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Supporting Information for:

Perivascular cells support folliculogenesis in the developing ovary

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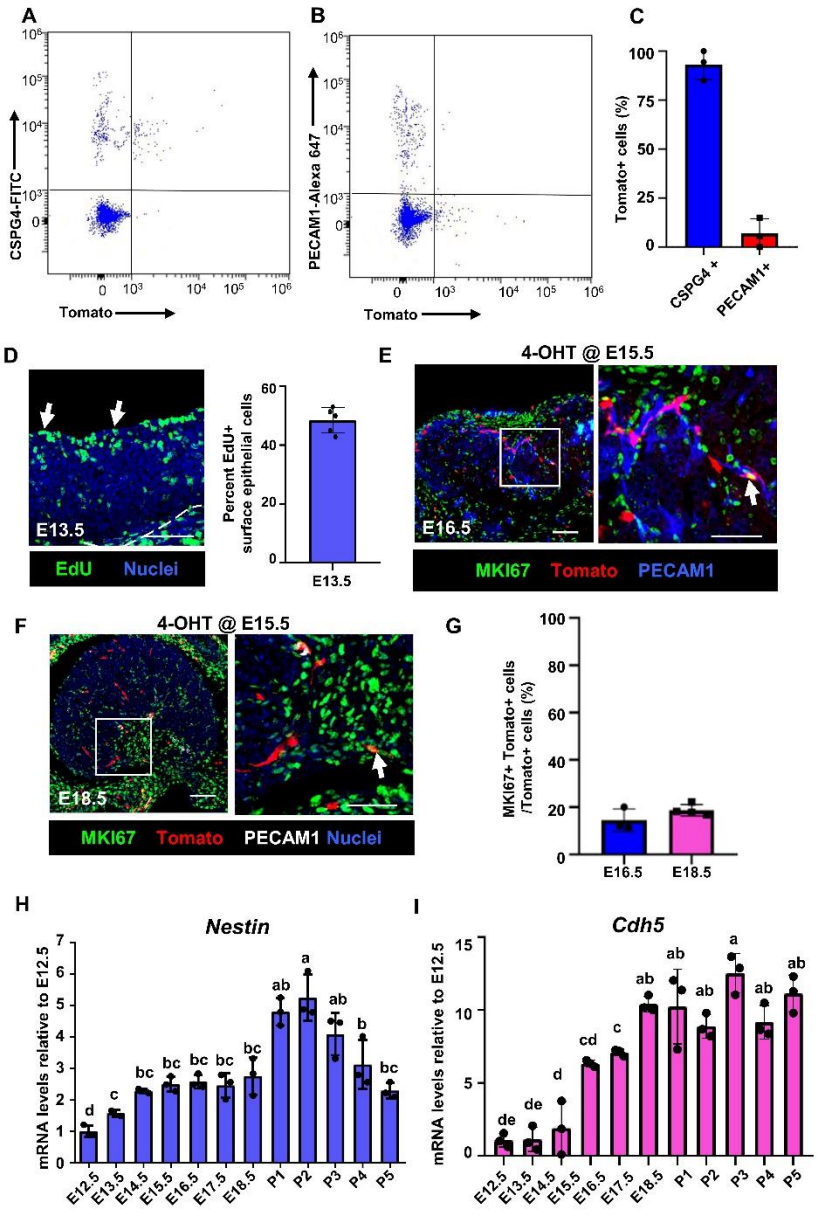

Fig. S1. Fetal Nestin-derived cells exhibit a low rate of proliferation. (A,B) Flow cytometry plots of E13.5 *Nestin*-CreER;*Rosa*-Tomato fetal ovaries exposed to 4-OHT at E12.5, showing that most Tomato+ cells expressed the pericyte/perivascular marker CSPG4 (also called NG2) (A), but not the endothelial marker PECAM1 (B). (C) Quantitative flow cytometric analyses of Tomato+ cells for CSPG4 and PECAM1 expression (*n*=3). (D) Immunofluorescence image highlighting EdU incorporation in coelomic surface epithelial cells (arrows) in E13.5 *Nestin*-CreER;*Rosa*-Tomato fetal ovaries exposed to 4-OHT at E12.5. Graph shows quantification of percent surface epithelial cells expressing EdU (*n*=5). (E,F) Immunofluorescence images of E16.5 (E; *n*=3) and E18.5 (F; *n*=4) *Nestin*-CreER;*Rosa*-Tomato fetal ovaries exposed to 4-OHT at E15.5, showing that few Tomato+ cells expressed MKI67 (arrows). (G) Quantification of percent Tomato+ cells expressing MKI67. Scale bars: 50 µm. Dashed line in D indicates gonad-mesonephros border. (H,I) Whole ovary qRT-PCR analyses of *Nestin* (H) and *Cdh5* (I) mRNA expression at various developmental stages (*n*=3). Letters indicate statistical differences via a two-tailed Student's t-test ($P<0.05$). Quantification values in C, D, and G and qRT-PCR values in H and I are presented as mean ± SD.

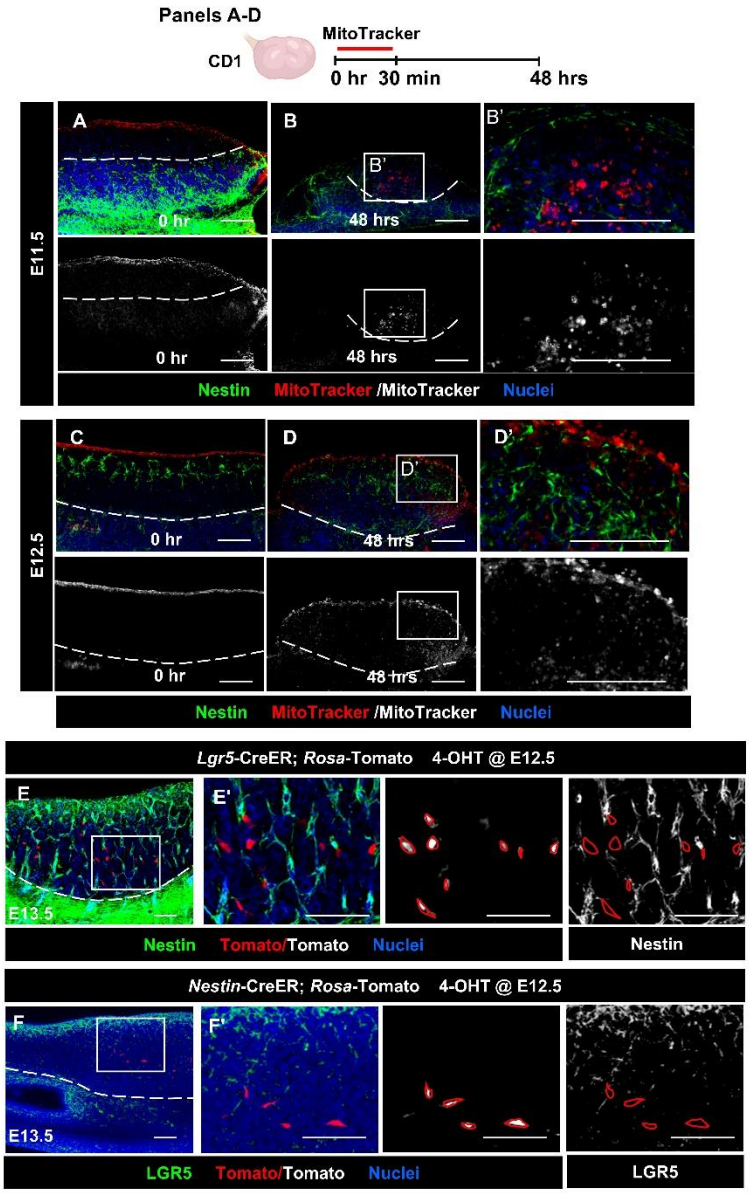

Fig. S2. Nestin+ cells in the fetal ovary are not derived from the coelomic epithelium. (A-D) Immunofluorescence images of E11.5 (A,B) or E12.5 (C,D) fetal ovaries labeled with MitoTracker and cultured *ex vivo*. (A) E11.5 gonad immediately after initial 30-minute incubation with MitoTracker dye, showing that dye labeling was limited to coelomic surface epithelial cells. (B) After 48-hour culture, coelomic epithelial cells labeled by MitoTracker migrated into the gonad. MitoTracker label did not overlap with Nestin (B'). (C) E12.5 gonad immediately after initial 30-minute incubation with MitoTracker dye, showing that dye labeling was limited to surface coelomic epithelial cells. (D) After 48-hour culture, epithelial cells labeled by MitoTracker migrated into the gonad. MitoTracker label did not overlap with Nestin (D'). (E) Immunofluorescence image of an E13.5 *Lgr5*-CreER;*Rosa*-Tomato fetal ovary exposed to 4-OHT at E12.5, showing that Nestin+ cells were Tomato-negative. (F) Immunofluorescence image of an E13.5 *Nestin*-CreER;*Rosa*-Tomato fetal ovary exposed to 4-OHT at E12.5, showing that Tomato+ cells did not express LGR5. B', D', E', and F' are higher-magnification images of the boxed regions in B, D, E, and F. Dashed lines indicate gonad-mesonephros border. Scale bars: 50 μm.

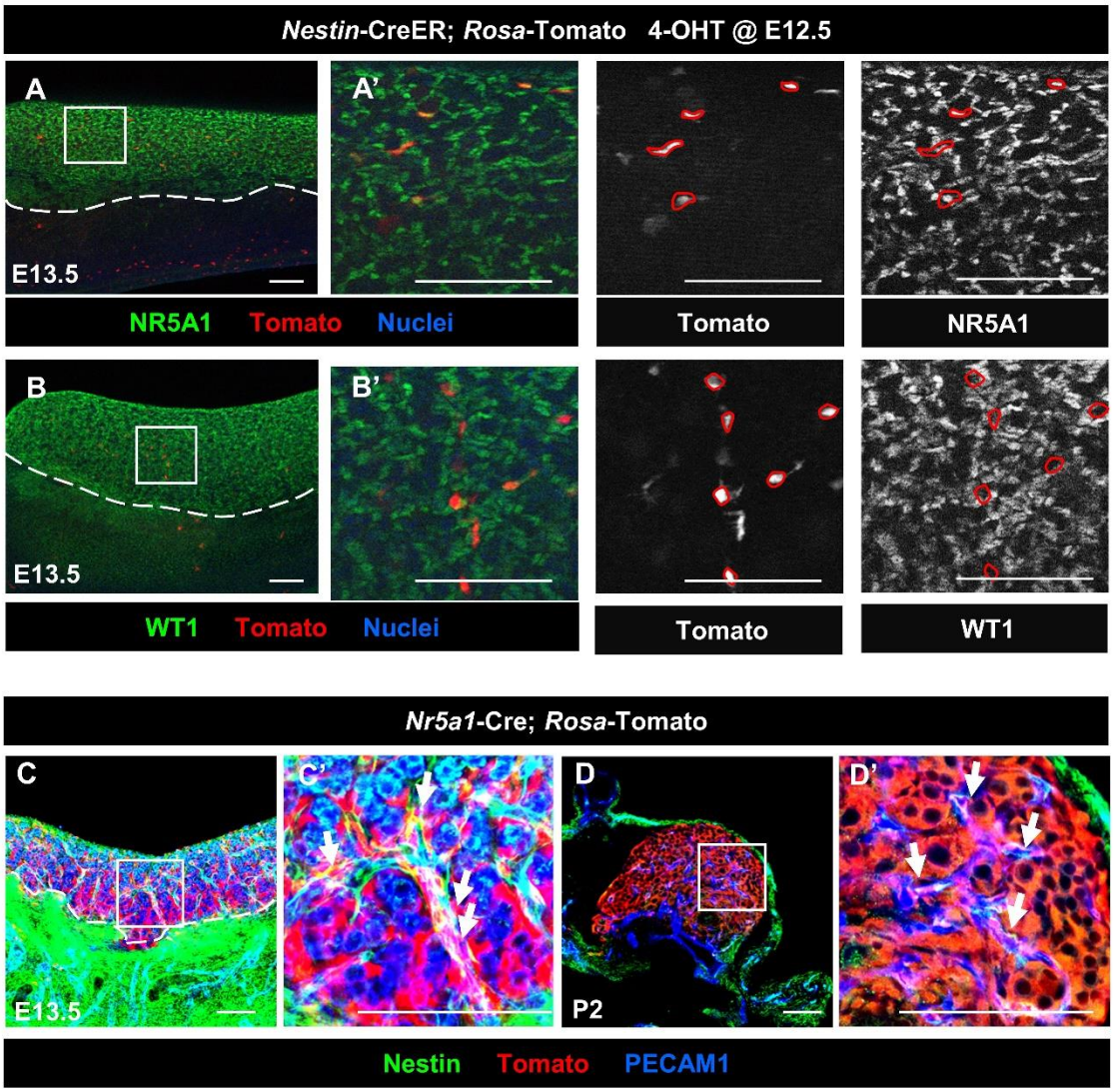

Fig. S3. Gonadal NR5A1+ cells are a source of Nestin+ cells in the fetal ovary. (A,B) Immunofluorescence images of E13.5 *Nestin*-CreER;*Rosa*-Tomato fetal ovaries exposed to 4-OHT at E12.5. Nestin+ cells are NR5A1-positive (A) but WT1-negative (B). (C,D) Immunofluorescence images of E13.5 (C) and P2 (D) *Nr5a1*-Cre;*Rosa*-Tomato fetal ovaries, showing that perivascular Nestin+ cells express Tomato. A'-D' are higher-magnification images of the boxed regions in A-D. Dashed lines indicate gonad-mesonephros border. Scale bars: 50 μm.

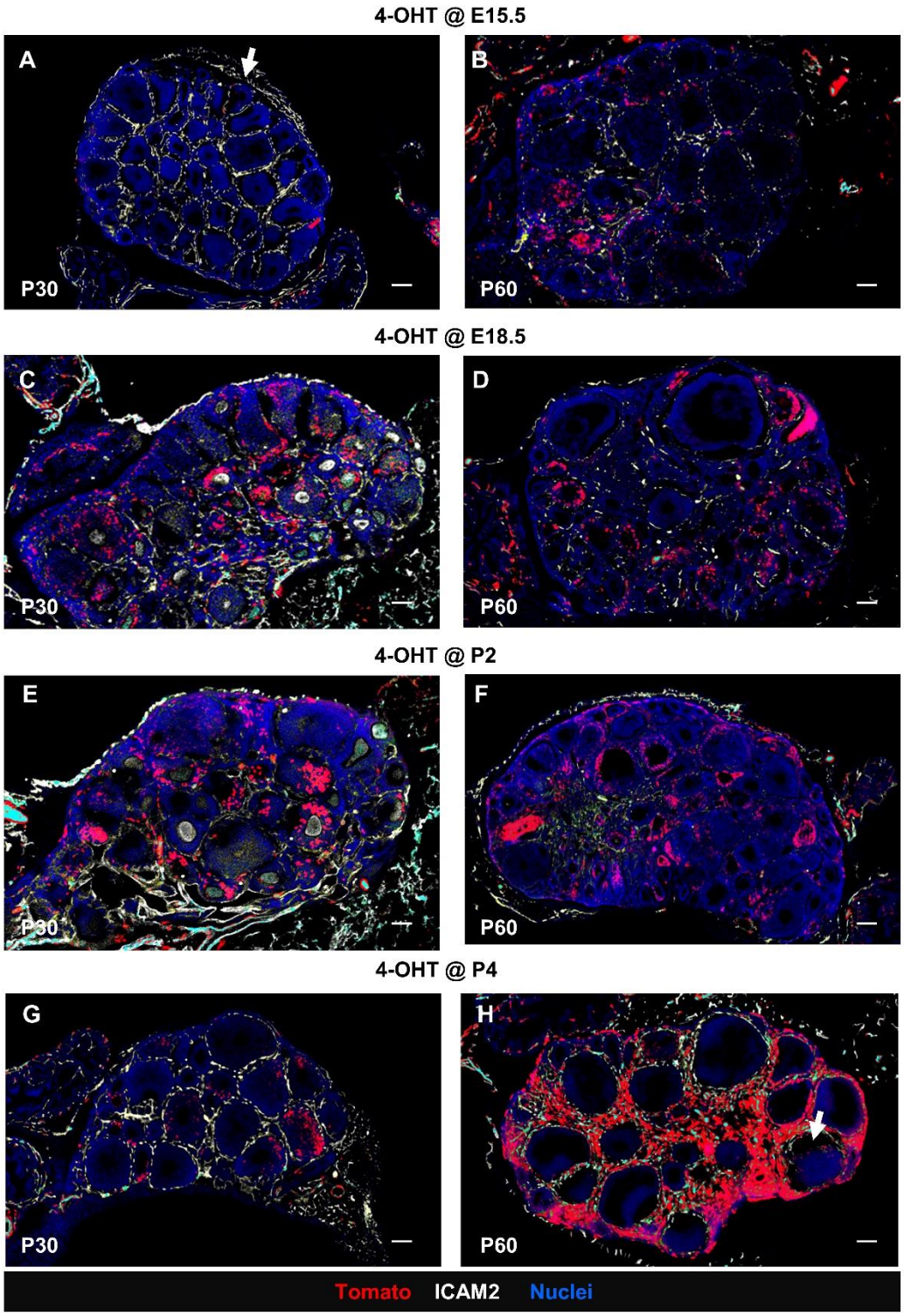

Fig. S4. Long-term *Nestin*-CreER-mediated lineage tracing reveals dynamic contribution of fetal and postnatal Nestin-derived cells in the juvenile and adult ovary. Low-magnification images showing long-term lineage tracing of fetal and postnatal Nestin+ cells in P30 juvenile (A,C,E,G) and P60 adult (B,D,F,H) *Nestin*-CreER;*Rosa*-Tomato ovaries exposed to 4-OHT at E15.5 (A,B), E18.5 (C,D), P2 (E,F), or P4 (G,H). Arrows indicate Tomato+ cells in granulosa cells. Scale bars: 100 μm.

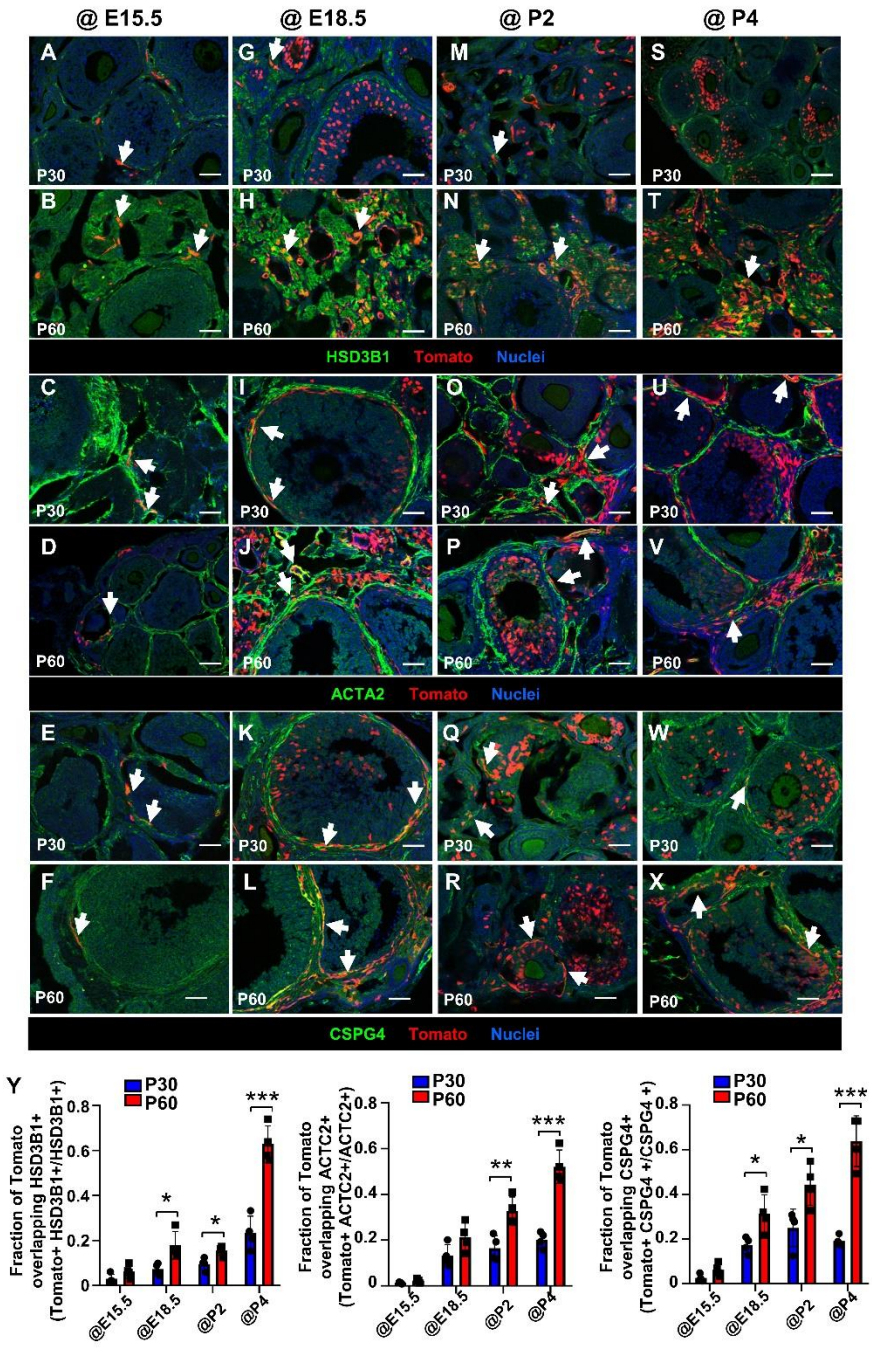

Fig. S5. Perivascular Nestin+ cells give rise to multiple ovarian cell types. (A-X) Long-term lineage-tracing of Nestin-derived cells in *Nestin*-CreER;*Rosa*-Tomato P30 juvenile and P60 adult ovaries, exposed to 4-OHT at E15.5 (A-F), E18.5 (G-L), P2 (M-R), or P4 (S-X). P30 and P60 ovaries were examined for expression of Tomato, steroidogenic cell marker HSD3B1, smooth-muscle cell marker ACTA2 (also called αSMA), and pericyte marker CSPG4. HSD3B1 was observed not only in the theca cell layer but also in interstitial cells. ACTA2 was expressed in the theca layer and in smooth muscle. CSPG4 was expressed in both the theca layer and in pericytes. Arrows indicate Tomato+ cells of interest labeled by each antibody. (Y) Fraction of Tomato expression overlapping with HSD3B1, ACTA2, or CSPG4 in P30 and P60 ovaries (n=4). *P<0.05; two-tailed Student's t-test. Values in Y are presented as mean ± SD. Scale bars: 50 μm.

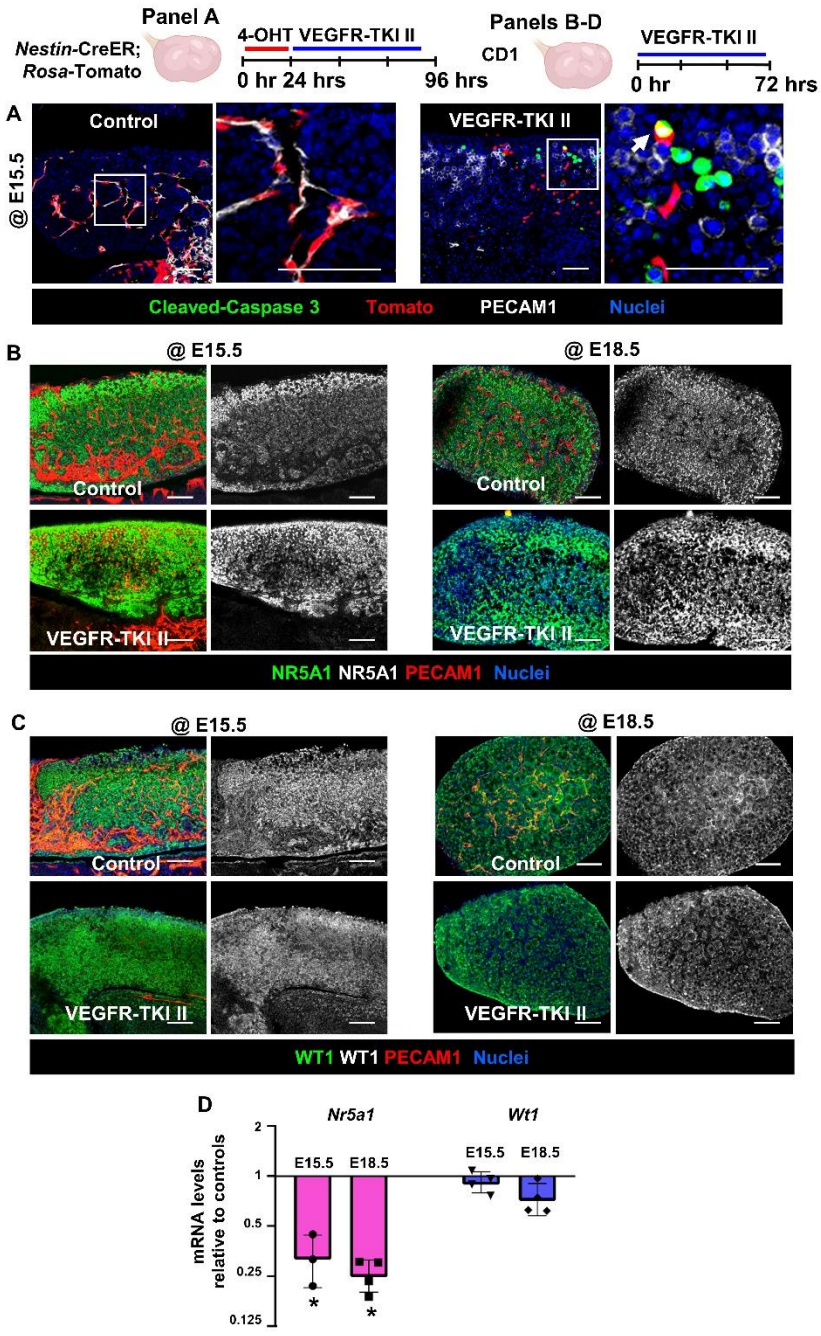

Fig. S6. Vascular disruption may affect the NR5A1+ lineage, but not does not affect the general WT1+ ovarian cell lineage. (A) Immunofluorescence images of E15.5 cultured *Nestin*-CreER;*Rosa*-Tomato fetal ovaries, showing a Tomato+ cell in a vascular-disrupted ovary co-expressing cleaved Caspase 3 (arrow). (B,C) Images of E15.5 and E18.5 cultured CD1 wild-type ovaries, showing that NR5A1 (B) and WT1 (C) expression patterns did not significantly change after vascular disruption. Scale bars: 50 μm. (D) qRT-PCR analyses showing fold change in *Nr5a1* and *Wt1* mRNA levels in E15.5 and E18.5 cultured ovaries after vascular disruption (n=3), indicating that *Nr5a1* levels were reduced while *Wt1* was unaffected. *P<0.05; two-tailed Student's t-test. qRT-PCR values in D are presented as mean ± SD.

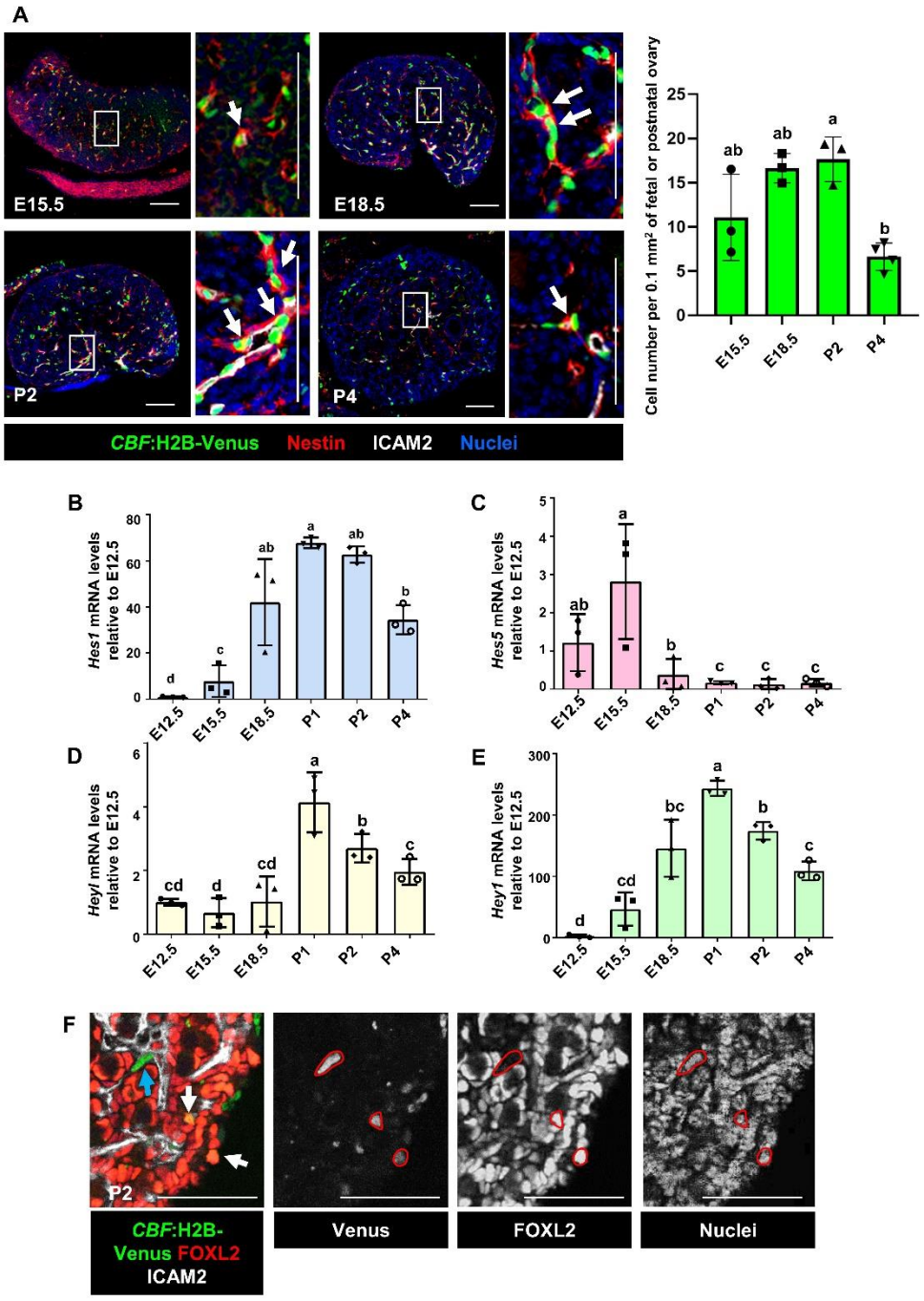

Fig. S7. Active Notch signaling shows a dynamic pattern in fetal and postnatal ovaries. (A) Images of E15.5, E18.5, P2, and P4 ovaries from *CBF*:H2B-Venus mice (active Notch signaling reporter). Arrows indicate perivascular Nestin+ cells co-expressing Venus. Graph shows quantification of Venus+Nestin+ double-positive cell number in fetal and postnatal ovaries (*n*=3 for E15.5, E18.5, and P2; *n*=4 for P4). (B-E) qRT-PCR analyses showing fold change in Notch target gene (*Hes1* (B), *Hes5* (C), *Heyl* (D), and *Hey1* (E)) mRNA levels in ovaries at various developmental stages (*n*=3). Different letters indicate statistically different values ($P<0.05$; two-tailed Student's t-test). Quantitative and qRT-PCR values in A-E are presented as mean ± SD. (F) Image of a P2 *CBF*:H2B-Venus ovary. Perivascular Venus+ cells did not express FOXL2 (blue arrow), while cortical Venus+ cells co-expressed FOXL2 (white arrows). Scale bars: 50 µm.

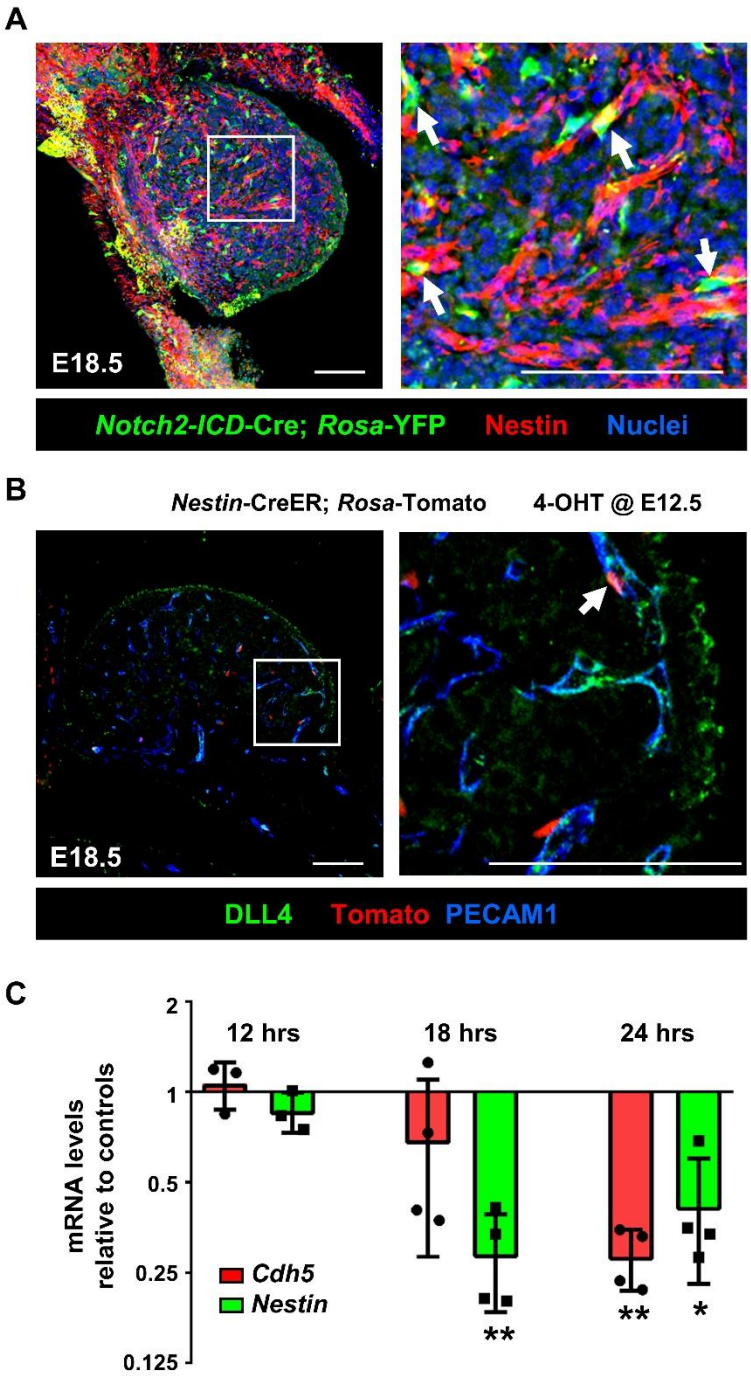

Fig. S8. Active NOTCH2 signaling, likely acting through vascular-expressed DLL4, occurs in fetal perivascular cells independently of other effects on endothelial cells. (A) Image of E18.5 *Notch2-ICD*-Cre;*Rosa*-YFP ovary, showing YFP expression in Nestin+ cells (white arrows). (B) Image of E18.5 *Nestin*-CreER;*Rosa*-Tomato fetal ovary exposed to 4-OHT at E12.5, in which perivascular Tomato+ cells are adjacent to endothelial cells expressing DLL4. Scale bars: 50 μm. (C) Whole-ovary qRT-PCR analyses showing fold change in *Cdh5* and *Nestin* mRNA levels after blockade of Notch signaling via DAPT treatment at E18.5 for 12 hours (n=3), 18 hours (n=4), or 24 hours (n=4) of *ex vivo* culture. *P<0.05, **P<0.01; two-tailed Student's t-test. qRT-PCR values in C are presented as mean ± SD.

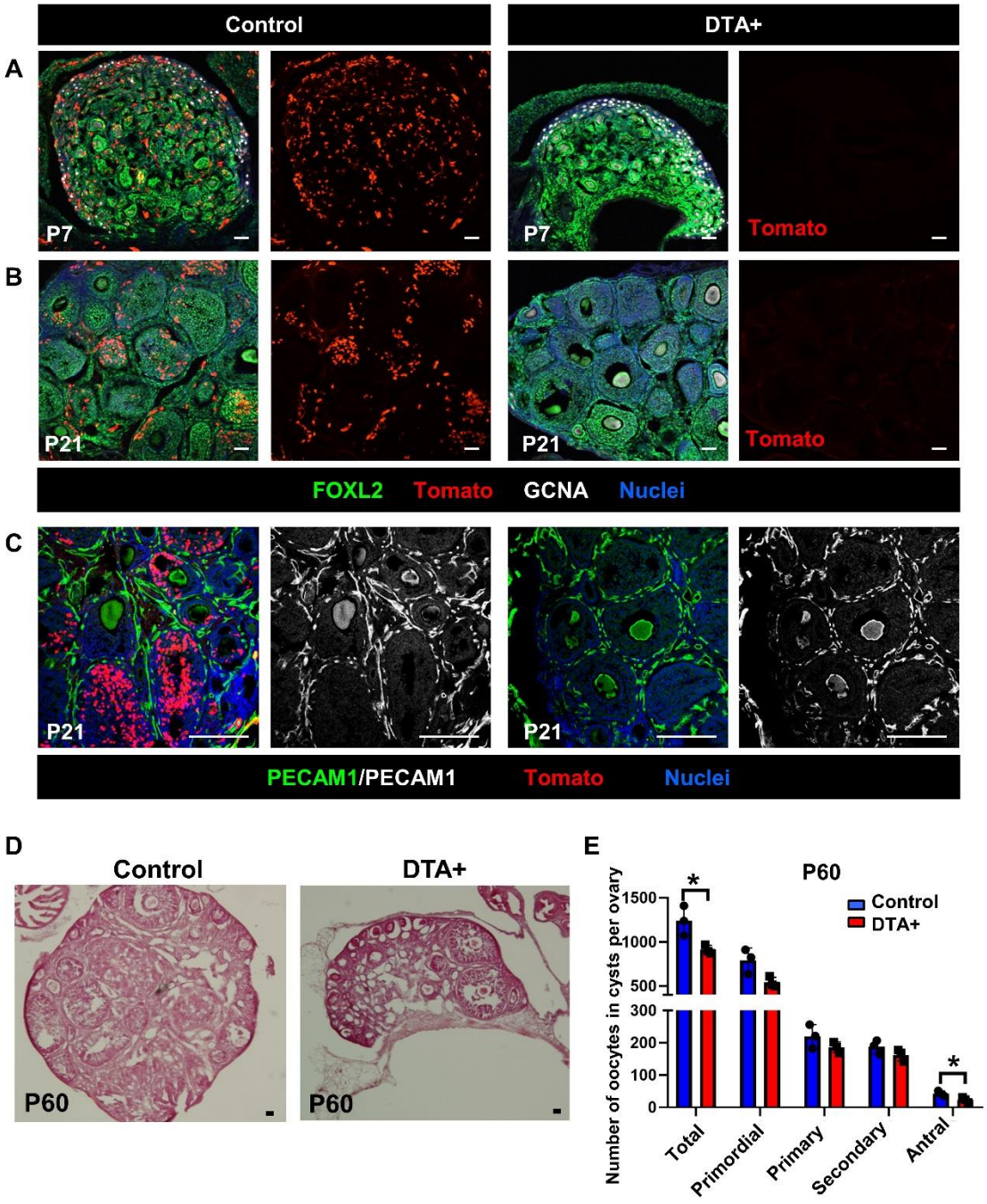

Fig. S9. *In vivo* ablation of Nestin+ cells in the postnatal ovary is highly efficient and does not impact ovarian vascularization. (A-C) Immunofluorescence images of P7 (A) and P21 (B,C) control (*Nestin*-CreER;*Rosa*-Tomato; left) and *Nestin*-CreER;*Rosa*-Tomato;*Rosa*-DTA (DTA+; right) postnatal ovaries exposed to 4-OHT at P2 and P4. In controls, Tomato+ cells co-expressed the granulosa cell marker FOXL2 in both P7 (A) and P21 (B) ovaries, whereas DTA+ ovaries exhibited a complete ablation of Tomato+ cells. GCNA (TRA98) labels germ cells. (C) P21 control and DTA+ ovaries showed similar vascularization as assessed by PECAM1 expression. (D,E) Hematoxylin staining (D) and quantification of follicles at different developmental stages (E) in P60 *Nestin*-CreER;*Rosa*-Tomato;*Rosa*-DTA ovaries (n=3). *P<0.05; two-tailed Student's t-test. Values in E are presented as mean ± SD. Scale bars: 50 μm.

Table S1. Primary antibodies used for immunofluorescence (IF) or flow cytometry (FC).
Unless otherwise noted, dilutions listed are for immunofluorescence.

Primary Antibody	Dilution	Source/Reference
Rabbit anti-ACTA2 (αSMA)	1:500	Abcam #ab5694
Rabbit anti-CSPG4 (NG2)	1:500 (IF); 1:1,000 (FC)	Millipore-Sigma #AB5320
Rabbit anti-FOXL2	1:500	D. Wilhelm (1)
Rat anti-GCNA (TRA98)	1:1,000	Abcam #ab82527
Rabbit anti-HSD3B1	1:500	Cosmo Bio #KAL-KO607
Rat anti-ICAM2 (CD102)	1:1,000	Bio-Rad #MCA2295
Rat anti-LGR5	1:250	NovusBio #NBP1-28904
Rabbit anti-MKI67 (Ki67)	1:500	GeneTex #GTX16667
Rabbit anti-Nestin	1:1,000	BioLegend #PRB-315C
Rat anti-NR5A1	1:200	Cosmo Bio #KAL-KO610
Rat anti-PECAM1	1:250 (IF); 1:500 (FC)	BD Pharmingen #553370
Rabbit anti-phospho-Histone H3 (Ser10)	1:500	Millipore-Sigma #06-570
Mouse anti-WT1 (F-6)	1:100	Santa Cruz #sc-7385
Goat anti-DLL4	1:100	R&D Systems #AF1389
Chicken anti-GFP	1:1,000	Aves #GFP-1020

Table S2. Sequences of primers used for qRT-PCR analyses.

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
Cdh5	TCCTCTGCATCCTCACTATCACA	GTAAGTGACCAACTGCTCGTGAAT
Nestin	GCTGGAACAGAGATTGGAAGG	CCAGGATCTGAGCGATCTGAC
Hes1	ATAGCTCCCGGCATTCCAAG	GCGCGGTATTTCCCCAACA
Hes5	AGTCCCAAGGAGAAAAACCGA	GCTGTGTTTCAGGTAGCTGAC
Hey1	GCGCGGACGAGAATGGAAA	TCAGGTGATCCACAGTCATCTG
Heyl	CAGCCCTTCGCAGATGCAA	CCAATCGTCGCAATTCAGAAAG
Foxl2	GCTACCCCGAGCCCGAAGAC	GTGTTGTCCCGCCTCCCTTG
Nr5a1	AGGTGTCGGGCTACCACTAC	CCACCCCGCATTCGATCAG
Wt1	AATGACCTCCCAGCTTGAATG	CCGTGGGTGTGTATTCTGTACT
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Table S3. Cell number sample sizes for cell counting and flow cytometry assays.

Figure	Cell type	Cell number
1F	Tomato+ cells	E13.5 (603); E18.5 (696)
1G	Tomato+ cells	E13.5 (77); E18.5 (212)
2G	Tomato+ cells	E16.5 (105); P1 (104); P3 (196); P5 (231)
2H	FOXL2+Tomato+ cells	E16.5 (3); P1 (2); P3 (45); P5 (34)
8B	Tomato+ cells	Control (221); *Rbpj*-flox (105)
S1A-C	Tomato+ cells	E13.5 (118)
S1D	Epithelial cells	E13.5 (202)
S1G	Tomato+ cells	E16.5 (133); E18.5 (140)
S7A	Venus+Nestin+ cells	E15.5 (105); E18.5 (104); P2 (196); P4 (231)

SI References

1. J. C. Polanco, D. Wilhelm, T. L. Davidson, D. Knight, P. Koopman, Sox10 gain-of-function causes XX sex reversal in mice: implications for human 22q-linked disorders of sex development. *Hum Mol Genet* **19**, 506-516 (2010).