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Supplemental Information

A *Gata2*-Dependent Transcription Network Regulates Uterine Progesterone Responsiveness and Endometrial Function

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SUPPLEMENTARY MATERIALS

Supplemental Figures

Supplemental Figure S1

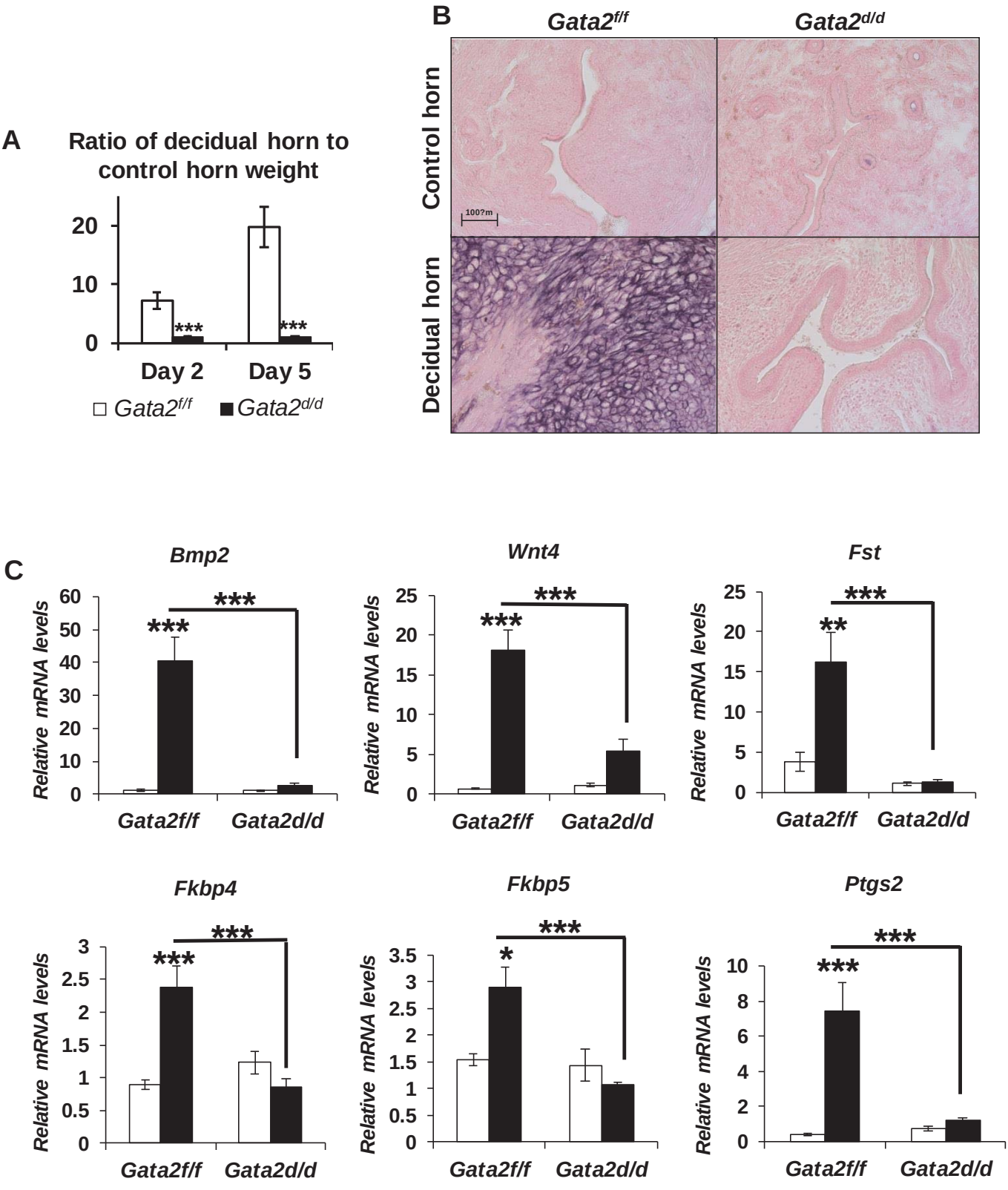


Figure S1. Related to Figure 1. Additional phenotyping results of Gata2 mutant mice. (A) Ratio of the decidual horn to the control horn weight of uteri (N = 5 for control and 6 for mutant). (B) Staining for alkaline phosphatase activities in uteri from control (unstimulated) and decidualized (stimulated) uteri 5 days after stimulation. (C) mRNA levels of genes critical for proliferation and differentiation of endometrial stromal cells in the *Gata2*$^{f/f}$ and *Gata2*$^{d/d}$ uteri 5 days after the decidual stimulus. Open and black bars represent the unstimulated and stimulated horns respectively. Error bars represent standard error of the mean. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by student's t-test.

Supplemental Figure S2

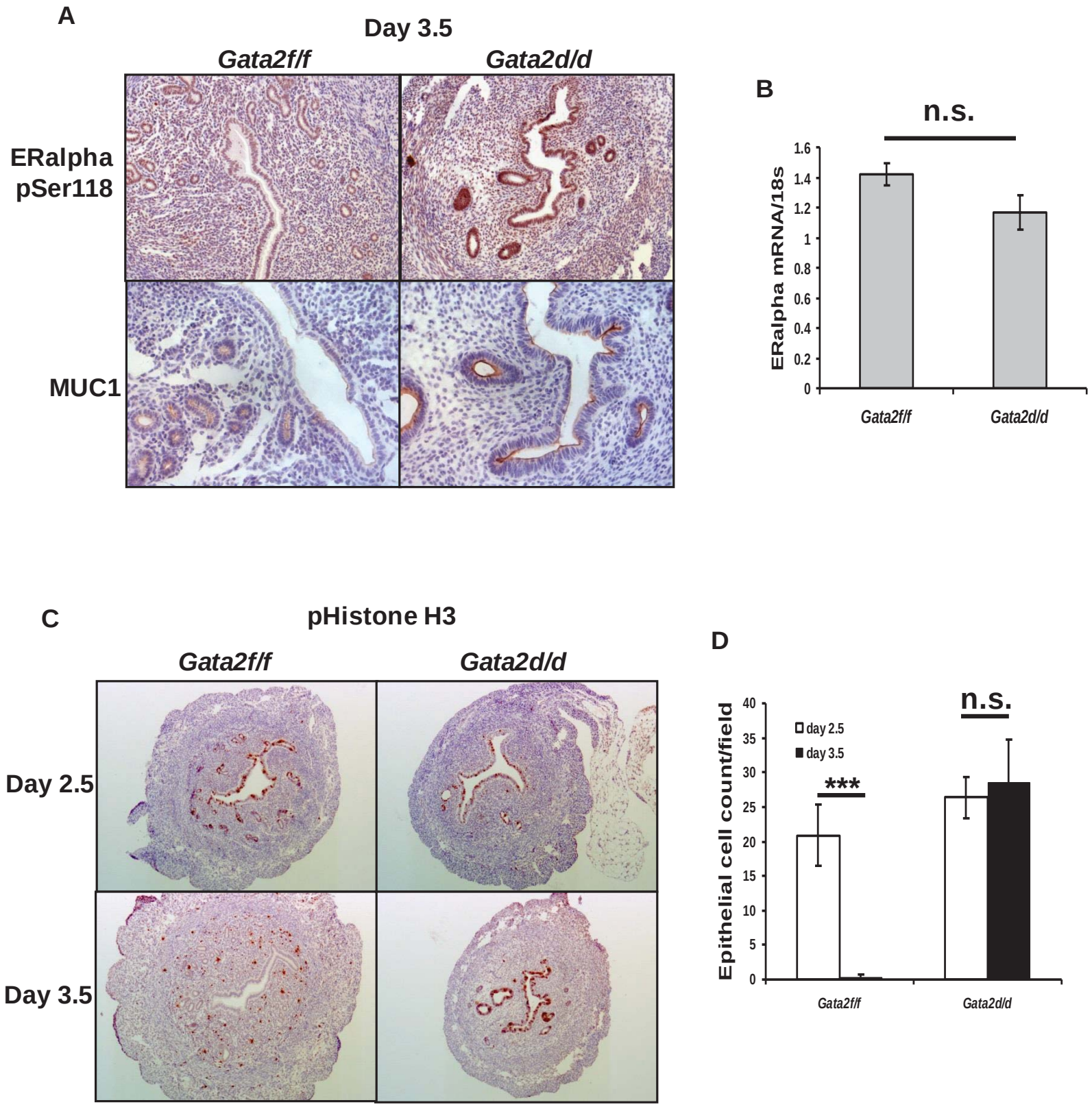
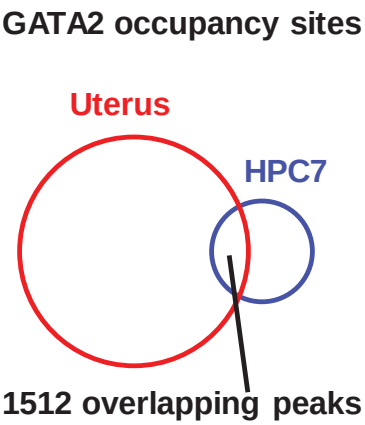


Figure S2. **Related to Figure 2. Dysregulated estrogen signaling in Gata2 deficient uterus.** Mice were subject to pseudopregnancy tests and tissues were examined at Day 2.5 or 3.5. (A) Levels of estrogen receptor alpha Ser118 phosphorylation and the MUC1 protein at Day 3.5. (B) mRNA levels of estrogen receptor alpha by qRT-PCR. (C-D) Mitotic index of uterine epithelial cells assayed by phosphohistone H3 immunostaining. Cell counts were conducted in 8 independent sections from 4 Gata2f/f mice at day 2.5, 10 independent sections from 5 Gata2d/d mice at day 2.5, 10 independent sections from 5 Gata2f/f mice at day 3.5 and 10 independent sections from 5 Gata2d/d mice at day 3.5. Hematoxylin serves as nuclear counterstaining. Error bars represent standard error of the mean. n.s., $p > 0.05$ by student's t-test. ***, $p < 0.001$.

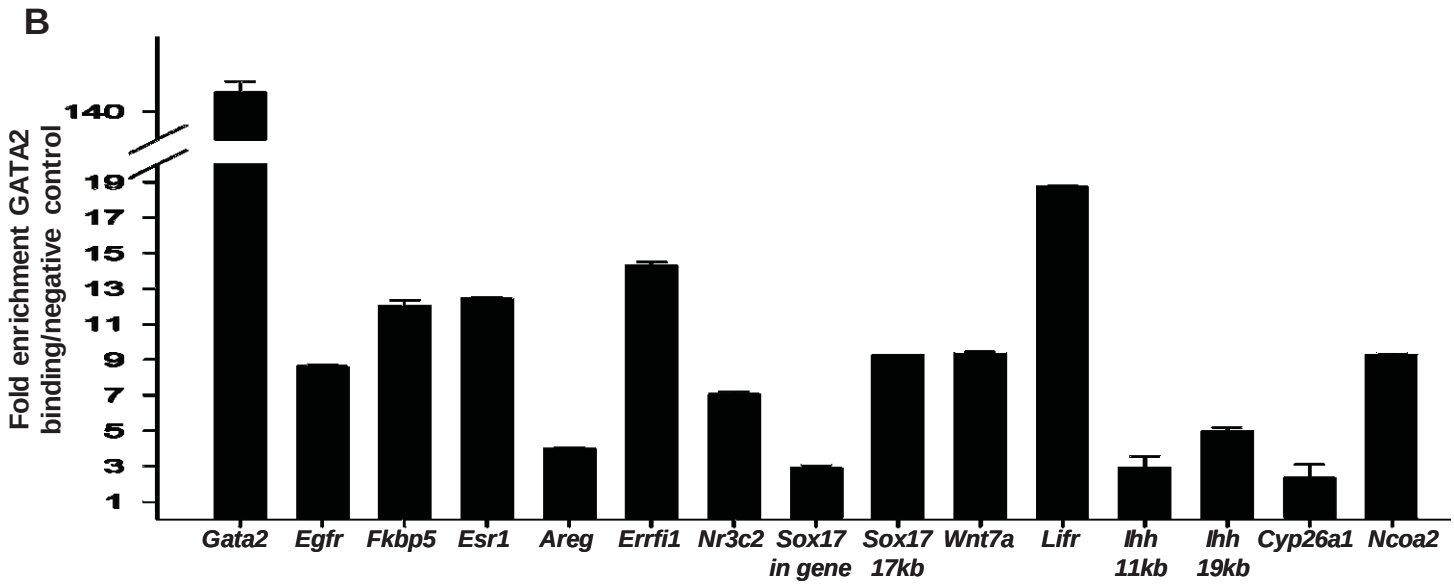
Supplemental Figure S3

A



	Total peaks	Fraction of unique peaks in total peaks
Uterus	46183	96.7%
HPC7	9243	83.3%

B



C

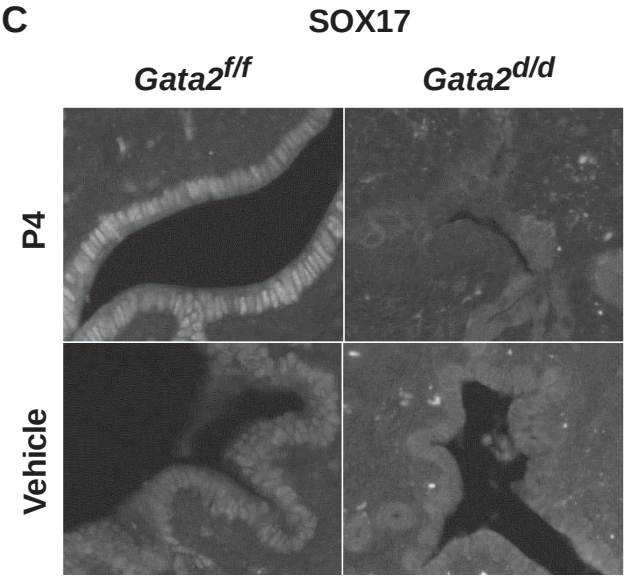


Figure S3. Related to Figure 4. Analyses of GATA2 cistrome. (A) GATA2 occupancy in the uterus and HPC7 hematopoietic progenitor cells. (B) ChIP-qPCR validation of GATA2 occupancy on progesterone target genes in uteri of mice treated with 1 mg P4 for 1 hour. Enrichment calculated as fold enrichment of GATA2 binding (IP/Input) over that of a negative control (gene deficient region). Error bars represent standard error of the mean. (C) Immunostaining of SOX17 in uteri of ovariectomized mice treated with vehicle or 1 mg P4 for 6 hours.

Supplemental Figure S4

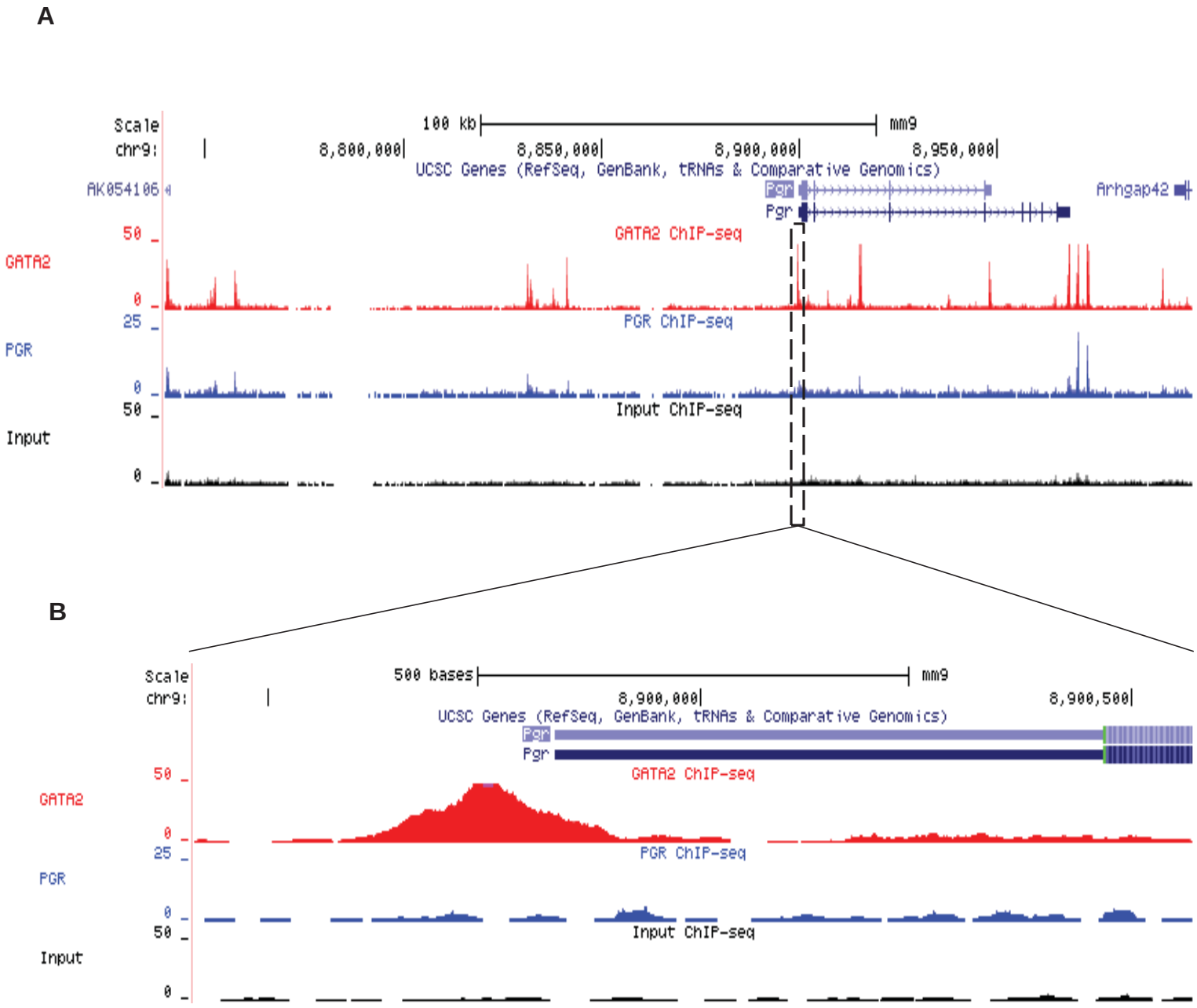


Figure S4. Related to Figure 4. GATA2 and PGR occupancy in the PGR locus. (A) Genomic regions of the PGR locus between the two closest upstream and downstream genes. (B) Occupancy patterns on the region that is used for the luciferase promoter analysis in Figure 3.

Supplemental Figure S5

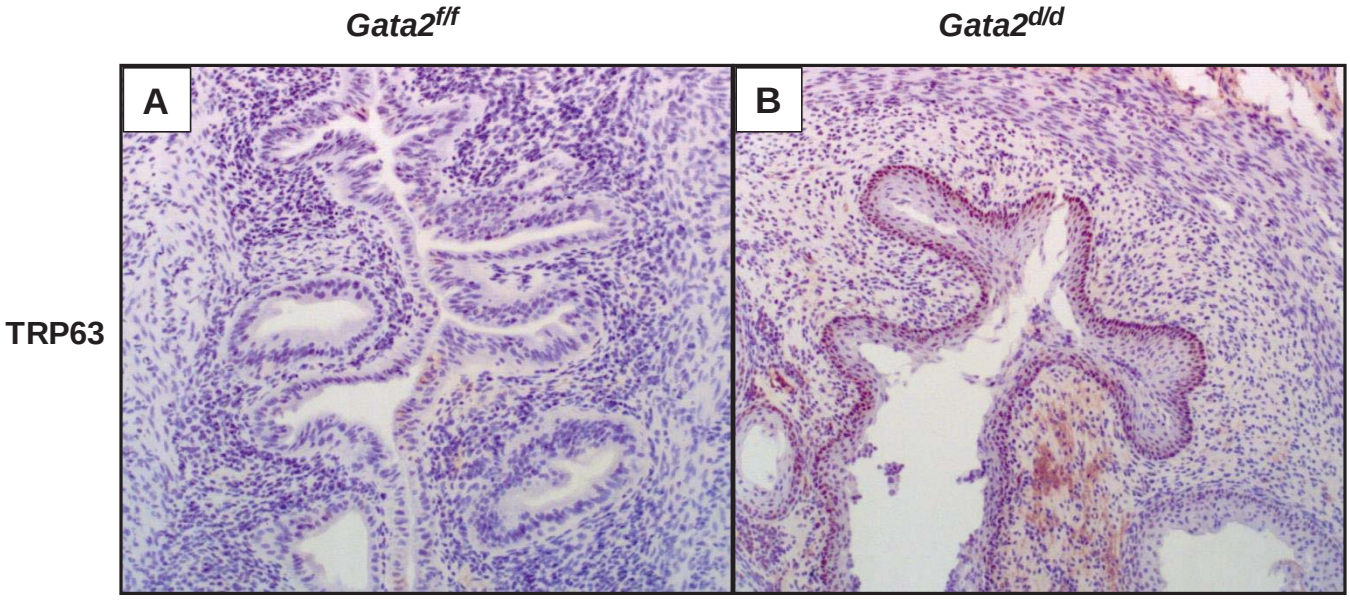


Figure S5. Related to Figure 5. Immunostaining of TRP63 in uteri of ovariectomized mice treated with estrogen for 2 months.

Supplemental Figure S6

A **GSE58144**

Gene	*r*	*p*
GATA2	**0.40**	**8.34×10^{-6}**
PGR	**0.49**	**1.87×10^{-8}**
PTCH1	**0.32**	**3.98×10^{-4}**
AREG	**0.24**	**9.29×10^{-3}**
FKBP4	**0.36**	**9.30×10^{-5}**
PTGS2	**0.47**	**1.41×10^{-7}**
SOX17	**0.39**	**1.79×10^{-5}**

B **GSE58144**

Model Fit Statistics

Model	CFI	TLI	SRMR	RMSEA Estimate
[GATA2]-[PGR]-[SOX17]	1.00	1.00	<0.001	<0.001
GATA2 signature-PGR signature-[SOX17]	1.00	1.00	<0.001	<0.001
[GATA2]-[PGR]-progesterone signature	1.00	1.00	<0.001	<0.001

CFI=Comparative Fit Index
TLI=Tucker-Lewis Fit Index
SRMR=Standardized Root Mean Square Residual
RMSEA=Root Mean Square Error of Approximation

Figure S6. Related to Figure 6. Additional information for signature and path analyses. (A) Statistical numbers of figure 6A. (B) Model fit statistics on GSE58144 for Figure 6 B-E.

Supplemental Tables

Table S1. Related to Figure 2. List of progesterone responsive genes in *Gata2*$^{f/f}$ mouse uteri.

Table S2. Related to Figure 2. List of progesterone responsive genes in *Gata2*$^{d/d}$ mouse uteri.

Table S3. Related to Figure 2. List of differentially expressed genes between *Gata2*$^{d/d}$ and *Gata2*$^{f/f}$ mouse uteri with acute treatment of exogenous progesterone.

Table S4. Related to Figure 4. Motif analyses of GATA2 occupying sites in uterus and HPC7 cells. Analysis of known motifs was conducted by using Homer with all GATA2 occupying sites in uterus and HPC7 cells.

Table S5. Related to Figure 5. List of differentially expressed genes between *Gata2*$^{d/d}$ and *Gata2*$^{f/f}$ mouse uteri with vehicle treatment.

Table S6. Related to Figure 6. List of differentially expressed genes between PRKO and wild type control mouse uteri with progesterone treatment.

Table S7. Related to Figure 2. Motif analysis on genes uniquely responded to progesterone or GATA2 in uteri. Promoter regions 2 kb upstream and downstream of the transcription start sites of individual group were used to search against known transcription factor binding motifs by Homer.

Supplemental Experimental Procedures

Animal fertility assay

Fertility was assessed by mating eight week old female mice with wild type male mice for 6 months and determining the number and size of litters delivered.

Superovulation

Superovulation was induced in 3 week old female mice by ip injection 5 international units (IU) of pregnant mare's serum gonadotropin (EMD Millipore, Billerica, MA) followed by 5 IU of human chorionic gonadotropin (Pregnyl, Merck & Co., Inc., Whitehouse Station, NJ) 48 hours later and placed with wild type male mice. The mice were sacrificed 24 hours later by cervical dislocation while under anesthetic, Avertin (2,2–tribromoethyl alcohol, Sigma–Aldrich, St. Louis, MO) and oocytes were flushed from the oviducts and counted.

Embryo implantation assessment

Embryo implantation was assessed by mating eight week old female mice with wild type male mice. The presence of the post-coital vaginal plug was designated d0.5, and embryo implantation was assessed on d5.5. Blood was collected and serum was isolated by centrifugation using serum separator tubes (BD Biosciences, San Jose, CA). The serum was sent to the University of

Virginia Center for Research in Reproduction Ligand Assay and Analysis Core for analysis of P4 by radioimmunoassay.

Artificial decidualization

The artificial induction of decidualization has been previously described. Briefly, six week old female mice were ovariectomized and treated with 3 daily sc injections of 100 ng E2 per mouse. After a 2 day rest, mice were then treated with daily sc injections of 1 mg P4 and 6.7 ng E2 per mouse for 3 days. Six hours after the last injection, one uterine horn was stimulated by the injection of 50 µl of sesame oil. Mice were given daily sc injections of 1 mg P4 and 6.7 ng E2 per mouse following the stimulation. Mice were sacrificed on day 2 and 5 after the stimulation. At the time of dissection, uterine tissues were placed in 4% paraformaldehyde (vol/vol) or flash frozen and stored at -80°C.

Alkaline Phosphatase Activity Assay

Uteri were fixed in 2% paraformaldehyde followed by sucrose gradients in PBS (15% and 30%) and embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA). Tissue sections were cut at 16 µm. Slides were post-fixed in 0.2% glutaraldehyde, washed in PBS, and incubated with a 100 mM Tris buffer (pH 9.5) containing chromogenic substrates for alkaline phosphatase (168.5 µl of 100 mg/ml nitro blue tetrazolium salt in dimethylformamide and 175 µl of 50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate/toluidinium salt in dimethylformamide added to 50 ml of the Tris buffer) (Roche, Boulder, CO). The development of a purple color is indicative of alkaline phosphatase activity. Tissues were counterstained with nuclear fast red.

Immunohistochemistry

Uteri were fixed overnight in 4% paraformaldehyde, followed by thorough washing in 70% ethanol, and tissues were processed and embedded in paraffin. Uterine sections were cut at 6 µm and mounted on silane-coated slides, deparaffinized, and rehydrated in a graded alcohol series. Sections were preincubated with 10% normal goat serum in PBS (pH 7.5) and then incubated with anti-PGR (1:500, A0098, DAKO, Carpinteria, CA) or anti-TP63 (1:600, sc-8431, Santa Cruz Biotechnology, Inc., Dallas, TX) in 10% normal goat serum in PBS overnight at 4°C. On the following day, sections were washed in PBS and incubated with biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. The immunoreactivity was detected using the Vectastain Elite ABC kit (PK-6100, Vector Laboratories, Inc., Burlingame, CA) followed with 3,3-diaminobenzidine substrate kit for peroxidase (SK-4100, Vector Laboratories, Inc., Burlingame, CA). The slides were counterstained with hematoxylin. The immunoreactivity was visualized as brown staining.

Quantitative real-time PCR

Total RNA was isolated from frozen uterine tissue using the Trizol reagent (Life Technologies, Grand Island, NY). One microgram of the RNA was reverse transcribed into cDNA with M-MLV (Life Technologies, Grand Island, NY). Expression levels of mRNA were

measured by qPCR TaqMan analysis using the ABI Prism 7500 Sequence Detector System according to manufacturer's instructions (Life Technologies, Grand Island, NY). The information of Taqman probes were shown in supplemental Table 1. All samples were run in triplicate and the threshold cycle was used for calculating relative mRNA levels of target gene by normalizing to 18S rRNA. Statistical analyses were performed using t test or one-way ANOVA followed by Tukey's post hoc multiple range test with the Instat package from GraphPad (GraphPad Software, La Jolla, CA).

Western blot

Western blot analysis was carried out using standard protocol. Uterine tissues were homogenized in protein lysis buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 50 mM NaCl, 0.1% NP40, 1 mM PMSF, 10 μg/ml aprotonin, 10 μg/ml leupeptin). Thirty micrograms of protein was electrophoresed on a 4-12% NuPAGE® Novex Bis-Tris Pre-Cast Gel (Life technologies, Carlsbad, CA) and transferred to PVDF membrane (EMD Millipore Corporation, Billerica, MA). Membranes were blocked in 5% nonfat dry milk which was dissolved in Tris-buffered saline/Tween solution (TBS/T; 20 mM Tris, 150 mM NaCl, pH 7.6, and 0.1% Tween-20) and then incubated with 1:1000 anti-PGR antibody (sc-7208, Santa Cruz Biotechnology, Inc., Dallas, TX) overnight at 4oC with gentle rocking. After washing, the membranes were probed with peroxidase-labeled goat anti-rabbit IgG (1:2000). The membranes washed with TBS/T and developed with ECL prime reagents (GE Healthcare Biosciences, Pittsburgh, PA), followed by exposure to X-ray film.

Chromatin immunoprecipitation

Six week old female C57BL/6 mice were ovariectomized and rested for two weeks. Mice were then administered 1 mg progesterone (P4) subcutaneously for 1 hour followed by extraction of the uterus. Mouse uteri were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by adding lysis buffer, followed by disruption with a hand-held mechanical homogenizer followed by 30 strokes with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300- 500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K, and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. An aliquot of chromatin (30 μg) was precleared with protein A agarose beads (Life Technologies, Grand Island, NY). ChIP was performed using an antibody against GATA2 (sc-9008, Santa Cruz Biotechnologies, Dallas, TX). After incubation at 4ºC overnight, protein A agarose beads were used to isolate the immune complexes. Complexes were washed, eluted from the beads with SDS buffer, and treated with RNase and proteinase K. Crosslinks were reversed by incubation overnight at 65ºC, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation. For ChIP-quantitative real time PCR (qPCR), primers were designed to amplify regions of GATA2 binding identified through ChIP-seq. qPCR reactions were carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad, Hercules, CA). The resulting signals were normalized to Input DNA.

ChIP-seq analysis

GATA2 and Input ChIPs were performed by Active Motif, Inc. (Carlsbad, CA) on mouse uteri treated with 1 mg P4 as described above. ChIP and input DNA were amplified using the Illumina ChIP-Seq DNA Sample Prep Kit (Illumina, Inc., San Diego, CA). Briefly, DNA ends were polished and 5'-phosphorylated using T4 DNA polymerase, Klenow polymerase and T4 polynucleotide kinase. Addition of 3' adenine to blunt ends using Klenow fragment (3'-5' exo minus), Illumina genomic adapters were ligated, and the sample was size-fractionated to 175–225 bp on a 2% agarose gel. After amplification for 18 cycles with Phusion polymerase, the resulting DNA libraries were tested by qPCR at the same specific genomic regions as the original ChIP DNA to assess quality of the amplification reactions. DNA libraries were sent to Illumina Sequencing Services for sequencing on a Genome Analyzer II. Sequences (35 bases) were aligned to the mouse genome (NCBI Build 37, July 2007) using Eland software. Aligns were extended in silico at their 3'-ends to a length of 110-200 bp and assigned to 32-nt bins along the genome. The resulting histograms were stored in BAR (Binary Analysis Results) files. Peak locations were determined by applying a threshold of 18 (5 consecutive bins containing .18 aligns). These files were analyzed using Genpathway proprietary software that provides comprehensive infaormation on genomic annotation, peak metrics and sample comparisons for all peaks (intervals).

Transfection and Luciferase Assay

Human endometrial epithelial cells (HEC-1A) were used for transfection according to manufacturer's instructions using Qiagen SuperFect reagent (Qiagen, Valencia, CA). For determine the regulation of Pgr by GATA2, a Pgr promoter driven luciferase reporter plasmid containing the mouse Pgr promoter (-384/+680) which includes the distal and proximal promoter regions (Sriraman et al., 2003) was cotransfected with expression plasmids for full length Gata2, as well as, a N-terminal zinc finger deleted (ΔNT) construct and a C-terminal zinc finger deleted (ΔCT) construct (Tong et al., 2000). The latter constructions were included because the Gata2d/d mouse was a result of ablation of the C-Terminal zinc finger and previous studies showed that the C-terminal zinc finger, but not the N-terminal finger is required for DNA binding and trans-activation function of Gata2 (Martin and Orkin, 1990; Shimizu et al., 2001; Yang and Evans, 1992).

To determine the coordination of PGR and GATA2 to regulate gene expression of Sex Determining Region Y-box 17 (Sox17) and Indian hedgehog homolog (Ihh), 200 ng of ~1 kb region of the mouse Sox17 and an Ihh +19kb region containing overlapping PGR and GATA2 interval locations fused to the pGL4.23 luciferase vector (Promega, Madison, WI) were cotransfected with 50 ng of human PGR-A, human PGR-B, human GATA2 or pcDNA empty vector control. The cells were treated with 10-8 M R5020 (Sigma, St. Louis, MO) or vehicle control (ethanol) in McCoy's 5A media (Life Technologies, Grand Island, NY) for 24 hours at 37ºC. The cells were harvested and lysed using the Promega Passive lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the Promega luciferase assay system (Promega, Madison, WI) in a Centro LB 960 luminometer (Berthold Technologies, Oak Ridge, TN). The luciferase activity was normalized to a Renilla luciferase internal control.

Microarray analysis

For microarray analysis, mice were ovariectomized at 6 weeks of age. Two weeks later, ovariectomized mice were injected with either vehicle (sesame oil) or P4 (in sesame oil, 1 mg per mouse in 100 μl) (Sigma-Aldrich, St. Louis, MO) and mice were sacrificed 6 hours after the injection, uterine tissues were snap frozen and stored at -80°C. Total RNA was extracted from uterine tissues using the Qiagen RNAeasy total RNA isolation kit (Qiagen, Valencia, CA). The RNA was pooled from the uteri of 3 mice per genotype. All RNA samples were analyzed with a Bioanalyser 2100 (Agilent Technologies, Wilmington, DE) before microarray hybridization. Microarray was performed by the Baylor College of Medicine Microarray Core Facility using Affymetrix murine genome 430 2.0 mouse oligonucleotide arrays (Affymetrix, Santa Clara, CA).