Retinoic acid controls body axis extension by directly repressing Fgf8 transcription

Sandeep Kumar and Gregg Duester*

ABSTRACT
Retinoic acid (RA) generated in the mesoderm of vertebrate embryos controls body axis extension by downregulating Fgf8 expression in cells exiting the caudal progenitor zone. RA activates transcription by binding to nuclear RA receptors (RARs) at RA response elements (RAREs), but it is unknown whether RA can directly repress transcription. Here, we analyzed a conserved RARE upstream of Fgf8 that binds RAR isoforms in mouse embryos. Transgenic embryos carrying Fgf8 fused to lacZ exhibited expression similar to caudal Fgf8, but deletion of the RARE resulted in ectopic trunk expression. The co-regulator RERE, the loss of which results in ectopic Fgf8 expression and somite defects, was recruited near the RARE by RA, but was released from the Fgf8 RARE by RA. Our findings demonstrate that RA directly represses Fgf8 through a RARE-mediated mechanism that promotes repressive chromatin, thus providing valuable insight into the mechanism of RA-FGF antagonism during progenitor cell differentiation.

KEY WORDS: Body axis extension, Somitogenesis, Neurogenesis, Ligand-induced repression, Retinoic acid, Raldh2, Fgf8, PRC2, RERE

INTRODUCTION
Vertebrate embryos develop in a head-to-tail fashion by extension of the body axis from a caudal progenitor zone containing axial stem cells that generate neuroectoderm and presomitic mesoderm (Tzouanacou et al., 2009; Wilson et al., 2009; Takemoto et al., 2011). Somitogenesis is a developmental process in which presomitic mesoderm exiting the caudal progenitor zone is sequentially segmented to form somites that later generate vertebrae and skeletal muscles (Dequéant and Pourquié, 2008). The location of segmentation lies just anterior to the expression domains of fibroblast growth factor 4 (Fgf4) and fibroblast growth factor 8 (Fgf8) in the caudal progenitor zone (Naiche et al., 2011; Boulet and Capеччи, 2012) in a region where fibroblast growth factor (FGF) activity is too low to maintain random cell migration, thus allowing epithelial condensation of presomitic mesoderm to form somites (Bénazéra et al., 2010). Whereas Fgf8 is required to position the somite front, ectopic administration of Fgf8 to one side of chick embryos has been shown to inhibit somite formation on that side (Dubrule et al., 2001). Alterations in the level of FGF signaling during embryogenesis have been linked to the etiology of congenital scoliosis, which is characterized by a lateral curvature of the spine caused by vertebral defects (Sparrow et al., 2012).

Retinoic acid (RA) produced by retinaldehyde dehydrogenase 2 (Raldh2; Aldh1a2) functions as a diffusible signal controlling vertebrate development (Duester, 2008; Niederreither and Dollé, 2008). Loss of RA synthesis in RA-deficient chick embryos and mouse Raldh2−/− embryos results in ectopic Fgf8 expression extending from the caudal progenitor zone into the trunk that disrupts somitogenesis and neurogenesis (Diez del Corral et al., 2003; Molotkova et al., 2005; Vernot et al., 2005; Vernot and Pourquié, 2005; Sirbu and Duester, 2006) as well as forelimb initiation (Zhao et al., 2009; Cunningham et al., 2013). Thus, RA functions as a diffusible signal that downregulates Fgf8 as cells exit the caudal progenitor zone. However, the mechanism through which RA downregulates caudal Fgf8 expression is unknown. Some clues have come from mutational studies on Rere encoding the transcriptional co-regulator RERE, a member of the Atrophin family of nuclear receptor co-regulators (Wang and Tsai, 2008); Rere mutants exhibited somite left-right asymmetry and asymmetric Fgf8 expression (Vilhais-Neto et al., 2010). Furthermore, previous studies tracking the position of the Fgf8 locus within the nucleus in the caudal progenitor zone and neural tube showed that it becomes more peripheral in the neural tube (a location associated with repression); however, this shift to the nuclear periphery was not observed in Raldh2−/− neural tube and was regulated by FGF signaling (Patel et al., 2013).

Signal-induced repression has been suggested to play a major role in developmental signaling (Affolter et al., 2008), but such a role for RA signaling has not been established. RA functions as a ligand for widely expressed nuclear RA receptors that bind as RAR/RXR heterodimers to RA response elements (RAREs) near target genes (Duester, 2008; Niederreither and Dollé, 2008). Although RAREs clearly control gene activation, relatively little is known about RA repression. Here, mutational and epigenetic studies were performed to investigate the biological function of a RARE upstream of Fgf8.

RESULTS AND DISCUSSION
Conserved RARE upstream of Fgf8
We investigated whether RA restriction of caudal Fgf8 expression is mediated by direct transcriptional regulation. Sequence comparisons identified a conserved RARE near the human, mouse, rat and chick Fgf8 genes located 4.1-4.5 kb upstream of the promoter for mammals and 3.2 kb upstream for chick (Fig. 1A). The Fgf8 RAREs all include the sequence AGTCTA in the downstream half-site, which is the most efficient variant for controlling RAR-binding specificity (Phan et al., 2010). Chromatin immunoprecipitation (ChiP) analysis of E8.25 (5-8 somite) whole mouse embryos revealed that the mouse Fgf8 RARE recruits all three RAR isoforms (Fig. 1A). We also performed ChiP on the 5′- and 3′-Hoxb1 RAREs that are required for Hoxb1 repression (Studer et al., 1994) and activation (Marshall et al., 1994), respectively, in different hindbrain domains at E8.25. The 5′-Hoxb1
RARE represses Hoxb1 in rhombomeres 3 and 5, but the mechanism of this repressive RARE remains unknown. Both the 3′- and 5′-Hoxb1 RAREs were able to recruit all three RARs (Fig. 1B). To further examine RAR binding to the Fgf8 RARE, we demonstrated that the wild-type (WT) Fgf8 RARE, but not a mutant version, binds RARs. For this, we used electrophoretic mobility shift assays with nuclear extracts from E8.25 WT whole mouse embryos and antibodies against RAR isoforms or IgG (control); input DNA (diluted 100-fold) and immunoprecipitated DNA were analyzed by PCR using primers for the RAREs or for nonspecific regions (NSR) indicated by arrows. IP, immunoprecipitation; M, molecular size markers.

RA directly represses caudal Fgf8 transcription through an upstream RARE

Using bacterial artificial chromosome (BAC) recombination-mediated genetic engineering (recombineering), we fused the E. coli lacZ gene in frame with the ATG start codon of mouse Fgf8 (contained in a 200.9 kb BAC) to create Fgf8-lacZ (Fig. 2A); the BAC used contains elements known to stimulate caudal RARE located at 2013). Further recombineering was performed to delete the 3′-a n d the ATG start codon of mouse 

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Histone methylation near Fgf8 RARE

Gene activation through RAREs functions through RA-dependent recruitment of transcriptional co-activators (Germain et al., 2002), but the mechanism through which RAREs repress transcription is unknown. Transcriptional repression is associated with histone-H3 lysine 27 trimethylation (H3K27me3) catalyzed by polycomb repressive complex 2 (PRC2), whereas transcriptional activation is linked with histone-H3 lysine 4 trimethylation (H3K4me3) catalyzed by trithorax [lysine (K)-specific methyltransferase 2A – Mouse Genome Informatics] (Schuettengruber et al., 2007). For regional ChIP assays, WT embryos and Raldh2−/− embryos (lacking RA synthesis) collected at E8.25 (5-8 somites) were dissected into head, trunk and caudal progenitor zone (CPZ) regions (Fig. 3A). For pooled WT trunks, in which Fgf8 is normally not expressed, we found a significant enrichment of the repressive H3K27me3 mark near the Fgf8 RARE compared with the activating H3K4me3 mark (Fig. 3B). Importantly, enrichment of H3K27me3 near the Fgf8 RARE was reduced more than 15-fold in Raldh2−/− trunks lacking RA activity (Fig. 3B,C). As a control, ChIP assays on pooled heads revealed that the 3′-Hoxb1 RARE required for gene activation exhibited much higher enrichment for H3K4me3 compared with H3K27me3, whereas the 5′-Hoxb1 RARE required for gene repression displayed much higher enrichment for H3K27me3 compared with H3K4me3; in both cases, enrichment was highly dependent upon Raldh2 function needed for RA synthesis (Fig. 3D-F). Our demonstration that RA stimulates accumulation of repressive chromatin marks near the Fgf8 and 5′-Hoxb1 RAREs provides further evidence that these RAREs function repressively in vivo.

Fig. 1. RAREs near Fgf8 and Hoxb1 bind RARs in mouse embryos. (A) Mouse Fgf8 RARE and alignment of sequences showing a highly conserved Fgf8 RARE; consensus RARE sequence is shown (Balmer and Blomhoff, 2005). (B) Hoxb1 gene harboring a repressive 5′-RARE and an activating 3′-RARE. ChIP was performed using chromatin from pooled E8.25 WT whole mouse embryos and antibodies against RAR isoforms or IgG (control); input DNA (diluted 100-fold) and immunoprecipitated DNA were analyzed by PCR using primers for the RAREs or for nonspecific regions (NSR) indicated by arrows. IP, immunoprecipitation; M, molecular size markers.

Fig. 2. RA directly represses caudal Fgf8 transcription through an upstream RARE. (A) BAC constructs used for generating Fgf8-lacZ and Fgf8−ΔRARE-lacZ transgenic embryos. Fgf8−ΔRARE-lacZ lacks the Fgf8 RARE, which was replaced by the 34 bp FRT-F3 site. (B) Transgenic 9-somite embryos carrying Fgf8-lacZ recapitulate endogenous caudal Fgf8 expression, whereas Fgf8−ΔRARE-lacZ exhibits ectopic expression in the three somites most recently generated (arrowheads, arrows) and presomitic mesoderm (psm; brackets). Left panels, dorsal view; right panels, lateral view. Scale bar: 100 µm.
RA-dependent recruitment of PRC2 near Fgf8 RARE

We examined whether RA recruits PRC2 that generates the H3K27me3 mark by performing ChIP for the PRC2 subunit SUZ12 (Gillespie and Gudas, 2007). In WT trunks we found a strong signal for enrichment of SUZ12 near the Fgf8 RARE, but this was greatly reduced in Raldh2−/− trunks lacking RA activity (Fig. 3G,H). The 5′-Hoxb1 RARE required for repression behaved similarly to the Fgf8 RARE, displaying greatly reduced binding of SUZ12 in Raldh2−/− head compared with wild type, whereas the 3′-Hoxb1 RARE did not recruit SUZ12 (Fig. 3I-K). These observations provide evidence that the Fgf8 and 5′-Hoxb1 RAREs control repression in vivo through RA-dependent recruitment of PRC2.

Regional recruitment of transcriptional repressors near Fgf8 RARE

We conducted ChIP assays to determine the relative regional occupancy of PRC2 components EZH2 and SUZ12, as well as the associated repressive factor histone deacetylase 1 (HDAC1), near the Fgf8 RARE in WT trunk and CPZ regions. HDAC1, EZH2 and SUZ12 were significantly enriched near the Fgf8 RARE in the trunk, where RA activity is high and Fgf8 expression is absent, but not in the CPZ, where RA activity is low and Fgf8 expression is high (Fig. 4A,B). ChIP performed on head tissue demonstrated a strong enrichment of HDAC1, EZH2 and SUZ12 in Raldh2−/− in the trunk compared with wild type (Fig. 4C-E). Taken together with our other findings, we conclude that the Fgf8 RARE functions in an RA-dependent and region-specific manner to repress Fgf8 transcription.

Localization of RERE during body axis extension

RERE is a nuclear receptor co-regulator that has been reported to function in mice as a co-repressor able to recruit HDAC1 (Zoltewicz et al., 2004; Wang and Tsai, 2008). Rere (om) mutant mice were found to exhibit somite left-right asymmetry and corresponding ectopic caudal Fgf8 expression on the right side (Vilhais-Neto et al., 2010). Rere(om);Raldh2−/− double mutants have a more severe somitogenesis defect than either single mutant, demonstrating a synergistic effect of these two mutations (Vilhais-Neto et al., 2010).
Rere (om) mutants exhibit reduced RA activity (RARE-lacZ expression) and RERE binds RAR/RXR at a RARE near the RARb gene to activate transcription in response to RA. This suggests that RERE controls somite symmetry by functioning as an RA-dependent co-activator that increases the amount of RA receptors (Vilhais-Neto et al., 2010). Thus, RERE might act as either a co-repressor or co-activator, depending on the particular gene involved.

As Rere mutants exhibit ectopic caudal Fgf8 expression, we investigated whether RERE might function as a co-repressor for Fgf8. Previous studies in cell lines showed that RERE functions as a co-activator for RARb through binding to a RARE located near the RARb promoter at −57 bp (Vilhais-Neto et al., 2010). Here, ChIP studies showed that binding of RERE near the RARb RARE in WT trunks was reduced approximately 3-fold in Raldh2−/− mutants, providing in vivo confirmation of RA-dependent binding of RERE near the RARb RARE (Fig. 4F,G). RERE binding was very low near the Fgf8 RARE in WT trunks, but a significant enrichment (approximately 10-fold) was observed in Raldh2−/− trunks (Fig. 4H,I). Thus, our findings demonstrate RA-dependent recruitment of RERE near the RARb RARE and RA-dependent release of RERE near the Fgf8 RARE, thereby suggesting that RERE affects somitogenesis not only by upregulating RARb but also by directly regulating Fgf8.

The previous observation that Rere(om);Raldh2−/− double mutants exhibit a more severe somite defect than either single mutant (Vilhais-Neto et al., 2010) can probably not be explained by a further reduction of RA signaling in Rere(om);Raldh2−/− mutants compared with Raldh2−/− mutants, as Raldh2−/− mutants already have no detectable RA activity (Vermot et al., 2005; Sirbu and Duester, 2006). However, synergism might be explained by our observation that recruitment of RERE to the Fgf8 RARE is greatly increased in Raldh2−/− embryos. Thus, loss of RA signaling in Raldh2−/− embryos might not totally de-repress caudal Fgf8 transcription due to recruitment of RERE (functioning as a co-repressor) to the Fgf8 RARE. However, simultaneous loss of RA and RERE could then further de-repress caudal Fgf8, resulting in more severe defects. RERE binds near the Fgf8 RARE in a manner similar to how other nuclear receptor co-repressors, such as SMRT or NCoR1, bind RAR/RXR heterodimers at RAREs in the absence of RA but are released upon binding of RA to RAR (Perissi et al., 2010). However, as RERE can also function as a co-activator, the mechanism of RERE action might be different when RERE is

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**Fig. 4. Mechanism of repressive Fgf8 RARE.**

(A-E) Regional ChIP assay from WT E8.25 embryos to examine recruitment of repressive proteins near Fgf8 and Hoxb1 RAREs. (F-I) ChiP assays showing recruitment of RERE to RARb and Fgf8 RAREs in WT or Raldh2−/− (KO) E8.25 trunk. See Fig. 3 legend for more details; data shown as percentage input, mean±s.e.m.; *P<0.05, **P<0.001, ***P<0.0001 (t-test). (J) Model for direct RA repression of caudal Fgf8 transcription through a RARE. Opposing gradients of RA and FGF8 along the body axis are shown. RA recruits a repressor complex near the Fgf8 RARE, containing PRC2 (EZH2, SUZ12) and HDAC1, that stimulates deposition of the repressive histone mark H3K27me3 (K27). RA also prevents binding of the co-repressor RERE near the Fgf8 RARE. DBD, DNA-binding domain; RAR, RA receptor; RXR, retinoid-X-receptor (heterodimer partner for RAR).
bound at an activating RARE compared with a repressive RARE. Together with Rere(om);Raldh2<sup>−/−</sup> genetic studies, our findings suggest a model in which RA binding to RAR at the repressive Fgf8 RARE releases RERE (Fig. 4J). It is unclear whether release of RERE is directly related to RA-mediated recruitment of PRC2 to the vicinity of the Fgf8 RARE, but it appears probable that additional regulatory proteins are involved in recruitment of PRC2 for RA-mediated Fgf8 repression.

Conclusions

Although the existence of causal RA-FGF8 antagonism as a fundamental mechanism needed for vertebrate body axis extension has been acknowledged for some time, the mechanism itself has remained elusive. Here, our deletion studies demonstrate that the RARE upstream of Fgf8 is required in vivo for repression of Fgf8 transcription at the border of the trunk and caudal progenitor zones. A repressive role for the Fgf8 RARE is further supported by our observation of RA-dependent recruitment of PRC2 and deposition of the repressive H3K27me3 mark to the vicinity of the Fgf8 RARE in trunk tissue (Fig. 4J). Our findings are consistent with the ability of RA to stimulate peripheral nuclear localization of Fgf8 (Patel et al., 2013), suggesting a mechanism involving recruitment of polycomb complexes (PRC1 and PRC2). These complexes are known to recruit PRC2 in general. Polycomb to vertebrate genes are more complex than those in lower organisms suggesting a mechanism involving recruitment of polycomb to vertebrate genes are more complex than those in lower organisms.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed on nuclear extracts from 32 pooled wild-type E8.25 embryos using biotin-labeled double-stranded oligonucleotide probes containing wild-type and mutant Fgf8 RARE sequences. Binding reactions were performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Thermo Scientific) according to the manufacturer’s instructions. Further details are available in Table S1 and Methods in the supplementary material.

Competing interests

The authors declare no competing financial interests.

Author contributions

S.K. carried out the experiments. S.K. and G.D. designed the study, analyzed the data and wrote the final manuscript.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.112367/-/DC1

References


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-\Delta RARE-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-\Delta RARE-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 mM 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


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.

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<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
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<td><strong>(A) Chromatin immunoprecipitation (ChIP) oligos:</strong></td>
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<tr>
<td>Fgf8-DR2 RARE-Fwd</td>
<td>5'-CAG CAC TCT GCC ATA CTG TCT TA-3'</td>
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<tr>
<td>Fgf8-DR2 RARE-Rev</td>
<td>5'-TCT GTC AGT CTT CAG CTT GTC TG-3'</td>
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<tr>
<td>Fgf8-NSR-Fwd</td>
<td>5'- TAG CAG CTG AAT GAG TGG CTC TA -3'</td>
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<tr>
<td>Fgf8-NSR-Rev</td>
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<tr>
<td>Hoxb1-5'-DR2 RARE-Fwd</td>
<td>5'-AGA CAA CTT TGG GCC CTT GAA GG-3'</td>
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<td>RARb-DR5 RARE-Rev</td>
<td>5'-CCC CCC TTT GGC AAA GAA TAG A-3'</td>
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<td><strong>(B) EMSA 5'-biotin labeled oligos (only sense strand is indicated; wild-type RARE sequence is in bold and mutated nucleotides are underlined):</strong></td>
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<td>Fgf8 RARE-S</td>
<td>5'-CCC CGG [<strong>GGT</strong>] CAG <strong>[CAG]</strong> TTC AGC AGT GTT GAT G-3'</td>
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<td>Mutant Fgf8 RARE-S</td>
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<td><strong>(C) BAC recombineering oligos:</strong></td>
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<tr>
<td>For wild-type Fgf8-lacZ BAC construct;</td>
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<tr>
<td>Fgf8-HA(A)-Ascl</td>
<td>5'‐ACTTC GGCGGC GCC AGGGT AGT GGG AGG CGC CCA CAC C-3'</td>
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<td>5'-CGATCAGCTAGT C GCT GCA ACT ACA TCC CAA CTA CC-3'</td>
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For mutant Fgf8ΔRARE-lacZ BAC construct:

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

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

Fgf8-ΔRARE-HA(B)-Frt (F3)-SalI  5’-GTATAC GTCGAC GAAGTTCCCTATTTTGTTTGAAGT 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.