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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). 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

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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). Among 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.

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 among 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