Published in final edited form as:

Dev Dyn. 2011 May ; 240(5): 1063–1086. doi:10.1002/dvdy.22605.

Pbx Homeodomain Proteins: TALEnted regulators of Limb Patterning and Outgrowth

Terence D. Capellini^{1,^}, Vincenzo Zappavigna², and Licia Selleri^{1,#}

¹Department of Cell and Developmental Biology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021, USA

²Department of Biology, University of Modena and Reggio-Emilia, Via Campi 213d, 41100 Modena, Italy

Abstract

Limb development has long provided an excellent model for understanding the genetic principles driving embryogenesis. Studies utilizing chick and mouse have led to new insights into limb patterning and morphogenesis. Recent research has centered on the regulatory networks underlying limb development. Here, we discuss the hierarchical, overlapping, and iterative roles of *Pbx* family members in appendicular development that have emerged from genetic analyses in the mouse. *Pbx* genes are essential in determining limb bud positioning, early bud formation, limb axes establishment and coordination, and patterning and morphogenesis of most elements of the limb and girdle. Pbx proteins directly regulate critical effectors of limb and girdle development, including morphogen-encoding genes like *Shh* in limb posterior mesoderm, and transcription factor-encoding genes like *Alx1* in pre-scapular domains. Interestingly, at least in limb buds, Pbx appear to act not only as Hox cofactors, but also in the upstream control of 5' HoxA/D gene expression.

Introduction

Limb development has long provided an excellent model for understanding the genetic and molecular principles driving embryogenesis. Studies utilizing both the chick and the mouse as model systems have led to new insight into fundamental biological processes that play important roles in limb patterning and morphogenesis, including cell fate specification, gene regulation, signal transduction, proliferation, migration, and adhesion (Niswander, 2003). Over the past two centuries, research in these two systems, coupled with studies in atypical models, has managed to address seminal questions of limb anatomy, function, and evolution (Stopper and Wagner, 2005). More recent work has centered on the genetic and transcriptional control of limb bud development, as well as the gene regulatory networks controlling appendicular skeleton and soft tissue formation (Bismuth and Relaix, 2010; Butterfield et al., 2010; Schweitzer et al., 2010; Zeller, 2010).

Limb and girdle anatomy

In vertebrates, each limb is connected to the body axis via a bony girdle along with associated musculature (Romer and Parsons, 1986), and it is during embryogenesis that the developmental integration of limb and girdles occurs. However, the integration of limbs to

[#]Corresponding author: Licia Selleri lis2008@med.cornell.edu Phone: 1-212-746-5009 Fax: 1-212-746-5596.

[^]Present Address: Terence D. Capellini Department of Developmental Biology Stanford University School of Medicine Beckman Center B300, 279 Campus Drive Stanford, CA 94305

their girdles has received less attention than deserved in the past. Therefore, in this review we will concentrate on both, given their developmental and functional interdependence.

In vertebrates, the limb proper consists of three main developmental domains: the stylopod (upper arm or humerus; thigh or femur), the zeugopod (forearm or radius and ulna; leg or tibia and fibula), and the autopod (hand or carpals, metacarpals, and digits; foot or tarsals, metatarsals and digits) (Zeller et al., 2009). In the forelimb, the humerus proximally articulates with the scapula, a large triangular shaped bone consisting of an expanded blade, head, and neck that in most tetrapods sits dorsolaterally on the thorax and is enveloped by musculature. The scapula, together with the clavicle, forms the pectoral girdle. In the hindlimb, the femur is proximally articulated via the hip or femoral-acetabular joint to the pelvic bone, which together with the sacral vertebrae form the complete pelvic girdle (Romer and Parsons, 1986). Limb and girdle development comprise all the processes by which the appendicular compartments form and their skeletal and soft tissue fates are realized.

Limb and girdle embryology: establishment of signaling centers

Limb development begins when a portion of the lateral plate mesoderm (LPM) at stereotypic positions along the embryonic axis undergoes lateral outgrowth. The mechanisms that control when and where this initial budding occurs remains mostly unknown. During this stage, the early limb bud or prominence consists of a proliferating mass of apparently undifferentiated mesenchymal cells enveloped by a layer of ectodermal cells (see Zeller et al., 2009). This prominence next emerges to form a true bud due to additional outgrowth and the establishment of several signaling centers, that act to define cell fates along the three main limb axes (Niswander et al., 1993; Niswander et al., 1994; Parr and McMahon, 1995; Loomis et al., 1996; Cygan et al., 1997; Towers and Tickle, 2009; Zeller et al., 2009). These axes are: the proximal-distal (P-D) axis (e.g. from shoulder to finger tip), the anterior-posterior (A-P) axis (e.g., from thumb to digit five), and the dorsal-ventral (D-V) axis (e.g. the back of the hand to the palm). Essential to the formation of the three axes, and the resulting skeletal elements that are specified within the bud, are the coordinated and controlled expression of numerous genes, most notably from the *Hox*, *Fgf*, *Shh*, *Wnt*, and *Bmp* gene families (Parr and McMahon, 1995; Zuniga et al., 1999; Sun et al., 2000; Sun et al., 2002; Khokha et al., 2003; Scherz et al., 2004; Zakany et al., 2004; Verheyden et al., 2008; Benazet et al., 2009; and reviewed in Zeller et al., 2009).

At the distal tip of the limb bud, a specialized epithelium, the apical ectodermal ridge (AER) signaling center forms and controls growth and patterning along the P-D axis via the production of growth factors belonging to the FGF family of signaling molecules (Niswander et al., 1993; Lewandoski et al., 2000; Mariani et al., 2008). Within the flank and proximal limb, *Hox* genes are also expressed, and together with proximalizing factors, such as retinoic acid, and distally expressed *Fgfs*, set up a P-D coordinate system that helps specify cellular identity along this axis (see Zeller et al., 2009). Concurrently, the A-P axis also develops due to the specification of a subset of cells along the posterior mesoderm of the limb, producing a second signaling center, the Zone of Polarizing Activity (ZPA) (Saunders and Gasseling, 1968). ZPA cells are the source of another signaling molecule, Sonic hedgehog (*Shh*), which plays a critical role in maintaining the A-P axis, preserving AER integrity (and thus P-D growth), and is essential for distal limb and digit patterning and morphogenesis (Echelard et al., 1993; Riddle et al., 1993). Along the dorso-ventral (D-V) axis, patterning is directed by the ectoderm lining the dorsal and ventral surfaces of the limb bud, which produce signaling molecules of the *Wnt* and *Bmp* families, respectively (Niswander et al., 1993; Niswander et al., 1994; Parr and McMahon, 1995; Loomis et al., 1996; Cygan et al., 1997). Gene expression within the three signaling networks is controlled by tightly coordinated and integrated feedback loops and this coordination leads to the

formation of the bony and soft tissue structures of the vertebrate limb (see Niswander, 2003; Zeller et al., 2009). Concomitant with the formation of the limb and its axes, the mesodermal portion of the somatopleure or medial portion of the LPM houses the cells that will give rise to each of the girdles (Huang et al., 2006; Malashichev et al., 2008). In the forelimb, cells from the dermomyotome of the somites migrate to the somatopleure, and together with nascent cells within this tissue form the scapula blade, head and neck (Huang et al., 2000; Valasek et al., 2010). In the hindlimb, it has recently been shown that the entire pelvis forms from the somatopleure proper with no somitic contribution (Malashichev et al., 2008), while the sacral vertebrae form from the sclerotome (Christ et al., 2000).

TALE homeodomain proteins

During the past few years, we have reported that Pbx members of the TALE (Three Amino acid Loop Extension) superfamily of atypical homeodomain-containing transcription factors (Bürglin, 1994; Bürglin, 1997) are essential for multiple developmental processes, including limb and girdle development (Figure 1). Vertebrate *Pbx* genes, together with their orthologous *Drosophila exd* and *C. elegans ceh-20* genes, belong to the gene family encoding the PBC subclass of TALE proteins, which sets itself apart for having a conserved protein-protein interaction domain, the PBC domain (Bürglin, 1998) that serves as an interaction surface with members of two other known subclasses of TALE proteins: the MEIS/MEINOX (Bürglin, 1997; Mukherjee and Bürglin, 2007) and the PREP (Fognani et al., 2002). Vertebrate genomes contain four *Pbx* genes: *Pbx1*, which was discovered first, due to its involvement in a chromosomal translocation in human pre-B cell acute lymphoid leukemia; *Pbx2* and *Pbx3*, which were subsequently identified because of their sequence homology to *Pbx1*; and *Pbx4*, which was initially isolated via a forward genetic screen for mutations disrupting the segmental patterns of gene expression in the developing zebrafish brain (Nourse et al., 1990; Monica et al., 1991; Kamps and Wright, 1994; Pöpperl et al., 2000). Since their discovery, TALE transcription factors and in particular Pbx proteins have been implicated in crucial developmental processes, largely from the perspective of their function as co-factors to members of the Hox family of transcriptional regulators (reviewed in Moens and Selleri, 2006; Laurent et al., 2008). Recently, though, a number of studies have indicated that Pbx proteins may act more broadly to include also Hox-independent functions in the control of pattern formation and organogenesis (see below).

Here, we will review recent findings on the function of *Pbx* genes in vertebrate limb and girdle development, as genetic analyses in the mouse have started to reveal critical roles for this gene family in appendage formation. Indeed, *Pbx* genes are essential in determining limb bud positioning, early bud formation, limb bud axis establishment and coordinated regulation, and patterning and morphogenesis of limb skeletal elements. In addition, Pbx transcription factors also co-regulate girdle formation and the integration of each girdle with the limb proper. Before discussing in detail the functions of Pbx proteins in limb and girdle developmental processes, we will review their transcriptional and biochemical features as well as illustrate the molecular landscape in which they act.

Pbx proteins

The perfect partners for many a situation

A wealth of studies over the past two decades has unveiled physical and functional interactions between Pbx proteins and a number of other transcription factors and transcriptional co-regulators. This has led to the conclusion that Pbx proteins are central to multiple gene regulatory networks. The most-studied interaction partners of Pbx proteins are Hox proteins. Hox proteins belong to a family of evolutionarily highly conserved transcription factors that control regional identities and cell fates along the A-P body and

limb axes (Alexander et al., 2009; Wellik, 2009; Mallo et al., 2010). Hox protein functions in development reveal an intriguing paradox. Indeed, while Hox display an exquisite degree of functional specificity *in vivo*, being able to selectively direct developmental programs in a gene-specific manner, their DNA binding capabilities, as analyzed *in vitro*, appear to be relaxed. In fact, most Hox proteins recognize different DNA sequences, characterized by a TAAT core consensus, with essentially the same binding affinity, and individual TAAT-containing sequences can be bound by different Hox proteins with similar efficiency. To explain this paradox, the intervention of co-factors was invoked. Co-factors, by physically interacting with Hox proteins, would raise their DNA-binding specificity/selectivity to *in vivo* standards (Chan et al., 1994; Van Dijk and Murre, 1994; reviewed in Mann, 1996). One of the first co-factors identified was the product of *extradenticle* (*exd*) in *D. melanogaster* (Rauskolb et al., 1993), the ortholog to the vertebrate *Pbx*. *exd* was shown to act genetically in parallel to Hox genes in patterning the segmental body plan of the fly (Peifer and Wieschaus, 1990), and its gene product was found to form stable heterodimers onto specific DNA sequences, whose complexity was considerably higher than that of the sites bound by the Hox or Exd proteins alone (Mann, 1996). Soon it was shown that Pbx proteins could also heterodimerize onto DNA with a large subset of vertebrate Hox proteins to enhance their target binding site selectivity (Chang et al., 1995; Knoepfler and Kamps, 1995; Phelan et al., 1995; Peltenburg and Murre, 1996).

Interactions between PBC and Hox proteins

PBC TALE proteins interact with their Hox partners by making contact with a small, evolutionarily conserved, tryptophan-containing motif, located at a variable distance N-terminal to the homeodomain of Hox proteins, which was variably christened “hexapeptide”, “pentapeptide”, or “YPWM” (Mavilio et al., 1986; Krumlauf et al., 1987; Johnson et al., 1995; Chan et al., 1996). PBC proteins form a hydrophobic pocket, comprising TALE family's characteristic three extra amino acids, which accommodates the “YPWM” motif, and thus stabilizes the physical interaction between the two proteins (Chang et al., 1995; Passner et al., 1999; Piper et al., 1999). Additional analyses, though, suggested that Hox-PBC interactions may be more versatile than previously thought. For example, *Exd* was shown to be able to heterodimerize with the *Ubx* and *Abd-A* Hox protein, also in the absence of a “YPWM” motif, which was also shown to be not sufficient to stabilize the Hox-PBC complex (Chan et al., 1994; Johnson et al., 1995), indicating that other contact surfaces may participate in the interaction between PBC and Hox proteins (Galant et al., 2002).

As mentioned above, the interaction between Hox and PBC proteins leads to the formation of DNA-bound heterodimers that recognize sequences with a higher complexity with respect to the single proteins. Site selection experiments, together with the identification of a number of naturally occurring regulatory elements within the promoters of target genes, have led to the definition of a consensus binding sequence for Hox-PBC complexes, 5'-TGATNNATNN-3', where the TGAT motif represents the core half site occupied by PBC proteins, while the remaining nucleotides vary in base composition according to the Hox partner entering the complex (Chan et al., 1994; Johnson et al., 1995; Phelan et al., 1995; Chang et al., 1996; Phelan and Featherstone, 1997; Passner et al., 1999; Piper et al., 1999). As to the capability of Hox-PBC heterodimers to regulate transcription, the transcriptional effector domain of the dimeric complex appears to be mainly contributed by the Hox (or other non-Hox, see below) partner, as in the case of the *Hoxb1-Pbx1* complex, which has been shown to activate transcription via an activation domain located within the *Hoxb1* protein N-terminal portion (Di Rocco et al., 1997). *Pbx* proteins, on the other hand, do not appear to have intrinsic capabilities to activate or repress transcription (Van Dijk et al., 1993; Lu et al., 1994; LeBrun et al., 1997), despite the fact that they are able to recruit

transcriptional co-repressors (Asahara et al., 1999; Saleh et al., 2000). The analysis of the transcriptional outcome (i.e. activation or repression) of Hox-PBC complexes binding at regulated target promoters has led to somewhat contradictory results. While earlier experiments suggested a simple scenario in which heterodimerization with PBC proteins converts Hox proteins from transcriptional repressors into activators (see Pinsonneault et al., 1997), more recent data suggest that the transcriptional effect of a given Hox-PBC heterodimer depends on several factors such as: the structure of the bound regulatory sequence, the presence of specific co-activators or co-repressors, and the activity of cell-specific signaling pathways (Kobayashi et al., 2003; Gebelein et al., 2004). Lastly, it was shown that for the fly proteins Sex Comb reduced (Scr) and Deformed (Dfd), Exd positions a normally unstructured portion of the Hox protein so that two basic amino acid side chains can insert into the minor groove of a Scr- or Dfd-specific DNA-binding site. Also, subtle differences in the way Scr or Dfd recognize their specific binding sites, in conjunction with non-DNA-binding domains, influence whether the target gene is transcriptionally activated or repressed (Joshi et al., 2010).

Furthermore, an additional level of complexity in Hox-PBC interactions was demonstrated in *Drosophila*. For example, it was demonstrated that *in vivo* specificity often depends on extended and unstructured regions that link Hox homeodomains to Exd. Using a combination of structure determination, computational analysis, and *in vitro* and *in vivo* assays, it was shown that Hox proteins recognize specific Hox-Exd binding sites via residues located in the extended regions that insert into the minor groove, but only when presented with the correct DNA sequence (Joshi et al., 2007). This work suggests that these specific residues, which are conserved in a paralog-specific manner, confer specificity by recognizing a sequence-dependent DNA structure instead of directly reading a specific DNA sequence. In summary, the concept has been put forth that PBC reveals a latent specificity that is then built into the Hox N-terminal arm/linker region.

Trimeric interactions of PBC, MEIS/PREP, and Hox proteins

An additional level of complexity was added when it was discovered that PBC proteins are almost always associated with members of two other sub-families of TALE proteins, MEIS and PREP (Bürglin, 1997; Fognani et al., 2002; Mukherjee and Bürglin, 2007). The MEIS sub-family of TALE proteins includes the products of the vertebrate *Meis1–3*, the fly *hth*, and the *C. elegans unc-62 (ceh-25)* genes, while the PREP sub-family includes the vertebrate Prep1 and Prep2. MEIS/PREP proteins interact in solution with PBC proteins via a conserved bipartite domain, the MEIS or HM domain, located N-terminal to the homeodomain (Bürglin, 1997; Rieckhof et al., 1997). This domain directly contacts the PBC domain (Moskow et al., 1995; Chang et al., 1997; Chen et al., 1997; Knoepfler et al., 1997; Berthelsen et al., 1998). As PBC proteins interact with Hox and MEIS/PREP proteins via different surfaces, it is recognized that heterotrimeric Hox-PBC-MEIS/PREP complexes can assemble on specific DNA sequences, adding a further level of binding selectivity to Hox proteins (Mann and Chan, 1996; Berthelsen et al., 1998; Mann and Affolter, 1998; Jacobs et al., 1999; Ryoo and Mann, 1999; Ferretti et al., 2000; Ebner et al., 2005). This interaction with MEIS/PREP proteins appears a means for controlling PBX protein function post-translationally. Specifically, both Exd and Pbx proteins have been shown to require MEIS/PREP for their nuclear import in specific cell contexts such as limb mesenchymal cells in vertebrates (see below) or limb imaginal disc cells in flies (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Mercader et al., 1999; Jaw et al., 2000; Kilstrup-Nielsen et al., 2003).

Interactions of PBC proteins with non-Hox homeodomain and non-homeodomain transcription factors

Pbx proteins have also been shown to interact with other homeodomain-containing and non-homeodomain DNA-binding proteins, demonstrating their involvement in the control of cellular processes other than regional cell fate specification. A number of non-Hox homeodomain-containing proteins indeed possess a "YPWM" motif, or variant forms of it, making them suitable candidates for Pbx interactions (Moens and Selleri, 2006). One of the best studied non-Hox Pbx heterodimerization partners is Pdx1 (Peers et al., 1995), which plays a critical role in pancreas development by regulating promoters of pancreas specific genes, such as those encoding somatostatin and insulin (Peers et al., 1995; Goudet et al., 1999). The *somatostatin* gene promoter is indeed bound by a Pdx1-Pbx1 heterodimer and becomes activated via a cooperative interaction between the Pbx1-Prep1 complex and Pdx1 bound at two adjacent regulatory elements (Peers et al., 1995; Goudet et al., 1999). Additionally, pancreatic acinar cell specific expression of the *elastase 1* gene (*ELA1*) is controlled by a trimeric Pdx1-Pbx1b-Meis2 complex (Swift et al., 1998; Liu et al., 2001). Recently, another non-Hox homeodomain protein, Emx2, was found to heterodimerize with Pbx1 and to control co-operatively with Pbx1 the expression of *Alx1*, a gene required for scapular blade development (see below) (Capellini et al., 2010).

Among non-homeodomain Pbx interaction partners, MyoD, of the bHLH family, is a notable example. Pbx1 has been shown to physically interact with several members of the bHLH transcription factor family, including myogenin, Myf-5, Mrf-4, and MyoD, via a tryptophan-containing motif, resembling the Hox "YPWM", located in proximity of the bHLH domain (Knoepfler et al., 1999). This interaction between MyoD and Pbx-Meis complexes has been shown to be important for the transcriptional regulation of the *Myogenin* gene, where MyoD is recruited through an interaction with a Pbx-Meis complex constitutively bound at a regulatory element within the *Myogenin* promoter (Berkes et al., 2004). Pbx proteins have also been found to physically interact with Smad transcription factors, which represent intracellular mediators of the TGF β family of signaling molecules. A Pbx1-Prep1 heterodimer was shown to form trimeric complexes with Smad proteins. Indeed, a Smad4-Pbx1-Prep1 complex regulates the promoter of the FSH β gene (Bailey et al., 2004).

Also members of the nuclear receptor superfamily of transcription factors have been reported to functionally interact with Pbx proteins. The malic enzyme gene, whose expression is activated by the thyroid hormone (T3), contains in its promoter a regulatory region, which is bound by several Pbx/Meis complexes, one of which contributes to its transcriptional activation. A physical interaction between the thyroid hormone receptor TR α , a nuclear receptor Zn-finger transcription factor, and Pbx1 has also been shown to be responsible for transcriptional activation, and the formation of a DNA-bound complex containing TR/RXR and Pbx1/Meis has been hypothesized (Wang et al., 2001). Likewise, an interaction between Pbx1 and another nuclear hormone receptor, the glucocorticoid hormone receptor (GR), was inferred to explain the glucocorticoid responsiveness, via a Hoxb1-Pbx1 complex, of the *Hoxb1*-ARE regulatory element (Pöpperl et al., 1995) in cultured cells (Subramaniam et al., 2003).

Lastly, a member of the forkhead transcription factor protein family, FoxC1, involved in the formation of neural crest-derived tissues and mesenchymal mesoderm, has also been shown to interact with Pbx1 (Berry et al., 2005). It has been reported that Pbx1 represses FoxC1 transcriptional activity and co-localizes with FoxC1 in heterochromatin-rich, transcriptionally silent regions of the cell nucleus (Berry et al., 2005).

Pbx proteins have distinct isoforms

Pbx proteins, more specifically Pbx1 and Pbx3, come in different flavours, as both can be found in distinct isoforms, a and b for Pbx1, and a through d for Pbx3, which arise via alternative splicing and differ in length and amino-acid composition at their N-terminal (Pbx3) and C-terminal (Pbx1 and Pbx3) ends (Monica et al., 1991; Milech et al., 2001). Pbx isoforms show tissue and/or stage specific expression patterns during development (Swift et al., 1998; Milech et al., 2001; Schnabel et al., 2001), which points at potentially differential activities. In practice, functional differences between Pbx isoforms have been only partially explored. However, the sparse findings available to the present day suggest that distinct isoforms have differential activities, adding further versatility to the Pbx universe. The Pbx3c and Pbx3d isoforms, for instance, which lack a portion of the N-terminal PBC domain, have been found to heterodimerize weakly with Meis1 and not with Prep1 (Ferretti et al., 1999). Also differences were reported between Pbx1a and Pbx1b as to their capability to modulate the transcriptional activity of Pdx1-Pbx1 heterodimers via the opposed recruitment of chromatin remodeling complexes (see below). Further work in this direction via selective inactivation of Pbx specific isoforms in mouse models is needed to uncover novel aspects of the complexity of Pbx-mediated gene regulation.

Pbx proteins and chromatin remodeling

Chromatin remodeling protein complexes have been involved in the process of transcriptional regulation since it was recognized that some transcriptional co-activators and corepressors function by inducing local changes in chromatin structure to favor or prevent gene transcription (Naar et al., 2001). Like numerous other transcription factors, Pbx proteins were shown to recruit chromatin remodeling complexes to promoters, thereby facilitating the binding of other transcription factors, thus enhancing or repressing transcription. For instance, Pbx1 was reported to physically interact, in the context of the *myogenin* promoter, together with MyoD, and with Brg1, a member of the SWI/SNF ATP-dependent chromatin remodeling complex (see above) (de la Serna et al., 2005). A Pbx isoform-specific recruitment of chromatin modifiers was also reported. A Pbx1b-Pdx1 complex was shown to recruit the histone acetyl transferase (HAT) CBP to activate transcription of the *somatostatin* promoter, while a Pbx1a-Pdx1 heterodimer recruited two co-repressor complexes NcoR and SMRT (Asahara et al., 1999). It was reported that recruitment of co-repressors by Pbx proteins can be controlled via cell signaling. PKA signaling, for instance, was shown to mediate the switch of the Hoxb1-Pbx1 complex from a transcriptional repressor to an activator (Saleh et al., 2000). In this context, repression was mediated via the association of the N-terminus of Pbx1 with a co-repressor complex containing histone deacetylases (HDACs), such as HDAC1 and HDAC3, NCoR/SMRT, and mSIN3b (Saleh et al., 2000). In summary, Pbx proteins could be considered as “pioneer factors” that poise tissue-specific loci for activation by downstream master regulators.

Our current view on PBC proteins: from ancillary co-factors to “equal partners”

The first evidence that Pbx proteins function as Hox co-factors in vertebrate development came with the identification of paired Hox/Pbx regulatory elements in the promoters of mammalian Hox genes themselves. Hox gene expression, particularly in the vertebrate hindbrain, is auto- and cross-regulated (reviewed in Alexander et al., 2009). For example, amongst the Hox paralog group 1 genes, *Hoxa1* is required for the normal onset of *Hoxb1* expression, while *Hoxb1* is required for maintaining its own expression (Pöpperl et al., 1995; Studer et al., 1998). In these and other examples of Hox-dependent gene regulation in vertebrates, the Hox recognition element consists of a paired Hox/Pbx binding site (often with a nearby Meis/Prep site), and mutating either the Hox or the Pbx-binding element prevents reporter expression in transgenic mice (Pöpperl et al., 1995; Maconochie et al., 1997; Ferretti et al., 2000; Manzanares et al., 2001; Samad et al., 2004). Additionally, *Pbx*

mutants in fish and mouse partially recapitulate Hox loss-of-function phenotypes. Indeed, zebrafish *pbx4* mutants were originally identified as resembling Hox mutants previously described in animal models (Pöpperl et al., 2000). They phenocopy mouse *Hoxb1* mutants (Goddard et al., 1996) and *hoxb1a* morpholino-injected zebrafish embryos (Studer et al., 1996; McClintock et al., 2002; Arenkiel et al., 2004). Furthermore, targeted mutations in *Pbx1*, *Pbx2* and *Pbx3* genes in the mouse cause less severe patterning defects than those of zebrafish *pbx4* or *pbx2*; *pbx4* mutants, indicating functional redundancy between the mouse genes at early developmental stages (see below). Interestingly, some aspects of mouse *Pbx1* homozygous mutants recapitulate Hox loss-of-function phenotypes. For example, *Pbx1*^{-/-} embryos do not display dramatic patterning defects in the hindbrain (F. Rijli, personal communication), but they exhibit morphological transformations of neural crest-derived second pharyngeal arch cartilages to structures similar to first arch cartilages (Selleri et al., 2001), a phenotype partially resembling that observed in *Hoxa2* mutants (Gendron-Maguire et al., 1993; Rijli et al., 1993). Phenotypic overlaps with Hox loss of function mutants are also observed in caudal pharyngeal pouch-derived organs of *Pbx1*^{-/-} embryos, such as thymus, parathyroids and ultimobranchial bodies. For example, the thymus in *Pbx1*^{-/-} embryos is mostly ectopic, the lobes do not fuse and do not descend into the mediastinum (Manley et al., 2004). A similar thymic phenotype is observed in mice that are compound mutants for three paralogous group 3 Hox genes (i.e. *Hoxa3*^{+/-}; *Hoxb3*^{-/-}; *Hoxd3*^{-/-} mutants) (Manley and Capecchi, 1998), consistent with a scenario in which Pbx1 acts together with multiple Hox proteins and in multiple cell types to regulate pharyngeal development. However, all of the above evidence for Pbx roles as *in vivo* Hox co-factors is circumstantial, as no clear-cut, unequivocal demonstration for such roles has been put forth in vertebrate development, with the only exception of the vertebrate hindbrain. Only in the hindbrain have Pbx functions as co-factors for 3' Hox proteins been demonstrated with some stringency in fish and mouse models (Pöpperl et al., 1995; Ferretti et al., 2000; Pöpperl et al., 2000; Samad et al., 2004). Interestingly, mice with mutations in the *Hoxa1* hexapeptide, which mediates Pbx contact, display hindbrain and cranial nerve defects reminiscent of those reported for *Hoxa1* loss-of-function (Remacle et al., 2004).

In light of the widespread presence of Pbx proteins in multiple developing tissues and organs (Schnabel et al., 2001; Selleri et al., 2001; Kim et al., 2002; Rhee et al., 2004; Selleri et al., 2004; Capellini et al., 2006, 2008, 2010; Di Giacomo et al., 2006), as opposed to the more restricted expression patterns of Hox interaction partners, a different perspective has emerged which posits that Hox should actually be considered as the co-factors, while Pbx proteins would play the leading role (Mann and Affolter, 1998; Laurent et al., 2008). This view, while perhaps appealing, does not stand up to the fact that at least some Pbx partners, such as select Hox proteins, have been reported to display patent PBC/MEIS-independent functions (Galant et al., 2002; Joshi et al., 2010). Not to mention that a subset of Hox proteins, such as those belonging to paralogy groups 11-13, appear unable to interact directly with PBC proteins (Chang et al., 1995; Knoepfler and Kamps, 1995; Phelan et al., 1995; Neuteboom and Murre, 1997). Thus, it would be too extreme to consider Hox proteins, and possibly other interaction partners, solely as co-factors to PBC and MEIS/PREP proteins. In sum, we believe that deciding who is the co-factor and who is the main player based on the extent of expression in embryonic development is a simplistic criterion that is ultimately misleading. This semantic issue set aside, it is becoming clear in light of mounting experimental evidence in animal models that Pbx homeoproteins and their multiple partners all exert both autonomous and co-operative functions in developmental programs and should thus be regarded as “equal partners” with equal dignity in their transcriptional relationship.

Roles of Pbx in Organogenesis

Commonalities of TALEs

Using a mouse line with global loss of *Pbx1* (*Pbx1*^{-/-}), we established that virtually every organ of *Pbx1*^{-/-} embryos is markedly hypoplastic and malformed leading to late-gestational lethality (Selleri et al., 2001). This observation led us to hypothesize that Pbx1 controls progenitor cell proliferation in mammalian embryonic development, possibly through direct control of the cell cycle. Indeed, S-phase Common Myeloid Progenitors (CMPs) are reduced in number (DiMartino et al., 2001) and proliferation rates are similarly diminished in chondrogenic, pancreatic, urogenital, adrenal, thymic, and splenic progenitors of *Pbx1*^{-/-} embryos (Selleri et al., 2001; Kim et al., 2002; Schnabel et al., 2003; Manley et al., 2004; Brendolan et al., 2005). Interestingly, *Pbx1* was originally identified in acute leukemia by the t(1:19) translocation, which results in *Pbx1* dysregulation leading to aberrant proliferation of hematopoietic progenitors and leukemic transformation (Kamps et al., 1990; Nourse et al., 1990). Similarly, the inappropriate expression of Hox genes encoding binding partners of Pbx proteins, such as *Hoxa9* (Shen et al., 1999; LaRonde-LeBlanc and Wolberger, 2003), was also implicated in leukemogenesis. Furthermore, the presence of Pbx/Meis as co-factors has been shown essential for Hox proteins to prevent differentiation or cause aberrant proliferation and transformation of cultured cells (Sauvageau et al., 1997; Krosi et al., 1998; Wang et al., 2010). Overall, these studies demonstrate a role for Pbx1 in promoting cellular proliferation, both in normal organ development and uncontrolled growth in cancer (Moens and Selleri, 2006) (Koss et al., submitted for publication). Interestingly, mice with global loss of *Pbx2* exhibited no overt developmental defects (Selleri et al., 2004) and mice with global loss of *Pbx3* succumbed to perinatal death due mainly to respiratory defects (Rhee et al., 2004), underscoring at least some unique and distinctive roles of Pbx homeoproteins in mammalian development.

Pbx Genes in Limb Development

Expression of Pbx and other TALE-encoding genes during axis and limb development

Research conducted in zebrafish suggested that the functional differences among *pbx* genes are likely to be due to differences in their expression rather than in their biochemical activities (Pöpperl et al., 2000). Indeed, ectopic expression of any of the zebrafish *pbx* genes was able to completely rescue the embryonic phenotype of *pbx4* mutants. As it turns out, *Drosophila exd* can accomplish the same rescue, underscoring the high degree of functional conservation across this protein family. Consistent with these findings, the *in vitro* DNA binding properties of different Pbx proteins appear very similar: intriguingly Pbx1, Pbx2 and Pbx3 exhibit identical DNA cooperative binding with a subset of Hox proteins *in vitro* (Chang et al., 1995). These findings predicted that where the expression of *Pbx* family members tightly overlaps, their functions may be partially redundant. Different Pbx proteins are expressed in temporally and spatially distinct patterns during organogenesis and in adult mice, and in these contexts mouse single mutant phenotypes have been extremely informative. However, overlapping expression patterns of more than one Pbx protein in multiple developing tissues and organs has been observed, including the appendage (Schnabel et al., 2001; Selleri et al., 2001; Wagner et al., 2001; Kim et al., 2002; Waskiewicz et al., 2002; Rhee et al., 2004; Selleri et al., 2004). Therefore, analysis of *Pbx* compound mutants was required to understand Pbx function in these contexts. The limb is a prime example (see below).

Like many transcription factor-encoding genes, *Pbx* and their TALE-encoding family members have dynamic expression patterns during development reflecting their multifaceted roles in embryogenesis. Within the developing limb, the dynamic nature of *Pbx* expression provides the backdrop in which to envisage the multiple genetic and molecular

interactions described above. Before discussing their expression patterns prior to and during limb and girdle development, it is important to highlight some general commonalities in *Pbx* expression in the vertebrate embryo. To begin, mRNA transcripts for *Pbx* and other TALE-encoding genes, such as *Meis* and *Prep*, are not restricted to a single germ layer (i.e., mesoderm, endoderm, or ectoderm), nor to a single histological type (e.g., mesenchyme *versus* epithelium), but present in all tissue types. Additionally, their expression is not confined to one developmental stage but found dynamically from early embryogenesis (e.g., gastrula) through organogenesis and into adulthood (G. Di Giacomo and L. Selleri, unpublished observations). *Pbx* and *Meis* family members often considerably overlap in their expression territories, likely reflecting their known function as Pbx heterodimers as described above. Accordingly, their expression domains can also, either separately or collectively, overlap with those of other homeodomain transcription factors, such as members of the Hox family, reflecting their ability, as discussed above, to form either heterodimeric (e.g., Pbx-Hox or Meis-Hox) or heterotrimeric (Pbx-Meis-Hox) complexes.

With regard to *Pbx* genes proper, *Pbx1-3* exhibit wide expression territories including the limb and girdle, while *Pbx4* is expressed solely in the testes (Wagner et al., 2001). However, despite *Pbx1-3* broad and often overlapping expression patterns in limb domains, the loss of any one *Pbx* gene does not substantially alter the expression of the others. This is specifically the case for the limb and girdle, as in these structures, single *Pbx* gene loss is not compensated for by spatial up-regulations in other *Pbx* genes (i.e., at least as assessed by *in situ* hybridization) (Capellini et al., 2006; Capellini et al., 2010). The expression of *Meis* was also examined in single *Pbx1* and *Pbx2*, as well as compound *Pbx1*^{-/-};*Pbx2*^{+/-}, mutant limbs and their expression remained unaltered spatially in the proximal forelimb and hindlimb in all mutant genotypes listed above (Selleri et al., 2001; Capellini et al., 2006).

Specific Pbx and Meis expression domains in the limb and girdle

Our previous studies established that *Pbx* genes are expressed throughout girdle and limb development in five general phases (Phases I-V). During Phase I, *Pbx1* and *Pbx2* show overlapping expression domains as early as E7.5 in the developing somites, in the Lateral Plate Mesoderm (LPM) along the embryonic axis, and through E9.0 in the forelimb bud prominences (Figure 1). In this phase, they also display overlapping expression in similar domains of the hindlimb, which are typically delayed in their development by approximately half a day (Figure 1). In contrast, *Pbx3* is not expressed in the LPM and is restricted solely to the early forelimb field (Di Giacomo et al., 2006). Less is known regarding *Meis* expression during this phase, although preliminary data indicate that *Meis1* and *Meis2* are also expressed in early somites and along the LPM, while *Prep* has a more restricted domain in the dermomyotome (unpublished results).

Phase II begins when the limb prominences become recognizable limb buds and ends at the first onset of a morphologically identifiable autopod. During this period, *Pbx* gene expression patterns dynamically change. As limb bud induction and initial outgrowth commence (E8.5-E9.5), *Pbx1* transcripts are restricted to LPM and its derivative medial somatopleuric and lateral proximal forelimb mesenchyme, and by E9.25 are lost from the distal mesenchyme (Figure 1). Henceforth, during this phase from E9.5-11, *Pbx1* transcripts remain in flank and proximal limb bud mesenchyme only (Figure 1). Conversely, during this same phase, *Pbx2* transcripts become localized distally once the forelimb bud is formed (E9.25). From E9.5 to E11, *Pbx2* expression remains distal, and as the bud matures it is confined only along the peripheral rim of the distal mesenchyme (Figure 1; unpublished data). However, *Pbx2* expression still partially overlaps with *Pbx1* (and *Pbx3* in forelimb only) in the anterior proximal limb field, a domain important for girdle development. During this phase, besides their mesenchymal expression, *Pbx1* and *Pbx2* display also overlapping expression in the developing AER (our unpublished results). Unlike *Pbx1* and *Pbx2*, *Pbx3*

expression is present in the flank of early (E9.5) embryos (Figure 1) but shortly thereafter becomes restricted to only the forelimb bud mesenchyme (until E11.0-11.5). Interestingly, *Pbx3* is not present in the AER (our unpublished observations). Regarding *Meis*, *Meis1-3* expression is typically restricted during these stages to the proximal fore- and hindlimb mesenchyme (Capellini et al., 2006; unpublished data), although *Prep* overlaps in its distal domain with *Pbx2* (our unpublished results). Overall, these early expression patterns for Phase I and Phase II suggest a scenario wherein *Pbx1* and *Pbx2* provide first an overlapping and then a complementary “code” along the early limb and girdle fields and in the entire limb bud (Figure 1). In contrast, *Pbx3* displays unique expression patterns that make this family member an excellent candidate for a highly specific role in early forelimb development (Di Giacomo et al., 2006). Experimental work on compound *Pbx1/Pbx3* mutants suggests this may be the case (see below).

Despite their dynamic, and unique expression domains from E9.5 to E11, later in development, *Pbx1-3* transcripts substantially overlap in their spatial extent. During Phase III in the forelimb, expression of all three *Pbx* (1–3) overlaps in the anterior and posterior mesenchyme proximal to the autopod (E11–11.75, Figure 1). In the hindlimb, transcripts for *Pbx1* and *Pbx2* are present at high levels within these domains, unlike *Pbx3*, which begins being expressed in the hindlimb only after E11. Phase IV occurs during handplate and footplate formation, when all three *Pbx* are co-expressed in the interdigital mesenchyme, suggesting that the three genes play important roles in later digit specification and morphogenesis (Di Giacomo et al., 2006). Lastly, during Phase V, once the skeletal elements of the limb are formed, *Pbx1*, *Pbx2*, and *Pbx3* are similarly expressed in proliferative chondrocytes of long bones, while their expression wanes in prehypertrophic and hypertrophic chondrocytes in endochondral ossification (Selleri et al., 2001).

Limb phenotypes of *Pbx* mutant mice

The multi-phase expression patterns described above suggest that *Pbx* family members have potentially multiple roles during limb positioning, induction, and outgrowth, as well as limb and girdle patterning, along with functions in digital patterning and endochondral ossification. The use of global mutant mice and the generation of compound mutant embryos, in which multiple *Pbx* alleles are concomitantly lost, have shed light on *Pbx* critical functions in these developmental processes.

With regard to the limbs and girdles of single *Pbx* mutant embryos, only *Pbx1* homozygotes exhibit phenotypes (Selleri et al., 2001), while *Pbx2* and *Pbx3* homozygotes exhibit phenotypically normal appendages (Rhee et al., 2004; Selleri et al., 2004). This finding, along with those obtained from the study of compound *Pbx* mutants, demonstrate the prime role of *Pbx1* in multiple developmental processes (Capellini et al., 2006; Capellini et al., 2008; Capellini et al., 2010). *Pbx1* mutants exhibit forelimbs with a superior-to-inferior reduced scapular blade that is fused at the scapular head to an adjoining humerus, which bears a detached and rudimentary deltoid tuberosity (Figure 2; Selleri et al., 2001). Their hindlimbs display a pelvis that often lacks an ilium or exhibits one that is detached from the pubis and ischium, and which is fused at the hip or femoral-acetabular joint to a truncated femur. Despite these proximal alterations, the distal fore- and hindlimbs of *Pbx1* homozygous embryos appear phenotypically normal (Figure 2; Selleri et al., 2001). *Pbx1* roles in limb development are also evident in zebrafish, in which loss of *Pbx1* ortholog *pbx4* leads to the reduction and absence of the pectoral fin in the *Lazarus* mutant (Pöpperl et al., 2000). The roles of other *pbx* paralogs in zebrafish remain unknown.

To date, analysis of available loss-of-function mouse models for the genes encoding the *Pbx* TALE dimerization partners *Meis* and *Prep*, has not been informative. Indeed, single *Meis1* and *Prep* mutants, along with compound *Meis1/Prep* mutants, do not reveal limb skeletal

phenotypes. All mutants listed above bear normal limbs, while *Meis2* mutants have not been obtained yet (Fernandez-Diaz LC, Torres, M and Blasi, F personal communication). Interestingly, work conducted in chick reported that *Meis* genes are involved in limb development, since over-expression of *Meis* in distal limb buds leads to proximalization of the limb (Capdevila et al., 1999; Mercader et al., 1999; Mercader et al., 2000; Mercader et al., 2005). While these data do indicate that *Meis* homeoproteins are important for proximal limb patterning, their requirements in mammalian limb development are still mostly unknown, as they cannot be inferred solely from over-expression experiments. In sum, to date there is no functional study in the mouse demonstrating that *Meis* genes are sufficient and/or required for limb bud outgrowth and patterning in mammals.

While the analyses of single *Pbx* mutants reveal a role for *Pbx1* in patterning proximal limb and girdle structures, by generating compound *Pbx* mutants we have addressed *Pbx* roles in the entire appendage and girdle. In this regard, we have begun to investigate issues of functional cooperation and redundancy within domains in which multiple *Pbx* genes are co-expressed. Below we address *Pbx* involvement in limb positioning, induction, and outgrowth, as well as limb and girdle patterning, along with their functions during endochondral ossification.

Pbx and limb bud positioning

One of the first events involved in limb formation is the specification of the axial levels where limbs form. It was demonstrated by the work of Harrison (1921) and Slack (1976), for example, using surgical manipulations of chick and salamander embryos that limb bud position was determined well before bud induction and outgrowth. Yet, despite the seminal work of the early days, the determination of limb bud position along the embryonic rostral-caudal axis still remains enigmatic. A sparse number of mouse mutant models exhibit shifts in the axial position of their limb buds allowing the study of critical factors required for positional determination. Such mouse mutants, as for example single and compound *Hox* mice, exhibit hindlimbs that are caudally shifted along the embryo (McIntyre et al., 2007). These studies are supported by work in the chick, demonstrating that the expression of *Hox9* paralogs along the axial LPM is important in maintaining the boundary conditions involved in limb positioning (Cohn et al., 1997). In these studies, however, little has surfaced regarding the molecular functions of these genes in axial specification. Interestingly, among *Polycomb* genes, compound loss of *Eed* and *Bmi* leads to modest rostral shifts in hindlimb axial position (Kim et al., 2006). As *Eed* and *Bmi* typically act as repressors of *Hox* in anterior axial domains, rostralization of the expression domains of multiple axial *Hox* genes is observed when these *Polycomb* genes are experimentally ablated (van der Lugt et al., 1994; Faust et al., 1995; Faust et al., 1998; Lessard et al., 1999; Kim et al., 2006). Thus, *Polycomb* may provide essential genetic control of limb positioning along the axis, via the modulation of *Hox* territories.

Importantly, *Pbx* loss from the embryonic axis (i.e., in *Pbx1*^{-/-};*Pbx2*^{+/-} mutants), from Phase I onwards, leads to reductions in the expression of the *Eed* and *Bmi* *Polycomb* genes and to simultaneous rostralizations in *Hox* expression territories within the somites and adjacent LPM (Capellini et al., 2008; Figure 3). These changes are indeed coincident with rostral shifts in the position of the hindlimb bud and limb skeleton in *Pbx* mutants (Capellini et al., 2008; Figure 3). For example, unlike single *Pbx1*^{-/-} and other compound *Pbx* mutant embryos (including compound triple *Pbx1*^{+/-};*Pbx2*^{+/-};*Pbx3*^{+/-} heterozygotes), *Pbx1*^{-/-};*Pbx2*^{+/-} embryos show rostral shift of the hindlimb buds along the somites and subsequently limb skeletons that are shifted rostrally by several lumbar-sacral vertebrae (Figure 3; and data not shown). These findings suggest that *Pbx1* and *Pbx2* cooperate in domains wherein the two genes exhibit overlapping expression to position the limb bud. The lack of axial shifts in examined compound *Pbx1/Pbx3* mutant embryos is in agreement with

our findings that *Pbx1* and *Pbx2* but not *Pbx3* are expressed in the posterior hindlimb embryonic field (Figure 1). Thus, hindlimb positioning along the embryonic axis is controlled specifically by genetic interactions of *Pbx1* and *Pbx2*, which may accomplish this task via their control of *Polycomb* gene expression. While this possibility is intriguing, it remains unclear whether Pbx also cooperate with Hox as co-factors in this process (see above). If this were the case, then the observed limb shifts in *Pbx* mutants could also be the result of a more marked and broad loss of Hox DNA-binding specificity within the LPM, coincidental with alterations in *Polycomb* gene expression.

Pbx and girdle development

Prior to and during initial limb bud induction, the LPM along the flank is partitioned into a medial portion, the mesodermal somatopleure, and a lateral portion, which gives rise to the limb proper. The mesodermal somatopleure, at the level of the respective limb fields, gives rise to specific structures of both the pectoral and pelvic girdles (Huang et al., 2000; Malashichev et al., 2008). In the forelimb, cells from specific axial levels of the dermomyotome migrate into this somatopleure, and via cues within this tissue, as well as from the paraxial mesoderm and ectoderm, become specified to form superior to inferior aspects of the scapular blade (Huang et al., 2000). Also, cells of the LPM are specified during the early limb bud stages to form the scapular head and neck, bony structures that directly articulate with the proximal humerus of the limb proper (Huang et al., 2006). In contrast, at the level of the hindlimb, only cells originating from the mesodermal somatopleure have been shown to give rise to the three main elements of each pelvic bone, i.e., a rostral oriented ilium, a ventral facing pubis, and a dorsal positioned ischium (Malashichev et al., 2008). These three elements fuse during development at the location of the hip joint, whereby each pelvic bone articulates with the proximal femur of the limb proper (Pomikal and Streicher, 2010). A number of genetic pathways are known to govern patterning of the bony elements of both pectoral and pelvic girdle during development, though knowledge of the genetic control of girdle development is still rudimentary in comparison to that of limb development (Kuijper et al., 2005; Huang et al., 2006). Below we will discuss *Pbx* contributions to the genetic control of girdle development and how *Pbx* loss during Phases I–III has marked phenotypic consequences on the development of each girdle. Importantly, like in axial development and limb positioning, *Pbx1*, *Pbx2*, and *Pbx3* appear to have distinct and differential functions in pectoral *versus* pelvic development, likely a consequence of differences in their respective expression patterns.

In the pectoral girdle, we have found that *Pbx1*^{-/-}, *Pbx1*^{-/-}; *Pbx2*^{+/-}, and *Pbx1*^{-/-}; *Pbx3*^{+/-} embryos exhibit alterations of the scapular blade and head. The latter two genotypes show markedly more severe phenotypes compared to the former (Capellini et al., 2010). While the scapular defects are more severe in *Pbx1*^{-/-}; *Pbx2*^{+/-} compared to *Pbx1*^{-/-}; *Pbx3*^{+/-} mutants, both bear reduced blades, fusions of the scapular head to the humerus and apparent duplications of the head/neck complex. We reported that blade patterning genes within the mesodermal somatopleure, but not within the dermomyotome, are severely down-regulated in compound *Pbx* mutants. Specifically, we identified marked down-regulation of genes that pattern the superior (*Alx4*, *Alx1*) (Kuijper et al., 2005), central (*Tbx15*) (Kuijper et al., 2005; Lausch et al., 2008), and inferior (*Gli3*) (Johnson, 1967; Kuijper et al., 2005) scapular blade. Also, within the mesodermal somatopleure (and LPM of the proximal limb bud) scapular head and neck markers, such as *Hoxc6* (Oliver et al., 1990), were severely reduced and mis-expressed in *Pbx* mutants, along with genetic pathways controlling mesenchymal condensation and chondrogenesis (Wright et al., 1995; Bi et al., 2001) (i.e., the *Emx2* → *Sox9* pathway; and *Col2a* expression) (Capellini et al., 2010). Importantly, while a major theme of research has focused on Pbx roles as Hox co-factors, we revealed that their functions in scapular development reside, at least in part, in their ability to form

heterodimeric complexes with homeodomain proteins other than Hox. Within the context of the complex morphogenesis of the scapula, we identified critical genetic interactions of *Pbx1* with *Emx2*, a gene responsible for scapular blade patterning, and established that both *Pbx1* and *Emx2* proteins form a heterodimeric complex capable of directly regulating *in vivo* and *in vitro* a conserved *Alx1* non-coding element (Capellini et al., 2010). Thus, in the forelimb pectoral field, *Pbx* homeoproteins cooperates with *Emx2* affecting the transcriptional activation of key pathways in scapular blade and head formation.

As for the pelvic girdle, we recently found that *Pbx1*^{-/-}; *Pbx2*^{+/-} mutants exhibit only one rudimentary ischial fragment that remains fused to the femoral head (Capellini et al. in this issue). Compared to our findings in the pectoral girdle, we did not observe a strong genetic interaction of *Pbx1* with *Pbx3* or *Emx2*, but found that *Pbx* genes lie upstream of *Emx2*, controlling its expression in the mesodermal somatopleure. We also established that *Pbx* homeoproteins hierarchically regulate multiple genes and pathways involved in the formation of each element of the pelvic girdle. Notably, we demonstrated that *Pbx* genes act upstream of *Pitx1*, *Tbx15*, and *Emx2*, genes that control ilium formation (Lanctot et al., 1999; Pellegrini et al., 2001; Marcil et al., 2003; Lausch et al., 2008); *Alx1*, *Prrx1*, and *Twist1*, genes that affect pubis development (ten Berge et al., 1998; Kuijper et al., 2005; Krawchuk et al., 2010); and *Pax1*, a marker of ischium formation (Timmons et al., 1994; LeClair et al., 1999). These results suggest that *Pbx* homeoproteins play critical roles in the early somatopleure, likely underlying the substantial down-regulation of genes involved in pelvic girdle development. Through the use of novel bioinformatic analyses (McLean et al., 2010) we additionally revealed that *Pbx* genes control girdle and hindlimb morphogenesis by potentially regulating multiple genes (including those listed above) with expression domains within the pelvic field/hindlimb. By this approach, we also found potential *Pbx1*/*Emx2* binding sites in non-coding, conserved regions adjacent to genes whose disruption (by loss of function mutations in mouse, or spontaneous mutation in humans) results in pelvic/hindlimb phenotypes, such *Prrx1* and *Tbx15* (Capellini et al., in current issue).

Pbx roles in developmental programs as cell fate specification have long been considered primarily as those of Hox cofactors (reviewed in Moens and Selleri, 2006). However, it is of note that no known single or compound Hox mutant mouse model displays scapular blade defects. Only *Hox5* mutants exhibit rostral blade shifts, with normal blade morphology (Capellini et al., 2010). Similarly, misexpression or loss of Hox genes result at best in mild alterations of the pelvic girdle, including defects in mice misexpressing *Hoxd12* in lateral plate derivatives; modest malformations of pelvic bones and sacrum in *Hoxc10* mutants; and lack of uterosacral ligaments in *Hoxa11* mutant mice (Capellini et al., in this issue). Therefore, it is unlikely that *Pbx* homeoproteins effect their roles in girdle formation solely as Hox cofactors, suggesting instead cooperation with other proteins, as *Emx2*, in scapula development. Thus, we cannot yet invoke a mechanism whereby loss of *Pbx* proteins, as Hox co-factors, leads to the girdle phenotypes of *Pbx* compound mutants via altered Hox function.

Roles of *Pbx* in limb P-D, A-P, and D-V axis formation, outgrowth, and skeletal patterning

When compared to single *Pbx* mutants, compound mutants exhibit significant alterations in most skeletal elements of each limb type (Capellini et al., 2006; Capellini et al., 2010). At E13.5, *Pbx1*^{-/-}; *Pbx2*^{+/-} limbs display marked exacerbations of the proximal limb phenotype of single *Pbx1*^{-/-} mutants as well as novel distal limb malformations (Figure 2). For example, *Pbx1*^{-/-}; *Pbx2*^{+/-} forelimbs have severely dysmorphic humeri that are fused to the girdle at the scapulohumeral joint (Capellini et al., 2010). Distally, these mutants exhibit a truncated and dysmorphic radius and ulna and bear substantial reductions in digits one and five (Capellini et al., 2006). On the other hand, *Pbx3* functions at this time are limited to the formation of the humerus and scapula, as *Pbx1*^{-/-}; *Pbx3*^{+/-} mutants forelimbs are affected

only proximally (Figure 2; (Capellini et al., 2010)). Compound *Pbx1/Pbx3* double homozygous mutants die during early limb formation, thus limiting a deeper assessment of *Pbx3* roles in this process.

The severity of appendicular phenotypes due to compound *Pbx* loss is more apparent in the hindlimb, wherein only *Pbx1* and *Pbx2* are expressed during limb outgrowth (Phase I and II; Figure 1). Therefore, in the hindlimb dosage reductions have more substantial effects, since loss of *Pbx1* and *Pbx2* enables a *Pbx*-null state. Accordingly, *Pbx1*^{-/-}; *Pbx3*^{+/-} hindlimbs do not present phenotypic exacerbations *versus* single *Pbx1* null mutants, and exhibit normal limb skeletons distal to the femur (Capellini et al., current issue). On the other hand, *Pbx1*^{-/-}; *Pbx2*^{+/-} hindlimbs at E13.5 exhibit a markedly truncated femur fused to a residual pelvic rudiment (Figure 2; Capellini et al., 2006). Distally, there is only one zeugopodial element, a moderately malformed but truncated tibia, and a single autopodial ray consisting of one digit, likely digit number one (Figure 2). This severe digit reduction is similar to that observed in *Sonic hedgehog* (*Shh*) null mutants (Chiang et al., 2001). The absence of distal limb elements and the drastic reduction in digit number, along with the severe proximal limb malformations, reveals that both *Pbx1* and *Pbx2* are required for patterning skeletal elements within all limb compartments. Despite the documented early gestational lethality of *Pbx1*^{-/-}; *Pbx2*^{-/-} homozygous mutants at E10.5, these embryos have forelimb buds that are highly dysmorphic and hindlimb buds that are barely detectable (Capellini et al., 2006).

Roles of *Pbx* in P-D outgrowth

The process by which the limb prominences undergo lateral, distal outgrowth occurs through the coordinated regulation of the three main P-D, A-P and D-V developmental axes (see Zeller et al., 2009). Disruptions in any one of these axes typically alters the proper development of the others and often leads to cessation of limb bud outgrowth and/or mis-patterning of soft tissue structures and skeletal elements. One of the earliest events in the establishment of the P-D axis is the induction of a fibroblast growth factor, *Fgf10*, in the early mesenchyme subjacent to the limb ectoderm of both limb prominences (E8.5–9.5) (Cohn et al., 1997; Ohuchi et al., 1997; Min et al., 1998; Sekine et al., 1999). While being induced by *Tbx4* and *Tbx5* in the forelimb and hindlimb fields, respectively (Agarwal et al., 2003; Minguillon et al., 2005; Koshiba-Takeuchi et al., 2006; Saito et al., 2006), *Fgf10* expression in these domains may also be under the control of collinear *Hox* gene expression in the early limb bud (Zakany et al., 2007). Once induced, however, *Fgf10* expression promotes the expression of *Fgf8* within a maturing strip of specialized ectodermal epithelium at the distal tip of the limb bud, the pre-AER and AER (Barrow et al., 2003). *Fgf8* expression in the AER then further promotes cellular outgrowth and patterning in the limb via a feedback mechanism on *Fgf10* expression in the subjacent mesenchyme (Figure 4) (Xu et al., 1998; Lizarraga et al., 1999). This *Fgf10*-*Fgf8* loop likely aids the early specification of the skeletal elements of the three limb compartments, and promotes and/or maintains the expression of multiple genes critical for limb development, such as *Hox*, *Wnt*, and *Shh* (Niswander et al., 1994)(reviewed in Niswander, 2003; Zeller et al., 2009). For example, *Hox* expression territories along the P-D axis are also under the influence of *Fgf* expression in the distal limb (Sun et al., 2002) and are essential for P-D limb outgrowth, as evinced by extreme distal limb truncations in mutants lacking entire *HoxA/D* cluster genes (Kmita et al., 2005). In these mutants, there is also a coordinated down-regulation and cessation of the *Fgf10*-*Fgf8* feedback loop demonstrating the complex circuitry between these factors.

As the proper spatio-temporal regulation of *Hox* genes and *Fgfs* is critical to the establishment of the P-D axis in the early limb prominence, we have identified essential roles for *Pbx* as upstream regulators of *Hox* gene expression in the early limb bud (Figure 4). Indeed, in *Pbx1*^{-/-}; *Pbx2*^{+/-} mutants, transcripts of both 5' *HoxA* and 5' *HoxD* genes are

substantially reduced and dysregulated in the forelimb bud, while they are absent in the early hindlimb bud specifically prior to and during initial P-D limb outgrowth (during Phase I and II; Figure 1) (Capellini et al., 2006). *Pbx1*^{-/-}; *Pbx2*^{+/-} embryos exhibit truncated and mispatterned proximal-to-distal hindlimbs, defects which partially phenocopy those observed when entire HoxA and HoxD clusters are simultaneously lost from the limb (Kmita et al., 2005). Furthermore, in addition to the observed reductions in Hox expression in the hindlimb, we also identified reductions in *Fgf10* and *Fgf8* expression in the distal limb mesenchyme and AER, keeping in mind that the residual expression of *Fgf10* and *Fgf8* in their respective domains likely results from the remaining copy of *Pbx2* in this mouse model (Capellini et al., 2006; data not shown). Accordingly, a hierarchical role for *Pbx* on P-D axis formation and the Fgf10-Fgf8 loop is evinced by the failure of hindlimb formation in compound *Pbx1*^{-/-}; *Pbx2*^{-/-} mutants (Capellini et al., 2006). Interestingly, in this mutant genotype *Fgf8* expression is completely lost in the hindlimb bud and extremely reduced in the forelimb bud (unpublished data; Figure 5). It is of note that loss of *Fgf8* expression is not observed in other tissues of these mutant embryos, and is maintained, for example, at the midbrain to hindbrain junction (Figure 5; unpublished data).

Roles of *Pbx* in limb A-P axis formation

Concurrent with the initiation of the Fgf10-Fgf8 feedback loop, early asymmetry in the A-P axis occurs due to inhibitory interactions in the anterior and posterior limb field mesoderm, specifically between *Gli3* and *Hand2*, respectively (Figure 4). This mutual antagonism ultimately leads to the restriction of *Hand2* expression to the posterior aspect of the early limb prominence (te Welscher et al., 2002). Simultaneously, collinear HoxA and HoxD gene expression patterns overlap at first in a wide A-P distributed pattern along this field. However, as the limb bud undergoes P-D outgrowth, transcripts of HoxA and HoxD genes become increasingly restricted to the distal and posterior limb bud (Zakany et al., 2004). This expression pattern, along with the presence of *Hand2* (Charite et al., 2000), leads to the activation of *Shh* along the posterior limb bud mesenchyme, in the ZPA (Figure 4) (Zakany et al., 2004), which is essential to distal limb and digital patterning (Riddle et al., 1993). *Shh* signaling in the ZPA mesenchyme promotes a cascade of gene expression leading to the induction of *Fgf4* in the AER (Chiang et al., 1996; Chiang et al., 2001). *Fgf4* further promotes ZPA function, maintains *Shh* and Hox gene expression along the A-P and P-D axes and thus facilitates further outgrowth and patterning of the distal limb (Niswander et al., 1993; Fallon et al., 1994; Niswander et al., 1994). In this regard, Hox genes serve to coordinate early development of the AER and ZPA via their effects of *Fgf10* and *Shh*, both of which then serve to maintain positive regulatory interactions in the limb bud along both A-P and P-D axes (Figure 4).

By showing that *Pbx* genes are required for regulating Hox gene expression in the limb, we have revealed a critical control of A-P axis formation by *Pbx*. Indeed, we have observed that the loss of *Pbx1* and *Pbx2* in the hindlimb field (i.e., in *Pbx1*^{-/-}; *Pbx2*^{-/-} mutants) leads to the complete failure of *Shh* induction in the ZPA, unlike in other tissues such as the cloaca and notochord wherein *Shh* is expressed at normal levels in these mutants (Capellini et al., 2006; Capellini et al., 2008) (Figure 4). Interestingly, in *Pbx1*^{-/-}; *Pbx2*^{+/-} hindlimbs, *Shh* absence is not coupled with marked reduction of *Fgf8* expression in the posterior AER at early days, or of *Hand2* expression in the mesenchyme, but is correlated with the near complete loss of all HoxA and HoxD transcripts in the pre-ZPA. Furthermore, the down-regulation of Hox and absence of *Shh* expression leads expectedly to the complete breakdown of the *Shh*-Fgf4 loop and the cessation of limb bud development. We have revealed that *Fgf4* is not expressed in *Pbx1*^{-/-}; *Pbx2*^{+/-} hindlimb AER and that all effectors of this feedback loop are substantially disrupted (for review see Zeller et al., 2009). Not surprisingly, the distal hindlimb zeugopodial and autopodial phenotype of compound

Pbx1^{-/-};*Pbx2*^{+/-} mutants phenocopies that observed in *Shh* null mutants (Chiang et al., 2001), while the striking proximal-to-distal phenotype across all limb compartments result at least in part from significant disruption of *HoxA/D* expression (Kmita et al., 2005). In the forelimb, *Hox* genes are down-regulated and mis-expressed, but not as severely as in the hindlimb, and *Shh* is only mildly down-regulated in the *Pbx1*^{-/-};*Pbx2*^{+/-} mutant genotype, likely a consequence of *Pbx3* expression in the forelimb. Notably, the importance of *Hox* control by *Pbx* in the pre-ZPA was further substantiated by our finding that both *Hox* and *Pbx* proteins are directly bound *in vivo* to a *Shh* long range limb enhancer (Capellini et al., 2006). This enhancer, when experimentally deleted in mice, recapitulates a global *Shh* null limb phenotype (Lettice et al., 2002; Lettice et al., 2003; Sagai et al., 2005). Thus, *Pbx* homeoproteins are essential upstream regulators of both P-D and A-P axes formation in the limb, in part via their coordinated control of *Hox* expression in the bud mesenchyme.

The severe alterations of 5' *HoxA/HoxD* gene expression, especially in posterior hindlimb bud domains of *Pbx1/2* mutant embryos, occurred in limbs that exhibited relatively normal early AP patterning. Therefore, at least in the limb, *Pbx1/Pbx2* act upstream of *Hox* genes and not solely as *Hox*-cofactors, in a manner that appears to occur independently from known molecular regulators required for the establishment of early AP asymmetry. Furthermore, this *Pbx*-mediated *Hox* gene control occurs early, perhaps in the limb field, because both 3' and 5' *HoxA/HoxD* genes were already altered at the onset of limb bud development. The mechanisms by which *Pbx* hierarchically control and maintain *Hox* genes spatial distribution in early limb mesenchyme remain elusive. *Pbx* cooperative roles with other transcription factors upstream of *Hox* genes (Berkes et al., 2004) (reviewed by Moens and Selleri, 2006) may have bearings. Indeed we cannot exclude the possibility that in the limb *Pbx* might directly regulate *Hox* gene colinearity, given their co-expression with *Hox* genes in the limb bud.

Roles of *Pbx* in limb D-V axis formation

Unlike for the P-D and A-P axes and their coordinated regulation, fewer molecular regulators of D-V axis formation have been identified. It is also unclear whether *Hox* genes have roles in D-V axis establishment. Two known genetic regulators of the D-V axis are *Engrailed1* (*En1*), expressed in the ventral ectoderm and essential for ventral fates, and *Wnt7a*, expressed in the dorsal ectoderm and critical for dorsal fates. Genetic studies using single and compound loss-of-function models for *En1* and *Wnt7a* demonstrated roles for these genes in D-V patterning and AER formation (Loomis et al., 1998). Specifically, *En1* has been shown to inhibit *Wnt7a* signaling ventrally, restricting it to the dorsal ectoderm, whereas *Wnt7a* spatially limits the expression of the transcription factor *Lmx1b* to the dorsal mesoderm (Loomis et al., 1996). In turn, *Lmx1b* has been shown to be critical for dorsal mesenchymal and ectodermal patterning (Riddle et al., 1995; Vogel et al., 1995; Chen et al., 1998). Interestingly, loss of proper D-V axis establishment, particularly via the disruption of *Wnt7a* in the dorsal ectoderm, does lead to the reduction of *Shh* expression in the ZPA and failure of A-P axis formation. This finding indicates that some degree of developmental coordination between these axes exist. Likewise, as *En1* and *Wnt7a* also genetically interact to direct proper AER development in the distal limb ectoderm (Loomis et al., 1998; Kimmel et al., 2000), they are important factors in P-D axis establishment and limb bud outgrowth (Figure 4).

Recent results in our laboratory (unpublished) suggest that *Pbx* genes may also have a role in the formation of the D-V axis. Indeed, we examined the expression of the key D-V regulators discussed above in *Pbx1*^{-/-};*Pbx2*^{+/-} embryos from E10 to E11 (note: we could not examine double *Pbx1*^{-/-};*Pbx2*^{-/-} embryos as they succumb *in utero* prior to these gestational days). In E10.25 *Pbx1*^{-/-};*Pbx2*^{+/-} forelimbs, we found that *En1* expression was reduced in the most anterior and posterior domains of the ventral ectoderm, while in

hindlimbs it was specifically down-regulated in the posterior aspect of the ventral ectoderm (Figure 5). However, despite *En1* reduction, we found that the expression of genes such as *Fgf8* remained mostly unperturbed in the posterior AER of the early bud (Capellini et al., 2006). The expression of *Lmx1b* was also examined. *Lmx1b* is normally expressed throughout the early mesenchyme prior to E9.5–10.5, but later becomes restricted to the dorsal mesenchyme. At E10.75, we found that *Lmx1b* expression was restricted to the dorsal mesenchyme of *Pbx1*^{-/-};*Pbx2*^{+/-} forelimbs as in wildtype littermates, indicating that the D-V axis was relatively well established in this mutant limb type. Furthermore, no overt skeletal or soft-tissue phenotypes which could reflect mis-patterning along the D-V axis were identified in the mutant forelimb. However, in *Pbx1*^{-/-};*Pbx2*^{+/-} hindlimbs, *Lmx1b* expression was up-regulated compared to somite-matched littermate controls (Figure 5). Specifically, this up-regulation was evident not only throughout the dorsal limb mesenchyme but also in the posterior ventral mesenchyme, suggesting a possible localized perturbation of Wnt7a signaling in the overlying ectoderm (Figure 5). However, we observed only a minor down-regulation of *Wnt7a* expression in this domain (data not shown). Interestingly, we found that *Lmx1b* was also expressed in ventral posterior and anterior domains in earlier staged wildtype hindlimb buds. This observation suggested that the ectopic *Lmx1b* expression in posterior *Pbx1*^{-/-};*Pbx2*^{+/-} hindlimbs potentially results from a developmental delay (Figure 5). Currently, it remains unclear if control of P-D and A-P axes formation by Pbx factors leads to a concurrent disruption in early D-V polarity, or if the latter D-V disruption is cell autonomous and results from *Pbx* loss in the ectoderm.

Overall, the loss of multiple *Pbx* genes from the limb substantially affects the establishment of all three developmental axes and disrupts major signaling centers. It is also highly probable, given the alteration of limb bud positioning and loss of key signaling centers in the early limb bud of *Pbx* compound mutants, that Pbx homeoproteins exert their roles in early development during limb initiation, prior to the formation of the A-P, P-D and likely D-V axes (Figure 4). Experiments by tissue-specific and temporally controllable *Pbx* deletion in the mouse are ongoing to address all of these open questions.

Pbx limb phenotypes and models of limb specification

We have established that the dosage of *Pbx1* is paramount, compared to that of *Pbx2* and *Pbx3*, in the developmental control of multiple organs, including the limb and girdle, (Capellini et al., 2006; 2008; 2010). We have also observed that *Pbx2* is the only *Pbx* family member that overlaps markedly with *Pbx1* in the early limb field (and ectoderm) and is the only *Pbx* expressed in the distal limb mesoderm after initial bud formation (Figure 1). Of all compound mutants analyzed, including *Pbx1*^{+/-};*Pbx2*^{+/-}, *Pbx1*^{+/-};*Pbx2*^{-/-}, *Pbx1*^{-/-};*Pbx3*^{+/-}, *Pbx1*^{+/-};*Pbx3*^{-/-}, *Pbx2*^{-/-};*Pbx3*^{+/-}, *Pbx2*^{+/-};*Pbx3*^{-/-}, and *Pbx1*^{+/-};*Pbx2*^{+/-};*Pbx3*^{+/-}, only compound *Pbx1*^{-/-};*Pbx2*^{+/-} and *Pbx1*^{-/-};*Pbx2*^{-/-} mutants have drastic P-D limb defects in which each compartment of the forelimb and hindlimb is markedly affected. Multiple known markers of early limb P-D and A-P axis formation are markedly perturbed in these mutants. These experimental findings are in conflict with our observations that single *Pbx2*^{-/-} and compound *Pbx1*^{+/-};*Pbx2*^{-/-} mutants have phenotypically normal limbs (Selleri et al., 2004; Capellini et al., 2006), and lack changes in limb gene expression. Taken together, these data constitute strong genetic evidence that Pbx1 and Pbx2 control early limb bud formation, in domains wherein they overlap and that Pbx1 plays a prime role in this interaction. We argue that they most likely govern specification and patterning of the limb skeletal elements early in development within the limb field proper.

As several models have been put forth over the last five decades describing how cells become specified to form the individual P-D segments of the limb skeleton (see (Benazet and Zeller, 2009) for a detailed review), each of these models (e.g., progress zone model,

early specification model, two signal gradient model, and differentiation front model) has specific predictions as to when and where cells of each of the limb compartments are specified, as well as to the roles that specific limb centers (i.e., proximal limb, PZ, AER) have during development. In this regard, it will be relatively straightforward to test against these temporal requirements using conditionally targeted *Pbx* alleles, together with specific Cre deleter lines that permit excision of *Pbx* at specific time points (prior to, or during, early limb field induction *versus* during limb bud formation) and in select limb spatial compartments (mesoderm *versus* AER). It is clear from predictions based on preliminary experimental evidence that two key features of *Pbx* expression (i.e., localization in mesoderm *versus* AER and differential temporal specificity) must be addressed in depth by genetic dissection. These critical experiments are currently underway. They will pinpoint *Pbx* functions in the limb and reveal when and where the dominant control of limb axes formation by *Pbx* homeoproteins occurs during development.

Pbx roles in limb endochondral ossification

Soon after generating the single *Pbx1* loss-of-function mouse model, we reported that the affected embryonic domains of limbs (and ribs) in these mutants displayed markedly diminished chondrocyte proliferation, associated to a notable increase in the number of hypertrophic chondrocytes, accompanied by premature bone ossification (Selleri et al., 2001). Intriguingly, the pattern of expression of genes known to regulate chondrocyte differentiation was not perturbed in *Pbx1*-deficient cartilage at early days of embryonic skeletogenesis. However, precocious expression of *Colla1*, a marker of bone formation, was found in single *Pbx1* homozygous mutant embryos. These initial studies established an iterative role for *Pbx1* in multiple developmental programs in skeletal formation and revealed a novel function in coordinating the extent and/or timing of proliferation with terminal differentiation during limb endochondral ossification. These later functions of *Pbx* impact on the rate of endochondral ossification and bone formation (Selleri et al., 2001).

Additionally, it was recently shown that *Pbx1* represses osteoblastogenesis by blocking *Hoxa10*-mediated recruitment of chromatin remodeling factors in mesenchymal cells. Conversely, targeted depletion of *Pbx1* by short hairpin RNA (shRNA) in cultured cells increased expression of osteoblast-related genes. Chromatin-associated *Pbx1* and *Hoxa10* were present at osteoblast-related gene promoters preceding gene expression, but only *Hoxa10* was associated with these promoters during transcription. These interesting results further revealed that *Pbx1* is associated with histone deacetylases normally linked with chromatin inactivation. Loss of *Pbx1* from osteoblast promoters in differentiated osteoblasts was associated with increased histone acetylation and CBP/p300 recruitment, as well as decreased H3K9 methylation. Based on these studies, conducted in cultured cells, it was proposed that *Pbx1* plays a central role in attenuating the ability of *Hoxa10* to activate osteoblast-related genes in order to establish temporal regulation of gene expression during osteogenesis (Gordon et al., 2010). It will now be important to corroborate these exciting findings in a system *in vivo*, possibly in animal models deficient for *Pbx* proteins.

Pbx function in evolution

Comparisons of gene sequence data for coding regions of *Pbx* orthologs indicate that they are evolutionarily conserved among Metazoan taxa (Moens and Selleri, 2006). For example, vertebrates such as mice and humans, and invertebrates, such as flies, share at least 90% identity in coding regions of *Pbx1* orthologs. This degree of conservation is even higher at the protein sequence level. This high conservation among *Pbx1* genes across different species is also present, though to a lesser extent, among the other orthologs (e.g., *Pbx2* across different vertebrates) and even between family members (e.g., between *Pbx1* and *Pbx2* in vertebrates). These findings suggest that *Pbx* proteins have remained conserved in

terms of general functionality over the course of Metazoan evolution (Moens and Selleri, 2006). However, it is possible that cis- and trans-regulation of *Pbx* has undergone evolutionary changes leading to differences in the spatio-temporal expression of these genes and their functional domains. Unfortunately, the regulatory elements for *Pbx* remain unknown and are the subject of current investigations. Regardless of this limitation, it is important to address if there exist functional differences in *Pbx* genes across different Metazoan taxa, and how such differences could relate to morphological divergence and specialization. At the same time, while comparative data on *Pbx* gene expression and function in different mammals remain unknown, knowledge of *Pbx* function during limb and girdle development can be used to address issues of modularity and the manners in which variation is generated by developmental systems. This knowledge can serve as a framework for interpreting evolutionary changes in limb and girdle morphology and function.

exd/Pbx and the evolution of invertebrate and vertebrate limbs

Comparisons of the expression of *Pbx* to its invertebrate ortholog, *exd*, indicate some similarities and differences that may stem from common heritage as well as from functional constraints during evolution (Figure 6). For example, *exd* is expressed in the wing and leg imaginal disc, ectodermal tissues critical for appendage patterning in flies (Rieckhof et al., 1997; Mercader et al., 1999). While *exd* is expressed in other fly embryonic tissues, it does not appear to be expressed in the epithelial-like cap surrounding the imaginal disc. These findings suggest that *exd* may not be required for regulating tissue-to-tissue interactions in the fly appendage. Conversely, *Pbx1* (and *Pbx2*) are expressed in both the vertebrate limb mesenchyme, a tissue of mesodermal origin, and in the surface ectoderm and AER of the limb and embryo proper (Schnabel et al., 2001; Selleri et al., 2001; and unpublished results). Thus, *Pbx1* may have essential roles for mesenchymal-epithelial interactions in vertebrate limb development. Other *Pbx* genes have been discovered only in vertebrates, and their additional domains of expression include both the proximal (*Pbx3*) as well as the distal (*Pbx2*) limb mesenchyme throughout limb development (Figure 1). These vertebrate innovations suggest that heterotopic shifts in the expression of TALE-encoding genes may have occurred during vertebrate evolution as *Pbx2* and *Pbx3* also play important roles in limb patterning and morphogenesis (Figure 6). In sum, we propose that *de novo* use of additional vertebrate *Pbx* homologs may have led to the evolution of the autopod and its acquisition of specialized functions.

Interestingly, the appearance of novel *Pbx* expression domains in vertebrate appendages, especially throughout limb development, is coupled with the novel expression of other genes encoding proteins that are critical for the proper function of Pbx factors. For example, as in the fly in which *hth* (the ortholog of vertebrate *Meis*) is expressed proximally together with *exd* in the larval imaginal disc (Rieckhof et al., 1995; Capdevila et al., 1999; Mercader et al., 1999), in the mouse, *Meis1* and *Meis2* are also expressed proximally in E10.5 to E12 limb mesenchyme with *Pbx1* (Mercader et al., 1999; Capellini et al., 2006). In addition, both *Meis* genes are expressed in the ectoderm and AER with *Pbx1* and *Pbx2* (unpublished results). Furthermore, in the mouse, *Pbx2* and *Prep1* proteins additionally co-localize in the distal limb mesenchyme at E10.5 (our unpublished data), while in flies during larval imaginal disc development and maturation, the distal appendage is devoid of nuclear *Exd* and *hth* protein (data not shown). These comparative data indicate that *Pbx/exd* and *Meis/hth* may have shared a common evolutionary heritage in the ectoderm and only later have appeared in the limb mesenchyme of vertebrates. Future work will have to clarify these relationships throughout evolution.

From an evolutionary point of view, the observed co-localization of both *Pbx/Exd* and *Meis/hth* indicates that their functions (and their underlying expression domains) may have co-evolved during Metazoan evolution due to constraints placed by their functional

cooperativity. For example, one of their conserved functions may involve *Meis/hth* ability to control the nuclear importation of *Pbx/Exd* (discussed above). In a similar fashion, since *Pbx/Exd* and *Meis/hth* co-localization results in their ability to form dimeric and trimeric complexes (with or without *Hox*) that are critical for the regulation of multiple target genes in developmental processes, such a constraint may have limited the functions of these proteins in domains where they overlap.

The findings on TALE gene functions indicate that the mechanisms of P-D and A-P limb patterning may be only partially conserved between invertebrates and vertebrates (Figure 6). There are indeed some interesting similarities and differences. First, unlike in vertebrates in which biphasic *Hox* gene expression underlies the development of distal limb domains (Capecchi, 1997; Freitas and Cohn, 2006; Freitas et al., 2007), fly leg P-D axis establishment develops independently of *Hox* (although the identity of nascent imaginal disc cells may require early *Hox* activity). Second, mutations in fly *exd* result in homeotic transformations without altering the expression patterns of *Hox* genes themselves; thereby suggesting that *exd* acts in parallel to *Hox* genes (Peifer and Wieschaus, 1990; Rauskolb et al., 1993; Rieckhof et al., 1997). However, in vertebrates loss of *Pbx* leads to alterations in *Hox* expression (Capellini et al., 2006), indicating an additional upstream role. Third, it was once thought that in the fly, *exd* and *hth* are restricted to proximal limbs throughout entire wing/leg development (Rieckhof et al., 1997; Capdevila et al., 1999; Mercader et al., 1999) and that *exd/hth* are required for specification of proximal, but not distal, limb identities (Gonzales-Crespo et al., 1998). However, McKay and colleagues (2009), found that the cells that give rise to the entire distal leg coexpress *hth* and *exd* in late embryos, and it is only later, during larval imaginal disc development, that *hth* is turned off from the distal leg. In this manner, both *Exd* and *hth* may be required for overall outgrowth prior to imaginal disc maturation. This may be similar to the early colocalization of *Pbx* and *Meis* in the limb bud at induction and initial outgrowth, the period during which *Pbx1* and *Pbx2* may cooperate to govern development of the entire limb via control of A-P and P-D axes. Fourth, in the fly, *exd* and *hth* are restricted to proximal limbs during late larval imaginal disc development (Rieckhof et al., 1997; Capdevila et al., 1999; Mercader et al., 1999) and accordingly *Exd* has been shown to be required for specification of proximal, but not distal, limb identities at this stage (Gonzales-Crespo et al., 1998). This process occurs partially through their distal repression, and thus restriction of *Distaless (Dll)* gene expression (Abu-Shaar and Mann, 1998). In vertebrates, such as mice, *Pbx* appear upstream of *Dlx*, homologs of *Dll*, as loss of *Pbx1* and *Pbx2* causes drastic alterations in the expression of *Dlx* genes in E10.5 and E11.5 embryos (our unpublished results). Fifth, despite their colocalization early, the roles of *hth* and *Exd* serve to antagonistically restrict hedgehog signaling to distal domains leading to P-D and D-V axis establishment (Abu-Shaar and Mann, 1998; Gonzales-Crespo et al., 1998) (Figure 6). In vertebrates, the situation is different, as *Pbx1* and *Pbx2* control *Hox* gene expression and *Shh* activation (Figure 6). This vertebrate innovation has in turn crucial bearings on A-P formation and digital patterning, as well as on the origins of morphological complexity of distal appendages in tetrapods.

Concluding Remarks and Future Directions

We have discussed the hierarchical, overlapping, and iterative functions of *Pbx* family members in appendicular skeleton development. *Pbx1* and *Pbx2* collaborate to control critical effectors of girdle and limb development along all three axes. We have found that such regulation affects many levels of girdle and limb patterning and morphogenesis, from directly controlling morphogens secreted by signaling centers, such as *Shh* from the limb ZPA, to downstream transcription factor-encoding genes, such as *Alx1* in pre-scapular domains. At least in the limb bud, *Pbx* proteins appear to act not only as *Hox* cofactors, but also in the upstream control of *5'HoxA/D* gene expression. Our work has additionally

established that *Pbx1/Pbx2* genetically regulate the development of all post-cranial axial and appendicular skeletal structures and that *Pbx1* is the linchpin of these genetic interactions. On the other hand, the unique expression patterns of *Pbx3* make this protein an excellent candidate for a highly specific role in early forelimb development. It is of note that *Pbx* primal control of multiple genetic pathways is not limited to the limb, but involves most developing organs. In many tissues, including the limb, Pbx proteins appear to have critical roles in the control of cell number, by regulating the expression of proliferation and/or apoptosis genes in populations whose fates have already been specified.

Concomitantly, Pbx factors may control directional cell movements in tissues such as the early limb field, where both segmental identity and shape depend on the growth and migration patterns of mesenchymal progenitors. Cutting edge technology such as live imaging (Nowotschin et al., 2010), lineage tracing in vertebrate models, and computer simulations (Boehm et al., 2010), have recently facilitated an unprecedented analysis of rapidly changing cellular dynamics and intercellular relationships during limb morphogenesis. For example, such technologies have uncovered the importance of oriented cellular behaviours and directional movements of mesenchymal cells migrating into the limb bud (Boehm et al., 2010; Wyngaarden et al., 2010). Application of these new technologies to the characterization of limb defects in *Pbx* loss-of-function models will shed light into the cellular behaviors that are governed by Pbx homeoproteins in early limb bud development.

In other tissues like muscle and pancreas, Pbx proteins have been shown to act as “pioneer” transcription factors that poise tissue-specific loci for activation by their downstream master regulators. Lastly, given the co-localization of multiple partner proteins with Pbx1–3, it is likely that the differential regulation of target genes by Pbx factors requires the formation of context-specific complexes, a process that is still poorly understood. As new imaging, molecular, and biochemical techniques are brought to bear, along with the use of *Pbx*, *Meis*, and *Prep* tissue-specific loss-of-function mouse models, the Pbx-dependent molecular circuitry in different limb compartments at specific developmental times, as well as their control of specific cellular behaviors in limb development, will hopefully gain clarity.

Acknowledgments

We are especially grateful to Robert Aho for the preparation of the artwork and to Richard Mann for critical reading of the manuscript and insightful comments. We are thankful to Giuseppina Di Giacomo for performing some of the experiments discussed in this review; Karen Handschuh and Xiao P. Peng for contributing ideas and thoughtful critiques; Liz Lacy, Ann Foley, Filippo Rijli, and James Sharpe for helpful discussions; Michael Cleary for anti-Pbx antibodies; and researchers of the Mouse Genetics Community for in situ probes that facilitated our research. T.C. was the recipient of the CUNY Carole and Morton Olshan Fellowship. All research that made this review possible was supported by grants from: the NIH (2RO1HD043997, 1RO1HD061403, and R21DE018031-02S1 to L.S.); March of Dimes and Birth Defects Foundation (#6- FY03-071 to L.S.); the Italian Association for Cancer Research and the ASM Foundation to V.Z. L.S. is an Irma T. Hirschl Scholar and recipient of research awards from The Alice Bohmfalk Trust and The Frueauff Foundation.

References

- Abu-Shaar M, Mann R. Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development*. 1998; 125:3821–3830. [PubMed: 9729490]
- Abu-Shaar M, Ryoo HD, Mann RS. Control of the nuclear localization of Extradenticle by competing nuclear import and export signals. *Genes And Development*. 1999; 13:935–945. [PubMed: 10215621]
- Agarwal P, Wylie JN, Galceran J, Arkhitko O, Li C, Deng C, Grosschedl R, Bruneau BG. Tbx5 is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo. *Development*. 2003; 130:623–633. [PubMed: 12490567]

- Alexander T, Nolte C, Krumlauf R. Hox genes and segmentation of the hindbrain and axial skeleton. *Annu Rev Cell Dev Biol.* 2009; 25:431–456. [PubMed: 19575673]
- Arenkiel BR, Tvrdik P, Gaufo GO, Capecchi MR. Hoxb1 functions in both motoneurons and in tissues of the periphery to establish and maintain the proper neuronal circuitry. *Genes Dev.* 2004; 18:1539–1552. [PubMed: 15198977]
- Asahara H, Dutta S, Kao HY, Evans RM, Montminy M. Pbx-Hox heterodimers recruit coactivator-corepressor complexes in an isoform-specific manner. *Mol Cell Biol.* 1999; 19:8219–8225. [PubMed: 10567547]
- Bailey JS, Rave-Harel N, McGillivray SM, Coss D, Mellon PL. Activin regulation of the follicle-stimulating hormone beta-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. *Mol Endocrinol.* 2004; 18:1158–1170. [PubMed: 14764653]
- Barrow JR, Thomas KR, Boussadia-Zahui O, Moore R, Kemler R, Capecchi MR, McMahon AP. Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev.* 2003; 17:394–409. [PubMed: 12569130]
- Bénazet JD, Bischofberger M, Tiecke E, Gonçalves A, Martin JF, Zuniga A, Naef F, Zeller R. A self-regulatory system of interlinked signaling feedback loops controls mouse limb patterning. *Science.* 2009; 323(5917):1050–3. [PubMed: 19229034]
- Benazet JD, Zeller R. Vertebrate limb development: moving from classical morphogen gradients to an integrated 4-dimensional patterning system. *Cold Spring Harb Perspect Biol.* 2009; 1:a001339. [PubMed: 20066096]
- Berkes CA, Bergstrom DA, Penn BH, Seaver KJ, Knoepfler PS, Tapscott SJ. Pbx marks genes for activation by MyoD indicating a role for a homeodomain protein in establishing myogenic potential. *Mol Cell.* 2004; 14:465–477. [PubMed: 15149596]
- Berry FB, O'Neill MA, Coca-Prados M, Walter MA. FOXC1 transcriptional regulatory activity is impaired by PBX1 in a filamin A-mediated manner. *Mol Cell Biol.* 2005; 25:1415–1424. [PubMed: 15684392]
- Berthelsen J, Kilstrup-Nielsen C, Blasi F, Mavilio F, Zappavigna V. The subcellular localization of PBX1 and EXD proteins depends on nuclear import and export signals and is modulated by association with PREP1 and HTH. *Genes And Development.* 1999; 13:946–953. [PubMed: 10215622]
- Berthelsen J, Zappavigna V, Ferretti E, Mavilio F, Blasi F. The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity. *EMBO J.* 1998; 17:1434–1445. [PubMed: 9482740]
- Bi W, Huang W, Whitworth DJ, Deng JM, Zhang Z, Behringer RR, de Crombrughe B. Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. *Proc Natl Acad Sci U S A.* 2001; 98:6698–6703. [PubMed: 11371614]
- Bismuth K, Relaix F. Genetic regulation of skeletal muscle development. *Exp Cell Res.* 2010; 316:3081–3086. [PubMed: 20828559]
- Boehm B, Westerberg H, Lesnicar-Pucko G, Raja S, Rautschka M, Cotterell J, Swoger J, Sharpe J. The role of spatially controlled cell proliferation in limb bud morphogenesis. *PLoS Biol.* 2010; 8(7):e1000420. [PubMed: 20644711]
- Brendolan A, Ferretti E, Salsi V, Moses K, Quaggin S, Blasi F, Cleary ML, Selleri L. A Pbx1-dependent genetic and transcriptional network regulates spleen ontogeny. *Development.* 2005; 132(13):3113–26. [PubMed: 15944191]
- Bürglin TR. The PBC domain contains a MEINOX domain: coevolution of Hox and TALE homeobox genes? *Development Genes and Evolution.* 1998; 208:113–116. [PubMed: 9569353]
- Bürglin TR. Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucleic Acids Res.* 1997; 25:4173–4180. [PubMed: 9336443]
- Bürglin, TR. A comprehensive classification of homeobox genes. In: Duboule, D., editor. *Guidebook to the homeobox genes.* Oxford University Press; Oxford, U.K.: 1994. p. 25-72.
- Butterfield NC, McGlinn E, Wicking C. The molecular regulation of vertebrate limb patterning. *Curr Top Dev Biol.* 2010; 90:319–341. [PubMed: 20691854]

- Capdevila J, Tsukui T, Rodriguez Esteban C, Zappavigna V, Izpisua Belmonte JC. Control of vertebrate limb outgrowth by the proximal factor *Meis2* and distal antagonism of BMPs by *Gremlin*. *Mol Cell*. 1999; 4:839–849. [PubMed: 10619030]
- Capecchi MR. Hox genes and mammalian development. *Cold Spring Harb Symp Quant Biol*. 1997; 62:273–281. [PubMed: 9598361]
- Capellini TD, Di Giacomo G, Salsi V, Brendolan A, Ferretti E, Srivastava D, Zappavigna V, Selleri L. *Pbx1/Pbx2* requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and *Shh* expression. *Development*. 2006; 133:2263–2273. [PubMed: 16672333]
- Capellini TD, Vaccari G, Ferretti E, Fantini S, He M, Pellegrini M, Quintana L, Di Giacomo G, Sharpe J, Selleri L, Zappavigna V. Scapula development is governed by genetic interactions of *Pbx1* with its family members and with *Emx2* via their cooperative control of *Alx1*. *Development*. 2010; 137:2559–2569. [PubMed: 20627960]
- Capellini TD, Zewdu R, Di Giacomo G, Asciutti S, Kugler JE, Di Gregorio A, Selleri L. *Pbx1/Pbx2* govern axial skeletal development by controlling Polycomb and Hox in mesoderm and *Pax1/Pax9* in sclerotome. *Dev Biol*. 2008; 321:500–514. [PubMed: 18691704]
- Chan SK, Jaffe L, Capovilla M, Botas J, Mann RS. The DNA binding specificity of ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. *Cell*. 1994; 78:603–615. [PubMed: 7915199]
- Chan SK, Pöpperl H, Krumlauf R, Mann RS. An extradenticle-induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J*. 1996; 15(10):2476–2487. [PubMed: 8665855]
- Chang CP, Shen WF, Rozenfeld S, Lawrence HJ, Largman C, Cleary M. *Pbx* proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Dev*. 1995; 9:663–674. [PubMed: 7729685]
- Chang CP, Brocchieri L, Shen WF, Largman C, Cleary ML. *Pbx* modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. *Mol Cell Biol*. 1996; 16:1734–1745. [PubMed: 8657149]
- Chang CP, Jacobs Y, Nakamura T, Jenkins NA, Copeland NG, Cleary ML. *Meis* proteins are major in vivo DNA binding partners for wild-type but not chimeric *Pbx* proteins. *Mol. Cell. Biol*. 1997; 17:5679–5687. [PubMed: 9315626]
- Charite J, McFadden DG, Olson EN. The bHLH transcription factor *dHAND* controls Sonic hedgehog expression and establishment of the zone of polarizing activity during limb development. *Development*. 2000; 127:2461–2470. [PubMed: 10804186]
- Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, Pepicelli CV, Gan L, Lee B, Johnson RL. Limb and kidney defects in *Lmx1b* mutant mice suggest an involvement of *LMX1B* in human nail patella syndrome. *Nat Genet*. 1998; 19:51–55. [PubMed: 9590288]
- Chen H, Rossier C, Nakamura Y, Lynn A, Chakravarti A, Antonarakis SE. Cloning of a novel homeobox-containing gene, *PKNOX1*, and mapping to human chromosome 21q22.3. *Genomics*. 1997; 41:193–200. [PubMed: 9143494]
- Chiang C, Litingtung Y, Harris MP, Simandl BK, Li Y, Beachy PA, Fallon JF. Manifestation of the limb prepattern: limb development in the absence of sonic hedgehog function. *Dev Biol*. 2001; 236:421–435. [PubMed: 11476582]
- Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature*. 1996; 383:407–413. [PubMed: 8837770]
- Christ B, Huang R, Wilting J. The development of the avian vertebral column. *Anat Embryol (Berl)*. 2000; 202:179–194. [PubMed: 10994991]
- Cohn MJ, Patel K, Krumlauf R, Wilkinson DG, Clarke JD, Tickle C. Hox9 genes and vertebrate limb specification. *Nature*. 1997; 387:97–101. [PubMed: 9139829]
- Cohn MJ, Tickle C. Developmental basis of limblessness and axial patterning in snakes. *Nature*. 1999; 399(6735):474–9. [PubMed: 10365960]

- Cygan JA, Johnson RL, McMahon AP. Novel regulatory interactions revealed by studies of murine limb pattern in Wnt-7a and En-1 mutants. *Development*. 1997; 124:5021–5032. [PubMed: 9362463]
- de la Serna IL, Ohkawa Y, Berkes CA, Bergstrom DA, Dacwag CS, Tapscott SJ, Imbalzano AN. MyoD targets chromatin remodeling complexes to the myogenin locus prior to forming a stable DNA-bound complex. *Mol Cell Biol*. 2005; 25:3997–4009. [PubMed: 15870273]
- Di Giacomo G, Koss M, Capellini TD, Brendolan A, Pöpperl H, Selleri L. Spatio-temporal expression of Pbx3 during mouse organogenesis. *Gene Expr Patterns*. 2006; 6:747–757. [PubMed: 16434237]
- Di Rocco G, Mavilio F, Zappavigna V. Functional dissection of a transcriptionally active, target-specific Hox-Pbx complex. *EMBO J*. 1997; 16:3644–3654. [PubMed: 9218805]
- DiMartino JF, Selleri L, Traver D, Firpo MT, Rhee J, Warnke R, O_Gorman S, Weissman IL, Cleary ML. The Hox co-factor and proto-oncogene Pbx1 is required for maintenance of definitive hematopoiesis in the fetal liver. *Blood*. 2001; 98:618–626. [PubMed: 11468159]
- Dudley AT, Ros MA, Tabin CJ. A re-examination of proximodistal patterning during vertebrate limb development. *Nature*. 2002; 418:539–544. [PubMed: 12152081]
- Ebner A, Cabernard C, Affolter M, Merabet S. Recognition of distinct target sites by a unique Labial/Extradenticle/Homothorax complex. *Development*. 2005; 132:1591–1600. [PubMed: 15753213]
- Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell*. 1993; 75:1417–1430. [PubMed: 7916661]
- Fallon JF, Lopez A, Ros MA, Savage MP, Olwin BB, Simandl BK. FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science*. 1994; 264:104–107. [PubMed: 7908145]
- Faust C, Lawson KA, Schork NJ, Thiel B, Magnuson T. The Polycomb-group gene *eed* is required for normal morphogenetic movements during gastrulation in the mouse embryo. *Development*. 1998; 125:4495–4506. [PubMed: 9778508]
- Faust C, Schumacher A, Holdener B, Magnuson T. The *eed* mutation disrupts anterior mesoderm production in mice. *Development*. 1995; 121:273–285. [PubMed: 7768172]
- Ferretti E, Marshall H, Pöpperl H, Maconochie M, Krumlauf R, Blasi F. Segmental expression of Hoxb2 in r4 requires two separate sites that integrate cooperative interactions between Prep1, Pbx and Hox proteins. *Development*. 2000; 127:155–166. [PubMed: 10654609]
- Ferretti E, Schulz H, Talarico D, Blasi F, Berthelsen J. The PBX-regulating protein PREP1 is present in different PBX-complexed forms in mouse. *Mechanisms Of Development*. 1999; 83:53–64. [PubMed: 10381567]
- Fognani C, Kilstrup-Nielsen C, Berthelsen J, Ferretti E, Zappavigna V, Blasi F. Characterization of PREP2, a paralog of PREP1, which defines a novel sub-family of the MEINOX TALE homeodomain transcription factors. *Nucleic Acids Res*. 2002; 30:2043–2051. [PubMed: 11972344]
- Freitas R, Zhang G, Cohn MJ. Biphasic Hoxd gene expression in shark paired fins reveals an ancient origin of the distal limb domain. *PLoS One*. 2007; 152(1):e754. [PubMed: 17710153]
- Freitas R, Cohn MJ. Genomic regulation of Hox collinearity. *Dev Cell*. 2006; 10(1):8–9. [PubMed: 16399073]
- Galant R, Walsh CM, Carroll SB. Hox repression of a target gene: extradenticle-independent, additive action through multiple monomer binding sites. *Development*. 2002; 129:3115–3126. [PubMed: 12070087]
- Gebelein B, McKay DJ, Mann RS. Direct integration of Hox and segmentation gene inputs during Drosophila development. *Nature*. 2004; 431:653–659. [PubMed: 15470419]
- Gendron-Maguire M, Mallo M, Zhang M, Gridley T. Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell*. 1993; 75:1317–1331. [PubMed: 7903600]
- Goddard JM, Rossel M, Manley NR, Capecchi MR. Mice with targeted disruption of Hoxb-1 fail to form the motor nucleus of the VIIth nerve. *Development*. 1996; 122:3217–3228. [PubMed: 8898234]

- Gonzales-Crespo S, Abu-Shaar M, Torres M, Martinez-Arias C, Mann R, Morata G. Antagonism between extradenticle function and Hedgehog signalling in the developing limb. *Nature*. 1998; 394:196–200. [PubMed: 9671305]
- Gordon JA, Hassan MQ, Saini S, Montecino M, van Wijnen AJ, Stein GS, Stein JL, Lian JB. Pbx1 represses osteoblastogenesis by blocking Hoxa10-mediated recruitment of chromatin remodeling factors. *Mol Cell Biol*. 2010; 30:3531–3541. [PubMed: 20439491]
- Goudet G, Delhalle S, Biemar F, Martial JA, Peers B. Functional and cooperative interactions between the homeodomain PDX1, Pbx, and Prep1 factors on the somatostatin promoter. *Journal Of Biological Chemistry*. 1999; 274:4067–4073. [PubMed: 9933599]
- Harrison RG. On relations of symmetry in transplanted limbs. *J Exp Zool*. 1921; 32:1–20.
- Huang R, Christ B, Patel K. Regulation of scapula development. *Anat Embryol (Berl)*. 2006; 211(Suppl 1):65–71. [PubMed: 17006658]
- Huang R, Zhi Q, Patel K, Wilting J, Christ B. Dual origin and segmental organisation of the avian scapula. *Development*. 2000; 127:3789–3794. [PubMed: 10934023]
- Jacobs Y, Schnabel CA, Cleary ML. Trimeric association of Hox and TALE homeodomain proteins mediates Hoxb2 hindbrain enhancer activity. *Molecular And Cellular Biology*. 1999; 19:5134–5142. [PubMed: 10373562]
- Jaw TJ, You LR, Knoepfler PS, Yao LC, Pai CY, Tang CY, Chang LP, Berthelsen J, Blasi F, Kamps MP, Sun YH. Direct interaction of two homeoproteins, homothorax and extradenticle, is essential for EXD nuclear localization and function. *Mech Dev*. 2000; 91:279–291. [PubMed: 10704852]
- Johnson DR. Extra-toes: anew mutant gene causing multiple abnormalities in the mouse. *J Embryol Exp Morphol*. 1967; 17:543–581. [PubMed: 6049666]
- Johnson FB, Parker E, Krasnow M. Extradenticle protein is a selective co-factor for the *Drosophila* homeotics: Role of the homeodomain and YPWM amino acid motif in the interaction. *Proc. Natl. Acad. Sci. USA*. 1995; 92:739–743. [PubMed: 7846045]
- Joshi R, Passner JM, Rohs R, Jain R, Sosinsky A, Crickmore MA, Jacob V, Aggarwal AK, Honig B, Mann RS. Functional specificity of a Hox protein mediated by the recognition of minor groove structure. *Cell*. 2007; 131(3):530–543. [PubMed: 17981120]
- Joshi R, Sun L, Mann R. Dissecting the functional specificities of two Hox proteins. *Genes Dev*. 2010; 24:1533–1545. [PubMed: 20634319]
- Kamps MP, Wright DD. Oncoprotein E2A-Pbx1 immortalizes a myeloid progenitor in primary marrow cultures without abrogating its factor-dependence. *Oncogene*. 1994; 9:3159–3166. [PubMed: 7936638]
- Khokha MK, Hsu D, Brunet LJ, Dionne MS, Harland RM. Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. *Nat Genet*. 2003; 34(3):303–7. [PubMed: 12808456]
- Kilstrup-Nielsen C, Alessio M, Zappavigna V. PBX1 nuclear export is regulated independently of PBX-MEINOX interaction by PKA phosphorylation of the PBC-B domain. *The Embo Journal*. 2003; 22:89–99. [PubMed: 12505987]
- Kim SK, Sella L, Lee JS, Zhang AY, Gu X, Jacobs Y, Cleary ML. Pbx1 inactivation disrupts pancreas development and in *Ipfl*-deficient mice promotes diabetes mellitus. *Nature Genetics*. 2002; 30:430–435. [PubMed: 11912494]
- Kim SY, Paylor SW, Magnuson T, Schumacher A. Juxtaposed Polycomb complexes co-regulate vertebral identity. *Development*. 2006; 133:4957–4968. [PubMed: 17107999]
- Kimmel RA, Turnbull DH, Blanquet V, Wurst W, Loomis CA, Joyner AL. Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Dev*. 2000; 14:1377–1389. [PubMed: 10837030]
- Kmita M, Tarchini B, Zakany J, Logan M, Tabin CJ, Duboule D. Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. *Nature*. 2005; 435:1113–1116. [PubMed: 15973411]
- Knoepfler P, Kamps M. The pentapeptide motif of Hox proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances DNA binding by Pbx1. *Mol Cell Biol*. 1995; 15:5811–5819. [PubMed: 7565734]

- Knoepfler PS, Bergstrom DA, Uetsuki T, Dac-Korytko I, Sun YH, Wright WE, Tapscott SJ, Kamps MP. A conserved motif N-terminal to the DNA-binding domains of myogenic bHLH transcription factors mediates cooperative DNA binding with pbx-Meis1/Prep1. *Nucleic Acids Res.* 1999; 27:3752–3761. [PubMed: 10471746]
- Knoepfler PS, Calvo KR, Chen H, Antonarakis SE, Kamps MP. Meis1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface disrupted in oncoprotein E2a-Pbx1. *Proceedings of the National Academy of Sciences of the United States of America.* 1997; 94:14553–14558. [PubMed: 9405651]
- Kobayashi M, Fujioka M, Tolkunova EN, Deka D, Abu-Shaar M, Mann RS, Jaynes JB. Engrailed cooperates with extradenticle and homothorax to repress target genes in *Drosophila*. *Development.* 2003; 130:741–751. [PubMed: 12506004]
- Koshiba-Takeuchi K, Takeuchi JK, Arruda EP, Kathiriya IS, Mo R, Hui CC, Srivastava D, Bruneau BG. Cooperative and antagonistic interactions between Sall4 and Tbx5 pattern the mouse limb and heart. *Nat Genet.* 2006; 38:175–183. [PubMed: 16380715]
- Krawchuk D, Weiner SJ, Chen YT, Lu BC, Costantini F, Behringer RR, Laufer E. Twist1 activity thresholds define multiple functions in limb development. *Dev Biol.* 2010
- Kros1 J, Baban S, Kros1 G, Rozenfeld S, Largman C, Sauvageau GU, et al. Cellular proliferation and transformation induced by HOXB4 and HOXB3 proteins involves cooperation with PBX1. *Oncogene.* 1998; 16:3403–3412. [PubMed: 9692548]
- Krumlauf R, Holland PW, McVey JH, Hogan BL. Developmental and spatial patterns of expression of the mouse homeobox gene, Hox 2.1. *Development.* 1987; 99:603–617. [PubMed: 2889591]
- Kuijper S, Beverdam A, Kroon C, Brouwer A, Candille S, Barsh G, Meijlink FU, et al. Genetics of shoulder girdle formation: roles of Tbx15 and aristaless-like genes. *Development (Cambridge, England).* 2005; 132:1601–1610.
- Lancot C, Moreau A, Chamberland M, Tremblay ML, Drouin J. Hindlimb patterning and mandible development require the Ptx1 gene. *Development.* 1999; 126:1805–1810. [PubMed: 10101115]
- LaRonde-LeBlanc NA, Wolberger C. Structure of HoxA9 and Pbx1 bound to DNA: Hox hexapeptide and DNA recognition anterior to posterior. *Genes Dev.* 2003; 17:2060–2072. [PubMed: 12923056]
- Laurent A, Bihan R, Omilli F, Deschamps S, Pellerin I. PBX proteins: much more than Hox cofactors. *Int J Dev Biol.* 2008; 52:9–20. [PubMed: 18033668]
- Lausch E, Hermanns P, Farin HF, Alanay Y, Unger S, Nikkel S, Steinwender C, Scherer G, Spranger J, Zabel B, Kispert A, Superti-Furga A. TBX15 mutations cause craniofacial dysmorphism, hypoplasia of scapula and pelvis, and short stature in Cousin syndrome. *Am J Hum Genet.* 2008; 83:649–655. [PubMed: 19068278]
- LeBrun DP, Matthews BP, Feldman BJ, Cleary ML. The chimeric oncoproteins E2A-PBX1 and E2A-HLF are concentrated within spherical nuclear domains. *Oncogene.* 1997; 15(17):2059–2067. [PubMed: 9366523]
- LeClair EE, Bonfiglio L, Tuan RS. Expression of the paired-box genes Pax-1 and Pax-9 in limb skeleton development. *Dev Dyn.* 1999; 214:101–115. [PubMed: 10030590]
- Lessard J, Schumacher A, Thorsteinsdottir U, van Lohuizen M, Magnuson T, Sauvageau G. Functional antagonism of the Polycomb-Group genes *ee* and *Bmi1* in hemopoietic cell proliferation. *Genes Dev.* 1999; 13:2691–2703. [PubMed: 10541555]
- Lettice LA, Heaney SJ, Purdie LA, Li L, de Beer P, Oostra BA, Goode D, Elgar G, Hill RE, de Graaff E. A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum Mol Genet.* 2003; 12:1725–1735. [PubMed: 12837695]
- Lettice LA, Horikoshi T, Heaney SJ, van Baren MJ, van der Linde HC, Breedveld GJ, Joosse M, Akarsu N, Oostra BA, Endo N, Shibata M, Suzuki M, Takahashi E, Shinka T, Nakahori Y, Ayusawa D, Nakabayashi K, Scherer SW, Heutink P, Hill RE, Noji S. Disruption of a long-range cis-acting regulator for Shh causes preaxial polydactyly. *Proc Natl Acad Sci U S A.* 2002; 99:7548–7553. [PubMed: 12032320]
- Lewandoski M, Sun X, Martin GR. Fgf8 signalling from the AER is essential for normal limb development. *Nat Genet.* 2000; 26:460–463. [PubMed: 11101846]

- Liu Y, MacDonald RJ, Swift GH. DNA binding and transcriptional activation by a PDX1.PBX1b.MEIS2b trimer and cooperation with a pancreas-specific basic helix-loop-helix complex. *J Biol Chem*. 2001; 276:17985–17993. [PubMed: 11279116]
- Lizarraga G, Ferrari D, Kalinowski M, Ohuchi H, Noji S, Kosher RA, Dealy CN. FGFR2 signaling in normal and limbless chick limb buds. *Dev Genet*. 1999; 25:331–338. [PubMed: 10570465]
- Loomis CA, Harris E, Michaud J, Wurst W, Hanks M, Joyner AL. The mouse *Engrailed-1* gene and ventral limb patterning. *Nature*. 1996; 382:360–363. [PubMed: 8684466]
- Loomis CA, Kimmel RA, Tong CX, Michaud J, Joyner AL. Analysis of the genetic pathway leading to formation of ectopic apical ectodermal ridges in mouse *Engrailed-1* mutant limbs. *Development*. 1998; 125:1137–1148. [PubMed: 9463360]
- Lu Q, Wright DD, Kamps MP. Fusion with E2A converts the Pbx1 homeodomain protein into a constitutive transcriptional activator in human leukemias carrying the t(1;19) translocation. *Mol. Cell. Biol*. 1994; 14:3938–3948. [PubMed: 7910944]
- Maconochie MK, Nonchev S, Studer M, Chan SK, Pöpperl H, Sham MH, Mann RS, Krumlauf R. Cross-regulation in the mouse *HoxB* complex: the expression of *Hoxb2* in rhombomere 4 is regulated by *Hoxb1*. *Genes Dev*. 1997; 11:1885–1895. [PubMed: 9242495]
- Malashichev Y, Christ B, Prols F. Avian pelvis originates from lateral plate mesoderm and its development requires signals from both ectoderm and paraxial mesoderm. *Cell Tissue Res*. 2008; 331:595–604. [PubMed: 18087724]
- Mallo M, Wellik DM, Deschamps J. Hox genes and regional patterning of the vertebrate body plan. *Dev Biol*. 2010; 344:7–15. [PubMed: 20435029]
- Manley NR, Capecchi MR. Hox group 3 paralogs regulate the development and migration of the thymus, thyroid, and parathyroid glands. *Dev Biol*. 1998; 195:1–15. [PubMed: 9520319]
- Manley NR, Selleri L, Brendolan A, Gordon J, Cleary ML. Abnormalities of caudal pharyngeal pouch development in *Pbx1* knockout mice mimic loss of *Hox3* paralogs. *Dev Biol*. 2004; 276:301–312. [PubMed: 15581866]
- Mann R. The specificity of homeotic gene function. *Bioessays*. 1996; 17:855–863. [PubMed: 7487967]
- Mann RS, Affolter M. Hox proteins meet more partners. *Curr Opin Genet Dev*. 1998; 8:423–429. [PubMed: 9729718]
- Mann RS, Chan SK. Extra specificity from *extradenticle*: the partnership between HOX and PBX/EXD homeodomain proteins. *Trends Genet*. 1996; 12:258–262. [PubMed: 8763497]
- Manzanares M, Bel-Vialar S, Ariza-McNaughton L, Ferretti E, Marshall H, Maconochie MM, Blasi F, Krumlauf R. Independent regulation of initiation and maintenance phases of *Hoxa3* expression in the vertebrate hindbrain involve auto- and cross-regulatory mechanisms. *Development*. 2001; 128:3595–3607. [PubMed: 11566863]
- Marcil A, Dumontier E, Chamberland M, Camper SA, Drouin J. *Pitx1* and *Pitx2* are required for development of hindlimb buds. *Development*. 2003; 130:45–55. [PubMed: 12441290]
- Mariani FV, Ahn CP, Martin GR. Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature*. 2008; 453:401–405. [PubMed: 18449196]
- Mavilio F, Simeone A, Giampaolo A, Faiella A, Zappavigna V, Acampora D, Poiana G, Russo G, Peschle C, Boncinelli E. Differential and stage-related expression in embryonic tissues of a new human homeobox gene. *Nature*. 1986; 324:664–666. [PubMed: 2879245]
- McClintock JM, Kheirbek MA, Prince VE. Knockdown of duplicated zebrafish *hoxb1* genes reveals distinct roles in hindbrain patterning and a novel mechanism of duplicate gene retention. *Development*. 2002; 129:2339–2354. [PubMed: 11973267]
- McIntyre DC, Rakshit S, Yallowitz AR, Loken L, Jeannotte L, Capecchi MR, Wellik DM. Hox patterning of the vertebrate rib cage. *Development*. 2007; 134:2981–2989. [PubMed: 17626057]
- McKay DJ, Estella C, Mann RS. The origins of the *Drosophila* leg revealed by the cis-regulatory architecture of the *Distalless* gene. *Development*. 2009; 136(1):61–71. [PubMed: 19036798]
- McLean CY, Bristor D, Hiller M, Clarke SL, Schaar BT, Lowe CB, Wenger AM, Bejerano G. GREAT improves functional interpretation of cis-regulatory regions. *Nat Biotechnol*. 2010; 28:495–501. [PubMed: 20436461]

- Mercader N, Leonardo E, Azpiazu N, Serrano A, Morata G, Martinez C, Torres M. Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature*. 1999; 402:425–429. [PubMed: 10586884]
- Mercader N, Leonardo E, Piedra ME, Martinez AC, Ros MA, Torres M. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. *Development*. 2000; 127:3961–3970. [PubMed: 10952894]
- Mercader N, Tanaka EM, Torres M. Proximodistal identity during vertebrate limb regeneration is regulated by Meis homeodomain proteins. *Development*. 2005; 132:4131–4142. [PubMed: 16107473]
- Milech N, Kees UR, Watt PM. Novel alternative PBX3 isoforms in leukemia cells with distinct interaction specificities. *Genes Chromosomes Cancer*. 2001; 32:275–280. [PubMed: 11579467]
- Min H, Danilenko DM, Scully SA, Bolon B, Ring BD, Tarpley JE, DeRose M, Simonet WS. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to *Drosophila* branchless. *Genes Dev*. 1998; 12:3156–3161. [PubMed: 9784490]
- Minguillon C, Del Buono J, Logan MP. Tbx5 and Tbx4 are not sufficient to determine limb-specific morphologies but have common roles in initiating limb outgrowth. *Dev Cell*. 2005; 8:75–84. [PubMed: 15621531]
- Moens CB, Selleri L. Hox co-factors in vertebrate development. *Dev Biol*. 2006; 291:193–206. [PubMed: 16515781]
- Monica K, Galili N, Nourse J, Saltman D, Cleary ML. *PBX2* and *PBX3*, new homeobox genes with extensive homology to the human proto-oncogene *PBX1*. *Mol. Cell. Biol*. 1991; 11:6149–6157. [PubMed: 1682799]
- Moskow J, Bullrich F, Huebner K, Daar I, Buchberg A. Meis1, a PBX1-related homeobox gene involved in myeloid leukemia in BXH-2 mice. *Mol. Cell. Biol*. 1995; 15:5434–5443. [PubMed: 7565694]
- Mukherjee K, Burglin TR. Comprehensive analysis of animal TALE homeobox genes: new conserved motifs and cases of accelerated evolution. *J Mol Evol*. 2007; 65:137–153. [PubMed: 17665086]
- Naar AM, Lemon BD, Tjian R. Transcriptional coactivator complexes. *Annu Rev Biochem*. 2001; 70:475–501. [PubMed: 11395415]
- Neuteboom ST, Murre C. Pbx raises the DNA binding specificity but not the selectivity of antennapedia Hox proteins. *Mol. Cell. Biol*. 1997; 17(8):4696–4706. [PubMed: 9234726]
- Niswander L. Pattern formation: old models out on a limb. *Nat Rev Genet*. 2003; 4:133–143. [PubMed: 12560810]
- Niswander L, Jeffrey S, Martin GR, Tickle C. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature*. 1994; 371:609–612. [PubMed: 7935794]
- Niswander L, Tickle C, Vogel A, Booth I, Martin GR. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell*. 1993; 75:579–587. [PubMed: 8221896]
- Nourse J, Mellettnin J, Galili N, Wilkinson J, Stanbridge E, Smith S, Cleary M. Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. *Cell*. 1990; 60:535–545. [PubMed: 1967982]
- Nowotschin S, Ferrer-Vaquer A, Hadjantonakis AK. Imaging mouse development with confocal time-lapse microscopy. *Methods Enzymol*. 2010; 476:351–77. [PubMed: 20691876]
- Ohuchi H, Nakagawa T, Yamamoto A, Araga A, Ohata T, Ishimaru Y, Yoshioka H, Kuwana T, Nohno T, Yamasaki M, Itoh N, Noji S. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development*. 1997; 124:2235–2244. [PubMed: 9187149]
- Oliver G, De Robertis EM, Wolpert L, Tickle C. Expression of a homeobox gene in the chick wing bud following application of retinoic acid and grafts of polarizing region tissue. *EMBO J*. 1990; 9:3093–3099. [PubMed: 1976508]
- Parr BA, McMahon AP. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature*. 1995; 374:350–353. [PubMed: 7885472]
- Passner JM, Ryoo HD, Shen L, Mann RS, Aggarwal AK. Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. *Nature*. 1999; 397:714–719. [PubMed: 10067897]

- Peers B, Sharma S, Johnson T, Kamps M, Montminy M. The pancreatic islet factor STF-1 binds cooperatively with Pbx to a regulatory element in the somatostatin promoter: importance of the FPWMK motif and of the homeodomain. *Mol. Cell. Biol.* 1995; 15:7091–7097. [PubMed: 8524276]
- Peifer M, Wieschaus E. Mutation in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. *Genes Dev.* 1990; 4:1209–1223. [PubMed: 1976570]
- Pellegrini M, Pantano S, Fumi MP, Lucchini F, Forabosco AU, et al. Agenesis of the Scapula in Emx2 Homozygous Mutants. *Developmental Biology.* 2001; 232:149–156. [PubMed: 11254354]
- Peltenburg LT, Murre C. Engrailed and Hox homeodomain proteins contain a related Pbx interaction motif that recognizes a common structure present in Pbx. *Embo J.* 1996; 15:3385–3393. [PubMed: 8698039]
- Phelan ML, Featherstone MS. Distinct HOX N-terminal arm residues are responsible for specificity of DNA recognition by HOX monomers and HOX.PBX heterodimers. *J. Biol. Chem.* 1997; 272:8635–8643. [PubMed: 9079695]
- Phelan ML, Rambaldi I, Featherstone MS. Cooperative interactions between HOX and PBX proteins mediated by a conserved peptide motif. *Mol. Cell. Biol.* 1995; 15:3989–3997. [PubMed: 7623795]
- Pinsonneault J, Florence B, Vaessin H, McGinnis W. A model for extradenticle function as a switch that changes HOX proteins from repressors to activators. *EMBO J.* 1997; 16:2032–2042. [PubMed: 9155029]
- Piper DE, Batchelor AH, Chang CP, Cleary ML, Wolberger C. Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of the hexapeptide and a fourth homeodomain helix in complex formation. *Cell.* 1999; 96:587–597. [PubMed: 10052460]
- Pomikal C, Streicher J. 4D-analysis of early pelvic girdle development in the mouse (*Mus musculus*). *J Morphol.* 2010; 271:116–126. [PubMed: 19658165]
- Pöpperl H, Bienz M, Studer M, Chan SK, Aparicio S, Brenner S, Mann RS, Krumlauf R. Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon *exd/pbx*. *Cell.* 1995; 81:1031–1042. [PubMed: 7600572]
- Pöpperl H, Rikhof H, Chang H, Haffter P, Kimmel CB, Moens CB. *lazarus* is a novel pbx gene that globally mediates hox gene function in zebrafish. *Mol Cell.* 2000; 6:255–267. [PubMed: 10983974]
- Pueyo JJ, Couso JP. Parallels between the proximal-distal development of vertebrate and arthropod appendages: homology without an ancestor? *Curr Opin Genet Dev.* 2005; 15:439–446. [PubMed: 15979300]
- Rauskolb C, Peifer M, Wieschaus E. *extradenticle*, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene *pbx1*. *Cell.* 1993; 74:1101–1112. [PubMed: 8104703]
- Rauskolb C, Smith KM, Peifer M, Wieschaus E. *extradenticle* determines segmental identities throughout *Drosophila* development. *Development.* 1995; 121:3663–3673. [PubMed: 8582279]
- Remacle S, Abbas L, De Backer O, Pacico N, Gavalas A, Gofflot F, Picard JJ, Rezsohazy R. Loss of function but no gain of function caused by amino acid substitutions in the hexapeptide of Hoxa1 in vivo. *Mol Cell Biol.* 2004; 24:8567–8575. [PubMed: 15367676]
- Rhee JW, Arata A, Selleri L, Jacobs Y, Arata S, Onimaru H, Cleary ML. Pbx3 deficiency results in central hypoventilation. *Am J Pathol.* 2004; 165:1343–1350. [PubMed: 15466398]
- Riddle RD, Ensini M, Nelson C, Tsuchida T, Jessell TM, Tabin C. Induction of the LIM homeobox gene *Lmx1* by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell.* 1995; 83:631–640. [PubMed: 7585966]
- Riddle RD, Johnson RL, Laufer E, Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell.* 1993; 75:1401–1416. [PubMed: 8269518]
- Rieckhof GE, Casares F, Ryoo HD, Abu-Shaar M, Mann RS. Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. *Cell.* 1997; 91:171–183. [PubMed: 9346235]

- Rijli FM, Mark M, Lakkaraju S, Dierich A, Dolle P, Chambon P. A homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2*, which acts as a selector gene. *Cell*. 1993; 75:1333–1349. [PubMed: 7903601]
- Romer, AS.; Parsons, TS. *The Vertebrate Body*. Saunders College Publishing; New York: 1986.
- Ryoo HD, Mann RS. The control of trunk Hox specificity and activity by Extradenticle. *Genes And Development*. 1999; 13:1704–1716. [PubMed: 10398683]
- Sagai T, Hosoya M, Mizushima Y, Tamura M, Shiroishi T. Elimination of a long-range cis-regulatory module causes complete loss of limb-specific *Shh* expression and truncation of the mouse limb. *Development*. 2005; 132:797–803. [PubMed: 15677727]
- Saito D, Yonei-Tamura S, Takahashi Y, Tamura K. Level-specific role of paraxial mesoderm in regulation of *Tbx5/Tbx4* expression and limb initiation. *Dev Biol*. 2006; 292:79–89. [PubMed: 16480709]
- Saleh M, Rambaldi I, Yang XJ, Featherstone MS. Cell signaling switches HOX-PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases. *Mol Cell Biol*. 2000; 20:8623–8633. [PubMed: 11046157]
- Samad OA, Geisen MJ, Caronia G, Varlet I, Zappavigna V, Ericson J, Goridis C, Rijli FM. Integration of anteroposterior and dorsoventral regulation of *Phox2b* transcription in cranial motoneuron progenitors by homeodomain proteins. *Development*. 2004; 131:4071–4083. [PubMed: 15289435]
- Sauvageau G, Thorsteinsdottir U, Hough MR, Hugo P, Lawrence HJ, Largman C, Humphries RK. Overexpression of *HOXB3* in hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. *Immunity*. 1997; 6:13–22. [PubMed: 9052833]
- Scherz PJ, Harfe BD, McMahon AP, Tabin CJ. The limb bud *Shh-Fgf* feedback loop is terminated by expansion of former ZPA cells. *Science*. 2004; 305(5682):396–9. [PubMed: 15256670]
- Schnabel CA, Selleri L, Cleary ML. *Pbx1* is essential for adrenal development and urogenital differentiation. *Genesis (New York, N.Y. : 2000)*. 2003; 37:123–130.
- Schnabel CA, Selleri L, Jacobs Y, Warnke R, Cleary ML. Expression of *Pbx1b* during mammalian organogenesis. *Mech Dev*. 2001; 100:131–135. [PubMed: 11118899]
- Schweitzer R, Zelzer E, Volk T. Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates. *Development*. 2010; 137:2807–2817. [PubMed: 20699295]
- Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, Kato S. *Fgf10* is essential for limb and lung formation. *Nat Genet*. 1999; 21:138–141. [PubMed: 9916808]
- Selleri L, Depew MJ, Jacobs Y, Chanda SK, Tsang KY, Cheah KS, Rubenstein JL, O'Gorman S, Cleary ML. Requirement for *Pbx1* in skeletal patterning and programming chondrocyte proliferation and differentiation. *Development (Cambridge, England)*. 2001; 128:3543–3557.
- Selleri L, DiMartino J, van Deursen J, Brendolan A, Sanyal M, Boon E, Capellini T, Smith KS, Rhee J, Pöpperl H, Grosveld G, Cleary ML. The TALE homeodomain protein *Pbx2* is not essential for development and long-term survival. *Mol Cell Biol*. 2004; 24:5324–5331. [PubMed: 15169896]
- Shen WF, Rozenfeld S, Kwong A, Köm ves LG, Lawrence HJ, Largman C. *HOXA9* forms triple complexes with *PBX2* and *MEIS1* in myeloid cells. *Molecular And Cellular Biology*. 1999; 19:3051–3061. [PubMed: 10082572]
- Slack JM. Determination of polarity in the amphibian limb. *Nature*. 1976; 261:44–6. [PubMed: 1272371]
- Stopper GF, Wagner GP. Of chicken wings and frog legs: a smorgasbord of evolutionary variation in mechanisms of tetrapod limb development. *Dev Biol*. 2005; 288:21–39. [PubMed: 16246321]
- Studer M, Gavalas A, Marshall H, Ariza-McNaughton L, Rijli FM, Chambon P, Krumlauf R. Genetic interactions between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning. *Development*. 1998; 125:1025–1036. [PubMed: 9463349]
- Studer M, Lumsden A, Ariza-McNaughton L, Bradley A, Krumlauf R. Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature*. 1996; 384:630–634. [PubMed: 8967950]

- Subramaniam N, Campion J, Rafter I, Okret S. Cross-talk between glucocorticoid and retinoic acid signals involving glucocorticoid receptor interaction with the homeodomain protein Pbx1. *Biochem J.* 2003; 370:1087–1095. [PubMed: 12487626]
- Summerbell D, Lewis JH, Wolpert L. Positional information in chick limb morphogenesis. *Nature.* 1973; 244:492–496. [PubMed: 4621272]
- Sun X, Mariani FV, Martin GR. Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature.* 2002; 418:501–508. [PubMed: 12152071]
- Sun X, Lewandoski M, Meyers EN, Liu YH, Maxson RE Jr, Martin GR. Conditional inactivation of *Fgf4* reveals complexity of signalling during limb bud development. *Nat Genet.* 2000; 25(1):83–6. [PubMed: 10802662]
- Swift GH, Liu Y, Rose SD, Bischof LJ, Steelman S, Buchberg AM, Wright CV, MacDonald RJ. An endocrine-exocrine switch in the activity of the pancreatic homeodomain protein PDX1 through formation of a trimeric complex with PBX1b and MRG1 (MEIS2). *Mol Cell Biol.* 1998; 18:5109–5120. [PubMed: 9710595]
- Tabin C, Wolpert L. Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes Dev.* 2007; 21:1433–1442. [PubMed: 17575045]
- te Welscher P, Zuniga A, Fernandez-Teran M, Ros M, Kuijper S, Drenth T, Goedemans H, Meijlink F, Zeller R. 1 Patterning the limb before and after SHH. *J Anat.* 2002; 201:417. [PubMed: 17103744]
- ten Berge D, Brouwer A, Korving J, Martin JF, Meijlink F. *Prx1* and *Prx2* in skeletogenesis: roles in the craniofacial region, inner ear and limbs. *Development.* 1998; 125:3831–3842. [PubMed: 9729491]
- Timmons PM, Wallin J, Rigby PW, Balling R. Expression and function of Pax 1 during development of the pectoral girdle. *Development.* 1994; 120:2773–2785. [PubMed: 7607069]
- Towers M, Tickle C. Growing models of vertebrate limb development. *Development.* 2009; 136:179–190. [PubMed: 19103802]
- Valasek P, Theis S, Krejci E, Grim M, Maina F, Shwartz Y, Otto A, Huang R, Patel K. Somitic origin of the medial border of the mammalian scapula and its homology to the avian scapula blade. *J Anat.* 2010; 216:482–488. [PubMed: 20136669]
- van der Lugt NM, Domen J, Linders K, van Roon M, Robanus-Maandag E, te Riele H, van der Valk M, Deschamps J, Sofroniew M, van Lohuizen M, et al. Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the *bmi-1* proto-oncogene. *Genes Dev.* 1994; 8:757–769. [PubMed: 7926765]
- Van Dijk MA, Murre C. *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell.* 1994; 78:617–624. [PubMed: 7915200]
- Van Dijk MA, Voorhoeve PM, Murre C. Pbx1 is converted into a transcriptional activator upon acquiring the N-terminal region of E2A in pre-B-cell acute lymphoblastoid leukemia. *Proc. Natl. Acad. Sci. USA.* 1993; 90:6061–6065. [PubMed: 8327485]
- Verheyden JM, Sun X. An Fgf/Gremlin inhibitory feedback loop triggers termination of limb bud outgrowth. *Nature.* 2008; 454(7204):638–41. [PubMed: 18594511]
- Vogel A, Rodriguez C, Warnken W, Izpisua Belmonte JC. Dorsal cell fate specified by chick *Lmx1* during vertebrate limb development. *Nature.* 1995; 378:716–720. [PubMed: 7501017]
- Wagner K, Mincheva A, Korn B, Lichter P, Pöpperl H. *Pbx4*, a new *Pbx* family member on mouse chromosome 8, is expressed during spermatogenesis. *Mechanisms of Development.* 2001; 103:127–131. [PubMed: 11335119]
- Wang Y, Yin L, Hillgartner FB. The homeodomain proteins PBX and MEIS1 are accessory factors that enhance thyroid hormone regulation of the malic enzyme gene in hepatocytes. *J Biol Chem.* 2001; 276:23838–23848. [PubMed: 11331288]
- Wang Z, Iwasaki M, Ficara F, Lin C, Matheny C, Wong SH, Smith KS, Cleary ML. GSK-3 promotes conditional association of CREB and its coactivators with MEIS1 to facilitate HOX-mediated transcription and oncogenesis. *Cancer Cell.* 2010; 17:597–608. [PubMed: 20541704]
- Waskiewicz AJ, Rikhof HA, Moens CB. Eliminating zebrafish pbx proteins reveals a hindbrain ground state. *Dev Cell.* 2002; 3:723–733. [PubMed: 12431378]

- Wellik DM. Hox genes and vertebrate axial pattern. *Curr Top Dev Biol.* 2009; 88:257–278. [PubMed: 19651308]
- Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, Greenfield A, Koopman P. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. *Nat Genet.* 1995; 9:15–20. [PubMed: 7704017]
- Wyngaarden LA, Vogeli KM, Ciruna BG, Wells M, Hadjantonakis AK, Hopyan S. Oriented cell motility and division underlie early limb bud morphogenesis. *Development.* 2010; 137:2551–2558. [PubMed: 20554720]
- Xu X, Weinstein M, Li C, Naski M, Cohen RI, Ornitz DM, Leder P, Deng C. Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development.* 1998; 125:753–765. [PubMed: 9435295]
- Zakany J, Kmita M, Duboule D. A dual role for Hox genes in limb anterior-posterior asymmetry. *Science.* 2004; 304:1669–1672. [PubMed: 15192229]
- Zakany J, Zacchetti G, Duboule D. Interactions between HOXD and Gli3 genes control the limb apical ectodermal ridge via Fgf10. *Dev Biol.* 2007; 306:883–893. [PubMed: 17467687]
- Zeller R. The temporal dynamics of vertebrate limb development, teratogenesis and evolution. *Curr Opin Genet Dev.* 2010; 20:384–390. [PubMed: 20537528]
- Zeller R, Lopez-Rios J, Zuniga A. Vertebrate limb bud development: moving towards integrative analysis of organogenesis. *Nat Rev Genet.* 2009; 10:845–858. [PubMed: 19920852]
- Zúñiga A, Haramis AP, McMahon AP, Zeller R. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature.* 1999; 401(6753):598–602. [PubMed: 10524628]

Figure 1. Phases of *Pbx* gene expression in limb mesenchyme during early bud development. As shown in the schemata, *Pbx1*, *Pbx2*, and *Pbx3* expression domains are highlighted at various stages of limb development. In the earliest phase (I) *Pbx1* and *Pbx2* genes exhibit overlapping expression domains in the LPM and early limb field (purple domains), as well as within the mesodermal component of the somatopleure that gives rise to the girdles. *Pbx3* is absent from the LPM and early limb field. During phase II, the domains of *Pbx1* and *Pbx2* expression diverge and only partially overlap in the most proximal domain of *Pbx2* expression during girdle and limb bud development. Interestingly, in phase II, *Pbx3* expression is restricted to only the forelimb bud mesenchyme (until E11.0). Phase III marks the return of *Pbx* overlapping expression domains, in the anterior and posterior limb mesenchyme, just proximal to the autopod. Abbreviations: FL, forelimb; HL, hindlimb. In panels E9-E11.5, proximal is to the left, anterior is up.

Figure 2.

Girdle and limb phenotypes of single and compound *Pbx* mutant embryos at E13.5. (FL) Cartilage preparations of forelimbs. *Pbx1* homozygous mutants exhibit scapula with superior-to-inferior reduced blade (red arrowhead), and fusions of the scapula to the humeral head with an expanded coracoid process (black arrowhead). *Pbx1*^{-/-}; *Pbx2*^{+/-} mutant forelimbs additionally show exceptionally hypoplastic scapular blades (red arrowhead in red inset) with heads that appear duplicated (double black arrowheads in red inset). Distally, they display contorted humeral shafts with deltoid tuberosities that are expanded and projected at an angle to the shaft (black arrowhead). Both the radius and ulna are truncated (red arrows) and there is a significant reduction to loss of digits 1 (empty red arrowhead) and 5 (empty black arrowhead). *Pbx1*^{-/-}; *Pbx3*^{+/-} mutant forelimbs display scapular blade hypoplasia (red arrowhead in inset), fusions and expansion in the proximal humerus (black arrowhead) and humeral head duplications (double black arrowheads in red inset). However, distal to the humerus, these mutant forelimbs appear grossly normal. (HL) Cartilage preparations of hindlimbs. *Pbx1* homozygous mutants exhibit reduced pelvic girdles, with an absent ilium (empty black arrowhead) and reduced pubis and ischium (red arrowheads) which are fused to the proximal femur. Distal to the femur, these mutants are grossly normal. *Pbx1*^{-/-}; *Pbx2*^{+/-} mutant hindlimbs bear pelvises with only one remaining element, likely an ischium (red arrowhead) that is fused to the shortened and malformed femur. They display only one zeugopodial element, likely a malformed tibia, lack the fibula (red arrow), and show one single autopodial ray (empty black arrowhead in inset). *Pbx1*^{-/-}; *Pbx3*^{+/-} mutants phenocopy single homozygous *Pbx1* mutants in all proximal girdle and femur abnormalities (red and empty black arrowheads), and like *Pbx1* mutants are grossly normal distal to the femur. Abbreviations: fe, femur; fi, fibula; FL, forelimb; HL, hindlimb; hu, humerus; pg, pelvic girdle; ra, radius; sc, scapula; ti, tibia; ul, ulna; WT, wildtype. In all panels, proximal is up and anterior is to the right.

Figure 3.

Pbx homeoproteins control limb bud and skeletal positioning during limb outgrowth. (A) Hierarchical role of *Pbx* on *Polycomb* (*Bmi* and *Eed*) gene expression along the rostral-caudal LPM and paraxial mesoderm (PM) in the axis. In *Pbx1*^{-/-}; *Pbx2*^{+/-} mutants, *Bmi* and *Eed* expression is downregulated or perturbed, such that their activity is reduced across the rostralcaudal axis. These changes may be responsible for the shifts in *Hox* expression along the entire axis, which in turn may account for the vertebral patterning defects also present across the entire axis. Concomitantly, loss of *Pbx* may dysregulate *Hox* activity independently of *Polycomb* (dashed arrow). Importantly, these shifts in *Hox* expression may be responsible for the rostralization in the position of the hindlimb bud to more anterior somite levels in *Pbx1*^{-/-}; *Pbx2*^{+/-} mutants compared to WT embryos (red line drawing/red somite numbers indicate shifts in the mutant condition, *versus* black lines/black somite numbers in the WT condition). (B) Cartilage preparations showing shifts in limb bud position leading to the more anterior or rostral location of the pelvic girdle and hindlimb skeleton in *Pbx1*^{-/-}; *Pbx2*^{+/-} mutants (right) compared to WT controls (left) (red dashed lines demarcate distance of girdle to the last rib-bearing thoracic vertebrae). Proximal is to the left; rostral is up. Adapted from (Capellini et al. 2008).

Figure 4.

Model of *Pbx* gene functions during early limb development. (E8.5–E9.0) During early limb field induction the proximal-to-distal (Pr-D) axis becomes established as *Pbx* genes control *Fgf10*–*Fgf8* signalling in limb mesoderm (black arrows) and/or possibly in pre-AER ectoderm (light blue arrow; light blue line represents pre-AER). (E9.0–E9.5) During Pr-D axis establishment, AER formation and limb outgrowth, *Pbx* continue to regulate *Fgf8*–*Fgf10* signaling, at least in part due to their upstream control of *Hox* expression in the early limb bud (black arrows) and possibly via their control of *Fgf8* in the AER (blue arrow; blue line represents AER). This upstream regulation leads to *Hox* expression throughout the limb bud and establishment of the pre-*Shh* ZPA domain (pink) in the posterior limb. (E10–E10.75) *Pbx* control of *Hox* expression leads to the direct regulation of *Shh* expression onset and maintenance (black arrows) in the ZPA (terracotta colored zone). *Shh* expression in turn promotes *Fgf* expression in the AER (blue) and maintains the ZPA-AER signalling network (black arrows). This genetic network leads to the proper control of *Shh*-dependent distal limb (digital) development and proximal-distal patterning and growth. Abbreviations: A, anterior; D, distal; P, posterior; Pr, proximal; ZPA, zone of polarizing activity.

Figure 5.

Pbx hierarchically regulate early limb P-D and D-V axis establishment. (Left-top) In situ hybridization at E11 reveals that *Fgf8* expression remains mostly unchanged (white arrowhead) at the mid-to-hindbrain junction in *Pbx1*^{-/-}*Pbx2*^{-/-} embryos (30 som.) compared to somite-matched E10 WT (30 som.) and littermate E11 WT (44 som.) embryos (black arrowheads). Littermate WT embryos are included to demonstrate that *Pbx1*^{-/-}*Pbx2*^{-/-} mutants are delayed in somitogenesis. (Left-middle) *Fgf8* expression is barely detectable in *Pbx1*^{-/-}*Pbx2*^{-/-} forelimb buds (red arrowhead) compared to somite-matched WT and littermate WT forelimb buds (black arrowheads). (Left-bottom) *Fgf8* expression is absent in *Pbx1*^{-/-}*Pbx2*^{-/-} hindlimb buds (blue arrowhead) compared to somite-matched WT and littermate WT hindlimb buds (black arrowheads). (Right-top) In situ hybridization at E10.25 reveals that *En1* expression is absent from the anterior and posterior ventral ectoderm in *Pbx1*^{-/-}*Pbx2*^{-/-} forelimbs (blue arrowheads) compared to controls. (Right-middle) In *Pbx1*^{-/-}*Pbx2*^{-/-} hindlimbs at E10.25, *En1* expression is only lost from the posterior ventral ectoderm (blue arrowhead) compared to controls. (Right-bottom) In hindlimbs at E10.75, *Lmx1b* expression is significantly up-regulated in the ventral domain in *Pbx1*^{-/-}*Pbx2*^{-/-} mutants (red arrowhead), although such up-regulation may reflect a delay in limb bud outgrowth (E10 limb bud on right). On left, panels show distal limb bud tip with anterior to the top and dorsal to the left; on right, panels show ventral limb bud with anterior to the top. Abbreviations: A, anterior; FL, forelimb; HL, hindlimb; P, posterior.

Figure 6.

Comparative expression of TALE-encoding genes and their functions in invertebrate and vertebrate appendage development. Schemata depict comparative Exd/Pbx-mediated invertebrate (left panels) and vertebrate (right panels) proximal-distal appendage development. Blue text denotes ectodermal gene expression, while black text denotes mesenchymal (mesoderm) gene expression. During larval imaginal disc development (top), Exd (and *hth*, not shown) restrict *Dll* signaling distally in the invertebrate ectoderm (imaginal disc) setting up distinct proximal and distal zones. This expression pattern is different in vertebrates, in which *Pbx* genes in the ectoderm are, at this time, upstream regulators of *Dlx* genes, which are *Dll* homologs. Furthermore, the entire vertebrate limb is *Pbx* positive, due to *Pbx2* in the distal limb at this stage (i.e., E10). During later-appendage development (middle panels), hedgehog (*Hh*) signaling in the invertebrate ectoderm acts to repress Exd (*hth*) to proximal domains and activates other genes distally (not shown), further establishing and compartmentalizing proximal and distal appendicular domains. Also, the fly leg P-D axis develops independently of *Hox*. In contrast, in vertebrates *Pbx* genes in the limb mesoderm hierarchically regulate *Hox* expression, which leads to *Shh* activation and promotes later distal limb patterning (i.e., at E10–E11). Lastly, once adult limb morphology is established, only the proximal domains of the invertebrate limb are specified by Exd (*hth*), as based on expression and experiments conducted during imaginal disc stages*, whereas the entire vertebrate limb requires *Pbx* function. * - see text for details. Proximal (left); anterior (top). Illustrations adapted from (Pueyo and Couso, 2005).