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# *Pbx1/Pbx2* requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and *Shh* expression

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Vertebrate limb development occurs along three cardinal axes – proximodistal, anteroposterior and dorsoventral – that are established via the organization of signaling centers, such as the zone of polarizing activity (ZPA). Distal limb development, in turn, requires a molecular feedback loop between the ZPA expression of sonic hedgehog (*Shh*) and the apical ectodermal ridge. The TALE homeoprotein Pbx1 has been shown to be essential for proximal limb development. In this study, we first uncover that *Pbx1* and *Pbx2* are co-expressed in the lateral plate and early limb field mesoderm. Later, *Pbx2* is expressed throughout the limb, unlike *Pbx1*, which is expressed only in the proximal bud. By exploiting a *Pbx1/Pbx2* loss-of-function mouse model, we demonstrate that, despite the lack of limb abnormalities in *Pbx2*-deficient (*Pbx2*$^{-/-}$) embryos, compound *Pbx1*$^{-/-}$*; Pbx2*$^{+/-}$ mutants, in addition to their exacerbated proximal limb defects, exhibit novel and severe distal abnormalities. Additionally, we reveal that *Pbx1*$^{-/-}$*; Pbx2*$^{-/-}$ embryos lack limbs altogether. Furthermore, we establish that, unlike in flies, where the leg develops independently of Hox and where the Pbx ortholog *Exd* is required for specification of proximal (but not distal) limbs, in vertebrates, distal limb patterning is *Pbx1/Pbx2* dependent. Indeed, we demonstrate that Pbx genetic requirement is mediated, at least in part, through their hierarchical control of Hox spatial distribution and *Shh* expression. Overall, we establish that, by controlling the spatial expression of Hox genes in the posterior limb and regulating ZPA function, *Pbx1/Pbx2* exert a primary hierarchical function on Hox genes, rather than behaving merely as Hox ancillary factors.

**KEY WORDS: *Pbx1/Pbx2*, Hox, *Shh*, Limb development, Distal limb patterning, Mouse**

## INTRODUCTION

Vertebrate limb development occurs along three cardinal axes: proximodistal (PD), anteroposterior (AP) and dorsoventral (DV) (reviewed by Niswander, 2003; Tickle, 2003). These axes are established through molecular networks, beginning when the limbs bud out of the flank and continuing with the organization of signaling centers, such as the zone of polarizing activity (ZPA) in the posterior limb mesenchyme (reviewed by Pearse and Tabin, 1998). Continued outgrowth and patterning of the distal limb requires a molecular feedback loop between the ZPA and a strip of specialized epithelium at the apex of the limb, known as the apical ectodermal ridge (AER). Crucial to this interaction is the expression of sonic hedgehog (*Shh*) in the ZPA (Riddle et al., 1993) and of fibroblast growth factors (Fgfs) in the AER (Sun et al., 2002). Shh signaling establishes the limb AP axis (Chiang et al., 2001) and leads to the formation of distal elements of the posterior zeugopod (i.e. ulna and fibula) and autopod (Harfe et al., 2004).

As of yet, the molecular triggers that activate *Shh* expression are mostly unknown, although a few candidate genes have been proposed. For example, *Hand2* (previously known as *dHand*) has been shown to act upstream of *Shh* as *Hand2*-deficient (hereafter, *Hand2*$^{-/-}$) embryos lack *Shh* expression (Charite et al., 2000). Hox genes (reviewed by Krumlauf, 1994; Capecchi, 1997; Deschamps and van Nes, 2005) have been more recently proposed as upstream regulators of *Shh* (Zakany et al., 2004) and of AP and PD axis formation (Kmita et al., 2005). For example, prior to *Shh* onset, early Hox colinear expression in limb mesenchyme leads to their transcript accumulation in the ZPA (Zakany et al., 2004; Tarchini and Duboule, 2006), while their ectopic expression leads to *Shh* transcription (Charite et al., 1994; Stratford et al., 1997; Knezevic et al., 1997). Finally, in vertebrates, functional ablation of multiple 5′ Hox leads to distal limb truncations partially mediated by *Shh* absence (Kmita et al., 2005).

Although early Hox colinearity appears essential for *Shh* onset, their dynamic expression throughout limb development aids in patterning elements along the PD axis. Indeed, patterning alterations occur in limb skeletal elements of specific developmental modules in mice where multiple paralogous Hox genes have been genetically ablated, suggesting a functional redundancy within paralogous groups (Condie and Capecchi, 1994; Davis et al., 1995; Davis and Capecchi, 1996; Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b; Wellik and Capecchi, 2003). These findings highlight Hox role as global regulators of patterning throughout limb development.

The present understanding of Hox function is that they act partially through the aid of co-factors, such as Pbx TALE homeoproteins (Burglin, 1997; Burglin, 1998), that increase Hox DNA-binding specificity and selectivity (reviewed by Mann and Chan, 1996; Mann and Affolter, 1998; Moens and Selleri, 2006). Pbx proteins, when forming complexes with Hox, are further known to transcriptionally regulate Hox genes themselves (Popperl et al., 1995; Maconochie et al., 1997; Jacobs et al., 1999; Ferretti et al.,

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2000). However, recent findings strongly suggest that Pbx can also function more broadly in Hox-independent manners (Knoepfler et al., 1999; Berkes et al., 2004) (reviewed by Mann and Morata, 2000; Moens and Selleri, 2006).

Recently, we elucidated roles of *Pbx* genes in skeletal development and found that although *Pbx1* is required for proximal limb patterning (Selleri et al., 2001), *Pbx2* or *Pbx3* loss does not determine skeletal or limb phenotypes (Selleri et al., 2004; Rhee et al., 2004). Specifically, in *Pbx1*$^{-/-}$ embryos, skeletal structures of girdles (i.e. scapula and pelvis) and proximal limb stylopod (i.e. humerus and femur) that normally express nuclear *Pbx1* at early developmental stages, are malformed, while their distal elements and joints appear normal. These findings parallel the role of *Exd* (the *Drosophila* Pbx ortholog) (Peifer and Weischaus, 1990; Rauskolb et al., 1993) in governing proximal domains of the fly appendage, where its expression is restricted (Mercader et al., 1999).

In this study, we first uncover that *Pbx1* and *Pbx2* are co-expressed in the early vertebrate limb field and that, later, *Pbx2* is expressed throughout the limb mesenchyme, while *Pbx1* is expressed only proximally. Next, by exploiting a *Pbx1/Pbx2* loss-of-function mouse model, we determine that, despite the lack of skeletal and/or limb abnormalities in *Pbx2*$^{-/-}$ embryos (Selleri et al., 2004), decreasing *Pbx2* dose in the absence of *Pbx1* does affect limb development more severely than the loss of *Pbx1* alone (Selleri et al., 2001). We demonstrate that compound *Pbx1*/*Pbx2* embryos, in addition to their proximal limb defects, exhibit novel and severe distal limb abnormalities; *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ embryos display loss of distal hindlimb elements, whereas *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ embryos lack hindlimbs altogether. We establish that in vertebrates distal limb patterning is genetically regulated by *Pbx1*/*Pbx2*, at least in part, through their hierarchical control of Hox spatial distribution and *Shh* expression.

## MATERIALS AND METHODS

### Mice

Intercrosses of *Pbx1*$^{+/-}$(Selleri et al., 2001) and *Pbx2*$^{+/-}$ (Selleri et al., 2004) were conducted to obtain *Pbx1*$^{+/-}$;*Pbx2*$^{+/-}$ mice. On a C57BL/6 background, the number of *Pbx1*$^{+/-}$;*Pbx2*$^{+/-}$ obtained was below the expected Mendelian ratio. To increase their number, we crossed C57BL/6 *Pbx1*$^{+/-}$;*Pbx2*$^{+/-}$ males to an outbred strain, Black Swiss [NIH-BL(S)], and observed a slight amelioration of the limb phenotype that remained fully penetrant. We then intercrossed these C57BL/6 females and mixed C57BL/6-Black Swiss males and analyzed the progeny for lethality and soft/hard tissue morphologies.

### Skeletal preparations

Differential staining of cartilage and bone in whole mouse embryos (E12.5 and E13.5) was visualized using Alcian Blue and Alizarin Red (Selleri et al., 2001).

### Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed on somite-matched embryos at different gestational days using digoxigenin or fluorescein-labeled antisense RNA probes as described (Di Giacomo et al., 2006). These analyses were performed on all compound genotypes, including key controls (*Pbx1*$^{+/-}$;*Pbx2*$^{-/-}$ and *Pbx1*$^{-/-}$;*Pbx2*$^{+/+}$), but only shown for those genotypes that displayed alterations in gene expression.

### Whole mount immunohistochemistry

Mouse embryos were dissected at E10.5, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and processed for immunohistochemistry to detect CD44 localization (Sherman et al., 1998). Embryos were permeabilized in 0.1%TritonX-100/PBS, blocked in 3%BSA/0.1%TritonX-100/PBS and then incubated in anti-CD44 (antibody IM7 rat monoclonal, Pharmingen) overnight. The embryos were washed in 0.1% TritonX-100/PBS and incubated with anti-rat Alexa 568 (Molecular Probes-Invitrogen) overnight. After rinsing, embryos were visualized with a fluorescence microscope.

### Chromatin immunoprecipitation (ChIP)

Formaldehyde cross-linking and ChIP (Aparicio et al., 1999) from E10.5 mouse limbs were performed according to described protocols (Orlando et al., 1997), with the following modifications: to disaggregate tissues, samples were forced through 18G needles, cells were fixed for 15 minutes with 1% formaldehyde at room temperature and reactions were quenched with 0.125 M glycine in PBS for 5 minutes. Crosslinked samples were sonicated for 15×25 seconds to obtain average fragment lengths of 500-1000 bp. Immunoprecipitation was performed with 10 μl of protein G-Agarose (KPL) and blocked twice with 1 μg/ml salmon sperm DNA (Sigma) and 1 μg/ml bovine serum albumin, for 2 hours, then overnight. Chromatin was precleared by adding 20 μl of protein G-Agarose for two hours, and incubated with 5 μg of the respective specific antisera, or with 5 μg of anti-Flag (F3165, Sigma) antibody as a control. Incubations were performed overnight at 4°C. These primers were used for PCR amplifications (42 cycles): mouse *Shh*E1, 5′-CTTTGATTTGAAGTCCTGGC-3′; mouse *Shh*E2, 5′-ACTGAGGGGAAAAGTCATC-3′; mouse *Shh*C1, 5′-TCAA-GAGAGATCAACAAAAG-3′; mouse *Shh*C2, 5′-TTGGACTCAAGT-CCAGAC-3′.

### Cell culture and transfection

P19 mouse embryonal carcinoma cells (McBurney and Rogers, 1982) were cultured in media (MEMA, GIBCO, Life Technologies) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Transfections were performed by $CaPO_4$ precipitation. In a typical experiment, reporter plasmid (5 mg), expression construct (2.5-5.0 μg) and CMV-βgal (Clontech) (0.1 μg) as an internal control, were used per 6 cm dish. Forty-eight hours after transfection, cells were washed, lysed and assayed for luciferase and β-galactosidase expression (Zappavigna et al., 1994).

## RESULTS

### Loss of *Pbx1*/*Pbx2* causes not only proximal but also distal limb defects

*Pbx1*$^{+/-}$;*Pbx2*$^{+/-}$ mice were intercrossed to generate progeny with different compound genotypes. Axial and limb skeletal abnormalities were evident only in *Pbx1*$^{-/-}$;*Pbx2*$^{+/+}$, *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ and *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ embryos. *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ embryos died at E9.5/10.5 during the period crucial for limb bud initiation, owing to multiple organogenesis defects, and therefore lacked limbs. Thus, our analyses focused on limb development in *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ (hereafter, *Pbx1/2* mutant) embryos (Fig. 1), which survived until E13.5/14.5. *Pbx1/2* mutant embryos exhibited drastic exacerbations of *Pbx1*$^{-/-}$ axial and proximal limb defects (Selleri et al., 2001) and novel distal phenotypes (Fig. 1A,B). Fore- and hindlimb development in *Pbx1/2* mutant embryos were differently affected. For example, although E9.5 *Pbx1/2* mutant forelimbs already appeared malformed, E10.5 hindlimbs still exhibited relatively normal morphology (Fig. 1A), despite the typical half-day delay in development. However, by E13.5, gross morphology of both limbs was severely affected; specifically, forelimbs displayed shortened zeugopodia and hypoplastic autopodia, whereas hindlimbs appeared rudimentary and lacked true autopodia (Fig. 1B).

Analysis of skeletal development in *Pbx1/2* mutant embryos demonstrated that in both limbs, proximal and distal elements were malformed and/or lacking (Fig. 2). Specifically, by E12.5, expression of *Sox9*, a marker for mesenchymal condensations (Wright et al., 1995), revealed that *Pbx1/2* mutant (Fig. 2B) forelimbs displayed only three rudimentary digital condensations, while hindlimbs (Fig. 2D) lacked digital mesenchyme altogether. Furthermore, in hindlimbs, condensations for fibulae were missing. Alcian Blue staining of early cartilaginous elements at E12.5 more clearly demonstrated proximal fore- and hindlimb defects (see Fig. 2E-H). By E13.5, *Pbx1/2* mutant forelimbs were dysmorphic, but possessed most of their elements, whereas their hindlimbs exhibited

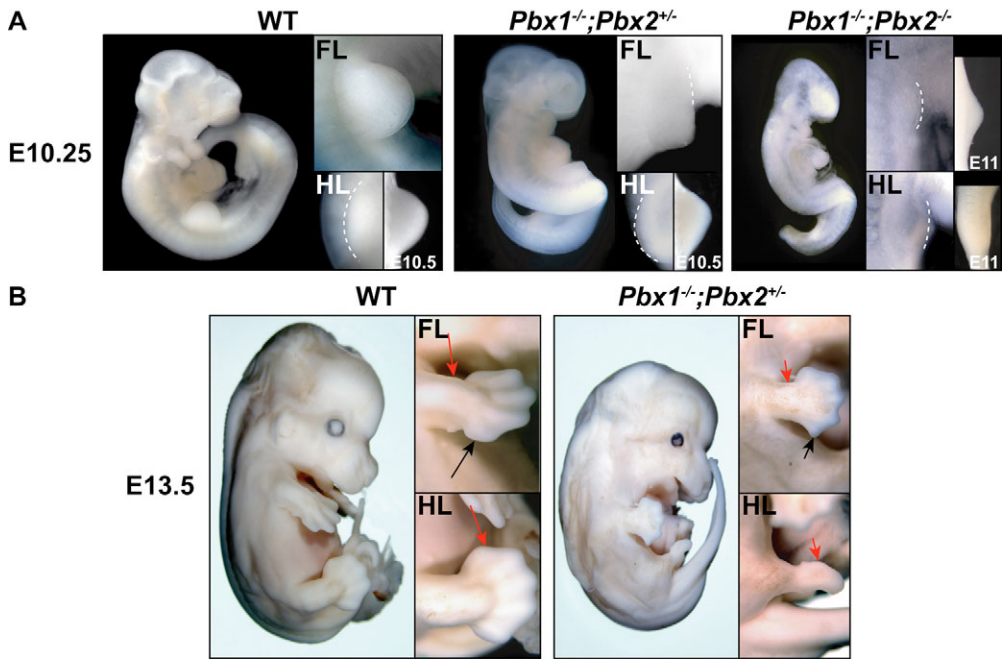


**Fig. 1. Abnormal morphology of *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ and *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ embryos.** (**A**) At E10.25 (and E10.5), *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ embryos exhibit abnormal morphology and appear delayed. Their forelimbs are PD shortened and AP dysmorphic, while their hindlimbs appear relatively normal compared with wild type. *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ embryos are severely delayed compared with wild type, exhibiting striking morphological abnormalities, including the lack of limbs. Enlargements of all wild-type and mutant limbs are shown on the right-hand side. In addition, both wild-type and *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ hindlimbs are shown at two gestational days in enlargements on the far right, whereas *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ limbs are shown at E11 to demonstrate rudimentary forelimb bud formation (lower magnification). (**B**) At E13.5, *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ embryos are smaller than wild type with abnormal morphology. Forelimbs display shorter zeugopodia (short red arrow) and dysmorphic autopodia (short black arrow) compared with wild type (long red and black arrows, respectively); hindlimbs exhibit rudimentary autopodia (short red arrow) compared with wild type (long red arrow). Proximal (left); anterior (top). FL, forelimb; HL, hindlimb.

striking abnormalities, including loss of distal elements (Fig. 2I-N). *Pbx1/2* mutant forelimbs exhibited mis-shapened scapulae that were fused to skeletal elements, probably representing duplicated proximal humeral heads. In addition, humeri had shafts that were thickened and contorted, with cartilaginous anlagen mimicking hypertrophic deltoid tuberosities, more severely than in *Pbx1*$^{-/-}$;*Pbx2*$^{+/+}$ (Fig. 2I-K). Although both forelimb zeugopod elements were present, albeit malformed, digits one and five were missing or rudimentary (Fig. 2K). In *Pbx1/2* mutant embryos, pelvic girdles were missing most elements except isolated anlagen reminiscent of ischia (Fig. 2L-N). These residual pelvic elements were fused to truncated thickened femurs more severely than in *Pbx1*$^{-/-}$;*Pbx2*$^{+/+}$ (Fig. 2L-N). The only remaining hindlimb zeugopodial element appeared as a dysmorphic tibia, whereas its fibula was absent, as were most autopodial elements (except for single rudimentary digital rays) (Fig. 2N).

### *Pbx1*/*Pbx2* early mesenchymal expression is overlapping and during later limb development is dynamic and, in part, complementary

To determine how specific spatiotemporal differences in Pbx limb expression underlie such abnormalities, we performed in situ hybridization on wild-type embryos with *Pbx1*- and *Pbx2*-specific probes. From E8.5-9.0, we detected both *Pbx1* and *Pbx2* in lateral plate-intermediate mesoderm (Fig. 3A,E,I) and early limb fields (Fig. 3B,F,J). From E9.25-E11, *Pbx1* was then present in the flank and forelimb proximal mesenchyme (Fig. 3C,K and Fig. 4A,C), while at E9.25 in the hindlimb field it still colocalized with *Pbx2* (Fig. 3D,H,L). By contrast, *Pbx2* remained throughout forelimb (E9.25-11; Fig. 3G,K and Fig. 4B,C) and hindlimb mesenchyme (E9.5-11; Fig. 4B,C), but was particularly intense distally. From E11 to E12, *Pbx1* and *Pbx2* expression then changed dynamically and became restricted to the anterior and posterior mesenchyme proximal to the autopod (Fig. 4A-C). We also analyzed *Pbx1* expression in *Pbx2*$^{-/-}$ limbs, and *Pbx2* expression in *Pbx1*$^{-/-}$ and *Pbx1/2* mutant limbs. *Pbx1* displayed a slightly broader expression domain but remained proximal (data not shown), while *Pbx2* (data not shown) expression remained unaltered in these experiments, thus indicating that *Pbx1* and *Pbx2* do not markedly control each other's expression.

### In *Pbx1/2* mutant limbs, early anterior and posterior mesenchymal patterning remains preserved

Starting at early gestational days prior to *Shh* onset, a mutual genetic antagonism has been shown to exist between *Gli3* in the anterior limb mesenchyme and *Hand2* in the posterior mesenchyme. In addition, *Hand2* has been shown to restrict *Alx4* anteriorly (te Welscher et al., 2002a; te Welscher et al., 2002b). These genetic interactions subdivide the early limb into anterior and posterior domains. At E10.5, we found normal *Alx4* and *Gli3* expression in *Pbx1/2* mutant hindlimbs (Fig. 5A), indicating that the anterior and posterior domains of the hindlimb mesenchyme were initially preserved. Similarly, *Hand2* expression resembled that of *Alx4* and *Gli3*, although it was slightly reduced proximally. Finally, although marked morphological alterations were observed

in *Pbx1/2* mutant forelimbs prior to E10.5 (Fig. 1A), no gross perturbations of *Alx4*, *Hand2* and *Gli3* expression were detected (data not shown).

### *Pbx1/Pbx2* are required for normal *Shh* expression in the limb bud ZPA

Given the similarities between the distal hindlimb phenotypes of *Pbx1/2* mutant and $Shh^{-/-}$ embryos (Chiang et al., 2001; Kraus et al., 2001), we examined *Shh* expression in *Pbx1/Pbx2* limbs. Strikingly, *Shh* was never detected in *Pbx1/2* mutant hindlimbs from E9.5 to E13.5, as represented at E10.5 and 11.5 (Fig. 5B). This absence could not be attributed to either a global *Shh* downregulation or to a delay in posterior embryonic development, as both *Pbx1/2* mutant cloaca and notochord developed normally and displayed normal *Shh* expression (see Fig. S1A in the supplementary material). Interestingly, we observed low levels of *Ptch1* and *Gli1* (readouts of hedgehog signaling) (reviewed by Niswander, 2003) at E10.75 (Fig. 5C), suggesting that a minimal or transient wave of *Shh* activity probably occurred. In contrast to *Pbx1/2* mutant hindlimbs, in forelimbs *Shh* expression was present, albeit at reduced levels, and *Ptch1 and Gli1* were expressed (see Fig. S1B in the supplementary material), indicating that *Shh* absence was hindlimb specific.

We next found a relatively preserved CD44 distribution (Sherman et al., 1998) in *Pbx1/2* mutant hindlimb AER, despite slight anterior reductions within smaller limbs (data not shown). This finding indicates that *Pbx1/2* mutant hindlimb AER development proceeded normally, at least until E10.5, and thus was unlikely to be responsible for *Shh* absence. This result was corroborated by our findings that AER-specific markers, such as *Dlx6* (Robledo et al., 2002), remained unperturbed in *Pbx1/2* mutant posterior hindlimbs (data not shown). Overall, these data demonstrate that the posterior AER

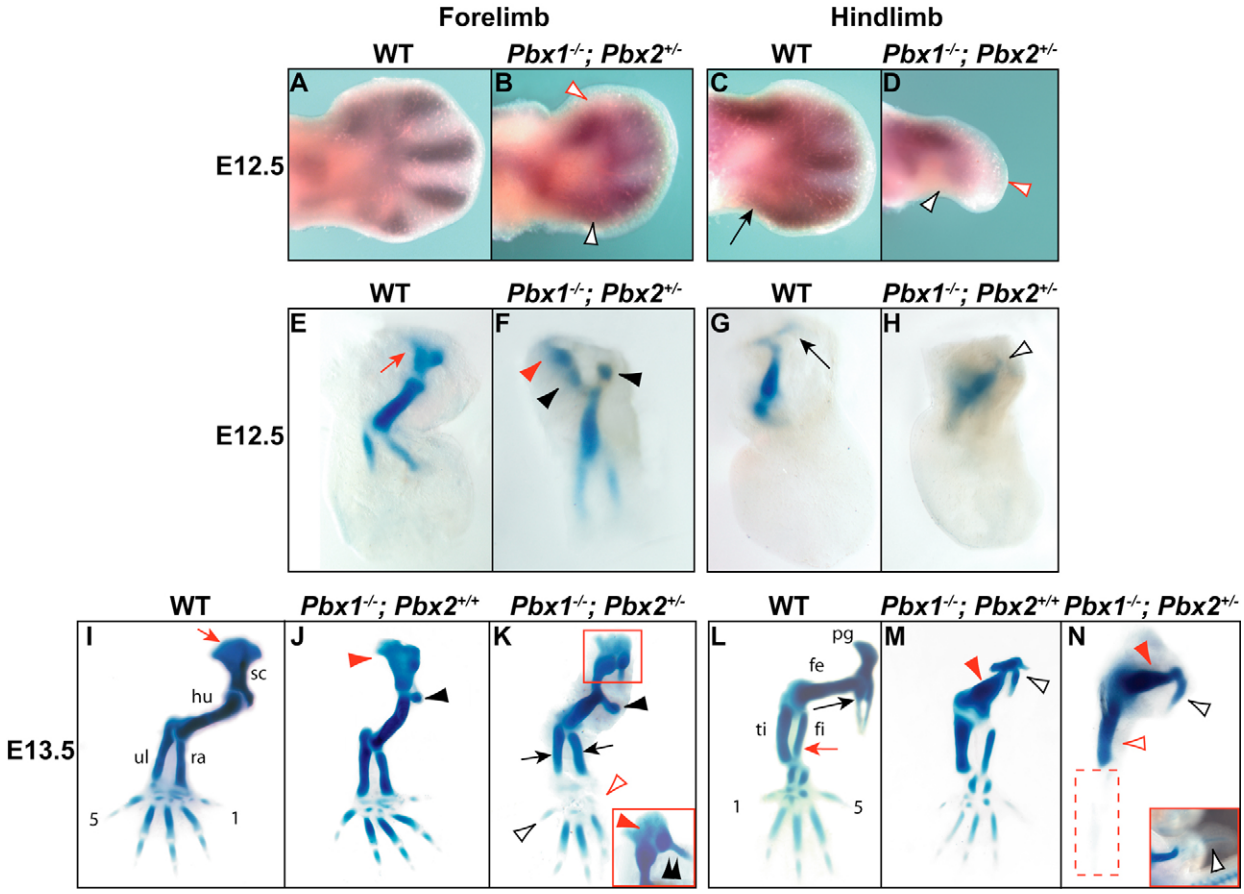


**Fig. 2. Limb skeletal phenotypes of *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ (mutant) embryos.** (**A-D**) At E12.5, *Sox9* expression indicates that mutant forelimb (B) mesenchymal condensations for digits 1 (empty red arrowhead) and 5 (empty black arrowhead) are rudimentary compared with wild type (A). In mutant hindlimbs (D), digit (red empty arrowhead) and fibula condensations (empty black arrowhead) are absent compared with wild type (C) (black arrow). (**E-H**) At E12.5, unlike wild type (E) (red arrow), mutant scapular (F) anlagen is reduced (red arrowhead) and the humerus is dysmorphic with a hypertrophic anlagen reminiscent of a deltoid tuberosity (F) (black arrowheads). In mutant (H), the pelvis is virtually absent, except for one element (empty black arrowhead) that may be a rudimentary ischium (G) (compare with wild type, long black arrow). (**I-N**) At E13.5, unlike wild type (I, red arrow), mutant scapula (K; inset) is dysmorphic (red arrowhead) and the humeral head is fused to it and duplicated medially (double black arrowhead), more severely than in *Pbx1*$^{-/-}$;*Pbx2*$^{+/+}$ mutants (J) (arrowheads). In mutant hindlimbs (N), only a rudimentary anlagen reminiscent of an ischium (open black arrowhead) remains fused to the dysmorphic femur (red arrow). This phenotype is more severe than in *Pbx1*$^{-/-}$;*Pbx2*$^{+/+}$ mutants (M) (arrowheads). The mutant forelimb (K) zeugopod (ra and ul) appears shortened (short black arrows), while the mutant hindlimb (N) zeugopod (ti and fi) lacks the fibula (open red arrowhead) when compared with wild type (I and L, respectively) (L, red arrow). The double mutant forelimb (K) autopod exhibits three central digits, with digits 1 (open red arrowhead) and 5 (open black arrowhead) absent or rudimentary. The mutant hindlimb (N) autopod lacks all digits (dashed red rectangle) except one rudimentary ray (open black arrowhead; inset) compared with wild type (L). fe, femur; fi, fibula; hu, humerus; pg, pelvic girdle; ra, radius; sc, scapula; ti, tibia; ul, ulna.

was preserved before *Shh* onset. Conversely at E11.5, after *Shh* initiation, *Fgf8* expression was patchy in *Pbx1/2* mutant hindlimbs (Fig. 5B) and resembled that reported for *Shh*$^{-/-}$ embryos (Chiang et al., 2001; Kraus et al., 2001).

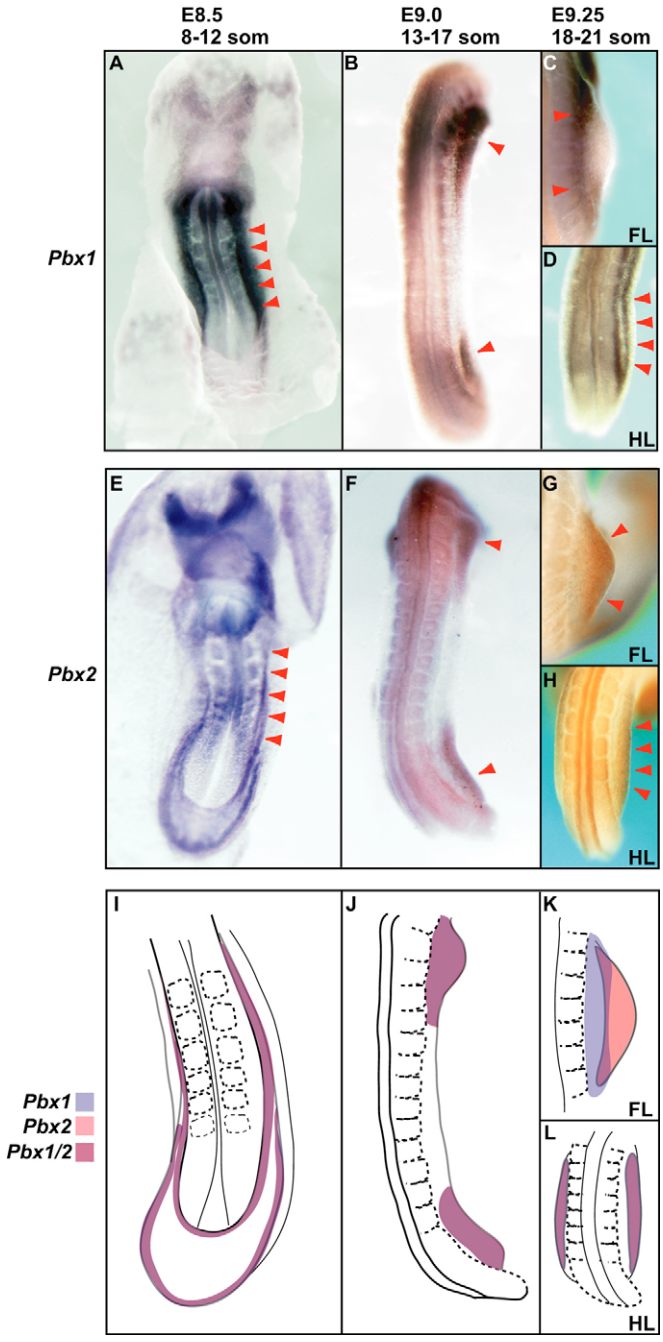


**Fig. 3. *Pbx1* and *Pbx2* colocalize in lateral plate-intermediate mesoderm and early limb fields.** (**A**,**E**,**I**) At E8.5, *Pbx1* (A) and *Pbx2* (E) colocalize (I) in lateral plate-intermediate mesoderm (red arrowheads). (**B**,**F**,**J**) At E9, *Pbx1* (B) and *Pbx2* (F) colocalize (J) in the early limb fields (red arrowheads). (**C**,**G**,**K**) At E9.25, although overlapping proximally (K), *Pbx1* (C) and *Pbx2* (G) forelimb expression domains become mostly complementary, being proximal and distal, respectively (red arrowheads). (**D**,**H**,**L**) At E9.25, *Pbx1* (D) and *Pbx2* (H) hindlimb expression domains remain overlapping (L) (red arrowheads). Proximal is leftwards; anterior is towards the top. som, somites.

Intriguingly, normal proximal limb identity and outgrowth were observed (at E11) in *Pbx1/2* mutant hindlimbs, as *Meis2*, a proximal mesenchymal marker (Capdevila et al., 1999), remained unchanged (Fig. 5C). Last, analyses of gremlin (Scherz et al., 2004), *Hand2* and *Gli3* (te Welscher et al., 2002a; te Welscher et al., 2002b) at later days (Fig. 5D) further confirmed *Shh* absence in *Pbx1/2* mutant hindlimbs, with the latter two expression patterns resembling those observed in *Shh*$^{-/-}$ (Chiang et al., 2001; Kraus et al., 2001) and *Hoxa/Hoxd*-deficient (Kmita et al., 2005) embryos. Overall, these findings establish that *Pbx1/Pbx2* are required in the limb mesenchyme for *Shh* expression.

### *Pbx* and *Hand2* probably control *Shh* through parallel pathways; *Shh* and *Pbx1/Pbx2* do not form a cross regulatory loop

*Shh* absence is intriguing, as *Hand2,* which has been shown to act upstream of *Shh* (Charite et al., 2000), remained unaltered in *Pbx1/2* mutant limbs (Fig. 5A). However, we found that *Pbx* expression remained mostly unperturbed in *Hand2*$^{-/-}$ forelimbs, although *Pbx1* was slightly broadened posteriorly (see Fig. S2A in the supplementary material). Accurate hindlimb analyses were hampered by the poor growth and posterior developmental delay in *Hand2*$^{-/-}$ embryos, as well as their lethality at E10.5 (Charite et al., 2000). Given that *Hand2*, *Shh* and Hox appear to form a regulatory loop in limb development, wherein *Hand2* lies up- and downstream of *Shh* (Zakany et al., 2004), we also examined if *Pbx1/Pbx2* and *Shh* form a similar regulatory loop. Analyses in *Shh*$^{-/-}$ embryos revealed relatively unperturbed *Pbx1/Pbx2* expression (see Fig. S2B in the supplementary material), despite their abnormal limb morphology and marked apoptosis in distal domains (Chiang et al., 2001).

### Hox gene expression, prior to and after, onset of *Shh* expression is spatially perturbed or absent in *Pbx1/2* mutant limbs

Recent findings have indicated that Hox expression in limb mesenchyme is crucial for *Shh* activation (Zakany et al., 2004). Given the lack of *Shh* in *Pbx1/2* mutant hindlimbs and its marked downregulation in forelimbs, we examined *Hoxa/Hoxd* expression in compound mutant limbs. Intriguingly, all analyzed Hox genes were severely reduced or absent in future *Shh*-expressing domains of early *Pbx1/2* mutant hindlimbs at E10.25-10.5, despite their relatively normal morphology (Fig. 6A; *Hoxd10* data not shown). By contrast, despite their abnormal expression, most Hox genes remained expressed in *Pbx1/2* mutant forelimb ZPA (data not shown). In addition, in *Pbx1/2* mutant forelimbs, multiple Hox genes were either up-regulated (e.g. *Hoxa9* and *Hoxd9*), severely downregulated, or absent (e.g. *Hoxa13* and *Hoxd13*), suggesting a multifaceted, hierarchical control of Hox by Pbx. Finally, Hox expression was unaltered in limbs of all other compound mutant genotypes (data not shown).

Given the suggested regulatory loop, wherein *Shh* regulates digit patterning via its control of autopod-specific 5′ Hox gene reverse colinearity, Hox gene expression was also examined at E11.5. Interestingly, 5′ Hoxd gene expression was reduced in *Pbx1/2* mutant hindlimb anterior domains, while 5′ Hoxa gene expression remained normal (Fig. 6B; *Hoxa10*, *Hoxd10* and *Hoxd12*, data not shown). This altered Hoxd gene expression pattern is similar to that reported in *Shh*-deficient limbs (Chiang et al., 2001). Additionally, *Pbx1/2* mutant forelimbs displayed only slight anterior alterations of 5′ Hoxd gene, but not 5′ Hoxa gene, expression (Fig. 6C; *Hoxa10*, *Hoxd10* and *Hoxd12*, data not shown), coincident with the

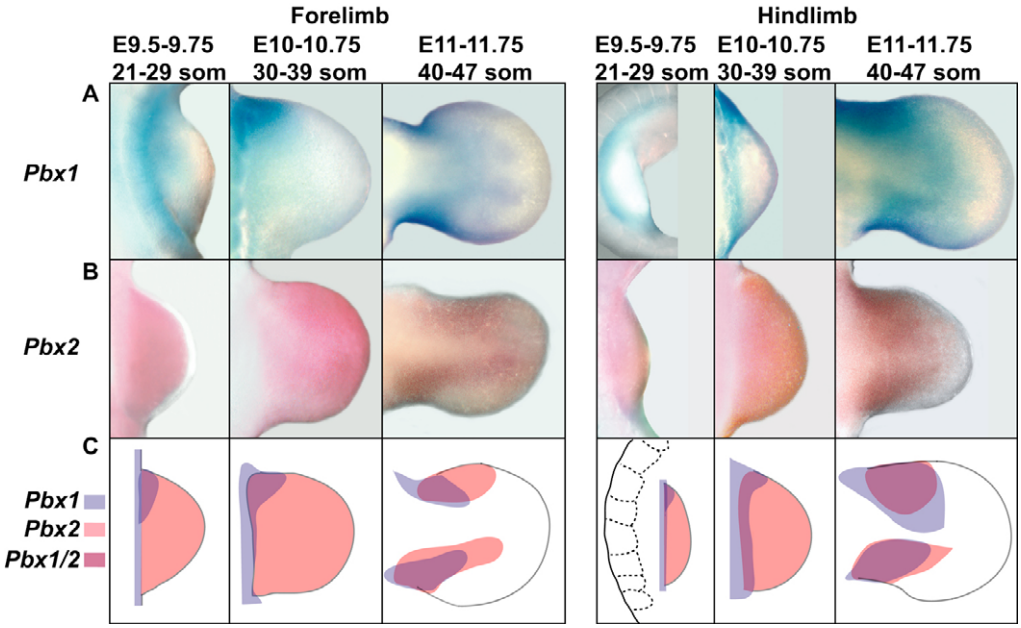


**Fig. 4. *Pbx1* and *Pbx2* mesenchymal expression in later limb development is dynamic and, in part, complementary.** (**A**,**B**) At E9.5-10.75, *Pbx1* is expressed in proximal forelimbs and hindlimbs, mostly anteriorly, whereas *Pbx2* is expressed distally and throughout the limb. At E11-11.75, *Pbx1* is still expressed proximally in limbs, but localized anteriorly and posteriorly in the mesenchyme proximal to the autopod. Similarly, *Pbx2* expression becomes restricted to this domain, albeit more broadly. (**C**) Diagrams depicting dynamic expression of *Pbx1*, *Pbx2* and overlapping domains of *Pbx1*/*Pbx2* at different gestational days. These schemes represent summaries of the expression patterns present at the gestational time-points indicated by the somite range at the top of each panel. Proximal is leftwards, anterior towards the top. som, somites.

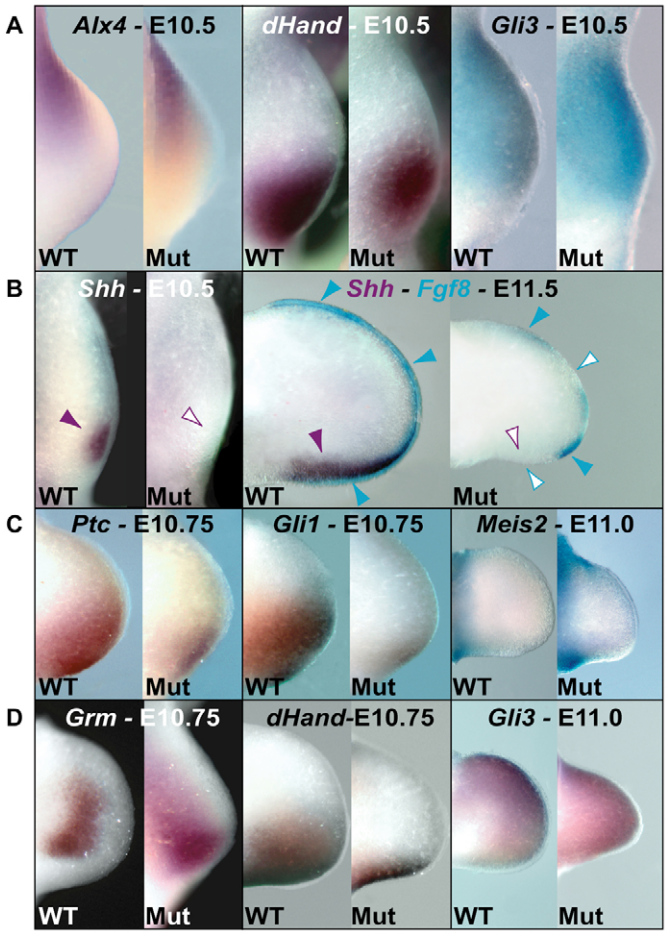


**Fig. 5. Mesenchymal gene expression in *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ (mutant) hindlimbs.** (**A**) Expression of *Alx4*, *Hand2* and *Gli3* is relatively unperturbed in mutant hindlimbs, before and/or at *Shh* onset, indicating that until E10.5 the mesenchyme is patterned similar to wild type. (**B**) *Shh* expression is absent throughout mutant hindlimb development at E10.5 and E11.5. At E11.5, *Shh* absence (empty purple arrowhead) in mutant hindlimbs is associated with a similar and expected alteration in *Fgf8* expression (empty blue arrowheads), compared with wild-type hindlimbs (normal *Shh* expression indicated by solid purple arrowhead and *Fgf8* by blue arrowheads). (**C**) Although reduced, expression of *Ptch1* and *Gli1* is present (E10.75), suggesting minimal and transient *Shh* activity. *Meis2* is unperturbed in mutant hindlimbs (E11.0), suggesting that proximal limbs remain relatively intact. (**D**) *Grm* is extended posterodistally in mutant hindlimbs (E10.75), indicating *Shh* absence in the ZPA (Scherz et al., 2004). *Hand2* expression is reduced (E10.75) and *Gli3* expression is diffused throughout mutant hindlimbs (E11), resembling expression patterns in mice that lack Hoxa/Hoxd (Kmita et al., 2005) and suggesting an AP patterning defect in the absence of sustained *Shh* activity. Proximal is leftwards; anterior is towards the top.

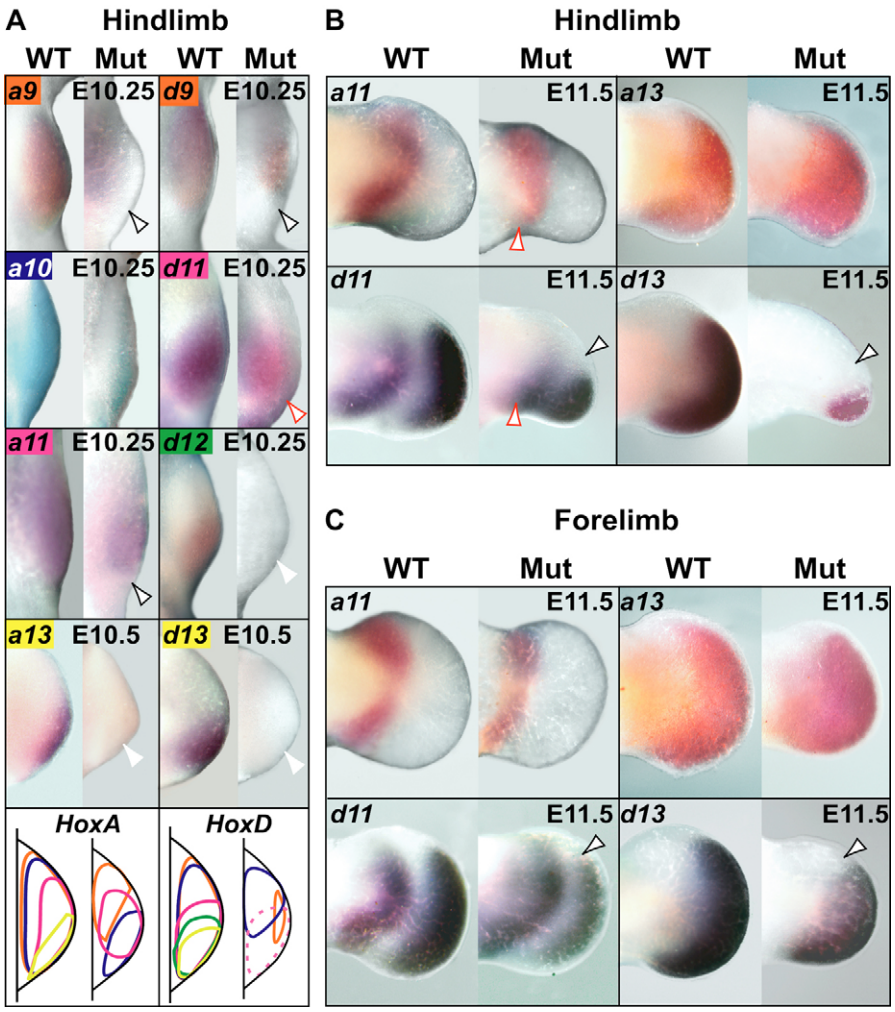


**Fig. 6. Hox gene expression is altered in *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ (mutant) limbs.** (**A**) Hox gene expression (E10.25-10.5) is absent or reduced in somite-matched mutant hindlimb posterior domains before, or at, *Shh* onset, despite their relatively normal morphology. Open black arrowheads indicate lack of posterior expression (*Hoxa9*, *Hoxa11*, *Hoxd9*). Open red arrowhead indicates reduced posterior expression (*Hoxd11*). White arrowheads indicate absence of expression (*Hoxa13*, *Hoxd12, Hoxd13*). Diagrams depict summaries of expression data across two time-points (E10.25 and E10.5): Hox gene expression domains are represented by the same colors displayed in their respective in situ hybridization panels. Dashed domains in mutant indicate reduced *Hoxd11* (pink) expression, whereas missing domains reflect complete absence of expression (*Hoxa13*, *Hoxd12, Hoxd13*). Blue domain in Hoxd diagram represents *Hoxd10* expression. (**B**) Distal 5′ Hoxa (*Hoxa11* and *Hoxa13*) expression (E11.5) remains relatively unperturbed in mutant hindlimbs, while Hoxd (*Hoxd11* and *Hoxd13*) expression is severely reduced anteriorly (open black arrowheads). Open red arrowheads indicate perturbed proximal posterior expression (*Hoxa11* and *Hoxd11*). (**C**) 5′ Hoxa (*Hoxa11* and *Hoxa13*) expression (E11.5) remains relatively unperturbed in mutant forelimbs, whereas 5′ Hoxd (*Hoxd11* and *Hoxd13*) expression is reduced anteriorly (open black arrowheads). Proximal is leftwards; anterior is towards the top.

observed reduction in *Shh* expression. Therefore, in the context of reduced *Pbx1/Pbx2* dose, the mechanisms underlying the spatial regulation of Hoxa versus Hoxd expression differed during autopod development.

## Hox proteins bind in vivo to, and activate transcription through, the *Shh* long-range limb enhancer

To ascertain whether *Shh* expression was directly regulated by Hox and/or Pbx proteins in developing limbs, we tested for their in vivo binding to an evolutionarily conserved *Shh* regulatory region (*Shh*E), which was shown to drive the correct spatiotemporal expression of *Shh* within developing mouse limbs (Lettice et al., 2003). We performed chromatin immunoprecipitation (ChIP) analyses (Orlando et al., 1997) on E10.5 mouse limb cells, using specific antisera against Hoxd10, Hoxd13 and Pbx (Fig. 7A). The enrichment for genomic DNA fragments containing the *Shh*E sequence was verified by PCR amplification. As shown (Fig. 7A), significant enrichment was observed for anti-Hoxd10 and anti-Hoxd13 antisera in forelimb and hindlimb immunoprecipitated chromatin, indicating that these Hoxd proteins are indeed bound to the *Shh*E in vivo. No enrichment was observed using a non-specific control antibody (Fig. 7A). Using an antiserum against all Pbx isoforms, Pbx protein was also found to bind in vivo to the *Shh*E in limb cells (Fig. 7A). The specificity of the Hoxd10, Hoxd13 and Pbx binding to the *Shh*E was further verified by the lack of enrichment on a genomic region located upstream to this enhancer (Fig. 7A). Finally, as an additional control, no binding was detected for Hoxd13 to the *Shh*E at E13.5 when *Shh* expression had ceased in limbs (Fig. 7B).

To determine which Pbx family member colocalized with *Shh* in limbs we performed in situ hybridization and immunohistochemistry. Notably, *Pbx2* was the only *Pbx* that colocalized with *Shh* in the ZPA, as it was present throughout the limb (Fig. 4B). By contrast, two-color in situ hybridization revealed that *Pbx1* did not colocalize with *Shh*, even in the absence of *Pbx2* (Fig. 7C). These results were corroborated by immunohistochemistry, whereby Pbx1 and Pbx2 proteins were detected on adjacent limb sections after *Shh* expression had been visualized by in situ hybridization (data not shown). Finally, we recently uncovered that *Pbx3* is not expressed in hindlimbs (Di Giacomo et al., 2006) and reported findings demonstrated that *Pbx4* is present only in testes (Wagner et al., 2001). In conclusion, *Pbx2* is the only *Pbx* family member that colocalizes with *Shh*, and thus is responsible for the observed binding to the *Shh*E.

The functional significance of the Hoxd10, Hoxd13 and Pbx2 binding to the *Shh*E was next tested in transient transfection assays using P19 embryonal carcinoma cells. A luciferase reporter construct (pT81*Shh*E) was generated, which contained a 745 bp fragment representing the complete *Shh*E (Lettice et al., 2003). Co-transfection of the *Shh*E reporter with Hoxd9, Hoxd10 and Hoxd13 expression constructs resulted in significant transcriptional

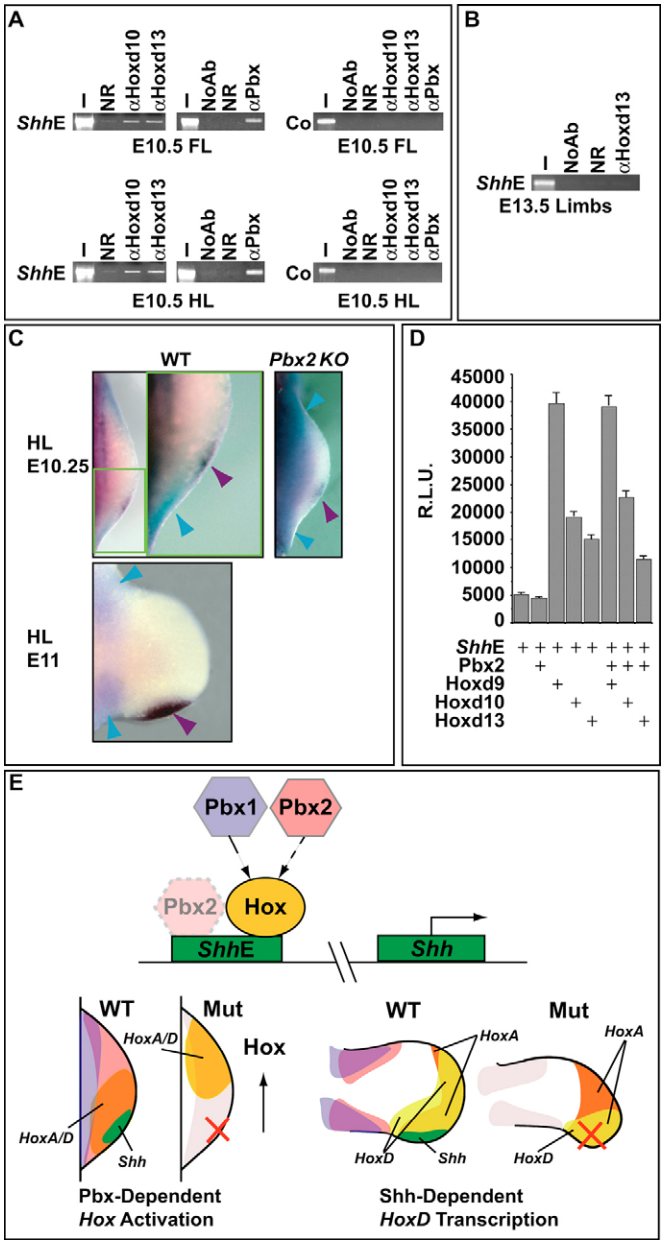


**Fig. 7. 5′ HoxD proteins are bound to and directly activate transcription from the *Shh* limb enhancer (*Shh*E).** (**A**) ChIP analyses on E10.5 mouse fore- and hindlimbs, using specific anti-Hoxd10 (αHoxd10), anti-Hoxd13 (αHoxd13) and anti-Pbx (αPbx) antisera, demonstrate their direct in vivo binding to the mouse *Shh*E. A 424 bp fragment of the *Shh*E was amplified by PCR. No PCR amplification of a 373 bp negative-control region (Co) located 1400 bp upstream of this enhancer was detected using E10.5 limb chromatin. Representative reactions of all PCR amplifications, carried out in triplicate, are shown. (**B**) ChIP on pooled E13.5 limbs using the αHoxd13 antiserum revealed no amplification of the *Shh*E. (**C**) At E10.25 and E11, *Pbx1* (blue) and *Shh* (purple) mRNA transcripts do not colocalize in wild-type and *Pbx2*$^{-/-}$ (KO) hindlimbs. (**D**) HoxD proteins can activate transcription from the *Shh*E (eightfold for Hoxd9, fourfold for Hoxd10 and threefold for Hoxd13) in P19 embryonal carcinoma cells. Luciferase activity, in arbitrary units (R.L.U.), was assayed from extracts of transiently transfected P19 cells. Co-transfection assays were performed in the presence (+) of the indicated expression vectors encoding Pbx2, Hoxd9, Hoxd10 and Hoxd13, and with a luciferase reporter construct (pT81m*Shh*E) containing the *Shh*E. Bars represent the mean±s.e.m. of at least four independent experiments. (**E**) Simplified model (top) depicts the overlapping genetic hierarchical roles of *Pbx1* and *Pbx2* in controlling Hox gene spatial distribution and Hox recruitment to the limb *Shh*E. In addition, Pbx2, albeit not functionally essential within this network, is also recruited to this enhancer in limb cells. Intensity of the hue of Pbx2 correlates with its functional relevance. Lower left diagram illustrates the requirement of *Pbx1* (violet), *Pbx2* (rose) and *Pbx1/Pbx2* (purple) for controlling the positioning of 5′ Hox genes (i.e. Hoxa/Hoxd, orange) to future *Shh*-expressing domain (green). In *Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$ (mutant) limbs, Hox gene expression remains external to the ZPA, and *Shh* activation does not occur (red cross). Lower right diagram depicts our refinement of a previous model (Zakany et al., 2004) and highlights that *Shh* loss, owing to early *Pbx1/Pbx2* perturbation, leads to partial disruption of 5′ Hoxd reverse colinearity (yellow), but not of 5′ Hoxa expression (deep orange). Proximal is towards the left; anterior is towards the top. Co, control; FL, forelimb; HL, hindlimb; I, input chromatin; NR, non-specific control antibody; NoAb, no antibody-control resin.

activation above the reporter's basal activity in this system (Fig. 7D). Conversely, Pbx2 co-transfection had virtually no effect on the reporter's activity (Fig. 7D) and its co-transfection with Hoxd9, Hoxd10 or Hoxd13 did not significantly alter their activation of the reporter (Fig. 7D). These findings are consistent with the non-essential function of Pbx2 throughout embryonic development (Selleri et al., 2004). Taken together, our results show that Hoxd proteins can bind in vivo to the limb *Shh*E and can activate transcription through this regulatory element in cultured cells, suggesting that they directly control *Shh* expression.

## DISCUSSION

### *Pbx1/Pbx2* are required for proximal and distal limb patterning

Our results establish that, although *Pbx1* is required for proximal limb patterning (Selleri et al., 2001), *Pbx1/Pbx2* share overlapping functions in proximal and distal limb development. In this respect, the fundamental roles of *Pbx1/Pbx2* are underscored by our finding that *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ mutants do not form limbs. Nevertheless, by decreasing *Pbx2* dose to one allele on a *Pbx1*-null background we were able to generate a mouse model where limb development could be studied in depth. Accordingly, *Pbx1/2* mutant embryos died earlier in utero than *Pbx1*$^{-/-}$ embryos and had limbs with more drastic skeletal defects. Furthermore, compared with those present in *Pbx1*$^{-/-}$ embryos, axial skeletal defects were also worsened in *Pbx1/2* mutant embryos (data not shown). Overall, these results establish that *Pbx1/Pbx2* genetically interacts in axial and appendicular skeletal development.

We observed that the distal *Pbx1/2* mutant forelimb and hindlimb morphologies varied. In the forelimb, the ulna and most digits were present, albeit malformed, whereas in the hindlimb, the fibula and most digits were lost. Although limb-specific expression levels of *Pbx1/Pbx2* could explain this variability, we never detected gross differences in their expression by in situ hybridization or immunohistochemistry. Instead, the less severe distal *Pbx1/2* mutant forelimb phenotype is more consistent with our finding that *Pbx3* is expressed only in forelimbs up to E11.5 (Di Giacomo et al., 2006). Indeed, in *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ mutants that survive to E11, rudimentary forelimbs, but not hindlimbs, develop (Fig. 1A), coincident with their *Pbx3* expression.

Of importance, except for *Pbx1*$^{-/-}$ (Selleri et al., 2001), *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ and *Pbx1/2* mutant embryos, all other compound genotypes lacked limb defects and alterations in gene expression. These findings highlight the primary role of *Pbx1* and the crucial impact that *Pbx1*/*Pbx2* spatiotemporal expression patterns have on limb development. Indeed, the early colocalization of *Pbx1* and *Pbx2* in lateral plate and limb field mesoderm (Fig. 3I,J,L), appears crucial for distal limb development. Concomitantly, the later proximal versus distal restrictions of *Pbx1* and *Pbx2*, respectively, suggest a scenario where both genes provide a dynamic spatiotemporal 'code' along the entire PD limb axis.

### *Pbx* requirement for distal limb patterning is, at least in part, mediated by *Shh* control

The *Pbx1/2* mutant distal limb phenotype markedly resembles those observed in embryos lacking *Shh* (Chiang et al., 2001; Krause et al., 2001) and Hoxa/Hoxd (Kmita et al., 2005). These similarities are particularly evident in *Pbx1/2* mutant hindlimbs, which initially exhibit relatively normal morphology and AP patterning (until E10.25-10.5), but subsequently lack *Shh* expression. *Shh* absence is further supported by *Gli3* posterior expansion and the severe reduction of 5′ Hoxd expression, as also documented in *Shh*$^{-/-}$ embryos (Chiang et al., 2001; Kraus et al., 2001). Recently, studies on the interaction of 5′ Hoxd and Gli3 proteins (Chen et al., 2004) suggest that their ratio across the limb regulates digit pattern and identity. Therefore, digital loss in *Pbx1/2* mutant hindlimbs appears mediated by *Shh* absence (Fig. 7E; right panel). This exact scenario does not hold for *Pbx1/2* mutant forelimbs, which already appear malformed early (E9.5-E10.5), but bear residual *Shh* expression throughout development. Thus, it is not surprising that forelimbs display less severe digit malformations and/or losses. In fact, digit five absence in *Pbx1/2* mutant forelimbs conforms to the expectation that the most posterior digits form under the influence of maximum and sustained Shh signaling (Ahn and Joyner, 2004; Harfe et al., 2004). Interestingly, digit one absence in *Pbx1/2* mutant forelimbs probably occurs via the disruption of *Shh*-independent pathways, as early forelimbs display morphological abnormalities prior to *Shh* onset.

Our findings additionally establish that *Pbx1*/*Pbx2* hierarchically control *Shh* expression and that this process may be independent of regulation of *Hand2* (i.e. *Pbx* and *Hand2* may control *Shh* through parallel pathways) (Charite et al., 2000). We cannot, however, rule out that Hand2 controls Pbx function at the post-transcriptional level and that, therefore, they may act on the same regulatory pathway. In light of the known regulatory interactions among *Hand2* and *Shh* in limb development, wherein *Hand2* lies both up- and downstream of *Shh* (see Zakany et al., 2004), we demonstrate that *Pbx1*/*Pbx2* are not downstream of *Shh* and thus that *Pbx/Shh* do not crossregulate each other in limb development.

### *Pbx1*/*Pbx2* hierarchical control of Hox gene spatial distribution mediates *Shh* expression in the limb

As it had been reported that *Shh* expression is triggered by Hox genes (Charite et al., 1994; Stratford et al., 1997; Knezevic et al., 1997; Zakany et al., 2004), we analyzed Hox gene expression prior to *Shh* onset in *Pbx1/2* mutant limbs. In *Pbx1/2* mutant hindlimbs, expression was severely perturbed for all 3′ and 5′ Hoxa/Hoxd genes analyzed, especially in posterior domains, while in forelimbs, the alterations varied depending on the Hoxa/Hoxd paralog. These alterations occurred in mutant limbs that exhibited relatively normal early AP patterning. Therefore, *Pbx1*/*Pbx2* act upstream of Hox genes in a manner that appears to occur independently from known molecular regulators required for the establishment of early AP asymmetry. Furthermore, this Hox gene control occurs early, perhaps in the limb field, because both 3′ and 5′ Hoxa/Hoxd genes are already altered at the onset of limb development.

The mechanisms by which *Pbx* hierarchically control and maintain Hox genes spatial distribution in early limb mesenchyme remain elusive. *Pbx* requirements in auto- and/or cross-regulatory interactions (Popperl et al., 1995; Popperl et al., 2000; Jacobs et al., 1999; Ferretti et al., 2000; Waskiewicz et al., 2002), or their cooperative roles with other transcription factors upstream of Hox genes (Berkes et al., 2004) (reviewed by Sagerstrom, 2004; Moens and Selleri, 2006) may have bearings. Indeed, with respect to the former, we cannot exclude the possibility that *Pbx* might directly regulate early Hox gene colinearity, given their co-expression with Hox genes in the early limb.

The absence of *Shh* expression in *Pbx1/2* mutant hindlimb ZPA is thus in keeping with the severe spatial perturbations of multiple Hox genes in posterior mutant hindlimbs (Fig. 7E; left panel). Conversely, in forelimbs, *Shh* regulation by Hox requires *Pbx1*/*Pbx2* only in part, as the reduction in *Shh* expression coincided with a less severe alteration in posterior Hox gene localization. Therefore, Hox gene localization to forelimb ZPA appears to require additional factors, with one potential candidate being *Pbx3*, the expression of which only in forelimbs partially overlaps with *Pbx1*/*Pbx2* in the mesenchyme (Di Giacomo et al., 2006).

We also found that several 5′ HoxD proteins bind in vivo to the *Shh* limb enhancer (Lettice et al., 2003) at a gestational stage when *Shh* is expressed, but not at a later stage when *Shh* expression has waned. Furthermore, we found that several HoxD proteins can activate transcription through this regulatory element in cell culture. These results provide evidence in favor of the proposed direct involvement of HoxD proteins in the control of *Shh* expression (Zakany et al., 2004; Kmita et al., 2005) and that such control may be exerted by Hox in an overlapping or synergistic manner (Fig. 7E; top schematic).

In agreement with Pbx2 non-essential function throughout embryonic development (Selleri et al., 2004), we found that despite its colocalization with *Shh* and its binding in vivo to the *Shh*E, Pbx2 does not activate transcription or cooperate with Hox proteins in transcriptional activation through this regulatory element in our cellular readout. Therefore, Pbx2 in vivo binding to the *Shh*E is functionally unessential within the *Pbx*-Hox-*Shh* regulatory network (Fig. 7E; top schematic). It may, however, represent a 'mark' for the possible recruitment of other yet uncharacterized transcription factors, as reported in the case of the myogenin promoter (Berkes et al., 2004) (reviewed by Sagerstrom, 2004). Thus, our data indicate that the primary role of *Pbx2* is exerted in concert with *Pbx1* in the early limb field via their genetic control of Hox gene spatial expression (Fig. 7E; top schematic).

Our findings that multiple Hox proteins are recruited to the limb *Shh*E are consistent with observations that single deletions of Hox genes do not produce dramatic phenotypes (Capecchi, 1997) that resemble those caused by the loss of *Shh*. Indeed, our *Pbx1/Pbx2* mutant mouse model reveals that multiple Hox gene transcripts must synergistically act in vivo in the ZPA for proper and sustained *Shh* activation, as corroborated by previous reports on mice with Hox gene cluster deletions (Zakany et al., 2004; Kmita et al., 2005). The findings that misexpression of single Hox genes (*Hoxb8* or *Hoxd12*) in anterior limbs caused ectopic *Shh* activation (Charite et al., 1994; Stratford et al., 1997; Knezevic et al., 1997), are not in contrast to our model, given the relaxed DNA-binding specificity of Hox proteins and the high expression levels obtained in misexpression

experimental settings. Overall, we establish that *Pbx1/Pbx2*-dependent Hox gene spatial perturbation in the posterior limb has an equally drastic effect on *Shh* onset as does the disruption of early temporal Hox gene colinearity and the complete ablation of Hoxa/Hoxd gene function (Kmita et al., 2005).

## Early mesenchymal interactions of *Pbx1/Pbx2* may drive mouse limb outgrowth

As Hoxa/Hoxd mutants bear limb truncations (Kmita et al., 2005) and Hox genes are known to synergistically pattern all limb elements (Davis et al., 1995; Davis and Capecchi, 1996; Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b; Wellik and Capecchi, 2003), they must govern limb outgrowth. Similarly, as *Pbx1/Pbx2* genetic interactions probably occur in early limb field mesenchyme, where they colocalize, they appear to have crucial bearings on overall limb outgrowth, especially in view of their control of Hox gene spatial distribution. Accordingly, stylopod, zeugopod and autopod elements are grossly mispatterned even at the onset of mesenchymal condensation, suggesting that early Hox-dependent patterning alterations underlie the *Pbx1/2* mutant defects. Furthermore, *Pbx1/Pbx2* homozygous loss results in the absence of hindlimb buds, suggesting that a complete disruption of the Hox gene code occurs in early limb development. In light of the early specification model for limb patterning (Dudley et al., 2002), we suggest that interactions of Pbx genes early in outgrowth may drive specification and patterning of distal limb elements. Limb mesenchyme-specific Pbx inactivation will test this scenario.

Despite similarities to Hoxa/Hoxd and *Shh* mutant phenotypes, the *Pbx1/2* mutant limb defects are indeed more severe. Therefore, our results do not completely negate other concomitant scenarios involving disruptions of different networks that govern limb patterning. For example, as suggested (Kmita et al., 2005), Hox genes may sustain distal limb outgrowth by ensuring proper AER function. Similarly, *Pbx1/Pbx2*, via control of Hox genes, may allow for the proper AER function required for *Shh* maintenance. Three factors, however, argue in favor of a primary role for Pbx in *Shh* control that is independent of AER function: (1) the relatively normal *Pbx1/2* mutant hindlimb AER morphology and observed gene expression prior to *Shh* onset; (2) the finding that ectopic Hox gene expression triggers *Shh* transcription (Charite et al., 1994; Stratford et al., 1997; Knezevic et al., 1997; Zakany et al., 2004); and (3) the strong resemblance of the *Pbx1/2* mutant hindlimb phenotype to those reported in *Shh* (Chiang et al., 2001; Kraus et al., 2001), as well as Hoxa/Hoxd absence (Kmita et al., 2005). However, *Pbx* roles in limb development may resemble those in spleen genesis, where *Pbx1* hierarchically controls all known pathways required for spleen development (Brendolan et al., 2005).

## *Pbx* roles in proximal-distal limb patterning are poorly conserved from flies to vertebrates

Evolutionarily, our findings indicate that the mechanisms of PD limb patterning are poorly conserved from insects to vertebrates. Unlike in vertebrates (Capecchi, 1997), the fly leg PD axis develops independently of Hox genes (Mann and Morata, 2000). In addition, in flies, *Exd* and *Hth* (the homolog of vertebrate *Meis*) are restricted to proximal limbs (Rauskolb et al., 1995; Capdevila et al., 1999; Mercader et al., 1999) and *Exd* is required for patterning of proximal identities (Gonzales-Crespo et al., 1998). By contrast, we demonstrate that in vertebrates distal limb patterning is also *Pbx1/Pbx2* dependent. Part of this vertebrate innovation may lie in *Pbx1/Pbx2* interaction in early limb field mesenchyme and possibly also in later *Pbx2* localization to distal limb domains. In addition, in flies, mutations in *Exd* result in homeotic transformations without altering Hox gene expression, thereby indicating that Exd can act as an ancillary co-factor to Hox proteins (Peifer and Weischaus, 1990; Rauskolb et al., 1993; Rauskolb et al., 1995). Conversely, loss of *Pbx1/Pbx2* causes drastic alterations in Hox gene expression, pointing to their primary role as upstream regulators of Hox genes in vertebrate limbs. This vertebrate novelty indeed has crucial bearings on *Shh* regulation, as well as on the origins of morphological complexity of distal appendages in tetrapods. Finally, we propose that Pbx1/Pbx2 proteins began to exert their functions differentially in hindlimbs versus forelimbs, because in vertebrates only hindlimb patterning is strictly dependent upon *Pbx1/Pbx2*-Hox-*Shh* regulation.

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### Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/11/2263/DC1

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