Supplemental Information

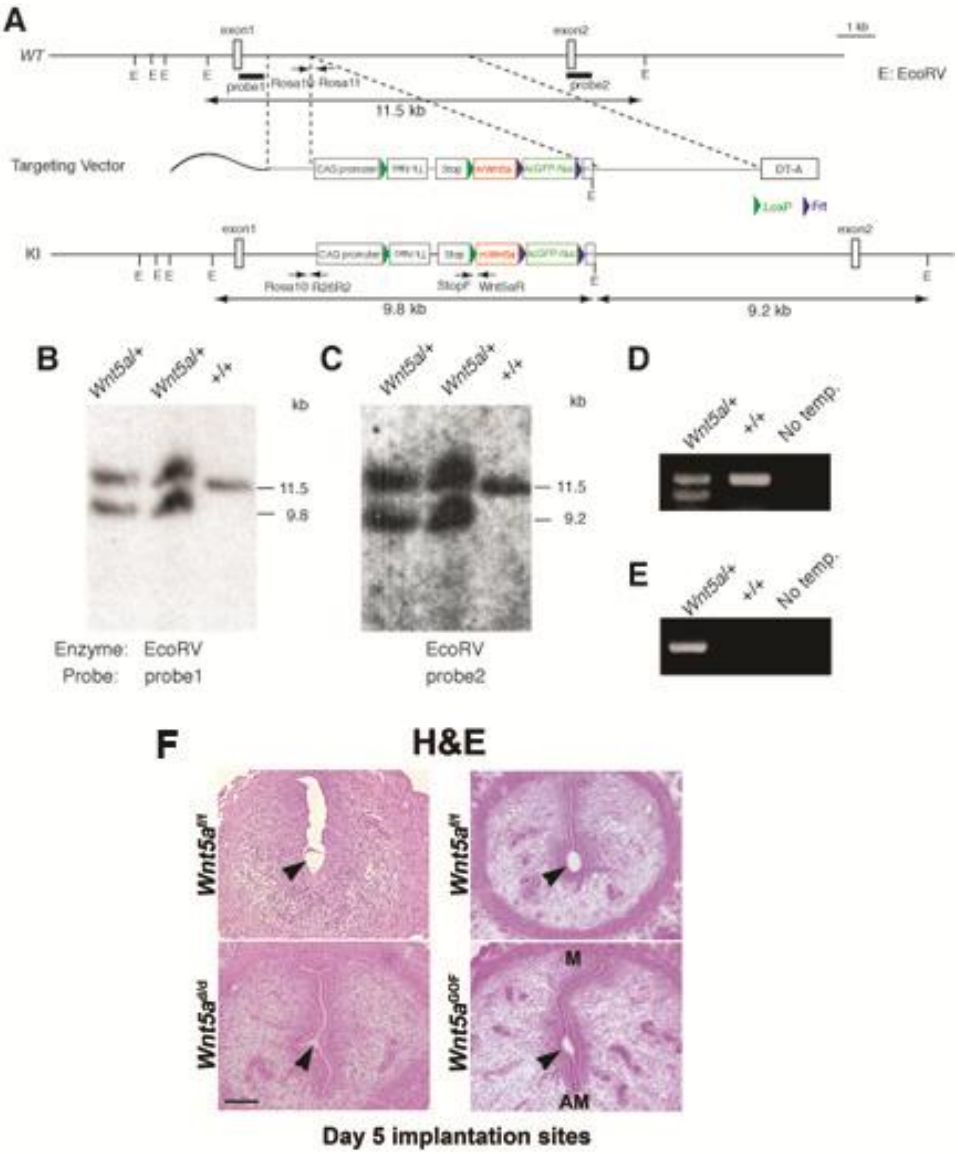


Figure S1

Figure S1. Construction of the *Rosa26*^{Wnt5a} allele, and histological analyses of day 5 *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} implantation sites, related to Figure 1. (**A**) Schematic representation of the targeting strategy for the *Rosa26* allele. *WT* Scheme: *Rosa26*

gene allele flanking exons 1 and 2. Insertion site of the targeting vector is shown below the *WT* Scheme. *KI* Scheme: Targeted allele. Probes for Southern blotting are shown as black bars and primers for PCR as arrows. E; EcoRV. (**B, C**) Representative results of Southern blotting analyses using genomic DNA of indicated genotypes of ES clones. (**D**) Representative genotyping PCR results using tail genomic DNA with Rosa10 + Rosa11 + R26R2 primers. (**E**) Representative results of PCR genotyping using tail genomic DNA with StopF + Wnt5aR primers. (**F**) Histology of representative cross-sections of *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} implantation sites showing aberrant luminal architecture and crypt formation compared to those of floxed littermates. Arrowheads denote location of blastocysts. M, mesometrial pole; AM, anti-mesometrial pole. Scale bar, 200 µm.

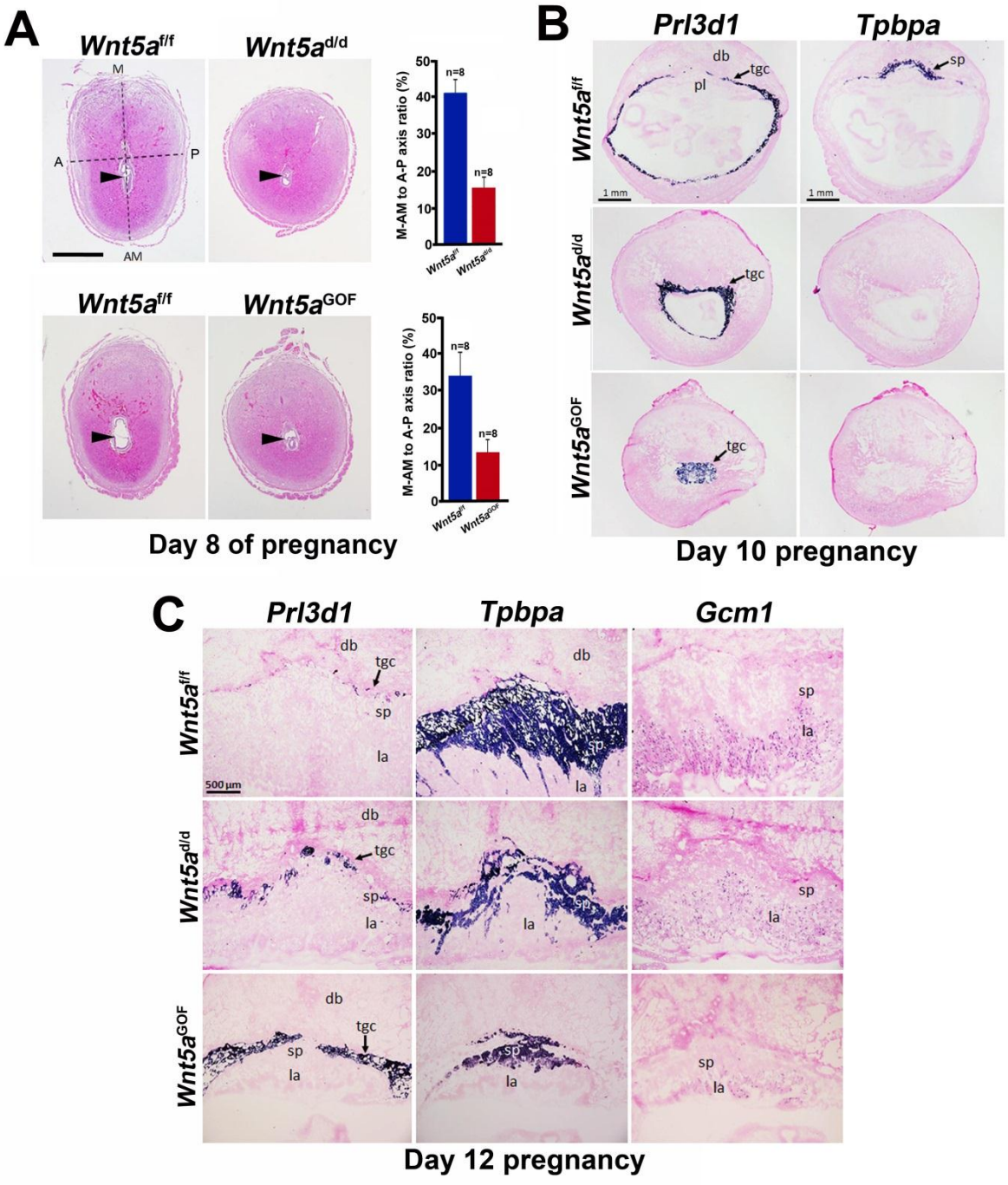
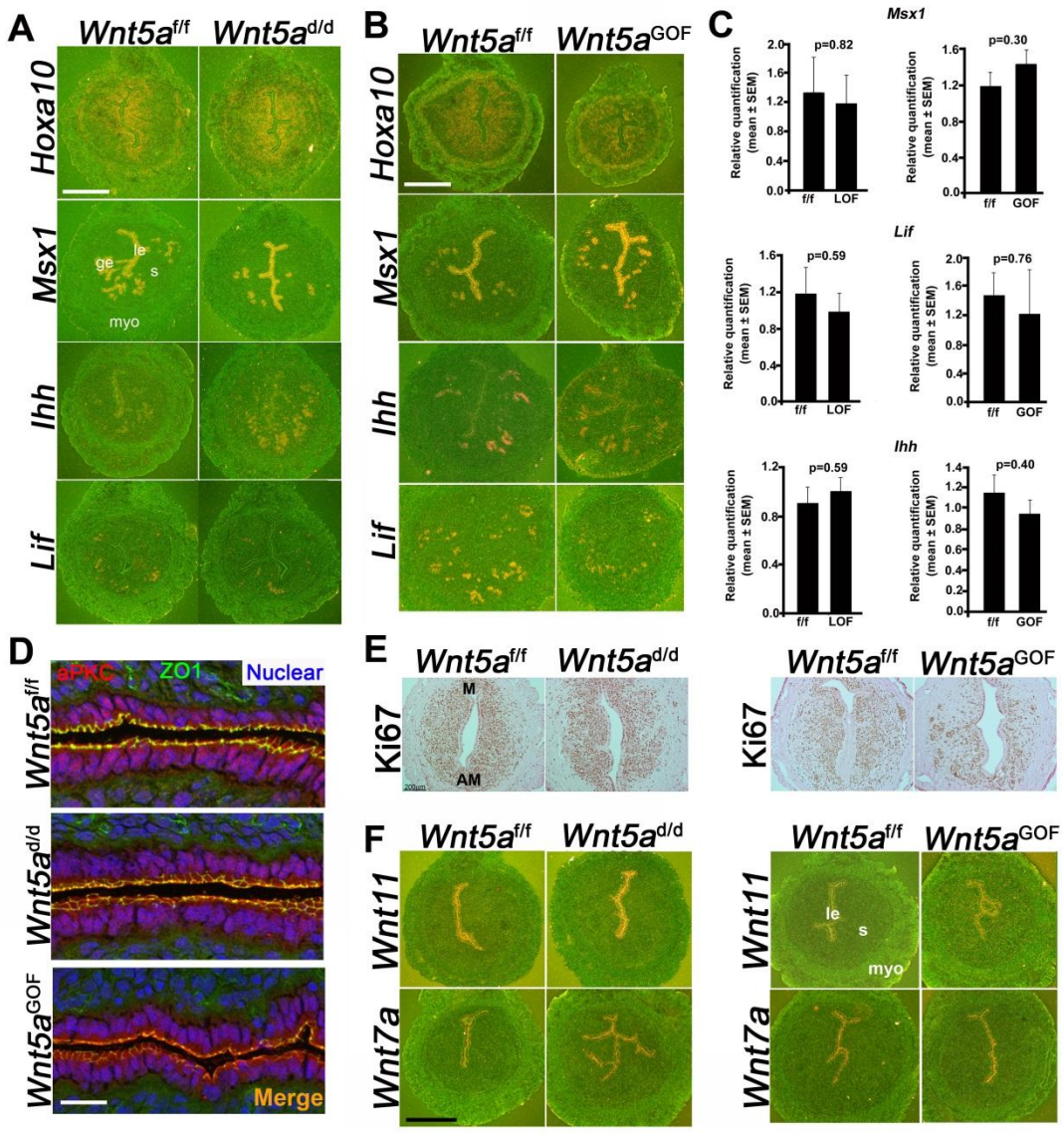

A
Wnt5a f/f
Wnt5a d/d
M
A
P
AM
M-AM to A-P axis ratio (%)
n=8
n=8
Wnt5a f/f
Wnt5a GOF
Day 8 of pregnancy
B
Prl3d1
Tpbpa
Wnt5a f/f
Wnt5a d/d
Wnt5a GOF
db
tgc
pl
sp
1 mm
Day 10 pregnancy
C
Prl3d1
Tpbpa
Gcm1
Wnt5a f/f
Wnt5a d/d
Wnt5a GOF
db
tgc
sp
la
500 µm
Day 12 pregnancy

Figure S2

Figure S2. Decreased ratio of the length of the M-AM axis to the A-P axis on day 8 and defective expression of trophoblast markers on days 10 and 12 in females with dysregulated uterine *Wnt5a,* related to Figure 2. (**A**) Histology of day 8 implantation sites of *Wnt5a*$^{f/f}$ and *Wnt5a*^{GOF} females compared to floxed littermates. Dotted lines indicate length of axes. Ratio of the length along M-AM axis to length along A-P axis in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} implantation sites compared to those of floxed littermates (mean ± SEM, n=number of implantation sites assessed). Arrowheads denote location of embryos. A, anterior; P, posterior; M, mesometrial pole; AM, antimesometrial. Scale bar, 500 μm. (**B**) Brightfield photomicrographs of DIG in situ hybridization results show reduced expression of trophoblast markers *Prl3d1* and *Tpbpa* in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} implantation sites. Scale bars, 1mm. (**C**) Persistent expression of trophoblast giant cell marker *Prl3d1* and reduced expression of trophoblast markers *Tpbpa* and *Gcm1* in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} implantation sites. Scale bar, 500 μm. Db, decidual basalis; tgc, trophoblast giant cells; pl, presumptive placenta; sp, spongiotrophoblast layer; la, labyrinth layer.



Day 4 of pregnancy

Figure S3

Figure S3. Dysregulated uterine *Wnt5a* expression is not reflected in aberrant expression of uterine receptivity markers, *Wnt11* or *Wnt7a*, related to Figure 3. In situ hybridization results show appropriate expression of uterine receptivity genes *Hoxa10*, *Msx1*, *Ihh*, and *Lif* in *Wnt5a*$^{d/d}$ (**A**) and *Wnt5a*^{GOF} (**B**) uteri and their floxed littermates on day 4 morning. Scale bars, 500 µm. (**C**) qPCR results showing comparable transcript levels of *Msx1*, *Ihh*, and *Lif* in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} uteri compared to floxed uteri on day 4 morning (n=3 females per genotype, mean ± SEM). (**D**) Immunofluorescence results for ZO1 (green) and αPKC (red) showing comparable apicobasal polarity in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} uteri compared to floxed uteri on day 4 morning. Scale bar, 50 µm. (**E**) The population of Ki67 positive cells in the luminal epithelium and stroma of *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} females was comparable to that of floxed littermates. Scale bar, 200 µm. (**F**) In situ hybridization results show comparable expression of *Wnt11*, another non-canonical Wnt ligand, and *Wnt7a* in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} uteri on day 4. Scale bars, 500 µm. M, mesometrial pole; AM, anti-mesometrial pole; le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium.

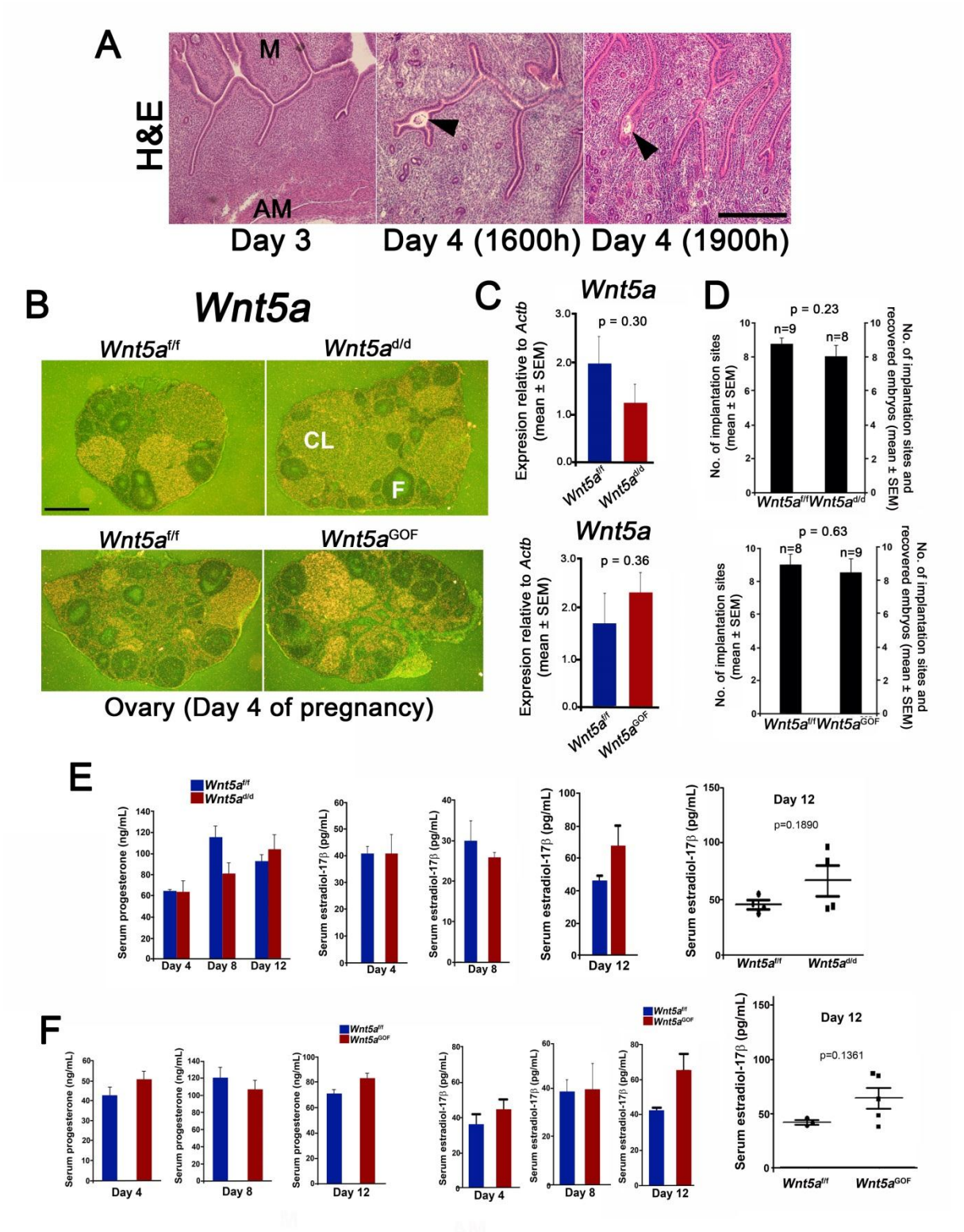

A
H&E
M
AM
Day 3
Day 4 (1600h)
Day 4 (1900h)
B
Wnt5a
Wnt5a^f/f
Wnt5a^d/d
CL
F
Wnt5a^f/f
Wnt5a^GOF
Ovary (Day 4 of pregnancy)
C
Wnt5a
Expresion relative to Actb (mean ± SEM)
p = 0.30
Wnt5a^f/f
Wnt5a^d/d
Wnt5a
p = 0.36
Wnt5a^f/f
Wnt5a^GOF
D
p = 0.23
n=9
n=8
No. of implantation sites (mean ± SEM)
No. of implantation sites and recovered embryos (mean ± SEM)
Wnt5a^f/f
Wnt5a^d/d
p = 0.63
n=8
n=9
Wnt5a^f/f
Wnt5a^GOF
E
Wnt5a^f/f
Wnt5a^d/d
Serum progesterone (ng/mL)
Day 4
Day 8
Day 12
Serum estradiol-17β (pg/mL)
Day 4
Day 8
Day 12
p=0.1890
Wnt5a^f/f
Wnt5a^d/d
F
Wnt5a^f/f
Wnt5a^GOF
Serum progesterone (ng/mL)
Day 4
Day 8
Day 12
Serum estradiol-17β (pg/mL)
Day 4
Day 8
Day 12
p=0.1361
Wnt5a^f/f
Wnt5a^GOF

Figure S4

Figure S4. Crypt formation progresses between days 3 and 4 of pregnancy, and fertility defects in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} females are not reflected in ovarian dysfunction, related to Figure 4. (**A**) H&E staining of longitudinal sections of uteri on day 3 (1000h) and day 4 (1600h and 1900h) of pregnancy showing progression of villi-like epithelial projections from the main lumen towards the AM pole in floxed mice. Arrowheads denote location of blastocysts. M, mesometrial pole; AM, anti-mesometrial pole. Scale bar, 250 µm. (**B**) In situ hybridization results show little lower and higher levels of *Wnt5a* expression in corporal luteal cells in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} ovaries, respectively, compared to floxed littermates on day 4. Scale bar, 500 µm. CL, corpus luteum; F, follicle. (**C**) Quantitative RT-PCR results of total ovarian RNA samples did not show significant changes in *Wnt5a* expression between floxed and experimental females on day 4 (mean ± SEM, n=4). (**D**) The number of implantation sites (blue bands) and recovered blastocysts in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} females were comparable to the number of implantation sites in floxed littermates on day 5 of pregnancy (mean ± SEM, n=number of females examined). (**E-F**) Serum levels of progesterone and estradiol-17β in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} females were comparable to floxed littermates on days 4, 8, and 12 of pregnancy (mean ± SEM; at least 4 independent samples per genotype and day of pregnancy were assessed). Variability seen in the levels of estradiol-17β on day 12 in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} females fall within the normal physiological range and were not significantly different from floxed littermates.

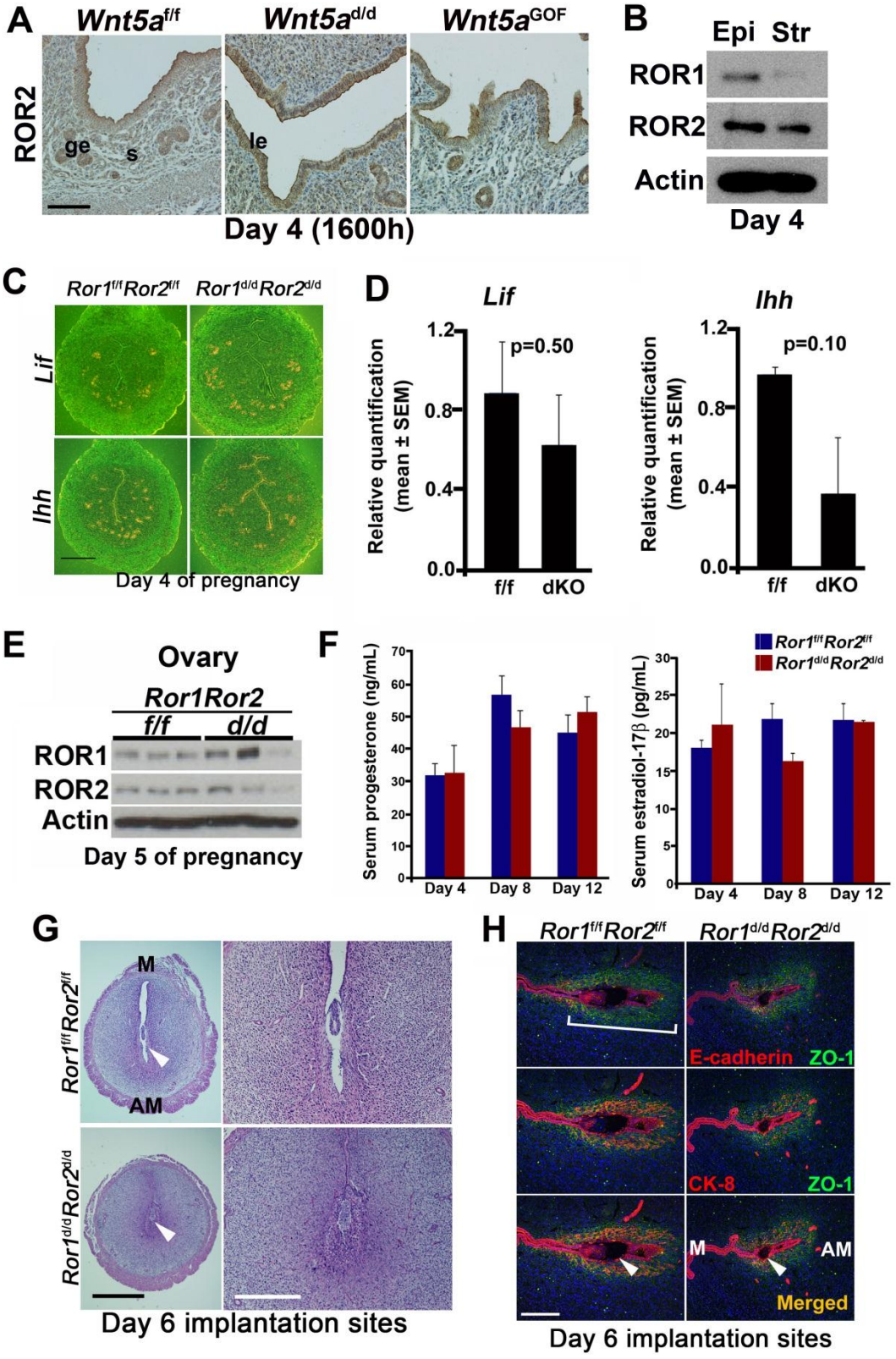

A
Wnt5a f/f
Wnt5a d/d
Wnt5a GOF
ROR2
ge
s
le
Day 4 (1600h)
B
Epi Str
ROR1
ROR2
Actin
Day 4
C
Ror1 f/f Ror2 f/f
Ror1 d/d Ror2 d/d
Lif
Ihh
Day 4 of pregnancy
D
Lif
Ihh
Relative quantification (mean ± SEM)
p=0.50
p=0.10
f/f
dKO
E
Ovary
Ror1Ror2
f/f
d/d
ROR1
ROR2
Actin
Day 5 of pregnancy
F
Serum progesterone (ng/mL)
Serum estradiol-17β (pg/mL)
Ror1 f/f Ror2 f/f
Ror1 d/d Ror2 d/d
Day 4
Day 8
Day 12
G
M
AM
Ror1 f/f Ror2 f/f
Ror1 d/d Ror2 d/d
Day 6 implantation sites
H
Ror1 f/f Ror2 f/f
Ror1 d/d Ror2 d/d
E-cadherin
ZO-1
CK-8
ZO-1
M
AM
Merged
Day 6 implantation sites

Figure S5

Figure S5. *Ror1*$^{d/d}$*Ror2*$^{d/d}$ females exhibit similar molecular signature during the periimplantation period as *Wnt5a*$^{d/d}$ females, related to Figure 5. (**A**) Immunohistochemistry of ROR2 in *Wnt5a*$^{f/f}$, *Wnt5a*$^{d/d}$, and *Wnt5a*^{GOF} uteri on day 4 (1600h). Note positive immunostaining primarily in the luminal epithelium at the apical surface with increases in *Wnt5a*$^{d/d}$ uteri. le, luminal epithelium; ge, glandular epithelium; s, stroma. Scale bar, 100 µm. (**B**) Western blotting results show epithelial enrichment of ROR1 and ROR2 in isolated primary uterine epithelial cells. Epi, epithelial cells; Str, stromal cells. (**C**) In situ hybridization results showed comparable levels of *Ihh* and *Lif* in *Ror1*$^{d/d}$*Ror2*$^{d/d}$ uteri on day 4. Scale bar, 500 µm. (**D**) qPCR results showing comparable expression of *Ihh* and *Lif* in *Wnt5a*$^{d/d}$ and *Wnt5a*^{GOF} uteri compared to floxed uteri on day 4 morning of pregnancy (n=3 females per genotype, mean ± SEM). (**E**) Western blotting results for ROR1 and ROR2 showed no appreciable changes in ovary on day 5 of pregnancy. Actin served as a loading control. (**F**) Serum levels of progesterone and estradiol-17β in *Ror1*$^{d/d}$*Ror2*$^{d/d}$ females are comparable to floxed littermates on days 4, 8, and 12 of pregnancy (mean ± SEM; n=3-4 independent samples per genotype and day of pregnancy were assessed except for Day 12 *Ror1*$^{d/d}$*Ror2*$^{d/d}$ samples, n=2). (**G**) Histology of day 6 implantation sites of *Ror1*$^{d/d}$*Ror2*$^{d/d}$ females compared to floxed littermates. Left panel scale bars: 500 µm; right panel scale bars: 250 µm. (**H**) Immunofluorescence of ZO1, E-cadherin, and cytokeratin shows epithelioid decidual cells at the PDZ (brackets) in day 6 implantation sites with embryo and luminal epithelium marked by E-cadherin. Scale bar, 200 µm. Arrowheads depict location of embryos. M, mesometrial pole; AM, anti-mesometrial pole.

Supplemental Experimental Procedures

Generation of conditional *Wnt5a* gain-of-function (Rosa26^{Wnt5a}) mice. The CTV vector (Xiao et al., 2007) was purchased from Addgene (Plasmid 15912), and IRES-eGFP cassette was replaced with IRES2-AcGFP-Nuc cassette (Clontech), then the mouse Wnt5a cDNA with Kozak sequence was inserted at the AscI site. Gene targeting was achieved using the Bruce-4 C57BL6 embryonic stem (ES) cell line. ES cells were cultured, electroporated with the SgfI-linearized targeting vectors and selected by G418. Targeted ES clones were screened individually by digesting genomic DNA with EcoRV and probing with probe1 and probe2 (**Figure S1A**). Southern blot probes were made using PCR DIG Probe Synthesis Kit according to manufacturer's protocol (Roche). Chimeric mice were obtained by injecting targeted ES cell clones into albino C57BL6 blastocysts, which were transferred into the uteri of pseudopregnant recipients. Male chimeras were mated with albino C57BL6 females. Genotypes of newborn mice and embryos were analyzed by PCR and confirmed by Southern blotting when necessary (**Figure S1B-E**). The primers used for the PCR analyses and PCR product sizes were as follows: Rosa10: 5'- CTCTGCTGCCTCCTGGCTTCT-3'; Rosa11: 5'-CGAGGCGGATCACAAGCAATA-3'; R26R2: 5'- GCGAAGAGTTTGTCCTCAACC-3'; StopF: 5'- CACACAGGCATAGAGTGTCTGCT-3'; Wnt5aR: 5'-AGAACTTGGAAGACATGGCACCT-3'. Product size from wild-type: Rosa10 + Rosa11 = 330bp, from KI: Rosa10 + R26R2 = 192bp, StopF+Wnt5aR = 500bp. Position of primers are shown in **Figure S1A**.

Analysis of Pregnancy Events. Female mice were mated with WT fertile males to induce pregnancy. The morning of finding a vaginal plug was considered day 1 of pregnancy. To examine receptivity and implantation, pregnant dams were euthenized on day 4 (0900-1000h), and day 5 (0900h), respectively. Implantation sites were visualized by intravenous injection of a Chicago blue dye solution, and the number of implantation sites — demarcated by distinct blue bands — was recorded. The number of blue bands on day 5 of pregnancy was compared between floxed and experimental animals as an index of ovulation, fertilization, and oviductal embryo transport and development. For litter size analysis, pregnant females were monitored from days 17 through 21 by observing mice daily for parturition and litter size.

Immunohistochemistry and histology. Immunohistochemistry of Ki67 (Neomarkers), Cox2 (custom made), and ROR2 (provided by Dr. Yasuhiro Minami, Kyoto University) was performed in paraffin-embedded sections using specific antibodies. Tissue sections from floxed and experimental groups were processed onto the same slide. A Histostain-Plus (diaminobenzidine) kit (Invitrogen) was used to visualize specific antigens. Paraffin sections (6 μm) were deparaffinized, hydrated and stained with hematoxylin and eosin for light microscopy analysis.

Immunofluorescence and confocal microscopy. Immunofluorescence for phospho-Smad1/5/8 (provided by Ed Laufer, Columbia University), ZO-1 (Invitrogen), E-cadherin (Cell Signaling), cytokeratin-8 (Developmental Studies Hybridoma Bank, University of Iowa), aPKC (Santa Cruz), were performed using Cy2-conjugated, Cy-3 conjugated,

and Cy-5-conjugated (Jackson Immuno Research) secondary antibodies. Nuclear staining was performed using Hoechst 33342. Tissue sections from floxed and experimental groups were processed onto the same slide.

RNA isolation and quantitative PCR. RNA samples were prepared from homogenized tissues using TRIzol reagent (Invitrogen). Quantitative PCR was performed using StepOnePlus Real-Time PCR System (Applied Biosciences). PCR was performed using the following primers: 5'-TTCATGAATGGGCCTTTTTC-3' and 5'-ACCCCTAAGTGGCTGGTTCT-3' for *Wnt5a* (product size 468 bp); 5'-GTACTCCTGTTGCTGCGTCA-3' and 5'- GAGAGTCCATTGGTCCCTGA-3' for *Lif* (product size 199 bp); 5'-CTCTAACCACTGCCCTCCTG-3' and 5'-ACGCTTGTCAGCTCAGTTCA-3' for *Ihh* (product size 200 bp); 5'-AAGTTCCGCCAGAAGCAGTA -3' and 5'- AGCCATCTTCAGCTTTTCCA -3' for *Msx1* (product size 220 bp); 5'-TCCATGACAACTTTGGCATTG-3' and 5'-CAGTCTTCTGGGTGGCAGTGA-3' for mouse *Gapdh* (product size, 72 bp). *Gapdh* served as an internal control.

***In Situ* Hybridization – Radiolabeled probes.** In situ hybridization was performed as previously described (Daikoku et al., 2011; Lim et al., 1997). Uterine cryosections (12 µm) were mounted onto poly-L-lysine-coated slides, fixed in cold 4% paraformaldehyde, acetylated and hybridized at 45°C for 4 h in formamide hybridization buffer containing ^{35}S-labelled cRNA probes. RNase A-resistant hybrids were detected by autoradiography with Kodak NTB-2 liquid emulsion using darkfield microscopy. To compare mRNA

localization, uterine sections from floxed and experimental groups were processed onto the same slides. Mouse-specific cRNA probes for *Msx1, Hoxa10, Ihh, Lif, Ptgs2, Bmp2, Wnt7a*, *Wnt11* and *Wnt5a* were used for hybridization.

In Situ **Hybridization – DIG-labeled probes.** In situ hybridization was performed as previously described (Simmons et al., 2008). Uterine cryosections were rehydrated in PBS and post-fixed in 4% PFA/PBS at 4°C. Sections were acetylated and hybridized with digoxigenin (DIG)-labeled cRNA probes for *Prl3d1*, *Tpbpa* and *Gcm1* overnight at 65°C. DIG-labeled probes were generated following the manufacturer's protocol (Roche). Slides were treated with RNase A, blocked in blocking solution, and incubated overnight at 4°C with anti-DIG antibody diluted in blocking solution. Sections were washed in appropriate buffers and developed using an NBT/BCIP kit (Promega).

Primary uterine epithelial and stromal cell culture. Uteri from day 4 pseudopregnant WT mice were split open longitudinally and cut into small pieces (2-3 mm long). Tissue pieces were incubated with pancreatin and dispase for 2 h on ice, followed by 30 min at room temperature and 10 min at 37ºC. Fragments of luminal epithelial sheets were liberated by pipetting the digests several times. The remaining tissue fragments were incubated in type II collagenase to free stromal cells. Stromal cells were suspended in Dulbecco's modified Eagle's medium:F12 Nutrient Mixture (DMEM:F12) containing 10% heat-inactivated charcoal-stripped fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 1.25 µg/ml fungizone. Cell suspensions were filtered through a nylon mesh (70 µm) to remove glands and clumps of epithelial cells. Stromal cells were plated

first followed by epithelial cells in 3.5 mm Petri dishes. Cells were allowed to attach for 48 h at 37 ºC under the atmosphere of 5% CO_2 in air. Protein lysates were collected 24 hours later in RIPA buffer for immunoblotting analysis.

Immunoblotting. Antibodies used included Wnt5a (R&D), ROR2 (Developmental Studies Hybridoma Bank, University of Iowa), ROR1 (provided by Henry Ho, UCSD), and actin (Santa Cruz). Bands were visualized by using an ECL kit (GE Healthcare). Actin served as a loading control. For gel-shift assay, proteins were separated in 7% Bis-acrylamide gel for 4 h at 70V for ROR1 and ROR2.

Immunoprecipitation. Snap frozen day 5 implantation sites were lysed in 500µL of lysis buffer (PBS at pH 7.4 containing phosphatase inhibitor cocktails 1 and 2 and protease inhibitor cocktail (Sigma)) on ice. Samples were incubated on ice for 30 min and centrifuged at 12,000g for 15 min. Protein lysates (1 mg) were added to 2 µg of ROR2 antibody or IgG (negative control) and rotated overnight at 4°C. Dynabeads coated with Protein G (Dynal Biotech) were washed and incubated with protein lysate for 2 hours at 24˚C. Immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting with phospho-Tyrosine antibody (BD Biosciences) or ROR2.

IP-Kinase assay. Ishikawa cells (ATCC) expressing endogenous ROR2 were lysed in a small volume of lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Sigma). Lysate was diluted in

2X kinase buffer (120mM Tris-HCl pH 7.4, 24mM $MgCl_2$ and supplemented with phosphatase inhibitors). Samples were preincubated with 200 ng/mL Wnt5a (R&D) (Gao et al., 2011) for 10 min on ice. Kinase reaction was initiated by adding 80 µM ATP at 30°C and terminated at indicated time points by 10 mM EDTA. Protein was precipitated with TCA on ice for 1 h. Samples were centrifuged at 13000 rpm for 5 min at 4°C and washed in a series of TCA/acetone washes to a final wash in 100% acetone. Pellets were resuspended in 50 µL of 50 mM Tris-HCl pH 7.4 and incubated with Dynabeads conjugated with phospho-tyrosine antibody (BD Biosciences). Protein-bead-antibody conjugates were placed on a shaker at 4ºC for 90 min, washed three times in 10 mM Tris-HCl pH 8.0 and resuspended in 40 µL 1X sample buffer. Samples were run on a 10% Bis-acrylamide gel and immunoblotted with ROR2 antibody.

Electron Microscopy. Small pieces (1mm thickness) of *Wnt5a*$^{f/f}$, *Wnt5a*^{GOF} and *Wnt5a*$^{d/d}$ uteri on day 5 of pseudopregnancy were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Tissues were then washed and fixed in 1% aqueous osmium tetroxide in 0.1 M sodium cacodylate buffer. Following dehydration with ethanol and propylene oxide, the tissues were embedded in 100% Embed 812 mixture. Sections (80 nm) were cut with a Leica UC-7 ultramicrotome, stained with 3% uranyl acetate and Sato's Lead, and examined under a JEOL JEM-1400 Transmission Electron Microscope at 80KV.

Supplemental References

Gao, B., Song, H., Bishop, K., Elliot, G., Garrett, L., English, M.A., Andre, P., Robinson, J., Sood, R., Minami, Y.*, et al.* (2011). Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. Developmental cell *20*, 163-176.

Xiao, C., Calado, D.P., Galler, G., Thai, T.H., Patterson, H.C., Wang, J., Rajewsky, N., Bender, T.P., and Rajewsky, K. (2007). MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell *131*, 146-159.