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Short communication

# The PBX1 lupus susceptibility gene regulates CD44 expression

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ABSTRACT

PBX1-d is novel splice isoform of pre-B-cell leukemia homeobox 1 (*PBX1*) that lacks its DNA-binding and Hox-binding domains, and functions as a dominant negative. We have shown that PBX1-d expression in CD4$^+$ T cells is associated with systemic lupus erythematosus (SLE) in a mouse model as well as in human subjects. More specifically, PBX1-d expression leads to the production of autoreactive activated CD4+ T cells, a reduced frequency and function of Foxp3+ regulatory T (Treg) cells and an expansion of follicular helper T (Tfh) cells. Very little is known about the function of PBX1 in T cells, except that it directly regulates the expression of miRNAs associated with Treg and Tfh homeostasis. In the present study, we show that PBX1 directly regulated the expression of *CD44*, a marker of T cell activation. Two PBX1 binding sites in the promoter directly regulated *CD44* expression, with PBX1-d driving a higher expression than the normal isoform PBX1-b. In addition, mutations in each of the two binding sites had different effects of PBX1-b and PBX1-d. Finally, we showed that an enhanced recruitment of co-factor MEIS by PBX1-d over PBX1-b, while there was no difference for co-factor PREP1 recruitment. Therefore, this study demonstrates that the lupus-associated PBX1-d isoform directly transactivates CD44, a marker of CD44 activation and memory, and that it has different DNA binding and co-factor recruitment relative to the normal isoform. Taken together, these results confirm that PBX1 directly regulates genes related to T cell activation and shows that the lupus-associated isoform PBX1-d has unique molecular functions.


## 1. Introduction

The pre-B-cell leukemia homeobox 1 (*Pbx1*) gene has been identified as the lupus susceptibility gene corresponding to the *Sle1a1* locus in the MZM2410 mouse model (Cuda et al., 2012). This locus leads to the production of activated autoreactive histone-specific CD4$^+$ T cells in a T-cell intrinsic manner (Chen et al., 2005; Cuda et al., 2012; Cuda et al., 2007). Sle1a1 also leads to a reduction of the frequency and function of Foxp3$^+$ regulatory T cells (Tregs) (Choi et al., 2016; Cuda et al., 2007) as well as an expansion of the follicular helper T cell compartment in the mLN (Tfh) (Choi et al., 2016). These three phenotypes are key features of lupus pathogenesis (Craft, 2012; Mohan et al., 1993; Ohl and Tenbrock, 2015). It is therefore important to understand how Pbx1 is responsible for these events and what molecular interactions drive the observed phenotypes in CD4$^+$ T cells.

Pbx1 is a member of the TALE family of homeodomain-containing transcription factors that modulates the DNA-binding function of Hox proteins (Mann and Chan, 1996; Berkes et al., 2004; Longobardi et al., 2014). Pbx1 plays a central role during development and organogenesis by integrating multiple signals through its interaction with numerous partners (Laurent et al., 2008), including Meis and Prep1 TALE proteins that regulate chromatin remodeling and co-activator access (Berkes et al., 2004; Longobardi et al., 2014). In the development of the immune system, Pbx1 is required to maintain the self-renewal of hematopoietic stem cells (Ficara et al., 2008), restraining myeloid maturation to preserve the differentiation potential of lymphoid progenitors (Ficara et al., 2013). *Pbx1*-deficient embryonic stem cells fail to generate common lymphoid progenitors, resulting in the absence of B and NK cells, as well as an impaired T cell development (Sanyal et al., 2007). Accordingly, we have shown that mesenchymal stem cells (MSC) from mice expressing *Sle1a1* show an accelerated lineage differentiation and expression of innate inflammatory genes, as well as an impaired immunoregulatory capacity (Lu et al., 2015), which corresponds to defects reported in MSCs from lupus patients as well as lupus-prone mice (Collins and Gilkeson, 2013). These complex regulatory networks regulated by reflect the ability of Pbx1 complexed with either Meis or Prep1 to recruit a wide array of Hox and non-Hox co-factors, many of which vary between cell types, and to direct either gene activation or repression (Laurent et al., 2008).

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Contrary to B cells (Sanyal et al., 2007), the function of Pbx1 in T cells is unknown, except for the requirement of Prep1/Pbx1 heterodimer DNA binding for thymic development of double negative T cells (Penkov et al., 2008). Out of the seven protein-coding splice isoforms known for Pbx1 in mice, $CD4^+$ T cells express *Pbx1-b*, which lacks exons 7 and 9 that are present in the full-length *Pbx1-a* (Cuda et al., 2012). In addition, NZM2410 and *Sle1a1* $CD4^+$ T cells express a novel splice isoform, *Pbx1-d,* which lacks exons 6 and 7 that encode the DNA and a HOX binding domains, respectively (Cuda et al., 2012). Pbx1 amino acid sequence is identical between human and mouse, and we found that *PBX1-d* is expressed at a significantly higher frequency in the $CD4^+$ T cells of lupus patients as compared to healthy controls, validating the role of this susceptibility allele in lupus (Cuda et al., 2012). *Pbx1-d* is the only known Pbx1 isoform lacking the DNA binding domain, suggesting that it functions as a dominant negative (DN) binding partner with reduced transcriptional function. We have validated the DN function of Pbx1-d showing that it is equivalent to a Pbx1 knock-down construct in activating MSC differentiation (Sengupta et al., 2012). At the single gene level, the DN function of Pbx1-d was also validated, both as a transcriptional activator of Sox3 in MSCs and a transcriptional repressor of CD44 in Jurkat T cells (Sengupta et al., 2012). We have started to characterize the molecular mechanism by which Pbx1 regulates T cell function, and shown that it directly transactivates the expression of miR-10a, miR-21, and miR-155, three miRNAs that have been implicated in Treg and Tfh homeostasis (Choi et al., 2016). We performed the current study to further define the role of Pbx1 in regulating T-cell specific genes and to assess the ability of the Pbx1-d isoform to recruit the Meis and Prep1 co-factors.

## 2. Materials and methods

### 2.1. CHiP-Seq and CHiP-qPCR

Chromatin immunoprecipitation was performed on Jurkat T cells with a CHiP assay kit (Milllipore, Billerica, MA, USA), according to manufacturer's instructions with an anti-PBX1 polyclonal antibody (P-20, Santa Cruz, CA, USA) or rabbit IgG (Santa Cruz) as negative control in triplicates. The final libraries (average size 350 bp) were quantitated with the Kapa SYBR Fast qPCR reagents (Kapa Biosystems, Wilmington, MA, USA) with an ABI7900HT real-time PCR system (Life Tech., Carlsbad, CA, USA). In preparation for sequencing, barcoded libraries were pooled equimolarly, and diluted to 9 pM for cluster generation on the cBOT (Illumina, San Diego, CA, USA). Samples were sequenced on a single flowcell lane on the HiSeq2000 instrument, using a $2 \times 101$ cycle multiplex, paired-end configuration. A typical sequencing run in the HiSeq2000 produced 300–400 million paired-end reads per lane. For ChIP sequencing 50–100 million reads provided sufficient depth for analysis. Peak calling was performed using CisGenome (www.biostat.jhsph.edu/~hji/cisgenome/). Data was averaged between the three replicates precipitated with anti-PBX1 or IgG control and compared by *t*-tests. For CHiP-PCR experiments, $10^7$ Jurkat cells were washed 2 times with cold PBS, and chromatin immunoprecipitation was performed following the cross-linking chromatin immunoprecipitation protocol (http://www.abcam.cn) with the same antibodies as for the CHiP-Seq experiment. Putative PBX1 binding sites in the *CD44* intron 1 and promoter were identified using Jaspar3 (http://jaspar.genereg.net/) set at a 75%score threshold. PCR primers are listed in Table S1 as CD44_ChIP P1, P2 and I1. SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) was used for quantification. Results were expressed as fold enrichment relative to the IgG control. *POL2* binding to *GAPDH* was used as positive control.

### 2.2. Luciferase assays

The *CD44* promoter region containing both P1 and P2 putative PBX1 binding sites (Fig. 1) was amplified with the CD44-PROM primers listed in Table S1, and the 912 bp fragment was ligated into the pGL4.23 luciferase reporter vector (Promega, Madison, WI, USA). HEK 293 cells were transfected with the CD44_PROM-pGL4.23 along with 0–1000 ng DNA of PBX1-b or PBX1-d expression plasmids using Lipofectamine® 2000 (Life Tech.). After 48 h, cells were lysed and the firefly and Renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. The results were presented as the ratio of Renilla to firefly luciferase activities normalized to the values for cells that were not transfected with PBX1 plasmids.

### 2.3. CD44 message expression

RNA was extracted from HEK 293 cells transfected with 1 ug PBX1-b or PBX1-d expression plasmids using the RNeasy mini kit (Qiagen, Germantown, MD, USA). After cDNA synthesis from 5 ug total RNA using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), real-time qPCR analysis of *CD44* expression was performed using Bio-Rad' SYBR® Green Supermix and its expression was normalized to *GAPDH*. Primer sequences are listed in Table S1 as CD44_qRT-PCR. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the *RQ* expression in cells transfected with an empty vector.

### 2.4. Flow cytometry

Jurkat T cells stably transfected with a lentiviral vector (LV) co-expressing either PBX1-b or PBX1-d and GFP (Sengupta et al., 2012) were stained with an anti-human CD44-Phycoerythrin (PE)-conjugated antibody (IM7, eBioscience, San Diego, CA, USA) or rat IgG2b-PE isotype control. At least 50,000 events were acquired per sample on a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA). Dead cells were excluded based on forward and side scatter characteristics. Analysis was conducted with the FCSExpress software (De Novo Software, Glendale, CA, USA) comparing CD44 expression in $GFP^+$ and $GFP^-$ cells.

### 2.5. Site directed mutagenesis

The PGL4.23 plasmid containing the two PBX1 P1 and P2 binding sites in the *CD44* promoter was amplified by the Q5® high fidelity DNA polymerase (New England Biolabs, Ipswich, MA) with either the Q5CD44M1 primers introducing a BafI restriction site in P1, or the Q5CD44M2 primers introducing a HpyCH4V restriction site in P2. An analysis with Jaspar3 predicted that the introduced mutations abrogated PBX1 binding. The primer sequences are listed in Table S1. The PCR fragments were cloned into pGL4.23 and mutations were confirmed by restriction enzyme analysis and Sanger sequencing.

### 2.6. Immunoprecipitation and western blotting

Immunoprecipitation (IP) and Western blotting (WB) was performed on two types of cells: 1) HEK 293 cells transfected with 1 ug PBX1-b or PBX1-d expression plasmids, along with FLAG-tagged MEIS or PREP-1 (a kind gift from Dr. F. Blasi, IFOM, Milano, Italy) and 2) Jurkat T cells transfected with either LV-PBX1-b-GFP or LV-PBX1-D-GFP (Sengupta et al., 2012). Cells were lysed in lysis buffer (150 mm NaCl, 1% NP-40, 50 μm Tris-HCl, pH 8.0) containing a protease inhibitor mixture (Roche, Branford, CT, USA) for 20 min on