Transcription factor TLX1 controls retinoic acid signaling to ensure spleen development


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The molecular mechanisms that underlie spleen development and congenital asplenia, a condition linked to increased risk of overwhelming infections, remain largely unknown. The transcription factor TLX1 controls cell fate specification and organ expansion during spleen development, and Tlx1 deletion causes asplenia in mice. Deregelation of TLX1 expression has recently been proposed in the pathogenesis of congenital asplenia in patients carrying mutations of the gene-encoding transcription factor SF-1. Herein, we have shown that TLX1-dependent regulation of retinoic acid (RA) metabolism is critical for spleen organogenesis. In a murine model, loss of Tlx1 during formation of the splenic anlage increased RA signaling by regulating several genes involved in RA metabolism. Uncontrolled RA activity resulted in premature differentiation of mesenchymal cells and reduced vasculogenesis of the splenic primordium. Pharmacological inhibition of RA signaling in Tlx1-deficient animals partially rescued the spleen defect. Finally, spleen growth was impaired in mice lacking either cytochrome P450 26B1 (Cyp26b1), which results in excess RA, or retinol dehydrogenase 10 (Rdh10), which results in RA deficiency. Together, these findings establish TLX1 as a critical regulator of RA metabolism and provide mechanistic insights into the molecular determinants of human congenital asplenia.

Introduction

The mammalian spleen is a secondary lymphoid organ that plays a central role in host defense. As a result, asplenia or hypoplasia and postsplenectomy patients often have an increased risk of overwhelming infections, particularly by encapsulated bacteria (1–5). Spleen development involves coordination of cell fate specification, migration, and proliferation to form a vascularized splenic primordium (6–9). In mice, these processes are coordinated by a limited set of transcription factors (10). Among these, T cell leukemia homeobox 1 (TLX1, also known as HOX11) (6, 11) acts downstream of the genetic cascade governing spleen development by promoting cell fate specification and organ expansion. At present, however, the precise downstream transcriptional networks and signaling pathways controlled by TLX1 remain unknown.

Tlx1-deficient mice are asplenic without any other abnormalities (6, 11), a phenotype resembling human isolated congenital asplenia (OMIM 271400), a condition in which the lack of the spleen exists as a sole organ defect (9, 12). Currently, only the ribosomal protein SA (RPSA) gene has been found mutated in isolated congenital asplenia patients (13); however, the precise role of RPSA during spleen development remains unknown. Congenital asplenia can be also associated with other abnormalities, such as laterality defects as observed in patients with Ivemark syndrome (OMIM 208530), patients with cardiac defects and transposition of great arteries (14), or patients with disorders of sexual development (OMIM 612965) carrying mutations in the gene encoding for steroidogenic factor 1 (SF-1/NR5a1) (15). Interestingly, a mutant form of SF-1 was recently shown to be defective in activating TLX1 expression in patients with disorders of sexual development and asplenia, thus providing the first evidence that perturbation of TLX1 expression may be implicated in human congenital asplenia (16).

TLX1 regulates cellular proliferation and differentiation in different cellular systems (6, 8, 17–22). During spleen development, loss of Tlx1 causes reduced proliferation of the splenic mesenchyme (SPM) and growth arrest (8, 23). Conversely, ectopic expression of Tlx1 in thymocytes blocks differentiation and promotes
leukemogenesis by altering the expression of genes involved in cell cycle regulation and thymocyte development (18, 19, 21, 24).

At the molecular level, TLX1 can act as both an activator and a repressor of gene transcription depending on the cellular context and its interaction with transcriptional cofactors (25). For example, retinaldehyde dehydrogenase 1 (Raldh1, also known as Aldh1a1) is a gene that encodes a retinoic acid–synthesizing (RA-synthesizing) enzyme (26), and in mouse embryonic fibroblasts, TLX1 activates Aldh1a1 expression (24, 25, 27). In contrast, in the developing mouse spleen, TLX1 represses Aldh1a1 expression (25). At present, however, it remains unknown whether TLX1 plays a role in regulating retinoid signaling during spleen development, and whether deregulation in this pathway affects spleen organogenesis.

RA, the active metabolite of vitamin A, is an essential molecule required for vertebrate patterning and embryogenesis (15, 26, 28–31). RA binds to nuclear receptors (RARs) and regulates critical developmental pathways governing cellular proliferation, differentiation, organogenesis, and tissue homeostasis (32, 33). In the developing embryo, the activities of RA-synthesizing (RDHs, ALDHs) and degrading enzymes of cytochrome P450 family 26 (CYP26) regulate RA metabolism (31). Notably, elevated RA signaling in Cyp26b1−/− mutants causes aberrant cellular proliferation and differentiation, leading to several organ abnormalities including lymphatic vascular defects and altered germ cell development (33–36). Notably, RA controls the fate of germ cells in mice while SF-1 regulates RA metabolism during germ cell development (15, 37). Furthermore, elevated RA signaling in the form of teratogenic doses of RA in mice, rats, and nonhuman primates has also been associated with organ growth abnormalities (38–43).

Herein, we set out to uncover the molecular mechanism by which TLX1 regulates spleen development. Using gene expression profile analysis, we found that loss of Tlx1 in the SPM causes upregulation of several genes involved in RA metabolism. Conversely, the expression of Cyp26b1, which encodes an enzyme involved in RA degradation, is markedly reduced in the embryonic splenic anlage of Tlx1 mutant mice. Analysis of Cyp26b1 or retinol dehydrogenase 10 (Rdh10) mutants, which respectively exhibit an excess or deficiency in RA signaling, revealed severe spleen hypoplasia or agenesis, demonstrating the importance of finely regulating RA metabolism to ensure proper spleen development. Interestingly, loss of Sf-1 during spleen development also reduced Tlx1 and Cyp26b1 expression. Genome-wide analysis indicated that TLX1 binds the regulatory regions of RA-associated genes through the AP-1 site and cooperates with the AP-1 family of transcription factors to regulate gene expression. Importantly, pharmacological inhibition of RA signaling partially rescued the spleen phenotype of Tlx1 mutants. Collectively, our findings unveil molecular interactions critical for spleen development and shed light onto the pathogenesis of congenital asplenia.

Figure 1. TLX1 controls RA signaling pathway. (A) GSEA enrichment plots and heat maps of differentially expressed genes belonging to the RA pathway associated with loss of Tlx1. The bar-code plot indicates the position of the genes on the expression data rank-sorted by its association with Tlx1 mutants, with red and blue colors indicating over- and underexpression in the mRNA. Validation of RA-associated genes by qPCR was performed on Tlx1+/− and Tlx1−/− embryonic spleens. The means of triplicates ± SD are shown. *P < 0.05, **P < 0.01 (2-tailed Student’s t test). Data are representative of 1 of 2 different validation experiments with 8–10 pooled spleens for each genotype. (B) TLX1 peak annotation relative to the indicated genomic feature. TLX1 ChIP-seq was performed in the eSMC line, and peak call was generated by comparison with an unrelated ChIP-seq experiment using rabbit IgG as control. MEME motif prediction of DNA sequences enriched in TLX1 ChIP-seq. ChIP-qPCR analysis of TLX1 binding in eSMC line. Positive (R1, R3, and R5) and negative (R2, R4, and R6) binding regions are indicated relative to transcription start site (TSS). Data are normalized to amplification of the input chromatin. Data are representative of 1 of 3 independent experiments.
Sequences 1–5). Moreover, we found that the AP-1 sequence was present in several RA-associated genes deregulated in our GSEA analysis (Figure 1A and Supplemental Table 1). Consistent with TLX1 acting as either a cotranscriptional repressor or activator of gene transcription, cotransfection of Tlx1 with Jdp2 or c-Jun — 2 AP-1 family members with opposite transcriptional functions — synergistically modulates the transactivation of an AP-1 luciferase reporter system (Supplemental Figure 2A). Expression of Jdp2 was strongly reduced in Tlx1 mutant as compared with control embryonic spleens (Supplemental Figure 2B), and, in agreement with these data, ChIP-seq analysis followed by ChiP-qPCR revealed direct binding of TLX1 to the Jdp2 locus (Supplemental Figure 2B). These findings raised the possibility that TLX1 and JDP2 may act in concert to regulate transcription. We therefore examined a possible physical association of TLX1 and JDP2, and found that these transcription factors interact in reciprocal coimmunoprecipitation assays (Supplemental Figure 2C), thus suggesting transcriptional coregulation of target genes, including RA-associated genes. Collectively, these findings demonstrate a direct transcriptional control of TLX1 on RA signaling during spleen organogenesis.

RA signaling and Tlx1 expression are mutually exclusive during spleen development. To determine whether and where RA signaling is active during normal spleen development, we analyzed WT RARE-LacZ transgenic embryos during the initial formation of the splenic anlage (Figure 2). In this mouse model, β-gal expression is under the control of an RA-responsive element (RARE), and, as a result, LacZ staining reveals the domains of active RA signaling (46). We found that at E11.5 RA activity is absent in the newly formed splenic anlage (Supplemental Figure 3). In contrast, RA activity is detectable at E13.5 and is confined to the outer mesothelial layer, whereas the inner SPM remains devoid of RA signaling (Figure 2A). Interestingly, staining of Tlx1<sup>−/−</sup> heterozygous embryos, in which LacZ marks the domain of Tlx1 expression, revealed that during normal spleen development, Tlx1 is expressed only in the SPM (Figure 2B). In contrast, TLX1 is excluded from the outer mesothelial region exhibiting RA signaling (Figure 2A), thus indicating that TLX1 and RA are present in mutually exclusive domains during spleen development. Altogether, these results indicate that TLX1 may repress RA signaling in the SPM, possibly through the control of Cyp26b1 expression and RA degradation.

Uncontrolled RA signaling causes spleen growth defects. Previous work has shown that Cyp26b1 deficiency causes developmental defects consistent with excessive RA activity (34, 35, 39), demon-

Results

Loss of Tlx1 deregulates the RA signaling pathway. We previously showed that loss of Tlx1 causes defects in specification and proliferation of spleen mesenchymal progenitors (8). However, the mechanisms by which TLX1 coordinates the initiation and expansion of the splenic anlage remain unknown. To identify deregulated genes and signaling pathways associated with loss of Tlx1, we performed gene expression analysis using mRNA obtained from Tlx1<sup>+/−</sup> heterozygous and Tlx1<sup>−/−</sup> homozygous embryonic spleens at E13.5 (Figure 1A). This time point was chosen because it coincides with the appearance of the spleen defect in Tlx1<sup>−/−</sup> homozygous embryos. Gene ontology analysis revealed statistically significant differences in the expression of genes related to developmental processes including spleen organogenesis (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI82956DS1). To identify deregulated pathways resulting from Tlx1 loss, we took advantage of the Gene Set Enrichment Analysis (GSEA) tool, a computational method that detects modest but coordinated changes in the expression of groups of functionally related genes (44). Remarkably, we found a highly significant deregulation of the RA pathway (FDR = 0.006) (Figure 1A). Quantitative PCR (qPCR) validation analysis revealed that genes encoding for the RA-synthesizing enzymes (i.e., Aldh1a1), RA nuclear receptors (i.e., Rara, Rarb), and vitamin A/RA transporters (i.e., Stra6/Crabp2) were all upregulated as a result of Tlx1 ablation (Figure 1A). Conversely, the expression of the RA-degrading enzyme Cyp26b1 was significantly reduced in the absence of Tlx1 (Figure 1A).

TLX1 binds genes associated with the RA pathway. To identify whether TLX1 binds RA-associated genes, we performed ChiP sequencing (ChiP-seq) in the embryonic spleen mesenchymal cell (eSMC) line. Analysis of the profile distribution revealed preferential binding of TLX1 to intergenic (44%) and intronic (38%) regions and, to a lesser extent, to promoter (14%) regions (Figure 1B). We then searched for putative TLX1-binding sites by motif discovery analysis using the multiple EM for motif elicitation (Meme) algorithm and found enrichment for an AP-1 site consensus element (Figure 1B), suggesting control of genes bearing the AP-1 motif (45). Confirming this finding, ChiP-qPCR analysis showed binding of TLX1 to Cyp26b1, Rara, and RXra genomic regions carrying the AP-1 sequence (Figure 1B and Supplemental Sequences 1–5). Moreover, we found that the AP-1 sequence was
revealed a large contribution of the YFP+ LPM mesodermal cells to the developing spleen as shown by LacZ staining of E14.5 spleens from Cyp26b1–/– embryos at E16.5 (Figure 3A) revealed the presence of only remnant splenic tissue when compared with littermate controls. Next, we crossed Rdh10fl/+;CreERT2;R26RYFP reporter mice to test the hypothesis that impaired CYP26B1 function causes spleen growth defects. Remarkably, analysis of Cyp26b1 mutant embryos at E16.5 (Figure 3A) revealed the presence of only remnants of splenic tissue when compared with littermate controls. We then hypothesized that excessive RA signaling due to loss of Cyp26b1 expression inhibits spleen development, and therefore sought to assess whether physiological levels of RA are required for the proper patterning of spleen descendants in the lateral plate mesoderm (LPM), from which the spleen is thought to arise. We first performed lineage-tracing experiments to unequivocally demonstrate the contribution of the LPM to the spleen by crossing R26YFP reporter mice to Hoxb6-CreER mice, in which tamoxifen administration causes a role for TLX1 as a repressor of RA signaling, we next demonstrated the critical role of CYP26B1 in regulating RA homeostasis during embryonic development. The finding that Cyp26b1 is expressed during spleen development (47) and that it is strongly reduced in Tlx1 mutant embryonic spleens (Figure 1A) prompted us to test the hypothesis that impaired CYP26B1 function causes spleen growth defects. Remarkably, analysis of Cyp26b1 mutant embryos at E16.5 (Figure 3A) revealed the presence of only remnants of splenic tissue when compared with littermate controls.

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Next, we crossed Rdh10fl/+ mice to the Cre-ERT2 tamoxifen-inducible transgenic line to conditionally ablate Rdh10, a critical enzyme involved in the first step of RA synthesis in the embryo (49, 50). Consistent with its expression in the LPM (Figure 3B), ablation of Rdh10 by a single dose of tamoxifen at E7.5 caused spleen agenesis in mutant embryos compared with littermate controls (Figure 3B). Conversely, despite expression of Rdh10 in developing spleen (Figure 3C), ablation of Rdh10 by administration of tamoxifen at E10.5 during initial formation of the spleen primordium did not affect organogenesis (Figure 3C). Collectively, these findings demonstrate a differential and precise spatiotemporal requirement for RA during spleen development.

CYP26B1 in spleen mesenchymal cells is required to prevent aberrant RA signaling. To formally demonstrate that increased RA activity in the Tlx1 mutant SPM is caused by reduced Cyp26b1 expression, we exploited an in vitro system by coculturing RA reporter F9-LacZ cells with an eSMC line that has been shown to mimic the native embryonic mesenchyme (9). Notably, silencing Tlx1 in eSMCs via shRNA recapitulates our in vivo data by causing deregulated expression of several RA-associated genes, including Cyp26b1 (Figure 4A and Supplemental Figure 4).

To assess the functional consequences of Tlx1 and Cyp26b1 deficiency for RA signaling, we cocultured RA reporter F9-LacZ cells with shRNA-Tlx1 or shRNA-Ctrl control eSMCs, and performed LacZ staining to measure RA activity (Figure 4B). In the absence of TLX1, the number of LacZ+ cells was significantly lower than among reporter cells cultured with shRNA-Ctrl control eSMCs (Figure 4B). Interestingly, rescue of Cyp26b1 expression reduced the number of blue cells seen among shRNA-Tlx1 eSMCs to none or a few, similar to what is seen in shRNA-Ctrl control eSMCs (Figure 4B). These findings demonstrate that loss of CYP26B1 function in Tlx1 mutants causes increased RA signaling in the SPM.

To corroborate the role of CYP26B1 in limiting RA content and signaling in the SPM, we measured the expression of RA target genes under condition of CYP26B1 inhibition. RA-treated eSMCs cultured in the presence of the CYP26B1 inhibitor R116010 (52) displayed increased expression of RA-responsive genes (Supplemental Figure 3), thus demonstrating the critical role of CYP26B1 in restricting RA signaling in spleen mesenchymal cells.

TLX1 blocks RA-induced differentiation. Since our data demonstrated a role for TLX1 as a repressor of RA signaling, we next evaluated the effect of RA on the differentiation of F9 cells, a cellular system previously used to study RA-induced differentiation (53, 54). We found that the expression levels of Lamb1 and Col4a1, 2 genes associated with RA-induced endodermal differentiation (53, 55), were significantly reduced in Tlx1-overexpressing F9 cells.
size revealed that RA exposure significantly inhibits growth, which is accompanied by reduced sprouting of mesenchymal cells into the collagen layer (Figure 5B, arrows). In line with these data, silencing of Tlx1 causes G1 cell cycle arrest and deregulation of the RA signaling pathway (Supplemental Figure 4A and Supplemental Figure 5B). Altogether, these findings demonstrate that excessive RA signaling inhibits spleen organ expansion by reducing spleen mesenchymal cell proliferation.

Increased RA signaling induces premature differentiation and reduced vasculogenesis. Retinoid excess in humans and mice causes a wide spectrum of malformations associated with patterning defects, premature differentiation, and organ growth arrest (29, 35, 36, 38–40, 56). Consistent with this phenomenon, our GSEA revealed enrichment of signatures associated with markers of lymphoid stromal cell maturation and extracellular matrix. Particularly, genes that belong to these pathways were induced in Tlx1 mutants as compared with control embryonic spleens (Figure 5A).

To assess the functional effects of RA exposure on spleen growth, we performed organotypic cultures. E13.5 WT littermate spleens were divided into 2 groups and cultured, on a collagen layer, in the presence of RA or vehicle. Quantitative analysis of spleen growth revealed that RA exposure significantly inhibits growth, which is accompanied by reduced sprouting of mesenchymal cells into the collagen layer (Figure 5B, arrows). In line with these data, silencing of Tlx1 causes G1 cell cycle arrest and deregulation of the RA signaling pathway (Supplemental Figure 4A and Supplemental Figure 5B). Altogether, these findings demonstrate that excessive RA signaling inhibits spleen organ expansion by reducing spleen mesenchymal cell proliferation.

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Tlx1 mutant SPM results in premature cellular differentiation, we evaluated the expression of Desmin, a marker associated with maturation of spleen mesenchymal cells (57–59). Desmin was strongly upregulated at both the mRNA and the protein level in E13.5 Tlx1 mutants as compared with control embryonic spleens (Figure 6A). Consistent with this observation, treatment of E14.5 primary spleen mesenchymal cells with RA induced a significant upregulation of Desmin expression (Figure 6A). In addition, we assessed the expression of ER-TR7, an antigen expressed by differentiating spleen and lymph node mesenchymal cells (60). Importantly, immunofluorescence analysis showed that ER-TR7 expression starts only postnatally in WT mice (Supplemental Figure 6B). However, ER-TR7 is prematurely expressed in Tlx1 mutants as compared with controls (Supplemental Figure 6C). Finally, GSEA analysis revealed a significant increase in the expression of Hox genes that are known to be under the control of retinoid signaling during embryonic development (32) (Supplemental Figure 6D). Altogether, these findings indicate that the premature differentiation occurring in Tlx1 mutants is caused, at least in part, by deregulation of RA signaling.

In addition to mesenchymal cells, the splenic anlage comprises endothelial cells that form an expanding vascular network. RA was shown to regulate the expression of Vegf-a (61), the major mitogen for endothelial cell proliferation and vessel network formation. Consistent with this principle, treatment of E14.5 primary spleen mesenchymal cells with RA reduced Vegf-a expression as compared with vehicle-treated cells (Figure 6B). Also, anlage and were more compact compared with untreated controls that exhibited multiple unjoined splenules/accessory spleens (Figure 7A). However, an evaluation of spleen size, as assessed by measurement of the surface of the LacZ+ anlagen, did not reveal significant differences between homozygous mutant spleens from BMS493- and vehicle-treated mice (Figure 7A). Furthermore, qPCR analysis revealed that BMS493 treatment induced a significant rescue in the expression of Vegf-a, whereas Desmin expression was not rescued (Figure 7B). Importantly, the expression of 2 RA target genes — Col4a1 and Lamb1 — which are negatively regulated by RA in the SPM of Tlx1 mutants, was completely recovered by RA signaling inhibition (Figure 7B). Similarly, the expression of Rarb, a gene positively regulated by RA, was also completely rescued by BMS493 treatment, thus demonstrating the presence of increased RA signaling in the absence of Tlx1. Altogether, these findings indicate that repression of RA signaling is required for condensation of spleen mesenchymal cells and organ morphogenesis.

SF-1 controls Tlx1 and RA metabolism in the SPM. Recent work by Zangen and collaborators reported that SF-1, which is required for human spleen development, transactivates the Tlx1 promoter (16). In addition, loss of Sf-1 in mice causes a severe spleen hypoplasia defect (64). To test the hypothesis that Sf-1 is genetically upstream of Tlx1, we performed gene expression analysis using mRNA obtained from Sf1+/− homozygous or control splenic anlagen at E14.5. Consistent with the hypothesis, we found a significant reduction of Tlx1 mRNA levels in Sf1−/− mutants as compared with controls (Figure 8A). The expres-
sion of the RA-degrading enzyme Cyp26b1 was also severely reduced in Sf1−/− mutants as compared with controls (Figure 8A). In agreement with this observation, we found that SF-1 transactivates the Cyp26b1 promoter in a dose-dependent manner (Figure 8B). We then tested the possibility that, similar to TLXI, SF-1 may counteract the differentiation effects of RA. To this end, we treated F9 cells, transiently transfected with Tlx1, SF-1, with RA and evaluated the expression of markers associated with cellular differentiation. Similarly to what we observed for Tlx1, overexpression of SF-1 significantly inhibited cellular differentiation as demonstrated by reduction of the expression of Lambl and Col4a1, 2 genes associated with RA-induced endodermal differentiation (53, 55) (Figure 8C). Moreover, the expression of Rarb, a gene positively regulated by RA, was also significantly lower in SF-1-expressing cells as compared with control cells (Figure 8C). Previous work showed that loss of SF-1 during spleen development causes reduced vasculogenesis (64). Consistent with this finding, we observed a significant reduction in Vegf-a mRNA levels in SF-1 mutants as compared with control embryonic splenic anlagen (Figure 8). Altogether these findings support a model in which SF-1 regulates Tlx1 expression and RA metabolism to ensure spleen development.

**Discussion**

The lack of a spleen is often associated with deadly infections in humans. At present, the molecular mechanisms underlying asplenia remain mostly elusive. In the absence of Tlx1, spleen mesenchymal cells fail to proliferate and to form a discrete splenic primordium after E13.5 (6, 8, 11, 65). Our findings demonstrate that expression of Tlx1 in the SPM is required to control excessive RA signaling in order to assure the proliferation of mesenchymal precursors and the formation of the splenic anlage. In support of this conclusion, we showed that loss of Tlx1 leads to an increase in RA activity. This effect is due to reduced RA degradation caused by deregulated Cyp26b1 expression and to increased nuclear receptor expression and activation. RA may diffuse or be transported within the nascent SPM, causing aberrant morphogenesis, premature differentiation, and vascular abnormalities. Furthermore, the finding that Cyp26b1 mutant embryos exhibit only a remnant of splenic tissue clearly demonstrates the importance of restricting RA activity within the SPM to ensure proper spleen development. In support of this notion, re-expression of Cyp26b1 in Tlx1-deficient mesenchymal cells restored RA signaling to control levels, indicating that uncontrolled RA signaling in Tlx1 mutants is due to a deficit in CYP26B1 activity. While exces-
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Cell cycle arrest in different cellular systems (67) suggests that RA may contribute to the proliferation defect observed in Tlx1 mutant spleen. Supporting this hypothesis is the finding that Cdkn2b/p15, a gene that we showed to be induced by RA treatment in primary eSMCs, is significantly increased in Tlx1-deficient splenic anlage. However, the spleen phenotype of Tlx1 mutants is only partially rescued upon inhibition of RA signaling in vivo. One explanation for this discrepancy may be the inefficient activity of the BMS493 inhibitor on RA signaling in vivo. In addition, TLX1 may control gene transcription and cellular proliferation independently of RA as previously demonstrated (17, 18). Indeed, while expression of Col4a1, Lamb1, and Rarb was significantly rescued upon RA signaling inhibition, Vegf-a transcription was only modestly reactivated. In addition, the expression of Desmin and Cdkn2b/p15 (not shown) was not rescued upon RA inhibition. At the molecular level, our ChIP-seq analysis revealed that TLX1 binds DNA preferentially through the AP-1 motif, a sequence found in the Vegf-a gene (61) and in several RA-associated genes, including Cyp26b1, Rara, and Rxra, which we found bound by TLX1. Among the TLX1 target genes, we also identified Jdp2, an AP-1 family transcription factor deregulated in the absence of Tlx1 and known to repress RA-induced differentiation (68). As with Jdp2, we showed that TLX1 also represses RA-induced differentiation and that TLX1 and JDP2 physically interact. These findings support a scenario in which reduced expression of these transcription factors within the SMP causes deregulation of genet-

Figure 7. Inhibition of RA signaling partially rescues the spleen phenotype. (A) Partial rescue of spleen morphogenesis in E14.5 Tlx1−/− embryonic spleens treated with BMS493 or control vehicle at E10.5. Number of unjoined splenules (outlined in white) and surface area measurements were normalized to littermate controls. Statistical significance in the number of unjoined splenules was calculated using χ², *P < 0.05. For the surface area measurement, the means of triplicates ± SD are shown. NS, not significant (2-tailed Student’s t test). Data are from 15 (BMS493) and 14 (DMSO) treated embryonic spleens from 3 different litters. HOM, homozygous; HET, heterozygous. (B) Validation of Vegf-a, collagen 4a1 (Col4a1), laminin B1 (Lamb1), and Rarb expression by qPRCs in E14.5 Tlx1+/− and Tlx1−/− embryonic spleens treated with BMS493 or control vehicle. The means of triplicates ± SD are shown, *P < 0.05, **P < 0.01, ***P < 0.001 (2-tailed Student’s t test). Data are representative of a pool of 12–16 embryonic spleens for each group from 4 different experiments.
ic programs controlled by RA. Thus, we propose that, in addition to controlling RA metabolism in the SPM, TLX1 directly regulates RA target genes containing the AP-1 sequence. Accordingly, our microarray and ChIP-seq data indicate that TLX1 represses the activation of nuclear RARs by binding to their regulatory regions. In the absence of TLX1, the transcription of RARs would be derepressed, while uncontrolled RA in the SPM would favor the conversion of RARs from corepressors into coactivators and the subsequent induction of c-Jun and other RA-regulated genes. In support of this idea, c-Jun, which is induced by RA in an AP-1-dependent manner, is strongly upregulated in Tlx1 mutant spleens compared with controls (not shown). In a similar manner, expression of Desmin, a marker associated with cellular differentiation positively regulated by RA and AP-1, is significantly increased in Tlx1 mutant spleens compared with controls.

In this scenario, our data further support the previous hypothesis by Zangen and collaborators (16) and implicate SF-1 as an upstream regulator of Tlx1 during spleen development. Our findings also indicate that SF-1 controls Cyp26b1 expression independently of TLX1. Thus, we propose a model in which SF-1 is required to activate Tlx1 transcription and both TLX1 and SF-1 independently control Cyp26b1 expression and RA metabolism (Figure 9). Supporting a role for SF-1 in the control of RA activity is the demonstration that SF-1 regulates Cyp26b1 expression during germ cell development (15). We showed that, similarly to what occurs with Tlx1 mutants, loss of SF-1 causes reduced Vegf-a expression. Given that RA affects Vegf-a expression in spleen mesenchymal cells, it is likely that the severe vasculogenesis defect observed in SF-1-deficient mice is caused, at least in part, by aberrant RA signaling (64).

Interestingly, although mice deficient for Cyp26b1 have severe spleen hypoplasia, it has recently been reported that 2 siblings homozygous for a null allele of CYP26B1 have normal spleen size (39) (S. Robertson, unpublished observations). At present, the reason for this discrepancy remains unclear, though it is possible that the human CYP26B1-null allele is associated with incomplete clinical penetrance or that other CYP family members compensate for CYP26B1 deficiency in humans. Nevertheless, it is tentative to speculate that perturbations of Tlx1 expression, both in mice and in humans, may deregulate critical downstream pathways, including retinoid signaling that affects spleen development. Interestingly, an excess of retinoids in animal models causes heterotaxy, a syndrome associated with congenital asplenia and vascular defects in humans (39, 40, 61). In conclusion, our findings reveal the important role of TLX1 in controlling RA signaling during spleen organogenesis and provide novel mechanistic insights underlying the pathogenesis of congenital asplenia.
**Methods**

*Mice.* Tlx1+/− (6), Cyp26b1−/− (33), Rdh10−/− (50), RARE-hs68-LacZ (46), Hoxb6-CreER (48), Rdh10/Geo (50), Sf−/− (69–71), and Cre-ERT2 mice have been previously described (72). R26RYFP mice were used as a reporter strain of Cre activity. C57BL/6N and CD1 mice were purchased from Charles River, Italy. Mice were bred and maintained at San Raffaele Scientific Institute in pathogen-free rooms under barrier conditions, with constant temperature, food and water ad libitum, and light/dark cycles of 12 hours. Tamoxifen (5 mg) and progesterone (1 mg) were dissolved in corn oil and administered via oral gavage to pregnant mice at the desired embryonic stage of development.

Pregnant Tlx1−/− or Tlx1+/− mice were treated with RAR antagonist BMS493 (Tocris Bioscience) or vehicle (DMSO; Sigma-Aldrich) at the dose of 5 mg/kg in corn oil. Mice were mated overnight, and the day of vaginal plug detection was marked as E0.5. Treatment was given by oral gavage twice daily in 10- to 12-hour intervals from E10.5 until E13.5.

**RNA isolation, qPCR, and microarray analysis.** For gene expression profile analysis, total RNA was extracted from embryonic spleens harvested at E13.5 and E14.5, primary spleen mesenchymal cells (E14.5), and the established esMC line with RNesy Mini isolation kit (Qiagen). Reverse transcription of 1 μg of total RNA was performed using the ImProm II Reverse Transcription System kit with random primers (Promega Corp.). qPCRs were performed using the Universal Probe Library system on a LightCycler 480 (Roche). The Ct of Rpl13a (housekeeping) was subtracted from the Ct of the target gene, and the relative expression was calculated as 2−ΔΔCt. qPCRs were performed in triplicate and mean ± SD represented as relative expression. Primer sequences are described in Supplemental Table 2. Microarray analysis was performed using GeneChip Mouse Gene 1.0 ST Array from Affymetrix (GSE68519). Data were processed and normalized (GC robust multi-array average [GCRMA]) using GenePattern (74).

**Chromatin immunoprecipitation.** ChIP experiments were performed using the esMC line according to a protocol described previously (8). In brief, 1 × 10⁵ cells were cross-linked with 1% formaldehyde at room temperature followed by addition of glycine at a final concentration of 0.125 M. Chromatin was fragmented by sonication with Sonicator Ultrasonic Processor XL (Misonix Inc.) with a 3/16-in. (4.8-mm) microtip (419A Tapered Microtip; Gilson Inc.) at 30% of maximum amplitude for 30 seconds and for 35 times with a 1-minute interval. Samples were immunoprecipitated with 4 μg of the antibodies anti-Tlx1 (rabbit polyclonal, SC-880X; Santa Cruz Biotechnology Inc.) and the normal rabbit serum IgG (ChromPure 011-000-003; Jackson Immunoresearch Laboratories Inc.). Immunoprecipitated DNA was purified using QiAquick PCR Purification kit (Qiagen), suspended in 45 μl of Tris-HCl 10 mM, and amplified by qPCR on a LightCycler 480 using TaqMan chemistries (Roche). Regions within Cyp26b1 (R1, R2), Rxrα (R3, R4), Rara (R5, R6), and Jdp2 (R7, R8, R9) were tested using primers designed using the UPL Assay Design Center (Roche) and are listed in Supplemental Table 3. For ChIP sequencing (ChIP-seq) analysis the purified DNA was quantified using Quant-iT PicoGreen dsDNA kit (Invitrogen). For each ChIP-seq assay 40 ng of DNA was used directly for cluster generation and sequencing analysis with the HiSeq 2000 following the protocol of the manufacturer (Illumina).

**Sequencing and data analysis.** Sequencing data (GSE81661) generated from the Illumina platforms were aligned to mouse reference genome (mm9) using Bowtie version 0.12.7. Only reads with unique alignment were retained for downstream analysis. Peak calling and bigWig files were generated using Model-Based Analysis for ChIP-seq (MACS) version 1.4. Only peaks with 10x –log P value ≥ 70 are considered for further processing. bigWig files were visualized using the University of California, Santa Cruz (UCSC) browser (http://genome.ucsc.edu). The list of mm9 annotated RefSeq genes used for the different analyses was downloaded from the UCSC database, and the data were processed by the MEME tool.

**Immunofluorescence staining.** E13.5 and E16.5 embryos and P1 spleens were harvested and fixed 30 minutes to 1 hour at 4°C with 4% (wt/vol) PFA (Sigma-Aldrich), then washed in PBS 1x and dehydrated overnight in 30% sucrose (Sigma-Aldrich) at 4°C. Samples were embedded in Tissue-Tek OCT compound (Bio-Optica) and frozen in an ethan- 
ol dry-ice bath. Eight- to ten-micrometer-thick sections were placed onto glass slides (Bio-Optica), fixed in cold acetone for 5 minutes, dried, and kept at −80°C until used. Slices were incubated 30 minutes with a blocking solution of PBS at 0.5% PBS and 0.5% Tween (PBS-T 0.05%), followed by anti-desmin (mouse IgG1; D1033, clone DE-U-10; 1:200 stock 8.8 μg/μl; Sigma-Aldrich), anti-β-gal (mouse IgG1; G8021, clone GAL-13; 1:100 stock 5 μg/μl; Sigma-Aldrich), anti-CD31/PECAM-1 (PE rat IgG2a; 553373, clone MEC 13.3; 1:100 stock 0.2 μg/μl; BD), and anti-ER-TR7 (rat IgG2a; BM4018, clone ER-TR7; 1:200 stock 0.4 mg/ml; Acris) specific antibodies. Secondary anti-mouse Alexa Fluor 488 (1:500 stock 2 μg/μl; A10684; Invitrogen) or anti-rat Alexa Fluor 488 (1:500 stock 2 μg/μl; A21208; Invitrogen) antibody was diluted in PBS-T 0.05% blocking solution and incubated for 30 minutes. Nuclei were visualized with DAPI (Fluka), and mounting was performed with Mowiol (Calbiochem). Images were acquired using an Ultraview Leica TCS SP2 laser confocal microscope. Digital images were recorded in separately scanned channels with no overlap in detection of emission from the respective fluorochromes. Final image processing was performed with Adobe Photoshop and Illustrator.

**IHC staining.** E13.5 embryos were harvested and fixed overnight at 4°C with 4% (wt/vol) PFA and processed into paraffin through graded alcohol series. Five- to eight-micrometer-thick sections were placed onto glass slides, dried overnight at 40°C–42°C, and kept at room temperature until used. Slices were deparaffinized, quenched for 5 minutes in 3% (vol/vol) hydrogen peroxide, and incubated with blocking solution of 3% of BSA (Sigma-Aldrich) in TBS (VWR) for 10 minutes at room temperature. Sections were stained with anti-CD31/PECAM-1 (rabbit polyclonal; 1:200 stock 200 μg/ml; RB-10333; Thermo Scientific) antibody overnight at 4°C. Immunolabeling was performed using Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions. Slides were counterstained in Gill’s Hema- 

toxyl (Sigma-Aldrich) and next mounted with xylene (Carlo Erba). Images were acquired using Aperio Scan Scope slide scanner (Leica Biosystems), and quantification of the microvessel density was performed with ImageJ software. Final image processing was performed with Adobe Photoshop and Illustrator.

**Cell cultures, silencing, and transfections.** The embryonic spleen mesenchymal cell (esMC) line, the F9-RARE-LacZ reporter cell line, and the embryonic endothelial cell line were previously described (9, 51, 75). Pri-
mary embryonic spleen mesenchymal cells were generated from E13.5 embryonic spleens. Cells were grown at 37°C, 5% CO2 in DMEM (Gibco) supplemented with 10% heat-inactivated FBS (Euroclone), 2 mM l-glutamine (l-Glu; Gibco), and 100 U/ml penicillin and 100 μg/ml strepto-
mycin (Pen/Strep; Gibco). 293T (71) and NIH3T3 (8) cells were grown in Iscove’s modified Dulbecco’s medium (IMDM; Gibco) or in DMEM (Gibco), respectively, supplemented with 10% heat-inactivated FBS,
L-Glu, and Pen/Strep. Embryonic endothelial cells were grown in 0.1% gelatin (Sigma-Aldrich) precoated dishes in DMEM supplemented with 20% FBS, L-Glu, Pen/Strep, 1 mM Na pyruvate (Gibco; Thermo Fisher Scientific), 100 μg/ml heparin (Sigma-Aldrich), and 5 μg/ml endothelial cell growth supplement (ECGS; Sigma-Aldrich). For silencing experiments, lentiviral particles were produced and used following the manufacturer’s instructions using the lentiviral plasmid pLKO.1-puro containing 2 different shRNA target sequences against Tlx1 (Sigma-Aldrich). The shRNA-Tlx1 sequence was CCGGGCGGTATGCAGATCTAATCTGATTTAGATTGCTG-TGCTGATCCGTTTTGG; the control shRNA-scrambled sequence was a non-target shRNA. Selection of infected cells was performed in puro-mycin (Sigma-Aldrich), and cells were kept under culture conditions described above during all experiments.

For transfection experiments, cells were transfected using Lipofectamine 2000 (Invitrogen) or Amaxa MEF2 Nucleofector Kit (Lonza) with the following vectors: pCMV-Cyp26b1 (76), pcdNA3-Tlx1 (8), pcdNA3-Jun (77), pcdNA3-SF-1 (78), pcDNA4-Jdp2, and pAPl-Luc (53, 55). The Cyp26b1 reporter vector was generated by cloning of 4,000 bp of the mouse Cyp26b1 proximal promoter isolated by PCR from genomic DNA into a pGL3 luciferase reporter vector (Promega Corp.). Sequencing was performed to verify accuracy of the insert. Luciferase activity was assayed with a dual luciferase assay system (Promega Corp.), and activities were standardized against the internal control, Renilla luciferase or β-gal.

In situ hybridization. Whole-mount in situ hybridization for Rdh10 was performed as previously described (49, 79) using digoxigenin-labeled probe with hybridization detected via NBT-BCIP or BM-purple (Roche).

Western blot analysis and immunoprecipitation assay. Cells were lysed in RIPA buffer (5 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% NaDOC) containing protease inhibitor cocktail (Roche). Protein concentration was determined by the Bradford assay using the Protein Assay Dye Reagent Kit (Bio-Rad). Proteins were transferred onto a PVDF membrane (Immobilon-O; Millipore) and blocked in 5% skim milk, and immunoblotting was performed with anti-Hox11/Tlx1 (1:500 stock 200 μg/ml; SC-880; Santa Cruz Biotechnology Inc.) and anti-vinculin (1:10,000; V4505, clone VIN-11-5; Sigma-Aldrich) primary antibodies. Binding of HRP-labeled anti-rabbit or -mouse secondary antibodies (NA994 or NA931, respectively; GE Healthcare) was detected with the SuperSignal West Pico Chemiluminescent kit (Thermo Scientific). Immunoprecipitation was performed as previously described (80, 81). In brief, cells were harvested using PRO-PREP Protein Extraction Solution (NIRON Biotechnology). The preparation of cell lysates, SDS-PAGE (8% or 10% gel), and Western blotting were performed as described elsewhere (80). In the case of sequential immunoprecipitation and Western blot analysis, 293T cells were transfected with pCDNA4-JDP2 or pCDNA3-Tlx1 by Lipofectamine 2000 (Invitrogen), and after 48 hours, the cell lysates of each transformant were prepared for the sequential immunoprecipitation and Western blotting as described elsewhere (80). Each supernatant was precleared with protein A/G beads (Millipore), and incubated with antibodies specific for JDP2 (2) or Tlx1 (1:550 stock 200 μl/ml; SC-880; Santa Cruz Biotechnology Inc.) at 4°C for 16 hours. The beads were preblocked with 1% BSA before being added to samples and then incubated at 4°C for 4 hours with rotation. The beads were pelleted, washed by chilled PBS, and loaded to Western blotting.
b-MeEtOH), 104 μl/well of 0.1 M sodium phosphate solution pH 7.5 (0.06 M Na2HPO4, 0.04 M NaH2PO4), and 34.6 μl/well of ONPG solution (3.6 mg/ml in 0.1 M sodium phosphate solution pH 7.5). The absorbance was measured with the Microplate Reader Model 680 (Bio-Rad) at 415 nm, and the data were analyzed using Microsoft Excel.

**Statistics.** Statistical analysis using a 2-tailed unpaired Student’s t test or 2-way ANOVA or χ² test, as indicated, was performed with GraphPad Prism and values expressed as mean ± SD. Differences were considered statistically significant at P less than 0.05.

**Study approval.** All animal experiments were conducted in accordance with protocols approved by the IACUC of San Raffaele Scientific Institute.

**Author contributions**
EL, DF, LC, F Bernassola, and AB designed and performed experiments. KW and KKY performed immunoprecipitation experiments. LL, NEBT, and PAT performed Rdh10 experiments. D Penkov, GL, D Pasini, and GT analyzed microarray and ChIP-seq data. NC and LLS, NEBT, and PAT performed ChIP-seq experiments. D Penkov, GL, DF, LC, F Bernassola, and AB designed and performed experiments. KW and KKY performed immunoprecipitation experiments. LL, NEBT, and PAT provided reagents. EL and AB analyzed data, prepared figures, and wrote the manuscript with the contribution of PAT, and AB directed the study.

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