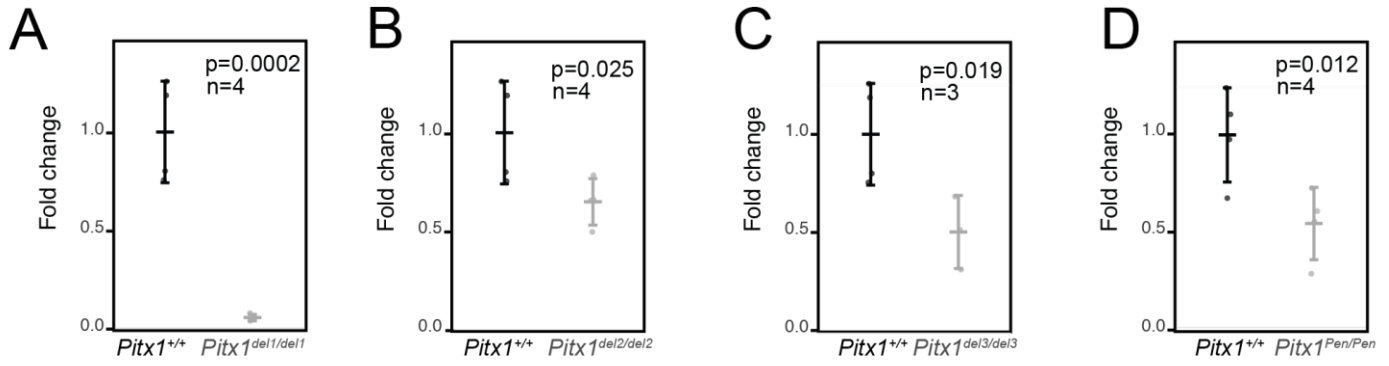


Supplementary Figure 1

Probing the regulatory sequences and activities of the *Pitx1* locus.

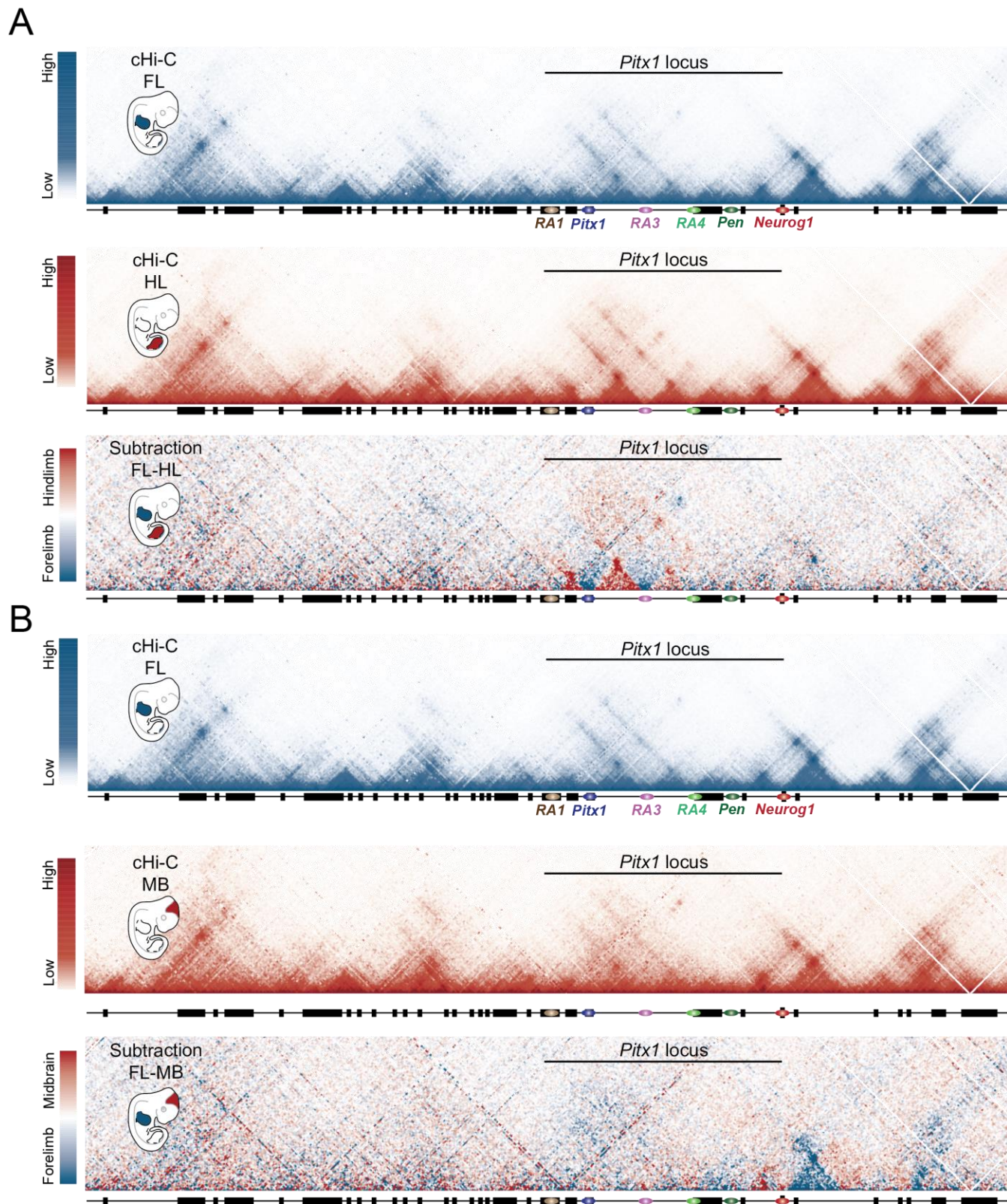
a, Table listing putative enhancer regions tested by *lacZ* reporter assay. Above is the *lacZ* reporter activity in E11.5 embryos. Numbers represent the number of embryos displaying the staining shown in the table. **b**, Table listing the genomic location of *lacZ* sensors 1 and 2 at the *Pitx1* locus (mm9) and *lacZ* reporter staining in E11.5 embryos.



Supplementary Figure 2

Quantification of *Pitx1* hindlimb transcription in several deletion mice.

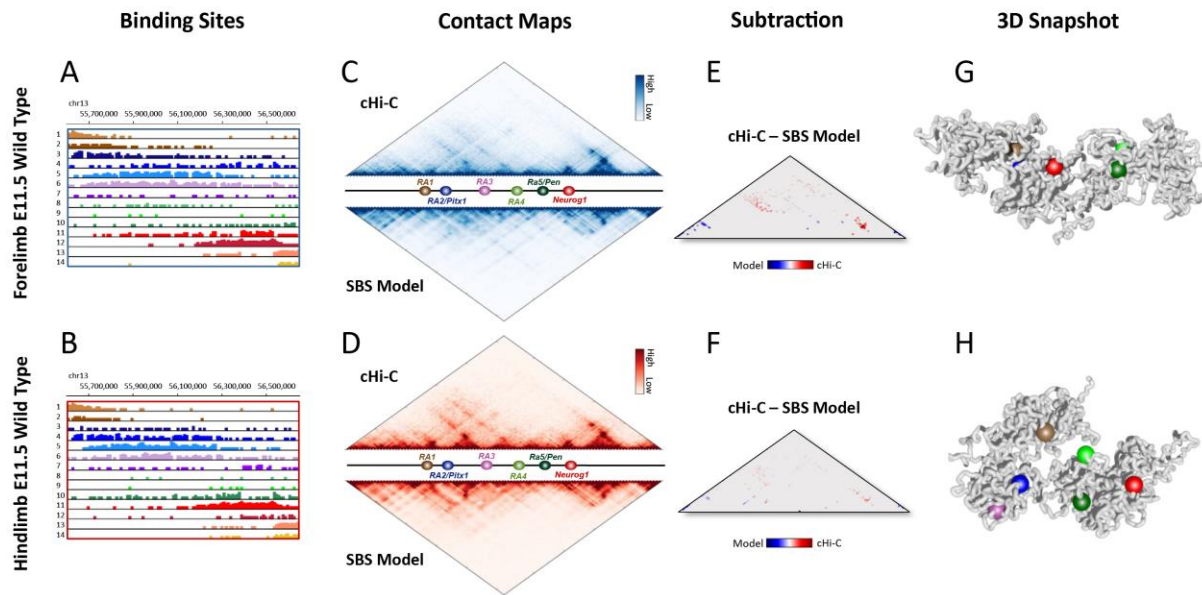
In all analyses, we used a one-sided *t* test to evaluate the significance of the decrease in *Pitx1* expression and *n* represents the number of wild-type and mutant hindlimb pairs assayed. The s.d. is displayed as error bars and the measure of the center corresponds to the average. **a**, qRT-PCR mRNA quantification of E11.5 *Pitx1*^{del1/del1} hindlimbs ($P = 0.0002$, $n = 4$). **b**, qRT-PCR mRNA quantification of E11.5 *Pitx1*^{del2/del2} hindlimbs ($P = 0.025$, $n = 4$). **c**, qRT-PCR mRNA quantification of E11.5 *Pitx1*^{del3/del3} hindlimbs ($P = 0.019$, $n = 3$). **d**, qRT-PCR mRNA quantification of E11.5 *Pitx1*^{Pen/Pen} hindlimbs ($P = 0.012$, $n = 4$).



Supplementary Figure 3

cHi-C of the extended *Pitx1* locus in wild-type forelimb, hindlimb and midbrain tissues.

a, cHi-C interaction map in E11.5 forelimb (blue) and hindlimb (red) tissues over a 3-Mb captured region. Bottom, subtraction of hindlimb and forelimb cHi-C whereby blue indicates a higher chromatin interaction frequency in forelimb and red a higher interaction frequency in hindlimb as compared to each other. Note that only the *Pitx1* locus displays clear changes in chromatin interactions within the entire captured region. **b**, cHi-C interaction map in E11.5 forelimb (blue) and E10.5 midbrain (red) tissues over a 3-Mb captured region. Bottom, subtraction of midbrain and forelimb cHi-C whereby blue indicates a higher chromatin interaction frequency in forelimb and red a higher interaction frequency in midbrain as compared to each other. Note the absence of chromatin interaction changes at the *Pitx1* locus, in contrast to the neighboring telomeric domain (see blue domain in subtraction map), which include *Cxcl14*, a gene transcriptionally repressed in midbrain and active in forelimb.

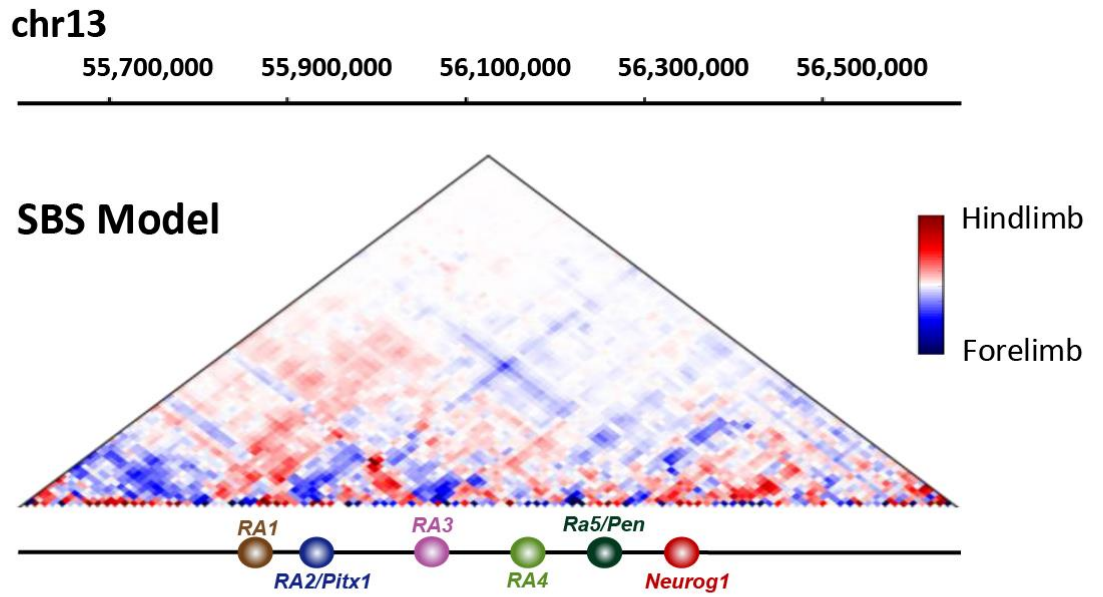


Supplementary Figure 4

Modeling of the *Pitx1* locus 3D architecture in wild-type forelimb and hindlimb.

a,b, Histograms displaying the position and abundance of 14 different types of binding sites (Methods) along the genome, in forelimbs (top) and hindlimbs (bottom) as derived from the E11.5 cHi-C data. Each binding site is displayed with a different color. **c,d**, Contact maps derived from cHi-C (above) and SBS model (below) display high similarity. The Pearson correlation, r , and the genomic-distance-corrected Pearson correlation, r' , between the cHi-C and SBS matrices ($105 \text{ bins} \times 105 \text{ bins} = 11,025$) are $r = 0.98$ and $r' = 0.84$ in forelimb and $r = 0.98$ and $r' = 0.82$ in hindlimb. **e,f**, Subtraction matrices between cHi-C and SBS model in wild-type forelimbs (top) and hindlimbs (bottom). Differences above random background are shown in red and blue. **g,h**, A representative 3D structure of the locus in forelimb (top) and hindlimb (bottom), selected from the ensemble of 'single-cell' model-derived conformations (Methods). In Fig. 4d,e, the corresponding coarse-grained versions are shown to highlight the position of genes and regulators.

Subtraction WT FL-HL

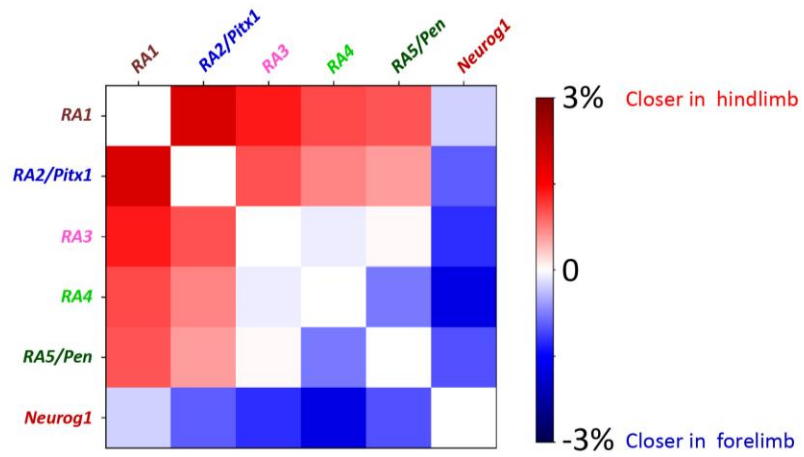


Supplementary Figure 5

Subtraction matrix between the SBS models of wild-type forelimb and hindlimb.

The corresponding chi-C data are shown in Fig. 4c.

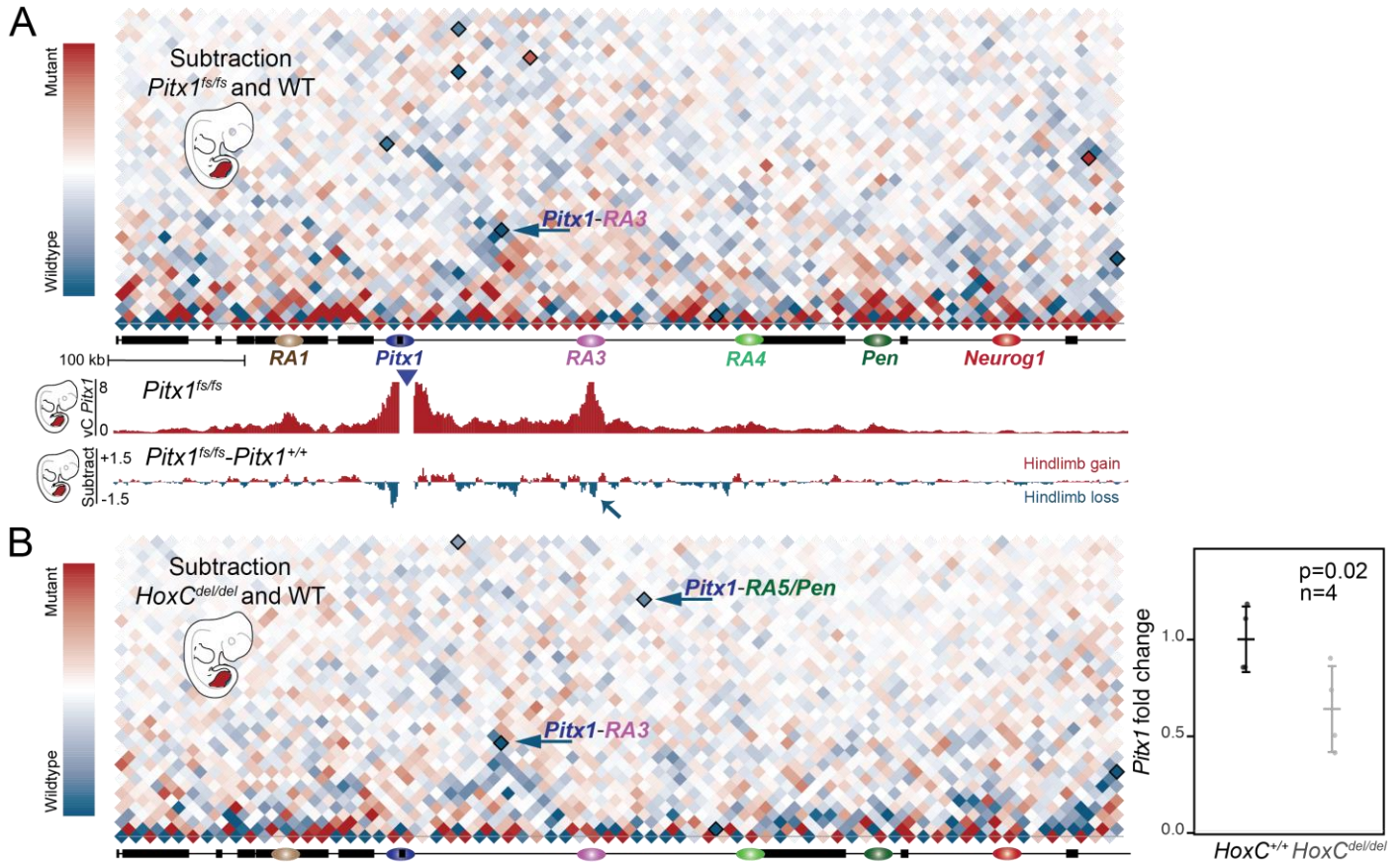
Average Relative Distance Changes



Supplementary Figure 6

Relative changes in physical distances between wild-type forelimb and hindlimb 3D structure.

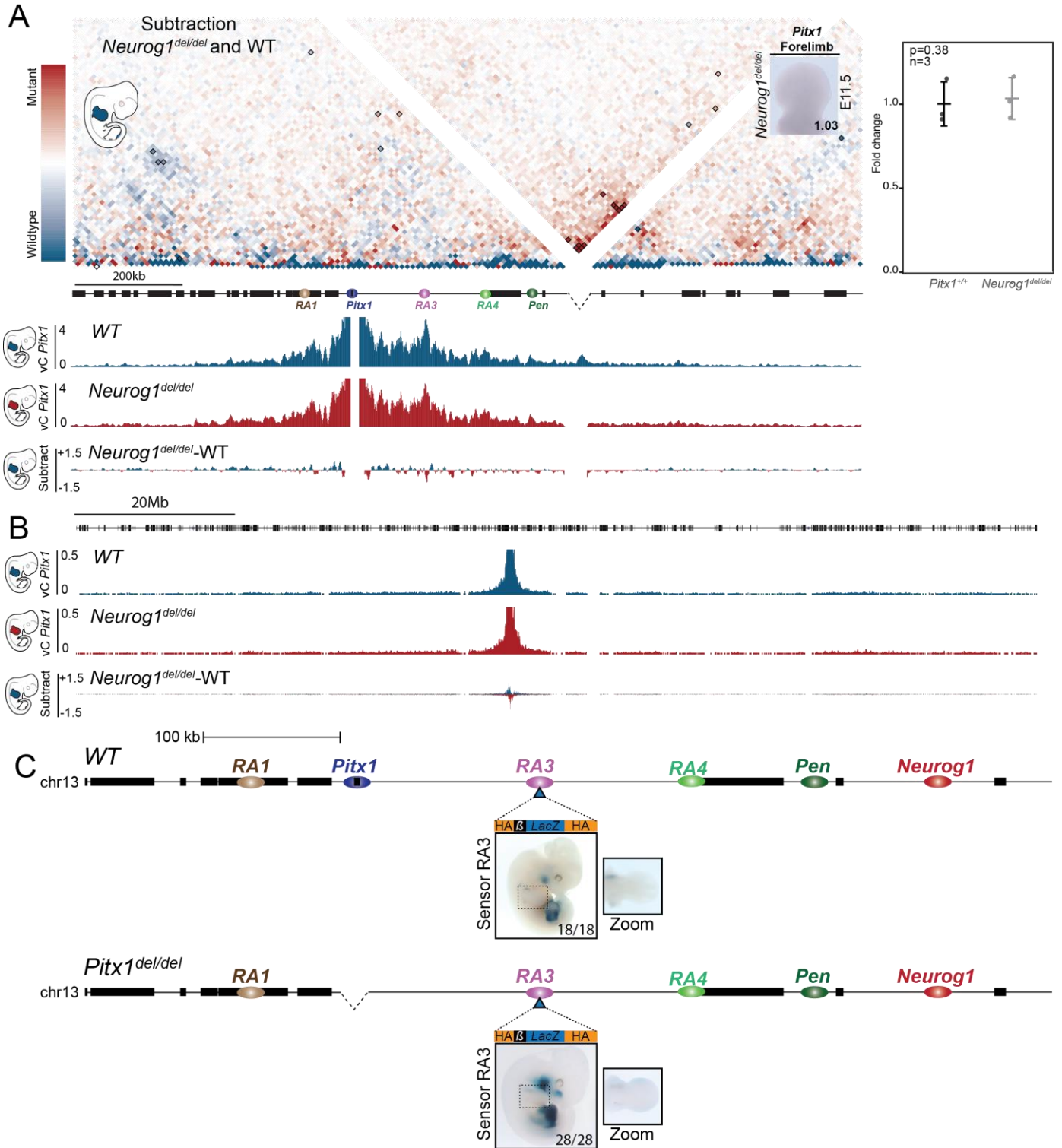
Heat map showing relative changes in physical distances between forelimb and hindlimb 3D structure as measured by the polymer model.



Supplementary Figure 7

Effect of *Pitx1* and *HoxC* loss of function on the 3D structure of the *Pitx1* locus in hindlimbs.

a, cHi-C subtraction between wild-type and *Pitx1^{fs/fs}* mutant hindlimb tissue at E11.5. Chromatin interactions more prevalent in mutant or wild-type hindlimb tissues are shown in red and blue, respectively. Significant changes in interactions are highlighted in black boxes (FDR = 0.05). Interactions significantly reduced between regulatory anchors are indicated with a blue arrow (*Pitx1-RA3* interaction). Derived viewpoint from cHi-C map, vC, using the *Pitx1* viewpoint is shown in red. Below is the subtraction track between wild-type and mutant hindlimb tissues using the respective viewpoint. **b**, cHi-C subtraction between wild-type and *HoxC^{del/del}* mutant hindlimb tissues at E11.5. Chromatin interactions more prevalent in mutant or wild-type hindlimb tissues are shown in red and blue, respectively. Significant changes are highlighted in black boxes (FDR = 0.05). Interactions significantly reduced between regulatory anchors are indicated with blue arrows (*Pitx1-RA3* and *Pitx1-Pen*). qRT-PCR quantification of *Pitx1* in *HoxC^{del/del}* mutant hindlimb tissues at E11.5 showed an average 36% reduction. (We used a one-sided *t* test to evaluate the significance of decrease in *Pitx1* expression and found $P = 0.02$; $n = 4$ wild-type and mutant hindlimb pairs; s.d. is displayed as error bars; the measure of the center is the average of the data points.)

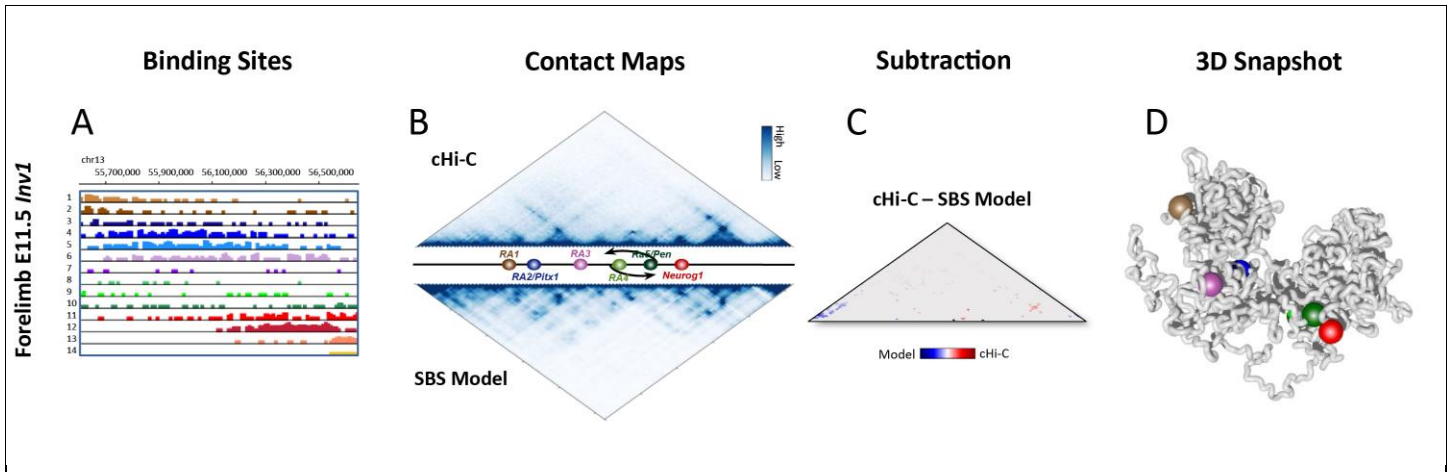


Supplementary Figure 8

Deletions of the *Pitx1* or *Neurog1* H3K27me3 domains are not sufficient to perturb the hindlimb-restricted regulatory activity of the locus.

a, cHi-C subtraction between wild-type and *Neurog1^{del/del}* mutant forelimb tissue at E11.5. Chromatin interactions more prevalent in mutant or wild-type forelimb tissues are shown in red and blue, respectively. Significant changes are highlighted in black boxes (FDR =

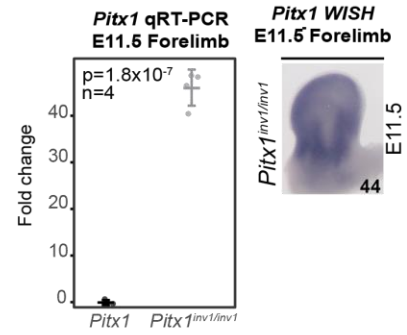
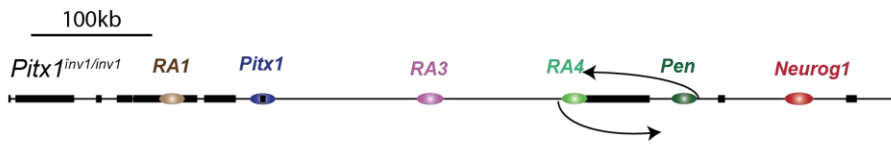
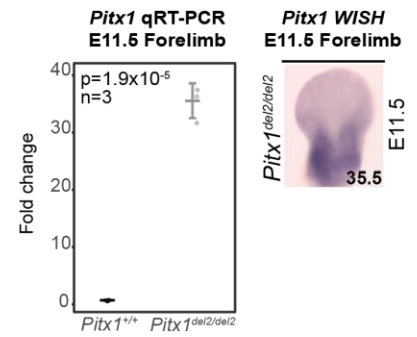
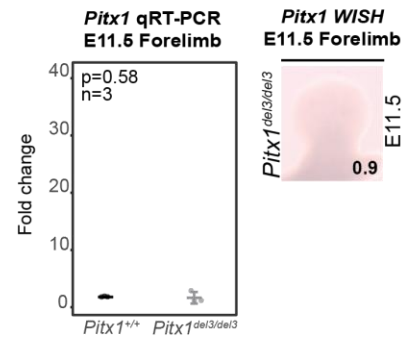
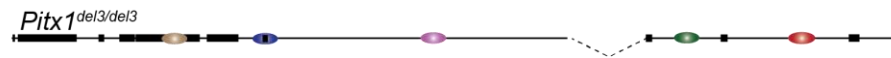
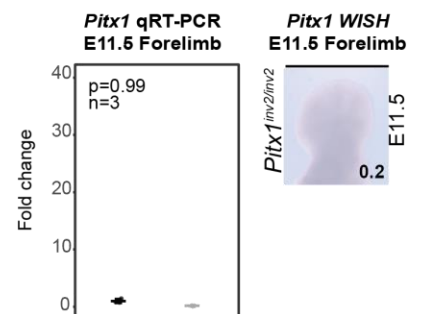
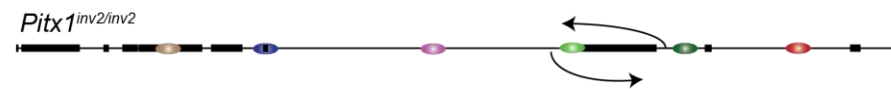
0.05). Right, *Neurog1*^{del/del} embryos do not show changes in *Pitx1* expression in E11.5 forelimbs as seen in WISH (photo) and quantified by qRT-PCR. (We used a one-sided *t* test to evaluate the significance of increased *Pitx1* expression and found $P = 0.38$; $n = 3$ wild-type and mutant limb pairs; the center is the average and the s.d. is displayed by the error bars.) Below, derived vC from the *Pitx1* viewpoint in wild-type and *Neurog1*^{del/del} forelimbs are shown in blue and red, respectively. Below is the subtraction track between wild-type and mutant forelimb tissue using the respective viewpoint. **b**, Whole chromosome 13 view of vC from the *Pitx1* viewpoint. Note that these profiles display the genomic region enriched in cHi-C as well as the non-enriched part of the chromosome. **c**, Staining of embryos with a *lacZ* sensor integrated in the *RA3* region. Wild-type (top) and *Pitx1*^{del/del} (bottom) staining display no obvious difference between fore- and hindlimb. Eighteen of 18 embryos displayed the same staining in the wild-type background, and 28 of 28 displayed the same staining in the *Pitx1*^{del/del} background.



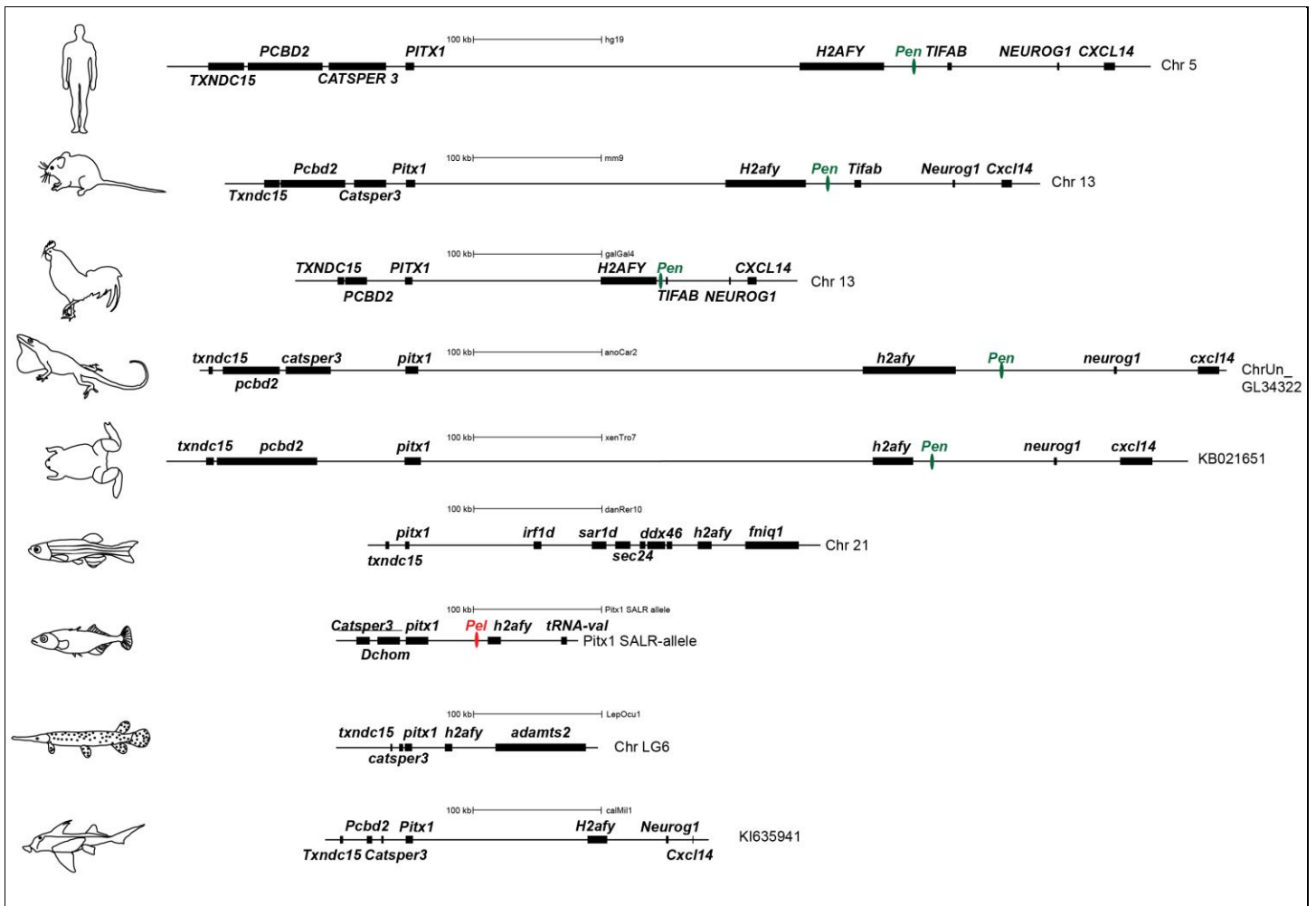
Supplementary Figure 9

Modeling of the *Pitx1* locus 3D architecture in *Pitx1*^{inv1/inv1} forelimbs.

a, Histograms displaying the position and abundance of 14 different types of binding sites (Methods) along the genome, in *Pitx1*^{inv1/inv1} forelimbs at E11.5. As in Supplementary Fig. 4a,b, each binding site is displayed with a different color. **b**, Contact maps derived from cHi-C (above) and SBS model (below) display high similarity. The Pearson correlation, r , and the genomic-distance-corrected Pearson correlation, r' , between the cHi-C and SBS matrices (105 bins * 105 bins = 11,025) are $r = 0.97$ and $r' = 0.74$. **c**, Subtraction matrix between cHi-C and SBS model in *Pitx1*^{inv1/inv1} forelimbs. Differences above random background are shown in red and blue. **d**, A representative 3D structure of the locus in *Pitx1*^{inv1/inv1} forelimbs, selected from the ensemble of 'single-cell' model-derived conformations (Methods). In Fig. 6e, the corresponding coarse-grained version is shown to highlight the position of genes and regulators.

A**B****C****D****Supplementary Figure 10**Quantification of *Pitx1* forelimb transcription in several deletion and inversion mice.

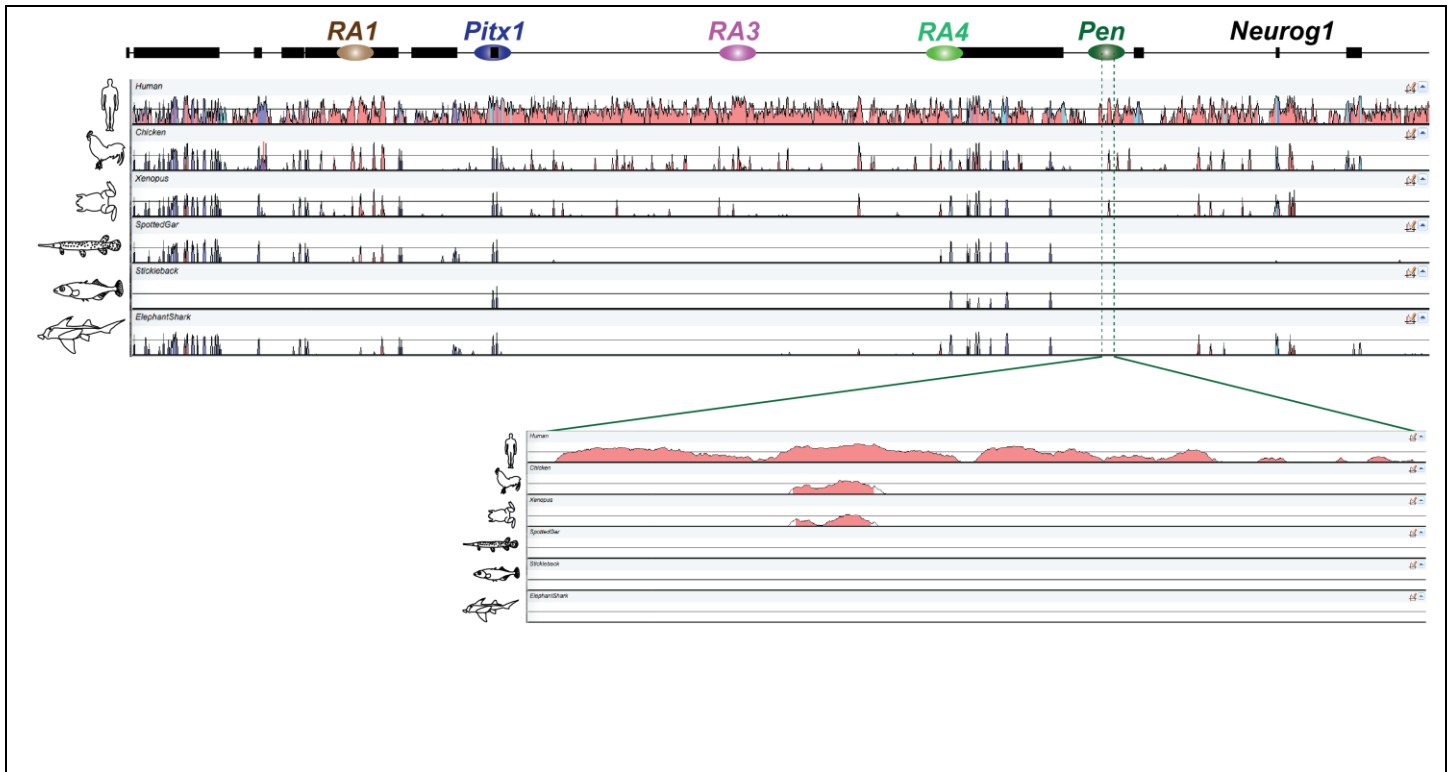
WISH and qRT-PCR mRNA quantification of deletions and inversion at the *Pitx1* locus. In all analyses, we used a one-sided *t* test to evaluate the significance of increased *Pitx1* expression and *n* represents the number of wild-type and mutant forelimb pairs assayed. The s.d. is displayed as error bars and the measure of the center corresponds to the average. **a**, WISH and qRT-PCR mRNA quantification of E11.5 *Pitx1*^{inv1/inv1} forelimbs ($P = 1.8 \times 10^{-7}$, $n = 4$). **b**, WISH and qRT-PCR mRNA quantification of E11.5 *Pitx1*^{del2/del2} forelimbs ($P = 1.9 \times 10^{-5}$, $n = 3$). **c**, WISH and qRT-PCR mRNA quantification of E11.5 *Pitx1*^{del3/del3} forelimbs ($P = 0.58$, $n = 3$). **d**, WISH and qRT-PCR mRNA quantification of E11.5 *Pitx1*^{inv2/inv2} forelimbs ($P = 0.99$, $n = 3$).



Supplementary Figure 11

Schematic representation of the *Pitx1* locus in several species.

Schematic representation of the *Pitx1* extended locus in several species, demonstrating the conserved synteny of the region. The species from top to bottom are human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), lizard (*Anolis carolinensis*), frog (*Xenopus (Silurana) tropicalis*), zebrafish (*Danio rerio*), stickleback (*Gasterosteus aculeatus*), spotted gar (*Lepisosteus oculatus*), and elephant shark (*Callorhynchus milii*). *Pen* (green) is found in tetrapods but not in fish. The previously characterized pelvic enhancer *Pel* (red) is displayed in red in stickleback.



Supplementary Figure 12

Conservation of sequences between vertebrates along the *Pitx1* regulatory landscape.

Conservation of sequences between vertebrates along the *Pitx1* regulatory landscape. A zoomed-in view of the *Pen* region shows that the element is conserved with human (*Homo sapiens*), chicken (*Gallus gallus*), and frog (*Xenopus (Silurana) tropicalis*), but not with bony or cartilaginous fishes (here stickleback (*Gasterosteus aculeatus*), spotted gar (*Lepisosteus oculatus*), and elephant shark (*Callorhynchus milii*)).