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

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Abstract

The sexual differentiation paradigm contends that the female pattern of the reproductive system is established by default, as the male reproductive tracts (Wolffian ducts) in the female degenerate due to a lack of androgen. Here, we discovered that female mouse embryos lacking *Coup-tfII* in the Wolffian duct mesenchyme became intersex that contained both female and male reproductive tracts. Retention of Wolffian ducts was not caused by ectopic androgen production or action. Instead, enhanced p-ERK signaling in Wolffian duct epithelium was responsible for the retention in an androgen-independent manner. These results support our hypothesis that elimination of Wolffian ducts in female embryos is actively promoted by COUP-TFII, which suppresses a mesenchyme-epithelium crosstalk responsible for Wolffian duct maintenance.

Sexually dimorphic establishment of reproductive tracts epitomizes the anatomical difference between males and females. This dimorphic establishment depends upon two concurrent events during embryogenesis: regression of one of the two primitive ducts (Müllerian and Wolffian ducts) and maintenance of the other. These two events ensure that the embryo retains only one reproductive tract that corresponds to its sex: Müllerian duct for the XX individual and Wolffian duct for the XY individual (1). In the1950s, Alfred Jost provided the first evidence for what became the foundation of the sexual differentiation paradigm: XY embryos retain Wolffian ducts through the action of testis-derived androgen whereas XX embryos lose Wolffian ducts as a result of a lack of androgens (2, 3).

The action of androgen on the Wolffian duct is mediated through androgen receptors in the mesenchyme surrounding Wolffian ducts (4, 5). It is well established that mesenchyme-derived factors govern the fate and differentiation of ductal epithelium (6). We identify the orphan nuclear receptor COUP-TFII (Chicken ovalbumin upstream promoter transcription factor II or NR2F2) as a mesenchyme-specific regulator in many developing organs,

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including the mesonephros where Wolffian ducts develop (7). COUP-TFII expression in Wolffian duct mesenchyme overlapped with Wilms' Tumor 1 (WT1; Fig. S1A), another mesenchyme-specific transcriptional factor (8). To investigate the role of COUP-TFII in Wolffian duct regression, we utilized the tamoxifen-inducible $Wt1^{CreERT2}$ mouse model that targeted $Coup$-$tfII$ deletion in $Wt1$+ mesenchymal cells (Fig. S1B). In the control ($Wt1^{CreERT2-}$; $Coup$-$tfII^{f/f}$) female, COUP-TFII remained in the mesenchymal cells of mesonephroi from E12.5 to E16.5 (Fig. S1, C–E), the developmental window that encompassed initiation (E14.5) and completion (E16.5) of Wolffian duct regression in XX embryos (Fig. 2SA). In the knockout ($Wt1^{CreERT2+}$;$Coup$-$tfII^{f/f}$) female, COUP-TFII ablation began twenty-four hours after the first tamoxifen injection (Fig. S1F) and completed by E14.5 (Figs. S1, G and H). Ablation of $Coup$-$tfII$ was further confirmed by RT-PCR (Fig. S2B). These results demonstrated an efficient ablation of $Coup$-$tfII$ in WT1-positive Wolffian duct mesenchyme in XX embryos.

The impact of $Coup$-$tfII$ ablation on XX mesonephroi was first examined at E18.5, when dimorphic development of reproductive tracts is completed. The control XX embryos contained only Müllerian ducts that were visualized by immunostaining of the epithelial marker PAX2 (Fig. 1A). Other control genotypes including $Wt1^{CreERT2+}$; $Coup$-$tfII^{f/+}$ and $Wt1^{CreERT2+}$; $Coup$-$tfII^{+/+}$ female embryos also developed normally with only Müllerian ducts (Fig. S3A). Knockout XX littermates, however, had both Müllerian and Wolffian ducts in the mesonephros (Fig. 1D). The identity of the Wolffian duct was confirmed by the presence of Wolffian duct epithelium marker AP-2α (5) (Fig. 1, B and E). $Coup$-$tfII$ knockout embryos died soon after birth. We therefor developed an organ culture system that allowed us to maintain E18.5 XX mesonephros with ovaries for 7 days to investigate whether Wolffian ducts remained present postnatally. At the end of culture, Wolffian ducts were still present in knockout tissues along with the components of female reproductive tracts (Fig. 1, C & F), indicating that Wolffian duct maintenance in knockout XX persisted after birth and was not a transient event.

Retention of Wolffian ducts in the $Coup$-$tfII$ knockout XX embryo points to a possible action of androgens based on the Jost paradigm (9). $Wt1^{CreERT2}$ targets $Coup$-$tfII$ deletion not only in mesonephroi but also somatic cells of the ovary (10), raising the possibility that the $Coup$-$tfII$ knockout ovary could synthesize androgens ectopically. We compared the transcriptomes between control and knockout ovaries at E14.5 and E16.5 during which Wolffian duct regression occurs. The transcriptome of the knockout ovary was not significantly different from the control ovary with the exception of 10 differentially expressed genes (including $Coup$-$tfII$) (Table S1). None of these genes were associated with androgen production. Besides, mRNA expression of two rate-limiting enzymes hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1($Hsd3b1$) and cytochrome P450 17A1 ($Cyp17a1$) for steroidogenesis was not different between control and knockout ovaries, and nearly undetectable compared to the wild-type fetal testis (a positive control; Fig. 2, A and B). A lack of androgen-producing capacity in the ovary was corroborated with unchanged anogenital distance (AGD), an androgen-sensitive parameter, between control and knockout XX at E18.5 (Fig. 2C). To exclude the possibility that androgens came from other resources in the knockout embryo, we removed the mesonephroi from XX embryos and cultured them for 4 days. After culture, Wolffian ducts regressed in control XX mesonephros as expected

whereas in knockout XX, either in the presence or absence of ovaries, Wolffian ducts were maintained (Fig. 2D and fig. S3B). These results indicated a lack of androgen production in the XX knockout ovaries, and led us to speculate that Wolffian duct retention in XX embryos could be due to ectopic activation of the androgen pathway in the absence of *Coup-tfII*. This possibility was excluded based on the finding that expression of androgen receptor (*Ar*) and two androgen-induced genes, folate hydrolase 1 (*Folh1*) and solute carrier family 26 member 3 (*Slc26a3*) (11, 12), was not different between control and knockout XX mesonephroi (Fig. 2, E–G). To rule out conclusively the involvement of androgens, we exposed the dam that carried control and knockout embryos to the androgen antagonist flutamide (13) (Fig. 2H). This regimen was sufficient to prevent Wolffian duct maintenance resulting from ectopic androgen action in XX embryos (14). Despite the verified action of flutamide (Fig. S4), Wolffian ducts were still retained in knockout XX embryos (Fig. 2H). Taken together, we conclude that the maintenance of Wolffian ducts in the *Coup-tfII* knockout XX embryo is not due to ectopic production or action of androgens.

To identify the androgen-independent mechanism underlying Wolffian duct retention in the *Coup-tfII* knockout female, we turned our attention to epidermal growth factor (EGF) and fibroblast growth factor (FGF) signaling pathways for their putative ability to promote Wolffian duct maintenance (15, 16). We first examined the expression of *Egf* and its receptor *Egfr* by RT-PCR. Their expression was not different between control and knockout XX mesonephroi (Fig. S5A). Components of FGF signaling, in contrast, exhibited unique changes in the absence of *Coup-tfII*. FGFR2 is the major FGF receptor in the Wolffian duct epithelium (17), and its binding ligands include FGF1, 3, 7, 10, 21, and 22 (18). mRNA expression of FGF receptors (*Fgfr1, Fgfr2-b*, and *Fgfr2-c*) and most ligands were unaltered (*Fgf1* and *Fgf3*) or undetectable (*Fgf21* and *Fgf22*) in knockout XX mesonephroi compared to the control (Fig. 3A). However, expression of *Fgf7 and Fgf10* was increased significantly in knockout XX mesonephroi at E14.5 (Fig. 3A) and E16.5 (Fig. S5B). To investigate whether FGF7 and FGF10 were capable of reproducing the Wolffian duct maintenance phenotype in the wild type female, we cultured E14.5 wild-type XX mesonephroi for 2 days in the presence of vehicle, FGF7, FGF10, or FGF7+FGF10. In the vehicle-treated group, Wolffian ducts regressed after 2-day culture, similar to the in vivo situation. In contrast, presence of FGF7, FGF10 or FGF7+FGF10 maintained the Wolffian duct in the wild-type XX mesonephroi (Fig. 3B).

FGF7 and FGF10 are expressed in the mesonephric mesenchyme, the same cellular compartment as COUP-TFII (19, 20). These FGFs bind FGFR2 in Wolffian duct epithelium and activate two intracellular signaling components, phosphorylated protein kinase B (p-AKT) and phosphorylated extracellular signal-regulated kinase (p-ERK) (18). Loss of *Coup-tfII* did not change p-AKT activation in Wolffian ducts (Fig. S5C). The presence of p-ERK, conversely, became detected in Wolffian duct epithelium of knockout XX compared to the control XX at E14.5 (Fig. 3C) and E16.5 (Fig. S5D). These results indicate that loss of mesenchymal *Coup-tfII* led to an enhanced activity of p-ERK signaling in Wolffian duct epithelium. We then tested whether elevated p-ERK signaling was the cause of Wolffian duct maintenance by culturing the *Coup-tfII* knockout mesonephroi with a p-ERK specific inhibitor PD0325901 (21). In the vehicle-treated group, Wolffian ducts in the knockout XX were maintained in culture. On the other hand, presence of the p-ERK inhibitor eliminated

Wolffian ducts in the knockout XX mesonephroi (Fig. 3D), indicating that enhanced p-ERK signaling was involved in Wolffian duct retention in the *Coup-tfII* knockout XX embryo.

Instead of a passive process occurring as a result of the absence of androgens, elimination of the male reproductive tract in the female embryo is actively promoted by COUP-TFII through its action in the Wolffian duct mesenchyme. COUP-TFII in the mesenchyme inhibits expression of FGFs, which otherwise activate the p-ERK pathway in the Wolffian duct epithelium for its maintenance. The function of COUP-TFII in facilitating Wolffian duct elimination is not restricted to XX embryos: when the testis was removed from the *Coup-tfII* knockout XY mesonephros, Wolffian ducts remained present despite of a lack of androgens (Fig. S6). These findings reveal unexpected mechanisms underlying the dimorphic development of the Wolffian ducts via COUP-TFII. In addition, maintenance of male reproductive tracts without androgens prompts a reassessment of the role of androgens in this process, which presumably is to antagonize the action of COUP-TFII.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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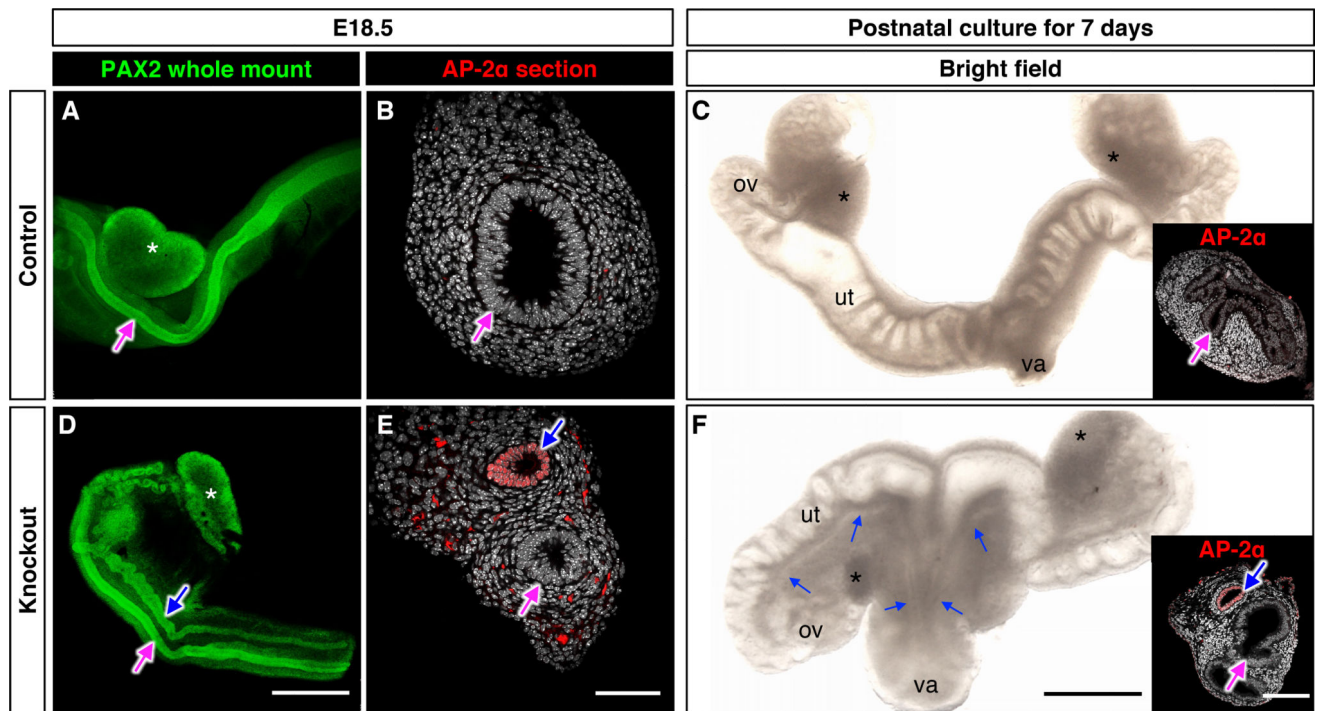
Fig. 1. *Coup-tfII* **ablation leads to Wolffian duct retention in the XX embryo**
Control and *Coup-tfII* knockout reproductive systems from E18.5 XX embryos were
analyzed by whole mount immunofluorescence for the epithelial marker PAX2 (A & D) or
AP-2a on frozen sections (B & E). Control and knockout mesonephroi from E18.5 XX
embryos were cultured for 7 days and analyzed by bright field microscopy (C & F) or AP-2a
immunofluorescence on frozen sections (insets in C & F). Blue arrows: Wolffian ducts;
Magenta arrows: Müllerian ducts; White asterisk: ovary; ov= ovary; ut= uterus; va= vagina.
Scales bars: 0.5 mm (A & D, C & F) and 50 μm (B, E and insets in C & F). N=14 in A; N=3
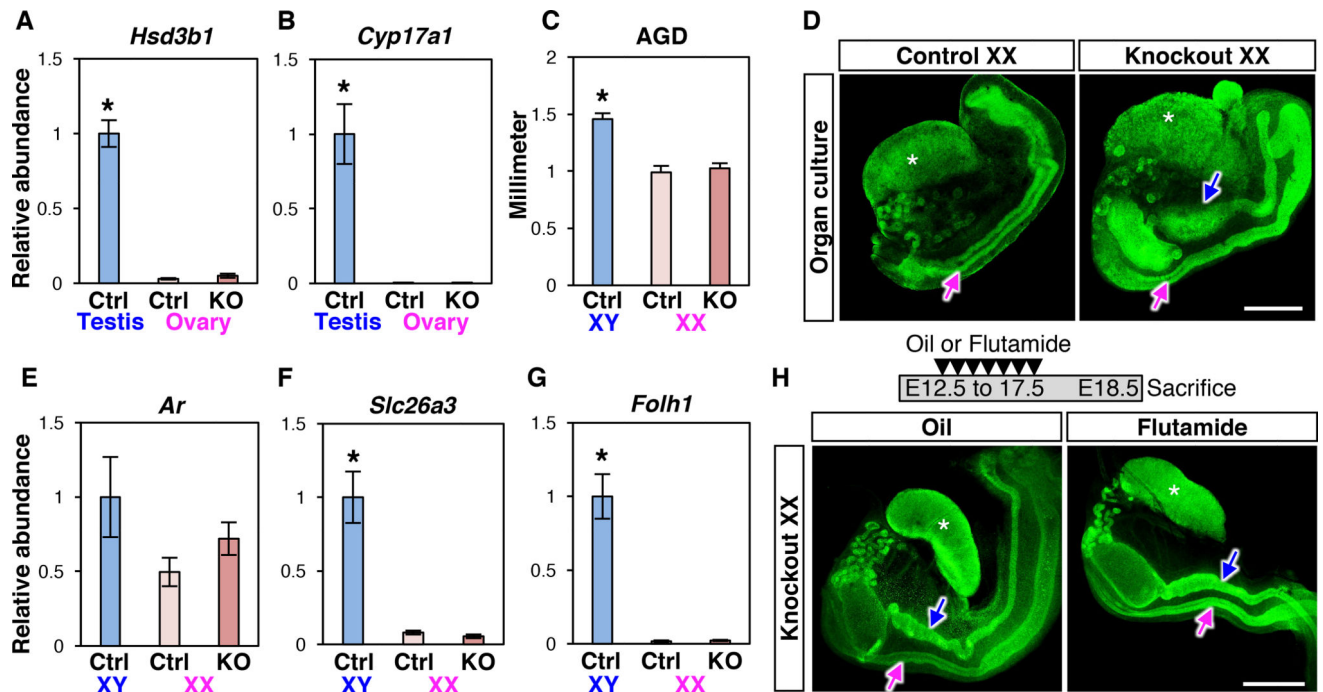per each genotype in B, C, E, F, and N=23 in D).

Fig. 2. Wolffian duct retention in *Coup-tfII* knockout XX embryo is independent of androgen production or action

(A & B) mRNA expression of two rate-limiting steroidogenic enzymes, *Hsd3b1* and *Cyp17a1*, in E14.5 control testis (light blue), control ovary (light pink), and knockout ovary (dark pink). (C) Anogenital distance (AGD) of the control and knockout embryos at E18.5. (D) PAX2 whole mount immunofluorescence of ovaries and mesonephroi after 4-day culture. N=7 per genotype. (E–G) mRNA expression of androgen receptor *Ar* and androgen-responsive genes (*Slc26a3* and *Folh1*) in control XY, control XX, and knockout XX mesonephroi. Results are shown as mean ± SEM. Asterisks in A–C, F and G represent statistical significance of p<0.05 compared to either control or knockout XX samples by one-way ANOVA followed by Tukey's test (N=8 per each group in A–B, N=8–11 in C; and N=8 in E–G). (H) Knockout XX embryos were exposed to either vehicle (oil) or androgen receptor antagonist flutamide in utero once daily from E12.5 to 17.5. Samples were collected at E18.5 and analyzed by PAX2 whole mount immunofluorescence. N=3 pe r genotype. Blue arrows: Wolffian ducts; Magenta arrows: Müllerian ducts. White asterisk: ovary. Scale bar in D & H: 0.5 mm.
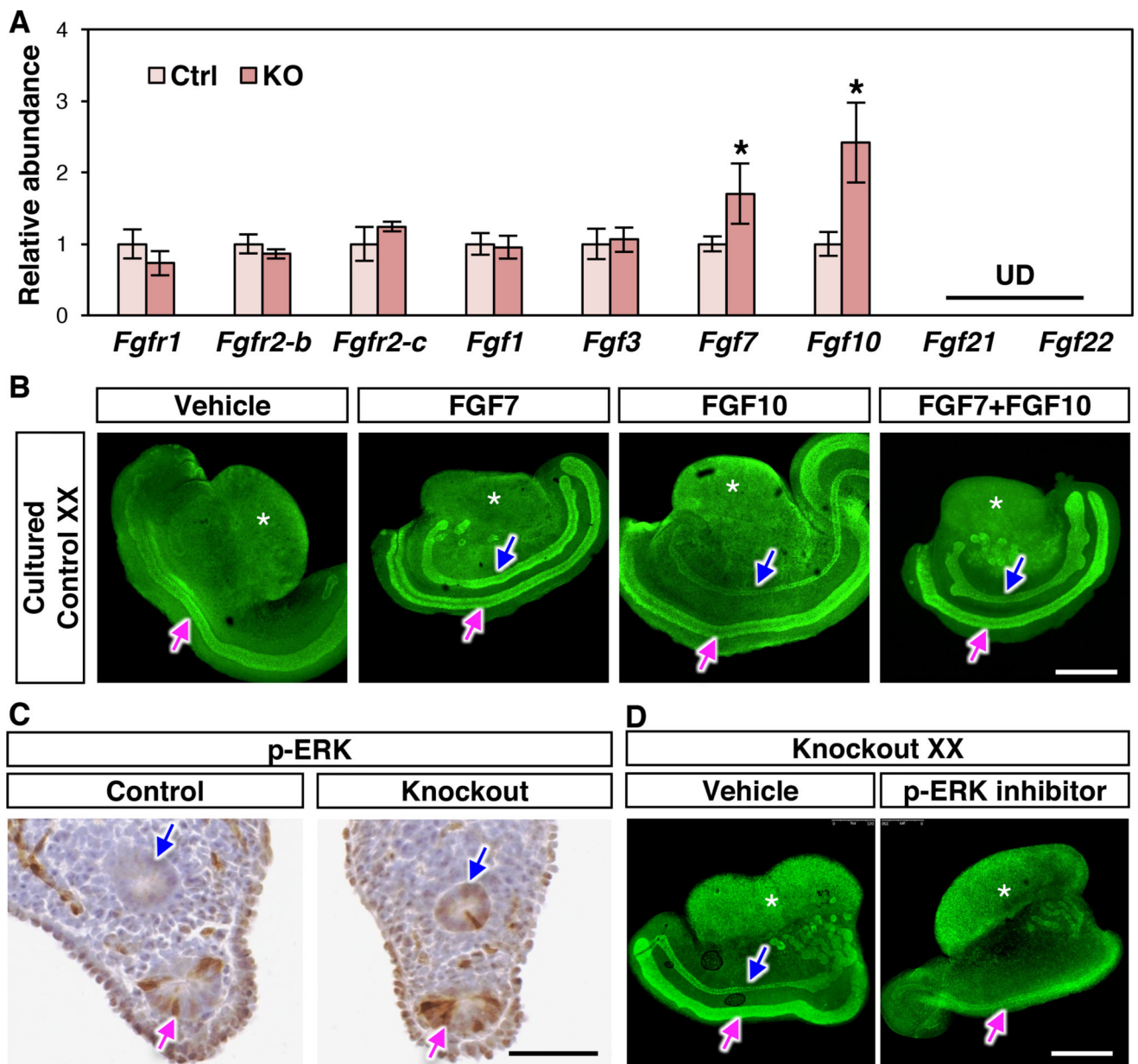
Fig. 3. Enhanced FGF signaling is involved in Wolffian duct retention in the absence of *Coup-tfII*
(A) mRNA expression of FGF receptors and ligands in the mesonephroi at E14.5. UD: undetected. Results are shown as mean ± SEM. Asterisks represent statistical significance of $p<0.05$ compared to control females using Student's t test (N=8 for each genotype). (B) Whole mount immunofluorescence of PAX2 of 2-day cultured E14.5 wild type XX mesonephroi in the presence of vehicle, or FGF7, or FGF10 or FGF7+FGF10. (C) p-ERK immunohistochemistry of control and knockout XX mesonephroi at E14.5. (D) PAX2 whole mount immunofluorescence in vehicle- or p-ERK inhibitor-treated knockout XX genital ridges. N=3 per group in B–D. Scale bars: 0.5 mm in B & D, and 50 μm in C. Blue arrows: Wolffian ducts; Magenta arrows: Müllerian ducts; White asterisk: ovary.