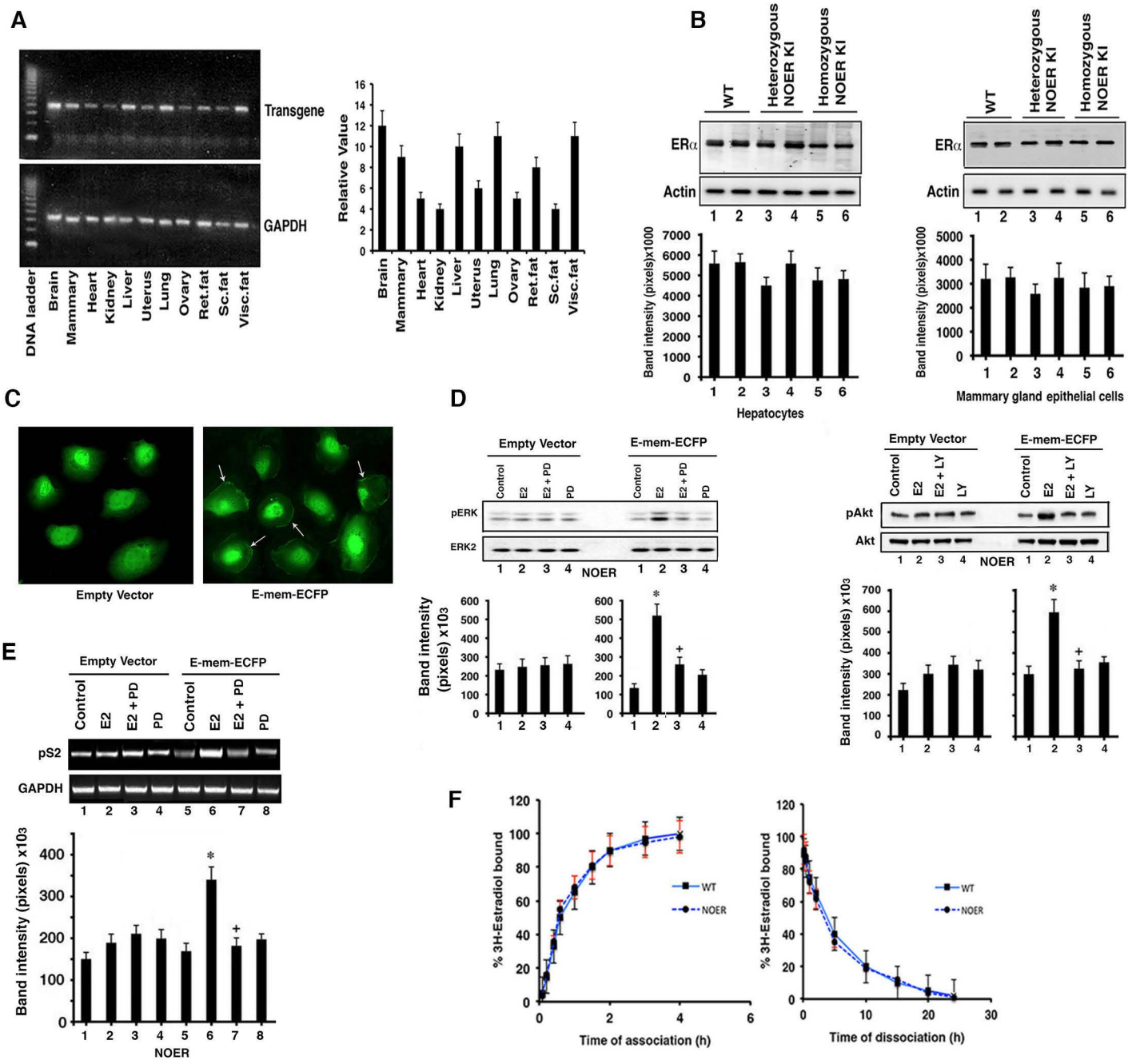
Supplemental Information

Inventory

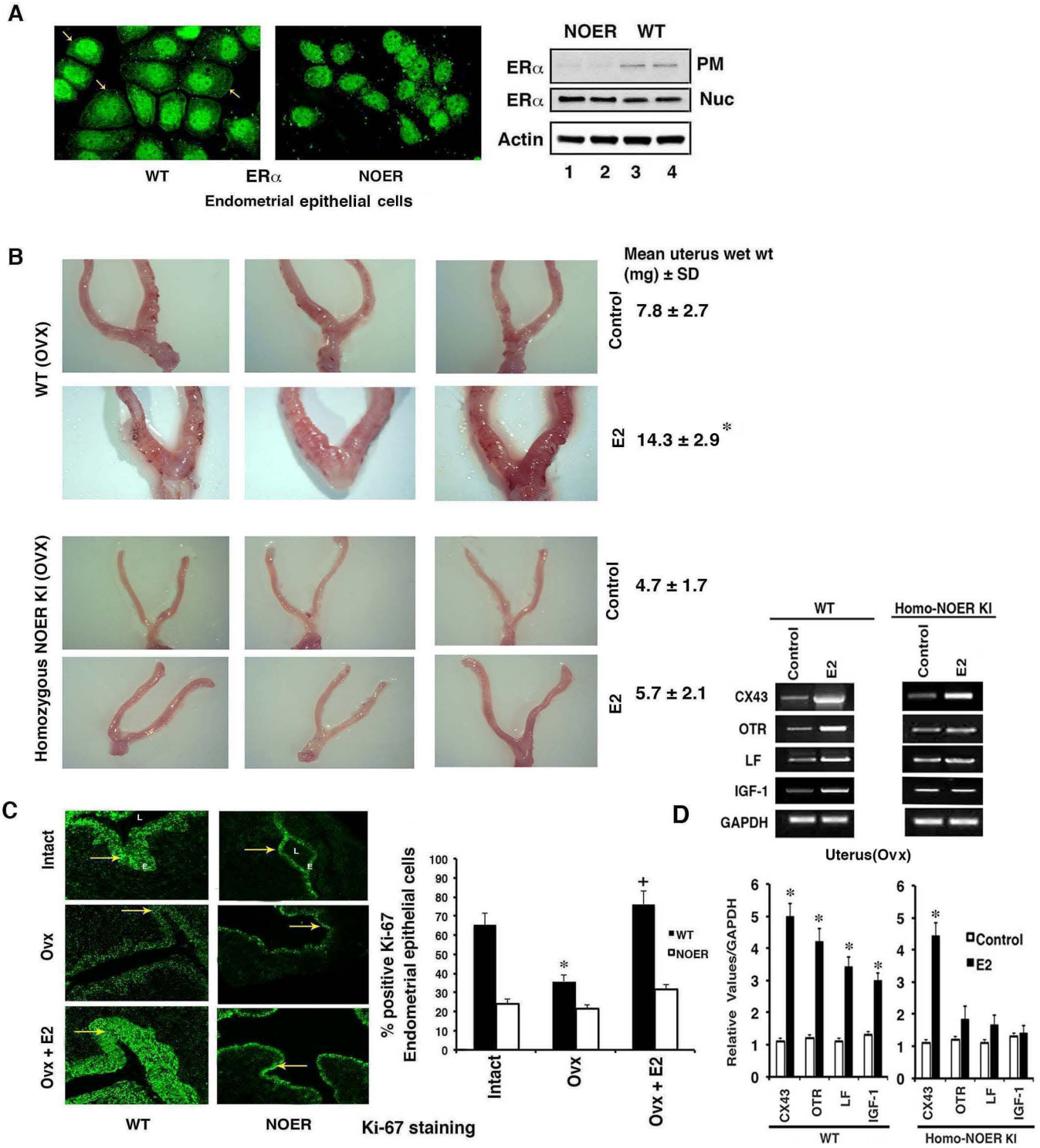
Figures S1-S4 Provide depth to Figures in the manuscript, validation of the model (S1), additional endometrial response to E2 studies (S2), and gene targets for organ development (S3). S4 shows the visceral fat phenotypes.

Table S1 Fertility data

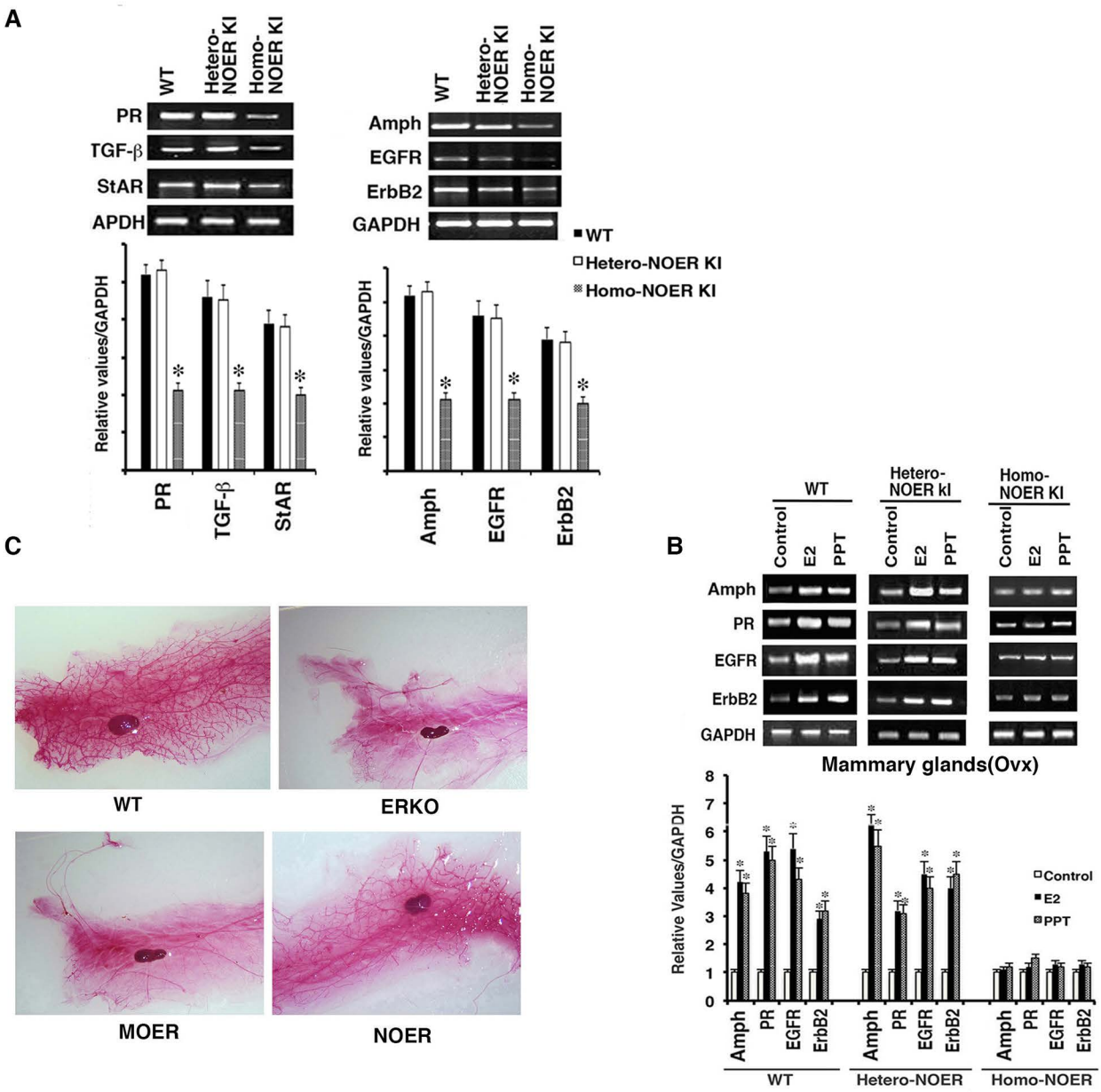
Supplemental Figures



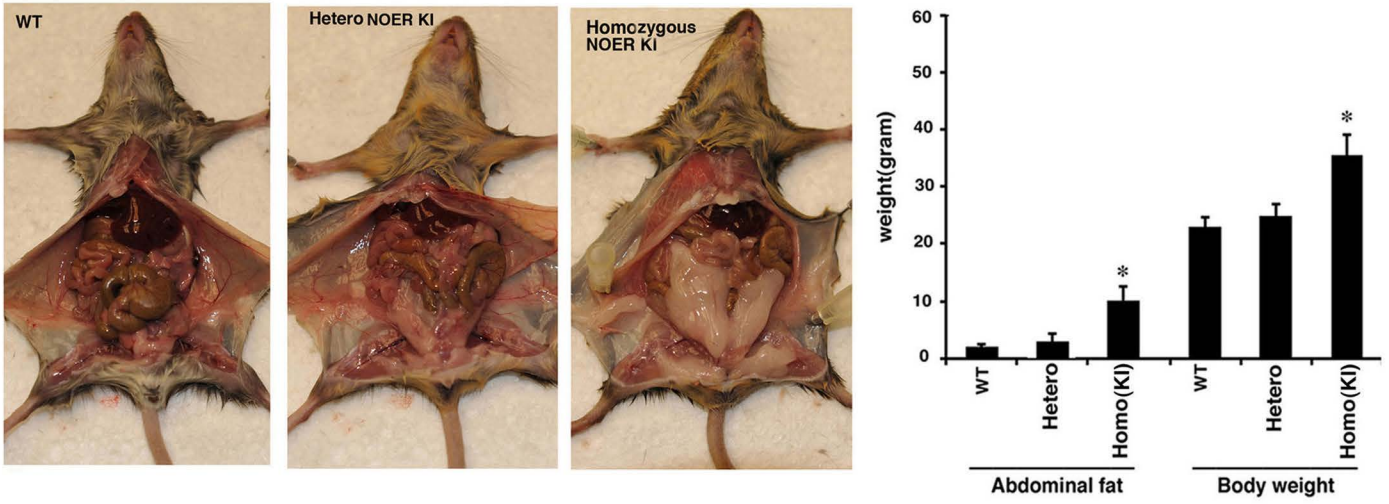
FigureS1 NOER mouse validation. (A)ERα mutant (C451A) mRNA expression in organs of the NOER homozygous female mouse by qRT-PCR. GAPDH is control. Ret is retro- peritoneal, Sc is subcutaneous, VISC is visceral. Graph is mean± SEM from 4 mice. (B)Total cell endogenous ERα protein expression from cultured hepatocytes and mammary gland epithelial cells. Graph is 5 females from each mouse type. Actin is control. (C) Expression of plasmid Emem-ECFP in homozygous NOER hepatocytes shows membrane localization (arrows) of the E domain of ERα, compared to control (ECFP) vector-transfected cells. ERα first antibody used for immuno-fluorescence was to the E/F domain (D) Phospho ERK and Ser473 AKT activation in response to E2 in Emem-ECFP expressing NOER hepatocytes. PD98059 is a MEK inhibitor, LY294072 is an AKT inhibitor. Graphs reflect triplicates in each of 3 exps. (E) PS2 mRNA expression is enhanced in response to E2 signaling to ERK (inhibited by the MEK inhibitor) in Emem-ECFP expressing NOER hepatocytes. Graph is cells from 5 individual homozygous NOER mice, studies done 3 times. *p<0.05 vs control, +p<0.05 vs E2. (F) Association and dissociation curves for labeled E2 binding in hepatocytes from 5 mice each. Each study was repeated. Related to Figure 1.



FigureS2- (A) Absence of membrane ERα protein in homozygous NOER endometrial epithelial cells. Whole cell immuno-fluorescent microscopy (left) and immuno-blots of membrane (PM) and nuclear fractions (right) are shown. (B) Uteri gross appearances from three representative ovariectomized control WT and homozygous NOER mice, and at day 12 after estrogen pellet insertion (n=5/group). All pictures were taken under the same microscopic magnification. Uterine wet weights at day 12 were determined (mean±SD).*p<0.05 for control versus E2-exposed (n=5). (C) Ki67 staining of endometrium from intact, ovexed and E2-treated mice. *p<0.05 for intact versus ovx, +p<0.05 for ovx versus ovx+E2-treated (n=5) (D) mRNA responses in endometrium of ovariectomized mice (n=5 each) at 12 days after E2 pellet insertion. GAPDH is loading control. *p<0.05 vs control. Related to Figure 2.



FigureS3 (A) Expression of key estrogen responsive mRNAs in samples of ovaries and mammary glands respectively from intact WT, hetero, and homozygous NOER mice (bar graph=5 mice each). *p<0.05 vs WT or heterozygous NOER organs (B) mRNA responses in mammary glands of ovariectomized mice (n=5 each) at 24 hours after in-vivo injections of saline (control), 100 ug E2 or PPT (right). GAPDH is loading control, *p<0.05 vs control.(C) Representative mammary gland tissue sections from WT, homozygous ERKO, MOER, and NOER mice (n=4 each). Related to Figures 2 and 3.



FigureS4 Visceral fat in the abdomen and whole body weights of 10-week old female WT, heterozygous and homozygous female NOER mice. Graphs are from 5 mice per group. Related to Figure 1.

Supplemental Table

TABLE S1- Breeding performances of NOER and WT mice. Related to Figure 2

Number of animals	Male X Female	Period between mating and first litter (days)	Number of pups in first litter	Weaning success In first litter (%)
10 x 10	WT/WTXWT/WT	21±0.4	9.1±1.0	92.4±0.5
25 x 25	WT/KI XWT/KI	30±3.0	7.4±0.6	76.4±1.2
18 x 18	KI/KI X KI/KI	0.00	0.00	0.00
20 x 20	WT/WT X KI/KI	0.00	0.00	0.00

Supplemental Experimental Procedures

Mouse construction The targeting vector was constructed by the UC-Davis Mouse Biology Program, via BAC recombineering, using bacterial strain EL350. The construct contains a 3.7 kb 5' arm of homology, a 3.8 kb 3'arm of homology, the C451A mutation, a diphtheria toxin A (DTA) cassette, and a neomycin-resistance cassette (neo) flanked by frt sites for selective deletion. The arms of homology were subcloned from the BAC clone, bMQ-339L19, obtained from BACPAC Resources at the Children's Hospital of Oakland Research Institute. The Sanger-bMQ BAC library was made with genomic DNA from AB2.2 embryonic stem (ES) cells, derived from the mouse strain, 129S7/SvEvBrd-Hprtb-m2.The targeting construct was linearized with AscI (New England Biolabs, Ipswich, MA), purified by ammonium acetate precipitation of the protein fraction followed by ethanol precipitation of the DNA, and electroporated into the E14TG2a line of ES cells, derived from the 129P2/OlaHsd mouse strain. In each of 4 cuvettes, 23 ug of linearized construct was mixed with 10 million ES cells and subjected to 800V at a capacitance of 10 uF (time constant = 0.1 msec) on a Bio-Rad Genepulser Xcell. Cells were immediately diluted in ES cell medium and plated on 10 cm gelatinized plates. ES cell medium consisted of GMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, sodium pyruvate, ESGRO (LIF), non-essential amino acids, L-glutamine, and beta-

mercaptoethanol. The day after electroporation the medium was supplemented with 300 ug/ml G418 to select for cells carrying the neo cassette. After 6 days of selection, the G418 concentration was lowered to 150 ug/ml. After an additional 3 days of selection, colonies were picked into 96-well plates, dispersed with trypsin, and cultured in ES cell medium with 75 ug/ml G418. Out of 192 colonies picked, one colony (C2) displayed the expected bands on Southern blots hybridized with probes specific to the 5' and 3' ends of the construct. The chromosomes of metaphase spreads prepared from clone C2 were counted and 85% of the spreads contained 40 chromosomes.

To make chimeric mice, cells from clone C2 were injected into blastocysts from C57BL/6NTac mice. A single injection session yielded 10 male chimeras with at least 75% ES cell contribution, as judged by coat color. Mating chimeras with C57BL/6NTac females and assaying the offspring for the presence of neo confirmed germ line transmission. Chimeras were crossed with Flpe-expressing females (stock #005703, Jackson Laboratories, Bar Harbor, ME) to delete the neo cassette in all tissues. Genotyping by PCR utilized the following primers that yielded a 400bp gel band corresponding to C451A ERα with the neo gene excised. Forward 5'-CTAAACAAGCTTCAGTGGCTCCTAG-3', Reverse 5'-ACCTGCAGGGAGAAGAGTTTGTGGC-3'.

Quantitative real-time PCR All experiments were approved by the Animal Use, and Research and Development Committees at the Department of Veteran's Affairs Long Beach facility. Mammary gland epithelial cells were isolated and grown in DMEM-F12 medium for epigenetic studies and PR expression (23). Cells were synchronized for 24 hours, washed and incubated with 10nm E2± inhibitors for 24 h. For mRNA(s) expression in the various organs, the organs from 5 mice per type were removed upon euthanasia, and total RNA was extracted using the Qiagen RNeasy Kit and pooled for the studies. cDNA was synthesized using the Improm-II reverse transcription system (Promega). qRT-PCR determined mRNA expression normalized to the housekeeping mRNA,

GAPDH. Primers were designed with Primer3 (http://frodo.wi.mit.edu/) and were blasted for specificity.

CX43	F 5′- ACT GTT CAT CAC CCC AAG CTG-3′
	R 5′- AGC ACC GAC AGC CAC ACC TTC-3′
OTR	F 5′- GGA CGT CAA TGC GCC CAA AGA AG-3′
	R 5′- ACT CGA GCT GCA ACG ACT CA-3′
LF	F 5'- AAA CAA GCA TCG GGA TTC CAG-3'
	R5' - ACA ATG CAG TCT TCC GTG GTG-3'
IGF1	F 5’- TCA TGT CGT CTT CAC ACC TCT TCT-3’
	R 5’- CCA CAC ACG AAC TGA AGA GCA T-3’
Amphiregulin	F 5’-TGT CAC TAT CTT TGT CTCTGC CAT-3’,
	R 5’- AGC CTC CTT CTT TCT TCT GTT TCT-3’
TGF-β1	F 5’-GGA TAC CAA CTA TTG CTT CAG CTC C-3’
	R 5’-AGG CTC CAA ATA TAG GGG CAG GGT C-3’
EGFR	F 5'-TCT TCA AGG ATG TGA AGT GTG-3',
	R 5'-TGT ACG CTT TCG AAC AAT GT-3')
ErbB2	F 5’- TGG CTG CAA GAA GAT CTT TG-3’
	R 5’-TGC AGT TGA CAC ACT GGG TG-3’
StAR	F 5’-TCT TCT TGG TTC TCA GCT GGA AGA C- 3’
	R 5’-CTT CTG CAG GAT CTT GAT CCT TCT TG -3’
pS2	F 5′-ATA CCA TCG ACG TCC CTC CA-3′
	R 5’-AAG CGT GTC TGA GGT GTC CG-3’
PR	F 5’-CGC GCT CTA CCC TGC ACT C-3’,
	R 5’-TGA ATC CGG CCT CAG GTA GTT-3’
GAPDH	F 5’-CCA CAG TCC ATG CCA TCA-3’
	R 5’-GGA TGA CCT TGC CCA CAG-3’

Primers were designed for annealing temperature of 60°C and to amplify regions of approximately 95-120bp. PCR amplicon sizes were confirmed by agarose gel electrophoresis prior to qRT-PCR analysis. For qRT-PCR, 500ng of cDNA was

used in a 50μl reaction consisting of 25μl of SYBR Green, qPCR Supermix (Invitrogen), 1μl of 10μM forward/reverse primer stocks and nuclease free water. Thermocycling was carried out using the iCycler (Biorad) with a melting curve temperature of 60°C.

ChIP (chromatin immunoprecipitation) assay ChIP assays were performed using Active Motif (Rixensart, Belgium) chip IT kit with some modifications. For each experimental condition, four confluent plates of mouse hepatocytes (10^7cells /dish) were used. After experiments for 24 hours, cells were washed with ice-cold PBS, 10mM sodium phosphate buffer, and 140mM NaCl, cross-linked for 5 min at room temperature with 1% formaldehyde. The reaction was stopped with glycine 125mM. Cells were scraped and washed with cold PBS containing proteinase inhibitor cocktail (Sigma Chemical) and 1mM PMSF. Preparation and enzymatic fragmentation of chromatin used the Enzymatic Shearing kit (Active Motif,) according to the manufacturer's instructions. An aliquot was used to verify fragmentation (average fragment length of 200 bp) on agarose gels and to quantify the amount of DNA. For ChIP assays, 50 μg digested chromatin was pre-cleared with protein A/G PLUS-Agarose beads saturated with salmon sperm DNA and equilibrated with 15 mM Tris/HCl, pH 8.0,167 mM NaCl, 3 mM Na_2EDTA, 0.1% SDS, 1% (w/v) Triton X-100 and protease inhibitors. After 3 h on a rocking platform at 4 °C, the beads were pelleted by centrifugation at 13,000*g* for 2 min at 20 °C, and supernatants were divided and incubated with 10μg of anti-ERa antibody (MC-20), rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or no antibody (mock condition), overnight at 4 °C under rotative agitation. Immuno-precipitated complexes were bound to protein A/G PLUS-Agarose beads for 3 h at 4 °C and sequentially washed in lysis buffer (10mM Tris/HCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 140 mM NaCl, 0.1% sodium deoxycholate and 1 mM PMSF, pH 7.4), LiCl buffer (1mM Tris/HCl, 250mM LiCl, 0.5% Nonidet P40, 0.5% sodium deoxycholate and 1mM EDTA, pH 7.4) and Tris–EDTA buffer (1 mM EDTA and 10mM Tris/HCl, pH 7.4). Chromatin complexes were eluted from washed beads by sequential

incubations at room temperature (twice for 15 min and once for 45 min) in 90 μl of elution buffer [100mM $NaHCO_3$ and 1% (w/v) SDS]. To reverse the cross-links and digest RNA, NaCl was added to a final concentration of 300mM with 30μg/ml RNase A and the samples were incubated at 67 °C for 5 h. Proteins were digested with 55 μg/ml of proteinase K at 55 °C for 12 h. The DNA was isolated by phenol/chloroform extraction and ethanol precipitation using 10 μg of glycogen as a carrier. The DNA precipitates were dissolved in 20μl of TE buffer diluted 1/3 in water. Samples of input chromatin were subjected to similar procedures for reversal of cross-links, digestion of RNA and protein, and isolation of DNA. The DNA concentration in input chromatin samples was assayed by measuring the light absorbance at 260 nm. The isolated DNA samples were subjected to PCR for amplification of pS2 mRNA using the JumpStart™ ReadyMix™ Taq system in 50 μl reactions. The sets of PCR primers used for the analysis of the pS2 promoter containing an estrogen response element (ERE) were 5'-CTA GAC GGA ATG GGC TTC AT-3' for sense and 5'-ATG GGA GTC TCC TCC AAC CT-3' for antisense. Primer sets for the analysis of the distal region of gene exon 4 (negative control) were 5′-GAC CTA CCA GTT GAA GAT TCC G-3′ for sense and 5′-AAG CGT AAC TCCA GCA CAC A-3′ for antisense. Samples of 10 ng of input DNA or 0.2 to 1 μl of immunoprecipitated DNA (normalized to the yield of DNA in chromatin input) were used as template. The PCR program was 30 cycles with denaturation at 95 °C, annealing at 57 °C and elongation at 72 °C, each for 30 s except for the initial three cycles during which denaturation was 45s, annealing 60s and the final cycle, elongation step, was 5 min. For analysis, the PCR products were electrophoresed in 2.5% agarose gels in 90 mM Tris, 90 mM boric acid, 2 mM EDTA containing 1 μg/ml ethidium bromide. Results were expressed as fold-enrichment relative to the value in control cells.

E2 binding studies Competition binding studies on hepatocytes were conducted as follows. 0.5 nM H^3-E2 was added to 10^6 cells, each in the absence or presence of 0.01nM-1μM unlabeled E2, and binding for 2 hour occurred at 23^0C. Binding data was subjected to Scatchard analysis using the LIGAND

computer program and K_d and Bmax were calculated. Each point was done in triplicate, the study repeated three times. Association binding was also done using H^3-E2 at 0.5 nM (total binding) and at different times, where another set of tubes also contained 100-fold excess unlabeled E2 (to calculate nonspecific binding). Time course for the dissociation of H^3-E2 after equilibrium binding (2 hours) was initiated by washing the cells in a large volume of buffer and determining binding at different time points.

ERα localization Hepatocytes, mammary epithelial cells, and endometrial epithelial cells were isolated from 5 each of 8-10 week old wild type, hetero and homozygous NOER female mice, acutely cultured, and characterized for sub-cellular localization of the steroid receptor by immuno-fluorescence microscopy and western blot, as described (2,23). The first antibody to ERα used was from Santa Cruz Biotechnology (c-terminus, MC-20) and FITC-conjugated second antibody was from Vector labs. Cells and antibody were also used for mRNA and protein expression studies. All in-vitro studies utilized random utilization of wells of cells for any experimental condition. Additionally, all samples from an experiment were run in the same gel, even if separated for figure presentation. For endometrial cells, To prepare uterine epithelial cells, uteri were removed, opened lengthwise, pooled, and incubated with 0.25% trypsin /2.5% pancreatin (Gibco-BRL/Invitrogen, Grand Island, NY) for 60 min at 4°C, then 60 min at 22°C. Following transfer to ice-cold (4°C) Hanks Balanced Salt Solution, digested uteri were vortexed to release sheets of epithelial cells. Uterine tissues were rinsed/vortexed an additional three times and resulting cell suspensions pooled. Epithelial sheets were recovered by passing cell suspensions through a 20-micron nylon mesh, collected, and centrifuged (500 × *g*). The resulting cells were re-suspended in complete Dulbecco Modified Eagle Medium (DMEM) (without phenol red)/Hams F-12 nutrient mixed 1:1 (Gibco/Invitrogen, Grand Island, NY) +10% fetal bovine serum (FBS; Hyclone, Logan, UT) supplemented with 20 mM Hepes, 100 µg/ml streptomycin, 100 U/ml penicillin, and 2 mM l-

glutamine (all from Gibco/Invitrogen). Cells were seeded on glass bottom dishes, incubated at 37°C/5% CO_2.

Kinase activities ERK activity was determined at 15 mins of cell incubation as phosphorylation of the protein isoform at the active site, tyrosine 202/ 204. Cells were exposed to various conditions and the cells were lysed, then separated by SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with antibodies to tyrosine 202/ 204 or with antibodies to determine total ERK protein (Santa Cruz). AKT activity at 15 mins was determined as Ser473 phosphorylation by immuno-blots from cells exposed to the various conditions, using phospho and total antibodies from Cell Signaling Inc. Studies were done 3 times.

Measurement of plasma hormone levels Blood samples were collected by cardiac puncture at time of euthanasia and placed in ice–cold tubes coated with sodium-EDTA and centrifuged at 3000 rpm for 10 min at 4^0C. The plasma was stored at -80^0C until the hormone assays. Hormone levels were measured in plasma using ELISA or RIA kit according to manufacture's instructions (Diagnostic Systems Lab, Webster,TX)(Cayman Chemical Company, Ann Arbor, MI). Available limits of sensitivity of the assays were 1ng/ml for FSH, 0.5 ng/ml for LH, 5pg/ml for E2, and 0.1 ng/ml for Progesterone.

Fertility studies 12-18 week male and female WT, heterozygous and homozygous NOER mice were used for multiple matings, and success rates, periods between first mating and first litters born, pups/ first litter were determined.

Histology and histochemistry Whole organs were obtained under anesthesia/euthanasia for mounts and thin tissue sections of uteri, ovaries, and mammary glands prepared as described (2). Hematoxylin and eosin staining (reproductive tract tissues) and carmine alum staining for mammary glands were done. Five mice per group were used for quantitation and representative sections are shown.

Uterine response to E2 10-12 wk old female mice (5 mice in each group) were ovariectomized and recovered for 1 week, estrogen pellets then inserted under

the skin for 21 days, the pellet producing physiological levels of E2 in mouse serum (Pedram et al., 2008)). The ~3mm, 5mg slow release pellets (60 days) were from Innovative Research of America, Sarasota FL and were used for the 21 day studies. Uterine epithelial thickness was measured on tissue sections. mRNA expression in intact WT, hetero and homozygous NOER mice was done, and also was determined for WT and homozygous NOER mice that were ovariectomized 1 week prior to the study then exposed to E2 for 12 days, uteri were removed upon euthanasia and processed for RT-PCR (n=5 per group). Uterine wet weight was measured at day 12 in E2- exposed ovariectomized WT and homozygous NOER mice; uterine measurements were to the nearest 0.1mg after paper blotting. The pellet used for the12 day studies delivered 0.5mg of E2 over 28 days. Total body weights of the mice at the start and conclusion of the 12 day experiments were comparable in the two groups.

E-mem-ECFP expression studies

Hepatocytes isolated from 5 homozygous female NOER mice were each transfected with a plasmid expressing only the E domain (ligand binding domain) of ERα, targeted exclusively to the membrane by paired myristylation sequences within the plasmid (E-mem ECFP) (2). As a control, some cells were transfected with the empty vector (ECFP). Cell distribution by fluorescent microscopy, signal transduction, pS2 mRNA expression and ChIP studies of nuclear ERα recruitment to the pS2 promoter/ERE were then performed. Duplicate determinations in cells from each mouse were carried out.

Epigenetic modulation studies Mammary gland epithelial cells from the three mice types were isolated and cultured. Epithelial cell monolayers were washed in ice-cold PBS supplemented with 5mM sodium butyrate. After centrifugation at 2000 rpm for 5 min in the cold, cell pellets were re-suspended in lysis buffer (10 mM Tris-HCl, pH 7–7.4; 10 mM NaCl; 3 mM $MgCl_2$; 1 mM phenylmethylsulfonylfluoride; 1 mM Na_3VO_4; 1 mM NaF; and Complete Protease Inhibitor Cocktail) with 0.5% NP-40 on ice. Cell lysates were centrifuged for 5 min at 6,000 rpm at 4^o C. Phosphorylation at serine 21 of the histone methyl

transferase EZH2 was also determined at 30 minutes of 10nME2 exposure to the cells. Some cells were also exposed to E2+10μMLY294002, a PI3K-AKT inhibitor. Pellets were re-suspended in media with 5mM $MgCl_2$ and 0.8M HCl, sonicated at low speed for 20 sec and incubated on ice for 1h. The solution of histone proteins was centrifuged at 14,000 rpm for 10 min at 4^oC. Supernatants were transferred to a new tube, and histones were precipitated with a 50% solution of trichloroacetic acid dissolved in ddH2O. Precipitated histones were collected via centrifugation for 20 min at 14,000 rpm at 4^o C. The resultant pellet was washed with ice-cold acetone, dried, and subsequently re-suspended with ddH2O (150 ml) and 1.5 M Tris-HCl, pH 8.8 (2 ml). Histone proteins were resolved by SDS-PAGE on a 10–20% Tris-tricine gel (Bio-Rad Laboratories), and changes in tri-methylation of histone 3 at lysine 27 relative to total histone 3 were investigated by immuno-blotting with antibodies from Abcam Inc. Total EZH2, and histone 3 proteins were determined as loading controls. PR expression was determined by RT-PCR after 24 hours of E2 treatment.

Mouse fat studies For abdominal visceral fat determination, the anesthetized mice were weighed, the abdomen was opened and pictures were taken, and fat was then dissected free from mouse abdominal viscera and weighed. The mice were then euthanized. Mean±SEM weights for each group were calculated, and resulting bar graphs were from 5 mice each,*$p<0.05$ for WT or heterozygous NOER versus homozygous NOER.

Statistical analysis Mean±SEM were calculated for 2-way ANOVA + Schefe's test comparison. $p<0.05$ was considered significantly different. Prospective statistical analysis after consultation with a statistician assumed a 50% difference between WT and homozygous NOER mice, resulting in the in-vivo mouse studies using 5 mice per group. The mice were randomly picked for experimentation after genotyping and assigned to receive either no E2 (control) or E2 pellets usning no systematic order.