SUPPLEMENTAL METHODS

Generation of *Fgf8* BAC transgenic mouse embryos

To generate the *Fgf8-lacZ* BAC construct, pHsp68-lacZ (Vokes et al., 2008) was used as a template to amplify *lacZ* which was then fused to an FRT-Kanamycin-FRT cassette (Gene Bridges) to generate a lacZ-FRT-Kanamycin-FRT cassette. Using lacZ-FRT-Kanamycin-FRT, we introduced ~500 bp of homology arms derived from sequences on either side of the ATG codon of the *Fgf8* coding sequence such that after recombination the ATG of *Fgf8* would be in frame with the *lacZ* coding region. Linearized targeting vector carrying the *Fgf8* homology arms and lacZ-FRT-Kanamycin-FRT cassette was electroporated into the *E.coli* DH10B, BAC host strain harboring *Fgf8* and subjected to kanamycin selection to generate *Fgf8-lacZ*. For the *Fgf8-ΔRARE-lacZ* deletion BAC construct, ~500 bp of homology arms surrounding the *Fgf8* RARE were PCR amplified using oligonucleotides containing mutant FRT-F3 sites (Nagy et al., 2003). During recombination, a 34 bp sequence including the *Fgf8* RARE was replaced by a single 34 bp mutant FRT-F3 site. Correct recombination and removal of the kanamycin cassette in *Fgf8-lacZ* and *Fgf8-ΔRARE-lacZ* BACs were tested by PCR, restriction enzyme digestion, and PCR product DNA sequencing. For oligonucleotide sequences used in recombineering see Table 1(C) in Supplemental Material.

For microinjection of these BAC constructs into fertilized mouse oocytes, the modified BACs were purified and isolated using Qiagen maxi purification kit (Qiagen). BAC-DNA was re-suspended and diluted in microinjection buffer (10 mM Tris pH 7.5, 0.5 mM EDTA, 30 nM spermine, 70 nM spermidine, 0.1 M NaCl) prepared in sterile endotoxin-free, ultra-pure water from Sigma (W1503, Sigma) and stored at 4°C for at least 5 days before the microinjection procedure to stabilize the BAC constructs.

Embryo Chromatin immunoprecipitation (ChIP) assay

Embryo ChIP was performed similar to previously described methods (Frank et al., 2001) according to the manufacturer's protocol (Active Motif).

For regional embryo ChIP assays we used pooled head, trunk and CPZ tissues from 67 wild-type or 67 *Raldh2*-/- E8.25 (5-8 somite) mouse embryos (separated by cutting just posterior to the heart and just posterior to the most recently formed somite). Tissues were cross-linked with 1% formaldehyde at room temperature for 15 min. Isolated nuclei (in 650 µl of shearing buffer) from these embryonic tissues were sonicated for 15-20 pulses of 10 sec each at 30% power output with 2 min rest between pulses using a Misonix Digital Sonicator 4000 equipped with a microtip probe (Cole-Parmer Instrument Company). Samples were sonicated on ice to shear DNA to an average size of 500-1000 bp followed by centrifugation at 13,000 rpm for 10 min. At this point, a small portion of supernatant containing sheared chromatin was stored as input control. For immunoprecipitation, 150 µl of sheared chromatin was mixed with 3 µg of antibodies specific for H3K4me3, H3K27me3, EZH2, or SUZ12 (all from Active Motif), HDAC1 (Genetex), RERE (Abgent), or control IgG antibody (Cell Signaling Technology), and then each ChIP reaction was incubated with 25 µl pre-blocked protein G-coated magnetic beads (Active Motif, Carlsbad, CA) for 4 h at 4°C. Beads were washed and eluted DNA-protein complexes were reverse cross-linked followed by purification using the Qiaquick PCR purification kit (Qiagen). Analysis of immunoprecipitated DNA was performed by PCR amplification. ChIP samples were subjected to PCR using primers flanking the mouse *Fgf8* RARE near -4.1 kb upstream or a non-specific region at -5.3 kb; the 5'-*Hoxb1* RARE at -1.6 kb, the 3'-*Hoxb1* RARE at +7 kb, or a non-specific region at -6 kb; the *RARb* RARE at -57 bp; for oligonucleotide sequences used in ChIP see Table 1(A) in Supplemental Material. For quantitation of ChIP results, enrichment of specific DNA fragments was measured by real-time qPCR using Mx3000P QPCR System (Agilent Technologies) and SYBR Advantage qPCR Premix (Clontech). Each ChIP analysis was repeated in at least in three independent experiments and results are reported as ± SEM; using the *t* test.

For whole-embryo ChIP, pooled wild-type E8.25 (5-8 somite) mouse embryos were subjected to the ChIP procedure detailed above, except immunoprecipitation was carried out with 3 µg of anti-

RARα (Santa Cruz Biotechnology), anti-RARβ (Affinity Bioreagents), anti-RARγ (Santa Cruz Biotechnology), or control IgG (Cell Signaling Technology), and PCR products were separated by 3% agarose gel electrophoresis and visualized using ethidium bromide staining. Each ChIP gel analysis was performed in triplicate and a representative example is presented.

Antibodies used (company and catalog number):

RERE (Abgent; AP9954a)

HDAC1 (GeneTex; GTX100513)

SUZ12 (Active Motif; 39357)

EZH2 (Active Motif; 39901)

H3K4me3 (Active Motif; 39159)

H3K27me3 (Active Motif; 39155)

IgG antibody (Cell Signaling Technology; 2729)

RARα (Santa Cruz Biotechnology; sc-551)

RARβ (Affinity Bioreagents; PA1-811)

RARγ (Santa Cruz Biotechnology; sc-550)

Electrophoretic Gel Mobility Shift Assay (EMSA)

Thirty-two wild-type E8.25 embryos were dissected and nuclear protein extracts were prepared as described (Dignam et al., 1983). Biotin-labeled double-stranded oligonucleotide probes containing wild-type and mutant *Fgf8* RARE sequences were bound to nuclear extracts. Binding reactions were performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Thermo Scientific) according to the manufacturer's instructions. Reaction mixtures were incubated for 20 min at room temperature. The binding reactions were mixed with 5x loading buffer and run on a 6% non-denaturing polyacrylamide gel in 0.5 X Tris-Borate-EDTA buffer for 90min at 100V on ice, then transferred onto Biodyne nylon membrane (Thermo Scientific) at 380mA for 1 hour in 0.5 X TBE on ice. The membrane was optimally UV-light cross-linked and detection was performed using a LightShift Chemiluminescent EMSA Kit (Pierce, Thermo Scientific) according to the manufacturer's instructions. For supershift analysis, nuclear extracts were incubated with 3 µg of anti-RARα (sc-551, Santa Cruz Biotechnology), anti-RARβ (PA1-811, Pierce, Thermo Scientific), or anti-RARγ antibodies (sc-550, Santa Cruz Biotechnology) for 20 min on ice before adding probe. For probe sequences, see Table 1 (B) in Supplemental Material.

REFERENCES

Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475-1489.

Frank, S. R., Schroeder, M., Fernandez, P., Taubert, S. and Amati, B. (2001). Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes Dev.* **15**, 2069-82.

Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, R. R. (2003) *Manipulating the Mouse Embryo, Third Edition*. Cold Spring Harbor: Cold Spring Harbor Press.

Vokes, S. A., Ji, H., Wong, W. H. and McMahon, A. P. (2008). A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. *Genes Dev.* **22**, 2651-63.

SUPPLEMENTAL FIGURE 1

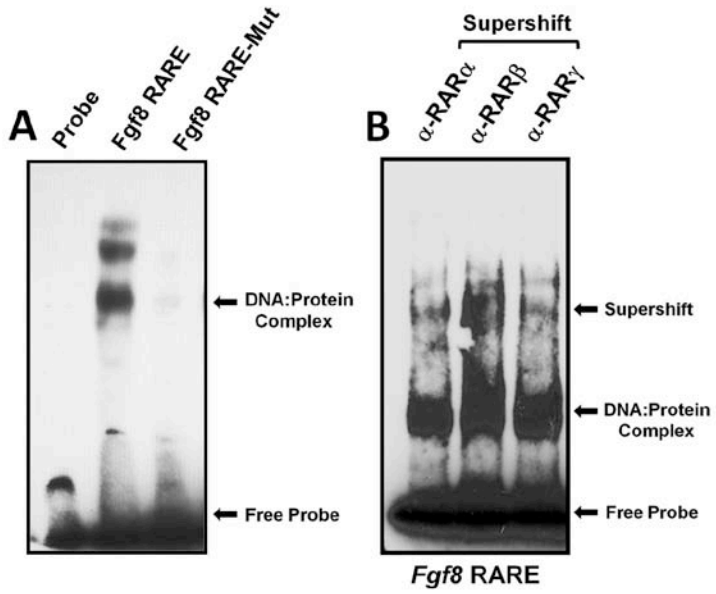

Figure S1. Analysis of *Fgf8* RARE DNA-binding activity.

(A) Electrophoretic Mobility Shift Assay (EMSA) using a nuclear extract from E8.25 whole mouse embryos was performed in the presence of wild-type and mutant *Fgf8* RARE biotinylated probes in the lanes indicated; left lane contains wild-type *Fgf8* RARE probe alone. Arrows indicate the free probe and specific DNA:protein complex shift.

(B) A supershift assay was performed by incubating nuclear extracts with RARα, RARβ, and RARγ antibodies prior to the addition of wild-type *Fgf8* RARE biotinylated probe. Arrows indicate the free probe, specific DNA:protein complex shift, and super-shift of the DNA:protein complex with RAR antibody.

SUPPLEMENTAL TABLE 1

List of oligonucleotides used for ChIP, EMSA, and BAC recombineering.

Oligonucleotides	Sequence
(A) Chromatin immunoprecipitation (ChIP) oligos:	
Fgf8-DR2 RARE-Fwd	5'-CAG CAC TCT GCC ATA CTG TCT TA-3’
Fgf8-DR2 RARE-Rev	5'-TCT GTC AGT CTT CAG CTT GTC TG-3’
Fgf8-NSR-Fwd	5'- TAG CAG CTG AAT GAG TGG CTC TA -3’
Fgf8-NSR-Rev	5'- GTA GCA AGC AGT TAC CTG ATC TG -3’
Hoxb1-5’-DR2 RARE-Fwd	5'-AGA CAA CTT TGG GCC CTT GAA GG-3’
Hoxb1-5’-DR2 RARE-Rev	5'-CTC CTC CAG ACT CTG AAG ACA AG-3’
Hoxb1-3’-DR5 RARE-Fwd	5'-CTT GGA GAT GAG GCA GCT CAG-3’
Hoxb1-3’-DR5 RARE-Rev	5'-CTG CCC TTC TGG CTT CAG CAA-3’
Hoxb1-NSR-Fwd	5'-AGA TAC AGT GTT ATG TGC CAG GAG-3’
Hoxb1-NSR-Rev	5'-GGA AGC TAT GCA GTA GGG TTC ATG-3’
RARb-DR5 RARE-Fwd	5’-TGG CAT TGT TTG CAC GCT GA-3’
RARb-DR5 RARE-Rev	5’-CCC CCC TTT GGC AAA GAA TAG A-3’
(B) EMSA 5’-biotin labeled oligos (only sense strand is indicated; wild-type RARE sequence is in bold and mutated nucleotides are underlined):	
Fgf8 RARE-S	5'-CCC CG**G GGT CAG CAG TTC A**GC AGT GTT GAT G-3’
Mutant Fgf8 RARE-S	5'-CCC CG**G** A**G**G **C**C**G CAG** AAA **A**GC AGT GTT GAT G-3’
(C) BAC recombineering oligos:	
For wild-type Fgf8-lacZ BAC construct;	
Fgf8-HA(A)-AscI	5’-ACTTC GGCGCGCC AGGGT AGT GGG AGG CGC CCA CAC C-3’
Fgf8-HA(A)-NcoI	5’-GCTGC CCATGG C GCG CGG CC-3’
Fgf8-HA(B)-BamHI	5’-GTATTGGATCC G CTG AGC TGC CTG TGA GTA CC-3’
Fgf8-HA(B)-SpeI	5’-CGATCACTAGT C GCT GCA ACT ACA TCC CAA CTA CC-3’

For mutant Fgf8ΔRARE-lacZ BAC construct:

Fgf8-ΔRARE-HA(A)-SphI 5'-AACTTC GCATGC TCC AGC CTG TTC CAT GTT CCT GC-3'

Fgf8-ΔRARE-HA(A)-Frt (F3)-NcoI 5'-GCATGC CCATGG *GAAGTTCCTATACTTCAAATAG AATAGGAACTTC* CAG TCC TCA CAC AAG CAA CCC C-3'

Fgf8-ΔRARE-HA(B)-Frt (F3)-SalI 5'-GTATAC GTCGAC *GAAGTTCCTATTCTATTTGAAGT ATAGGAACTTC* TGG GTT GGG ATG GGT GGG GAT-3'

Fgf8-ΔRARE-HA(B)-NsiI 5'-CGATCT ATGCAT AGG GCG CTA GTT CCA CCT TCC-3'

Note: HA(A) and HA(B) represents oligos used for PCR amplification of homology arm A and B for BAC recombineering. Restriction enzyme sites are underlined and letters in italics indicate the sequence of mutant Frt-F3 site.