comparison, *noi* (*pax2a*$^{-/-}$) embryos lack all midbrain-derived structures. The isthmus is complete absent and the tectum and cerebellum are unrecognizable. Anterior is to the left; panels A–D are lateral views and panels A′– D′ are dorsal views.

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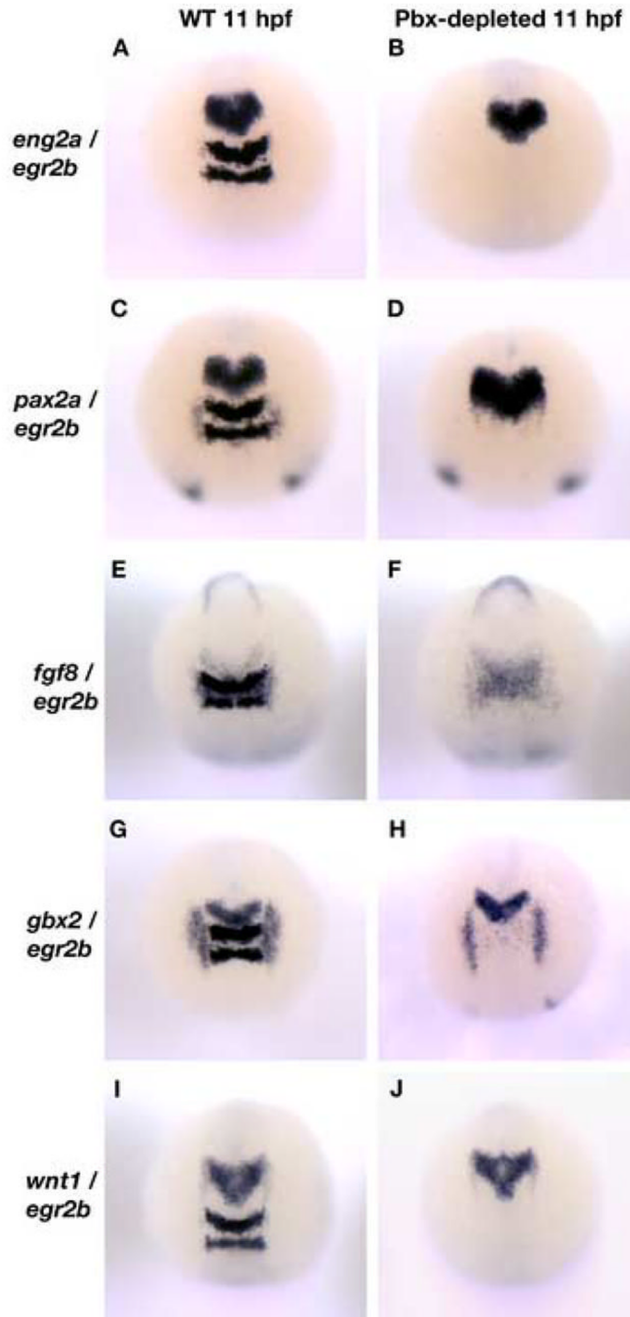

Fig. 2.

The establishment of the midbrain region of the neural tube is normal in Pbx-depleted embryos. (A – F): *eng2a* (A, B), *pax2a* (C, D), *fgf8* (E, F), *gbx2* (G, H) and *wnt1* (I, J) expression at the MHB is normal in both wild type (WT) and Pbx-depleted embryos at 11 hpf. The absence of *egr2b* (*krox20*) expression in the rhombomeres 3 and 5 of the presumptive hindbrain was used as an indicator of Pbx loss-of-function. All embryos are shown in dorsal view.

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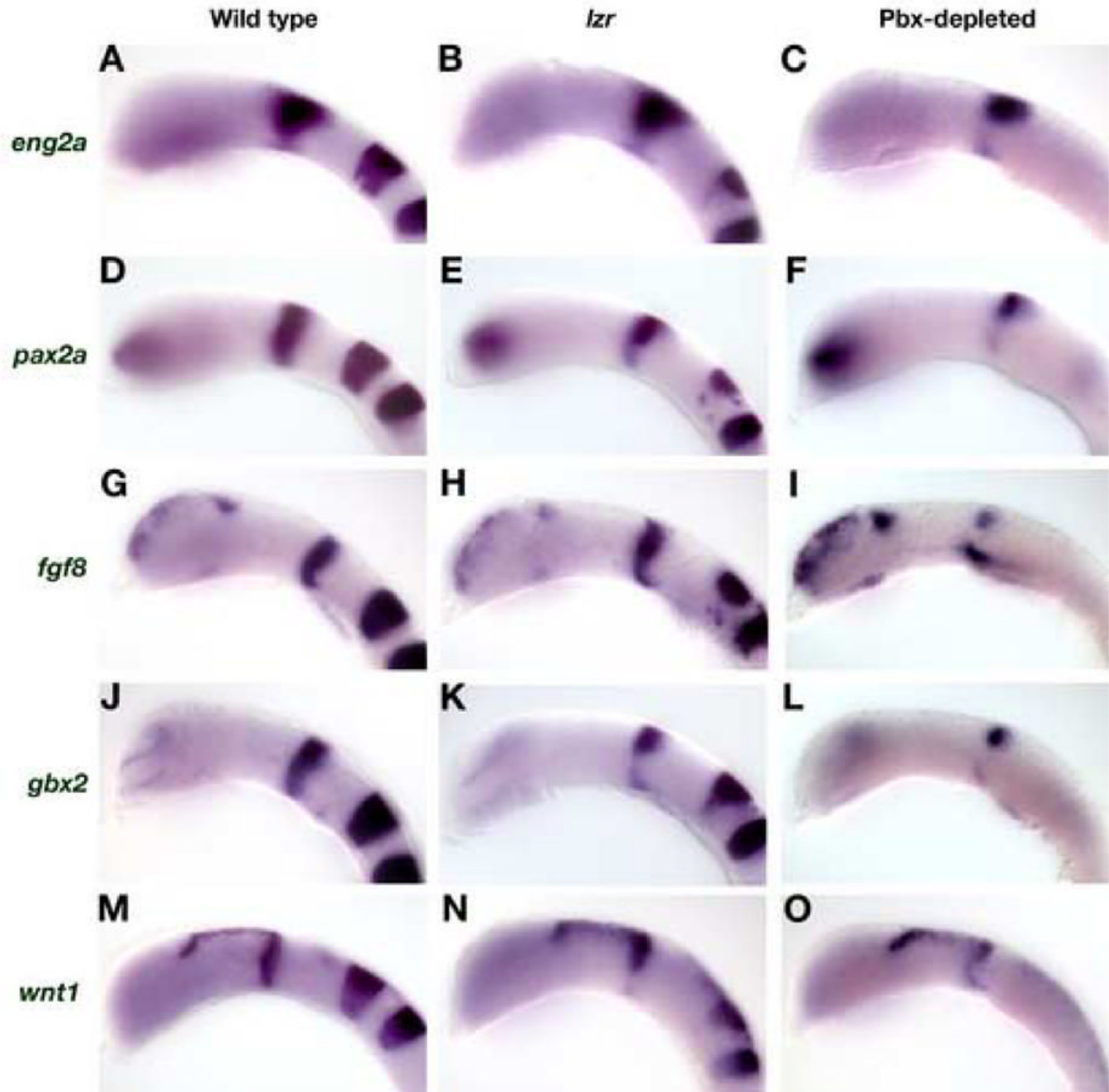

Fig. 3.
Pbx-depleted embryos do not maintain gene expression at the MHB. (A–C) *eng2a*: In 18 hpf wild type embryos, *eng2a* is expressed broadly across the MHB in a wedge-shaped domain (A). *eng2a* expression is decreased slightly in *lzr* embryos (B). In Pbx-depleted embryos, the ventral expression of *eng2a* is greatly decreased and the rostral domain is diminished. (D–F) *pax2a*: At 18 hpf, *pax2a* expression at the MHB is normally restricted to a narrow stripe with approximately equal expression over the dorsal-ventral axis (D). In *lzr* embryos, *pax2a* expression is decreased in the ventral domain (E). In Pbx-depleted embryos, the ventral expression of *pax2a* is also completely absent (F). (G–L) *fgf8* and *gbx2*: Both *fgf8* and *gbx2* are expressed in the caudal half of the MHB at 18 hpf (G, J). *fgf8* expression is normal in *lzr* embryos (H), whereas the ventral domain of *gbx2* expression is decreased (K). In Pbx-depleted embryos, the medial domain of *fgf8* expression is lost, while the dorsal domain is decreased and the ventral domain is expanded caudally (I). The effect of Pbx-depletion on *gbx2* expression is more severe with only a residual dorsal patch remaining (L). (M–O) *wnt1*: In 18 hpf wild type embryos, *wnt1* is expressed in the rostral half of the MHB and the dorsal diencephalons

(M). The ventral domain of wnt1 expression at the MHB is decreased in *lzr* embryos, whereas the dorsal domains remain unchanged (N). In Pbx-depleted embryos, *wnt1* expression is decreased at the MHB and expanded caudally, but is expressed in the dorsal diencephalon at near normal levels. All embryos are shown in lateral view with anterior to the left.

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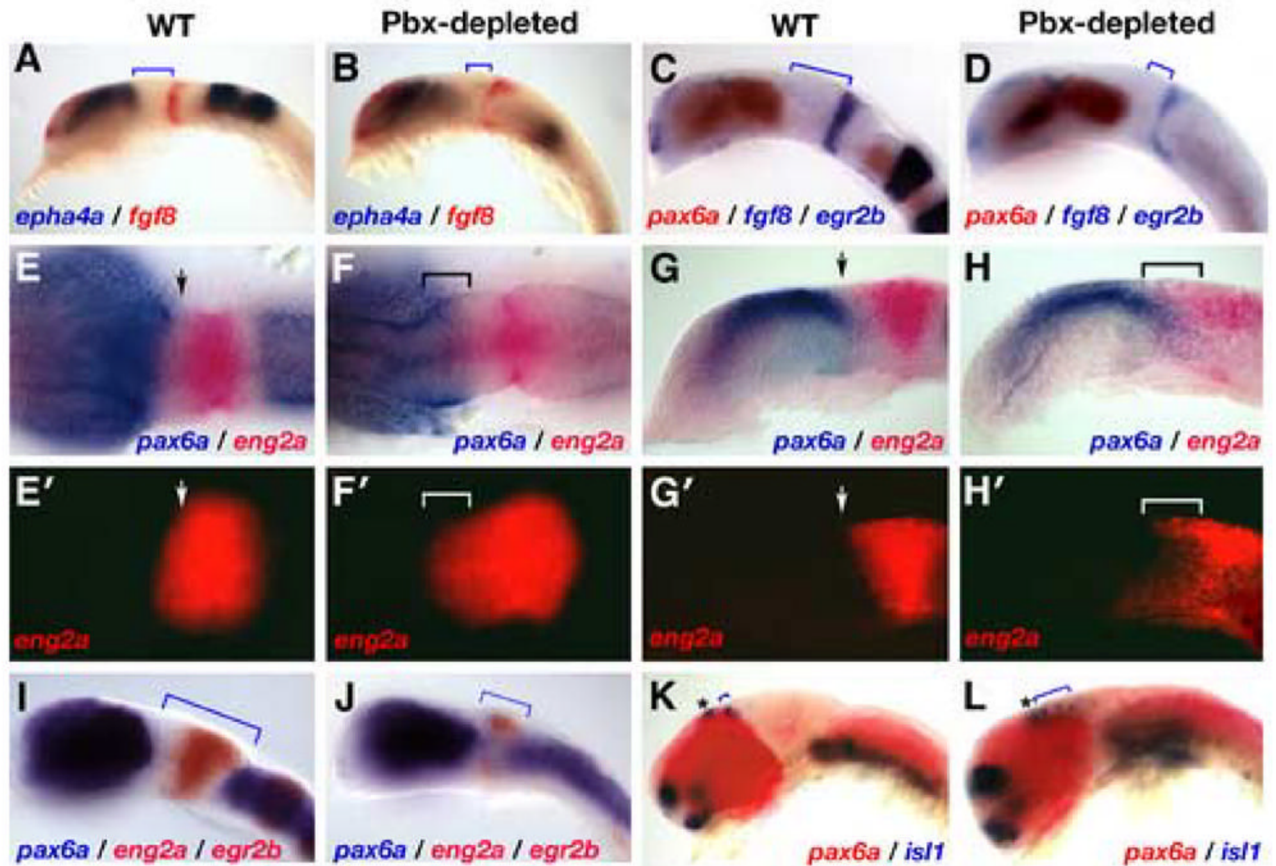

Fig. 4.

The boundary between diencephalon and mesencephalon (DMB) is not formed properly in zebrafish embryos lacking Pbx proteins. (A–D): We examined the size of forebrain and midbrain domains in 16.5 hpf (A, B) and 18 hpf (C, D) Pbx-depleted embryos. The expression domains of *epha4a* (A, B) and *pax6a* (C, D) are expanded caudally at the expense of midbrain territory, as indicated by the blue brackets. (E–H′): Forebrain and midbrain cells no longer exist as separate populations in Pbx-depleted embryos. In wild type (WT) 16.5 hpf embryos, *pax6a* (blue) and *eng2a* (red) expressing cells are separated by a sharp boundary at the DMB (arrow heads E, E′, G, G′). 16.5 hpf Pbx-depleted embryos exhibit a loss of DMB integrity, a caudal expansion of *pax6a* and a rostral expansion of *eng2a* expression (F, F′, H, H′). There is a region of overlap between these two cell populations as indicated by the brackets in F, F′, H, and H′. Note that identical embryos are shown in E–H and E′–H′. (I – L): Midbrain territory is lost in older Pbx-depleted embryos. Wild type 20 hpf embryos have well defined forebrain and hindbrain *pax6a* domains separated by *eng2a* positive cells of the midbrain. In 20 hpf Pbx-depleted embryos, the forebrain and hindbrain domains of *pax6a* expression have moved into the mesencephalic region (compare brackets in I and J) while *eng2a* expression is limited to residual dorsal and ventral patches. To determine the state of the DMB in 28 hpf Pbx-depleted embryos, we analyzed the expression of *pax6a* (red) and visualized the position of the epiphysis (marked with an asterisk) and posterior commissure interneurons by analyzing expression of *isl1* (blue). In wild type embryos, the *isl1*-positive neurons of the posterior commissure are tightly grouped (blue brackets in K). In 28 hpf Pbx-depleted embryos, the position of the posterior commissure neurons is caudally expanded (marked by blue brackets in L), while the *isl1*-positive neurons of the epiphysis are unaffected. Embryos were imaged either using DIC microscopy (A–H, I–L)) or using fluorescent emission of Fast-Red stain (E′–H′). All embryos

are deyolked and shown as either lateral or dorsal views with anterior to the left, except the embryos shown in G, G′, H, and H′, which are saggital sections.

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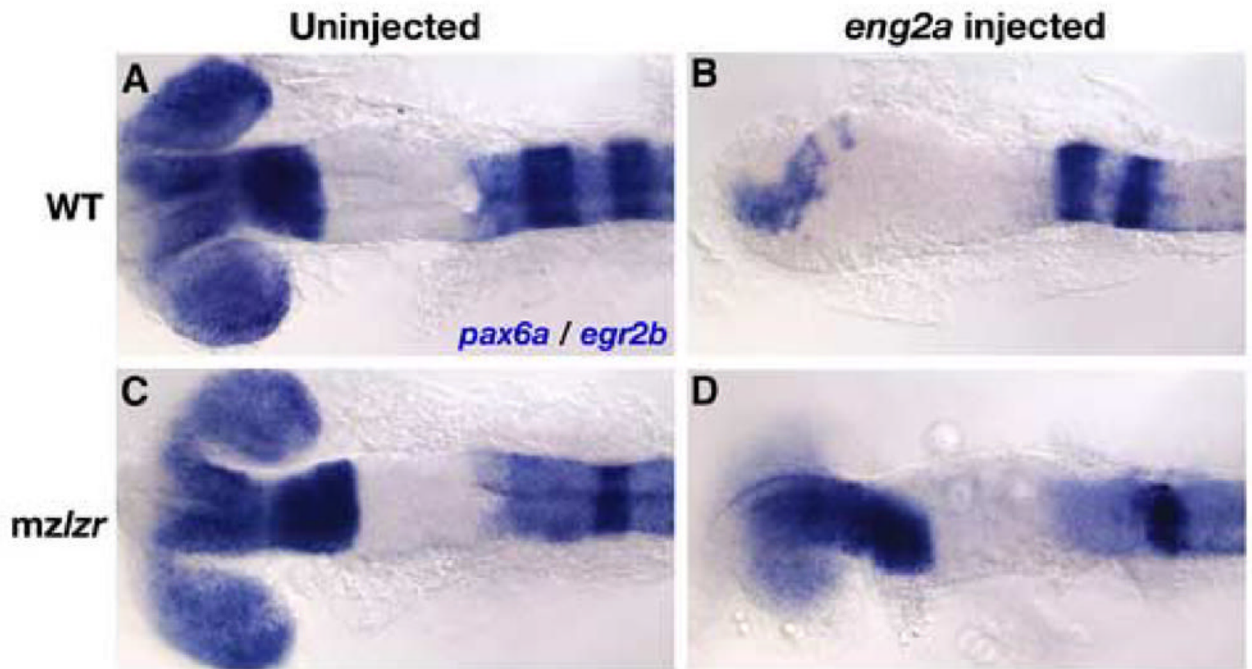

Fig. 5.
Activity of ectopically expressed *eng2a* mRNA is dependent on presence of Pbx4 protein. We analyzed the expression of *pax6a* by in situ hybridization to visualize the effect of ectopic *eng2a* overexpression in both 18 hpf wild type (WT) (A,B) or mz*lzr* embryos (C,D). We also analyzed the expression of the rhombomere 3 and 5 marker *egr2b* to distinguish which embryos had the mz*lzr* genotype (lack of r3 *egr2b* expression). Injection of 50 pg *eng2a* mRNA causes profound defects in the formation of the forebrain, including a reduction in *pax6a* expression and the loss of eye formation (compare A and B). These effects are strongly attenuated in embryos lacking Pbx4 (compare C and D). All embryos are deyolked and mounted dorsally with anterior to the left.

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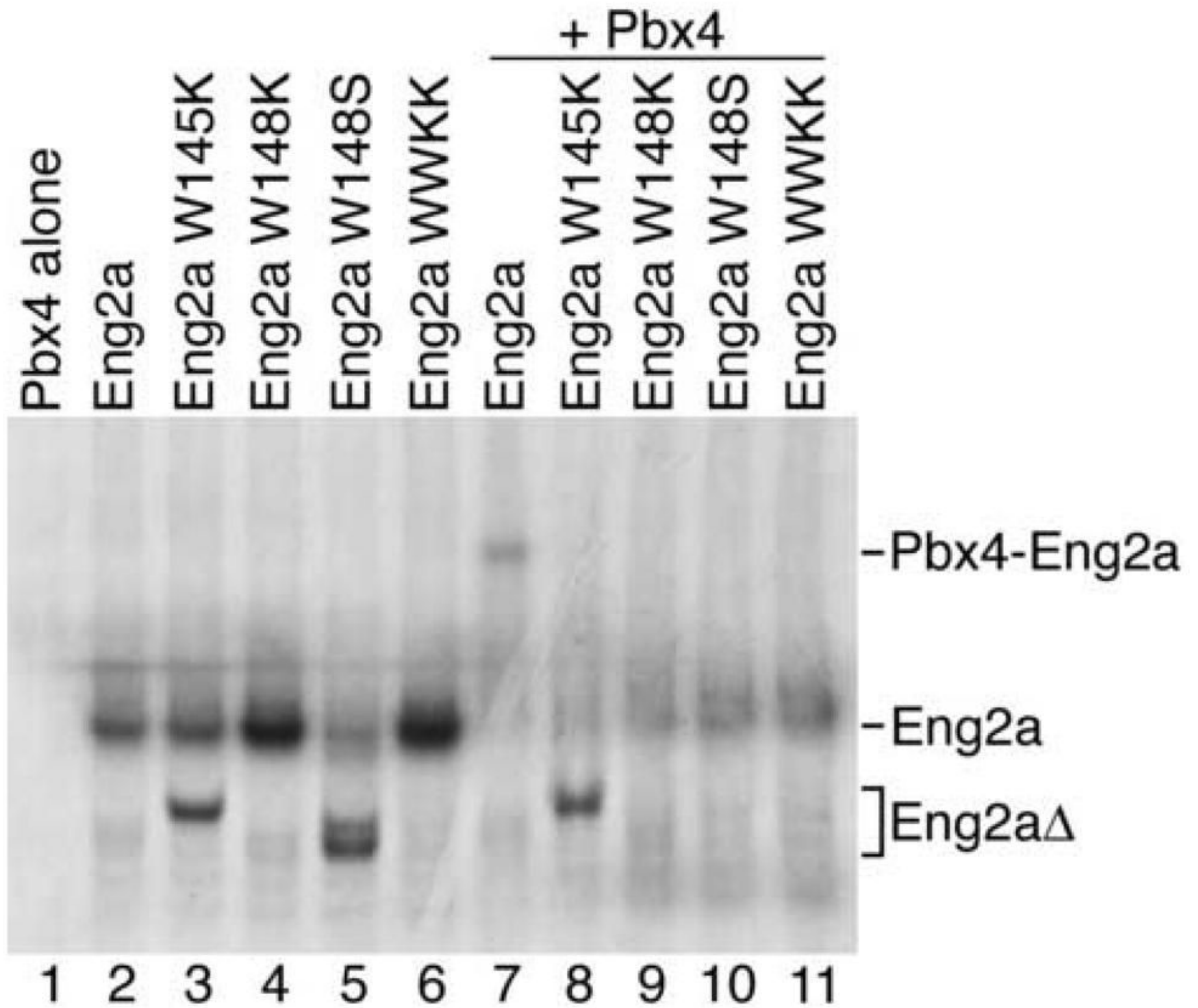

Fig. 6.

Eng2a requires a functional Pbx-binding hexapeptide to bind Pbx4 in vitro. Zebrafish Pbx4 and myc-Eng2a were synthesized using a wheat germ in vitro transcription and translation system. Crude in vitro translated proteins were mixed with a ^{32}P-labeled oligo containing both Pbx and Eng consensus binding sites: 5'-GTCAATTAAATGATCAATCAATTTCG-3' (van Dijk et al., 1995). myc-Eng2a proteins with mutations in the hexapeptide motif were also tested for cooperative binding with Pbx4. By site-directed mutagenesis, zebrafish Eng2a tryptophan residues W145 and W148 were changed to either lysine (W145K, W148K, WWKK), or a serine (W148S) residues. In lanes containing Pbx4 (Lanes1, 7–11), only the sample containing both Pbx4 and myc-Eng2a was capable of binding the ^{32}P-labeled oligo (Lane 7). Mutations in the Eng2a hexapeptide abrogated cooperative Eng2a-Pbx4 binding to the oligo (Lanes 8–11).

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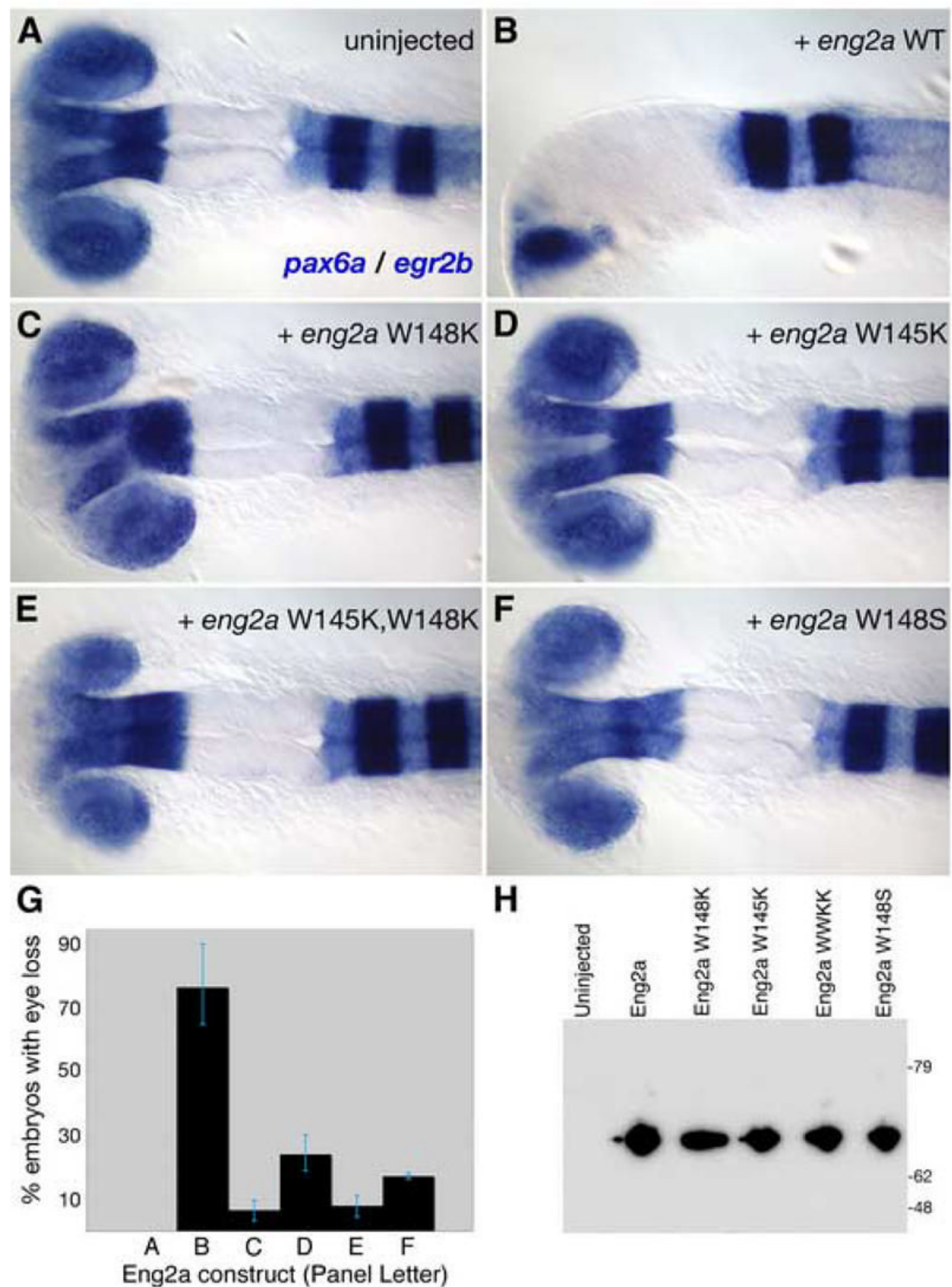

Fig. 7.

Eng2a constructs with point mutations in the hexapeptide exhibit attenuated *pax6a*-reducing activity. To determine whether mutations in the Eng2a hexapeptide affected the in vivo activity of overexpressed *eng2a*, we injected single-cell zebrafish embryos with mRNAs coding for the same myc-Eng2a proteins that we used in our EMSA assay and assayed for *pax6a* and *egr2b* expression by in situ analysis. (A, B) Uninjected wild type embryos never exhibited eye loss, while the majority of embryos injected with *eng2a* mRNA displayed a loss of eye formation and greatly reduced *pax6a* expression. (C–F) Mutations in the Eng2a hexapeptide, W148K (C), W145K (D), W145KW148K (WWKK) (E), W148S (F), resulted in strongly reduced biological activity compared to the wild type Eng2a (B). (G) Biological effects of each

construct were quantified and are shown with the error bars denoting the range of values from two separate experiments. Compared to the activity of wild type Eng2a, mutations in the hexapeptide caused a 3-11X reduction in activity. (H) To rule out the possibility that the reduced biological activity of the Eng2a point mutants was due to inefficient translation or preferential degradation of the protein products, we performed a Western analysis using a monoclonal antibody (9E10) against the myc-epitope to which the various *eng2a* coding regions were fused. All Eng2a constructs were full length and present at similar levels, showing that the point mutations introduced into the *eng2a* coding region did not affect the translation or stability of the protein product.

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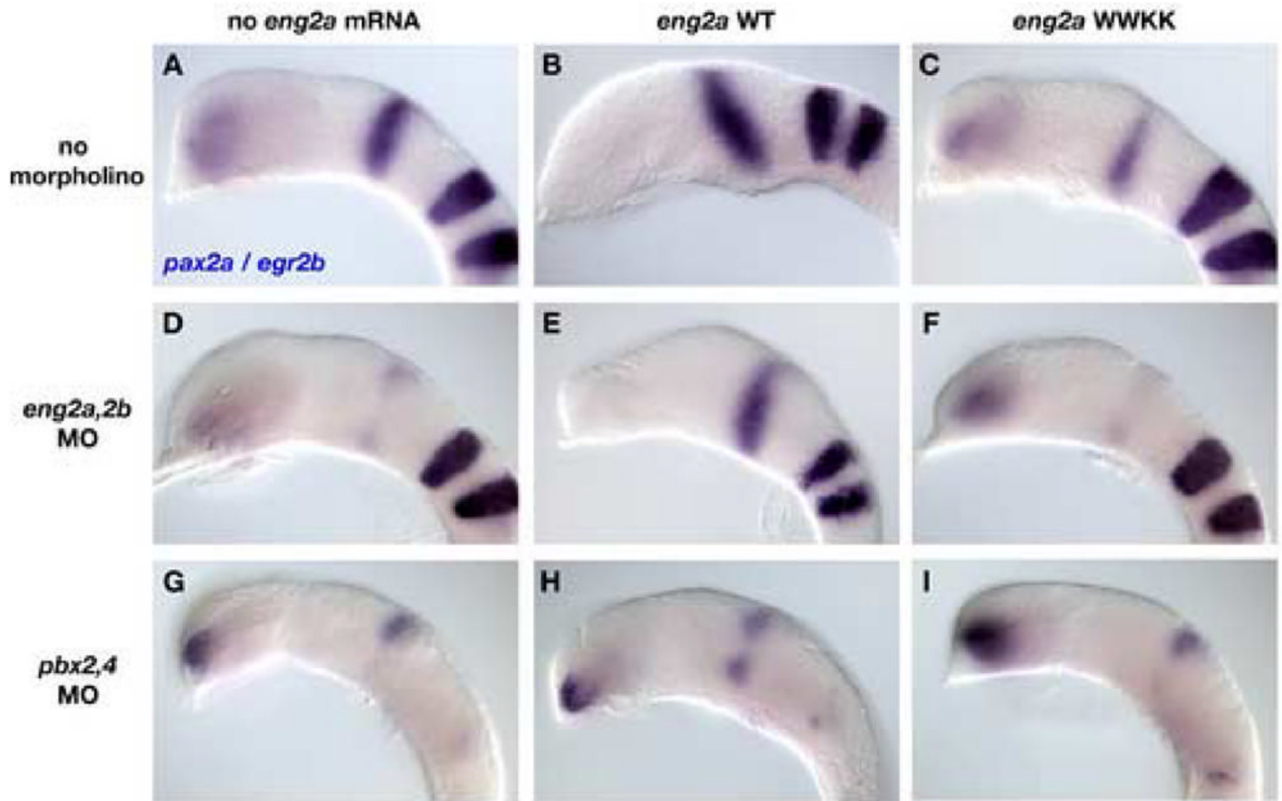

Fig. 8.

Engrailed and Pbx cooperatively regulate midbrain-hindbrain boundary development. (AC) We used *pax2a* as a marker for the MHB in wild type, *eng2a* WT, and *eng2a* WWKK injected embryos. Overexpression of wild type Eng2a causes a slight expansion of the MHB, together with a loss of eye formation (compare A and B). Overexpression of a hexapeptide mutated form of Eng2a (WWKK) has a dominant negative effect on MHB development, as shown by the decrease in *pax2a* expression (compare A and C). (D–F) *eng2a,2b* morphant embryos (D) can be rescued by injection with wild type *eng2a* mRNA (E), but not by *eng2a* WWKK (F). (G–I) The MHB defect in Pbx-depleted embryos (G) cannot be rescued by injection of either *eng2a* WT (H) or *eng2a* WWKK (I). All embryos are deyolked and mounted laterally with anterior to the left.