

# Dual hindlimb control elements in the *Tbx4* gene and region-specific control of bone size in vertebrate limbs

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The *Tbx4* transcription factor is crucial for normal hindlimb and vascular development, yet little is known about how its highly conserved expression patterns are generated. We have used comparative genomics and functional scanning in transgenic mice to identify a dispersed group of enhancers controlling *Tbx4* expression in different tissues. Two independent enhancers control hindlimb expression, one located upstream and one downstream of the *Tbx4* coding exons. These two enhancers, hindlimb enhancer A and hindlimb enhancer B (HLEA and HLEB), differ in their primary sequence, in their precise patterns of activity within the hindlimb, and in their degree of sequence conservation across animals. HLEB is highly conserved from fish to mammals. Although *Tbx4* expression and hindlimb development occur at different axial levels in fish and mammals, HLEB cloned from either fish or mouse is capable of driving expression at the appropriate position of hindlimb development in mouse embryos. HLEA is highly conserved only in mammals. Deletion of HLEA from the endogenous mouse locus reduces expression of *Tbx4* in the hindlimb during embryogenesis, bypasses the embryonic lethality of *Tbx4*-null mutations, and produces viable, fertile mice with characteristic changes in the size of bones in the hindlimb but not the forelimb. We speculate that dual hindlimb enhancers provide a flexible genomic mechanism for altering the strength and location of *Tbx4* expression during normal development, making it possible to separately modify the size of forelimb and hindlimb bones during vertebrate evolution.

**KEY WORDS:** *Tbx4*, Hindlimb, cis-regulatory element, Enhancer

## INTRODUCTION

Tetrapod limbs show great morphological diversity (Flower, 1876; Mariani and Martin, 2003). Although a common pattern of skeletal structures can be seen in different species, the position of limbs, and the size, morphology and number of bones within limbs are extensively modified in organisms adapted to running, hopping, flying or swimming. Differences between forelimbs and hindlimbs of a single species can be just as striking as the differences exhibited among highly diverged species. For example, digit bones show striking elongation in flying animals owing to a local increase in skeletal growth in forelimbs but not hindlimbs. Conversely, pelvic structures are lost or greatly reduced in marine mammals, an alteration that affects the hindlimbs without disrupting normal forelimb development.

Numerous signaling and transcription factor pathways have now been identified that participate in limb development (Capdevila and Izpisua Belmonte, 2001; Mariani and Martin, 2003; Tickle, 2003). Most of these factors are expressed in both forelimbs and hindlimbs. However, particular members of the T-box family of DNA-binding proteins are expressed specifically in forelimbs or hindlimbs of many different animals (Chapman et al., 1996; Gibson-Brown et al., 1998; Logan et al., 1998; Tamura et al., 1999; Ruvinsky et al., 2000; Takabatake et al., 2000; Tanaka et al., 2002). *Tbx5* is expressed in the forelimb field prior to forelimb outgrowth and continues to be expressed specifically in the forelimb mesenchyme as limb development proceeds. In humans, haploinsufficiency for *TBX5* results in reduced and malformed forelimbs, as well as heart defects in Holt-Oram syndrome (Basson et al., 1997; Li et al., 1997; Agarwal et al., 2003; Rallis et al., 2003). Likewise, loss of *Tbx5*

function in mice and zebrafish completely prevents outgrowth of forelimbs and pectoral fins, respectively (Ahn et al., 2002; Garrity et al., 2002).

A related gene, *Tbx4*, is specifically expressed in the lateral plate mesoderm of the early hindlimb field and in the developing hindlimb bud (Chapman et al., 1996). Haploinsufficiency for *TBX4* in humans results in the hindlimb-specific defects of small-patella syndrome (SPS), including incomplete ossification of the pelvis and small or reduced kneecaps (Bongers et al., 2004). Complete loss of mouse *Tbx4* function also disrupts hindlimb development, although detailed studies have been difficult because of mid-gestation lethality due to vascular defects (Naiche and Papaioannou, 2003; Naiche and Papaioannou, 2007).

Although *Tbx4* and *Tbx5* are among the earliest markers of the prospective forelimb and hindlimb fields (Gibson-Brown et al., 1996), it is not yet clear how their forelimb or hindlimb specificity is established, or what causes the genes to turn on at different axial levels in animals whose forelimbs and hindlimbs form at different positions along the body. Previous studies suggest that the homeodomain transcription factor *Pitx1* may act upstream of *Tbx4*. *Pitx1* also shows hindlimb-specific expression, and can induce *Tbx4* expression when overexpressed in chick forelimbs (Logan and Tabin, 1999). The related paralog, *Pitx2*, has similar trans-activating properties and is initially co-expressed with *Pitx1* in the lateral plate mesoderm of the early hindlimb field. However, *Tbx4* expression is reduced, but not eliminated, in *Pitx1*<sup>-/-</sup> or *Pitx1*<sup>-/-</sup> *Pitx2*<sup>+/-</sup> embryos (Lancot et al., 1999; Szeto et al., 1999; Marcil et al., 2003). Although *Tbx4* levels in *Pitx1*<sup>-/-</sup> *Pitx2*<sup>-/-</sup> embryos have not been examined owing to early embryonic lethality, *Tbx4* expression is maintained in *Pitx1*<sup>-/-</sup> *Pitx2*<sup>+/-</sup> embryos at stages of hindlimb development at which *Pitx2* is not expressed (Marcil et al., 2003). Together, these results suggest that *Pitx1* and *Pitx2* are important upstream regulators of *Tbx4*, and that additional factors might also contribute to hindlimb-specific expression of *Tbx4*.

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Here we report a large-scale enhancer survey of the *Tbx4* locus. Our transgenic and comparative studies show that two different cis-regulatory elements contribute to hindlimb expression. Targeted knockout of one of these elements bypasses the lethality seen in *Tbx4*-knockout mice, and reveals an essential role for this enhancer in regulating bone size in developing hindlimbs.

## MATERIALS AND METHODS

### Transgenic mice

Transgenic mice were generated by pronuclear injections of FVB or C57BL6/CBA F2 embryos (Stanford Transgenic Research Facility and Xenogen Biosciences). BAC and plasmid DNAs were purified as previously described (DiLeone et al., 2000). Multiple independent embryos were scored for each transgene and only reproducible patterns were scored as significant (see Table S1 in the supplementary material).

### Hsp68LacZ transgenes

Potential enhancer regions were amplified using primers (Table 1) containing *NotI* restriction sites and cloned into the *NotI* site of p5'-Not-HspLacZ (DiLeone et al., 1998). In cases where multiple tandem copies were desired, products were amplified with primers containing *XbaI* and *SpeI* sites, cloned into a modified pBS KS+ plasmid with two *NotI* sites flanking an *XbaI* site, excised with *NotI* and cloned into p5'-Not-HspLacZ.

### BAC modifications

BACs RP24-376P4, RP24-84E15 and RP23-136J3 were identified by electronically searching mouse BAC end sequences (Zhao et al., 2001), and were modified by homologous recombination in bacteria (Lee et al., 2001). A *Tbx4* targeting cassette was generated by amplifying 5' and 3' homology arms and cloning them into pIPTGfTet, which contains an IRES-βGeo cassette and an FRT-flanked tetracycline resistance gene (TetR) (Chandler et al., 2007). 5' homology primers: *Tbx4*-IBG1, 5'-AGCTTGCTAG-CACATGTGGTCAGAGAGCATC-3' and *Tbx4*-IBG2, 5'-ATGAACTC-GAGTAATGGTGGACTCGGCTCCT-3'. 3' homology primers: *Tbx4*-IBG3, 5'-TTGACACTAGTCCCTGGGTGGGATAGAAGTA-3' and *Tbx4*-IBG4, 5'-AGTTATGCGGCCGCAAGAAAGGCCCTCCCTGTG-3'. Modified BACs contained an IRES-βGeo cassette 245 bp downstream of the *Tbx4* stop codon. The FRT-flanked TetR was removed by transient expression of FLPe recombinase.

BAC deletions were generated by amplifying TetR with primers possessing homology to regions flanking particular enhancers. HLEA: HLEA-DeIF, 5'-CCATGGAGCTCCAGGCTGCTTGGGGGAGGAGG-CCGAAGAGAGGGAACCCAAGATCTATGATTCCTTTGTC-3' and HLEA-DeIR, 5'-GGCTAGAAGTGTGACTTCTCCAAGAGTCAAC-AGGCCCTAGACTGGACTTATGAGAATAGGAACCTCAAGCT-3'. HLEB: HLEB-DeIF, 5'-AATGCGCATTTCCTCACAAAGTCAAC-CAATAAGTTGGCTCAGGAGACTAGATCTATGATTCCCTTTGTC-3'

and HLEB-DeIR, 5'-CGAGCATGGGTTGTGTGCTCTGCATTCGCA-TGAGACATAACCCGTGTACTAGAGAATAGGAACCTCAAGCT-3'. TetR was subsequently removed from modified BACs by negative selection on fusaric acid after re-targeting using homology arms identical to those used to create the initial TetR targeting cassette, but which lacked intervening TetR sequence (Yang et al., 1997). Retargeting fragments were generated from overlapping primers: HLEA-TetRDeIF, 5'-CCATGGAGCTCCAG-GCTGCTTGGGGGAGGAGGCCGAAGAGAGGGAACCCAAGAGT-CCAGT-3' and HLEA-TetRDeIR, 5'-GGCTAGAAGTGTGACTTCT-CCAAGAGTCAACAGGCCCTAGACTGGACTCTTGGGTTCCCT-3'; HLEB-TetRDeIF, 5'-CCATGGAGCTCCAGGCTGCTTGGGGGAGG-AGGCCGAAGAGAGGGAACCCAAGAGTCCAGT-3' and HLEB-TetRDeIR, 5'-GGCTAGAAGTGTGACTTCTCCAAGAGTCAACAG-GCCCTAGACTGGACTTCTTGGGTTCCCT-3'.

### lacZ staining and in situ hybridization

Whole-mount *lacZ* staining and in situ hybridization were performed as described (Wilkinson, 1992; DiLeone et al., 1998). The *Tbx4* riboprobe was generated by amplifying a 959 bp fragment from mouse embryonic hindlimb cDNA, cloning into pCR4Blunt-TOPO vector (Invitrogen), digesting with *NotI*, and transcribing with T3 RNA polymerase. *Tbx4* primers: *Tbx4*-PIF, 5'-CAAGGAGTATCCCGTGATCT-3' and *Tbx4*-P2R, 5'-CACATTC-TGAAATACCTTTCCATG-3'.

### Quantification of lacZ staining

Forelimbs and hindlimbs of E12.5 embryos carrying transgenes pHLEA-768 and pPitx1Mut were measured for area of *lacZ* staining using ImageJ (Abramoff et al., 2004). Ratios of hindlimb to forelimb staining for different constructs were compared using ANCOVA in S-Plus (6.0) (Insightful Corporation).

### Comparative sequence analysis

Sequences from *Mus musculus*, *Homo sapiens*, *Felis catus*, *Canis familiaris*, *Bos taurus*, *Dasyurus novemcinctus*, *Loxodonta africana*, *Monodelphis domestica*, *Ornithorhynchus anatinus*, *Gallus gallus*, *Anolis carolinensis*, *Danio rerio*, *Gasterosteus aculeatus* and *Callorhynchus milii* were obtained from NCBI, aligned with Multi-LAGAN and analyzed with VISTA (Brudno et al., 2003; Frazer et al., 2004).

### Generation of hindlimb enhancer-knockout mice

An HLEA targeting vector was generated by amplifying 5' and 3' homology arms from mouse strain 129P2 (5' arm: genome assembly mm9; Chr11: 85,687,031-85,689,553; 3' arm: Chr11: 85,690,623-85,696,175) and cloning into pLoxPNT flanking a floxed Neo cassette (Shalaby et al., 1995). The HLEA sequence with a 3' flanking loxP site was then inserted between the floxed Neo and the 3' homology arm to create targeting vector pHwHLEAKO. Homology arms were sequenced to verify the absence of mutations.

**Table 1. Hsp68LacZ transgene primers**

Transgene	Forward primer (5' to 3')	Reverse primer (5' to 3')
pDBM2	AGTTATGCGGCCGCGGTGGGCACACTATGGTACA	AGTTATGCGGCCGCATGCTCTTGGGGTCTACA
pDBM3	AGTTATGCGGCCGCTGATAGAAGGGATGAT	AGTTATGCGGCCGCAAACCCCTCTCCACTACA
pDBM5	ATCATTCTAGAATGAGGTATGAGCTCTTTTGGGA	ATCATACTAGTCTGTCAATATTTCTTGTGAG
pDBM7	CTAATGCGGCCGCTAATTAAGCCTATACCCA	TGTAAGCGGCCGCTCTTGTGCTTTCTGAAGC
pDBM40	AATAAGCGGCCGCTAGGGGAGGAACCAAGGATC	ATTAAGCGGCCGCTCATGTTGCTTAGAGCACT
pDBM42	AATAAGCGGCCGCTAGACGCCAGCTTTTACAGCTC	AAATAGCGGCCGCACTGTGTAGATTCTCTCTAGG
pDBM43	AATAAGCGGCCGCTACCTGGACTGTTTCTTTGC	AATATGCGGCCGCAAGGCTGTAGATGGCAGC
pDBM44	AATAAGCGGCCGCAAGAGCTCCAAACGATGC	ATTAAGCGGCCGCTCATGTTGCTTAGAGCACT
pDBM45	AATATGCGGCCGCTTATCAGACCGGTCACCTG	TTATTGCGGCCGCTAAAGGATACAAAAAGAGGCCCTGAG
pDBM50	AATAAGCGGCCGCTAATATTAGGATTGTTGGGAAG	CCACACCACTAGTTATTTTATGTTTTACAGATGC
pDBM51	AATATGCGGCCGCTTATCAGACCGGTCACCTG	
pDBM20 (stickleback HLEB)	ATCATTCTAGAATTTATGTTATCTAACTTTCCAC	
pHLEA-768*	CTAATGCGGCCGCTAATTAAGCCTATACCCA	TGGGTATAGGCTTAATTAGCGGCCGCATTAG
pPitMut*	CTAATGCGGCCGCTAATTAAGCCTATACCCA	TGGGTATAGGCTTAATTAGCGGCCGCATTAG

\*pHLEA-768 and pPitMut are identical with the exception that a putative Pitx1 site has been mutated from TAATCC to GACTAC in pPitMut. The following primers were used to introduce the mutation by PCR (mutated bases underlined): 5'-AGCCGCTGCCAGGGTAGTCTGTCGCCGCTGTTGGTGG-3' and 5'-ACTACCTGGCGACGGGCTGGGGTGGCGTCTGGGAGT-3'.

Homologous targeting was performed on ES cell line CGR8.8 (strain background 129P2) by the Stanford Transgenic Facility using standard protocols (Joyner, 1993; Townley et al., 1997). ES cell colonies were screened for correct targeting at the 5' end by PCR. PCR-positive clones were verified by Southern blot using a 3' probe. Chimeras were bred to C57BL/6J females resulting in germline transmission from two ES cell clones. Heterozygous mice were bred to mice carrying a CMV:Cre transgene (mouse strain DBA/2Tg-2.6I, maintained on a C57BL/6J background, provided by David Anderson, California Institute of Technology, Pasadena, CA) to generate the *Tbx4*<sup>AHLEA</sup> allele. Mice were maintained on a mixed background of 129P2 and C57BL/6J.

Mice were genotyped using a three-primer multiplex PCR that produces a wild-type product of 164 bp and *Tbx4*<sup>AHLEA</sup> product of 263 bp: HLEA1F, 5'-AACCTGGCTGAAGACTCCTG-3'; HLEA2F, 5'-AGGACATGTT-TCTGAACGAGC-3'; and HLEA1R, 5'-GCTGTCCGAGGAATGCC-ATG-3'.

Animals were handled using protocols approved by the Stanford University Animal Care and Use Committee.

#### Allele-specific expression

Levels of *Tbx4* expression from wild-type and *Tbx4*<sup>AHLEA</sup> alleles were measured in E11.5 F1 hybrid embryos generated by crossing DBA/2J males and 129P2 or *Tbx4*<sup>AHLEA</sup> females. The second coding exon of *Tbx4* was amplified from lung and hindlimb RNA using RT-PCR with forward primer 5'-CGGAGCAGACCATCGAGAA-3' and reverse primer (5'-biotinylated, HPLC-purified) 5'-TGCCTGCCTCGTGGAACTT-3'. Pyrosequencing was performed using primer 5'-CCATCGAGAACATCAA-3' to analyze the relative levels of mouse SNP rs29454648 derived from DBA/2J and 129P2 *Tbx4* alleles (EpigenDx, Worcester, MA). Statistical analyses were performed by arcsine transforming the percentage of DBA/2J and 129P2 transcripts from each tissue sample and performing a two-tailed *t*-test assuming unequal variance.

#### Skeletal preparations and measurements

Skeletal preparations of 8-week-old male mice were prepared with Alcian Blue and Alizarin Red staining as described (Lufkin et al., 1992) and measured under a dissecting microscope with an eyepiece reticule ( $n=14$  wild-type,  $n=15$  *Tbx4*<sup>AHLEA</sup> heterozygous and  $n=15$  *Tbx4*<sup>AHLEA</sup> homozygous animals). Body lengths and forelimb and hindlimb bone sizes were compared using a two-tailed *t*-test assuming unequal variation. No statistically significant differences were seen for body length or forelimb

bone sizes between wild-type and mutant animals, and normalization of hindlimb measurements to body length or forelimb bone size produced similar results.

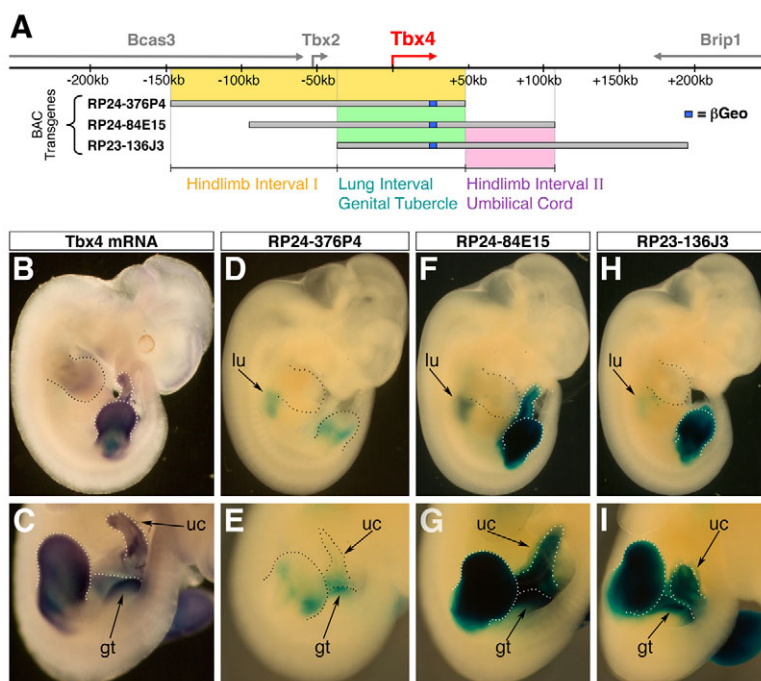
## RESULTS

### A BAC scan across the *Tbx4* locus localizes cis-regulatory elements

To screen large regions upstream and downstream of *Tbx4* for limb control elements, we identified three mouse BACs that span a 344 kb region (Fig. 1A). Each BAC contains the entire coding region of *Tbx4* plus varying amounts of flanking sequence. We inserted an IRES- $\beta$ Geo reporter into the *Tbx4* 3' UTR of each clone, and generated transient transgenic embryos that were stained for  $\beta$ -galactosidase activity with X-Gal (*lacZ* staining).

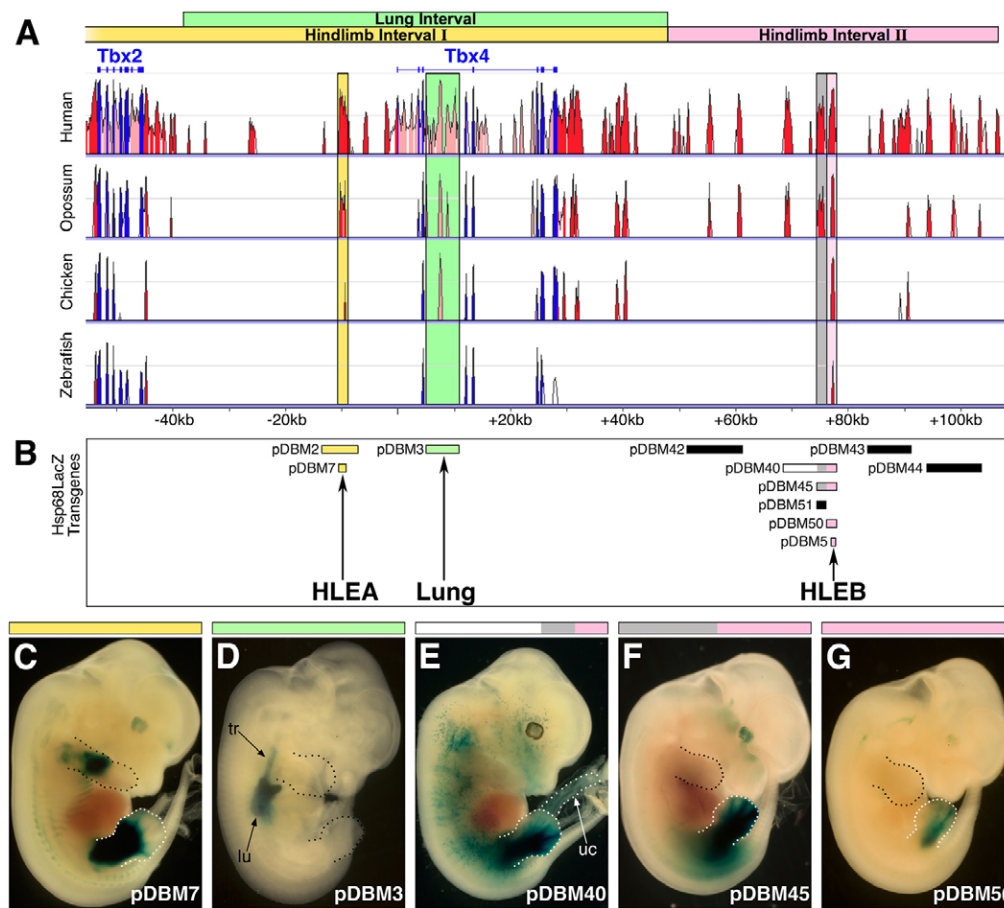
The medial and distal BACs, RP24-84E15 and RP23-136J3, generated essentially identical patterns of *lacZ* staining in embryos at day 12.5 (E12.5) of development. Robust expression was observed in hindlimbs and in the lung, anterior genital tubercle and umbilical cord (Fig. 1F-I). This pattern closely resembles the endogenous pattern of *Tbx4* expression, suggesting that these BACs contain most of the major *Tbx4* cis-regulatory elements required at E12.5 (Fig. 1B,C) (Chapman et al., 1996). By contrast, the proximal BAC, RP24-376P4, exhibited staining in the lung and anterior genital tubercle, but minimal staining in the umbilical cord and incomplete staining in the hindlimb with no expression observed in the distal half of the autopod (Fig. 1D,E).

The similar lung and genital tubercle expression seen with all three BACs suggests that lung and genital tubercle regulatory elements are located in an 86 kb region shared by all three clones. We refer to this region as the lung interval (Fig. 1A). The weak hindlimb expression driven by the proximal BAC suggests that some hindlimb control elements map to the region contained within this BAC (hindlimb interval I, Fig. 1A). The much more complete hindlimb and umbilical cord staining observed with the medial and distal BACs suggests that a distal shared 59 kb region contains additional hindlimb elements, as well as umbilical cord elements (hindlimb interval II, Fig. 1A).



**Fig. 1. A BAC enhancer scan across the mouse *Tbx4* region.** (A) Schematic of genomic region encompassing *Tbx4*. Arrows denote the direction of transcription of *Tbx4* and flanking genes. Gray bars show BAC locations with the inserted IRES- $\beta$ Geo cassette in blue. Mapped cis-regulatory intervals are shaded: yellow, hindlimb interval I; green, lung interval; pink, hindlimb interval II. (B,C) Whole-mount in situ hybridization for *Tbx4* mRNA in E12.5 mouse embryos. (D-I) Whole-mount *lacZ* staining of transgenic embryos carrying RP24-376P4, RP24-84E15 or RP23-136J3. Dotted lines denote boundaries of limbs, umbilical cord (uc) and genital tubercle (gt). lu, lung.





**Fig. 2. Comparative genomics reveals locations of candidate cis-regulatory elements.**

(A) Sequence comparison of mouse with human, opossum, chicken and zebrafish *Tbx4*. Colored bars (top) denote location of cis-regulatory intervals; blue boxes indicate position of *Tbx2* and *Tbx4* exons. All regions with >70% identity over a 100 bp window are colored: blue, exons; red, conserved intergenic sequence; pink, conserved intronic sequences). Block-shaded portions of plots indicate positions of confirmed enhancers (see B): yellow, hindlimb enhancer A (HLEA); green, lung enhancer; pink, hindlimb enhancer B (HLEB); gray, umbilical cord. (B) Genomic regions assayed for enhancer activity. Black bars represent fragments that did not drive consistent expression patterns at E12.5. (C-G) Whole-mount *lacZ* staining of E12.5 transgenic mouse embryos illustrating expression patterns seen with the different constructs. tr, trachea; lu, lung; uc, umbilical cord.

### Isolation and characterization of *Tbx4* enhancers

Previous studies suggest that evolutionarily conserved sequences often correspond to tissue-specific enhancers (Fortini and Rubin, 1990; Mortlock et al., 2003; Woolfe et al., 2005; Pennacchio et al., 2006). Alignment of mouse and human *Tbx4* region sequences revealed numerous evolutionarily conserved regions (ECRs) with 70% or higher sequence identity over at least 100 bp (Fig. 2A). We cloned several of these ECR-containing regions in front of an *Hsp68* minimal promoter and a *lacZ* reporter, and tested whether individual fragments were capable of driving reporter gene activity at consistent locations in transgenic mice.

A 6.3 kb fragment within the hindlimb I interval drove reproducible *lacZ* staining in the hindlimbs of transient transgenic E12.5 embryos (Fig. 2B, transgene pDBM2, and data not shown). A 1073 bp subregion of this sequence showed high sequence identity between placental mammals and opossum, and exhibited identical enhancer activity to the full-length fragment (Fig. 2B,C, transgene pDBM7). We designated this 1073 bp fragment hindlimb enhancer A (HLEA). HLEA produced strong expression in the proximal portion of the hindlimb, but was generally excluded from the distal half of the autopod. Sections through hindlimbs showed that HLEA exhibits strong activity throughout the proximal limb mesenchyme at E12.5 (see Fig. S1 in the supplementary material). Lower and more-restricted enhancer activity was also observed in the forelimb (Fig. 2C).

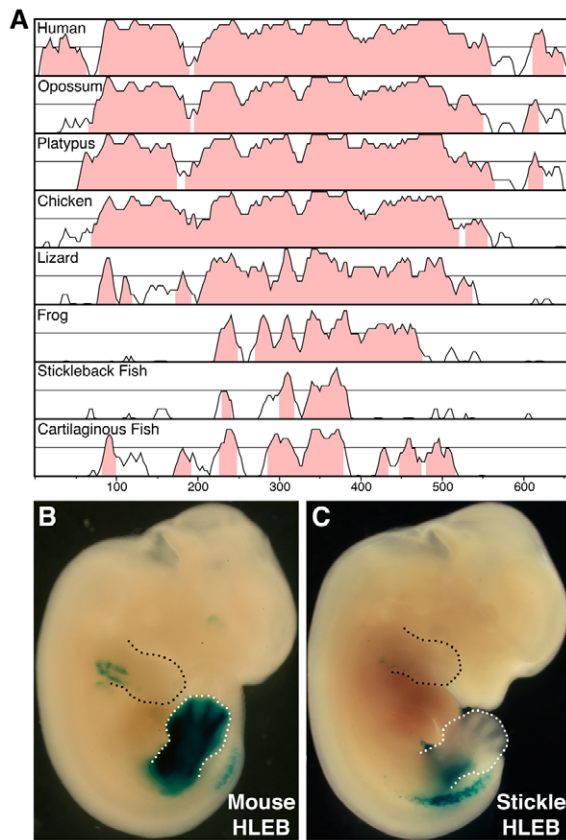
Within the lung interval defined by BAC scanning, the third intron of *Tbx4* contains the highest concentration of ECRs, including sequences with high conservation to chicken (Fig. 2A). A 5.5 kb

segment from this region drove consistent expression in the developing lung and trachea of transgenic mouse embryos at E12.5 (Fig. 2B,D, transgene pDBM3).

The 59 kb hindlimb II interval showed numerous regions conserved between mouse and human. We designed four transgenes that together enabled us to test 94% of these conserved sequences for enhancer activity (Fig. 2A,B). Only one of the four transgenes, pDBM40, drove consistent reporter expression at E12.5, with expression clearly observed in the hindlimb, umbilical cord and anterior genital tubercle (Fig. 2E and data not shown). The hindlimb staining exhibited a clear posterior bias, which was evident both in whole-mount and sectioned material (Fig. 2E and see Fig. S1 in the supplementary material). Reporter expression was also reproducibly observed in scattered cells throughout the torso and facial region, but this pattern did not resemble endogenous *Tbx4* expression.

A 3.5 kb subregion of the original 9.2 kb pDBM40 fragment drove hindlimb, genital tubercle and umbilical cord expression but had lost the scattered torso/facial activity (Fig. 2B,F, transgene pDBM45, and data not shown). To further define the hindlimb control sequences, we subdivided the 3.5 kb fragment into two parts. The 3' half (pDBM50) retained the genital tubercle and hindlimb enhancer activity, albeit at somewhat reduced levels (Fig. 2B,G). Neither half reproducibly drove umbilical cord expression, suggesting that both regions are required for umbilical enhancer activity. The 3' portion of pDBM50 contains a region with high sequence conservation from mice to cartilaginous fish (Fig. 3A). We cloned a 654 bp region from mouse that encompassed all the sequences conserved in fish and placed four tandem copies upstream of *Hsp68LacZ* (Fig. 2B, pDBM5). This construct drove extremely





**Fig. 3. HLEB is conserved from mammals to cartilaginous fish.**

(A) VISTA plot (Frazer et al., 2004) comparing the 654 bp mouse HLEB sequence against sequences from human, opossum, platypus, chicken, lizard, frog, teleost fish (stickleback) and cartilaginous fish. Percentage sequence identity [ $y$ -axis, ranging from 40% to 100% (lower to upper thick line in each panel)] is shown in a sliding window across the region ( $x$ -axis, bp). Pink shading indicates regions of 20 bp or more that are  $\geq 65\%$  identical. (B,C) Whole-mount *lacZ* staining of E12.5 transgenic mouse embryos. (B) Hsp68LacZ transgene pDBM5 with four copies of mouse HLEB. (C) Hsp68LacZ transgene pDBM20 with four tandem copies of stickleback HLEB.

intense *lacZ* expression throughout the hindlimb and genital tubercle (Fig. 3B and data not shown). We designated this 654 bp fragment hindlimb enhancer B (HLEB).

Because of the extreme conservation of HLEB, we also tested whether the orthologous sequence from fish exhibited similar enhancer activity to mouse HLEB when injected into mouse embryos. We cloned four copies of HLEB from a marine threespine stickleback, a fish with large spine-like pelvic fins (Bell and Foster, 1994), upstream of Hsp68LacZ. Stickleback HLEB is 56% identical to mouse HLEB over a 300 bp region. Mouse embryos that carried the stickleback HLEB transgene exhibited strong *lacZ* expression in the posterior portion of the hindlimb at E12.5 (Fig. 3C, pDBM20). However, enhancer activity dropped off dramatically at the boundary of the autopod. Of fifteen independent F0 transgenic embryos, eleven completely lacked expression in the autopod. In the remaining four embryos, *lacZ* staining extended into the autopod, but this staining was weaker than that in the proximal hindlimb (Fig. 3C and data not shown). At E9.5 and E10.5, expression was also posteriorly restricted, but to a lesser degree than at E12.5 (see Fig. S2 in the supplementary

material). In addition, expression at E10.5 extended into the distal portion of the limb bud. Thus, fish HLEB also drives hindlimb expression, but with less autopod expression than seen with the mouse element.

### Developmental time course of HLEA and HLEB

We examined the activity of mouse HLEA and HLEB at additional stages by generating stable transgenic lines carrying either the 1073 bp HLEA element or four copies of the 654 bp HLEB element upstream of Hsp68LacZ. Both enhancers were active in the hindlimb field before the onset of hindlimb bud outgrowth and continued to drive strong expression at E10.5 and E11.5 in patterns that strongly resembled endogenous *Tbx4* mRNA expression (Fig. 4). HLEA demonstrated fairly uniform activity throughout the hindlimb, before losing activity in the distal half of the autopod at E12.5 (Fig. 2C and Fig. 4). HLEA demonstrated varying levels of activity in the forelimb, but in all embryos forelimb staining was less intense and less extensive than in hindlimbs. By comparison, HLEB was much more hindlimb-specific. Both enhancers were active in hindlimbs during late embryogenesis. However, by E17.5, HLEA activity was restricted to knee and ankle bones (see Fig. S3 in the supplementary material). By E16.5, HLEB activity was strongest in knees, including the distal femur, proximal tibia and patellar ligament (see Fig. S3 in the supplementary material).

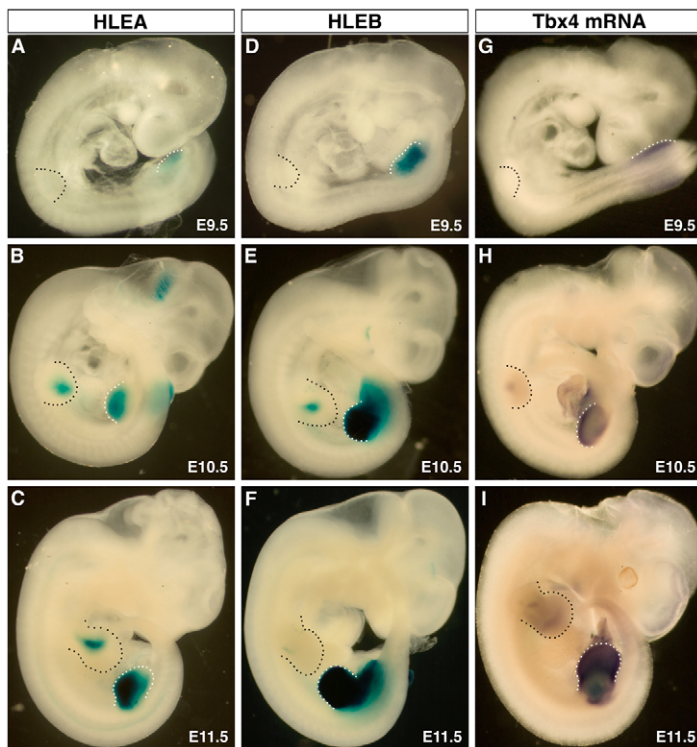
### HLEA and HLEB are essential for *Tbx4* hindlimb expression

We tested the relative importance HLEA and HLEB by examining the effects of deleting them, individually and in combination, from BAC RP24-84E15 (Fig. 5A-C). Deletion of HLEA dramatically reduced hindlimb expression, although expression was still seen in the posterior region of the hindlimb (Fig. 5D,E). Expression in the lungs, genital tubercle and umbilical cord was unaltered. The remaining hindlimb expression seen from the HLEA-deleted BAC (which still retains HLEB) appeared weaker than that seen when HLEB itself was cloned immediately upstream of a heterologous promoter (Fig. 2F). This difference might be due to the fact that in the BAC clone, HLEB is located over 75 kb downstream of the *Tbx4* promoter. Deletion of only HLEB from RP24-84E15 reduced expression in the most proximal and distal regions of the hindlimb, but the loss of expression was less dramatic than that seen with the deletion of HLEA (Fig. 5F,G).

When we deleted both HLEA and HLEB from RP24-84E15, hindlimb *lacZ* staining was undetectable or barely detectable in transgenic embryos (Fig. 5H,I). Expression in lung, genital tubercle and umbilical cord was not impaired, showing that HLEA and HLEB are required primarily for limb expression. We conclude that both HLEA and HLEB are required for robust hindlimb expression. Moreover, the patterns observed when HLEA and HLEB are deleted individually are not complementary, suggesting that these enhancers act synergistically rather than in a purely additive manner.

### Mutation of a putative Pitx1 binding site reduces HLEA activity

*Pitx1* expression in chicken forelimbs can induce *Tbx4* (Logan and Tabin, 1999). We therefore examined mouse HLEA for Pitx1/Pitx2 consensus binding sites [TAA(T/G)C(C/T) (Lamonerie et al., 1996; Tremblay et al., 2000)]. Three sites were found in a 768 bp subregion of the original 1073 bp HLEA that was sufficient to drive strong hindlimb expression in transgenic embryos (Fig. 6C, transgene HLEA-768). One predicted binding site is conserved in 17 of 18 placental mammals; a second is perfectly conserved in

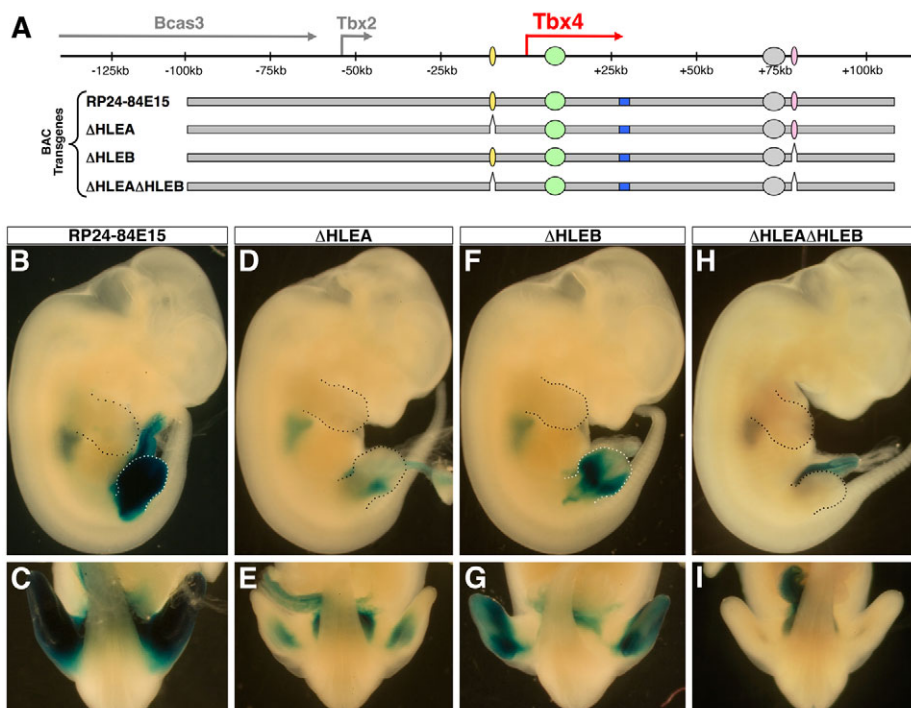


**Fig. 4. Developmental time course of HLEA and HLEB activity compared with the endogenous expression of *Tbx4*.** (A-F) Whole-mount *lacZ* staining of mouse embryos from stable transgenic lines carrying HLEA transgene pDBM7 (A-C) or HLEB transgene pDBM5 (D-F) show that these enhancers are active in the early hindlimb field (A,D) and developing hindlimb bud (B,C,E,F). (G-I) *Tbx4* mRNA as detected by whole-mount in situ hybridization shows similar patterns of expression. Embryonic stages as indicated.

placental mammals, marsupials and monotremes; and a third is found only in mouse (Fig. 6A and see Fig. S4 in the supplementary material).

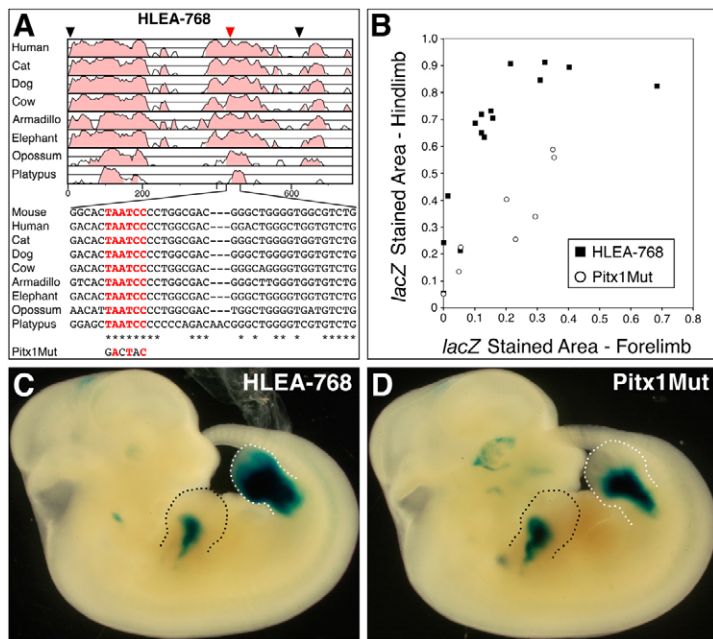
We mutated three bp in the single perfectly conserved Pitx1 binding site, creating a sequence that should be incapable of binding Pitx1/Pitx2 (from TAATCC to GACTAC, Fig. 6, transgene Pitx1Mut) (Lamonerie et al., 1996; Tremblay et al., 2000; Lamolet

et al., 2001). The Pitx1Mut enhancer still drove *lacZ* expression in transgenic embryos. However, expression in hindlimbs appeared lower than that seen with the wild-type enhancer (Fig. 6C,D). Quantitation of *lacZ* staining areas confirmed a significant reduction in hindlimb relative to forelimb staining in Pitx1Mut-LacZ compared with control transgenic embryos ( $P=0.012$ ) (Fig. 6B). Thus, the perfectly conserved Pitx1 binding site is required



**Fig. 5. HLEA and HLEB are both required for robust hindlimb expression of *Tbx4*.** (A) Schematic of mouse *Tbx4* genomic region. BAC transgene RP24-84E15 and derivatives are represented by gray bars. Yellow ovals, HLEA; green circles, lung enhancer; gray circles, umbilical cord element; pink ovals, HLEB; blue box, IRES- $\beta$ Geo. (B-I) Lateral (B,D,F,H) and ventral-posterior (C,E,G,I) views of whole-mount *lacZ* staining of E12.5 transgenic mouse embryos carrying BAC transgenes RP24-84E15 (B,C),  $\Delta$ HLEA (D,E),  $\Delta$ HLEB (F,G) or  $\Delta$ HLEA $\Delta$ HLEB (H,I).





**Fig. 6. Mutation of a conserved putative Pitx1 binding site in HLEA results in reduced hindlimb enhancer activity.**

(A) VISTA plot comparing 768 bp subregion of mouse HLEA against human, cat, dog, cow, armadillo, elephant, opossum and platypus sequences. Horizontal panels show sequence identity across the region, ranging from 50-100%. Pink shading indicates regions of 20 bp or more that are  $\geq 65\%$  identical. Arrowheads indicate positions of putative Pitx1 binding sites, including one that is perfectly conserved (red arrowhead). The accompanying sequence alignment (beneath) shows perfectly conserved bases (asterisks), the sequence of the wild-type Pitx1 site (red), and the sequence of the mutated site in Pitx1Mut. (B) Plot of the area of *lacZ* staining in forelimbs and hindlimbs of transgenic mouse embryos carrying wild-type (HLEA-768) or mutant (Pitx1Mut) enhancers. Each data point represents an individual transgenic embryo. (C, D) Whole-mount *lacZ* staining of E12.5 transgenic mouse embryos carrying wild-type (C) and mutant (D) enhancers.

for full hindlimb activity, but additional sequences must also contribute to the remaining forelimb and hindlimb activity of HLEA. These additional sequences might include additional Pitx1 binding sites, or sites for additional factors that also influence limb expression.

### Knockout of HLEA results in reduced *Tbx4* expression

To rigorously test the function of a putative *Tbx4* hindlimb control region, we used homologous recombination to precisely excise HLEA from the endogenous mouse *Tbx4* locus. We focused on HLEA because removal of this element from the BAC had the largest effect on hindlimb expression. We anticipated that removal of HLEA would generate a hypomorphic regulatory allele that would bypass the embryonic lethality observed with *Tbx4*-null embryos (Naiche and Papaioannou, 2003), and thereby allow us to both determine the functional role of HLEA and observe the effects of reduced *Tbx4* expression on normal hindlimb development. We created mice carrying a floxed HLEA allele via homologous recombination in ES cells (Fig. 7A,B). We bred heterozygous mice carrying the floxed allele to a Cre deleter strain to generate the *Tbx4* <sup>$\Delta$ HLEA</sup> allele in which HLEA has been completely deleted from the locus, leaving a single loxP site in its place (Fig. 7A,C). We refer to this allele as  $\Delta$ HLEA.

Animals heterozygous or homozygous for the  $\Delta$ HLEA allele were viable and fertile and occurred at normal Mendelian ratios in crosses between heterozygous carriers (50  $+/+$ , 98  $+/ \Delta$ HLEA, 58  $\Delta$ HLEA/ $\Delta$ HLEA). At E9.5, whole-mount in situ hybridization revealed no obvious differences in *Tbx4* expression between  $\Delta$ HLEA/ $\Delta$ HLEA and wild-type embryos (see Fig. S5 in the supplementary material). However, by E10.5,  $\Delta$ HLEA/ $\Delta$ HLEA embryos had significantly less *Tbx4* hindlimb bud expression (Fig. 7E-H). This altered expression was most apparent in the anterior portion of hindlimb buds, and was also observed at E11.5 and E12.5 (Fig. 7I,J and data not shown). In wild-type embryos, low levels of *Tbx4* mRNA were routinely found in forelimb buds. This forelimb expression was reduced in  $\Delta$ HLEA/ $\Delta$ HLEA E10.5 and E11.5 forelimb buds, but by E12.5 forelimb expression was

less affected (Fig. 7E,F and data not shown). As expected, umbilical cord and genital tubercle expression did not appear to be affected.

We used an allele-specific expression assay to more precisely quantify the impact of deleting HLEA on *Tbx4* expression. The *Tbx4* transcript contains SNPs that differ between 129P2 and DBA mouse strains. Since the  $\Delta$ HLEA allele was generated on a 129P2 genetic background, we generated mice that carried one DBA allele and either the wild-type 129P2 or  $\Delta$ HLEA 129P2 *Tbx4* allele. We performed RT-PCR on E11.5 hindlimbs and lungs and quantified the relative levels of the DBA and 129P2 allele-specific SNPs using pyrosequencing. In both hindlimbs and lungs, the wild-type DBA and 129P2 alleles were expressed at equivalent levels (Fig. 7D). By contrast, the  $\Delta$ HLEA allele was expressed at levels comparable to the DBA allele in the lungs but at  $\sim 3$ -fold lower levels in the hindlimbs (129/DBA,  $n=18$ ; 129 $\Delta$ HLEA/DBA,  $n=16$ ;  $P=2.3 \times 10^{-21}$ ).

Previous work showed that conditional inactivation of *Tbx4* at E9.5 results in perturbed expression of *Fgf8*, *Hand2* and *Alx4* and in smaller hindlimbs (Naiche and Papaioannou, 2007). No differences in expression of these genes were seen in  $\Delta$ HLEA mutant embryos by in situ hybridization (see Fig. S6 in the supplementary material).

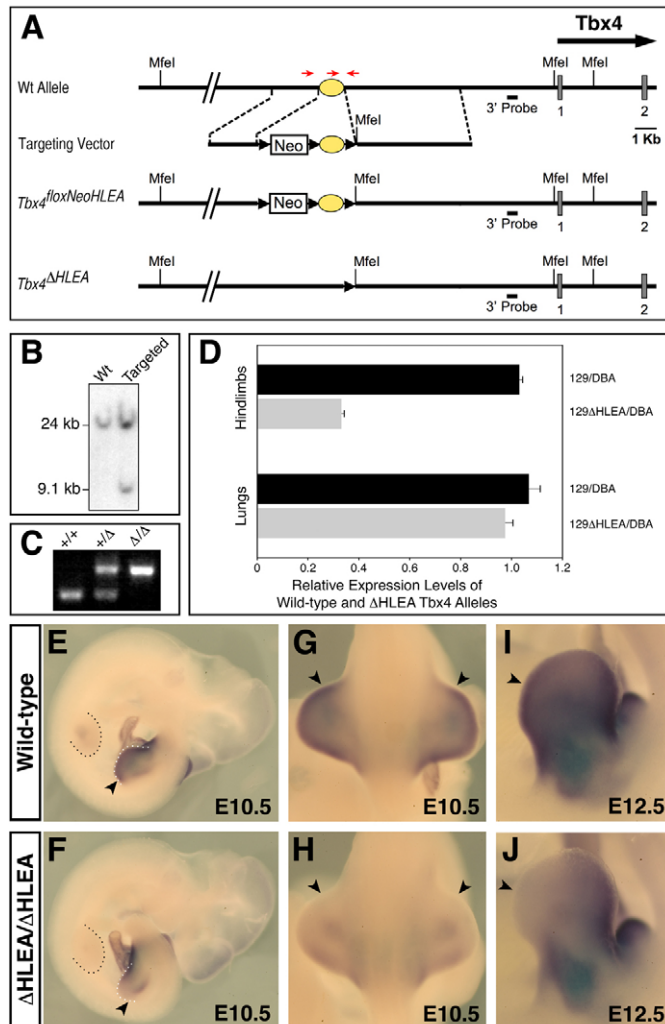
### Skeletal alterations in $\Delta$ HLEA mice

Adult animals homozygous for the HLEA deletion showed small, but significant, reductions in the size of multiple bones in the hindlimb, including the pelvis, femur, tibia and patella (Fig. 8A). The hindlimb bones of heterozygous adults were also affected and were intermediate in size between the wild type and homozygous mutants (see Table S2 in the supplementary material). In the feet of homozygotes, anterior digits were more severely reduced than were posterior digits, and distal-most elements were more reduced than were proximal elements (Fig. 8C and see Table S3 in the supplementary material).

The peroneal process on the head of the fibula was missing in all mutants, and there were fusions between tarsal bones of the ankle. Kneecaps were also displaced in 33% of animals (see Fig. S7 in the supplementary material). By contrast, no significant



change was seen in average body length of wild-type and mutant mice, and length and width measurements for forelimb bones were similar, confirming that HLEA acts as a quantitative element for hindlimb but not forelimb growth during normal development (Fig. 8B).



**Fig. 7. Targeted knockout of HLEA reduces expression of *Tbx4* in hindlimbs.** (A) Schematic showing homologous targeting of the *Tbx4* locus in mouse ES cells to create *Tbx4*<sup>flloxNeoHLEA</sup>, a floxed HLEA knock-in allele, and *Tbx4* <sup>$\Delta$ HLEA</sup>, where the floxed HLEA allele has been deleted using Cre recombinase. Gray bars, first and second exons of *Tbx4*; yellow ovals, HLEA; triangles, loxP sites; red arrows, genotyping primers; Neo, neomycin resistance cassette. (B) Southern blot of ES clones digested with *MfeI* shows a wild-type (Wt) band of 24 kb and a targeted *Tbx4*<sup>flloxNeoHLEA</sup> band of 9.1 kb with an external 3' probe (see A). (C) Mice carrying the *Tbx4*<sup>flloxNeoHLEA</sup> allele were crossed to a Cre deleter strain to generate the *Tbx4* <sup>$\Delta$ HLEA</sup> allele. Genotyping by PCR confirmed the presence of heterozygous and homozygous animals. (D) The relative expression levels of wild-type 129P2 and  $\Delta$ HLEA 129P2 *Tbx4* alleles were compared with a wild-type DBA *Tbx4* allele in E11.5 hindlimbs and lungs. Black bars, expression ratio of wild-type 129P2 to DBA; gray bars, ratio of  $\Delta$ HLEA 129P2 to DBA. (E–J) Whole-mount in situ hybridization for *Tbx4* mRNA in wild-type and homozygous  $\Delta$ HLEA knockout embryos. Lateral views of E10.5 embryos (E,F) and dorsal views of E10.5 hindlimb buds (G,H) and E12.5 hindlimbs (I,J). Arrowheads indicate anterior side of hindlimb buds.

To determine whether the size reduction observed in adults is apparent during embryogenesis, we also measured the widths of forelimbs and hindlimbs of E11.5 wild-type and homozygous mutant embryos. When normalized to crown-rump length, we found that hindlimbs of homozygous mutants were ~7% narrower (+/+,  $n=12$ ;  $\Delta$ HLEA/ $\Delta$ HLEA,  $n=11$ ;  $P=4.1 \times 10^{-5}$ ), whereas forelimbs of the wild type and mutants did not differ significantly ( $P=0.20$ ). These results show that small, but significant, effects on hindlimb growth begin at the limb bud stage, before overt differentiation and ossification of skeletal elements.

## DISCUSSION

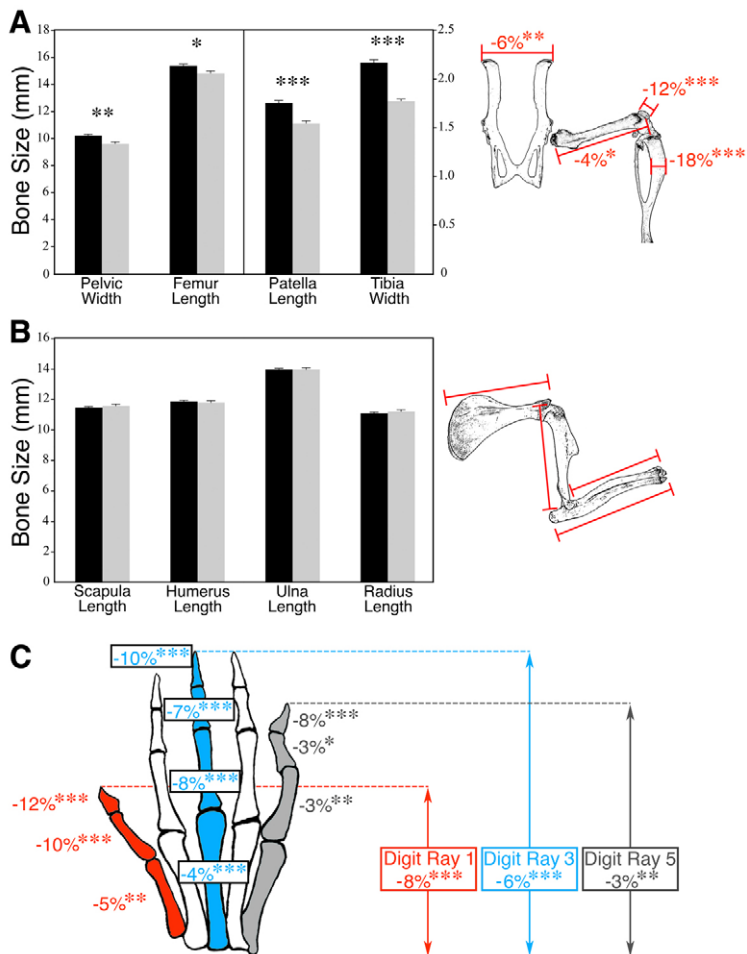
Our analysis of cis-regulatory sequences controlling *Tbx4* shows a dispersed group of enhancers spanning upstream, downstream and intronic regions of the locus, including two distinct regions controlling hindlimb expression. The hindlimb enhancers differ in their sequence, precise patterns of activity within limbs, position relative to the *Tbx4* promoter region, and their degree of conservation. Our BAC deletion analysis shows that both enhancers are required for robust hindlimb expression, and suggests that these two enhancers might act synergistically to achieve this expression.

### Conservation of cis-regulatory sequences from fish to mammals

*Tbx4* expression in developing hindlimbs/fins has been faithfully conserved among most vertebrates with paired appendages. Are the factors regulating this expression also well conserved? HLEB is conserved at the sequence level from mice to cartilaginous fish. This indicates that despite dramatic alterations in the location and morphology of limbs in different animals, at least some mechanisms regulating hindlimb expression of *Tbx4* have been maintained across ~500 million years (Blair and Hedges, 2005).

In order to determine the extent of HLEB functional conservation, we examined enhancer activity of the HLEB from a fish in the context of a developing mouse embryo. At E9.5, the HLEB from the threespine stickleback drives patterns of hindlimb-specific expression that closely resemble those of mouse HLEB (compare Fig. 4D with Fig. S2A in the supplementary material). The functional conservation of this enhancer is noteworthy given that sticklebacks, as well as other spiny-rayed teleosts, have pelvic fins that develop at a far more anterior position than those of other vertebrates (Nelson, 1994). Previous studies have shown *Tbx4* is expressed at the level of the developing hindfin in sticklebacks and so has also undergone an anterior shift (Cole et al., 2003). The different axial levels of *Tbx4* expression and hindlimb development could have occurred through alterations in the cis-regulatory sequences of *Tbx4* in different animals or through shifts in the expression domains of upstream trans-acting factors. Since the stickleback HLEB drives expression in the hindlimb of mouse embryos, both the fish and mammal enhancers presumably respond to similar trans-acting factors. These results suggest that shifts in *Tbx4* expression and hindlimb position in different animals occur by changes in the expression domains of shared upstream factors, rather than by modification of *Tbx4* cis-acting sequences.

Whereas at early stages of mouse hindlimb development the stickleback HLEB drives expression extending into the distal-most portion of the mouse hindlimb bud, by E12.5 the activity of the stickleback enhancer is sharply decreased at the boundary of the autopod (Fig. 3C and see Fig. S2 in the supplementary material). Thus, during early phases of mouse hindlimb bud outgrowth, stickleback HLEB behaves much more like mouse HLEB than at later phases of hindlimb development. This functional difference in



**Fig. 8. Homozygous  $\Delta$ HLEA mice show reduced size of hindlimb bones.** (A,B) Bar charts showing mean sizes of wild-type (black bars) and homozygous  $\Delta$ HLEA mutant (gray bars) bones in hindlimbs (A) and forelimbs (B) of adult mice. Error bars indicate s.e.m. Red brackets in diagrams to right show positions of measurements. (C) Metatarsals and phalanges of digit rays 1 (red), 3 (blue) and 5 (gray) were measured in the feet of the wild type and homozygous mutants. Shown are percent decreases in the sizes of individual bones, or in the combined length of digit rays, in mutants compared with wild type. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 5 \times 10^{-5}$ .

enhancer activity is interesting when considered in the context of fish fin and tetrapod limb development and morphology. The digits of mice and of other tetrapods are believed to be novel distal structures without clear homologs in fish, whereas sticklebacks have a spine-like pelvic fin formed from the fusion of enlarged dermal fin rays. The stickleback enhancer might lack crucial binding sites for autopod-specific or distally restricted trans-acting factors that are required to maintain activity in the distal portion of the mouse hindlimb at E12.5. This absence of crucial sites in stickleback HLEB could be due to a gain of transcription factor binding sites in the HLEB of tetrapods. Alternatively, sticklebacks, and possibly other teleosts, might have lost binding sites in HLEB that are important in maintaining distal enhancer activity. Given that the HLEB of elephant shark, a cartilaginous fish, demonstrates significantly better conservation to mammals than does the HLEB of sticklebacks and other teleosts (our unpublished observations), we favor the second possibility (Fig. 3). This loss in teleosts would also be consistent with recent studies that have shown that teleosts have lost the later phases of Hox gene expression in their fins, whereas this expression has been retained in more-basal bony fish and cartilaginous fish (Davis et al., 2007; Freitas et al., 2007).

### A second hindlimb enhancer highly conserved in mammals

In addition to the HLEB enhancer shared with fish, mammals have a second enhancer highly conserved from placental mammals to monotremes. HLEA is located over 86 kb from HLEB, and shows

no obvious sequence homology with HLEB, suggesting that HLEA did not arise by simple duplication of an ancestral element. It is possible that some relationship exists between the two enhancers that cannot be detected by simple sequence alignment. Previous studies have shown that the sequence of enhancer elements can change to the point that global alignment is lost, even though the divergent enhancers still drive similar expression patterns and may respond to similar upstream factors (Ludwig et al., 2005; Fisher et al., 2006). Several features of *Tbx4* regulation, however, suggest that the two enhancers have somewhat different functions. First, the detailed expression patterns driven by HLEA and HLEB clearly differ, and this difference becomes more pronounced as expression is traced to later stages of development (see Fig. S3 in the supplementary material). Second, the patterns of residual expression following deletion of HLEA or HLEB from a BAC transgene also differ, confirming that some regions of the hindlimb are more dependent on one or the other of the two enhancers for *Tbx4* expression (Fig. 5). Third, the morphological effects of deleting HLEA are not uniform across the hindlimb. Anterior digits are more affected than posterior digits, a morphological effect that parallels the loss of *Tbx4* expression in the most anterior portion of the autopod when HLEA is deleted (compare Fig. 7E-J with Fig. 8C).

Deletion of HLEA and HLEB from a BAC transgene results in loss of *Tbx4* expression in distinct hindlimb domains. Anterior expression is completely lost from hindlimbs when HLEA is deleted. By contrast, deletion of HLEB results in reduced expression in proximal and distal regions (Fig. 5). These patterns of residual

expression are not complementary and suggest that these two enhancers interact synergistically. Consistent with our BAC results, deletion of HLEA from the endogenous locus (with retention of a single 34 bp loxP site at the corresponding location) results in reduced *Tbx4* expression in the anterior portion of mutant hindlimbs. However, the loss of anterior expression is less severe than that observed with our HLEA-deleted BAC transgene (compare Fig. 5D with Fig. 7J), perhaps because additional cis-regulatory information is present in the endogenous locus that is absent from the BAC clone.

The factors acting upstream of HLEA and HLEB are not yet known. Previous studies have shown that ectopic *Pitx1* expression is sufficient to trigger *Tbx4* expression (Logan and Tabin, 1999). In addition, *Pitx1*-knockout mice show reduced, but not absent, expression of *Tbx4* in the hindlimb, suggesting that *Pitx1* is one of the endogenous factors that controls *Tbx4* expression (Lancot et al., 1999; Szeto et al., 1999). We found that mutation of a highly conserved *Pitx1* binding site in HLEA reduces but does not eliminate hindlimb *Tbx4* expression. The remaining enhancer activity might be due to additional binding sites for *Pitx1*, or to sites for additional factors that bind in the several-hundred-bp conserved HLEA enhancer region.

### ***Tbx4* and hindlimb morphology**

Early studies of *Tbx4* focused on the possibility that *Tbx4* expression helped specify forelimb versus hindlimb identity (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). More recent studies have shown that *Tbx4* expression is capable of rescuing forelimb outgrowth defects in a *Tbx5* mutant mouse (Minguillon et al., 2005). The resulting limbs have morphological features characteristic of typical forelimbs, not hindlimbs, suggesting that *Tbx4* functions in limb initiation, rather than limb identity. Conditional inactivation studies also show that early loss of *Tbx4* leads to severe truncation of hindlimb outgrowth, whereas *Tbx4* inactivation at later stages produces milder limb defects (Naiche and Papaioannou, 2007).

Our genetic knockout of HLEA provides a new method to bypass viability problems associated with loss-of-function mutations in the *Tbx4* gene. Animals missing HLEA survive, are fertile, and have normal forelimb structures. Loss of HLEA leads to quantitative changes in the size of several different hindlimb bones, however, confirming that HLEA functions as a hindlimb enhancer and that *Tbx4* is required for regional growth of specific skeletal structures in the vertebrate skeleton. The phenotypic effects of deleting HLEA are milder than the effects seen when disrupting all *Tbx4* function in limbs beginning at E9.5-10.5 (Naiche and Papaioannou, 2007). The milder phenotypes seen in  $\Delta$ HLEA mice might be due to remaining *Tbx4* expression and function in limbs driven by HLEB, a possibility that can be tested in the future by similar knockouts of HLEB, or of both HLEA and HLEB, from the mouse *Tbx4* locus.

Comparative studies have long suggested that vertebrates can independently control the size and shape of specific bones in forelimbs and hindlimbs (Flower, 1876; Hinchliffe and Johnson, 1980). Bats show striking elongation of digits in forelimbs but not hindlimbs. By contrast, kangaroos show robust development of hindlimb structures, and proportionately much shorter bones in forelimbs. Striking species-specific variation is also seen for individual bones within the hindlimb. The first digit ray of the human foot is unusually robust, giving rise to a characteristic big toe that supports a bipedal gait. By contrast, the anterior-most and posterior-most digits are nearly lost in horses, with weight-bearing transferred almost entirely onto a central hoofed digit.

The multiple independent hindlimb enhancers in the *Tbx4* gene might provide a flexible genomic mechanism for influencing the quantitative size of skeletal elements in vertebrate limbs. Our genetic studies show that even heterozygous loss of one copy of HLEA can produce significant changes in the size of hindlimb bones (see Table S2 in the supplementary material). Fine-scale changes in the size of hindlimb skeletal elements can thus arise from quantitative changes in *Tbx4* expression, and these expression levels are themselves controlled by at least two different hindlimb enhancer regions. Recent studies suggest that regulatory alterations in key developmental control genes, such as *Shh*, *Pitx1* and *Prx1* (*Prrx1*), play an important role in limb modifications in naturally occurring species (Sagai et al., 2004; Shapiro et al., 2004; Cretekos et al., 2008). In each case, null mutations in the corresponding gene are lethal, whereas regulatory mutations are viable and fertile. Our results show that mutations in limb-specific enhancers of *Tbx4* can also provide a way to bypass the lethal, pleiotropic effects seen when the coding regions of the *Tbx4* gene are disrupted. Based on the localized skeletal phenotypes seen in the current studies, we hypothesize that structural changes in HLEA and HLEB enhancer regions might be one of several factors that contribute to quantitative modifications in the size of hindlimb bones during vertebrate evolution. This possibility can now be tested by comparative sequencing of HLEA and HLEB in a range of organisms with different hindlimb morphologies, followed by functional tests in transgenic and knock-in mice (Cretekos et al., 2008).

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### **Supplementary material**

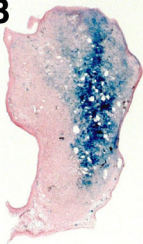
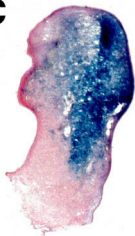
Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/15/2543/DC1>

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**A****pDBM7****B****pDBM40****C****pDBM45**

Wild-type

$\Delta$ HLEA/ $\Delta$ HLEA

**A**

**B**

*Fgf8*

*Fgf8*

**C**

**D**

*Hand2*

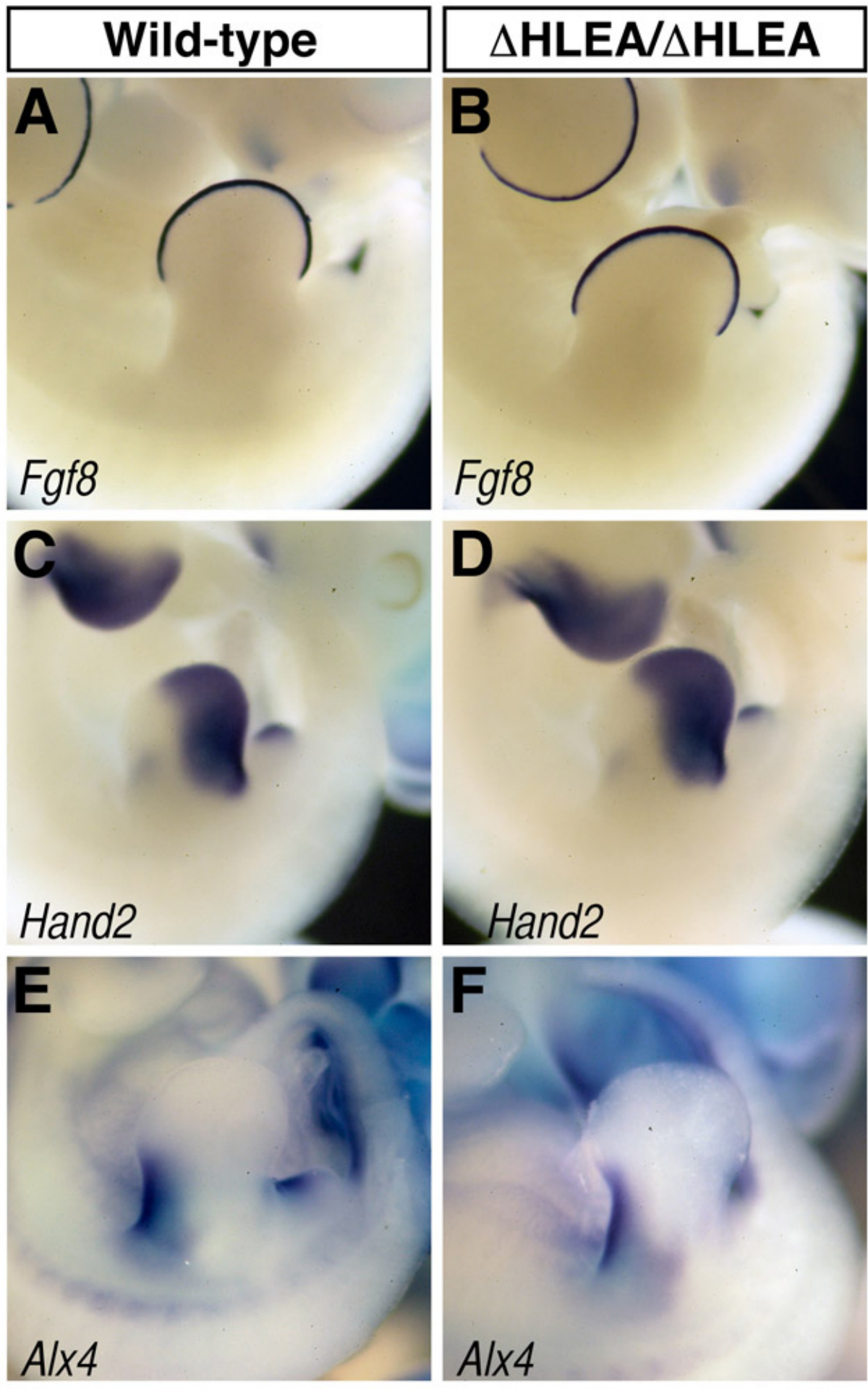
*Hand2*

**E**

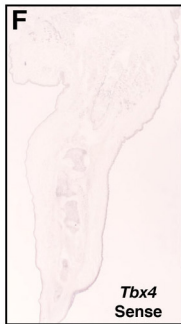
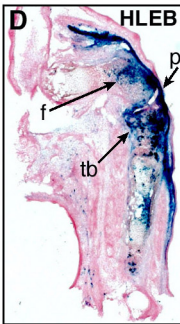
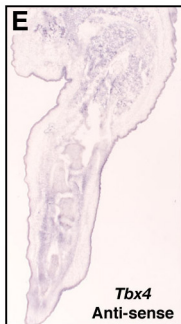
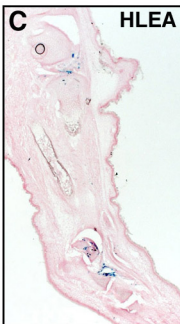
**F**

*Alx4*

*Alx4*







Pitx1

Mouse GCTAATTAAGCCTA--TACCACAAGCGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Kangaroo Rat GCTAATTAAGCCTA--TACCATGAGTGAATGTGG--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CTCCACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Guinea Pig GCTAATTAAGCCTC--TACCATGAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Rabbit GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Pika GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Human CCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Macaque CCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Tarsier CCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
European Hedgehog ACTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Eurasian Shrew ACTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Dog GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Cat GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Flying Fox GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Horse GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Cow GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Armadillo GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 100
Elephant ACTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Hyrax GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 110
Opossum AATAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 115
Platypus GCTAATTAAGCCTC--TACCACAGTGAATGTGA--GGCTAATAAGGCGGCCCAATTCAGGGTCCCT--CCACACCAAGC--CAGCCGGCTGCCTGTTTATTGCCCGTATGGAGA 112
\* \* \* \* \*

Mouse TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAGACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 220
Kangaroo Rat TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACA--GGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Guinea Pig TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 222
Rabbit TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Pika TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Human TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Macaque TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Tarsier TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
European Hedgehog TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGGT--- 223
Eurasian Shrew TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 223
Dog TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Cat TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Flying Fox TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Horse TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 221
Cow TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 222
Armadillo TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGA--- 211
Elephant TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGGGAG 225
Hyrax TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGGGG 223
Opossum TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---GGAGCTT 235
Platypus TCATTAATGCTCCTGACACCGGGTCACTCCGGGTCATCCGAGGCTGCTGCAAAAACAAGGGGAAATGAACTTCCAGC--ACATGTGCCCGAGGCTGACG---G--- 215
\* \* \* \* \*

Mouse GGGTGGG---GGTTCAG--TCACCCCACTCTAGAAGT--GATC---TGCACCTCCACC---CCAGTCACTGAGACCTCTCTTCTC---CTCAAGCAAGC--- 315
Kangaroo Rat GGGGACTCCGATCACCC--ACACTTTAGCCTGGGAGA--GATC---AGCACCTCCAGCAGTCAAGACCCCTGAAACAACTCCCTGCCCAATTCGTGTCTGACAGGGAGGG--- 330
Guinea Pig GGGGATTTCTGTCACCC--ACACTCTGACCTCTGGGAGA--GATC---CAGCAGC---CAAGCAGTCAAGATCC---AAACAAATCC--- 302
Rabbit GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGACAC---CAAGCAGTCAAGACCC---AAACAAATCC--- 300
Pika GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 311
Human GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 299
Macaque GGGGACTCTGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 299
Tarsier GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 299
European Hedgehog GGGGTTCTGTTACCC--TCACTCAGCAGTCTGGGAGA--GATC---AGCACC---CAAGTGTAGGACCCCT---AAACAAATCC--- 299
Eurasian Shrew GGGGTTCTGTTACCC--TCACTCAGCAGTCTGGGAGA--GATC---AGCACC---CAAGTGTAGGACCCCT---AAACAAATCC--- 299
Dog GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 301
Cat GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 299
Flying Fox GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 296
Horse GGGGACTTCCATCACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 299
Cow GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 299
Armadillo GGGGACTCCGTTACCC--ACACTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCAGTCAAGACCC---AAACAAATCC--- 287
Elephant GGGGTTCTGTTACCC--ACCCCTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCTTTCAGACCCCA---AAACAAATCC---C 305
Hyrax GGGGTTCTGTTACCC--ACCCCTCAGCTCTGGGAGA--GATC---AGCACC---CAAGCTTTCAGACCCCA---AAACAAATCC---C 303
Opossum GGGCAAGTCCGACTCTGCCCACCCCTGAGAAATGATC---CTCCCTAATAC---CAAATGGCAGACCC---AAGAAATCC--- 319
Platypus GGGCAAGTCCGACTCTGCCCACCCCTGAGAAATGATC---CTCCCTAATAC---CAAATGGCAGACCC---AAGAAATCC--- 283
\* \* \* \* \*

Mouse CCTGCTCCATA---CTGCTCTGAAAG---AAGGA---AGTCTATGGAAGGCG---GAAA---TGGCGACCCCTTGCTCG---CCGGG---CAACCCCACTGA 398
Kangaroo Rat GAGGGGGGAT---GGGCAAGGAAGGGCGAGGA---ATCTCGGGAAGGCG---GAAA---TGGAGGCCCTTGCTGACAGCAGG---AA---CAACCCCACTGA 418
Guinea Pig GAGGCCCATG---CTGCTCTAAGGG---AGGGA---GTCTCGGGAAGGCG---GAAG---TGGAGGCCCTTGCTGACAGCAGG---AA---CAACCCCACTGA 386
Rabbit CTCCGCACAGC---CTGACCTGGGGAG---GAGGG---GTCTCGGGAAGGCGAGG---TGGAGGCTTGCTGCTGACAGCAGG---AA---CAACCCCACTGA 408
Pika TTCTCGAAGCAACTCTGACCTCCGGAG---GAGGG---GTCTCGGGAAGGCGATGAAAGAGA---TGGAGGCTTGCTGCTGACAGCAGG---AA---CAACCCCACTGA 377
Human TTCTGCCATG---CTGACCTCAGGGGG---GGTG---GTCTCGGGAAGGCG---GAGG---TGGAGGCCCTTGCTGACAGCAGG---AA---CAACCCCACTGA 384
Macaque TTCTGCCATG---CTGACCTCAGGGGG---GGTG---GTCTCGGGAAGGCG---GAGG---TGGAGGCCCTTGCTGACAGCAGG---AA---CAACCCCACTGA 384
Tarsier CTCTGCCATG---CTGACCTCAGGGGG---GGTG---GTCTCGGGAAGGCG---AAGG---TGGAGGCCCTTGCTGACAGCAGG---AA---CAACCCCACTGA 384
European Hedgehog -TCTGTCCAGG---TGGACTCAGGGGGTGGAGG---GTTCTGTTGGAAGGCG---GAAG---GGAGGCCCTCTCTGACAGCAGG---AA---CAACCCCACTGA 387
Eurasian Shrew CCTGTCCCAAG---CTGTGCGCGCGAGT---GGTGGGA---GTCTCGGGAAGGCG---GCCGG---GGAGGCCCTC---CCGCGCCCGGG---AAACCCCACTGA 389
Dog CTCTGCCCAG---CTGACTCTGGGGA---AGGG---GTCTCGGGAAGGCGT---GAAG---GGAGGCCCTCTCTGACAGCAGG---AAACCCCACTGA 384
Cat CTCTGCCCAG---CTGACTCTGGGGA---AGGG---GTCTCGGGAAGGCGT---GAAG---GGAGGCCCTCTCTGACAGCAGG---AAACCCCACTGA 382
Flying Fox GCTGTGCCAG---CTGACTCTGGGGA---AGGG---GTCTCGGGAAGGCG---AAGG---GTAGGCCCTCTGCTGACAGCAGG---AAACCCCACTGA 381
Horse CCTGTCCAC---CTGACTCTGGGGA---AATG---GTCTCGGGAAGGCG---GAAAG---GGAGGCCCTCTGCTGACAGCAGG---AAACCCCACTGA 384
Cow TTCTGCCAGG---TGGGCTCTGAGA---GATG---GTGTT---GGAAGGCGA---AAGG---GGAGGCCCTCTGCTGACAGCAGG---AAACCCCACTGA 384
Armadillo CCTCTCTTAG---CTGACTCTGGGCTCTGGGGTGGGGT---GTCTGGAAGGCG---GAAG---TGTAGTCTCTGACAGCAGG---AAACCCCACTGA 383
Elephant TCCACACTAG---CGGACTCTGGGAGGGGTA---ATCTCGGGAAGGCG---GAAG---TGGAGGCCCTCTGCTGACAGCAGG---AAACCCCACTGA 393
Hyrax TACACACTA---CGGAGACTCTGGGAGGGGCA---GTCTCGGGAAGGCG---GAAG---TGTAGTCTCTGACAGCAGG---AAACCCCACTGA 391
Opossum ---ATTCTATT---TGGGAGATGTGT---AGCT---CTGTGAGAAATAAAGG---GGAGGCCCTCTGCTT---CTGCA---TCCACTCA 395
Platypus CCTCTCCAT---TGTAGCGGGAGCTGAGGGG---GCTCCAGAGGGAGCTGAGGATCATTC---TCATCCCAAGGCAAGCAGCTGGGGATCCGGCTACCCCTGGCA 388
\* \* \* \* \*

Pitx1

Mouse GGCTGTCCCCACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 500
Kangaroo Rat GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 520
Guinea Pig GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 488
Rabbit GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 490
Pika GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 510
Human GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 486
Macaque GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 486
Tarsier GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 486
European Hedgehog GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 495
Eurasian Shrew GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 494
Dog GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 483
Cat GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 485
Flying Fox GGCTTTCACCAAGATGAGC---GATCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 478
Horse GGCTGTCCCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 483
Cow GGCTTTCACCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 489
Armadillo GGCGGACCAACCAACAGC---GGTCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 485
Elephant GGCTTTCACCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 493
Hyrax GGCTTTCACCAACCAACAGC---GGGCAATAATCCCTGGGAGC---GGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---ACAGGGGCCAG---ACTACC---AGCCGC 495
Opossum GAGGCTCCCAAGTATAGACT---AATCAATAATCCCTGGGAGC---TGGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---CCAGGAGCAAGCCCGCTCTCCAT 500
Platypus GGACCCCGCCGGAAGAGAA---GGGAGCTAATCCCTGGGAGC---AAGCGCTGGGGTGGCTCTGGGAGTCTGACGTGACGCTCG---TTCAGGAGCCAGCCCTCCGCTGCT 503
\* \* \* \* \*

Mouse AGGT---GGCCGGGAAGACAC----GGGCCT-----GA-GGCCACGTG9GT-GCGCATGCTGGGCCTCCG-CTCCACCCTGCT-CTGCC-----TTTTTTTTTATCAGT--- 587  
 Kangaroo Rat AGGT---GGCCGGGAAGGCAC----TGGCCC-----GGCCACCACTG9T---CACATGCTCTGGGCCTTAG-CCCACCCTTCTCCAGT-----TTCCTCCTCTGTGAT--- 607  
 Guinea Pig AGGT---GGCCGGGAAGGCAC----TGGCCT-----GGTCCACAGTGGT---TTCCGGCCAGAGCT-AG-CCCTTTC---CAATC-----AGTGAITTTGGGGTCT--- 568  
 Rabbit AGGT---GGCCGGGAAGGCAC----AGGCCC-----GGCGCCACCTG9G---CACACAGCAGAGTCTCTAG-CCCGCCCTTCCAGCCAGT-----TCTTAGTCACTGAT--- 577  
 Pika AGGT---GGCCGGGAAGGCAT---GAGCCA-AGC-----CCTTGG-CCCTGCCCTCACCOCGT-----CCTAGTGGGC-ATT--- 572  
 Human AGGT---GGCCGGGAAGGCAC----AGGCCT-----GTTGGCCACTG9G---CGCAGCTTGGGCCTTGG-CCCCACCCTCACCOCGT-----TCCCCCTCTGTGAT--- 573  
 Macaque AGGT---GGCCGGGAAGGCAC----AGGCCT-----GTTGGCCACTG9G---CGCAGCTTGGGCCTTGG-CCCCACCCTCACCOCGT-----TCCCCCTCTGTGAT--- 573  
 Tarsier AGGT---GGCCGGGAAGGCAC----AG-CCC-----GGCCGCCACCTG9G---CGCAGCTTGGGCCTTGG-CCCCACCCTCACCOCGT-----TCCGGTGTGAT--- 561  
 European Hedgehog AGGT---GGCCGGGAAGGCAC----AGACTC-----GAGCACCCCGG---GCAACC-AGCCTTGG-CCCCACCCTCCCGGGCT-----TGCCTCTGTGAT--- 576  
 Eurasian Shrew AGGT---GGCCGGGAAGGCAC----CA-----CAGCCTATG9G---CGCGAA-ATGGCCCTCCA-CCCCACCACACTGGCC-----GCCTCTCCGTGAT--- 573  
 Dog AGGT---GGCCGGGAAGGCAC----AGTGC-----G-CGCGCACCTG9G---CTCGAGCGCGGCCTCA-CCCCACCCTCACCOCGT-----TCCCGAGGCTGAT--- 569  
 Cat AGGT---GGCCGGGAAGGCAC----AGTGC-----GCGCGCACCTG9G---CATTAGCGCGGCCTCT-CCCCACCCTCACCOCGT-----TCCCGCGCCATCC--- 568  
 Flying Fox AGGT---GGCCGGGAAGGCCT---GGGCCA-----AGGGGCCACTG9G---CTCGAGCGCGGCCTTGG-GGCCACCCTCATTCAC-----TCCCGAGGCTGAT--- 565  
 Horse AGGT---GGCCGGGAAGGCAC----AGGCCCC-----GGCGGCCACTG9G---CGCGAAGCTGGGGAATGGCCCCCGCCCTCAGCCTCT-----TCCCCCTCTGTGAT--- 573  
 Cow AGGT---GGCCGGGAAGGCAC----AGGCCCC-----A-CGCGCACCTG9G---CAGCAGCTGCAGACCCGG-CCCCACTCC-CCGCT-----TCCACCGCATGAT--- 573  
 Armadillo AGGT---GGCCGGGAAGGCAC----AGGCCCC-----CSCGCTCAGTGGG---CGCGAGCCGGGGCCTTGG-CCCGAGCCCTCC-CCGCT-----TCCCTCTGTGTGAT--- 571  
 Elephant AGGT---GGCCGGGAAGGCAC----AGGCCCC-----GGCAGCCACTG9G---CGTGGCCCGGGCCTTAA-CCCGCCCTTAA-CCACT-----TCCCGCTCCAGAT--- 583  
 Hyrax AGGT---GGCCGGGAAGGCAC----AGGCCCC-----GGCAGCCACTG9G---CGGGAACCCGAGCCCTTGA-CCCGCCGCT---CCGCG-----TCCCTCTCCCGCCG--- 577  
 Opossum AGGT---GTTGGAGGGGGG---CGAAGC-----AGGGCCTGAGTGGG---CATGTGGCTTCTCTCTGTTCCTCCCTCTCTCTCT-----TCTCTGTCTCCCTTCT--- 591  
 Platypus GTACTTGGCCAGGAATCACCA-CAGGCTCCTGG-GTGGTGGTGGTGGAGAGGATCAGAACTCATTTACTTCCCTTCTCTCTCCCCAGACGACA-TCATTTGGGATGTTTGGT 618

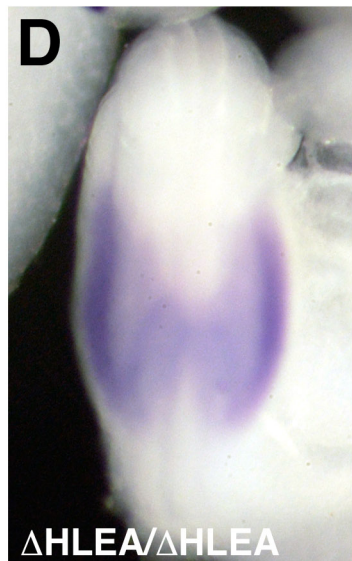
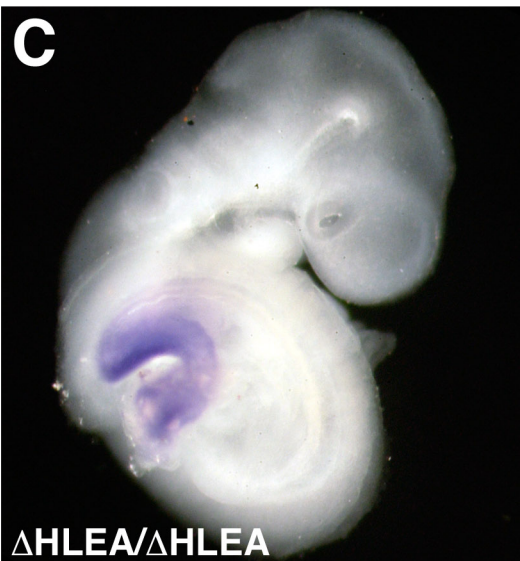
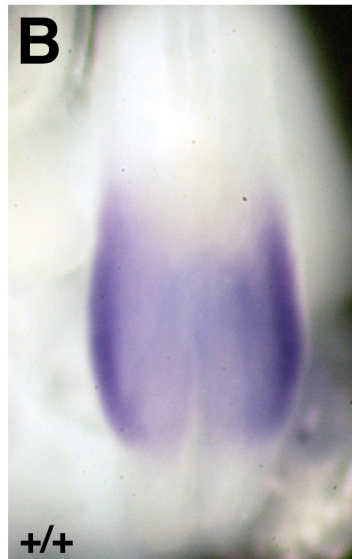
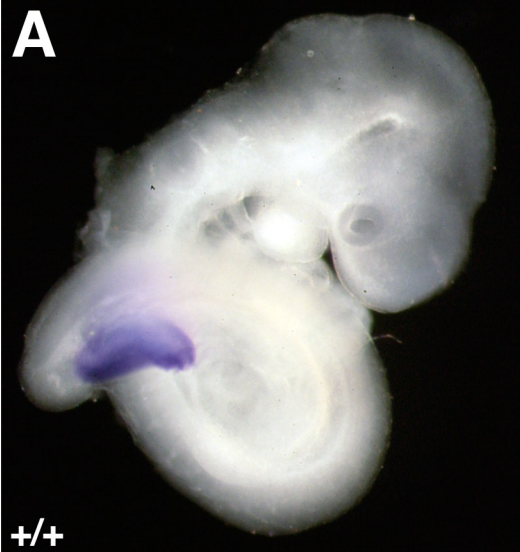
**Pitx1**

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 Kangaroo Rat --GG-----GCCCCAGGC-CCTCAGAATCCAAGATGGAGTGTATAGG-CGGGGCTCTGGACCACTCAGAGCTC-TGATGG--TGGAGTCTCAGACTCTTGAACCT--GAAGG 706  
 Guinea Pig --TG-----CC-----C-CTCCAAATGGAAGATGGCGGGGAGGGGGCGGAGTTCAGAGTATGATCGGAGCTA-TGATGG--TGGAGGTGAGCTCTTTGAGCT--GAAG 661  
 Rabbit --GG-----GGCCTTCGCGCTCAGAGTCAAGATGGCGAGGGGGG-CGGGGCTCCAGGCAGATGGGAGCTC-GGATGG--TGAAG-CCAGCTCTTTGAGT--GAAGG 675  
 Pika --GG-----GCTCTTCGCTCCAGATTCGAAGATGGCGAGATGGG-CAGGTTTCCAGGCAGATGGGAGTTG-T-----TGAAA-ACAACACTCTC----- 655  
 Human --GG-----GCCCTTCCCCTCCGGAATCCAAGATGGTGGGGGGGGGGGGCTTCCAGGCAGATCGGAGCTT-CAATGG--TGAAGTGCAGGCCCTTTGAGCT--GAAG 674  
 Macaque --GG-----GCCCTTCCCCTCCGCA-TCCAAGATGGCGAGGGGGG-CGGGTTTCCATGGCATCAGAGCTT-CAATGG--TGAAGTGCAGATCTTTGAGCT--GAAG 672  
 Tarsier --GG-----ACCCCTGCCACTCCGGAATCCAAGATGGTGGGAGGGG-CGGGGCACCAGCCGAGCCGCGCTC-GAATGG--TGAAGCCCGGAGCTTTGAGCT--GAAG 665  
 European Hedgehog --GG-----GCTCTTGTCCCTCAGAAATCCAAGATGGCGGGTGGGA-CGGTGTCCAGCCGAGCCGAACTA-CAATGG--TGAAGGGCCGCTCTT-GAATA- 676  
 Eurasian Shrew --GG-----GCTCTGGGGCGCTCCGCGTCCAAGATGGCGGGCGAGGGGGGTTCCGGTCCAGCCGCGCTG-TGATGG--CGAGACTCAGGCTCCAGCCG-G-GATGG 673  
 Dog --GG-----GGCCCCCTTACCCTCCGGA-TCCAAGATGGCGAGAGGGG-CGGGGTTCGCGGCCAAGCAAGCTG-CGATGG--TGAAGCCCAAGGCTTTGAGCT--GAAG 670  
 Cat --GG-----GGTGTGATGGGGCCCTTACCCTCCGGA-TCCAAGATGGAGTAT-GGGG-CGGGGTTCAGGCCAAGCAAGCTG-TGATGG--TGAAGCCCAAGGCTTTGAGCT--GAAG 676  
 Flying Fox --GG-----GTCTGTACCCTCTCGAATCCAAGATGGCGAGGGGG-CGGGTTTCCAGGCCAAGGGAAGCTG-CGATGG--TGAAGTGCAGGCTTTGAGCT--GAAG 664  
 Horse --GG-----GGCCTTACCCTCCGGAATCCAAGATGGCGAGGGGG-CGGGGTTCGCGGCTAAGCTAAGCTG-TGATGG--TGAAGCCCAAGGCTTTGAGCT--GAAG 673  
 Cow --GG-----GCCCTTACCCTCCGGAATCCAAGATGGCGAGGGGG-CGGGGCTCCTGGCCAAGCAAGCTG-CGATGG--TGAAGCCCAAGGCTTTGAGCT--GAAG 673  
 Armadillo --GG-----GCCGTTCCCTCCCGGA-TCCAAGATGGCGAGAGGGG-CGTGCTTCCGCTCCACCAGGCTT-CAATGG--TGTACTCTGGCTCTGTGAGCT--GAAG 670  
 Elephant --GG-----ACCCCTGCTCTCCGGAATCCAAGATGGCGAGAGGGG-TGGGGTCCGGAACAACCTGAAGCAG-CGATGG--TGGCTCAGGCTCTTCTAGCT--GAAG 683  
 Hyrax --GG-----GATCCGCTCCCTCCGGAATCCAAGATGGCGAGAGGGG-GAATCCAGATGGCAGAGAGGGG-AGGATCTCCAGGGAAACAGAGCTGGAGATGG--TGGATACAGGACCACAGGCCA-GATGG 731  
 Opossum -TCAGCTTCAAAGAGCCCTGAGGAGTCTCTTTCAAATCCCAAAATATTCTCTTCCCAAGTCCAGCTTGAATTC-AAGATGGCAGAGGAGGAGGGTTGGGGAACAATAGGAG 709  
 Platypus TTCGGTCTCCAC-AATTCCTCCCTCGGATAGTTCAGATGGTGGATGGGG-AGGATCTCCAGGGAAACAGAGCTGGAGATGG--TGGATACAGGACCACAGGCCA-GATGG 731

Mouse ACAGACTCAGCAGCCACTCAGGGCACT--CCTAG-TGATGGG-----GTGGCA-----GCTGCGGAGG---TGGCTGTAAACTCACCACCTACTCTTGG 743  
 Kangaroo Rat ACAGCCTCAGTAGCCACTCAGGGAGT--CCTCA-CGGTGGAG-----GTGG-TG-----GCTCGCCATC---TCACTTACCCTCGGACTTAGCAGCGGA 792  
 Guinea Pig ACAGCACCAGTAGCCACTCAGGGCCCA--CCTGG-TTGTAT-TG-----GCGGGT-----GCTCAGCCACT---TTCCTCTGTTTAACCTCAAAGCAGG 746  
 Rabbit ACATTCTCAGTAGCCACTCAGGGAGCA--CCCGG-TGGTGGTG-----CTCGCG-----CCTTAGTCAAC---TTACCTCACACTTAAGCAGCCGAGCGCG 761  
 Pika -CAGTATCAGTGGCTCTCAGAGAATGA--CCTGG-TGGGGGTG-----CTGCCT-----GCTCAGTATG---TGTCTCTACACTTCAAGTACCTGCCCAAG 740  
 Human ACAGCCTCAGTAGCCACTCAGGGAGCT--CATCG-TGGTGGT-----GTGGTGGG---GCTCAAGCCACT---TTACCTCACACTTAAGCAGCCGAGGTGAC 763  
 Macaque ACAGCCTCAGTAGCCACTCAGGGAGCT--CTTCG-TGGTGGT-----GTGGTGGG---GCTCAAGCCACT---TTACCTCACACTTAAGCAGCCGAGGTGAC 761  
 Tarsier ACAGATTCAAGTAGCCACTCAGAGAATG--CCTGG-TGGTGGT-----ATGGTGGTCA--GCTCAAGCCACT---TCACTCACACTTAAGCAGCCGAGGGCG 753  
 European Hedgehog ATGTTTTAGTAGCCACTCAGAGAATGTTT-CTCAC-CAGCAACACACCAC-CACCACCAGGAGTGGTGGTGGTGGTCTTCCAGG---TCAACCCATTAAGCAGCCAGGGCGG 788  
 Eurasian Shrew ACAGCCTCAGTAGCCACTCAGGGAGCT--CTGG-CGGTGGT-----GCGGGT-----CCGCTGCT---TCATTTCAGACTTTCAGCAACCCAGGGCGAG 756  
 Dog ACAGCCTCAGTAGCCACTCAGGGAGCT--CCCGG-AGTGGGG-----GTGGCA-----GTGAGCGGCT---TCACTCAGACTTTCAGCAACCCAGGGCTC 756  
 Cat ACAGCCTCAGTAGCCACTCAGGGAGCT--CCTGG-AGTGGT-----GTGGCA-----CCTAGCCGCT---TCACTCAGACTTTCAGCAACCCAGGGCGG 752  
 Flying Fox ACAGCCTCAGTAGCCACTCAGGGAGCT--CCTGG-CAGTGGT-----GTGGCA-----CTCAGCCCT---TTACCTCACACTTTCAGCAACCCAGGGCGG 750  
 Horse ACAGTTCAGTAGCCACTCAGGGAGCT--CCTGG-CGTTGGG-----GTGGGCT---CTCAGCCGCT---TTACCTCACACTTTCAGCAACCCAGGGCGG 759  
 Armadillo ACAGCCTCAGTAGCCACTCAGGGAGCT--CCTGG-CGTTGGG-----GTGGGCT---CTCAGCCGCT---TTACCTCACACTTTCAGCAACCCAGGGCGG 758  
 Elephant ATGGCTCAGTAGCCACTCAGGGAGCT--CCTGG-CGTTGG---GCGAGCTG---GAGCCGCC---TTACCTCACACTTTCAGGGCCAGGCTGCT 754  
 Hyrax ACAGCCTCAGTAGCCACTCAGGGAGCT--CCAA-TGTAGCA---CCGCGTGG---CTGCGCAGCT---TTCCTCGCTTTCAGTAGGGAGTCCAG 771  
 Hyrax ACAGCCTCAGTAGCCACTCAGGGAGCT--CCAA-TGTAGCA---CCGCGTGG---CTGCGCAGCT---TTCCTCGCTTTCAGTAGGGAGTCCAG 771  
 Opossum CTACGATTTGGTGCAGACTTGGTCCCT--CCAAAGTGTATGACAGCCAG--TGAACCACTCAAAGAGCCGCTTGGGCTGGCACAC--TCATTTGGGCAAGTGGGGGATTTAGTCTG 822  
 Platypus GCACGCTCTTAGGCCACTCAGAGGAGCGGGCCAGGCTGGCA.CCAGGAGGCCACTGAGTTGTCACTAGGCCAAATGGTTCCACAGGTCATGGCCCTATTGGCACTATACTCCCCACC 851

Mouse A-----CGTAAAGCACTGAGG-----CCCTCTGAG 768  
 Kangaroo Rat GCGAGCCTCAA AAAAATCTCAG-----CCTTAAAG 822  
 Guinea Pig G-----TTACAAATCCA-----TCTTTGGCTG 768  
 Rabbit C-----TCTTGAATCCAT-----GTCCCGCAA 783  
 Pika T-----TCTTCAGTCCAT-----CTCTCGCAG 762  
 Human C-----TCAGGAATCGGT-----TCCCGCAG 785  
 Macaque C-----TCAGGAATCGGT-----TCCCGCAG 783  
 Tarsier C-----CCTCAGTCTGG-----CTCAGTGG 775  
 European Hedgehog T-----TTTTAGACTCCGGCTTT--CTCTCACTG 815  
 Eurasian Shrew C-----TCTCCATCCCT-----CTGTCTGGG 778  
 Dog C-----GCACAGATGGT-----CTCTCCAG 778  
 Cat C-----TCGCAAGTCCAA-----ATCTCGCAT 784  
 Flying Fox T-----TTACGAATCCC-----CTCTCCAG 772  
 Horse C-----CCACGGAGCCGT-----CTCTGGCAG 781  
 Cow -----TCACCAATCTGC-----CTCTCCGAG 779  
 Armadillo C-----TCCGAAATCGT-----TCCCGTAG 775  
 Elephant C-----TTATGAATTTGTG-----TCCCTGAG 794  
 Hyrax C-----TTCTGGATTTGCG-----CCCTGAG 771  
 Opossum CATCGGAGCTTTGGGCTGGCCCTG-GCCTCAAGT 856  
 Platypus C-CTACTCCACCAGTTCAGAGAAATCCCAATGG 885





**Wild-type**

**$\Delta$ HLEA/ $\Delta$ HLEA**

**A**

**B**

*Fgf8*

*Fgf8*

**C**

**D**

*Hand2*

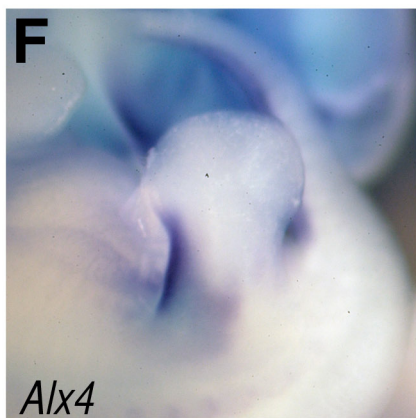
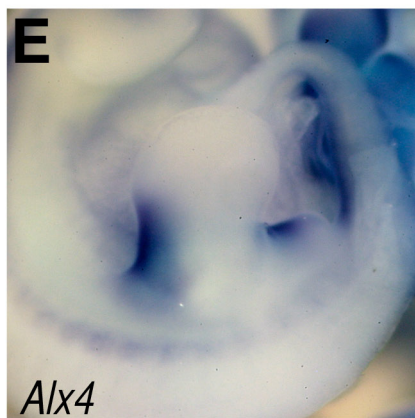
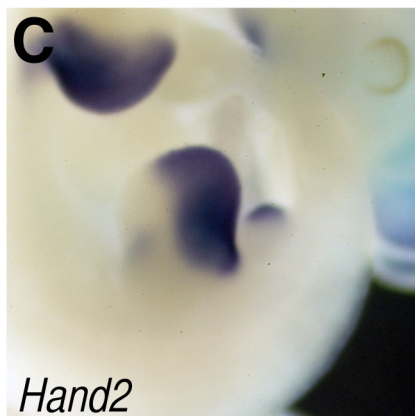
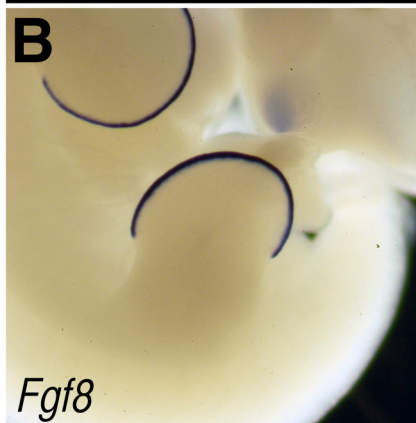
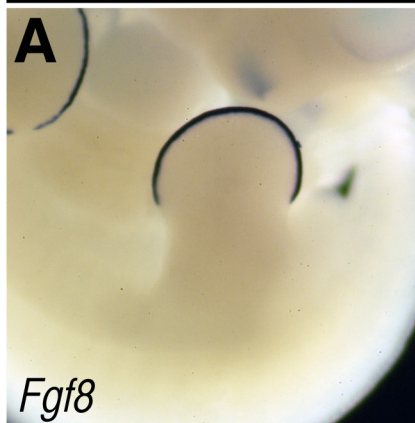
*Hand2*

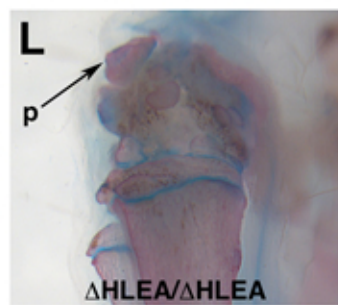
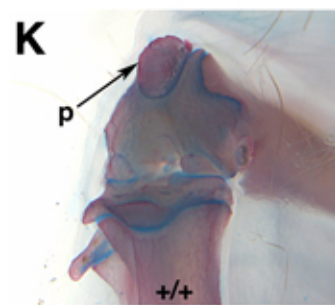
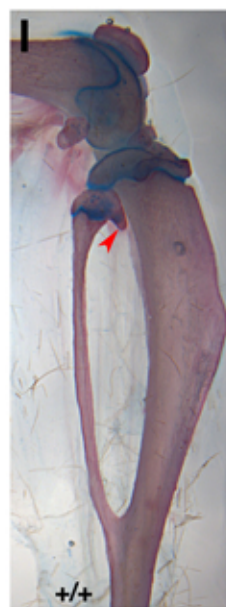
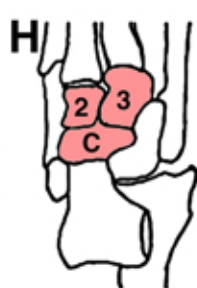
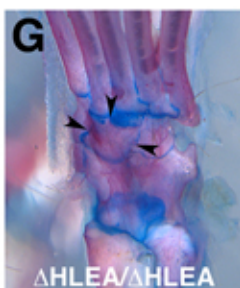
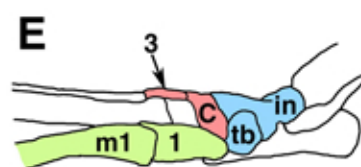
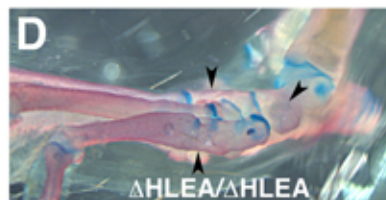
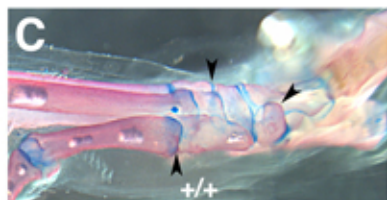
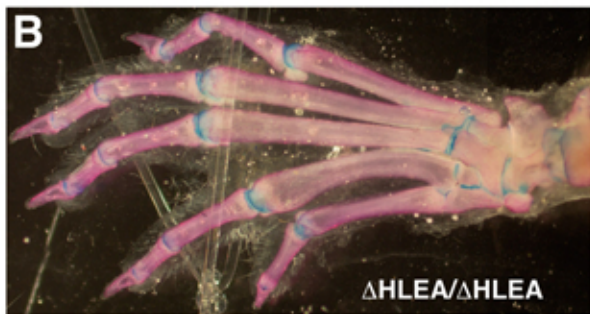
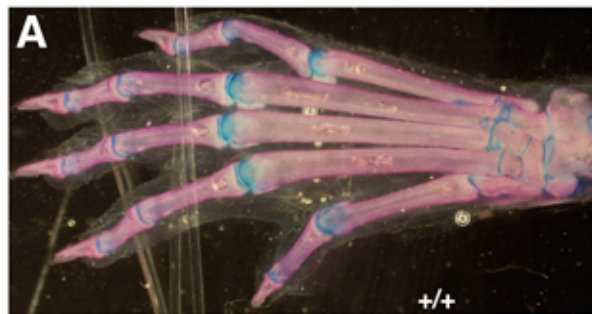
**E**

**F**

*Alx4*

*Alx4*





**Table S1. Number of independent founders for each transgene**

Transgene	Number of independent transgenic founders	Number of transgenics with staining pattern
RP24-376P4	2	Hindlimbs: 2/2 (weak) Lungs: 2/2 Genital tubercle: 2/2 Umbilical cord: 0/2
RP24-84E15	4	Hindlimbs: 4/4 Lungs: 4/4 Genital tubercle: 4/4 Umbilical cord: 4/4
RP23-136J3	3	Hindlimbs: 3/3 Lungs: 3/3 Genital tubercle: 3/3 Umbilical cord: 3/3
ΔHLEA	4	Hindlimbs: 4/4 (note: posterior only) Lungs: 4/4 Genital tubercle: 4/4 Umbilical cord: 4/4
ΔHLEB	8	Hindlimbs: 8/8 (note: reduced staining in distal half of autopod) Lungs: 8/8 Genital tubercle: 8/8 Umbilical cord: 8/8
ΔHLEA/ΔHLEB	5	Hindlimbs: 1/5 Lungs: 5/5 Genital tubercle: 5/5 Umbilical cord: 5/5
pDBM2	15	Hindlimb: 14/15
pDBM3	10	Lung: 9/10
pDBM5	6	Hindlimb: 6/6 Genital tubercle: 6/6 Umbilical cord: 1/6
pDBM7	6	Hindlimb: 5/6
pDBM36	16	Hindlimb: 15/16
pDBM46	11	Hindlimb: 8/11
pDBM40	3	Hindlimb: 3/3 Genital tubercle: 3/3 Umbilical cord: 3/3 Scattered cells in torso/face: 3/3 (note: ectopic pattern)
pDBM42	9	No clear pattern (genital tubercle: 4/9)
pDBM43	6	No clear pattern (limbs: 2/6)
pDBM44	9	No clear pattern
pDBM45	14	Hindlimb: 10/14 Genital tubercle: 13/14 Umbilical cord: 6/14
pDBM50	10	Hindlimb: 7/10 Genital tubercle: 8/10 Umbilical cord: 2/10
pDBM51	3	Hindlimb: 0/3 Genital tubercle: 0/3 Umbilical cord: 0/3
pDBM20 (stickleback HLEB)	19	Hindlimb: 17/19 Genital tubercle: 18/19



**Table S2. Relative hindlimb bone size averages of wild-type, +/ $\Delta$ HLEA and  $\Delta$ HLEA/ $\Delta$ HLEA littermates**

Measurement	+/+ (n=14)	+/ $\Delta$ HLEA (n=15)	$\Delta$ HLEA/ $\Delta$ HLEA (n=15)	WT-Het <i>P</i> -value	WT- $\Delta$ HLEA <i>P</i> -value
Patella length	1.0 $\pm$ 0.012	0.95 $\pm$ 0.0095	0.88 $\pm$ 0.012	0.0018	1.4 $\times$ 10 <sup>-07</sup>
Femur length	1.0 $\pm$ 0.0044	0.98 $\pm$ 0.0044	0.96 $\pm$ 0.0049	0.0040	3.8 $\times$ 10 <sup>-06</sup>
Tibia length	1.0 $\pm$ 0.0032	1.0 $\pm$ 0.0045	0.98 $\pm$ 0.0053	NS	0.013
Tibia width	1.0 $\pm$ 0.012	0.93 $\pm$ 0.013	0.82 $\pm$ 0.0075	9.4 $\times$ 10 <sup>-4</sup>	5.5 $\times$ 10 <sup>-12</sup>
Fibula length	1.0 $\pm$ 0.0031	1.0 $\pm$ 0.0041	0.98 $\pm$ 0.0060	NS	0.030
Pelvis length	1.0 $\pm$ 0.0056	0.99 $\pm$ 0.0064	0.99 $\pm$ 0.0061	NS	NS
Pelvis width (anterior)	1.0 $\pm$ 0.0091	0.97 $\pm$ 0.0097	0.94 $\pm$ 0.012	0.040	2.7 $\times$ 10 <sup>-4</sup>
Pelvis width (center)	1.0 $\pm$ 0.0067	0.98 $\pm$ 0.0067	0.96 $\pm$ 0.0085	0.013	0.0018
Pelvis width (posterior)	1.0 $\pm$ 0.017	1.0 $\pm$ 0.017	1.1 $\pm$ 0.020	NS	5.8 $\times$ 10 <sup>-4</sup>

Hindlimb bone sizes have been normalized relative to forelimb bone lengths and the normalized wild-type (WT) average for each hindlimb bone has been arbitrarily set to 1. The s.e.m. of each normalized average is indicated. The WT-Het and WT- $\Delta$ HLEA *P*-value columns refer to comparisons between +/+ and +/ $\Delta$ HLEA and between +/+ and  $\Delta$ HLEA/ $\Delta$ HLEA, respectively.

NS, not significant.

**Table S3. Average lengths of metatarsal and phalangeal elements of the first, third and fifth digit rays from wild-type and  $\Delta$ HLEA/ $\Delta$ HLEA littermates**

		+/+ (n=14)	$\Delta$ HLEA/ $\Delta$ HLEA (n=15)	WT- $\Delta$ HLEA P-value
<b>Digit-ray 1</b>	Metatarsal 1	4.30 $\pm$ 0.033	4.07 $\pm$ 0.063	0.0017
	Phalanx 1	2.14 $\pm$ 0.010	1.93 $\pm$ 0.020	2.5 $\times$ 10 <sup>-09</sup>
	Phalanx 2	1.09 $\pm$ 0.032	0.96 $\pm$ 0.047	2.9 $\times$ 10 <sup>-09</sup>
<b>Digit-ray 3</b>	Metatarsal 3	7.23 $\pm$ 0.034	6.93 $\pm$ 0.053	5.5 $\times$ 10 <sup>-05</sup>
	Phalanx 1	2.98 $\pm$ 0.025	2.74 $\pm$ 0.023	9.1 $\times$ 10 <sup>-08</sup>
	Phalanx 2	1.81 $\pm$ 0.014	1.69 $\pm$ 0.015	2.3 $\times$ 10 <sup>-06</sup>
	Phalanx 3	1.50 $\pm$ 0.017	1.34 $\pm$ 0.014	1.3 $\times$ 10 <sup>-07</sup>
<b>Digit-ray 5</b>	Metatarsal 5	6.42 $\pm$ 0.044	6.32 $\pm$ 0.046	NS
	Phalanx 1	2.55 $\pm$ 0.020	2.47 $\pm$ 0.019	0.0029
	Phalanx 2	1.45 $\pm$ 0.014	1.41 $\pm$ 0.015	0.032
	Phalanx 3	1.14 $\pm$ 0.013	1.04 $\pm$ 0.011	5.5 $\times$ 10 <sup>-06</sup>

Lengths are in millimeters. The s.e.m. of each average is indicated.

NS, not significant.