**SUPPLEMENTAL INFORMATION**

**Experimental Procedures:**

**Derivation of the ESC Lines.** We derived RARγ-KO ESCs by crossing RARγ/RARβ heterozygous male and female mice (Ghyselinck et al., 1997; Lohnes et al., 1993), isolating blastocysts, and establishing ESC lines in culture from these blastocysts (Kawase et al., 1994). Wild type (WT) ESCs were also established by this method at the same time. Genotypes were verified by Southern blotting. The RARγ-KO ESC line (RARβ+/-, RARγ-/-) retains neomycin resistance genes within the knocked out RARβ and RARγ genes (see refs. above for maps of the constructs). The Hoxa1 3' RARE enhancer knockout ESC line was derived from WT ESCs by knocking out a small region (about 280 bp) containing the Hoxa1 3' RARE. The targeting vector containing the neomycin resistance gene (NeoR) is shown in Figure 1. WT ESCs were transfected with the RARE-targeting vector and cloned following two rounds of G418 selection to knock out both the alleles. Several independent ESC clones were expanded and Southern blots were used to identify six of the lines with one or both RARE copies replaced by the knockout construct containing NeoR. One of these lines with homozygous knockout of the RARE was chosen for analysis (RARE-KO). The NeoR gene expression is driven by the pgk promoter, which is situated in approximately the same position relative to the Hoxa1 gene as the RARE in the WT ESC line. This allows us to compare the WT ESCs, which contain an RA-responsive RARE 3' of the Hoxa1 gene (Langston and Gudas, 1992), to the RARE-KO line, which contains a constitutively active pgk promoter that is not responsive to RA.

**Culturing ESCs.** WT, RARE-KO, and RARγ-KO ESC lines were cultured as described (Chen and Gudas, 1996). All experiments were performed in the presence of LIF (Millipore, Billerica, MA). All-*trans* retinoic acid (RA) was from Sigma Chemical Co., St. Louis, MO.

**ChIP and ChIP-Chip.** ESCs ($2.5 \times 10^6$ cells) were plated in 150 mm gelatin coated tissue culture dishes approximately 48 hr prior to formaldehyde fixation. RA treatment was staggered such that all the plates with varying time points (0, 1, 4, 8 and 24 hours) were cross-linked and harvested at the same time. An additional plating of cells was used for counting purposes to normalize for the number of cells per immunoprecipitation. Cells were fixed by adding 37% formaldehyde directly to the culture media in the plates to a final concentration of 1%. Plates were incubated at room temperature with gentle shaking for 10 min and then quenched by adding 1.25 M glycine to a 200 mM final concentration. Cells were washed 4 times with cold Cell Wash Buffer (0.1% BSA, 2 mM EDTA in phosphate saline buffer, PBS) and cell pellets were stored at -70°C until use. All ChIP experiments

were performed at least three times, starting with the cultured ESCs, as described (Gillespie and Gudas, 2007a, b).

Pellets were incubated 10 min in Nuclei Release Buffer (50 mM Hepes, pH7.4, 140 mM NaCl, 1 Mm EDTA, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100) at 4°C on a rotating wheel. Nuclei were pelleted and then lysed for 10 min in SDS Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH8). Chromatin was pelleted by centrifugation at 2,000g for 1 min, and then resuspended in 500 µl of Sonication Buffer (50 mM Tris, pH8, 100 mM NaCl, 2 mM EDTA, 0.5% Sarcosyl). Chromatin was fragmented by sonication (15 sec pulses with 15-sec rest for a total processing time of 20 min using a Misonix 3000 Sonicator). Insoluble material was pelleted by centrifugation and fragmented chromatin (DNA fragment size 200 to 500 bp) was recovered in the supernatant.

For each immunoprecipitation, 10 µg of the indicated antibody were incubated with 250 µl of pre-blocked Dynal Protein G beads (Invitrogen Dynal AS, Oslo, Norway) in Bead Blocking Buffer (0.5% BSA in PBS) overnight at 4°C. Beads were then washed in Bead Blocking Buffer and resuspended in 1 mL ChIP Dilution Buffer (12.5 mM Tris, pH8, 162.5 mM NaCl, 2 mM EDTA, 1.25% Triton X-100, 0.625% BSA). Cross-linked chromatin (2 µg) in a total volume of 250 µl ChIP Sonication Buffer was added to each tube containing beads prior to overnight incubation at 4°C with continuous rotation. An aliquot of the fragmented chromatin was reserved as "Input" for DNA extraction. Beads were then washed in Low Salt Buffer (1% Triton X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH8), High Salt Buffer (1% Triton X-100, 0.1% SDS, 2 mM EDTA, 500 mM NaCl, 20 mM Tris, pH8), LiCl2 Wash Buffer (1% NP40, 1% Deoxycholate, 1 mM EDTA, 250 mM LiCl, 10 mM Tris, pH8) and TE (10 mM Tris, 1 mM EDTA pH8) and resuspended in 100 µl of Proteinase K/SDS Lysis Buffer. After reversal of crosslinks overnight at 65°C, DNA was prepared by phenol-chloroform extraction and ethanol precipitation. Purified DNA was resolubilized and ends repaired by incubating for 12 min at 12°C in the presence of 1.5 units of T4 DNA polymerase (Promega) and 100 µM dNTPs. DNA was then purified using MinElute PCR Cleaning Kit (Qiagen) and linkers were ligated to the blunt ends using 200 units of T4 Ligase (BioLabs, New England) and 1 µM LM-PCR Duplex (Annealed LM-PCR1, 5'- GCG GTG ACC CGG GAG ATC TGA ATT C-3' and LM-PCR2 5'-GAA TTC AGA TC-3'). DNA was then amplified by PCR using Pfu Turto Hotstart Master Mix (Stratagene) and 1 µM LM-PCR1 primer. After purification using the MinElute Kit, a secondary amplication was performed using Pfu Turto Hotstart Master Mix (Stratagene), 1 µM LM-PCR1 primer, 200 µM dNTPs and 2.5 Units of Supplemental Pfu Turbo Hotstart DNA Polymerase (Stratagene). DNA was purified again using the MinElute kit. Immunoprecipitated DNA and Input

DNA samples were then used for analysis on Agilent Chip-on-Chip Array. Custom tiling arrays with oligonucleotides tiled across all clustered Hox genes were designed and produced with eArray (Agilent). Oligonucleotides were tiled to a median interprobe distance of 43 bp.

**ChIP-chip Data Analysis**. Two-color microarray data was normalized using a weighted lowess curve fit to the control probes on the microarrays and interarray normalization was performed essentially as described (Song et al., 2007). To identify regions of enrichment, we first identified clusters of probes with normalized ratio above a chosen threshold. To guard against false discovery, we selected the threshold value so that the number of "peaks" identified when the signal probe signals were randomly distributed across the probe locations (noise) divided by the number of peaks identified in the original data set (signal + noise) was below a pre-defined false discovery rate (FDR). We accepted peaks that spanned at least two probes and at least 300 bp (modal fragment length) with a signal above a threshold that ensured a FDR < 0.1.

**Microarray Expression Profiling.** WT, RARE-KO and RARγ-KO ESCs were plated at a density of $1\times 10^6$ cells in gelatin coated 100 mm tissue culture dishes. Cells were treated with RA for various times as described above prior to harvesting. Total RNA was isolated using Trizol. RNA quality was assessed using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent). Samples with a 28S/18S ribosomal peak ratio of 1.8–2.0 were considered suitable for labeling. 200 ng of total RNA from each sample was labeled using the Illumina Total Prep RNA Amplification kit (Ambion), according to the manufacturer's instructions. Labeled and fragmentated cRNA (3 μg) were then hybridized to the mouse-ref8 array (Illumina), which incorporates 22,000 transcripts of known mouse genes. Custom R language programs and Bioconductor packages (lumi, limma, GOstats) were used to normalize and analyze the expression data (Du et al., 2008; Gentleman et al., 2003; Smyth, 2004). GeneSpring 7 software was also used for microarray data analysis.

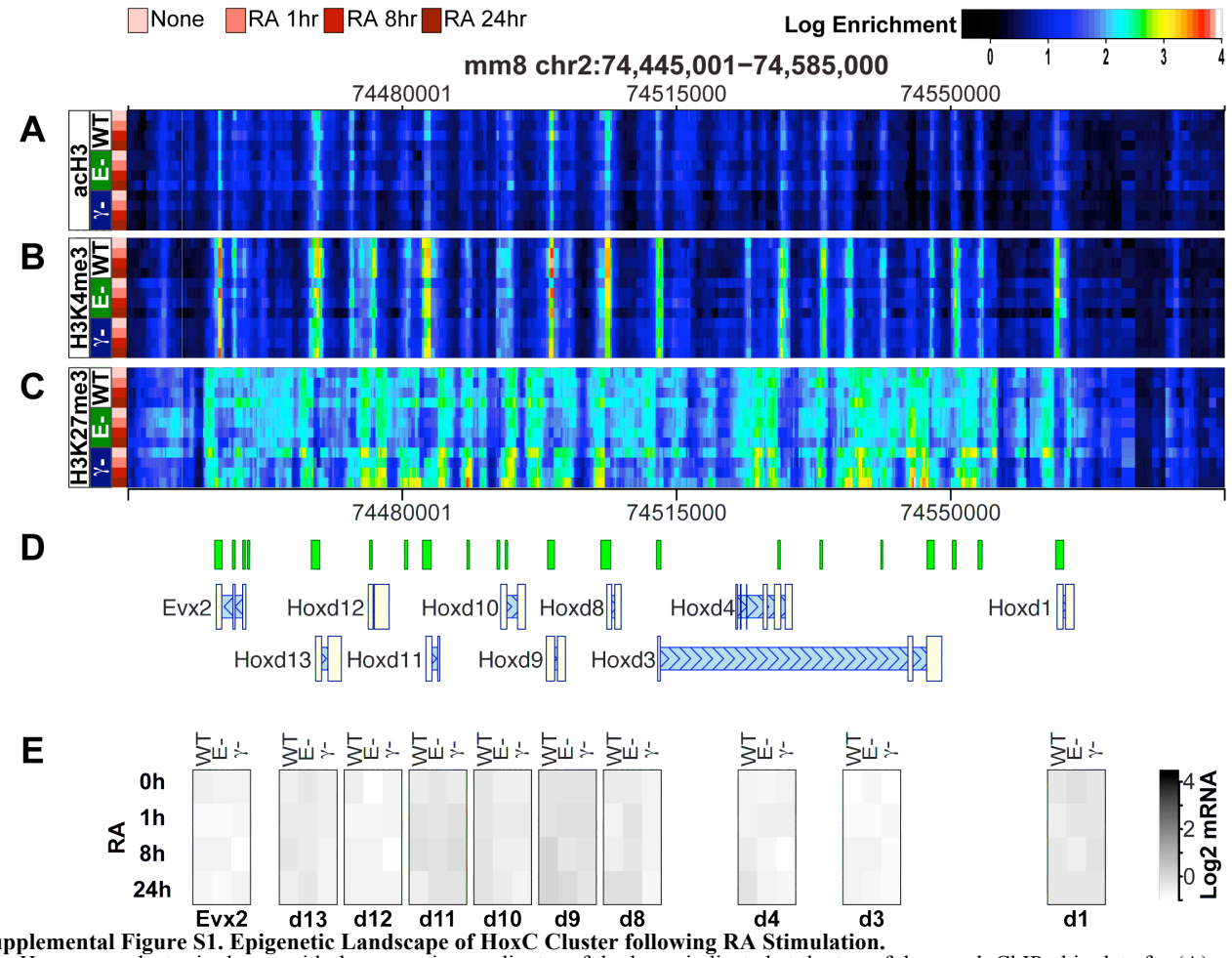
**Quantitative PCR and Northern Blots.** Quantitative PCR (qPCR) was carried out on DNA purified from ChIP samples using iQ SYBR Green supermix (BioRad, CA) on a MyiQ single color PCR detection system (BioRad, CA). Purified ChIP DNA (2 μl) was used as template in a total volume of 20 μl as described (Keenen and de la Serna, 2009). Cycle thresholds (Ct) were converted to percent enriched by interpolation from a standard curve generated with serially diluted input samples ranging from concentrations equivalent to 3% input to 0.01% input. Northern blots were performed as described (Boylan et al., 1993). Primers are listed in Table S1.

**Table S1: Primers used in qPCR analyses. All primers are designed for the mouse genome.**

| Location | Forward | Reverse | Amplicon Size (bp) |
|---|---|---|---|
| **Hoxa1 RARE** | TCTTGCTGTGACTGTGAAGTCG | GAGCTCAGATAAACTGCTGGGACT | 268 |
| **Hoxa1 PP** | ATTGGCTGGTAGAGTCACGTG | GAAAGTTGTAATCCCATGGTCAGA | 276 |
| **Hoxb1 RARE** | GGAGAACTGTCTGTCCAG | ATATTAGCCCAGGTCCATG | 183 |
| **Hoxb1 PP** | TTTAGAGTACCCACTTTGTAACC | GGCTGCTGGACAGGATAC | 168 |
| **Cyp26a1 R1 RARE** | CCCGATCCGCAATTAAAGATGA | CTTTATAAGGCCGCCCAGGTTAC | 87 |
| **Cyp26a1 R2 RARE** | TTCACTGAGATGTCACGGTCC | TTCCCAATCCTTTAGCCTGA | 64 |
| **Hoxa1 mRNA** | TTCCCACTCGAGTTGTGGTCCAAGC | TTCTCCAGCTCTGTGAGCTGCTTGGTGG | 220 |
| **36B4 mRNA** | AGAACAACCCAGCTCTGGAGAAA | ACACCCTCCAGAAAGCGAGAGT | 448 |
| **RARβ2 mRNA** | GATCCTGGATTTCTACACCG | ACTGACGCCATAGTGGTA | 247 |

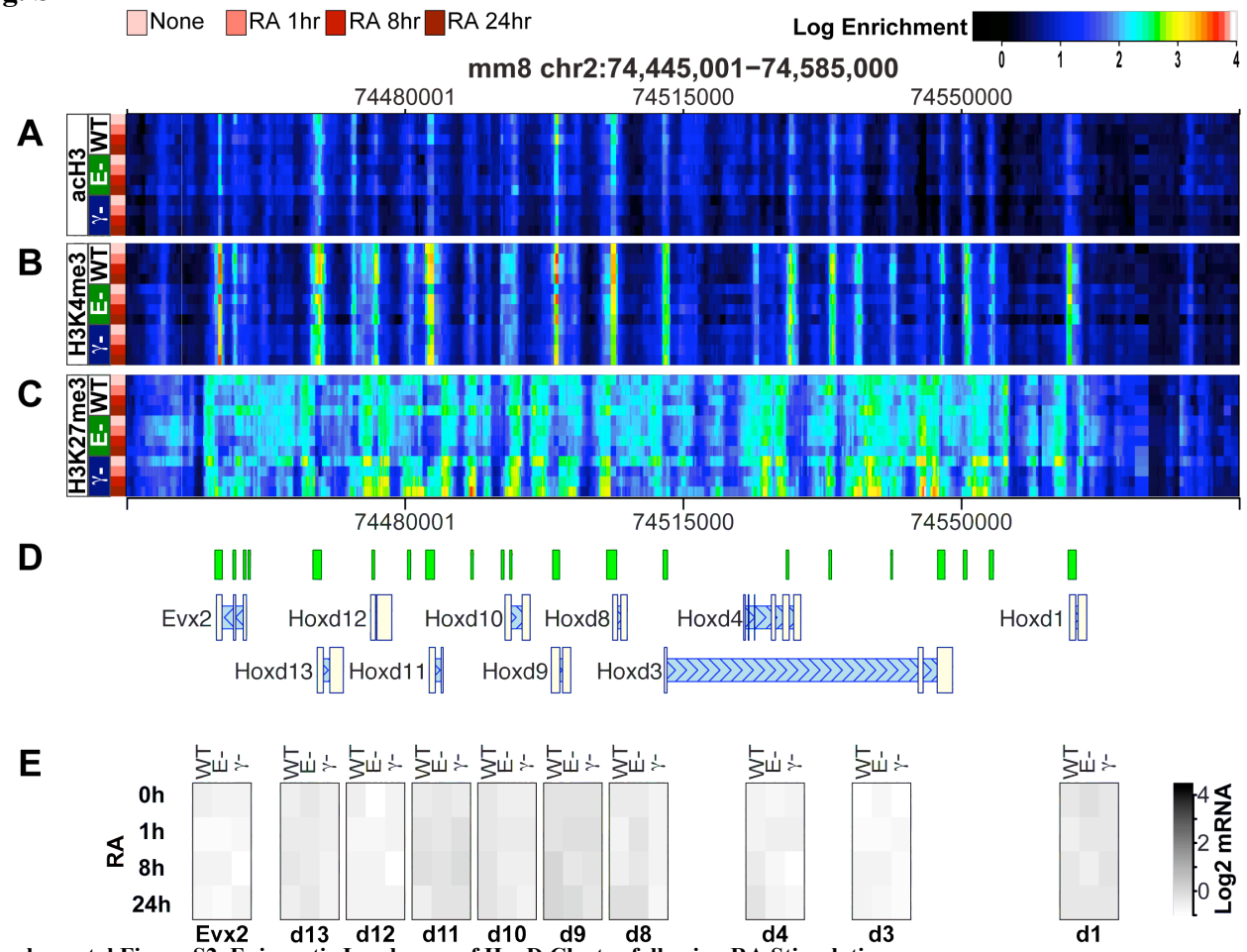
## Supplemental Figures

## Fig. S1:



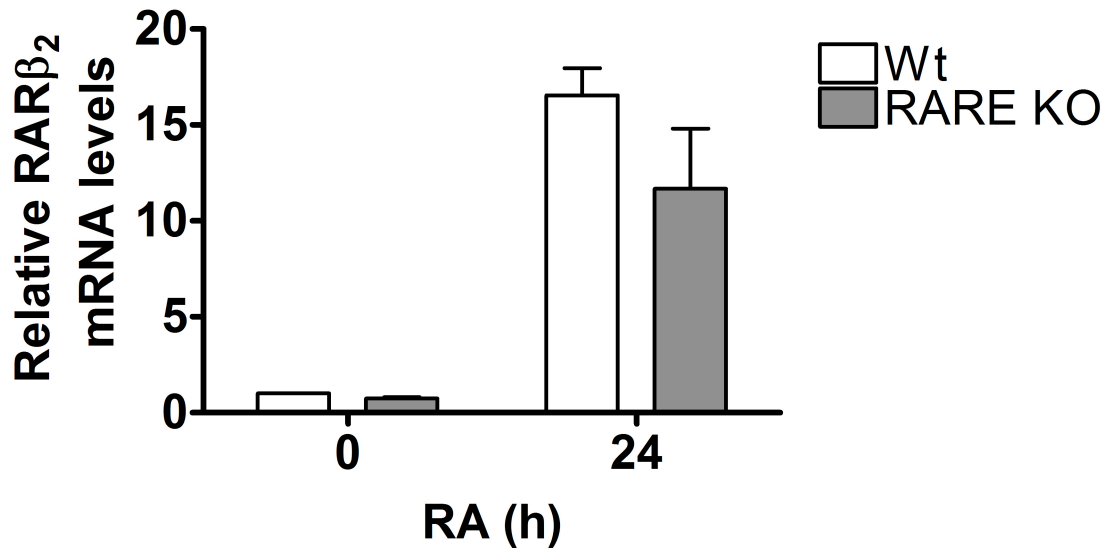

**Supplemental Figure S1. Epigenetic Landscape of HoxC Cluster following RA Stimulation.**
The Hoxc gene cluster is shown with the genomic coordinates of the locus indicated at the top of the panel. ChIP-chip data for (A) acH3, (B) H3K4me3 and (C) H3K27me3 is presented as heatmaps with rows representing ChIP-chip data sets (replicate means), columns indicating genomic loci, and color representing log2-transformed ChIP enrichment. ChIP-chip was performed for three ESC lines: (WT) wild-type, (E-) RARE-KO; and (γ-) RARγ-KO. ESCs were cultured +/- 1μM RA for 1 h, 8 h and 24 h, as indicated. The color scale of the log2 enrichment is indicated at the top of the figure. The red bars indicate regions enriched for the indicated histone modification. (D) The gene locations (blue tones) and CpG Islands (green tones) are indicated schematically. The direction of transcription for each gene is indicated by light blue hashes and proceeds from left to right. (E) The log2-transformed average (triplicate) mRNA microarray expression signal is represented for each gene in the locus as a grayscale level. The expression scale is indicated in the right hand portion of the panel. At lease three biological replicates were performed for each experiment.

**Fig. S2**




**Supplemental Figure S2. Epigenetic Landscape of HoxD Cluster following RA Stimulation.**
The Hoxd gene cluster is shown with the genomic coordinates of the locus indicated at the top of the panel. ChIP-chip data for (A) acH3, (B) H3K4me3 and (C) H3K27me3 is presented as heatmaps with rows representing ChIP-chip data sets (replicate means), columns indicating genomic loci, and color representing log2-transformed ChIP enrichment. ChIP-chip was performed for three ESC lines: (WT) wild-type, (E-) RARE-KO; and (γ-) RARγ-KO. ESCs were cultured +/- 1μM RA for 1 h, 8 h and 24 h, as indicated. The color scale of the log2 enrichment is indicated at the top of the figure. The red bars indicate regions enriched for the indicated histone modification. (D) The gene locations (blue tones) and CpG Islands (green tones) are indicated schematically. The direction of transcription for each gene is indicated by light blue hashes and proceeds from left to right. (E) The log2-transformed average (triplicate) mRNA microarray expression signal is represented for each gene in the locus as a grayscale level. The expression scale is indicated in the right hand portion of the panel. At lease three biological replicates were performed for each experiment.

**Fig. S3**




**Supplemental Figure S3:** Relative RARβ$_2$ transcript levels prior to and 24 hrs after 1 μM RA in WT and RARE-KO ESCs. Quantitative RT-PCR was used to quantify transcripts normalized to a 36B4 reference control (mean ± SE).

## REFERENCES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