Fig. S1. Details of the ZRS targeting constructs and representation of the correctly inserted into the locus before and after Cre-recombinase. (A) The three constructs which were targeted into the endogenous ZRS (shown as a grey rectangle in the bottom line, with HindIII (H) sites at either end). These constructs comprise a 7kb region of genomic DNA flanked by NotI and SalI restriction sites (N and Sl respectively), carrying a bacterial LacZ gene driven by a minimal β-globin promoter (inserted into a SmaI site (S)) and a Neomycin resistance (neo) resistance cassette driven by a PGK promoter. The mutated ETS site in ZRS^{ETS} is indicated by the black rectangle. (B) The loci after correct targeting, which was screened for by PCR using primers 1f and 1r and 2f and 2r across homology arms 1 and 2 respectively. Mice carrying these chromosomes were genotyped for the presence of lacZ using primers Lf and Lr and for the presence of $neomycin^R$ using Nf and Nr. The unmodified wildtype chromosome was screened for using Wf and R. (C) The result of cre mediated excision of the lacZ and $neomycin^R$ cassettes which leaves only a single loxP site. Genotyping of mice with Wf and R or Df and R allows the modified and wildtype chromosomes to be distinguished because of the size difference created by the remaining lox site.

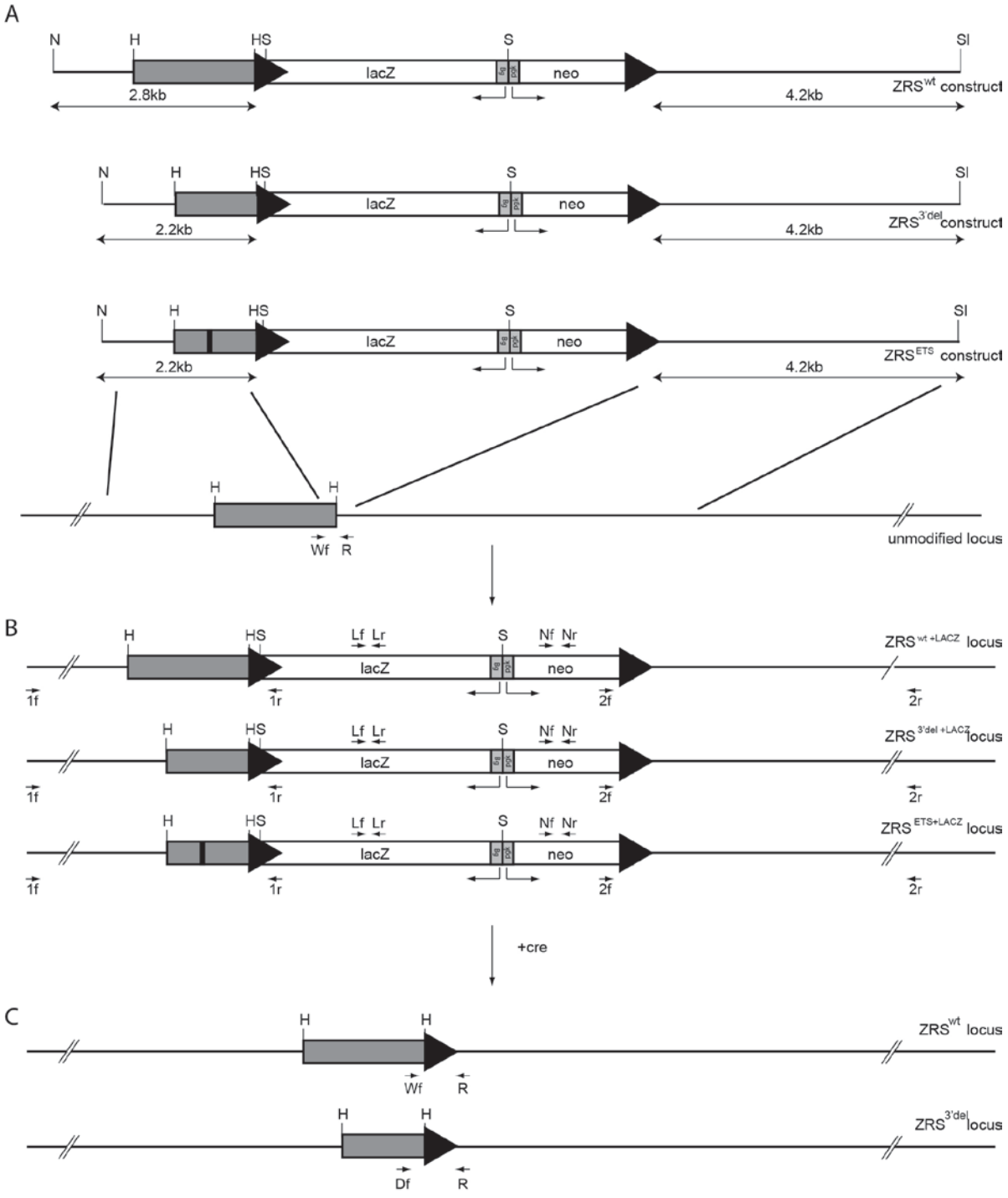
A
N
H
HS
S
Sl
lacZ
neo
2.8kb
4.2kb
ZRS^wt construct
2.2kb
ZRS^3'del construct
ZRS^ETS construct
Wf
R
unmodified locus
B
Lf
Lr
Nf
Nr
1f
1r
2f
2r
ZRS^wt +LACZ locus
ZRS^3'del +LACZ locus
ZRS^ETS+LACZ locus
+cre
C
ZRS^wt locus
Df
ZRS^3'del locus

Table S1. Primer sequences

Constructs		
DelA	F- GAATTCTCAAGTATAAGAGAACATAG R- gatcat**AAGCTT**AACAAGCAAAAATAAGAAAG	
DelB	F- GAATTCTCAAGTATAAGAGAACATAG R- gatcat**AAGCTT**TAAGATGGAGGCCTGAGAC	
DelC	F- GAATTCTCAAGTATAAGAGAACATAG R- gatcat**AAGCTT**GTCATCTGGTCATAAAATACAG	
DelD	F- GAATTCTCAAGTATAAGAGAACATAG R- gatcat**AAGCTT**CATATTAAAACGTACTTAGTTC	
3' END	F- gatcat**AAGCTT**TTTCTATCCTGTGTCACAGTTTG R- TACTAAGAAGGTGGCAACAGAC	
Core	F- gatcat**AAGCTT**AGTCCTGGCATAAAACTTAAC R- gatcat**AAGCTT**TAAGATGGAGGCCTGAGAC	
Mutagenise Hx	F- GCTTGTTTTTTTTGCCACTAATGATCCATAAATTGTTGGAAATGAG R- CTCATTTCCAACAATTTATGGATCATTAGTGGCAAAAAAAACAAGC	
Mutagenise M100081	F- GTGACCTTGTACTGTGTTTTATGACCAGATGACTTTTCCCCTCAG R- CTGAGGGGAAAAGTCATCTGGTCATAAAACACAGTACAAGGTCAC	
-ETS	F- GCACAAAATCTGAGGTCACTTGAACTCTTAATTAGTTGCACTGACCAGG R- CCTGGTCAGTGCAACTAATTAAGAGTTCAAGTGACCTCAGATTTTGTGC	
Mutagenise REP3A	F- GCTTGTTTTTTTTGCCACCAGTGATCCATAAATTGTTGGAAATGAGC R- GCTCATTTCCAACAATTTATGGATCACTGGTGGCAAAAAAAACAAGC	
Mutagenise REP3B	F- GCTTGTTTTTTTTGCCACAATTGATCCATAAATTGTTGGAAATGAGC R- GCTCATTTCCAACAATTTATGGATCAATTGTGGCAAAAAAAACAAGC	
Flip80 Region flipped is underlined	Arm 1 gatcat**AAGCTT**TTTAATGCCTATCATTTGATTTGAAGTC and ATAAATTGTTGGAAATGAGCGATTCAGGAAGTGCTGCTTAGTGTTAGTGGCAAATGCGCTAACAAGCAA AAATAATGAAAGAATC Arm 2 AAGCAGCACTTCCTGAATCGCTCATTTCCAACAATTTATGGATCATCAGTGGCAAAAAAAACTCAGTCT GGTTCTGCTG and gatcat**AAGCTT**ACATAGCAACAGTTAGTGAGATATG	
Flip80 +Hx	Arm 1 gatcat**AAGCTT**TTTAATGCCTATCATTTGATTTGAAGTC and ATAAATTGTTGGAAATGAGCGATTCAGGAAGTGCTGCTTAGTGTTAGTGGCAAATGCGCTAACAAGCAA AAATAATGAAAGAATC Arm 2 AAGCAGCACTTCCTGAATCGCTCATTTCCAACAATTTATGGATCATTAGTGGCAAAAAAAACTCAGTCT GGTTCTGCTG and gatcat**AAGCTT**ACATAGCAACAGTTAGTGAGATATG	
Flip80+Rep3A	Arm 1 gatcat**AAGCTT**TTTAATGCCTATCATTTGATTTGAAGTC and	

	ATAAATTGTTGGAAATGAGCGATTCAGGAAGTGCTGCTTAGTGTTAGTGGCAAATGCGCTAACAAGCAA AAATAATGAAAGAATC Arm 2 AAGCAGCACTTCCTGAATCGCTCATTTCCAACAATTTATGGATCACTGGTGGCAAAAAAAACTCAGTCT GGTTCTGCTG and gatcat**AAGCTT**ACATAGCAACAGTTAGTGAGATATG	
FLIP49 Region flipped is underlined	Arm 1 gatcat**AAGCTT**TTTAATGCCTATCATTTGATTTGAAGTC and CGTTCATTGGATTCTTTCATTATTTTTGCTTGTTTTTTTTTGCCACTGATCTCATGGAGTCCCAGGC Arm 2 AACAAGCAAAAATAATGAAAGAATCCAATGAACGGATCCATAAATTGTTGGAAATG and gatcat**AAGCTT**ACATAGCAACAGTTAGTGAGATATG	
FLIP49+REP3A	Arm 1 gatcat**AAGCTT**TTTAATGCCTATCATTTGATTTGAAGTC and CGTTCATTGGATTCTTTCATTATTTTTGCTTGTTTTTTTTTGCCACCTGTCTCATGGAGTCCCAGGC Arm 2 AACAAGCAAAAATAATGAAAGAATCCAATGAACGGATCCATAAATTGTTGGAAATG and gatcat**AAGCTT**ACATAGCAACAGTTAGTGAGATATG	
REP5	Arm1- GAATTCTCAAGTATAAGAGAACATAG and CATTATTTTTGCTTGTTTTTTTTTGCCTGCAGTGATCCATAAATTGTTGGAAATGAGC Arm 2- GCTCATTTCCAACAATTTATGGATCACTGCAGGCAAAAAAAACAAGCAAAAATAATG and TACTAAGAAGGTGGCAACAGAC	
Del41	Arm 1 gatcat**AAGCTT**TTTAATGCCTATCATTTGATTTGAAGTC and GTACTGTATTTTATGACCAGATGACAGAGAGTAGGAAGTCCAGCCTGG Arm 2 CCAGGCTGGACTTCCTACTCTCTGTCATCTGGTCATAAAATACAGTAC and gatcat**AAGCTT**ACATAGCAACAGTTAGTGAGATATG	
Shh promoter ATG is underlined	F- gatcat**GTCGAC**TAAACTGGAAGCCTCAGGTG R- gatcat**GGTACC**ATCTCGTCCGCGGAACCTGAG	
ES cell targeting screening		
Arm 1	1f- ATGGCACTGAAGTGTTGACTG 1r- CACTGCATTCTAGTTGTGG	
Arm 2	2f- ATCGCATTGTCTGAGTAGGTG 2r- GACCAATAGTACTCCAAGCAAG	
Genotyping		Allele size
lacZ	LF- CAACTTAATCGCCTTGCAGCAC LR- CGCTGATTTGTGTAGTCGGTT	+lacZ - 550bp
Neo	NF- TGTTCCGGCTGTCAGCGCAG NR- GATATTCGGCAAGCAGGCATC	+neo - 480bp
ZRS^{Del}	DF- CCAAGCAACATGACAGCAC R- CACACACGGATACTAAGACGG	Wt- 950bp ZRS^{Del} – 440bp
ZRS^{Wt}	WF- GGAATGCATGCAGGAACTCAG R- CACACACGGATACTAAGACGG	Wt- 290 bp ZRS^{Wt} – 380bp

qRT-PCR		
Shh	F- ACCCCGACATCATATTTAAGGA R- TTAACTTGTCTTTGCACCTCTGA	
B-gal	F- ATGGATGAGCAGACGATGG R- CGGCGTTAAAGTTGTTCTGC	

Table S2. Fosmid Probes

Region	Whitehead (Sanger) Name	Ensemble name	Coordinates Start	End	Size (bp)
Shh	WI1-574O18	G135P64333A4	28754458	28795879	41421
ZRS	WI1-1047E14	G135P600929F6	29611727	29653695	41968

Names are Ensembl (r 45) (http://jun2007.archive.ensembl.org/Mus_musculus/index.html). Mouse genome assembly number: NCBI m37.

Table S3. Co-localisation frequency of the *Shh* probe with the ZRS and *LacZ* reporter construct probes for E11.5 limb bud sections

Limb region	***Shh*-ZRS (847 kb)**	***Shh*-LacZ (847 kb)**	**Probe pairs**	**ZPA**	**Proximal**
	Co-localisation frequency (%)			**Co-localisation frequency (%)**	
ZPA	35.1	11.9	***Shh*-ZRS**	35.1	22.7
Proximal	22.7	19.3	***Shh*-LacZ**	11.9	19.3
	$p = 0.04$	$p = 0.29$		$p = 0.0002$	$p = 0.63$

Statistical analysis of data for Figure 5J- *p*-values from Fisher's Exact Tests.

Table S4. Frequency of Shh-ZRS and *Shh-LacZ* probes separated by ≥ 400nm for E11.5 limb bud sections

Probe pairs	ZPA	Proximal
	Frequency (%) ≥ 400 nm	
***Shh*-ZRS (847 kb)**	18.3	11.1
***Shh*-LacZ (847 kb)**	39.5	30.5
	$p = 0.0011$	$p = 0.0012$

Statistical analysis of data for Figure 5J- *p*-values from Fisher's Exact Tests

Table S5. Co-localisation frequency of the *Shh* probe with the ZRS probe for E11.5 limb bud sections derived from the fore limbs of $ZRS^{wt/wt}$ and $ZRS^{3'del/3'del}$ homozygous embryos

Forelimb	***Shh*-ZRS in $ZRS^{wt/wt}$ (847 kb)**	***Shh*-ZRS in $ZRS^{3'del/3'del}$ (847 kb)**	**Probe pairs**	**ZPA/ Distal posterior**	**Proximal**
	Co-localisation frequency (%)			**Co-localisation frequency (%)**	
ZPA/Distal posterior	38	20	***Shh*-ZRS in $ZRS^{wt/wt}$**	38	21
Proximal	21	20	***Shh*-ZRS in $ZRS^{3'del/3'del}$**	20	20
	$p = 0.01$	$p = 1$		$p = 0.008$	$p = 1$

Statistical analysis of data for Figure 5J- *p*-values from Fisher's Exact Tests

Table S6. Co-localisation frequency of the *Shh* probe with the ZRS probe for E11.5 limb bud sections derived from the hind limbs of ZRS$^{wt/wt}$ and ZRS$^{3'del/3'del}$ homozygous embryos

Hindlimb	*Shh*-ZRS in ZRS$^{wt/wt}$ (847 kb)	*Shh*-ZRS in ZRS$^{3'del/3'del}$ (847 kb)	Probe pairs	ZPA	Proximal
	Co-localisation frequency (%)			Co-localisation frequency (%)	
ZPA	39.1	26.9	***Shh*-ZRS in ZRS$^{wt/wt}$**	39.1	24.2
Proximal	24.2	22.5	***Shh*-ZRS in ZRS$^{3'del/3'del}$**	26.9	22.5
	p = 0.009	p = 0.45		p = 0.04	p = 0.88

Statistical analysis of data for Figure 5J- p-values from Fisher's Exact Tests