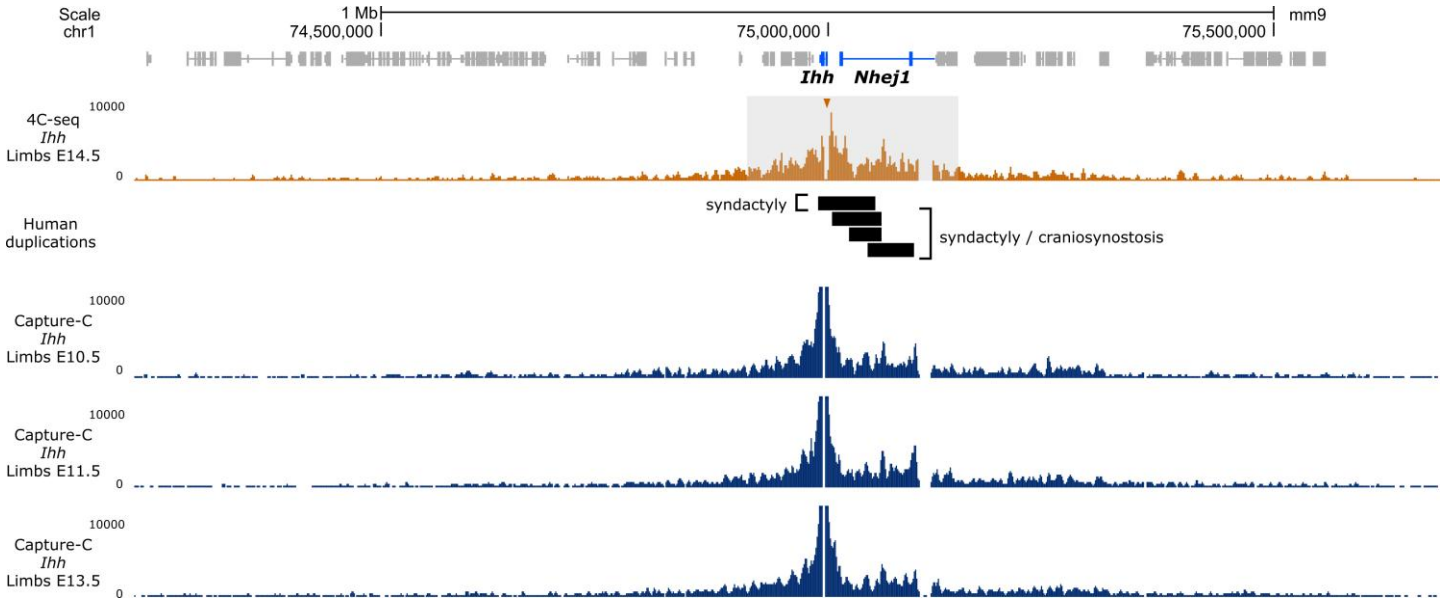
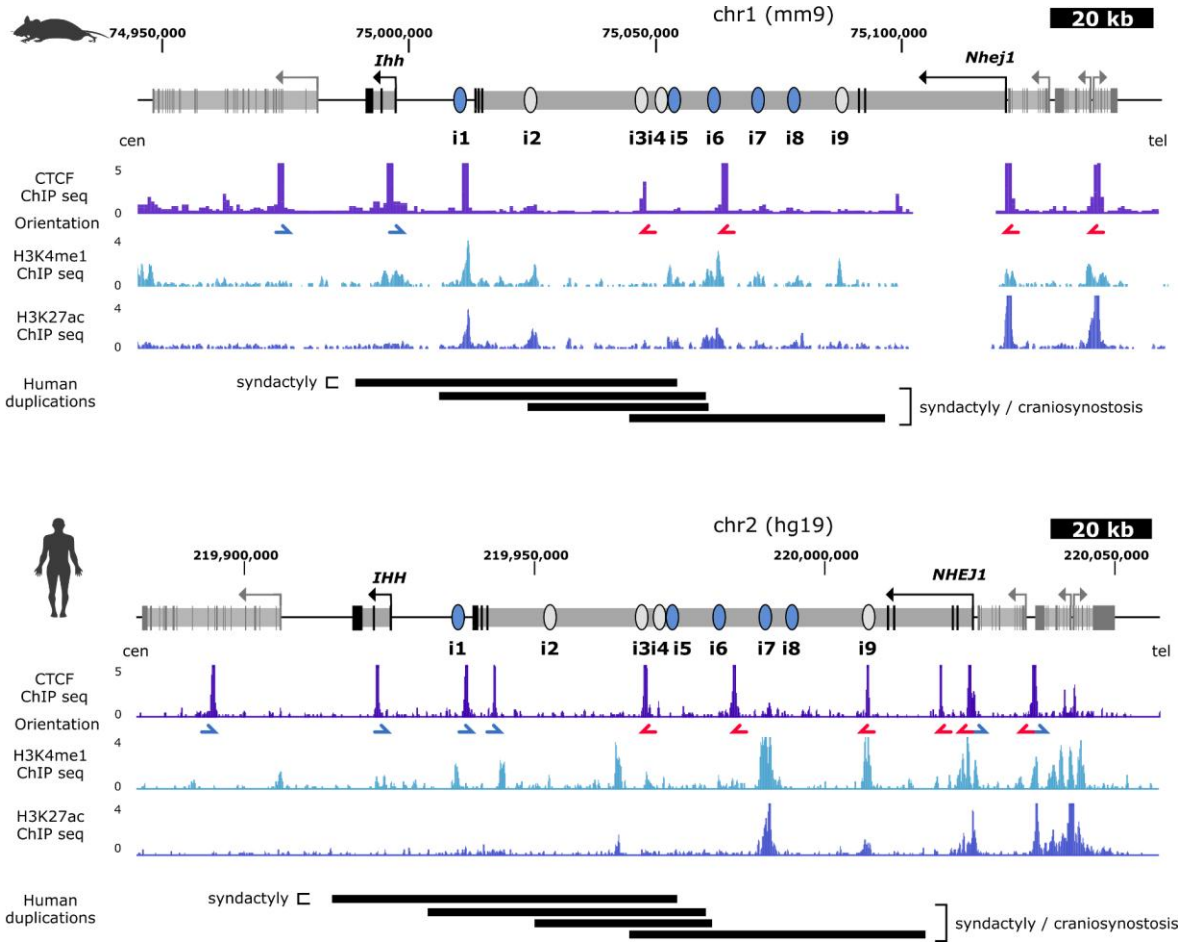


Supplementary Figure 1

***Ihh* interacts preferentially with its upstream neighboring gene *Nhej1*.**

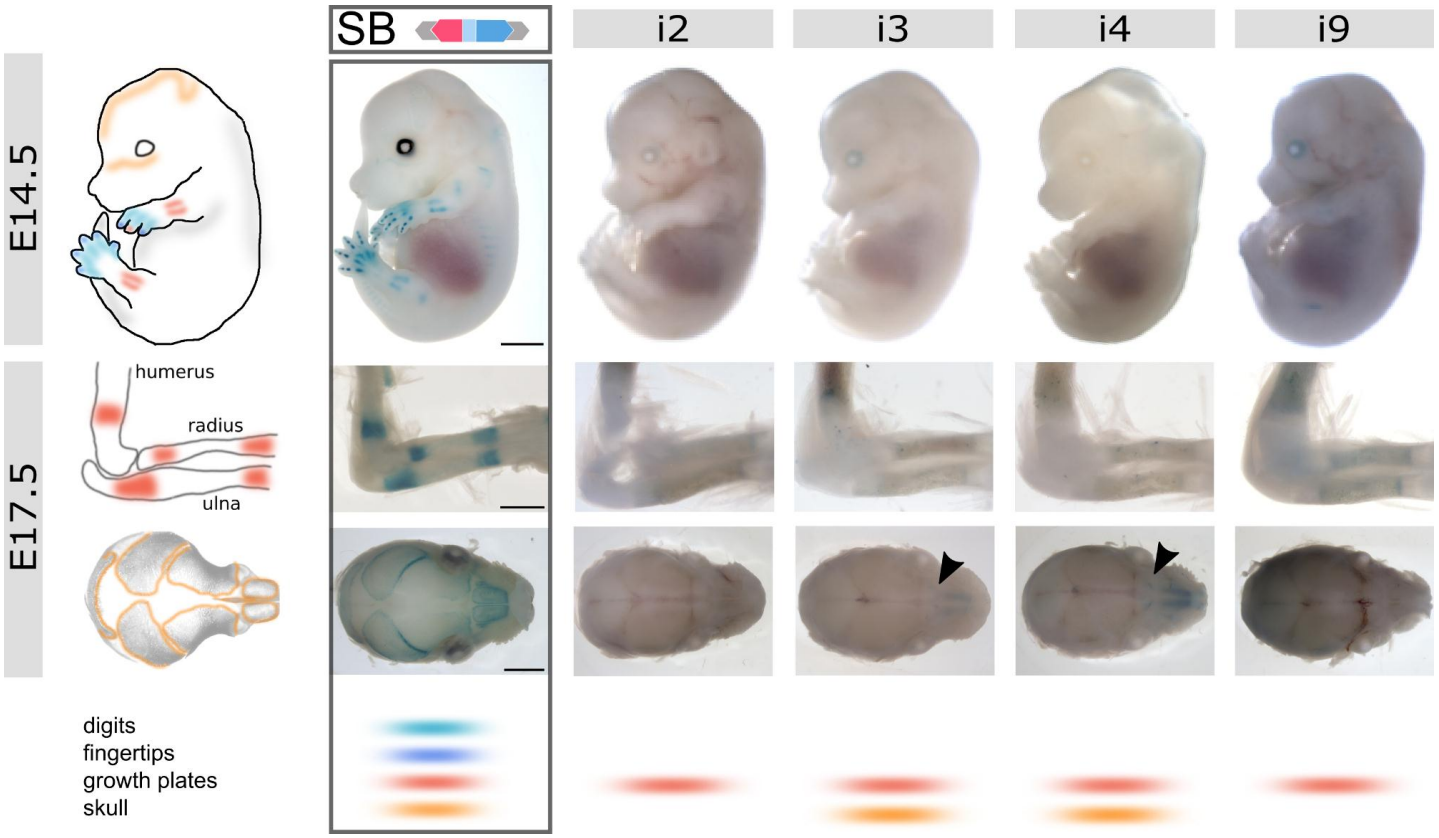
Genes are indicated by gray lines, and *Ihh* and *Nhej1* are highlighted in blue. 4C–seq performed in E14.5 limbs using the *Ihh* promoter as the viewpoint is shown below. Note the increased interactions with intron 3 of the adjacent *Nhej1* gene. The gray line indicates the zoomed region displayed in **Figure 1**. Black bars indicate the size and position of human duplications converted to mouse genome coordinates that overlap with the regulatory landscape of *Ihh*. Below, Capture-C data from Andrey *et al.* (2017) at different developmental time points. Chromatin organization is maintained during limb development.



Supplementary Figure 2

Conservation of the *IHH* locus between mouse and human.

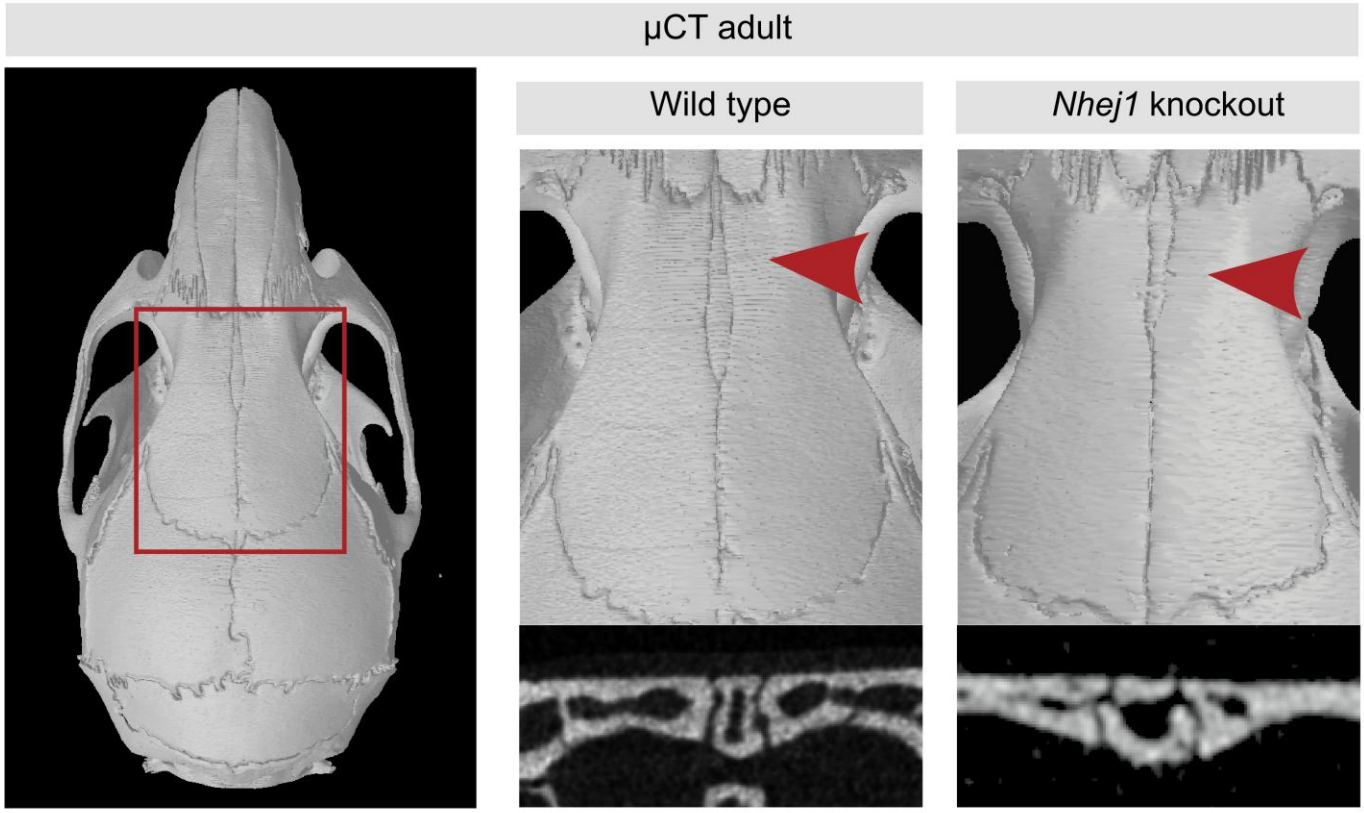
The upper panel shows a representation of the mouse locus with positions indicated for the genes and regulatory elements investigated (blue and gray ovals). Below, ChIP–seq tracks for CTCF with corresponding motif orientation as well as ChIP–seq tracks for active enhancer elements (H3K4me1 and H3K27ac); all experiments were performed in developing limbs at E14.5 (ENCODE). The equivalent positions of human pathogenic duplications are shown at the bottom. The lower panel shows a representation of the human locus with the positions of genes and equivalent positions of the regulatory elements investigated in mouse (blue and gray ovals). Below, ChIP–seq tracks for CTCF with corresponding motif orientation as well as ChIP–seq tracks for active enhancer elements (H3K4me1 and H3K27ac); all are ENCODE data sets for osteoblasts. Note that the convergent orientation of CTCF at the locus is conserved between mouse and human, as well as the presence of active enhancers. The equivalent positions of human pathogenic duplications are shown at the bottom.



Supplementary Figure 3

Transgenic reporter assay (LacZ) of elements positive at E17.5.

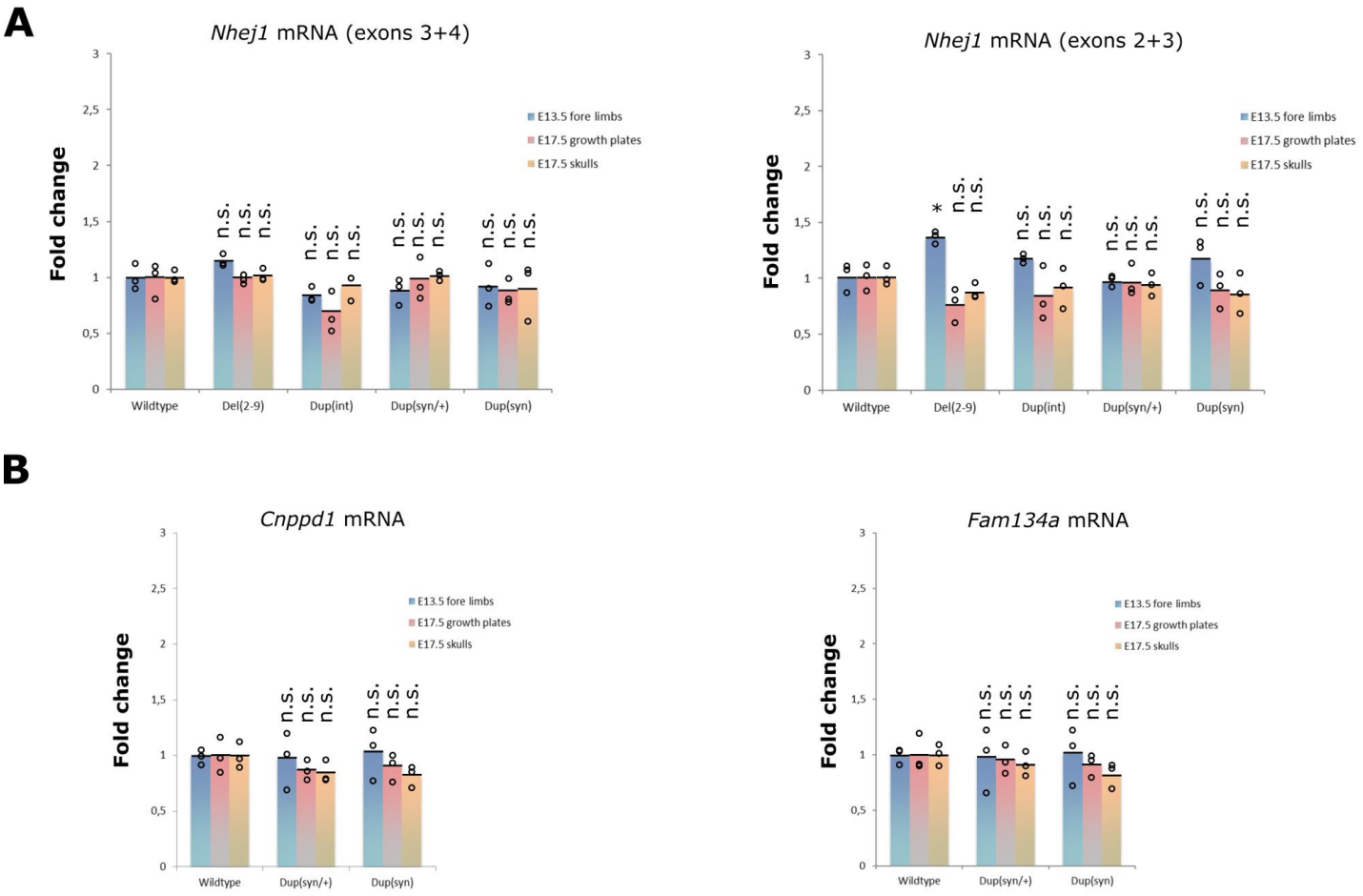
Each element displays a lateral view of the embryo at E14.5 (scale bar, 2,000 μm), a dorsal view of the forelimbs (scale bar, 1,000 μm) and a top view of the skull at 17.5 (scale bar, 2,000 μm) together with tissue specificity scoring (bottom). All tested elements appear positive at E17.5 but not at E14.5 and are marked in **Figure 1** in gray. An arrowhead indicates positive staining in the skull. The regulatory activity of the region as indicated by the inserted *lacZ* reporter (SB; black outline) is also displayed.



Supplementary Figure 4

***Nhej1*-knockout mice have normal skulls.**

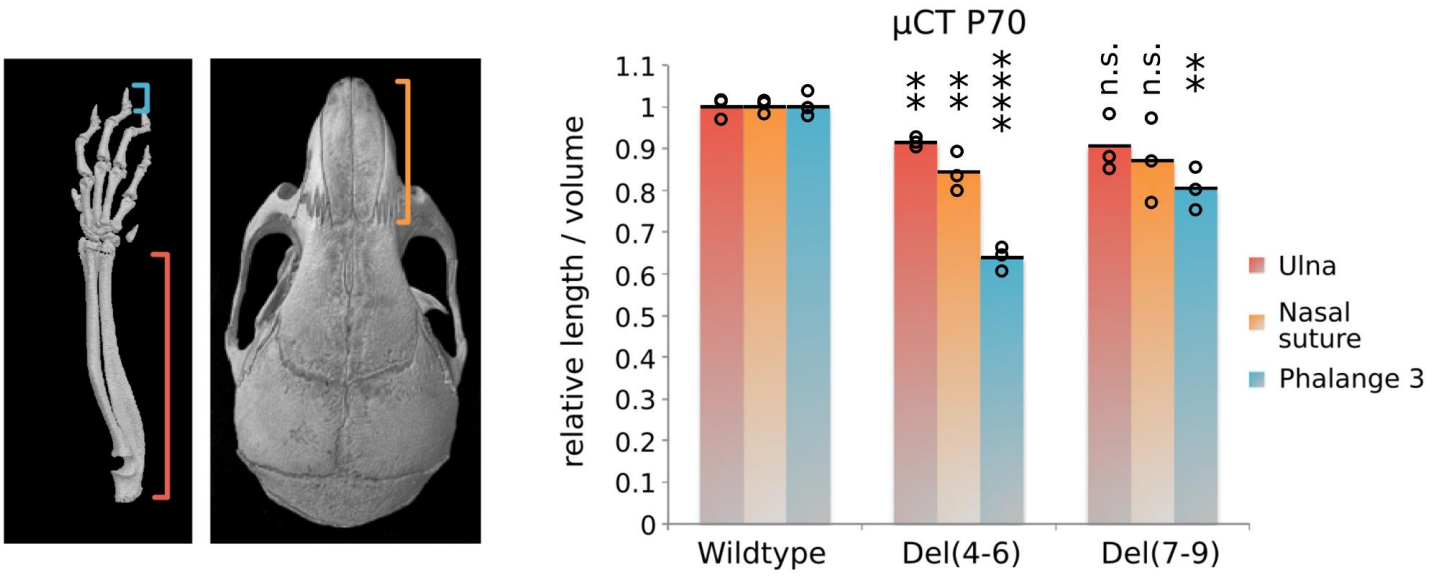
µCT analysis of adult skulls. The red square indicates enlargement of the metopic suture region, shown on the right. An enlargement of the corresponding cross-section (red arrow) of the metopic sutures is shown below. Note the normal development of sutures in *Nhej1*-knockout mice as compared to wild-type controls.



Supplementary Figure 5

Quantitative expression analysis (qPCR) of mutants at different tissues and stages.

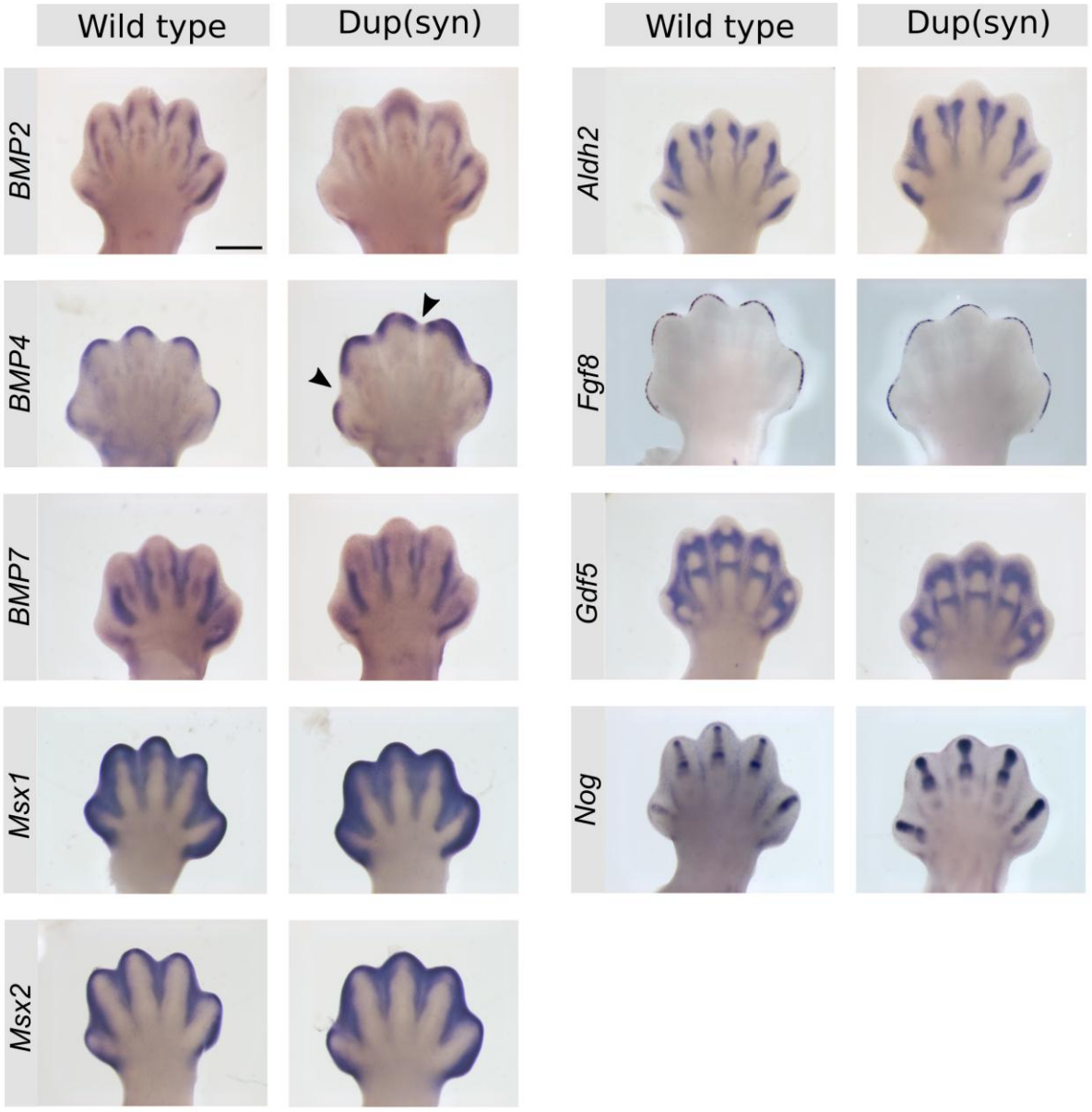
(**a**) Expression analysis of *Nhej1*. Note that manipulations of the intronic region of the *Nhej1* gene do not cause alterations in expression levels overall. (**b**) Expression analysis of *Cnppd1* and *Fam134a*. Note that the increased contacts observed in 4C–seq experiments for Dup(syn) mutants (**Fig. 4b**, asterisk) do not cause any alteration in the expression levels of the genes. Bars represent the mean of n = 3 different individuals (circles). Two-sided Student's t test, $^*P < 0.05$; ns, not significant.



Supplementary Figure 6

Enhancer deletions result in delayed skull ossification and reduced bone length.

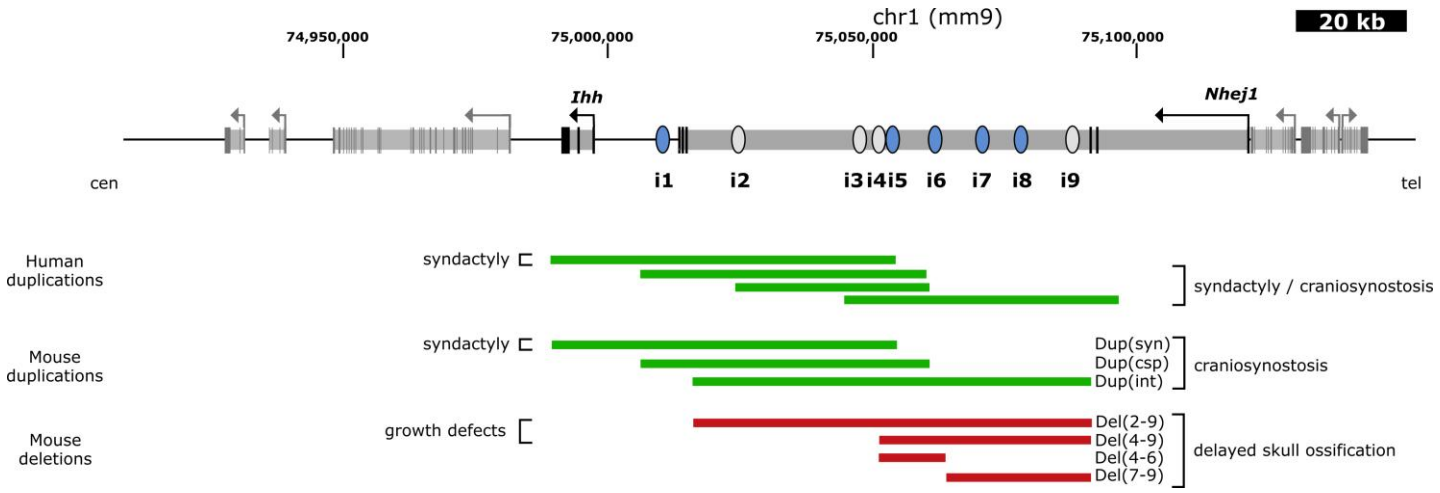
Left, µCT scan of wild-type mouse forelimb and skull displaying the different regions used for measurement. Right, bone measurements for Del(4–6) and Del(7–9) mutants and wild-type age-matched controls (P70). Note the reduction in ulna and nasal suture length for Del(4–6). Del(4–6) shows a more severe effect on digit length than that observed in Del(7–9) mutants. Bars represent the mean of n = 3 different individuals (circles). Two-sided Student's t test, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant.



Supplementary Figure 7

Expression analysis of genes involved in syndactyly/interdigital cell death.

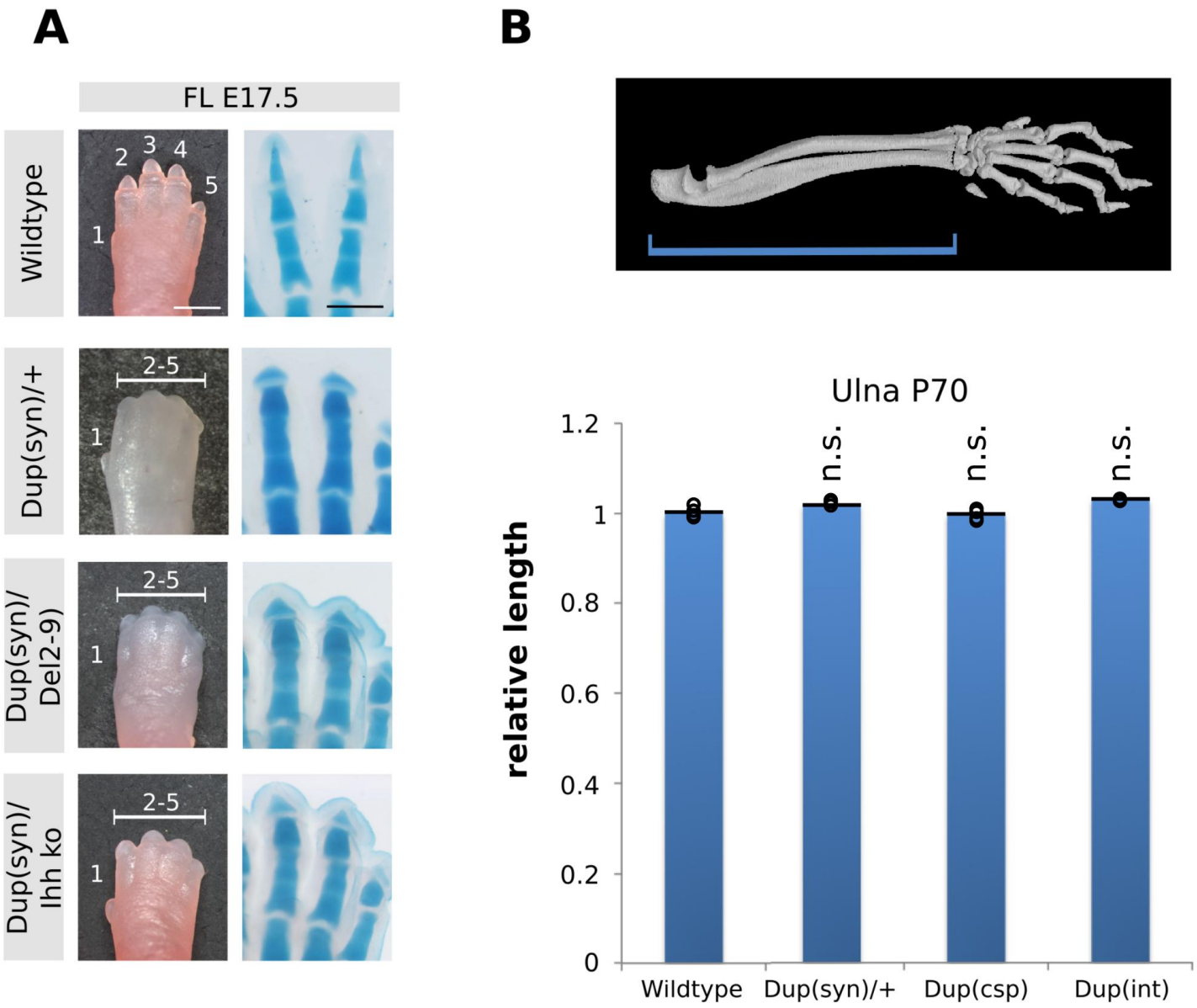
In situ hybridization analysis were performed in E14.5 forelimbs from Dup(syn)/+ mutants and corresponding wt controls. Note increased expression for *Bmp4* and *Nog* as well as expansion of *Bmp4* expression in the interdigital space (arrows). Bars represent 200μm.



Supplementary Figure 8

Pathogenic structural variants associated to the *Ihh* locus.

Schematic of the mouse locus with coordinates of structural variants indicated by colored bars and associated phenotypes. Positions of human duplications were transformed to the mouse genome. Enhancer elements are displayed with ovals. Duplications are depicted in green and deletions in red. All human variants are heterozygous, all mouse variants are homozygous.



Supplementary Figure 9

Limb abnormalities of Dup(syn) mice do not result from increased copies of *Ihh* gene.

(**A**) Forelimb morphology of duplications. Dup(syn)/+ mice (3 copies of *Ihh* gene) display 2/5 syndactyly. Skeletal stainings (right) show short and broad terminal phalanges. Dup(syn) mice were crossed to Del(2-9) or *Ihh* ko in order to have only 2 functional copies of *Ihh,* both in the duplicated allele. In both cases compound heterozygous displayed the same phenotypical effects. Bars represent 1000µm for P7 and 500µm for E17.5 autopods. (**B**) µCT analysis of wt mouse forelimb at P70 displaying the different regions used for measurement. None of the mutant mice displayed alterations in bone length. Bars represent mean of n ≥ 3 different individuals (circles). Two-sided Student's *t* test, $*P< 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$; ns, not significant.

SUPPLEMENTARY TABLES

Construct	E14.5			E17.5		
	Embryos analysed	Fingertips	Digits	Embryos analysed	Skulls	Growth Plates
i1	9	0	1	8	**3**	**7**
i2	9	0	0	10	1	**3**
i3	7	0	0	5	**2**	**4**
i4	6	0	0	11	**2**	**10**
i5	5	**3**	**3**	7	**3**	**5**
i6	12	0	**9**	7	1	**7**
i7	7	**2**	**3**	6	1	**6**
i8	7	1	**4**	10	**5**	**10**
i9	5	0	0	6	1	**5**

Supplementary Table 1: Tissue-specific activity of enhancer elements.

Each stage shows total number of embryos analyzed for each construct as well as those displaying positive staining for the corresponding tissue. Positive scoring is indicated in bold.

Construct	Genomic Position	Size	Guide	Sequence
Del(2-9), Dup(int)	Chr1:75,015,710-75,091,187	75kb	N2-L1	gagacacgtggagaattcgc-agg
			N2-R1	gttacccacactactacgtt-agg
Del(4-6)	Chr1:75,050,992-75,063,567	13kb	N4-L2	ggacacgactttcataacac-tgg
			N4-R2	aatttcgggtagggcgttgg-agg
Del(7-9)	Chr1:75,063,567-75,091,187	24kb	N4-R2	aatttcgggtagggcgttgg-agg
			N2-R1	gttacccacactactacgtt-agg
Del(4-9)	Chr1:75,050,992-75,091,187	37kb	N4-L2	ggacacgactttcataacac-tgg
			N2-R1	gttacccacactactacgtt-agg
Dup(syn)	Chr1:74,989,792-75,055,634	65kb	N9-L9	agcgtggggcttttaaccgt-ggg
			N9-R6	ttagacacaccagtatacgg-agg
Dup(csp)	Chr1:75,005,921-75,060,430	54kb	N10-L4	ggggcaatctgatatagtgg-ggg
			N10-R5	tggcccctgacccgtaggat-tgg

Supplementary Table 2: Genomic rearrangements generated using CRISPR/Cas9 genome editing.

Two sgRNAs flanking the target region were used to generate the genomic rearrangement.

Construct	Genomic Position	Size	Primer	Sequence
centromeric homologous arm				
SB-HR-L1	Chr1:75,055,877-75,058,875	3kb	HR3a1-f-SalI	tata***gtcgac***caaagtccttgtaaggaacagcagt
			HR3a1-r-ClaI	tata***atcgat***gacatgcctctgctgtacatagttt
SB-HR-L2	Chr1:75,058,877-75,060,875	2kb	HR3a2-f-F3-ClaI	tata***atcgat***tacaagctttacgaagttcctattcttcaaatagtat aggaacttcagcaactcaggaagaattcctaacac
			HR3a2-r-F3-SacII	tata***ccgcgg***gtagaagttcctatactatttgaagaataggaactt cttgcagccctcctatagaaaatgga
telomeric homologous arm				
SB-HR-R	Chr1:75,060,877-75,063,875	3kb	HR3b-f-XhoI	tata***ctcgag***tctataagaacacacaacaatgtgccag
			HR3b-r-NotI	tata***gcggccgc***actgttctgggtgaaccagaaatctt

Supplementary Table 3: Homologous arms cloned for the insertion of the SB cassette.

Cloning of the centromeric arm (total size 5kb) was performed in two steps (constructs SB-HR-L1 and SB-HR-L2). Restrictions sites are shown in italic/bold.

Element	Vista ID	Primer forward, reverse	Genomic Position (mm9)	Size (bp)
i1	mm1142	ctcagtgtctcaaccacttgaa, ctctgccatgacttcttgtgta	chr1:75,008,008-75,012,847	4840
i2	mm1143	ggtgggattaatctctcgactg, ggtgatgaacagcagtatggaa	chr1:75,023,290-75,026,536	3247
i3	mm1148	tctcccagaccaaaatgcttat, aaccttgccctcatgaagttta	chr1:75,046,263-75,049,025	2763
i4	mm1144	cagactggagttcacagagtgc, actcaggcacaagtctagcaca	chr1:75,051,762-75,053,663	1902
i5		cctctgtgctcttgagttagactac, cctccttgctagttcttacctaaaga	chr1:75,053,880-75,055,928	2045
i6	mm1145	tccttgagagactccagaaagg, tcccccatatcagatgtttacc	chr1:75,059,085-75,064,020	4936
i7	mm1146	gtactgggaaaaatggcaagag, ctgaaaggggttagaaggact	chr1:75,068,299-75,072,430	4132
i8		ttgaggcagaaggattgtcata, agccagaggtcaacatttgagt	chr1:75,075,786-75,080,268	4483
i9	mm1439	gctgagatgaatgacagtgagg, gtcacacctgatgatctgcatt	chr1:75,085,302-75,089,234	3933

Supplementary Table 4: Genomic regions tested for enhancer activity.

Primer	Sequence
Gapdh-F	GGGAAGCCCATCACCATCTT
Gapdh-R	CGGCCTCACCCCATTTG
Ihh-F	GCCGACCGCCTCATGAC
Ihh-R	CATGACAGAGATGGCCAGTGA
Nhej1-F (exon3+4)	TGAAGACAGAGCCATTTGAAGA
Nhej1-R (exon3+4)	GCTTTCCATCACCAACAGCA
Nhej1-F (exon2+3)	CATTGCTTCGGATGAAGGACC
Nhej1-R (exon2+3)	TCAATCGACTTCGGCTCAG
Cnppd1-F	CCTATTCGCCGACTCCAGAAA
Cnppd1-R	CATTCGTCATTGAAGACCTCCTC
Fam134a-F	CACAAACATGACAAGAGAAAGCG
Fam134a-R	AGCTCAGAGTCTGTAATAGCCA

Supplementary Table 5: qPCR primer sequences.

Viewpoint	1st primer (5′ – 3′)	Genomic Position	2nd primer (5′ – 3′)	Genomic Position
Ihh promoter	ACAGCTGGGGACCCTATAC	chr1:74,998,765-74,998,783	CCCGTCAGGAGGACAATC	chr1:75,059,837-75,059,854

Supplementary Table 6: 4C-seq primer sequences.

For reference, the mm9 mouse genome was used.