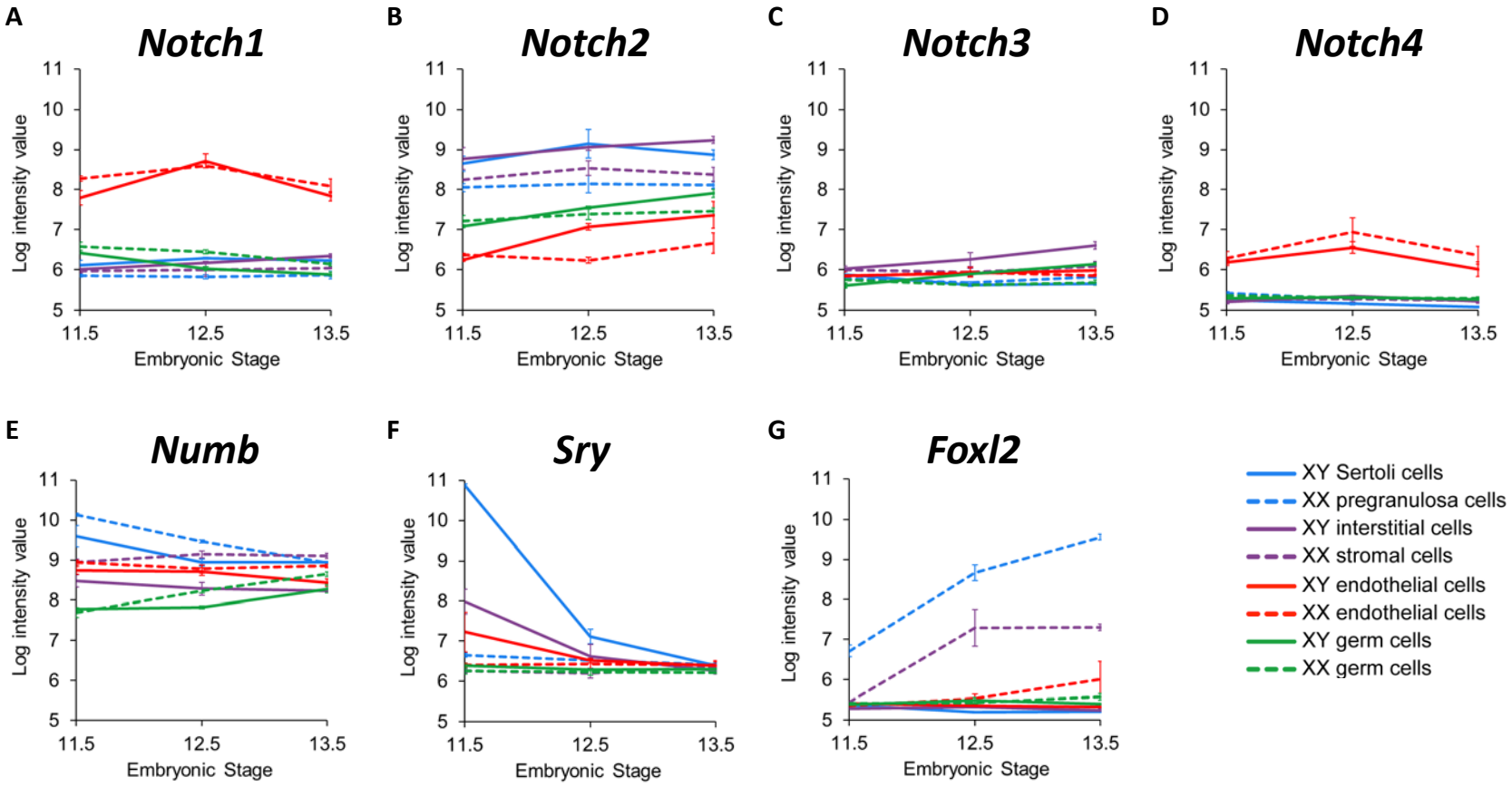

A
Notch1
B
Notch2
C
Notch3
D
Notch4
E
Numb
F
Sry
G
Foxl2
Log intensity value
Embryonic Stage
11.5
12.5
13.5
XY Sertoli cells
XX pregranulosa cells
XY interstitial cells
XX stromal cells
XY endothelial cells
XX endothelial cells
XY germ cells
XX germ cells

Fig. S1. Temporal transcriptional profiling of *Notch* receptor genes and *Numb*. Microarray analysis [data from (Jameson et al., 2012)] of supporting (blue, Sertoli in XY gonads and granulosa in XX gonads), interstitial (purple, interstitial in XY gonads and stromal in XX gonads), endothelial (red), and germ cells (green) from E11.5 – E13.5 embryonic gonads reveal the differential expression patterns of *Notch1, Notch2, Notch3, Notch4,* and *Numb* (*Numbl* was not detected in this microarray dataset). (A) *Notch1* is highly expressed in the endothelial lineage, but its expression is very low in other lineages in both XX and XY gonads at all three stages. (B) *Notch2* is expressed most abundantly in the Sertoli, granulosa, interstitial and stromal lineages of XY and XX gonads at all three stages. Germ cell and endothelial cell lineages have lower *Notch2* expression levels in both sexes. (C) The expression of *Notch3* is very low in all cell lineages throughout all three stages. (D) *Notch4* is specifically expressed in the endothelial cell lineage in both XX and XY gonads. (E) *Numb* is abundantly expressed in all cell lineages from stage E11.5 to E13.5. The Sertoli cell and granulosa cell lineages have the highest expression level at E11.5. (F,G) Plots for *Sry* (specific to the male supporting cell lineage), and *Foxl2* (specific to the female supporting cell and stromal lineage) are shown for comparison. Log intensity values 6 or lower are usually considered very low or background.

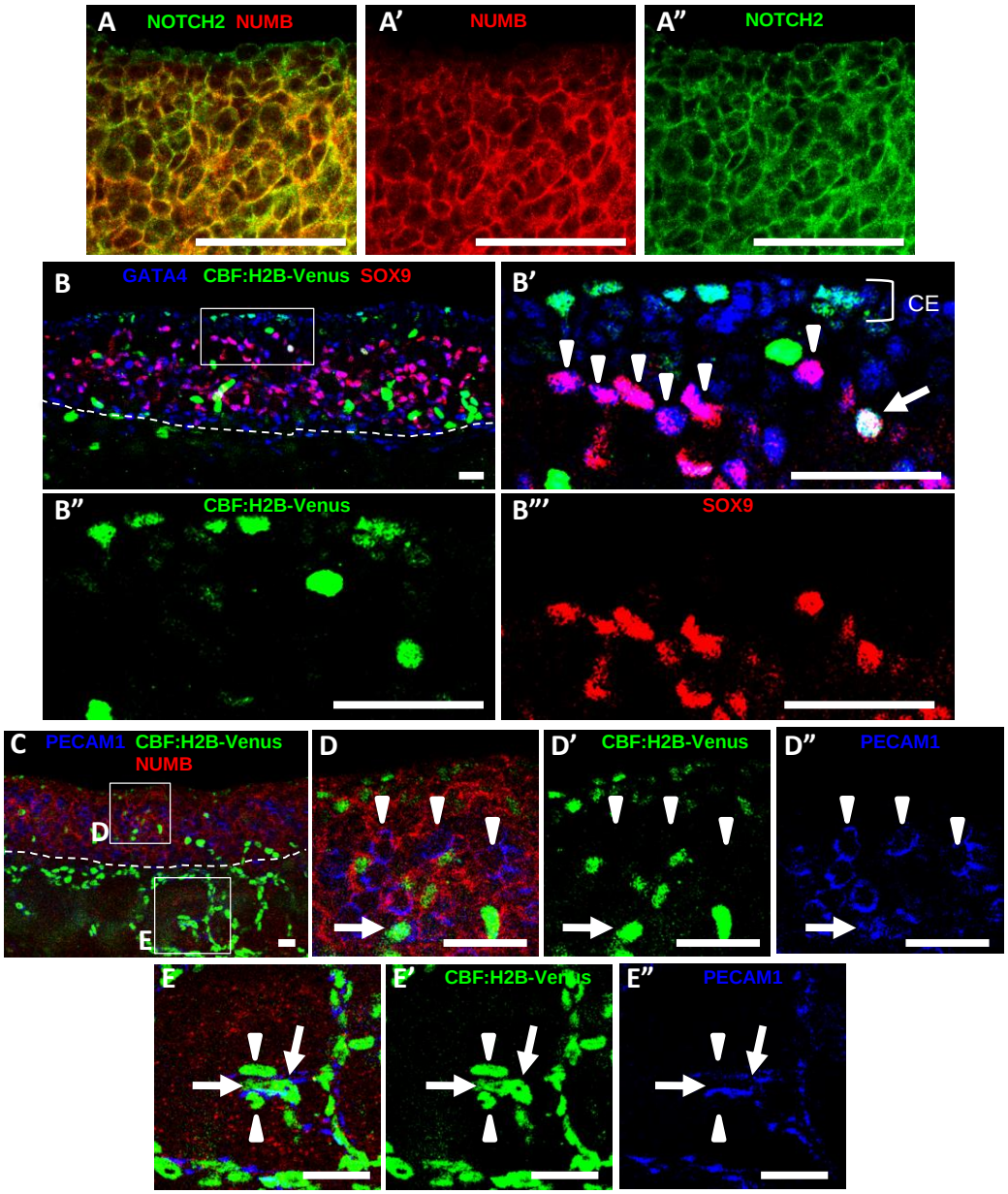


Fig. S2. Notch activity is absent from Sertoli and germ cells, but is observed in the coelomic epithelium and vascular/perivascular cells in the E11.5 gonad. Immunofluorescent images of E11.5 XY control (A) or E11.5 XY *CBF:H2B-Venus* (B-E) gonads, which report canonical Notch activity via the expression of a nuclear-localized YFP variant driven by multiple RBPJ (CBF1) binding sites (Nowotschin et al., 2013). B'-B''', D-D'' and E-E'' are higher-magnification images of the boxed regions in B and C. (A) NOTCH2 and NUMB expression are widespread throughout the gonad, likely in multiple cell types. (B) Notch activity, as reported by H2B-Venus, is observed in the coelomic epithelium ("CE" in B'), but is absent in SOX9-positive Sertoli cells (arrowheads), except for rare cells (arrow) that are likely newly-born Sertoli cells in which Venus expression persists. (C-E) Notch activity is also absent from PECAM1-positive germ cells (arrowheads in D-D''), but is observed in PECAM1-positive vascular endothelial cells (arrow in E). E-E'' shows that endothelial cells (arrows) and perivascular cells (arrowheads) throughout the mesonephros strongly express Venus, which is consistent with our previous reports of Notch signaling activity in the gonad using a Transgenic Notch Reporter GFP (TNR-GFP) mouse line (Defalco et al., 2013). Therefore, these data suggest that these two distinct Notch reporter lines have similar expression patterns in the fetal gonad. Scale bars in all images=25 µm.

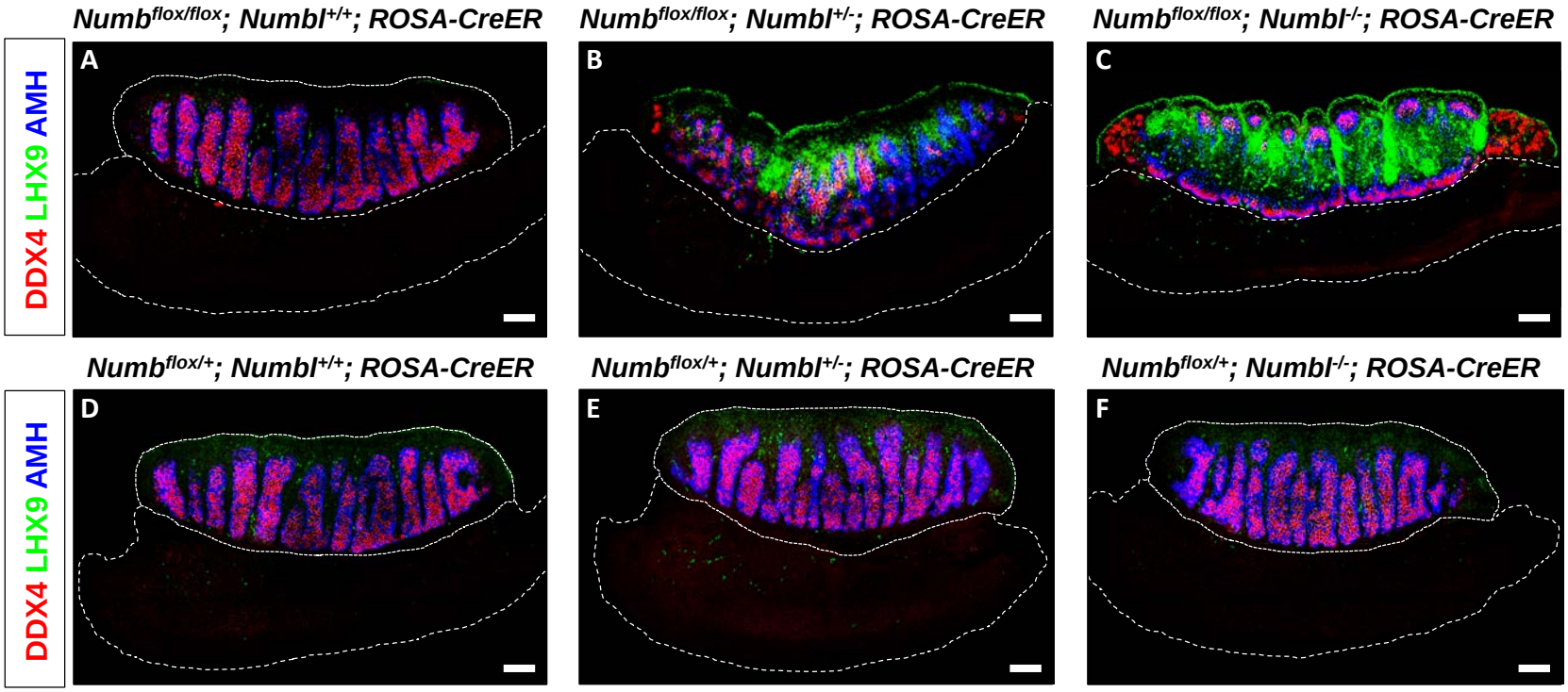


Fig. S3. E13.5 *Numb*$^{flox/flox}$*;Numbl*$^{+/-}$*;ROSA-CreER* and *Numb*$^{flox/flox}$*;Numbl*$^{-/-}$*;ROSA-CreER* XY gonads display mutant phenotypes, whereas other segregating genotypes do not. Immunofluorescent images of E13.5 XY RosaCreER-positive gonads with *Numb*$^{flox/flox}$ or *Numb*$^{flox/+}$ alleles in various combinations with wild-type (*Numbl*$^{+/+}$), heterozygous (*Numbl*$^{+/}$), or homozygous (*Numbl*$^{-/-}$) genotypes. Only *Numb*$^{flox/flox}$ animals with heterozygous or homozygous mutation in *Numbl* presented mutant phenotypes as measured by the presence of LHX9-positive patches and reduction of both DDX4-positive germ cells and AMH-positive Sertoli cell populations (B and C). All other allelic combinations presented phenotypically normal gonads at E13.5. Scale bars=100 µm.

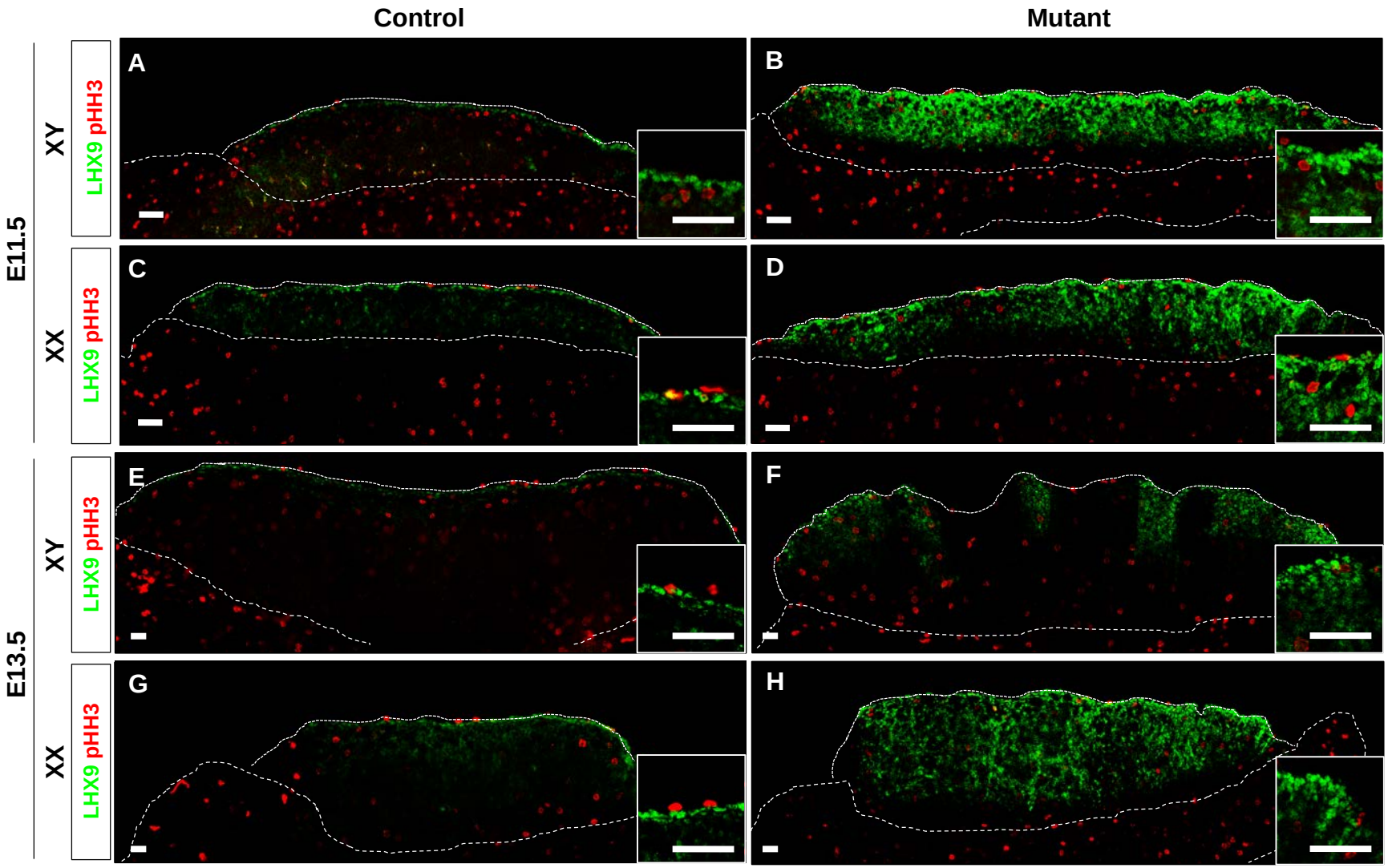
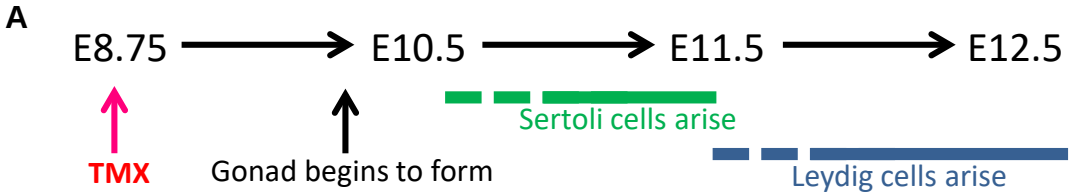
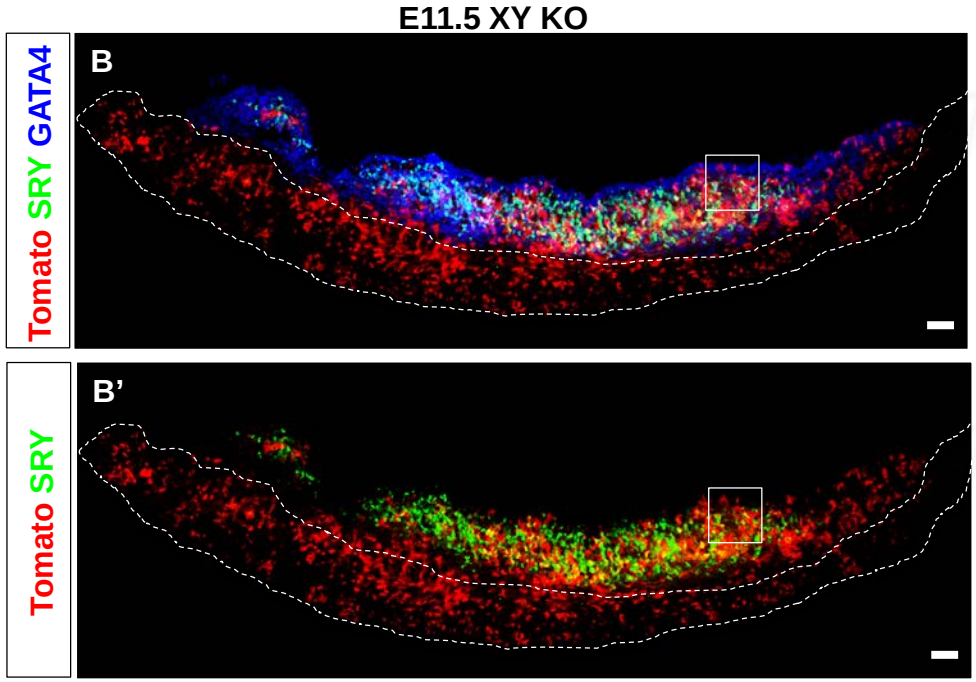


Fig. S4. No significant cell proliferation differences were observed between XX and XY E11.5 and E13.5 control and mutant gonads. Control and mutant gonads showed similar numbers of pHH3-positive cells in the CE domain and in the gonad field at both E11.5 and E13.5. pHH3-positive cells were absent in LHX9-positive patches in mutant gonads (B,D,F and H). Scale bars in all images=50 µm.





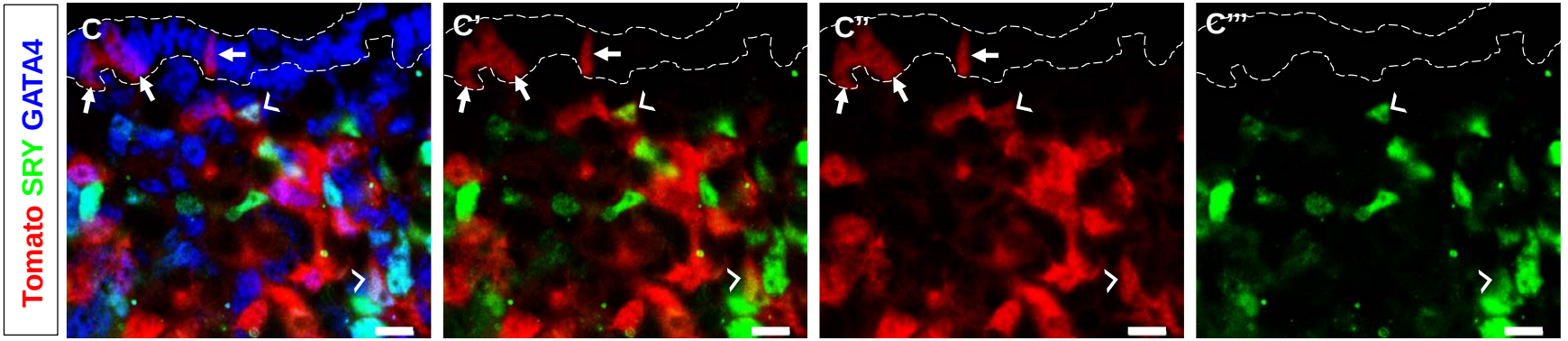


Fig. S5. Tamoxifen injection at E8.75 led to activation of ROSACreER in ~50% of gonadal cells, unevenly distributed across the gonad field at E11.5. (A) Schematic diagram outlines the time of tamoxifen administration relative to the formation of the gonad and the time of Sertoli and Leydig progenitor specification. Tamoxifen was administered at E8.75, ~1.5 days before the initial formation of the gonad. The specification of the Sertoli cell lineage occurs between gonad formation and E11.5, followed by the specification of interstitial cells that are progenitors of the Leydig cell lineage. (B) The ROSA-Tomato reporter (RTR) was crossed onto the *Numb*$^{flox/flox}$*/Numbl*$^{-/-}$*;ROSACre-ER* background. The expression of Tomato reports Cre recombinase activity in individual cells of E11.5 XY mutant gonads after tamoxifen induction at E8.75. Gonads were co-stained with antibodies against RFP (Tomato), SRY (Sertoli progenitor marker, green) and GATA4 (pan somatic cell marker, blue). Mesonephroi are outlined with white dashed lines. (C, C', C", C'") Higher magnification images of boxed region in B. Cre was active in some cells (arrows) in the CE (outlined with dashed lines), but most were negative for Tomato. In a few SRY-positive cells, Tomato reported CRE activity (white carets). The CE is outlined with white dashed lines. Scale bars in B=50 µm. Scale bars in C-C"'=10 µm.

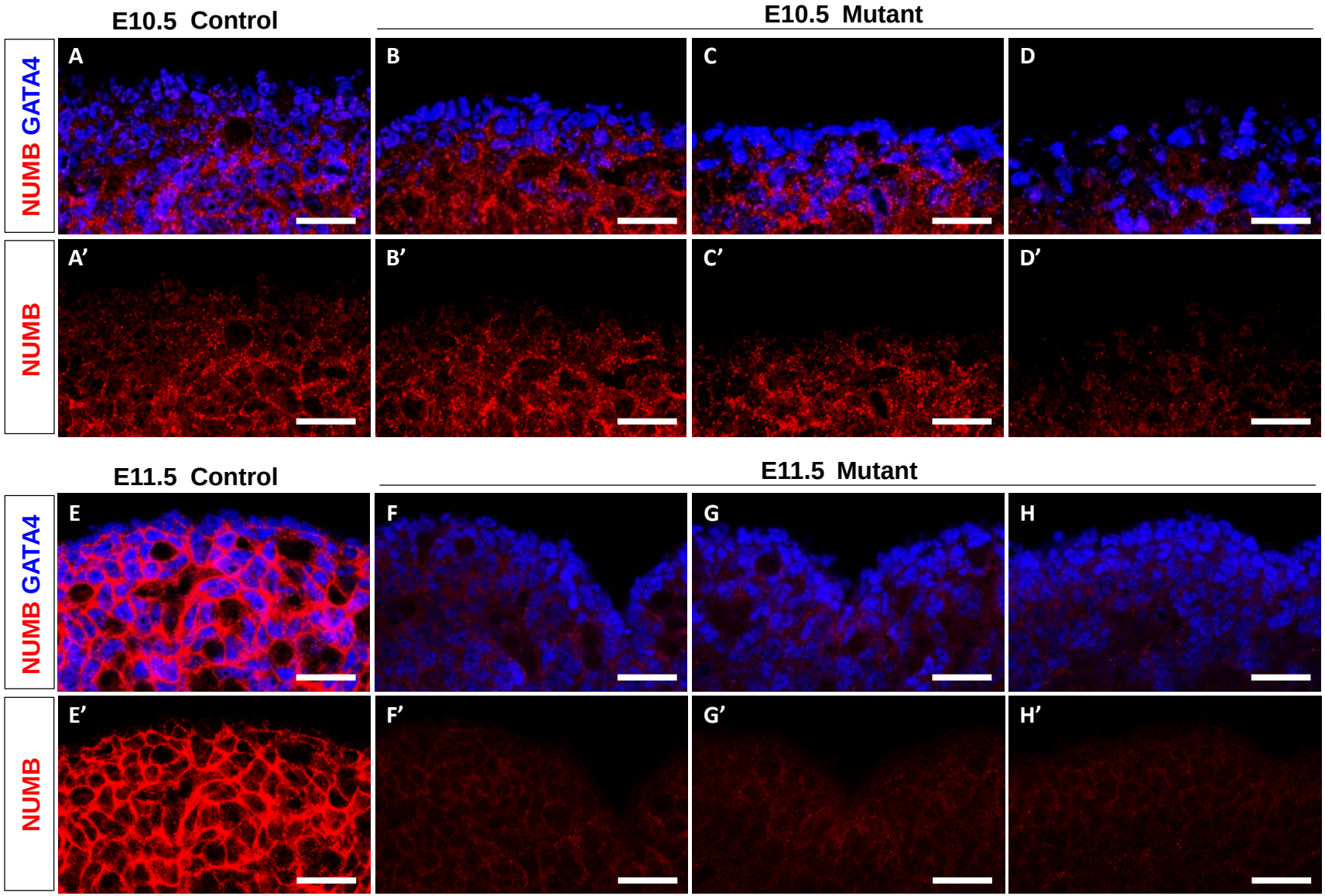


Fig. S6. Levels of NUMB protein declined between E10.5 and E11.5 after tamoxifen injection at E8.75. (A and E) In control E10.5 and E11.5 gonads, NUMB (red) was detected in almost all gonadal cells. Somatic cells were co-labeled with GATA4 (blue). (B-D) At E10.5, *Numb/Numbl* mutant gonads showed abundant NUMB protein across the gonad field in the majority of samples. (F-H) At E11.5, levels of NUMB protein were strongly reduced in *Numb/Numbl* mutant gonads. Three examples (showing some variability) are shown for each stage. Lower images for each stage (A'-H') show isolated NUMB signal for the merged images above (A-H). Scale bars=25 µm.

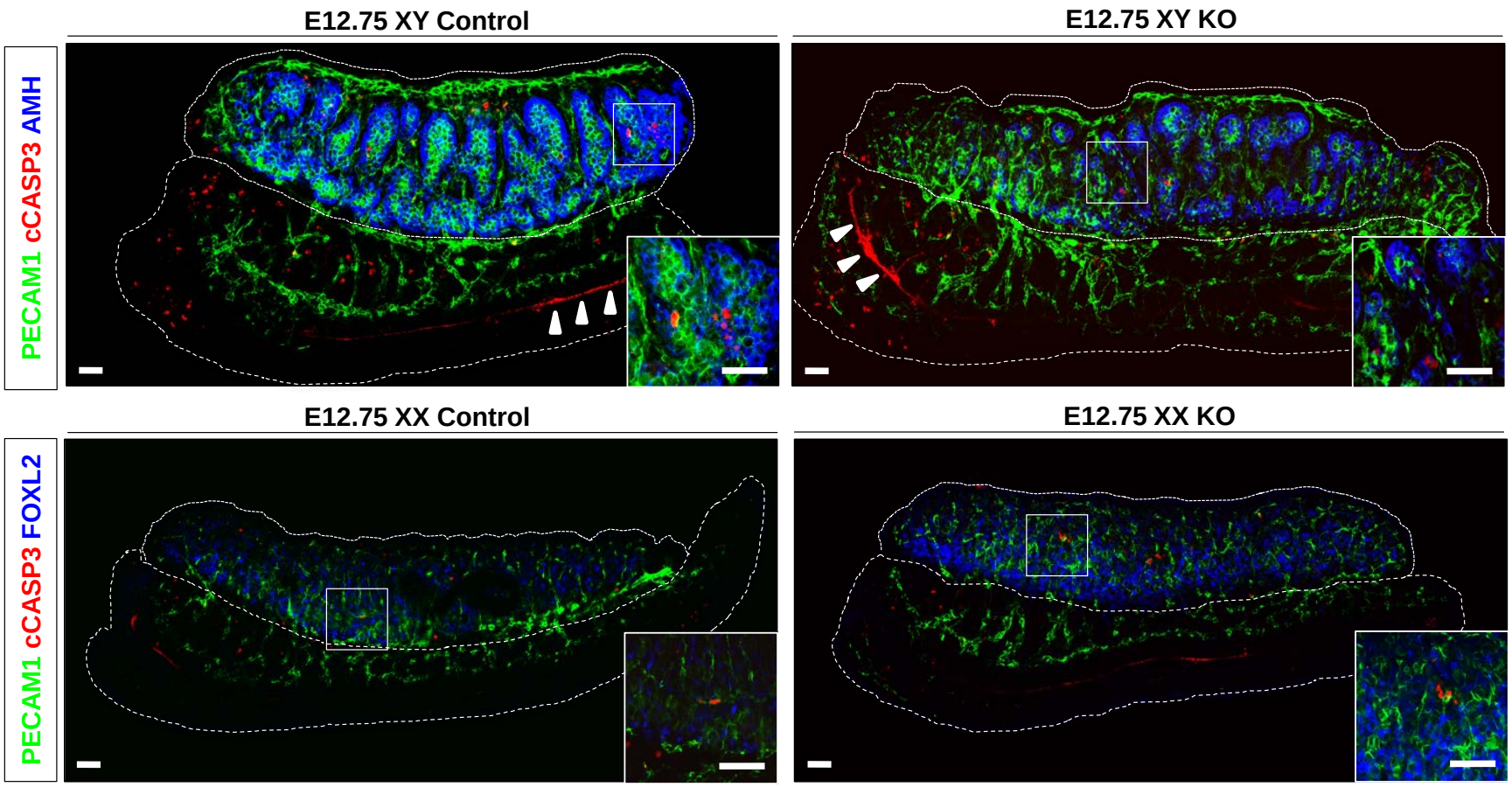


Fig. S7. Up-regulation of cell death pathways is unlikely to explain the loss of Sertoli or granulosa cells in mutants. Few cCASP3-positive cells were observed in XX or XY control or mutant gonads at E12.75. A few positive cells are shown at higher magnification in the boxed region of each frame as a control for the antibody, which also labels the degenerating Mullerian duct in XY samples (arrowheads). Scale bars in all images=50 µm.

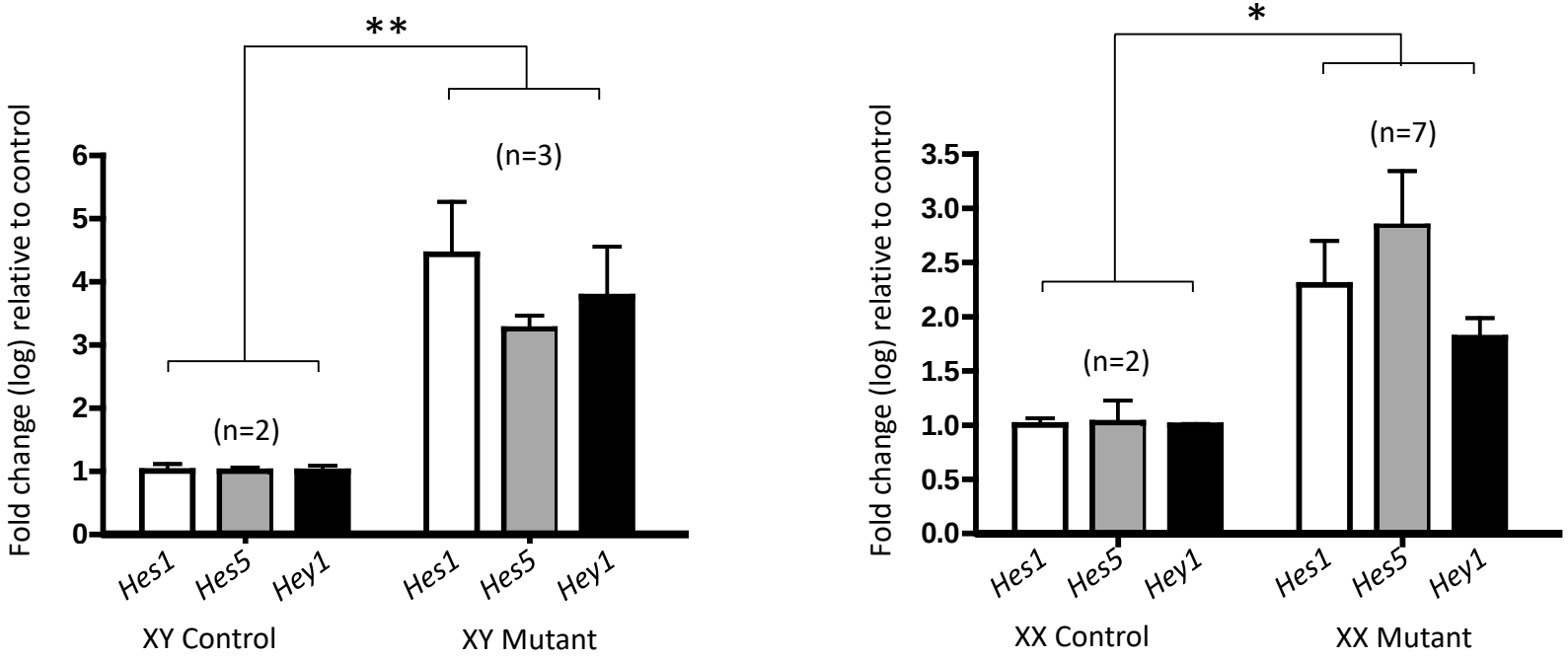


Fig. S8. Three Notch downstream target genes are upregulated in E13.5 XY and XX mutant gonads. Quantitative RT-PCR of *Hes1*, *Hes,5* and *Hey1* showed the elevation of Notch downstream signaling target genes in XY and XX mutant gonads. Statistical significance was determined by unpaired *t*-tests. *P* value in XY control versus mutant is 0.0012. *P* value in XX control versus mutant is 0.0117. Asterisks apply to all three genes.

REFERENCES

Defalco, T., Saraswathula, A., Briot, A., Iruela-Arispe, M.L., and Capel, B. (2013). Testosterone levels influence mouse fetal Leydig cell progenitors through notch signaling. *Biol Reprod* **88**, 91.

Jameson, S.A., Natarajan, A., Cool, J., DeFalco, T., Maatouk, D.M., Mork, L., Munger, S.C., and Capel, B. (2012). Temporal transcriptional profiling of somatic and germ cells reveals biased lineage priming of sexual fate in the fetal mouse gonad. *PLoS Genet* **8**, e1002575.

Nowotschin, S., Xenopoulos, P., Schrode, N., and Hadjantonakis, A.K. (2013). A bright single-cell resolution live imaging reporter of Notch signaling in the mouse. *BMC Dev Biol* **13**, 15.

Table S1. List of primary antibodies.

Primary antibodies	Species	Cat#	Dilution	Source
NUMB	Rabbit	ab-14140	1:100	Abcam, Cambridge, MA
NOTCH2	Goat	sc-7423	1:250	Santa Cruz Biotechnology, Santa Cruz, CA
GATA4	Goat	sc-1237	1:500	Santa Cruz Biotechnology, Santa Cruz, CA
VCAM1	Goat	AF-643	1:1000	R&D Systems
LHX9	Rat		1:50	kind gift of Thomas Jessell, Columbia University, New York, NY
LHX9	Rat		1:50	kind gift of Ken-Ichirou Morohashi, Kyushu University, Fukuoka, Japan
DDX4	Rabbit	ab13840	1:250	Abcam, Cambridge, MA
SRY	Rabbit		1:250	kind gift of Dagmar Wilhelm, University of Melbourne, Parkville, Australia
Laminin	Rabbit		1:500	kind gift of Harold Erickson, Duke University Medical Center, Durham, NC
RFP	Rat	5F8	1:500	Chromotek, Germany
WT1	Rabbit	sc-192	1:100	Santa Cruz Biotechnology, Santa Cruz, CA
NR5A1	Rabbit		1:1500	kind gift of Ken-ichirou Morohashi, Kyushu University, Fukuoka, Japan
β1-Integrin	Rat	MAB1997	1:200	Millipore, Billerica, MA
3β-HSD	Goat	sc-30820	1:250	Santa Cruz Biotechnology, Santa Cruz, CA
FOXL2	Goat	NB100-1277	1:250	Novus Biologicals, Littleton, CO
MKI67	Rabbit	RM-9106-S	1:500	Neomarkers, Thermo Scientific, Waltham, MA
PECAM1	Rat	553370	1:500	BD Pharmingen, San Diego, CA
SOX9	Rabbit	AB5535	1:2000	Millipore, Billerica, MA
cCASPASE3	Rabbit	9661S	1:250	Cell Signaling Technology, Danvers, MA
phospho-Histone H3 (S10)	Rabbit	9701S	1:250	Cell Signaling Technology, Danvers, MA
CADHERIN1	Rat	13-1900	1:500	Novus Biologicals, Littleton, CO
GFP	Chicken	GFP-1020	1:2000	Aves, Tigard, OR