Supplementary Materials for

Elimination of the male reproductive tract in the female embryo is actively promoted by COUP-TFII

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Materials and Methods

Animals

Wt1^{CreERT2} knock-in mice on mixed genetic backgrounds (C57BL/6, Swiss Webster, and B6129SF1/J) were purchased from Jackson Laboratories (Bar Harbor, ME, Stock # 010912). Generation of the floxed *Coup-tfII* mice was described in our previous work (*5*). CD-1 mice were obtained from the established colony at NIEHS. Timed mating was produced by housing two females with a male. Vaginal plugs were checked daily and the day when the vaginal plug was found was designated as embryonic day E0.5. All animal procedures were approved by the National Institute of Environmental Health Sciences (NIEHS) Animal Care and Use Committee and are in compliance with a NIEHS-approved animal study proposal and public laws.

Tamoxifen treatment

In the *Wt1*^{CreERT2}*; Coup-tfII*$^{f/f}$ mouse model, CreER^{T2} activity in the fetal embryos was induced by intraperitoneal injection of tamoxifen (T-5648, Sigma) at the dose of 2 mg/10 g body weight per dam once daily on E11.5 and E12.5. For the vehicle control, an equivalent volume of corn oil was injected. The *Wt1*$^{Cre-}$*; Coup-tfII*$^{f/f}$ and *Wt1*$^{Cre+}$*; Coup-tfII*$^{f/f}$ embryos were designated as the control and knockout, respectively. Gonads and mesonephroi were collected on E14.5, E16.5 or E18.5.

Flutamide treatment

Flutamide (250 mg, Sigma, catalog #: F9397) was dissolved in 1 ml ethanol (200 proof), then mixed with 9 ml corn oil, and incubated at 60°C for 2 hours with periodic vortexing. Dams were injected at the dose of 100 mg/kg body weight or an equivalent volume of vehicle once daily from E12.5-E18.5. The dams were sacrificed at E18.5.

Anogenital distance measurement

The anogenital distance (from the base of the genital tubercle to ventral edge of the anus) of E18.5 embryos was measured using a 4" pocket digital caliper under the dissecting microscope.

Immunofluorescence of whole mount tissues and sections

Mesonephros and gonad complexes were fixed in 4% paraformaldehyde at 4°C overnight. Briefly, for whole mount immunofluorescence, fixed tissue was washed three times in PBST (1xPBS with 0.1% Triton X-100) and incubated with blocking solution (5% Donkey serum in PBST) for 1 hour. The tissue was then washed in PBST and incubated with primary antibody in blocking solution overnight at 4°C. After washing three times in PBST, the tissue was incubated with 2nd antibodies, washed, mounted with DAPI mounting reagent and cover slipped for imaging. For immunofluorescence of frozen sections, tissues were dehydrated, embedded, and cyrosectioned at 10 μm. For paraffin sections, tissues were processed, embedded in paraffin, sectioned at 5 μm, deparaffinized, and rehydrated. The sections were treated for antigen retrieval using commercial antigen unmasking solution (H-3300, VECTOR) and underwent immunostaining procedures. The following primary antibodies were used: chicken anti-βGAL (1:500, ab9361, Abcam), rabbit anti-PAX2 (1:200, PRB-276P, Covance), mouse

anti-COUP-TFII (1:300, PP-H7147-00, R&D systems), rabbit anti-WT1 (1:300, ab15249, Abcam), mouse anti-AP-2α (1:50, 3B5, DSHB). The secondary antibodies conjugated with different fluorescent dyes were used (1:200): Cy™3 donkey anti-chicken IgY (Jackson ImmunoResearch), Alexa Fluor@ 488 and 568 donkey anti-mouse IgG, Alexa Fluor@ 488 and 568 donkey anti-rabbit IgG (Invitrogen). All the results were imaged under a Leica DMI4000 confocal microscope.

Immunohistochemistry

Frozen or paraffin sections were treated for antigen retrieval using commercial antigen unmasking solution (H-3300, VECTOR). Endogenous peroxidase was inactivated with 3% H_2O_2 (H325, Fisher Scientific). Sections were incubated with the blocking reagent and followed with primary antibody mouse anti-p-ERK (M8159, Sigma), or rabbit anti-p-AKT (4060, Cell Signaling) at 4 °C overnight. Sections were washed three times and then incubated with biotinylated donkey anti-mouse (BA-9200, VECTOR) or anti-rabbit secondary antibody (#94583, Jackson ImmunoResearch) for 30 min at room temperature. Sections were then incubated with ABComplex/HRP (Vectastain ABC kit, VECTOR) and DAB substrate (SK4100, VECTOR), counterstained with hematoxylin (HHS16, Sigma), and mounted for imaging.

Real-time PCR

Gonads and mesonephroi were collected on E14.5 and E16.5 and snap-frozen. RNA extraction and first strand cDNA synthesis from 150 ng RNA were performed using PicoPure RNA Isolation kit (Life technologies, USA) and the Superscript II cDNA synthesis kit (Invitrogen, USA), respectively, according to manufacturer's protocols. Taqman gene-expression probes (*Coup-tfII, Mm00772789_m1; Ar, Mm00442688_m1; Cyp17a1, Mm00484040_m1; Hsd3b1, Mm01261921_mH; Gapdh, mm99999915_g1; Slc26a3, Mm00445313_ml; Folh1, Mm0089655_ml)* and SYBR primers (Table S2) were used to run thermal cycles in the Bio-Rad CFX96 Real-Time PCR Detection system. All samples were analyzed in duplicates and normalized to the housekeeping gene *Gapdh*. The relative expression was reported as a ratio of the expression of genes in knockout mice relative to those of wild type mice.

Microarray analysis

Gene expression analysis was conducted using Affymetrix Mouse Genome 430 2.0 GeneChip® arrays (Affymetrix, Santa Clara, CA). One hundred ng of total RNA was amplified as instructed in the Affymetrix 3' IVT Express kit protocol. Amplified biotin-aRNAs (12.5 μg) were fragmented and 10 μg were hybridized to each array for 16 hours at 45°C in a rotating hybridization oven using the Affymetrix Eukaryotic Target Hybridization Controls and protocol. Array slides were stained with streptavidin/phycoerythrin utilizing a double-antibody staining procedure and then washed for antibody amplification according to the GeneChip Hybridization, Wash and Stain Kit and user manual. Arrays were scanned in an Affymetrix Scanner 3000 and data were obtained using the GeneChip® Expression Console Software (AGCC; Version 1.2) and the MAS5 algorithm to generate .CHP files. The resulting data were processed using Partek Genomic Suite Software.

Ex vivo organ culture

The organs were cultured at 37°C with 5% CO_2/95% air on MilliCELL-CM culture plate insert 0.4 μm filters (Millipore) in Dulbecco's Minimal Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal calf serum (Hyclone), and 100 U/ml Penicillin-Streptomycin. A series of culture were performed:

1) For postnatal culture, E18.5 control and knockout XX mesonephroi with ovaries attached were cultured for 7 days. After 7-day culture, reproductive tracts were collected for imaging and immunofluorescence for the Wolffian duct marker AP-2α.

2) E14.5 control and knockout XX mesonephroi with or without ovaries from the *Wt1*^{CreERT2}*; Coup-tfII*$^{f/f}$ mouse model were cultured for 4 days.

3) E14.5 wild type XX mesonephroi with ovaries attached were cultured with 250 ng/ml of FGF7 (R&D systems, Catalog #: 5029-KG-025), 1000 ng/ml FGF10 (R&D systems, Catalog #: 707-MF-050), FGF7 (250 ng/ml) plus FGF10 (1000 ng/ml), or an equivalent volume of vehicle (PBS with 1% BSA) for 2 days.

4) E14.5 knockout XX mesonephroi were cultured in the presence of an equivalent volume of vehicle (DMSO), or p-ERK inhibitor PD0325901 (2 μM, SellekChem) for 2 days.

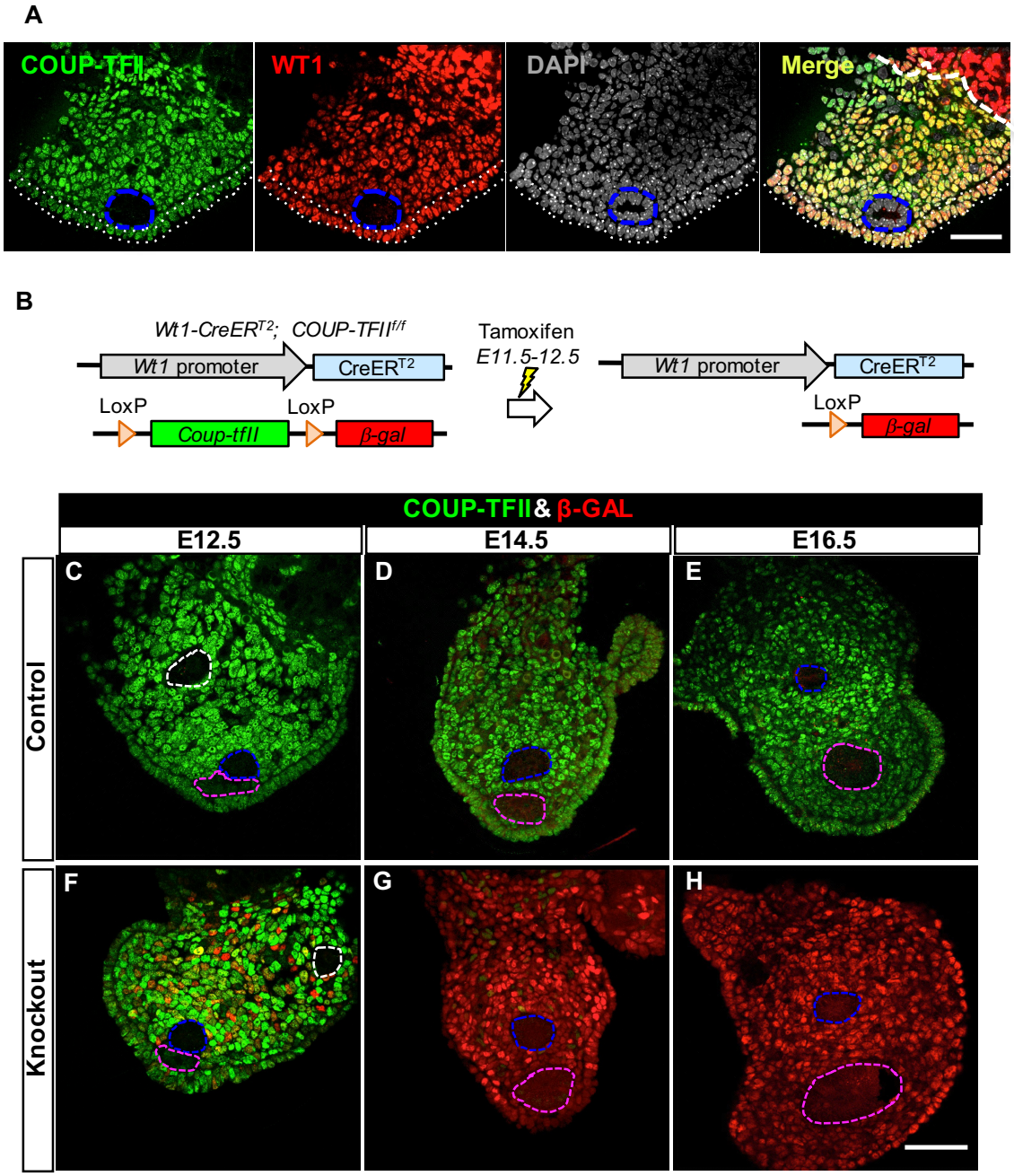
5) For XY mesonephroi culture, we cultured E12.5 XY mesonephroi without testis for 5 days.

For culture experiments (2-5) cultures, the presence of the Wolffian duct was identified by whole mount PAX2 immunofluorescence. Media was changed every other day for 1), 2) and 5) cultures, and daily for 3) and 4).

Statistical analyses

Quantitative data is presented as mean ± SEM. Sample sizes and statistical tests were indicated in the figure legends. The significant level was set at $p<0.05$.

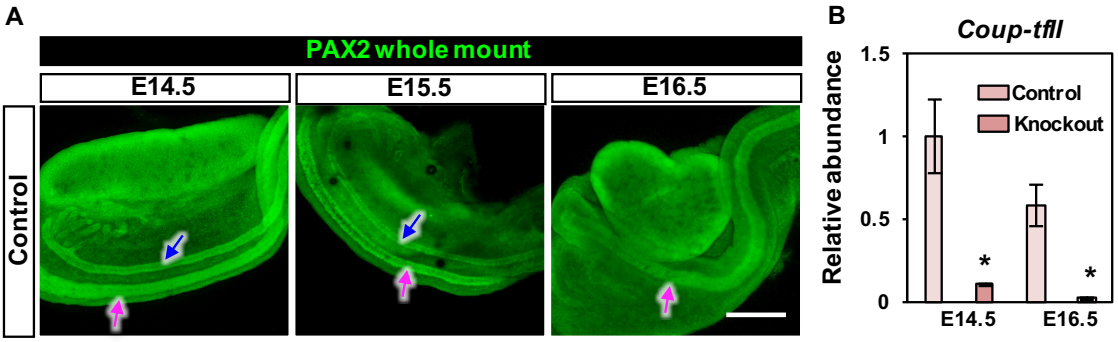
Fig. S1: COUP-TFII expression in the mesonephros and its ablation in the tamoxifen-induced *Wt1*^{CreERT2}; *Coup-tfII*$^{f/f}$ mouse model.



(A) Detection of COUP-TFII (green), WT1 (red), DAP counterstain (grey), and merged channels (yellow) by immunofluorescence in the mesenchyme of E12.5 XX mesonephros. The thick white dash line indicates the boundary between mesonephros and ovary and the thin dotted line indicates the coelomic epithelium. (B) The scheme illustrates the tamoxifen-induced *Wt1*^{CreERT2}; *Coup-tfII*$^{f/f}$ mouse model. (C-H) Double immunofluorescence of COUP-TFII (Green) and β-GAL (red) in the mesonephroi of control (C-E) and *Coup-tfII* knockout (F-H) XX embryos at E12.5, 14.5, and 16.5. N=3

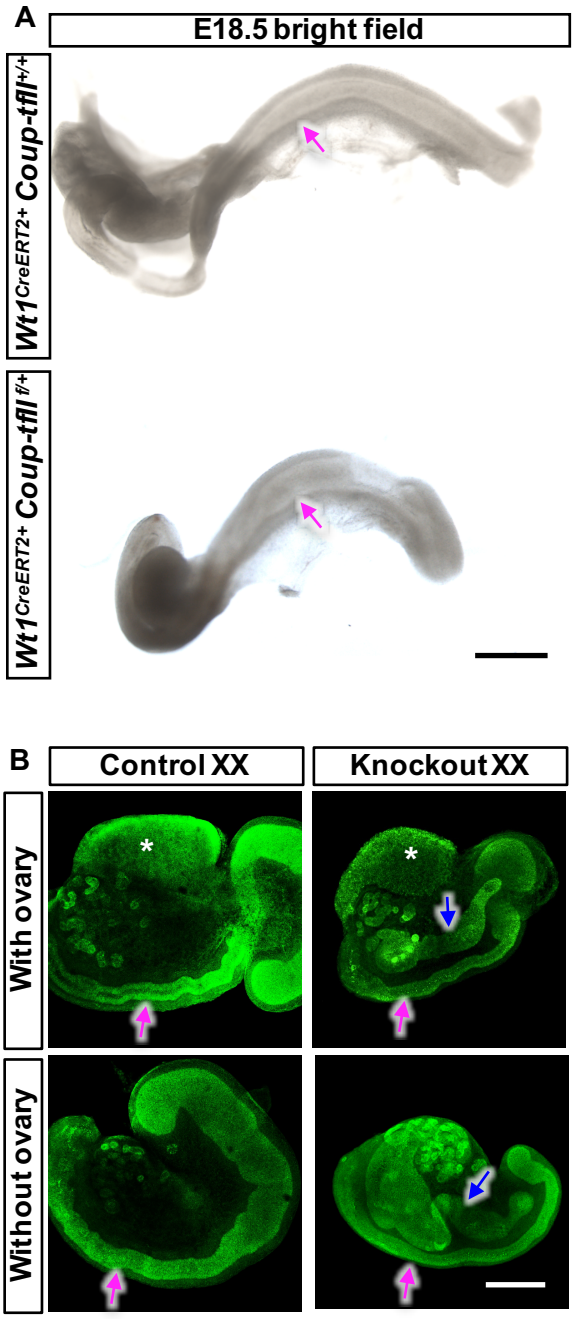
per group in A and C-H. The blue, magenta and white dotted circles indicate Wolffian ducts (remnants at E16.5), Müllerian ducts, and mesonephric tubules, respectively. Scale bar: 50 μm.

Fig. S2: Time-course analysis of Wolffian duct regression and *Coup-tfII* mRNA expression in the XX embryo.



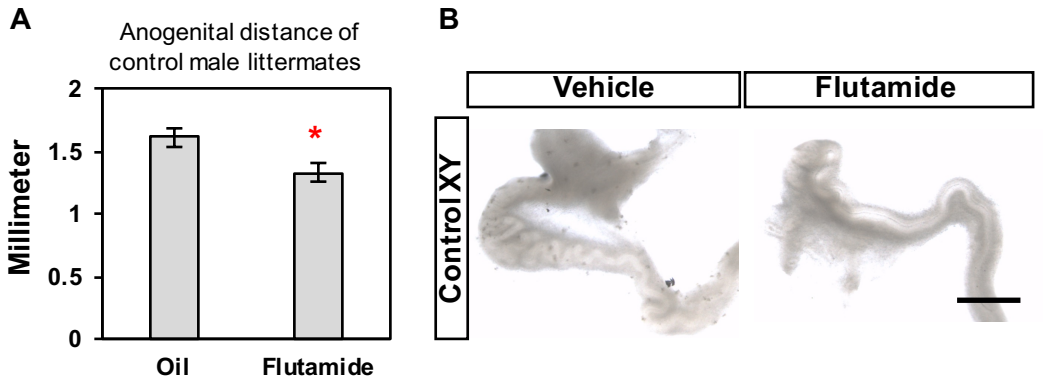
(A) PAX2 whole mount immunofluorescence of control XX embryos at E14.5, 15.5, and 16.5. Blue arrows: Wolffian ducts; Magenta arrows: Müllerian ducts. Scale bar: 0.25 mm. N=3 per time point. (B) mRNA expression of *Coup-tfII* in control and knockout XX mesonephroi at E14.5 and E16.5. Asterisks represent statistical significance of $p<0.05$ compared to control XX samples using Student's t test (N=8 for each group). Results are shown as mean ± SEM.

Fig. S3: Female reproductive tract development in *Wt1*$^{CreERT2+}$ *Coup-tfII*$^{+/+}$ or *Wt1*$^{CreERT2+}$ *Coup-tfII*$^{f/+}$ mice and ex vivo organ culture of E14.5 XX mesonephroi with or without ovaries.



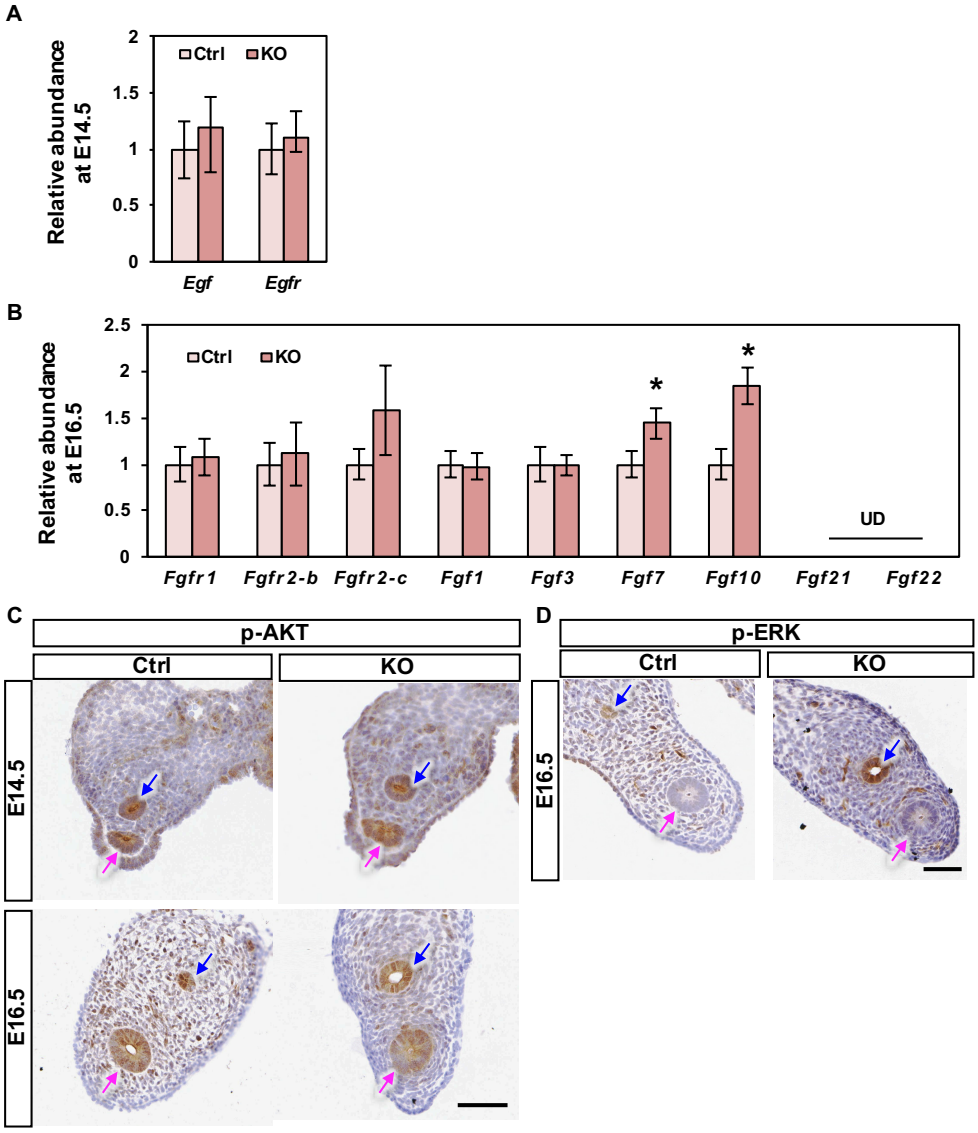
(A) Bright field images of the reproductive tracts in the *Wt1*$^{CreERT2+}$ *Coup-tfII*$^{+/+}$ or *Wt1*$^{CreERT2+}$ *Coup-tfII*$^{f/+}$ XX embryos at E18.5. Magenta arrows: Müllerian ducts. N=6 for *Wt1*$^{CreERT2+}$ *Coup-tfII*$^{+/+}$ and N=10 for *Wt1*$^{CreERT2+}$ *Coup-tfII*$^{f/+}$ genotype. (B) PAX2 whole mount immunofluorescence of control and knockout XX mesonephroi with or without ovaries after 4-day ex vivo culture. Blue arrows: Wolffian ducts; Magenta arrows: Müllerian ducts. N=9 for each genotype. Scale bar: 0.5 mm.

Fig. S4: Efficacy of the flutamide treatment.



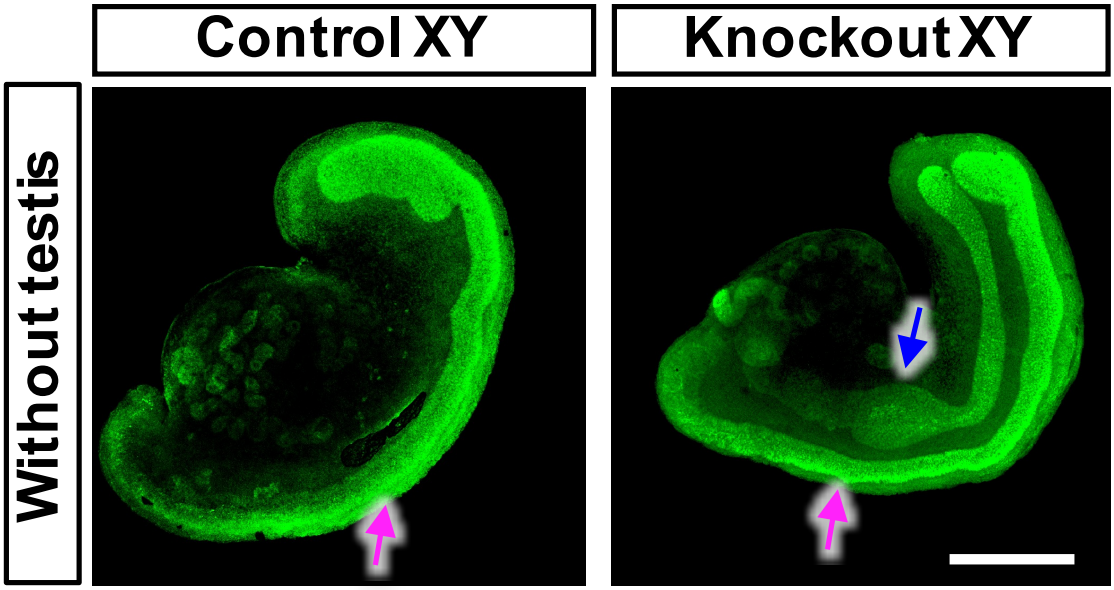
The efficacy of flutamide was verified by the reduction of anogenital distance (A) and epididymal coiling (B) in the wild type male compared to the vehicle-treated male**.** Asterisks represent statistical significance of $p<0.05$ compared to control females using Student's t test (N=11 for vehicle-treated WT males, N=5 for flutamide-treated WT males). Results in A are shown as mean ± SEM. Scale bar in B: 1 mm.

Fig. S5: Changes of *Egf*, *Fgf*, and *Fgf* receptor mRNA expression and p-AKT and p-ERK proteins in control and knockout XX mesonephroi.



(A) Relative mRNA expression of *Egf* and its receptor *Egfr* at E14.5. N=4 per genotype. (B) Relative mRNA expression of FGF receptors and FGF ligands for epithelial FGFR2 at E16.5. UD: undetected. Results are shown as mean ± SEM. Asterisks represent statistical significance of $p<0.05$ compared to control females using Student's t test (N=8 for each genotype). (C) Immunohistochemistry of p-AKT in the mesonephroi at E14.5 and E16.5. (D) Immunohistochemistry of p-ERK in the mesonephroi at E16.5. Scale bars C & D: 50 μm. N=3 for each group in C & D. Blue arrows: Wolffian ducts; Magenta arrows: Müllerian ducts.

Fig. S6: Organ culture of E12.5 XY mesonephroi without testis



PAX2 whole mount immunofluorescence of XY mesonephroi after a 5-day culture. Blue arrows: Wolffian ducts; Magenta arrows: Müllerian ducts. N=3 for each genotype. Scale bar: 0.5 mm.

Table S1: A list of genes that were differentially expressed in the knockout ovary compared to the control ovary at E16.5.

Gene	**Full name**	**Fold change (KO/WT)**
Nr2f2	Coup-TFII, nuclear receptor subfamily 2, group F, member 2	-5.96009
Ajap1	adherens junction associated protein 1	-3.1997
Wnt5a	wingless-related MMTV integration site 5A	-2.70982
Ajap1	adherens junction associated protein 1	-2.6044
Slc6a7	solute carrier family 6 (neurotransmitter transporter, L-proline), member 7	-1.89055
Lsp1	lymphocyte specific 1	-1.58917
B4galt6	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6	1.53885
Jmjd7 /// Pla2g4b	jumonji domain containing 7 /// phospholipase A2, group IVB (cytosolic)	2.00401
Masp1	mannan-binding lectin serine peptidase 1	2.2632
Ccng1	cyclin G1	2.37517
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	2.78257
Pvt1	plasmacytoma variant translocation 1	3.40263

Table S2: Primers used for real time PCR analyses

Primer	**Sequence**
Fgfr2-b F	AACGGTCACCACACCGGC
Fgfr2-b R	AGGCAGACTGGTTGGCCTG
Fgfr2-c F	AACGGTCACCACACCGGC
Fgfr2-c R	TGGCAGAACTGTCAAC
Fgfr2-F	AATCTCCCAACCAGAAGCGTA
Fgfr2-R	CTCCCCAATAAGCACTGTCCT
Fgfr1-F	TAATACCACCGACAAGGAAATGG
Fgfr1-R	TGATGGGAGAGTCCGATAGAGT
Fgf1-F	CAGCTCAGTGCGGAAAGTG
Fgf1-R	TGTCTGCGAGCCGTATAAAAG
Fgf3-F	TACAACGCAGAGTGTGAGTTTG
Fgf3-R	CACCGACACGTACCAAGGTC
Fgf7-F	CTCTACAGGTCATGCTTCCACC
Fgf7-R	ACAGAACAGTCTTCTCACCCT
Fgf10-F	TTTGGTGTCTTCGTTCCCTGT
Fgf10-R	TAGCTCCGCACATGCCTTC
Fgf21-F	CTGCTGGGGGTCTACCAAG
Fgf21-R	CTGCGCCTACCACTGTTCC
Fgf22-R	CCAGGACAGTATAGTGGAGATCC
Fgf22-R	AGTAGACCCGCGACCCATAG

Gapdh-F	*TTCACCACCATGGAGAAGGC*
Gapdh-R	*GGCATGGACTGTGGTCATGA*