Erratum

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Fig. S1 in the supplementary material accompanying this paper was published incorrectly when the paper first appeared on-line. This has now been rectified and we apologise to the authors and to readers for this mistake.

A *Pbx1*-dependent genetic and transcriptional network regulates spleen ontogeny

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Summary

The genetic control of cell fate specification, morphogenesis and expansion of the spleen, a crucial lymphoid organ, is poorly understood. Recent studies of mutant mice implicate various transcription factors in spleen development, but the hierarchical relationships between these factors have not been explored. In this report, we establish a genetic network that regulates spleen ontogeny, by analyzing asplenic mice mutant for the transcription factors Pbx1, Hox11 (Tlx1), Nkx3.2 (Bapx1) and Pod1 (capsulin, Tcf21). We show that *Hox11* and *Nkx2.5*, among the earliest known markers for splenic progenitor cells, are absent in the splenic anlage of *Pbx1* homozygous mutant ($^{-/-}$) embryos, implicating the TALE homeoprotein Pbx1 in splenic cell specification. *Pbx1* and *Hox11* genetically interact in spleen formation and loss of either is associated with a similar reduction of progenitor cell proliferation and failed expansion of the splenic anlage. Chromatin immunoprecipitation assays show that Pbx1 binds to the *Hox11* promoter in spleen mesenchymal cells, which co-express *Pbx1* and *Hox11*. Furthermore, Hox11 binds its own promoter in vivo and acts synergistically with TALE proteins to activate transcription, supporting its role in an auto-regulatory circuit. These studies establish a *Pbx1-Hox11*-dependent genetic and transcriptional pathway in spleen ontogeny. Additionally, we demonstrate that while *Nkx3.2* and *Pod1* control spleen development via separate pathways, *Pbx1* genetically regulates key players in both pathways, and thus emerges as a central hierarchical co-regulator in spleen genesis.

Key words: Spleen ontogeny, Organogenesis, *Pbx1*, *Hox11* (*Tlx1*), *Nkx2.5*, *Nkx3.2* (*Bapx1*), *Pod1* (capsulin, Tcf21), *Wt1*, Transcriptional regulation, Pbx1 targets, Proliferation, Mouse

Introduction

In vertebrates, the spleen is a lymphoid organ that serves important roles in hematopoiesis and the generation of primary immune responses, as well as acting as a filter that removes and processes aged and abnormal blood cells (VanRooijen et al., 1989; Zapata and Cooper, 1990). During development, the spleen originates from splanchnic mesoderm (mesenchyme that surrounds the gut endoderm), which arises from splitting of the lateral plate mesoderm into somatic mesoderm (body wall) and splanchnic mesoderm (Funayama et al., 1999). The latter undergoes gut-specific mesodermal differentiation (Apelqvist et al., 1997; Ramalho-Santos et al., 2000) in response to signals from the endoderm that gives rise to the epithelium of the gut and associated organs. Histological studies using frog, chick and mammalian embryos indicate that development of the splenic anlage is first detectable at approximately gestational day (E) 11, as progenitor cells derived from splanchnic mesoderm form a single condensation within the dorsal mesogastrium (Dm; the mesenchymal sheet that attaches the stomach to the dorsal body wall), adjacent to the stomach and dorsal pancreas (Thiel and Downey, 1921; Manning and Horton, 1969; Sty and Conway, 1985; Vellguth et al., 1985; Yassine et al., 1989).

In vertebrates, the mesodermally derived spleen normally displays left-handed asymmetry (Boorman and Shimeld, 2002) and has been considered to be a landmark organ for detecting laterality defects (Aylsworth, 2001). But, interestingly, mice with left-right (LR) asymmetry defects, such as the *Inv/Inv* (Yokoyama et al., 1993) and *ActRIIB* (Oh et al., 2002) mutant mice exhibit either normally developed spleens or, infrequently, splenic hypoplasia. Furthermore, asplenic mouse models, such as the *Dh* spontaneous mutant (Green, 1967) and the *Nkx3.2* (*Bapx1* – Mouse Genome Informatics) mutant mouse (Lettice et al., 1999; Tribioli et al., 1999), appear to exhibit only regional perturbations of LR asymmetry in the primordial splenopancreatic mesoderm (Hecksher-Sorensen et al., 2004). Other asplenic mouse models, such as those mutant for *Hox11* (*Tlx1* – Mouse Genome Informatics) (Roberts et al., 1994; Dear et al., 1995), display asplenia as the sole organ abnormality. Likewise, in humans, asplenia may present as the sole organ anomaly, without perturbations of LR asymmetry

(Rose et al., 1975; Waldman et al., 1977). Overall, these findings underscore the notion that, both in mice and humans, mechanisms other than the regulation of LR asymmetry must be responsible for the control of splenic cell fate specification and morphogenesis.

Recent advances in mouse genetics have led to the discovery of novel genes required for early spleen ontogeny. These include *Hox11* (Roberts et al., 1994; Dear et al., 1995; Kanzler and Dear, 2001), *Nkx3.2* (Lettice et al., 1999; Tribioli et al., 1999), *Pod1* (Quaggin et al., 1999; Lu et al., 2000) and *Wt1* (Herzer et al., 1999); however, the hierarchical relationships among these genes remain unknown. This limited collection of genes also includes *Pbx1* (Nourse et al., 1990; Kamps et al., 1990), which encodes a TALE class (Burglin, 1997) homeodomain protein, the absence of which results in embryonic asplenia with 100% penetrance (Selleri et al., 2001). Although the role of Pbx1 in spleen development is undefined, its reported biochemical in vitro interaction with homeodomain protein Hox11 through the hexapeptide motif (Shen et al., 1996) raises the possibility that these two homeoproteins may cooperate in spleen ontogeny, as Hox11 is also required for spleen formation (Roberts et al., 1994; Dear et al., 1995).

Heterodimers of Pbx and other TALE proteins of the Meinox family, such as Meis (Bischof et al., 1998; Chang et al., 1997) and Pknox/Prep1 (Berthelsen et al., 1998; Knoepfler et al., 1997; Fognani et al., 2002), form stable nuclear complexes, and biochemical analyses suggest that these complexes regulate several genes (Swift et al., 1998). Indeed, we have found that loss of Pbx1 causes multiple organogenesis defects in the mouse and lethality in utero at E15.5 (Selleri et al., 2001). These defects include abnormalities in patterning and development of the skeleton (Selleri et al., 2001), in pancreas morphogenesis and function (Kim and Selleri et al., 2002), in adrenal/urogenital development (Schnabel et al., 2003a; Schnabel et al., 2003b), and in caudal pharyngeal pouch-derived organ formation and patterning (Manley and Selleri et al., 2004), as well as impaired hematopoiesis (DiMartino and Selleri et al., 2001). These findings underscore the notion that Pbx1 serves as a key developmental regulator, although the crucial genetic and transcriptional pathways underlying its specific developmental roles have not been established.

In this report, we investigated spleen ontogeny by analyzing asplenic mouse models lacking *Pbx1*, *Hox11*, *Nkx3.2* or *Pod1* (capsulin). Our studies define a genetic hierarchy in which Pbx1 serves a central and crucial role as a common co-regulator in spleen ontogeny.

Materials and methods

Mice

Asplenic knockout mice for *Hox11* (Roberts et al., 1994), *Hox11^{lacZ}* (*lacZ* insertion into the *Hox11* locus) (Dear et al., 1995), *Pbx1* (Selleri et al., 2001) and *Pod1* (Quaggin et al., 1999) have been described and were genotyped according to previously published protocols. *Nkx3.2* (*Bapx1*) asplenic knockout mice were generated and kindly provided by Drs W. Zimmer and R. Schwartz (unpublished). *Pbx2* knockout mice, which develop normally, were genotyped as described previously (Selleri et al., 2004).

Histological analysis, immunohistochemistry and in situ hybridization

Embryos from E10.5 to E15.5 were harvested and fixed overnight at 4°C in phosphate-buffered saline (PBS) containing 4% (w/v) paraformaldehyde (PFA). For histological analysis and immunohistochemistry with a mouse anti-Pbx1b primary antibody (Jacobs et al., 1999), protocols were followed as described (Selleri et al., 2001). Single-stranded sense and antisense riboprobes for in situ hybridization on frozen sections were specific for *Hox11* (Dear et al., 1995), *Nkx3.2* (Tribioli et al., 1997), *Nkx2.5* (Lyons et al., 1995), *Pbx1* (3′ UTR), *Pod1* (Quaggin et al., 1998) and *Wt1* (Herzer et al., 1999).

Assessment of β-galactosidase activity

Embryos heterozygous for *Hox11^{lacZ}* were collected at E11.5 and E12.5 and stained for β-galactosidase as described previously (Dear et al., 1995).

Staining of germinal centers

Mice 6-8 weeks old were immunized by intravenous (i.v.) injection of 5×10^7 sheep red blood cells (SRBC) in PBS. Mice were sacrificed and the spleens processed for immunohistochemistry with biotinylated lectin peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA) as described (Inada et al., 1998).

BrdU analysis

Pregnant *Pbx1$^{+/-}$* and *Hox11$^{+/-}$* female mice, carrying embryos at E12.5 and E13.5, respectively, were injected intraperitoneally with 5-bromo-2-deoxy-uridine (BrdU) (50 μg/g of body weight) and BrdU incorporation was assayed as previously described (Selleri et al., 2001). The number of BrdU-positive cells (dark brown nuclei) within the developing spleen were counted in six to eight sagittal sections (10 μm thickness) for each genotype. Quantitative analysis of BrdU immunoperoxidase-stained sections was made on a Nikon microscope equipped with a video camera. TUNEL assays were performed as described by Gavrieli et al. (Gavrieli et al., 1992).

Cell culture and immunostaining

Embryonic spleens at day E16-17 were dissected and trypsinized (0.25% final concentration) for 10-15 minutes at 37°C. The cell suspension was washed twice and cultured in Dulbecco's Modified Eagle Medium (D-MEM), supplemented with 10% fetal calf serum (Celbio), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) in humidified 5% CO_2, and used as a primary cell culture for chromatin immunoprecipitation (ChIP) assays. Primary spleen cultures were immunophenotyped by using an α-smooth muscle actin Ab (ASMA; Santa Cruz Biotech), which stains mesoderm-derived cells. To generate immortalized spleen embryonic cell lines from *Pbx2$^{-/-}$* embryos, the NIH 3T3 protocol (Todaro et al., 1963) was used.

Western blot analysis

Western blot analysis was performed as described previously (Berthelsen et al., 1996; Jacobs et al., 1999). The following antibodies were used: anti-Hox11 (1:1,000) (Santa Cruz Biotech, CA), anti-Pbx1b (1:1,500) (Jacobs et al., 1999) and anti-Prep 1 (1:1,500; Upstate Biotechnology).

In vitro transcription assays

NIH 3T3 cells were cultured in D-MEM supplemented with 5% fetal calf serum and 5% delipidated fetal calf serum. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For *Hox11* promoter analysis, the following constructs were used: a luciferase reporter vector (pGL2); a pGL2 construct carrying an *Eco*RI-*Sal*I 0.9 kb fragment, corresponding to the *Hox11* promoter region (pGL2-540) (Arai et al., 1997); pcDNA3 constructs containing the cDNA of Pbx1a or Prep1 (Berthelsen et al., 1998) and a pBlueScript-Hox11 construct containing the full-length cDNA of Hox11 (obtained from Dr N. Hoehler) (Koehler et al., 2000). Cells were lysed 40-45 hours after transfection and assayed for luciferase activity (Benasciutti et al.,

2004). Values were normalized for β-gal activity. Data represent means of triplicate values from a representative experiment. All transfections were independently performed three times.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed as described (Berthelsen et al., 1996; Jacobs et al., 1999) using nuclear extracts from spleen cells. The following oligonucleotides, spanning Pbx1-binding sites within the *Hox11* promoter (AB 000681) (Arai et al., 1997), were employed in EMSA reactions: 5′-CCAAAGGCTTGTGA-CTGCTTTTCAGG-3′ PX1 and 5′-CCAAAGGCTTGTCG-ACGCTTTTCAGG-3′ PX1 mutated.

The following antibodies were used: rabbit polyclonal antibodies specific for Pbx1 (P-20) and Hox11 (C-18; Santa Cruz Biotech, CA).

Chromatin immunoprecipitation assay

Formaldehyde crosslinking and chromatin immunoprecipitation were performed as described (Frank et al., 2001). Samples were immunoprecipitated overnight at 4°C with the following antibodies: mouse monoclonal specific for Pbx1b (Jacobs et al., 1999), rabbit polyclonal specific for Hox11 (Santa Cruz Biotech, CA), mouse monoclonal anti-Green Fluorescent Protein (anti-GFP; Santa Cruz Biotech, CA) and normal rabbit serum (Covenge Research). Immune complexes were recovered by adding 30 µl of blocked protein G beads and incubated for 2 hours at 4°C. Beads were washed and eluted, and crosslinks were reversed as described (Aparicio et al., 1999). The eluted DNA was resuspended in 30-60 µl water. A region within the *Hox11* promoter containing the Pbx1-binding sites (–359 s/–115 as and –258 s/–54 as) and a control region within the same promoter (–1170 s/–891 as) were amplified by PCR using specific primer pairs. One primer pair that amplifies a region of the *Bmp4* promoter was also used as additional negative control: 5′-ACGCACTTCCCTGA-TTCTCGTC-3′ (–359 s) and 5′-AGCAGTCACAAGCCTT-TGGATTAC-3′ (–115 as) product size 244 bp; 5′-TCTCACAAACCCAGAGCCATTC-3′ (–258 s) and 5′-TAG-CAGCCACTCCAACTCAGTCTC-3′ (–54 as) product size 204 bp; 5′-TGAGAACAACTACCTGCTTCGTGC-3′ (–1170 s) and 5′-TGGAGACTTGACTTGCCCAACC-3′ (–891 as) product size 279 bp; and 5′-AATGAACAAACACCACT-CTCCCTC (*Bmp4* s) and 5′-AACACCAGACCGAAAAGAT-GACTG (*Bmp4* as) product size 350 bp.

Results

Loss of *Pbx1* causes agenesis of the spleen

Analysis of *Pbx1*$^{-/-}$ embryos at E15.5 demonstrated that they are asplenic (Fig. 1B,D), compared with wild-type controls, which exhibited a fully developed ribbon-shaped spleen (Fig. 1A,C). The defect in spleen development was evident at E13.5 because of the absence of the splenic anlage (Fig. 1F), which is easily recognizable in close proximity to the stomach on histological sections of wild-type embryos (Fig. 1E). Earlier in development, at E11-12.5, histological and in situ analyses (Fig. 2) indicated that the splenic primordium, which starts to be recognizable as a condensation of mesenchymal cells within the normal Dm, lateral to the stomach (Thiel and Downey, 1921), was hypoplastic in *Pbx1*$^{-/-}$ embryos (Fig. 2). At even earlier stages of organogenesis (E10-10.5), however, the differentiation of the splanchnic mesoderm into the epithelial-like plate of cells that is well distinguished from the surrounding unorganized mesenchyme (Green, 1967), was completely preserved in *Pbx1*$^{-/-}$ embryos (Fig. 1G-J). In fact, at this developmental stage, the general architecture of the splanchnic mesoderm that surrounds the stomach enlargement (Fig. 1G-J) and midgut primordia (not shown) within the coelomic cavity did not appear morphologically abnormal in *Pbx1*$^{-/-}$ embryos. This contrasts with other cases of murine asplenia, which exhibit marked abnormalities of the splanchnic mesoderm, such as that documented in the *Dh* mutant, which lacks the epithelial-like plate of cells at E10-10.5 (Green, 1967; Hecksher-Sorensen et al., 2004).

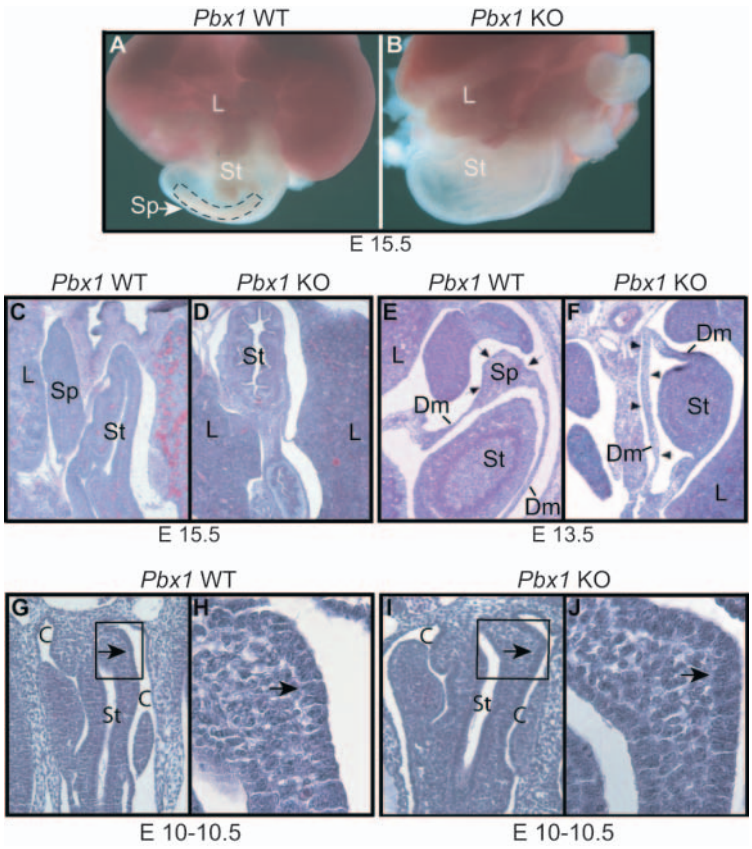

Fig. 1. Agenesis of the spleen in *Pbx1*$^{-/-}$ embryos. (A,B) Comparative whole-mount preparation of upper abdominal organs at E15.5 shows the lack of a spleen, together with liver hypoplasia, in *Pbx1*$^{-/-}$ embryos (B). The spleen forms lateral to the stomach, and appears as a reddish ribbon-shaped organ in wild-type embryos (black dashes in A). (C,D) Histology of Hematoxylin and Eosin-stained transverse sections at E15.5 show agenesis of the spleen in *Pbx1*$^{-/-}$ embryos compared with wild-type littermates. (E,F) Histology of Hematoxylin and Eosin-stained transverse sections of E13.5 wild-type and *Pbx1*$^{-/-}$ embryos. A visible spleen primordium forms as a mesenchymal condensation within the dorsal mesogastrium (Dm, arrows) in wild-type embryos (E). In *Pbx1*$^{-/-}$ littermates (F), no mesenchymal condensation is detectable within the Dm (arrowheads). (G-J) Histology of Hematoxylin-stained transverse sections of wild-type and *Pbx1*$^{-/-}$ embryos in the region of the stomach enlargement at E10-10.5. The splanchnic mesoderm lateral to the stomach enlargement (G,I) consists of a thick epithelial-like plate of cells (arrows) that encloses unorganized mesenchyme. The black boxes indicate the regions magnified (40×) in H,J, which highlight the organized epithelial-like cellular structure of the splanchnic mesoderm. C, coelomic cavity; Dm, dorsal mesogastrium; L, liver; Sp, spleen; St, stomach.

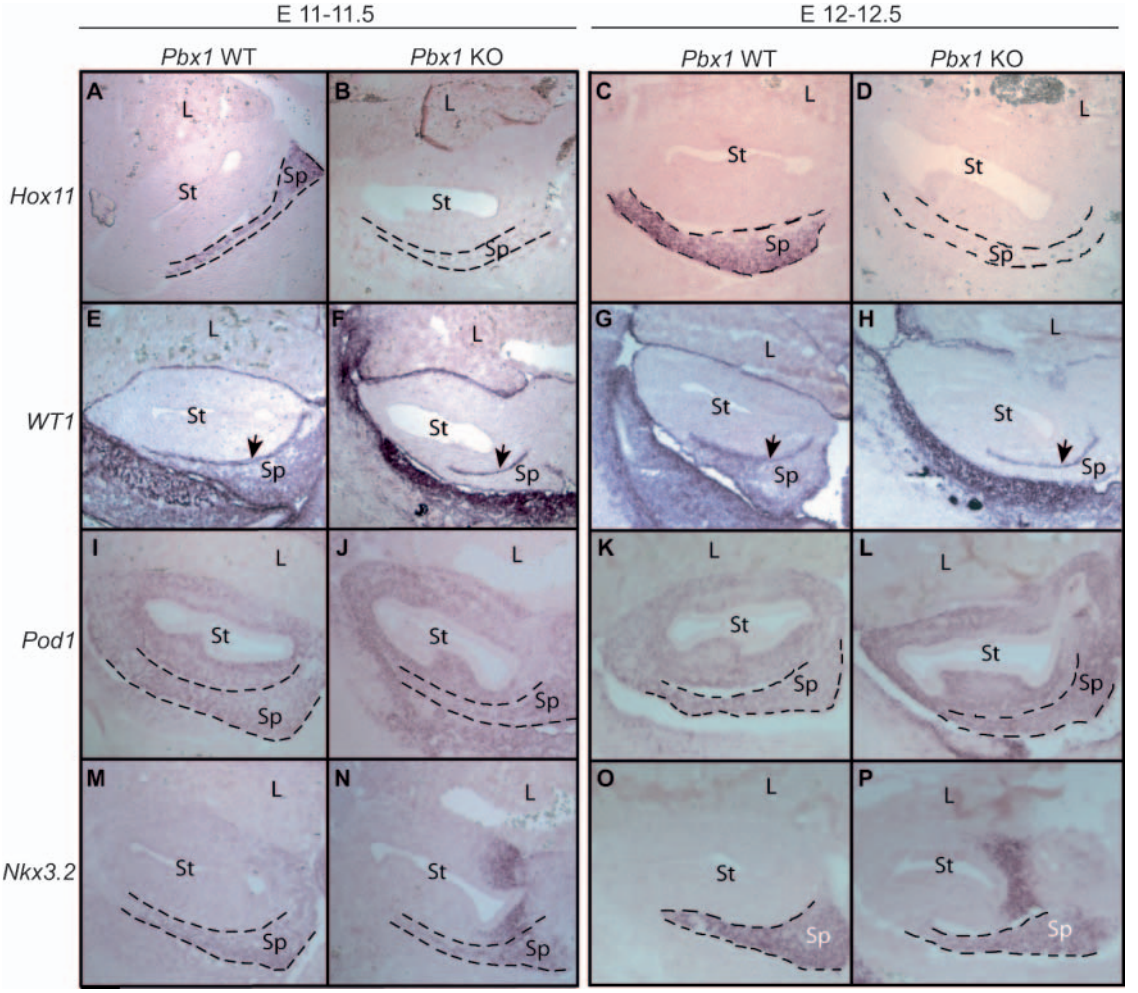

Fig. 2. Perturbed expression of *Hox11* and *Wt1*, known regulators of spleen development, in *Pbx1*$^{-/-}$ embryos. Transcripts for the spleen regulators *Hox11*, *Wt1*, *Pod1* and *Nkx3.2* were detected by in situ hybridization to frozen sagittal sections of wild-type and *Pbx1*$^{-/-}$ upper abdominal organs at E11-11.5 and E12-12.5, as indicated. The splenic primordium is outlined by black dashes. (A-D) Both at E11-11.5 and E12-12.5 *Hox11* expression is absent in the spleen primordia of *Pbx1*$^{-/-}$ embryos, compared with wild-type littermates. (E-H) *Wt1* expression is also absent in spleen anlage of *Pbx1*$^{-/-}$ embryos compared with wild-type littermates both at E11-11.5 and E12-12.5. It is noteworthy that *Wt1* expression is still present in the mesothelial lining that surrounds the mesenchyme of the splenic anlage (arrow). (I-L) *Pod1* and (M-P) *Nkx3.2* are normally expressed within the developing spleen primordia of *Pbx1*$^{-/-}$ embryos compared with wild-type littermates, both at E11-11.5 and E12-12.5. L, liver; Sp, spleen; St, stomach.

Pbx1 is required for onset of *Hox11* (*Tlx1*) and *Nkx2.5* gene expression in the splenic anlage

Visualization of gene transcripts known to be present in the condensing splenic mesenchyme within the Dm was conducted to trace spleen ontogeny at early embryonic stages (E11-12.5) and to establish hierarchical requirements for specific transcription factors in splenic cell fate specification and morphogenesis. Using in situ hybridization, we assessed expression of *Hox11* (Koehler et al., 2000; Kanzler and Dear 2001) (Fig. 2A-D) and *Nkx2.5* (Patterson et al., 2000; Hecksher-Sorensen et al., 2004), which are first observed in Dm mesenchyme between E10.5 and E11 and are regarded as the earliest known markers for splenic cell fate.

By E11, *Nkx2.5* is normally detectable in a domain of the murine Dm (Figs 2 and 3) that overlaps with the expression of *Hox11*, lateral to the stomach (Hecksher-Sorensen et al., 2004). In *Pbx1*$^{-/-}$ embryos, however, *Hox11* and *Nkx2.5* were absent in the condensing splenic mesenchyme of the Dm from E11 to E12.5 (*Hox11*, Fig. 2A-D; *Nkx2.5*, Fig. 3K,L; analysis of *Nkx2.5* at E11-11.5 is not shown). *Hox11* expression was not detected up to E13.5 by in situ hybridization in Dm condensing splenic mesenchyme of *Pbx1*$^{-/-}$ embryos (not shown), indicating a complete absence of *Hox11*, rather than a temporal delay of its expression in the spleen anlage. Thus, *Hox11* expression is completely absent in splenic condensing mesenchyme and later in the spleen anlage. Conversely, *Hox11* expression was unperturbed in other organ primordia, such as the developing pancreas at E12.5 and the branchial arches (not shown). By contrast, no alterations of *Pbx1* expression were observed in the Dm splenic condensing mesenchyme of *Hox11*$^{-/-}$ embryos (Fig. 3A,B). Taken together, these results indicate that the onset and continued expression of *Hox11* in the splenic anlage, unlike in non-splenic domains, is *Pbx1* dependent, whereas *Pbx1* expression is not dependent on the

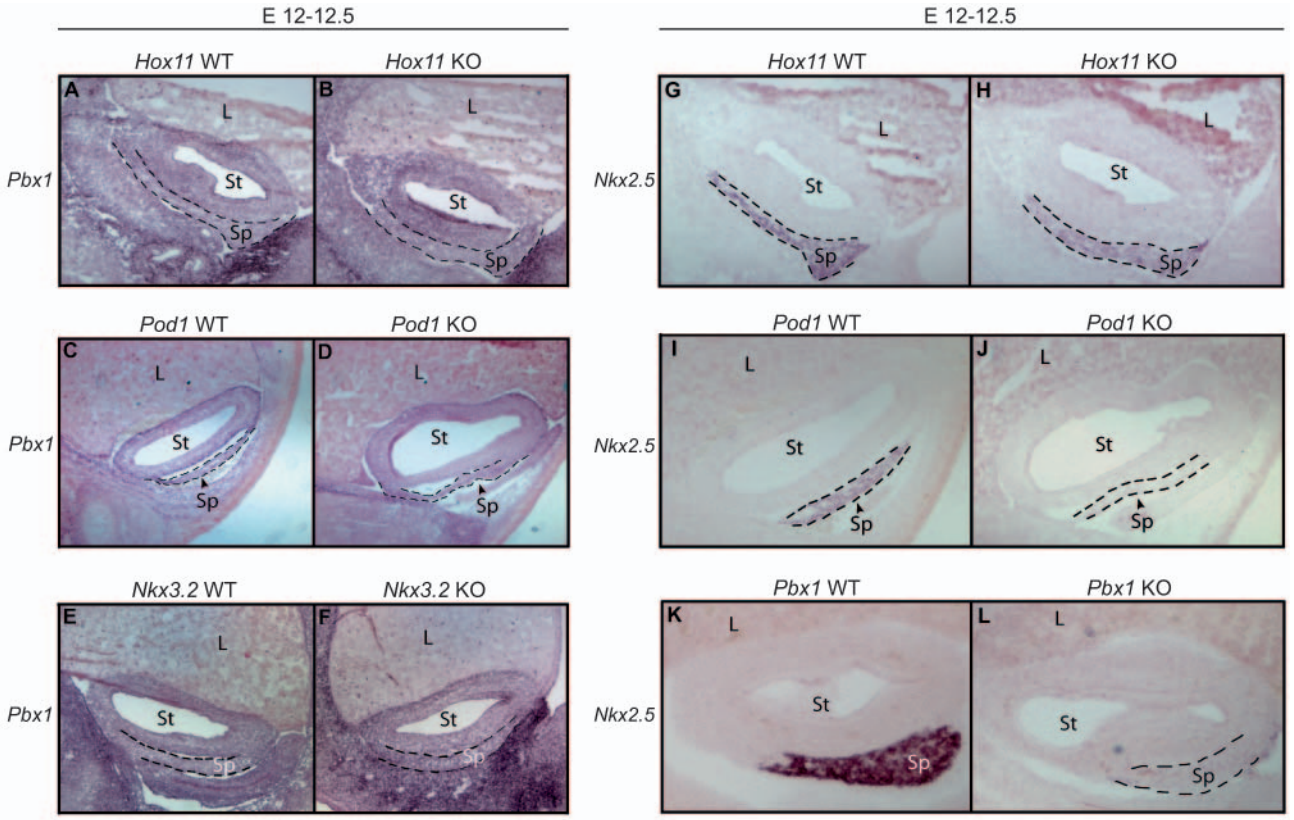

Fig. 3. Analysis of *Pbx1* and *Nkx2.5* gene expression during early spleen organogenesis. In situ hybridization was performed on frozen sagittal sections of E12-12.5 embryonic upper abdominal organs. (A-F) Normal expression of *Pbx1* within the developing splenic anlage of *Hox11*$^{-/-}$, *Pod1*$^{-/-}$ and *Nkx3.2*$^{-/-}$ embryos. (G-L) Perturbed expression of *Nkx2.5* in *Pod1*$^{-/-}$ and *Pbx1*$^{-/-}$ embryos. *Nkx2.5* expression is unperturbed in the developing splenic anlage of *Hox11*$^{-/-}$ embryos (H), while it is absent in *Pod1*$^{-/-}$ (J) and *Pbx1*$^{-/-}$ (L) embryos, compared with their wild-type littermates (G,I,K). L, liver; Sp, spleen; St, stomach.

presence of Hox11 protein. Furthermore, our findings also suggest that, in the absence of *Pbx1*, splenic cell fate specification is, at least in part, compromised, as two of the earliest known markers of splenic cell fate, *Nkx2.5* and *Hox11*, are absent in Dm condensing mesenchyme. Conversely, expression of other genes that mark splenic condensing mesenchyme, such as *Pod1* (Fig. 2I-L) and *Nkx3.2* (Fig. 2M-P) is maintained in *Pbx1*$^{-/-}$ embryos, demonstrating that condensing mesenchymal cells are still present in Dm of *Pbx1*$^{-/-}$ embryos and that splenic gene expression is not globally impaired by *Pbx1* loss.

Pbx1 regulates *Wt1* gene expression in splenic condensing mesenchymal cells, but not in the outer mesothelial lining

Wt1 is expressed during early spleen development in the mouse (Rackely et al., 1993) and is required for spleen development (Herzer et al., 1999). It is normally expressed in a domain of the Dm that, by E11, overlaps with that of *Hox11* within the splenic condensing mesenchyme lateral to the stomach (Fig. 2E,G). *Wt1* is regulated by *Hox11* in spleen development, as its expression is diminished in *Hox11*$^{-/-}$ embryos (Koehler, 2000) (data not shown). In addition, *Wt1*, like *Pbx1*, is highly expressed in the outer mesothelial lining of the splenic anlage (Fig. 2E,G) that will give rise to the splenic capsule (Sadler, 1995). By contrast, *Hox11* is not expressed in the mesothelial lining of the developing spleen (Fig. 4A, arrowhead).

Expression of *Wt1* was severely down regulated in the condensing mesenchyme of *Pbx1*$^{-/-}$ Dm, when compared with wild-type littermates. However, its expression was well maintained in the outer mesothelial lining of the *Pbx1*$^{-/-}$ splenic primordium, both at E11-11.5 and E12-12.5 (Fig. 2E-H). As *Hox11* is absent in *Pbx1*$^{-/-}$ embryos, and *Wt1* is regulated by *Hox11* (Koehler, 2000) (data not shown), these findings suggest a genetic hierarchy whereby *Pbx1* regulates *Hox11*, which in turn regulates *Wt1* in condensing splenic mesenchyme. Nevertheless, while *Wt1* expression in Dm splenic mesenchyme is hierarchically dependent upon *Pbx1*, probably through *Hox11*, its expression is independent of *Pbx1* in the developing splenic capsule.

Pbx1 and *Pod1* independently regulate *Nkx2.5* in the splenic anlage

Expression of *Pod1* (Robb et al., 1998; Lu et al., 2000) (Fig. 2I-L) and *Nkx3.2* (Fig. 2M-P) overlaps with *Hox11* and *Nkx2.5* in the condensing splenic mesenchyme. In addition, gene targeting studies have shown that *Pod1* (Quaggin et al., 1999; Lu et al., 2000) and *Nkx3.2* (Lettice et al., 1999; Tribioli et al., 1999) are required for spleen development. *Pod1* (Fig. 2I-L) and *Nkx3.2* (Fig. 2M-P) expression is maintained in *Pbx1*$^{-/-}$ embryos, and *Pbx1* expression is unperturbed in the

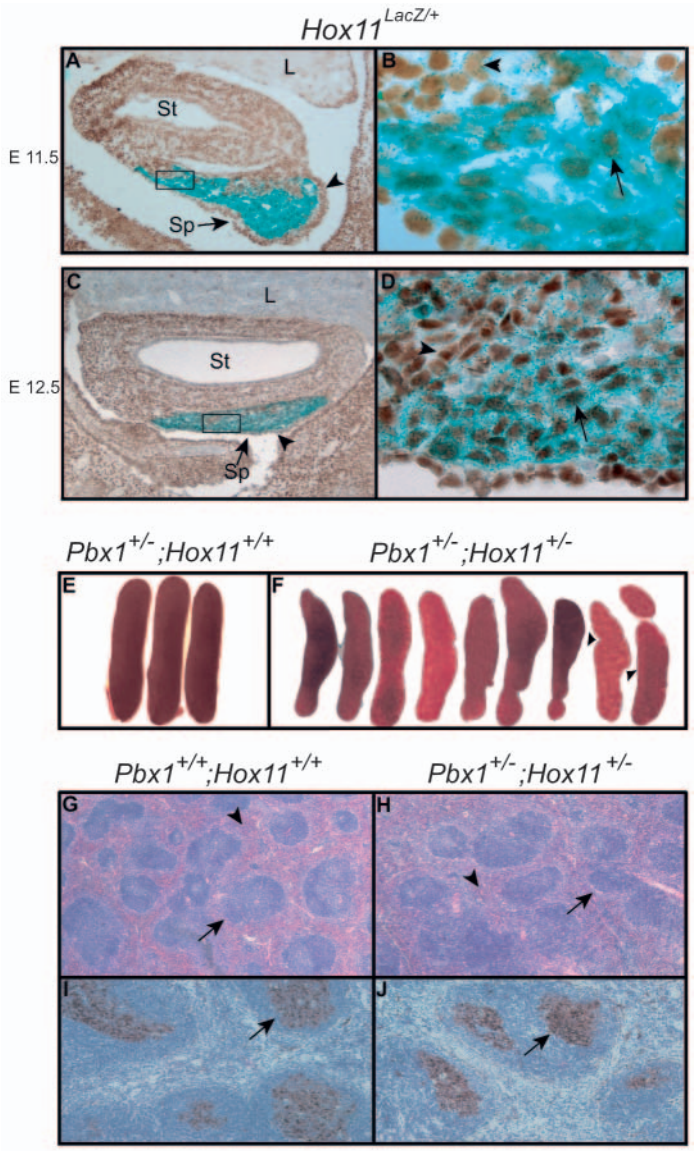

Fig. 4. Genetic interaction of *Pbx1* and *Hox11* during spleen organogenesis. (A-D) Immunostaining of sagittal sections of E11.5 (A,B) and E 12.5 (C,D) $Hox11^{lacZ/+}$ embryonic upper abdominal organs. Arrowheads in A-D indicate cells expressing Pbx1 but not Hox11. Pbx1 is visualized in the splenic anlage with DAB (brown staining), and *Hox11* by β-galactosidase staining (blue). (B,D) Enlargements of the black rectangle depicted on the splenic anlage in A,C. Arrows in B,D indicate cells in which Pbx1 and Hox11 colocalize. (E,F) The spectrum of malformations of double heterozygous spleens (F) is compared with the normal gross morphology of $Pbx1^{+/-}$ spleens (E). The $Pbx1^{+/-};Hox11^{+/-}$ double heterozygous spleens are hypoplastic (F), and display sickle shapes, indentations, tubercles and nodules, as well as fusions of two spleens (arrowheads). All spleens were isolated from 6- to 8-week-old mice. (G,H) Hematoxylin and Eosin-stained spleen sections reveal no abnormalities in splenic structure of $Pbx1^{+/-};Hox11^{+/-}$ double heterozygous mice. Distribution of white (arrows) and red (arrowheads) pulp is normal within the spleen parenchyma of double heterozygous mice (H) compared with $Pbx1^{+/-}$ (not shown), $Hox11^{+/-}$ (not shown) and wild-type (G) littermates. (I,J) PNA-stained spleen sections of mice immunized with sheep red blood cells (SRBC). Formation of germinal centers (GC; arrows), as indicated by PNA staining (brown), appears normal in $Pbx1^{+/-};Hox11^{+/-}$ double heterozygous mice (J) compared with $Pbx1^{+/-}$ (not shown), $Hox11^{+/-}$ (not shown) and wild-type (I) littermate controls. L, liver; Sp, spleen; St, stomach.

condensing mesenchyme of both $Pod1^{-/-}$ (Fig. 3C,D) and $Nkx3.2^{-/-}$ (Fig. 3E,F) embryos at E12-12.5. Thus, *Pbx1* expression in Dm condensing mesenchyme is not dependent on the presence of either Pod1 or Nkx3.2. We also confirm that *Nkx3.2* is expressed in the condensing mesenchyme of $Pod1^{-/-}$ embryos at E12-12.5 (see Fig. S1A,B in the supplementary material), as already reported by others (Lu et al., 2000). Additionally, $Pod1^{-/-}$ expression is unperturbed in $Nkx3.2^{-/-}$ embryos at E12-12.5 (see Fig. S1C,D in the supplementary material). Thus, the *Pod1* and *Nkx3.2* pathways appear to be separate in early spleen development. Furthermore, our present studies (Fig. 2A-D) and work by Lettice et al. (Lettice et al., 1999) reveal that *Pbx1* and *Nkx3.2* independently regulate *Hox11* in a hierarchical fashion. Therefore, *Hox11* expression is dependent on both Pbx1 (Fig. 2A-D) and Nkx3.2 (Lettice et al., 1999). Moreover, no expression of *Nkx2.5* was observed in the condensing splenic mesenchyme of $Pod1^{-/-}$ or $Pbx1^{-/-}$ embryos (Fig. 3I,J; K,L). Conversely, we demonstrated that *Nkx2.5* is still expressed in the condensing splenic mesenchyme of $Nkx3.2^{-/-}$ embryos (see Fig. S1E,F in the supplementary material) at E12-12.5. Thus, *Nkx2.5* expression is dependent on both Pbx1 (Fig. 3K,L) and Pod1 (Fig. 3I,J), although it is not dependent on Nkx3.2 (see Fig. S1E,F in the supplementary material). Taken together, these results suggest that *Pbx1* impinges on the control of the separate *Pod1* and *Nkx3.2* pathways, both essential for spleen development, by functioning upstream of *Hox11* and *Nkx2.5*, respectively.

Genetic interaction of *Pbx1* and *Hox11* during spleen organogenesis

Given the dependence of *Hox11* expression on Pbx1, the potential colocalization of these two transcription factors was assessed in splenic mesenchymal cells. Sections from E11.5 and E12.5 $Hox11^{lacZ/+}$ embryos (in which the *lacZ* gene was inserted into the *Hox11* locus) (Dear et al., 1995) were immunostained with an anti-Pbx1b monoclonal antibody (Jacobs et al., 1999) and simultaneously stained for β-galactosidase activity. In these mice, *lacZ* expression faithfully recapitulates expression of *Hox11* in the splenic anlage (Kanzler and Dear, 2001). Pbx1 (visualized by brown staining) and Hox11 (visualized by blue staining) were present and colocalized (Fig. 4B,D; arrow) in cells of the condensing splenic mesenchyme. However, not all Pbx1-positive cells expressed *Hox11* (Fig. 4B,D; arrowhead), which was notably absent in the thickened mesothelial lining that surrounds the mesenchyme of the splenic anlage (Fig. 4A,C; arrowhead).

Given the requirement for both *Hox11* (Roberts et al., 1994; Dear et al., 1995) and *Pbx1* in spleen development and their cellular colocalization in the splenic anlage, their potential genetic interaction during spleen ontogeny was assessed. $Pbx1^{+/-}$ and $Hox11^{+/-}$ mice were intercrossed and offspring were examined at 6 to 8 weeks of age (Table 1). A high percentage (80%) of $Pbx1^{+/-};Hox11^{+/-}$ double heterozygous mice displayed hypoplastic and malformed spleens, compared with wild-type or single heterozygous littermates (Fig. 4E,F; Table 1), of which a very low percentage exhibited splenic

Table 1. Genetic interaction of *Pbx1* and *Hox11* in vivo during spleen organogenesis

Spleen phenotype	*Pbx1*$^{-/-}$; *Hox11* wild type	*Pbx1* wild type; *Hox11*$^{-/-}$	*Pbx1* wild type; *Hox11* wild type	*Pbx1*$^{+/-}$; *Hox11* wild type	*Pbx1* wild type; *Hox11*$^{+/-}$	*Pbx1*$^{+/-}$; *Hox11*$^{+/-}$
Asplenia	100%	100%	0%	0%	0%	0%
Spleen malformations			4% Indentations	9% Indentations	6% Indentations	80% Sickle form, indentations, tubercles, nodules, protuberances, fusion of two spleens

Percentage of mice exhibiting the indicated spleen phenotype. Data are from 30-60 mice (6 to 8 weeks old) of each genotype.

morphological abnormalities such as minor indentations (Table 1). The spectrum of malformations of double heterozygous spleens (Fig. 4F) comprised sickle shapes, presence of indentations, tubercles and nodules, as well as fusions of two spleens (polysplenia). Thus, *Pbx1* and *Hox11* genetically interact in spleen development. Despite the observed morphological abnormalities, *Pbx1*$^{+/-}$;*Hox11*$^{+/-}$ double heterozygous mice exhibited normal splenic architecture (Fig. 4G,H), germinal center (GC) (Dent et al., 1997) formation (Fig. 4I,J) and primary immune function (not shown).

Mesenchymal cell proliferation is similarly impaired in the splenic anlage of embryos that lack *Pbx1* or *Hox11*

Early expansion of the splenic anlage, prior to hematopoietic invasion at E14.5 (Sasaki and Matsumura, 1988), is dependent primarily on proliferation of splenic mesenchymal progenitor cells. Evaluation of mesenchymal cell proliferation by BrdU in vivo labeling showed a marked reduction in the percentage of S-phase cells in *Pbx1*$^{-/-}$ splenic mesenchyme compared with wild-type controls at E13.5 (Fig. 5A,B). No significant reduction of S-phase cells in *Pbx1*$^{-/-}$ splenic mesenchyme was found at E12.5 (not shown). Conversely, no detectable increase of apoptosis was found in *Pbx1*$^{-/-}$ splenic mesenchyme by TUNEL assay (Gavrieli et al., 1992), either at E12.5 or at E13.5 (not shown). Taken together, these results indicate that although loss of *Pbx1* does not affect mesenchymal cell survival, by E13.5 it severely affects cell proliferation and, therefore, expansion of the splenic anlage.

Given the in vivo genetic interaction of *Pbx1* and *Hox11* in spleen development and their cellular colocalization, BrdU in vivo labeling was also performed in E13.5 *Hox11*$^{-/-}$ embryos. The splenic anlage of *Hox11*$^{-/-}$ embryos (Fig. 5C,D) was remarkably similar to that of *Pbx1*$^{-/-}$ embryos (Fig. 5A,B), with a reduction in the percentage of S-phase cells of ~50% in both mutants. Interestingly, the splenic primordium of E13.5 *Hox11*$^{-/-}$ embryos, unlike that of *Pbx1*$^{-/-}$ embryos, also exhibited a modest increase in apoptosis, mostly localized to the mesothelium surrounding the mesenchyme of the splenic primordium (not shown). In sum, loss of either *Pbx1* or *Hox11* presents a comparable phenotype, affecting the

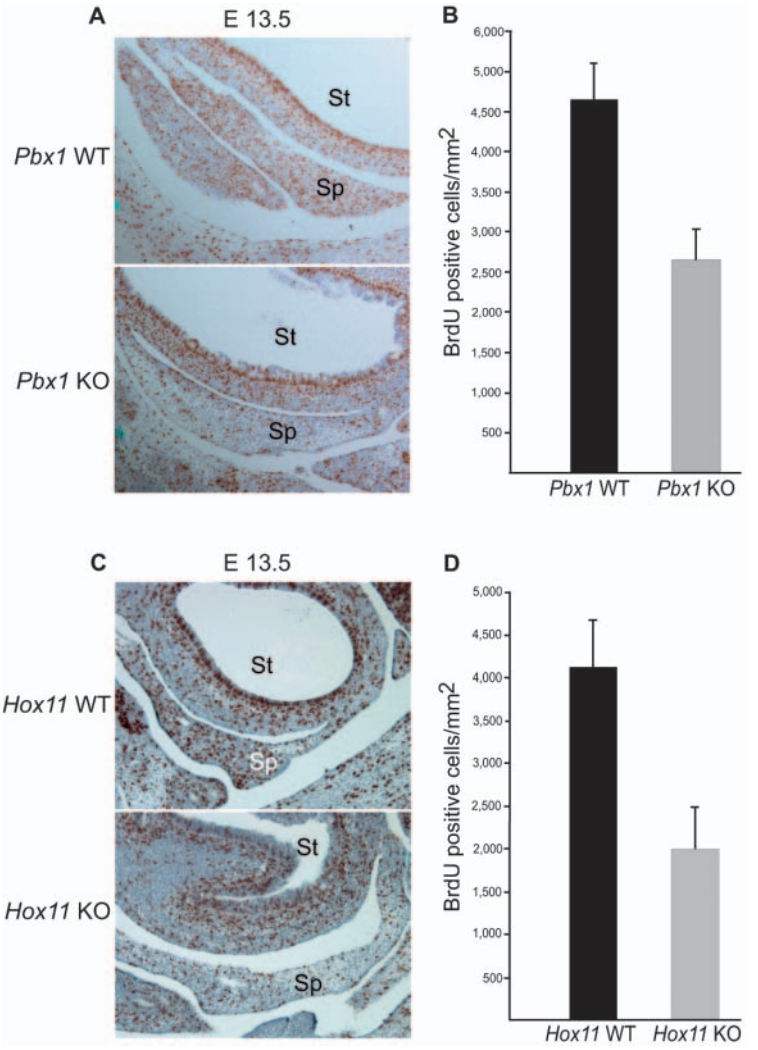

Fig. 5. Similar reduction of spleen progenitor cell proliferation in *Pbx1*$^{-/-}$ and *Hox11*$^{-/-}$ embryos. (A,C) BrdU in vivo labeling of sagittal sections of upper abdominal organs shows a striking difference in the percentage of BrdU-positive nuclei (brown) within the spleen anlage of *Pbx1*$^{-/-}$ and *Hox11*$^{-/-}$ embryos, compared with wild-type littermates at E13.5. (B,D) Proliferation of spleen mesenchymal progenitors is reduced by ~50% in *Pbx1*$^{-/-}$ and *Hox11*$^{-/-}$ embryos, respectively, when compared with wild-type littermates. The results are expressed as total BrdU-positive cells per mm^2 of spleen anlage. Data are mean±s.e.m. of four E 13.5 embryos analyzed for each genotype. Black bars, wild type; grey bars, *Pbx1*$^{-/-}$ and *Hox11*$^{-/-}$ embryos; Sp, spleen; St, stomach.

proliferation of mesenchymal splenic progenitor cells and preventing normal expansion of the splenic anlage.

Hox11 is a direct in vivo target of Pbx1 in spleen ontogeny

To investigate the possibility that Pbx1 may directly regulate *Hox11* expression, sequences of the *Hox11* promoter that are conserved between the mouse and human genes were examined for Pbx-binding sites. A potential Pbx-binding site (PX1) was identified within the 540 bp *Hox11* region that displays promoter activity (Arai et al., 1997), as indicated in Fig. 6A. To determine if the PX1 element could support the formation of a Pbx1 DNA-binding complex, EMSA assays were performed using nuclear extracts from primary embryonic spleen cells. A slow-migrating band containing Pbx1, as demonstrated by its specific competition with an anti-Pbx1 antibody, was observed to form on an oligonucleotide containing the PX1 site (Fig. 6B: left panel, lanes 1 and 2).

Hox11 was also present in this DNA-binding complex, as indicated by the finding that an anti-Hox11 antibody inhibited complex formation (Fig. 6B: right panel, lane 2). The complex was not observed to form on a PX1 oligo mutated within the Pbx1 site (mPX1) (Fig. 6B: middle panel, lane 1), confirming the specificity of Pbx1-Hox11 binding. Additionally, complex formation was inhibited by excess unlabeled wild-type PX1 oligo, but not by its mutated form mPX1 (not shown), further confirming binding specificity. These results demonstrate that an endogenous Pbx1-Hox11 heterodimer assembles on the PX1 site of the *Hox11* promoter in vitro.

Possible in vivo *Hox11* promoter-specific binding by Pbx1 and Hox11 was examined by chromatin immunoprecipitation (ChIP) performed on primary cell cultures established from murine embryonic spleens at E16. These primary murine spleen

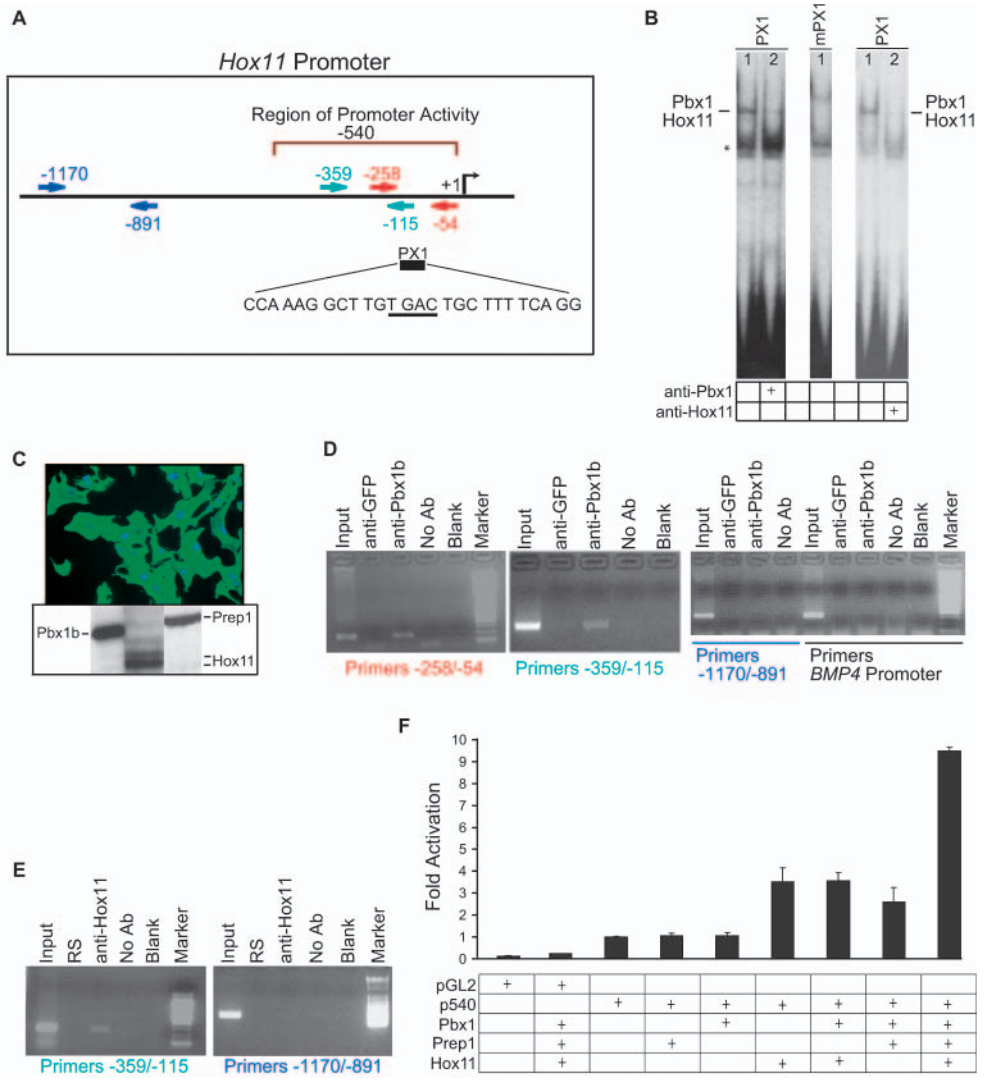

Fig. 6. Recruitment of Pbx1 and Hox11 to the mouse *Hox11* promoter. (A) Schematic illustration of 1.2 kb of the *Hox11* genomic segment with known promoter activity that contains Pbx1-binding sites, and a 5′ upstream region. Primers used for PCR analysis are indicated by arrows (each pair in a different color) and the oligoprobe (PX1) used for EMSA is indicated by a black box. (B) Binding of Pbx1 and Hox11 to the (PX1) oligo within the *Hox11* promoter. Nuclear extracts derived from wild-type primary embryonic spleen cells were subjected to EMSA with a radiolabeled PX1 probe containing a *Pbx1* wild-type (PX1) or mutated (mPX1) core binding site (underlined), as indicated above gel lanes. Asterisk indicates non-specific band. (C) Primary cells isolated from embryonic spleen stained in culture for the mesodermal marker α smooth muscle actin (green fluorescence). Western blot analysis (panel below) demonstrates that these cells produce Pbx1, Prep1 and Hox11 proteins. Two isoforms of Hox11 are present in embryonic spleen, as indicated (Yamamoto et al., 1995). (D,E) For ChIP analysis, chromatin was subjected to immunoprecipitation (IP) using antibodies specific for Pbx1b (anti-Pbx1b) (D) or Hox11 (anti-Hox11) (E). As negative controls, IPs were also performed with an anti-GFP antibody, rabbit serum (RS) or no antibody (No Ab). A primer pair that amplifies a region within the Bmp4 promoter was used as an additional negative control. (F) Synergistic activation of the *Hox11* promoter by Pbx1, Prep1 and Hox11 proteins. Luciferase activity was assayed from transiently transfected NIH 3T3 cells. Co-transfection assays were performed in the presence (+) of the indicated expression vectors encoding Pbx1, Prep1 or Hox11, and with a vector containing the promoter regulatory region of *Hox11* (p540), or with vector alone (p-GL2). Data are expressed as the fold activation over the p540 basal luciferase activity. Bars represent the mean of three independent transfections (performed in duplicate)±s.e.m. normalized for β-galactosidase activity (internal control) within each experiment.

cultures, which uniformly expressed the mesodermal marker alpha-smooth muscle actin (green fluorescence; Fig. 6C: top panel), exhibited high levels of Pbx1b, Hox11 and the Meinox co-factor protein Prep1, as detected by western blot analysis (Fig. 6C: bottom panel). The binding of Pbx1 and Hox11 was examined at the region of the *Hox11* promoter (Arai et al., 1997; Fig. 6A) that contains the PX1 site, where assembly of the Pbx1-Hox11 heterodimer had been detected by EMSA. Two different pairs of primers within this region (spanning regions –258 to –54, depicted in red, and –359 to –115, depicted in teal) amplified sequences within the *Hox11* promoter that had been immunoprecipitated by the anti-Pbx1b antibody (Fig. 6D: left and middle panels). As a control for Pbx1b antibody specificity, two different sets of primers (one on the *Hox11* promoter: –1170 to –891; depicted in blue; and one on the *Bmp4* promoter) did not amplify their respective intervening sequences after immunoprecipitation (Fig. 6D: right panel).

The potential binding of Hox11 at its own promoter in vivo in embryonic murine spleen cells was also examined by ChIP assay. Similar to Pbx1, Hox11 was present at the region of *Hox11* promoter activity (Fig. 6E), as detected by both primer pairs that revealed Pbx1 on the *Hox11* promoter (Fig. 6E: left panel and data not shown). Overall, these results indicate recruitment of both Pbx1 and Hox11 on the *Hox11* promoter in vivo in spleen embryonic cells. Thus, *Hox11* is a direct target of Pbx1. The simultaneous binding of Hox11 to its own promoter suggests that it may contribute to an auto-regulatory circuit in spleen development.

Hox11 autoregulates its own promoter with Pbx1

The functional consequences of potential interactions of Pbx1 and Hox11 for *Hox11* expression were tested in transient transcription assays using NIH 3T3 fibroblasts. For these studies, we employed a luciferase reporter construct containing the promoter regulatory region of *Hox11* (p540) (Arai et al., 1997), which spans the PX1 site. When the p540 reporter gene was co-transfected with the Pbx1 construct no activation above background was observed (Fig. 6F). Co-expression of Pbx1 and a representative Meinox family protein, Prep1 (highly expressed in spleen mesenchyme; Fig. 6C: bottom panel), resulted in transcriptional activation of two- to threefold above background levels (Fig. 6F). Significantly, co-transfection of Hox11 with Pbx1 and Prep1 resulted in an eight- to ninefold increase in transcription above the baseline (Fig. 6F). These results demonstrate that synergistic activation of the *Hox11* promoter is achieved by the association of the three homeodomain proteins, consistent with a Pbx1-dependent auto-regulatory role for Hox11 to enhance and/or maintain its own expression during spleen development.

Discussion

In this report we investigated the genetic and transcriptional control of cell fate specification, morphogenesis and expansion of the spleen, using various asplenic mouse models. Our results establish a genetic network that regulates spleen ontogeny and is dependent upon *Pbx1*, which encodes a TALE class homeodomain protein that serves a reiterative role in early splenic morphogenesis as well as later anlage expansion. A crucial part of the Pbx1 role in spleen development is due to its genetic and transcriptional interaction with Hox11, which autoregulates its own promoter with Pbx1 in spleen progenitor cells. However, *Pbx1* genetically regulates key genes downstream of both *Nkx3.2* and *Pod1*, which we demonstrate to control spleen development via separate pathways. These results reveal a broad and crucial role for *Pbx1* as a central hierarchical co-regulator in spleen genesis.

Loss of *Pbx1* does not affect LR decisions or development of the splanchnic mesoderm

Regional perturbations of LR asymmetry have been associated with spleen agenesis in *Dh* spontaneous mutants and *Nkx3.2*$^{-/-}$ embryos (Hecksher-Sorensen et al., 2004). However, our studies demonstrate that asplenia in *Pbx1*$^{-/-}$ embryos is not the result of perturbations of LR asymmetry, providing further evidence that asplenia is not always associated with LR asymmetry defects. This notion is best supported by the presence of spleen agenesis as the sole organ abnormality, without perturbations of LR asymmetry, in mice that lack *Pod1* (Quaggin et al., 1999) or *Hox11* (Roberts et al., 1994; Dear et al., 1995) (N. Dear, personal communication). Similarly, *Pbx1*$^{-/-}$ embryos do not exhibit anomalies of LR asymmetry in the heart (C. P. Chang, unpublished), lungs (B. Hogan, personal communication), liver or stomach (data not shown). Although the *Pbx1*$^{-/-}$ pancreas displays an anteroposterior (AP) patterning defect, it does not present LR asymmetry abnormalities (Kim and Selleri et al., 2002).

Furthermore, at E10-10.5, the architecture of the splanchnic mesoderm, which normally exhibits an epithelial-like cellular organization, remains well preserved in *Pbx1*$^{-/-}$ embryos. This contrasts with *Dh* mutant embryos, in which the splanchnic mesoderm is replaced by unorganized mesenchyme, and *Nkx3.2*$^{-/-}$ embryos, in which it is in part defective (Green, 1967; Hecksher-Sorensen et al., 2004). Thus, loss of *Pbx1* does not result in perturbations of very early developmental choices, such as LR position and differentiation of the splanchnic mesoderm.

Reiterative requirement for Pbx1 in early splenic morphogenesis and later anlage expansion

In vertebrates *Pbx1* is a key developmental regulator required for the ontogeny of most organ systems (Selleri et al., 2001). In this study, we demonstrate that *Pbx1* is reiteratively required in spleen development. First, it affects, at least in part, the fate of condensing mesenchymal cells during early organogenesis, at E11-11.5, as demonstrated by the absence of *Nkx2.5* and *Hox11* in *Pbx1*$^{-/-}$ Dm. Therefore, *Pbx1* genetically regulates both *Hox11* and *Nkx2.5* in early spleen morphogenesis. *Nkx2.5* is regarded as one of the earliest known markers for spleen progenitor cells (Patterson et al., 2000), and its expression overlaps with *Hox11*, although its requirement in spleen genesis has not yet been shown since *Nkx2*.5 loss causes lethality in utero at E9-10, before development of the splenic anlage (Lyons et al., 1995). Second, at later stages of organogenesis, by E13.5, *Pbx1* is required again for splenic progenitor cell proliferation, as detected by BrdU incorporation experiments, underscoring the essential role of *Pbx1* in organ expansion. Thus, *Pbx1* exhibits temporally distinct roles in spleen ontogeny, reminiscent of its dual contributions to skeletal development (Selleri et al., 2001).

Regulation of another marker of early splenic anlage, *Wt1*, is also dependent on *Pbx1*, possibly through regulation of *Hox11*, in the splenic condensing mesenchyme. It is of interest

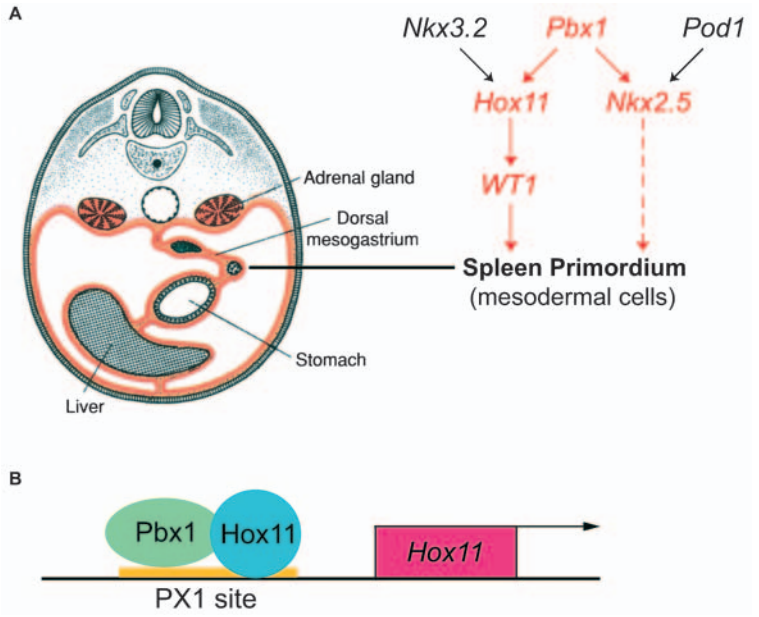

Fig. 7. *Pbx1*-dependent genetic network and transcriptional pathway regulate spleen ontogeny. (A) Transverse section of abdominal organs schematically illustrates the location of the spleen primordium during vertebrate development. Adapted, with permission, from Sadler (Sadler, 1995). To the right, the Pbx1-Hox11 transcriptional hierarchy regulating spleen ontogeny is depicted, with the *Pbx1*-dependent pathway in red. The *Nkx2.5* downstream pathway is illustrated with a broken arrow as the requirement for *Nkx2.5*, an early marker of splenic progenitor cells, has not yet been demonstrated in spleen development. (B) Within the Pbx1-Hox11 transcriptional pathway, Pbx1 directly regulates *Hox11* in spleen progenitor cells and Hox11, in turn, regulates its own promoter together with Pbx1.

that regulation of *Wt1* expression is independent of *Pbx1* in the outer mesothelial lining of the splenic anlage, which normally does not express *Hox11*, and will give rise to the splenic capsule. Taken together, these results support a scenario where *Pbx1* regulates *Hox11*, which in turn regulates *Wt1* in the splenic mesenchyme, whereas in the mesothelial lining of the developing spleen regulation of *Wt1* is uncoupled from *Pbx1*. Thus, *Pbx1* can be considered as the uppermost known genetic regulator within the *Hox11-Wt1* pathway in the non-mesothelial splenic mesenchyme (Fig. 7A).

Expression of other genes that mark early condensing splenic mesenchyme, such as *Pod1* and *Nkx3.2*, is well maintained in $Pbx1^{-/-}$ embryos, demonstrating that splenic condensing mesenchymal cells are still present in Dm of $Pbx1^{-/-}$ embryos and splenic gene expression is not globally impaired by *Pbx1* loss. Thus, requirement for *Pod1* and *Nkx3.2* in spleen ontogeny is not dependent upon Pbx1. Finally, the findings by Lu et al. (Lu et al., 2000), confirmed by our present studies, that *Nkx3.2* is expressed in the condensing mesenchyme of $Pod1^{-/-}$ embryos at E12-12.5, and our results that $Pod1^{-/-}$ expression is unperturbed in $Nkx3.2^{-/-}$ embryos, indicate that the Pod1 and Nkx3.2 transcription factors use separate pathways to regulate early spleen development.

Although previous work has demonstrated asplenia in *Hox11* mutants (Roberts et al., 1994; Dear et al., 1995), until now the cellular basis of this defect was mostly unknown. Indeed, apoptosis was not detected in $Hox11^{-/-}$ splenic primordium in a previous study (Roberts et al., 1995), while it was documented in another report that used a different *Hox11*-deficient model (Dear et al., 1995). In the present study, we observed a modest increase of apoptosis in $Hox11^{-/-}$ splenic primordium. The apoptotic cells were mostly localized to the outer mesothelial lining of the splenic anlage, which gives rise to the spleen capsule and does not normally express *Hox11*. Thus, it appears that such a subtle increase of apoptosis cannot be responsible for the complete lack of spleen development in *Hox11*-deficient embryos. Conversely, our finding that, by E13.5, $Hox11^{-/-}$ spleen progenitor cells exhibit a marked defect in cellular proliferation comparable with that in $Pbx1^{-/-}$ embryos is consistent with the hypoplasia of $Hox11^{-/-}$ splenic anlage (Roberts et al., 1994; Dear et al., 1995) and the demonstrated involvement of *Hox11* in cellular proliferation and cell cycle control (Kawabe et al., 1997; Hough et al., 1998; Owens et al., 2003). Finally, the finding of a common cellular defect (i.e. impaired progenitor cell proliferation) in spleen development of $Pbx1^{-/-}$ and $Hox11^{-/-}$ embryos further corroborates the observation that *Pbx1* genetically regulates *Hox11*.

In sum, the requirement for *Pbx1* in spleen ontogeny appears to be reiterative. This reiterative role can account for the complete absence of the spleen, which would not otherwise be explained either by a partial impairment of splenic cell fate specification or by a 50% decrease in progenitor cellular proliferation in the splenic anlage, but probably results from the summation of these defects.

Genetic interaction of *Pbx1* and *Hox11* in spleen ontogeny

The finding of a high percentage (80%) of $Pbx1^{+/-};Hox11^{+/-}$ double heterozygous mice displaying severely hypoplastic and malformed spleens (Fig. 4E,F; Table 1), compared with single heterozygotes, demonstrates that *Pbx1* and *Hox11* genetically interact in vivo in spleen development. The wide spectrum of malformations of $Pbx1^{+/-};Hox11^{+/-}$ double heterozygous spleens, which includes fusions of two spleens, mimics polysplenia, a human congenital condition. In polysplenia two or more splenic masses, hypoplastic and irregularly shaped (splenules), are present lateral to the stomach (Lodewyk et al., 1972). Unlike human asplenia, which involves life-threatening infections in children (Waldman et al., 1977), polysplenia is associated with normal splenic function (Lodewyk et al., 1972). Despite their morphological abnormalities, $Pbx1^{+/-};Hox11^{+/-}$ double heterozygous mice exhibit normal splenic architecture, germinal center formation and primary immune function (not shown), thus closely modeling the human polysplenic condition.

Hox11 is a direct in vivo target of Pbx1 and auto-regulates its own promoter with Pbx1 in spleen ontogeny

Despite the growing understanding of Hox and TALE homeoprotein functions in development (Krumlauf, 1994; Mann and Affolter, 1998; Popperl et al., 2000; Selleri et al.,

2001; Waskiewicz et al., 2002; Hisa et al., 2004; Selleri et al., 2004) and their functional interactions (Popperl et al., 1995; Maconochie et al., 1997; Jacobs et al., 1999; Ferretti et al., 2000; Manzanares et al., 2001; Samad et al., 2004), to date, only a few direct target genes have been reported (Rauskolb et al., 1993; Graba et al., 1997; Bromleigh and Freedman, 2000; Theokli et al., 2003). In this study, we provide the first in vivo evidence that *Pbx1* directly regulates *Hox11* in embryonic spleen cells. Interestingly, at E9.5, *Pbx1* is already expressed in the mid-gut mesenchyme (Schnabel et al., 2001), from which the spleen is derived, well before the onset of *Hox11* expression (Kanzler and Dear, 2001). And indeed, *Pbx1* controls the onset of *Hox11* expression at E11 within the Dm, as demonstrated by our in situ hybridization experiments. Although *Pbx1* expression starts to decrease in the splenic anlage after E13.5 (data not shown), *Hox11* persists in the spleen until birth (Kanzler and Dear, 2001), suggesting that Pbx1 is required for the onset of *Hox11* expression and for its continued expression in early spleen development, until E13.5, although it is not necessary for *Hox11* maintenance in later phases of organogenesis.

Hox11 is one of the earliest known markers for spleen cell progenitors (Dear et al., 1995). A useful tool to monitor *Hox11* transcription in the developing spleen is provided by $Hox11^{lacZ}$ mice (Dear et al., 1995), in which *lacZ* expression is dependent on *Hox11* regulatory sequences and faithfully recapitulates *Hox11* expression. Analysis of $Hox11^{lacZ/lacZ}$ embryos previously demonstrated that *lacZ* expression is normally initiated in the absence of *Hox11* (Dear et al., 1995), suggesting that the Hox11 protein is not required for initiation of its own transcription in the splenic mesenchyme. These findings indicate that other factors might be necessary for the onset of *Hox11* transcription. Here, we identify Pbx1 as one such factor that activates *Hox11* transcriptional onset and early expression in the splenic anlage until E 13.5.

Pbx1 and Hox11 bind to a potential Pbx-binding site (PX1) within the *Hox11* promoter, as shown by EMSA assays conducted on embryonic spleen primary cells. Additionally, Pbx1 and Hox11 bind the *Hox11* promoter in vivo in embryonic spleen cells, as revealed by ChIP assays. Regulatory interactions of Hox genes, such as the induction of *Hoxb1* segmental expression by Hoxb1 and Hoxa1 through auto- and cross-regulatory loops, have been documented in developmental processes (Popperl et al., 1995; Studer et al., 1996; Studer et al., 1998). Here, we reveal an autoregulatory loop for an orphan Hox gene, *Hox11*, which is non-clustered but bears a hexapeptide motif (Shen et al., 1996). Taken together, our findings establish that *Hox11* is a direct target of Pbx1 and that, simultaneously, it regulates its own promoter. Significantly, co-transfection of Hox11 with Pbx1 and Prep1 resulted in a striking increase in transcription above baseline, demonstrating that synergistic activation of the *Hox11* promoter is achieved by the association of the three homeoproteins, consistent with a Pbx1-dependent autoregulatory role for Hox11 to enhance and/or maintain, at least in part, its own expression during spleen development. Interestingly, additional potential Pbx-Meinox-binding sites were identified within the *Hox11* promoter downstream of the PX1 site (not shown), suggesting that multiple binding sites within close proximity might be used, simultaneously or at different times, for a complex, multi-faceted transcriptional regulation of *Hox11* in spleen development.

Establishment of a *Pbx1*-dependent genetic and transcriptional network that regulates spleen ontogeny

In addition to establishing that *Pbx1* is the most upstream known direct regulator of Hox11 in spleen ontogeny (Fig. 7A), our studies demonstrate an even broader role for *Pbx1* in spleen development. *Pbx1* regulates key genes downstream of *Nkx3.2* and *Pod1*, which we show to control spleen development through separate genetic pathways (Fig. 7A). Both *Nkx3.2* (Lettice et al., 1999; Tribioli et al., 1999) and *Pod1* (Quaggin et al., 1999; Lu et al., 2000) are essential for spleen development, and their expression in condensing splenic mesenchyme overlaps with *Hox11* and *Nkx2.5*. The specific mechanisms and cellular behaviors by which the Nkx3.2 transcription factor regulates spleen development are as yet unknown, while Pod1 has been proposed to control splenic cell survival (Lu et al., 2000). Interestingly, Lu et al. have reported that the spleen primordium of $Pod1^{-/-}$ embryos does not further expand after E12.5 and starts to undergo apoptotic cell death. As a result, after E12.5, expression of all splenic markers, including *Nkx3.2*, disappears from the degenerating splenic primordium of $Pod1^{-/-}$ embryos (Lu et al., 2000). Our studies demonstrate that *Pbx1* expression is not dependent on the presence of either Nkx3.2 or Pod1 in the splenic mesenchyme (Fig. 7A). Likewise, the requirement for both of these transcription factors in splenic development is *Pbx1* independent (Fig. 7A). In addition, our findings that *Nkx3.2* is expressed in the condensing mesenchyme of $Pod1^{-/-}$ embryos at E12-12.5, and that *Pod1* expression is also unperturbed in $Nkx3.2^{-/-}$ embryos, indicate that the Pod1 and Nkx3.2 transcription factors use separate pathways to regulate early spleen development.

Furthermore, our studies reveal that *Pbx1* and *Nkx3.2* independently regulate *Hox11* in a hierarchical fashion (Fig. 2M-P; Fig. 3E,F) (Lettice et al., 1999). And, in a similar scenario, *Pbx1* and *Pod1*, but not *Nkx3.2* (see Fig. S1E,F), independently control *Nkx2.5* gene expression in a hierarchical fashion (Fig. 7A). Thus, Pbx1 impinges on the separate *Nkx3.2* and *Pod1* pathways by genetically regulating key players in both of these pathways, i.e. *Hox11* and *Nkx2.5* (Fig. 7A). As a result, *Pbx1* emerges as a central hierarchical co-regulator in spleen ontogeny (Fig. 7A). It will be of interest to determine the roles of additional transcription factors required for spleen development, such as Sox11 (Sock et al., 2004) and Nkx2.3 (Pabst et al., 1999; Wang et al., 2000; Tarlinton et al., 2003), within the genetic pathways established by our study.

In conclusion, we demonstrate here the essential role of the Pbx1-Hox11 transcriptional pathway in spleen ontogeny. We provide evidence that *Pbx1* is reiteratively required during spleen development, as it is implicated, at least in part, in splenic cell fate specification and morphogenesis, and then is essential again, later in organogenesis, for anlage expansion through control of progenitor cell proliferation. Finally, we demonstrate that spleen ontogeny is dependent on the orchestration of a complex network of transcription factors, among which Pbx1 emerges as a central, master co-regulator. Overall, our study takes a significant first step towards

understanding the genetic and transcriptional control of spleen development.

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Supplementary material

Supplementary material for this article is available at
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