Expanded View Figures

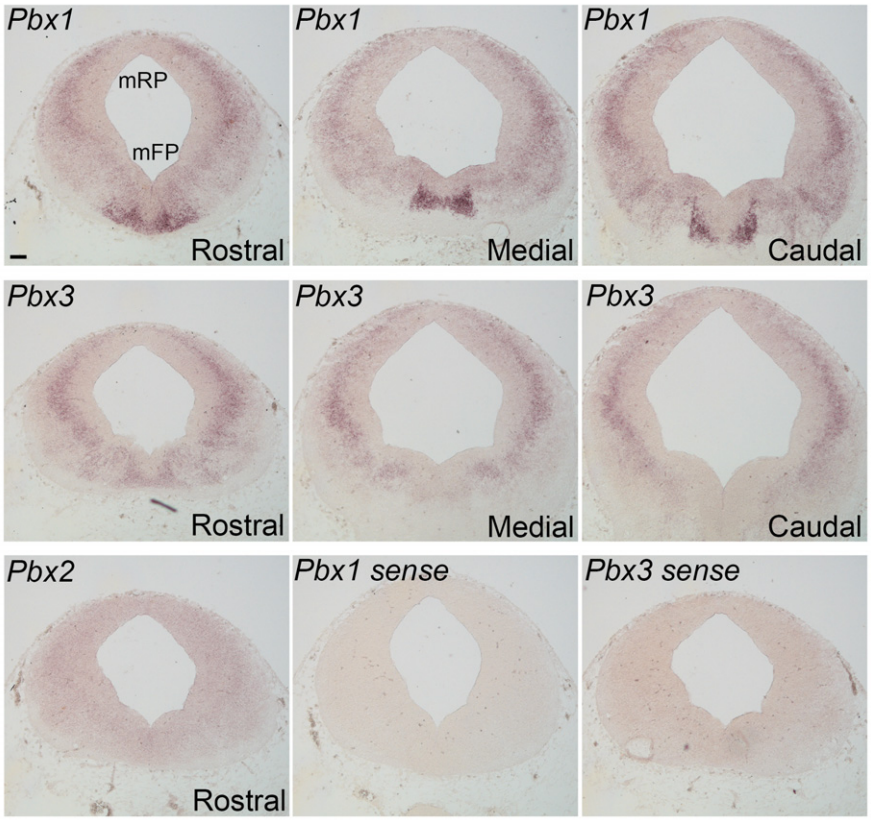

Figure EV1. *Pbx1* and *Pbx3*, but not *Pbx2*, are expressed in the mouse midbrain.
Pbx1 is expressed at high levels in the VM, at rostral, medial, and caudal levels at E12.5. Weak expression of *Pbx3* is found only in rostral levels of the VM. *Pbx2* was not detected in the midbrain. *Pbx1* and *Pbx3* sense controls show the specificity of the antisense probe. Scale bar, 80 μm.

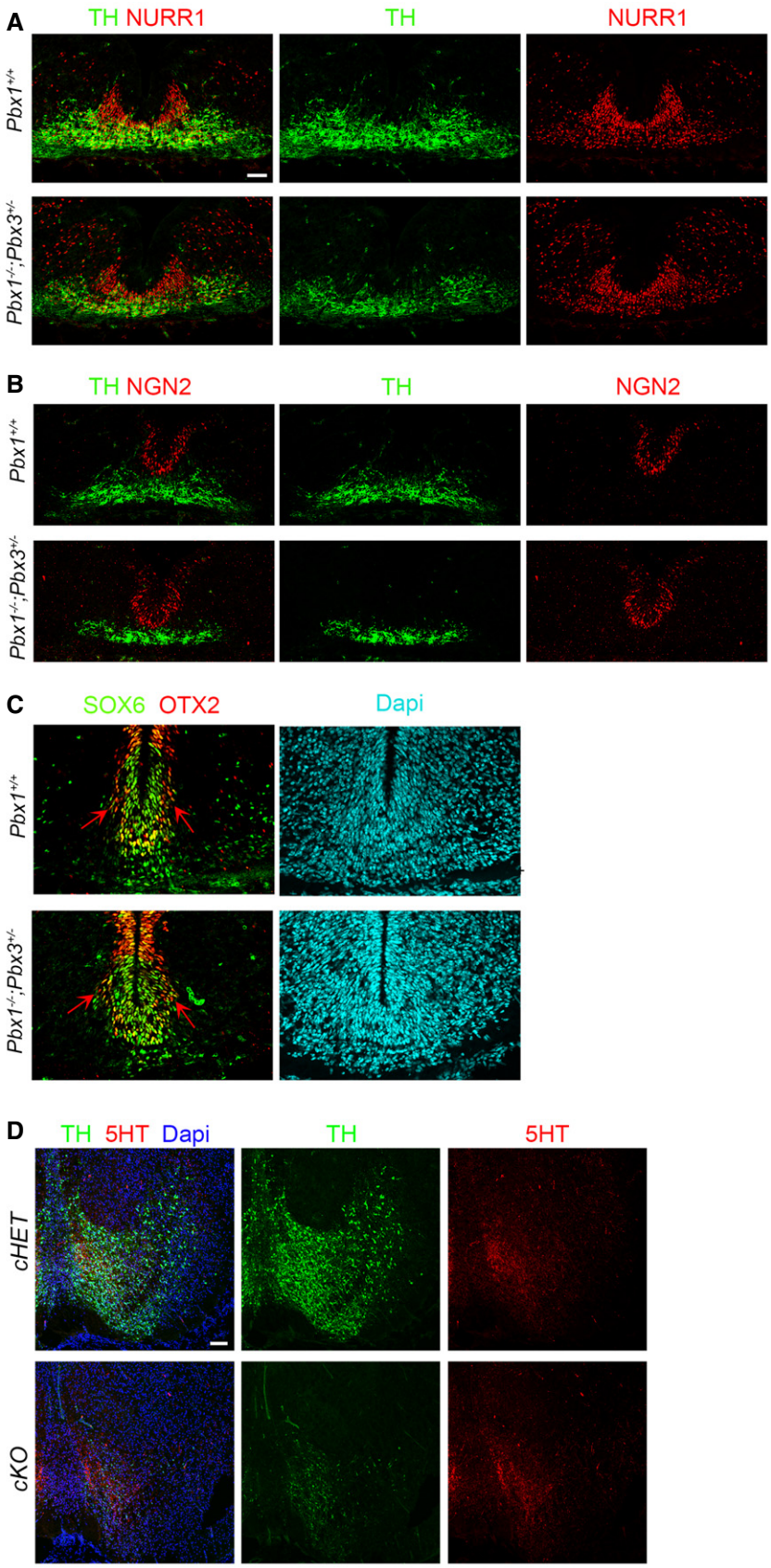

Figure EV2. Analysis of the VM phenotype of *Pbx1*$^{-/-}$;*Pbx3*$^{+/-}$ and cKO embryos.

A No alteration in the number of NURR1^{+} cells and a reduction in TH^{+} cells is detected in *Pbx1*$^{-/-}$;*Pbx3*$^{+/-}$ embryos at E12.5.

B Immunostaining for NGN2, a transcription factor required for mDA neurogenesis, was not altered in *Pbx1*$^{-/-}$;*Pbx3*$^{+/-}$ embryos at E12.5.

C Immunostaining for SOX6 and OTX2, two transcription factors involved in SN vs. VTA subtype specification, was not different in *Pbx1*$^{-/-}$;*Pbx3*$^{+/-}$ compared to *Pbx1*$^{+/+}$ embryos at E12.5. Red arrows indicate the lateral OTX2^{+} domain.

D Staining for 5HT (serotoninergic neurons) showed no major differences in cKO vs. *cHET* embryos at E18.5, indicating that there is no misspecification of mDAn into serotoninergic neurons.

Data information: All scale bars, 40 μm.

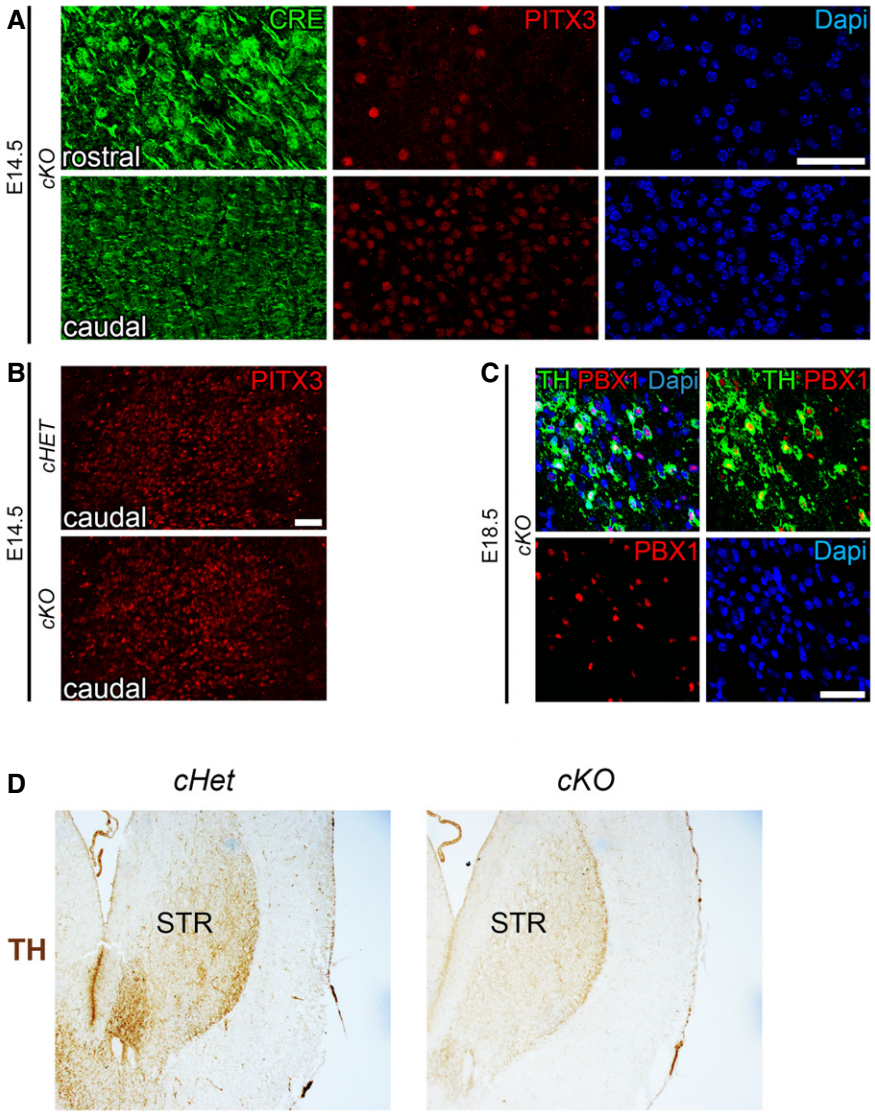

Figure EV3. CRE recombination does not take place in all mDA TH$^+$ cells of cKO embryos.

A *Pbx1$^{flox/flox}$;Pbx3$^{-/-}$;Th-IRES-Cre-ERT* (cKO) embryos treated with tamoxifen at E12.5 and E13.5 showed a reduction of PITX3 at E14.5 in rostral VM levels, where a strong nuclear localization of CRE was detected, but not in caudal VM, where diffuse low levels of CRE were found.

B PITX3 levels were not affected in caudal levels of E14.5 cKO embryos compared to cHET.

C At E18.5, CRE recombination still did not occur in a few cells in the most medial and caudal aspect of the VM of cKO embryos, which remained PBX1$^+$TH$^+$.

D Immunohistochemistry (DAB) for TH indicates that the loss of TH$^+$ neurons in the VM was also reflected by a loss of TH immunoreactive fibers in the striatum (STR) of cKO at E18.5, compared to cHET.

Data information: Scale bars, 40 μm.

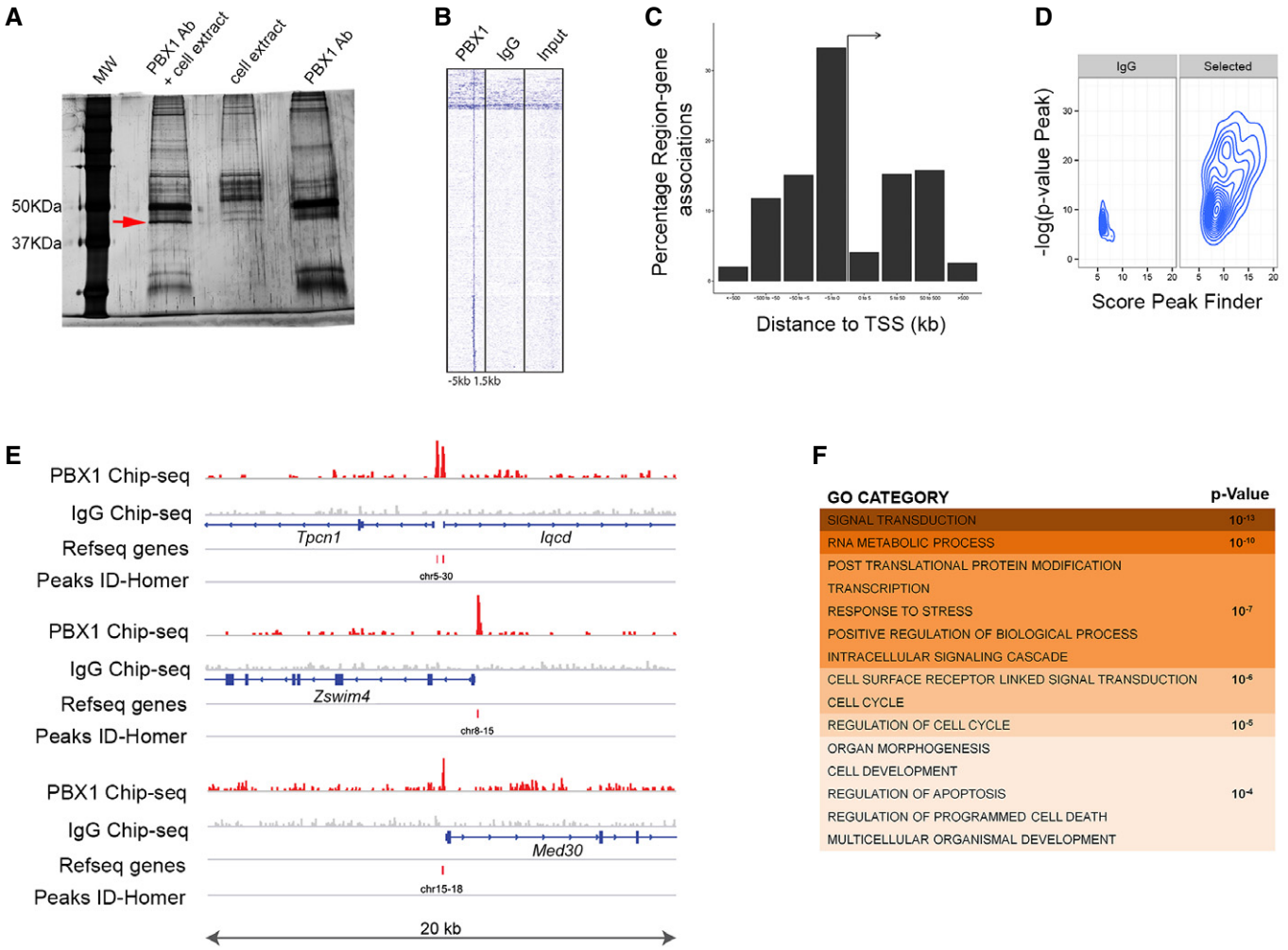

Figure EV4. PBX1-chromatin immunoprecipitation.

A Validation of the antibody against PBX1 (rabbit anti-PBX1, #43428, 1:500, Cell Signaling) to immunoprecipitate PBX1 from cell extracts obtained from SN4741 cells transfected with a *psg-PBX1* plasmid. The red arrow indicates the band identified as PBX1 by mass spectrometry.

B Heatmap enrichment of the peaks found within the ChIP-seq for PBX1 and IgG, in a window of −5 kb to +1.5 kb from the transcription starting site (TSS).

C Summary of the localization of the detected peaks according to their distance to the TSS.

D Quality representation of the peaks detected with HOMER in IgG and PBX1 (selected) ChIP-seq when statistical significance and peak scores are plotted. The values for peak score represent adjusted number of reads per peak detected.

E Schematic of PBX1 tracks compared to control IgG ChIP-seq on different representative loci. PBX1 peaks were identified with HOMER. Each locus represents a 20-kb chromosome region.

F Gene Ontology terms of the enriched categories identified by GSEA.

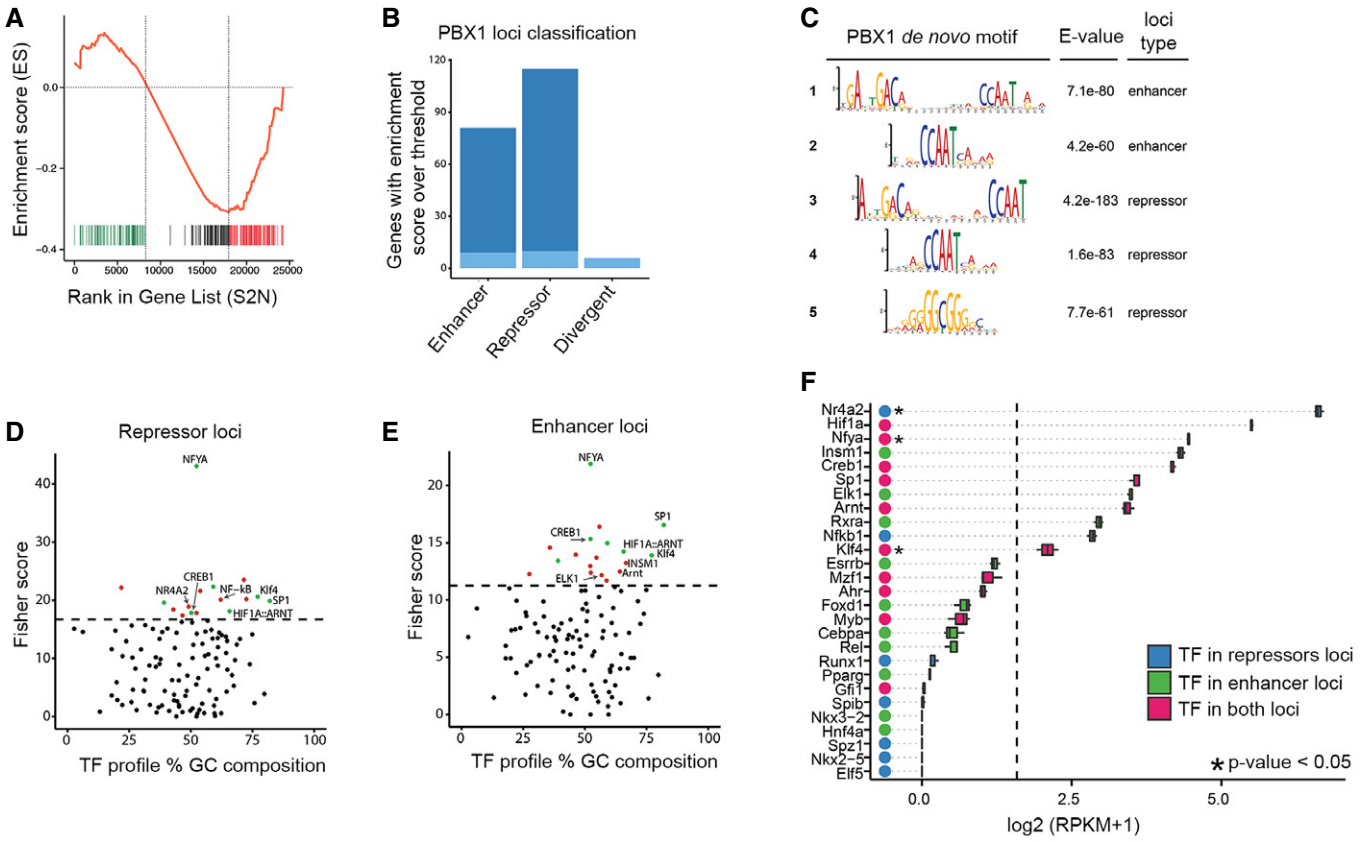

Figure EV5. Dual role of PBX1 in transcription.

A GSEA curve showing genes that are enriched (green) or depleted (red) in the E12.5 VM.

B Analysis of PBX1 peaks in the proximity of two neighbors TSS (as described in Materials and Methods). Dark blue indicates transcriptional regulation of only one gene of the two neighbors. Light blue indicates regulation of both genes. Peaks were classified as divergent if one gene was upregulated and the other downregulated.

C Motifs identified *de novo* on regions of 200 bp centered on PBX1 peaks.

D, E Overrepresented transcription factors binding sites found on neighboring loci of PBX1 using the oPOSSUM-3 software. In green, transcription factor binding sites common in both loci. In red, transcription factor binding sites found only in one locus. Threshold of Fisher score (dashed line) is mean plus standard deviation.

F Boxplot of the expression value of transcriptions factors associated with the enriched TFBS at ventral midbrain at E12.5. Color of the dot indicates the type of loci in which the transcription factor is found. DEG genes in the VM at E12.5 are annotated with an asterisk. Dashed line at RPKM = 2.

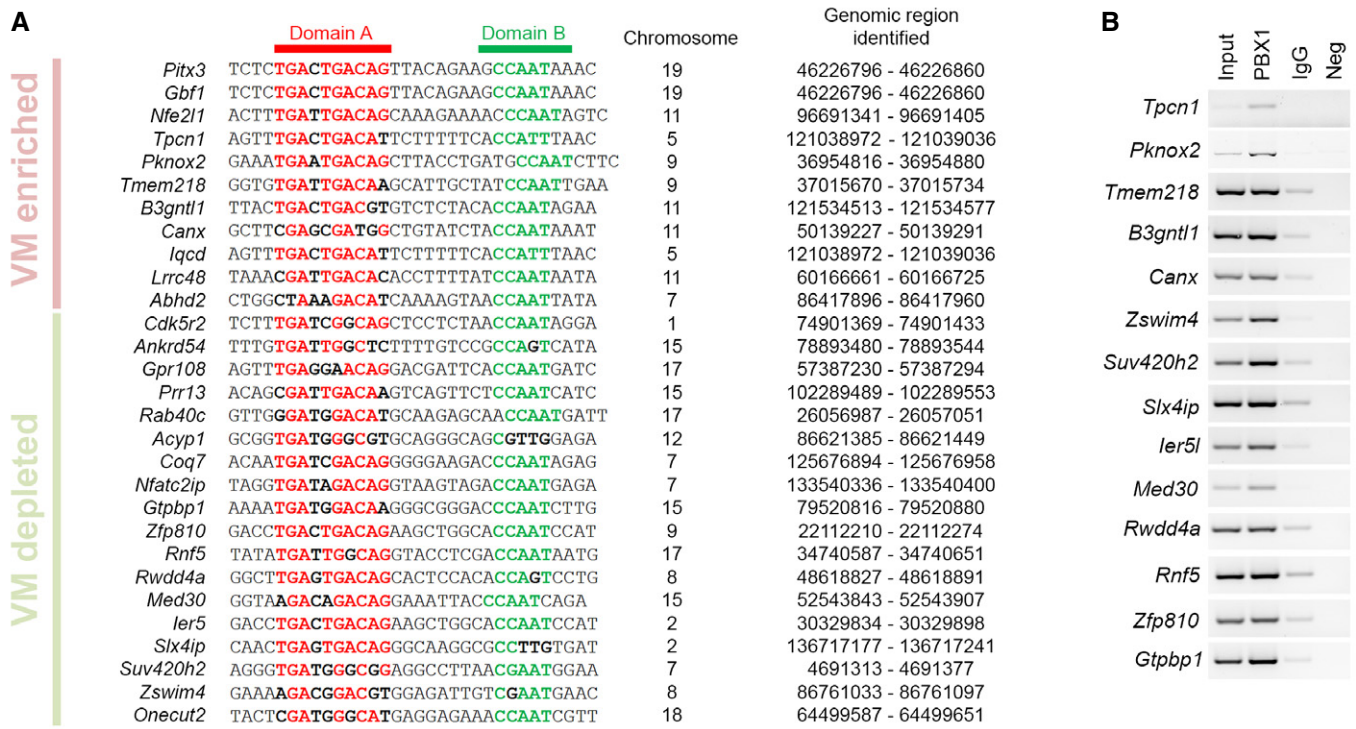

Figure EV6. Multiple comparison of PBX1 binding sites located in different regulatory regions.

A Our analysis identified a PBX1 binding site composed of a 10-nucleotide motif (domain A, red) separated by 8–11 nucleotides from a 5-nucleotide motif (domain B, green). Interval numbers indicate the genomic regions identified in our ChIP experiments. Inside each domain, highly conserved amino acids are in bold red or green, and low conserved amino acids are in bold black.

B PBX1 ChIP in E12.5 mouse VM followed by PCR confirmed that PBX1 binds to multiple regions.