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## Control of Pelvic Girdle Development by Genes of the Pbx Family and *Emx2*

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### Abstract

Genes expressed in the somatopleuric mesoderm, the embryonic domain giving rise to the vertebrate pelvis, appear important for pelvic girdle formation. Among such genes, Pbx family members and *Emx2* were found to genetically interact in hindlimb and pectoral girdle formation. Here, we generated compound mutant embryos carrying combinations of mutated alleles for *Pbx1*, *Pbx2*, and *Pbx3*, as well as *Pbx1* and *Emx2*, to examine potential genetic interactions during pelvic development. Indeed, Pbx genes share overlapping functions and *Pbx1* and *Emx2* genetically interact in pelvic formation. We show that in compound *Pbx1;Pbx2* and *Pbx1;Emx2* mutants, pelvic mesenchymal condensation is markedly perturbed, indicative of an upstream control by these homeoproteins. We establish that expression of *Tbx15*, *Prrx1*, and *Pax1*, among other genes involved in the specification and development of select pelvic structures, is altered in our compound mutants. Lastly, we identify potential Pbx1-Emx2-regulated enhancers for *Tbx15*, *Prrx1*, and *Pax1*, using bioinformatics analyses.

### Introduction

In most mammalian tetrapods, the pectoral and pelvic girdles are crucial proximal limb structures involved in weight bearing and propulsion during locomotion (Romer and Parsons, 1986). The pelvic girdle consists of three segments. Ventrally, there are two pelvic bones, each of which comprises of a rostrally facing ilium, a ventrally oriented pubis, and a dorso-laterally positioned ischium (Fig. 1, representation of the hindlimb (HL) axes within red inset; Fig. 2). Dorsally, there is the sacrum, which in different mammal species bears a variable number of vertebrae. In the pelvic girdle, the two pubic elements articulate ventrally

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at the pubic symphysis, while each ilium (or iliac blade) articulates dorsally at the sacroiliac joint with the sacral vertebrae. The three main bones of the pelvis also form a laterally facing socket called the acetabulum, whereby the head of the femur articulates (Romer and Parsons, 1986).

While the girdles have been the subjects of anatomical (Romer and Parsons, 1986), functional (Young, 2006), and evolutionary (Sanchez-Villagra and Maier, 2002) studies, their embryological derivation and underlying genetic networks have largely been neglected. To date, research conducted mainly in the chick (Huang et al., 2006) but also in the mouse (Pomikal and Streicher, 2010; Valasek et al., 2010), has partially elucidated the tissue origins for each girdle. Notably, the scapula has been shown to develop from multiple tissues, such as the dermomyotome of the somites, the mesodermal portion of the somatopleure (a domain of the lateral plate mesoderm or LPM) and neural crest cell-derived mesenchyme (Huang et al., 2000; Matsuoka et al., 2005; Wang et al., 2005). However, only the mesodermal portion of the somatopleure has been shown to give rise to each pelvic bone (Malashichev et al., 2008; Pomikal and Streicher, 2010), while sclerotome gives rise to sacral vertebrae (Christ et al., 2000).

Only a sparse number of genes have been identified so far that control girdle formation (Niswander, 2003; Huang et al., 2006). Specifically, more is known of the genetic pathways that govern the development of the scapular blade, head, and neck *versus* the pelvic girdle (Kuijper et al., 2005; Capellini et al., 2010). Interestingly, of the genes involved in scapular blade morphogenesis few have only minor roles in pelvic development. For example, *Tbx15* regulates the murine scapular blade, through its genetic interactions with *Gli3*, but does not singularly or cooperatively control the formation of the pelvic girdle (Kuijper et al., 2005). However, in humans, mutations in *TBX15* do produce pelvic iliac defects, suggesting potential differences in the genetic control of girdle development across species (Lausch et al., 2008). Notably, the genetic interactions identified between *Aristaless* family genes for the formation of the scapular blade were also identified in the pelvis. Specifically, compound *Alx4;Alx1* mutants exhibit a reduction of the superior scapular blade and the loss of the anterior pubic ramus (also somewhat evident in single *Alx4* homozygous mutants and *Alx3;Alx4* double homozygous mice) (Kuijper et al., 2005). These pelvic defects, however, are not exacerbated by the compound loss of *Tbx15* (Kuijper et al., 2005).

One emergent finding is that the somatopleuric mesoderm constitutes a domain that is important to both pectoral and pelvic girdle development (Malashichev et al., 2008; Pomikal and Streicher, 2010), as it is specifically involved in progenitor cell fate specification, patterning, and condensation formation (Kuijper et al., 2005; Capellini et al., 2010). Not surprisingly, of the genes required for pelvic development in the mouse, most are expressed in this specific domain. Compound null mutations in *Prrx1* and *Prrx2* (ten Berge et al., 1998), genes of the *Aristaless* family that are expressed in the mesodermal portion of the somatopleure, result in the loss of the pubic symphysis. Likewise, levels of *Twist1* activity in the somatopleure and LPM are known to control pubis formation (Krawchuk et al., 2010). Furthermore, ilium reduction is observed when *Pitx1* or *Fgf10* are lost from the early HL bud LPM and somatopleure, as well as when *Tbx4* is conditionally inactivated from the proximal HL LPM (Lanctot et al., 1999; Sekine et al., 1999; Naiche and Papaioannou, 2007).

*Emx2*<sup>-/-</sup> and *Pbx1*<sup>-/-</sup> embryos exhibit pelvic defects, as both have reduced ilia that are detached from the pubis and ischium (Pellegrini et al., 2001; Selleri et al., 2001). Previous studies have further implicated Pbx Three-Amino acid-Loop-Extension (TALE) homeoproteins as regulators of HL development (Selleri et al., 2001; Capellini et al., 2006). Lastly, *Emx2* and *Pbx1* were shown to interact genetically and molecularly in scapular blade

formation (Capellini et al., 2010) within the mesodermal portion of the somatopleure. Here, we demonstrate that *Pbx1* and *Pbx2* share overlapping functions in the somatopleure and LPM during pelvic development. Compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutant embryos, but not *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup> mutants, present exacerbations of the iliac defects of single *Pbx1*<sup>-/-</sup>, as well as novel caudal pelvic phenotypes absent from *Pbx1*<sup>-/-</sup> embryos (Capellini et al., 2006). Conversely, single loss of *Pbx2* or *Pbx3* does not cause pelvic phenotypes (Rhee et al., 2004; Selleri et al., 2004). We reveal that Pbx family members act as upstream regulators of multiple genes that are critical for ilium and pubis formation. Additionally, we demonstrate that in pelvic development *Pbx1* interacts with *Emx2*, albeit less markedly than in the pectoral girdle. Lastly, we independently reveal potential regulatory elements that are putative targets of Pbx1/Emx2 in the proximity of genes expressed and involved in pelvis formation, such as *Tbx15*, *Prrx1*, and *Pax1*. Of note, these latter genes are down-regulated in Pbx compound mutant embryos. Our results delineate previously unknown genetic networks in pelvic girdle formation.

## Materials and Methods

### Mice

Intercrosses between *Pbx1*<sup>+/-</sup> (Selleri et al., 2001), *Pbx2*<sup>+/-</sup> (Selleri et al., 2004), *Pbx3*<sup>+/-</sup> (Rhee et al., 2004) and *Emx2*<sup>+/-</sup> (Pellegrini et al., 2001) mice were performed to obtain *Pbx1*<sup>+/-</sup>;*Pbx2*<sup>+/-</sup>, *Pbx1*<sup>+/-</sup>;*Pbx3*<sup>+/-</sup>, *Pbx2*<sup>+/-</sup>;*Pbx3*<sup>+/-</sup> and *Pbx1*<sup>+/-</sup>;*Emx2*<sup>+/-</sup> mutants. On a C57BL/6 background, the double heterozygous numbers obtained were below expected Mendelian ratios. To increase litter numbers and sizes, C57BL/6 double heterozygous males for each mutant line were crossed to a Black-Swiss strain [NIH-BL(S)]. Next, double heterozygous C57BL/6 females and mixed double heterozygous C57BL/6-Black-Swiss males were intercrossed and their progeny analyzed for skeletal phenotypes. On more out-crossed mixed genetic backgrounds, marked ameliorations of C57BL/6 pelvic skeletal and gene expression phenotypes were observed leading us to use only progeny from the first C57BL/6-to-Black-Swiss intercross in our in situ hybridization experiments. However, some mutants, obtained from parents that had been out-crossed to the Black Swiss strain more than once, were used in our analysis of skeletal phenotypes and are highlighted as such in the text. To generate more complex Pbx compound genotypes, *Pbx1*<sup>+/-</sup>;*Pbx2*<sup>+/-</sup> and *Pbx1*<sup>+/-</sup>;*Pbx3*<sup>+/-</sup> mutants were intercrossed to obtain triple *Pbx1*<sup>+/-</sup>;*Pbx2*<sup>+/-</sup>;*Pbx3*<sup>+/-</sup> mice. However, in utero lethality of Pbx triple heterozygotes prevented further intercrossing.

### Skeletal preparations

Skeletons of E13.5–E14.5 mouse embryos were harvested according to standard protocols following IUACC guidelines, then dehydrated in ethanol, treated next with acetone, stained with Alcian Blue/Alizarin Red S and cleared using KOH and glycerol (McLeod, 1980; Depew et al., 1999). At least four embryos per genotype were analyzed.

### Optical Projection Tomography

As described in detail (Sharpe et al., 2002; Sharpe, 2003), fixed specimens from skeletal preparation were washed in PBS and briefly rinsed with distilled H<sub>2</sub>O before being embedded in 1% LMP agarose in H<sub>2</sub>O. The agarose block was then trimmed to a pyramidal shape around the specimen, leaving about 5 mm on the base. The embedded specimens were dehydrated in 100% methanol (MetOH) (2×2–4 hours, 1× overnight) and cleared in BABB (1:2 mixture of Benzyl Alcohol and Benzyl Benzoate), overnight or until transparent, at room temperature and protected from light. The cleared specimens were glued by the base to a magnetic mount and placed into the OPT scanner. Light source and intensity, exposure, filters, position and focus were adjusted to maximize the dynamic range but avoiding

saturation. Four hundred tiff images of each specimen were captured during a 360-degree single axis rotation.

Using the OPT Recon software (NRecon v1.6.2.0 ©Skyscan 2009) to correct alignment and signal intensity thresholds, the individual images were then reconstructed to produce a 3D voxel data-set of the specimen, which could be visualized with both OPT Viewer and Imaris software (BiOPTonics © Viewer V.1.6.1 and Imaris ×64 6.3.1 ©Bitplane AG). From these data, using the Imaris software, iso-surface 3D representations of the specimens were produced.

### Section and whole mount in situ hybridization

For both section and whole mount in situ hybridization, harvested embryos were first somite-matched to control for developmental variations within each litter, then rinsed in PBS1X, fixed overnight at 4 C, and processed accordingly. As compound mutants from several distinct Pbx and Emx2 lines were acquired using multiple, different genetic crosses, each experiment was performed using specific littermate and somite-matched controls. For this reason, we have, for sake of scientific accuracy, displayed the proper controls for each experiment in the appropriate figures in this text. At least three mutant embryos per genotype per experimental assay were analyzed. For section in situ hybridization, embryos were embedded in Tissue Tek OCT compound, stored at -80 C, and sectioned at 10 μm. Section in situ hybridization was conducted as described (Di Giacomo et al., 2006). For whole mount in situ hybridization, embryos were passed through a methanol dehydration series, stored at -20 C until their use in in situ protocols (see (Selleri et al., 2001)). For both types of in situ hybridization experiments, digoxigenin-labeled RNA probes were generated as described (Di Giacomo et al., 2006) for the following probes: *Alx1* (Kuijper et al., 2005), *Alx4* (Beverdam and Meijlink, 2001), *Col2a* (Cheah et al., 1991), *Emx2* (Pellegrini et al., 2001), *Gli3* (Beverdam and Meijlink, 2001), *Pax1* (Chalepakis et al., 1991), *Pbx1* (Brendolan et al., 2005), *Pbx2* (Selleri et al., 2004), *Pbx3* (Di Giacomo et al., 2006), *Pitx1* (Lanctot et al., 1999), *Prrx1* (Beverdam and Meijlink, 2001), *Scx* (Brent and Tabin, 2004), *Sox9* (Wright et al., 1995), *Tbx15* (Kuijper et al., 2005) and *Twist1* (Krawchuk et al., 2010).

### Bioinformatic analysis

To assess the functionality of Emx2-Pbx1 binding events genome-wide by a bioinformatic approach, we utilized the Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010). GREAT evaluates sets of cis-regulatory elements by assigning each element to its likely target gene(s). The annotations for each gene are then associated with each cis-element and statistical enrichment is assessed using two complementary statistical tests, which account for biases in the distribution of genes across the genome. We entered into GREAT 2,826 conserved instances of the Emx2-Pbx1 dimer motif in the mouse genome (mm9). We used default input parameters and default output display settings available at the GREAT website (url: <http://great.stanford.edu/>) to identify “hindlimb morphogenesis” and “cell fate specification” as the most significantly enriched Gene Ontology (GO) terms.

## Results

### Pbx1, Pbx2, and Emx2 are expressed in the mesodermal portion of the somatopleure and proximal hindlimb

Pelvic girdle formation begins approximately at the time of initial HL bud outgrowth (in mouse at E9.5–10), when cells of the mesodermal portion of the somatopleure are specified as pelvic progenitors (Malashichev et al., 2005; Malashichev et al., 2008). This process leads to the formation of a single mesenchymal mass (at around E11.5) that undergoes

chondrogenesis (E12.5–E13.5) and osteogenesis (E14.5) to form each pelvic bone (Pomikal and Streicher, 2010). Previous studies established that *Pbx1*, *Pbx2*, and *Emx2* are expressed in mesodermal tissues that undergo endochondral ossification (Pellegrini et al., 2001; Selleri et al., 2001; Capellini et al., 2006; Capellini et al., 2010). Additionally, *Pbx1* and *Pbx2*, but not *Pbx3*, are co-expressed within the LPM of the posterior embryo together with *Emx2* during early HL bud outgrowth (Fig. 1A; Pellegrini et al., 2001; Capellini et al., 2006). However, once HL buds elongate, *Pbx1* remains localized proximally with *Emx2* in the pelvic field within the mesodermal portion of the somatopleure and LPM, while *Pbx2* becomes restricted distally (Fig. 1A; Capellini et al., 2006). Thus, at E9.5–10.5, *Pbx1* is localized to the proximal HL and mesodermal portion of the somatopleure and *Pbx2* is present in the distal HL bud, as revealed by immunohistochemistry (Fig. 1B; Capellini et al., 2006). At E11–11.5, while *Pbx1*, *Pbx2*, and *Emx2* maintain the expression patterns described, *Pbx3* transcripts emerge, albeit at low levels, in the mesodermal portion of the somatopleure (Fig. 1C). The expression patterns of *Pbx1*, *Pbx2*, and *Emx2*, which initially overlap in the mesodermal portion of the somatopleure and early HL bud, and then are partially co-expressed with *Pbx3* in the proximal HL bud after E11, suggest roles in pelvic girdle formation.

### **Pbx1 and Pbx2 genetically interact to regulate pelvic development**

We tested for cooperative roles of genes of the Pbx family in the patterning and morphogenesis of the pelvic girdle by generating *Pbx1*, *Pbx2*, and *Pbx3* single and compound mutant genotypes. Morphological analyses by skeletal preparation and optical projection tomography (OPT) of single *Pbx1*<sup>-/-</sup> embryos reveal: (1) absence of ilia, or presence of truncated and flattened ilia (Fig. 2A; red asterisk in red inset) that are separated from the remaining pelvis as single rudimentary elements (Fig. 2A, red inset); (2) presence of pubic and ischial structures that are slightly shortened rostral-caudally and detached from each other along their caudal rami (Fig. 2A); and (3) failure of the hip joint to form as the femoral head remains fused to the acetabulum (Fig. 2A, blue arrowhead in red inset). OPT of these skeletons at E13.5 (Fig. 2B) shows hypoplastic and detached ilia and pubic and ischial elements with some vestige of normal morphological identity and position.

The severity of these defects becomes more pronounced when a single allele of *Pbx2* is removed from a *Pbx1* null background. Of note, due to the lethality of double homozygous *Pbx1*;*Pbx2* embryos at E10 (Capellini et al., 2006), pelvic development could not be examined. In E13.5 *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> embryos, the only remaining pelvic element appears as a small rudiment that exhibits some apparent morphological and positional similarity to an ischium, as highlighted by OPT (Fig. 2B; Supp. Movie S1). When *Pbx1*;*Pbx2* double heterozygous mice (on a C57BL/6 genetic background) were crossed to an outbred Black Swiss strain more than once, a marked amelioration of the phenotype was observed in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> progeny with the appearance of a small skeletal element of uncertain identity, approximately 180 degrees opposite to the forming ischium (Fig. 2A, blue arrowhead in lower red inset). *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants also exhibit a truncated femur that is fused to a single remaining pelvic element. The malformations observed in these compound mutants were not observed in other, even complementary genotypes, such as *Pbx1*<sup>+/-</sup>;*Pbx2*<sup>+/-</sup>, *Pbx1*<sup>+/-</sup>;*Pbx2*<sup>-/-</sup> and *Pbx2*<sup>-/-</sup> embryos (Selleri et al., 2004), all of which exhibit normal pelvic girdles.

Similar experiments were performed to evaluate potential genetic interactions between *Pbx1* and *Pbx3*, as well as *Pbx2* and *Pbx3*, in pelvic development. No significant genetic interactions were identified in any of the obtained compound genotypes at E13.5. In *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup> embryos the abnormalities and hypoplasia of the pelvic structures and of the femur appeared to closely resemble the defects of single *Pbx1*<sup>-/-</sup> mutants (Fig. 2A and red inset), as clearly revealed by OPT (Fig. 2B; Supp. Movie S2). It was also apparent that



in *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup> embryos, HL skeletal elements distal to the femur (which is thicker, shorter, and dysmorphic as in single *Pbx1*<sup>-/-</sup> mutants) appear normal (Fig. 2A). Since double homozygous *Pbx1*;*Pbx3* and *Pbx2*;*Pbx3* embryos die early in gestation, prior to HL development, pelvic development could not be assessed. In all other double compound genotypes (i.e., *Pbx1*<sup>+/-</sup>;*Pbx3*<sup>+/-</sup>, *Pbx1*<sup>+/-</sup>;*Pbx3*<sup>-/-</sup>, *Pbx2*<sup>+/-</sup>;*Pbx3*<sup>+/-</sup>, *Pbx2*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup>, and *Pbx2*<sup>+/-</sup>;*Pbx3*<sup>-/-</sup>) as well as triple (i.e., *Pbx1*<sup>+/-</sup>;*Pbx2*<sup>+/-</sup>;*Pbx3*<sup>+/-</sup>) mutants, no pelvic phenotype was detected (data not shown).

### **Pbx1;Emx2 compound mutants exhibit abnormal pelvic skeletal structures**

Given the overlapping expression patterns of *Pbx1* and *Emx2* in the mesodermal portion of the somatopleure and proximal HL, as well as their reported genetic and molecular interactions in scapular development (Capellini et al., 2010), pelvic structures were assessed in single and compound *Pbx1* and *Emx2* mutants.

As for single *Emx2* mutants, it was confirmed that at E14.5 *Emx2*<sup>+/-</sup> heterozygotes lack skeletal defects (Fig. 3A) (Pellegrini et al., 2001) and *Emx2*<sup>-/-</sup> homozygotes exhibit either absent or reduced iliac blades that are separated from the caudal pelvic elements (Fig. 3A). This pelvic phenotype only partially resembles that of single *Pbx1*<sup>-/-</sup> mutants in the iliac defects, because *Pbx1*<sup>-/-</sup> mutants have additional abnormalities caudally (Fig. 3A). *Pbx1*<sup>+/-</sup>;*Emx2*<sup>+/-</sup> compound mutants, which displayed modest alterations in the scapular blade (Capellini et al., 2010), did not exhibit pelvic phenotypes (data not shown). Additionally, in *Pbx1*<sup>+/-</sup>;*Emx2*<sup>-/-</sup> embryos, the loss of one allele of *Pbx1* on an *Emx2* null background did not lead to either exacerbated or novel pelvic phenotypes compared to those observed in single *Emx2*<sup>-/-</sup> mutants (Fig. 3A). Indeed, in these compound mutants the ilium is reduced and detached from the other pelvic elements, while the pubis and ischium remain intact.

Similar to the situation described for *Pbx2*, the reduction of functional *Emx2* alleles on a *Pbx1* null background leads to more severe pelvic malformations. At E14.5, *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> mutants resemble single *Pbx1*<sup>-/-</sup> mutants in that all three pelvic elements are reduced; the ilium absent or detached; and the pubis and ischium partially truncated (Fig. 3A–B; Supp. Movie S3). However, compound *Pbx1*<sup>-/-</sup>;*Emx2*<sup>-/-</sup> mutants exhibit a more pronounced phenotype (Fig. 3A–B), with a single well-formed skeletal element, partly reminiscent of an ischium, and a smaller pubic rudiment (Fig. 3A, red arrow). OPT reveals a truncated, rounded pubis (Fig. 3B, red arrowheads; Supp. Movie S4). Also, the pelvis is fused to a truncated femur (Fig. 3B). Due to the lethality of compound *Pbx1*;*Emx2* mutants and also *Pbx1*;*Pbx2* mutants, it was not possible to produce triple *Pbx1*;*Pbx2*;*Emx2* mutants to study their pelvic morphogenesis.

### **Genes of the Pbx family control Emx2 expression in the pelvic field**

To understand the hierarchy in the control of pelvic development by genes of the Pbx family and *Emx2*, *Pbx1*, *Pbx2*, and *Pbx3* expression was assessed in *Emx2*-deficient embryos, and *Emx2* expression was evaluated in compound mutants deficient for different Pbx genes (Fig. 4). Gene expression was examined by whole mount *in situ* hybridization from E10 to E11.5, although only select gestational days are displayed here. In E10.5 *Emx2*<sup>-/-</sup> embryos, the only detectable down-regulation of Pbx genes was observed for *Pbx1*, whose mRNA was slightly reduced in the proximal-to-distal aspect of the HL bud (Fig. 4A). *Pbx1* expression, however, persisted along the anterior-posterior boundary of the proximal flank (Fig. 4A). This modest spatial alteration of *Pbx1* expression was not observed later at E11.5 (data not shown). In contrast, in both single *Pbx1*<sup>-/-</sup> mutants and in compound *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup> mutants, *Emx2* expression was reduced in the proximal flank and HL field (Fig. 4B). In *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, *Emx2* expression was substantially down-regulated and nearly

absent from domains of the proximal HL flank from E10 to E11.5 (Fig. 4B and data not shown), while being unperturbed in the more rostral flank domains (Fig. 4B).

### Genes of the *Pbx* family and *Emx2* act upstream of pelvic mesenchymal condensation and early skeletogenesis

Given the observed skeletal defects in single *Pbx1* and *Emx2*, as well as in select *Pbx* compound mutants and *Pbx1;Emx2* mutants, gene expression analysis of mesenchymal markers was performed using whole mount *in situ* hybridization. For simplicity, hereafter, only those mutants that exhibit detectable changes in gene expression will be shown, while all other genotypes will be left for supplementary figures, or discussed as data not shown. Mesenchymal condensation and cartilage differentiation were evaluated by gene expression of *Sox9* and *Col2a*, markers for both of these processes, respectively (Cheah et al., 1991; Wright et al., 1995). From E11–E12, *Sox9* was reduced in single *Pbx1* and *Emx2* homozygous mutants (Fig. 5A). Likewise, *Sox9* expression was down-regulated along the flank and proximal HL of *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup>, *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup>, *Pbx1*<sup>+/-</sup>;*Emx2*<sup>-/-</sup>, and *Pbx1*<sup>-/-</sup>;*Emx2*<sup>-/-</sup> mutants (Fig. 5A; data not shown). Additionally, at E12, *Col2a* expression was unchanged or slightly reduced in most of these mutants compared to WT (data not shown). Intriguingly, in *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> mutants, *Col2a* persisted along the flank and proximal HL, albeit slightly up-regulated. As expected, in complementary genotypes for these mutants, along with *Pbx1*<sup>+/-</sup>;*Pbx2*<sup>-/-</sup> embryos, *Sox9* and *Col2a* were unchanged (data not shown). In E12 compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, which exhibit the most severe pelvic phenotypes, *Sox9* was nearly absent from the pelvic field (Fig. 5A). A substantial down-regulation of *Col2a* at E12 was also detected in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (Fig. 5B). These latter findings indicate that the pelvic abnormalities of *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants may likely result from earlier perturbations of pelvic progenitor cell specification and patterning.

### Pubis, ilium, and ischium marker gene expression is disrupted in *Pbx* compound mutants and in *Pbx1;Emx2* mutants

Unlike for pectoral girdle development (Huang et al., 2006), it remains mostly unclear when and in which tissues the pelvic girdle is specified. For these reasons, we initially examined the expression of numerous genes whose individual or compound loss affects the formation of the entire pelvis or of one of its components. We performed these analyses at murine embryonic stages E10–E11.5, when specification of the pelvic girdle is thought to occur based on comparable studies in chick (Malashichev et al., 2005; Malashichev et al., 2008). The genes analyzed by *in situ* hybridization comprised those whose loss most affects the pubis (*Alx4*, *Alx1*, *Twist1*) (Kuijper et al., 2005; Krawchuk et al., 2010); the pubic symphysis (*Prrx1*) (ten Berge et al., 1998); or the ilium (i.e., *Pbx1*, *Emx2*, *Pitx1*) (Lanctot et al., 1999; Pellegrini et al., 2001; Selleri et al., 2001). No known gene has yet been reported to specify uniquely the ischium (i.e., no single or compound homozygous mouse mutant exhibits a specific ischial reduction), although *Pax1* is expressed in the early pelvic HL field and in the ischium cartilaginous rudiment (Timmons et al., 1994; LeClair et al., 1999).

In E10 and E11.5 HL of all *Pbx1;Pbx2*, *Pbx1;Pbx3*, and *Pbx1;Emx2* mutants, expression of *Alx4*, a gene whose loss results in pubic reduction (Kuijper et al., 2005), was unperturbed versus normal littermates (Fig. 6A, Supp. Fig. S1A, and data not shown). Next, expression of *Alx1* (formerly *Cart1*), which aids in the specification of the pubis (Kuijper et al., 2005), was unperturbed in all compound mutants listed above, except in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> embryos, in which it was lost from the ventral proximal somatopleure (Fig. 6B). *Twist1*, which contributes to pubic formation (Krawchuk et al., 2010), was found down-regulated only in the proximal posterior HL of E10 *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> embryos (Fig. 6C). Lastly, expression of *Prrx1* (ten Berge et al., 1998) was reduced at E10.5 (Fig. 6D) and E11.5 (Fig. 6E; Supp. Fig. S1B) in both the dorsal and ventral domains of the proximal mesodermal portion of the

somatopleure in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, while it remained unperturbed in *Pbx1*<sup>-/-</sup>;*Emx2*<sup>-/-</sup> embryos (Fig. 6E; Supp. Fig. S1B).

We also examined expression of ilium markers such as *Pbx1*, *Emx2*, and *Pitx1* in our mutants (Lancot et al., 1999; Pellegrini et al., 2001; Selleri et al., 2001). As mentioned, we observed a significant reduction of *Emx2* expression in select Pbx compound mutants and only modest changes of *Pbx1* expression in single *Emx2* homozygous mutants (Fig. 4). In compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants only, and not in *Pbx1*;*Emx2* genotypes, *Pitx1* expression was significantly reduced in the pre-pelvic and proximal HL field (Fig. 7A). We also detected *Pitx1* down-regulation at later stages in *Pbx1*;*Pbx2* mutants with the most severe pelvic phenotypes, but not in all other genotypes (Fig. 7A and Supp. Fig. S1C).

We next assessed whether overall posterior proximal HL development is markedly altered prior to limb outgrowth in compound mutants for different Pbx genes and in *Pbx1*;*Emx2* mutants. *Tbx15*, a marker of pre-skeletogenic mesenchyme in the early mouse limb (Singh et al., 2005), with known roles in human pelvic defects (Lausch et al., 2008), was expanded posteriorly in E10.5 HL buds of all compound mutants bearing two *Pbx1* mutant alleles (Fig. 7B). Additionally, in compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> HL buds, *Tbx15* expression was substantially reduced (Fig. 7B). Later, at E11.5, a posterior expansion was also observed in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> embryos, while no changes of *Tbx15* expression were observed in other mutant genotypes compared to controls (Supp. Fig. S2A). The degree of abnormal specification and cellular disorganization in pelvic domains of single *Pbx1*<sup>-/-</sup> and compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants was further highlighted by the posteriorization of transcripts for *Scleraxis* (*Scx*), a tendon marker (Perez et al., 2003) in E10–11.5 HL buds (Fig. 7B; Supp. Fig. S2B). Abnormal *Scx* expression was not detected in other mutants (Supp. Fig. S2B).

Lastly, *Pax1*, a marker for ischium condensation and pelvic domains (Timmons et al., 1994; LeClair et al., 1999), was down-regulated in E11.5 single *Pbx1*<sup>-/-</sup> embryos, as well as in other mutants lacking both *Pbx1* alleles, such as *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup>, *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> and *Pbx1*<sup>-/-</sup>;*Emx2*<sup>-/-</sup> embryos (Fig. 7C; Supp. Fig. S3; and data not shown). In *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, *Pax1* was completely absent from the proximal HL bud (Fig. 7C). Importantly, no detectable down-regulation of *Pax1* was observed in any complementary genotypes, including single *Emx2*<sup>-/-</sup> mutants (Supp. Fig. S3).

### Identification by bioinformatic GREAT analysis of Emx2-Pbx1 TF binding site enrichments in conserved, non-coding regions of the mouse genome

Given the remarkable phenotypes of Pbx compound mutants and *Pbx1*;*Emx2* mutants, we analyzed whether *Pbx1* and *Emx2* can regulate, as heterodimers, genes involved in pelvic and proximal HL development. We previously determined the optimal DNA-binding consensus sequence of the *Emx2*-*Pbx1* heterodimer (EP site; Capellini et al., 2010) and identified *in vivo* an *Emx2*-*Pbx1* binding site within the *Alx1* locus.

Here, based on those sequences, we generated a position weight matrix (PWM) (Supp. Fig. S4) to search for predicted *Emx2*-*Pbx1* binding sites across a genome-wide multiple alignment of 32 eutherian mammals. By this approach, we identified 2,826 conserved *Emx2*-*Pbx1* heterodimer binding sites in the mm9 version (July/2007) of the mouse genome (data not shown). We next used the Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010) to assess functional enrichments of these predicted binding sites within the mouse genome (see Materials and Methods).

The top hits obtained from the analysis by GREAT were further examined for their Gene Ontology (GO) Biological Process annotations, revealing that our predicted *Emx2*-*Pbx1*



binding sites are enriched near genes involved in “HL morphogenesis” (Table 1). Indeed, of the 33 genes annotated by GO as “HL morphogenesis” genes, 16 have instances of the Emx2-Pbx1 binding motif in their regulatory domains. Significant enrichment was also observed for sites present near genes involved in cell-fate specification (Table 1), according to GO.

### **In silico identification of potential Emx2-Pbx1 regulated enhancers near MGI-annotated genes involved in pelvic development**

We next examined all of the conserved genome-wide Emx2-Pbx1 predictions to verify their occurrence near genes expressed and/or involved in pelvic development. To accomplish this goal, we extracted from the MGI database (<http://www.informatics.jax.org/>) all genes whose expression or phenotype matched the search term “pelvic girdle”. We then inspected approximately 200 Kb upstream and downstream of the transcriptional start site (TSS) of these genes for the presence of highly conserved Emx2-Pbx1 heterodimeric binding sites, using the UCSC genome browser (Kent et al., 2002). Of the 2,826 predicted sites, we found 53 sites located near 43 “pelvic girdle” genes (Table 2). These findings indicate that the identified conserved regions may indeed represent functional Emx2-Pbx1 regulatory elements for the nearby genes. Two specific examples are shown in Figure 8. It is of note that one site, approximately 5 Kb upstream of *Tbx15*, overlaps with a previously published binding site for the p300 co-activator identified through p300 ChIP-Seq analyses in limb tissue at E11.5 (Visel et al., 2009) (Fig. 8).

## **Discussion**

### **Among Pbx genes, Pbx1 plays a primary role in pelvic development**

In this study, we examined pelvic development using compound Pbx and *Pbx1;Emx2* mutant mice. In all compound Pbx mutants bearing two *Pbx1* null alleles, save compound *Pbx1<sup>-/-</sup>;Pbx2<sup>+/-</sup>* mutants whose phenotype is strikingly severe, the ilium is absent or reduced and only minor malformations of the pubis and ischium are present (Fig. 9). In compound *Pbx1<sup>-/-</sup>;Emx2<sup>+/-</sup>* mutants, ilium hypoplasia is likely due to the loss of *Pbx1* function, since single *Emx2<sup>+/-</sup>* and compound *Pbx1<sup>+/-</sup>;Emx2<sup>+/-</sup>* mutants are normal. In complementary Pbx compound mutant genotypes, such as *Pbx1<sup>+/-</sup>;Pbx2<sup>-/-</sup>*, *Pbx1<sup>+/-</sup>;Pbx3<sup>-/-</sup>*, and triple *Pbx1<sup>+/-</sup>;Pbx2<sup>+/-</sup>;Pbx3<sup>+/-</sup>*, pelvic skeletal phenotypes are absent. All of these findings not only establish that *Pbx1* plays a paramount role in pelvic formation, but also that, overall, *Pbx1-2-3* gene dosage does not additively regulate pelvic girdle development. Importantly, *Pbx1* dominant role over its family members has also been reported for the development of limb (Capellini et al., 2006), scapula (Capellini et al., 2010), and axial (Capellini et al., 2008) skeletons.

### **Pbx family members control Emx2 expression in the mesodermal portion of the somatopleure. Collaboratively, Pbx genes and Emx2 direct ilium formation by modulating Sox9 expression and mesenchymal cell condensation**

Analyses performed in chick and mouse have illustrated that the skeletal elements of each pelvic bone form only from the somatopleure (Chevallier et al., 1977; Malashichev et al., 2008). Additional studies have demonstrated that the three adjoining pelvic elements (i.e., ilium, pubis, and ischium) arise from one single mesenchymal mass, which then undergoes chondrogenesis and osteogenesis in separate centers (Pomikal and Streicher, 2010). In this context, it has also been reported that: (1) *Sox9* serves as a marker for mesenchymal condensations of the pectoral and pelvic girdles (Malashichev et al., 2005; Wang et al., 2005; Malashichev et al., 2008); and (2) *Sox9* haploinsufficient mutant mice exhibit defects in most skeletal elements, including the pelvis, which is mostly affected in the ilium (Bi et al., 2001). Lastly, *Emx2* expression has been shown prior to *Sox9* activation in the

developing girdles (Malashichev et al., 2005; Malashichev et al., 2008; Capellini et al., 2010), and may instruct programs for the establishment of the iliac segment of the pelvic mesenchymal condensation (Malashichev et al., 2008).

In single *Pbx1* and *Emx2* homozygous mutants, the marked down-regulation of *Sox9* in the pelvic field (Fig. 5) precedes the reduction in ilium size (Fig. 9). Additionally, other mutants such as *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup>, *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup>, and *Pbx1*<sup>+/-</sup>;*Emx2*<sup>-/-</sup>, exhibit corresponding reductions in *Sox9* expression and ilium size. Strikingly, *Sox9* expression is virtually absent in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants that lack most of the pelvis. Thus, *Pbx1*-2-3 and *Emx2* act upstream of *Sox9* in the posterior mesodermal portion of the somatopleure (Fig. 9B) and likely govern ilium formation, at least in part, through control of *Sox9*.

Importantly, we also observed *Emx2* down-regulation in single *Pbx1*<sup>-/-</sup> and compound *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup> mutants and a more drastic reduction of *Emx2* expression in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, indicating that Pbx family members control *Emx2* in pelvic formation (Fig. 9B). Therefore, ilium hypoplasia in Pbx compound mutants is mediated, at least in part, through their genetic control of *Emx2* in the pelvic field, although concomitant regulation of *Sox9* expression by Pbx family members cannot be excluded. Likewise, the reduction of the ilium in single *Emx2* homozygotes, as well as in compound *Pbx1*<sup>+/-</sup>;*Emx2*<sup>-/-</sup> and *Pbx1*<sup>-/-</sup>;*Emx2*<sup>-/-</sup> mutants, is likely due to down-regulation of *Sox9* as a result of *Emx2* loss (Fig. 9B). Similar hierarchical relationships among *Pbx1*-2-3, *Emx2*, and *Sox9* genes were observed in pectoral somatopleuric mesenchyme (Capellini et al., 2010).

### **Pbx family members control ilium patterning genes *Pitx1* and *Tbx15* in early proximal HL bud, supporting early specification for pelvic girdle progenitors**

While it has been shown that the pelvic girdle forms from a single mesenchymal condensation (Pomikal and Streicher, 2010), little is known about the primary cues that specify progenitor somatopleure cells to form one single mass and then separate into three distinct pelvic bones. In chick, signals from the paraxial mesoderm (Malashichev et al., 2008) and overlying ectoderm (Malashichev et al., 2005) are important in triggering mesodermal portion of the somatopleure gene expression and specifying the pelvic rudiments during initial HL outgrowth (Malashichev et al., 2008). Additionally, genes expressed in the mesodermal portion of the somatopleure that likely specify all three pelvic bones respond differentially to signals provided by the ectoderm (Malashichev et al., 2005). If the findings in the chick can be applied to murine pelvic girdle development, then these patterning and morphogenetic events likely occur at early stages of mouse embryogenesis during initial HL bud development (Malashichev et al., 2005).

In homozygous *Pitx1* mutants, as well as in compound *Pitx1*;*Pitx2* mutants (Lanctot et al., 1999; Marcil et al., 2003), the ilium is either missing or detached from the remaining girdle. We observed strikingly similar defects in Pbx compound and *Pbx1*;*Emx2* mutants. Importantly, in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, we also detected down-regulation of *Pitx1* (Fig. 7) in the early proximal HL bud (e.g. E10–10.5), while *Pitx1* reduction was less severe distally and at later stages when condensations form (E11.25; Fig. 7; and Supp. Fig. S1C). These findings suggest that ilium loss in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants is at least partially mediated by down-regulation of *Pitx1*. Given the persistence of *Pitx1* distal expression in the limb proper of E10.5–11.5 *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, we contemplate the possibility that Pbx family members may control *Pitx1* in the proximal mesodermal portion of the somatopleure (i.e., the pelvic progenitor field only), wherein both *Pbx1* and *Pbx2* expression patterns overlap (Fig. 9). These data also suggest that the timing of specification for the pelvic girdle likely takes place prior to, or at, early HL bud outgrowth, as in the chick, and

support the hypothesis of an early specification model for pelvic progenitors (Dudley et al., 2002; Malashichev et al., 2005).

While *Tbx15* homozygous mutant mice do not exhibit pelvic skeletal defects, human patients suffering from Cousin Syndrome bear *TBX15* mutations and have dysmorphic and reduced iliac blades (Lausch et al., 2008). Notably, we observed a substantial down-regulation and spatial perturbation of *Tbx15* expression in E10.5 *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutant proximal HL fields (Fig. 7). These changes are also present, but not as severe, in genotypes with homozygous loss of *Pbx1* alone, (Fig. 7) placing Pbx family genes upstream of *Tbx15* and supporting a model whereby the early proximal HL field may be incapable of specifying ilium progenitors in Pbx mutants.

### **Pbx family members control pubic (*Twist1*, *Alx1*, and *Prrx1*) and ischial (*Pax1*) genes in early proximal HL bud**

Only in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, which exhibit complete pubic loss, besides perturbation of genes involved in specifying the ilium, we also observed down-regulation of *Twist1*, *Alx1*, and *Prrx1* at E10.5. *Twist1* functions during pelvic (and limb) development have been revealed (Bialek et al., 2004; Krawchuk et al., 2010), as reducing *Twist1* activity yields pubis loss (Krawchuk et al., 2010). In *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, we observed that *Twist1* expression is partially down-regulated in the posterior HL. In Pbx compound mutants (Fig. 6 and Supp. Fig. S1), we also detected marked reduction of expression of *Alx1*, but not *Alx4*, which participate in pubic development (Kuijper et al., 2005). *Alx1* down-regulation is intriguing, since *Twist1* is also known to act upstream of *Aristaless* family genes, including *Alx4*, during limb development (Loebel et al., 2002; O'Rourke et al., 2002). However, while reductions in *Alx4* expression were not identified in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, it has to be noted that *Twist1* is still normally expressed in the proximal anterior domain wherein *Alx4* is actively transcribed.

In *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants, we also observed significant down-regulation of *Prrx1*, a gene involved in the development of the pubic symphysis (ten Berge et al., 1998)(Fig. 6D–E). These results suggest that either Pbx genes regulate *Prrx1* expression directly (see below), or that perturbation of as yet unknown Pbx-dependent upstream pathways result in *Prrx1* loss (Fig. 9). Since compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants die at E13.5, prior to symphysis formation, conditional gene targeting will help clarify whether ablation of Pbx genes in the pubic field results in abnormal symphyseal development.

Lastly, in all mutants analyzed, including the *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> genotype that is most severely affected, a rudimentary ischium remains, although it is significantly truncated in the latter genotype. We have found that the expression of *Pax1*, which is expressed in the proximal HL field and later in the ischium condensation (Timmons et al., 1994; LeClair et al., 1999), is reduced early in all mutants lacking two copies of *Pbx1* and is absent in compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants. However, *Pax1* absence cannot explain ischium reduction in these mutants, since single *Pax1* homozygotes or compound *Pax1*;*Pax9* homozygous mutant mice lack ischial abnormalities (Peters et al., 1999). Ischium reduction in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mice may alternatively result from *Sox9* down-regulation and reduced mesenchymal formation in these mutants, or from disruption of earlier, as yet unknown, pathways (see below).

### **Pbx family members are prime regulators of pelvic development**

Our results establish that Pbx family members are critical upstream regulators of multiple genes involved in the specification, patterning, and morphogenesis of the murine pelvic girdle. We have previously demonstrated that Pbx homeoproteins control cell fate

specification and morphogenesis of the HL proper (Capellini et al., 2006), the axial skeleton (Capellini et al., 2008), the pectoral girdle (Capellini et al., 2010), and the splenic anlage (Brendolan et al., 2005), among other organs (Moens and Selleri, 2006). In the pectoral girdle, we presented evidence that Pbx1 and Emx2 are bound *in vivo* to a putative regulatory region of *Alx1*, a scapular blade effector gene, and drive transcriptional activation of a luciferase reporter in cultured cells. Likewise, it will be important to evaluate if cell fates of specific progenitors for the ilium, ischium, and pubis are also controlled directly by Pbx homeoproteins within the early mesodermal portion of the somatopleure or during mesenchymal condensation.

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, misexpression or loss of Hox genes result in mild alterations of the pelvic girdle, including defects in mice misexpressing *Hoxd12* in lateral plate derivatives (Knezevic et al., 1997); modest malformations of pelvic bones and sacrum in *Hoxc10* mutants (Hostikka et al., 2009); and lack of uterosacral ligaments in *Hoxa11* mutant mice (Connell et al., 2008). Therefore, it is unlikely that Pbx homeoproteins effect their roles in pelvis formation solely as Hox cofactors, suggesting instead cooperation with other proteins, e.g. Emx2.

Using GREAT (McLean et al., 2010), a new bioinformatics tool, we found that potential Emx2-Pbx1 regulated enhancers, defined by the presence of strongly conserved Emx2-Pbx1 binding sites, are more often enriched in the vicinity of genes involved in HL morphogenesis and cell fate specification than predicted by chance. Furthermore, we identified specific Emx2-Pbx1 dimerization motifs in well conserved non-coding sequences near (at least) three genes involved in ischium, pubis, and ilium development (i.e., *Pax1*, *Prrx1*, and *Tbx15*, respectively). Based on their conservation and close proximity to nearby genes, these sequences may represent functional regulatory elements. For the ischium, we identified the presence of a strongly conserved Emx2-Pbx1 dimerization site approximately 99kb from the TSS of *Pax1* (Fig. 8A), which is down-regulated in compound *Pbx1;Emx2* mutants and lost in *Pbx1;Pbx2* mutants (Fig. 7). For *Prrx1*, a gene patterning the pubis, we identified a motif conserved to *Medaka*, inside a block of approximately 230 bp of sequence homology within 1.5 kb of *Prrx1* TSS (data not shown). Of note, *Prrx1* expression is also strikingly reduced in E10.5–11.5 *Pbx1;Pbx2* mutants (Fig. 6), which have no pubis. Finally, our prediction for a functional enhancer of *Tbx15*, a gene involved in ilium formation in humans, is of exceptional interest, not only considering its close proximity to the *Tbx15* TSS (~5 kb) (Fig. 8B), but also given its overlap with a separately identified target of p300 in limb cells at E11.5 (Visel et al., 2009). Independently, we observed down-regulation and spatial alteration of *Tbx15* *in vivo*, in E10.5–11.5 *Pbx1;Pbx2* and *Pbx1;Emx2* compound mutants (Fig. 7) that lack the ilium. While these *in silico* findings provide strong associations, future experiments will be required to verify transcriptional regulation by Pbx and Emx2 homeoproteins via these potential enhancers identified *in silico* (Table 2). Among these potential regulatory elements, some may control cell fate specification and patterning of specific pelvic structures such as the pubis, ilium, and ischium in development.

In summary, our studies reveal that Pbx family members genetically interact and that concomitantly *Pbx1* interacts with *Emx2* to control pelvic development in mammalian embryogenesis. To perform these functions, Pbx genes regulate effector genes involved in the specification and development of the ilium (*Pitx1* and *Emx2*), ischium (*Pax1*), and pubis (*Alx1*, *Twist1*, *Prrx1*), as well as genes responsible for mesenchyme formation and condensation (*Sox9* and *Col2*). In the context of this overarching control by Pbx homeoproteins, our work highlights multiple potential target genes either known for their involvement in pelvic development, or of unknown function. Thus, our studies open the path for new research in an under-studied area of vertebrate embryonic development.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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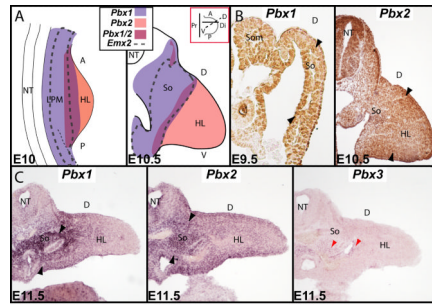
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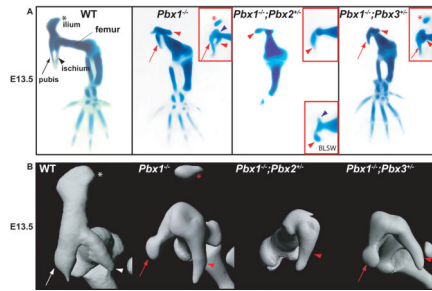
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**Fig. 1.**

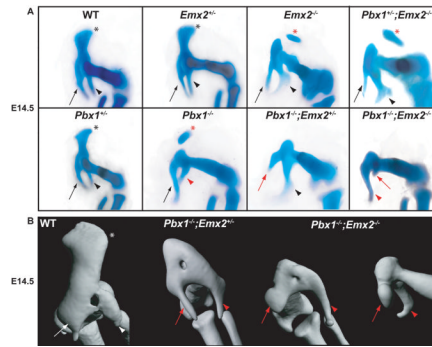
Pbx family member and *Emx2* expression during pelvic development via in situ hybridization and immunohistochemistry. **A:** Summary illustration of *Pbx1* and *Pbx2* co-expression with *Emx2* during early HL and pelvic development (see also Capellini et al., 2006; Di Giacomo et al., 2006). At E10 (left) to E10.5 (right), all three genes overlap within the LPM and mesodermal portion of the somatopleure of the proximal HL bud. Red inset shows orientations along plane of section used in the larger illustration below and the *Pbx2* immunohistochemistry results in (B). **B:** Immunohistochemistry of *Pbx1* (left) at E9.5 and *Pbx2* (right) at E10.5 in the HL mesodermal portion of the somatopleure (black arrowheads). **C:** At E11.5, *Pbx1* (left, black arrowheads) and *Pbx2* (middle, black arrowheads) are strongly co-expressed in the mesodermal portion of the somatopleure, while *Pbx3* is only weakly expressed (right, red arrowheads). Abbreviations: A, anterior; D, dorsal; Di, distal; HL, hindlimb; LPM, lateral plate mesoderm; NT, neural tube; P, posterior; Pr, proximal; So, somatopleure; Som, somite; V, ventral.



**Fig. 2.**

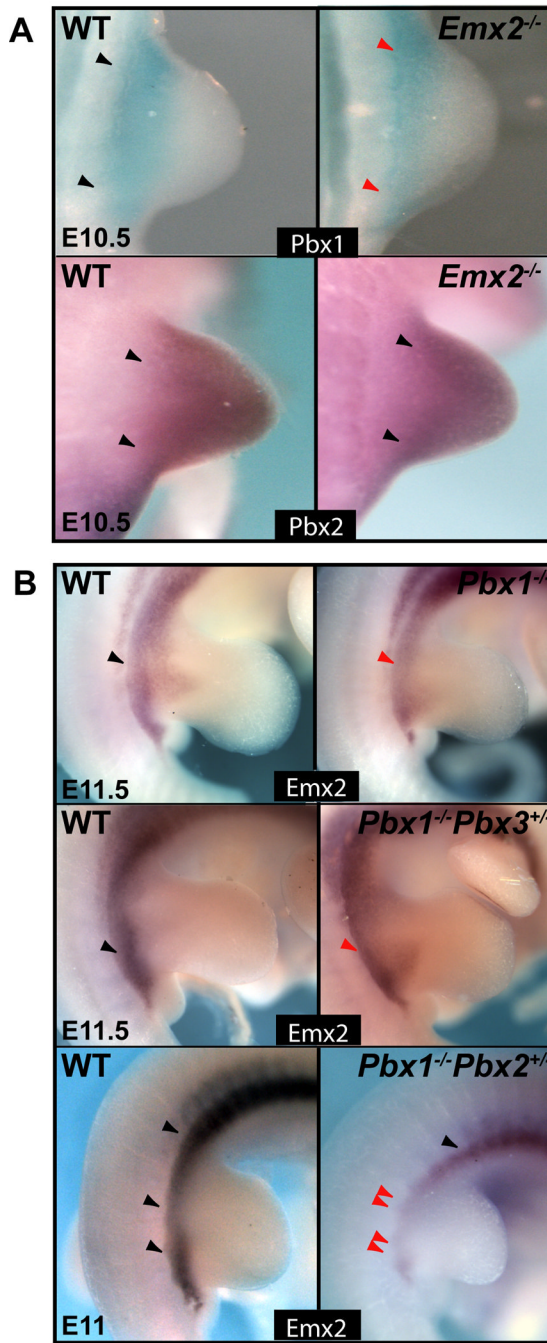
Pelvic skeletal phenotypes of compound Pbx family member mutants at E13.5 as assessed using Alcian blue and Alizarin red staining and OPT. **A:** Compared to WT HL (left panel): *Pbx1*<sup>-/-</sup> embryos exhibit either absent or detached ilia (red inset, red asterisk), and reduced pubis (red arrow) and ischia (red arrowhead); *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants lack ilia and pubis and display rudimentary ischia (red arrowheads in main panel and insets). Insets show additional embryos acquired via breeding schemes described in Materials and Methods and Results. Lower red inset depicts the presence of a small tissue rudiment (blue arrowhead), often found in mutants repeated out-crossed to Black Swiss (BLSW); *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup> mutants phenocopy single *Pbx1* homozygotes in all defects (red asterisk, arrows, and arrowheads), and reveal that *Pbx3*, in this mutant model, is not required for distal HL development. **B:** OPT of WT (white symbols) and select Pbx family member mutants highlights defects illustrated above (red symbols), and that *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants exhibit only truncated ischial rudiments (red arrowhead).



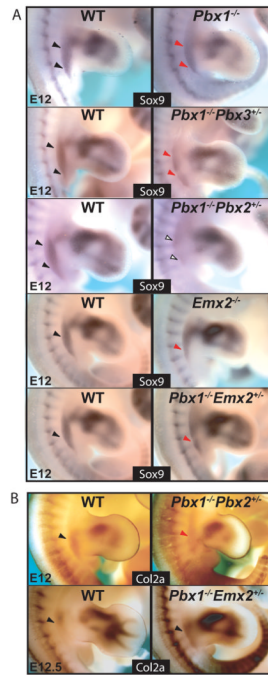


**Fig. 3.**

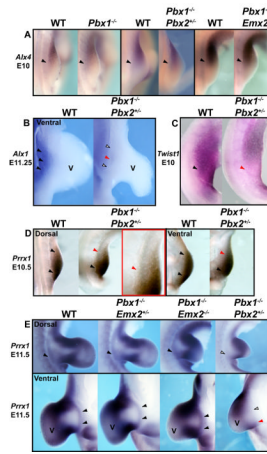
Pelvic skeletal phenotypes of single and compound *Pbx1;Emx2* mutants at E14.5 as assessed using Alcian blue and Alizarin red staining and OPT. **A:** Compared to WT HL (left panel): single *Pbx1*<sup>+/-</sup> and *Emx2*<sup>+/-</sup> mutants display normal pelvis (black symbols); single *Emx2*<sup>-/-</sup> and compound *Pbx1*<sup>+/-</sup>;*Emx2*<sup>-/-</sup> mutants phenocopy the rostral *Pbx1*<sup>-/-</sup> mutant pelvic morphologies with either absent or detached ilia (red asterisk); compound *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> embryos additionally show modestly truncated pubis (red arrow); and compound *Pbx1*<sup>-/-</sup>;*Emx2*<sup>-/-</sup> embryos exhibit well formed ischia (red arrowhead), and truncated pubis (red arrow). **B:** OPT reveals that compared to WT (left panel; white symbols), and compound *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> mutants, *Pbx1*<sup>-/-</sup>;*Emx2*<sup>-/-</sup> mutants show morphologically identifiable ischia and substantially reduced and rounded pubis (red arrows and arrowheads in both views, respectively).



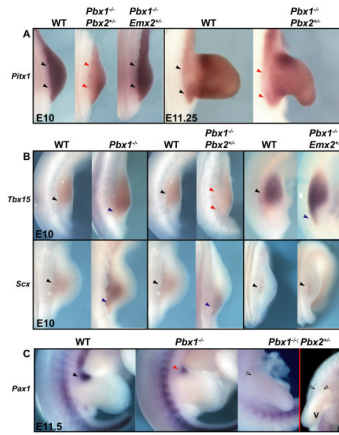
**Fig. 4.** Expression of Pbx family members and *Emx2* in respective single *Emx2* homozygous and compound *Pbx1-3* mutants via in situ hybridization. **A:** Compared to WT (black arrowhead); in E10.5 *Emx2*<sup>-/-</sup> mutants, *Pbx1* expression (top) is modestly spatially disrupted in its proximal-to-distal domain, but remains in the proximal HL (red arrowhead); and *Pbx2* expression (bottom) is unaffected (black arrowhead). **B:** Compared to WT (black arrowheads): at E11.5, *Emx2* expression is slightly reduced in single *Pbx1*<sup>-/-</sup> and compound *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup> mutant proximal HL (red arrowheads), but is severely reduced-to-absent in compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutant proximal HL (double red arrowheads).



**Fig. 5.** Expression of *Sox9* and *Col2a*, markers of mesenchymal condensation and skeletal development, in single and compound Pbx family member and *Pbx1;Emx2* mutants via in situ hybridization. **A:** Compared to WT (black arrowheads), at E12, in single *Pbx1*<sup>-/-</sup> and *Emx2*<sup>-/-</sup> mutants and compound *Pbx1*<sup>-/-</sup>;*Pbx3*<sup>+/-</sup> and *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> mutants (red arrowheads), *Sox9* expression is reduced, while in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (empty arrowheads) it is nearly absent. **B:** Compared to WT (black arrowheads), in E12 *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (red arrowheads) *Col2a* expression is markedly reduced, while at E12.5, *Col2a* is modestly up-regulated in *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> mutants (black arrowheads).



**Fig. 6.** Expression of genes involved in pubis development in single and compound Pbx family member and *Pbx1;Emx2* mutants via in situ hybridization. **A:** Compared to WT (black arrowheads), at E10, expression of *Alx4* remains unperturbed in single *Pbx1*<sup>-/-</sup> and compound *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> and *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> mutants (black arrowheads). **B:** Compared to WT (black arrowhead), at E11.25, expression of *Alx1* is absent or severely reduced from the ventral somatopleure of the proximal HL bud in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (empty and red arrowheads). **C:** Compared to WT (black arrowhead), at E10, expression of *Twist1* is reduced from the posterior margin of the HL bud in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (red arrowhead). **D:** Compared to WT at E10.5 (black arrowheads), expression of *Prrx1* is reduced from the anterior margin of the dorsal and ventral somatopleuric domains of the proximal HL bud in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (red arrowheads). High magnification red inset shows the proximal anterior HL reduction in *Prrx1* expression in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (red arrowhead). **E:** Compared to WT and all other mutants shown (black arrowheads), at E11.5, expression of *Prrx1* is absent or significantly reduced from the dorsal (top) and ventral (bottom) somatopleure of the proximal HL bud in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (empty and red arrowheads). Abbreviation: V, ventral.

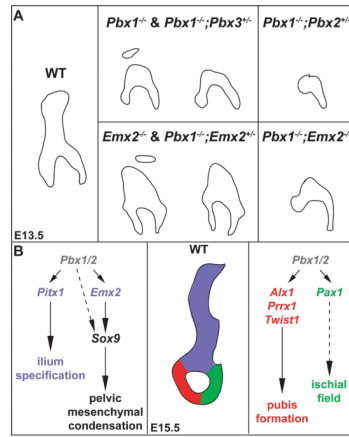


**Fig. 7.** Expression of genes involved in ilium specification and hindlimb development in single and compound Pbx family member and *Pbx1*;*Emx2* mutants via in situ hybridization. **A:** Compared to WT (black arrowheads), at E10, expression of *Pitx1* is markedly reduced in its proximal domain but present in its distal limb domain in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants only (red arrowheads). At E11.25, *Pitx1* expression is modestly reduced from the proximal HL of this same mutant genotype (red arrowheads). **B:** (top) Compared to WT (black arrowheads), at E10, expression of *Tbx15* is posteriorly expanded in single *Pbx1*<sup>-/-</sup> and compound *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> mutants (blue arrowheads) and greatly posteriorly expanded and reduced in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (red arrowheads). (bottom) Compared to WT (black arrowheads), at E10, expression of *Scx*, a tendon marker, is slightly posteriorly expanded in single *Pbx1*<sup>-/-</sup> mutants (blue arrowhead), but markedly posteriorly shifted in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> mutants (red arrowheads), and unchanged in *Pbx1*<sup>-/-</sup>;*Emx2*<sup>+/-</sup> mutants (black arrowheads). **C:** Compared to WT (black arrowhead), at E11.5, expression of *Pax1*, an ischium marker, is markedly reduced in single *Pbx1*<sup>-/-</sup> mutants (red arrowheads) and absent in *Pbx1*<sup>-/-</sup>;*Pbx2*<sup>+/-</sup> HL (empty arrowheads in left dorsal view and right superior-anterior view). Proximal is to the left in all panels. Abbreviation: V, ventral.





**Fig. 8.** Bioinformatic analysis of two putative enhancers near *Pax1* and *Tbx15*. **A:** (top – red box) UCSC browser window depicting the genomic location of *Pax1* (right) in the mouse genome (mm9) relative to known sequence conservation across forty-four vertebrates (green peaks). (bottom – orange box) Zoom-in UCSC browser view of the specific location of the Emx2-Pbx1 heterodimeric TF prediction, showing sequence conservation to opossum. A dot in the alignment denotes an identical base to the reference species. **B:** (top – red box) UCSC browser window depicting the genomic location of *Tbx15* in the mouse (mm9) genome relative to known sequence conservation across forty-four vertebrates (green peaks). (bottom – orange box) Zoom-in UCSC browser view of the specific location of the Emx2-Pbx1 heterodimeric TF prediction, showing conservation to opossum, and incomplete sequence conservation to chicken. This specific motif overlaps with a predicted P300 limb enhancer identified at E11.5 in mouse embryos (limb.405 line, above; horizontal black line, below) (see text for details). Red bars represent GREAT calculated regulatory domains for the genes listed to the left.



**Fig. 9.** Summary and model of Pbx family member and *Emx2* genetic functions during pelvic development. **A:** Illustrations depicting the phenotypes of the compound Pbx family member and *Pbx1;Emx2* mutants analyzed in this study (see text for details). **B:** Model of Pbx family member and *Emx2* genetic functions during pelvic girdle formation. (center panel) An E15.5 WT pelvis demonstrating the three distinct bony elements in color (blue – ilium, red – pubis, green – ischium). These colors reflect the genes involved in the formation of each structure, as depicted in the left and right panels. (left panel) *Pbx1/Pbx2* hierarchically regulate *Pitx1* and *Emx2* during the specification and condensation of the ilium (blue), respectively. Dashed arrow indicates a potential control of *Sox9* directly by Pbx family members. (right panel) *Pbx1/Pbx2* hierarchically regulate genes involved in pubis development (red), such as *Alx1*, *Prrx1*, and *Twist1*, and ischial field expression (dashed green arrow), such as *Pax1*.

**TABLE 1**

Results of the analysis by great\*

Term	Binomial P-value	Fold enrichment	Genes with annotation	Genes hit	Number of binding sites
Hindlimb morphogenesis	7.3e-5	2.3	33	16	29
Cell fate determination	9.6e-5	2.1	30	15	33
Embryonic hindlimb morphogenesis	1.9e-4	2.3	25	12	24

\* McLean *et al.*, 2010

TABLE 2

Emx2-Pbx1 dimerization motifs within 200 Kb of pelvic genes

Gene	Chr.	Motif Start	Motif End	Strand	To TSS (bp)	MGI Term: TS: Description
<i>Ahr4</i>	chr2	93642898	93642908	+	+160308	Phenotype: small pubis
<i>C77370</i>	chrX	101551207	101551217	-	-154752	Expression: TS23: embryo:skeleton;pelvic girdle
<i>C77370</i>	chrX	101552028	101552038	-	-155573	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Cd59a</i>	chr2	103962170	103962180	-	+26161	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Cdkn3</i>	chr14	47220880	47220890	+	-159363	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Chrdll</i>	chrX	139677009	139677019	-	+151708	Expression: TS21: embryo:skeleton;pelvic girdle
<i>Chrdll</i>	chrX	139837753	139837763	-	-9027	Expression: TS21: embryo:skeleton;pelvic girdle
<i>Chst8</i>	chr7	35475321	35475331	+	+122364	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Col11a1</i>	chr3	113834894	113834904	-	+101437	Expression: TS23: vertebral muscle; pelvic girdle
<i>Col9a1</i>	chr1	24209022	24209032	+	+24484	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Coph1</i>	chr7	121280770	121280780	+	+117414	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Ctgf</i>	chr10	24132107	24132117	-	-183196	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Ctgf</i>	chr10	24156973	24156983	-	-158330	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Dpyd</i>	chr3	118088581	118088591	+	-176505	Expression: TS23: vertebral muscle; pelvic girdle
<i>Ext1</i>	chr15	53028807	53028817	-	+148921	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Fat3</i>	chr9	16135872	16135882	-	+46793	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Fat3</i>	chr9	16268418	16268428	+	-85744	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Foxl1</i>	chr11	34014004	34014014	-	+94075	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Foxl1</i>	chr11	34032581	34032591	-	+75498	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Foxp1</i>	chr6	98976797	98976807	+	+136216	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Gl3</i>	chr13	15508916	15508926	-	-46630	Phenotype: abnormal pubis morphology
<i>Gl3</i>	chr13	15708609	15708619	-	+153054	Phenotype: abnormal pubis morphology
<i>Hhip</i>	chr8	82763655	82763665	+	-181764	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Hmg2</i>	chr10	119881607	119881617	+	+32374	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Hpse</i>	chr5	101180712	101180722	-	-32011	Expression: TS23: embryo:skeleton;pelvic girdle
<i>Irx5</i>	chr8	94916857	94916867	-	+35163	Expression: TS23: embryo:skeleton;pelvic girdle

Gene	Chr.	Motif Start	Motif End	Strand	To TSS (bp)	MGI Term: TS: Description
<i>Ippr3</i>	chr17	27275740	27275750	+	+81260	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Kend2</i>	chr6	21194367	21194377	+	+28259	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Lmx1b</i>	chr2	33653044	33653054	-	-157014	Phenotype: abnormal ilium morphology
<i>Magi1</i>	chr6	94286552	94286562	+	-52655	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Ndufv2</i>	chr17	66388461	66388471	-	+62360	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Ndufv2</i>	chr17	66392826	66392836	-	+57995	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Nr1</i>	chr6	29954775	29954785	-	-43203	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Pax1</i>	chr2	147091657	147091667	+	-99086	Expression: TS21: embryo:skeleton:pelvic girdle
<i>Pbx1</i>	chr1	170333299	170333309	-	+29080	Phenotype: abnormal pelvic girdle morphology
<i>Pik3c3</i>	chr18	30314187	30314197	-	-118353	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Pik3c3</i>	chr18	30542253	30542263	+	+109704	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Plekhh1</i>	chr7	107780622	107780632	+	+30247	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Prrx1</i>	chr1	165245123	165245133	-	-1343	Phenotype: abnormal pubis morphology
<i>Ptch1</i>	chr13	63555761	63555771	-	+111057	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Rasl1b</i>	chr5	74572329	74572339	+	-19012	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Rg9md2</i>	chr3	137794428	137794438	+	-12064	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Runx2</i>	chr17	45008461	45008471	+	-56886	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Sarb2</i>	chr1	56955591	56955601	+	+72577	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Tbx15</i>	chr3	98992619	98992629	-	-65054	Phenotype: abnormal pelvic girdle morphology
<i>Tbx15*</i>	chr3	99052908	99052918	-	-4765	Phenotype: abnormal pelvic girdle morphology
<i>Tbx4</i>	chr11	85760419	85760429	+	+56855	Phenotype: abnormal pelvic girdle morphology
<i>Tnmd</i>	chrX	130290990	130291000	-	-94547	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Trp63</i>	chr16	25783048	25783058	+	-18970	Phenotype: abnormal pelvic girdle morphology
<i>Trps1</i>	chr15	50674966	50674976	-	+46611	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Unc5c</i>	chr3	141241263	141241273	-	+112736	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Unc5c</i>	chr3	141303308	141303318	-	+174781	Expression: TS23: embryo:skeleton:pelvic girdle
<i>Unc5c1</i>	chr17	48752190	48752200	-	+157943	Expression: TS23: embryo:skeleton:pelvic girdle

\* This specific motif overlaps a previously identified p300 limb element at E11.5 in mouse (Visel et al., 2009).