REVIEW

'Building a perfect body': control of vertebrate organogenesis by PBX-dependent regulatory networks

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***Pbx* genes encode transcription factors that belong to the TALE (three-amino-acid loop extension) superclass of homeodomain proteins. We have witnessed a surge in information about the roles of this gene family as leading actors in the transcriptional control of development. PBX proteins represent a clear example of how transcription factors can regulate developmental processes by combinatorial properties, acting within multimeric complexes to implement activation or repression of transcription depending on their interaction partners. Here, we revisit long-emphasized functions of PBX transcription factors as cofactors for HOX proteins, major architects of the body plan. We further discuss new knowledge on roles of PBX proteins in different developmental contexts as upstream regulators of *Hox* genes—as factors that interact with non-HOX proteins and can work independently of HOX—as well as potential pioneer factors. Committed to building a perfect body, PBX proteins govern regulatory networks that direct essential morphogenetic processes and organogenesis in vertebrate development. Perturbations of PBX-dependent networks can cause human congenital disease and cancer.**

The purpose of this review is to discuss the knowledge that has been gained in past years on the contributions of PBX homeodomain-containing transcription factors (TFs) to vertebrate embryonic development. While in the first part of the review we provide a summary of the biochemical and transcriptional properties of PBX proteins, the body of the review is devoted to illustrating PBX-dependent regulatory networks that direct morphogenetic processes and organogenesis in vertebrates. In the early 1990s, it was reported that PBX1 is the product of a proto-oncogene targeted by chromosomal translocations in human hematologic malignancies and that the *Drosophila* ortholog of vertebrate PBX1, Exd, acts as a Hox cofactor in embryonic body (EB) segmentation (see Kamps et al. 1990; Nourse et al. 1990; Peifer and Wieschaus 1990; Cleary 1991; Rauskolb et al. 1993). Since those days, it has been established that PBX1 and its family members are protagonists of diverse and essential developmental processes, also in *Hox*-less embryonic domains (for reviews, see Moens and Selleri 2006; Capellini et al. 2011b).

Combinatorial transcriptional regulation of embryonic development and evolution

The vertebrate body originates from a few pluripotent stem cells within the blastocyst (for reviews, see Zhu and Huangfu 2013; McCracken et al. 2016; Morgani et al. 2017). Despite the heterogeneity of seemingly uniform populations of stem cells and multipotent progenitors (for review, see Simon et al. 2018), lineage decisions result in tissues and organs of defined size and shape with consistent proportions of differentiated cell types. Organ development and growth (for reviews, see Tam and Loebel 2007; Vichas and Zallen 2011; LeGoff and Lecuit 2015; Williams and Solnica-Krezel 2017) and their integration into a functional organism (for reviews, see Hogan 1999; Gavrilov and Lacy 2013; Hubaud and Pourquié 2014; Campàs 2016) depend on the precise expression of genes in space and time (for review, see Zeller 2010), which in turn is subject to large-scale regulation and modeled by the chromatin state (for review, see Deschamps and Duboule 2017). Research over the past three decades has elucidated that many genetic pathways, including signaling molecules and TFs, that control organogenesis are similar to those deployed in earlier stages of development and are used iteratively by the embryo. Moreover,

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transcription of genes in distinct embryonic domains can involve shared enhancer-containing landscapes and can use the same regulatory topology (TADs) (Lonfat et al. 2014). Also, a number of genetic networks that control developmental processes are evolutionarily conserved, including the collinearity of the *Hox* code (i.e., the sequential activity of *Hox* genes based on their positions within their clusters), which is preserved throughout bilateralia (for review, see Darbellay and Duboule 2016). For example, while nonvertebrate chordates lack hindbrain (HB) segmentation, they exhibit nested *Hox* expression domains that are established partly by retinoic acid (RA) (for review, see Parker and Krumlauf 2017).

Vertebrate organisms have evolved with great complexity of shapes and forms, while their genomes and gene-encoded products have not expanded proportionally (for review, see Villar et al. 2014). Thus, within each species, the embryo requires a method to create distinct and differently specialized tissue types and organ structures from a single genome. Differences in gene regulation have long been recognized as major contributors to phenotypic diversity (see Britten and Davidson 1969; Carroll 2008). Specifically, enhancers have been shown to play central roles in orchestrating spatiotemporally precise gene expression programs during development. Divergence in enhancer sequence and activity is an important mediator of interspecies and intraspecies phenotypic variation (Prescott et al. 2015; Long et al. 2016). Indeed, evolutionary alteration of transcriptional *cis*-regulatory modules can underpin evolutionary diversification and changes in organ morphology among different species (Lopez-Rios et al. 2014). Notably, distant-acting tissue-specific enhancers vastly outnumber protein-coding genes in vertebrate genomes. Functional enhancer redundancy, a widespread feature of genomes, might provide a regulatory buffer during development, preventing deleterious phenotypic consequences upon loss of individual enhancers (Osterwalder et al. 2018). Concomitantly, combinatorial regulatory mechanisms lay the foundations of both the complexities of the vertebrate body and species-specific differences by deploying the same factors in different combinations within elaborate transcriptional networks, which are used at different times and in different domains of the developing embryo. The PBX family constitutes a clear and well-documented example of how proteins can regulate morphogenesis and developmental processes by using combinatorial transcriptional properties; accordingly, PBX TFs act within multimeric complexes and can activate or repress transcription depending on their binding partners.

Biochemical characteristics of TALE (three-amino-acid loop extension) PBC TFs

In the animal kingdom, the TALE superclass of TFs is characterized by the insertion of a three-amino-acid loop in their homeodomain, which forms a flexible linker generating a hydrophobic pocket (Mukherjee and Bürglin 2007; Bürglin and Affolter 2016). This superclass of TFs consists of multiple families, including (1) Iroquois (IRO), (2) TGIF, (3) MEIS/PREP, and (4) PBC. The latter family comprises vertebrate PBX1-4, *Drosophila melanogaster* extradenticle (Exd), and *C. elegans* ceh-20. PBX proteins share remarkable sequence homology (Monica et al. 1991) that extends beyond the homeodomain and encompasses two other domains located at the protein N terminus, which are critical for heterodimerization with MEIS/PREP factors (for review, see Moens and Selleri 2006).

Partnerships of PBC TFs

The PBC three-amino-acid loop moiety mediates the interaction with the tryptophan-containing hexapeptide (HX) motif (IYPWMK) found N-terminal to the homeodomain of most HOX proteins, major architects of the body plan (see Mann et al. 2009; Saadaoui et al. 2011; Ladam and Sagerström 2014; Longobardi et al. 2014). During development, HOX proteins can bind similar AT-rich DNA sequences in vitro (for reviews, see McGinnis and Krumlauf 1992; Gehring et al. 1994; Lemons and McGinnis 2006). HOX proteins can heterodimerize with PBX/Exd proteins (see Saadaoui et al. 2011; Ladam and Sagerström 2014; Merabet and Mann 2016; Ortiz-Lombardia et al. 2017) and can also pair with other classes of TFs for function; for example T-box factors, as recently described in limb development (Jain et al. 2018). Through the years, heterodimerization with PBX/Exd has been proposed as a mechanism through which HOX proteins acquire DNA-binding selectivity and specificity. However, it remains challenging to envisage how factors such as PBX proteins with widespread presence can confer functional specificity to HOX proteins, which exhibit domain-restricted localization in vivo. Of note, recent studies have demonstrated that HOX proteins do in fact bind specific sequences in vivo and drive the expression of different target genes at different times in different tissues, thus executing distinct developmental programs (Alexander et al. 2009; Crocker et al. 2015; Beccari et al. 2016; Sheth et al. 2016; Jerkovic et al. 2017; Parker et al. 2018).

The role of the HOX HX motif in the interaction with PBX TFs has been revised recently. Indeed, it has been reported that all HOX proteins, except paralogous groups 1 and 2, can interact with PBX/MEIS in the absence of the HX moiety. Novel HOX paralog-specific TALE-binding sites were identified, which are used in a cell context-specific manner (Dard et al. 2018). The MEIS/PREP (MEINOX) class of TALEs can also regulate HOX activity by forming trimeric DNA-bound HOX/PBC/MEINOX complexes. It is of note that MEIS/PREP can interact directly with a subset of HOX proteins independently of the HX motif (for review, see Moens and Selleri 2006). In addition to forming heterodimers with HOX proteins and with TALE partners MEIS/PREP, PBX proteins can form multimeric complexes with other TFs, such as MYOD, EN, and PDX1 (Berkes et al. 2004; Longobardi et al. 2014). PBX/Exd can also confer specificity to their binding partners in part by regulating their nuclear localization and stability. Unlike PREP/MEIS, PBX TFs have nuclear localization and nuclear export signals (Berthelsen et al. 1999; Kilstrup-

Nielsen et al. 2003). Last, PBX proteins can regulate transcription by interacting with basic transcription regulators, such as histone acetyltransferases (HATs) and CBP coactivators, and with histone deacetylases (HDACs) and the corepressor N-CoR/SMRT (for reviews, see Ladam and Sagerström 2014; Longobardi et al. 2014). Thus, PBX TFs can be part of activating or repressing transcriptional complexes.

In zebrafish blastulas, Pbx–Meis heterodimers displace HDACs from the *hoxb1a* promoter, but *hoxb1a* is not transcribed until the gastrula stage, when binding of newly synthesized Hoxb1b protein to TALE factors on the *hoxb1a* promoter triggers its transcription (Choe et al. 2009). This would indicate that, in this context, TALEs access promoters during early embryogenesis, facilitating chromatin accessibility of transcriptionally inactive genes, but Hox proteins are subsequently required to initiate transcription (Choe et al. 2014). Accordingly, it was proposed that during early development, PBX proteins can mark genes for transcriptional activation as part of multimeric complexes, suggesting their potential roles as "pioneer factors" (for review, see Grebbin and Schulte 2017). However, a currently held definition of pioneer factors specifies that their binding to a DNA element is not dependent on pre-existing histone modifications that are indicative of gene activation (for reviews, see Iwafuchi-Doi and Zaret 2014; Donaghey et al. 2018). Of note, the findings discussed above in zebrafish demonstrate that the *hoxb1a* promoter is marked with the active histone modification H3K27ac and thus is primed for transcription prior to Pbx binding. Recent studies have also established that PBX1 controls early neurogenesis partly via the direct regulation of the target gene doublecortin (*Dcx*) (Grebbin et al. 2016). In undifferentiated neural progenitor cells in culture, PBX1 binds the promoter/proximal enhancer of *Dcx* while the latter is still compacted to histone H1 and apparently before it is significantly marked by activating histone modifications, long before *Dcx* is expressed. Once differentiation is induced, MEIS associates with chromatin-bound PBX1, it recruits poly-ADP-ribose polymerase 1 (PARP1) and then initiates PARP1-mediated eviction of H1 from chromatin. These findings delineate a sequence of events by which a PBX/MEIS complex facilitates chromatin accessibility of transcriptionally inactive genes in neural progenitor cells (Hau et al. 2017). Overall, it has been reported that PBX1 primes genes for activation in different systems and can bind to its target sites when chromatin is still compacted. However, caution must be used in attributing universal pioneer factor roles to PBX1.

DNA binding by PBX factors—a glimpse from whole-genome studies

Genome-wide studies of TF binding, three-dimensional genome organization, and transcriptomes have radically changed our views on how TFs regulate gene expression (for reviews, see Spitz and Furlong 2012; Denker and de Laat 2016). PBX TFs bind preferentially to the DNA hexameric sequence TGACAG when they dimerize in vitro with both PREP and MEIS, while PBX/HOX dimers bind the DNA octameric motif TGATNNAT, in which the variable sequence is determined by the HOX protein involved. Ternary complexes such as those comprising PBX/HOX/MEIS or PBX/HOX/PREP can also bind the octameric motifs (for reviews, see Mann et al. 2009; Longobardi et al. 2014). In *Drosophila*, Hox proteins gain novel recognition properties when they bind DNA with Exd/homothorax (Hth) complexes, suggesting that emergent properties in DNA recognition, revealed by interactions with cofactors, contribute to TF-binding specificities in flies (Slattery et al. 2011). Analyses based on ChIP-seq (chromatin immunoprecipitation [ChIP] combined with high-throughput sequencing) of TALE proteins in embryonic day 11.5 (E11.5) mouse embryos revealed the majority of TALE-bound DNA sequences and bona fide target genes (Penkov et al. 2013) and uncovered similarities to the SELEX-seq (systematic evolution of ligands by exponential enrichment combined with deep sequencing) analyses performed in *Drosophila* (Slattery et al. 2011). Thus, PBX DNA-binding motifs appear to be conserved throughout evolution but show varying DNA-binding properties dependent on the binding partners. Of note, ultrabithorax (Ubx) in complex with Exd binds selectively to clusters of low-affinity sites in enhancers of the *Drosophila shavenbaby* gene (Crocker et al. 2015). Multiple low-affinity sites are required to confer both specific and robust expression in embryos developing in variable environments. Natural selection appears to work in this context at the level of the enhancer, underscoring the concept that changes in enhancer sequence contribute to morphological variation.

In mice, PBX1 forms dimers mainly with PREP1, while, when heterodimerizing with MEIS, it forms multimeric complexes with HOX proteins. Furthermore, PBX/PREP dimers bind preferentially to promoters, whereas PBX/MEIS dimers bind to enhancers, intergenic regions, and intragenic regions (Penkov et al. 2013). Gene ontology analysis indicated that PBX1/MEIS-bound genes are enriched for functions related to various aspects of development, such as A/P pattern specification, heart and vascular morphogenesis, and nervous system development, while PBX1/PREP-bound genes are instead annotated to basal cell functions, such as DNA and histone modification, protein transport, and signal transduction (Penkov et al. 2013). Additional ChIP-seq analyses exploring genome-wide binding of PBX1, MEIS1/MEIS2, and HOXA2 in E11.5 murine branchial arch 2 (BA2), revealed strong similarities in the distribution of MEIS, PBX, and HOXA2 peaks, suggesting that these proteins form multimeric complexes in BA2 development. This research highlighted that HOXA2 acts as a BA2-specific TF that selectively enhances MEIS binding to drive transcription of BA2 target genes, thus directing BA2 identity during head development (Amin et al. 2015). Recent research investigated the genome-wide co-occupancy of HOXA1 with key TALE members (De Kumar et al. 2017), showing that almost all genomic sites occupied by HOXA1 are bound by one or more TALEs. This study defined potential distinct classes of HOXA1 targets, each characterized by the occupancy of a discrete combination of TALE factors

and correlated with different biological processes. It is also unclear whether TALE proteins act by the same mechanisms throughout embryogenesis. In this context, it was reported that in zebrafish blastulas, TALEs occupy genomic DECA motifs with nearby sites for the nuclear transcription factor Y (NF-Y) and form complexes with NF-Y, thus regulating the chromatin state at genes of a key regulatory network that drives anterior embryonic development. However, at subsequent segmentation stages, TALE occupancy expands to include HEXA motifs near PBX-HOX sites (Ladam et al. 2018). This important research highlights that throughout anterior zebrafish embryogenesis, TALE proteins control critical regulatory networks by using distinct DNA motifs and protein partners at different developmental stages.

Overall, the binding properties observed for multimeric complexes that comprise various members of the PBX, MEIS, and HOX families of TFs resonate with the concept of combinatorial transcriptional regulation. However, the exact nature of these interactions remains to be elucidated, and no general rules have emerged as yet for how particular combinations of cofactors mediate transcriptional outcomes. Given that PBX TFs are present in most embryonic tissues, while HOX proteins exhibit precise temporal and spatial localization, it could be envisaged that HOX factors drive specificity, while PBX proteins modify the HOX effect. Overall, knowledge is still rudimentary regarding how distinct TALEs combine with different HOX and non-HOX partners to identify unique sets of target genes in vivo in different tissues and how they execute specific functions at different developmental stages of the vertebrate embryo. Deeper discussions of the biochemical properties of TALE factors are found in relevant articles (for reviews, see and Selleri 2006; Mann et al. 2009; Ladam and Sagerström 2014; Longobardi et al. 2014; Merabet and Mann 2016).

Control of embryonic development and onset of disease under PBX laws

Pbx genes are conserved in vertebrates and invertebrates (see Moens and Selleri 2006; Blassberg et al. 2013; Chen et al. 2013). Vertebrate *Pbx* genes are widely expressed during embryogenesis (Monica et al. 1991; Ferretti et al. 1999; Schnabel et al. 2001; Selleri et al. 2001, 2004; Wagner et al. 2001; Di Giacomo et al. 2006). In zebrafish, functional differences among *pbx* genes are due to differences in their expression rather than in their biochemical activities, since ectopically expressing any of the zebrafish *pbx* genes rescued the phenotype of *pbx4* mutant embryos (Pöpperl et al. 2000). *Pbx*, *Meis*, and *Hox* genes share expression domains in multiple tissues, reflecting their ability to form heterodimeric or heterotrimeric complexes. Despite frequent overlapping patterns of *Pbx1–3* (Capellini et al. 2006, 2010), different *Pbx* genes also exhibit a certain degree of tissue- and organ-specific expression in the mouse embryo. For example, while *Pbx3* is expressed in the nervous system, forelimb (but not hindlimb) mesenchyme, and ovaries (Monica et al. 1991; Rhee et al. 2004; Di Giacomo et al. 2006), *Pbx4* is expressed mainly in the testes (Wagner et al. 2001). In contrast, *Pbx1* transcripts are present in multiple tissues (Selleri et al. 2001), with decreasing levels in late gestation (Ferretti et al. 1999; Koss et al. 2012). Last, *Pbx2* expression is widespread throughout development and in the adult (Monica et al. 1991; Selleri et al. 2004). Thus, with the exception of PBX4, PBX proteins are present in most vertebrate embryonic tissues, in stark contrast to tissue-specific TFs.

As demonstrated by loss of function (LOF) in mice, different *Pbx* genes play fundamental and pleiotropic roles in organogenesis. *Pbx1* homozygous mutant embryos ($Pbx1^{-/-}$) die in utero with dramatic abnormalities in multiple organs (Selleri et al. 2001; DiMartino et al. 2001; Kim et al. 2002; Manley et al. 2004; Brendolan et al. 2005; Capellini et al. 2006, 2008, 2010, 2011a; Stankunas et al. 2008; Ferretti et al. 2011; Vitobello et al. 2011; Koss et al. 2012; Hurtado et al. 2015; Grebbin et al. 2016; Villaescusa et al. 2016; Losa et al. 2018; McCulley et al. 2018; Welsh et al. 2018). In contrast, $Pbx2^{-/-}$ mice do not display detectable abnormalities (Selleri et al. 2004), while $Pbx3^{-/-}$ mutants die perinatally, from central respiratory failure (Rhee et al. 2004). Of note, compound *Pbx1;Pbx2*- or *Pbx1;Pbx3*-deficient embryos die earlier in utero than single $Pbx1^{-/-}$ mutants and display drastic phenotypic exacerbations; for example, in the axial and appendicular skeletons, together with the appearance of novel craniofacial and distal limb defects that are absent in single $Pbx1^{-/-}$ mutants (Capellini et al. 2006, 2008, 2010, 2011a; Ferretti et al. 2011; Vitobello et al. 2011; Koss et al. 2012; Golonzhka et al. 2015; Hanley et al. 2016). These findings emphasize that different PBX TFs execute overlapping functions during development with collaborative regulatory roles on common targets in tissues where these genes are coexpressed.

Like PBX TFs, MEIS/PREP proteins are also present broadly in vertebrate embryos, and their overlapping localization is a likely source of genetic redundancy. In mice, *Prep1* and *Meis1* mutants die in utero, exhibiting hematopoietic and angiogenic defects with hypoplasia of some organs, reminiscent of *Pbx1* mutations. Hypomorphic mutation of *Prep1* causes a major reduction of PBX and MEIS proteins, pointing to an essential role of PREP1 within a PBX–MEIS network that regulates embryonic development (Hisa et al. 2004; Azcoitia et al. 2005; Ferretti et al. 2006; Cai et al. 2012). Constitutive inactivation of *Meis2* also results in embryonic lethality with massive hemorrhaging, while its conditional inactivation in neural crest reveals defects in cranial and cardiac neural crest derivatives (Machon et al. 2015). Given that mutant mice for compound loss of *Meis* or *Meis*/*Prep* genes have not yet been generated, it is not known whether *Meis* and *Meis*/*Prep* family members have overlapping roles in mammalian development similar to *Pbx* genes.

There is a strong association between dysregulation of PBX-directed gene networks identified in *Pbx* LOF mice and human congenital defects, including cleft lip/palate (CL/P) (Ferretti et al. 2011; Losa et al. 2018; Welsh et al. 2018), congenital asplenia (Koss et al. 2012), and diabetes (Kim et al. 2002; Muharram et al. 2005). De novo deleterious sequence variants of *PBX1* were discovered recently

in children affected by a new syndrome characterized by pleiotropic developmental defects that mimick the phenotypes of *Pbx1*$^{-/-}$ embryos. Functional studies of the *PBX1* sequence variant proteins in cell culture revealed alterations of PBX1/PREP1-dependent transactivation ability and altered nuclear translocation of PBX1, suggesting abnormal interactions between mutant PBX1 proteins and other TALE or HOX cofactors (Slavotinek et al. 2017). These perturbations likely affect transcription of PBX1 target genes with a severe impact on human development.

In regard to cancer, PBX1 was identified upon cloning the product of the human (1:19) chromosomal translocation, which results in fusion of the transactivation domain of E2A to the homeodomain of PBX1, forming an E2A-PBX1 chimeric protein in pre-B acute lymphoblastic leukemias (Kamps et al. 1990; Nourse et al. 1990). In the context of solid tumors, it was also reported that PBX1 directs estrogen receptor transcriptional activity in human breast cancers (Magnani et al. 2011). PBX proteins can be envisaged as transcriptionally silent but achieving effective regulation of gene expression via the recruitment of tissue-specific TFs that orchestrate tissue morphogenesis and organ development. An elegant study described that PBX1 cooperates with PREP1 to trigger TGFβ-induced epithelial-to-mesenchymal transition (EMT) in human lung adenocarcinoma cells by regulating SMAD3 (Risolino et al. 2014). Similarly, *PBX1* was associated with EMT in hepatocellular carcinoma with poor survival (Kodama et al. 2016). Last, it was observed that high levels of PBX1 correlate with resistance to platinum-based therapy in ovarian cancer (Jung et al. 2016). There is evidence that TALE and HOX proteins are dysregulated in a wide range of human cancers and that their interactions as heterodimers or multimeric complexes can be exploited as a therapeutic target in solid and haematological malignancies (Morgan et al. 2017).

'Heads-up': HOX-depedent and HOX-independent roles of PBX in brain and craniofacial morphogenesis

The pervasive and dynamic expression of *Pbx*, *Meis*, and *Prep* genes in the mouse embryonic head, in domains patterned by *Hox* genes, and also in *Hox*-less territories (for reviews, see Alexander et al. 2009; Schulte and Frank 2014) suggested critical roles for these TFs in brain and cranium development. Here we discuss three paradigmatic examples of PBX-depedent processes in head morphogenesis.

PBX homeoproteins drive activation of the Hox gene cascade in the HB

In the vertebrate embryo, the HB is transiently subdivided into distinct units called rhombomeres (Rs) and lateral outpocketings that form the brachial arches (BAs), which are populated by migrating neural crest cells (NCCs) (Fig. 1; Parker et al. 2018). Unique combinations of HOX proteins confer segmental identity along the HB (Fig. 1), and their perturbation results in changes of rhombomere identity, known as homeotic transformations (see Alexander et al. 2009). PBX and HOX proteins form multimeric complexes that restrict *Hox* gene products to specific rhombomeres, reinforcing *Hox* gene segmental expression by cross-regulatory, pararegulatory, and autoregulatory loops (for reviews, see Alexander et al. 2009; Schulte and Frank 2014) and providing positional information along the HB anterior–posterior (A/P) axis. In zebrafish, inactivation of *lazarus* (homologous to mammalian *Pbx4*) and *pbx2* results in homeotic transformations whereby all HB rhombomeres acquire a homogeneous ground state identity, that of R1 (Pöpperl et al. 2000). Furthermore, it was shown that PBX factors interact with HOX paralog group 1 proteins to specify HB segment identities, pointing to primary roles of PBX proteins as HOX partners to modify the ground state identity during HB development in zebrafish (Waskiewicz et al. 2002). *Pbx1/Pbx2* double-homozygous mutant mouse embryos (*Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$) exhibit abnormal HB segmentation and forebrain development and hypoplastic posterior BAs as well as somite and vertebral patterning defects. These anomalies resemble those of embryos with deficiency of RA, which is essential for the establishment of the restricted pattern of *Hox* gene expression during HB segmentation (Vitobello et al. 2011). In *Pbx1*$^{-/-}$;*Pbx2*$^{-/-}$ and *Pbx1*$^{-/-}$;*Hoxa1*$^{-/-}$ mutants, levels of *Raldh2*, the enzyme that synthetizes RA, were not maintained in the lateral plate mesoderm (LPM), resulting in caudal shift of rhombomere segmentation. PBX proteins, together with MEIS2 and HOXA1, bound to a HOX–PBX bipartite element that drives transcriptional activity only in *Raldh2*-positive domains of E8.5 embryonic trunks (Vitobello et al. 2011). These findings established a molecular feed-forward mechanism linking HOX/PBX-dependent RA synthesis in axial patterning with the establishment of spatially restricted HOX/PBX activity in HB patterning. The key message from this study is that PBX TFs are critical not only for maintaining segmental *Hox* gene expression but also for the initiation of HB segmentation (Fig. 1). In the latter instance, however, PBX proteins cooperate with select HOX factors in the mesoderm to initiate HB segmentation at least in part via a RA signal from the mesoderm to the HB.

Revisiting potential functions of PBX proteins as HOX cofactors in patterning BA-derived craniofacial structures

Developmental defects in *Pbx*-deficient mouse embryos affect organs (e.g., axial and appendicular skeletons, hematopoietic system, and thymus) that are shaped by specific HOX proteins (for review, see Moens and Selleri 2006). It is noteworthy that craniofacial phenotypes that we described years ago in *Pbx1*$^{-/-}$ embryos (Selleri et al. 2001) and abnormalities subsequently reported in mice with cranial neural crest-specific deletion of the *Hoxa* cluster (Minoux et al. 2009) are similar, with the appearance of BA1-like morphologies in BA2-derived elements (see details in Fig. 1). While loss of *Pbx1* alone resembles LOF of the *Hoxa* cluster, which is more striking than LOF of *Hoxa2* alone (Rijli et al. 1993), it does not alter 3′

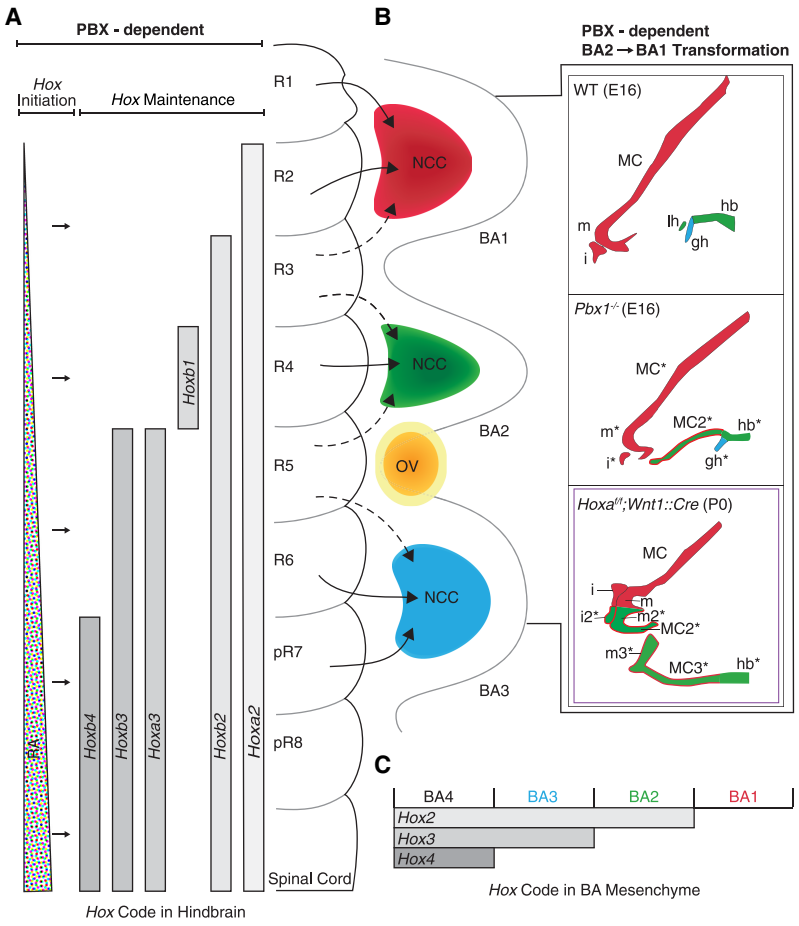

Figure 1. PBX proteins drive activation of the *Hox* gene cascade in the HB and may function as HOX cofactors in patterning BA-derived craniofacial structures. (*A*) Graded RA activity decreases rostrally (dotted triangle), diffuses into the neuroepithelium (arrows), and drives *Hox* gene activation, establishing the HB *Hox* code (PBX-dependent *Hox* initiation) (Vitobello et al. 2011). HB divided into six rhombomeres (R1–R6) and pseudorhombomeres (pRs; pR7 and pR8 shown) (Gray 2013; Kratochwil et al. 2017). Boxes of different gray hues represent expression domains of individual *Hox* genes. PBX and MEIS/PREP form transcriptional complexes with HOX proteins to maintain *Hox* gene expression in HB (PBX-dependent *Hox* maintenance). (*B*) Rhombomeres drive pathways of cranial NCC migration (curved arrows) into the BAs. In mice, NCC contribution from R3 and R5 into BAs is sparse (dashed curved arrows). BA1–BA3 cores of NCCs. (Red) BA1; (green) BA2; (azure) BA3. *Pbx1* and *Hoxa* pattern BA2-derived skeletal elements. Sketches of skeletal structures derived from BA1 (red), BA2 (green), and BA3 (azure) in E16 wild-type embryos (WT; *top right*), E16 $Pbx1^{-/-}$ embryos (*middle right*), and postnatal day 0 (P0) embryos with NCC-specific inactivation of the *Hoxa* cluster ($Hoxa^{f/f}$;*Wnt1::Cre*) (*bottom right*). Asterisks indicate abnormal structures in $Pbx1^{-/-}$ and $Hoxa^{f/f}$;*Wnt1::Cre* embryos. BA2-derived craniofacial structures transformed into elements mirroring BA1 derivation are green with red outline. *Pbx1* LOF shows anteriorization of BA2 NCC-derived structures (Selleri et al. 2001) with hyoid bone lesser horn (lh) acquiring features resembling BA1-derived Meckel's cartilage (MC2*). Similar to *Pbx1* LOF, NCC-specific inactivation of *Hoxa* cluster results in anteriorization of BA2-derived structures, with appearance of two additional elements resembling MC (MC2* and MC3*), two elements similar to the malleus (m2* and m3*), and duplicated incus (i2*) (Minoux et al. 2009). (Gh) Greater horn of hyoid; (hb) hyoid body; (i) incus; (m) malleus; (OV) otic vesicle. (*C*) *Hox* gene code in BA NCC-derived mesenchyme. Domain of *Hox* paralogous expression; the same shades of gray as in *A* are used. BA1 mesenchyme is *Hox*-less.

Hoxa gene expression in this context, supporting the notion that PBX1 may act as a cofactor for 3′ HOXA proteins to direct the developmental programs that shape BA2 and posterior BAs, where these genes are coexpressed. As further support, HOXA2 DNA-binding profiles overlap those of PBX in BA2 (Amin et al. 2015). However, PBX1 and 3′ HOXA proteins must also execute critically independent functions in BA2, as morphologic transformations and craniofacial defects present in the two mouse mutants are not a complete phenocopy.

A HOX-independent, PBX-dependent regulatory circuit directs midface morphogenesis

In mice PBX proteins execute morphogenesis of the *Hox*-less midface, which comprises the upper lip, primary palate, and nose and requires growth and fusion of the frontonasal and maxillary processes (for review, see Dixon et al. 2011) at a three-way seam named the lambdoidal junction (λ) (Compagnucci et al. 2011). During mouse development, *Pbx1-3* genes are expressed in both the cephalic epithelium and mesenchyme at the λ (Ferretti et al. 2011). For fusion of the facial prominences to occur, all epithelial cells at the λ must be removed to allow coalescence of the mesenchymal cores, while persistence of the epithelium results in orofacial clefting. We established that compound loss of *Pbx* genes results in fully penetrant orofacial clefting. Depending on the combinations of *Pbx* mutant alleles, abnormalities of the midfacial complex in these mice manifest as unilateral CL/P, bilateral CL/P, or cleft palate only (CPO) (Ferretti et al. 2011; Welsh et al. 2018), providing unique models for these human birth defects. Cephalic epithelium-specific loss of *Pbx1* on a *Pbx2*- or *Pbx3*-deficient background results in fully penetrant CL/P, demonstrating the critical role of the epithelium in the pathogenesis of this abnormality. We uncovered that in normal development, at least two mutually exclusive cellular behaviors—apoptosis and EMT—mediate removal of the epithelium during prominence fusion. We demonstarted that, partnering with PREP, PBX proteins direct parallel tissue-specific regulatory axes at the *Hox*-less λ within a complex network that converges on control of epithelial apoptosis via regulation of WNT canonical (WNT^{Can}) signaling and EMT through transactivation of

Snail1 (Fig. 2). Pertubation of this PBX-dependent circuit results in concomitant loss of apoptosis and EMT at the λ with ensuing CL/P. By reactivation of WNT^{Can} in the cephalic epithelium of compound *Pbx* mutant embryos, we restored apoptosis and rescued CL/P in all mutants analyzed, opening new avenues for in utero tissue repair of this birth defect (Ferretti et al. 2011; Dong et al. 2017; Losa et al. 2018). As PBX1 was identified as a driver of EMT in lung and liver cancer (Risolino et al. 2014; Kodama et al. 2016), this research highlights PBX-dependent interconnected cellular behaviors in midface morphogenesis and tumor metastasis.

Development of axial, girdle, and appendicular skeletons under PBX-directed rules

The spatial and temporal patterns of *Pbx* and *Meis* transcription in somites and LPM and then later in girdle and limb development are a prototypical illustration of their dynamic expression. These expression patterns suggest that *Pbx1* and *Pbx2* provide first an overlapping code along the flank, girdle, and limb fields and then a complementary code in limb bud morphogenesis. In contrast, *Pbx3* is present in the flank of the early embryo but thereafter becomes restricted to only the forelimb mesenchyme (for review, see Capellini et al. 2011b). We uncovered that *Pbx1* and *Pbx2* cooperate to pattern the axial skeleton at least in part via the upstream control of *Polycomb* and *Hox* gene spatial domains along the axis (Fig. 3). We also reported that in limb patterning, PBX homeoproteins do not appear to act as HOX cofactors but may execute critical roles in the upstream control of *5′ HoxA/D* expression (Fig. 4). We envisage mechanisms whereby PBX TFs may regulate *Hox* collinearity, given their coexpression with specific *Hox* genes in limb bud domains. However, it is still unclear whether PBX factors initiate *Hox* collinearity or reinforce it once it has been initiated. Deeper discussions on the roles of PBX TFs in axial patterning, limb bud positioning, and girdle and limb development can be found in relevant original studies (Selleri et al. 2001; Capellini et al. 2006, 2008, 2010, 2011a) and reviews (Capellini et al. 2011b; Young et al. 2019).

Once the skeletal elements are formed, *Pbx1/2/3* are similarly expressed in proliferative chondrocytes of long bones, while their expression wanes in prehypertrophic and hypertrophic chondrocytes during endochondral ossification. The affected domains of $Pbx1^{-/-}$ limbs and ribs exhibited diminished chondrocyte proliferation, with increased numbers of hypertrophic chondrocytes and precocious ossification. These studies

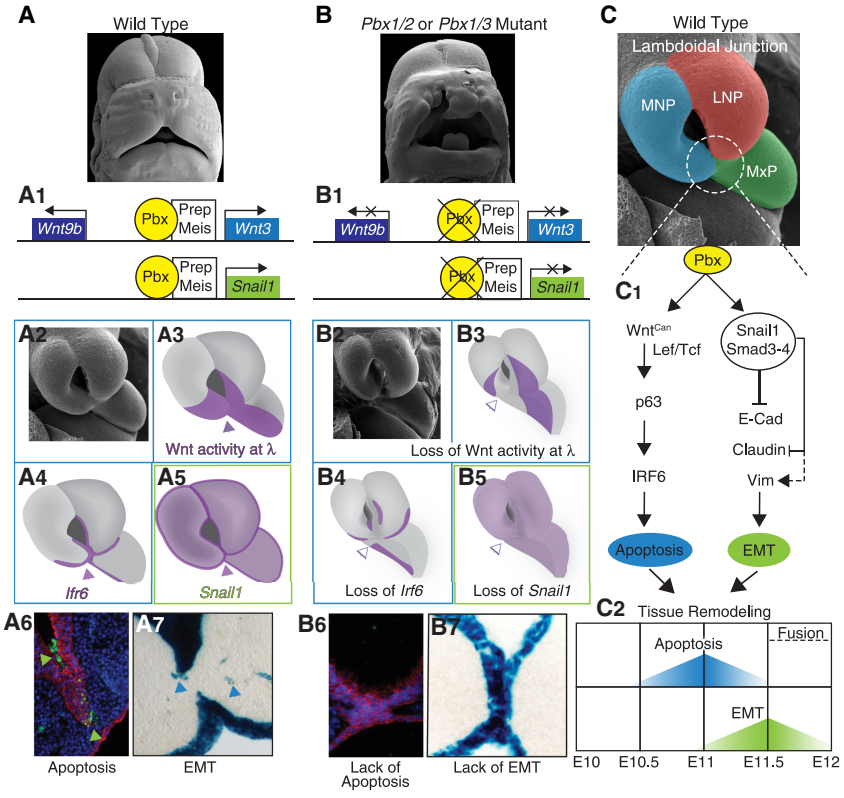

Figure 2. PBX-dependent regulatory networks direct apoptosis and EMT in *Hox*-less midface epithelium. (*A*,*B*) Scanning electron microscopy (SEM) of E12.5 midfaces from wild-type mouse embryos (*A*) and *Pbx1/2* or *Pbx1/3* mutant embryos (*B*). (*C*) E11.5 wild-type midface λ (dashed white circle) in which medial nasal process (MNP; azure), lateral nasal process (LNP; salmon), and maxillary process (MxP; green) fuse. (*A1*) PREP/MEIS–PBX complexes bind *Wnt3*–*Wnt9b* (blue and azure boxes, respectively) and *Snail1* (green box) regulatory elements at wild-type λ. (Arrows) Transcription start site (TSS); (yellow circle) PBX proteins; (white square) PREP/MEIS cofactors. (*A2*) SEM of E11.5 murine wild-type λ. Sketches illustrating WNT activity (*A3*) and *Ifr6* (*A4*) and *Snail1* (*A5*) expression (spatial domains in purple; filled arrows point to λ). (*A6*) Immunofluorescence of LNP/MNP epithelial fusion at λ with breaking down of E-cadherin (red) and the presence of green apoptotic cells (green arrows). (*A7*) Cells of epithelial descent (blue) migrating into MNP/LNP λ mesenchymal cores (blue arrows). (*B1*) In *Pbx* compound mutant embryos, loss of PBX binding (cross on PBX) abolishes transcription of *Wnt3*, *Wnt9b*, and *Snail1* at λ (cross on TSS). (*B2*) SEM of E11.5 dysmorphic λ in *Pbx* compound mutant. Drawings depicting down-regulation or loss of WNT activity (*B3*) and *Ifr6* (*B4*) and *Snail1* (*B5*) expression at mutant λ (empty arrows indicate loss of gene activity/expression). (*B6*) Immunofluorescence at λ reveals the persistence of E-cadherin-positive cells (red) and the absence of apoptosis (lack of green signal). (*B7*) The absence of blue cells within MNP/LNP mesenchyme. (*C1*) PBX-dependent regulatory networks controlling apoptosis (blue) and EMT (green) at wild-type λ (Ferretti et al. 2011; Losa et al. 2018). (Solid arrows) Direct transcriptional control; (dashed arrows) indirect activation; (flat heads) transcriptional repression. (*C2*) Apoptosis and EMT mediate tissue remodeling, promoting facial prominence fusion at λ by E12.

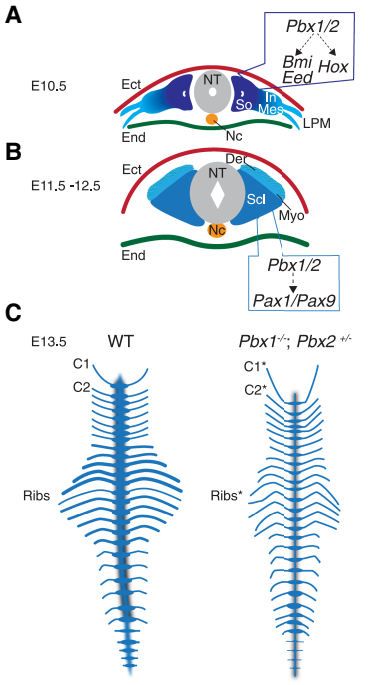

Figure 3. PBX proteins govern axial patterning via control of domain-specific regulatory modules. (*A*) Sketch of E10.5 mouse embryonic trunk (transverse section). Within somites, *Pbx1/Pbx2* direct expression of *Polycomb* genes *Bmi/Eed* and control *Hox* spatial domains (genetic network in purple box) (Capellini et al. 2008). (*B*) At E11.5–E12.5, *Pbx1/Pbx2* modulate expression of sclerotome markers *Pax1/Pax9* (blue box). (*C*) Schemes of axial malformations in E13.5 *Pbx1/2* mutant embryos (*right*; $Pbx1^{-/-}$; $Pbx2^{+/-}$) compared with wild type (*left*). Perturbation of the genetic networks in *A* and *B* results in axial skeletal defects (asterisks) in compound mutants. (Der) Dermatome; (Ect) ectoderm; (End) endoderm; (In Mes) intermediate mesoderm; (Myo) myotome; (Nc) notochord; (NT) neural tube; (scl) sclerotome; (So) somite.

uncovered novel roles for PBX1 in coordinating the extent and/or timing of chondrocyte proliferation with terminal differentiation, which in turn impacts the rate of endochondral ossification (Selleri et al. 2001). It was subsequently reported that in cultured mesenchymal cells, PBX1 represses osteoblastogenesis by blocking HOXA10-mediated recruitment of chromatin remodeling factors and that depletion of PBX1 increases expression of osteoblast-related genes, histone acetylation, and CBP/p300 recruitment (Gordon et al. 2010). This research underscores iterative roles for PBX1 in early and late skeletal developmental programs.

Organogenesis programs guided by TALEnted PBX architects

Roles of PBX proteins in the development of skeletal muscle, the heart, and the lungs

PBX TFs have established roles in the transcriptional control of skeletal muscle differentiation, which is regulated by the basic helix–loop–helix (bHLH) proteins MYOD, MYF5, MRF4, and Myogenin (for reviews, see Hernandez-Hernandez et al. 2017; Sartorelli and Puri 2018). MYOD acts as a master regulator that is capable of converting fibroblasts into skeletal muscle (see Weintraub et al. 1989). MYOD activates *Myogenin* in cooperation with PBX and MEIS proteins: PBX is prebound to the *Myogenin* promoter, enabling the binding of MYOD to atypical E-box motifs, which are juxtaposed to PBX/MEIS-binding sites (Berkes et al. 2004; Maves et al. 2007). The interaction of PBX/MEIS with MYOD on the *Myogenin* promoter facilitates the recruitment of HATs, PRMT5 arginine methyltransferase, and the SWI/SNF chromatin remodeling complex (Heidt et al. 2007; Cho et al. 2015). It was reported that PBX proteins, with the coactivators BRG1 and PRMT5, are bound to the *Myogenin* promoter in caudal somite-enriched tissues from E9.5 mouse embryos, where MYOD is not present and *Myogenin* is transcriptionally silent. Taken together, these findings demonstrate that PBX alone is not sufficient for *Myogenin* transcription and suggest that, while PBX is constitutively present at this promoter, it is not able to reorganize it from a repressed to an active state. Overall, these studies also underscore that PBX proteins mark the promoter of *Myogenin* and of other MYOD target genes for transcription and that they modulate MYOD-driven programs of muscle differentiation in diverse species, such as zebrafish and mice (Maves et al. 2007; Yao et al. 2013; Cho et al. 2015). In addition, key roles for Exd and Hth were described in

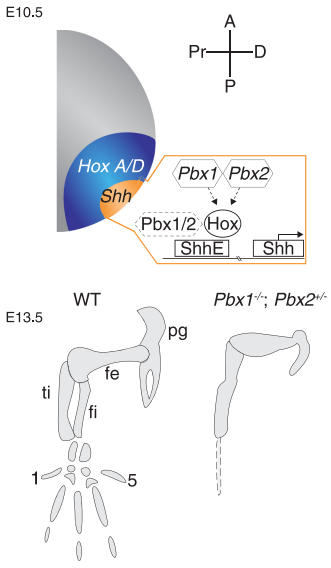

Figure 4. *Pbx1/Pbx2* control transcription of *Shh* in posterior limb mesenchyme. (*Top*) Sketch of E10.5 limb bud in which *Pbx1/Pbx2* control *Hox* gene spatial distribution (blue) and HOX recruitment to *Shh* limb enhancer in posterior mesenchyme (orange). The PBX-directed regulatory network is shown within the orange box. (*Bottom*) At E13.5, loss of *Pbx1/Pbx2* ($Pbx1^{-/-}$;$Pbx2^{+/-}$; *right*) results in proximal and distal limb defects versus wild type (*left*) (Capellini et al. 2006). Digits 1 and 5 are indicated in wild type. (fe) Femur; (fi) fibula; (pg) pectoral girdle; (ti) tibia.

the specification of *Drosophila* muscle fiber fates. In flight muscles, Exd and Hth act genetically upstream of *salm*, a muscle identity gene, and are direct transcriptional regulators of the flight muscle gene *Actin88F* (Bryantsev et al. 2012), highlighting evolutionary conservation of PBX/Exd- and MEIS/Hth-dependent skeletal muscle regulatory networks. While zebrafish *pbx* morphants exhibit compelling muscular phenotypes, muscle abnormalities have not been described yet in *Pbx*-deficient mice. However, *Pbx1* LOF affects diaphragm development in mice, pointing to *PBX1* as a candidate causative gene for human congenital diaphragmatic hernia (CDH) (Russell et al. 2012).

In both mice and zebrafish, PBX TFs are essential for the development of the heart, a vital muscular organ. Nonsynonymous exonic sequence variants in *PBX3*, *PREP1*, *MEIS1*, and *MEIS3* were identified in human congenital cardiac disease (Arrington et al. 2012). Furthermore, functional testing of a human *PBX3* sequence variant in zebrafish revealed a modifier role in congenital heart defects (Farr et al. 2018). Together, these findings point to the involvement of *PBX/MEIS* genes in cardiac birth defects. Mutations in *PBX1* were also reported in children with a new developmental syndrome with heart anomalies (Slavotinek et al. 2017). In the murine lung, *Pbx1* mesenchyme–specific deletion on a *Pbx2*-deficient background resulted in misexpression of genes encoding both vasoconstrictors and vasodilators in pathways converging to increased phosphorylation of myosin in vascular smooth muscle. This led to vasoconstriction with ensuing lethal pulmonary hypertension after birth (McCulley et al. 2018). Thus, PBX TFs play critical roles in lung development and function. We refer the interested reader to additional relevant literature on roles of PBX TFs in heart and lung development (Chang et al. 2008; Stankunas et al. 2008; Maves et al. 2009; Li et al. 2014).

PBX-directed control of thymus, pancreas, and spleen development

Caudal pharyngeal pouch-derived organs of single $Pbx1^{-/-}$ mouse embryos exhibit disorganized patterning of the third pharyngeal pouch, which results in defects of thymus and parathyroids. $Pbx1^{-/-}$ thymic phenotypes comprise hypomorphic thymic lobes that remain localized in the neck, fail to descend into the mediastinum, and do not fuse; unilateral lack of one thymic lobe; and complete absence of the thymus. PBX1 loss was associated with perturbed expression of thymic differentiation markers such as *Pax1*, *Tbx1*, *Foxn1*, and *Gcm2* (Fig. 5) and reduced proliferation of the epithelium (Manley et al. 2004). Notably, $Pbx1^{-/-}$ thymic defects phenocopy the abnormalities reported in embryos with deficiency of three paralogous 3′ *Hox* genes ($Hoxa3^{+/-};b3^{-/-};d3^{-/-}$ mutants), which exhibit separated thymic lobes being retained in the neck (Manley and Capecchi 1998), and the anomalies described in mice with a single null mutation of *Hoxa3*, which are athymic (Manley and Capecchi 1995). In summary, the thymic phenotypes observed in $Pbx1^{-/-}$ embryos phenocopy *Hox3* mutant phenotypes, although some of the defects in $Pbx1^{-/-}$ mutants do not exhibit full penetrance, likely due to redundant roles of *Pbx2* and *Pbx3*, which are also expressed in the thymus. In addition, 3′ *Hox* gene expression is unchanged in $Pbx1^{-/-}$ embryos, suggesting that in this context, PBX1 acts in parallel with 3′ HOX proteins as a cofactor directing pharyngeal organ morphogenesis through the regulation of critical markers for pouch differentiation as well as tissue migration.

PBX1 loss results also in mouse embryonic pancreatic defects, which recapitulate abnormalities observed in mutants for PDX1 (Kim et al. 2002). In vitro PDX1, a non-HOX homeoprotein with critical roles in pancreas development, binds with PBX1 as heterodimers to promoters that direct expression of pancreatic-specific genes, such as somatostatin, insulin, and elastase (Arda et al. 2013). Furthermore, in exocrine cells, trancription of *elastase1* takes place via the formation of a trimeric complex consisting of PBX1/PDX1/MEIS2 (for review, see Cerdá-Esteban and Spagnoli 2014), which in turn activates gene expression by cooperating with the pancreatic TF PTF1a and the E protein HEB. Of note, it was reported that in transgenic mice, PDX1/PBX complexes are dispensable for glucose homeostasis and differentiation of ductal, endocrine, and acinar lineages; however, it was shown that they are essential for expansion of these populations during pancreatic development (Dutta et al. 2001). $Pbx1^{-/-}$ embryos exhibit hypoplastic pancreas (Fig. 5) with abnormalities in exocrine and endocrine cell differentiation. PBX1 regulates pancreatic cell fate by controlling *Isl1* and *Neurogenin3* as well as production of insulin and glucagon (Fig. 5). Moreover, compound $Pbx1^{+/-};Pdx1^{+/-}$ mice develop overt late-onset diabetes, unlike either single heterozygous mutant (Kim et al. 2002), demonstrating that *Pdx1* and *Pbx1* genetically interact in mice. This research underscores that cell lineage-specific activities of pancreatic TFs such as PDX1 depend at least in part on the availability of TALEs. Notably, partial overlap of *Pbx1* and *Pdx1* expression patterns in the embryonic and adult pancreas suggest that PBX1 has both PDX1-dependent and PDX1-independent pancreatic functions (Kim et al. 2002). In contrast, besides directing *Pax6* expression in the pancreas (Zhang et al. 2006), the function of MEIS proteins in the development of this organ are unknown in mice (Hisa et al. 2004; Azcoitia et al. 2005). However, in zebrafish, *meis3* acts upstream of *shh* to negatively regulate pancreatic fate (for review, see Cerdá-Esteban and Spagnoli 2014), and PREP1 controls insulin glucoregulatory function in the mouse liver (Oriente et al. 2011). Research in animal models is warranted to dissect the respective contributions of *Pbx* genes to the development of different pancreatic tissues, where *Pbx1–3* are all expressed (Kim et al. 2002; Selleri et al. 2004; Di Giacomo et al. 2006), and the underlying mechanisms. While sequence variants in human *PBX1* were identified in patients with type 2 diabetes (Duesing et al. 2008) and metabolic syndrome (Ban et al. 2008), the involvement of PBX1 in human pancreatic disease is still debated.

Given the association of dorsal pancreatic mesenchyme and spleen primordium in early development, it is not surprising that *Pbx1* LOF causes also splenic defects in mice. Constitutive $Pbx1^{-/-}$ embryos exhibit fully penetrant

asplenia (Brendolan et al. 2005, 2007), a phenotype that phenocopies spleen agenesis of *Tlx1*$^{-/-}$ mice (Roberts et al. 1994; Dear et al. 1995). *Tlx1* (also known as *Hox11*) encodes a nonclustered homeodomain protein that binds DNA, dimerizing with PBX1 via a conserved HX motif (Shen et al. 1996). While we demonstrated that *Tlx1* is a direct in vivo target of PBX1 in the murine spleen analage, the *Tlx1*-encoded protein in a complex with PBX1 concomitantly autoregulates its own gene promoter during spleen development (Brendolan et al. 2005). In addition, *Pbx1* and *Tlx1* genetically interact in spleen formation, since *Pbx1*$^{+/-}$;*Tlx1*$^{+/-}$ mice develop hypoplastic and dysmorphic spleens, compared with single heterozygotes, which have normal spleens. Splenic mesenchyme-specific inactivation of *Pbx1* does not cause asplenia or defects in splenic cell fate specification but results instead in abnormal morphogenesis and growth of the spleen, which is exacerbated in mutants also lacking *Pbx2*. This phenotype is due to cell-autonomous and direct transcriptional repression of *NKX2.5*, an effector of spleen organogenesis, and transactivation of *p15Ink4b*, a cell cycle inhibitor, in splenic mesenchymal cells. Strikingly, removal of *p15Ink4b* in *Pbx1* spleen-specific mutant embryos partially rescued organ growth (Koss et al. 2012). This research established a PBX–NKX2.5–p15 regulatory network that is required for mammalian spleen organogenesis and growth. PBX target genes within this network were critical to guide whole-exome sequencing analysis of a kindred with isolated congenital asplenia (ICA), a life-threatening birth defect due to pervasive bacterial infections. In affected individuals from this kindred, a heterozygous missense mutation was identified in the PBX target gene *NKX2.5* (Koss et al. 2012). It was subsequently reported that ~50% of ICA patients exhibit mutations of *RPSA*, the gene encoding ribosomal protein SA (Bolze et al. 2013, 2018). Of note, ICA patients bearing the *NKX2.5* mutation described above also carried a mutation in *RPSA*, suggesting that the two mutations may act synergistically in the causation of asplenia.

PBX functions in adrenal and urogenital development

Pbx1$^{-/-}$ embryos lack adrenal glands as a result of reduced cellular proliferation of adrenogenital precursors and genital ridges (Schnabel et al. 2001, 2003b; Zubair et al. 2006), consistent with decreased progenitor cell proliferation in other organs (Selleri et al. 2001; Kim et al. 2002; Manley et al. 2004; Brendolan et al. 2005; Koss et al. 2012). These mutants also exhibit mispositioned and hypoplastic kidneys or unilateral renal agenesis (Schnabel et al. 2003a). Of note, most patients with *PBX1* mutations also exhibit urogenital defects (Slavotinek et al. 2017). In mice, *Pbx1* is expressed in renal vascular mural cell (VMC) progenitors prior to their up-regulation of VMC markers. Renal

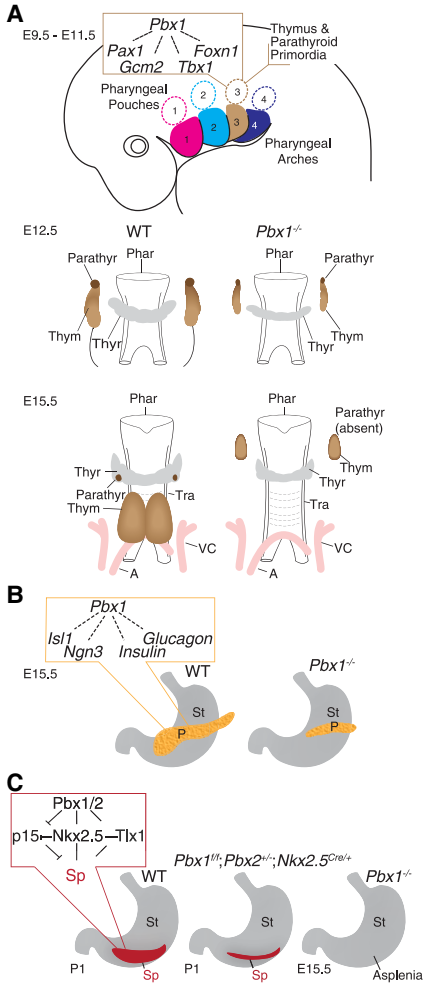

Figure 5. PBX proteins direct organogenesis of the thymus, pancreas, and spleen by regulation of effectors of cell fate specification, cell differentiation, and cell cycle progression. (*A*, *top*) Sketch of E9.5–E11.5 mouse pharyngeal (or branchial) arches (BAs) 1–4 (colored outpocketings) and pouches between BAs (dashed circles). Expression of markers for the third pharyngeal pouch, which gives rise to the thymus (Thym; light brown) and parathyroids (Parathyr; dark brown), is controlled by a *Pbx*-directed genetic network (dashed arrows in brown box). (*Middle*) E12.5 *Pbx1*$^{-/-}$ embryos (*right*) exhibit reduced thymus and parathyroid primordia versus wild type (*left*), with hypoplastic thyroid (Thyr; gray). In wild type, arrows indicate thymic lobe descent into mediastinum. (*Bottom*) In E15.5 *Pbx1*$^{-/-}$ embryos, hypoplastic thymic lobes rostral to the thyroid do not descend; parathyroids are absent, and the thyroid is hypoplastic (Manley et al. 2004). (*B*) *Pbx1* controls expression of *Isl1* and *Ngn3* as well as insulin and glucagon, in pancreatic development (genetic network in yellow box). E15.5 *Pbx1*$^{-/-}$ embryos (*right*) exhibit hypoplastic pancreas versus wild type (*left*) (Kim et al. 2002). (*C*) Mouse neonates (P1) with Nkx2.5-specific *Pbx1* loss in spleen progenitors on a *Pbx2*-deficient background (*Pbx1*$^{f/f}$;*Pbx2*$^{+/-}$;*Nkx2.5*$^{Cre/+}$; *middle*) show splenic hypoplasia versus wild-type (*left*). (*Right*) E15.5 *Pbx1*$^{-/-}$ embryos lack the spleen (asplenia). PBX1/2–TLX1–NKX2.5–p15 regulatory module controlling splenic fate specification, morphogenesis, and expansion (in red box) (Koss et al. 2012). (Pointed arrows) Transcriptional activation; (blunted arrowheads) transcriptional repression; (solid line) NKX2.5 and TLX1 act cooperatively to transactivate target genes in spleen mesenchyme; (A) aorta; (P) pancreas; (Phar) pharynx; (Sp) spleen; (St) stomach; (Tra) trachea; (VC) vena cava.

VMC-specific *Pbx1* LOF revealed that this TF governs the architecture of the kidney arterial tree by direct transcriptional repression of *PDGFR*β, a master initiator of VMC–endothelial association and vessel maturation (Hurtado et al. 2015). Similar to the reported roles of PBX TFs as regulators of lung vascular smooth muscle cells (McCulley et al. 2018), PBX1 plays critical functions also in kidney VMC progenitors. Together, these findings support a key requirement for PBX factors in patterning and maturation of the mesenchymal progenitors that will give rise to smooth muscle cells and pericytes, which surround the endothelial tubes and have vital functions for organ vascular development and stability.

PBX wardens safeguard normal hematopoiesis, embryonic stem cell (ESC) pluripotency, and tissue regeneration

PBX roles in hematopoieisis

Multiple regulatory proteins that control hematopoiesis (for reviews, see Seita and Weissman 2010; Costa et al. 2012; Nakamura-Ishizu et al. 2014) are TFs that were discovered as the products of proto-oncogenes targeted by chromosomal aberrations in hematologic malignancies (for review, see Cleary 1991). Among these TFs is PBX1. Constitutive *Pbx1* LOF resulted in impaired production of common myeloid progenitors (CMPs) (DiMartino et al. 2001) and common lymphoid progenitors (CLPs) as well as perturbed B-cell commitment (Fig. 6; Sanyal et al. 2007). Conditional inactivation of *Pbx1* in Tie2-positive compartments (i.e., in hematopoietic stem cells [HSCs] and endothelial cells) caused loss of long-term repopulating HSCs (LT-HSCs), quiescent residents of the bone marrow with the potential for long-term engraftment and clonal expansion (for review, see Nakamura-Ishizu et al. 2014). In *Pbx1* conditional mutants, LT-HSCs inappropriately entered the cell cycle, initiated differentiation, and progressively exhausted themselves (Fig. 6; Ficara et al. 2008), demonstrating that PBX1 acts as a positive regulator of HSC quiescence. In the absence of PBX1, multipotent progenitor (MPP) and CMP pools were reduced due to premature maturation (Ficara et al. 2013), and, concomitantly, PBX1-deficient CMPs aberrantly expressed B-lymphoid and HSC markers. In addition to their intrinsic roles in HSCs, PBX factors can also effect non-cell-autonomous functions within the splenic mesenchymal niche that contribute to the control of extramedullary hematopoiesis partly via the control of KitL/SCF and Cxcl12/SDF-1 (Zewdu et al. 2016). PBX1 further governs macrophage functions by transcriptional activation of *Interleukin-10* (*IL-10*) in a complex with PREP1/MEIS1 (Chung et al. 2007). MEIS1 and PREP1 are also critical regulators of murine and zebrafish hematopoiesis (Hisa et al. 2004; Azcoitia et al. 2005; Di Rosa et al. 2007; Pillay et al. 2010), pointing to conserved roles of TALEs in hematopoiesis during vertebrate evolution. In fish, *pbx* genes control *gata1*, an essential gene for erythrocyte development (Fig. 6). Of note, HOX proteins execute similar functions in hematopoiesis (for reviews, see Lawrence et al. 1996; Rawat et al. 2012). For example, mice with *Hoxa9* homozygous LOF exhibit reduced numbers of granulocytes and lymphocytes as well as committed progenitors, with smaller spleens and thymuses (Lawrence et al. 1997). In contrast, the *Hoxb* cluster genes normally expressed in c-Kit E14.5 fetal liver cells are dispensable for hematopoiesis (Bijl et al. 2006). However, in *Hoxb* mutant fetal livers, *Hoxa* and *Hoxc* genes exhibited substantial changes in expression levels, indicating the existence of complex cross-regulatory interactions and compensatory mechanisms within *Hox* clusters in the control of hematopoiesis. Overall, while it is established that PBX and other TALE and HOX proteins have critical roles in vertebrate hematopoiesis, we lack knowledge of the target genes, cross-

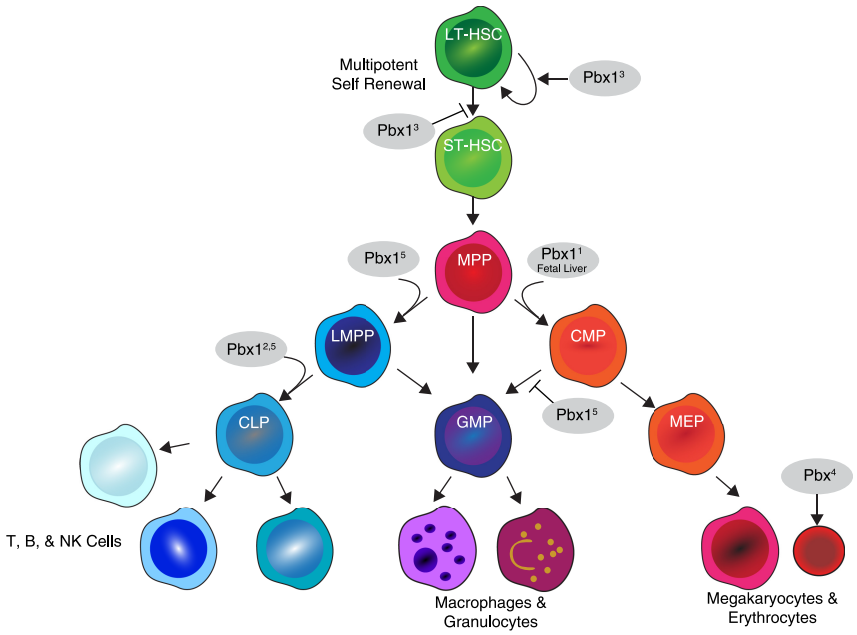

Figure 6. From hematopoietic stem cells to mature blood cells: pleiotropic roles of PBX homeoproteins. *Pbx* genes (within gray ovals) contribute to various steps of blood cell development. The superscript numbers indicate references. (1) DiMartino et al. (2001); (2) Sanyal et al. (2007); (3) Ficara et al. (2008); (4) Pillay et al. (2010); (5) Ficara et al. (2013). (Pointed arrows) Positive effects; (blunted arrowheads) repressive roles; (GMP) granulocyte macrophage progenitor; (LMPP) lymphoid-primed multipotent progenitor; (MEP) megakaryocyte erythrocyte progenitor; (ST-HSC) short-term repopulating HSC.

regulatory networks, and molecular mechanisms underlying potential TALE–HOX interactions in this process.

PBX functions in ESC pluripotency and regeneration

PBX proteins are present in both human and mouse ESCs (hESCs and mESCs, respectively) (Gemel et al. 1999; Chan et al. 2009; Jürgens et al. 2009). In ESCs, PBX TFs must have HOX-independent roles, since *Hox* genes are expressed only later in development. Maintenance of pluripotency and self-renewal is guaranteed by a set of TFs, including NANOG, OCT4, and SOX2, which activate pluripotency genes and repress differentiation genes (for review, see Cerdá-Esteban and Spagnoli 2014). In hESCs, PBX1 and KLF4 synergistically bind the *NANOG* promoter and regulate its expression in cooperation with OCT4 and SOX2 (Chan et al. 2009; Bjerke et al. 2011). Furthermore, in EBs PBX1 and Engrailed together regulate expression of *Fgf8* (Gemel et al. 1999), which executes crucial roles in ESC pluripotency and differentiation together with *Wnt* genes (Villegas et al. 2010; Sokol 2011). It is not known whether PBX1 can direct lineage commitment and cell type-specific differentiation. While RA treatment of *Pbx1*-null EBs suggested that PBX1 is dispensable for neuronal differentiation (Jürgens et al. 2009), it was proposed that PBX1 directs adipocyte lineage commitment (Monteiro et al. 2011). Polymorphisms within *PBX1* were associated with obesity (Ban et al. 2008), corroborating a possible involvement of PBX1 in adipogenesis. As we discussed, PBX1 is essential for preventing precocious differentiation of progenitors (Selleri et al. 2001; Gordon et al. 2010; Hurtado et al. 2015), and, similarly, *Pbx1* loss in HSCs causes impaired self-renewal and premature cell differentiation (Ficara et al. 2008, 2013). It is of note that *Pbx1* and *Pbx2* are highly expressed in mESCs and hESCs. In addition, mouse *Pbx1/Pbx2* and *Pbx1/Pbx3* double homozygous mutant embryos die in utero before E7.0 on C57bl6 background (E Ferretti, TD Capellini, and L Selleri, unpubl.). While these results together point to critical collaborative roles of PBX TFs at earliest developmental stages, likely in stem and pluripotent cells, our knowledge of the underlying mechanisms is sorely lacking.

PBX factors promote cell proliferation and tissue growth in multiple embryonic organs (DiMartino et al. 2001; Selleri et al. 2001; Manley et al. 2004; Ficara et al. 2008; Koss et al. 2012). However, it is unknown whether they also control vertebrate tissue regeneration (Nacu and Tanaka 2011; Tanaka 2016). In planaria, metazoans that have the capacity to replace missing structures, regeneration occurs employing neoblasts, adult somatic stem cells that express *pbx*. In neoblasts, *pbx* is required for the expression of polarized markers that control head and tail regeneration (Blassberg et al. 2013; Chen et al. 2013). The planarian ortholog *prep* is also essential for anterior pole regeneration (Felix and Aboobaker 2010), suggesting that in planaria, pbx acts together with prep in this process. Additionally, in salamanders, vertebrates that are also able to regenerate body parts, *Meis* overexpression during limb regeneration relocates distal blastema cells proximally (for review, see Capellini et al. 2011b). It is tempting to speculate that PBX–MEIS proteins may control the molecular events that restore positional identity and that mediate regeneration of body parts.

Concluding remarks and perspectives

Here we reviewed the overlapping, hierarchical, and iterative functions of PBX TALE proteins within regulatory networks that guide morphogenesis of different tissues and organs during vertebrate development. PBX-directed control of target genes and regulatory networks has critical bearings on the morphogenesis of most, if not all, vertebrate tissues and organs. However, while we have acquired knowledge of the roles of PBX factors in mid and late murine gestation, little is known of their collaborative functions in stem and pluripotent cells during early development. Available literature based on primary findings from our and other groups supports the concept that in select vertebrate developmental processes, such as patterning of BA2 as well as posterior BAs and pouches in the mouse embryo, PBX proteins act as HOX cofactors in vivo, a role that was long emphasized based on studies conducted in vitro and in *Drosophila*. In addition, we uncovered novel roles for PBX TFs in the activation of the *Hox* gene cascade during HB segmentation, whereby PBX homeoproteins cooperate with select HOX factors in the mesoderm via a RA signal from the mesoderm to the HB. We also reported that PBX TFs may execute prime functions in the upstream genetic control of *5′ HoxA/D* expression in the limb. We further established that PBX proteins can function independently of HOX, partnering with other cofactors in the morphogenesis of the *Hox*-less midface. Last, it was suggested that PBX proteins can act as "pioneer factors" that recognize target binding sites in compacted chromatin, thus increasing DNA access to other TFs and poising specific loci for transcriptional activation or repression (for review, see Grebbin and Schulte 2017). However, caution should be used before attributing general and broad pioneer factor roles to PBX TFs. Additional mechanistic studies based on stringent and comprehensive criteria are needed to unequivocally clarify these potential functions.

PBX TFs regulate the transcription of critical developmental effectors, including morphogen-encoding genes such as *Shh* in the limb mesoderm and genes that encode WNT^{Can} signaling components in the *Hox*-less midface. They act pleiotropically and direct both TF-encoding genes, such as *Nkx2.5*, and cell cycle genes, such as *p15Ink4b*, in spleen mesenchymal progenitors. In multiple organs, PBX proteins also control cell number and tissue growth by directing the expression of proliferation and/or apoptosis genes in different cell populations. The PBX family therefore constitutes a linchpin of regulatory interactions in the embryo, at the top of multiple cell fate hierarchies. In the embryo, PBX1–3 proteins colocalize with different TALE partners in a tissue-specific manner. As a result, a tight PBX-dependent regulation of individual target genes and gene networks requires the formation of different context-specific combinatorial

complexes that guide distinct developmental programs, a process poorly understood. As high-throughput technology (for reviews, see Spitz and Furlong 2012; Villar et al. 2014; Denker and de Laat 2016), live imaging (for reviews, see Saiz et al. 2015; de Medeiros et al. 2016), single-cell transcriptomics (see Sahakyan and Plath 2016; Regev et al. 2017), and system-level approaches (Du et al. 2014; Regev et al. 2017) are brought to bear, PBX-dependent networks and molecular circuitries will be illuminated in specific morphogenetic contexts and in different cell types at different developmental stages. Ultimately, the availability of encyclopedias of regulatory elements (Vierstra et al. 2014; Yue et al. 2014) in the genome of different species, together with tissue-specific and temporally controllable LOF animal models to guide functional studies in vivo, will enable a deeper understanding of how various organisms use combinations of regulatory factors and pathways to assume all of the beautiful forms that they display.

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