Xpbx1b and *Xmeis1b* play a collaborative role in hindbrain and neural crest gene expression in *Xenopus* embryos

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Pbx1 is a homeodomain protein that functions in complexes with other homeodomain-containing proteins to regulate gene expression during embryogenesis and oncogenesis. Pbx proteins bind DNA cooperatively as heterodimers or higher order complexes with Meis family members and Hox proteins and are believed to specify cell identity during development. Here, we present evidence that Pbx1, in partnership with Meis1b, can regulate posterior neural markers and neural crest marker genes during *Xenopus* development. A *Xenopus* homolog of the Pbx1b homeodomain protein was isolated and shown to be expressed throughout embryogenesis. *Xpbx1b* expression overlaps with *Xmeis1* in several areas, including the lateral neural folds, caudal branchial arch, hindbrain, and optic cup. When ectopically expressed, Xpbx1b can synergize with Xmeis1b to promote posterior neural and neural crest gene expression in ectodermal explants. Further, a physical interaction between these two homeodomain proteins is necessary for induction of these genes in embryonic tissue. In addition, coexpression of Xmeis1b and Xpbx1b leads to a prominent shift in the localization of Xmeis1b from the cytoplasm to the nucleus, suggesting that nuclear transport or retention of Xmeis1b may depend upon Xpbx1b. Finally, expression of a mutant construct in which Xpbx1b protein is fused to the repressor domain from *Drosophila* Engrailed inhibits posterior neural and neural crest gene expression. These data indicate that Xpbx1b and its partner, Xmeis1b, function in a transcriptional activation complex during hindbrain and neural crest development.

In *Xenopus*, formation of the anteroposterior axis in the prospective neurectoderm is induced during gastrulation on the dorsal side of the embryo (1). Neural patterning has been proposed to be a two-step process where neurectoderm is first "activated" (anterior state) and subsequently "transformed" or respecified into posterior neurectoderm. Several antagonists of bone morphogenic proteins have been identified which may play a role in the "activation" state (2). Several secreted molecules have "transforming" activity and may be involved in reprogramming this tissue to more posterior cell fates, such as hindbrain and spinal cord (2). Neural crest tissue is induced at the border between the neural plate and epidermis. These cells eventually begin to migrate throughout the embryo and give rise to most of the peripheral nervous system, epidermal pigment cells, and craniofacial cartilage (3). Rhombomeric generation of neural crest cells is observed along the dorsal part of the hindbrain, where they migrate ventrally and give rise to cranial sensory ganglia and populate the pharyngeal arches. The multipotent cells ultimately contribute to the formation of neural, muscular, skeletal, and vascular structures (3). *Hox* genes are segmentally expressed in the developing vertebrate hindbrain, neural crest cells, and pharyngeal arches, demonstrating an important role in patterning these structures (3).

An array of transcriptional cofactors, such as the homeodomain proteins of the EXD/PBX (PBC; ref. 4) and MEIS/PREP (MEINOX) families regulate the transcriptional activity of HOX proteins during development (5–7). These homeodomain cofactors do not encode any obvious transcriptional activator or repressor domains but they do play important roles during embryonic development. For example, the *Drosophila Meis* homolog, *homothorax* (*Hth*), cooperates with a *Drosophila PBX* homolog termed *extradenticle* (*Exd*). Together, these two homeodomain proteins control antenna determination (8), patterning of the embryonic fly PNS (9, 10), and suppression of eye development (11). The interaction between Hth and Exd triggers the nuclear localization of Exd, thus allowing for proper function of the protein complex (8, 10–14). In vertebrates, aberrant *Meis1* gene expression has been shown to be involved in the pathogenesis of murine myeloid tumors and human leukemias (15–17), but new information on the developmental role of these proteins is beginning to emerge. For example, a *Xenopus* homolog of the mammalian *Meis3* gene has been implicated in the caudalization of neural tissue (18, 19), and recent studies in chicken suggest that restriction of *Meis1* to proximal regions of the limb is essential for the specification of cell fates along the proximal-distal axis of the limb (20, 21). Also, until recently, little was known about *Pbx* function in vertebrates.

Pbx1 was identified as a fusion partner with E2A in a translocation breakpoint found in human pre-B cell leukemias (22, 23). Mutations in the *Drosophila Exd* gene cause homeotic transformations, and *Caenorhabditis elegans Pbx* mutants display ectodermal and even some mesodermal phenotypes (24, 25). In vertebrates, the expression of *Pbx1* and the formation of Pbx/Hox complexes are found in developing neural tissue and in areas of mesenchyme-epithelial interaction (6, 7, 26, 27). Recently, a zebrafish *Pbx* gene was isolated (28, 29), and null mutants demonstrated that *Pbx* was critical to segmentation of the hindbrain and pharyngeal pouches (28). Moreover, zebrafish Pbx was shown to function in the same pathway as Meis during hindbrain development (30, 31).

We recently isolated a *Xenopus* homolog of *Meis1b*, an alternatively spliced form of *Xmeis1*. In ectodermal explants, overexpression of Xmeis1b induces expression of neural markers and neural crest marker genes in the absence of mesoderm. Moreover, misexpression of Xmeis1b in developing *Xenopus* embryos induces ectopic expression of neural markers and neural crest markers along the antero-posterior axis of the neural tube (32). Here, we describe a *Xenopus* Pbx1b gene, *Xpbx1b*, which can synergize with *Xmeis1b* to promote posterior neural markers and neural crest markers in embryonic tissue. We also show that a physical interaction between these two homeodomain proteins is necessary for posterior neural gene-marker induction in ectodermal explants. Moreover, coexpression of Xpbx1b and Xmeis1b leads to a relocation of the Xmeis1b protein from the cytoplasm to the nucleus. Finally, we demonstrate that introduction of the Engrailed repressor domain

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fused to the Xpbx1b protein inhibits posterior neural and neural crest gene expression during embryogenesis.

Materials and Methods

Isolation and Sequencing of the *Xenopus* Pbx1b cDNA and Generation of Mutants. A cDNA-encoding *Xenopus Pbx1b* was isolated from a *Xenopus laevis* stage-30 head cDNA library by using human P*bx1a* as a probe. From a full-length clone, a *Xmeis1b*-ΔM1 mutant (lacking amino acids 71–96), ΔM2 (lacking amino acids 148–161), and ΔM1/M2 (lacking amino acids 71–96 and 148–161) mutants were generated by PCR and inserted into pCS2+. *Engrailed* fusions were made by inserting either the *Xmeis1b* or *Xpbx1b* coding region into the 3′ end of the Engrailed repressor domain construct in pCS2+. Flag-tag was added at the C-terminal end of the *XPbx1b* or *Xmeis1b* coding regions by PCR and then inserted into pCS2+.

Embryos and Explants. Wild-type or albino *X. laevis* embryos were obtained by artificial insemination after induction of female with 300 units of human chorionic gonadotropin and microinjected as described (32). *Xenopus Pbx1b* and *XMeis1b* RNA were synthesized and injected, and explants were prepared and cultured, as described (32).

Northern Analysis. RNA from staged embryos was prepared with Trizol, as suggested by the manufacturer (Life Technologies, Rockville, MD). Seven micrograms of total RNA was separated in an agarose/formaldehyde gel (15). Radiolabeled probes were generated representing a 0.8-kb *Pst*I/Eco0109I restriction fragment of Xpbx1b coding region or a 1.8-kb restriction length fragment from the 3′UTR region of the *Xpbx1b* cDNA. An 18S ribosomal subunit template (Ambion) was used to generate a control probe.

Whole-Mount *in Situ* Hybridization. An N-terminal fragment lacking the homeodomain (277–972) of *Xpbx1b* was generated by PCR and was subcloned into pCS2+. The digoxygenin-labeled riboprobe was synthesized with T7 RNA polymerase. Plasmids containing *Xmeis1a* (32), *XNrp-1* (33), *Krox-20*, *XAp-2* (34), *Xslug* (35), *Xzic3* (36), and *Otx2* were linearized, and digoxygenin-labeled riboprobes were synthesized. *In situ* hybridization was performed as described (32). Photographs were taken with a dissecting microscope (Nikon SMZ 1500) and a charge-coupled device camera (Sony). For transverse sections, the embryos were embedded in paraffin after whole-mount *in situ* hybridization and dehydration, and 30-μm sections were cut and mounted on sialynated slides without counterstain.

Reverse Transcriptase (RT)-PCR Assay. Extraction of total RNA and RT-PCR assay, primer sequences, and conditions were performed as described (32), with the exception of *XAG-1* (37). These experiments were repeated three times for consistency.

Immunocytochemistry. The embryos injected with *Flag-tagged Xpbx1b* or *Flag-tagged Xmeis1b* RNA were cultured to stage 9 and fixed with MEMFA (0.1 M Mops, pH 7.4/2 mM EGTA/1 mM $MgSO_4$/4% paraformaldehyde) at 4°C overnight and dehydrated in ethanol. Paraffin-embedded blocks were serially sectioned at 7 μm. Slides were incubated in xylene followed by ethanol dehydration and PBS. FLAG M2 antibody at 1:500 was overlaid on the slides for 1 h at 37°C. After washing in PBS + 0.1% Tween-20 (PTW) for 1 h, peroxidase-conjugated anti-mouse antibody (1:500) was added, and slides were incubated for 1 h at 37°C. After washing in PTW, slides were immunostained with PBS + 0.2 mg/ml diaminobenzidine.

Immunoprecipitation and Western Blot Analysis. A peptide corresponding to the amino terminal 14 amino acid of Xpbx1b was synthesized, conjugated to keyhole limpet hemocyanin and used to immunize rabbits (Macromolecular Resources, Fort Collins, CO).

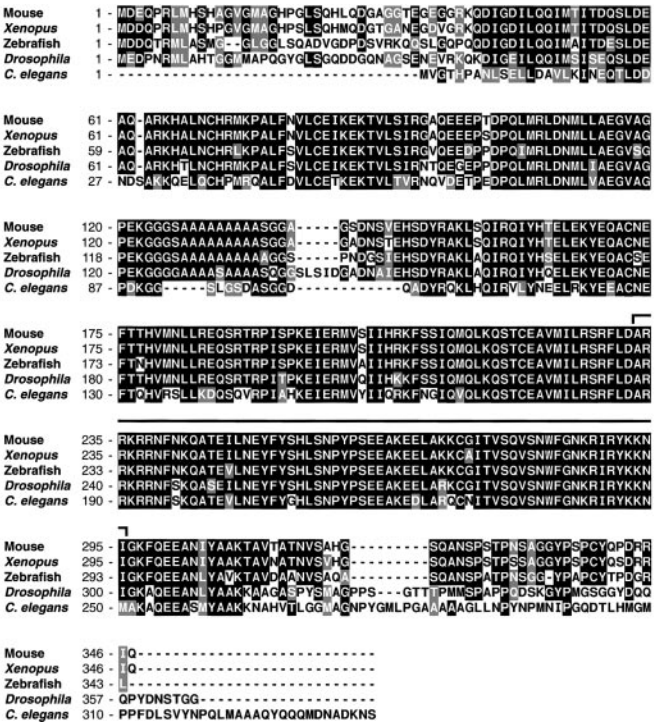

Fig. 1. Xpbx1b encodes a TALE family homeodomain-containing protein. Amino acid comparison of the coding sequences of *Xenopus* Pbx1b, Mouse Pbx1b, *Drosophila* extradenticle, Zebrafish Pbx 4(Lazarus), and *C. elegans* Pbx (Ceh-20). Identical residues are shaded in black, conservative differences are shaded in gray, and white represents nonhomology. The bracketed area above the sequence denotes the homeodomain region.

RNA-injected embryos were cultured until stage 10 and solubilized with lysis buffer [10 μl per embryo: 137 mM NaCl/20 mM Tris·HCl, pH 8.0/2 mM EDTA/1% (vol/vol) Nonidet P-40-containing protease inhibitors (Calbiochem)]. Immunoprecipitation analysis was performed on lysates from 15 embryos per sample, and immune complexes were separated by SDS/10% PAGE. Western analysis was performed as described (38) by using the indicated primary rabbit polyclonal antibodies at 1:1,000 dilution.

Results

Isolation and Expression Pattern of Xpbx1b. Previous results demonstrated that overexpression of Xmeis1b could induce ectopic expression of hindbrain and neural crest marker genes in ectodermal explants and developing *Xenopus* embryos. Because these data suggest that Xmeis1b may contribute to the execution of a posterior neural developmental program, we isolated the proposed *Xenopus* partner gene *Xpbx1b*. A human P*bx1a* cDNA was used to probe an embryonic stage-30 head cDNA library, and sequence analysis revealed that a full-length *Xpbx1b* cDNA was obtained. The *Xpbx1b* gene is extremely well conserved, displaying 95% and 80% amino acid identity to two vertebrate proteins, Pbx1b (mouse) and Lazarus (zebrafish Pbx4), respectively. Less homology was evident with regard to the invertebrate proteins *Drosophila* Exd (70% identity) and *C. elegans* Ceh-20 (60%). In contrast, the Pbx homeodomains displayed over 90% identity among all of the species (Fig. 1). We next examined the temporal pattern of *Xpbx1* mRNA expression during development with Northern blot analysis. A low level of *Xpbx1* RNA was observed in unfertilized eggs (data not shown), but subsequent stages revealed an expression pattern similar to *Xmeis1*, with increased expression during late gastrula through neurula and tailbud stages (Fig. 2*A*).

To determine the spatial expression of *Xpbx1* during devel-

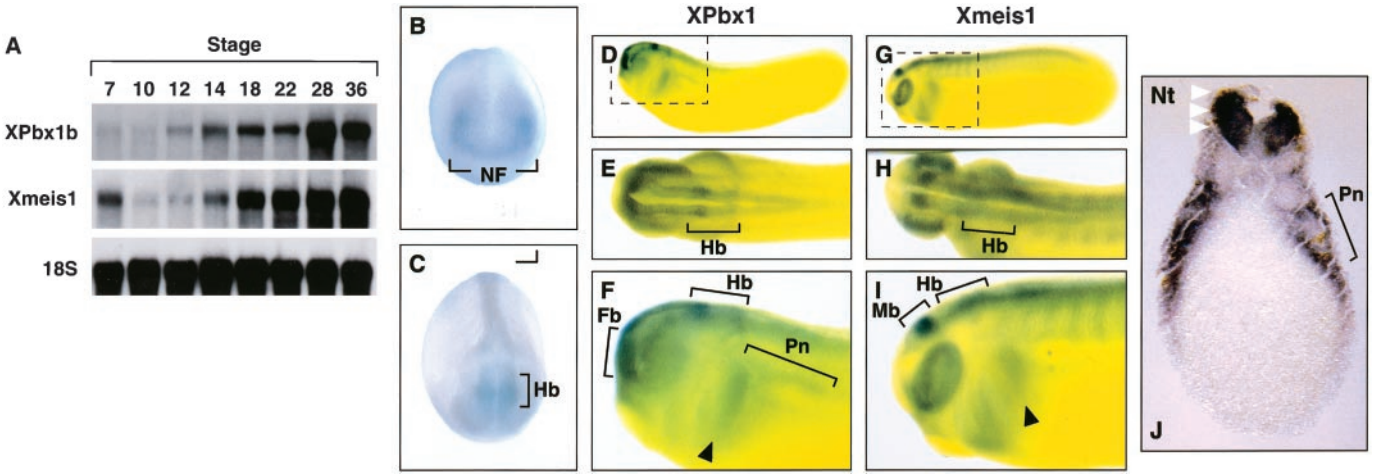

Fig. 2. Temporal and spatial distribution of *XPbx1b* and *Xmeis1* RNA expression during embryogenesis. (*A*) Northern blot analysis of RNA extracted from embryos at the indicated stages with *Xpbx1b* and *Xmeis1* specific probe. The 18S RNA probe was used as a loading control. Whole mount *in situ* hybridization analysis of the tissue distribution of *XPbx1b* (*B–F*) and *Xmeis1* (*G–I*) transcripts in *X. laevis* embryos. (*B*) Stage 15, anterior view. Note expression in lateral neural folds. (*C*) Stage 21, dorso-anterior view. Note strong staining in the presumptive hindbrain and along neural folds. (*D*) Lateral view of cleared stage-26 embryo. (*E*) Stage 26 dorsal view. (*F*) Enlarged lateral view of enclosure from *D*. Note the distinct *Xpbx1* staining in the forebrain, hindbrain, and caudal branchial arch. (*G*) Lateral view of cleared stage-25 embryo. (*H*) Stage 25 dorsal view. (*I*) Enlarged lateral view of enclosure from *G*. Note strong expression of *Xmeis1* in the midbrain, hindbrain, caudal and rostral branchial arches, and somites. (*J*) Transverse section through the pronephros of a stage-28 embryo. Note the strong *Xpbx1* staining in lateral edge of neural tube (white arrowheads); expression also is observed in the lateral mesoderm surrounding the pronephritic anlage. (*B–J*) Black arrowheads indicate caudal branchial arch. Black brackets denote the indicated tissues. Fb, forebrain; Hb, hindbrain; Mb, midbrain; NF, neural fold; Nt, neural tube; Pn, pronephros.

opment, whole-mount *in situ* hybridization was performed. Very faint broad expression was detected from the blastula through gastrula stages. At stage 14/15, *Xpbx1* expression is diffuse, appearing as a broad arc that will give rise to the forebrain and eyes (Fig. 2*B*). More intense staining is observed in the lateral neural folds (presumptive neural crest), and expression is also apparent as horizontal stripes in the posterior neural plate that will give rise to the hindbrain (Fig. 2*B*). Although *Xmeis1* expression at this stage has significant overlap with *Xpbx1, Xmeis1* is less broad, with more restricted expression in the lateral neural folds and presumptive hindbrain (32). As development proceeds, staining progresses posteriorly along the neural folds. At stage 21, expression is pronounced within the prospective hindbrain (Fig. 2*C*). A gap in *Xpbx1* expression is observed where the caudal portion of the hindbrain meets the rostral portion of the neural fold (Fig. 2*C*). Above this gap, expression is found in the lateral region of the neural folds, where migratory neural crest resides (Fig. 2*C*). At later stages (stage 26), *Xpbx1* expression becomes intense within the dorsal portion of the forebrain. Staining also is observed within the optic cup, caudal branchial arch, peripheral to the pronephric anlage, and in the dorsal anterior half of the spinal cord (Fig. 2 *D–F*, *J*). *Xpbx1* expression remains robust throughout the hindbrain but gradually becomes more restricted. At stage 26, two more intense stripes of expression are observed within the hindbrain, where one is more anterior and the other more posterior above the otic vesicle (Fig. 2 *D–F*). At stage 25, *Xmeis1* is found in the optic cup, somites, branchial arches, as well as in the midbrain, hindbrain, and the length of the spinal cord (Fig. 2 *G–I*). At late stages of development (stage 25/26), both *Xmeis1* and *Xpbx1* display divergent patterns of expression in the forebrain (*Xpbx1*), midbrain (*Xmeis1*), rostral branchial arches and somites (*Xmeis1)*, but also display distinct areas of overlap in the hindbrain, optic cup, caudal branchial arch, and rostral portion of the spinal cord (Fig. 2 *D–I*). Transverse sections through late stage-28 embryos confirm *Xpbx1* expression in the dorsal lateral portion of the neural tube (Fig. 2*J*). *Xpbx1* expression also is observed in the somatic layer of the lateral plate mesoderm that surrounds the pronephritic anlage (Fig. 2*J*). Collectively, the expression pattern suggests a possible interaction between *Xpbx1* and the *Xmeis1* binding partner in the patterning of posterior neural and neural crest-derived tissue in embryos.

Xpbx1b and Xmeis1b Cooperate to Induce Neural Markers in Explants. Pbx and Meis have been shown to form dimers, and these interactions are thought to regulate the activity of transcriptional complexes and thus affect developmental programs (4). Therefore, we tested whether Xpbx1b and Xmeis1b could have a functional influence on cell fate in embryonic ectodermal explants. We examined the expression of neural and mesodermal tissue markers by RT-PCR in animal pole explants from embryos injected with *Xpbx1b* or *Xmeis1b* RNA or both. Whereas the injection of 2.5 ng of *Xmeis1b* RNA was previously shown to induce posterior neural and neural crest markers (32), injection at low concentrations of *Xmeis1b* RNA (0.5 ng) does not induce any of these markers. Expression of *Xpbx1b* at high or low concentrations (0.5–2.5 ng) also was unable to induce mesodermal or neural markers. In contrast, when Xpbx1b was coexpressed with Xmeis1b, several neural and neural crest markers were induced. These markers included *N-CAM* (pan-neural marker), *N-tubulin* (pan-neuron marker), *Xtwist* (neural crest marker), *Krox-20* (hindbrain marker), *Hoxb9* (posterior neural marker; Fig. 3*A*), and *Xzic3* (a proneural gene that also promotes the earliest steps in neural crest development; ref. 36). Ectopic *Krox-20*, *Xslug*, and *N-tubulin* expression also was observed in whole embryos (data not shown). In contrast to the prominent induction of posterior neural and neural crest markers, only a very modest effect on *XAG-1* (cement gland) and *Otx-2* (anterior neural) expression was observed in animal caps (Fig. 3*A*). Neither *Xbrachyury* (early mesoderm) nor *muscle actin* (late mesoderm) transcripts were induced by these products (data not shown, Fig. 3*A*). These results indicate that the interaction between Xpbx1b and Xmeis1b leads to the induction of posterior neural cell fate markers in the absence of mesoderm.

Physical Interaction Between Xpbx1b and Xmeis1b Is Critical for Inductive Activity. To test whether a direct interaction between Xmeis1b and Xpbx1b was necessary for the conversion of ectodermal tissue to a posterior neural cell fate, we generated

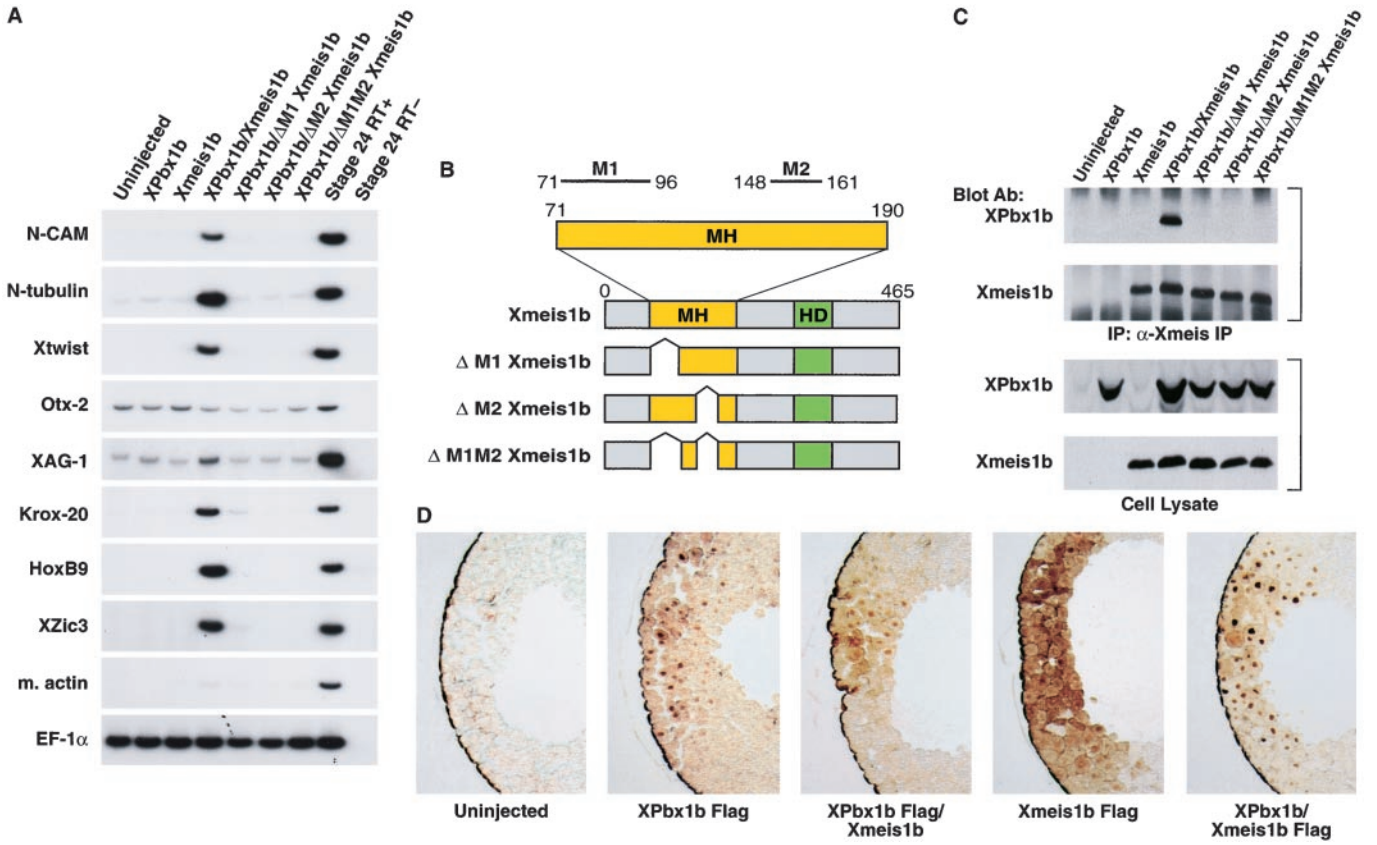

Fig. 3. Interaction of *XPbx1b* and *Xmeis1b* induces posterior neural and neural crest markers in animal cap explants in the absence of mesoderm. (*A*) The animal pole region of two-cell stage embryos were injected with either *XPbx1b* RNA (1.0 ng per embryo), *Xmeis1b* RNA (0.5 ng per embryo), or both RNAs. Animal pole explants were excised at stage 9 and cultured until stage 26. RT-PCR gene analysis was performed for *N-CAM* (pan-neural), *N-tubulin* (pan-neuronal), *Xtwist* (neural crest), *Otx-2* (forebrain), *XAG-1* (cement gland), *Krox-20* (hindbrain), *HoxB9* (spinal cord), *Xzic3* (proneural and early neural crest marker), *muscle actin* (dorsal mesoderm), and *EF-1a* (loading control). Stage-24 embryonic RNA with or without reverse transcriptase (RT+ or RT−) also was used as a positive and negative reaction control. Note that coinjection of *XPbx1b* and *Xmeis1b* RNA induced the expression of posterior neural and neural crest cell markers. (*B*) Schematic representation of the Xmeis1b mutants. Wild-type Xmeis1b consists of a Meis-Homothorax domain (MH, yellow box) and homeodomain (HD, green box). MH domain possesses two subdomains: M1 box (amino acids 71–96) and M2 box (amino acids 148–161) that are important for Pbx binding. Deletions of one or more subdomains are indicated. (*C*) XPbx1b and Xmeis1b physically interact. The animal pole region of two-cell stage embryos were injected with either *XPbx1b* (2.5 ng per embryo) or *Xmeis1b* (2.5 ng per embryo) RNA alone or were coinjected with *XPbx1*b and *Xmeis1b* wild-type and mutant RNAs. Embryos were cultured until stage 9, and embryonic extracts were prepared and either directly immunoblotted (*Lower*) or immunoprecipitated with an anti-Xmeis1 antibody before immunoblotting (*Upper*). Anti-Meis1 antibody or anti-Pbx1b antibody was used to detect the indicated protein. Note: the Xpbx1b protein was coimmunoprecipitated with the wild-type Xmeis1b but not with any of the Xmeis1b mutants. (*D*) Nuclear localization of the Xmeis1b protein depends upon the XPbx1b protein in frog embryonic cells. The animal pole region of two-cell stage embryos was injected with either *Flag tagged XPbx1b* (2.5 ng per embryo) or *Flag-tagged Xmeis1b* RNA (2.5 ng per embryo) alone or in combination with *Xmeis1b* or *XPbx1b* RNA (2.5 ng per embryo). Injected embryos were fixed at stage 9, embedded, sectioned, and immunostained with anti-Flag antibody. Note that Xmeis1b protein was in the cytoplasm when expressed alone but was localized in the nuclei in the presence of XPbx1b. In contrast, XPbx1b was localized in the nuclei regardless of whether exogenous Xmeis1b was present.

forms of Xmeis1b with reduced binding activity to Xpbx1b (Fig. 3*B*). These mutants are termed ΔM1, ΔM2, and ΔM1/M2, depending upon whether the first, second, or both N-terminal Pbx1-binding sites have been removed (Fig. 3*B*). Coexpression of the mutant RNAs with *Xpbx1b* RNA in ectodermal explants did not induce any of the neural genes examined. One exception was ΔM1; it displayed a very weak ability to induce the pan-neural gene *N-CAM*, the posterior neural gene *Krox-20*, and the neural crest gene *Xtwist* (Fig. 3*A*). These data strongly suggest that the physical binding of Xpbx1b and Xmeis1b is necessary for the neural and neural crest inductive activities of these two proteins.

To confirm that the three Xmeis1 mutants were impaired in their ability to physically interact with Xpbx1b, immunoprecipitation and Western analysis was performed on the cell lysates from embryos expressing these proteins (Fig. 3*C*). Immunoprecipitation with an Xmeis1 polyclonal antibody revealed that Xpbx1b coimmunoprecipitated only with the wild-type Xmeis1b molecule and not with the M1, M2, and M1/M2 mutants (Fig. 3*C*). These data are consistent with the idea that Xpbx1b and Xmeis1b must be stably associated to affect neural cell fate (Fig. 3*C*). Further, whereas the interaction between Hth and Exd is essential for the mutual stabilization of both proteins in *Drosophila*, western analysis shows that this interaction is not necessary for the accumulation of either Xpbx1b or Xmeis1b when expressed in *Xenopus* embryos (Fig. 3*C*).

Xpbx1b Affects Localization of Xmeis1b. In *Drosophila,* the interaction between Hth and Exd triggers the nuclear localization of Exd, thus allowing for functional activation of the protein complex (8, 10–14). Because the Pbx1 protein is nuclear in proximal cells of the mouse limb and cytoplasmic in distal cells (20, 39), we examined whether a similar mechanism was present in *Xenopus* embryos (Fig. 3*D*). *Xenopus* embryos were injected with RNA encoding a *Flag-tagged* version of *Xpbx1b*, either alone or along with *Xmeis1b* RNA. Immunocytochemistry showed that Xpbx1b localized to the nucleus in the absence or presence of

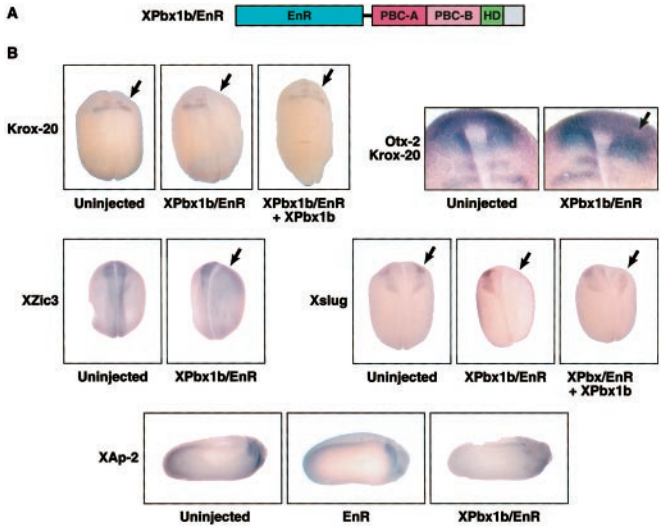

Fig. 4. XPbx1b/EnR suppresses posterior neural and neural crest markers during embryonic development. (*A*) Schematic representation of XPbx1b protein fused with an Engrailed repressor domain. The three major conserved domains with vertebrate Pbx1 and *C. elegans* Ceh-20; PBC-A, PBC-B, and the homeodomain (HD) also are indicated. (*B*) One blastomere of two-cell stage embryos was either injected with *XPbx1b/EnR* RNA (0.75 ng per embryo) alone or coinjected with wild-type *XPbx1b* RNA (2.5 ng per embryo), as indicated. Engrailed control embryos were injected with *EnR* RNA (2.5 ng per embryo) as noted. Embryos were cultured until stage 16 (*Xslug* images), stage 18 (*Krox-20*, *Krox-20*/*Otx-2,* and *Xzic3* images), or stage 24 (*Xap-2* images). Embryos were fixed, and *in situ* hybridization was performed by using the indicated probe. Arrow indicates injected side of embryo. Note that XPbx1b/EnR suppressed *Krox-20* (hindbrain), *Xzic*3 (proneural and neural crest), *Xslug* (neural crest), and *Xap-2* (neural crest) expression on the injected side, but *Otx-2* (anterior neural) expression remained. Also note that coexpression of wild-type Xpbx1b rescued *Xslug* and *Krox-20* expression.

exogenously expressed Xmeis1b. The reciprocal experiment also was performed where *Flag-tagged Xmeis1b* RNA was injected either alone or with *Xpbx1b* RNA. Interestingly, when Xmeis1b is expressed alone, it localizes mostly in the cytoplasm, with only a small fraction in the nucleus. In contrast, coexpression of Xmeis1b and Xpbx1b leads to very distinct and prominent nuclear localization of Xmeis1b. These data suggest that nuclear transport or retention of Xmeis1b may depend upon Xpbx1b.

Xpbx1b Fused to the Engrailed Repressor Inhibits Expression of Posterior Neural Markers and Neural Crest Markers. Interactions between Pbx, Meis, and Hox homeodomain-containing proteins have been postulated to play key roles in their activities as transcriptional regulators. Although Hox proteins contain transcriptional activation or repressor domains, Meis (5, 6) and Pbx1 (40) seem to lack such domains. To determine whether the posterior neural and neural crest gene induction observed by the synergistic action of Xpbx1b and Xmeis1b were the result of transcriptional activation or repression, Engrailed–repressor domain fusions were generated (Fig. 4*A*). One blastomere of two-cell embryos was injected with RNA (2.5 ng) encoding wild-type and/or *Engrailed* fusion versions (/EnR) of *Xpbx1b. In situ* hybridization analysis showed that ectopic expression of Xpbx1b induced a modest expansion of *Krox-20* and *Xslug* expression (data not shown) but to a lesser degree than previously reported for Xmeis1b alone (32). In contrast, Xpbx1b/EnR protein resulted in the loss of *Krox-20* mRNA expression in the hindbrain (78%; $n = 46$), whereas the expression of *Otx-2* (an anterior neural marker) in the forebrain remained unaffected (Fig. 4*B*). Various neural markers were examined in early (stages 16 and 18) and late stage (stage 24) embryos expressing Xpbx1b/EnR. *Xzic3* (proneural and neural crest) and *Xslug* (a transcriptional repressor critical for neural crest development; refs. 41 and 42) were both inhibited on the injected side of the embryos [Fig. 4*B*; 72% ($n = 32$) and 81% ($n = 46$), respectively]. The uninjected side displayed a normal *Xzic3* or *Xslug* expression pattern in the neural folds of the embryo and thus acts as a control. In stage-24 embryos, Xpbx1b/EnR markedly reduced *XAp-2* (cranial neural crest) expression and showed a low level of *XAp-2* staining in the remaining dysmorphic neural crest-derived branchial arches. A similar inhibition of posterior neural and neural crest markers also was observed with an *Xmeis1b/EnR* construct (data not shown). Embryos expressing only the Engrailed repressor domain showed normal *XAp-2* expression and pharyngeal structure, demonstrating the specificity of the effect. Collectively, these data indicate that the inhibitory effects induced by Xpbx1b/EnR show specificity toward posterior neural markers and neural crest markers but not an anterior neural marker.

Rescue experiments were undertaken as an additional test of the specificity of the Xpbx1b/EnR repressor activity (Fig. 4*B*). Embryos injected with only *Xpbx/EnR* RNA (0.75 ng) displayed a loss of *Krox-20* (53%, $n = 25$) or *Xslug* expression (64%, $n = 25$). In contrast, coinjection of wild-type *Xpbx1b* RNA (2.5 ng) significantly reduced the loss of *Krox-20* (18%, $n = 28$) and *Xslug* (15%, $n = 27$) expression induced by *Xpbx/EnR*. Collectively, these data show that Xpbx1b is a member of a transcriptional activator complex that is important for proper expression of posterior neural and neural crest genes.

Discussion

In this report, we isolate the *Xenopus Pbx1b* cDNA and show that it has broad expression in neural tissue during embryonic development. Although *Xpbx1* expression overlies the reported *Xmeis1* pattern in the neural fold (Fig. 2 *B* and *C*), there is also overlap at later stages in the hindbrain, optic cup, caudal branchial arch, and dorsal lateral portions of the neural tube (Figs. 2 *D–J*; ref. 32). The *Xpbx1b* and *Xmeis1b* expression patterns along with the coexpression studies reveal a possible link to hindbrain and neural crest development. There are also locations where these two proteins are less likely to interact. For example, although *Xmeis1* and *Xpbx1* are both expressed quite strongly in the prospective hindbrain region, only *Xmeis1* is expressed prominently in the midbrain, and only *Xpbx1* is expressed robustly in the forebrain. Thus, a role in hindbrain patterning or posterior neural development is consistent with the opportunity for interaction between these two homeodomain proteins. *Lazarus* mutants (*Pbx4*) in zebrafish have hindbrain patterning defects and display defects in cranial neural crest segmentation (28). The *Pbx1* knockout mouse also shows severe phenotypic effects in the caudal branchial arches (43).

Although *Xpbx1b* and *Xmeis1b* can induce some of the early players in neural crest development (*Xslug, Xzic3*), it is possible that they play a role later in neural crest development. For example, *Xpbx1b* and *Xmeis1b* may exert their influence as the crest cells populate the caudal branchial arches, where strong expression is observed. In addition to presenting evidence that Xpbx1 and Xmeis1 may have the opportunity to interact, we show that the interaction between Xpbx1b and Xmeis1b is essential for the induction of posterior neural and neural crest markers in ectodermal explants. Xmeis1b mutants lacking the M1 (amino acids 71–96) and M2 (amino acids 148–161) boxes were unable to form stable interactions with Xpbx1b in embryos or induce posterior neural markers in explants. The ability of the Xpbx1b and Xmeis1b proteins to form heterodimers allows for complex formation by using various combinations of homeodomain proteins and thus an increased level of complexity in gene regulation of developmental processes. Recent work on the *D. rerio Pbx4* gene (*lazarus)* supports a role for Pbx in neural crest development, where *lazarus* mutants have defects in the segmentation of cranial neural crest (28). Recent evidence indicates

that expression of the Meis protein partially rescues the mutant *lazarus* phenotype in zebrafish, suggesting Meis functions in the same pathway as Pbx (31). Although it is clear that Xpbx1b and Xmeis1b are collaborative partners in the phenotypic and cell-fate effects described here, it is unclear exactly how their interaction facilitates these events.

In *Drosophila* (8, 10–14) and mouse (39), Hth or Meis are required to retain Exd or Pbx1 in the nucleus. In our experiments, immunocytochemistry demonstrated that exogenously expressed Xpbx1b localized to the nucleus in the absence of ectopic Xmeis1b. These data suggest that *Xenopus* Pbx1b may not require interaction with Xmeis1b for nuclear localization. In contrast, coexpression of Xmeis1b and Xpbx1b leads to a dramatic shift in Xmeis1b localization from the cytoplasm to the nucleus. This result is consistent with a similar transport mechanism reported for zebrafish Pbx4 and Meis3 (30). Collectively, these data suggest that Xpbx1b may function as a nuclear transporter of Xmeis1b, and that the synergistic induction of neural markers by the coexpression of Xpbx1b and Xmeis1b in animal caps may depend on this nuclear transport activity. A recent study in zebrafish also reports that coexpression of Meis and Pbx4 causes mutual stabilization of both proteins (31). We have not observed a significant increase in Xmeis1b accumulation when these proteins are coexpressed in *Xenopus* embryos, but there is a small reproducible increase in Xpbx1b (Fig. 3*C*). We also have observed an Xmeis1-induced stabilization of Xpbx1b protein in *Xenopus* oocytes (data not shown), suggesting that such a mechanism may exist in a context-dependent manner.

Although the coexpression of Xpbx1b and Xmeis1b induced posterior neural markers in ectodermal explants and embryos, it was still unclear whether the two proteins were functioning through a transcriptional activator or repressor complex. Therefore, an Engrailed repressor fusion construct was generated for Xpbx1b and then expressed in developing embryos. Xpbx1b/EnR blocks neural crest and posterior neural markers, but not an anterior marker (*Otx-2*; Fig. 4*B*) nor a pan-neural marker (*Nrp-1*; data not shown). These data support the idea that Xpbx1b plays a role in posterior neural development and argues against possible secondary effects caused by inhibiting central nervous system development. The Xpbx1b/EnR repressor activity is rescued by coexpression of wild-type Xpbx1b, demonstrating the specificity of the effect. These data are consistent with the Xpbx1b protein having a role in posterior neural development rather than anterior neurogenesis during early development. Moreover, the Xmeis1b/Xpbx1b complex along with unknown Hox partners may function as a transcriptional activator in this process. A model has been proposed in which the Hox-Pbx complex can act as a repressor or activator of transcription by means of association with corepressors or coactivators (39). These associations are suggested to be a direct determinant of Hox-Pbx function in the patterning of the animal embryo (39). Clarification of how the Xpbx1b/Xmeis1b complex plays a role in the process of hindbrain and neural crest development awaits the isolation of cooperating factors and direct target genes of the Xmeis1b/Xpbx1b complex.

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