Supporting Information

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SI Materials and Methods

Generation of ERβ-Floxed and ERβ-Δex3 Mice. A genomic P1 clone from the mouse ERβ locus was isolated from a 129/SvJ library by Incyte Genomics using primers corresponding to exon 3. A 12-kb KpnI fragment from this clone was subcloned into pBS-KS (Stratagene) and used to make the targeting construct. Briefly, a loxP site was cloned into the BamHI site 5′ of exon 3 and a fragment containing a loxP flanked neocassette, from the plasmid pL2-neo, was cloned into the BamHI site 3′ of exon 3. A TK cassette was cloned into the SalI site 5′ of exon 2. The targeting construct was linearized by NotI and electroporated into RW-4 129SvJ embryonic stem (ES) cells and selected with geneticin (G418) and ganciclovir on embryonic fibroblast feeder cells. Resistant clones were isolated and analyzed for homologous recombination by Southern blot analysis after KpnI digestion with a 5′ external probe (KpnI/SalI fragment) as illustrated in Fig. S1*A*. The neo selection marker was deleted by partial Cre-mediated recombination in ES clones to generate ES clones with a "floxed" allele of ERβ. One clone in which partial Cre-recombination had occurred was injected into C57BL/6J blastocysts that were implanted into pseudopregnant females. Chimeric male mice were bred to C57BL/6J females and germ line transmission of the targeted allele was examined in the agouti offspring by Southern blot, PCR analysis (Fig. S1*B*) and sequence determination of the PCR products (data not shown). The name of the generated heterozygous floxed ERβ mouse line according to the Institute for Laboratory Animal Research (ILAR) nomenclature is B6.129 × 1-*Esr2*^{tm1Gust}. Mice with a deleted ERβ allele were generated by crossing ERβ$^{flox/+}$ with transgenic CMV-Cre deleter or Rosa-Cre deleter mice (Taconic) as described previously (1, 2). Correct recombination and deletion of exon 3 in the offspring was confirmed by PCR (Fig. S1*C*) and sequence determination of the PCR products (data not shown). After backcrossing into C57BL/6J mice, mice that lacked both exon 3 and the Cre transgene were selected for maintaining the colony. ERβ-Δex3 mice were generated by heterozygous breeding of ERβ$^{+/-}$ mice. The name of the ERβ-Δex3 mice according to the ILAR nomenclature is B6.129 × 1-*Esr2*$^{tm1.1Gust}$. All mice analyzed in this study were on a congenic C57BL/6J genetic background, backcrossed into C57BL/6J for 10 generations or more. Animals were maintained on a 14-h light, 10-h dark cycle and given a continuous supply of standard mouse chow and water. The animal studies were approved by the Stockholm South ethical review board and the local Animal Experimentation Ethics Committee for animal experimentation (University of Houston animal protocol 09-036).

Original ERβ$^{-/-}$ Mice (Smithies ERβ$^{-/-}$). The original ERβ$^{-/-}$ mice were generated as previously described (3).

Genotyping of Mice. DNA from tail or ear biopsies were used as templates in PCR reactions using primers P3 (ISP: 5′-AAAGGTATGCTTATGTCCGGTGGG-3′) and P4 (BIASP: 5′-GTGGATGCCTATGATCACTGTGGA-3′), which detect the WT allele as a 160-bp product and the floxed allele as 300 bp, whereas primers P1 (B1-2SP: 5′- ATCAGCCCATGGGCAGAGTGTG-3′), P2 (CIASP: 5′-CCAGATGCATAATCACTGCAGACG-3′), and P4 were used to detect the WT (size: 984 bp and 423 bp) and deleted (size: 195 bp) alleles. Presence of Cre was assayed with primers Cre-sense: 5′-CCAATTTACTGACCGTACACC-3′ and Cre-antisense: 5′-GTTTCACTATCCAGGTTACGG-3′.

RNA Isolation, RT-PCR, and DNA Sequencing. Ovaries and ventral prostate were freshly collected and RNA was isolated using an E.Z.N.A. kit (Omega Bio-Tek) or Qiagen RNeasy mini kit, respectively. Ovary cDNA was synthesized using random hexamers and SuperScript II reverse transcriptase (Invitrogen), whereas ventral prostate cDNA was obtained using M-MLV reverse-transcriptase (Invitrogen) and oligo dT (Integrative DNA Technologies, IDT). PCR was done with high-fidelity Taq DNA polymerase (Fermentas) with the following primers: ERβ-5′: 5′-GCCAATCATCGCTTCTCTAT-3′ (exon 2) with mERβ exon5R: 5′-GGCACTTCTCTGTCTTCGTA-3′ or ESR2R1: 5′-AGTGGTCTAGAAGACACCAT-3′ (exon 9). PCR products were cloned by TA cloning using pGEM-T Easy vector (Promega) and sequenced at Macrogen (Ovary cDNA) or Lone Star Labs (VP cDNA).

Morphologic Classification of Growing Follicles and Corpora Lutea. Four ERβ-Δex3 mice and six WT female mice at 7–8 mo old were photographed and analyzed. Whole ovaries were embedded in paraffin and cut in 4-μm thicknesses. Sections were taken at intervals of 40 μm and mounted on slides. Routine hematoxylin and eosin staining was performed for histological examination under a light microscope. The proportion of follicles and corpora lutea from all obtained sections was evaluated. Follicle types in ovarian cross-sections were defined as previously described (3, 4).

Tissue Preparation. Mice were killed by CO_2 asphyxiation. The prostates, lungs, and ovaries were collected, and the ventral lobe of the prostate was separated from dorsal, anterior, and lateral lobes under a dissecting microscope. For immunohistochemical studies, tissues were fixed in 4% (wt/vol) paraformaldehyde for 24 h and embedded in paraffin using a Tissue Processor Excelsior processing machine (Fisher Scientific). For protein studies, tissues were snap frozen in liquid nitrogen and kept at −80 °C for further analysis.

Protein Extraction. For preparation of the whole cell extracts, ventral prostates were kept on ice and homogenized in 1× PBS containing protease inhibitors (Complete Protease Inhibitor Mixture, Roche), PMSF 2 mM, and Na_3VO_4 1 mM using a tissue lyser Polytron PT 1200 E (Kinematica). Homogenates were centrifuged at 13,400 × *g* for 30 min at 4 °C and protein content of the supernatant was determined by Bradford assay in a Victor X4 multilabel spectrophotometer (Perkin-Elmer). For preparation of the nuclear extracts, prostates were homogenized in hypotonic buffer (Hepes 10 mM, pH 7.9; Nonidet P-40 0.1%; $MgCl_2$ 1.5 mM; KCl 10 mM; EGTA 1 mM and EDTA 1 mM, pH 8) containing protease inhibitors, DTT 0.05 mM and PMSF 0.4 mM and incubated for 40 min on ice. Cellular homogenates were centrifuged at 3,100 × *g* for 4 min at 4 °C and nuclear pellet was resuspended in hypertonic buffer [Hepes 20 mM, pH 7.9; glycerol 25% (vol/vol); $MgCl_2$ 1 mM; NaCl 420 mM and EDTA 0.2 mM] supplemented with protease inhibitors, DTT 0.05 mM and PMSF 0.4 mM and incubated for 1 h on ice. Nuclear homogenates were finally centrifuged at 16,100 × *g* for 4 min at 4 °C and protein content of the supernatant was assayed by Bradford assay. Protein extracts were stored at −80 °C.

In Vitro Translation. ERβ cDNA from WT (WT-ERβ), WT-INS (WT-ERβINS), or mutant (ERβ-Δex3) mouse ovaries were used as template in PCR reactions and cloned into a pGEM-T Easy expression vector (Promega). ERβ INS domain corresponds to the insertion of 18 additional amino acids in position 364 of ERβ amino acid sequence. Plasmids were prepared, sequenced, and

used to perform in vitro translations with SP6 RNA polymerase. cDNAs to be transcribed and translated were added as plasmid DNAs to a coupled transcription translation rabbit reticulocyte lysate (TnT Quick Coupled Transcription/Translation System, Promega) according to the manufacturer's instructions. Transcription required SP6 RNA polymerase and was carried out in the presence of 2 μL of Transcend Biotin-Lysyl-tRNA (Promega). Transcription/translation reactions were performed for 60 min at 30 °C after which time equal amounts of labeled reticulocyte lysates were resolved on a 4–20% gradient SDS polyacrylamide gel. Translation products were visualized with the Transcend Non-Radioactive Translation Detection Systems kit (Promega), according to the manufacturer's instructions.

Immunofluorescence. Five-micrometer paraffin-embedded sections were dewaxed in xylene, rehydrated, and processed for antigen retrieval with 10 mM citrate buffer (pH 6.0) for 10 (caveolin-1 staining) or 15 min (DACH-1 staining) in a Lab Vision PT module (Thermo Scientific). Then, the cooled sections were rinsed with 1× PBS and tissues were permeabilized with 0.5% Triton X-100 for 30 min at room temperature (DACH-1 staining). Unspecific binding was blocked by incubating the slides in 3% (wt/vol) BSA in PBS for 1 h at 4 °C. Then, the tissues were subjected to immunofluorescence staining with a caveolin-1 antibody (1:1,000 dilution) or an anti–DACH-1 (1:600 dilution) antibody in 1% BSA with 0.1% Nonidet P-40 in PBS overnight at 4 °C. The sections were then washed with 1× PBS three times for 5 min each and incubated with Cy3-labeled goat anti-rabbit secondary antibody (1:200 dilution) at room temperature for 2 h. For dual immunofluorescence staining, cells were incubated with anti–DACH-1 and anti-ERβ 503 (1:100) antibodies overnight at 4 °C. After washing with 1× PBS, sections were incubated with Cy3-labeled goat anti-rabbit (1:200) and FITC-conjugated donkey anti-chicken (1:200) secondary antibodies at room temperature for 2 h. The tissue sections were counterstained with DAPI (Sigma-Aldrich), mounted with UltraCruz mounting medium (Santa Cruz Biotechnology), and examined by fluorescence microscopy (Olympus XM10 camera).

Antibodies and Preparation of Preabsorbed Antibodies to ERβ. The chicken polyclonal ERβ 503 IgY used for immunohistochemical stainings was raised against the N-terminally truncated human ERβ1 with dominant epitope at the N terminus of the protein and was prepared as previously described (5). The rabbit polyclonal anti-ERβ–LBD antibody was directed against the LBD of human ERβ (amino acids 320–527), and prepared as previously described (5). All anti-ERβ antibodies have been tested for their specificity for ERβ, their lack of interaction with ERα, and loss of signals on Western blots and immunohistochemistry after preabsorption with the antigen against which they were raised. Preabsorbed ERβ–LBD antibody was prepared by incubating ERβ–LBD antibody for 12 h at 4 °C with ERβ protein coupled to activated Sepharose (Sigma-Aldrich). The mouse monoclonal anti-ERβ N-terminal antibody was raised against amino acids 1–153 of ERβ1 and purchased from GeneTex (GTX70174). Rabbit antiandrogen receptor (N-20, sc-816), rabbit anti-ERα (MC-20, sc-542), and biotinylated goat anti-chicken (sc-2430) antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-Ki67 (ab 15580) antibody was purchased from Abcam and biotinylated goat anti-rabbit antibody (656140) was from Invitrogen. Rabbit anti–DACH-1 (10914-1) antibody was purchased from Proteintech Group.

1. Schwenk F, Baron U, Rajewsky K (1995) A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res* 23(24):5080–5081.
2. Antonson P, Omoto Y, Humire P, Gustafsson JA (2012) Generation of ERα-floxed and knockout mice using the Cre/LoxP system. *Biochem Biophys Res Commun* 424(4): 710–716.
3. Krege JH, et al. (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci USA* 95(26):15677–15682.
4. Britt KL, et al. (2000) An age-related ovarian phenotype in mice with targeted disruption of the Cyp 19 (aromatase) gene. *Endocrinology* 141(7):2614–2623.
5. Saji S, et al. (2000) Estrogen receptors alpha and beta in the rodent mammary gland. *Proc Natl Acad Sci USA* 97(1):337–342.

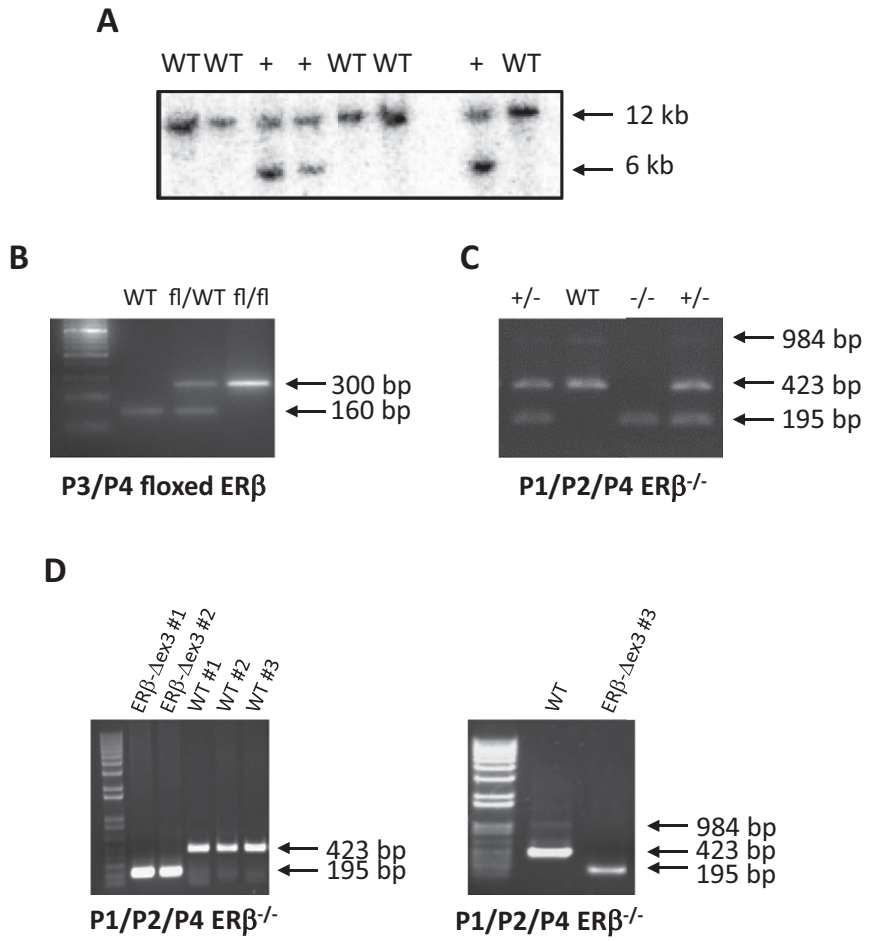

Fig. S1. Targeted disruption of the mouse ERβ gene. Resistant clones (+) were analyzed for homologous recombination by Southern blot analysis after KpnI digestion with a 5′ external probe (KpnI/SalI fragment). The WT allele produces a 12 kb after KpnI digestion, whereas the targeted allele generates a 6-kb band (*A*). Germ-line transmission of the targeted allele was examined by PCR genotyping of mouse DNA using primers P3 and P4 after partial Cre-mediated recombination (*B*). The top band represents the ERβ floxed allele (300 bp) and the bottom band the WT allele (160 bp). PCR genotyping after complete Cre recombination was performed using P1, P2 and P4 primers to check correct recombination and removal of exon 3 in the offspring (*C*). The top bands represent the WT allele (984 bp and 423 bp) and the bottom band the deleted allele (195 bp). PCR genotyping after complete Cre recombination for the ERβ-Δex3 mice used for protein analysis presented in Fig. 3 is shown in (*D*). These ERβ-Δex3 mice are ERβ homozygous knockouts.

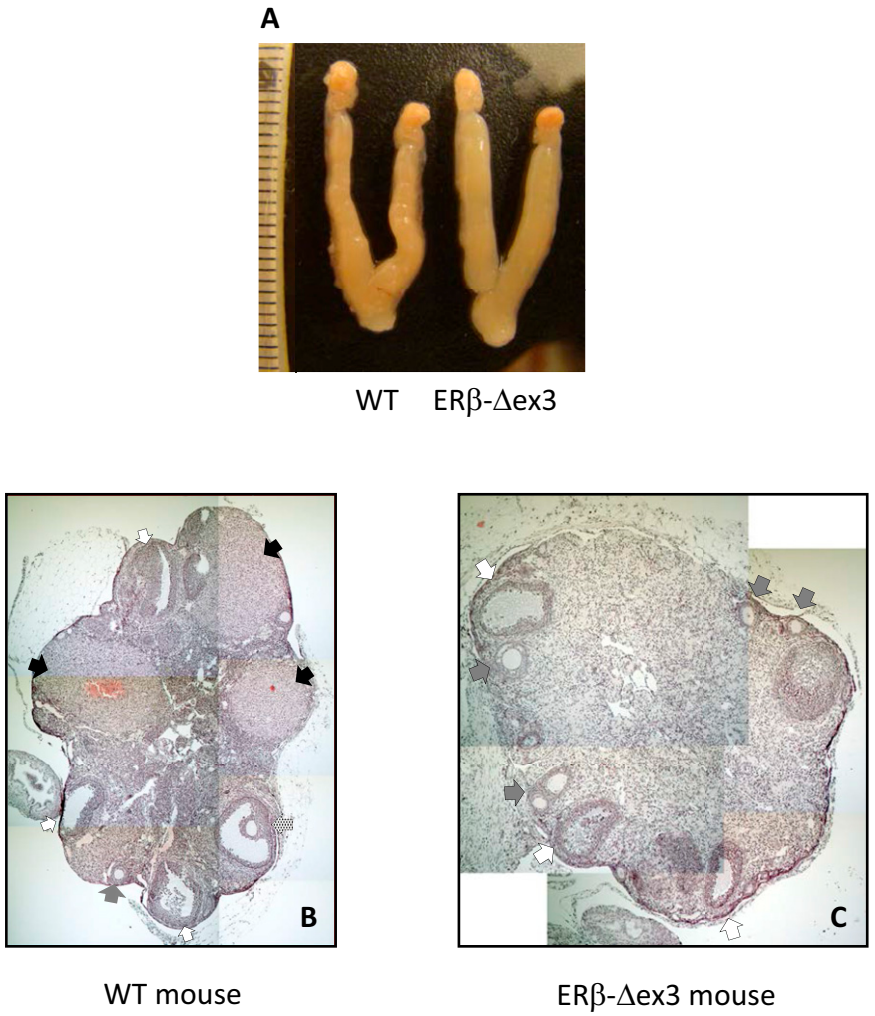

Fig. S2. Morphologic classification of growing follicles and corpora lutea in WT and ERβ-Δex3 ovaries. 7- to 8-mo-old ovary and uterus were collected and photographed (*A*). Ovarian cross-sections were stained with H&E and analyzed under light microscope. Growing follicles and corpora lutea were classified as described under Supplemental Materials and Methods. In WT mouse (*B*), there were many corpora lutea (black arrow) and follicles at different stages of growth. Healthy early antral follicles (gray arrow), healthy preovulatory follicles (dotted arrow), and atretic follicles (white arrow) are present. In the ERβ-Δex3 infertile mouse (*C*), there were many preantral follicles and a few early antral follicles (gray arrow). Most late antral follicles are atretic (white arrow). There was no corpus luteum. In conclusion, ERβ-Δex3 mouse ovaries display more atretic follicles and less corpora lutea than WT mice. There was no significant difference in the number of developing follicles between the WT and the mutant mice.

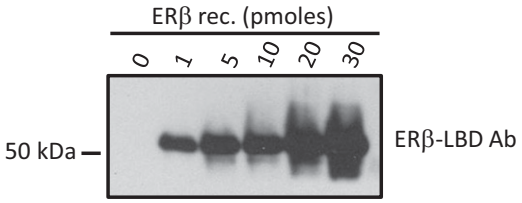

Fig. S3. Sensitivity of the ERβ–LBD antibody. The sensitivity of the ERβ–LBD antibody was assessed by loading increasing amounts of ERβ recombinant protein (ERβ rec.) on an SDS/PAGE and detecting it with an anti-ERβ–LBD antibody.

1 MSICASSHKDFSQLRPTQDMEIKNSPSSLTSPASYNCSQSILPLEHGPIYIPSSYVESRHEYSA
MTFYSPAVMNYSVPSSTGNLEGGPVRQTASPNVLWPTSGHLSPLATHCQSSLLYAEPQKSP
WCEARSLEHTLPVNRETLKRKLGGSGCASPVTSPSAKRDAHFCAVCSDYASGYHYGVWSCE
GCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMVKCGSRRERCGYRIV
RRQRSASEQVHCLNKAKRTSGHTPRVKELLLNSLSPEQLVLTLLEAEPPNVLVSRPSMPFTEAS
MMMSLTKLADKELVHMIGWAKKIPGFVELSLLDQVRLLESCWMEVLMVGLMWRSIDHPG
KLIFAPDLVLDRDEGKCVEGILEIFDMLLATTARFRELKLQHKEYLCVKAMILLNSSMYPLATAS
QEAESSRKLTHLLNAVTDALVWVISKSGISSQQQSVRLANLLMLLSHVRHISNKGMEHLLSM
KCKNVVPVYDLLLEMLNAHTLRGYKSSISGSECCSTEDSKSKEGSQNLQSQ 549

1-180: N-terminal sequence used to raise the ERβ N-terminal antibody (1-153 in human ERβ).
144-146: Trypsin cleavage site which could result in the WT mouse protein having the same molecular weight as the ERβ-Δex3 protein.
163-244: Exon3 (DNA binding domain) which was targeted for deletion.
282-517: Ligand binding domain (LBD) which was used to raise the ERβ-LBD antibody.

Fig. S4. Amino acid sequence of WT mouse ERβ. Full-length amino acid sequence of wild-type mouse ERβ is shown in capital letters (1–549). Protein sequences corresponding the DBD, LBD, and sequences used to raise the antibodies used in this study are indicated.

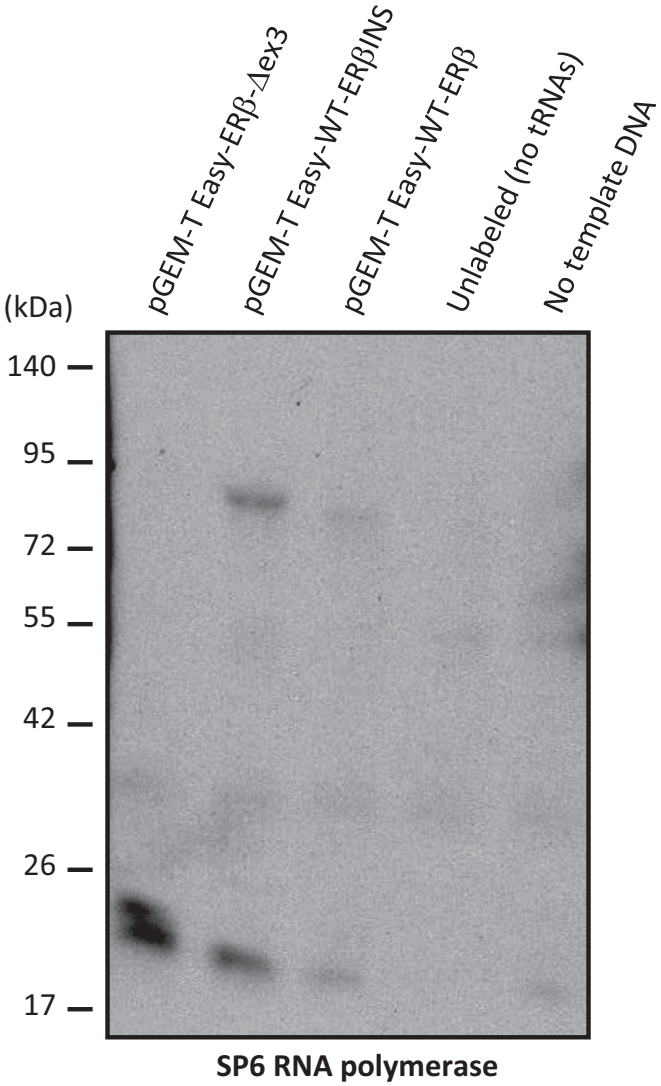

Fig. S5. In vitro transcription/translation of ERβ mRNA extracted from WT, WT-INS, or ERβ-Δex3 mouse ovaries. WT-ERβ, WT-ERβINS, and ERβ-Δex3 cDNAs were cloned into a pGEM-T Easy plasmid and transcribed and translated in vitro using the SP6 RNA polymerase, as described in *SI Materials and Methods*. As expected, the WT-ERβINS cDNA produces an in vitro protein presenting a slightly higher molecular weight than the WT-ERβ cDNA. In contrast, ERβ-Δex3 cDNA produces a shorter in vitro protein whose apparent molecular weight is around 20 kDa.

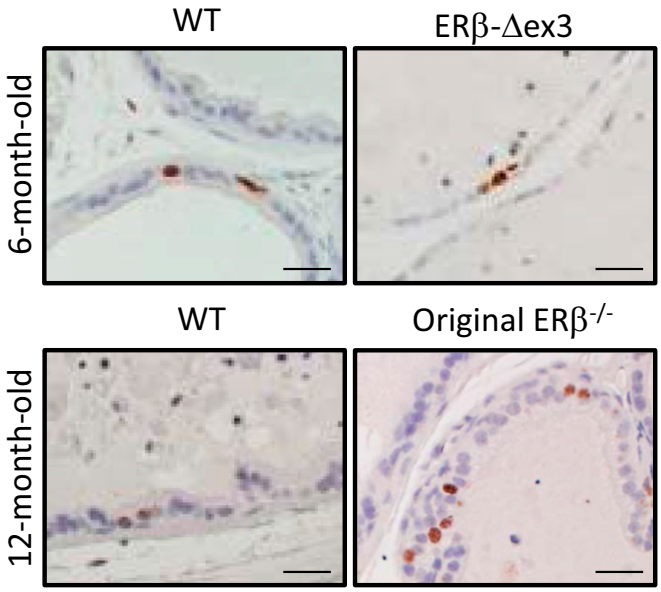

Fig. S6. Measurement of epithelial cellular proliferation by Ki67 staining in WT and ERβ-Δex3 mouse ventral prostates. Proliferation of epithelial cells located in actively secreting and inactive ventral prostate ducts was evaluated by Ki67 immunostaining. At 6 mo of age, the number of Ki67-positive cells was similar in WT and ERβ-Δex3 mice. In 12-mo-old mice, the proliferation rate of the prostatic epithelium is very low and few Ki67-positive cells were detectable. Ki67-positive staining was clearly higher in the original $ER\beta^{-/-}$ mouse VP. These data show that ERβ regulation of epithelial ventral prostate cellular proliferation does not involve the first zinc finger of the DBD. (Scale bar, 25 μm.)

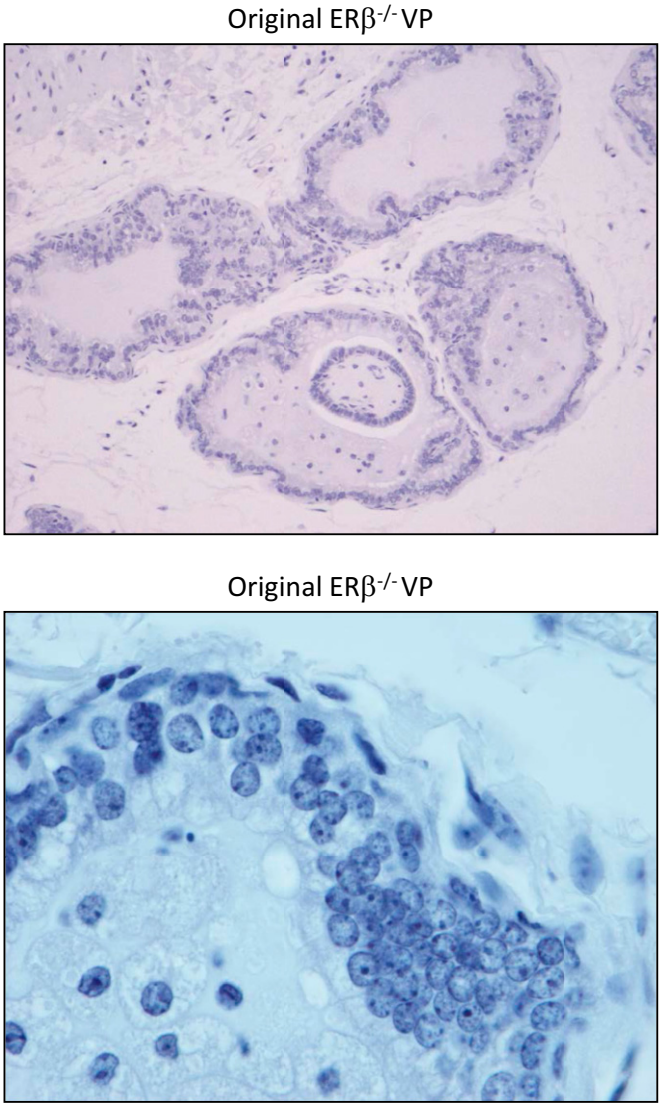

Fig. S7. Histological structure of original mouse ventral prostate. Representative sections of the ventral prostate collected from aged original ERβ$^{-/-}$ mice were stained with hematoxylin. In the original ERβ$^{-/-}$ mouse, the prostatic epithelium was disrupted and the ducts were filled with multiple layers of proliferative epithelial cells.

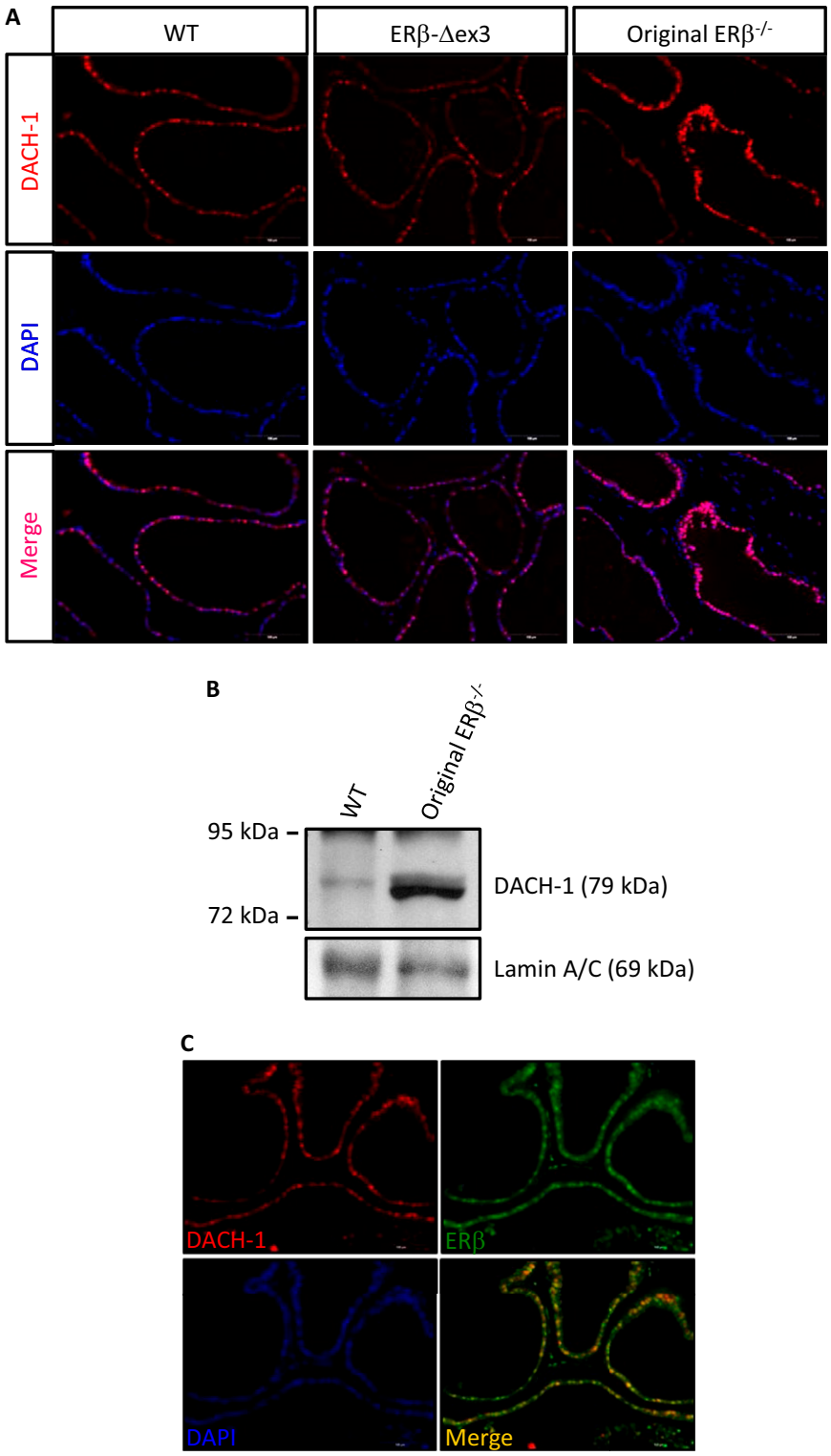

Fig. S8. Detection of DACH-1 and ERβ protein expression and colocalization in WT, ERβ-Δex3, and original ERβ$^{-/-}$ mouse ventral prostates by Western blot and immunofluorescence. The expression of DACH-1 was increased in the ventral prostate of 7- to 8-mo-old original ERβ$^{-/-}$ mice (*A*). In contrast, prostatic epithelium from WT and ERβ-Δex3 mice showed similar DACH-1 stainings. Indeed, no significant changes in the intensity or the number of positive cells were observed in the ERβ-Δex3 mouse ventral prostate compared with the littermate WT. DACH-1 protein was specifically expressed in the nuclei of the epithelial cells. (Scale bar, 100 μm.) Whole cell extracts of ventral prostate of 12-mo-old (WT) or 20-mo-old mice (original ERβ$^{-/-}$) were examined by Western blotting (*B*). Western blot analysis using DACH-1 antibody shows that DACH-1 expression is increased in the original ERβ$^{-/-}$ mouse ventral prostate extracts. The 7-mo-old WT ventral prostates were processed for double immunofluorescence, using anti–DACH-1 and anti-ERβ503 antibodies (*C*). Representative fluorescent images of ventral prostate stained with anti–DACH-1 antibody (red) and anti-ERβ (green) are shown. DAPI indicating cell nuclei is shown in blue. Merge panel represents overlapping images of DACH-1 and ERβ panels. Results show that DACH-1 and ERβ colocalize in the nuclei of the prostatic epithelium. (Scale bar, 100 μm.)

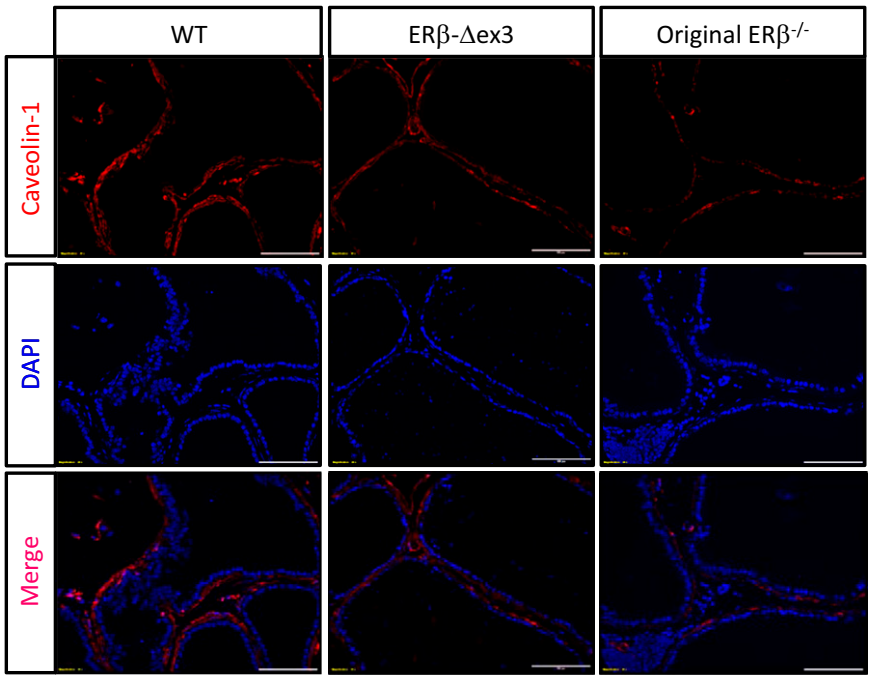

Fig. S9. Protein expression and localization of caveolin-1 in WT, ERβ-Δex3, and original $ER\beta^{-/-}$ mouse ventral prostates. The 9- to 12-mo-old WT, ERβ-Δex3, and original $ER\beta^{-/-}$ ventral prostates were processed for immunofluorescence, using anti–caveolin-1 antibody. The expression of caveolin-1 was strongly decreased in the ventral prostate of original $ER\beta^{-/-}$ mice. In contrast, only a slight decrease in the intensity or the number of positive cells was observed in the ERβ-Δex3 mouse ventral prostate compared with the littermate WT. Caveolin-1 protein was specifically expressed at the plasma membrane of the epithelial and stromal cells. Representative fluorescent images of ventral prostate stained with anti–caveolin-1 antibody (red) are shown. DAPI indicating cell nuclei is shown in blue and merge panel represents overlapping images of caveolin-1 and DAPI panels. (Scale bar, 100 μm.)