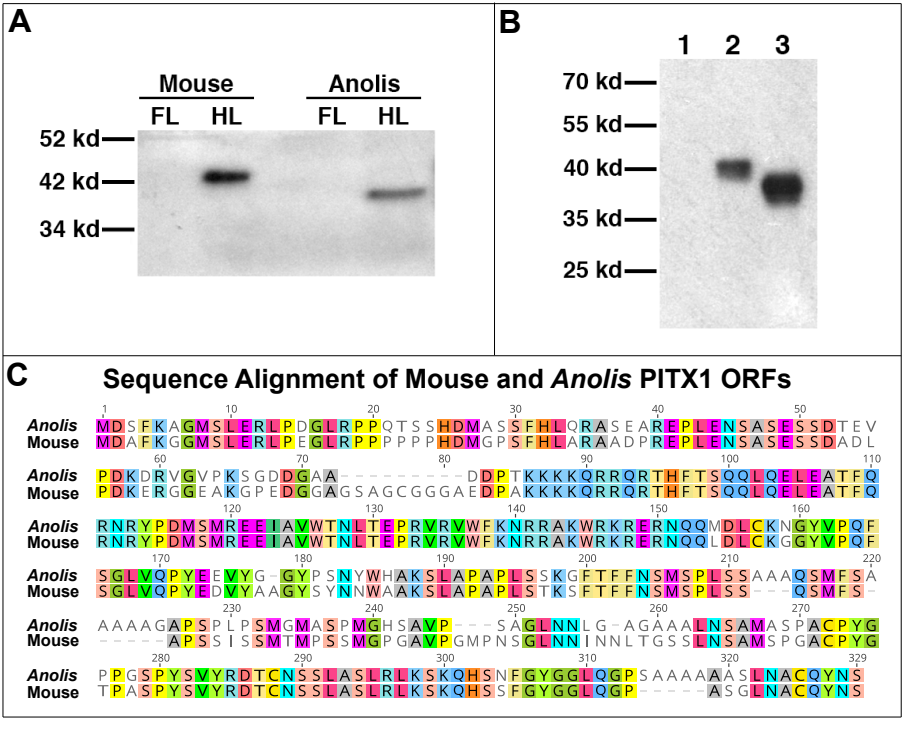

Figure S1. PITX1 antibody specificity and sequence alignment of mouse and Anolis PITX1. A) Western blot analysis of PITX1-specific antibody against protein extracts from mouse and *Anolis* hindlimbs. The antibody recognizes a hindlimb-specific protein in the embryonic hindlimbs of both species. 20 micrograms of protein were loaded onto each lane. **B)** Western blot using PITX1 antibody on HEK293T cells transformed with expression constructs containing GFP (lane 1) or the full ORF of Mouse PITX1 (lane 2) or *Anolis* PITX1 (lane 3). Mouse and *Anolis* PITX1 proteins exhibit different migration sizes despite having similar molecular weights. 10 micrograms of protein were loaded onto each lane. **C)** Sequence alignment of predicted mouse and *Anolis* PITX1 open reading frames (ORFs). The overall amino acid identify is 73%. Identical amino acids are highlighted in color.

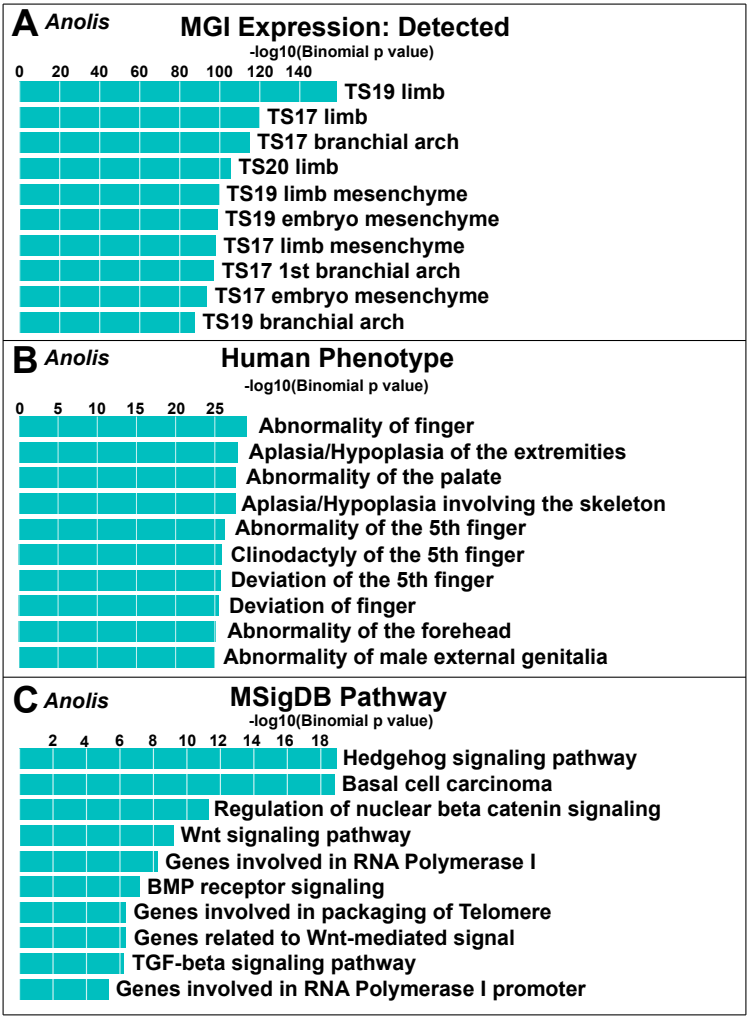

Figure S2. Gene ontology associations of PITX1 binding sites in *Anolis* hindlimbs. The top 10 GO terms associated with the subset of *Anolis* PITX1 peaks that have detectable sequence conservation in the mouse genome for the following gene ontology databases: **A)** Mouse Genome Informatics (MGI) Expression, **B)** Human Phenotype and **C)** MSigDB (Molecular Signature Database) Pathway. Analysis was performed using GREAT (McLean et al., 2010).

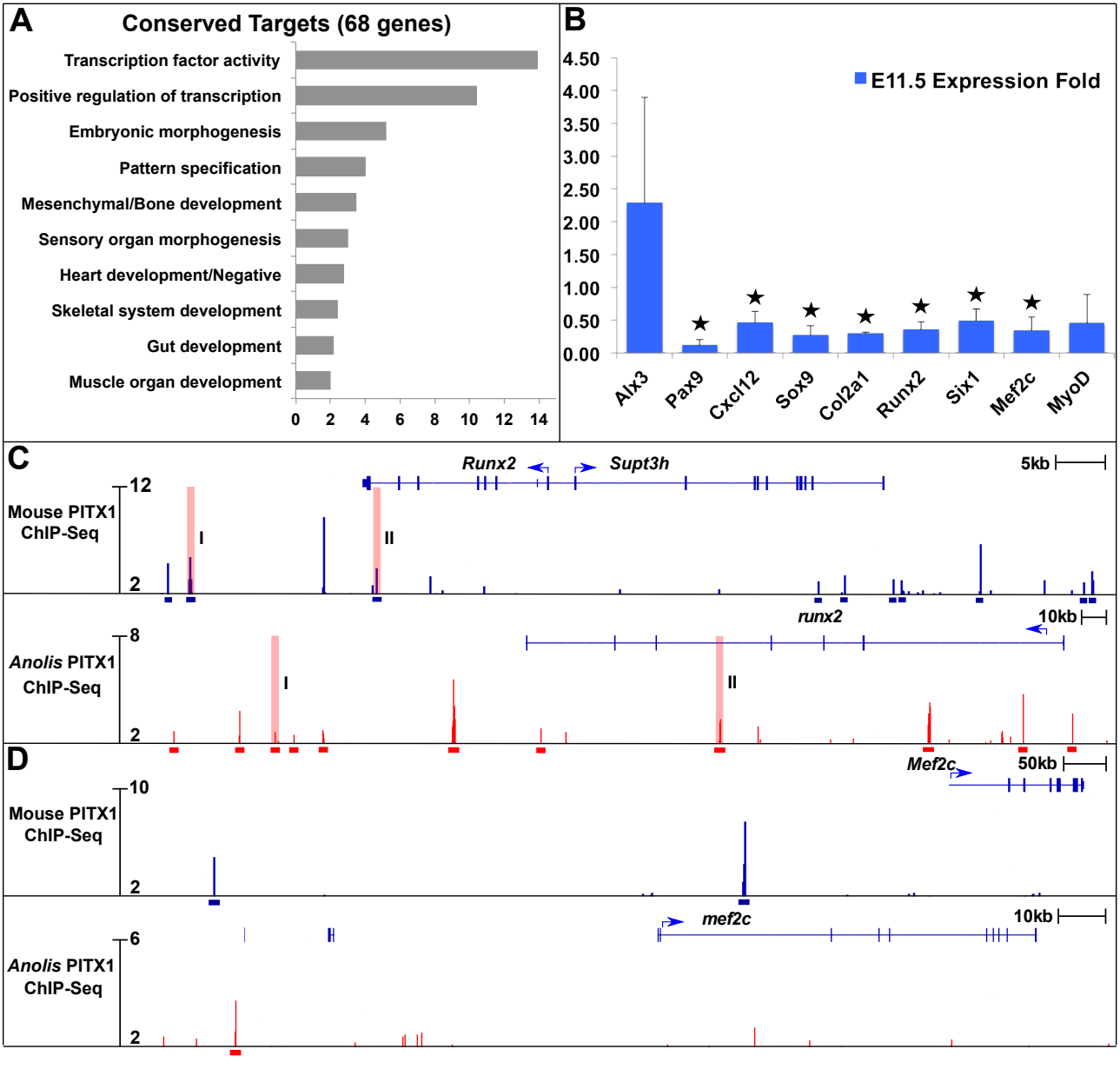

Figure S3. The binding events and expression analyses of PITX1 putative direct targets. A) The top 10 enriched clusters associated with conserved targets of PITX1 in developing mouse hindlimbs. **B)** qRT-PCR of limb patterning, chondrogenesis- and myogenesis-related genes in wide-type versus *Pitx1*$^{-/-}$ mouse hindlimbs at E11.5. Asterisks indicate $p < 0.05$ (Two-tailed t-test). C-D) Comparison of PITX1 binding profiles in mouse and *Anolis* hindlimbs showing the positions of mouse, mouse/*Anolis* conserved and *Anolis* PITX1 peaks in the neighboring regions of putative direct targets **C)** *Runx2* and **D)** *Mef2c*. Blue bars underline identified mouse PITX1 peaks and red bars underline identified *Anolis* PITX1 peaks. Conserved peaks identified in both species are highlighted in pink. I and II are used to distinguish two different pairs of conserved peaks at the *Runx2* and *Mef2c* loci.