Supplemental Table and Figure Legends

Table S1. *Gli3* and *Gremlin*$^{\Delta GRE1}$ genetically interact. Summary of digit phenotypes in E18.5 littermates generated by compound crosses with Gli3 and *Gremlin*$^{\Delta GRE1}$. The presence of nubs was assayed visually upon dissection. All other phenotypes were obtained by inspecting processed skeletal preparations.

Table S2. Schematic summary of results. Schematics show representative data for the main experimental results of this study. Arrows indicate polydactylous nubs or digits in skeletal preparations. See Table S1 a complete description of skeletal phenotypes in *Gli3*$^{+/-}$;*Gremlin*$^{\Delta GRE1/\Delta GRE1}$ compound crosses.

Figure. S1. *Gli1* is significantly reduced in *Gli3*$^{-/-}$ forelimbs. Datapoints indicate relative gene expression assayed by qRT-PCR for pairs of forelimbs from single embryos normalized to a single 32 somite wild-type sample for Wild-type (n=6) and G*li3*$^{-/-}$ littermates (n=5). All embryos were between 32-34 somites. (A) The presence of an asterisk (red) indicates that *Gli1* expression is significantly reduced in *Gli3*$^{-/-}$ forelimbs (Mann-Whitney U Test; U=1.000, p = 0.0087). (B) In the same samples, *Shh* levels tend to be reduced although not to statistically significant levels (Mann-Whitney U Test; U=6.000, p = 0.1255). The bars indicate the mean and standard error of mean.

Figure. S2. *GRE1* enhancer activity in various genetic backgrounds at E11. *GRE1LacZ*$^{+/-}$ forelimbs stained for β-galactosidase activity. Limb buds in A,D,E,F are from embryos that were 40 somites, B from 38 somites, C from 42 somites. 'a.', anterior; 'p.', posterior.

Figure. S3. GRE1 enhancer activity is not negatively regulated by FGF. E11.5 *GRE1LacZ*$^{+/-}$ forelimbs (45-48 somites) were cultured in vehicle-containing control media (0.1% DMSO) (A) while their contralateral forelimbs were cultured in 10μM SU5402 8 hours (B) and stained for β-galactosidase activity. The normalized distance of the β-galactosidase domain from the distal (C) and anterior (D) limb (schematized as red lines in (A)) is not significantly altered in SU5402-treated embryos (Mann Whitney U Test). Horizontal lines indicate the mean and standard error of mean. (E,F) Consistent with previous reports, inhibiting FGF signaling results in an increase in distal anterior *Gremlin* expression (n=2). (G,H) Contralateral hindlimbs from the same embryos shown in panels A and B show a reduction in the FGF target gene *Sprouty4* in SU4202 cultured limb buds (H) compared to contralateral limb buds cultured in control media (G). Images A,B,G,H are from a 48 somite embryo. Images E,F are from a 45 somite embryo.

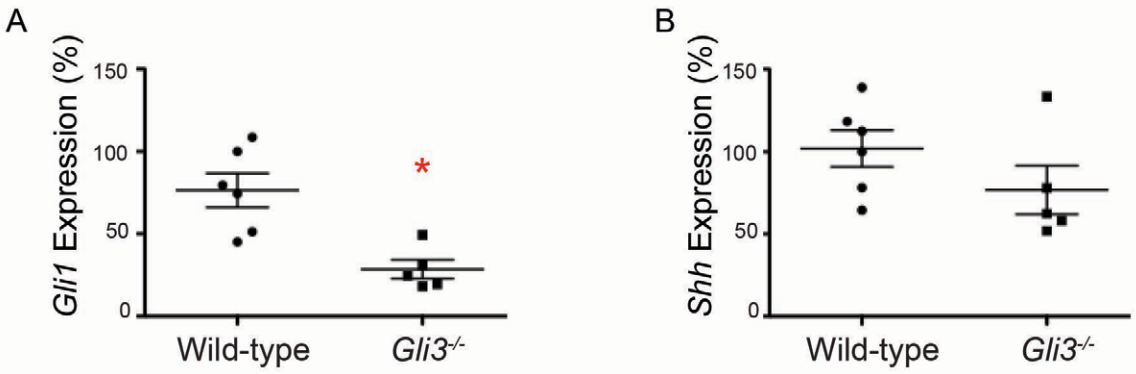
Figure S4. Generation of *Gremlin*$^{\Delta GRE1}$ mice. (A) A 7.5kb genomic fragment containing the *Gremlin* CRM (chr2:113,637,382-113,644,893) was digested from BAC #RP23-113H17 with *Xma1* and *Kpn1* and cloned into a pBluescript upstream of a diphtheria toxin A (DTA) negative selection cassette. A single loxP site and then an FRT-Neo-FRT-LoxP cassette were inserted immediately upstream and downstream, respectively, of the CRM (chr2:113640843-113641280). The targeting vector was linearized with KpnI and electroporated into AV3 ES cells (obtained from Dr. Andy McMahon's laboratory). Approximately 200 colonies were screened by Southern blot. DNA was digested with with *Bgl I* and hybridized with a 5' probe corresponding to chr2:113636128-113636494 and a 3' probe corresponding mm10| chr2:113,648,199-113,648,558. We identified two correctly targeted colonies by Southern blot, which were used to generate chimeric mice. Germline-transmitting chimeras were crossed with a Cre deleting strain,

Sox2Cre, to generate a deletion allele, *Gremlin*$^{\Delta GRE1}$ (MGI:5486166). (B) Mice that were wild-type, heterozygous and homozygous null mice were determined by genotyping with primers flanking the CRM (indicated by arrows underneath targeting constructs) that amplify a 582bp fragment in the wild-type allele and a 210bp fragment in the deletion allele. The sequences of the primers are: 5'-GCTAAACACAAAGAACTTTTAATGG-3' and 5'-GCAGCAGCAGTATTTTTCAGA-3'.

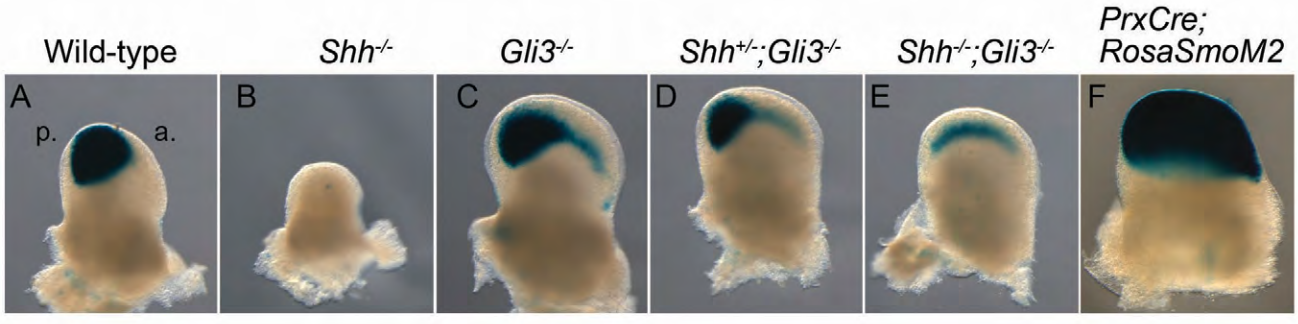
Figure S5. A single copy of the *Gremlin* with GRE1 is sufficient for limb development. Embryos containing a null allele of *Gremlin* and the other allele with a deletion of the CRM have normal limb skeletal patterning. The *Gremlin*$^{-}$ allele is *Grem1*^{tm1Rmh}. Images depict forelimbs and hindlimbs from the same embryo at E18.5 stained for bone (Alizarin Red) and cartilage (Alcian Blue). The numbers of skeletons that were analyzed for each genotype are indicated below.

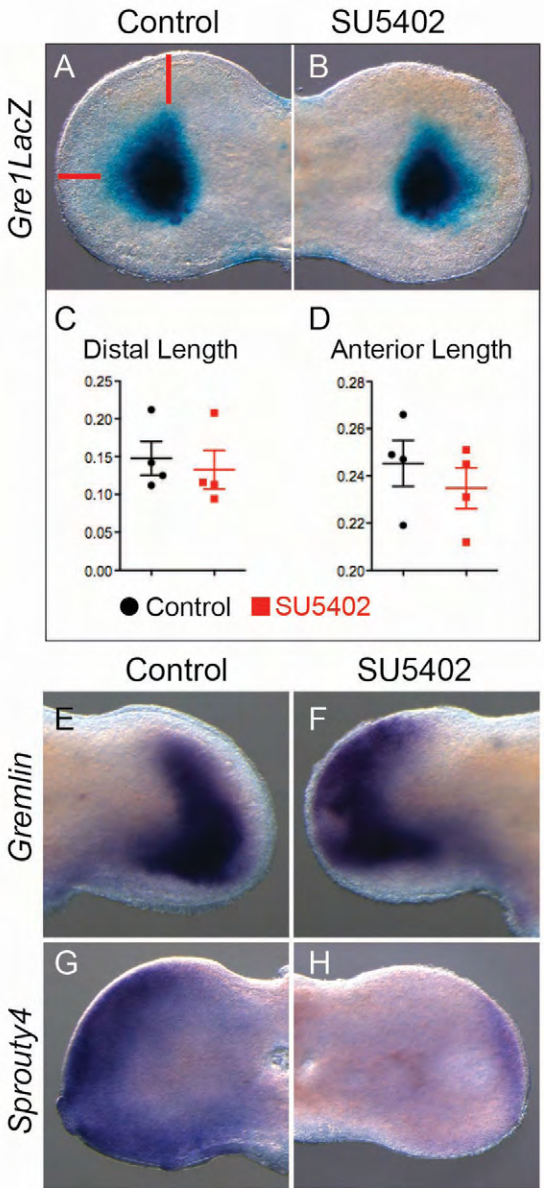
Figure S6. The *Gremlin*$^{\Delta GRE1}$ allele does not genetically interact with Shh. *Gremlin*$^{\Delta CRM/+}$;*Shh*$^{+/-}$ males were crossed to *Gremlin*$^{\Delta GRE1/+}$ females and their litters were analyzed by skeletal preparations at E18.5 stained for bone (Alizarin Red) and cartilage (Alcian Blue). Representative hands from forelimbs are shown on the top row. The second row shows representative feet. The numbers of skeletons that were analyzed for each genotype are indicated below. All skeletal preparations were normal with the exception of a small, fleshy nub observed on one hindlimb of a *Gremlin*$^{\Delta GRE1/\Delta GRE1}$ skeleton (something we have never otherwise observed).

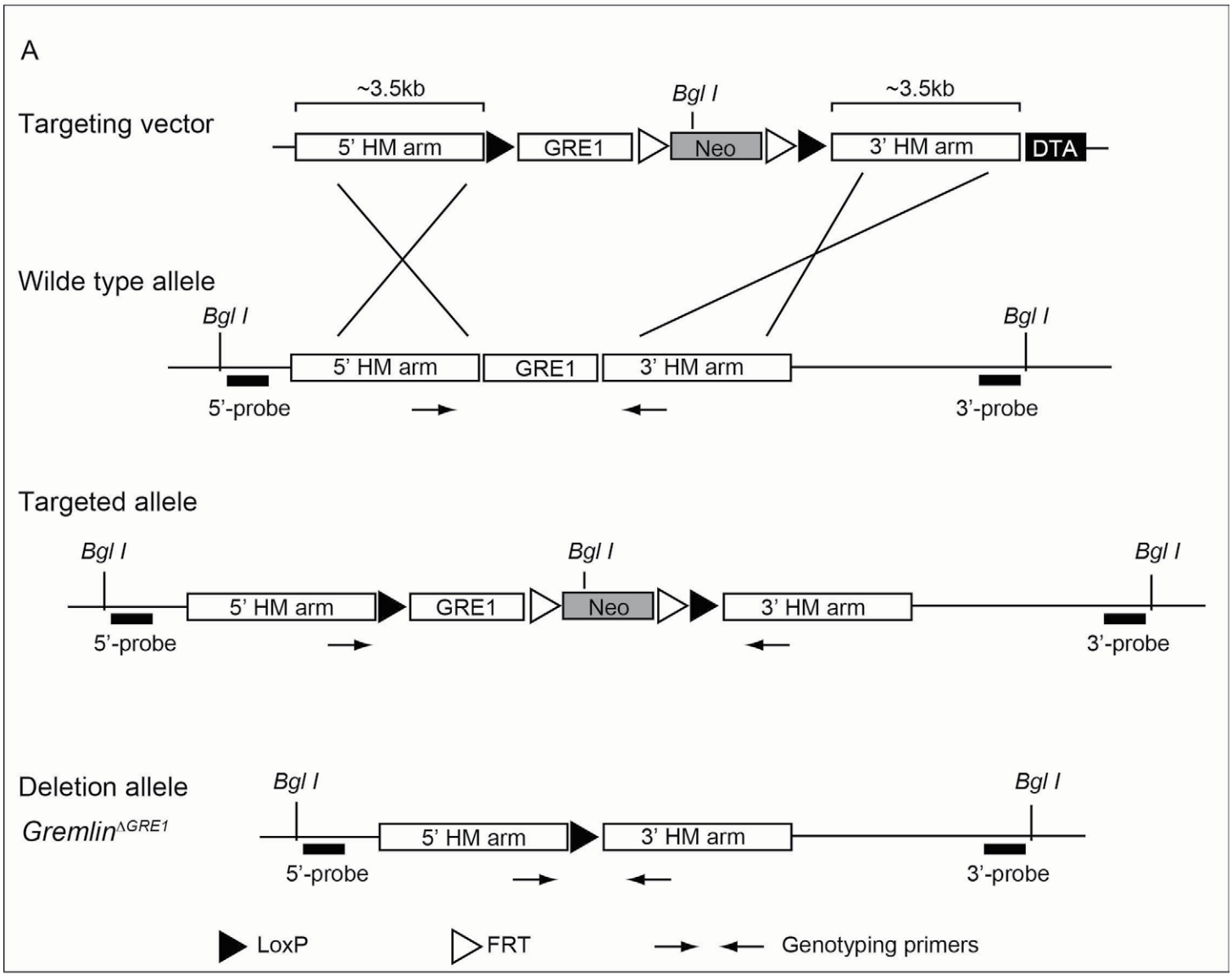
Li et al., Figure S1

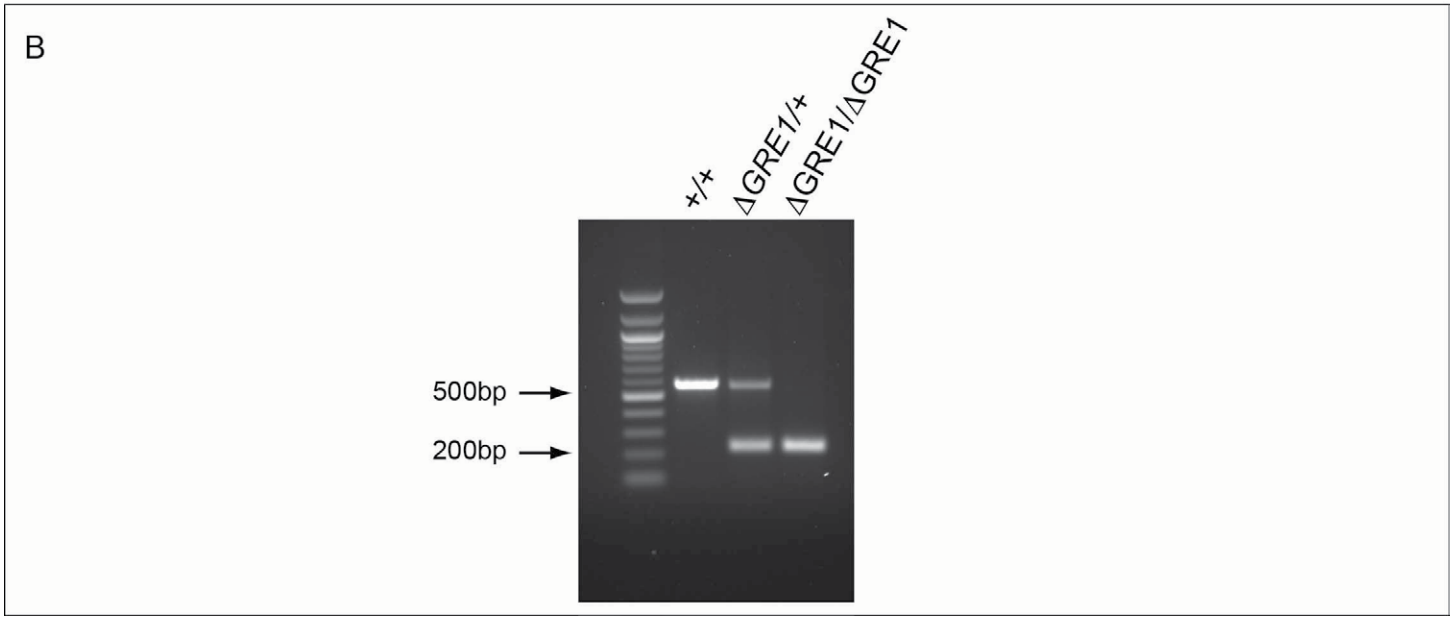


Li et al., Figure S2

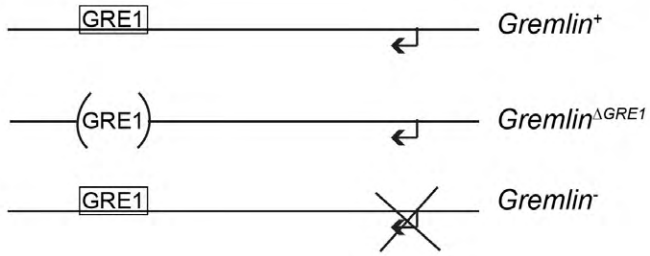


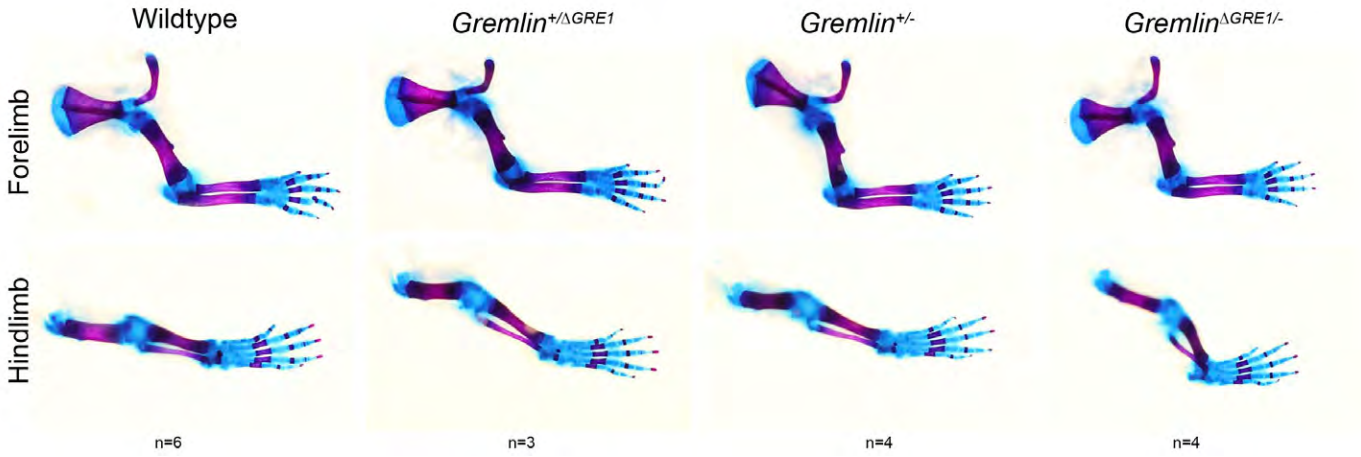

Control
SU5402
Gre1LacZ
A
B
C
Distal Length
0.25
0.20
0.15
0.10
0.05
0.00
D
Anterior Length
0.28
0.26
0.24
0.22
0.20
Control
SU5402
Control
SU5402
Gremlin
E
F
Sprouty4
G
H


A
Targeting vector
~3.5kb
Bgl I
~3.5kb
5' HM arm
GRE1
Neo
3' HM arm
DTA
Wilde type allele
Bgl I
Bgl I
5' HM arm
GRE1
3' HM arm
5'-probe
3'-probe
Targeted allele
Bgl I
Bgl I
Bgl I
5' HM arm
GRE1
Neo
3' HM arm
5'-probe
3'-probe
Deletion allele
Gremlin^ΔGRE1
Bgl I
Bgl I
5' HM arm
3' HM arm
5'-probe
3'-probe
LoxP
FRT
Genotyping primers

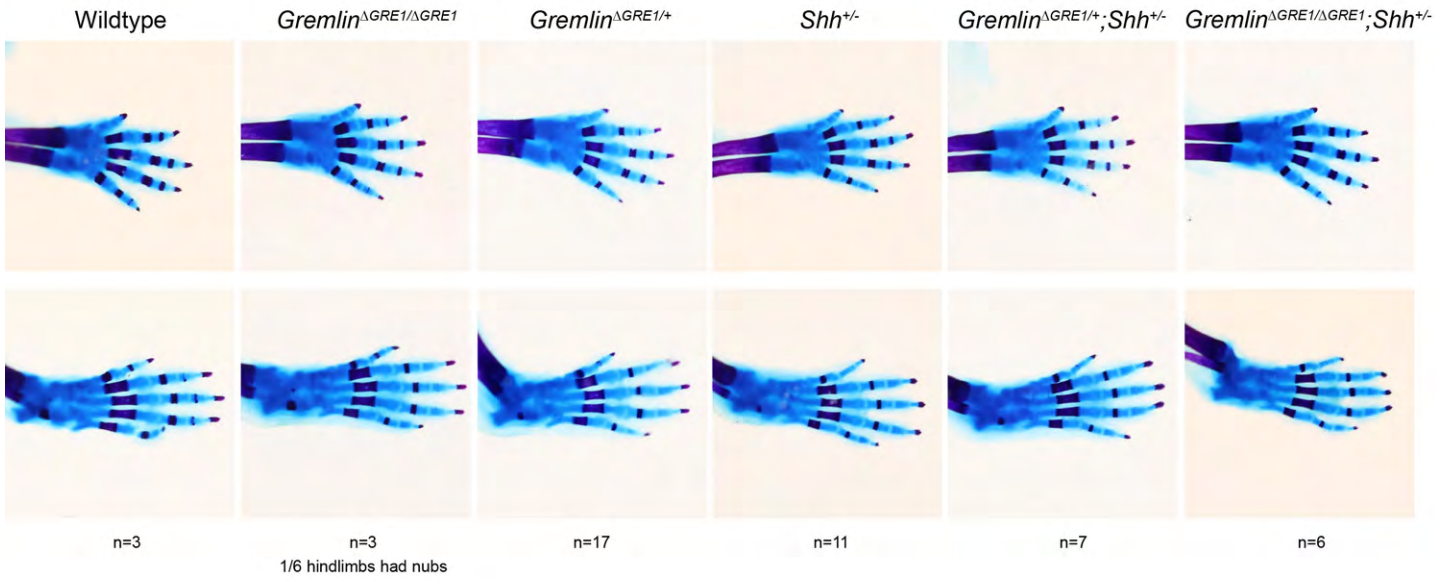

B
+/+
ΔGRE1/+
ΔGRE1/ΔGRE1
500bp
200bp

Li et al., Figure S5

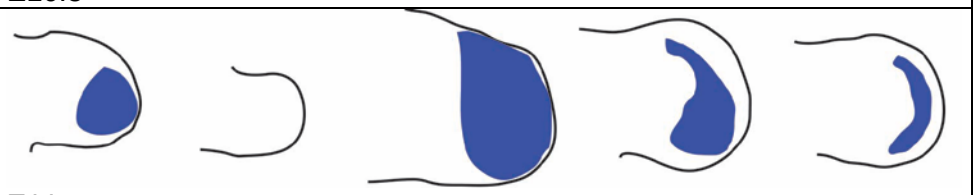
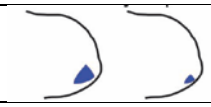
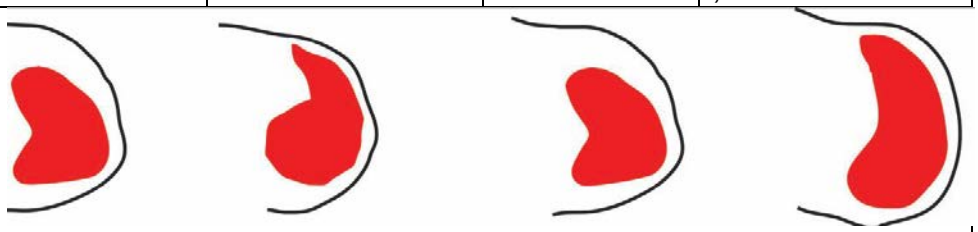
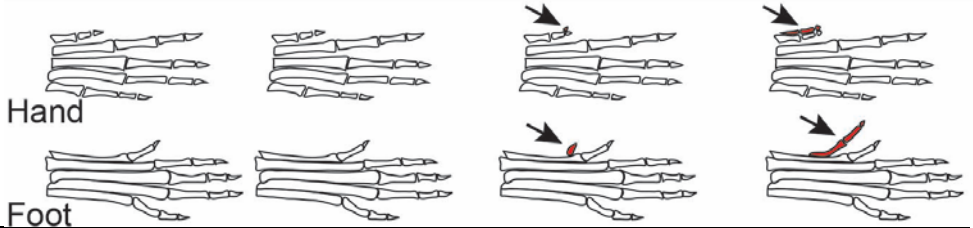




Li et al., Figure S6



	Wild-Type	*Gremlin*$^{\Delta GRE1/+}$	*Gremlin*$^{\Delta GRE1/\Delta GRE1}$	*Gli3*$^{+/-}$	*Gremlin*$^{\Delta GRE1/+}$ *;Gli3*$^{+/-}$	*Gremlin*$^{\Delta GRE1/\Delta GRE1}$ *;Gli3*$^{+/-}$
Forelimbs						
Polysyndactyly	0	0	0	4	23	8
Distal Bifurcation	0	0	0	3	3	0
Broad Thumb	0	0	0	3	2	0
Normal	8	38	14	7	0	0
Total	8	38	14	17	28	8
Hindlimbs						
Polydactyly	0	0	0	0	1	3
Nub	0	0	0	18	13	5
Normal	8	38	14	0	0	0
Total	8	38	14	18	14	8

<table>
<tr><td colspan="6">Enhancer Activity</td></tr>
<tr><td></td><td>Wild-type</td><td>Shh^{-/-}</td><td>RosaSmoM2</td><td>Gli3+/-</td><td>Shh^{-/-};Gli3^{-/-}</td></tr>
<tr><td>GRE1LacZ (GRE1::minimal promoter::LacZ</td><td colspan="5"> 
E10.5</td></tr>
<tr><td></td><td colspan="5"> 
E11</td></tr>
<tr><td></td><td colspan="5">Enhancer activity requires sustained Hh signaling  </td></tr>
<tr><td colspan="6">BAC Transcriptional Reporter</td></tr>
<tr><td rowspan="2">BAC with LacZ in Gremlin transcript</td><td>Wild-type</td><td>GRE1 Deletion</td><td>GRE1-GliMut</td><td></td><td></td></tr>
<tr><td colspan="3"> 
E11</td><td></td><td></td></tr>
<tr><td colspan="6">GRE1 deletion phenotypes</td></tr>
<tr><td></td><td>Wild-type</td><td>Gremlin^{ΔGRE1/ΔGRE1}</td><td>Gli3^{+/-}</td><td colspan="2">Gremlin^{ΔGRE1/ΔGRE1};Gli3^{+/-}</td></tr>
<tr><td>In situ hybridization for Gremlin</td><td colspan="5"> 
E10.5</td></tr>
<tr><td>Skeletal preparations</td><td colspan="5"> 
Hand
Foot
Skeletal elements are normal in Gremlin^{ΔGRE1/-} and Gremlin^{ΔGRE1/ΔGRE1};Shh^{+/-} embryos</td></tr>
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