Numb regulates somatic cell lineage commitment during early gonadogenesis in mice

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ABSTRACT

During early gonadogenesis, proliferating cells in the coelomic epithelium (CE) give rise to most of the somatic cells in both XX and XY gonads. Previous dye-labeling experiments showed that a single CE cell could give rise to additional CE cells and to both supporting and interstitial cell lineages, implying that cells in the CE domain are multipotent progenitors, and suggesting that an asymmetric division is involved in the acquisition of gonadal cell fates. We found that NUMB is asymmetrically localized in CE cells, suggesting that it might be involved. To test this hypothesis, we conditionally deleted Numb on a Numbl mutant background just prior to gonadogenesis. Mutant gonads showed a loss of cell polarity in the surface epithelial layers, large interior cell patches expressing the undifferentiated cell marker LHX9, and a loss of differentiated cells in somatic cell lineages. These results indicate that NUMB is necessary for establishing polarity in CE cells, and that asymmetric divisions resulting from CE polarity are required for commitment to differentiated somatic cell fates. Surprisingly, germ cells, which do not arise from the CE, were also affected in mutants, which may be a direct or indirect effect of loss of Numb.

KEY WORDS: NUMB, NOTCH, Mouse gonad, Cell polarity, Asymmetric cell division, Coelomic epithelium

INTRODUCTION

The undifferentiated gonadal primordia arise within the intermediate mesoderm. Beginning about embryonic day (E) 9.5, proliferation of cells in the region of the coelomic epithelium (CE) that overlies the mesonephric ducts, leads to thickening of tissue to form the gonad. Prior to E10.5, the gonads are molecularly indistinguishable between genotypically XX and XY embryos. However, at E10.5, some somatic cells of the XY gonad activate the Y-chromosome gene *Sry* (sex-determining region of the Y-chromosome), which initiates the male pathway and commits the gonad to testis fate (Bullejos and Koopman, 2001). Conversely, in XX gonads or XY gonads that lack the *Sry* gene, the female pathway dominates and directs ovary development (Gubbay et al., 1990).

Proliferating cells in the CE give rise to most of the somatic cells in both XX and XY gonads, including the supporting cells in direct contact with germ cells (Sertoli cells in males and granulosa cells in females) and other interstitial/stromal cells that include the steroidogenic lineages (DeFalco et al., 2011; Karl and Capel, 1998; Liu et al., 2016; Mork et al., 2012; Schmahl and Capel, 2003). Dye-labeling experiments suggested that a single CE cell could give rise to both supporting and interstitial cell lineages, implying that cells in the CE domain are multipotent progenitors, and suggesting that an asymmetric division is involved in the acquisition of gonadal cell fates (Karl and Capel, 1998). However, the mechanism underlying asymmetry in CE cells has not been explained.

Notch and Numb are obvious candidates for mediating asymmetric division of cells in the CE. *Notch1*, *Notch2*, *Notch4* and *Numb* are expressed in the early gonad (Defalco et al., 2013; Jameson et al., 2012b; Tang et al., 2008). Deletion of *Notch2* using *Nr5a1*-CRE, which is expressed in the CE and in most somatic cells of the gonad beginning ~E11.5 (Liu et al., 2016; Tang et al., 2008), showed that Notch signaling maintains the Leydig cell progenitor population. Blocking Notch signaling with a γ-secretase inhibitor or through loss of function of the downstream target *Hes1* resulted in differentiation of the precursor population into mature Leydig cells (Tang et al., 2008). However, whether NUMB was involved in cell fate determination decisions in the embryonic gonad was not clear.

NUMB, the monomeric PTB-containing adaptor protein, is a known antagonist of Notch signaling. Activation of Notch signaling involves ligand and receptor binding, followed by a series of proteolytic cleavage events that release the Notch intracellular domain (NICD), which enters the nucleus and associates with the transcriptional repressor RBPJ (recombination signal binding protein for immunoglobulin kappa J region, also known as CBF or CBF-1) (Allman et al., 2002; Artavanis-Tsakonas et al., 1995; Callahan and Raafat, 2001). In association with the transcriptional co-activator mastermind-like 1 (MAML1), NICD converts CBF-1 to a transcriptional activator, thereby initiating expression of target genes such as *Hes1*, *Hes5* and *Hey1* (Fischer et al., 2004; Wu et al., 2000). NUMB acts as an antagonist by preventing NOTCH localization to the cell membrane, thereby suppressing Notch signaling (O'Connor-Giles and Skeath, 2003).

During development, NUMB often acts as a cell fate determinant (reviewed by Knoblich, 2001, 2010). During the asymmetric cell division of *Drosophila* sensory organ precursor cells, NUMB protein is asymmetrically allocated to only one of the two daughter cells. In the cell that inherits NUMB, Notch signaling is silenced, leading to the differentiation of a pIIb signal-sending cell; the other daughter cell, which lacks NUMB, becomes a pIIa signal-receiving cell (Uemura et al., 1989). There are two Numb homologs in mice, encoded by *Numb* and numb-like (*Numbl*), which often act redundantly (Petersen et al., 2002), and are nearly ubiquitously expressed during embryogenesis (Zhong et al., 1997). In this study, we investigated the role(s) of Numb during fetal gonadogenesis. Using a ubiquitous Cre under the temporal control of tamoxifen induction, we triggered the conditional deletion of *Numb* on a

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Numbl mutant background beginning at E8.75, just prior to gonad formation. We found that polarity of CE cells was disrupted and multiple cell lineages were lost or under-represented, including supporting cells and Leydig cells. Surprisingly, germ cell numbers were also reduced, which could be a direct or indirect effect of loss of *Numb* and *Numbl*. These results reveal a new function for Numb and Notch signaling during mammalian gonadogenesis.

RESULTS

Notch and Numb are expressed during early gonadogenesis

The development of the bipotential gonad begins with thickening of the CE domain overlying the mesonephric ducts. Daughter cells from proliferation of the CE ingress into the interior of the gonad and give rise to both the supporting and interstitial/stromal cell lineages, suggesting that CE cells are multipotent progenitors and that an asymmetric division is involved. To address the possibility that the Numb and the Notch pathway play a role in CE proliferation and the subsequent ingression and/or differentiation of cell lineages, we first investigated the expression pattern of Numb and the Notch receptors during early gonadogenesis.

Based on microarray data from four sorted cell populations from XX and XY gonads at stages E11.5, E12.5 and E13.5 (Jameson et al., 2012b; Nef et al., 2005), *Numb* is expressed in all cell lineages, with higher expression levels at E11.5 in the supporting cell lineage in both XX and XY gonads. *Notch2* is expressed at high levels in both male and female supporting cell and interstitial/stromal cell lineages, whereas male and female germ cells and endothelial cells expressed *Notch2* at slightly lower levels. *Notch1* and *Notch4* are specifically expressed in the endothelial lineages (Brennan et al., 2002), whereas *Notch3* expression is low in all tested lineages (Fig. S1).

Notch2 expression was previously analyzed using a *Notch2* reporter line (*Notch2^{lacZ}*) (Hamada et al., 1999). Using this line, *Notch2* expression was detected at the CE and in most somatic cells of the XY gonad at E11.5, localized to the Sertoli cells at E12.0, and shifted to interstitial cells at E13.5 (Tang et al., 2008). We re-investigated this pattern using antibodies against NOTCH2. Consistent with the microarray data (Fig. S1B), NOTCH2 protein showed a broad expression pattern in gonadal cells (Fig. 1A,B; Fig. S2A,A″). By immunofluorescence, NUMB was also detected in almost all cell lineages at varying levels (Fig. S1E). However, whereas NOTCH2 was distributed evenly in the CE cells (Fig. 1A,B), NUMB was asymmetrically allocated to the basolateral domain of CE cells in both E11.5 XX and XY gonads (Fig. 1C,D; Fig. S2A,A′). This asymmetric distribution suggested that NUMB might be involved in polarity of the CE cells and/or in the determination of supporting and interstitial cell fates.

To determine where Notch signaling was active, we used a transgenic mouse line, *CBF:H2B-Venus* (Nowotschin et al., 2013), which reports Notch signaling activity via a nuclear-localized Venus fluorescent protein downstream of four copies of a consensus CBF binding site. In *CBF:H2B-Venus* Notch reporter mice, activation of Notch signaling by any of the Notch receptors is reflected by expression of Venus protein. Despite wide expression of NOTCH2 in most gonadal cells (Fig. 1A-B′; Fig. S2A,A″), Notch signaling was activated in relatively few cells, including many cells of the CE in which NUMB protein is present at low levels and/or polarized to the basolateral domain (Fig. 1C-D′; Fig. S2A,A′). Deeper inside the gonad field, where NUMB protein is present at high levels (Fig. 1E-F″), Notch signaling was activated in a small number of cells, very few of which are positive for the Sertoli cell marker SOX9 (*Sry*-box 9) (Fig. S2B-B′″). Occasional

cells double positive for the Notch reporter and SOX9 could be newly born Sertoli cells in which H2B-Venus has not yet degraded. Many H2B-Venus-positive cells also express the endothelial marker PECAM1 (platelet/endothelial cell adhesion molecule 1) (Fig. S2C-E″).

Temporal deletion of *Numb* in a *Numbl* mutant background led to severe morphological defects in the gonad

Homozygous *Numb^{−/−}* embryos die at E11.5 from central nervous system defects (Zhong et al., 2000), precluding an analysis of potential gonadal defects. To elucidate the role of *Numb* in gonadogenesis, we deleted a conditional allele of *Numb* driven by a ubiquitously expressed, tamoxifen-inducible ROSA-CreER at E8.75. The central nervous system defects were bypassed and viability was restored with this conditional strategy. Numb-like (*Numbl*) has been reported to compensate for loss of *Numb* (Petersen et al., 2002). For this reason, Cre-mediated deletion of *Numb* was performed on a *Numbl* homozygous or heterozygous mutant background.

Compared with control littermates, E13.5 and E14.5 XX and XY mutant gonads with conditional deletion of *Numb* and also mutant or heterozygous for *Numbl* (hereafter termed *Numb/Numbl* mutants) showed an irregular CE surface as well as variable morphological defects (Fig. 2A,B; Fig. S3). Antibodies against typical gonadal somatic cell markers, including PECAM1 (labels vasculature and germ cells), NR5A1 (nuclear receptor subfamily 5, group A, member 1, also known as steroidogenic factor 1, SF1; labels Sertoli cells, granulosa cells and steroidogenic cells) and VCAM1 (vascular cell adhesion molecule 1; labels interstitial/stromal cells), failed to label large patches of cells within mutant gonads of both sexes (Fig. 2C-F).

To determine whether these patches contained cells that retained markers of undifferentiated fate, we stained for LHX9, a transcription factor in the LIM homeobox domain gene family. LHX9 is a marker of undifferentiated cells within the CE (Birk et al., 2000; Mazaud et al., 2002). It is typically downregulated in cells within the gonad as differentiation occurs (DeFalco et al., 2011; Mazaud et al., 2002). In controls, the LHX9-positive domain was restricted to the CE (Fig. 2G,I), whereas in mutant gonads LHX9 labeled cells throughout the gonad that lacked expression of markers for specific gonadal lineages, such as SOX9 (Sertoli cells), VCAM1 (interstitial progenitors) (Fig. 2G,H), FOXL2 (forkhead box L2; granulosa cells) and DDX4 [DEAD (Asp-Glu-Ala-Asp) box polypeptide 4, also known as MVH or VASA; germ cells] (Fig. 2I,J). These results suggested that LHX9-positive patches observed in mutant gonads are composed of undifferentiated cells.

Deletion of *Numb* in a *Numbl* mutant background disrupted cell polarity in the CE

NUMB is involved in establishment of cell polarity in various systems (Wang et al., 2009; Wirtz-Peitz et al., 2008). Laminin and ITGB1 (β1-integrin) are proteins that define the basolateral domain of polarized cells (Barczyk et al., 2010; Domogatskaya et al., 2012; Durbeej, 2010; Hynes, 2009; Kadler et al., 2008). Because NUMB can regulate integrins (Nishimura and Kaibuchi, 2007), we tested whether NUMB is required for establishing cell polarity in CE cells by characterizing the subcellular distribution patterns of laminin and ITGB1 in E11.5 *Numb/Numbl* mutant gonads compared with control littermates (Fig. 3). In controls, both laminin and ITGB1 were located exclusively in the basolateral domain of CE cells (Fig. 3A,C). However, in *Numb/Numbl* mutants, laminin and ITGB1 were no longer restricted to the basolateral domain of surface
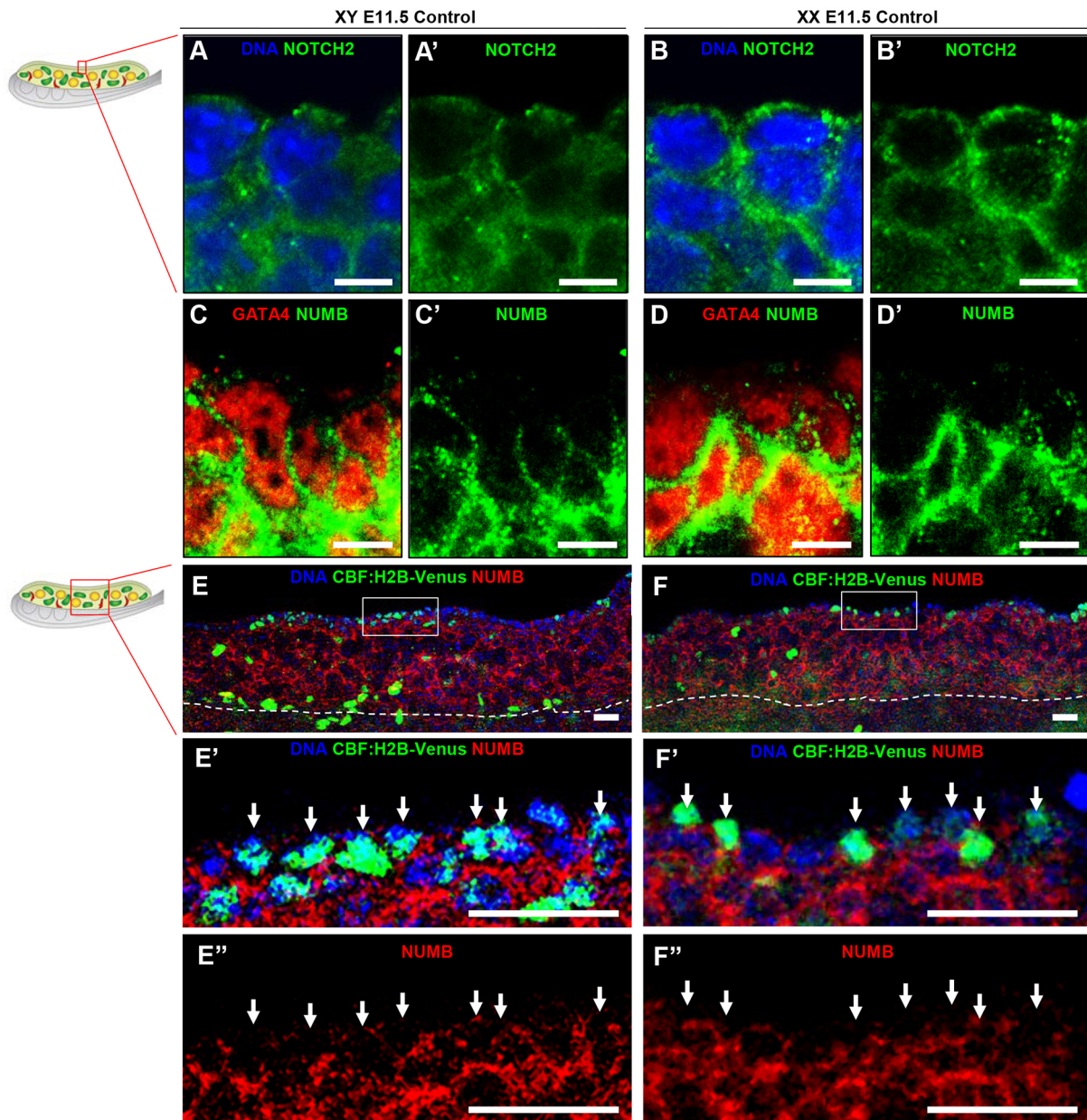
Fig. 1. NUMB is basolaterally localized, and Notch activity is high in CE cells of E11.5 gonads. (A,B) NOTCH2 (green) is evenly distributed in the cytoplasm of somatic and germ cells throughout XY (A) and XX (B) gonads at E11.5; nuclei are labeled by Hoechst staining (blue). (A′,B′) NOTCH2 signal alone. (C,D) NUMB (green) is asymmetrically located in the basolateral domain in both XY (C) and XX (D) CE cells at E11.5; GATA4 (red) labels nuclei of somatic cells. (C′,D′) NUMB signal alone. (E,F) A Notch reporter transgene (*CBF:H2B-Venus*; green) reveals high active Notch signaling in CE cells in XY (E) and XX (F) gonads (arrows in E′,E″,F′,F″). E′,E″ and F′,F″ show higher magnifications of the boxed regions in E and F; E″ and F″ show the NUMB channel alone. Schematics show the region of the gonad captured in the image. Scale bars: 5 µm (A-D′); 25 µm (E-F″).

epithelial cells. Instead, laminin and ITGB1 were distributed to all CE cell surfaces, including the apical domain (Fig. 3B,D). These results indicated that depletion of NUMB disrupts the establishment of CE apical-basal polarity.

LHX9-positive patches are derived from the CE
To determine whether the LHX9-positive patches in mutant gonads arose via de-differentiation *in situ* or were derived from the proliferating CE cells that ingress into the gonad, we performed a MitoTracker tracing experiment. MitoTracker is a dye that labels the mitochondria of cells with which it comes in direct contact, providing a short-term marker to trace the progeny of dye-labeled

cells (Brennan et al., 2003; DeFalco et al., 2011; Mork et al., 2012). *Numb/Numbl* mutant and control gonads were collected at E11.5, surface-labeled with MitoTracker, and transferred to organ culture as previously described (Martineau et al., 1997).

Only the first layer of the CE was labeled by MitoTracker after 1 h (Fig. 4A,C,E,G). Note that even in *Numb;Numbl* mutant gonads, in which the polarity of the CE was disrupted, MitoTracker did not immediately diffuse into the interior of the gonad, but was restricted to CE cells. In control gonads cultured for 24 h (Fig. 4B), the labeled cell population had expanded to the four or five top cell layers of the gonad, consistent with previous findings that proliferation of the CE contributes to thickening of the gonad
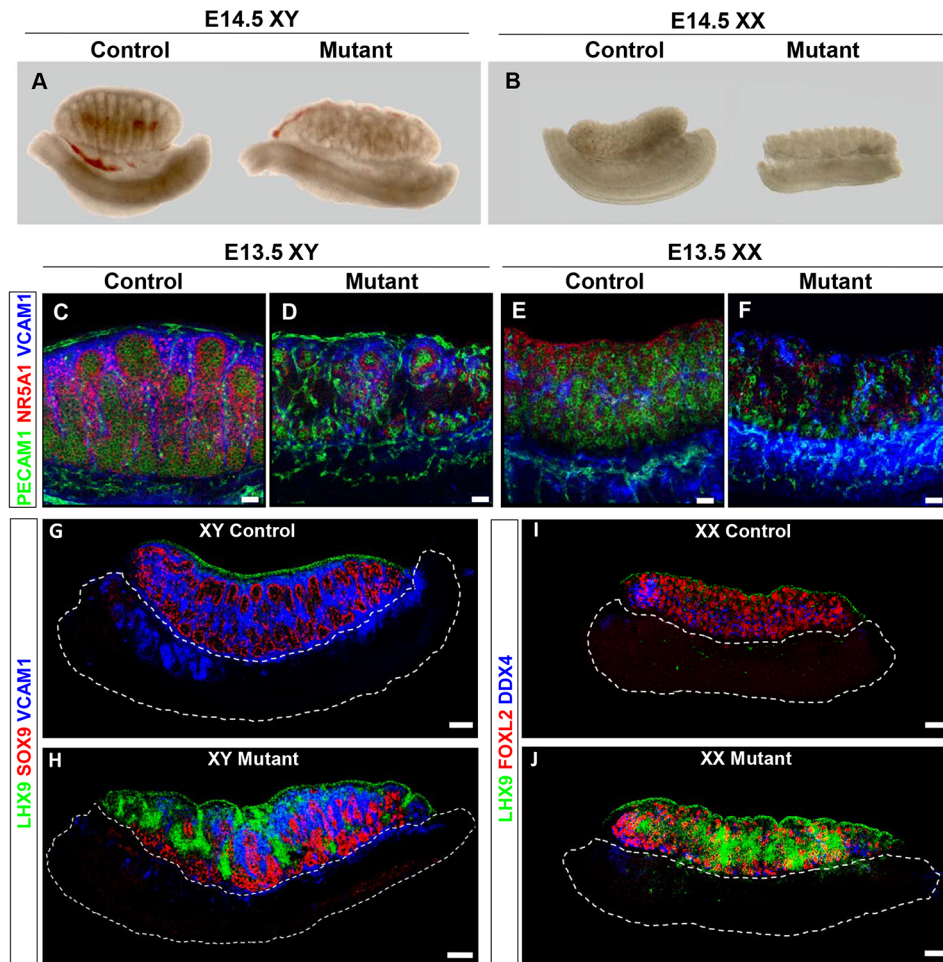
Fig. 2. *Numb/Numbl* mutant gonads have an irregular surface and interior pockets of undifferentiated cells. (A,B) At E14.5, both XY (A) and XX (B) *Numb/Numbl* mutant gonads were smaller and had an irregular surface. Disrupted testis cord structure was evident in XY mutants compared with controls. (C,D) In the E13.5 control XY gonad (C), PECAM1 antibody labeled germ cells and endothelial cells; NR5A1 antibody labeled Sertoli cells in regular cord structures as well as some interstitial cells, and VCAM1 antibody labeled interstitial mesenchymal cells (DeFalco et al., 2011). In mutant XY gonads (D), germ cell (PECAM1 positive, green) and Sertoli cell (NR5A1 positive, red) numbers were reduced. Some regions retained VCAM1 (blue) and NR5A1 (red) positive interstitial cells. However, several domains in the mutant gonad were negative for all tested differentiation markers. (E,F) Control XX gonads (E) contained PECAM1-positive germ cells (green), interstitial and granulosa cells (VCAM1 positive, blue, and NR5A1 positive, red, respectively). Similar to XY gonads, mutant XX gonads (F) showed a significant reduction of germ cells and other differentiated cell types and the presence of regions negative for differentiation markers. (G-J) In control XY and XX gonads (G,I), LHX9 is restricted to undifferentiated cells in the CE. However, in mutants (H,J), LHX9-positive domains are present in the gonad interior. SOX9 and VCAM1 label Sertoli and interstitial cells in G,H; FOXL2 and DDX4 label granulosa and germ cells in I,J. Throughout the figures, 'Mutant' refers to gonads from *Numb^{flox/flox};Numbl^{−/−};ROSA-CreER* or *Numb^{flox/flox};Numbl^{+/−};ROSA-CreER* embryos injected with tamoxifen at E8.75. 'Control' refers to all other genotypes resulting from the cross in which no phenotype was evident. Scale bars: 50 μm (C-F); 100 μm (G-J).

(Karl and Capel, 1998). LHX9-positive cells were restricted to the CE domain as shown previously (Fig. 4A,B,E,F). In contrast, in mutant gonads, MitoTracker-positive cells were not distributed in layers, but occupied expanded irregular domains, and many cells in the gonad interior retained LHX9 expression (Fig. 4C,D,G,H). This result supported the idea that the undifferentiated LHX9-positive cell patches in the *Numb/Numbl* mutants arose from the CE. Note also that cells in the CE in both controls and mutants retained the MitoTracker label, suggesting that one division product of the original labeled cell is left behind in the CE.

Cells in the CE are highly proliferative between E11.5 and E13.5 (Schmahl et al., 2000). To investigate whether cells in the undifferentiated LHX9-positive patches are also in active cell cycle, we stained gonads with anti-MKI67 antibody (also known as KI67), a marker of all stages of the cell cycle except G0 (Fig. 4I-P). In both E13.5 (Fig. 4I-L) and E11.5 (Fig. 4M-P) mutant and control gonads, CE cells were all MKI67 positive. However, to our surprise,

the LHX9-positive patches in the mutant gonads were negative for MKI67. To confirm this finding, we compared levels of phospho-histone H3 (Ser10) (pHH3), a marker of M phase of the cell cycle, between mutant and control samples. We found no elevation of pHH3 in LHX9-positive patches or elsewhere in mutant gonads relative to controls (Fig. S4).

Numb/Numbl mutants showed a 60% reduction in Sertoli cells

To determine whether gonadal cell defects occurred early and affected Sertoli progenitors, we stained E11.5 XY gonads from mutant and control littermates with antibodies against LHX9, GATA4 (expressed in all gonadal somatic cells) and SRY (specific to Sertoli cells) (Fig. 5A-I). At E11.5, some somatic cells expressed SRY and seemed to adopt their fate normally in mutant gonads. However, most LHX9-expressing cells did not also express SRY, although occasional exceptions were seen in higher magnification
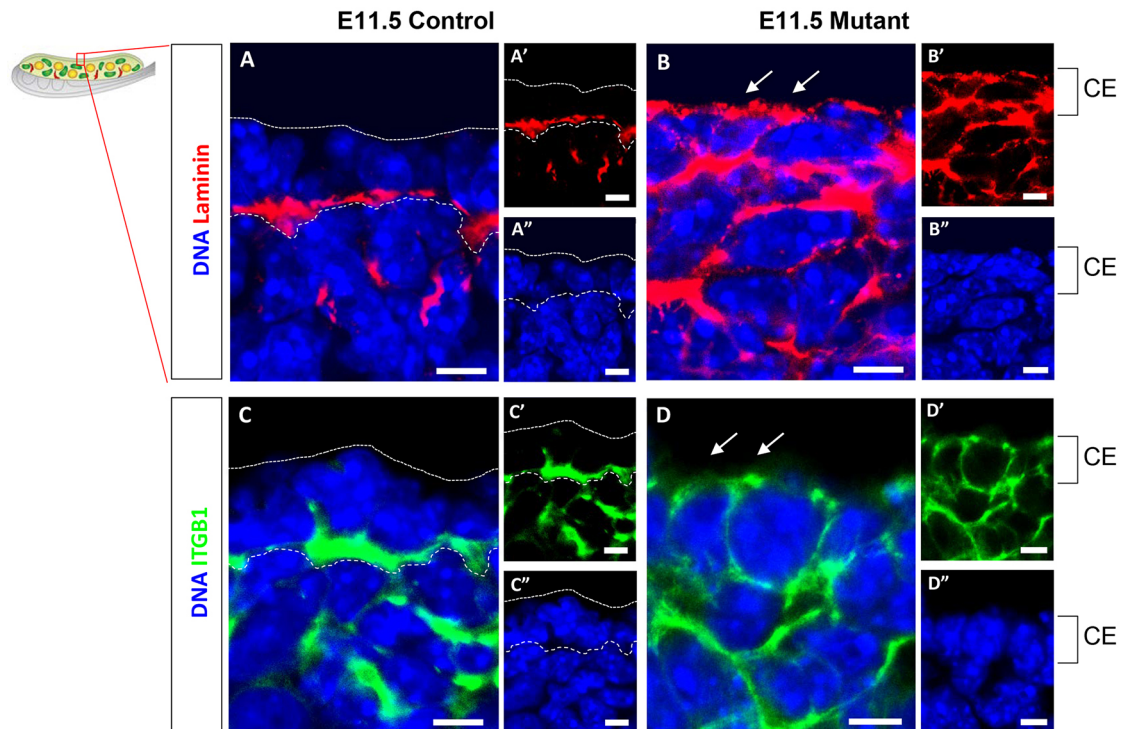
DEVELOPMENT

Fig. 3. Polarity of CE cells in *Numb/Numbl* mutant gonads was disrupted. (A-D″) Laminin (A,B, red) and ITGB1 (C,D, green) are localized to the basolateral domain of CE cells in control gonads (A,C). However, in *Numb/Numbl* mutant gonads (B,D), both laminin and ITGB1 are also detected in the apical domain of CE cells (arrows). A′,A″,B′,B″,C′,C″ and D′,D″ show individual channels. The CE is delineated by a dotted line at the surface and a dashed line beneath the basement membrane in A and C. Expected CE domain is marked by a bracket in B′,B″ and D′,D″. Nuclei are labeled by Hoechst staining (blue). Schematic shows the region of the gonad captured in the image. Scale bars: 5 μm.

images (Fig. 5I, arrowheads). To quantify whether the SRY-expressing cell population was reduced in mutants, we determined the proportion of SRY-positive cells relative to the total cell number in each *z*-section. These data revealed a 60% reduction of SRY-expressing cells in mutant versus control gonads (Fig. 5G).

Although this result suggested that Sertoli differentiation was strongly affected, it was not completely blocked. We speculated that this might be related to the efficiency or timing of *Numb* deletion. To identify the cells in which the Cre recombinase had been activated, we crossed a strong Cre reporter in the *ROSA* locus (ROSA-flox-STOP-flox Tomato, RTR) onto the *ROSACreER; Numb^{flox/flox}; Numbl^{−/−}* background. As shown in Fig. S5, ~50% of gonadal cells (in a patchy distribution across the gonad) were Tomato positive by E11.5, 3 days post-tamoxifen injection. Although the majority of SRY-expressing cells were Tomato negative, a few were double positive (Fig. S5C-C‴, arrowheads). It is unclear whether activation of the Cre reporter occurred before or after the activation of *Sry*.

However, expression of the RTR reporter involves recombination at only one allele, whereas loss of *Numb* activity requires recombination at two alleles in these crosses, and could show different kinetics. To obtain a direct measure of NUMB depletion in gonadal cells, we stained mutant and control gonads with an antibody against NUMB. At E10.5, NUMB protein was present in most mutant gonads at levels similar to controls (Fig. S6A-C), but some samples already showed significant loss of NUMB (Fig. S6D). NUMB protein was strongly reduced in all mutants relative to controls by E11.5, although levels varied slightly among samples and across the field of the gonad (Fig. S6E-H). These findings indicate that NUMB protein is lost between E10.5 and E11.5 in most mutants.

Numb/Numbl mutants had reduced supporting cell numbers and a near-complete loss of Leydig cells

In E13.5 XX and XY mutant gonads, some of the SOX9-positive (Sertoli) and FOXL2-positive (granulosa) cells adopted their fates normally. However, a reduction in supporting cells in XX and XY gonads, and a near-complete loss of Leydig cells in XY gonads (based on a marker for the steroidogenic enzyme HSD3B1, also known as 3β-HSD) was evident by immunocytochemistry (Fig. 2; Fig. 6A-F). To quantify the loss of specific cell lineages in *Numb/Numbl* mutants at E13.5, we compared the number of cells positive for SOX9, FOXL2 and HSD3B1 relative to the total cell number in each *z*-section (Fig. 6G-I). In XY mutant gonads, the Sertoli cell population was reduced by ~50% compared with control littermates. In XX mutants, the number of granulosa cells was reduced by ~60% relative to controls. Leydig cells were the most affected cell lineage, showing a ~90% reduction in the mutant gonad. There is no marker that distinguishes the steroidogenic population in the XX gonad at this stage, thus comparable analysis of the XX population could not be done. Cleaved caspase 3 (cCASP3, a marker of apoptosis) staining of E12.75 XX and XY gonads revealed no major changes in apoptotic cell number in mutant gonads compared with controls (Fig. S7).

Germ cell numbers were reduced upon loss of *Numb/Numbl*

The germ cell lineage was also dramatically affected with the DDX4-positive population reduced by 50-60% at E13.5 in both XX and XY mutants (Fig. 7A-F). This reduction was evident as early as E11.5 (Fig. 7G-L). To investigate whether this early loss of germ cells was associated with widespread apoptosis (as in *Fgf9* mutants; DiNapoli et al., 2006), we stained gonads at this stage with
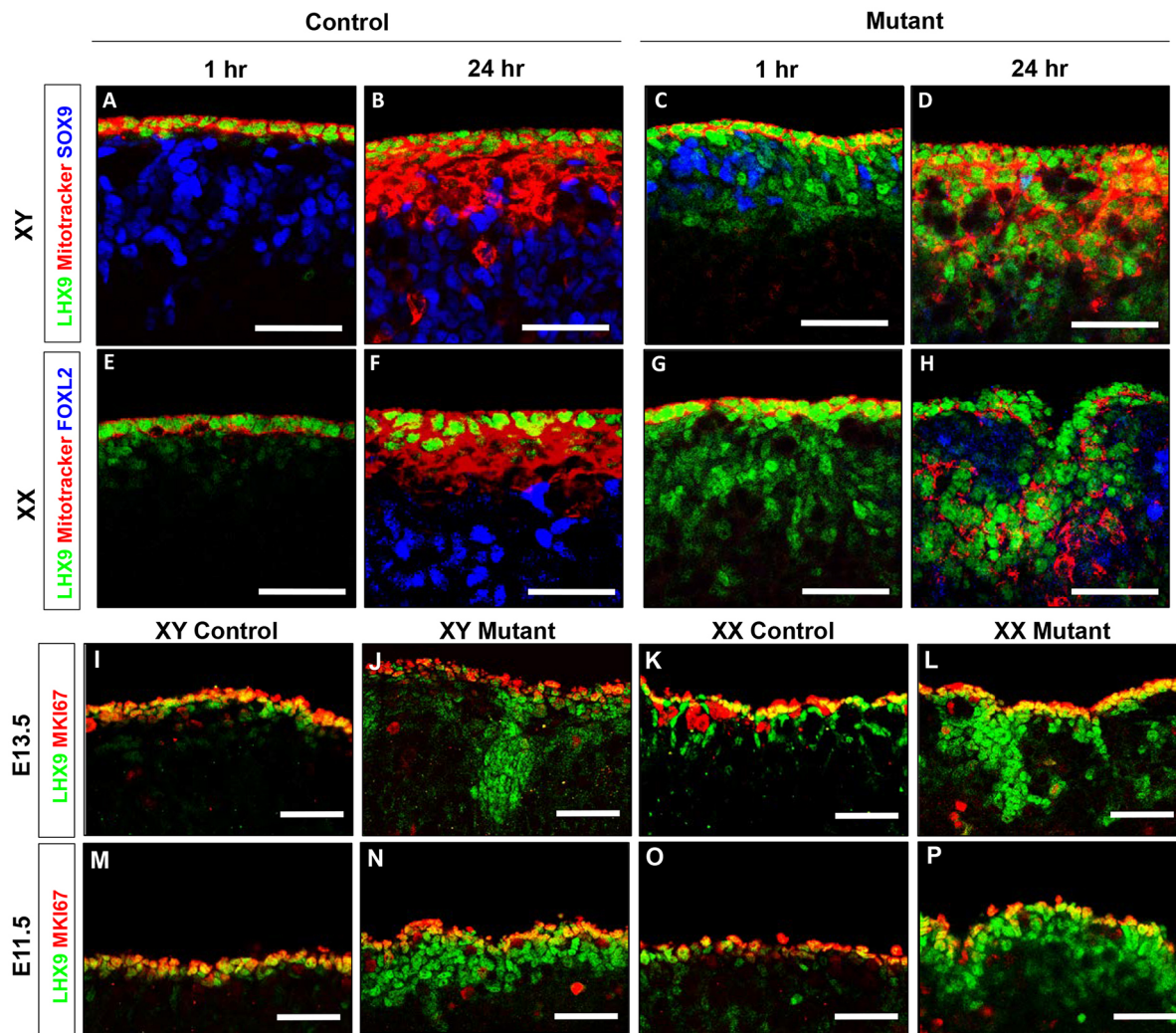
Fig. 4. Undifferentiated cells in *Numb/Numbl* mutant gonads derive from the CE, but are not in active cell cycle. (A-H) The CE of XY and XX E11.5 control and mutant gonads was labeled with MitoTracker (red). One gonad of the pair was cultured for 1 h, fixed and stained to confirm that MitoTracker had not penetrated beneath the CE layer during the labeling step (A,C,E,G). The other member of the pair was cultured for 24 h prior to fixation (B,D,F,H). Gonads were stained for SOX9 or FOXL2 (blue, Sertoli or granulosa cells) and LHX9 (green). After 24 h of culture, several layers of MitoTracker-positive cells were present within control gonads (B,F). MitoTracker-positive cells inside control gonads were negative for LHX9, and some co-labeled with SOX9 or FOXL2. In mutant gonads, MitoTracker was still restricted to the CE after 1 h of culture (C,G) but showed irregular labeling of cells deep within the gonad after 24 h of culture. Most MitoTracker-positive cells in mutants retained LHX9 expression and did not co-express SOX9 or FOXL2. (I-P) LHX9-positive cells are not in active cell cycle. E13.5 (I-L) and E11.5 (M-P) control and mutant gonads were labeled with MKI67 to identify cells in active cell cycle. MKI67-positive cells were restricted to the CE at both stages in control and mutants. Scale bars: 50 µm.

antibodies against cCASP3 and the germ cell markers cadherin 1 (CDH1, also known as E-Cad) (Fig. 7M,N), PECAM1 and SOX2 (data not shown). Although occasional cCASP3 signals were detected in the mesonephros, there was no overlap with germ cell markers or evidence for significant apoptosis within the gonad, suggesting that germ cells are lost prior to this stage.

Blocking Notch signaling rescued the *Numb/Numbl* mutant phenotype

The canonical function of NUMB is to antagonize Notch signaling. However, other functions have been reported for NUMB that are independent of this role (Gulino et al., 2010). To investigate whether loss of *Numb* led to elevation of Notch signaling, we performed qPCR (quantitative RT-PCR) for three Notch target genes, *Hes1*, *Hes5* and *Hey1*, all of which were significantly upregulated in both XX and XY mutants relative to controls at E13.5 ($P=0.0012$ in XY

control versus mutant, $P=0.0117$ in XX control versus mutant; asterisks apply to all three genes) (Fig. S8).

In a second line of experiments to test whether the accumulation of LHX9-positive cells within the *Numb/Numbl* mutant gonad was due to overactivation of Notch signaling, we blocked Notch signaling in mutants using DAPT, an inhibitor of γ-secretase, the enzyme responsible for releasing NICD (Cheng et al., 2003). Gonads were collected at E11.5 and cultured with 100 µM DAPT or DMSO for 48 h (Fig. 8). Control gonads treated with DAPT contained supporting and germ cells, although germ cell numbers were consistently reduced after culture with DAPT (Fig. 8A,B,E,F). Although mutant XX or XY gonads treated with DAPT showed variable levels of rescue, the patches of undifferentiated LHX9-positive cells in *Numb/Numbl* mutants decreased in all samples, suggesting that these patches were the result of overactivation of Notch signaling. Mutant gonads cultured with DAPT often had
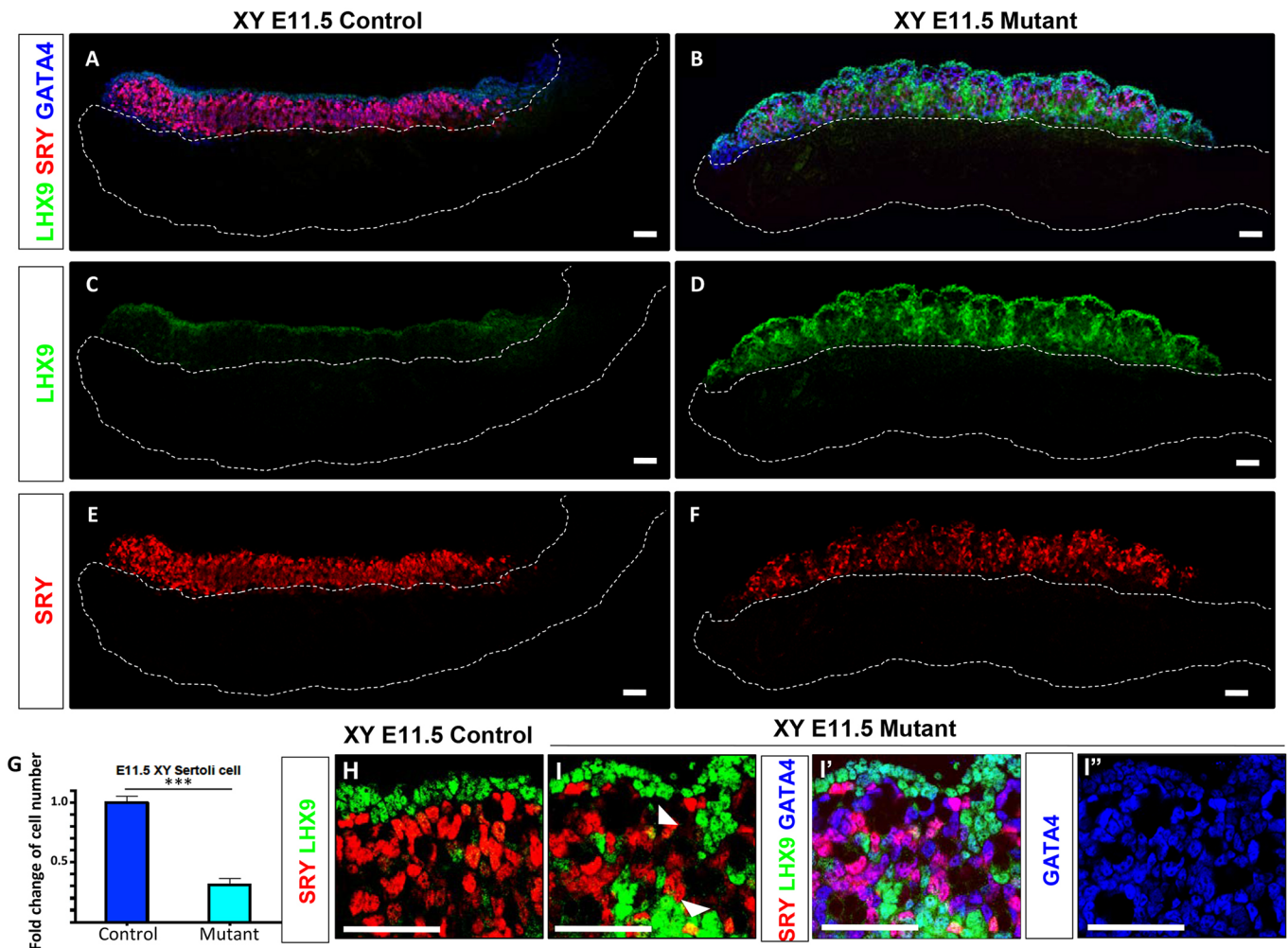
Fig. 5. *Numb/Numbl* mutant XY gonads are disrupted at E11.5, with fewer Sertoli cells. Control and mutant XY gonads were collected and stained for SRY (Sertoli cells, red), GATA4 (somatic cells, blue) and LHX9 (green). (A-F) In controls (A,C,E), abundant SRY-positive Sertoli cells were present in the gonad (A,E) along with other somatic cells labeled by GATA4 (A). LHX9-positive cells were restricted to the CE (C). Mutant gonads (B,D,F) had an irregular surface, multiple patches of LHX9-positive cells (D) and fewer SRY-positive Sertoli cells (F). The mesonephros is outlined by dashed lines in A-F. (G) Approximately 30% of the normal number of SRY-expressing cells were specified in mutants. Error bars represent s.e.m.; ***$P<0.001$. (H,I) In control gonads, all SRY-positive cells were negative for LHX9 (H). In mutant gonads, most SRY-positive cells were negative for LHX9; however, a few cells were double-labeled by SRY and LHX9 (I, arrowheads). All LHX9-positive cells were GATA4 positive (I′,I″). Scale bars: 50 μm.

more SOX9- or FOXL2-positive cells than those cultured with DMSO, but this result was also variable. Interestingly, LHX9 expression in the CE was reduced in both controls and mutants (Fig. 8A-H), which could imply that *Lhx9* is a target of Notch signaling.

DISCUSSION
Proliferation of CE cells in the early gonad gives rise to somatic progenitors that remain in the CE and also to progenitors of at least two distinct somatic lineages within the gonad, including supporting cells that differentiate as Sertoli cells in the testis or granulosa cells in the ovary, and interstitial cells. Here, we show that deletion of *Numb* and *Numbl* disrupted CE cell polarity in both XX and XY gonads, and led to the formation of large patches of undifferentiated LHX9-positive cells within the gonad. This phenotype was coupled with severe defects in the specification of supporting and steroidogenic cell lineages, which could be partially reversed by blocking Notch signaling. These results indicate that *Numb* is required for the specification of cells competent to differentiate as male or female somatic lineages. Surprisingly, germ

cell numbers were also reduced at early stages of gonadogenesis, suggesting an independent role for *Numb* in the germ cell lineage, or possibly a secondary result of supporting cell loss.

Cells in the gonadal CE are a rapidly dividing population (Schmahl et al., 2000). Previous work using dye-labeling approaches indicated that single CE cells can give rise to both Sertoli cells and interstitial cells (Karl and Capel, 1998; Liu et al., 2016). More recently, Liu et al. used a lineage-tracing strategy to show that the WT1-positive progenitor cell pool at the CE contributes to at least three distinct lineages: HES1-negative Sertoli cells, HES1-negative interstitial progenitor cells and HES1-positive interstitial progenitor cells (Liu et al., 2016). CE cells express LHX9, a protein that is expressed in undifferentiated cells and is required for gonadogenesis (Birk et al., 2000; Mazaud et al., 2002). In control E11.5 gonads, LHX9 expression is mostly restricted to the CE. As cells leave the CE and enter the gonad field, they adopt asymmetric fates coincident with downregulation of LHX9 and upregulation of differentiation markers such as SOX9 and FOXL2. However, the mechanisms governing this process are not understood.
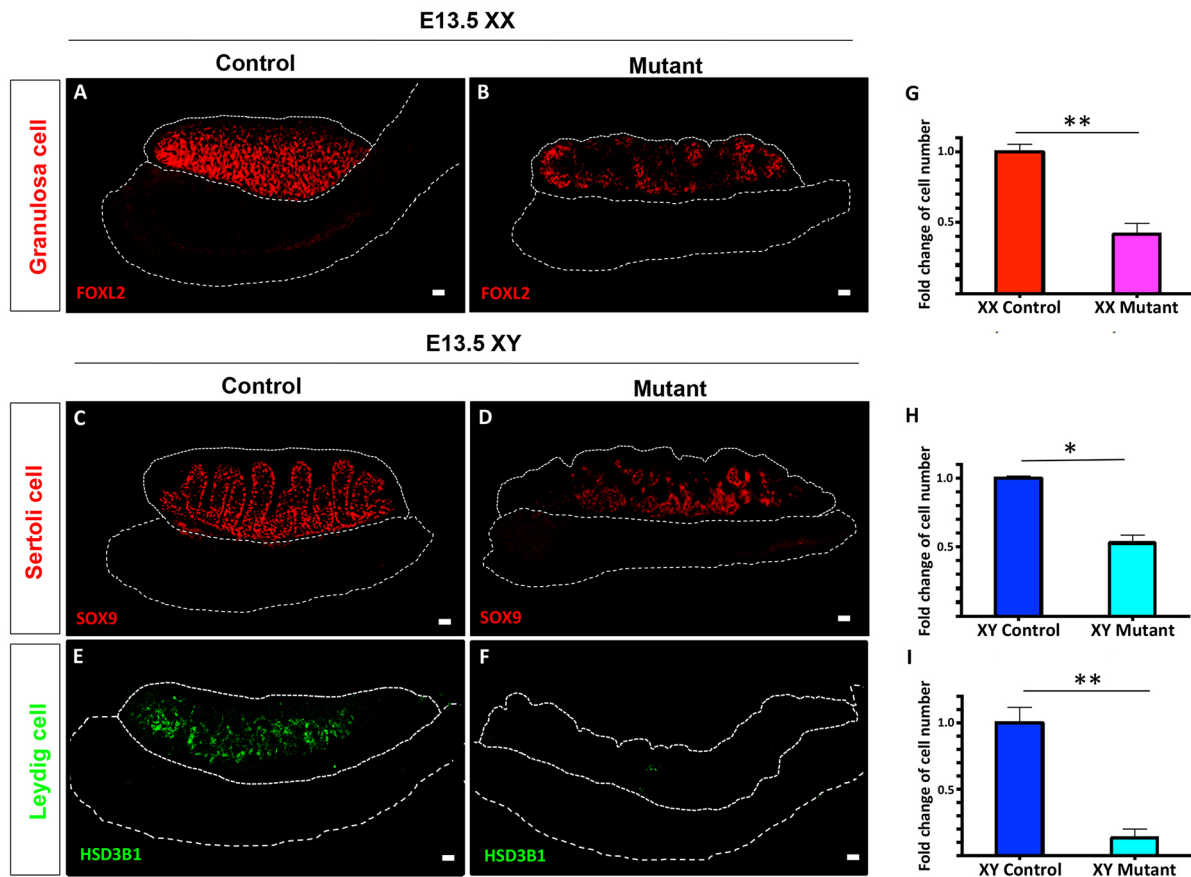
Fig. 6. Numbers of supporting cells in both XX and XY, and steroidogenic cells in XY E13.5 *Numb/Numbl* mutant gonads were reduced. (A,B,G) At E13.5, granulosa cell numbers were reduced by ~60% in mutant XX gonads, based on staining for FOXL2 (red). (C,D,H) Based on SOX9 staining (red), Sertoli cell numbers were reduced to ~50% of controls. (E,F,I) Based on HSD3B1 staining (green), the Leydig cell population was the most severely affected in mutant gonads, reduced to ~10% of controls. Error bars represent s.e.m.; *$P<0.05$, **$P<0.01$. Scale bars: 50 μm.

Several lines of evidence suggested that Notch and Numb signaling were involved. Although transcription of *Notch2* and *Numb* is ubiquitous in most cells of the gonad (Jameson et al., 2012b), NUMB protein is asymmetrically localized to the basolateral domain of CE cells. Consistent with this pattern, a reporter for Notch signaling revealed high levels of Notch signaling in the CE cells, but reduced levels deeper within the gonad. These findings prompted us to investigate whether Notch and Numb are involved in regulating asymmetric outcomes of CE divisions.

A model we first considered was that CE cells exit the CE as they divide, and the allocation of NUMB to one of the two daughter cells leads to specification of supporting or interstitial cell fate. However, when CE cells were uniformly labeled with MitoTracker, and gonads were cultured for 24 h, many cells within the gonad were labeled, but all cells in the CE domain also remained MitoTracker positive, suggesting that one labeled daughter cell of each division was left in the CE. Another model to explain how a single CE progenitor can give rise to diverse progeny is that the division of a CE cell gives rise to one cell that remains in the CE and a progenitor that inherits NUMB and acquires the competence to differentiate as either a supporting cell or an interstitial cell (Fig. 9). Our evidence based on deletion of *Numb* and *Numbl* is consistent with this hypothesis.

Conditional deletion of *Numb* on a *Numbl* mutant background, beginning at E8.75, just prior to gonad formation, led to the presence of large patches of LHX9-positive cells within the gonad. We anticipated that these patches would be actively proliferating,

but this was not the case, suggesting that NUMB is not required to escape from the rapidly proliferating status of cells in the CE. LHX9-positive patches were evident as early as E11.5 and correlated with reduced numbers of differentiated Sertoli, granulosa and Leydig cells. The granulosa cell population was more severely affected than the Sertoli cell population, and the Leydig population was most severely affected. As this hierarchy reflects the sequence of specification of these lineages, it might be related to the timing with which NUMB protein is lost in precursor cells. Experiments to determine when Cre is active in gonadal cells following tamoxifen injection at E8.75 revealed variability across the gonad at E11.5. High levels of NUMB protein were still detectable in most samples at E10.5, but lost by E11.5. The timing of *Numb* deletion in different samples could account for the variability in the extent of the phenotype. The window of time in which *Sry* expression can trigger Sertoli cell specification ends at E11.25 (Hiramatsu et al., 2009). However, granulosa cells, interstitial and steroidogenic lineages in both XX and XY gonads continue to arise from the CE until E12.5 in XY gonads, and E14.5 in XX gonads (Mork et al., 2012). Thus, the gradual loss of NUMB would be expected to affect the lineages specified later more severely than those that arise earlier. Consistent with this model, cells expressing differentiated markers (SOX9 or FOXL2) are usually located deeper in the gonad in mutants, whereas the LHX9-positive patches are typically located nearer the CE (Fig. 2).

Another possibility that is not mutually exclusive is that *Numb* also is required at a second step during Leydig cell differentiation. In
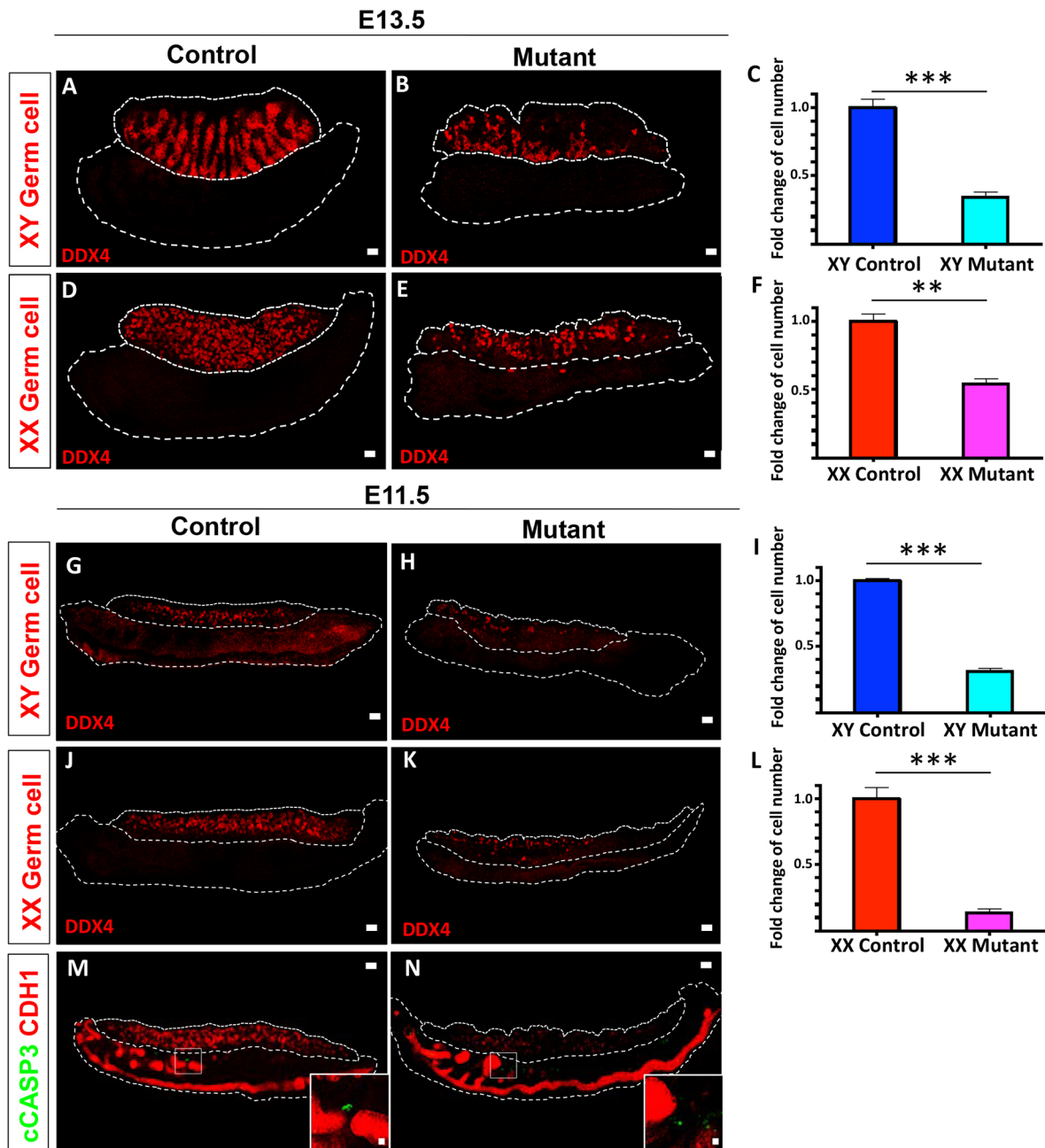
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Fig. 7. Germ cells were lost in *Numb/Numbl* mutants as early as E11.5, but not via cell death within the gonad or mesonephros. (A-F) At E13.5, numbers of germ cells labeled by DDX4 (red) were reduced by 50-70% in XY and XX gonads compared with controls. (G-L) The germ cell population was even more strongly reduced at E11.5, where numbers were 10-30% of controls. (M,N) cCASP3 staining (green) labeled very few cells in control gonads and in mutants (insets). Germ cells were labeled with antibodies against CDH1 (red), and nuclei in insets were labeled with Hoechst 33342. The gonads and mesonephroi are outlined by dashed lines. Error bars represent s.e.m.; **$P<0.01$, ***$P<0.001$. Scale bars: 50 µm.

a previous study, we used a *Hes1* mutant mouse model, as well as DAPT treatments, to show that Notch signaling maintains Leydig progenitor cells, and inhibition of Notch increases the Leydig cell population (Tang et al., 2008). These results were recently confirmed using different approaches (Liu et al., 2016). In our current study, overactivation of Notch signaling in *Numb/Numbl* mutants could increase the number of undifferentiated steroidogenic progenitors and reduce the number of differentiated Leydig cells. In fact, the *Numb/Numbl* mutants showed a much more severe Leydig cell reduction phenotype than in previous work. This could be due to disruption of Notch/Numb signaling earlier than in the previous

studies, or to the fact that Leydig cell fate determination relies on signals from Sertoli cells, which were also affected in the *Numb/Numbl* mutants.

The fact that Notch downstream targets were elevated and the mutant phenotype could be partially rescued by culturing with DAPT strongly suggests that the defect in *Numb* mutant gonads is due to an imbalance of Notch/Numb signaling. Although rescue was variable among samples, mutant gonads had reduced LHX9-positive patches and frequently showed rescue of both the supporting cell population and the Leydig cell population (data not shown, but previously reported; Tang et al., 2008). We note that
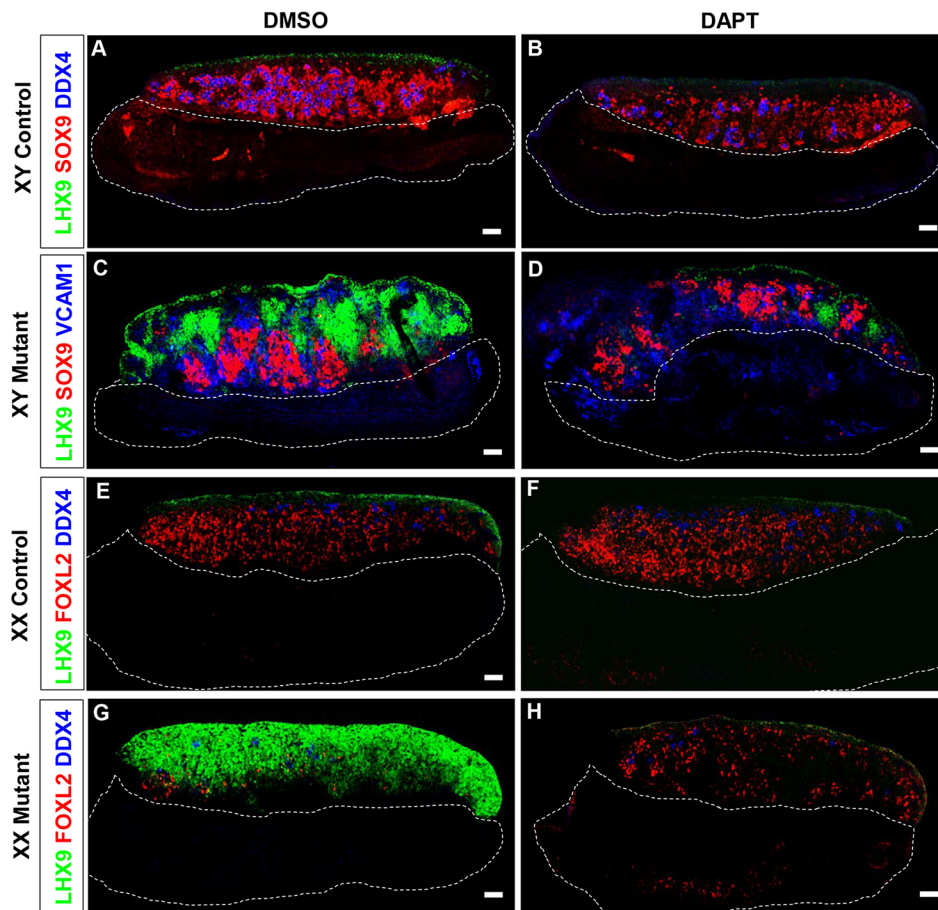
Fig. 8. Culture with DAPT rescued some aspects of the *Numb/Numbl* mutant phenotype. E11.5 control and mutant gonads were dissected and cultured with DMSO or 100 µM DAPT for 48 h. (A,B) Control XY gonads treated with DMSO (A) or DAPT (B) specified their Sertoli cell lineage normally based on SOX9 expression (red). Levels of LHX9 in the CE and germ cell numbers were reduced after culture with DAPT in control gonads. (C,D) Mutant XY gonads treated with DMSO (C) retained large LHX9-positive domains. DAPT treatment of mutant gonads (D) led to a reduction in LHX9-positive domains coupled with a variable increase in the SOX9-positive population (red). (E,F) Similar results were seen in XX gonads, in which granulosa cells and germ cells were observed in control XX gonads treated with DMSO or DAPT, although germ cell numbers were reduced by DAPT treatment (F). (G,H) LHX9-positive domains were present in DMSO-treated XX mutant gonads (G). With DAPT treatment, reduction of LHX9-positive domains was associated with an increase in FOXL2-positive cells (red); however, germ cells were not rescued (H). Scale bars: 50 µm.

treatment with DAPT reduced the germ cell population, even in control gonads. It is unclear whether this reflects a direct role for Notch in germ cell survival during gonadal stages, or a toxic effect of DAPT, which is likely to be the case because active Notch signaling represented by a transgenic Notch reporter (TNR) was not detected in fetal germ cells in a previous study (Defalco et al., 2013) nor in this study using a CBF:H2B-Venus reporter line.

We did not anticipate defects in the germ cell lineage. Germ cells migrate to the gonad via the gut epithelium and move into the gonad through the mesonephros (Molyneaux and Wylie, 2004). The expression of *Numb* in both XX and XY gonadal germ cells is lower than all other lineages (Fig. S1E) (Jameson et al., 2012b), yet we found severe reductions in germ cell numbers as early as E11.5. One possibility is that defects in mutant somatic cells disrupt the gonadal signals that attract germ cells or support their viability. We hypothesized that if this were the case, we would find many germ cells remaining in the mesonephros and/or undergoing apoptosis. However, we did not find significant numbers of germ cells delayed in the mesonephros, nor were significant numbers of germ cells undergoing apoptosis in the gonad or mesonephros as in previous studies in which gonadal signals were disrupted (DiNapoli et al., 2006). These findings are consistent with the possibility that *Numb*
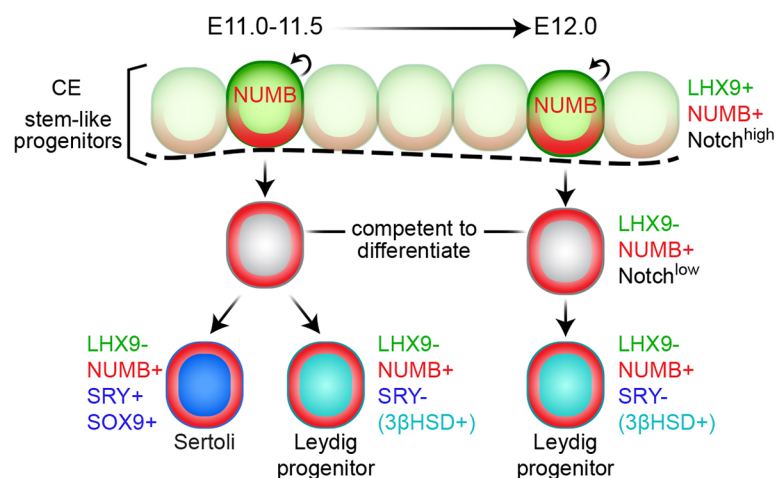

Fig. 9. NUMB is essential for asymmetric division of CE cells during gonadogenesis. LHX9^{+} embryonic CE cells are the stem-like progenitors of Sertoli and interstitial cells, polarized by asymmetric allocation of NUMB to the basolateral domain. Asymmetric divisions give rise to one daughter that remains at the CE, and one daughter that inherits NUMB and the competence to differentiate. Sertoli cells arise from this progenitor until ~E11.5. After E11.5, only interstitial cells are generated. The more severe loss of Leydig cells in XY mutants might be related to their later specification (when loss of NUMB is nearly complete), or to a secondary requirement of NUMB in the Leydig lineage. Only the XY gonad is modeled because information is more complete (Karl and Capel, 1998; Schmahl and Capel, 2003; Tang et al., 2008). Specification of XX supporting cells is likely to be similar, but continues until E14.5 (Mork et al., 2012).

plays a direct role during germ cell migration. NUMB can affect cell migration through regulation of adhesion pathways (Nishimura and Kaibuchi, 2007). Cell type-specific deletion of *Numb* and *Numbl* in germ cells will be required to distinguish a cell-autonomous from a non-cell-autonomous role.

Our current working model is that the asymmetric distribution of NUMB in proliferating CE cells regulates their polarity and results in asymmetric products of the division. One of the daughter cells remains at the CE and maintains LHX9 expression and 'stem-like' properties, whereas the other daughter cell, which inherits NUMB, gains competence to differentiate as a supporting or interstitial cell (Fig. 9). Both supporting and interstitial cells can arise from a CE cell dye-labeled at E11.5. However, it remains unclear whether the cell that inherits NUMB can divide to produce cells of different lineages, or whether these cells produce different lineages at different stages of development. Although *Numb* is required to produce these progenitors, it is possible that an additional stage-limited factor confers competence to activate *Sry* and specify the Sertoli lineage. Another possibility is that lateral inhibition pathways are involved in regulating the proportion of Sertoli and interstitial cells that differentiate. Earlier experiments driving *Sry* expression in all cells of the gonad resulted in a normal number of Sertoli cells (Hiramatsu et al., 2009), further implying that one of these two mechanisms could be involved in restricting the fates of these gonadal lineages.

MATERIALS AND METHODS
Mouse strains, staging, genotyping, and tamoxifen administration
The mouse line carrying a floxed allele of *Numb*, *Numb*^{tm1Ynj} (*Numb*^{flox}) (Zhong et al., 2000), and a null allele for numb-like, *Numbl*^{tm1Wmz} (*Numbl*$^{-}$) (Petersen et al., 2002), was kindly provided by C. T. Kuo (Department of Cell Biology, Duke University, NC, USA) and maintained on a mixed background (B6/129). The ROSA-CreER [B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$/J; JAX Stock No: 008463] line was obtained from Jackson Laboratories. CBF:H2B-Venus mice (Nowotschin et al., 2013) were obtained from Jackson Laboratories [Tg(Cp-HIST1H2BB/Venus)47Hadj/J; JAX Stock No: 020942]. RTR mice were a gift from Fan Wang [Duke University Medical Center; also available from Jackson Laboratories: B6;129S6-Gt(ROSA) 26Sor$^{tm9(CAG-tdTomato)Hze}$/J; JAX Stock No: 007905]. Timed matings were performed by placing *Numb*$^{flox/flox}$;*Numbl*$^{+/-}$, *Numb*$^{flox/flox}$;*Numbl*$^{-/-}$, *Numb*$^{flox/+}$;*Numbl*$^{+/-}$ or *Numb*$^{flox/+}$;*Numbl*$^{-/-}$ females in cages with *Numb*$^{flox/flox}$;*Numbl*$^{+/-}$;*ROSA-CreER*, *Numb*$^{flox/flox}$;*Numbl*$^{-/-}$;*ROSA-CreER* males after 16.00 h each day. The females were inspected and separated from the male the next morning. The presence of a vaginal plug was designated as E0.5. Tamoxifen (T5648; Sigma-Aldrich) was dissolved in corn oil and administered orally to pregnant females at a dose of 0.75 or 1 mg tamoxifen/10 g body weight at E8.75 to induce activity of ROSA-CreER. Phenotypes were only detected in embryos that carried *ROSA-CreER*, were *Numb*$^{flox/flox}$, and carried at least one null allele for *Numbl*. These mice are designated 'mutants', regardless of whether they were *Numbl*$^{+/-}$ or *Numbl*$^{-/-}$. All other segregants of the cross showed no phenotype, and were designated 'controls'. For each experiment, at least five mutant animals and five control animals were examined. Mice were housed in accordance with National Institutes of Health guidelines, and experimental protocols were approved by the Institutional Animal Care and Use Committee of Duke University Medical Center.

Gonad culture, DAPT treatment and MitoTracker staining
Embryonic gonads were dissected from embryos at E11.5 and cultured in agar blocks for 24 h as previously described (Martineau et al., 1997). For DAPT treatment, DAPT {N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-*t*-butyl ester; 65784, Calbiochem; 100 µM in DMSO} or an equal volume of DMSO was added to the medium. For MitoTracker staining, dissected gonads were incubated for 30 min in 1 µM MitoTracker

Orange (CMTMRos, Invitrogen), then washed extensively in culture medium, and cultured for 24 h as described above. To ensure that MitoTracker only labeled the first layer of the CE, sample gonads were collected after 1 h of culture, stained for LHX9, and imaged. Remaining gonads were cultured for 24 h prior to labeling and imaging. In all cases, gonads were fixed in 4% paraformaldehyde at room temperature for 1 h and processed for immunofluorescence.

Immunofluorescence
Embryonic gonads at E11.0-E14.5 were dissected from embryos and fixed in 4% paraformaldehyde for 1-2 h at room temperature. Fixed gonads were washed 3×20 min with TBS-Triton X-100 (0.1%), and stored in methanol at −20°C until use. Samples were rehydrated and whole-mount immunostaining was performed as previously described (Jameson et al., 2012a). Antibodies used in this study are listed in Table S1. Secondary antibodies used included Alexa 647- and 488-conjugated secondary antibodies (Molecular Probes), applied at 1:500, and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch) used at 1:500. Hoechst 33342 (Invitrogen) was also used to label nuclei. Samples were mounted in 2.5% DABCO (Sigma-Aldrich) in 90% glycerol.

Cell counts
Cells were labeled by anti-DDX4 or anti-PECAM1 (for germ cells), anti-SOX9 (for Sertoli cells), anti-FOXL2 (for granulosa cells) and anti-HSD3B1 (for Leydig cells) antibodies and three to seven biological replicates for each category were counted using the Fiji software suite. For E13.5 samples, two central *z*-sections separated by 50 µm were imaged on a Zeiss 710 inverted confocal microscope. For the E11.5 Sertoli and germ cell counts, three separate *z*-sections were counted and averaged. Cells expressing lineage markers were counted and divided by the total cell counts in each *z*-section (estimated by selecting the gonad area then dividing by the average area of one cell in the gonad. The average area of one cell was defined by averaging the cell area of 20 randomly selected cells labeled by Hoechst). Statistical significance was determined by unpaired *t*-tests.

Quantitative RT-PCR
Isolated gonads were subjected to an RNA preparation protocol or frozen at −80°C. RNA was extracted as previously described (Munger et al., 2009). Verso cDNA synthesis kit (Thermo Fisher Scientific) was used for cDNA synthesis and RT-qPCR was performed using LightCycler technology (Roche Diagnostics). Each cDNA sample was run in technical triplicate on a StepOnePlus Real-time PCR system (Applied Biosystems) for 45 cycles of 95°C for 15 s, 59°C for 30 s and 72°C for 30 s. Threshold cycle (Ct) values were calculated using StepOne software (version 2.2.2; Applied Biosystems). ΔCt values were calculated using the housekeeping gene *Canx* as an internal control (van den Bergen et al., 2009). Normalized expression values from biological replicates were averaged to calculate mean normalized expression (MNE). Significant differences in normalized expression between genotypes were determined using Student's *t*-tests.

Primer sequences are as follows: *Canx* F: 5′-GACATGACTCCTCCT-GTAAACCCT-3′, R: 5′-CGTCCATATCCTCATCCCAATCCT-3′; *Hes1* F: 5′-ATAGCTCCCGGCATTCCAAG-3′, R: 5′-GCGCGGTATTTCCC-CAACA-3′; *Hes5* F: 5′-GCTCCGCTCGCTAATCGCCT-3′, R: 5′-CCG-GCTTCCGCAGTCGGTTTTT-3; *Hey1* F: 5′-GCGCGGACGAGAATG-GAAA-3′, R: 5′-TCAGGTGATCCACAGTCATCTG-3′.

Author contributions
Conceptualization: Y.-T.L., L.B., T.D., B.C.; Methodology: Y.-T.L., L.B., T.D.; Software: Y.-T.L.; Validation: Y.-T.L.; Formal analysis: Y.-T.L.; Investigation: Y.-T.L.;

Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.149203.supplemental

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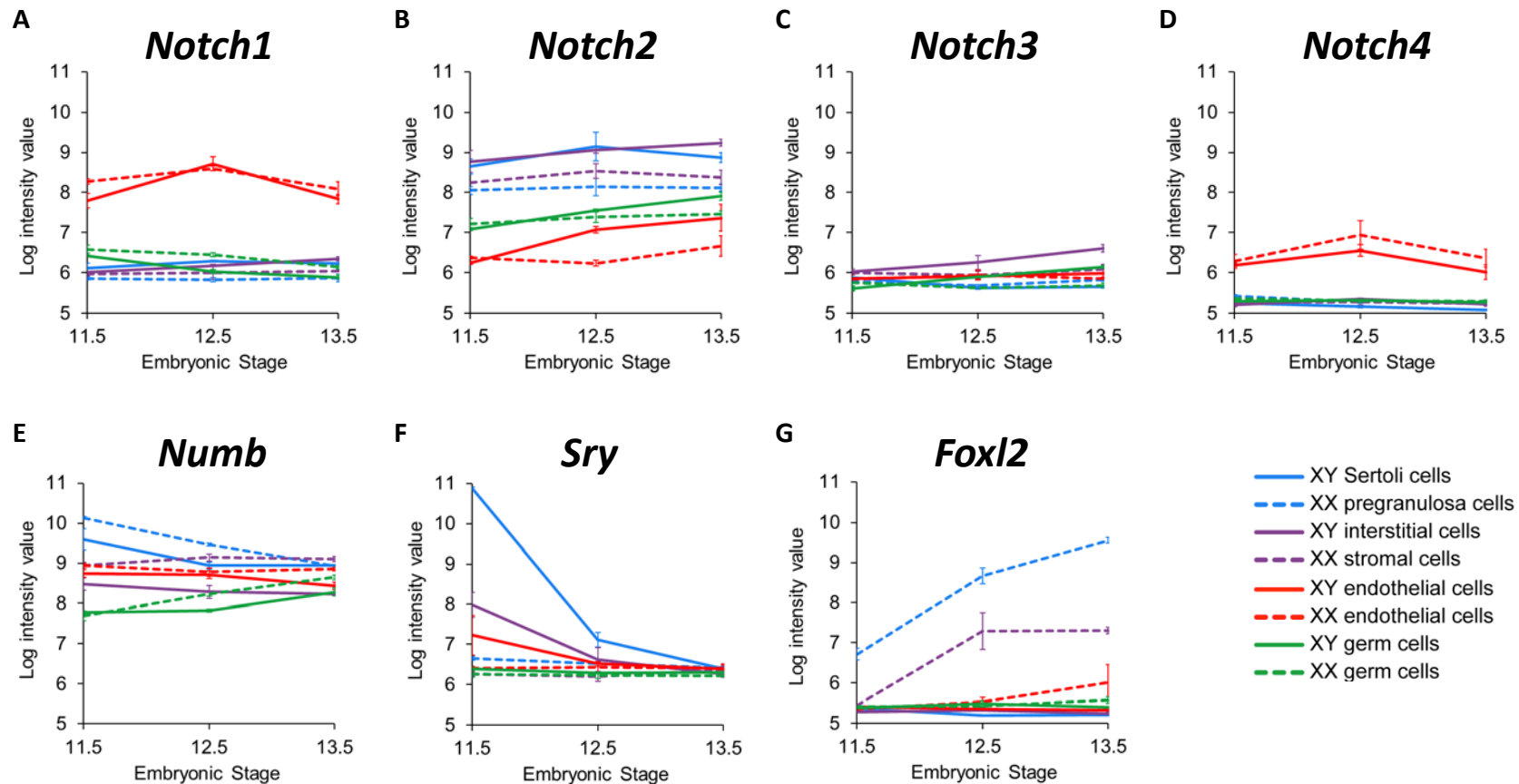
DEVELOPMENT

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 *Sr*y (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.
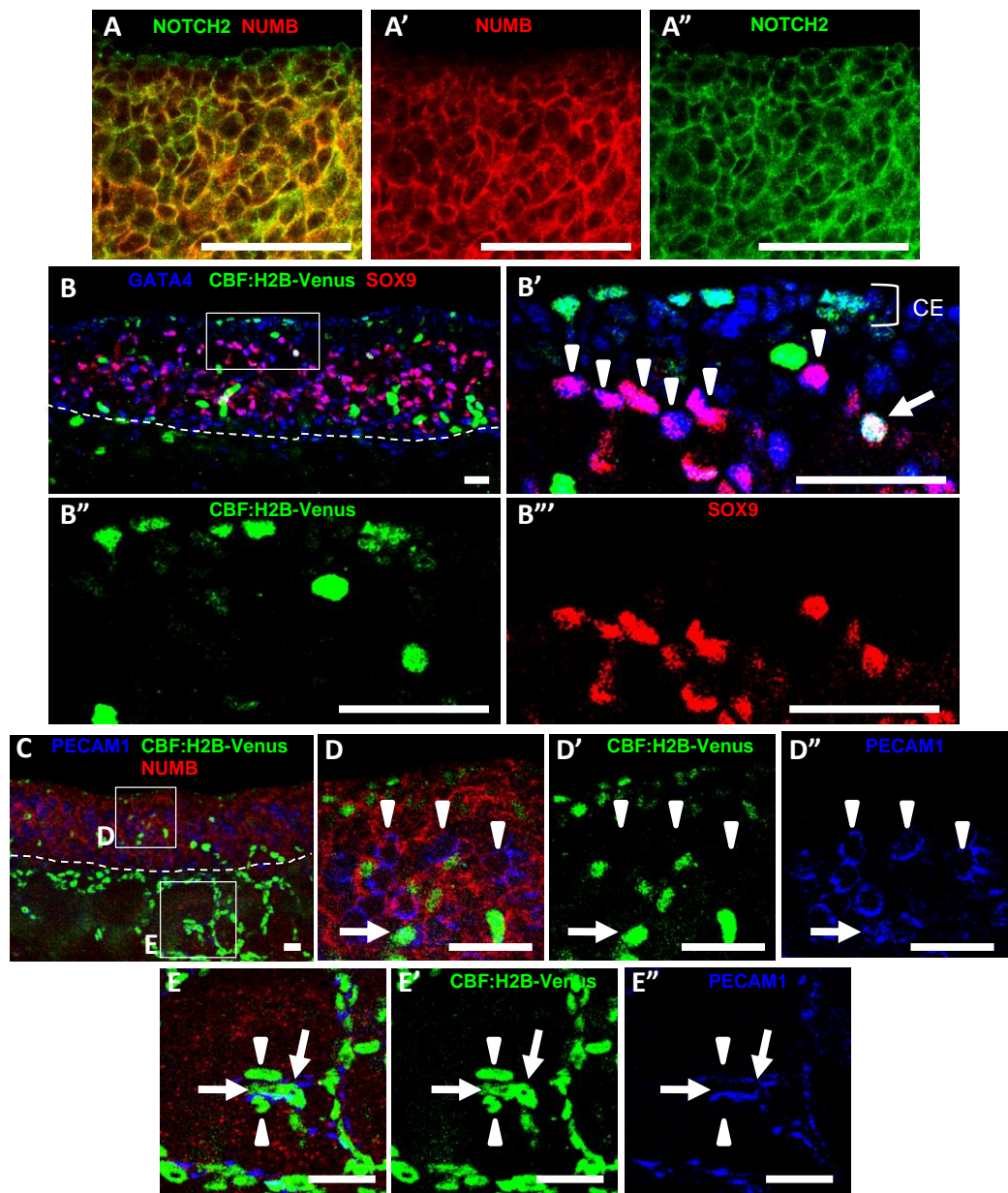
Fig. S2. Notch activity is absent from Sertoli and germ cells, but is observed in the coelomic epithelium and vascular/perivascular cells in the E11.5 gonad. Immunofluorescent images of E11.5 XY control (A) or E11.5 XY *CBF:H2B-Venus* (B-E) gonads, which report canonical Notch activity via the expression 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.
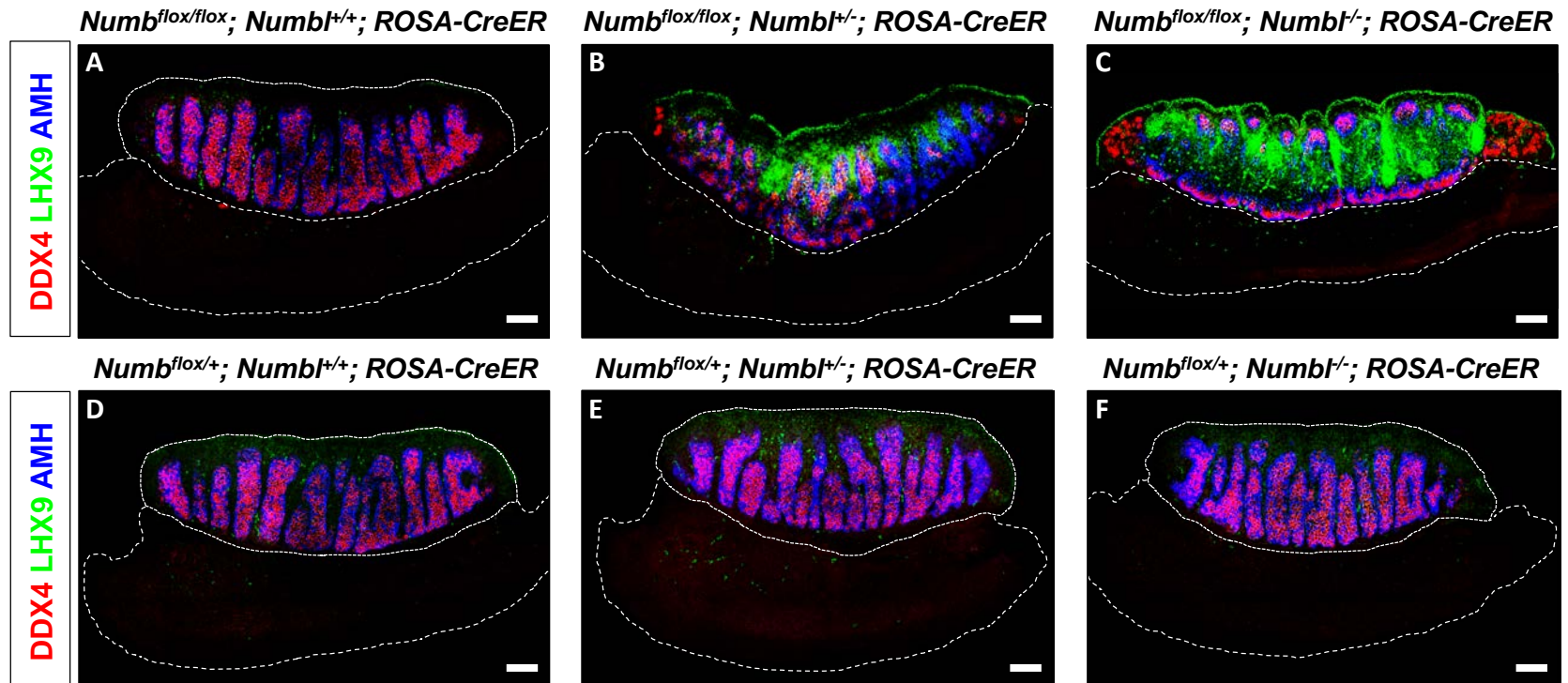
Fig. S3. E13.5 *Numb^flox/flox;Numbl^+/-;ROSA-CreER* **and** *Numb^flox/flox;Numbl^-/-;ROSA-CreER* **XY gonads display mutant phenotypes, whereas other segregating genotypes do not.** Immunofluorescent images of E13.5 XY RosaCreER-positive gonads with *Numb*^flox/flox or *Numb*^flox/+ alleles in various 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.
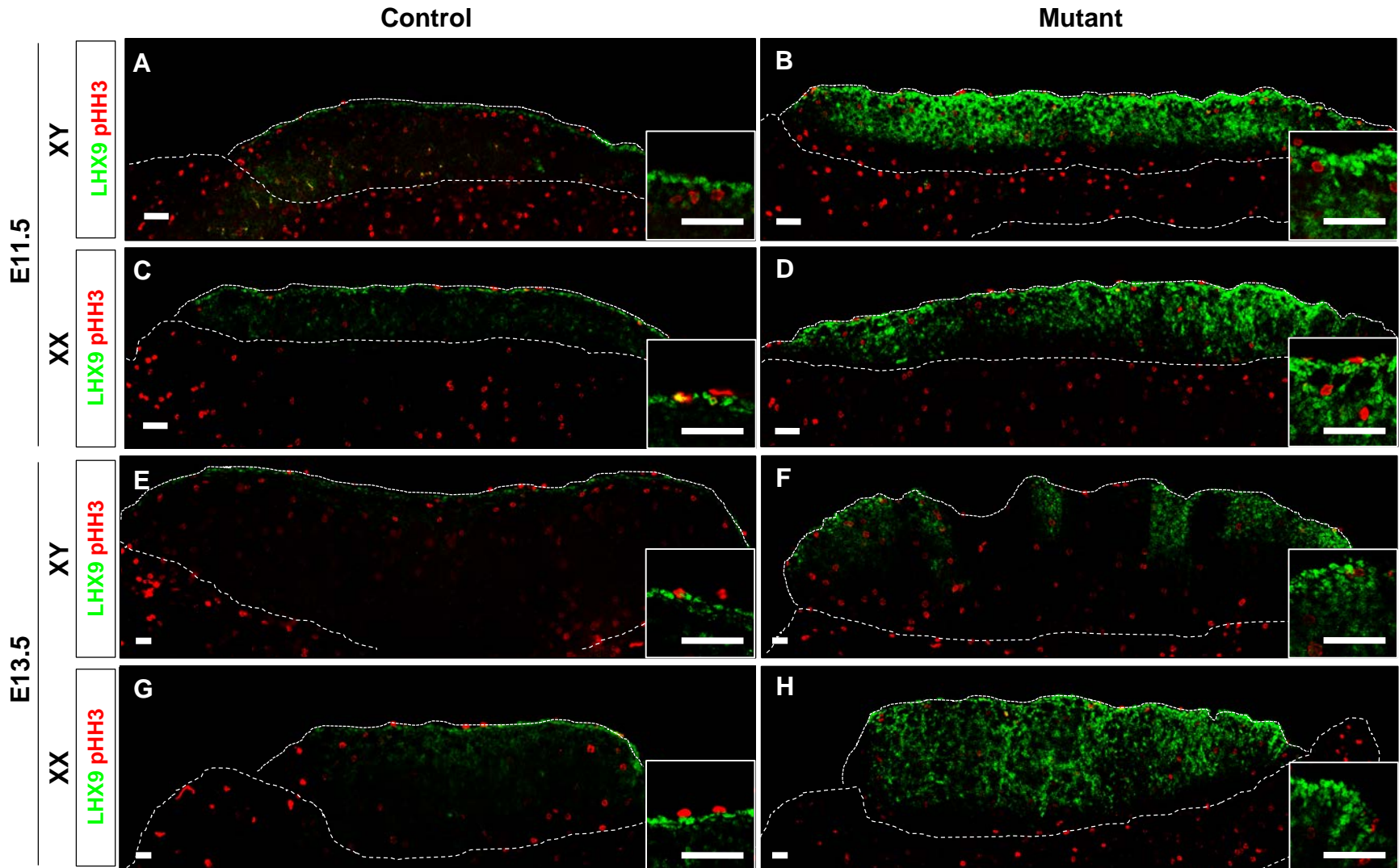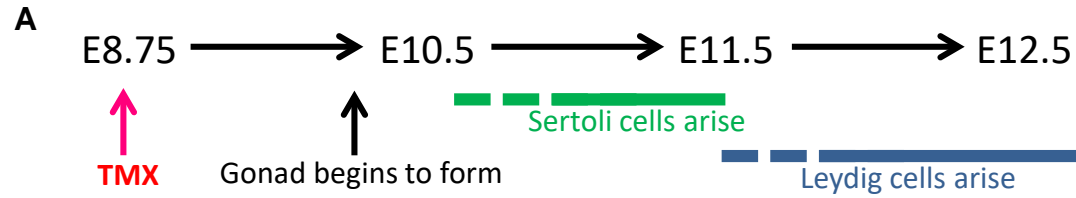
Fig. S4. No significant cell proliferation differences were observed between XX and XY E11.5 and E13.5 control and mutant gonads. Control and mutant gonads showed similar numbers of pHH3-positive cells in the CE domain and in the gonad field at both E11.5 and E13.5. pHH3-positive cells were absent in LHX9-positive patches in mutant gonads (B,D,F and H). Scale bars in all images=50 µm.
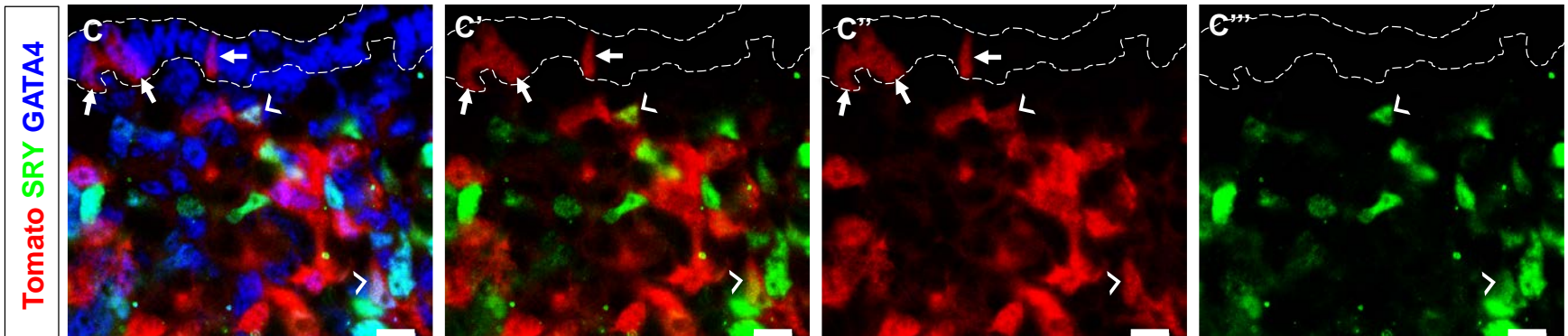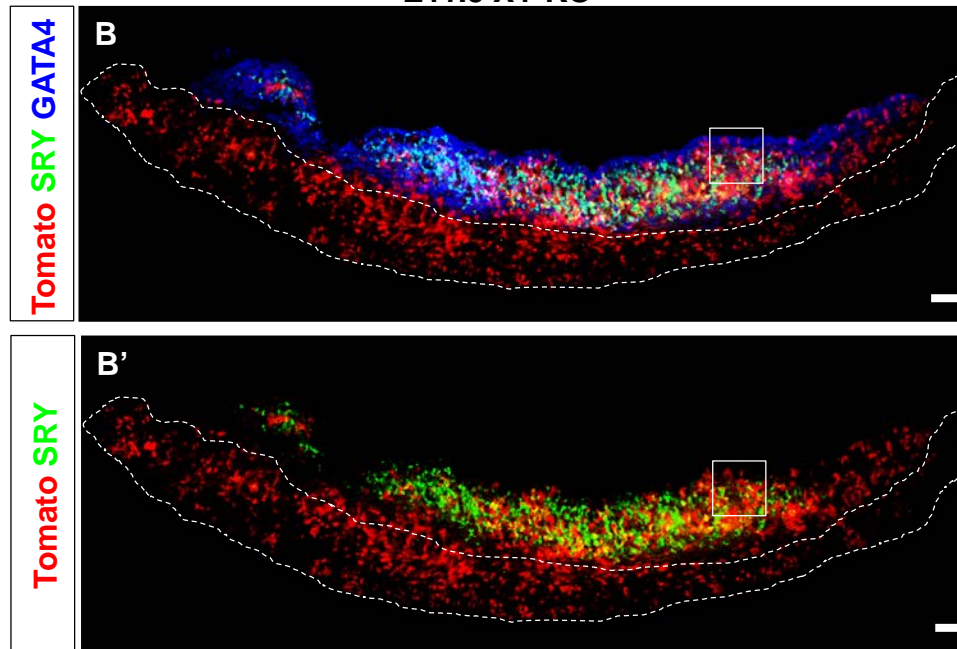
A

E8.75 → E10.5 → E11.5 → E12.5

TMX

Gonad begins to form

Sertoli cells arise

Leydig cells arise

E11.5 XY KO

B Tomato SRY GATA4

B' Tomato SRY

C Tomato SRY GATA4   **C'**   **C''**   **C'''**

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.
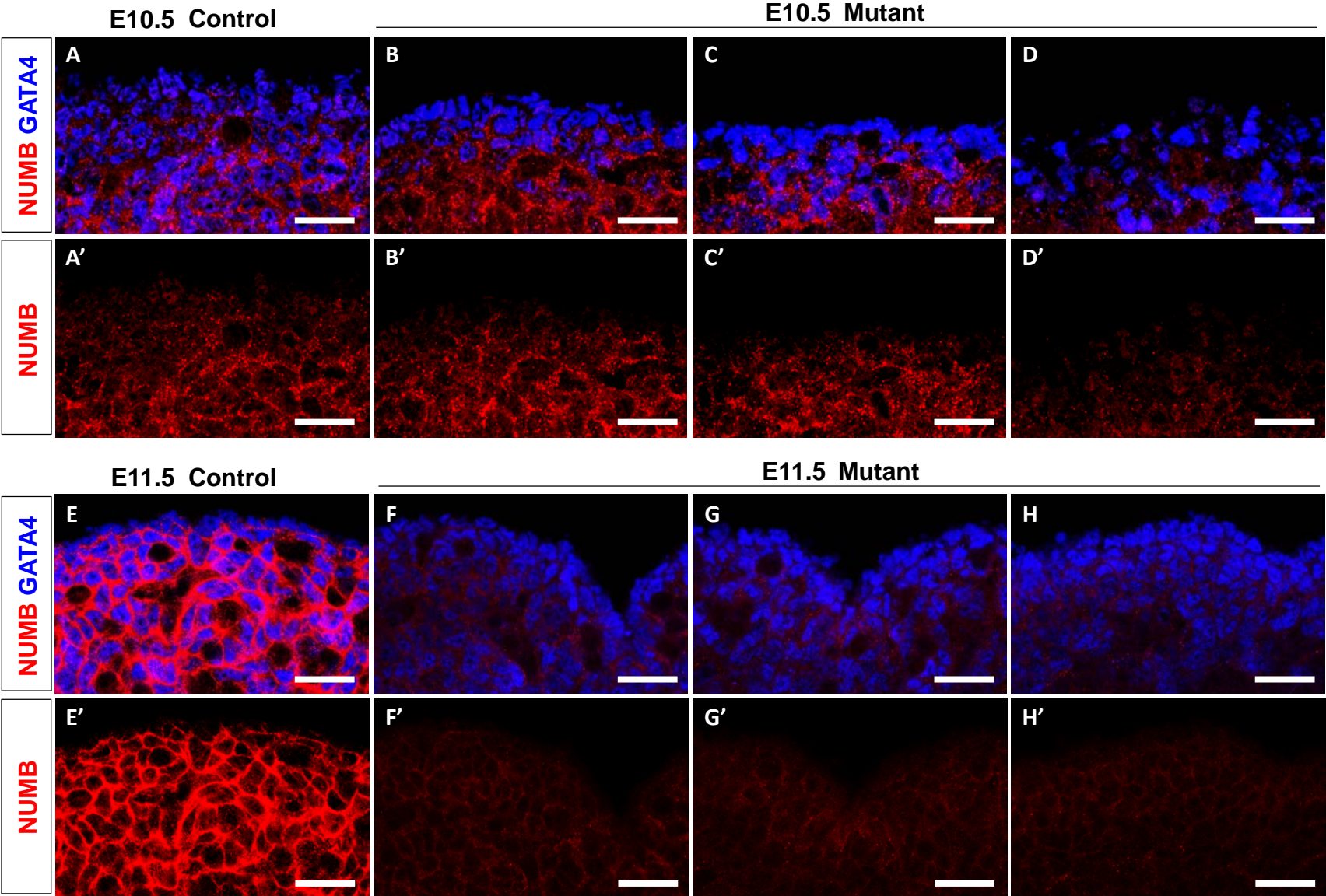
Fig. S6. Levels of NUMB protein declined between E10.5 and E11.5 after tamoxifen injection at E8.75. (A and E) In control E10.5 and E11.5 gonads, NUMB (red) was detected in almost all gonadal cells. Somatic cells were co-labeled with GATA4 (blue). (B-D) At E10.5, *Numb/Numbl* mutant gonads showed abundant 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.
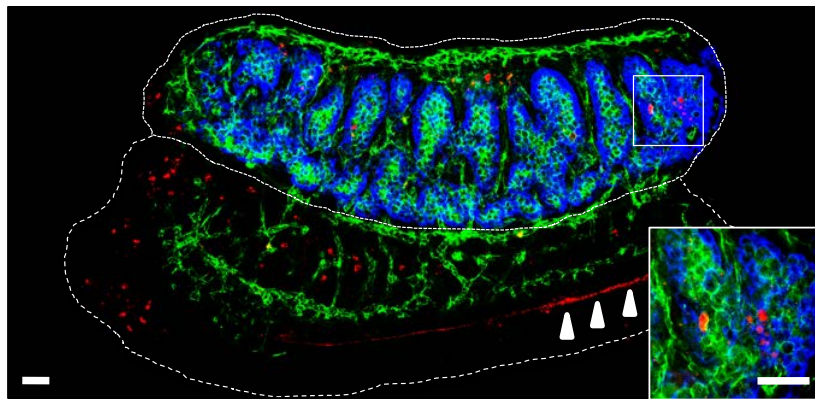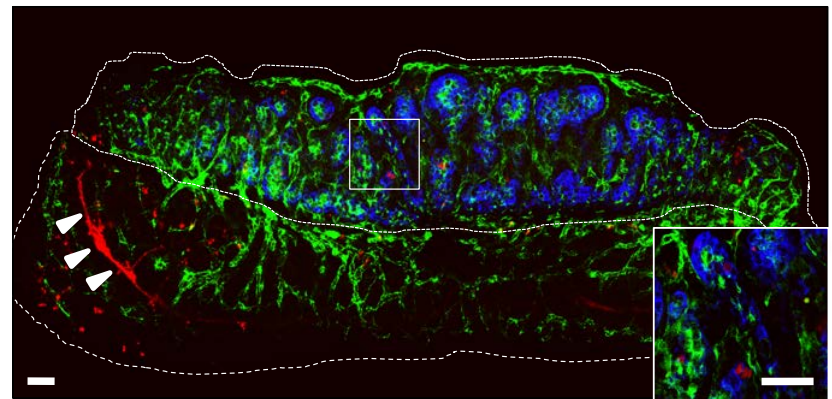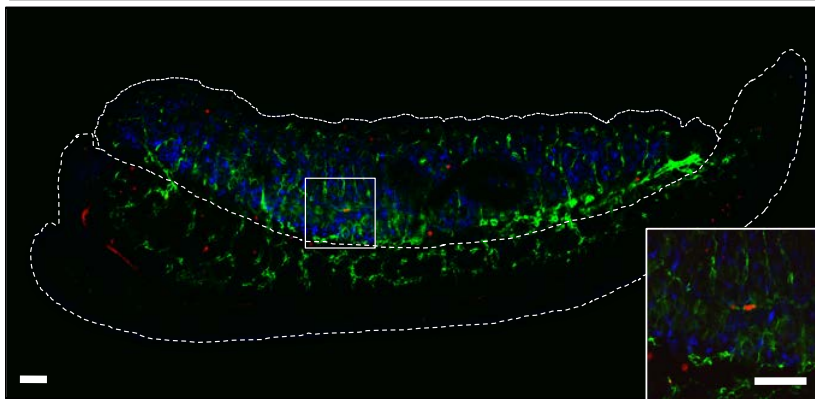
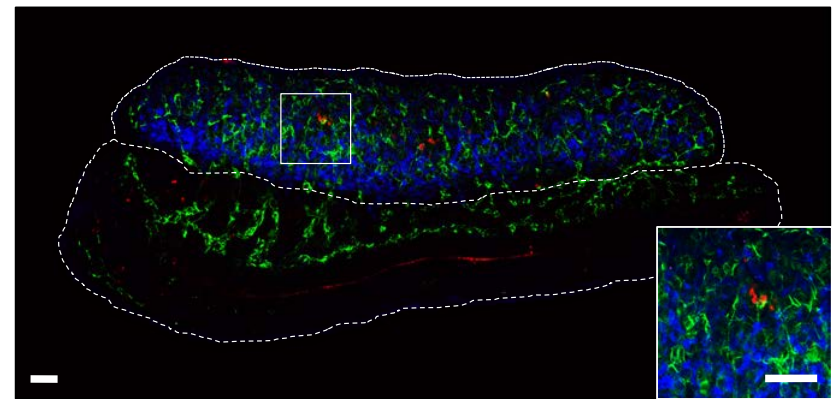**Fig. S7. Up-regulation of cell death pathways is unlikely to explain the loss of Sertoli or granulosa cells in mutants.** Few cCASP3-positive cells were observed in XX or XY control or mutant gonads at E12.75. A few positive cells are shown at higher magnification in the boxed region of each frame as a control for the antibody, which also labels the degenerating Mullerian duct in XY samples (arrowheads). Scale bars in all images=50 μm.
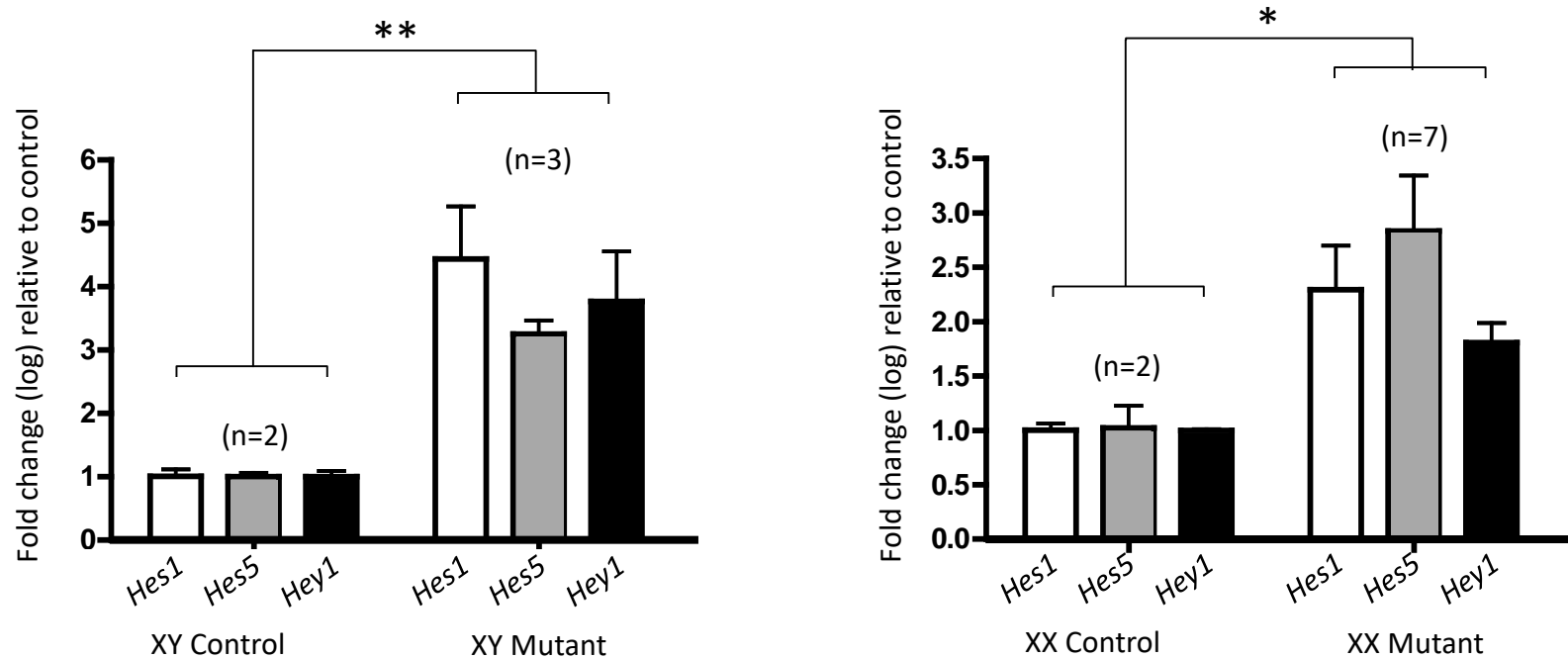
Fig. S8. Three Notch downstream target genes are upregulated in E13.5 XY and XX mutant gonads. Quantitative RT-PCR of *Hes1*, *Hes,5* and *Hey1* showed the elevation of Notch downstream signaling target genes in XY and XX mutant gonads. Statistical significance was determined by unpaired *t*-tests. *P* 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.

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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